# Studies on Drug-Induced Lipodosis (V). Changes in the Lipid Composition of Rat Liver and Spleen Following the Administration of 4,4′-Diethylaminoethoxyhexestrol

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## ABSTRACT

Administration of 4,4′-diethylaminoethoxyhexestrol increased total phospholipids and free cholesterol in rat liver. An elevation of a peculiar glycerophospholipid, lysobisphosphatidic acid, was marked both in liver and spleen. An increase in phosphatidylinositol was also noticed in spleen. However these changes in rats were not so marked as in human cases. There was an accumulation of a substance in rat tissue which was identified as a derivative of the drug, while the intact drug accumulated in large amount in human cases. Differences in the effect of 4,4′-diethylaminoethoxyhexestrol on lipid metabolism between human and rat seem to be related to the difference in drug digesting ability between these two animal species.

## INTRODUCTION

Administration of 4,4′-diethylaminoethoxyhexestrol caused a peculiar syndrome histologically characterized by the appearance of foamy histiocytes and an accumulation of intracellular particles with myelin-like structure (1-4). Similar changes occurred also in rats by administration of the compound (5,6). The present investigation gives information on the effect of 4,4′-diethylaminoethoxyhexestrol on lipid composition of rat liver and spleen. There will be discussion of the mechanism of the elevation of a peculiar glycerophospholipid, lysobisphosphatidic acid, by comparing changes in lipid composition between human and rat and comparing the metabolism and accumulation of the drug.

## MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain weighing 150-180 g were fed on an ordinary stock diet. 4,4′ -diethylaminoethoxyhexestrol dihydrochloride was administered at a level of 20 mg/kg body wt/day. In short term experiments for a period less than 2 weeks the drug was given through a stomach tube in 1% aqueous solution. In long term experiments rats were fed 4,4′-diethylaminoethoxyhexestrol dihydrochloride mixed in the stock diet. Each rat consumed an average of ca. 3.5-4 mg daily. Rats were sacrificed at 2 days, 1, 2, 3, 4, 8 and 12 weeks of feeding. Another group of rats

TABLE I

Effect of 4,4′-Diethylaminoethoxyhexestrol on the Lipid Composition of Rat Liver[a]

| Subject | Weight of liver, g | Phospholipid | Free sterol | Esterified sterol | Triglyceride |
|---|---|---|---|---|---|
| Control | 7.9 ± 0.5 | 3.13[b] ± 0.21 | 0.18 ± 0.06 | 0.06 ± 0.02 | 0.37 ± 0.17 |
| 1 Week[c] | 7.2 ± 1.1 | 3.14 ± 0.08 | 0.19 ± 0.02 | 0.12 ± 0.02 | 0.46 ± 0.12 |
| 2 Weeks | 8.2 ± 0.4 | 4.68 ± 0.52 | 0.27 ± 0.02 | 0.08 ± 0.02 | 0.43 ± 0.18 |
| 3 Weeks | 7.5 ± 0.4 | 4.18 ± 0.75 | 0.30 ± 0.04 | 0.09 ± 0.02 | 0.49 ± 0.19 |
| 4 Weeks | 7.5 ± 0.4 | 4.14 ± 0.71 | 0.26 ± 0.03 | 0.15 ± 0.03 | 0.32 ± 0.19 |
| 8 Weeks | 7.2 ± 0.8 | 3.84 ± 0.63 | 0.24 ± 0.04 | 0.09 ± 0.03 | 0.90 ± 0.32 |
| 12 Weeks | 7.2 ± 0.7 | 3.74 ± 0.17 | 0.27 ± 0.04 | 0.21 ± 0.03 | 0.94 ± 0.38 |
| 8 Weeks after withdrawal of the drug[d] | 8.3 ± 0.7 | 3.24 ± 0.57 | 0.21 ± 0.02 | 0.13 ± 0.05 | 0.56 ± 0.16 |

[a]Values are the average of five rats (± standard deviation); Body weight of rats at the end of the feeding period was 220-280 g.

[b]Per cent of wet weight of tissue.

[c]Weeks of administration of 4,4′-diethylaminoethoxyhexestrol.

[d]4,4′-Diethylaminoethoxyhexestrol administered for preceding 8 weeks.

TABLE II

Effect of 4,4'-Diethylaminoethoxyhexestrol on the Phospholipid Composition of Rat Liver[a]

| Subject | LBPA | CL | PE | PC | Sph | PI | PS | LPC | PA |
|---|---|---|---|---|---|---|---|---|---|
| Control | 0.0[b] | 5.9 ±0.3 | 26.8 ±0.6 | 50.3 ±1.3 | 4.9 ±0.2 | 7.3 ±0.6 | 3.7 ±0.5 | 0.6 ±0.2 | 0.1 ±0.1 |
| 1 Week[c] | 1.3 ±0.4 | 4.9 ±0.2 | 25.1 ±0.7 | 51.1 ±0.9 | 4.5 ±0.7 | 7.8 ±0.4 | 3.8 ±0.3 | 1.0 ±0.4 | 0.1 ±0.1 |
| 2 Weeks | 1.4 ±0.4 | 4.8 ±0.4 | 25.1 ±0.7 | 50.4 ±0.7 | 4.6 ±0.3 | 7.9 ±0.4 | 3.9 ±0.3 | 1.3 ±0.2 | 0.1 ±0.1 |
| 3 Weeks | 4.2 ±0.4 | 5.0 ±0.6 | 25.4 ±0.2 | 47.7 ±0.9 | 4.7 ±0.8 | 7.6 ±0.5 | 3.8 ±0.7 | 1.0 ±0.1 | 0.1 ±0.2 |
| 4 Weeks | 5.4 ±0.8 | 4.8 ±0.4 | 23.6 ±1.0 | 48.0 ±0.7 | 4.3 ±0.5 | 8.8 ±1.0 | 3.5 ±0.7 | 0.9 ±0.1 | 0.1 ±0.1 |
| 8 Weeks | 7.1 ±2.5 | 4.8 ±0.6 | 25.3 ±1.6 | 44.5 ±3.1 | 5.5 ±0.7 | 7.5 ±0.5 | 3.7 ±0.7 | 0.8 ±0.2 | 0.0 |
| 12 Weeks | 6.1 ±0.7 | 4.7 ±0.7 | 24.0 ±0.8 | 46.3 ±0.7 | 5.0 ±0.7 | 8.4 ±0.6 | 3.5 ±0.7 | 1.3 ±0.4 | 0.1 ±0.1 |
| 8 Weeks after withdrawal of drug | 0.7 ±0.6 | 5.4 ±0.4 | 26.5 ±0.7 | 50.0 ±1.0 | 4.7 ±0.2 | 7.1 ±0.5 | 3.5 ±0.4 | 1.3 ±0.2 | 0.2 ±0.1 |

[a]Values are the average of five rats (± standard deviation). Abbreviations: LBPA, lysobisphosphatidic acid; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserin; LPC, lysophosphatidylcholine; PA, phosphatidic acid.

[b]Per cent of total phospholipids.

[c]Weeks of administration of 4,4'-diethylaminoethoxyhexestrol.

were fed on the stock diet without drug after the drug had been administered for 8 weeks. These rats were sacrificed at 8 weeks after withdrawal of the drug.

Rats were sacrificed by exsanguination. Liver and spleen were removed and homogenized with 20 volumes of chloroform-methanol (C/M) 2:1 and washed by the method of Folch et al. (7). Free cholesterol, cholesterol ester and triglyceride were determined by thin layer chromatography (TLC) → colorimetric assay procedures as previously described (8,9). Colorimetric assay of cholesterol was performed by the method of Hanel and Dam, modification of Tschugaeff reaction (10). By using this method, desmosterol gave almost the same color yield as cholesterol. Separation and determination of cholesterol and desmosterol were carried out on gas liquid chromatography (GLC) using a 3 mm x 6 ft column of 1% $QF_1$ or XE-60 on 80-100 mesh Gaschrom Q (11). Shimadzu GC-4A apparatus equipped with hydrogen flame ionization detector was used for GLC. Phospholipid analysis was carried out on TLC essentially according to the method of Rouser et al. (3,12).

Identification of 4,4'-diethylaminoethoxyhexestrol and its derivatives was performed on TLC followed by IR spectroscopy and GLC. TLC was developed with chloroform-methanol-aqueous ammonia (28%) 190:10:1.6 into the first dimension and with chloroform-acetone-methanol-acetic acid-water 100:40:30:20:12 into the second dimension. Spots were detected by charring with sulfuric acid or by spraying Dragendorff reagent. For identification and determination of 4,4'-diethylaminoethoxyhexestrol, total lipids extracted from the tissue were applied on TLC and developed with C/M/aqeous ammonia 190:10:1.6. Standard substance was applied on one side on the plate and the spot of this standard was visualized by Dragendorff reagent. The area of samples corresponding to the standard was scraped and extracted with C/M 2:1 added with a small amount of 28% aqueous ammonia 100:3. The extract was evaporated to dryness under nitrogen stream, the residue dissolved in carbon disulfide and analyzed on GLC using 1% XE-60 or SE-30 on 80-100 mesh Gaschrom Q packed in a glass column. Column temperature was 220 C and nitrogen was used as a carrier gas at a pressure of 2.0 kg/cm$^2$.

For extraction and purification of a major component of derivatives of 4,4'-diethylaminoethoxyhexestrol from rat liver, total lipids extracted from 8 g of livers were treated with 30 ml of 0.3 N NaOH in 95% ethanol at 50 C for 1 hr. An excess amount of ethylacetate was

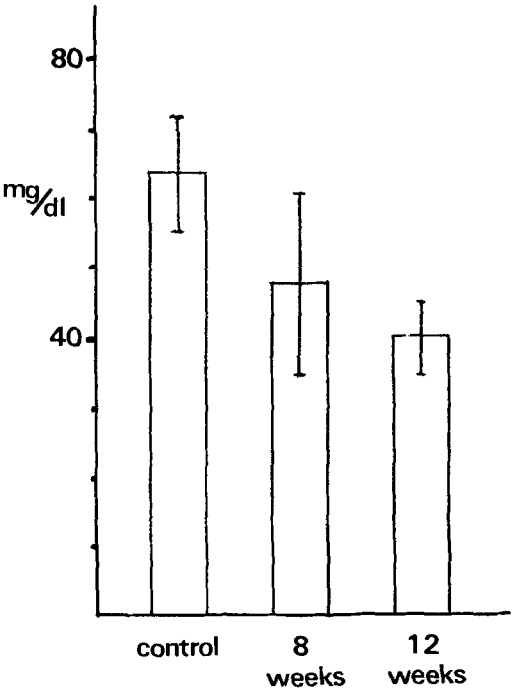


FIG. 1. Effect of 4,4'-diethylaminoethoxyhexestrol on the cholesterol level in blood serum of rat.

added and the sample was dried under nitrogen stream. 5 ml of 1 N HCl were added to the residue and fatty acids were extracted twice with 5 ml of petroleum ether. After addition of 20 ml of C/M 2:1 to the water phase with a fluff floating on the top, the sample was partitioned between C/M/$H_2O$. The lower phase was discarded after reextraction with pure upper phase. The combined upper phase was evaporated to dryness under nitrogen stream and the sample was then partitioned between C/M/diluted aqueous ammonia. The lower phase was taken, evaporated to dryness and purified on TLC. The sample obtained from TLC was analyzed on IR specroscopy.

## RESULTS

### Lipid Composition of the Liver

There was a significant increase in total phospholipids after 2 weeks of administration of 4,4'-diethylaminoethoxyhexestrol (Table I). However further increase in concentration of phospholipid was not observed after this period up to 12 weeks. Free sterols (cholesterol plus desmosterol) showed the same tendency as total phospholipids. The concentration of triglycerides in the liver showed no change until 8 weeks when there was promptly a marked

TABLE III

Effect of 4,4'-Diethylaminoethoxyhexestrol on the Phospholipid Composition of Rat Spleen[a]

| Subject | LBPA | CL | PE | PC | Sph | PI | PS | LPC | PA |
|---|---|---|---|---|---|---|---|---|---|
| Control | 0.4[b] ±0.2 | 1.9 ±0.5 | 26.2 ±0.9 | 47.4 ±0.9 | 7.3 ±0.3 | 4.9 ±0.3 | 9.3 ±0.3 | 1.3 ±0.3 | 0.3 ±0.2 |
| 2 Days[c] | 1.1 ±0.1 | 1.7 ±0.3 | 26.5 ±0.2 | 47.5 ±0.4 | 6.8 ±1.4 | 5.3 ±0.8 | 9.2 ±0.2 | 1.3 ±0.2 | 0.2 ±0.1 |
| 1 Week | 2.6 ±0.3 | 1.5 ±0.2 | 25.2 ±0.9 | 45.1 ±1.1 | 7.5 ±0.1 | 5.7 ±0.6 | 9.4 ±0.4 | 1.9 ±0.5 | 0.4 ±0.1 |
| 2 Weeks | 2.8 ±0.5 | 1.3 ±0.8 | 24.8 ±0.9 | 46.4 ±0.6 | 7.5 ±0.2 | 5.0 ±1.1 | 9.2 ±0.9 | 1.4 ±0.2 | 0.4 ±0.2 |
| 3 Weeks | 3.5 ±1.0 | 2.2 ±0.4 | 25.3 ±0.6 | 46.3 ±0.7 | 6.7 ±0.7 | 5.5 ±0.6 | 8.5 ±0.6 | 1.3 ±0.3 | 0.2 ±0.2 |
| 4 Weeks | 5.3 ±1.2 | 2.1 ±0.5 | 23.7 ±1.8 | 44.6 ±1.9 | 6.1 ±0.4 | 6.9 ±0.4 | 7.7 ±0.5 | 1.5 ±0.3 | 0.3 ±0.2 |
| 8 Weeks | 5.7 ±1.1 | 1.9 ±0.5 | 21.7 ±0.8 | 42.9 ±1.8 | 8.6 ±1.4 | 8.0 ±0.8 | 7.0 ±0.8 | 2.1 ±0.6 | 0.1 ±0.1 |
| 12 Weeks | 5.6 ±0.8 | 1.9 ±0.6 | 23.4 ±0.8 | 45.7 ±0.8 | 6.8 ±0.6 | 6.5 ±0.7 | 7.6 ±1.1 | 1.1 ±0.2 | 0.1 ±0.1 |
| 8 Weeks after withdrawal of drug | 1.5 ±0.3 | 1.7 ±0.2 | 25.8 ±0.5 | 47.2 ±1.0 | 7.9 ±0.3 | 5.0 ±0.4 | 8.8 ±0.8 | 1.4 ±0.1 | 0.3 ±0.1 |

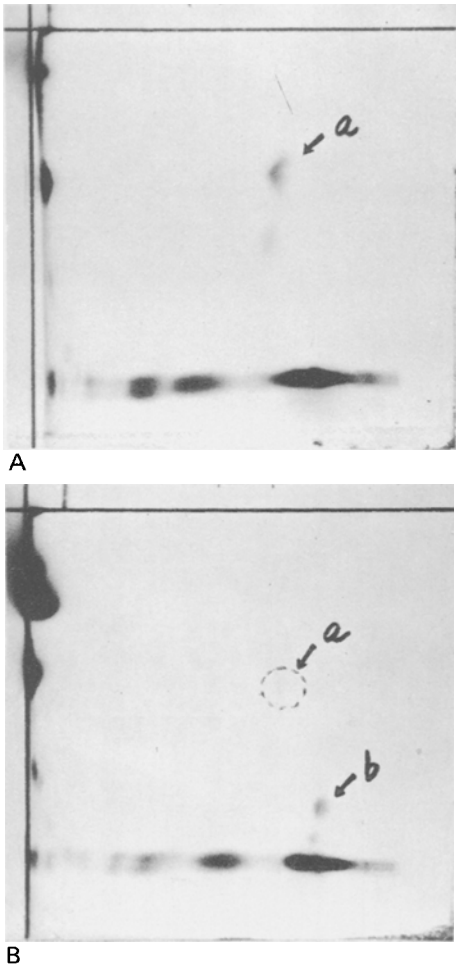


FIG. 2. Accumulation of 4,4′-diethylaminoethoxyhexestrol in the liver (2D-thin layer chromatography of total liver lipids). A, human; B, rat.

increase. Sterol esters also showed a slight but significant increase at this stage.

After stopping administration of the drug for 8 weeks, concentrations of total phospholipids, free sterols, sterol esters and triglycerides were all recovered to the control level.

#### Phospholipid Composition of the Liver

Phospholipid analysis showed that there was a gradual increase in lysobisphosphatidic acid in the liver following administration of 4,4′-diethylaminoethoxyhexestrol (Table II). At 8 weeks of feeding, the concentration of lysobisphosphatidic acid attained its maximum and occupied 6-7% of the total phospholipid. After withdrawal of the drug for 8 weeks a slight increase in this glycerophospholipid was still observed. There were slight decreases in concentration of cardiolipin and phosphatidylethanolamine during administration of the drug.

#### Phospholipid Composition of the Spleen

An increase in concentration of lysobisphosphatidic acid was observed in spleen already at 2 days of administration of the drug. (Table III) Lysobisphosphatidic acid per cent of total phospholipids in early stages of the feeding was larger in spleen than in liver, while the later increment was more prominant in liver. There was also an increase in concentration of phosphatidylinositol in spleen at 8 weeks of feeding, when the concentration of lysobisphosphatidic acid reached its maximum. At 8 weeks after withdrawal of the drug, concentration of phosphatidylinositol was almost in normal range, while concentration of lysobisphosphatidic acid was still significantly higher than normal value.

#### Changes in Cholesterol Concentration in Blood Serum

In contrast to human cases, 4,4′-diethylaminoethoxyhexestrol showed a cholesterol lowering effect in rats (Fig. 1). The decrease in serum cholesterol concentration became most significant after 12 weeks of feeding.

#### Accumulation of 4,4′-Diethylaminoethoxyhexestrol and Derivatives in Tissues

On two dimensional TLC of total lipids from liver and spleen developed with C/M/28% ammonia 190:10:1.6 and C/acetone/M/acetic acid/$H_2O$ 100:40:30:20:12, a spot (Spot a, Fig. 2) corresponding to the drug itself was detected. However the spot was not so intense in rats as in human cases. Another spot (Spot b) showing a smaller rate of migration was observed clearly in rats but was not detected in human cases. Each of these two spots was accompanied by a small additional spot which migrated closed to the parent spot.

IR spectrum of the substance which was extracted from the spot peculiar to rat tissue (Spot b) showed absorption at 1240, 1530 and 1620 ($cm^{-1}$) characteristic of 4,4′-diethylaminoethoxyhexestrol (Fig. 3).

#### Presence of Desmosterol in Liver, Spleen and Blood Serum

Desmosterol was determined in control and treated rats at 3 weeks of feeding. GLC analysis showed that desmosterol occupied 9.4% and 9.3% of total sterols in liver and in spleen in the treated rats but was not detected in control animal.

#### Changes in Lipid Composition of Rat Livers Following Administration of Triparanol

Triparanol has a chemical structure some-

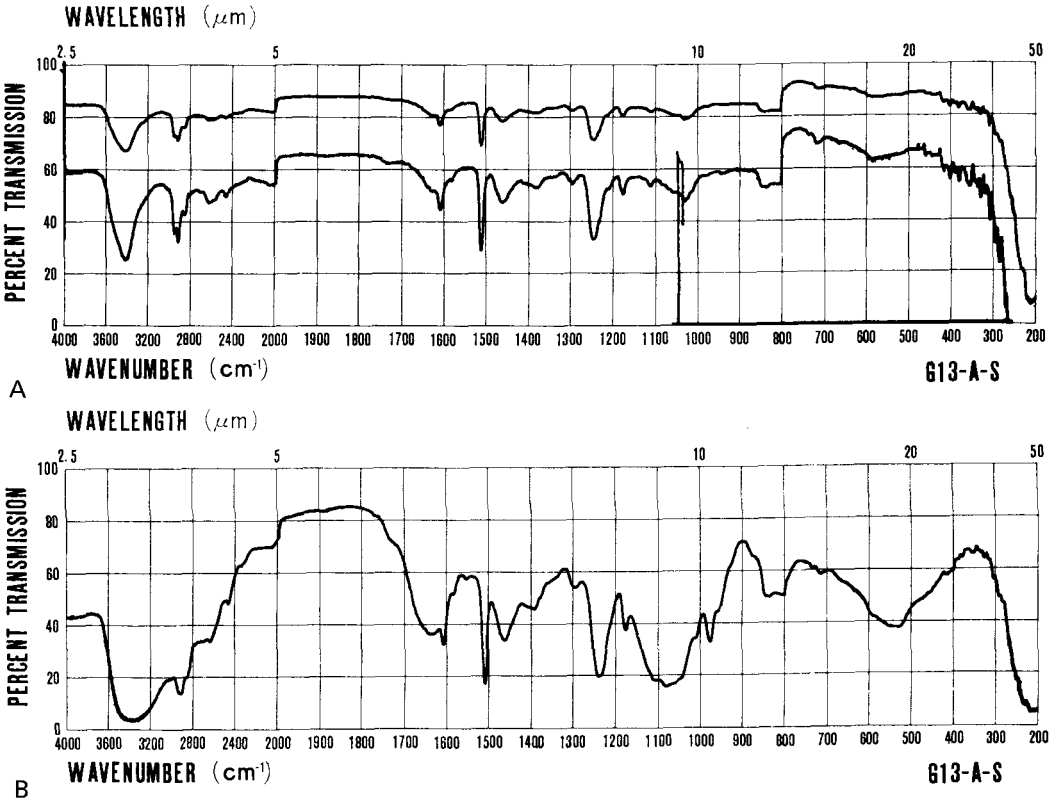


FIG. 3. IR spectrum of 4,4'-diethylaminoethoxyhexestrol and its derivative formed in rat liver. A, 4,4'-diethylaminoethoxyhexestrol dihydrochloride: B, partially purified derivative of the drug obtained from livers of rats treated with 4,4 -diethylaminoethoxyhexestrol.

what resembling 4,4'-diethylaminoethoxyhexestrol. Both drugs show a cholesterol lowering effect on rats. However triparanol gave no change in phospholipid composition of the liver when it was administered at a level of 20 mg/kg body wt for 2 weeks. Considerable increase in lysobisphosphatidic acid was noticed after 1 week of administeration when the drug was given at a level of 250 mg/kg per day (Fig. 4).

Effect of triparanol on cholesterol metabolism was clearly demonstrated even when the drug was given in a smaller dose, and the concentration of desmosterol in total sterols attained 50% in blood serum.

## DISCUSSION

Comparison of clinical and experimental data on drug-induced lipidosis (4,6,11) shows that the effect of 4,4'-diethylaminoethoxyhexestrol on lipid metabolism and cellular structure differs in several points between human and rat. (1) There was a 70% increase in total phospholipids and free cholesterol in the liver in humans, while the rate of increase was less than 50% in rats. (2) The concentration of lysobisphosphatidic acid in total phospholipids attained 25% in humans, while it reached a maximum at a level of 6-7% of total phospholipids in rats. There was a marked increase in phosphatidylinositol in human cases. However phosphatidylinositol showed no increase in rat liver. (3) There was marked elevation of cholesterol level in blood serum in humans. However the drug showed a cholesterol-lowering effect in rats (13). (4) Hepatosplenomegaly was one of the most remarkable signs in drug-induced lipidosis in humans. In rats there was no significant increase in the weight of liver and spleen in the drug-treated group. (5) Accumulation of intracellular particles with laminated structure was commonly seen in human cases and in animal experiments. However the change was more marked in reticuloendothelial cells in rat liver and the formation of the concentrically laminated structure was not so marked in hepatocytes in rat as in human livers. Formation of vacuoles which was observed in histiocytes and leucocytes was rather prominent in rat liver.

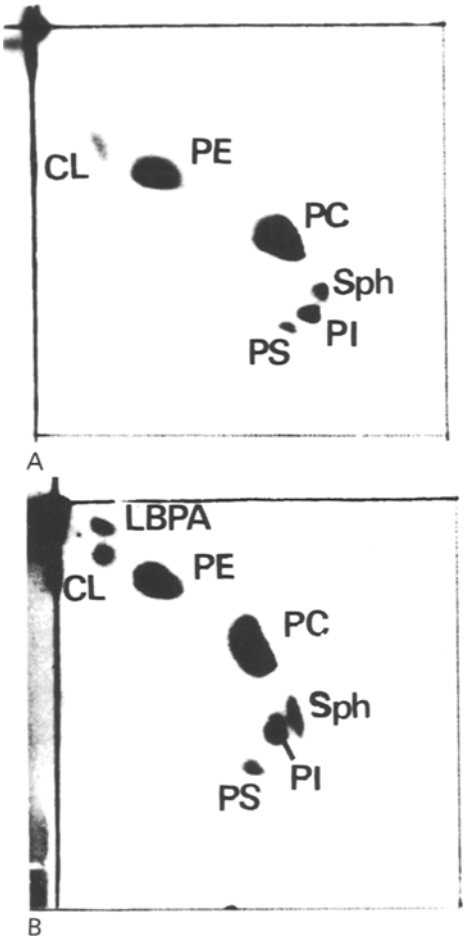


FIG. 4. Effect of triparanol on the phospholipid composition of rat livers. A, 4 mg/day for 2 weeks; B, 50 mg/day for 1 week.

A long term experiment (longer than 6 months) may be necessary to make the comparison more accurate, because patients claimed their symptoms after 6 months of administration of the drug (about 3 mg/kg body wt daily). However a search for the accumulation of the drug and its derivatives in tissues seems to give enough evidence to explain these differences between human and rat.

The amount of the drug which accumulated in the liver in one of our cases was 0.3% of the wet weight of the tissue. In rats the major component of the storage substance (spot b, Fig. 2) seems to be more polar than the original compound, 4,4'-diethylaminoethoxyhexestrol. The metabolite was not detected in human liver and spleen. The result shows that the drug is metabolized in rats whereas intracellular digestion of the drug does not take place in human liver. Species difference in the ability to digest a drug has been reported (14).

Recovery test gave further evidence of differences in drug metabolism. In rats, changes in lipid composition were almost completely recovered by withdrawal of the drug for 8 weeks. Human cases showed very slow recovery. Even at 1.5 years after stopping administration of the drug, the syndrome composed of accelerated blood sedimentation rate, hepatosplenomegaly, hyperlipidemia, increases in total phospholipids and lysobisphosphatidic acid in the liver, the presence of foamy histiocytes and the accumulation of myelin-like structure was still noticeable. Further observations on the clinical course and measurement of the amount of the drug accumulating in the liver of patients are still in progress in our laboratory.

Lysobisphosphatidic acid seems to have some relationship to accumulation of the drug. Elevation of this phospholipid was first observed in Niemann-Pick disease (15). Acid phosphatase activity was histochemically demonstrated on the intracellular particles both in Niemann-Pick disease (16) and in the present lipidosis (17). These results seem to support a hypothesis that the intracellular inclusions are of lysosomal origin and lysobisphosphatidic acid is a naturally occurring substance which is proper to some kind of intracellular structure participating in the metabolism and excretion of foreign substances. Probably the phospholipid showed an adaptive increase in Niemann-Pick disease as well as in the present cases. The speculation seems reasonable because acidic glycerophospholipids with similar structure show peculiar distribution pattern among cell organellae, as cardiolipin specific to mitochondria and phosphatidylglycerol to plant chloroplast.

Triparanol shows a strong hypocholesterolemic effect in rats in a small dose (18). It was reported that triparanol induces a formation of myelin-like structure when administered in large amount (19). Data of our present investigation agree with results of previous authors and showed that formation of the myelin-like structure coincides with accumulation of lysobisphosphatidic acid.

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# Fat Metabolism in Higher Plants XLIX Fatty Acid Biosynthesis by Subcellular Fractions of Higher Plants[1]

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## ABSTRACT

A method is described for the rapid and comprehensive subcellular fractionation of plant tissue using a combination of differential and discontinuous Ficoll gradient centrifugation. The procedure has been used to study the synthesis of fatty acids from acetate-1-$^{14}$C or malonyl CoA-1,3-$^{14}$C, by fractions of germinating pea and lupin seeds and developing avocado fruit, castor bean and safflower seeds. Particle free supernatants of seeds synthesize fatty acids from $^{14}$C-malonyl CoA in the presence of added cofactors. Since acetyl CoA carboxylase activity is absent the utilization of $^{14}$C-acetate by these fractions is minimal. Other particulate fractions show different activities depending on seed types. Active fractions include the low speed particulate of pea and lupin, the pea microsomes, the avocado mesocarp chloroplasts, and the fat fractions of castor bean and safflower. Individual fractions produce characteristic patterns of acids; especially noteworthy is oleic acid biosynthesis by soluble enzymes of castor bean and safflower from $^{14}$C-malonyl CoA. Some characteristics of the avocado supernatant, pea supernatant, and castor bean fat fraction synthesizing systems are compared. As a result of these studies, generalizations derived from work with mammalian or bacterial systems cannot be applied to higher plants.

## INTRODUCTION

Localization of the site of mammalian fatty acid synthesis has now been achieved with a number of tissues. Generally the fatty acid synthetase complex is soluble, while the mitochondria contain elongation enzymes and the microsomes the desaturases (1,2). In contrast much less is known of plant systems. Several workers have studied isolated fractions, but detailed analysis of intracellular distribution was rarely undertaken. With the development of a rapid method for plant cell fractionation, it has become possible to analyze the problem in detail.

Early work with isolated plant organelles was, not surprisingly, with chloroplasts. Smirnov (3) reported rapid incorporation of $^{14}$C-acetate into long chain fatty acids by spinach chloroplasts. This organelle has also been used for further studies by Brooks and Stumpf (4) who implicated acyl-carrier protein (ACP) and by Nagai and Bloch (5) who examined the stearyl-ACP desaturase. Later work on the control of oleic acid synthesis by spinach chloroplasts has also been reported (6).

Apple microsomes synthesized fatty acids, particularly polyunsaturated, from acetate (7), and a soluble fatty acid synthetase from potato has been examined (8). Pea mitochondrial, microsomal and supernatant fractions were all active in fatty acid synthesis, but with differences of detail (9). Soybean cotyledons contain soluble fatty acid synthesizing systems (10), particularly desaturases (11). Following reports of particulate systems from avocado mesocarp (12), Yang and Stumpf (13) showed that this tissue also contained a soluble enzyme capable of using malonate but not acetate. Recently Weaire and Kekwick (14) have concluded that the particulate activity is owing to the intact chloroplast, and soluble activity to leakage of stromal proteins.

Perhaps the most extensive research has involved the developing castor bean. Canvin's original analysis of endogeneous lipid (15) indicated that a sequence of synthesis of different acids occurred during development, and this was confirmed by work with tissue slices (16). The hydroxylation of oleic acid to ricinoleic acid was studied with extracts (17) and later with microsomal fractions (18). A supernatant system using malonyl CoA synthesized mainly saturated acids (19), while oleic acid synthesis by a particulate preparation (20) was later localized in a specific nonmitochondrial particle (21).

In order to clarify the sites of fatty acid synthesis in plants, we have examined the synthesis of fatty acids from $^{14}$C-acetate and $^{14}$C-malonyl CoA in well-characterized subcellular fractions from two low lipid (pea, lupin) and two high lipid (avocado, castor bean) tissues.

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

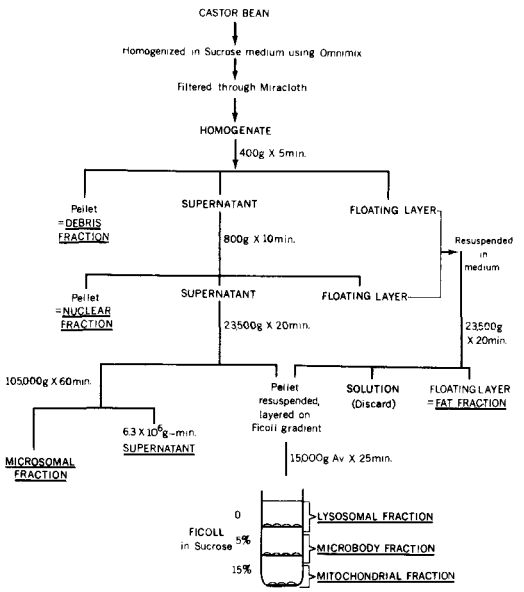


FIG. 1. Fraction scheme for castor bean. Final fractions are underlined. See Experimental Procedures for details.

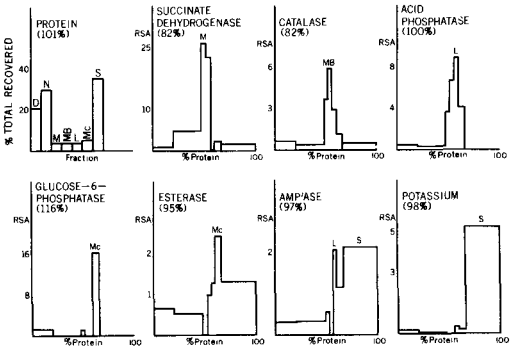


FIG. 2. Subcellular fractionation of lupin. Results are the average of two fractionations and expressed as relative specific activity (RSA) in relation to homogenate. Percentage recovered activity is indicated in parentheses. Homogenate values for: Succinate dehydrogenase = 0.79 nmoles/min/mg protein; catalase = 278; acid phosphatase = 2.19; glucose-6-phosphatase = 0.81; esterase = 2,73; AMPase = 3.15; potassium = 11.05 μg/mg protein. Fraction designations: D = debris, N = nuclear, M = mitochondrial, MB = microbodies, L = lysosomal, Mc = microsomal, S = supernatant.

## EXPERIMENTAL PROCEDURES

### Materials

Pea seeds, *Pisum sativum L.* Alaska, were obtained from the Asgrow Seed Co., New Haven, Conn.; castor bean, *Ricinus communis L.* from the Department of Horticulture of this University; and avocado, *Persea americana* from the local supermarket. Safflower, *Carthamus tinctorius* variety Hela, seeds were kindly donated by P. Knowles, Department of Agronomy; and Lupin, *Lupinus angustifolius* seeds by E.E. Conn of this department.

Malonic-2-$^{14}$C acid (14.5 mc/mM) was obtained from New England Nuclear. Acetyl CoA-1-$^{14}$C was synthesized by the method of Simon and Shemin (22). Acetate-1-$^{14}$C, Malonyl CoA-1,3-$^{14}$C and ACP were obtained as previously cited (23).

Ficoll was obtained from Pharmacia and silicic acid for chromatography from E. Merck, Darmstadt, Germany; L-malic acid was obtained from Eastman Organic Chemicals; titanium sulphate from K and K Laboratories Inc.; *p*-Nitrophenylacetate from Mann Chemicals; guaiacol from Matheson, Coleman and Bell; and iodonitrotetrazolium violet, β-glycerophosphate, *p*-nitrophenylglucuronide, glucose-6-phosphate, NADP, NADPH, NADH, UDP, AMP, ATP, glyceraldehyde-3-phosphate, and Horse Radish peroxidase from Sigma.

### Germination

Pea and lupin seeds were germinated in dionized water containing 50 μg/ml chloramphenicol (to prevent bacterial growth) at room temperature for 30 hr.

### DNA Estimation

DNA, which was used as a nuclear marker, was estimated by the method of Burton (24) after extraction as described by Schneider (25).

### Chlorophyll

The method of Arnon (26) employing 80% acetone was used.

### Triglyceride

Triglyceride was extracted by the method of Bligh and Dyer (27) and separated by thin layer chromatography (TLC) on Silica Gel G using diethyl ether-petroleum ether 1:9 as solvent. The purified lipid was scraped from the plate, extracted into diethyl ether-methanol 4:1 and measured as ester by the method of Stearn and Shapiro (28).

### Protein

This was estimated by the Lowry et al. (29) or Gornell et al. (30) procedures. Ether extractions were necessary to clear high lipid-containing fractions.

### Enzyme Assays

Assays for mitochondria were for succinate dehydrogenase (EC 1.3.99.1) (31), fumarase (EC 4.2.1.2) (32) and cytochrome oxidase (EC 1.9.3.1) (33). Catalase (EC 1.11.1.6) was used

TABLE I

Subcellular Fractionation of Developing Avocado Tissue[a]

| Estimate | Homogenate | Fractions | | | | | | | | Number Fractionation tested |
|---|---|---|---|---|---|---|---|---|---|---|
| | | N | F | P/C | M | MB | L | Mc | S | |
| Protein | 94 ± 1.9% Recovery | 5.9 ±1.4 | 22.2 ±4.6 | 3.4 ±1.0 | 7.1 ±0.1 | 5.7 ±0.9 | 3.1 ±0.2 | 5.1 ±1.3 | 47.5 ±5.2 | 4 |
| DNA | 91% Recovery 116 μg DNA-Pi/gm protein | 7.41 | n.d. | 1.43 | n.d. | n.d. | n.d. | n.d. | 1.11 | 1 |
| Succinate dehydrogenase | (106 ± 10.2%) Recovery 3.66 ± 1.32 mμmoles/min/mg protein | 1.49 ±0.16 | 2.63 ±0.18 | 1.25 ±0.06 | 2.21 ±0.43 | 2.07 ±0.36 | 1.15 ±0.05 | 0.53 ±0.07 | 0.01 ±0 | 3 |
| Chlorophyll | (85%.0 ± 6.0%) Recovery 2.05 ± 1.5 μg/mg protein | 2.71 ±1.51 | 1.52 ±0.41 | 10.39 ±4.61 | n.d. | n.d. | 0.21 ±0.26 | 0.38 ±0.14 | n.d. | 3 |
| RDP Carboxylase | (98%) Recovery | 1.96 | 1.50 | n.d. | n.d. | n.d. | n.d. | 1.51 | 1.26 | 1 |
| Catalase | (96.0 ± 6.2%) Recovery 12480 ± 2500 mμmoles/min/mg protein | 1.29 ±0.19 | 0.89 ±0.43 | 1.12 ±0.10 | 0.86 ±0.15 | 1.30 ±0.30 | 1.50 ±0.36 | 1.82 ±0.55 | 1.12 ±0.18 | 3 |
| Acid phosphatase | (113.0 ± 4.0%) Recovery 35.9 ± 8.1 mμmoles/min/mg protein | 1.05 ±0.60 | 0.52 ±0.43 | 0.15 ±0.10 | 0.28 ±0.19 | 0.65 ±0.05 | 1.02 ±0.17 | 1.58 ±0.35 | 2.20 ±0.20 | 2 |
| Glucose-6-phosphatase | (118.0 ± 1.0%) Recovery 9.1 ± 2.6 mμmoles/min/mg protein | 0.68 ±0.20 | 0.29 ±0.12 | 0.11 ±0.08 | 0.09 ±0.06 | 0.70 ±0.33 | 0.56 ±0.14 | 1.20 ±0.02 | 1.95 ±1.40 | 2 |
| Esterase | 91% Recovery 10.8 mμmoles/min/mg protein | 0.42 | 0.50 | 0.08 | 0.28 | 0.32 | 0.42 | 1.19 | 0.79 | 1 |
| UDPase | 129% Recovery 38.5 mμmoles/min/mg protein | 0.65 | 0.30 | 0.57 | 0.98 | 0.13 | 0.47 | 2.30 | 2.78 | 1 |
| AMPase | 123% Recovery 4.1 mμmoles/min/mg protein | 2.92 | 0.56 | 2.72 | 0.78 | 2.83 | 5.63 | 1.26 | 0.48 | 1 |
| Peroxidase | 128% Recovery 45.7 mμmoles/min/mg protein | 0.82 | 0.52 | 0.11 | n.d. | 0.63 | 0.62 | 0.72 | 2.69 | 1 |
| Triglyceride | 91% Recovery 12.30 meq ester/mg protein | 0.20 | 3.46 | n.d. | n.d. | n.d. | 0.06 | 0.13 | n.d. | 1 |
| Potassium | 121% Recovery 66.3 μgK/mg protein | 0.24 | 0.06 | 0.03 | 0.02 | 0.08 | 0.38 | 0.75 | 2.22 | 1 |

[a]Results are expressed as relative specific activity (RSA) of homogenate values. Means ± SEM (where available). For nomenclature of fractions see Figure 2. F = fat fraction, P/C = proplastic-chloroplast fragments, n.d. = not detectable. Mesocarp and endocarp layers analyzed. Protein is given as percentage recovered in each fraction ± SEM.

to measure microbodies and was estimated by the method of Michell et al. (35) as was lysosomal β-glucuronidase (EC 3.2.1.31). Acid phosphatase (EC 3.1.3.2) was also used as a lysosomal marker. This enzyme and the rough endoplasmic reticulum glucose-6-phosphatase (EC 3.1.3.9) were measured by the method of Hubscher and West (36). Esterase (EC 3.1.1.2) using *p*-nitrophenylacetate for substrate was assayed according to the method of Bier (37) and the smooth endoplasmic reticulum UDPase (EC 3.6.1.6) by the method of Heppel et al. (38). AMPase (EC 3.1.3.5), a plasma membrane enzyme, and potassium were measured as previously (31). Ribulose 1,5 diphosphate carboxylase, a chloroplast stromal enzyme, was estimated according to Anderson et al. (39).

#### Fatty Acid Biosynthesis

Incubations of fractions with acetate, acetyl CoA or malonyl CoA, and lipid extractions were carried out as previously described (9). Analysis of individual samples by TLC or gas liquid chromatography (GLC) was as described before (23), but 15% HI-EFF-2BP columns (Applied Science Labs.) were also used.

#### Subcellular Fractionation

Pea and lupin seeds (30 hr germination) or castor bean seeds (20-60 DAF) were blended for 10 sec at 90 v in an Omnimix blender in 0.32M sucrose-2mM Tris-HCl pH 7.4- 0.5mM dithiothreitol. The ratio of tissue to medium was about 1:2 w/w. Avocado fruit was homogenized in a Potter homogenizer of ca. 0.20 mm radial clearance using 500 rpm and five complete strokes. Crude homogenates were filtered through two layers of Miracloth to give the starting homogenate. Further fractionation was as follows: (a) Lupin and pea: Centrifugation was carried out at 1-4 C. The homogenates were spun at 400 g x 5 min in a Sorvall RC-2B supercentrifuge. The pellet was designated the debris fraction and the supernatant spun at 800 g x 10 min to yield the nuclear pellet. The supernatant was then centrifuged at 23,500 g x 20 min. The post-23,500 g supernatant was spun at 105,000 g x 60 min in a Spinco model L ultracentrifuge to yield the microsomal and the 6.3 x $10^6$/min supernatant fractions. The 23,500 g pellet was resuspended at 5-20 mg protein/ml of the original sucrose medium using a Potter homogenizer. The suspension was layered on an equal volume gradient of 5:15% Ficoll in sucrose medium and the gradients spun at 15,000 g Av. x 25 min. in a swing-out bucket rotor. The top phase and 0:5% interface were designated the lysosomal layer, the 5:15% interface the micro-body layer, and the denser particles the mitochondrial fraction. The fractions were removed with a pasteur pipette, and resuspended with a Potter homogenizer. (b) Castor bean and safflower: Centrifugation was carried out in the above manner except that a floating lipid layer was formed during the debris and nuclear spins. This layer was removed by a spatula and washed with .5 original volume of sucrose medium before spinning at 23,500 g x 20 min. The floating layer was again removed and resuspended in sucrose medium to give the fat fraction while the remaining solution was discarded. This fractionation is depicted in Figure 1. (c) Avocado: The initial homogenate was spun at 800 g x 10 min to give the nuclear pellet, supernatant and floating lipid layer. The latter was removed and resuspended in half the original volume of sucrose medium. The suspension and the post nuclear supernatant were each spun at 23,500 g x 20 min. The floating lipid layers were removed, resuspended and designated as the fat fraction. The pellets were resuspended at about 5 mg protein/ml medium and layered in a Ficoll gradient as described above. The gradient was then spun at 10,500 g Av. x 20 min. and the resulting layers designated as follows: loading volume = lysosomal; 0-5% interface = microbody; 5-15% interface = mitochondrial; pellet = chloroplast (fragments).

Conditions for high speed centrifugation were as described for the pea. Mesocarp and endocarp layers were analyzed both separately and together. When endocarp alone was used, the pellet from the gradient was designated proplastid membrane fraction.

#### Ficoll Gradients

Continuous Ficoll gradients were set up using a Multiple Sucrose Gradient Marker (Hoefer Scientific Instruments, San Francisco). All gradients were allowed to stand at 4 C for 15 min before use. One milliliter fractions were collected from the centrifuged gradients by displacement using the most dense Ficoll solution and a syringe. Discontinuous Ficoll gradients were made up using precooled (4 C) solutions. After standing at room temperature for 15 min they were placed at 4 C for 30 min, and after sample loading used immediately.

#### Electron Microscopy

Samples for electron microscope examination were fixed in glutaraldehyde and stained with osmic acid. After embedding in Araldite, the sections were examined with a RCA-EMU-3G electron microscope.

TABLE II

Subcellular Fractionation of Developing Castor Bean[a]

| Estimate | Homogenate | Fractions | | | | | | | | Number fractionations tested |
|---|---|---|---|---|---|---|---|---|---|---|
| | | D | N | f | M | MB | L | Mc | S | |
| Protein | 99 ± 2% Recovery | 18.5 ±3.2 | 3.9 ±1.1 | 16.0 ±9.1 | 3.5 ±1.4 | 5.8 ±2.4 | 5.2 ±3.2 | 3.2 ±0.8 | 43.9 ±4.8 | 4 |
| Succinate dehydrogenase | 86.0 ± 3.1 Recovery 3.73 ± 0.36 mμmoles/min/mg | 0.43 ±0.38 | 6.63 ±4.90 | 6.30 ±4.34 | 10.98 ±4.73 | 6.90 ± .31 | 1.19 ±0.90 | 2.20 ±1.60 | n.d. ± | 3 |
| Catalase | 81.3 ± 3.9% Recovery 4580 ± 1960 mμmoles/min/mg protein | 0.45 ±0.25 | 1.24 ±1.14 | 0.54 ±0.17 | 1.52 ±0.81 | 1.92 ±1.12 | 1.43 ±1.00 | 2.74 ±0.50 | 1.03 ±0.36 | 3 |
| Acid phosphatase | 86.7 ± 5.0% Recovery 16.56 ± 3.75 mμmoles/min/mg protein | 0.22 ±0.10 | 0.44 ±0.14 | 0.17 ±0.09 | 0.85 ±0.42 | 0.85 ±0.62 | 1.55 ±0.16 | 1.92 ±0.80 | 1.45 ±0.23 | 3 |
| Glucose-6-phosphatase | 75.0 ± 6.3% Recovery 1.55 ±0.32 mμmoles/min/mg protein | 0.23 ±0.22 | 0.34 ±0.22 | 0.36 ±0.21 | 0.13 ±0.08 | 0.14 ±0.08 | 0.88 ±0.12 | 2.94 ±1.51 | 0.74 ±0.38 | 3 |
| Esterase | 80.5 ± 2.5% Recovery 1.14 ± 0.73 mμmoles/min/mg protein | 0.52 ±0.50 | 0.79 ±0.80 | 0.48 ±0.43 | 0.18 ±0.17 | 0.25 ±0.01 | 0.89 ±0.32 | 1.04 ±0.33 | 1.29 ±0.39 | 2 |
| UDPase | 99% Recovery 31.4 mμmoles/min/mg protein | 0.58 | 0.60 | n.d. | 0.28 | 0.26 | 0.60 | 0.86 | 1.28 | 1 |
| AMPase | 80% Recovery 3.05 mμmoles/min/mg protein | n.d. | n.d. | 0.49 | 3.74 | 3.74 | 5.01 | 5.91 | 0.75 | 1 |
| Peroxidase | 78.0 ± 5.9 Recovery 1.21 ± 0.32 mμmoles/min/mg protein | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.50 ±0.50 | 2.20 ±0.34 | 3 |
| Potassium | 114% Recovery 14.7 μgk/mg protein | 0.38 | 0.02 | 0.32 | n.d. | n.d. | 0.63 | 0.62 | 3.00 | 1 |
| Triglyceride | 70% Recovery 2.915 meq ester/mg protein | 0.24 | 0.21 | 1.61 | n.d. | 0.07 | 0.09 | n.d. | n.d. | 1 |

[a]Results are expressed as relative specific activity in relation to homogenate values given. Fraction designations are as for Figure 2 and F = fat fraction. n.d. = not detectable.

**Solubilization of Castor Bean Fat Fraction**

The fat fraction was prepared in the usual way and 1 ml portions were treated at 4 C with Triton or Lubrol (0.1%, 1%), 1 M KCl or resuspended in 2 mM Tris buffer containing $10^{-4}$ M dithiothreitol only. After standing for 15 min. at 4 C the solutions were spun at 105,000 g x 60 min. and the resulting supernatant taken as "soluble" protein. For acetone solubilization treatments a 1 ml suspension of fat fraction was treated with 10 volumes of acetone at -15 C. The result was sucrose buffer. This suspension was then spun at 105,000 g and the supernatant taken as "soluble" protein as before.

**Preparation of Antibodies to Avocado Chloroplast Lamella**

Chloroplasts were prepared from avocado

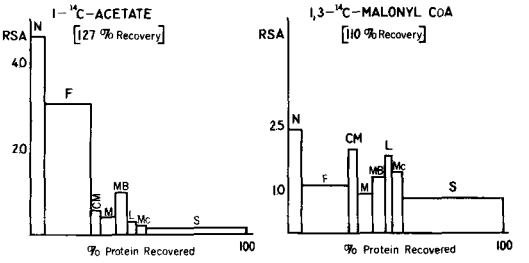


FIG. 3. Fatty acid synthesis by developing avocado fractions. Read in conjunction with Table I. Percentage recovered activity is indicated in parentheses. See Figure 2 for fraction designations and Experimental Procedures for incubation details. CM = chloroplast membranes, F = fat fraction. Intact chloroplasts are found in fractions N and F. Homogenate activity = 71 pmoles/min/mg protein (acetate), 176 pmoles/min/mg (Malonyl CoA).

TABLE III

Fatty Acids Synthesized by Maturing Avocado Fractions[a]

| Fraction used | Fatty acids | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | <10 | 10:0 | 12:0 | 14:0 | 14:1 | 16:0 | 16:1 | 18:0 | 18:1 |
| Mesocarp | | | | | | | | | |
| Homogenate | Trace | 6 | 3 | 17 | Trace | 42 | 3 | 32 | 3 |
| Supernatant | 5 | 5 | 7 | 21 | | 49 | Trace | 12 | Trace |
| Endocarp | | | | | | | | | |
| Homogenous | 6 | 5 | 10 | 21 | | 42 | | 16 | |
| Nuclear | | | 7 | 26 | | 40 | | 12 | 15 |
| Microbody | 2 | 8 | 14 | 15 | 4 | 36 | Trace | 10 | 11 |
| Supernatant | 10 | 11 | 10 | 22 | | 31 | | 16 | |

[a]Incubations as in Experimental Procedures with Malonyl CoA-1,3-$^{14}$C Substrate. Results are expressed as % of total fatty acids. Refer to Figure 3 for relative specific activities of each fraction.

TABLE IV

Release of Fatty Acid Synthetase From Avocado Mescocarp Chloroplasts[a]

| Preparation | Treatment | Protein, % solubilized | Fatty acid synthesis, % solubilized | Ribulose 1,5 diphosphate-carboxylase, % solubilized | Ratio synthetase-carboxylase |
|---|---|---|---|---|---|
| Chloroplast | None | 0 | 0 | 0 | 1.00 |
| Chloroplast | 10 Sec blend | 52(115%) | 49(110%) | 53(104%) | 1.19 Membrane<br>0.98 Soluble |
| Chloroplast | 30 Sec blend | 57(117%) | 60(102%) | 85(99%) | 2.72 Membrane<br>0.72 Soluble |
| Lamellar membranes | None | | | | 3.05 |
| Particle free supernate | None | | | | 0.10 |

[a]Intact chloroplast cpm/mg protein = 230,000 for fatty acid synthesis, and 1,610 for ribulose diphosphate-carboxylase ratio taken as 1.0. Figures in brackets represent recoveries. Preparation of chloroplast and supernatant fractions are detailed in Methods. After blending, the chloroplast preparation was spun at 6000 g x 10 min to yield membrane and soluble fractions. Lamellar membranes were prepared by osmotically shock treatment of purified chloroplast (see Methods).

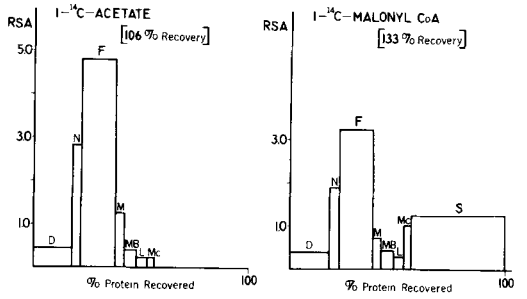


FIG. 4. Fatty acid synthesis by developing castor bean fractions. See Figures 2 and 3 for details of fractions. Homogenate activity = 41 pmoles/min/mg protein (acetate), 295 pmoles/min/mg (Malonyl CoA).

mesocarp on a sucrose gradient by the method of Leech (41). They were then shocked osmotically with water and spun at 6000 g x 10 min. This was repeated three times and the final lamellar preparation made up in 0.09% sodium choride solution. Rabbits were injected simultaneously with the lamellar solution together with Fruends Adjuvant. After three weeks a booster injection (iv) of lamellae was given, and samples of serum were taken a week later.

## RESULTS AND DISCUSSION

### Subcellular Fractionation

Subcellular fractionation of plant tissues has

TABLE V

Effect of Rabbit Antilamella Serum on Avocado Fatty Acid Biosynthesis[a]

| Addition | Sites of fatty acid synthesis | | |
|---|---|---|---|
| | Fraction used | | |
| | Chloroplast membranes | Particle free supernatant | Homogenate |
| None | 100 | 100 | |
| 0.1 ml Control serum | 35.2 ± 2.2 | 123 ± 32 | 100 |
| 0.2 ml Control serum | 33.4 ± 3.3 | 87 ± 38 | |
| 0.1 ml Control serum (boiled) | 104 ± 2.5 | 114 ± 2 | |
| 0.1 ml Antilamella serum | 15.8 ± 4.2 | 107 ± 35 | 46 ± 8 |
| 0.2 ml Antilamella serum | 9.1 ± 0.1 | 102 ± 47 | |
| 0.1 ml Antilamella serum (boiled) | 69 ± 15 | 126 ± 42 | |

[a]Values expressed as % of control. Controls = 2,460 cpm (Homog.), 15,100 cpm (Chloroplast (Memb.), 6,060 (P.F. Sup.). Fractions prepared as in Materials and Methods.

TABLE VI

Fatty Acids Synthesized by Developing Castor Bean[a]

| Fraction | Seed age | Substrate | Fatty acids, % 5o5al | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | <16 | 16:0 | 16:1 | 18:0 | 18:1 | 20:0 | Other |
| Homogenate | 25 DAF | Malonyl CoA-1,3-$^{14}$C | | 5 | 3.5 | 53 | 36.5 | 2 | |
| | 40 DAF | Malonyl CoA-1,3-$^{14}$C | | 1 | | 92 | | 7 | |
| | 45 DAF | Malonyl CoA-2-$^{14}$C | 1.5 | 4 | 1 | 69 | 4 | 16 | 4.5[b] |
| Fat fraction | 25 DAF | Malonyl CoA-1,3-$^{14}$C | | 3 | | 48 | 44 | 5 | |
| | 40 DAF | Malonyl CoA-1,3-$^{14}$C | | | | 97 | | 3 | |
| Supernatant | 25 DAF | Malonyl CoA-1,3-$^{14}$C | | 12 | 2 | 35 | 51 | | |
| | 40 DAF | Malonyl CoA-1,3-$^{14}$C | | | | 88 | 7 | 5 | |
| | 50 DAF | Malonyl CoA-2-$^{14}$C | 33 | 19 | | 36 | 12 | | |
| Homogenate | 25 DAF | Acetate-1-$^{14}$C | | 17 | | 18 | 65 | | |
| | 40 DAF | Acetate-1-$^{14}$C | | 14 | Trace | 62 | 24 | | |
| Fat fraction | 25 DAF | Acetate-1-$^{14}$C | | 7 | | 22 | 71 | | |
| | 40 DAF | Acetate-1-$^{14}$C | | 8 | 2 | 57 | 33 | | |

[a]See Figure 4 for specific activities of Fractions. DAF = Days after flowering.

[b]Ricinoleic acid being synthesized but not measured.

often employed differential centrifugation (31,40). While this procedure is useful in the isolation of a number of organelles such as chloroplasts (41), spherosomes (42) or microsomes, the crude mitochondrial fraction is usually very heterogeneous. This has led to the development of methods for subfractionation of the mitochondrial fraction by discontinuous (43-45) or continuous (46,47) sucrose density gradient centrifugation.

However since sucrose gradients required a lengthy spin time, a more rapid method was needed in order to assay the fractions for fatty acid synthetase activity without storage. To achieve this Ficoll gradients were used which, although not producing homogeneous subcellular fractions, gave sufficient enrichment of organelles for localization of fatty acid synthesis to be estimated.

Initial experiments were conducted by centrifuging particulate fractions using continuous 0-20% Ficoll gradients. On the basis of enzyme marker distributions (see Experimental Procedures), Ficoll concentrations were selected for discontinuous gradient separation. Figure 1 shows the method applied to developing castor bean seeds. In this tissue as with maturing avocado, the high concentration of storage lipid allowed the isolation of a fat fraction by flotation. In low lipid seeds, such as lupin and pea, this fraction was not obtained.

The activity of marker enzymes in the fractions isolated from avocado, castor bean and lupin are shown in Tables I, II and Figure 2, respectively. Recoveries of all enzymes, protein, potassium and triglyceride were satisfactory.

In general clean fractions of all plant tissue organelles are difficult to achieve simultaneously. However enrichment of succinate dehydrogenase, catalase and acid phosphatase was usually obtained in different fractions from the Ficoll gradient. For convenience these were termed the mitochondrial, microbody and lysosomal fractions, respectively. Similar enrichment could be seen by electron microscopic study of the fractions. Certain of the enzyme markers do not always show a unimodal distribution. For instance, with the exception of lupin (Fig. 2), we found considerable soluble catalase activity which agrees with other workers (45,48). Peroxidase was mainly soluble (49) but activity was associated with the low speed particulate fraction in avocado (Table I), possibly in the cell wall (50). The question of lysosomes as such in plants is a debatable one, but the lightest fractions from the Ficoll gradient contained many membrane-bound vesicles together with latent acid phosphatase

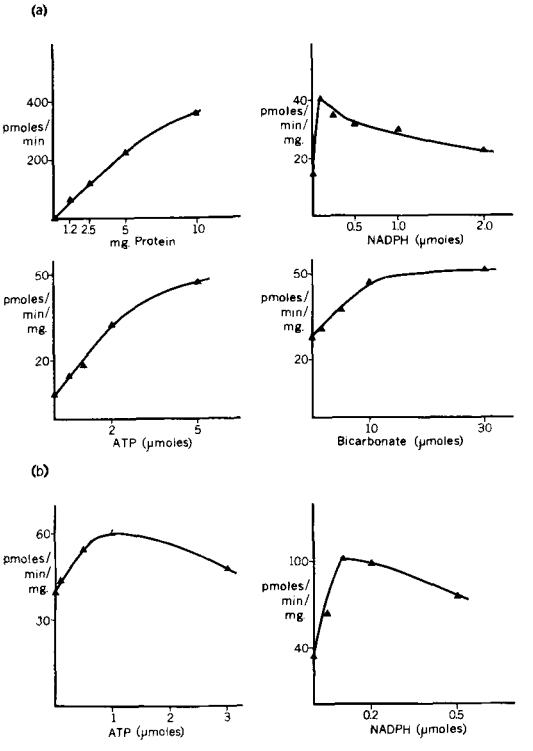


FIG. 5. Characteristics of fatty acid synthetase. See Experimental Procedures for details. (a) Castor bean fat fraction was incubated with 1-$^{14}$C-acetate and (b) avocado supernatant with 1,3-$^{14}$C-Malonyl CoA.

activity, thus fulfilling two basic qualifications of a lysosome (51). Lysosomal enzymes are also frequently found in the supernatant as well (52), and we obtained similar results with avocado and castor bean (Tables I and II). Varying amounts of plant glucose-6-phosphatase have been found to be soluble (53) but to a lesser degree than unspecific esterase (52), making the former a preferential rough endoplasmic reticulum marker.

#### Localization of Fatty Acid Synthesis Activities

*Avocado:* In Figure 3 the synthesis of fatty acids by fractions from developing avocado (endocarp plus mesocarp) is shown. Separate homogenates of endocarp or mesocarp had approximately the same specific activity with relation to protein. In addition distribution of activity in fractions prepared from mesocarp or endocarp was similar, except that the fractions from mesocarp containing chloroplasts, indicated by both chlorophyll and ribulose, 1,5 diphosphate carboxylase activity (Table I), were more active than corresponding endocarp fractions. With 1-$^{14}$C-acetate as substrate, fatty acid synthesis was almost entirely confined to

TABLE VII

Solubilization of Castor Bean Fatty Acid Synthetase From Fat Fraction

| Treatment | Protein, % solubilized | Total fatty acid synthetase, % solubilized | Recovery of FA synthetase |
|---|---|---|---|
| 0.1% Triton-X-100 | 55 | 18 | 118 |
| 1% Triton-X-100 | 65 | 0 | 49 |
| 0.1% Lubrol | 45 | 13 | 100 |
| 1% Lubrol | 68 | 38 | 29 |
| Acetone powder | 60 | 91 | 174 |
| KCl Addition | 55 | 1 | 99 |
| Osmotic shock | 53 | 17 | 81 |

those mesocarp fractions containing intact chloroplasts, which agrees with the suggestion of Weaire and Kekwick (14) that fatty acid biosynthesis occurs in the chloroplast. However similar fractions from endocarp, which contain neither marker enzyme nor chlorophyll, effectively synthesized fatty acids 1-$^{14}$C from acetate. In addition the supernatant accounted for approximately half the total fatty acid synthesis when $^{14}$C-malonyl-CoA was the substrate. Fractions containing AMPase, and therefore likely to contain surface membranes, also showed good activity with malonyl-CoA.

With $^{14}$C-acetate as substrate, the avocado mesocarp synthesized long chain fatty acids (actual figures were 60% palmitic, 20% stearic, 20% oleic). With $^{14}$C-malonyl-CoA as substrate (Table III) saturated acids from lauric upwards were made. The particular fractions also made appreciable amounts of oleic acid. Individual percentages of, for example, palmitic or stearic acids varied somewhat from preparation to preparation.

The synthesizing ability of the avocado mesocarp chloroplast was studied further. Chloroplasts were purified by the method of Leech (41) and then subjected to blending. As Table IV shows, this treatment released increasing amounts of soluble ribulose 1,5,diphosphate carboxylase and protein with time. The percentage of carboxylase (a stromal marker enzyme) can be used as a measure of chloroplast intactness. Comparison of fatty acid synthesis from 1,3-$^{14}$-malonyl CoA with $CO_2$ fixation by the carboxylase revealed that considerable synthetase activity remained in the particulate fraction, although some release took place on homogenization. Purified lamellae prepared by osmotic shock showed considerable fatty acid synthesis but little carboxylase activity (Table IV). The amounts of total soluble ribulose 1,5- diphosphate carboxylase and soluble fatty acid synthesis in an average fractionation were approximately equal (Table I and Fig. 3). Thus after deducting the contribution of the lamellae it could be seen that the chloroplast stromal proteins could not account for all soluble fatty acid synthesis as suggested (14).

To test further the localization of the avocado synthetase, we prepared antibodies to the purified chloroplast lamellae. Table V shows the inhibition produced by the anti-lamella serum on various avocado fractions.

TABLE VIII

Cofactor Requirements for Fatty Acid Synthesizing Systems[a]

| Fraction substrate | Pea supernatant $^{14}$C-malonyl CoA | Avocado supernatant $^{14}$C-malonyl CoA | Castor bean fat fraction $^{14}$C-acetate |
|---|---|---|---|
| Complete system[b] | 100 | 100 | 100 |
| -Acyl carrier protein | 22 | 1 | 68 |
| -ATP | 85 | 66 | 10 |
| -$MnCl_2$ | 86 | 104 | 73 |
| -NADPH | 15 | 12 | 65 |
| -NADH | 78 | 83 | 56 |
| -GSH | --- | 77 | 38 |

[a]Actual values for complete system = 16.4 pmoles fatty acids/min/mg protein (pea); 82.5 pmoles fatty acids/min/mg protein (avocado); 44 pmoles fatty acids/min/mg protein (castor bean).

[b]Complete system contained 0.1 mM CoA.

TABLE IX

Fatty Acid Synthesis by Pea and Lupin Fractions[a]

| Plant | Substrate | D | N | M | MB | L | Mc | PFS | Recovery, % |
|---|---|---|---|---|---|---|---|---|---|
| Pea | Acetate | 30 | 24 | 6 | 22 | 8 | 2 | 8 | 111 |
| | Malonyl CoA | 3 | 8 | 5 | 5 | 6 | 8 | 65 | 95 |
| Protein | | 13 | 11 | 8 | 8 | 12 | 8 | 40 | 99 |
| Lupin | Acetate | 34 | 29 | 7 | 3 | 1 | 2 | 4 | 90 |
| | Malonyl CoA | 12 | 20 | 2 | 3 | 6 | 6 | 51 | 95 |
| Protein | | 20 | 28 | 3 | 4 | 4 | 5 | 36 | 101 |

[a]Figures as % of total recovered. See Figure 2 for nomenclature of fractions. Homogenate activities for pea = 0.40 pM/min/mg protein (acetate), 8.00 pM/min/mg protein (malonyl CoA); for lupin = 0.96 pM/min/mg protein (acetate), 3.60 pM/min/mg protein (malonyl CoA).

Significant inhibition of fatty acid synthesis by the lamellae occurred on addition of control serum alone. As this effect was abolished by boiling the serum, it may be caused by unspecific binding of synthetase to a protein such as albumin. However the effect of the antilamellae serum was considerably more, and almost complete inhibition of synthesis occurred in the chloroplast membrane fraction.

Interestingly the supernatant activity was unaffected by the addition of control or antilamella serum. These results are taken to indicate the existence of at least two separate types of fatty acid synthetase enzymes in avocado mesocarp. One is associated with lamella fragments and the other(s) with stromal or cytoplasmic proteins. As could be predicted the homogenate was partly inhibited by the antilamellae serum over control values. While synthetase activities can be found in the chloroplast stroma and lamellae, a functional organelle is not necessary. Indeed in the case of the endocarp where there is no measurable ribulose 1,5 diphosphate carboxylase, the location of particulate synthetase must be in an organelle which does not have chloroplast properties.

*Maturing castor bean seeds:* The maturing castor bean has been studied by a number of groups because of the unusual nature of ricinoleic acid as its principal storage acid. The synthesis of this acid is confined to a specific period of seed development (15) and has been studied using a particulate fraction (18). We did not consider its synthesis in the present investigation but measured only saturated and unsaturated acids. Figure 4 shows that acetate incorporation was confined almost entirely to the fat fraction. This paralleled triglyceride distribution exactly and was not owing to adsorption of membranes or organelles onto the oil droplets. Extensive washing, which removed all measurable succinate dehydrogenase and AMPase activity and which gave a homogeneous preparation as judged by electron microscopy, failed to reduce the fatty acid synthesizing ability. When $^{14}$C-malonyl CoA was employed, the usual particle free supernatant activity was observed. Young seeds, which had not begun to form oil droplets, showed very low activity.

Further investigations (54) have revealed that the oil droplets do not contain a bounding membrane but only membranous inclusions. The fatty acid synthesizing activity of these droplets is undiminished after washing and is distinct from the spherosome system of Jacks et al. (42). It was suggested (54) that the membranous inclusions of the droplets are the site of fatty acid synthesis. The oil droplets originate in the maturing castor bean seed around a cluster of enzymes in the ground substance of the cell involving both de novo fatty acid biosynthesis and triglyceride formation.

The pattern of acids synthesized by the most active fractions at different stages of development is shown in Table VI. The production of ricinoleic acid which has previously been extensively examined (18) is not detailed, but it occurred at a late stage (40 DAF) of development in unfrozen fractions. It is interesting to note that its precursor, oleic acid, was present in high amounts at 25 DAF but then declined. The patterns of acids produced at each developmental stage by the fat fractions, supernatant and homogenate are very similar, suggesting that the same enzyme complex with bimodal location could be involved. It is of considerable interest to note the synthesis of oleic acid by a soluble fraction and this is being further examined in our laboratory. Canvin's group have also studied the production of oleic from $^{14}$C-malonyl CoA or $^{14}$C-acetyl CoA in castor bean. The highest specific activity was found in a heavy particle (20) later suggested to be a proplastid (21). The fat fraction was not assayed, however, and the supernatant was measured with $^{14}$C-acetyl CoA which is an ineffective substrate for soluble plant systems.

TABLE X

Acyl Carrier Protein Requirement for Plant Fatty Acid Synthesis

| System | Substrate | +ACP, cpm | -ACP, cpm | Difference, % |
|---|---|---|---|---|
| Castor bean fat fraction | Malonyl CoA | 41,400 | 5430 | -87 |
| Castor bean PFS | Malonyl CoA | 8000 | 1800 | -78 |
| Pea PFS | Malonyl CoA | 4160 | 563 | -86 |
| Avocado homogenate | Malonyl CoA | 37,850 | 1030 | -97 |
| Avocado PFS | Malonyl CoA | 31,400 | 362 | -99 |

The specific activities obtained in the present investigation for homogenate were about 3-5 nmoles/mg protein/15 min, which is higher than reported in Canvin's papers. While the specific activities of our mitochondrial and microbody fractions are approximately the same as reported for the isolated particles (20), the fat fraction is very much higher (Fig. 4).

In Table VII it can be seen that the fat fraction activity was not easily solubilized. Only complete defatting with acetone resulted in an active solubilized enzyme system. Although this solubilized enzyme was very active, the production of oleic acid had disappeared.

The fat fraction of developing safflower also showed considerable synthesis of fatty acids from malonyl CoA. In addition this plant contains a soluble system producing $^{14}$C-oleic, among other acids from $^{14}$C-malonyl CoA.

Some characteristics of the fat fraction synthetase were compared with avocado and pea supernatant enzymes (Table VIII and Fig. 4). ACP and NADPH requirements are typical of all three systems. In addition ommission of reduced glutathione results in decreased activity indicating a functional sulphydryl group at or close to the active center(s). NADH omission also caused some lowering of activity whereas NADH was very essential in the particulate enzyme preparation examined by Drennen and Canvin (20). ATP was necessary in the castor bean fat fraction since $^{14}$C-acetate was the substrate. The pH optima between 7 and 8, and inhibition by high neutral detergent concentrations were common to all three systems. The effect of changing the malonyl CoA-acetyl CoA ratios or altering the ATP concentration in the avocado supernatant incubations were also tested. No significant effects were seen, although ATP caused a slight stimulation of activity (optimum 1mM) accompanied by an increase in the stearate-palmitate ratio.

*Low lipid-containing seeds:* The low lipid seeds, pea and lupin, were also examined. These seeds showed much lower specific activities than the high lipid tissues. $^{14}$C-malonyl CoA was always more effective than $^{14}$C-acetate, and the latter was not incorporated by the soluble fraction for any appreciable extent (Table IX). $^{14}$C-acetyl CoA incorporation gave similar results to $^{14}$C-acetate. The lack of conversion of these substrates to fatty acids by the plant supernatant could be related to one of two reasons. Either there is a lack of acetyl CoA carboxylase, or alternatively there is an inhibitor present which prevents carboxylase activity. Avocado supernatant will inhibit purified wheat germ carboxylase (J.L. Harwood, unpublished observations) and several other plant systems contain a soluble inhibitor (55).

Both pea and lupin have low speed particulate fractions, possibly proplastids, which synthesize fatty acids including monounsaturated from $^{14}$C-acetate or $^{14}$C-malonyl CoA (Table IX). Both also form palmitate and stearate in the supernatant from $^{14}$C-malonyl CoA. The pea microsomes were found to be the site of very long chain fatty acid synthesis from malonyl CoA, confirming the results of Macey and Stumpf (9).

A noticeable feature of the plant systems studied is their stimulation by added acyl carrier protein, as further detailed in Table X. This stimulation was most marked in the supernatant where ACP was limiting, presumably because of dilution. The avocado particulate free supernatant in particular had very low activity in its absence.

In conclusion the localization of fatty acids synthesis in seeds is dependent on the species (and even age) chosen. Generalizations derived from work with bacterial or mammalian systems certainly cannot be applied. Active particulate fractions usually gave rise to monounsaturated as well as saturated fatty acids, but synthesis of linoleic or linolenic acids was not observed in vitro. All particle free supernatants examined synthesized fatty acids, mainly palmitate and stearate, from $^{14}$C-malonyl CoA in the presence of added cofactors such as NADPH, reduced glutathione and acyl carrier protein. A block in the carboxylation of acetyl CoA in the supernatant prevented its incorporation. The soluble protein fraction generally accounted for about half of the total $^{14}$C-malonyl CoA incorporation.

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# The Effects of Biliary Obstruction on the Rates of Uptake, Synthesis and Secretion of $^{14}$C-Labeled Cholesterol by Isolated Perfused Rat Livers

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## ABSTRACT

The isolated perfused rat liver technique was utilized to study the effect of biliary obstruction on the rates of uptake, synthesis and secretion of $^{14}$C-labeled cholesterol in an effort to elucidate the mechanism of hypercholesterolemia in this condition. By perfusing normal and biliary obstructed livers with a medium containing $^{14}$C-labeled acetate or $^{14}$C-labeled lipoprotein cholesterol and by measuring cholesterol biosynthesis in vitro, it was possible to clarify some of the questions concerning these aspects of lipid metabolism. The results of these studies revealed that the uptake of cholesterol was not altered in biliary obstruction. Instead the hypercholesterolemia of biliary obstruction arises from an increased rate of secretion of cholesterol from liver, which is accompanied by an increased rate of hepatic cholesterolgenesis. Evidence is also presented to suggest that a substance is present in biliary obstructed blood which is capable of initiating increased cholesterolgenesis in normal livers.

[1]Deceased.

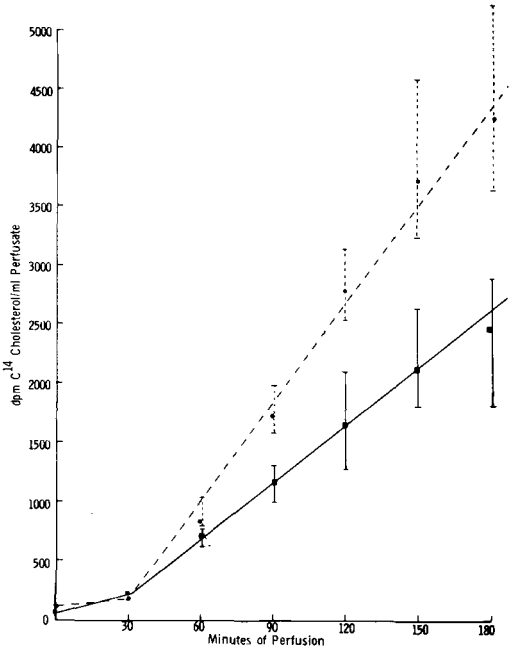


FIG. 1. Release of $^{14}$C-labeled free cholesterol into perfusate by biliary obstructed livers perfused with biliary obstructed perfusate. (■——■) Normal livers perfused with normal perfusate. The bile ducts were cannulated throughout the perfusions. (●- - ●) Livers from rats with bile ducts ligated for 48 hr, perfused with perfusate from similarly treated rats. Each point on the curves is the mean result obtained in three perfusions.

## INTRODUCTION

The sequence of events leading to the hypercholesterolemia of biliary obstruction appears to be well established (1-3). Obstruction of the bile duct is followed by an immediate increase in the concentration of cholate in blood. This hypercholatemia is followed by and is primarily responsible for the ensuing hyperphospholipidemia. The hypercholesterolemia occurring after biliary obstruction is secondary to the hyperphospholipidemia, and is in fact induced by the elevated phosphatides present in the blood. However the mechanism of the phosphatide-induced hypercholesterolemia has not yet been elucidated. Byers et al. (3) have suggested that the probable direct effect of the hyperphospholipidemia is to shift cholesterol from tissues to blood either by mobilization of cholesterol from tissues or by interference with the usual rate of egress of cholesterol from plasma to tissues.

The present study, employing the isolated rat liver perfusion technique using blood and liver from biliary obstructed rats, was undertaken in an attempt to clarify the mechanism of this hypercholesterolemia occurring in biliary obstruction. In particular it was an attempt to elucidate whether the accumulation of excess cholesterol in blood resulted from a decreased uptake of cholesterol from blood by liver or from an increased secretion of cholesterol from liver into blood.

## METHODS AND MATERIALS

Adult male rats of the Sprague-Dawley strain, weighing from 350-500 gm, were used as blood and liver donors for all perfusions. These rats were fed a Purina rat chow diet and water ad libitum.

Biliary obstruction was induced for 48 hr in rats by ligation of their common bile ducts. The ligature did not obstruct pancreatic flow. Confirmation of the obstruction was made by visual observation when the rats were sacrificed. In each instance the common bile duct was dilated above the ligature and showed no indications of leakage. The existence of biliary obstruction was also confirmed by noting an elevated concentration of both cholesterol and bilirubin in plasma. When these livers were used in perfusion studies, the bile ducts were severed distal to the ligature at the time of surgery and were continually checked for leakage throughout the length of perfusion. If leakage occurred the experiment was terminated and the study discarded.

Each of 10-15 rats to be used as blood donors were lightly anesthetized with ether, and blood was obtained from the dorsal aorta by means of a 20 ml syringe containing heparin as an anticoagulant. Approximately 75-100 ml of pooled rat blood was obtained for each experiment, strained through a surgical cotton gauze pad to remove small clots and mixed with an equal volume of sterile Tyrode solution. The final volume of the perfusate varied between 180 and 200 ml. Ten $\mu$c (25 $\mu$moles) of 2-$^{14}$C-acetate (New England Nuclear) was thoroughly mixed with 100 ml of perfusate; the pH was recorded and the solution was placed in the reservoir of the perfusion apparatus. The perfusate was circulated through the apparatus for 30 min before the liver was placed in the system to insure complete mixing and oxygenation.

In uptake experiments, perfusate containing $^{14}$C-labeled lipoprotein cholesterol was prepared according to a modification of the method of Sodhi and Kalant (4). Eight $\mu$c (25 $\mu$moles) of 1-$^{14}$C-mevalonate as the dibenzylethylenediamine (DBED) salt was injected ip into either biliary obstructed or normal rats 2 hr before they were used as blood donors. The blood, then containing the newly synthesized $^{14}$C-labeled lipoprotein cholesterol, was collected and the perfusate prepared as previously described.

The operative procedure and perfusion apparatus used were essentially those of Miller et al. (5). A basal aliquot (2.0 ml) of perfusate was drawn from the apparatus immediately before the liver was introduced into the system.

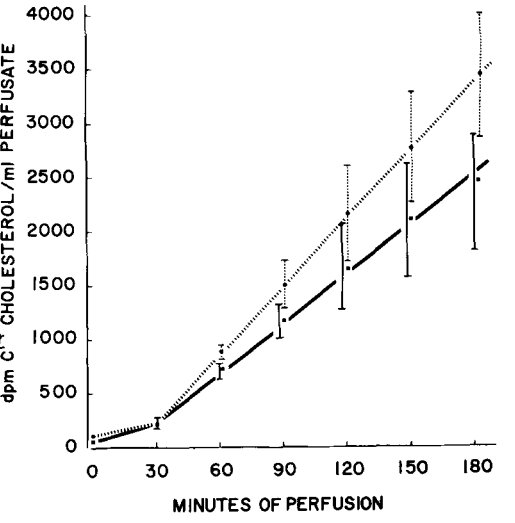


FIG. 2. Release of $^{14}$C-labeled free cholesterol into perfusate by normal liver perfused with biliary obstructed perfusate. (■——■) Normal livers perfused with normal perfusate. The bile ducts were cannulated throughout the perfusion. Each point on the curve represents the mean result obtained in three perfusions. (●- - ●) Normal livers perfused with perfusate from rats with bile ducts ligated for 48 hr. Each point on the curve represents the mean result obtained in two perfusions.

Perfusate samples (2.0 ml) were drawn every 30 min throughout the 3 hr of perfusion. These aliquots were used for the determinations of $^{14}$C-labeled free and esterified cholesterol. Perfusate withdrawn for analysis was replaced with Tyrode solution in order to maintain a constant volume throughout the experiment. Weights were obtained on livers before they were placed on the perfusion apparatus. Blood flow, bile flow and gross appearance of the liver were used as criteria of viability of the liver. The average blood flow was approximately 26 ml/min. If all lobes of the liver did not develop a pink appearance in 10 min, the liver was discarded. If any signs of anoxia developed in the lobes during perfusion, the experiment was terminated. Bile flow in the perfusions averaged 1.7 ml/3 hr (1.4-2.5 ml/3 hr). If bile flow stopped at any time during the perfusion, the experiment was discontinued. The pH of the blood was monitored throughout the perfusion and was maintained at 7.35.

After termination of the perfusions, the livers were immediately frozen and analyzed the next day for free and esterified $^{14}$C-cholesterol. The livers were cut into small pieces and homogenized with 100 ml of acetone-ethanol-ether 4:4:1. The homogenate was spun at 6000 rpm for 10 min and the supernatant removed.

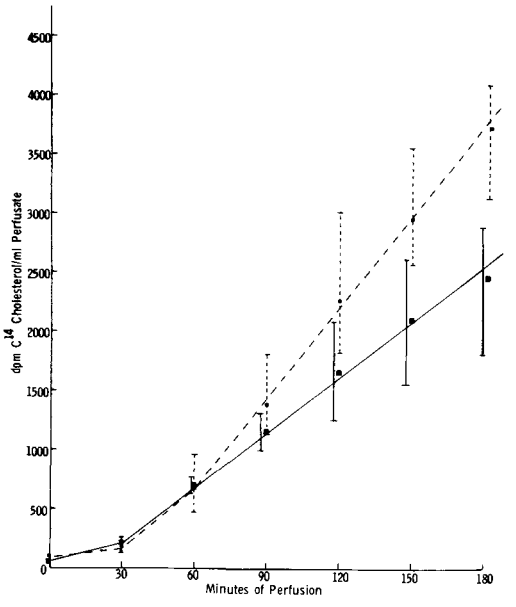


FIG. 3. Release of $^{14}$C-labeled free cholesterol into perfusate by biliary obstructed livers perfused with normal perfusate. (■——■) Normal livers perfused with normal perfusate. The bile ducts were cannulated throughout the perfusions. Each point on the curve represents the mean result obtained in three perfusions. (●- -●) Livers from rats with their bile ducts ligated for 48 hr perfused with normal perfusate. Each point on the curve represents the mean result obtained in two perfusions.

Ten milliliter aliquots were used for the determination of both free and esterified $^{14}$C-labeled cholesterol. Each liver sample was analyzed in triplicate according to the method of Kabara et al. (6).

One milliliter aliquots of the perfusate were hemolyzed with 4.0 ml of distilled $H_2O$ to facilitate the extraction of lipid material. Twenty milliliters of acetone-ethanol-ether 4:4:1 was added and the cholesterol was extracted at room temperature. The precipitated protein was separated by centrifugation at 6000 rpm for 15 min and the supernatant decanted. If the supernatant was not clear, it was filtered through Whatman No. I filter paper. Two milligrams of carrier cholesterol was added to a 10.0 ml aliquot of the supernatant, and the analysis of $^{14}$C-labeled free and esterified cholesterol was conducted in duplicate according to the method of Kabara et al. (6).

Bile was collected at hourly intervals throughout the period of the perfusion. The volume of bile obtained at each hour was recorded and the samples were frozen until analysis of $^{14}$C-labeled cholesterol could be conducted. A 0.1 ml aliquot of bile was added to 2 mg of carrier cholesterol, and the cholesterol was precipitated as the tomatinide according to the method of Kabara et al. (6). All determinations were done in triplicate.

The cholesterol tomatinides obtained from the above procedures were dissolved in 2.0 ml of glacial acetic acid. Fifteen milliliters of scintillation fluid [3 g of 2,5 diphenyloxazolyl (PPO) and 150 mg of 1,4 (bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter] were added and the samples counted in a Packard liquid scintillation spectrophotometer, model 314. The amount of quenching that occurred in each sample was determined by the addition of an internal standard to each vial.

Throughout the study when cholesterol levels were determined in livers and perfusate following perfusion, the spectrophotometric method of Abell et al. (7) was used. In studies involving the comparison of cholesterol synthesis in perfused livers and cell-free homogenates of liver the in vitro cholesterol synthesizing enzyme system reported by Eskelson (8) was employed.

## RESULTS

Perfusion of livers from rats whose bile ducts had been ligated for 48 hr with blood from similarly treated animals resulted in a striking increase in the release of free $^{14}$C-labeled cholesterol into the perfusate (Fig. 1). The increase was observed even when a normal liver

TABLE 1

The Effect of Biliary Obstruction on the Specific Activity of Cholesterol in Perfused Rat Livers

| Conditions of perfusion | | | Number of perfusions | Specific activity, dpm/mg | |
|---|---|---|---|---|---|
| Liver | Bile duct | Blood | | Free cholesterol, mean range | Esterified cholesterol, mean range |
| Normal | Cannulated | Normal | 3 | 550 (400-720) | 27 (21-38) |
| Normal | Cannulated | Obstructed[a] | 2 | 532 (467-596) | 18 (15-21) |
| Obstructed[a] | Ligated | Normal | 3 | 616 (527-795) | 23 (15-33) |
| Obstructed[a] | Ligated | Obstructed[a] | 3 | 674 (465-880) | 22 (14-31) |

[a]Liver or blood was obtained from rats with their bile ducts ligated for 48 hr.

TABLE II

The Effect of Biliary Obstruction on Cholesterol Synthesis in Cell-Free Rat Liver Homogenates

| Condition of liver | Number of experiments | Substrate: $^{14}$C-Acetate Mean dpm per flask | $^{14}$C-Acetate SD | $^{14}$C-Mevalonate Mean dpm per flask | $^{14}$C-Mevalonate SD |
|---|---|---|---|---|---|
| Normal | 5 | 722 | 154 | 20,656 | 1680 |
| Biliary obstructed | 5 | 4396[a] | 527 | 20,904 | 1848 |

[a]Significant at P value > 98%.

was perfused with blood from a biliary obstructed rat (Fig. 2) or when a biliary obstructed liver was perfused with normal blood (Fig. 3).

The accumulation of $^{14}$C-labeled esterified cholesterol in perfusates of control perfusions was 380 dpm/ml at the end of 3 hr of perfusion and was not altered in any of the other experiments.

As seen in Table I the specific activity of cholesterol remained unchanged in normal or biliary obstructed livers, if they were perfused with normal blood perfusate or blood prepared from biliary obstructed animals. However when the incorporation of $^{14}$C-labeled acetate into cholesterol was studied in liver homogenates according to the method of Eskelson (8), different results were obtained. Table II shows that under these conditions the incorporation was significantly increased in the biliary obstructed rat liver when compared to normal livers. The rate of cholesterol synthesis was not altered when $^{14}$C-labeled mevalonate was used as the substrate.

Byers and Friedman (9) have shown that the biliary excretion of cholesterol in the rat is a function directly dependent upon the rate of hepatic synthesis of cholesterol and is independent of the concentration of cholesterol in plasma. When biliary obstructed perfusate containing $^{14}$C-labeled acetate was perfused through normal livers, the secretion of $^{14}$C-labeled cholesterol from liver into bile was noticeably increased (Table III) and is interpreted as additional evidence for increased rate of cholesterolgenesis in biliary obstruction.

Experiments were conducted to study the uptake of $^{14}$C-labeled lipoprotein cholesterol from perfusate by liver in biliary obstruction. These studies revealed that the uptake of cholesterol by normal or biliary obstructed livers perfused with either normal or biliary obstructed perfusate were similar (Fig. 4). No $^{14}$C-labeled cholesterol was secreted into the bile in any of the uptake studies. The basal concentration of cholesterol in normal perfusate (following dilution with Tyrode solution) was 23 (17-28) mg/100 ml, while the basal concentration of cholesterol in biliary obstructed perfusate was 57 (48-66) mg/100 ml. The concentration of cholesterol in the perfusate did not change throughout any of the perfusions conducted and are in agreement with the previously reported data of Roheim et al. (10). Similarly the concentration of cholesterol in perfused livers from normal and biliary obstructed rats was the same (Table IV).

## DISCUSSION

The hypercholesterolemia observed in

TABLE III

The Effect of Biliary Obstructed Perfusate on the Rate of Secretion of $^{14}$C-Labeled Cholesterol From Normal Perfused Livers Into Bile

| Perfusate | Number of perfusions | $^{14}$C-Labeled cholesterol secreted in 0.1 ml of bile, dpm: Mean range 1 hr | Mean range 2 hr | Mean range 3 hr |
|---|---|---|---|---|
| Normal | 3 | 750 (635-832) | 254 (164-420) | 133 (58-237) |
| Biliary obstructed[a] | 2 | 3587 (2450-4715) | 1109 (826-1392) | 825 (193-1058) |

[a]Perfusate prepared from blood donated by rats with their bile ducts ligated for 48 hr.

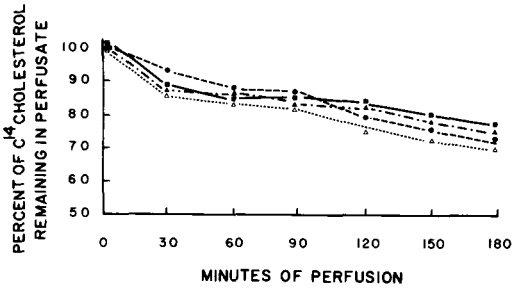


FIG. 4. The effect of biliary obstruction on the rate of uptake of $^{14}$C-labeled lipoprotein cholesterol by isolated perfused livers (■——■) Normal livers perfused with normal perfusate. The bile ducts were cannulated throughout the perfusion. (▲----▲) Livers from rats with their bile ducts ligated for 48 hr perfused with normal perfusate. (●- - ●) Livers from rats with their bile ducts ligated for 48 hr perfused with perfusate from similarly treated rats. Each point on these curves represents the mean result obtained in three perfusions. (△· · · △) Normal livers were perfused with perfusate from rats whose bile ducts were ligated for 48 hr. Each point on the curve represents the mean results obtained in two perfusions. In all of the above experiments, the perfusates contained $^{14}$C-labeled lipoprotein cholesterol prior to the start of the perfusion.

human or experimental biliary obstruction is mainly the result of an accumulation of free cholesterol in plasma. This accumulation of excess cholesterol in plasma is secondary to and initiated by a rise in plasma phospholipids (3). Even though the mechanism of the phosphatide-induced hypercholesterolemia has not been elucidated, Byers et al. (3) have shown that an increased rate of cholesterolgenesis does occur after phosphatide-induced hypercholesterolemia. The increased cholesterolgenesis is probably an indirect effect of phosphatides, since the hypercholesterolemia is mainfested long before the rate of cholesterolgenesis is increased. The direct effect of phospholipids would appear to involve a shift in cholesterol from tissues to blood, either by increasing the mobilization of cholesterol from tissues or by decreasing the egress of plasma cholesterol to tissues. This investigation demonstrated that the uptake of $^{14}$C-labeled lipoprotein cholesterol was the same when biliary obstructed livers were perfused with normal or biliary obstructed blood, or when normal livers were perfused with normal blood. Thus the hypercholesterolemia of biliary obstruction does not appear to be the result of a decreased rate of uptake of cholesterol from blood by liver. Similar observations have been reported by Sodhi and Kalant (4) in their studies on the hypercholesterolemia occurring in nephrosis.

Since the rate of uptake of $^{14}$C-labeled lipoprotein cholesterol from blood by liver is not altered in biliary obstruction, the accumulation of $^{14}$C-labeled free cholesterol observed in biliary obstructed blood must have resulted from an increased secretion of $^{14}$C-labeled free cholesterol by biliary obstructed liver into blood. Furthermore the increased secretion of cholesterol in biliary obstruction is accompanied by an increased rate of cholesterolgenesis in biliary obstructed livers. Three lines of evidence support this observation of increased cholesterolgenesis: (a) The specific activity of cholesterol in normal and obstructed livers at the end of the perfusions was the same. However the accumulation of $^{14}$C-labeled cholesterol in biliary obstructed perfusate was markedly increased when compared to control perfusate. Since the concentration of endogenous cholesterol in perfusate did not change throughout the length of any of the perfusions, the specific activity of cholesterol in biliary obstructed perfusates was increased when compared to the specific activity of cholesterol in control perfusates. These results must be interpreted to signify an elevated rate of cholesterol synthesis in the obstructed liver, since only elevated synthesis could account for the normal specific activity of cholesterol in obstructed liver in the face of its increased secretion. Alternatively if the rate of cholesterolgenesis

TABLE IV

The Effect of Biliary Obstruction on the Concentration of Cholesterol in Perfused Livers

| Conditions of perfusion | | | Number of perfusions | Liver cholesterol, mean range mg/liver |
|---|---|---|---|---|
| Liver | Bile duct | Blood | | |
| Normal | Cannulated | Normal | 3 | 31 (29-34) |
| Normal | Cannulated | Obstructed[a] | 2 | 35 (31-38) |
| Obstructed[a] | Ligated | Normal | 3 | 35 (31-39) |
| Obstructed[a] | Ligated | Obstructed[a] | 3 | 33 (31-39) |

[a]Liver or blood obtained from rats with their bile ducts ligated for 48 hr.

was not increased in the obstructed liver, the specific activity of cholesterol in this tissue should have decreased when compared to control, as a result of the increased secretion of $^{14}$C-cholesterol from obstructed liver into perfusate. (b) Byers and Friedman (9) have shown that the biliary excretion of cholesterol in the rat is directly dependent upon the rate of hepatic synthesis of cholesterol and is independent of the concentration of cholesterol in plasma. The increased specific activity of cholesterol in bile observed in the cross-perfusions in this investigation support the above findings, and in addition add further support to the concept of an increased rate of cholesterolgenesis in biliary obstructed livers. (c) Employing an in vitro cholesterol synthesizing enzyme system prepared from cell-free homogenates of biliary obstructed rat livers has also confirmed the increased cholesterolgenesis. Since the increased rate was found with acetate as substrate and not with mevalonate, it would appear that the rate of conversion of β-hydroxy-β-methyl glutaryl CoA to mevalonic acid is increased in biliary obstruction. The increased cholesterolgenesis could have resulted as a compensatory response initiated by a phosphatide-induced shift of cholesterol from tissues to blood. This may have occurred as a result of removal of the feed-back inhibition of cholesterol, per se, at some regulatory site in the liver, as was previously suggested by Byers et al. (3). On the other hand a phosphatide-induced hypercholesterolemia of biliary obstruction could stem from an increased rate of secretion of cholesterol from liver to blood, which in turn arises directly from an increased rate of cholesterolgenesis in unobstructed liver. The mechanism responsible for the increased rate of cholesterol biosynthesis occurring in biliary obstruction, therefore, still remains obscure.

The use of cross-perfusion in this investigation would suggest that there is some substance present in biliary obstructed blood that is capable of initiating an increased cholesterolgenesis in normal liver. When normal livers were perfused with biliary obstructed perfusate containing $^{14}$C-labeled acetate, an increased rate of secretion of $^{14}$C-labeled cholesterol from liver into bile was noted (Table III). Uptake studies using a similar cross-perfusion system containing $^{14}$C-labeled cholesterol instead of $^{14}$C-labeled acetate also revealed that none of the $^{14}$C-labeled cholesterol could be detected in bile. Therefore the increased secretion of $^{14}$C-labeled cholesterol from liver into bile in the cross-perfusion experiments must have arisen from an increased rate of cholesterolgenesis from $^{14}$C-labeled acetate. The increased rate of cholesterolgenesis observed in normal liver must have been initiated by a substance present in the blood from biliary obstructed animals. The increased rate of biosynthesis observed in such a cross-perfusion is reflected by an increased accumulation of $^{14}$C-labeled cholesterol in perfusate observable in the final hour of perfusion. It is felt that longer perfusion times would reflect a more marked accumulation of cholesterol in perfusate.

Although the results of this study do not reveal the nature of the substance present in biliary obstructed blood that is capable of inducing the increased rate of cholesterolgenesis in normal livers, it would seem reasonable to assume from the studies of Friedman and Byers (1,2) and Byers et al. (3) that this substance is phospholipid or some substance having its physical or chemical properties modified by excess phospholipid, e.g., lipoproteins.

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# The Phospholipid Composition of Human Placenta, Endometrium and Amniotic Fluid: A Comparative Study

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## ABSTRACT

The contents of lecithin in human lung, liver, spleen, kidney, heart muscle, skeletal muscle, placenta, endometrium, amniotic flud, and brain gray and white matter are 52.1-54.2%, 44.2-45.8%, 42.6-44.5%, 35.1-37.2%, 42.5-43.0%, 48.0-50.0%, 45.5-46.4%, 42.0-43.0%, 65.0-66.0%, 23.9-24.3% and 24.7-24.9% respectively in total phospholipids. The level of diphosphatidyl glycerol is much lower in placenta, lung and spleen, and almost absent in amniotic fluid and brain; the heart muscle has 8.6%. The content of phosphatidyl serine is high in endometrium and brain. The content of sphingomyelin is high in lung, spleen, kidney and brain as compared to liver, heart muscle, skeletal muscle and endometrium. It is suggested that the phospholipid composition of various human tissues is characteristic for that class.

## INTRODUCTION

The two dimensional thin layer chromatography (GLC) (1-4) or two dimensional chromatography on silica gel loaded filter paper (5) are usually employed for the separation of phospholipids. In a recent study, Simon and Rouser (6) have analyzed the phospholipid composition of various tissues in human, rat, mouse, frog, bovine, lobster, scallop, pink abalone and sea urchin. The present communication describes a method for the determination of the percentage composition of phospholipid components of human lung, liver, spleen, kidney, heart muscle, skeletal muscle, placenta, endometrium, brain and amniotic fluid. A comparative study of the phospholipid composition is described.

## EXPERIMENTAL PROCEDURES

The specimens of human tissue were obtained at autopsy as soon as possible after death and, when necessary, frozen at -20 C until they could be analyzed. Mature adult brain tissue, white and gray matter, were carefully dissected. Amniotic fluid was taken from a patient by artificial rupture of the membrane. All solvents were of AR grades and redistilled before use. The tissues were homogenized in chloroform-methanol 2:1 v/v for 5 min in a Waring blender. Lipids were extracted with chloroform-methanol 2:1 v/v following the method of Folch et al. (7). The solvent was removed under a stream of nitrogen.

### Isolation of Phospholipids

For the isolation of the phospholipid mixtures, the lipid sample was submitted to TLC separation of Silica Gel G, the developing medium being 98% acetone + 2% petroleum ether v/v as presented previously (8). The first fraction of low mobility, which was eluted from the gel portion with chloroform-methanol, contained the phospholipids. The phospholipids were also isolated by the Florisil column chromatography as advanced by Carroll (9).

### Separation of Phospholipids

The reference phosphatides were obtained from Applied Science Laboratories. The phospholipid mixture was resolved into individual components by the methods outlined by Singh and Gerishbein (4). The extraction procedure, preparation of the plates, solvents, the procedure for phosphorous determination, and a typical TLC of phospholipids have been previously described (4). By two dimensional TLC, ascending chromatography was conducted for 30 min in the medium chloroform-methanol-6N ammonium hydroxide of 65:30:5, and the dried plates introduced for 30 min in the system containing chloroform-methanol-6N ammonium hydroxide at 30:65:5. The cardiolipin spot occurred above the phosphatidyl ethanolamine zone as depicted in Figure 1 of the above reference. The individual gel portions were scraped from the plates and the resulting pools eluted with two volumes each of methanol, chloroform-methanol 1:1, and finally with chloroform followed by vacuum concentration. The recovery of the phosphatides is 99-100% from TLC. The analysis of phospholipid distribution was accurately accomplished in the presence of silica gel. The results are presented in Tables I and II.

TABLE I

Phospholipid Composition of Various Human Tissues[a]

| Sample number | Placenta | | Endometrium | | Amniotic[b] fluid | | Brain gray matter | | Brain white matter | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Phosphatidyl choline | 46.4 ±0.5 | 45.5 ±0.5 | 42.0 ±0.4 | 43.0 ±0.5 | 65.0 ±0.5 | 66.0 ±0.6 | 23.9 ±0.2 | 24.3 ±0.3 | 24.7 ±0.3 | 24.9 ±0.4 |
| Phosphatidyl ethanolamine | 26.5 ±0.3 | 26.9 ±0.3 | 23.0 ±0.3 | 22.0 ±0.4 | 16.0 ±0.3 | 14.8 ±0.3 | 35.2 ±0.3 | 35.0 ±0.3 | 34.5 ±0.4 | 36.7 ±0.4 |
| Phosphatidyl serine | 5.0 ±0.2 | 5.8 ±0.2 | 16.0 ±0.2 | 14.2 ±0.3 | 7.0 ±0.2 | 8.1 ±0.2 | 22.7 ±0.2 | 22.9 ±0.4 | 19.7 ±0.2 | 19.0 ±0.2 |
| Phosphatidyl inositol | 4.0 ±0.2 | 3.8 ±0.1 | 6.0 ±0.1 | 6.5 ±0.1 | 2.0 ±0.1 | 2.0 ±0.1 | 2.3 ±0.1 | 2.6 ±0.1 | 2.5 ±0.2 | 2.0 ±0.1 |
| Phosphatidic acid | 0.9 ±0.1 | 1.2 ±0.1 | 1.0 ±0.1 | 1.0 ±0.1 | 2.0 ±0.1 | 1.8 ±0.1 | 1.1 ±0.1 | 1.0 ±0.1 | 1.2 ±0.1 | 1.1 ±0.1 |
| Lysophosphatidyl choline | 2.0 ±0.1 | 2.0 ±0.1 | 2.0 ±0.1 | 1.9 ±0.1 | 2.0 ±0.1 | 1.3 ±0.1 | 1.1 ±0.1 | 1.0 ±0.1 | 1.3 ±0.1 | 1.1 ±0.1 |
| Diphosphatidyl glycerol | 1.1 ±0.1 | 0.6 ±0.1 | 2.0 ±0.1 | 2.4 ±0.1 | Trace | Trace | Trace | Trace | Trace | Trace |
| Spingomyelin | 14.01 ±0.3 | 14.2 ±0.2 | 8.0 ±0.1 | 8.8 ±0.2 | 6.0 ±0.2 | 6.0 ±0.1 | 13.7 ±0.3 | 12.8 ±0.3 | 16.1 ±0.3 | 15.0 ±0.2 |

[a]As percentage of the total lipid phosphorous ± standard deviation.

[b]Samples 1 and 2 are different.

[c]Amniotic fluid is also included in the table.

TABLE II

Phospholipid Composition of Various Human Tissues[a]

| | Lung | | Liver | | Spleen | | Kidney | | Heart muscle | | Skeletal muscle | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample number | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Phosphatidyl choline | 52.1 ±0.3 | 54.2 ±0.5 | 45.8 ±0.4 | 44.2 ±0.3 | 44.5 ±0.4 | 42.6 ±0.5 | 35.1 ±0.5 | 37.2 ±0.2 | 42.5 ±0.6 | 43.0 ±0.3 | 48.0 ±0.5 | 50.0 ±0.6 |
| Phosphatidyl ethanolamine | 19.6 ±0.2 | 17.9 ±0.3 | 29.1 ±0.3 | 28.5 ±0.2 | 26.1 ±0.4 | 26.5 ±0.4 | 29.7 ±0.4 | 29.4 ±0.4 | 28.7 ±0.5 | 28.0 ±0.4 | 27.0 ±0.5 | 27.0 ±0.3 |
| Phosphatidyl serine | 7.6 ±0.2 | 8.0 ±0.2 | 3.2 ±0.1 | 3.7 ±0.1 | 8.7 ±0.3 | 8.9 ±0.2 | 7.7 ±0.2 | 6.6 ±0.1 | 3.2 ±0.2 | 4.0 ±0.1 | 4.0 ±0.1 | 3.0 ±0.1 |
| Phosphatidyl inositol | 4.4 ±0.1 | 3.6 ±0.2 | 9.3 ±0.2 | 9.0 ±0.2 | 4.3 ±0.2 | 4.6 ±0.1 | 6.6 ±0.1 | 6.2 ±0.2 | 6.4 ±0.2 | 7.0 ±0.2 | 9.0 ±0.3 | 8.0 ±0.1 |
| Phosphatidic acid | 1.1 ±0.1 | 1.0 ±0.1 | 1.0 ±0.1 | 1.3 ±0.2 | 1.1 ±0.1 | 1.0 ±0.1 | 1.1 ±0.1 | 1.0 ±0.1 | 1.0 ±0.1 | 1.0 ±0.1 | 1.0 ±0.1 | 1.2 ±0.1 |
| Lysophosphatidyl choline | 2.2 ±0.1 | 3.1 ±0.1 | 2.1 ±0.1 | 3.1 ±0.1 | 1.1 ±0.1 | 2.0 ±0.2 | 2.2 ±0.1 | 2.3 ±0.1 | 4.2 ±0.2 | 3.0 ±0.1 | 2.0 ±0.1 | 2.5 ±0.1 |
| Di-phosphatidyl glycerol | 1.1 ±0.1 | 1.0 ±0.1 | 4.2 ±0.2 | 3.9 ±0.1 | 1.1 ±0.1 | 1.3 ±0.1 | 3.3 ±0.1 | 3.5 ±0.1 | 8.6 ±0.3 | 8.6 ±0.2 | 5.0 ±0.2 | 3.2 ±0.1 |
| Spingomyelin | 11.9 ±0.2 | 10.9 ±0.3 | 5.2 ±0.2 | 6.0 ±0.1 | 13.0 ±0.3 | 12.8 ±0.2 | 14.2 ±0.3 | 13.5 ±0.3 | 5.3 ±0.2 | 5.4 ±0.1 | 4.0 ±0.1 | 4.9 ±0.2 |

[a]Same as in Table I.

## RESULTS AND DISCUSSION

The two dimensional TLC method employed in this study has facilitated a fairly comprehensive analysis of the phospholipid. This report is one of a series in which data are presented for variation of phospholipid class distribution among various tissues. The greater emphasis is given to placenta, amniotic fluid and endometrium phospholipid composition. It is possible that the phospholipid composition of each tissue is characteristic for that class. It is observed from the table that lecithin and phosphatidyl ethanolamine are the main components of the phospholipids. Table I shows that amniotic fluid, placenta and endometrium phospholipids contain 14.8-16.0%, 26.5-26.9% and 22.0-23.0% phosphatidyl ethanolamine. Thin layer chromatography indicates that lysolecithin is present, although it is impossible to determine whether or not it is an artifact of the isolation procedure. The sphingomyelin content of endometrium and amniotic fluid is low, while in placenta it is high. Endometrial tissue contains 14.2-16.0% phosphatidyl serine, although placenta and amniotic fluid contain low amounts. It is noted from Table I that the content of phosphatidic acid is about 1% in the tissues studied. The mean value of phosphatidyl inositol in human placenta, endometrium and amniotic fluid is 3.9%, 6.3% and 2.0% respectively of total phospholipids.

Our results for phospholipids of other human tissues agree fairly closely with those reported by other workers (6,10-14). The most striking difference in the phospholipid composition of various tissues is in their diphosphatidyl glycerol content. Tables I and II show that it increases in the following order: amniotic fluid + brain < lung + spleen + placenta < endometrium < kidney < liver < skeletal muscle < heart muscle.

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# The Distribution of Cyclopropane and Cyclopropene Fatty Acids in Higher Plants (Malvaceae)

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## ABSTRACT

The occurrence and distribution of cyclopropane and cyclopropene fatty acids has been investigated in seeds, leaves and other tissues of several species of Malvaceae. Sterculic and malvalic acids and their dihydro-analogs were present in all the plant tissues examined, the highest proportions generally being in immature seeds. The cyclic acids were mainly concentrated in the neutral lipids with lesser proportions in phospholipid or glycolipid classes. The occurrence of these acids in all tissues of some species offered the attractive possibility of establishing callus tissue cultures capable of synthesizing cyclopropane and cyclopropene fatty acids and thereby providing a more convenient and reproducible system for biosynthetic studies. Substantial proportions of these cyclic acids were found in callus tissue cultures propagated from two *Malva* species.

## INTRODUCTION

Cyclopropane and cyclopropene fatty acids are not widely distributed in higher plants but they do occur in the seed oils of many species of the plant order Malvales (1,2). The cyclopropene acids, sterculic acid (I) and malvalic acid (II), are specific to higher plants and are usually accompanied by minor amounts of the corresponding cyclopropane acids, dihydrosterculic acid (III) and dihydromalvalic acid (IV). Cyclopropane fatty acids on the other hand occur widely as constituents of bacterial lipids (1,2) especially lactobacillic acid (V), the positional isomer of dihydrosterculic acid, and its lower homolog (VI) but in bacteria they are never accompanied by cyclopropene acids.

(I) $CH_3(CH_2)_7C = C(CH_2)_7CO_2H$

(II) $CH_3(CH_2)_7C = C(CH_2)_6CO_2H$

(III) $CH_3(CH_2)_7CH-CH(CH_2)_7CO_2H$

(IV) $CH_3(CH_2)_7CH-CH(CH_2)_6CO_2H$

(V) $CH_3(CH_2)_5CH-CH(CH_2)_9CO_2H$

(VI) $CH_3(CH_2)_5CH-CH(CH_2)_7CO_2H$

The distribution of cyclopropane acids among and within individual phospholipid classes of bacteria has been well established (3,4). Less is known however about the distribution of cyclopropane and cyclopropene acids in plant lipids. In mature seeds of Bombacaceae, Malvaceae and Sterculiaceae species they occur largely but not necessarily entirely as components of the triglycerides (2,5,6).

Shenstone and Vickery (7,8) observed that these acids frequently constitute significant proportions of the fatty acids derived from leaves, stems and roots, as well as from seeds, of several species of Malvaceae but their distribution among the various lipid classes of these somatic lipids was not established.

Because of our general interests in pathways of fatty acid biosynthesis in plants and in the inhibitory action of exogenous cyclopropene acids on fatty acid desaturations in plant (9) and animal (10,11) systems, we have studied the occurrence and the distribution of cyclopropane and cyclopropene fatty acids in seeds and other tissues of a number of species of Malvaceae, in order to define a suitable biosynthetic system. The occurrence of these acids in all tissues of some species would offer the attractive possibility of establishing callus tissue cultures capable of synthesizing cyclopropane

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TABLE I

Equivalent Chain Lengths, on GLC on PEGA and SE-30 Stationary Phases, of Methyl Esters of Cyclopropane Acids and of Methyl Branched Saturated Acids Derived From Cyclopropene Acids

| Stationary phase | 18:CPA[a] | 18:CPE(br-18:0) | 19:CPA | 19:CPE(br-19:0) |
|---|---|---|---|---|
| 15% PEGA | 18.4 | 17.3 | 19.4 | 18.3 |
| 3% SE-30 | 17.8 | 17.4 | 18.8 | 18.4 |

[a]Abbreviations denote the total number of carbons in the fatty acid (18, 19, etc.), number of double bonds (:0, :1, etc.) or, for cyclic acids, whether they are cyclopropane (:CPA) or cyclopropene (:CPE). Thus 19:CPE = sterculic, 18:CPE = malvalic, 19:CPA and 18:CPA are their dihydro analogs, 18:0 = stearic, 18:2 = linoleic, etc.

and cyclopropene acids. Such callus cultures could provide a more reproducible tissue for biosynthetic studies, capable of being used for prolonged incubation studies without the danger of contaminating microorganisms.

## MATERIALS AND METHODS

Mature seeds of *Malva moschata* and *M. sylvestris* were collected in the wild, seeds of *M. parviflora* were provided by A.R. Johnson, Division of Food Preservation, CSIRO, Ryde, N.S.W., Australia, and seeds of *M. alcea fastigiata* and *Lavatera olbia rosea* were purchased from Thomson and Morgan Ltd., Ipswich. All of these species were grown from seed in the greenhouses of the Plant Products Division of this laboratory.

Callus tissue cultures were established from a 1 cm piece of hollow stem internode excised from near the tip of a main shoot of a mature *M. parviflora* plant and from germinating seed of *M. sylvestris*. The piece of stem and the seeds were surface sterilized by immersing for 5 min in dilute hypochlorite solution (1% available chlorine) containing two drops of Teepol detergent to aid wetting, after which they were washed three times in fresh changes of sterile distilled water. Before transferring to 100 ml conical flasks containing 25 ml of the agar medium described below, the surface tissues of the stem explant were cut away and the seeds were plated out in Petri dishes containing a mineral agar with glucose, any seeds showing signs of infection within 48 hr being discarded. The flasks were plugged and incubated at 25 C under controlled lighting conditions and after 2-3 weeks callus formation was evident. After

TABLE II

The Cyclopropane and Cyclopropene Acid Content of Tissues From *Malva* Species

| Species | Tissue | Weight % of total fatty acids 18:CPE[a] | 19:CPE[b] | 19:CPA | Total cyclic acids |
|---|---|---|---|---|---|
| *M. parviflora* | Leaf (winter) | 3.8 | 1.4 | 0.5 | 5.7 |
| | Immature seed | 20.0 | 9.8 | 4.3 | 34.1 |
| | Stem | 1.6 | 3.6 | 0.4 | 5.6 |
| | Root | 1.9 | 6.1 | 0.3 | 8.3 |
| | Callus culture | 6.2 | 4.5 | 3.9 | 16.8 |
| *M. sylvestris* | Leaf (winter) | 8.1 | 1.7 | tr | 9.8 |
| | Leaf (summer) | 1.5 | 0.6 | tr | 2.1 |
| | Immature seed | 4.5 | 2.5 | 0.8 | 7.8 |
| | Stem | 3.7 | 4.9 | tr | 8.6 |
| | Callus culture | 4.3 | 10.2 | 5.5 | 20.0 |
| *M. alcea fastigiata* | Leaf (winter) | 3.5 | 2.2 | 0.4 | 6.1 |
| | Leaf (summer) | tr | tr | tr | <1.0 |
| | Immature seed | 9.4 | 2.6 | 4.3 | 16.3 |
| *M. moschata* | Leaf (winter) | 4.1 | 1.6 | 1.0 | 6.7 |
| | Immature seed | 15.5 | 2.9 | 1.4 | 19.8 |

[a]Abbreviations as in Table I.

[b]Small proportions of 18:CPA are included in these figures; tr = 0.2% or less, but detectable.

TABLE III

Fatty Acid (wt. %) Composition of *M. parviflora* Seed Lipids at Various Stages of Seed Maturation

| Seed maturity[a] | Total saturated | 18:1[b] | 18:2 | 18:3 | 18:CPE | 19:CPE[c] | 19:CPA | Total cyclic acids |
|---|---|---|---|---|---|---|---|---|
| Very immature | 30.0 | 13.6 | 19.6 | 18.0 | 7.4 | 6.4 | 5.0 | 18.8 |
| Immature | 25.8 | 11.3 | 16.8 | 12.0 | 20.0 | 9.8 | 4.3 | 34.1 |
| Intermediate maturity | 23.5 | 10.3 | 51.8 | 6.3 | 3.8 | 3.0 | 1.3 | 8.1 |
| Mature | 22.8 | 7.5 | 65.1 | 2.5 | 0.8 | 1.3 | tr | 2.1 |

[a]See text for explanation of terms employed.

[b]Abbreviations as in Table I.

[c]Small proportions of 18:CPA are included in these figures.

about 4 weeks quantities of soft, pale brown callus were separated from the original explant or seed and transferred to fresh medium. Subsequently portions were subcultured in fresh medium at approximately 4-6 week intervals for *M. parviflora* callus and approximately 3 week intervals for the faster growing *M. sylvestris* callus.

The medium used for callus tissue culture, subculture and incubation with labelled substrates contained: $Ca(NO_3)_2.4H_2O$, 660 mg; $KNO_3$, 410 mg; $MgSO_4.7H_2O$, 200 mg; $KH_2PO_4$, 70 mg; NaCl, 60 mg; glycine, 3.0 mg; nicotinic acid, 0.5 mg; thiamine hydrochloride, 0.1 mg; pyridoxine, 0.1 mg; glucose, 25 g; agar, 6 g; 0.01% napthalene acetic acid solution, 2 ml; coconut milk, 100 ml; and trace amounts of $H_3BO_3$, $MnCl_2$, $CuCl_2$, $Na_2MoO_4$, $ZnCl_2$ and Fe-EDTA, made up to 1 liter with deionized water and adjusted to pH 5.5. The medium was sterilized by autoclaving under 15 psi for 20 min.

All chemicals were analytical grade and solvents were redistilled.

#### Extraction and Fractionation of Lipids

Plant tissues were macerated with approximately 200 volumes of iso-propanol and, after filtration, the residue was re-extracted with chloroform-methanol (2:1, v/v) at room temperature for 30 min and again filtered. The iso-propanol and chloroform-methanol extracts were combined, the solvent was removed under vacuum in a rotary evaporator and the residue was redissolved in a small volume of chloroform-methanol (2:1) and washed by the method of Folch et al. (12) to remove water soluble materials. Fractionation of the lipid extracts was obtained by a combination of column chromatography on DEAE-cellulose (acetate form) and thin layer chromatography (13).

#### Determination of Fatty Acid Composition

Fatty acids were obtained from total lipid extracts or separated lipid fractions after hydrolysis with 10% methanolic KOH under reflux for 1 hr and methyl esters were prepared by treatment of a methanol-ether solution of the fatty acids with ethereal diazomethane. The more common fatty acid methyl esters were analyzed directly by gas liquid chromatography (GLC) on both polyethyleneglycol adipate (PEGA) (15%) and silicone elastomer (SE-30) (3%) stationary phases. Cyclopropane and cyclopropene fatty acid methyl esters were also determined by GLC, following reaction of the mixed methyl esters with silver nitrate and hydrogenation of the resultant mixture according to the method of Johnson and coworkers (14). This treatment results in a mixture, readily separated and identified by GLC, of normal saturated fatty acids, cyclopropane acids which are not affected, and methyl-branched fatty acids derived from cyclopropene acids in the original mixture. The equivalent chain lengths or carbon numbers of these various fatty acid esters on the two stationary phases are listed in Table I.

## RESULTS

#### Fatty Acid Compositions

The proportions of cyclopropane and cyclopropene fatty acids in the lipid extracts from different tissues of various Malva species are given in Table II. In all but one species the immature seed extracts contained the highest proportion of these acids relative to the other tissues.

The finding that cyclopropane and cyclopropene acids were present in appreciable proportions in all the *Malva* plant tissues examined encouraged us to establish callus cultures from two species. The cyclic acid contents of these are included in Table II and indicate that substantial proportions of these acids were present in each case.

The differences in cyclic acid content of

TABLE IV

Distribution (wt. %) of Cyclopropane and Cyclopropene Fatty Acids Between the Major Lipid Classes of Immature Seeds of *Lavatera olbia rosea*

| Lipid class | 18:CPE[a] | 19:CPE[b] | 19:CPA |
|---|---|---|---|
| Total lipids | 10.8 | 5.6 | 10.3 |
| Neutral lipids | 14.7 | 6.5 | 14.0 |
| Phosphatidyl choline | 6.8 | 4.8 | 5.6 |
| Phosphatidyl ethanolamine | 0.2 | 1.0 | 0.5 |
| Phosphatidyl inositol | 0.3 | 2.4 | 1.0 |
| Digalactosyl diglyceride | 1.2 | 2.5 | 1.8 |

[a]Abbreviations as in Table I.

[b]The methyl branched stearate peak, derived from 19:CPE, masked smaller quantities of 18:CPA which are therefore included in these figures.

leaves in *M. sylvestris* and *M. alcea fastigiata* taken from greenhouse grown plants in summer (July-August) and in winter (December-February) are of interest. No such marked seasonal variations were evident in the cyclic acid content of seeds or of callus tissues.

Changes in fatty acid composition relative to maturity of seeds are shown in Table III for *M. parviflora*. Maturation of these seeds took about 35 days from flowering. "Very immature" seeds were from seed heads of less than 2 mm in diameter taken 5-10 days after flowering, "immature" seeds from seed heads of about 2 mm diameter at 15 days, "intermediate maturity" seeds from 4 mm diameter seed heads at 25 days, and "mature" seeds from seed heads about 8 mm across at about 35 days after flowering. Table III shows that there was a rapid buildup of cyclic acids during the initial stages of seed formation but that the proportion of these then decreased markedly as the seeds matured.

This decrease in the proportion of cyclic acids during the period of active triglyceride synthesis and accumulation as seeds developed toward maturity seems to be general and was found by Johnson and co-workers in all the species they examined (5). For this reason all seed lipid analyses listed in Table II are of immature seeds and, in general, immature seeds were used subsequently for incubations with labelled substrates (15).

### Fatty Acid Distribution in Lipids

The distribution of individual fatty acids between the different lipid classes of immature seeds as exemplified by those of *Lavatera olbia rosea* is given in Table IV, which shows that the highest proportion of cyclic acids was in the neutral lipid fraction. There was a lesser but still substantial proportion of these acids in the phosphatidylcholine fraction but only small amounts in the other polar lipid classes. Similar patterns were obtained with seeds of the various *Malva* species and Table V indicates that in leaves these acids are also primarily located in the neutral lipid classes.

TABLE V

The Total Cyclic Acid Content (as wt.% of Mixed Acids) of Total Lipid Extract and the Neutral Lipid Fraction of Leaves of *Malva* Species

| Species | CPA + CPE acids (wt.%) in Total lipid | CPA + CPE acids (wt.%) in Neutral lipid |
|---|---|---|
| *M. parviflora* | 5.7 | 20.5 |
| *M. sylvestris* | 9.8 | 47.3 |
| *M. alcea fastigiata* | 6.1 | 23.1 |
| *M. moschata* | 6.7 | 38.7 |

## DISCUSSION

The analytical studies herein reported confirm the occurrence of cyclopropane and cyclopropene fatty acids in a wide range of species of the family Malvaceae. More significantly the wide distribution of these acids not only in the seeds but throughout the tissues of these plants as originally reported by Shenstone and Vickery (7) has been confirmed. Indirect evidence as to the generality of their distribution within these plants has also been provided by their occurrence in significant proportions in the dedifferentiated cells of callus tissue cultures of two species.

There are some quantitative differences, however, between our results and those of the Australian workers. They never found less than 10% of cyclopropene acids in their extracts from leaves of *M. parviflora* and occasionally up to 30% of the fatty acids in their leaf extracts were cyclopropenes (7,8), whereas in only one case did we find the total of cyclopropane and cyclopropene acids to approach 10% of a total leaf extract. We believe these quantitative differences between our results and those of Shenstone et al. are due to the different extraction procedures employed and to the fact (Table V) that the cyclic acids are concentrated in the neutral lipid fraction of total leaf lipids. Our procedure extracts essentially all of the lipids from plant tissues whereas the petroleum fraction they used is likely to have extracted only the neutral lipid fraction (triglycerides, diglycerides, free acids and so on) and not the phospholipids and glycolipids, which comprise the major proportion of leaf lipids and which contain only minor amounts of cyclic acids.

The wide distribution of cyclopropane and cyclopropene acids in the plant tissues suggests that they are unlikely to have any function more specific than the normal fatty acids, particularly as they are concentrated largely in the triglycerides. Bacterial systems are different in that the cyclopropane acids are restricted to the phospholipids and especially to phosphatidyl ethanolamine (3). A further difference is that cyclopropane acids tend to accumulate in bacteria after the active growth phase (16-18) whereas in our seeds both cyclopropane and cyclopropene acids are produced largely during the early period of seed maturation and, at least proportionally, their synthesis decreases with increasing maturity of the seeds.

These analytical studies were a necessary and successful prelude to the biochemical studies we had planned and which are reported in the following paper (15). They demonstrated that both leaf and seed tissues were likely to be suitable for incubation studies, that quite immature seeds were likely to be more active in biosynthesizing cyclopropane and cyclopropene acids than more mature seeds, and finally, that the callus cultures that had been established would provide a novel and potentially advantageous system for biochemical studies.

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# The Biosynthesis of Cyclopropane and Cyclopropene Fatty Acids in Higher Plants (Malvaceae)

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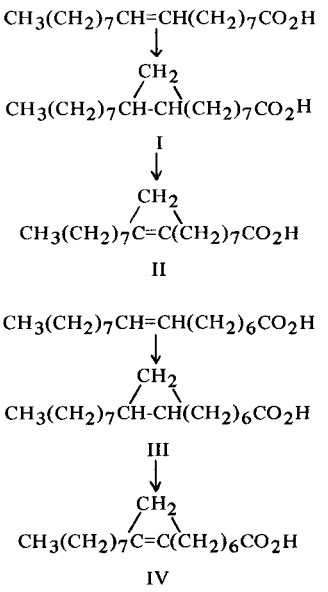
## ABSTRACT

The biosynthesis of cyclopropane and cyclopropene fatty acids has been investigated in immature seeds, leaves and callus tissue cultures of several species of Malvaceae. Chemical characterization of labeled cyclopropane and cyclopropene fatty acids obtained from incubations with L-[$^{14}CH_3$]methionine confirmed that the ring methylene group was derived from the methyl group of methionine. The variation with time in the distribution of radioactivity in the products of incubations with [$^{14}CH_3$]methionine and [2-$^{14}$C]acetate suggested that the pathway involved initial formation of dihydrosterculic acid from oleic acid with subsequent desaturation to sterculic acid and α-oxidation to malvalic and dihydromalvalic acids. Direct evidence in favor of this pathway was provided by the conversion of [1-$^{14}$C]oleic acid to dihydrosterculic and sterculic acids and by the desaturation of [1-$^{14}$C]dihydrosterculic acid to sterculic acid, the first time that these processes have been demonstrated in higher plants. No conversion of [1-$^{14}$C]stearolic acid to sterculic acid could be obtained under the same conditions. The presence of an active fatty acid α-oxidation system was demonstrated in the callus cultures.

## INTRODUCTION

The mechanism of the biosynthesis of cyclopropane fatty acids in bacteria and the substrate specificities of the synthetase enzyme have now been substantially defined, largely by the work of Hofmann and of Law and their respective coworkers (1-8), and have been reviewed (9,10). However the pathways of biosynthesis, of cyclopropane and, more particularly, of cyclopropene fatty acids in higher plants are less well understood.

Wilson et al. (11) demonstrated the co-occurrence of sterculic and malvalic acids and the corresponding cyclopropane acids in various seeds and suggested that methylene addition to oleic acid gave rise to dihydrosterculic acid (I) which was desaturated to sterculic acid (II), and that 8-heptadecenoic acid was similarly the precursor of dihydromalvalic acid (III) and malvalic acid (IV) (the symbols used for these acids in the Tables and Figures are 19:CPA = I, 19:CPE = II, 18:CPA = III, and 18:CPE = IV):




Smith and Bu'Lock (12) demonstrated that in *Hibiscus* seedlings the chains of sterculic and malvalic acids, but not the ring methylene carbon, were derived from acetate. They also showed that the labeling pattern in malvalic acid was the same as that in sterculic acid minus the carboxyl carbon, and concluded that a chain shortening by α-oxidation must occur at some stage in the biogenesis of malvalic acid.

Hooper and Law (13), also working with *Hibiscus* seedlings, established that the ring methylene carbon of both cyclopropane and cyclopropene acids was derived from the methyl group of methionine and suggested, from the distribution of label, that the pathway was oleic→dihydrosterculic→sterculic acids.

Smith and Bu'Lock, however, had proposed

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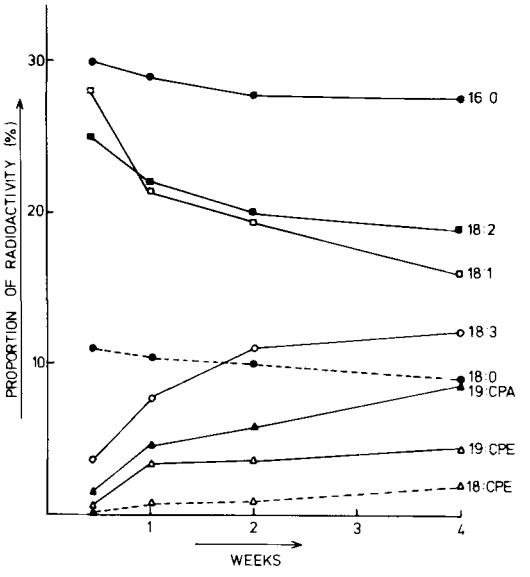



FIG. 1. Variation with time in the distribution of radioactivity incorporated from [2-$^{14}$C]acetate into fatty acids of *Malva sylvestris* callus tissue cultures.

(12) that sterculic acid was derived directly by methylene addition across the 9,10-triple bond of stearolic acid (9-octadecynoic acid). When these authors subsequently reported the occurrence of minor amounts of stearolic and 8-heptadecynoic acids in seeds containing sterculic and malvalic acids, they implied that this pathway of cyclopropene acid biogenesis was thereby established (14).

In a more recent study (15), Johnson and coworkers showed that, of several $^{14}$C-labeled compounds, L-methionine was the most efficient precursor of the ring methylene. From the variations in the labeling pattern with duration of incubations, they concluded that the probable pathway was oleic→dihydrosterculic→sterculic acid and that Bu'Lock's suggestion of acetylenic precursors for the cyclopropene acids was not supported by their results.

Our present studies of the biosynthesis of cyclopropane and cyclopropene fatty acids in higher plant tissues were initiated to resolve this question and because of our general interest in pathways of fatty acid biosynthesis in plants. A further reason for our interest lay in the apparent anomaly that, whereas exogenous cyclopropene fatty acids have a marked inhibitory action on fatty acid desaturations both in animal tissues (16,17) and in plants (18,19), no such inhibition of desaturation appears to occur in the seeds and leaves of many malvaceous species where cyclopropene acids and unsaturated acids are synthesized and exist together.

The previous paper (20) describes analytical studies which indicated that both leaf and immature seed tissues of a number of Malvaceae species were likely to be suitable for studies of the biosynthesis of cyclopropane and cyclopropene fatty acids. It was also demonstrated that callus cultures established from two of these species contained substantial proportions of the various cyclic acids and were likely to provide a novel and potentially advantageous system for biochemical studies.

## EXPERIMENTAL PROCEDURE

### Materials

Plant materials were obtained and callus cell cultures were established and cultured as described in the preceding paper (20).

All chemicals were of analytical grade and solvents were redistilled.

L-[$^{14}CH_3$]Methionine, [2-$^{14}$C]acetate, [1-$^{14}$C]oleic acid, [U-$^{14}$C]stearic acid and [U-$^{14}$C]palmitic acid were purchased from The Radiochemical Centre, Amersham, England.

[1-$^{14}$C]Stearolic acid was synthesized by bromination in diethyl ether solution at 0 C of [1-$^{14}$C]oleic acid (100 $\mu$c), admixed with oleyl alcohol (100 mg) as carrier, followed by dehydrobromination of the product with potassium *t*-butoxide in refluxing *t*-butanol. The product was treated with ethereal diazomethane and the labeled ester product was separated from the alcohol carrier by preparative thin layer chromatography (TLC) and purified by argentation-TLC (21). The ester was completely pure, chemically and radiochemically, as judged by TLC, argentation-TLC and radiochemical-gas liquid chromatography (GLC) (22) on both polyethyleneglycol adipate (PEGA) and silicone elastomer (SE-30) stationary phases. Hydrolysis yielded [1-$^{14}$C]stearolic acid (29 $\mu$c).

Methyl [1-$^{14}$C]dihydrosterculate was synthesized from [1-$^{14}$C]oleate (100 $\mu$c), mixed with 1-pentadecene (100 mg) as carrier, by reaction with methylene iodide over zinc-copper couple essentially as described by Christie and Holman (23). The dihydrosterculate product was isolated from the carrier and unreacted oleate by TLC and argentation-TLC. It was shown by radiochemical-GLC to be *cis*-dihydrosterculate with a small (<5%) proportion of the *trans*-isomer. Hydrolysis yielded [1-$^{14}$C]dihydrosterculic acid (30 $\mu$c).

The specific activities of the [1-$^{14}$C]stearolic and [1-$^{14}$C]dihydrosterculic acid were of course the same as the [1-$^{14}$C]oleic acid from which they were derived (57 $\mu$c/$\mu$mole).

All the radioactively labeled substrates were

TABLE I

Effect of L-Methionine on the Incorporation of [2-$^{14}$C]Acetate Into Total Lipids and Into Cyclopropane and Cyclopropene Fatty Acids in *M. parviflora* Callus Tissue[a]

| Concentration of added L-methionine in medium | Per cent of added $^{14}$C in total lipid | Per cent of total fatty acid radioactivity | | | |
|---|---|---|---|---|---|
| | | 19:CPA | 19:CPE | 18:CPE | Total CPA + CPE |
| 0 | 16.0 | 2.4 | 1.9 | Trace | 4.5 |
| $4 \times 10^{-4}$M | 17.7 | 4.7 | 5.7 | 3.0 | 13.4 |
| $2 \times 10^{-3}$M | 11.8 | 5.2 | 2.9 | 1.5 | 9.6 |
| $1.2 \times 10^{-2}$M | 11.1 | 3.1 | 3.7 | 1.2 | 8.0 |

[a]2 weeks incubation.

dissolved or suspended in distilled water at a concentration of 10 μc/ml, the dispersion of the long chain acids being assisted by the addition of sodium carbonate (ca. 1 mg/ml) and Tween 80 (ca. 0.1 mg/ml) and sonication for 30 sec.

### Methods

*Incubations:* Immature seeds and young leaves were washed thoroughly in 0.1% aqueous chloramphenicol solution, sliced finely with a scalpel, weighed and added to small conical flasks containing 0.2 M phosphate buffer (pH 7.4, 2.0 ml), the $^{14}$C-labeled substrate (2-10 μc), and water to make the volume up to 5.0 ml. Between 0.5-1.0 g chopped leaves or 1.5-2.0 g sliced seeds were used in each incubation. The flasks were plugged with cotton wool and incubated aerobically, with shaking at 27 C under illumination by 4 x 40 W daylight fluorescent tubes at a distance of 30 cm, for 2-48 hr.

For the biochemical experiments using callus tissue cultures, 25 ml of the growth medium described above was placed in each 100 ml conical flask, $^{14}$C-labeled substrate (25 μc of acetate or methionine or 2 or 5 μc of a long chain fatty acid precursor) was added and the flask and its contents were sterilized in an autoclave at 15 psi for 20 min. When the agar had set on cooling, 0.5-1.0 g wet weight of callus tissue was transferred under sterile conditions from the subculture flask to the surface of the medium containing the precursor. Each flask was plugged and maintained aerobically under sterile conditions and controlled temperature (25 C) in subdued light for periods of up to 5 weeks, as in normal tissue subculture. During these periods of incubation, callus cell growth results in an increase in the wet weight of tissue by a factor of 2 to 4.

After the appropriate incubation time, the entire seed or leaf incubation mixture or the mass of callus tissue, removed from the medium, was macerated in excess chloroform-methanol 2:1 v/v mixture. The lipid extracts were worked up and fatty esters were prepared for analysis as described in the preceding paper (20). The fatty acid composition, the distribution of radioactivity, and the specific activities of individual components of the mixed esters from these incubations were determined as described in the preceding paper (20) but using radiochemical-GLC (22) on PEGA or SE-30 stationary phases, or both.

## RESULTS

### Incubations With [2-$^{14}$C] Acetate

The distribution of radioactivity among the fatty acids of *M. sylvestris* callus tissue after incubation with [2-$^{14}$C] acetate in the growth medium for periods of 3 days to 4 weeks is summarized in Figure 1. The total proportion of added substrate incorporated into total lipids, predominantly into fatty acids, increased linearly with time to about 16% at 2 weeks and thereafter remained essentially constant. The pattern illustrated in Figure 1 shows that the fatty acids most rapidly produced from labeled acetate were palmitic, oleic, linoleic and, to a lesser extent, stearic acids. The formation of linolenic acid was slower, as is common in many plant tissues, and increased steadily with time relative to the other common fatty acids. The synthesis of labeled dihydrosterculic (19:CPA) and sterculic acids (19:CPE) was still slower but was apparent after only 3 days, whereas formation of labeled malvalic acid (18:CPE) was not apparent until 1 week had elapsed. The proportion of label in each of these cyclopropane and cyclopropene fatty acids, like linolenic acid, then increased steadily with time.

Time studies of incorporation of [2-$^{14}$C] acetate into fatty acids by *M. parviflora* callus and by various seed and leaf tissue incubations were qualitatively similar to the *M. sylvestris* callus tissue results, but with less efficient

TABLE II

Distribution of Radioactivity in the Lipid Fractions After Incubation With L-[$^{14}CH_3$] Methionine

| Fate of label | *M. parviflora* callus grown | | *M. moschata* seed slices incubated | |
|---|---|---|---|---|
| | 1 week | 3 weeks | 3.5 hr | 15 hr |
| Per cent added $^{14}C$ incorporated into total lipids | 4.7 | 13.1 | 3.4 | 13.3 |
| Specific activity of total lipids, dpm/mg | 9900 | 21,400 | 1200 | 4100 |
| Per cent of total lipid activity in | | | | |
| Fatty acids | 13.1 | 24.4 | 17.4 | 29.0 |
| Unsaponifiable fraction | 49.5 | 44.0 | 43.8 | 49.4 |
| Water-soluble fraction | 29.6 | 18.7 | 20.7 | 7.5 |

production of labeled cyclopropane and cyclopropene acids, relative to the common fatty acids, in the chopped seed and leaf incubations after incubation times up to 48 hr.

The effect of unlabeled L-methionine on the biosynthesis of cyclopropane and cyclopropene fatty acids from [2-$^{14}C$]acetate in *M. parviflora* callus tissue is summarized in Table I. The lowest concentration of added methionine may have slightly stimulated incorporation of acetate into fatty acids but higher concentrations apparently inhibited incorporation by about 25%. All three concentrations of added methionine, however, have stimulated the production of labeled cyclic acids, relative to normal fatty acids, with the lowest concentration investigated ($4 \times 10^{-4}$ M) exerting the optimum effect. Methionine concentration therefore seems to be rate limiting for the biosynthesis of cyclic acids in callus tissue cultures in the medium used.

### Incubations With L-[$^{14}CH_3$] Methionine

The extent of incorporation of radioactivity from L-[$^{14}CH_3$]methionine into total lipids and the distribution of the label among different products after incubation with *M. parviflora* callus tissue and with *M. moschata* seed slices is shown in Table II. With each tissue the extent of total incorporation was essentially linear with time over the period studied. Approximately half of the incorporated label was associated in each case with the unsaponifiable fraction of the lipid extract. This fraction consisted mainly of sterols and quinones, which have not been characterized in this work, and the labeled methionine has presumably acted as donor for the well known side chain methylations of such compounds (9,24). A further substantial proportion of the total recovered activity was associated with water soluble hydrolysis products. This activity was presumably in choline, derived from phosphatidylcholine, which is also known to be synthesized, at least in part, by a pathway involving methylation of ethanolamine with methionine as the methyl donor (25). The remaining portion of recovered radioactivity was incorporated in the fatty acid fraction. The label was exclusively associated with the cyclopropane and cyclopropene fatty acids and the increase with time in the proportion of radioactivity in these fatty acids indicated their continued active synthesis over the periods studied.

TABLE III

Specific Activities[a] of the Methyl Esters of the α-Oxidative Degradation Products From Dihydrosterculic Acid (19:CPA)[b]

| Product | Specific activity | |
|---|---|---|
| | Calculated | Found |
| 19:CPA (residual) | --- | 2.04 |
| 18:CPA | 2.14 | 2.16 |
| 17:CPA | 2.24 | 2.28 |
| 16:CPA | 2.36 | 2.40 |
| 15:CPA | 2.49 | 2.54 |
| 14:CPA | 2.64 | 2.76 |
| 13:CPA | 2.80 | 2.93 |
| 12:CPA | 2.98 | 3.15 |
| br-11:0 | 3.16 | 3.18 |

[a]Calculated and determined by radiochemical-GLC.
[b]Biosynthesized from L-[$^{14}CH_3$]methionine.

That these labeled cyclic acids were dihydrosterculic, sterculic and malvalic acids, with the $^{14}C$-label from methionine on the carbon of the methylene bridge, was determined by radiochemical-GLC of the products of chemical α-oxidation (22) of the cyclopropane acid and of the branched chain acids derived from each of the cyclopropene acids by the silver nitrate-hydrogenation treatment (15,20). The chemical α-oxidation product from the cyclopropane acid consisted of residual ester (19:CPA) and a whole series of shorter chain cyclopropane ester homologues (18:CPA, 17:CPA, 16:CPA, etc.)

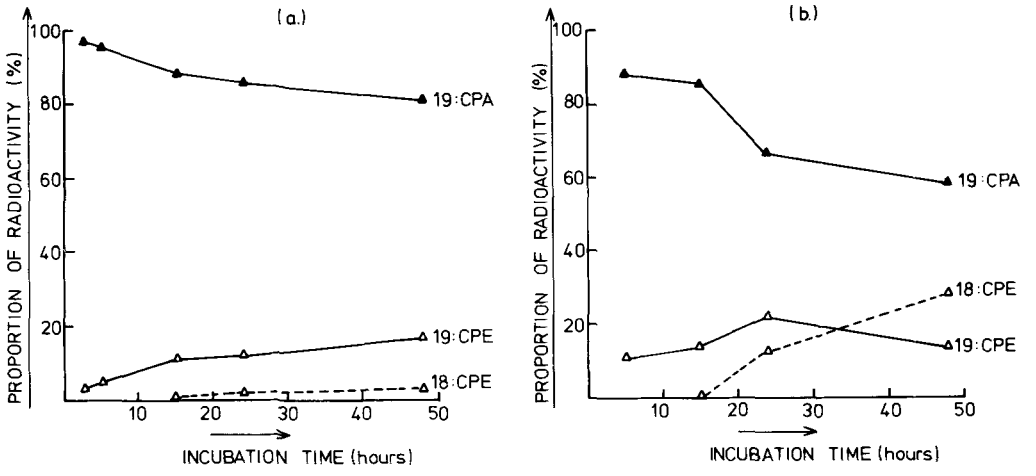



FIG. 2. Variation with time in the distribution of radioactivity incorporated from L-[$^{14}CH_3$]methionine into fatty acids after incubation of slided seed tissue of (a) *Malva sylvestris* and (b) *Malva moschata*.

down to the $C_{12}$ homologue, all of which were labeled and had steadily increasing specific activities (Table III). The next lower product had the retention characteristics of 2-methyldecanoate and the only other shorter chain product with any trace of radioactivity was 2-decanone. The products of chemical α-oxidation of the methyl-branched derivatives from the two cyclopropene acids contained residual methyloctadecanoate and methylheptadecanoate, respectively, and shorter methyl-branched homologues, again of steadily increasing specific activities, down to the $C_{11}$ component (methyldecanoate). Two other labeled compounds, corresponding to 2-undecanone and 2-decanone, were present; but there was no sign of activity in any shorter chain materials. Chemical α-oxidation of the pair of methyloctadecanoic acids derived from the cyclopropane acid, by hydrogenation over Adams catalyst in glacial acetic acid (cf. 10), gave the same mixture of labeled methyl-branched homologues plus methyl ketones. These results proved that the structures of the cyclic acids labeled from methionine were dihydrosterculic (9,10-methyleneoctadecanoic) acid, sterculic (9,10-methylene, 9-octadecenoic) acid, and malvalic (8,9-methylene, 8-heptadecenoic) acid with the label in each case on the methylene carbon.

The variation in the distribution of radioactivity in fatty acids with time in incubations of *M. sylvestris* and *M. moschata* seeds with L-[$^{14}CH_3$]methionine is shown in Figure 2. The major labeled acid in both seeds and at all time intervals was dihydrosterculic acid, but the preponderance of label in this acid was most marked after the shortest incubation times. The proportion of the label in dihydrosterculic acid then declined, steadily in *M. sylvestris* seed but abruptly between 15-24 hr in *M. moschata* seed, while the proportion of label appearing in sterculic acid and later in malvalic acid increased. The specific activity of dihydrosterculic acid increased rapidly to a high value after 5 and 15 hr respectively, and thereafter changed relatively little. The specific activity of sterculic acid increased later, and that of malvalic acid increased later still. Neither approached the specific activity values of dihydrosterculic acid and the value for malvalic acid, the major cyclic acid constituent of both these seeds, remained very low.

A time study of the incorporation of L-[$^{14}CH_3$]methionine into the fatty acids of *M. parviflora* callus tissue provided an even clearer picture of the probable sequence of biosynthesis of the cyclic fatty acids. Figure 3 provides a summary of the distribution of radioactivity, the total activity and the specific activities of the individual cyclic fatty acids after different periods of growth on medium containing labeled methionine. At the shortest incubation time, the proportion of radioactivity was highest in dihydrosterculic acid. This decreased as the proportion of activity in sterculate first increased and then dropped off slightly, and the proportion of activity in malvalic acid increased steadily. In addition to these acids there was a small but steady incorporation of label into a $C_{17}$ cyclopropene acid. (This was identified on the basis of radioactivity peaks corresponding to methylhexadecanoate on radiochemical-GLC of the products of the silver nitrate-hydrogenation treatment (15,20) on both PEGA and SE-30

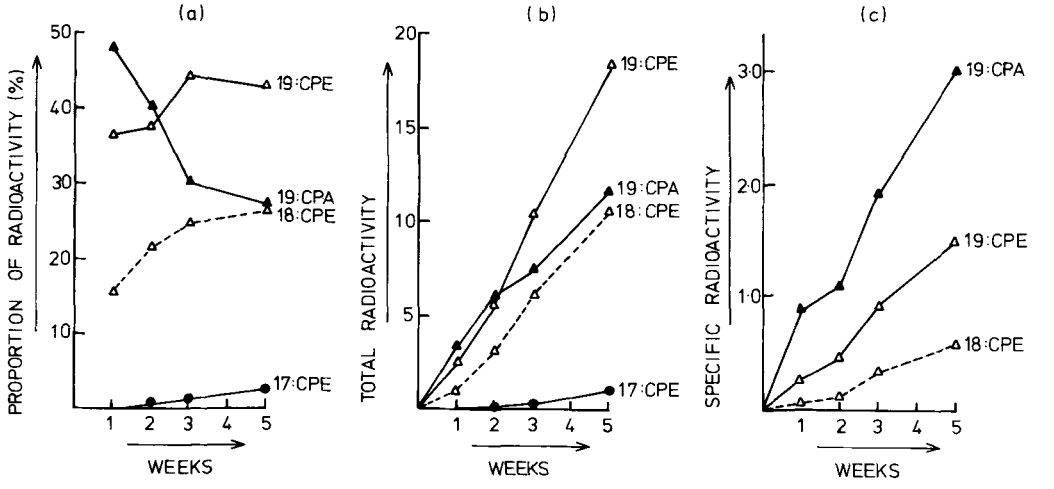



FIG. 3. Variation with time of (a) distribution of radioactivity, (b) total radioactivity, and (c) specific activities of cyclic fatty acids in *Malva parviflora* callus tissue cultured with L-[$^{14}CH_3$]methionine.

stationary phases, i.e., with equivalent chain length of 16.3 and 16.4, respectively (20). No significant mass was apparent and not enough of this material was formed to permit more rigorous characterization.)

The rate of incorporation of label into dihydrosterculic acid was essentially constant (Fig. 3b). For the first 2 weeks the rates of incorporation into sterculic and malvalic acids were slower, but after that time they became faster than the rate of incorporation into dihydrosterculic acid. The rate of labeling of the $C_{17}$-cyclopropene acid also increased after the third week. The specific activities of these acids all increased steadily with time, as would be expected where extensive new cell growth is involved. (The cells of these tissue cultures doubled or trebled during the period of a 5 week incubation.) The crossover of the sterculic and dihydrosterculic acid plots of total radioactivity was not found with the specific activity plots because of the higher proportion of sterculic than dihydrosterculic acid in the tissue.

Figure 2 shows that there was a considerable difference between the two seeds in the extent of incorporation of radioactivity from methionine into sterculic acid and, more particularly, into malvalic acid. *M. parviflora* seed gave a very similar pattern to *M. sylvestris* seed (Fig. 2a), while time course studies with leaves gave patterns quite similar to that illustrated for *M. moschata* seed (Fig. 2b). The callus tissue time study (Fig. 3) gave yet another pattern with far higher levels of incorporation into the two cyclopropene acids relative to dihydrosterculic. These differences are undoubtedly due largely to species or tissue differences but the difference between the two seed systems of Figure 2 may also be due, at least in part, to the maturity of the seeds used from each species. In general, young seeds were used for all of these incubations but it was very much a subjective assessment of which seeds were "young" and the degree of maturity of seeds taken for incubations could easily have varied somewhat, particularly from species to species.

The maturity of seeds had a considerable effect on the proportions of methionine label incorporated into the various cyclic fatty acids as shown by Table IV, which records the distribution of activity in fatty acids of young

TABLE IV

Effect of Maturity of *M. alcea fastigiata* Seeds on the Distribution of Radioactivity Incorporated Into Cyclic Acids From L-[$^{14}CH_3$]Methionine After 15 hr Incubation

| Seed maturity | Radioactivity, % | | | | |
|---|---|---|---|---|---|
| | 19:CPA | 19:CPE | 18:CPA | 18:CPE | 17:CPA |
| Immature | 70.2 | 15.2 | 5.2 | 3.0 | 6.0 |
| Fairly mature | 68.2 | 31.8 | < 0.1 | < 0.1 | < 0.1 |

TABLE V

Effect of Oxygen on the Incorporation of L-[$^{14}CH_3$]Methionine Into Dihydrosterculic, Sterculic and Malvalic Acids in *M. alcea fastigiata* Seeds After 14 hr Incubation

| Atmosphere | Radioactivity, % | | |
|---|---|---|---|
| | 19:CPA | 19:CPE | 18:CPE |
| Oxygen | 71.9 | 18.4 | 9.7 |
| Air | 88.9 | 11.0 | 0.1 |

and fairly mature seeds of *M. alcea fastigiata* after 15 hr incubation with [$^{14}CH_3$]methionine. In the mature seeds, approximately two-thirds of the label was in dihydrosterculic acid and the other third in sterculic acid, with effectively no conversion to the shorter chain cyclic acids. In the younger seeds, the proportion of label in dihydrosterculic acid was nearly the same, but only half of the remaining radioactivity was incorporated in sterculic acid and the other half was distributed between dihydromalvalic and malvalic acids and also $C_{17}$-cyclopropane acid. In these young *M. alcea fastigiata* seeds, a significant proportion of the radioactivity from methionine was incorporated into dihydromalvalic acid. Labeling of this acid was not generally significant in other seeds or in leaves or callus, even when appreciable labeling of the cyclopropene analogue, malvalic acid was effected. The appearance of labeled methylenehexadecanoic acid (17:CPA) in these young seeds may reflect some bacterial contamination which had escaped sterilization by the chloramphenicol wash or some β-oxidation of dihydrosterculic acid or some further α-oxidation of dihydromalvalic acid or synthesis from hexadecenoic acid (certainly present in young seeds). Insufficient labeled material was obtained for the position of the methylene bridge to be ascertained.

An investigation of the effect of an oxygen atmosphere, relative to air, on the pattern of incorporation of methionine label into cyclic acids in *M. alcea fastigiata* seeds is summarized in Table V. Under oxygen, the proportion of label in dihydrosterculic acid was reduced from that of the normal aerobic control, while incorporation into sterculic and malvalic acids was considerably enhanced. The stimulation by oxygen of incorporation into malvalic acid was particularly significant in that only a trace of label was found in this product from the control aerobic incubation.

Because an α-oxidation step is implicated in the biogenesis of malvalic acid, both from its structure relative to sterculic acid and from the pattern of labeling with acetate (12), and because imidazole is a known specific inhibitor of plant α-oxidation systems (26), a study was made of the incorporation of labeled methionine into the cyclic acids of *M. parviflora* callus tissue grown on media containing several concentrations of added imidazole. The results are summarized in Table VI. The proportion of radioactivity incorporated into malvalic acid (18:CPE) decreased markedly with increasing concentrations of added imidazole while the proportion in sterculic acid (19:CPE) remained fairly constant. The ratios of proportions of radioactivity incorporated into these two acids clearly show the effect of imidazole. This effect was evident also on the macroscale, in the fatty acid composition of the *M. parviflora* callus tissue grown in the absence and in the presence of $10^{-3}$M imidazole in the culture medium. The total cyclic acid content (13.1% and 14.6%, respectively) and the total cyclopropene acid content (9.1% and 10.7%) were fairly similar in these two callus tissues but the ratio of malvalic acid to sterculic acid content was 1.40 in the absence of imidazole but only 0.63 in the presence of $10^{-3}$M imidazole.

TABLE VI

Effect of Imidazole on the Distribution of Radioactivity From L-[$^{14}CH_3$]Methionine Into Cyclopropane and Cyclopropene Fatty Acids in *M. parviflora* Callus Tissue After 3 Weeks Incubation

| Imidazole in medium | Radioactivity, % | | | |
|---|---|---|---|---|
| | 19:CPA | 19:CPE | 18:CPE | 18:CPE/19:CPE Ratio |
| 0 | 23.2 | 40.8 | 36.0 | 0.88 |
| $10^{-4}$M | 26.6 | 42.0 | 31.4 | 0.75 |
| $10^{-3}$M | 46.5 | 34.5 | 19.0 | 0.55 |
| $10^{-2}$M | 48.4 | 44.4 | 7.2 | 0.16 |

TABLE VII

Conversion of [1-$^{14}$C] Oleic Acid Into Linoleic, Linolenic and Dihydrosterculic Acids in Seeds and Leaves of *M. parviflora*

| Incubation time, hr | Seeds | | | Leaves | | | |
|---|---|---|---|---|---|---|---|
| | Radioactivity, % | | | Radioactivity, % | | | |
| | 18:1 | 18:2 | 19:CPA | 18:1 | 18:2 | 18:3 | 19:CPA |
| 3 | 94.0 | 2.5 | 3.5 | 95.0 | 14.0 | 1.0 | --- |
| 8 | 92.5 | 3.0 | 4.5 | 79.0 | 16.0 | 5.0 | Trace |
| 24 | 90.0 | 4.0 | 6.0 | 70.0 | 17.5 | 9.0 | 1.5 |
| 48 | 87.0 | 3.5 | 9.5 | 66.5 | 23.5 | 8.0 | 2.0 |

**Incubations With [1-$^{14}$C] Oleic Acid**

Table VII summarizes the distribution of radioactivity in the fatty acids of seed slices and chopped leaves of *M. parviflora* after incubation for various times with [1-$^{14}$C] oleic acid. (These data are derived from radiochemical-GLC of intact mixed methyl esters; traces of radioactivity associated with decomposition products of methyl sterculate, emerging after the dihydrosterculate peak, were not estimated. No analyses after silver nitrate-hydrogenation treatment were performed to measure reliably any conversion to sterculic acid, but see also below.) In seeds conversion of oleic acid to dihydrosterculic acid was faster than desaturation to linoleic acid and increased steadily to 9.5% of total radioactivity after 48 hr. In leaves this conversion was very much slower and amounted to only 2% of total activity after 48 hr.

Conversion of [1-$^{14}$C] oleic acid to both dihydrosterculic and sterculic acids was investigated in incubations with *M. parviflora* callus tissue, and the results are shown in Table VIII. Oleic acid was converted into dihydrosterculic and linolenic acids at roughly comparable rates, but formation of labeled sterculic acid was only significant during the second half of the incubation time period.

The methyl dihydrosterculate from these incubations was isolated by preparative-GLC, hydrolyzed and subjected to chemical α-oxidation. The esterified product consisted of a homologous series of cyclopropane fatty acid esters of which only the residual dihydrosterculate contained any $^{14}$C-label. The direct conversion of oleic acid to dihydrosterculic acid, and presumably also to sterculic acid in the tissue culture, was thereby established.

**Incubations With Racemic [1-$^{14}$C] Dihydrosterculic Acid**

The conversion of dihydrosterculic to sterculic acid was studied in both leaves and seeds of *M. parviflora*. In view of the effect of oxygen, described above, on incorporation of labeled methionine into cyclopropene acids, duplicate incubations of each tissue were made, one under air enriched to 42% oxygen and the other under nitrogen. In both leaf and seed tissues there was significant conversion to sterculic acid (4.1% and 5.5%, respectively) in the presence of oxygen but less conversion (2.9% and 1.8%, respectively) under nitrogen, particularly in the seeds where the conversion was inhibited by approximately 70%. In the leaves this inhibition by the nitrogen atmosphere was more limited (ca. 30%), presumably because of the production of oxygen by photosynthesis in the tissue. The seeds were also quite green and may have produced enough oxygen by photosynthesis to account for the small degree of desaturation obtained anaerobically. Only residual substrate and the sterculic acid product were radioactive, so that there is no possibility

TABLE VIII

Distribution of Radioactivity Among Fatty Acids of *M. parviflora* Callus Tissue[a]

| Incubation time, weeks | Radioactivity, % | | | | |
|---|---|---|---|---|---|
| | 18:1 | 18:2 | 18:3 | 19:CPA | 19:CPE |
| 1 | 84.0 | 12.5 | 3.0 | 0.5 | Trace |
| 2 | 76.5 | 15.5 | 4.5 | 3.5 | Trace |
| 3 | 72.0 | 17.5 | 4.5 | 5.5 | 0.5 |
| 5 | 59.5 | 23.0 | 10.5 | 6.0 | 1.0 |

[a]Isolated after incubation on a medium containing [1-$^{14}$C] oleic acid.

TABLE IX

Products of Incubation of *M. sylvestris* Callus Tissue for 3 Weeks With [U-$^{14}$C] Palmitic Acid and [U-$^{14}$C] Stearic Acid

| Precursor | Radioactivity, % | | | | | |
|---|---|---|---|---|---|---|
| | 14:0 | 15:0 | 16:0 | 17:0 | 18:0 | 20:0 |
| [U-$^{14}$C] 16:0 | 2.6 | 23.5 | 71.4 | -- | 2.5 | --- |
| [U-$^{14}$C] 18:0 | 1.2 | 1.6 | 3.4 | 8.0 | 81.5 | 4.1 |

that β-oxidation and resynthesis contributed in any way to this conversion. These results establish the direct desaturation of dihydrosterculic to sterculic acid and suggest strongly that, like normal plant desaturations, oxygen is an obligatory cofactor.

### Incubations With [1-$^{14}$C] Stearolic Acid

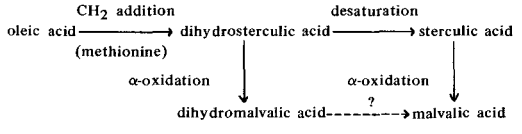
Seeds and callus tissue of *M. parviflora* were each incubated with [1-$^{14}$C]stearolic acid, for 24 hr and 3 weeks respectively, and parallel incubations with [1-$^{14}$C] oleic acid were carried out as controls. In both tissues labeled dihydrosterculic acid was formed in significant amounts from oleic acid (6.3% in seeds and 5.8% in callus), with lesser proportions of sterculic acid (3.8% and 0.9%, respectively) also being formed. Under identical conditions, however, no conversion of stearolic acid to sterculic acid could be detected in either seeds or callus, and the loads applied to the radiochemical-GLC were such that even 0.1% incorporation of label into sterculic acid would have been detected.

### Incubations With [U-$^{14}$C] Palmitic and [U-$^{14}$C] Stearic Acids

Analysis of fatty acids isolated from callus tissue incubated with [U-$^{14}$C] palmitic or [U-$^{14}$C] stearic acid showed that there was substantial degradation of precursor to lower homologues in one carbon steps, as shown in Table IX. There was also a small amount of elongation to stearic and eicosanoic acids, respectively, but as expected little or no sign of any direct desaturation to monoenoic acids was evident.

## DISCUSSION

These labeling studies with the various tissues of *Malva* species indicate the following sequence for the biosynthesis of cyclopropane and cyclopropene acids:




We have confirmed that methionine, presumably as S-adenosyl methionine, is the methylene donor and that in the callus tissue cultures, in the medium used, methionine concentration may be rate limiting. The conversions of oleic acid to dihydrosterculic and sterculic acids, and of dihydrosterculic acid to sterculic acid have been directly demonstrated, confirming the suggestions of Hooper and Law (13) and of Johnson and coworkers (15). The pathway of cyclopropene acid formation by methylene addition to an acetylenic intermediate, suggested by Smith and Bu'Lock (12,14), could not be demonstrated by similar direct experimentation and is therefore unlikely.

The bacterial cyclopropane synthetase has as its substrate unsaturated acyl phospholipid, in general phosphatidyl ethanolamine (3,6-8). The present studies do not provide any information as to the nature of the enzyme substrate for cyclopropane or cyclopropene fatty acid biosynthesis, or both, in plant tissues, i.e., whether it is acyl-S-CoA, acyl-S-ACP, phospholipid or even free fatty acid.

The α-oxidation pathways in seed and leaf tissues of other plants (26,27) appear to require no activation of fatty acids and in the various tissues of these Malvaceae, free fatty acids are the likely substrates for the α-oxidation steps.

The precise point(s) in the biosynthetic sequence at which the α-oxidation step operates has not been directly established. The time studies of incorporation of acetate and of methionine suggests that α-oxidation occurs after ring formation. This suggestion is supported by the finding that neither in terms of mass nor of labeling from acetate was there any significant accumulation of odd number homologues of the normal straight chain acids. In seeds (see Table IV) the α-oxidation system is quite active at early stages of maturity but this activity is lost, relative to dihydrosterculic and sterculic acid biosynthesis in more mature seeds.

The finding of an active α-oxidation system in the callus tissues, which can operate not only on the biosynthesized cyclic acids but also on

exogenous normal fatty acids, is of considerable interest. Indeed from our studies it would seem to be the major or even the only pathway for oxidative degradation of fatty acids in these callus tissues. Thus in no case of incubation of the callus tissue cultures with 1-$^{14}$C-labeled long chain substrates was there any label incorporated into, e.g., palmitic acid, to indicate any β-oxidation followed by resynthesis. Similarly the degradation of U-$^{14}$C-labeled palmitic and stearic acids provided shorter chain metabolites in steadily decreasing proportions, with no preference for even chain metabolites to indicate a contribution from β-oxidation, and no label appeared in, e.g., oleic and linoleic acids, which again would be the case if there were acetyl-CoA being formed via β-oxidation.

Although exogenous cyclopropene compounds markedly inhibit desaturations of long chain fatty acids in animal systems (16,17) and in algal and higher plant systems (18,19), no such inhibitory effect is apparent in the various tissues of these Malvaceae species where the cyclopropene acids and the normal unsaturated acids co-exist. Thus, for example, a concentration of 3 mM sterculic acid or 1,2-dihydroxysterculene was sufficient to inhibit the desaturation of added stearic acid totally and of added oleic acid by 75% in *Chlorella vulgaris* cells and linoleic acid formation was similarly inhibited in leaf tissue preparations (18). Yet the concentration of cyclopropene compounds in the tissues, and particularly the seeds, of these *Malva* species must be substantially greater than 3 mM. An explanation based simply on the compartmentalization of the normal desaturase enzymes and the cyclopropene-containing lipids seems unlikely because appreciable proportions of cyclopropene acids are esterified to the membrane phospholipids and glycolipids (20) and therefore presumably in close association with the desaturases. A more likely explanation is that advanced previously (18,19): namely that the desaturase enzymes per se are not inhibited by cyclopropene compounds but that the inhibition is directed towards an acyl transferase enzyme or enzymes concerned in the activation of exogenous long chain fatty acid substrates or their transfer to the desaturase. Clearly the desaturase enzymes in these *Malva* tissues are not inhibited (Fig. 1, Tables VII and VIII) but clearly also exogenous oleic acid (Tables VII and VIII) and dihydrosteaculic acid are handled by the appropriate enzyme systems—even in the presence of endogenous cyclopropene compounds—effectively enough to permit substantial desaturation to linoleic and linolenic acids and to sterculic acid, respectively. Presumably therefore both explanations must apply in part, and sterculic and malvalic acids acylated to lipids, whether in the triglyceride store or in the ordered array of membrane polar lipids, are not available to effect inhibition. More work will be required to fully define the situation.

Finally if the enzyme which desaturates dihydrosterculic acid in these tissues has a stereospecificity similar to all the fatty acid desaturases so far examined (28,29, and L.J. Morris, R. Bickerstaffe, D. Brett and W. Kelly, unpublished)—namely that only the hydrogen atoms of the D-absolute configuration are removed on desaturation—then one can predict that the absolute configuration of the methylene group of dihydrosterculic acid and dihydromalvalic acid in these plant systems is L.

ACKNOWLEDGMENTS

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# Accumulation of Cardiac Fatty Acids in Rats Fed Synthesized Oils Containing $C_{22}$ Fatty Acids

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## ABSTRACT

Synthesized oils containing a high proportion of oleic, eicosenoic or docosenoic acid were fed to weanling rats as 20% w/w of the diet. After 1 week, a high intake of eicosenoate produced cardiac fat droplets detected histologically, whereas erucate (22:1 Δ 13) or cetoleate (22:1 Δ 11) caused an appreciably greater accumulation of cardiac lipid characterized by the dietary fatty acids.

## INTRODUCTION

It is known that cardiac fat accumulates within a few days in the young rat fed rapeseed oil (1-3). The lipid deposited in the heart within a week of experimental feeding was found to contain high levels of dietary monoenoic acids including erucic acid (2). To determine the relative effects of oleic acid (18:1 Δ 9), eicosenoic acid (20:1 Δ 11) and erucic acid (22:1 Δ 13) with low and moderately high level of saturated fatty acids, and of erucic acid vs. cetoleic acid (22:1 Δ 11), random triglycerides of tailored fatty acid composition were fed to rats.

## MATERIALS AND METHODS

Weanling male rats, specific pathogen-free, were obtained from Charles River Breeding Laboratories, Mass., and were immediately fed a test diet for 1 week. The diet contained w/w 20% casein, 30% cornstarch, 20% sucrose, 1% vitamin mixture (4), 4% U.S.P. XIV salt mixture, 5% alphacel and 20% test oil.

The triglycerides were prepared as follows: rapeseed, sunflower, soybean, olive and herring oils were converted to fatty acid ethyl esters and fractionally distilled under reduced pressure from a Podbeilniak Heli-Grid column. The fatty acid composition of fractions was determined by gas liquid chromatography (GLC). Ethyl palmitate and stearate were prepared as a source of saturated fatty acids and mixed with selected fractions of distilled ethyl esters to achieve specified fatty acid compositions. The mixed ethyl esters were filtered in a sintered glass funnel containing successive layers of Celite 545, activated charcoal and activated alumina to remove unknown impurities that interfered with the quantitative conversion to triglycerides. Ethyl esters were converted to triglycerides by interesterification with freshly distilled triacetin in the presence of sodium ethoxide as catalyst. The reaction mixture was dissolved in petroleum ether, bp 60-68 C; excess acetic acid was added to neutralize the catalyst; the mixture was washed five times with water, dried over anhydrous sodium sulfate, filtered, decolorized with activated charcoal and the solvent removed under reduced pressure. The resultant oil was steam-vacuum deodorized in an all glass apparatus, and analyzed by GLC for unreacted ethyl ester. The conversion to triglycerides was 97-99%.

The fatty acid composition of each oil is shown in Table I. Of the first six dietary oils, 1, 2 and 3 contained equivalent quantities of saturated fatty acids and 18:2 but varied in the monoenoic fatty acid, as did oils 4, 5 and 6 which contained a higher level of saturated fatty acids. Oils 1 and 4 had a high proportion of oleic acid, oils 2 and 5 of eicosenoic acid and oils 3 and 6 of erucic acid. Oils 7 and 8 contained similar levels of erucic acid but differed in the levels of oleic and saturated fatty acids. The difference between oils 9 and 10 was the position of the double bond in the docosenoic acid.

Each oil was fed to 15 rats, 5 of which were randomly selected for chemical analysis of the heart and 10 for histological examination. Oils 1-6 were tested using a randomized block design of individual cages, then oils 7 and 8 were tested simultaneously, and then oils 9 and 10. On the seventh day, the rats were fasted for at least 5 hr, anesthetized with ether and bled from the abdominal aorta. Hearts and livers were stored in saline at -20 C until the lipids were extracted or in buffered 10% formalin until sections were prepared for staining with Oil Red O or hematoxylin-phloxine-saffron.

The cardiac fatty acids were extracted by the procedure of Bligh and Dyer (5) as modified by Hanson and Olley (6) and Beare-Rogers et al. (2). A sample of methyl esters containing a known proportion of total cardiac lipids and 0.5 μmole of methyl lignocerate was gas-chromatographed in a Hewlett Packard instru-

ment, model 7620A, equipped with hydrogen-flame detectors and 6 ft 1/8 in. OD stainless steel columns packed with 10% butanediol succinate on Anachrom ABS (80/90 mesh). The inlet, column and detector temperatures were 250 C, 190 C and 250 C, respectively. Peak areas of the chromatograms were obtained from a 3370A Hewlett Packard integrator.

## RESULTS

As shown in Table II, weight gain was related to food intake, and in the groups 1 to 6, was lowest with diet 3 which contained the high level of erucic acid and the low level of saturated fatty acids. The greatest deposition of cardiac fatty acids occurred with the highest intake of erucic acid, from diets 3 and 6, but hepatic fatty acids exhibited no such alteration. Varying the level of saturated acids with approximately 32% erucic acid as in diets 7 or 8, or changing the position of the double bond by two carbon atoms as in diets 9 and 10, had no effect on food intake and weight gain in the comparisons made.

The amount of erucic acid found in the hepatic tissue was low (Table III). Eicosenoic acid, on the other hand, was elevated in the liver of rats fed a high level of that fatty acid.

The gross appearance of randomly selected whole hearts from groups 1-6, as shown in Figure 1, demonstrated the fatty heart obtained with the high level of erucic acid and the low level of saturated fatty acids.

In the heart, eicosenoic acid from diets 2 and 5 significantly increased but not to the extent that erucic acid from diets 3 and 6 accumulated (Table IV). The striking difference between the fatty acids of the liver and those of the heart in rats fed high levels of erucic acid was the large amount of that long chain monoenoic acid in the cardiac tissue. As shown in Figure 2, the increases in the total fatty acids and in the erucic acid of the heart were closely related. The cardiac palmitic acid and $C_{18}$ acids including linoleic acid also increased when erucic acid did, but arachidonic acid remained unchanged (Table IV). The level of saturated fatty acids used in diets 7 and 8 did not significantly alter the extent of deposition of erucic acid. Nor was there a significant difference detected between the effect of erucic and cetoleic acids in diets 9 and 10.

The histopathological results are shown in Figure 3. Compared to oleic acid, a high level of dietary eicosenoic acid, particularly with the low level of saturated fatty acids (diet 2), increased the prevalence of myocardial fat droplets. Considerably more cardiac lipid was

TABLE I

Fatty Acid Composition of Synthesized Oils[a] in Each Diet

| Diet | Fatty acids, % | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 20:2 | 22:1Δ13 | 22:1Δ11 |
| 1 | 2.8 | --- | 1.6 | <u>71.9</u> | 23.2 | 0.6 | --- | --- | --- | --- | --- |
| 2 | 2.8 | --- | 1.0 | 4.9 | 18.2 | --- | 4.1 | <u>65.8</u> | 0.8 | 2.4 | --- |
| 3 | 2.8 | --- | 1.1 | 4.7 | 17.2 | --- | --- | 2.5 | --- | <u>72.6</u> | --- |
| 4 | 18.8 | --- | 4.9 | <u>54.8</u> | 21.5 | --- | --- | --- | --- | --- | --- |
| 5 | 16.4 | 0.1 | 5.6 | 4.7 | 19.7 | --- | 2.2 | <u>49.5</u> | --- | 0.9 | --- |
| 6 | 16.5 | --- | 4.9 | 5.0 | 20.9 | --- | --- | 1.8 | --- | <u>50.8</u> | --- |
| 7 | 3.5 | 0.2 | 0.9 | 44.4 | 18.7 | 0.6 | --- | 0.1 | --- | <u>31.6</u> | --- |
| 8 | 19.1 | 0.1 | 4.9 | 24.9 | 18.6 | --- | --- | 0.3 | --- | <u>32.2</u> | --- |
| 9 | 2.6 | --- | 1.2 | 37.7 | 21.3 | 6.8 | --- | --- | --- | <u>30.4</u> | --- |
| 10 | 3.0 | --- | 1.7 | 35.8 | 21.3 | 7.3 | --- | --- | --- | --- | <u>30.7</u> |

[a]The principal fatty acid of interest in each synthesized oil is underlined.

TABLE II

Food Intake, Weight Gain, Heart and Liver Weights, and Fatty Acid Content

| Diet | Food intake, g/week | Weight gain, g/week | Heart wt., mg | Liver wt., g | Total fatty acids, mg/g | |
|---|---|---|---|---|---|---|
| | | | | | Heart | Liver |
| 1 | 55 ± 3 (15)[a] | 33 ± 2 (15) | 425 ± 27 (5) | 4.36 ± 0.34 (5) | 15.5 ± 1.1 | 33.2 ± 7.3 |
| 2 | 58 ± 3 | 38 ± 3 | 449 ± 16 | 4.49 ± 0.40 | 23.5 ± 3.0 | 37.1 ± 4.4 |
| 3 | 49 ± 2 | 24 ± 2 | 466 ± 33 | 4.33 ± 0.30 | 68.9 ± 9.8 | 35.0 ± 2.9 |
| 4 | 61 ± 2 | 37 ± 2 | 460 ± 25 | 4.05 ± 0.20 | 14.9 ± 0.3 | 29.9 ± 2.6 |
| 5 | 56 ± 3 | 33 ± 3 | 400 ± 32 | 3.85 ± 0.33 | 18.2 ± 0.2 | 31.4 ± 3.0 |
| 6 | 55± 2 | 32 ± 2 | 433 ± 55 | 4.27 ± 0.45 | 46.5 ± 10.4 | 36.9 ± 1.6 |
| 7 | 57 ± 3 | 31 ± 3 | 465 ± 16 | --- | 42.7 ± 7.0 | --- |
| 8 | 61 ± 2 | 34 ± 2 | 459 ± 19 | --- | 40.7 ± 9.0 | --- |
| 9 | 43 ± 2 | 24 ± 2 | 361 ± 38 | --- | 39.0 ± 3.9 | --- |
| 10 | 39 ± 1 | 20 ± 1 | 321 ± 8 | --- | 40.8 ± 2.8 | --- |

[a]Mean ± standard error of the mean. Number of rats in parentheses.

TABLE III

Principal Fatty Acids of Liver Lipids

| Diet | Fatty acids, mg/g | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 | 20:1 | 20:4 | 22:1 | 22:6 |
| 1 | 5.2 ± 1.1[a] | 5.6 ± 1.2 | 9.5 ± 2.3 | 2.7 ± 0.9 | 0.1 ± 0.1 | 7.1 ± 1.4 | --- | 1.2 ± 0.3 |
| 2 | 4.5 ± 0.6 | 3.7 ± 0.4 | 10.0 ± 1.5 | 4.3 ± 0.6 | 5.6 ± 0.9 | 5.5 ± 0.5 | 0.1 ± 0.1 | 1.2 ± 0.2 |
| 3 | 5.2 ± 1.1 | 3.5 ± 0.2 | 10.8 ± 1.1 | 4.5 ± 0.7 | 1.1 ± 0.2 | 4.9 ± 0.2 | 2.3 ± 0.7 | 1.0 ± 0.2 |
| 4 | 6.2 ± 0.7 | 4.8 ± 0.3 | 7.9 ± 0.8 | 3.9 ± 0.4 | 0.1 ± 0.1 | 5.3 ± 0.3 | ---- | 1.0 ± 0.1 |
| 5 | 5.5 ± 0.5 | 4.4 ± 0.4 | 6.3 ± 0.9 | 4.2 ± 0.5 | 3.3 ± 0.3 | 5.1 ± 0.5 | --- | 1.1 ± 0.1 |
| 6 | 7.1 ± 0.6 | 4.5 ± 0.2 | 10.2 ± 0.5 | 4.7 ± 0.3 | 1.0 ± 0.1 | 5.1 ± 0.3 | 1.7 ± 0.3 | 0.9 ± 0.1 |

[a]Mean ± standard error of the mean.

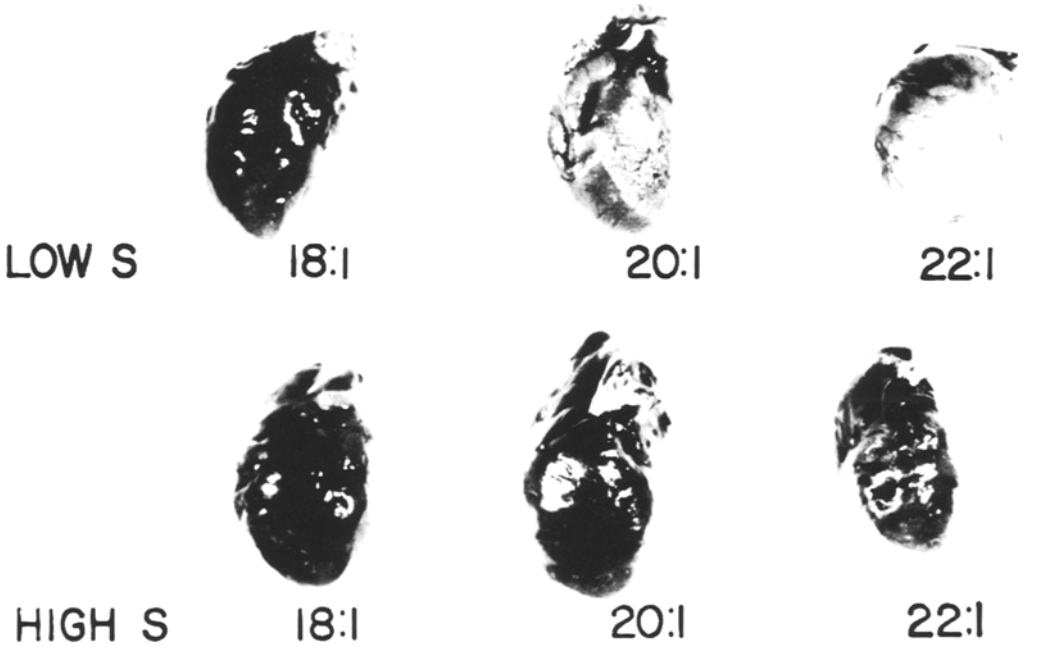


FIG. 1. Photograph of a randomly selected heart from each experimental diet, 1-6.

deposited with the high levels of erucic acid from diets 3 and 6. The combination of a high level of erucic acid and a low level of saturated acids (diet 3) was associated with myocytolysis and necrosis. No cytolytic effects were observed with diets 1, 6, 7, 8 or 10. There appeared to be a small histological difference between the effects obtained with diets 9 and 10, in that 2 of the 10 rats examined from those fed erucic acid showed a high degree of fat deposition and of myoctolysis.

## DISCUSSION

The dietary docosenoic acids, erucic and cetoleic, were associated with a pronounced accumulation of cardiac lipid after 1 week of experimental feeding. That the heart can readily incorporate long chain fatty acids from the diet was demonstrated with cod liver oil by Rieckehoff et al. (7). More recently the occurrence of fatty hearts in rats fed rapeseed oil containing erucic acid has been documented (1-3). Also the increased content of erucic acid in the heart decreased the rate of ATP synthesis in the mitochondria (8).

An increased fat deposition from a high intake of eicosenoic acid was detected histologically. Ventricular sections showed the positive histopathology whereas the entire heart was extracted for fatty acid analysis. From the total fatty acid components no distinction was apparent in the degree of fat deposition produced by erucic acid or cetoleic acid, but from the histological assessment there appeared to be somewhat lesser lesions with the Δ 11 isomer. The top histopathological scores were obtained with the two highest levels of erucic acid, but the total amount of fatty acids varied with the dietary intake of this fatty acid.

There has been speculation that erucic acid alters the metabolism of the essential polyunsaturated acids. On the basis of relative percentages of total fatty acids, when erucic acid increased, linoleic acid was unchanged and arachidonic acid decreased (3,9,10). According to the actual amounts deposited, linoleic acid increased and arachidonic acid was unchanged. It appears that in the heart all dietary acids present with the erucic acid accumulated and that there was no interference with the synthesis of arachidonic acid.

The effect of saturated fatty acids in modifying the action of erucic acid has been observed (9,11) but as found here, it cannot always be reproduced. There was a histological indication that the low level of saturated acids

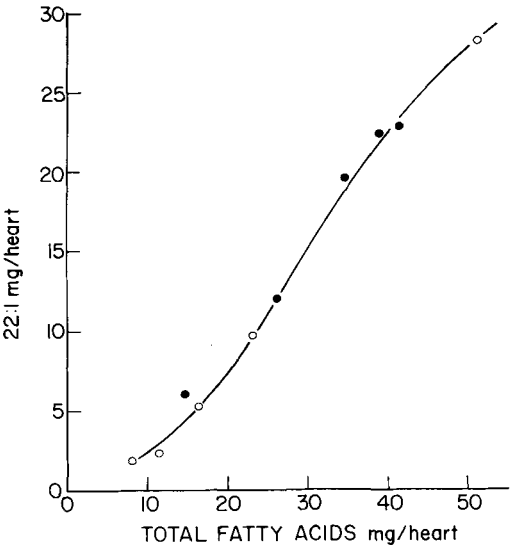


FIG. 2. Relationship between total fatty acids and erucic acid in the heart of rats fed diet 3 (•) or diet 6 (○).

TABLE IV

Principal Fatty Acids of Cardiac Lipids

| | Fatty acids, mg/g | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Diet | 16:0 | 18:0 | 18:1 | 18:2 | 20:1 | 20:4 | 22:1 | 22:6 |
| 1 | 1.7 ± 0.1[a] | 3.3 ± 0.2 | 3.2 ± 0.2 | 2.5 ± 0.2 | 0.1 ± 0.1 | 3.2 ± 0.2 | — | 0.9 ± 0.1 |
| 2 | 1.7 ± 0.2 | 2.5 ± 0.1 | 2.2 ± 0.3 | 3.3 ± 0.5 | 7.9 ± 2.0 | 3.7 ± 0.4 | 0.4 ± 0.1 | 0.5 ± 0.2 |
| 3 | 4.0 ± 0.4 | 3.7 ± 0.1 | 8.1 ± 1.1 | 8.2 ± 0.9 | 1.6 ± 0.2 | 3.7 ± 0.1 | 36.2 ± 6.9 | 1.4 ± 0.3 |
| 4 | 2.1 ± 0.1 | 3.4 ± 0.1 | 2.2 ± 0.2 | 2.7 ± 0.2 | — | 3.2 ± 0.2 | — | 0.9 ± 0.1 |
| 5 | 2.3 ± 0.1 | 3.2 ± 0.1 | 1.4 ± 0.1 | 3.2 ± 0.1 | 2.8 ± 0.2 | 3.5 ± 0.2 | 0.2 ± 0.1 | 1.0 ± 0.2 |
| 6 | 5.3 ± 0.7 | 5.1 ± 0.5 | 5.1 ± 0.9 | 6.4 ± 0.8 | 1.3 ± 0.4 | 3.6 ± 0.2 | 17.5 ± 6.8 | 1.1 ± 0.1 |
| 7 | 2.8 ± 0.2 | 2.9 ± 0.2 | 14.7 ± 3.1 | 5.2 ± 0.7 | 0.4 ± 0.1 | 3.4 ± 0.2 | 12.4 ± 2.7 | 0.6 ± 0.1 |
| 8 | 5.4 ± 1.2 | 4.7 ± 0.5 | 8.7 ± 2.5 | 6.0 ± 0.9 | 0.5 ± 0.2 | 3.4 ± 0.2 | 10.6 ± 3.6 | 0.9 ± 0.1 |
| 9 | 2.6 ± 0.3 | 3.8 ± 0.2 | 10.6 ± 1.7 | 6.1 ± 0.5 | 0.5 ± 0.7 | 3.4 ± 0.1 | 9.8 ± 1.2 | 1.1 ± 0.1 |
| 10 | 2.8 ± 0.2 | 4.7 ± 0.1 | 11.0 ± 1.1 | 6.4 ± 0.4 | 1.0 ± 0.1 | 3.6 ± 0.1 | 9.1 ± 1.0 | 1.0 ± 0.1 |

[a]Mean ± standard error of the mean.

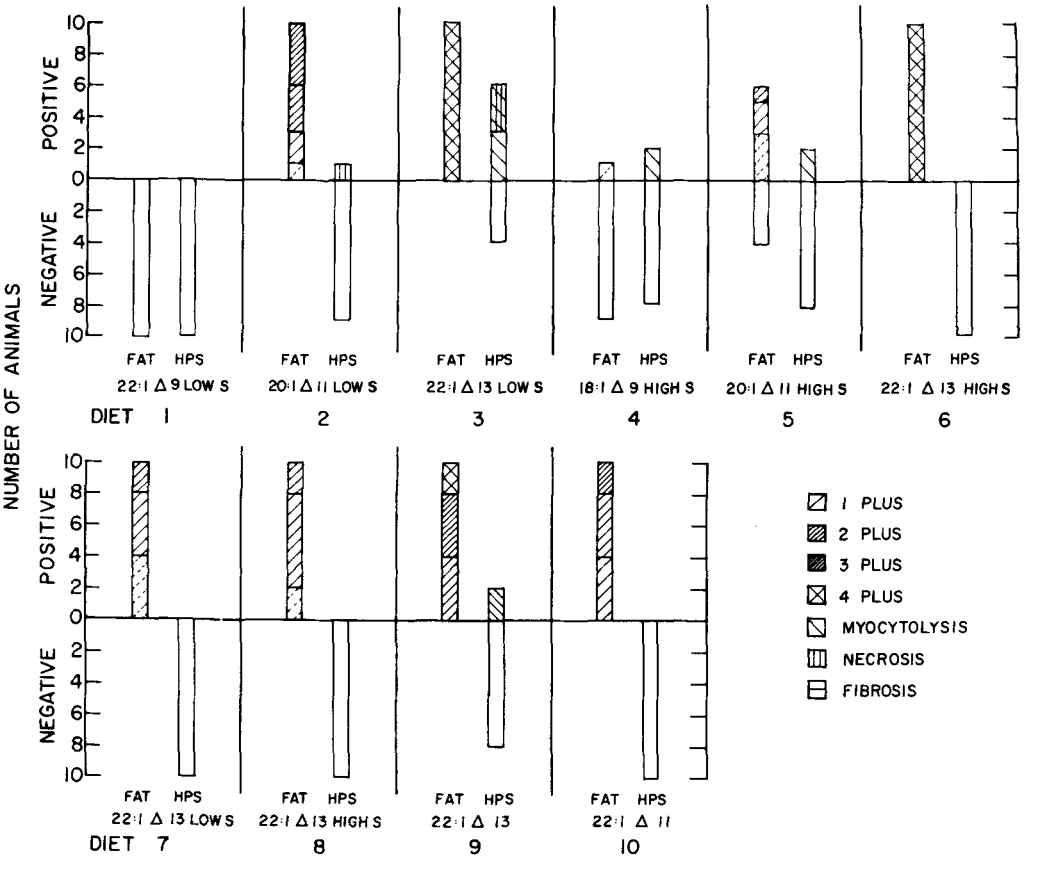


FIG. 3. Histological assessment of hearts on the basis of fat by the Oil Red O stain and by hematoxylin-phloxine-saffron stain (HPS).

aggrevated the fat deposition with high levels of eicosenoic acid and the development of necrosis with high levels of erucic acid. The effect of the synthesized oils containing about 30% erucic acid, similar to that of the rapeseed oil previously fed (2), were not significantly modified by changing the level of saturated fatty acids.

The most important factor in producing the accumulation of cardiac lipids was the dietary intake of a docosenoic acid.

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# Metabolism of Odd-Numbered Fatty Acids in *Ochromonas danica*

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**ABSTRACT**

The lipids of the protozoon, *Ochromonas danica*, contain very small amounts of odd-numbered fatty acids. Nevertheless the organism incorporates into its lipids odd- as well as even-numbered acids from the media. Unsaturated odd acids such as 9-17:1 or 6,9,12-17:3 were efficient substrates for elongation and desaturation in the proximal and the former acid also in the distal part of the chain. In contrast none of these conversions was observed with 7-17:1, 10-17:1 and 10,13-19:2 acids, although these substrates were taken up by the organism. Elongation and desaturation of the proximal part of already unsaturated fatty acid chains appear to be subject to the same structural requirements in *O. danica* as in the rat, and the same effects apply to desaturation in the distal part of the chain.

Dienoic and trienoic odd-numbered fatty acids are efficiently converted in the rat to more highly unsaturated acids, provided their double bonds are, in reference to the carboxyl group, in positions as they are in the common next higher homologous even-numbered acid (1,2). For example 9,12-17:2 and 6,9,12-17:3 acids were converted to 5,8,11,14-19:4 by essential fatty acid (EFA) deficient rats to the same extent as was 9,12-18:2 to 5,8,11,14-20:4. In contrast to this, 10,13-19:2 yielded such small amounts of higher conversion products that they were detected and identified only after prolonged feeding (2,3). It was the purpose of the work reported here to extend the comparison of odd- and even-numbered fatty acids to organisms which are capable of desturations between the methyl group and the double bond closest to it (distal part of the chain), in addition to elongation and desaturations in the proximal part which are typical for vertebrates.

The photosynthetic protozoon *Ochromonas danica* was selected for this study since it synthesizes in lipid-free medium the common saturated and unsaturated fatty acids, including linoleic, α- and γ-linolenic, arachidonic, 8,11,14,17-20:4 and 4,7,10,13,16-22:5 (4,5). Moreover the organism contains only a very small amount of odd-numbered straight chain acids in its lipids, and branched acids are not detectable at all. *O. danica* absorbed the common fatty acids from the media, and converted them to longer chain and more highly unsaturated acids. Odd-numbered fatty acids, including unsaturated ones having appropriate structures, were also utilized, and yielded appreciable amounts of odd chain products corresponding in structure to the even ones. However the odd chain acids 7-17:1, 10-17:1 and 10, 13-19:2 did not yield conversion products in significant amounts, although they were taken up by the organism without adverse effects on growth. It appears that the double bond position exerts the same influence on desaturation in the distal part of the chain as it does on the conversions of the proximal part.

## EXPERIMENTAL PROCEDURES

### Fatty Acids

The acids 15:0, 17:0, 10-17:1 and 10,13-19:2 were obtained by Arndt-Eistert synthesis from the lower homologs (6,7); 7-17:1 was synthesized from 7-iodoheptanoate and decanal by the Wittig reaction (8). The acids 9-17:1 and 6,9,12-17:3 were isolated from mullet oil (3,9). Fatty acids uniformly labeled with $^{14}C$ were available from earlier experiments with *O. danica* (5).

The samples were obtained as methyl esters and their identity and purity were checked by gas liquid chromatography (GLC), thin layer chromatography (GLC), ozonization and IR spectroscopy. They contained less than 2% contaminants of other chain length or double bond position, and less than 5% *trans* double bonds.

### Incubations

*Ochromonas danica* was grown in 1 liter or in 100 ml of media, as previously described (5,10), at 26 C and constant illumination of 150 ft candles. The fatty acids were added, either dissolved in 80% ethanol or as aqueous sodium or potassium soaps. Complexing of fatty acids with bovine plasma albumin did not prove advantageous. Radioactive acids ($5 \times 10^6$ dpm) were used at concentrations of approximately 0.025 mmolarity in the media. They were added in appropriate amounts on the fourth day after inoculation of the media and

TABLE I

Even- and Odd-Numbered Fatty Acids of *Ochromonas danica*

| Even acids | Per cent | Odd acids | Per cent |
|---|---|---|---|
| 14:0 | 5.8 | | |
| 16:0 | 2.2 | 15:0 | 0.08 |
| 9-16:1 | 0.3 | 15:2 | 0.01 |
| 16:2 | 0.3 | 15:2 | 0.01 |
| 16:3 | 0.1 | 15:3 | 0.01 |
| 16:4 | 0.1 | | |
| 18:0 | 2.1 | 17:0 | 0.16 |
| 9-18:1 | 3.2 | 17:1 | 0.04 |
| 6,9-, 9,12-18:2 | 10.4 (1:30)[a] | 17:2 | 0.03 |
| 6,9,12-, 9,12,15-18:3 | 5.7 (1:4)[a] | 17:3 | 0.01 |
| 6,9,12,15-18:4 | 3.2 | 17:4 | 0.01 |
| 20:0 | 1.2 | 19:0 | 0.02 |
| 20:1 | 0.2 | 19:1 | 0.03 |
| 8,11-, 11,14-20:2 | 2.2 (1:1)[a] | 19:2 | 0.06 |
| 8,11,14-20:3 | 8.5 | 19:3 | 0.15 |
| 5,8,11,14-, 8,11,14,17-20:4 | 22.7 (20:1)[a] | 19:4 | 0.07 |
| 22:0 | 3.7 | | |
| 22:1 | 0.2 | | |
| 22:3 | 0.1 | 21:3 | 0.02 |
| 7,10,13,16-22:4 | 4.4 | 21:4 | 0.01 |
| 4,7,10,13,16-22:5 | 13.5 | | |

[a]Ratio of isomers.

incubated for three days. Nonradioactive acids were used at 1 mMolar concentration. They were added to the media at the time of inoculation and incubated for 17 days, by which time the logarithmic phase of growth has ended. When applying radioactive together with nonradioactive acids, the respective concentrations were kept the same as in the experiments with single substrates. The mixtures were incubated as outlined for nonradioactive acids.

Cells were harvested by centrifugation. They were treated with HCl and then KOH for saponification (5), and the unsaponifiable lipids were extracted. The fatty acids were esterified with diazomethane for further analyses.

TABLE II

Conversion of Saturated Fatty Acids Under Different Conditions

| Millimolarity of substrate fatty acids | Conversion[a] after 3 Days | Conversion[a] after 17 Days |
|---|---|---|
| 0.025 [U-$^{14}$C] 14:0 | 14 | |
| 1.0 15:0 | | 17[b] |
| 0.025 [U-$^{14}$C] 16:0 | 26 | |
| 0.025 [U-$^{14}$C] 18:0 | 15 | 34 |
| 0.025 [U-$^{14}$C] 18:0 (in presence of 1.0 18:0) | | 6 |
| 0.025 [U-$^{14}$C] 18:0 (in presence of 1.0 17:0) | | 13 |
| 0.025 [U-$^{14}$C] 18:0 (in presence of 1.0 9,12-18:2) | | 25 |

[a]Per cent of $^{14}$C found in acids other than the substrate acid except for 15:0.

[b]Per cent of total odd acids other than the substrate odd acid.

### Analyses

Samples containing radioactive esters were analyzed by GLC at 220 C on the polar phase, cycloheptaamylose acetate (11). Fractions were collected (5) and their activity determined by liquid scintillation counting. Nonradioactive samples up to 50 mg were fractionated according to chain length by GLC at 233 C on the low polarity phase, cycloheptaamylose valerate (2). Fractions containing esters of equal chain length were collected and further analyzed by GLC at 180 C on the polar phase, ethylene glycol succinate. Quantifications were made by the triangulation method from the polar phase chromatograms and from the relative amounts of each chain length as determined by GLC of the hydrogenated total esters. Hydrogenations were carried out in methanol over $PtO_2$ with $H_2$ at 6 atm pressure.

Larger amounts of esters were fractionated first by liquid-liquid chromatography on silicone oil with aqueous acetonitrile as solvent (5) and the superimposing components separated by preparative GLC.

Fatty esters were identified by their equivalent chain length (ECL) in GLC, by comparing them with authentic esters (2,3,9). In many cases structures were determined and confirmed by isolation, ozonization-reduction and GLC of the resulting aldehydic compounds.

The distribution of $^{14}$C in arachidonic acid was determined by ozonization of the methyl

TABLE III

Conversion Products of Even-Numbered Fatty Acids[a]

| Fatty acids recovered | U-$^{14}$C Fatty acid fed | | |
|---|---|---|---|
| | 14:0 | 16:0 | 9,12-18:2 |
| 14:0 | (85.8) | 0.5 | 0.2 |
| 16:0 | 3.1 | (73.7) | |
| 16:1 | 0.1 | 1.3 | |
| 18:0 | 1.0 | 3.9 | |
| 9-18:1 | 2.7 | 6.9 | |
| 6,9-,9,12-18:2 | 3.2 | 6.3 | (81.0) |
| 6,9,12-18:3 | 0.7 | 1.2 | 2.8 |
| 9,12,15-18:3 | 0.7 | 1.6 | 3.9 |
| 6,9,12,15-18:4 | 0.2 | 0.4 | 0.8 |
| 8,11-, 11,14-20:2 | 0.1 | 0.2 | 0.7 |
| 8,11,14-20:3 | 0.5 | 1.0 | 3.2 |
| 5,8,11,14-20:4 | 1.1 | 1.9 | 4.9 |
| 8,11,14,17-20:4 | 0.1 | < 0.1 | 0.3 |
| 22:3 | < 0.1 | 0.1 | 0.1 |
| 7,10,13,16-22:4 | 0.1 | 0.2 | 0.3 |
| 4,7,10,13,16-22:5 | 0.3 | 0.4 | 1.0 |

[a]Per cent of total radioactivity in fatty acids recovered.

ester and oxidation of the ozonides with $H_2O_2$ (12,13). The resulting acids were esterified with diazomethane and dimethyl glutarate; malonate and methyl caproate were separated by GLC, collected, weighed and counted (12).

## RESULTS

Table I gives the composition of even and odd acids characteristic of *O. danica*. Under normal conditions the level of total odd acids was below 1%, so that none of them was available in amounts that would allow determination of their double bond structure by chemical degradation. However in the two step separation procedure, the chain lengths are known from the first chromatogram so that interpretations of ECL from the second chromatogram are more certain. Positional isomerism of double bonds in polyunsaturated fatty acids markedly affects ECL. The data indicate that the positions of double bonds in the odd-numbered polyunsaturated acids are the same as in the homologous even-numbered acids of equal unsaturation. The acids are listed in Table I.

It was necessary to incubate nonradioactive odd-numbered acids at high concentration for 17 days in order to obtain enough material for chemical identification of conversion products. Labeled even-numbered acids, however, were incubated at low concentration for only three days to minimize distribution of $^{14}$C to acids other than those produced by direct conversions. The effect of these different conditions was evaluated by numerous incubations from which Table II lists some representative examples. Conversions were obvious in all cases, but the data from stearic acid indicated that conversion may be impeded by high concentration of certain fatty acids.

Details on conversions of uniformly labeled 14:0, 16:0 and 18:2 acids are given in Table III. Between 50-70% of the radioactivity offered was recovered as fatty acids from the lipids of the organism. The radioactivities in anabolic products from 16:0 and 18:2 are higher than in acids shorter or more saturated than the added precursors and this indicates direct conversion rather than partial degradation or de novo synthesis via radioactive acetate. In further investigation of this point, arachidonic acid, that had been isolated from *O. danica* incubated with U-$^{14}$C labeled myristic acid, was chemically degraded to locate $^{14}$C in the chain. Average radioactivities per meq C in the fragments were as follows: glutarate (C1-5), 1080 dpm; malonate (C6-14), 4440 dpm; caproate (C15-20), 3980 dpm. Carbons 1-6 were derived from the acetate pool, and therefore approximately 10% of the radioactivity in arachidonic acid has entered the molecule this way. The labeled myristic acid had been incubated for three days and it appeared advisable to limit all radioactive incubations to such period.

Conversion products of odd-numbered acids are listed in Table IV in percentages of the total fatty acids to enable comparison with the control data. In all cases the precursor acid is the major odd chain component, but their conversion products amount to 6-18% of the total. These values are significantly higher than in the blank culture.

In contrast to the conversions shown in

TABLE IV

Conversion Products of Odd-Numbered Fatty Acids[a]

| Fatty acids recovered | Fatty acid fed | | | |
|---|---|---|---|---|
| | None | 15:0 | 9-17:1 | 6,9,12-17:3 |
| 15:0 | 0.08 | (30.10) | 0.06 | 0.18 |
| 15:1 | 0.07 | | 0.16 | 0.04 |
| 15:2 | 0.01 | | 0.06 | 0.04 |
| 15:3 | 0.01 | | | 0.04 |
| 17:0 | 0.16 | 3.24 | + | 0.10 |
| 9-17:1 | 0.04 | 1.74 | (22.91) | 0.10 |
| 6,9-17:2 | 0.03 | 0.10 | 1.38 | |
| 9,12-17:2 | | 0.86 | 2.76 | |
| 6,9,12-17:3 | 0.01 | 0.04 | 0.01 | (19.90) |
| 9,12,15-17:3 | | 0.06 | | |
| 19:0 | 0.02 | 0.11 | 0.01 | |
| 11-19:1 | 0.03 | 0.06 | 1.63 | |
| 8,11-19:2 | 0.06 | 0.14 | 0.39 | |
| 11,14-19:2 | | | 0.31 | |
| 8,11,14-19:3 | 0.15 | 0.14 | 0.44 | 12.67 |
| 5,8,11,14-19:4 | 0.07 | 0.12 | 0.92 | 4.22 |
| 21.3 | 0.02 | + | + | 0.29 |
| 21:4 | 0.01 | + | + | 0.26 |
| 21:5 | | | | 0.05 |

[a]Per cent of total fatty acids recovered.

Table IV, no significant elongations or desaturations of any kind were evident from 7-17:1, 10-17:1 and 10,13-19:2. GLC of hydrogenated samples showed that the level of the bis-homologous esters was not elevated. In regard to desaturation without concurrent elongation, GLC of the respective nonhydrogenated $C_{17}$ and $C_{19}$ fractions indicated that dienoic or trienoic esters did not exceed the background level.

Products of partical degradation were detected but their amounts were not significantly different from acids which would or would not convert. It may be mentioned that in similar experiments, *O. danica* did not elongate 14-methylhexadecanoic (14) nor 2-methyllinoleic (15) acids.

## DISCUSSION

Straight chain odd-numbered fatty acids are quite common in lipids of microorganisms, and the level of less than 1% odd acids in *O. danica* (Table I) is unusually low. Structures of the unsaturated odd components seem to conform with many of those found in *Euglena gracilis* (16) and in mullet (9) where odd chains may represent more than 25% of the total acids.

The low background of odd acids makes investigation of their metabolism in *O. danica* particularly interesting. The organism incorporates and converts odd as well as even long chain fatty acids from the media (Tables II-IV), although experiments with labeled propionic acid (not described here) showed that only minimal amounts of this potential precursor are taken up and converted into long chain acids. Pentadecanoic acid is converted into 9-17:1, and the same double bond structure is probable for the small amount of 17:1 found in *O. danica* under normal conditions. The same structure, 9-17:1, has also been reported as resulting from 17:0 in *Chlorella vulgaris*, and it leads there to some 9,12-17:2 (17). Oleic acid is the crucial precursor for the majority of polyunsaturated even acids and 9-17:1 seems to have the same role for odd acids of higher unsaturation.

Chain elongation, desaturation in the proximal, and desaturation in the distal part of the chain are obvious in Table IV, for the odd-numbered unsaturated substrates. The former two types of conversion are exemplified with 6,9,12-17:3 which, as in EFA deficient rats (1,2), gives rise to 5,8,11,14-19:4. This and the closely related acid 8,11,14-19:3 are formed in appreciable amounts, although *O. danica*, in contrast to the rats, had not been depleted of the even polyunsaturated acids. The same type conversions were found also with a mixture, obtained from mullet oil, of 5,8,11,14-19:4, 8,11,14,17-19:4, and related $C_{19}$ acids of lower unsaturation. When supplied to the culture, they yielded 5,8,11,14,17-19:5 together with $C_{21}$ and $C_{23}$ acids. GLC analysis of the hydrogenated mixture showed that the latter chain lengths were considerably above the background level.

Desaturation of odd numbered acids in the distal moiety is clearly demonstrated by the

products from 15:0 and in particular from 9-17:1. This substrate, 17:1ω8, was converted to 17:2ω8 and 17:2ω5 and sequential products (Table IV). These desaturations are analogous to those of 18:2ω6 to 18:3ω6 and 18:3ω3 (Table III) which have also been reported by Nichols and Appleby (18). However 17:3ω2 was not found from 17:1ω8, nor from 17:2ω5 which was also offered as substrate. The latter acid yielded 19:3ω5 and 19:4ω5 as did 17:3ω5. The lack or low level of conversion of odd acids to the ω2 type may not be restricted to *O. danica*. Euglena contains fewer odd ω2 acids in much smaller amounts than ω2 acids (16), and the same is true in mullet (9,19) where the polyunsaturated acids can be considered derived from a great variety of dietary microbial sources.

The lack of any kind of conversion of 7-17:1, 10-17:1 and 10,13-19:2 is in strong contrast to the conversions that have been encountered with the odd acids of common double bond structure. The unusual acids are foreign to *O. danica*, but they are incorporated and are not toxic to the organism, as has been experienced with *Tetrahymena pyriformis* and certain fatty acids not normally found in this organism (20). Our results show that in *O. danica* desaturation in the distal moiety of monoenoic and dienoic acids is subject to the same effects of structure, as are elongation and desaturation in the proximal moiety.

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# Stimulation in Vitro of Galactocerebroside Galactosidase by N-Decanoyl 2-Amino-2-Methylpropanol

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## ABSTRACT

Amides resembling ceramide (fatty acyl sphingosine) were synthesized and tested in vitro for their effects on the rat brain β-galactosidase which hydrolyzes galactosyl ceramide. The N-decanoyl derivative of 2-amino-2-methyl-1-propanol was most effective, giving a 34% stimulation at 0.15 mM concentration and a 60% stimulation at maximal levels. Addition of a hydroxyl group in the 3 position reduced the degree of stimulation, as did increasing or decreasing the length of the fatty acid portion. Omission of the branched methyl group resulted in inhibition instead of stimulation. Kinetic analysis indicates that the stimulator does not affect the binding of substrate to enzyme, but does speed the rate of hydrolytic action. Stimulation was also observed with the cerebrosidase in spleen and kidney. It is suggested that the stimulators act on an enzyme site other than the substrate-active site.


## INTRODUCTION

Galactosyl ceramide β-galactosidase (cerebrosidase or galactocerebrosidase) is a specific enzyme which acts on one of the major lipids of brain (2,3,6). It appears to require a bile acid for activity in vitro and the addition of fatty acid raises the activity additionally (4). Suzuki and Suzuki (10,11) have shown that the enzyme is lacking in the hereditary disorder, Krabbe's disease, and have postulated that excessive acculumation of its substrate provokes the appearance in brain of globoid cells. These cells, in massive infiltration, apparently act to damage the brain fatally.

While the serum of one patient with Krabbe's disease was found to contain no detectable cerebrosidase, a small amount of activity was found inleukocytes,fibroblasts and brain (11). It is possible that the enzyme activity present in these patients, about 5-10% of normal, could be stimulated sufficiently by administration of a suitable drug, so that normal cerebroside metabolism could be maintained. Cerebrosidase activity normally rises considerably during myelination (4) and it is conceivable that normal development of a Krabbe child would ensue if it could be maintained during the critical stage of relatively rapid cerebroside turnover during early myelination (7,9).

In the course of an attempt to synthesize compounds which could inhibit cerebroside hydrolysis (1), we found certain compounds which exerted a stimulatory effect. These compounds resemble ceramide, the product of cerebrosidase action, but differ in omission of the lipoidal side chain of sphingosine and

TABLE I

Stimulation of Cerebrosidase Produced by Fatty Acid Amides of Aminopropanols[a]

| Fatty acid chain length | 2-Amino-2-methyl-1-propanol | | 2-Amino-2-methyl-1,3-propanediol | |
|---|---|---|---|---|
| | Above controls, % | Melting point | Above controls, % | Melting point |
| 18 | 12,8 | 64-65 | 14,11 | 73-74 |
| 16 | 16,14 | 72-73 | 11,7 | 69-70 |
| 14 | 30,26 | 48-49 | 18,13 | 61-62 |
| 12 | 28,29 | 36-37 | 20,16 | 46-47 |
| 10 | 59,50 | Oil | 24,26 | Oil |
| 8 | 12,9 | Oil | 24,20 | Oil |

[a]Stimulations shown are from two separate experiments, each run in duplicate. The assay medium contained enzyme derived from 3 mg of rat brain, 0.1 M citrate pH 4.5, 0.1 mg of labeled cerebroside, 1 mg of Tween, 0.5 mg of Myrj, 0.37 mg of Tris oleate and 2 mg of Na taurocholate in 1 ml. Incubations ran for 3 hr at 37 C. Control activities (without amides) were about 8000 cpm, corresponding to about 23 nmoles. The substrate specific activity was about 250 cpm/nmole and the amount of amide in the incubation tubes was 0.3 μmole.

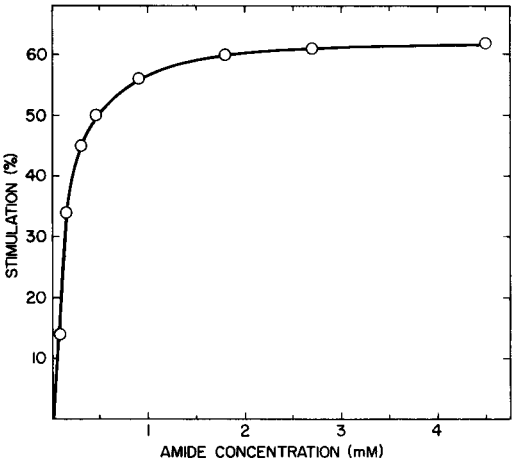


FIG. 1. The effect of increasing concentration of N-decanoyl 2-amino-2-methylpropanol on rat brain cerebrosidase. Conditions as in Table I.

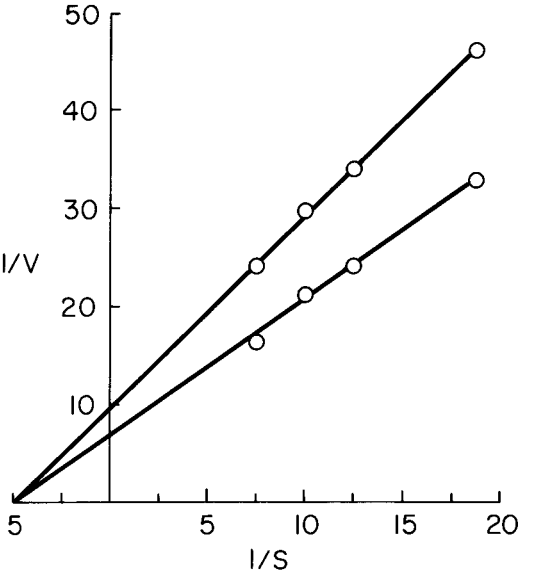


FIG. 2. The effect of changing the substrate concentration on the rate of hydrolysis. The concentrations of Tween, Myrj and oleate were changed too, in proportion to the cerebroside concentration. Upper line: control tubes; lower line: 0.3 mM decanoyl aminomethylpropanol added. The unit of cerebroside concentration is $10^{-3}$ M and the rate of reaction is in millimoles hydrolyzed in 3 hr. Incubation conditions were as in Table I.

inclusion of a branched methyl group.

## METHODS AND MATERIALS

The enzyme was prepared in crude form from the brains of 3-month-old rats as described before (4), and assayed by a simplified procedure (8). The assay medium contained galactose-labeled stearoyl psychosine emulsified with crude Na taurocholate, Tris oleate, Tween 20 and Myrj 59.

The synthetic compounds were made by acylation of amino alcohols with fatty acyl chlorides and characterized by thin layer chromatography, which showed a single spot (sometimes with a trace second spot) with each amide. IR spectra showed amide and alcohol peaks, but no ester peak. Details of the syntheses are described separately (1). The melting points of the compounds, which appear to be new, are given in Table I. Each compound was tested by evaporating a solution containing a weight sufficient to give a concentration in the medium of 0.3 mM. To this was added the substrate, diluent, citrate buffer and enzyme, in that order.

## RESULTS

### N-Fatty Acyl Amides of 1-Amino-2-Methyl-1-Propanol and 2-Amino-2-Methyl-1,3-Propanediol

The degree of stimulation for derivatives of fatty acids of different chain length is shown in Table I. The most effective compound was the decanoyl derivative of the methyl propanolamine. A sharp decrease in effectiveness resulted when a shorter or longer fatty acid was used. At each chain length, except with stearate, the insertion of a second alcohol group reduced the stimulatory effect. When the branching methyl group was omitted (2-amino-1-propanol), inhibition rather than stimulation was observed (1). The free amines and decanoic acid, which could conceivably arise by enzymatic hydrolysis of the amides, were slightly inhibitory.

A study with the two decanoate stimulators showed that preincubation of enzyme with buffer and amide, prior to addition of the substrate, did not materially affect the extent of stimulation. For the aminomethylpropanol derivative, the stimulation amounted to 60, 51 and 54% with the preincubation periods of 60, 30 and 0 min, respectively. For the aminomethylpropanediol derivative, the corresponding stimulations were 25, 27 and 22%. The preincubation of the controls did not affect their activity.

To investigate the possibility that the stimulation was due to nonenzymatic catalysis of cerebroside hydrolysis, we incubated substrate with the decanoyl propanol derivative in the absence of enzyme. No increase above the blank value was observed.

TABLE II

Stimulation of Cerebrosidase of Lyophilized Tissues by N-Decanoyl Amides

| Tissue[a] | Control activity, cpm | Aminomethylpropanol Above control, % | Aminomethylpropanediol Above control, % |
|---|---|---|---|
| Rat brain | 2020 | 20 | 9 |
| Rat spleen | 2677 | 16 | 11 |
| Rat kidney | 4099 | 29 | 23 |
| Rat liver | 831 | 7 | 2 |
| Rabbit gray matter | 377 | 5 | 0 |
| Rabbit white matter | 481 | 7 | 3 |

[a]Tissues from rats were from 18-day-old animals; a brain from a 10-day-old rabbit was also used. In these assays the tissue was homogenized in a small volume of water and lyophilized. The powder was rehomogenized in benzene, aliquots containing 2 mg of powder were dried down with a stream of nitrogen, and the incubation components were added as in Table I. The amides were added in solution and dried down prior to addition of the enzyme.

#### Effect of Stimulator Concentration

The data in Figure 1 show that decanoyl aminomethylpropanol produced a distinct stimulation at a concentration as low as 75 $\mu$M, and saturation of the effect appeared at about 1.8 mM and 60% stimulation. The saturation phenomenon is not due to limited solubility of the amide (which is somewhat lipoidal), since this would show up as a sharp discontinuity in Figure 1; the curve would suddenly flatten out at the solubility concentration. Moreover the amide must dissolve and combine rapidly with the enzyme, since a time study (15-180 min) showed that the control and stimulated reactions proceeded at constant rates.

#### Kinetic Analysis of the Stimulation

Evaluation of the stimulation effect at various concentrations of substrate yielded a Lineweaver-Burk plot (Fig. 2) which indicates that the $K_m$ of the enzyme-substrate complex was unchanged by the stimulator.

#### Tests with Other Tissues and Compounds

Although galactocerebroside is very rare outside the central nervous system, its hydrolase has been found in every tissue examined (5,6,10,11). To test the possibility that extraneural cerebrosidase differs from the neural enzyme, we compared the effect of the two decanoyl amide stimulators on the enzyme activity in several tissues (Table II). Stimulation was seen in rat kidney and spleen, two major sources of the enzyme (6). The specific activity of the enzyme in liver was low, as noted before with freshly homogenized liver (6), and the activity in rabbit brain was even lower. However a more realistic comparison of rat and rabbit brain cerebrosidase activities must await a detailed comparison at different ages, since marked changes occur with age.

It is not clear why the stimulatory effect of the amides with dried whole brain was so much less than with the purified enzyme.

The N-phenylacetyl amide of aminomethylpropanol (mp 129-130 C) yielded a stimulation of only 5% in the assay with purified cerebrosidase, and the corresponding diol (a semisolid) had even less effect (1%). However the $\alpha$-bromo derivative of the former compound ($\alpha$-bromophenylacetyl 2-amino-2-methyl-1-propanol, mp 73-74 C) produced 29% stimulation at 0.3 mM concentration.

## DISCUSSION

The ability of miscellaneous substances to increase the activity of an enzyme is an often-noted phenomenon. In this instance special interest comes from the rather sharp structural specificity involved, and the fact that the stimulator resembles the natural substrate structurally. Related compounds, which differ from the stimulators in possessing a lipoidal side chain and lacking the branched methyl group (thereby resembling natural ceramide more closely), act as noncompetitive inhibitors (1), and it may be that both groups of amides act by attachment to the same enzyme site.

It is likely that the stimulators act by combining in a reversible manner with the enzyme, producing a conformational change which increases the speed of enzyme action ($V_{max}$). This idea is supported by the results of the Lineweaver-Burk plot (Fig. 2), which showed that the increase in enzyme activity is not owing to an effect on the strength of the enzyme-substrate binding ($K_m$). If the stimulators act by an irreversible process, as by a chemical reaction with the enzyme, the reaction must be quite rapid, for we did not see any increased stimulation as the result of preincubation with stimulator, nor did the amount of

stimulation increase with incubation duration. Moreover a chemical reaction of a simple amido alcohol with an enzyme seems unlikely.

Whether a stimulating substance can be made which will be therapeutically useful in Krabbe's disease remains to be seen. The 60% stimulation observed in our in vitro experiments could not be a sufficient effect, and we plan a search for more active compounds. From the results reported here, it would appear that a branching group at the 2 position is essential and that a substituent such as Br or OH in the acyl group is helpful. The latter is of particular interest as 2-hydroxy acyl groups occur primarily in galactocerebrosides, and the substituent might increase the specificity of the stimulator with respect to galactosyl ceramide (as opposed to glucosyl ceramide). A second hydroxyl group weakens the stimulator effect and an aromatic substituent at the 3 position produces inhibition (1). It remains to be seen, also, whether the same structural features are relevant to the cerebroside-hydrolyzing enzyme found in Krabbe's disease.

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# The Phospholipid and Fatty Acid Composition of Platelets in Patients With Primary Defects of Platelet Function

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**ABSTRACT**

Platelet phospholipid and fatty acid composition was determined in nine normal subjects and in 11 patients with primary defects in platelet function. Two of the patients had thrombasthenia (Glanzmann) and nine had various types of abnormalities in platelet aggregation and platelet factor 3 availability attributed to impairment of the platelet release reaction. The values observed for platelet lipids in the normal subjects were similar to those reported by others. Four of the patients with a disturbance in the platelet release reaction were in the same family and showed the same abnormal pattern of platelet lipid composition. Phospholipid analysis showed a decrease in the relative amount of phosphatidyl ethanolamine (PE) and an increase in lecithin. Abnormalities in fatty acids consisted of an increase in the relative amounts of 18:1ω9, 20:0 and 20:1ω9 and a decrease in the 22:4ω6 + 24:1 fraction. Similar changes in PE and 18:1ω9 were also observed in another patient with a similar defect in platelet function. In this patient the relative amount of platelet sphingomyelin was also increased. The platelet lipid composition in the other six patients and in one normal subject given aspirin was essentially normal.

Phospholipids constitute a major component of cellular membranes and hence are assumed to play an important role in maintaining the structural and functional integrity of the cell and its organelles. The specific role which the membranous structures of platelets may play in the hemostatic properties of these cells has been reviewed (1). In the initial stages of hemostasis, platelets contribute to the primary arrest of bleeding by adhering to collagen (2)

[1]Career Scientist of the Health Research Council of the City of New York.

TABLE I

Platelet Function in Patients Studied

| Subjects | Age and sex | References[a] | Degree of platelet aggregation[b] | | | | Platelet factor-3 sec |
|---|---|---|---|---|---|---|---|
| | | | ADP $2\times10^{-6}$M 20 C | Connective tissue | Epinephrine, 5 μM 1st Wave | Epinephrine, 5 μM 2nd Wave | |
| Normal patients[a] | 20-60,MF | (12,13,17) | 3-4+ | 3-4+ | 1-2+ | 3-4+ | 34-51 |
| L.W. | 25,M | (12,15) | 0 | 0 | 0 | 0 | 68 |
| M.M. | 22,M | (16) | 0 | 0 | 0 | 0 | 60 |
| L.G. | 26,F | (18) | 4+ | 1-2+ | 2+ | 2+ | 40 |
| E.P. | 27,F | (18) | 4+ | ± | 2+ | 1-2+ | 48 |
| S.K. | 60,F | (12,17,18) | 3+ | ± | 1+ | 0 | 60 |
| F.A. | 62,F | (12,17,18) | 4+ | 1-2+ | 2+ | 0-2+ | 61 |
| S.N. | 25,F | (12,17,18) | 3+ | ± | 2+ | 1-2+ | 58 |
| D.C. | 28,F | (13,18,19) | 3+ | ± | 2+ | 0 | 51 |
| R.C. | 8,F | (13,19) | 3+ | 0 | 2+ | 0 | 60 |
| S.C. | 6,M | (13,19) | —[d] | ± | —[d] | —[d] | 61 |
| W.C. | 34,M | (13,19) | 3+ | ± | ± | 0 | 58 |
| Aspirin[c] | 30,M | | 4+ | 2+ | 2+ | 0 | 40 |

[a]Studies on these patients have been published in the references cited.

[b]Degree of aggregation expressed on a 0-4+ scale; details of quantitative methods are described elsewhere (12,14,17,18).

[c]Normal subject 2 hr after ingesting 1.5 gm aspirin.

[d]Not analyzed.

by a process presumably involving the plasma membrane. Subsequently the platelets release adenosine diphosphate (ADP) (3) which, in turn, causes them to aggregate (4). Recent studies by Holmsen et al. (5) have shown that this "released" ADP is derived from a metabolically inactive pool which may be localized within specific platelet granules of high electron density. The initial loose platelet aggregate is converted into a firm hemostatic plug by the subsequent evolution of thrombin, a process requiring both the plasma clotting factors and the participation of the platelets themselves. The clotting activity of platelets is not present in intact platelets (6) but is only uncovered after the platelets have been "activated" by freeze-thawing (6), kaolin (7) or suspensions of connective tissue (8). This activity, which has been called platelet factor 3 (PF-3), has been found in both platelet membranes and granules (9) as well as in a nonsedimentable form (10), and has been attributed to phospholipids, particularly phosphatidyl serine and phosphatidyl ethanolamine (PE) (11).

In some patients with congenital bleeding disorders, a variety of abnormalities in platelet function have been described, including defective release of platelet ADP, decreased PF-3 availability, and impaired aggregation by either ADP, collagen, or both (12,13). Because of the methodological problems inherent in studying the role of individual platelet phospholipids in normal subjects, a study of the platelet phospholipids and fatty acids in these "experiments of nature" appeared worthwhile, and the results obtained on 11 such patients are reported herein.

## MATERIALS AND METHODS

### Patients and Platelet Function Studies

Studies were performed on a heterogeneous group of patients with a lifelong history of bleeding in whom a variety of abnormalities in platelet function were found (Table I). The methods used for measuring platelet aggregation by ADP, collagen (connective-tissue suspension) and epinephrine, and for assaying kaolin-induced PF-3 availability have been described in detail previously (12-14). Patient L.W., reported previously (12,15), and Patient M.M. (16) showed the typical findings of absent ADP-induced platelet aggregation characteristic of Glanzmann's thrombasthenia. The other nine patients showed various abnormalities in platelet aggregation and PF-3 availability, as reported on more extensively elsewhere (12,13,17,18). All showed diminished collagen-induced aggregation of varying degree. The

TABLE II

Phospholipid Composition of Platelets

| Component[a] | Normal subjects[b] | Patients | | | | | | Aspirin[c] | |
|---|---|---|---|---|---|---|---|---|---|
| | | L.W. | M.M. | L.G. | G.P. | S.K. | F.A. | S.N. | Before | After |
| μg Lipid P/$10^9$ platelets | 16.8 ± 3.9 | 12.7 | 18.0 | 16.4 | 16.4 | 20.1 | 15.5 | 14.0 | --[d] | --[d] |
| g PL/g dry platelet x 100 | 15.0 ± 3.0 | 16.0 | 22.5 | 13.7 | 12.8 | 16.6 | 13.1 | 13.5 | --[d] | --[d] |
| PL distribution, moles/100 moles of P recovered | | | | | | | | | | |
| Phosphatidyl ethanolamine | 28.1 ± 0.6 | 28.9 | 28.7 | 28.5 | 28.0 | 26.3 | 27.7 | 27.4 | 28.2 | 28.3 |
| Phosphatidyl serine | 11.0 ± 0.5 | 11.1 | 10.9 | 11.3 | 10.0 | 11.0 | 11.3 | 11.7 | 10.4 | 10.6 |
| Lecithin | 41.3 ± 1.1 | 40.7 | 42.0 | 40.4 | 43.1 | 41.2 | 42.2 | 42.2 | 40.4 | 41.0 |
| Sphingomyelin | 18.0 ± 0.7 | 17.3 | 16.8 | 17.7 | 17.7 | 20.2 | 17.0 | 17.5 | 19.9 | 19.2 |
| Lysolecithin | 1.2 ± 0.3 | 1.9 | 1.6 | 2.0 | 0.8 | 1.1 | 1.8 | 1.2 | 1.2 | 0.8 |
| Recovery of P after TLC, % | 98.6 ± 3.8 | 98.8 | 100.6 | 96.7 | 94.4 | 93.3 | 105.9 | 100.2 | 94.9 | 94.9 |

[a]Abbreviations: P, phosphorus; PL, phospholipid; TLC, thin layer chromatography.
[b]Values shown are mean ± standard deviation in nine normal subjects.
[c]See Table I for details.
[d]Not analyzed.

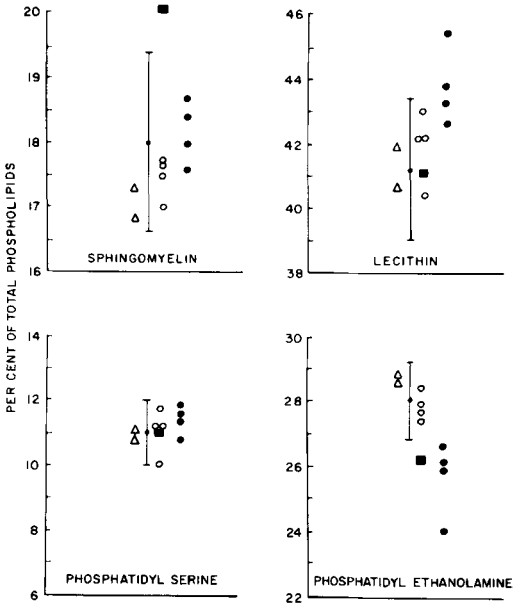


FIG. 1. Phospholipid distribution in platelets. ϕ, Normal subjects shown as mean ± 2 S.D.; △, patients with thrombasthenia (L.W. and M.M.); •, Family C (D.C., R.C., S.C. and W.C.); ▪, Patient S.K.; ○, other nonrelated patients with platelet aggregation defects (L.G., E.P., F.A. and S.N.).

second wave of epinephrine-induced aggregation at 37 C was either absent or significantly diminished while primary aggregation by ADP at ambient temperature was consistently normal. These findings have been interpreted previously as owing to an abnormality in the platelet release reaction (12,13,18) and in one large family (Family C), four of whose members are described herein [D.C., R.C., S.C. and W.C., previously (13) designated III-8, IV-4, IV-5 and III-3, respectively], this was shown to be owing to a lack of the storage pool of adenine nucleotides (19) which are selectively extruded from the platelets during this reaction. A similar abnormality has been demonstrated in Patients L.G. and S.N. (18,20). PF-3 availability was impaired in the two thrombasthenic patients and in seven of the nine other patients. In all but one (S.K.) of the patients in whom PF-3 availability was abnormal, freeze-thawing of the platelets rendered PF-3 "available" to the same extent as in normal subjects. The effect of aspirin ingestion was studied in one normal subject in whom ingestion of 1.5 g of aspirin produced the characteristic defects in platelet aggregation described previously (14).

#### Normal Subjects

Control studies were obtained on nine apparently normal subjects, aged 20-46.

Patients and normal subjects indicated that they had complied with the request to abstain from drug ingestion for at least 1 week prior to study.

#### Preparation of Platelets

A total of 54-108 ml of venous blood was mixed with 0.1 volume of 1% EDTA in isotonic saline and centrifuged to obtain platelet-rich plasma, as described previously (12,14). The platelets were sedimented by centrifugation at 12,000 g and 4 C for 20 min and were then suspended in 7 ml of 0.05 M imidazole-buffered saline, pH 7.3 (14), containing 0.1% EDTA (IBS-EDTA). They were sedimented once in a McNaught tube for 20 min at 2,500 g to permit separation of the platelets from any remaining red cells, washed twice more with IBS-EDTA, and finally suspended in 12 ml of this solution. A small aliquot of this suspension was removed for cell counts, and the platelets were then sedimented by centrifugation at 12,000 g and 4 C for 30 min. The supernatant was discarded and the remaining pellet was analyzed for lipids. Red cells and platelets were counted by standard phase microscopy. The percentage of red cells which still remained in the final suspension ranged from 0.01-0.23%. Red cells were previously found to contain 12.4 ± 0.5 μg of lipid P per $10^9$ cells (21), while a value of 16.8 ± 3.9 was obtained for platelets in the present study (see Results). Since the total amount of phospholipid per cell appears to be very similar in platelets and red cells, despite the difference in their size, and since the lipid compositions of these two cells are similar (21,23), the contribution of this red cell contamination to the determination of platelet phospholipids and fatty acids could be ignored.

#### Lipid Studies

Platelet pellets were obtained from patients and normal subjects on the same day and analyzed in parallel. Two to four pellets, at least one of which was from a normal subject, were prepared on each occasion, and lipid extraction was begun immediately using a modification (21) of the method of Folch et al. (24). Five ml of methanol were added to the platelets in a 40 ml centrifuge tube, and the suspension was mixed well and then allowed to stand for 30 min. Five ml of chloroform were added, again with mixing and standing for 30 min, and the suspension centrifuged. The supernatant was transferred to another 40 ml centrifuge tube, and the residue was extracted twice more in the same fashion with 0.5 volume of the solvents. The three extracts were pooled and 10 ml of chloroform were added to make

TABLE III

Phospholipid Composition of Platelets in Family C

| Component[a] | Normal subjects[b] | Normal child | D.C. | R.C. | S.C. | W.C. |
|---|---|---|---|---|---|---|
| μg Lipid P/$10^9$ platelets | 16.8 ± 3.9 | 19.6 | 17.0 | 15.4 | 17.2 | 18.6 |
| g PL/g dry platelet x 100 | 15.0 ± 3.0 | 11.4 | 18.0 | 15.7 | 15.0 | 15.2 |
| PL distribution, moles/100 moles of P recovered | | | | | | |
| Phosphatidyl ethanolamine | 28.1 ± 0.6 | 27.9 | 24.1 | 26.7 | 26.2 | 26.0 |
| Phosphatidyl serine | 11.0 ± 0.5 | 11.7 | 11.6 | 10.8 | 11.3 | 11.8 |
| Lecithin | 41.3 ± 1.1 | 41.6 | 45.5 | 43.9 | 42.6 | 43.3 |
| Sphingomyelin | 18.0 ± 0.7 | 17.6 | 17.6 | 18.0 | 18.4 | 18.6 |
| Lysolecithin | 1.2 ± 0.3 | 1.1 | 1.1 | 0.5 | 1.2 | 0.8 |
| Recovery of P after TLC, % | 98.6 ± 3.8 | 99.2 | 90.4 | 98.8 | 94.0 | 92.0 |

[a]Abbreviations: see Table II.

[b]Values shown are mean ± standard deviation in nine normal subjects.

the final volume 30 ml with a chloroform-methanol ratio of 2:1. To this was added 6 ml of 0.05 M KCL. The mixture was emulsified and centrifuged and the supernatant discarded. The infranatant, containing the lipid extract, was dried at 40 C (water bath) with a nitrogen jet and dissolved in chloroform for chromatographic studies. The platelet residue, following the third extraction, was dried in a vacuum oven at 40-50 C and weighed. The dried platelet residue from a normal subject and patient were pooled, heated at 110 C for 18 hr in 5 ml of 2 N HCl, and the hydrolysate was then extracted with pentane. The entire pentane extract showed only a trace of free fatty acid by silicic acid thin layer chromatography (TLC), indicating that the extraction of platelet lipid was essentially complete. To calculate the gram phospholipid per gram dry platelet (Tables II and III), the factor 25 was used to convert lipid P to phospholipid, and the weight of the dry platelet was taken as the sum of the weights of the dry lipid-free platelet residue and the total lipid, assuming that phospholipid comprised 80% of the total lipid (25,26).

Duplicate aliquots of the lipid extract were removed for total lipid phosphorus determination. Duplicate aliquots were also spotted on silicic acid thin layer plates for separation of the individual phospholipids with chloroform-methanol-acetic acid-water 25:10:4:2 (27) as solvent. The fatty acid composition of the platelet lipid extract was determined using gas liquid chromatography, which was performed with a Barber-Colman instrument, model 5000, equipped with paired 8 ft columns of EGSS-X (an ethylene glycol succinate polyester combined with a silicone) 8% on Gas-Chrome-P, 100-120 mesh (Applied Science Laboratories, Inc., State College, Pa.), and dual flame ionization detectors. Details of these methods have been described previously (21).

All of the chloroform and methanol used contained 0.005% 2,6 di-*tert*-butyl-*p*-cresol (BHT) (Ionol, CP; Shell Chemical Co., New York, N.Y.) as an antioxidant (28).

## RESULTS

The phospholipid composition of the platelets of the patients and normal subjects is shown in Tables II and III. The amount of phospholipid per platelet in the patients appeared to be within the normal range; so did the amount of phospholipid per gram dry weight of platelet. The relative amounts of the individual phospholipids, however, showed abnormalities which, although small, were significant statistically. Patient S.K. (Table II) had an elevation in sphingomyelin with a decrease in PE; these same abnormalities were demonstrated on a second platelet sample obtained from the patient 7 months later. Patients D.C., R.C., S.C. and W.C. (Table III), who were members of Family C with similar abnormalities of platelet function, showed a decrease in PE. In addition the relative amount of the lecithin fraction was increased in Patients D.C. and R.C., and suggestively increased in the other two. Thus the molar ratio of PE/lecithin was significantly decreased ($p > 0.02$) in each of these four patients. The distribution of the individual phospholipids in the patients compared to normal is depicted in Figure 1.

The distribution of fatty acids in the total platelet lipid of the patients and normal subjects is shown in Table IV. Patients D.C., R.C., S.C. and W.C., who had similar abnormalities in phospholipid distribution, also showed essentially the same abnormal pattern of fatty acid distribution. The relative amounts of 20:0 and 20:1ω9 (in this abbreviation of fatty acids, the

TABLE IV

Fatty Acid Composition of Total Lipid of Platelets

| Components[a] | Distribution, g/100 g of fatty acid | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normal subjects[b] | Normal child | Patients | | | | | | | | | | Aspirin[c] |
| | | | L.W. | M.M. | L.G. | S.K. | F.A. | S.N. | D.C. | R.C. | S.C. | W.C. | Before | After |
| 16:0 | 16.2 ± 0.9 | 17.5 | 14.8 | 16.2 | 16.6 | 17.6 | 17.6 | 14.3 | 15.0 | 16.1 | 16.4 | 15.3 | 15.3 | 15.5 |
| 16:1ω9 | 0.68 ± 0.34 | 0.54 | 1.2 | 0.87 | 1.3 | 1.2 | 1.2 | 0.44 | 0.48 | 1.1 | 0.60 | 0.57 | 0.43 | 0.42 |
| 18:0 | 19.8 ± 1.4 | 19.4 | 17.9 | 18.1 | 18.4 | 17.2 | 18.4 | 18.8 | 18.9 | 17.8 | 18.6 | 17.1 | 20.5 | 20.2 |
| 18:1ω9 | 16.7 ± 1.0 | 17.4 | 19.3 | 15.6 | 17.5 | 20.3 | 17.0 | 17.1 | 19.6 | 18.2 | 19.9 | 18.3 | 17.1 | 17.3 |
| 18:2ω6 | 5.6 ± 0.6 | 5.4 | 5.1 | 4.8 | 5.2 | 6.5 | 5.2 | 6.5 | 6.6 | 5.8 | 5.6 | 5.4 | 5.2 | 4.8 |
| 20:0 | 1.9 ± 0.2 | 1.6 | 1.4 | 1.5 | 2.0 | 1.4 | 1.9 | 2.2 | 2.9 | 2.5 | 2.5 | 2.6 | 2.0 | 2.0 |
| 20:1ω9 | 0.86 ± 0.15 | 0.78 | 0.62 | 0.61 | 0.71 | 0.86 | 0.97 | 1.4 | 1.6 | 1.1 | 1.5 | 1.4 | 0.88 | 0.70 |
| 20:2ω6;20:2ω9; 21:0 | 0.20 ± 0.11 | 0.24 | ---[d] | 0.06 | 0.16 | 0.20 | 0.26 | 0.49 | 0.09 | 0.19 | 0.14 | 0.37 | 0.10 | 0.11 |
| 20:3ω6;22:0 | 4.0 ± 0.5 | 4.0 | 3.7 | 4.0 | 3.6 | 3.7 | 4.0 | 4.7 | 4.4 | 4.4 | 4.0 | 4.0 | 4.3 | 4.3 |
| 20:4ω6 | 23.9 ± 0.8 | 21.8 | 25.1 | 26.0 | 22.7 | 20.7 | 22.5 | 26.6 | 22.3 | 23.5 | 21.8 | 24.6 | 25.2 | 25.6 |
| 22:1ω9 | 0 51 ± 0.33 | 0.22 | 0.70 | 0.51 | 0.72 | 0.50 | 0.57 | 0.32 | 1.0 | 0.29 | 0.42 | 1.0 | 0.19 | 0.21 |
| 23:0;20 | 0.33 ± 0.11 | 0.29 | 0.21 | 0.31 | 0.22 | 0.27 | 0.26 | 0.19 | 0.35 | 0.43 | 0.28 | 0.37 | 0.38 | 0.39 |
| 22:2ω6;20:5ω3 | 0.31 ± 0.11 | 0.11 | 0.26 | 0.37 | 0.62 | 0.34 | 0.22 | 0.12 | 0.41 | 0.19 | 0.18 | 0.35 | 0.26 | 0.21 |
| 24:0 | 1.3 ± 0.4 | 1.5 | 1.3 | 2.0 | 2.0 | 1.8 | 0.79 | 1.7 | 1.2 | 1.1 | 1.0 | 0.87 | 1.6 | 1.4 |
| 22:4ω6;24:1ω9 | 4.7 ± 0.4 | 5.6 | 4.9 | 4.6 | 3.3 | 4.5 | 4.7 | 4.5 | 3.0 | 3.4 | 3.4 | 4.0 | 4.3 | 4.9 |
| 22:5ω6 | 0.44 ± 0.16 | 0.39 | 0.73 | 1.1 | 0.83 | 0.33 | 0.62 | 0.35 | 0.12 | 0.29 | 0.49 | 0.42 | 0.26 | 0.35 |
| 22:5ω3 | 1.2 ± 0.3 | 1.3 | 0.94 | 1.8 | 1.2 | 1.2 | 1.6 | 0.94 | 0.92 | 1.1 | 0.92 | 1.9 | 1.0 | 1.1 |
| 22:6ω3 | 1.4 ± 0.4 | 1.8 | 1.1 | 1.2 | 2.4 | 1.9 | 1.7 | 0.95 | 0.97 | 1.5 | 1.4 | 1.1 | 0.93 | 1.0 |

[a]This shorthand designation for fatty acids is explained in the text.

[b]Values shown are mean ± standard deviation in eight normal subjects.

[c]See Table I for details.

[d]Not analyzed.

first two digits state the number of carbon atoms, the third digit states the number of double bonds, and the digit after the omega states the end-carbon chain length, which is the number of carbon atoms from the methyl end of the acyl chain to the middle of the nearest double bond) were increased and 22:4ω6 + 24:1ω9 depressed. 18:1ω9 was increased in Patients D.C. and S.C. and may have been increased in the other two. The consistency of these phospholipid and fatty acid changes within the same family strongly supports their validity. Since Patients R.C. and S.C. were children while the normal subjects to whom they were compared in Tables III and IV were adults, values obtained on a normal child are also included in these tables for comparison. As can be seen, the phospholipid and fatty acid distributions of this child were very similar to those of the normal adults and showed none of the abnormalities noted in Patients R.C. and S.C. In order to determine whether the lipid changes observed were confined to the platelets, the phospholipid and fatty acid compositions of the red cells of Patient D.C. were analyzed. The distribution of individual phospholipids was normal. The fatty acid analysis, however, showed an increase in the relative amounts of 20:0 and 20:1ω9. The possible independence of the platelet functional defect and the lipid changes was tested by analyzing the platelets of an unaffected brother of Patient D.C.'s affected father; platelet function, total phospholipid content and phospholipid distribution (PE-27.1%, lecithin-41.1%) were normal.

Isolated abnormalities (>3 SD from the normal mean) were seen in other patients. Patient S.K. had an elevation in the relative amount of 18:1ω9, Patient S.K. a decrease and S.N. an increase in 20:4ω6; Patient M.M. had an increase in 22:5ω6, and Patient L.G. a decrease in 22:4ω6 + 24:1ω9.

The platelet lipids of one normal subject were studied before and after he ingested 1.5 g of aspirin. No effect of aspirin on the platelet phospholipid or fatty acid composition was observed (Tables II and IV).

## DISCUSSION

The amount of lipid phosphorus per platelet and per gram dry weight of platelet observed in the normal subjects is in general agreement with that found by others (26,29,30). The distribution of individual platelet phospholipids is also similar to that found by others (22,31) and agrees very closely with the values reported recently by Marcus et al. (25) and Cohen and Derksen (23). Phosphatidyl inositol, which makes up about 3-5% of the platelet phospholipid (22,23,25,31), was included with the lecithin fraction in the present study and accounts for the difference in the relative amount of lecithin in this compared with previous studies (23,25). The distribution of fatty acids in the total platelet lipid of the normal subjects compares favorably with the values calculated from the results of previous investigators (23,25) who carried out this determination on the major individual phospholipids only. Since platelet fatty acid appears to be almost entirely in the phospholipid fraction (25), this comparison seems valid.

Four of the patients in the present study whose abnormal platelet function has been attributed to a specific type of defect in the release reaction (19) were in the same family and showed multiple changes in platelet lipid distribution. Although the platelet lipid abnormalities were small, they were significant statistically. That the four patients showed essentially the same pattern of lipid change, furthermore, supported its validity. The disturbance in phospholipid distribution observed consisted of a decrease in the relative amount of PE with an increase in the relative amount of lecithin; the latter finding could have been owing to a change in the amount of the phosphatidyl inositol included in the lecithin fraction in the analysis. The changes in the relative amounts of fatty acids consisted of increases in 18:1ω9, 20:0 and 20:1ω9, and a decrease in the 22:4ω6 + 24:1ω9 fraction. Since each platelet phospholipid has a characteristic fatty acid composition (23,25,31), the possibility arises that the abnormal fatty acid pattern was secondary to the change in phospholipid distribution. Although the fatty acid changes observed were in a direction to be consistent with this possibility (31), the lack of change in certain other fatty acids, such as 16:0 and 18:2ω6, suggests another explanation. The increase in the relative amounts of 20:0 and 20:1ω9 with a normal phospholipid distribution in the red cells of the one patient (D.C.) in whom the red cells were studied also suggests that the phospholipid and fatty acid abnormalities were independent. If the lipid composition of platelets reflects dietary intake as does red cells in man (32), the lipid abnormalities observed could have possibly resulted from an unusual diet in these patients. No history of such a diet was obtained in any of the patients and the fact that the unaffected husband of Patient D.C. had a normal phospholipid distribution and one of the affected family members (W.C.) lived in a different household argues against this explanation. A different type of

platelet lipid abnormality was obtained in Patient S.K. In this patient, in addition to a decrease in the relative amounts of PE and 20:4ω6, sphingomyelin and 18:1ω9 were increased. The lipid patterns in the other patients were essentially normal or showed isolated fatty acid changes.

The possible mechanisms by which the lipid changes described could produce the functional abnormality of the platelets are not clear. Nine of the 11 patients studied had abnormalities in platelet aggregation and PF-3 availability which may be attributed to impairment of the platelet release reaction. Although no inferences regarding a possible genetic defect can necessarily be drawn in the unrelated patients who were studied, the consanguinity of the patients in Family C indicates that their platelet abnormalities are due to the same genetic defect. Recent studies on affected members of this family demonstrated a defect in the storage pool of adenine nucleotides, which may be located within specific granules and which are extruded from the platelets during the release reaction (19). That this type of defect may be unrelated to an abnormality in platelet lipid composition is indicated by the observation of a similar defect (18,20) in two patients (L.G. and S.N.) with essentially normal platelet lipid compositions. Marcus et al. (25) have reported recently that platelet granules and membranes have different phospholipid compositions. Since the abnormal platelet phospholipid pattern observed in Family C resembles that of membranes more closely than of granules, a decrease in the number of granules could explain this pattern. That an abnormal phospholipid pattern might result in decreased PF-3 availability is suggested by previous findings that certain phospholipids possess this clotting activity (11). Phosphatidyl serine (PS) has been shown to be the single phospholipid most active in promoting clotting in vitro (11). Although a decrease in PF-3 availability was observed in the patients in Family C, as well as in three other unrelated patients with abnormalities in the release reaction, no abnormality in the amount of platelet PS was found. In a previous study it was found that the unavailability of PF-3 activity was due to a decreased release of platelet ADP, and that the associated defects of platelet aggregation provided a more reasonable explanation for the bleeding tendency in these patients than an abnormality in platelet clotting activity (12). Hence the finding of a normal PS in these patients is not surprising. A comparison of PF-3 availability and the amount of platelet PS may be specious, moreover, since platelet membrane has more activity than the amount of phospholipid contained therein (9). Interestingly, Patient S.K., whose lipid abnormalities were unique, was the only one in the group of patients studied whose platelets released PF-3 activity only minimally even after freeze-thawing. Finally the possibility that the lipid abnormalities and functional defects in the patients' platelets may be unrelated must be considered. The lipid abnormalities could represent an uncommon and previously undescribed genetic variation not associated with functional abnormality. The independent segregation of lipid abnormalities and the Chediak-Higashi syndrome in a family has been demonstrated previously (33). The one unaffected sibling of an affected member of Family C that was studied showed no platelet lipid abnormality. In five patients with an abnormal release reaction, as well as in a normal subject who ingested aspirin, platelet lipids were essentially normal, while the lipid abnormalities in Patient S.K. and Family C were different from each other. These observations suggest that the lipid abnormalities and functional defects of the platelets were unrelated. Alternatively the functional defects in Family C and Patient S.K. could have been secondary to the lipid abnormalities, if the defects in the other patients were on different genetic bases. That different genetic defects could account for the same apparent defect in platelet function has been suggested (19).

Two patients with thrombasthenia (L.W. and M.M.), both of whom showed the typical finding of absent ADP-induced platelet aggregation, were also studied. The platelets of these two patients showed essentially normal lipid compositions.

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# Triton-Induced Hyperlipidemia in Rats as an Animal Model for Screening Hypolipidemic Drugs

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## ABSTRACT

We describe a screening test for hypolipidemic agents in which compounds are administered orally to fasted rats after a single intravenous injection of 225 mg Triton WR-1339/kg and serum cholesterol and triglycerides are measured 43 hr post-Triton. Conditions for the screen were established by studying interrelationships between serum cholesterol, triglycerides and Triton levels during the post-Triton period and the effects of Triton dose, route of administration and fasting on serum lipid levels and drug hypocholesterolemic activity. The test detects compounds which inhibit lipid biosynthesis or stimulate lipid catabolism. Several drugs with different mechanisms of action which are hypolipidemic in man, including nicotinic acid, *D*-thyroxine, triparanol, nafoxidine HCl and clofibrate are active in this system. Results with standard hypolipidemic agents are reproducible and conform well to performance levels of the screen predicted from statistical analysis.

## INTRODUCTION

A single parenteral administration of Triton WR-1339 to adult rats produces a hyperlipidemia in which cholesterol, triglycerides and phospholipids increase to a maximum in about 20 hr and decrease thereafter. The majority of experimental evidence (1-8) supports the concept that Triton physically alters very low density lipoproteins, rendering them refractive to the action of lipolytic enzymes of blood and tissue. This prevents or delays their removal from blood and secondarily stimulates the synthesis of lipid, enhancing the hyperlipidemia.

Garattini et al. (9) and Paoletti (10) suggested the use of Triton-induced hyperlipidemia as an approach to screen for or to differentiate the mechanism of action of hypolipidemic drugs. Their tests were of two kinds. In the "first phase" test the drug was given ip at the same time as Triton, and a decrease in hyperlipidemia 8 hr later compared to controls was taken as evidence of activity resulting from an inhibition of increased synthesis of cholesterol

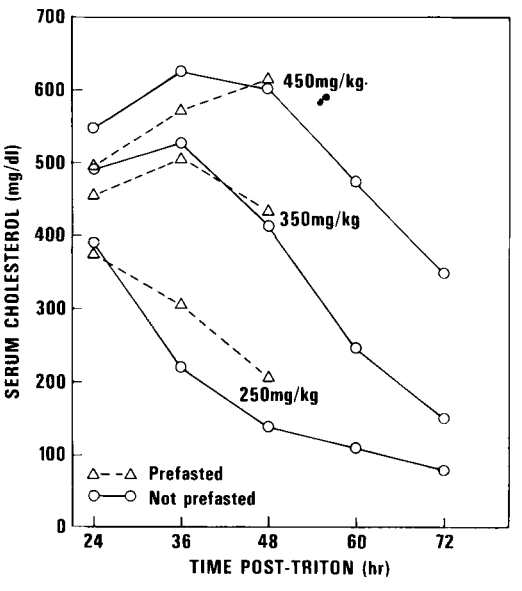


FIG. 1. Effects of Triton dose and fasting on serum cholesterol.

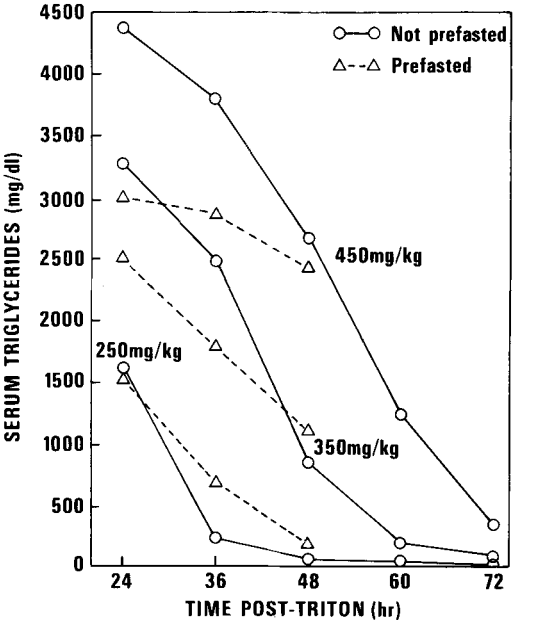


FIG. 2. Effects of Triton dose and fasting on serum triglycerides.

TABLE I

Effects of Triton Dose and Fasting on Serum Cholesterol and Triglycerides

| Time post-Triton, hr | | Serum cholesterol, mg/dl[a] Triton dose, mg/kg 250 | 350 | 450 | Serum triglycerides, mg/dl[a] Triton dose, mg/kg 250 | 350 | 450 |
|---|---|---|---|---|---|---|---|
| 24 | Prefasted | 374±18 | 455±18 | 496±4 | 1526±15 | 2504±8 | 2944±9 |
| | Not prefasted | 389±15 | 490±7 | 545±8 | 1615±36 | 3270±23 | 4386±17 |
| 36 | Prefasted | 302±15 | 504±22 | 572±8 | 695±34 | 1808±14 | 2841±12 |
| | Not prefasted | 220±24 | 527±8 | 626±10 | 234±61 | 2473±24 | 3810±18 |
| 48 | Prefasted | 205±20 | 436±21 | 614±6 | 183±53 | 1106±52 | 2420±10 |
| | Not prefasted | 141±19 | 413±15 | 604±9 | 64±58 | 853±54 | 2667±15 |

[a]Mean ± % standard deviation.

and fatty acids. In the "second phase" test the drug was given 22-24 hr after Triton, and decreased blood lipids 8 hr later compared to controls was interpreted as an indication of the drug's ability to accelerate lipid removal.

In this communication we describe a screening test for hypolipidemic drugs in which the agents were administered orally to rats immediately following and 20 hr after intravenous injection of Triton, and activity was determined by measuring serum cholesterol and triglycerides 43 hr post-Triton. Thus one test detects compounds which affect lipid synthesis, removal, or both. The influence of Triton dose, route of administration and fasting on hyperlipidemic response of the animals, their sensitivity to cholesterol-lowering drugs, and the ability of the screen to detect several drugs previously demonstrated to be hypolipidemic in experimental animals or man by different mechanisms of action were investigated.

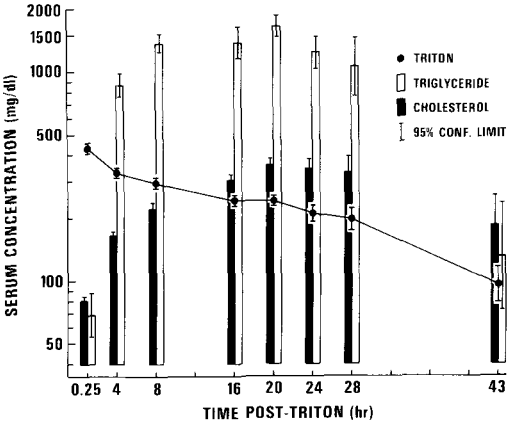


FIG. 3. Serum levels of cholesterol, triglycerides and Triton following Triton administration.

## EXPERIMENTAL PROCEDURES

### Animals and Diet

Male albino rats derived from the Sprague-Dawley strain obtained from the Upjohn rodent colony (Upj:TUC(SD)spf) were housed in groups of five animals and allowed free access to food and water for at least 21 days before being distributed by weight into experimental groups. The diet of Phillips and Berg (11) was used, with 10% coconut oil substituted for corn

TABLE II

Cholesterol Lowering Activity of U-10,387 and U-23,469 in Triton-Induced Hyperlipidemic Rats

| Triton WR-1339 dose, mg/kg | Control | Serum cholesterol, mg/dl U-10,387, (5 mg/kg) | U-23,469, (25 mg/kg) | Standard deviation, % |
|---|---|---|---|---|
| 200 | 116 | 74[a] | 75[a] | 18 |
| 250 | 247 | 157[a] | 181[a] | 35 |
| 300 | 340 | 279 | 307 | 27 |
| 350 | 509 | 414[a] | 471 | 13 |
| 400 | 583 | 508[a] | 527 | 16 |
| 450 | 619 | 545[a] | 592 | 7 |

[a]Significantly different from control.

TABLE III

Comparison of Hypocholesterolemic Activities of U-10,387 and U-23,469 in Rats Given Triton by Parenteral Routes

| Route of Triton administration | Treatment | Serum cholesterol, mg/dl | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Study no. | | | | | Summary | | |
| | | 1 | 2 | 3 | 4 | 5 | Mean log | Antilog | SD, % |
| Intravenous | Control | 181 | 163 | 168 | 183 | 162 | 2.2330 | 171 | 30 |
| | U-10,387 (3.3 mg/kg) | 98[a] | 106[a] | 96[a] | 112[a] | 109[a] | 2.0170 | 104 | 21 |
| | U-10,387 (1.1 mg/kg) | 129[a] | 121[a] | 111[a] | 114[a] | 110[a] | 2.0682 | 117 | 27 |
| | U-10,387 (0.37 mg/kg) | 153 | 117[a] | 124[a] | 131[a] | 125[a] | 2.1139 | 130 | 24 |
| | U-23,469 (10 mg/kg) | 118[a] | 133 | 85[a] | 104[a] | 104[a] | 2.0334 | 108 | 25 |
| | Na carbonate (30 mg/kg) | 227[a] | 173 | 165 | 169 | 167 | 2.2528 | 179 | 33 |
| Intraperitoneal | Control | 154 | 133 | 151 | 163 | 170 | 2.1847 | 153 | 47 |
| | U-10,387 (3.3 mg/kg | 90[a] | 80[a] | 88[a] | 137 | 110[a] | 1.9956 | 99 | 33 |
| | U-10,387 (1.1 mg/kg) | 129 | 101 | 89[a] | 130 | 123 | 2.0531 | 113 | 34 |
| | U-10,387 (0.37 mg/kg) | 131 | 139 | 182 | 180 | 164 | 2.1987 | 158 | 42 |
| | U-23,469 (10 mg/kg) | 103[a] | 105 | 109[a] | 114 | 142 | 2.0569 | 114 | 47 |
| | Na carbonate (30 mg/kg) | 171 | 130 | 139 | 131 | 167 | 2.1673 | 147 | 54 |

[a]Significantly different from control.

TABLE IV

Comparison of Hypotriglyceridemic Activities of U-10,387 and U-23,469 in Rats Given Triton by Parenteral Routes

| Route of Triton administration | Treatment | Serum triglycerides, mg/dl | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Study no. | | | | | Summary | | |
| | | 1 | 2 | 3 | 4 | 5 | Mean log | Antilog | SD, % |
| Intravenous | Control | 144 | 124 | 129 | 152 | 114 | 2.1206 | 132 | 71 |
| | U-10,387 (3.3 mg/kg) | 55[a] | 63[a] | 51[a] | 54[a] | 65[a] | 1.7559 | 57 | 56 |
| | U-10,387 (1.1 mg/kg) | 72[a] | 69[a] | 64[a] | 54[a] | 60[a] | 1.7993 | 63 | 78 |
| | U-10,387 (0.37 mg/kg) | 109 | 70[a] | 68[a] | 86[a] | 67[a] | 1.8976 | 79 | 62 |
| | U-23,469 (10 mg/kg) | 109 | 136 | 57[a] | 77[a] | 77 | 1.9395 | 87 | 50 |
| | Na carbonate (30 mg/kg) | 265[a] | 147 | 133 | 130 | 115 | 2.1790 | 151 | 80 |
| Intraperitoneal | Control | 111 | 87 | 103 | 129 | 128 | 2.0414 | 110 | 115 |
| | U-10,387 (3.3 mg/kg) | 43[a] | 44 | 32[a] | 88 | 61 | 1.6990 | 50 | 78 |
| | U-10,387 (1.1 mg/kg) | 81 | 42[a] | 35[a] | 81 | 84 | 1.7782 | 60 | 92 |
| | U-10,387 (0.37 mg/kg) | 88 | 96 | 154 | 179 | 127 | 2.0934 | 124 | 99 |
| | U-23,469 (10 mg/kg) | 80 | 69 | 90 | 96 | 160 | 1.9777 | 95 | 88 |
| | Na carbonate (30 mg/kg) | 103 | 100 | 82 | 77 | 122 | 2.0212 | 105 | 123 |

[a]Significantly different from control.

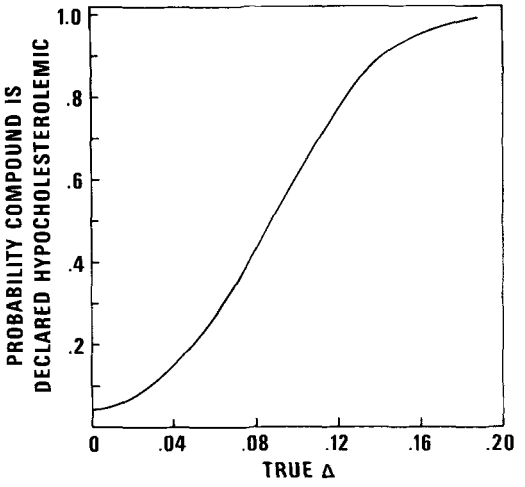


FIG. 4. Cholesterol operating characteristic curve.

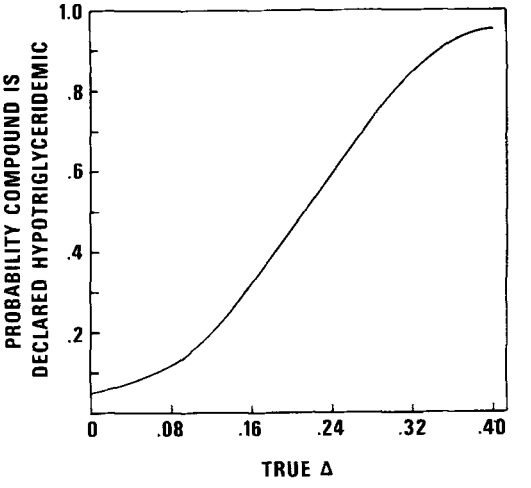


FIG. 5. Triglyceride operating characteristic curve.

oil, and 18% casein and 0.2% methionine as the protein source.

#### Screening Procedure

Rats of average body weight 270 g (range 240-300 g) were fasted for 24 hr and were injected via a tail vein with 225 mg Triton WR-1339 (oxyethylated tertiaryoctylphenol-formaldehyde polymer, Winthrop Laboratories, N.Y.)/kg dissolved in 0.15 *M* NaCl at a concentration of 62.5 mg/ml. A screening test consisted of up to 48 groups of five rats each. At random six of these groups received vehicle (0.25% aqueous methylcellulose) and pairs of the remaining groups received test compounds. Compounds were dispersed in vehicle using a glass tissue grinder at a concentration of 2 mg/ml, which provided the total screening dose of 30 mg/kg for a 270 g rat in 4 ml. Each rat received two 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. Blood was drawn 43 hr after Triton administration from the abdominal aorta under sodium cyclopal anesthesia (80 mg/kg, ip), allowed to clot and serum was obtained by centrifugation.

Most of the studies reported were carried out in conjunction with a screening test performed according to these procedures. Exceptions are described.

#### Serum Analyses

Serum cholesterol was determined from 1:10 isopropanol extracts of individual samples using ferric choloride-sulfuric acid reagent (12) in the AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N.Y.). Serum triglycerides were measured by the method of Royer and Ko (13). Triton levels were determined by measuring absorption at 278 mμ (14) in 1:10 isopropanol extracts.

#### Statistical Analyses

Serum lipid values were transformed to logarithms to achieve more homogeneous within-group variance. The antilogs of log means are reported. Differences between control means and means of each drug-treated group were compared using single degree of freedom F tests (15) at the 0.05 level of significance.

## RESULTS AND DISCUSSION

#### Effects of Triton Dose and Fasting on Hyperlipidemia

To establish the effects of dose and fasting on the characteristics of Triton-induced hyperlipidemia, rats were administered iv 250, 350 or 450 mg Triton/kg. Some groups were fasted 24 hr before injection and bled 24, 36 and 48 hr later. Other groups were fasted when injected and bled 24, 36, 48, 60 and 72 hr later. Hypercholesterolemia increased with Triton dose, reaching maximum levels at about 24, 36 and 48 hr after 240, 350 and 450 mg/kg doses, respectively, and decreased thereafter (Fig. 1). In contrast the marked hypertriglyceridemia induced by these Triton doses reached maximum levels by 24 hr and began to decrease almost immediately (Fig. 2). In general the relative magnitude of the animal to animal standard deviation (expressed as per cent) decreased as lipid levels increased with Triton

TABLE V

Effects of Hypolipidemic Agents in the Triton Test

| Treatment | Drug dose, mg/kg | Serum cholesterol mg/dl | Serum cholesterol T/C | Serum triglycerides mg/dl | Serum triglycerides T/C |
|---|---|---|---|---|---|
| Control | 0 | 163 | | 115 | |
| Nafoxidine HCl | 3.3 | 97[a] | .60 | 63[a] | .55 |
| | 1.1 | 117[a] | .72 | 75 | .65 |
| | 0.37 | 105[a] | .64 | 69[a] | .60 |
| Triparanol | 90 | 145 | .89 | 135 | 1.17 |
| | 30 | 122[a] | .75 | 69[a] | .60 |
| | 10 | 140 | .85 | 89 | .77 |
| D-Thyroxine Sodium | 3.3 | 100[a] | .61 | 95 | .83 |
| | 1.1 | 101[a] | .62 | 74 | .64 |
| | 0.37 | 117[a] | .72 | 88 | .77 |
| Nicotinic Acid | 270 | 98[a] | .60 | 89 | .77 |
| | 90 | 136 | .83 | 114 | .99 |
| | 30 | 148 | .91 | 116 | 1.01 |
| Clofibrate | 270 | 105[a] | .64 | 74 | .64 |
| | 90 | 140 | .86 | 94 | .82 |
| | 30 | 165 | 1.01 | 104 | .90 |
| Standard Deviation, % | | 30 | | 69 | |

[a]Significantly different from control.

dose (Table I). These data illustrate the advantage of using prefasted rats in the screening procedure. Prefasted rats removed cholesterol and triglycerides at a slower rate than rats fasted at the time Triton was injected (Figs. 1,2). Variance in lipid levels, particularly triglycerides, also was less in prefasted animals (Table I).

#### Triton Blood Levels and Hyperlipidemia

Interrelationships between serum cholesterol, triglycerides and Triton levels during the post-Triton period are shown in Figure 3. Ten rats, fasted 24 hr prior to receiving 225 mg Triton/kg iv, were sacrificed at each time interval. Triglycerides increased rapidly, accompanied by a slower increase in serum cholesterol, both reaching peak levels at 20-24 hr. Triton concentration decreased rapidly for the first 4 hr, followed by a slower decline continuing to 43 hr. Friedman and Byers (5) reported similar relative responses of cholesterol and triglyceride levels at higher Triton doses, but their relationship to Triton serum levels has not been reported previously.

In screening, test animals are sacrificed routinely at 43 hr post-Triton in order to detect compounds which affect either the waxing or waning phases of the Triton-induced hyperlipidemia, or both.

#### Effects of Triton Dose on Assessment of Drug Hypocholesterolemic Activity

Three groups of 10 rats each fasted 24 hr received Triton at doses of 200-450 mg/kg. At each dose level one control group was administered methylcellulose vehicle and the other two groups were treated with a total of 5 mg U-10,387 (3-methyl-5-isoxazolecarboxylic acid)/kg or 25 mg U-23,469 (3-[p-(1,2,3,4-tetrahydro-3-methoxy-2-phenyl-1-naphthyl) phenoxy]-1,2-propanediol)/kg in divided doses 0 and 20 hr post-Triton. These compounds were selected as standards because of the consistent hypocholesterolemic response which they produce in the Triton test. Although variance expressed as per cent standard deviation was less with high Triton doses, the activity of the standard compounds also was decreased (Table II). Both compounds were hypocholesterolemic in rats given 200 or 250 mg Triton per kg, but only U-10,387 was active in rats given higher Triton levels. However that activity was minimal and inconsistent. Thus since high doses of Triton masked the activity of hypolipidemic agents, 200-250 mg/kg was selected as the most appropriate Triton dose range for the test system.

#### Route of Triton Administration

Although Triton was routinely administered iv, preliminary studies indicated that serum lipids also were elevated following Triton ip. Since animals could be injected more quickly by this route, its use would increase the number of rats which could be included in each screening test. Therefore the responses to standard hypolipidemic agents were compared in

TABLE VI

Comparison of Hypolipidemic Activities of Polysacchrides Administered Orally and Parenterally in the Triton Test

| Treatment | Route of administration: ip[a] Cholesterol mg/dl | ip[a] Triglycerides mg/dl | po Cholesterol mg/dl | po Triglycerides mg/dl |
|---|---|---|---|---|
| Control[b] | 130 | 89 | 119 | 63 |
| Methylcellulose (40 mg/kg)[c] | 107[d] | 61 | 105 | 56 |
| Zymosan (30 mg/kg) | 90[d] | 69 | 132 | 86 |
| Amylose (30 mg/kg) | 99[d] | 52[d] | 114 | 46 |
| Chitin (30 mg/kg) | 106[d] | 65 | 122 | 77 |
| Standard deviation, % | 18 | 43 | 24 | 61 |

[a]Administered ip in two 1 ml doses instead of the usual two 2 ml doses administered po.
[b]Sterile water.
[c]Equivalent to usual dose administered as methylcellulose vehicle.
[d]Significantly different from control.

animals receiving 225 mg/kg Triton iv or ip. In each group 30 rats served as controls (vehicle alone) and groups of 10 rats each were given 0.37, 1.1 or 3.3 mg U-10,387/kg, 10 mg U-23,469/kg or 30 mg sodium carbonate/kg.

In general, lipid levels in control animals were lower in the groups which had been given Triton ip (Tables III and IV). In these animals 3.3 mg U-10,387/kg reduced cholesterol in four out of five studies, the 1.1 mg/kg dose was active in one out of five and the 0.37 mg/kg dose was not active in any. The 10 mg/kg dose of U-23,469 lowered cholesterol in two out of five studies. In contrast to these results, in animals given Triton iv both 3.3 mg/kg and 1.1 mg/kg doses of U-10,387 reduced cholesterol in five out of five studies and 0.37 mg U-10,387 and 10 mg U-23,469/kg were active in four out of five studies. A significant elevated response above control levels was observed with the sodium carbonate negative standard in one out of five studies. The groups given Triton ip consistently exhibited greater response variation in cholesterol levels than the animals in which Triton was administered iv.

Similar results were observed when triglyceride levels were compared. In animals given Triton ip both the 3.3 mg/kg and 1.1 mg/kg levels of U-10,387 were active in two out of five studies. No other significant responses were found. The two larger doses of U-10,387 were active in five out of five studies when the animals were given the Triton iv. The 0.37 mg/kg dose was active in four out of five studies and U-23,469 was active in two out of five. Increased triglyceride levels with the negative standard also were observed in the group with increased cholesterol levels. As with cholesterol, the variation in triglyceride levels was much greater in animals administered Triton ip.

**Quality Control**

The test system in its final form consists of animals fasted for 24 hr and injected with 225 mg Triton per kg iv. Each screening test consists of 30 controls, 10 rats treated with positive standard (1.1 mg U-10,387/kg) and 10 rats given negative standard (30 mg sodium carbonate/kg). The remaining rats are used for test compounds. The activity of each compound is evaluated by comparing its mean response with that of the controls as described above.

The data in Table III from animals receiving Triton iv was used to determine the expected performance of the test system. The operating characteristic curve for cholesterol (Fig. 4) gives the probability of declaring a test drug active as a function of its true but unknown activity. These curves were constructed by the methods provided by Pearson and Hartley (16) and from tables given by Tiku (17). Since all analyses are conducted on logarithms of the original lipid concentrations, the activity scale is expressed as the difference ($\Delta$) between the logarithms of the drug and control means. This curve shows that the probability of declaring a drug active which has a true $\Delta$ of 0.16 is 0.95. The level of activity obtained with 1.1 mg U-10,387 per kg in the five studies reported in Table III was 2.2330-2.0682 or $\Delta$=0.1648. The probability of declaring test substances active which have no effect on serum cholesterol ($\Delta$=0) is 0.05.

The operating characteristic curve for triglyceride levels (Fig. 5) shows that for this endpoint greater levels of activity are required for a compound to be declared active. The probability of declaring a drug active which has a true $\Delta$ of 0.32 (the value observed with 1.1 mg U-10,387/kg) is approximately 0.85. The decreased sensitivity is mainly a reflection of

greater variation in triglyceride levels between animals.

Control charts were maintained for the positive and negative standards and the controls to monitor performance of the test system. Comparison of expected responses (Fig. 4) with actual responses from 60 consecutive tests showed that the positive standard U-10,387 was declared hypocholesterolemic in 95% of the tests, and the negative standard sodium carbonate was declared active in 7%. For serum triglycerides the detection rate of the positive standard was 75%, somewhat less than predicted (Fig. 5). Sodium carbonate was declared active in 5% of the tests.

#### Effects of Known Hypolipidemic Drugs

In addition to U-10,387 and U-23,469, several other compounds with previously demonstrated serum lipid lowering activity in experimental animals, in man or both were used to evaluate the Triton test system (Table V). Nicotinic acid, like U-10,387, depresses plasma FFA levels by inhibiting lipolysis (18). *D*-Thyroxine (19), nafoxidine hydrochloride (U-11,100A) (20) and U-23,469 probably exert their hypocholesterolemic effects by enhancing cholesterol excretion. Clofibrate inhibits cholesterol biosynthesis prior to mevalonate (21) and triparanol inhibits conversion of desmosterol to cholesterol (22). These compounds with different mechanisms of actions all lowered serum cholesterol in the Triton-induced hyperlipidemic rat. Nicotinic acid and clofibrate would not have been detected at our normal screening dose of 30 mg/kg, but the data in Table IV indicate that the system is responsive to compounds with these types of activity. Although results are given for lower doses of nafoxidine hydrochloride and *D*-thyroxine, these agents also are active at 30 mg/kg.

In their tests Garattini et al. (9) determined activity of hypolipidemic drugs administered ip. In our Triton test system we have chosen to screen compounds orally in order to approximate more closely the desired dosage form to be used in man. Although lipid lowering drugs administered ip are active in Triton-induced hyperlipidemic rats, it appears that less specificity for intrinsic hypolipidemic activity is obtained using the parenteral route. We have found that substances with no apparent direct effects on lipid metabolism such as amylose, chitin, zymosan and methylcellulose do not affect serum lipids in Triton-treated rats when administered orally, but reduce both cholesterol and triglycerides when administered ip (Table VI), presumably by stimulating the reticuloendothelial system (23,24) and enhancing lipoprotein clearance.

Thus our standardized procedure for induction of hypercholesterolemia and hypertriglyceridemia in rats with triton WR 1339 provides a screening method for the detection of new lipid lowering agents with a wide variety of activities. The Triton test is sensitive to compounds which have been shown to be active in man, and our results with these standard hypolipidemic agents show that the test is reproducible and conforms well to performance levels predicted from statistical analysis.

ACKNOWLEDGMENTS

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# 2-Alkylcyclobutanones From the Radiolysis of Triglycerides


## ABSTRACT

A cyclic compound was isolated from the radiolytic products of each of the simple triglycerides containing $C_6$, $C_8$, $C_{10}$, $C_{12}$, $C_{14}$, $C_{16}$ and $C_{18}$ fatty acids. In each case the compound was identified as the 2-alkylcyclobutanone of the same carbon number as the precursor fatty acid. A mechanism is proposed for the production of these compounds which involves the formation of a six-membered ring intermediate, cyclization and cleavage at the acyl-oxy bond.


In the course of a recent investigation on the radiolysis of tricaproin, an odorous compound was isolated which eluted after *n*-hexanal on SE-30 gas chromatograph (GC) columns, and gave rise to a mass spectral pattern very similar to that of hexenal. Aldehydes with the same chain length as the triglyceride fatty acids and possessing an additional double bond had been tentatively identified by Dubravcic and Nawar (1) in seven higher triglycerides which were irradiated at 6 Mrads and 25 C. However when attempts were made to determine the position of unsaturation by ozonolysis in the compound isolated from tricaproin, it was discovered that this compound did not contain a double bond. The compound was finally identified conclusively as 2-ethylcyclobutanone. Its retention on two different GC columns, and its IR and mass spectra (shown in Figure 1) were identical to those of an authentic sample synthesized in the laboratory by the method of Hanach and Herterich (2).

The corresponding compounds were conse-

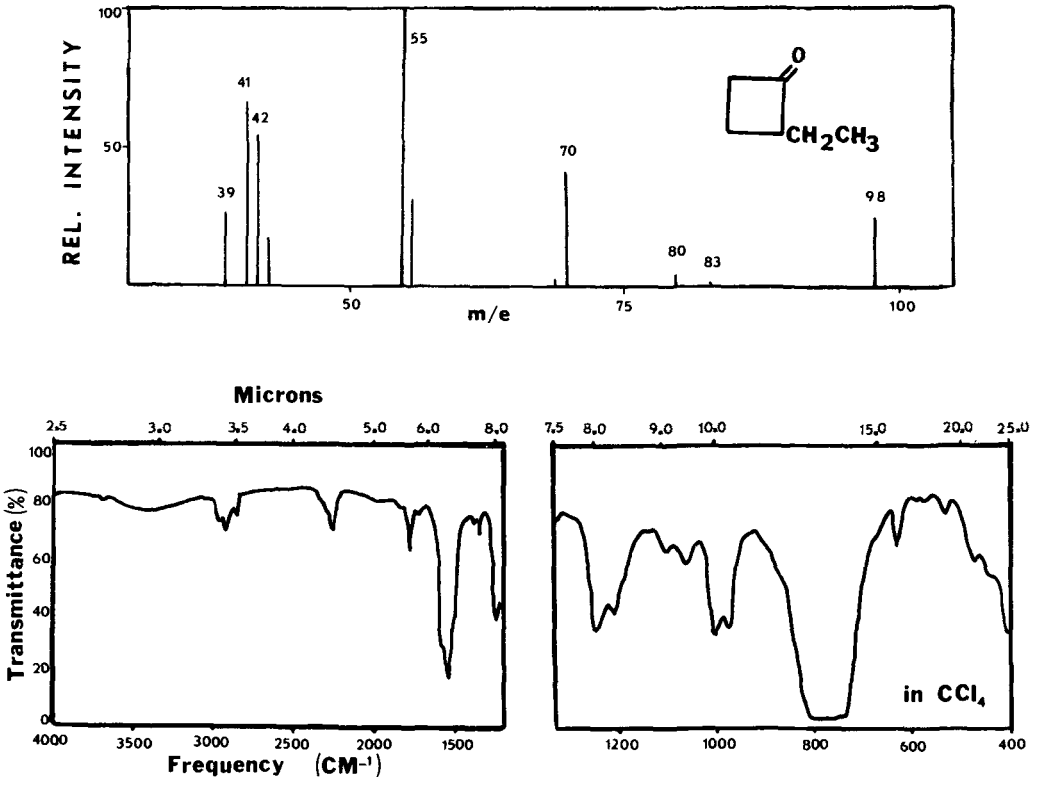
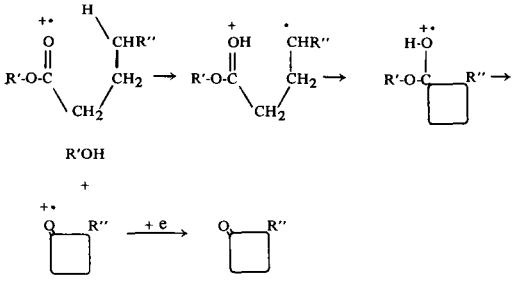



FIG. 1. Mass and IR spectra of 2-ethylcyclo-butanone.

quently isolated from the radiolytic products of the simple triglycerides tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin and tristearin. The triglycerides were irradiated and analyzed as previously described by Nawar et al. (3), and in each case the compound eluting on SE-30 immediately following the *n*-alkanal of the same chain length as the triglyceride fatty acid was subjected to IR and mass spectral analyses. In addition the compound recovered from tripalmitin was analyzed by NMR and that recovered from trimyristin was analyzed by high resolution mass spectrometry. All of these compounds, which were previously suspected of being unsaturated aldehydes, were found to be 2-alkylcyclobutanones of the same carbon number as the esterified fatty acid. Tributyrin did not yield a cyclobutanone upon irradiation.

It is proposed that these compounds may result from cleavage at the acyl-oxy bond via the formation of a six-membered ring intermediate as follows:




R' is the glyceryl moiety plus two acyl groups, and R'' is the remainder of the fatty acid chain. This pathway is similar to the mechanism leading to the formation of methylcyclobutanol in the photolysis of 2-pentanone (4).

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[Received October 18, 1971]

# Phospholipids of Fish Gills

**ABSTRACT**

Lipids were extracted from gill filaments of nine species of fish. Individual phospholipids as a percentage of the total were determined. These data revealed a pattern which did not appear to vary between pre- and postspawning salmon or among fish living at greatly differing ocean depths.

During two expeditions of the R/V Alpha Helix we had the opportunity to analyze individual phospholipids in gills of several species of fish. The gill primarily functions in respiration and osmoregulation, i.e., transport of gases and ions across membranes. Pink and coho salmon *(Oncorhynehus gorbuscha* and *O. kisutch)* were netted in coastal waters of British Columbia. Kelp bass *(Paralabrax clathratus)*, sand bass *(P. maculatofasciatus)*, whitefish *(Caulolatilus princeps)*, and sheepshead *(Pimelometopon pulchrum)* were caught with hook and line in 25-50 m water off Isla de Guadalupe (Mexico). Pacific rattail *(Corphaenoides acrolepis)*, flatnose codling *(Antimore rostrata)*, and sablefish *(Anaplopoma fimbria)* were caught by sunken traps or lines which were subsequently released to the surface from depths of 2000-3000 m. Sablefish were caught off San Diego, the other two species near Isla de Guadalupe.

Gills were removed immediately from the freshly caught fish. Gill filaments were clipped from the arches with surgical scissors and then extracted with chloroform-methanol 2:1 v/v by the method of Folch et al. (1). Phospholipids in the extracts were isolated by silicic acid column

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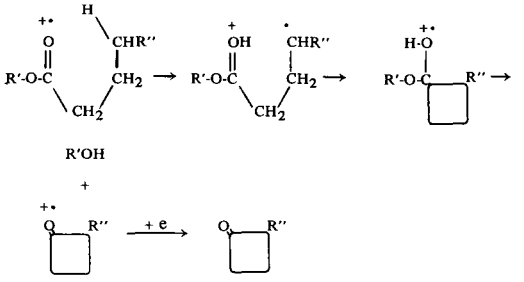


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TABLE I

Composition of the Phospholipids in Gill Filaments of Some Species of Fish, %

| Phospholipid[a] | Coho salmon (2) | Pink salmon (2) | Kelp bass (1) | Sand bass (1) | White-fish (3) | Sheeps-head (2) | Pacific rattail (1) | Flatnose codling (1) | Sable-fish (3) |
|---|---|---|---|---|---|---|---|---|---|
| PC | 56.1 | 52.9 | 62.3 | 61.8 | 56.6 | 58.6 | 73.8 | 57.6 | 46.9 |
| PE | 24.9 | 27.1 | 23.2 | 17.6 | 15.7 | 23.3 | 5.7 | 21.1 | 22.0 |
| Sp | 2.5 | 5.0 | 2.7 | 6.4 | 15.4 | 7.2 | 10.6 | 8.0 | 9.7 |
| PS | 8.4 | 7.2 | 7.0 | 9.7 | 7.2 | 6.4 | 1.6 | 7.9 | 11.4 |
| PI | 3.3 | 5.3 | 4.9 | 5.7 | 5.1 | 4.6 | 4.5 | 3.1 | 4.0 |
| PA | Trace | Trace | Trace | nd | nd | nd | nd | nd | 2.3 |
| DPG | 2.4 | 2.7 | Trace | Trace | Trace | Trace | Trace | Trace | 4.5 |
| LPC | 2.4 | Trace | nd | nd | nd | nd | 4.0 | 1.8 | Trace |

[a]Values are mean percentages of total lipid phosphorus for the number of animals indicated in parentheses; trace $<1.5\%$; nd, not detected; abbreviations: PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; Sp, sphingomyelin; PS, phosphatidyl serine; PI, phosphatidyl inositol; PA, phosphatidic acid; DPG, diphosphatidyl glycerol; LPC, lysophosphatidyl choline.

chromatography (2). The individual phospholipids were resolved on two dimensional thin layer chromatography (TLC) plates (3) and identified by use of selective spray reagents and co-chromatography with authentic references as previously employed at this laboratory (3-6). The individual phospholipid components isolated by two dimensional TLC were scraped from the plates and quantified by phosphorus analysis (7).

Replicate TLC separations (3-6) of each sample were analyzed. The standard deviations of the analyses did not exceed 10% of the analyzed value for major components (10% of the total lipid phosphorus) or more than 25% of the value for minor components (1.5-10% of total lipid phosphorus). The total lipid content of gill filaments on a wet weight basis was 1-2%.

In 9 of the 16 fish for which data are given in Table I, total phospholipids were quantified and averaged 69.5% of total gill lipids. The range was from 59.8-76.4%. Thus it would appear that phospholipids are the dominant lipid class in the gill filament. Free cholesterol, identified by $R_f$ and specific color reaction on TLC plates, was observed to be the principal component of the neutral lipid fraction. The proportions of individual phospholipids as a function of total phospholipids of gill filaments from the various fish are presented in Table I. To our knowledge such data have not been collected previously. They have a rather uniform pattern with the exception of the Pacific rattail which showed relatively low levels of phosphatidyl ethanolamine and phosphatidyl serine. Phosphatidyl choline and phosphatidyl ethanolamine account for two-thirds or more of the gill phospholipids in all species. The proportion of sphingomyelin in the phospholipids exhibited the greatest variation. All of the spots detectable on TLC plates with iodine vapor contained phosphorus.

We had thought that such stresses as spawning migration of the salmon into fresh water or pressure effects of great depths (rattail, codling and sablefish) might be reflected in different composition of gill membranes; however no profound differences were observed. Pre- and postspawning pink salmon yielded data for gill phospholipid composition which were identical within the experimental error of the method. Moreover perfusion of the gills with water to remove blood had no significant effect on the percentage composition of phospholipids in the gill filaments. Investigations of the several cell types (8) in the gill and more refined analyses of the quantities of the various gill lipids and their fatty acid compositions may reveal discrete effects of the variables mentioned. The lipid composition of gills or organelle membranes of gills have been studied in a few species of fresh water fish (9-11). The findings indicate that in the goldfish *(Carassius auratus)* differences in temperature acclimation produce small but significant variations in the proportions and fatty acid composition of individual phospholipids in gill mitochondria (9,10). It is also possible that the effects of such factors as temperature, depth or salinity on gill function are adjusted at the level of enzyme regulation rather than by changes in tissue or membrane lipid composition.

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[Revised manuscript received November 29, 1971]

# Further Evidence for the Interconversion of Monophosphoinositides in Vivo

**ABSTRACT**

The incorporation of $^{3}$H-inositol into the molecular species of rat liver monophosphoinositides was studied as a function of time. At early time intervals following intraperitoneal injection of the tracer, the specific activity of the monoenoic and dienoic species exceeded that of the total and tetraenoic inositides by 7- and 14-fold. From 90 min to 9 hr, the specific activity of the unfractionated phosphatide remained nearly constant while the pronounced decrease in activity of the monoenoic and dienoic subfractions was concomitant with an increase in the radioactivity of the tetraenoic species, as found previously with $^{32}$P- and $^{14}$C-glycerol. The present results suggest that the entire glycerophosphorylinositol backbone originally associated with the monoenoic and dienoic monophosphoinositides is converted into tetraenoic species with remarkable conservation of label in vivo.

Recently we reported (1) on the differential incorporation of orthophosphate-$^{32}$P and glycerol-2-$^{14}$C among individual molecular species of phosphatidylinositol of rat liver in vivo. The results suggested that the $\alpha$-glycerophosphate moiety originally associated with the monoenoic and dienoic species was being transferred to the tetraenoic species with time. Since only a small per cent of the total lipid radioactivity was located in the inositides at all times (1), it was possible that the label accumulating in the tetraenoic fraction was derived from other glycerophosphatides by transphosphatidylation. It remained to be established that the interconversion of the molecular species of monophosphoinositides (MPI) in vivo also occurred with radioactive myo-inositol which is a specific precursor for MPI.

The myo-inositol-2-$^{3}$H (3470 $\mu$c/$\mu$M) was purchased in high purity (>99%) from the New England Nuclear Corp., Boston, Mass. For injection, the labeled inositol (0.013 mg in 0.250 ml of ethanol-water 3:2 v/v) was diluted with 0.9% NaCl to give 40 $\mu$c/ml solutions. The animals used in the study were male Wistar rats (310-330 g) which had been maintained on a Purina Chow diet for 1 week prior to experimentation. After a 4 hr access to water alone, the animals were injected intraperitoneally at 12 noon with 20 $\mu$c of the radioactive inositol and sacrificed in pairs at 15, 90, 300 and 540 min. The specific activity of the MPI was determined after separation of the intact molecules according to degree of unsaturation (1).

The incorporation of the administered radioactivity into the chloroform soluble hepatic lipids is given in Table I. It is seen that an average of 2.0, 4.7, 5.1 and 5.6% of the injected dose of $^{3}$H-inositol was recovered at 15, 90, 300 and 540 min, respectively. Thus maximum incorporation was reached by 90 min, after which the total radioactivity showed little change. At all times studied, 92-95% of the activity was associated with the monophospho-

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TABLE I

Distribution of Radioactivity Among Rat Liver Lipids[a] After Administration of Inositol-$^3$H

| Fraction[b] | Total radioactivity, % | | | |
|---|---|---|---|---|
| | 15 min. | 90 min. | 300 min. | 540 min. |
| Neutral lipid | 1.4 | 1.5 | 0.7 | 0.6 |
| PE | 0.5 | 0.9 | 0.7 | 0.8 |
| MPI | 94.3 | 93.4 | 92.3 | 94.0 |
| PC | 3.0 | 3.8 | 3.5 | 4.0 |
| Sph, LPC | 0.8 | 0.4 | 2.8 | 0.6 |
| Total,[c] % ID | 1.96 | 4.72 | 5.07 | 5.56 |

[a]Lipids were separated by thin layer chromatography according to Skipski et al. (2). Each value is an average from two animals.

[b]Abbreviations: PE, phosphatidylethanolamine; MPI, monophosphoinositide; PC, phosphatidylcholine; Sph, sphingomyelin; LPC, lyso-phosphatidylcholine. The neutral lipid fraction contains cardiolipin and MPI contains phosphatidylserine.

[c]ID was 20 μc of myo-inositol-$^3$H.

inositide. The amount of the tritium label associated with the glycerol or fatty acid moieties was negligible as evidenced by only traces of radioactivity present in the neutral lipids.

Figure 1 gives the per cent distribution of radioactive inositol among the various molecular species of rat liver MPI. At 15 min, the monoenes plus dienes, trienes, tetraenes and polyenes contained an average of 36.7, 4.2, 41.2 and 17.9% of the total incorporated radioactivity, although the mass distribution of the species was 5.5, 6.5, 80.7 and 7.3%, respectively. The proportion of total activity recovered in the monoenes plus dienes decreased with time (90 min to 9 hr) to about 8% while that of the tetraenes rose steadily and had reached 72% by the end of the experiment.

Evidence for a transfer of label from the monoenic and dienoic into the tetraenoic species is supported by the specific activity data plotted in Figure 2. Initially the specific activity of all the molecular species was found to be increasing, as was that of the total unfractionated MPI. However over the period of 90 min to 9 hr, the specific activity of the monoenoic and dienoic inositides showed a drastic decrease (3.9-fold) concomitant with an increase in the activity of the tetraenoic fraction (2.2-fold). Furthermore the specific activity of the total unfractionated MPI remained essentially constant over this same time interval. The specific activity of the monoenoic plus dienoic inositides was initially 7- and 14-fold the total and tetraenoic species, respectively. This difference was only 1.5- and 1.7-fold by

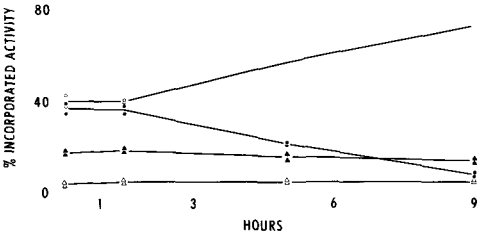


FIG. 1. Per cent distribution of radioactivity among molecular species of rat liver MPI after intraperitoneal injection of myo-inositol-$^3$H. •, Monoenes + Dienes; △, Trienes; ○, Tetraenes; ▲, Polyenes. Each point represents separate animals.

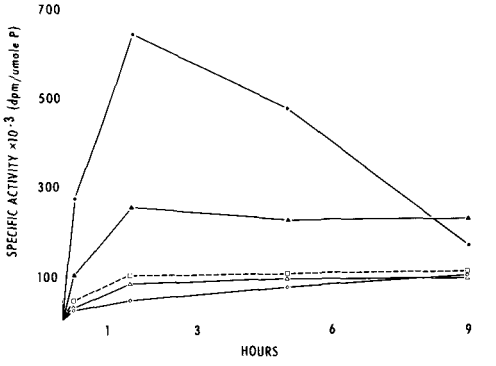


FIG. 2. Specific activities of the molecular species of rat liver MPI after intraperitoneal injection of myo-inositol-$^3$H. □, unfractionated MPI; symbols for species as given in legend to Figure 1. The concentrations of the MPI in μmoles per liver were as follows: Total, 21.42 ± 3.02; Monoenes + Dienes, 1.17 ± 0.21; Trienes, 1.40 ± 0.57; Tetraenes, 17.28 ± 0.79; Polyenes, 1.57 ± 0.63 (values are means ± SD, $n$ = 5).

the ninth hour. The specific activity of the monoenoic and dienoic approached that of the tetraenoic MPI when the latter neared their maximum; this would be expected if the former were precursors for the latter (3). In contrast, the initial specific activities of the trienoic and pentaenoic plus hexaenoic molecules showed only minor changes during the 9 hr period.

On the basis of previous work with glycerol, it would be expected that the initial activity in the dienes would be largely associated with the palmitoyl linoleate species. At the end of the experiment, however, the label would be expected largely in the stearoyl arachidonate in agreement with the mass distribution. This could occur by a stepwise transformation via palmitoyl arachidonoyl or stearoyl linoleoyl intermediates, since both stearate and arachidonate readily enter glycerophosphatides via acyl transfer (4-6). At present there is no method for separating intact MPI into the

palmitoyl and stearoyl species which would be required for an identification of the intermediates.

The potential operation of a deacylation-reacylation cycle in vivo had been previously indicated from in vitro studies on the phosphatidylcholines and phosphatidylethanolamines (6,7) as well as MPI (8,9). Differences in the labeling of the MPI species with $^{3}$H-inositol and $^{14}$C-glycerol (1) at early time periods may reflect the entry of free inositol into MPI by simple exchange and de novo synthesis (10). The biological significance of the above demonstrated interconversion of molecular species awaits elucidation.

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# Fatty Acid Interrelationships in Plasma, Liver, Muscle and Adipose Tissues of Cattle Fed Safflower Oil Protected From Ruminal Hydrogenation

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## ABSTRACT

Steers were given diets containing formaldehyde-treated casein-safflower oil supplements, in which the constituent 18:2 was protected from ruminal hydrogenation. A similar group was given unsupplemented diets. The fatty acid compositions of plasma, liver, muscle and adipose tissue lipids were determined in both groups of cattle after 0, 2, 4 and 8 weeks of experimentation. The proportion of 18:2 in the triglycerides was markedly increased on feeding the supplement and the rate of incorporation into the plasma triglycerides was higher than that in the triglycerides of muscle and adipose tissue. Associated with this increase there were compensatory decreases in the proportions of 16:0 and 18:1 but no consistent change in the proportion of 18:0. The proportion of 18:2 in the plasma phospholipids and cholesteryl esters was initially much higher than in the triglycerides and this was further increased by feeding the safflower oil supplement. A linear relationship existed between the proportion of 18:2 in the phospholipids and cholesteryl esters of plasma. The supplement also caused substantial increases in the proportion of 18:2, both in phospholipids from liver and muscle and in cholesteryl esters from liver, and there were compensatory decreases in the proportions of other unsaturated fatty acids, e.g., 18:1, 18:3, 22:6. These studies demonstrate that when ruminal hydrogenation was circumvented by feeding formaldehyde-treated casein-safflower oil particles, the linoleic acid was absorbed and the pattern of incorporation into plasma and tissue lipids was similar to that in nonruminants.

## INTRODUCTION

Rumen microorganisms are responsible for the lipolysis of dietary fats and the hydrogenation of their constituent $C_{18}$ polyunsaturated fatty acids (1-4). Thus only small amounts of polyunsaturated fatty acids are absorbed from the small intestine and ultimately incorporated into the tissue lipids of ruminants (5,6). On the other hand, the products of the hydrogenation reactions, e.g., *trans* 11, *cis* 15 octadecadienoic, *trans* 11 octadecenoic, *cis* 9 octadecenoic and octadecanoic acids, are absorbed from the small intestine in substantial amounts, and are incorporated into the triglycerides and phospholipids of ruminant species (5,6). However polyunsatu-

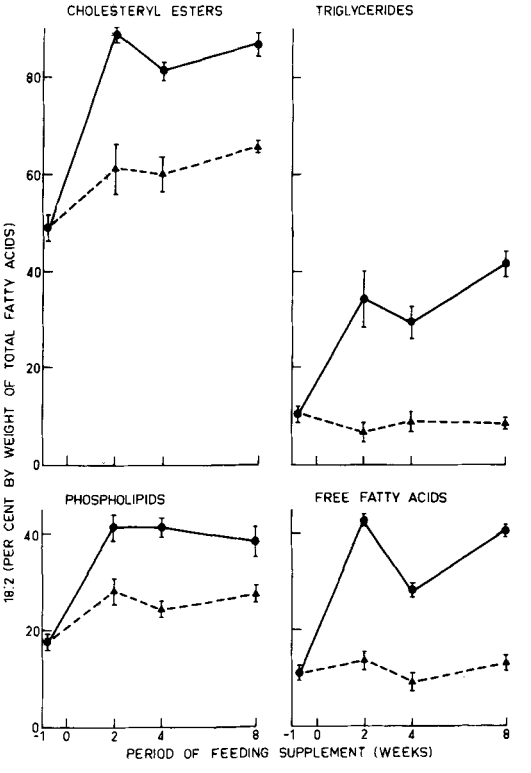


FIG. 1. Incorporation of dietary 18:2 into plasma lipids. ●——● supplemented diet, ▲- - - -▲ unsupplemented diet. Mean values (with standard errors) for three animals on each diet.

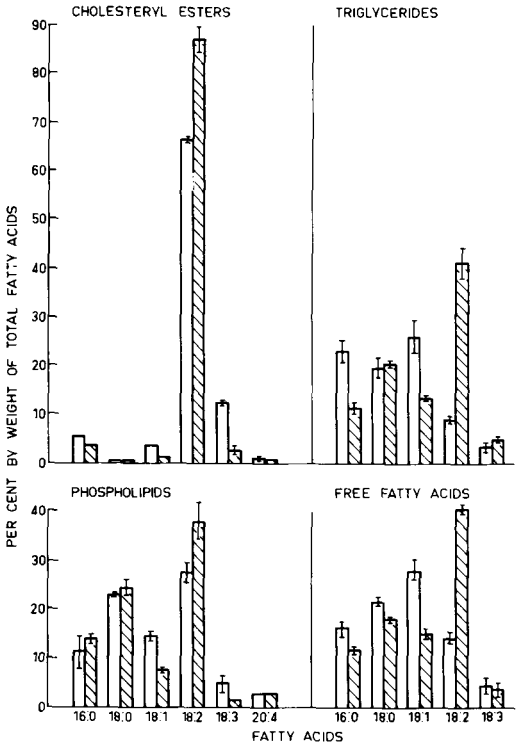


FIG. 2. Fatty acid composition of plasma lipids from cattle fed supplemented ▨ and unsupplemented □ diets. Mean values (with standard errors) for three animals after 8 weeks of feeding the maximal level of supplement.

rated fatty acids, administered directly into the abomasum or duodenum (7,8) or fed to ruminants in the form of formaldehyde-treated protein-oil particles (9-11), avoid ruminal hydrogenation and are absorbed and incorporated into tissue and milk lipids. This circumventing of hydrogenation in ruminants presumably creates a pattern of lipid metabolism which resembles that in nonruminants, i.e., the glycerides in an unchanged form are hydrolyzed by pancreatic lipases in the small intestine to form monoglycerides and free fatty acids, and these components are used in the synthesis of chylomicron triglycerides by the intestinal mucosal cells (12,13). The chylomicra are transported throughout the body and the constituent fatty acids are used in the biosynthesis of tissue and plasma lipids (14). Thus when the diets of monogastric animals and young suckling ruminants contain large proportions of polyunsaturated lipids, the fatty acids, e.g., linoleic, are incorporated into the tissue and plasma lipids and there are compensatory changes in the proportions of other acids (5, 15-21).

The present experiments were designed to examine the fatty acid patterns and the interrelationships that exist between plasma, liver, muscle and adipose tissue from ruminants fed large amounts of linoleic acid protected from ruminal hydrogenation.

## EXPERIMENTAL PROCEDURES

### Animals and Diets

The animals used in this study comprised 20 9-month-old Friesian steers (mean wt 186 kg), which had previously been grazing a rye grass-clover pasture. Two of these animals were slaughtered prior to the start of the experiment to provide initial tissue and blood samples. The 18 remaining steers were randomly divided into two groups of animals of similar body weight. One group (unsupplemented) was offered 8 kg/day of a ration containing rolled barley and chopped alfalfa hay 5:3 w/w, for the first 2 weeks of the experiment. Thereafter this group was offered a ration (8 kg) containing rolled barley, chopped alfalfa hay and peanut meal 95:57:10 w/w/w. The other group (supplemented) was given a ration (8 kg) containing rolled barley, chopped alfalfa hay and formaldehyde-treated casein-safflower oil 5:3:2 w/w/w. During the first 6 days the amount of formaldehyde-treated supplement was gradually increased to the desired level (20% of the total diet). The supplement (containing equal parts by weight of casein and safflower oil) was prepared using spray drying techniques as previously described (10). It was tested in vitro and found to be resistant to ruminal hydrogenation (10,11). The safflower oil used in the preparation of the supplement contained 75% 18:2. The animals on the supplemented diet consumed 60-80% of the ration offered; further details of the dietary intakes and live weight gains are reported elsewhere (22).

### Collection of Samples

Three animals from each group were slaughtered 2, 4 and 8 weeks after feeding the maximal level of supplement. Samples (2 g) of liver, muscle (*M. psoas major*) and subcutaneous (hind leg), perirenal and omental fats were collected immediately postslaughter, frozen on solid $CO_2$ and stored in chloroform-methanol 2:1 v/v under nitrogen at -17 C. Muscle tissue was dissected free of adhering adipose tissue prior to freezing with solid $CO_2$. Blood (50 ml) was collected from animals at intervals of two weeks by jugular puncture into heparinized bottles and, after centrifugation, the plasma was removed and stored at -17 C.

### Lipid Extraction and Fatty Acid Analyses

All lipids were extracted using the procedure of Folch et al. (23). Plasma, liver and muscle lipids were further fractionated by thin layer chromatography on silica gel (Adsorbosil-1) using a solvent system of petroleum ether (BP 40-60 C):diethyl ether:acetic acid (86:14:1 by volume). The thin layer chromatograms were sprayed with a solution of 2,7-dichlorofluorescein (0.2% w/v) in ethanol and the lipid components were visualized using an ultraviolet lamp. The areas of gel containing the cholesteryl esters, triglycerides and free fatty acids were removed from the plate and the lipids were eluted with diethyl ether. The phospholipids were eluted from the corresponding areas of gel with a solution of chloroform: ethanol:water:acetic acid (65:50:10:1 by volume). The lipids were saponified and the fatty acid methyl esters were prepared using diazomethane (24). Analysis of the fatty acid composition was carried out by gas chromatography (24).

## RESULTS

### Fatty Acid Composition of Plasma and Liver Lipids

The addition to the diet of formaldehyde-treated casein-safflower oil supplement markedly increased the proportion of 18:2 in all plasma lipids (Fig. 1). This effect was apparent within two weeks of feeding the maximal level of supplement and was maintained throughout the 8 week period. The proportion of 18:2 in the plasma triglycerides and free fatty acids at the commencement of feeding was 8-10% and this was increased to 30-40% by supplementation. The proportion of 18:2 in the plasma phospholipids, and more particularly in the cholesteryl esters, was initially much higher than in the triglycerides and free fatty acids and was further increased by feeding the supplement (Fig. 1). There were also small increases in the proportions of 18:2 in the cholesteryl esters and phospholipids of unsupplemented animals; this effect may have been due to the change from pasture to the alfalfa-barley diet (25).

Figure 2 shows the proportion of fatty acids in the four major plasma lipids from supplemented and unsupplemented animals after 8 weeks of feeding the maximal level of supplement (in this and Figs. 3, 5 and 7 only the major fatty acids are included; minor components, e.g., 14:0 and 16:1, have been omitted for clarity of presentation). The safflower oil-containing supplement caused substantial increases in the proportions of 18:2 together with decreases in the proportion of 18:1 in the plasma lipid classes. Supplementation also resulted in reduced proportions of 18:3 (in cholesteryl esters and phospholipids) and 16:0 (in triglycerides, free fatty acids and cholesteryl esters). The proportion of 18:0 in the plasma lipid fractions was not markedly altered by feeding the safflower oil supplement (Fig. 2).

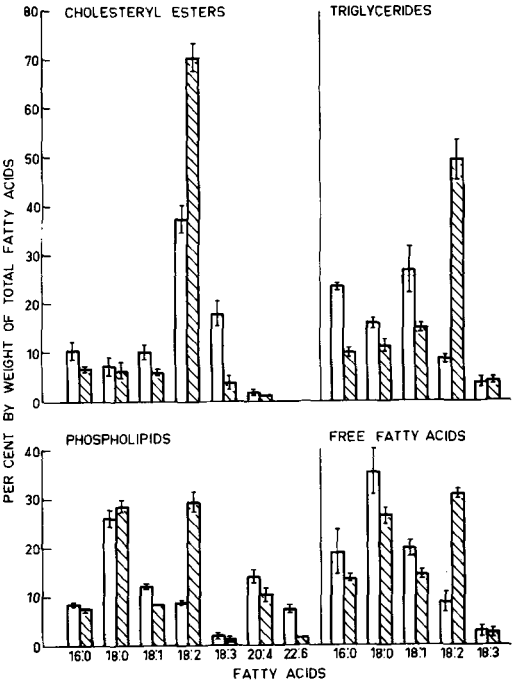


FIG. 3. Fatty acid composition of liver lipids from cattle fed supplemented ▨ and unsupplemented □ diets. Mean values (with standard errors) for three animals on each diet after 8 weeks of maximal supplementation.

The animals that had been given the formaldehyde-treated supplement also showed significant alterations in the fatty acid composition of the liver lipids after 2, 4 and 8 weeks of experimentation, and the 8 week results are shown in Figure 3. This response was similar to that observed in the corresponding lipids of plasma, i.e., the supplement caused marked increases in the proportions of 18:2 and decreases in the proportions of 18:1. Supplementation also resulted in reduced proportions of 18:3 (in cholesteryl esters), 16:0 (in triglycerides) and 22:6 (in phospholipids). The liver cholesteryl esters and phospholipids from unsupplemented steers (Fig. 3) contained lower proportions of 18:2 than did the corresponding plasma lipids (Fig. 2).

### Fatty Acid Composition of Perirenal, Omental and Subcutaneous Fats

Figure 4 shows the proportions of 18:2 in

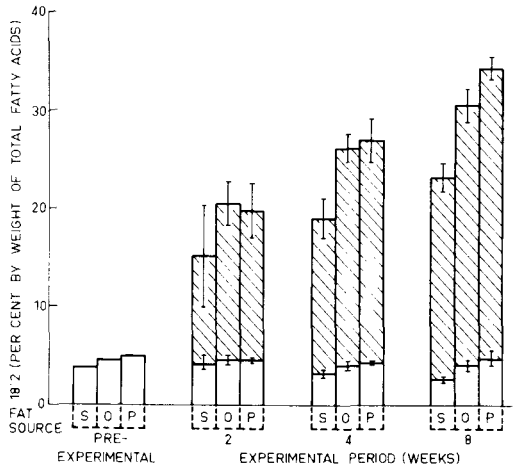


FIG. 4. Incorporation of dietary 18:2 into subcutaneous (S), omental (O), and perirenal (P) fats, ▪ supplemented diet; □ unsupplemented diet. Pre-experimental values are the means for two animals; other values are the means (with standard errors) for three animals on each diet.

the subcutaneous, omental and perirenal fats throughout the period of experimentation. The supplement caused significant increases in the proportions of 18:2 in all three depot fats. The proportion of this acid in the internal body fats (omental and perirenal) appeared to be greater than in the external (subcutaneous) fats (Fig. 4). The proportions of 18:2 in the depot fats from the unsupplemented animals remained at relatively low levels (3-5%) throughout the experiment.

Figure 5 shows the fatty acid composition of the depot fats from the supplemented and unsupplemented animals at the 8 week period. These data clearly illustrate that the increases in the proportions of 18:2 in the supplemented animals were associated with corresponding decreases in the proportions of 16:0 and 18:1; there was little change in the proportions of 18:0. These substitution effects were analogous to those observed in plasma and liver triglycerides (see Figs. 2 and 3).

The data in Figure 5 also demonstrate the characteristic differences in the relative proportions of 18:1 in the fat from different regions of the body. Thus subcutaneous fat from both supplemented and unsupplemented animals contains more 18:1 relative to 18:0 than do the deeper body fats (omental and perirenal).

### Fatty Acid Composition of Muscle Lipids

The proportions of 18:2 in the *M. psoas major* triglycerides and phospholipids were increased by feeding the maximum level of supplement for 2 weeks and there were further increases after 4 and 8 weeks (Fig. 6). The proportions of 18:2 in muscle triglycerides from the unsupplemented steers remained at relatively low values (4-6%) throughout the period of feeding. In contrast, the proportions of 18:2 in muscle phospholipids of unsupplemented steers were maintained at relatively high levels (27%) throughout the experimental period.

Figure 7 shows the fatty acid composition of the muscle triglycerides and phospholipids from both groups of steers after 8 weeks of experimentation. The pattern of alteration in the muscle triglyceride fatty acid spectrum was similar to that observed in the plasma and liver triglycerides (Figs. 2 and 3) and in depot fats (Fig. 5), i.e., the safflower oil supplement increased the proportions of 18:2 and decreased the proportion of 16:0 and 18:1. Likewise the pattern of alteration in the muscle phospholipid fatty acid composition (Fig. 7) was analogous to that observed in the plasma phospholipids (Fig. 2), i.e., the supplement caused an increase in the proportion of 18:2 and corresponding decreases in the proportions of 18:1. There was no apparent effect on the proportion of 20:4 in either plasma or tissue phospholipids.

## DISCUSSION

In ruminants, as in nonruminants, the fatty acids that are absorbed from the small intestine are resynthesized to form triglycerides, incorporated into chylomicra and transported via the lymph to appear subsequently in plasma (6,12,13). In the fed state the chylomicron triglycerides are a major fraction of the total plasma triglycerides (14). Thus in ruminants fed conventional diets, the low proportions of $C_{18}$ polyunsaturated fatty acids in the plasma triglycerides is due to the hydrogenation of the dietary 18:2 and 18:3 in the rumen prior to entering the small intestine (6). However by protecting dietary safflower oil (which contains 75% 18:2) from this hydrogenation and thereby increasing the amount of 18:2 available for intestinal absorption, we have been able to markedly increase the proportion of this acid in the plasma triglycerides of growing cattle (Fig. 1). This response is similar to that observed when oils containing 18:2 were administered directly into the abomasum of sheep (26).

The formaldehyde-treated supplement not only caused an increase in the proportion of 18:2 in plasma triglycerides but also markedly increased the proportion of this acid in the plasma free fatty acids. The proportion of 18:2

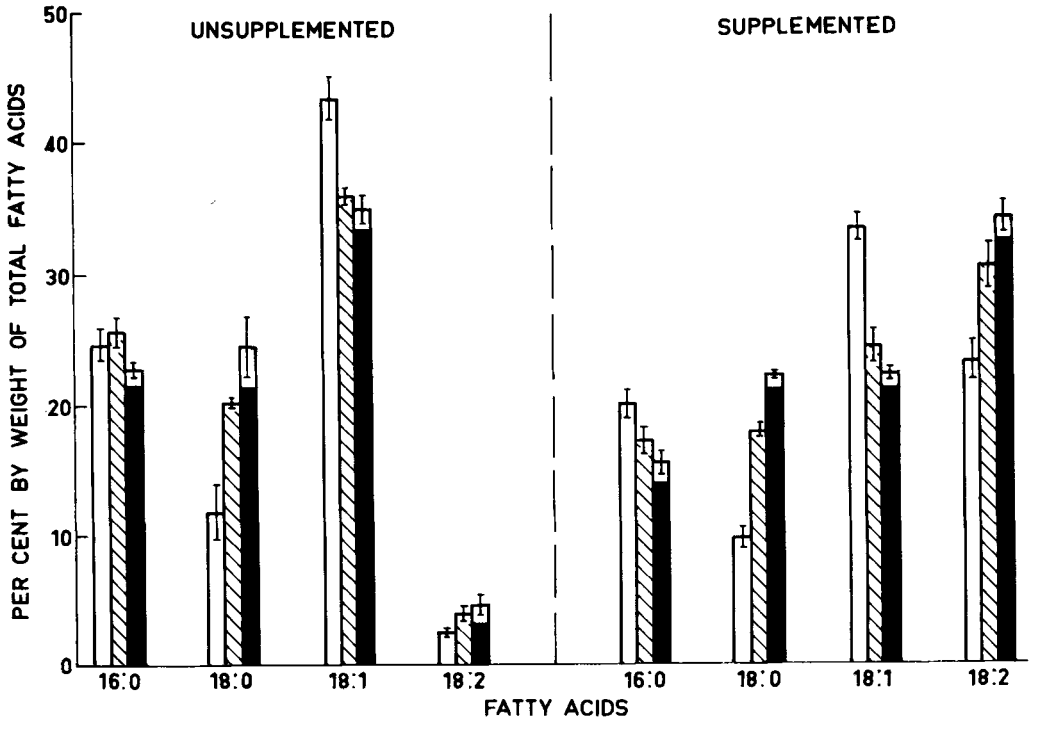


FIG. 5. Fatty acid composition of subcutaneous □, omental ▨, and perirenal ■ fats from cattle fed supplemented and unsupplemented diets. Mean values (with standard errors) for three animals on each diet after 8 weeks of maximal supplementation.

in the latter plasma lipid fraction after 2 weeks of feeding the maximum level of supplement was approximately twice that in the depot fats at this time (Figs. 1 and 4), and was similar to that occurring in the plasma triglycerides (Fig. 1). These results suggest that during this initial period the 18:2 in the free fatty acid fraction was largely derived from hydrolysis of the plasma chylomicra glycerides (14). However one cannot completely exclude the possibility that some of the 18:2 may have been derived from specific lipolysis of depot fats. The proportions of 18:2 in the triglycerides of depot fats and muscle were substantially increased by feeding the formaldehyde-treated supplement (Figs. 4 and 7), but the rate of this increase was less than that observed with the plasma triglycerides (Fig. 1). On the other hand the proportion of 18:2 in the triglycerides of liver was similar to that in the plasma triglycerides (Figs. 2 and 3), and was considerably higher than in the muscle and depot fats. These observations are consistent with the concept that plasma triglycerides are important in the transport of absorbed fatty acids and in the biosynthesis of tissue triglycerides (14,27,28). Furthermore the differences in the content of

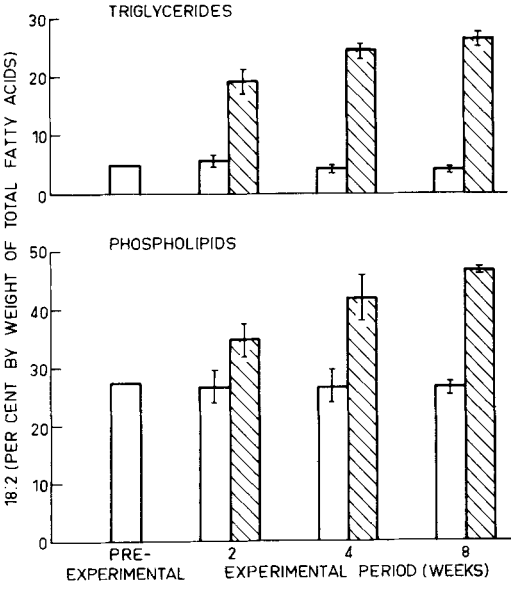


FIG. 6 Incorporation of dietary 18:2 into the triglycerides and phospholipids of *M. psoas major;* ▨ supplemented diet; □ unsupplemented diet. Pre-experimental values are the means for two animals; other values are the means (with standard errors) for three animals on each diet.

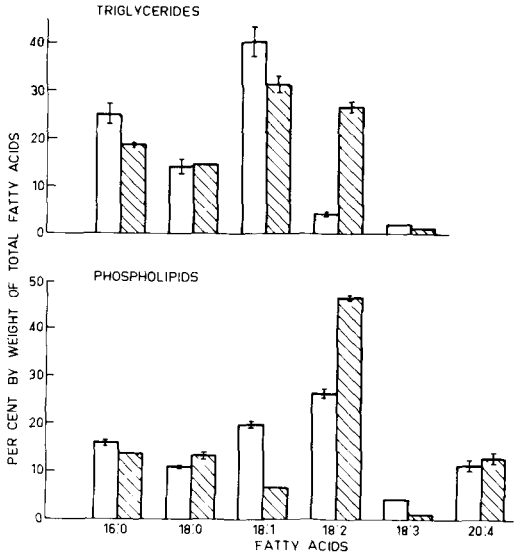


FIG. 7. Fatty acid composition of triglycerides and phospholipids of *M. psoas major.* from cattle fed supplemented ▣ and unsupplemented □ diets. Mean values (with standard errors) for three animals on each diet after 8 weeks of maximal supplementation.

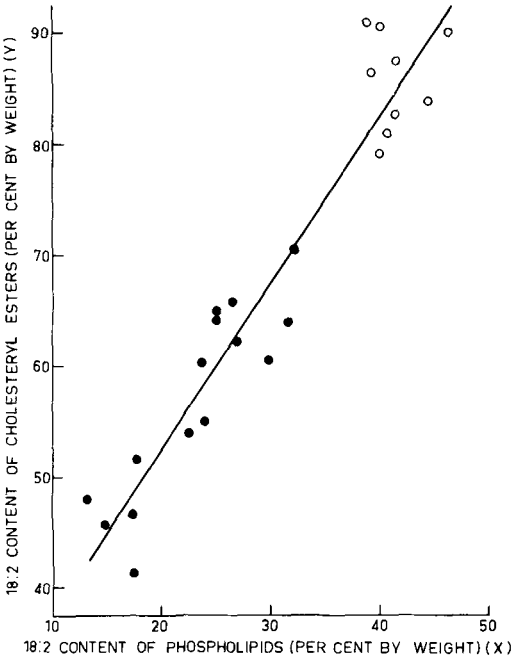


FIG. 8. Linear relationship between 18:2 content of cholesteryl esters and phospholipids of plasma from cattle fed supplemented (○) and unsupplemented (●) diets. Regression equation Y = 22.43 + 1.51 X; correlation coefficient 0.96.

18:2 between liver and other tissues, e.g., depot fats and muscle, suggest that in ruminants, as in nonruminants, the liver is an important organ in the clearing of chylomicra from the blood (28-30).

The phospholipids and cholesteryl esters of plasma from ruminants receiving conventional diets contain relatively high proportions of 18:2 or 18:3, or both (Fig. 1) (25,26). This implies that some of the dietary $C_{18}$ polyunsaturated fatty acids escape hydrogenation in the rumen, and that these acids are incorporated into phospholipids, e.g., lecithin; these acids would then be esterified to cholesterol via the lecithin-cholesterol-acyl transferase reaction proposed by Glomset (31). The feeding of the formaldehyde-treated supplement further increased the proportion of 18:2 in both of these plasma lipid components (Figs. 1 and 2), and there was a linear relationship between the 18:2 contents of plasma phospholipids and cholesteryl esters (Fig. 8). This linear relationship is consistent with the mechanism of cholesterol esterification (vide supra) and has previously been observed both in ruminants fed conventional diets (25) and in nonruminants (32). The phospholipids and cholesteryl esters of liver and the phospholipids of muscle from steers receiving control diets also contained relatively high proportions of 18:2, and the proportion of this acid was also increased on feeding the supplement (Figs. 3, 6 and 7). This increased incorporation of 18:2 was associated with compensatory decreases in the proportions of other unsaturated fatty acids (18:1, 18:3 and 22:6). This pattern of substitution in plasma and tissue phosphoglycerides suggests that 18:2 competes with other unsaturated fatty acids for esterification to the β-position of the phospholipid molecule (25).

Despite the increased proportions of 18:2 in the phospholipids, there were no corresponding increases in the proportions of 20:4; this suggests that in the unsupplemented steers the availability of 18:2 was not limiting the biosynthesis of 20:4 (33). Similarly in nonruminants, e.g., pigs and rats, when the dietary content of 18:2 was increased above the levels which were necessary to prevent essential fatty acid deficiency there were no corresponding increases in the proportion of 20:4 in tissue phospholipids (17,18).

Although the incorporation of 18:2 into the plasma and tissue triglycerides caused compensatory decreases in the proportions of 18:1 similar to that observed in phospholipids, there were also decreases in the proportion of 16:0 (Figs. 2, 3, 5 and 7); these substitution effects are analogous to that observed in pigs, mice and rats (15,17,18).

In conclusion, the present studies definitively show that when ruminants are fed formaldehyde-treated casein-safflower oil particles, the constituent polyunsaturated fatty acid i.e., 18:2, is protected from ruminal hydrogenation and is readily incorporated into plasma and tissue lipids. Moreover the interrelationships between plasma and tissue lipids and the nature of the compensatory changes in the fatty acid spectrum are similar to that observed in non-ruminants.

ACKNOWLEDGMENTS

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# Fatty Acid Alterations During Migration and Early Sea Water Growth of Chum Salmon *(Oncorhynchus keta)*[1]

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## ABSTRACT

Lipid and fatty acid characteristics were examined in chum salmon fry *(Oncorhynchus keta)* in freshwater, at various stages in their early growth in sea water, and 7 days after their artificial introduction from freshwater into sea water. The average lipid content of salmon fry in freshwater decreased from 2.5% in newly emerged fry (30-40 mm fork length) to 1.7% in older fry (40-50 mm). Lipid content did not appear to change during migration, but steadily declined during the first 4-6 weeks of sea water growth to 1.7%. Within 7 days after artificial introduction into sea water, lipid content of the salmon had declined to 1.4%. Extended residence in freshwater, e.g., 2-3 weeks, resulted in a fatty acid pattern more characteristic of freshwater fish, whereas residence in sea water resulted in patterns more characteristic of marine fish. Salmon artificially introduced into sea water incurred substantial diminutions in polyunsaturated fatty acids. This response did not occur in wild fry in the marine environment and may therefore be due to the stresses of capture, handling, anesthesia, partial starvation and confinement to which the artificially reared fish were exposed.

## INTRODUCTION

Environmental changes are known to affect the fatty acids of fish and other marine organisms. A substantial diversity in polyunsaturated fatty acid composition occurs among the three major groups of fish—marine, freshwater and anadromous (1). Poeciliid teleosts have been observed to store more unsaturated fatty acids at lower temperatures than at higher temperatures (2). Long term starvation in fish usually results in a marked depletion of polyunsaturated fatty acids, whereas short term starvation is characterized by their conservation (3-5). Size, since it is associated with metabolic rate, will modify the rate of depletion (6).

Juvenile coho salmon *(Oncorhynchus kisutch)*, when forced to swim at a water velocity (59 cm/sec) greater than that which could be sustained, were found to be depleted of 20:5 and 22:6, while at a velocity of 52 cm/sec, 16:0, 16:1 and 18:1 were the major fatty acids utilized. Apparently excessively high water velocities acted in this instance as a stress which disrupted fatty acid metabolism (7). The fatty acid composition of Atlantic Coast cod *(Gadus morhua)* has been demonstrated to change seasonally (8).

Studies were conducted in May and June 1968, to determine fatty acid characteristics and lipid contents of chum salmon fry *(O. keta)* during their early development in freshwater, seaward migration and early sea water growth. During these stages of life these fish would experience predation, fasting, changes in water velocity, adaptation to sea water, and accompanying changes in diet and environment.

## MATERIALS AND METHODS

### Collection and Handling of Fish

Chum salmon fry taken from Big Beef Creek, Kitsap County, Washington, prior to migration into sea water, and chum salmon fry taken from Hood Canal, Washington, after their entry into sea water were used in the studies.

The fry ($n$=107) were collected from Big Beef Creek (13-16 C) immediately after yolk sac absorption and emergence from the gravel, and were divided into two groups. One group ($n$=53) was killed by asphyxiation and frozen in dry ice. The other ($n$=54) was lightly anesthetized with tricaine methanesulfonate, transported to a floating laboratory (9) anchored in Seabeck Bay, Hood Canal, and held for 7 days without food in an aquarium supplied with fresh sea water. Some zooplankton were available to the fish from the sea water system. Of these fry, 37 (69% survival) lived through the holding period and were used for lipid analysis. The temperature of Hood Canal ranged from 13-16 C and the salinity was approximately 26 ppt.

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[1]Presented at the ISF-AOCS World Congress, Chicago, September 1970. Contribution No. 357, College of Fisheries, University of Washington.

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TABLE I

Mean Fork Lengths, Body Weights and Lipid Contents of Chum Salmon Fry[a]

| Length range | Group | Sample size | Fork length, mm | Body wt, g | Lipid content, % |
|---|---|---|---|---|---|
| 30-40 | FW | 28 | 37 | 0.31 | 2.5 |
| 30-40 | SW | 11 | 38 | 0.44 | 2.4 |
| 30-40 | FW→SW | 20 | 38 | 0.29 | 2.0 |
| 40-50 | FW | 25 | 44 | 0.54 | 1.7 |
| 40-50 | SW | 9 | 45 | 0.84 | 2.0 |
| 40-50 | FW→SW | 26 | 45 | 0.47 | 1.4 |
| 50-60 | SW | 9 | 52 | 1.40 | 1.7 |
| 50-60 | FW→SW | 8 | 55 | 0.99 | 1.4 |

[a]Residing in fresh water (FW), after migration into sea water (SW), and following artificial introduction into sea water (FW→SW).

Two weeks after the first collection, additional chum salmon fry were taken from Hood Canal near the entrance to Big Beef Creek with a beach seine.

The collections were grouped by fork length, as follows: 30-40 mm, 40-50 mm and 50-60 mm. The majority of fry emerged from the gravel with an average length ranging between 30 and 40 mm and migrated immediately to Hood Canal; observations over the past 4 years indicated that only about 2% of the population remain in freshwater for an extended period of time. Of the wild salmon captured in Hood Canal, those in the length range 30-40 mm had been in sea water less than 7 days. Fish 40-50 mm in length, whether in freshwater or sea water, had resided in their respective environments approximately 2-3 weeks, whereas fish 50-60 mm in length had been in sea water for ca. 4-6 weeks (Koski, unpublished data).

### Lipid Analysis

The juvenile salmon were pooled to make a sample weight of approximately 3 gm. Thus with the three size ranges, different numbers of fish were required. Lipids were extracted from the juvenile salmon with a mixture of chloroform and methanol according to the method of Bligh and Dyer (10). Total lipid content was determined gravimetrically from an aliquot of the lipid extract. The fatty acid composition was determined from a methylated aliquot of the lipid extract (11), by means of a Hewlett-Packard research gas chromatograph (model 5750) equipped with a dual hydrogen flame detector. The column used was 6 ft x 1/8 in. aluminum packed with 15% ethylene-glycol-succinate on Chromosorb P. Oven temperature was 190 C and helium flow rate 60 ml/min. After gas chromatographic analysis, the fatty acid methyl ester mixture was evaporated and the residue weighed on an analytical balance. This procedure allowed determination of the amount of total fatty acid methyl esters used for gas chromatographic analysis.

The fatty acid methyl esters extracted from the fish tissues were identified by comparison with known, commercially available standards. Additional identification of both the saturated and unsaturated fatty acids was accomplished by a plot of the log of the retention times against the number of carbons in the chain (12,13). The amount of fatty acids present in the mixture was proportional to the retention time multiplied by the peak height of the component fatty acids (14,15). In this manner the percentage contribution of each individual fatty acid to the total fatty acids in the sample was obtained. Additional information was obtained by comparison with fatty acid methyl esters extracted from other salmonids (16).

## RESULTS

The fork lengths, body weights and lipid contents of chum salmon collected in freshwater, those collected in sea water, and those that had been collected in freshwater and artificially transplanted into sea water are compared in Table I. Most of the chum salmon migrated from freshwater into sea water at a size averaging 30-40 mm in fork length, soon after completing yolk sac absorption. The percentages of total saturated, total monounsaturated and total polyunsaturated fatty acids in the fish's fatty acids are listed in Table II. After their entry into Hood Canal some of their fatty acids changed, while their total lipid content decreased. In contrast all major fatty acids diminished in chum salmon artificially transplanted into sea water and kept there for 7 days.

TABLE II

Mean Concentrations and Percentages[a] of Total Saturated, Total Monounsaturated and Total Polyunsaturated Fatty Acids of Chum Salmon Fry[b]

| Length range | Group | Sample size | Saturated fatty acids, % | Monounsaturated fatty acids, % | Polyunsaturated fatty acids, % | Saturated fatty acids, mg/g | Monounsaturated fatty acids, mg/g | Polyunsaturated fatty acids, mg/g |
|---|---|---|---|---|---|---|---|---|
| 30-40 | FW | 28 | 32.5 | 32.6 | 34.7 | 5.01 | 5.24 | 5.90 |
| 30-40 | SW | 11 | 31.2 | 25.5 | 43.3 | 4.68 | 3.84 | 6.51 |
| 30-40 | FW→SW | 20 | 36.0 | 31.7 | 32.3 | 3.64 | 3.48 | 3.56 |
| 40-50 | FW | 25 | 45.7 | 29.8 | 24.4 | 3.86 | 2.50 | 2.13 |
| 40-50 | SW | 9 | 30.7 | 25.3 | 43.9 | 3.88 | 3.33 | 5.65 |
| 40-50 | FW→SW | 26 | 42.0 | 26.8 | 31.1 | 3.18 | 2.10 | 1.71 |
| 50-60 | SW | 9 | 32.0 | 23.0 | 45.0 | 3.92 | 2.79 | 5.56 |
| 50-60 | FW→SW | 8 | 44.9 | 31.8 | 23.1 | 3.82 | 2.69 | 1.77 |

[a]Relative to total fatty acids.
[b]See Table I.

Twenty-eight fatty acids were found. The fatty acids contained from 8-24 carbons and from 0-6 double bonds. Eight of the fatty acids were selected for detailed examination because they accounted for approximately 90% of the total fatty acids present in the groups of salmon (Table III).

A dramatic change occurred in fatty acid composition when the chum salmon remained in freshwater for a period of time greater than that spent by the majority of chum fry. The percentage of total saturated fatty acids for fish that migrated when they averaged between 30 and 40 mm in length was 32.5%, while it was 45.7% for fish that remained in freshwater until they ranged between 40-50 mm in fork length. This increase was accompanied by a reduction in the total polyunsaturated fatty acids from 34.7% to 24.4%, respectively.

Changes in fatty acid composition and lipid content of salmon during and following their entry into sea water were examined in all groups of fish except the 40-50 mm group from freshwater, since they differed physiologically and behaviorally from the main population of chum salmon fry. This group will be discussed separately. Salmon in the 30-40 mm group from Hood Canal were presumed to have been in sea water less than 7 days; hence it is thought that their lipid characteristics would be a more precise index of fatty acid changes occurring during migration from those of other fish. During this period there occurred a small increase in size, a negligible change in lipid content, an abrupt decrease in the proportion of total monounsaturated fatty acids, and an increase in the proportion of total polyunsaturated fatty acids, notably 22:6. During the ensuing period of marine growth, there was a steady increase in size concomitant with diminishing lipid content. Proportions of monounsaturated fatty acids continued to fall slowly, while polyunsaturated fatty acids continued to rise gradually.

The percentage contributions for the eight major fatty acids are presented in Table III, while the fatty acids weights (in mg/g body wt), indicating the amount actually contained in the growing fish, are given in Table IV.

During the chum fry's initial 4-6 week period of marine growth, there were reductions in lipid content, gradual declines in the proportion of total monounsaturated fatty acids, and slight decreases in the percentage of total polyunsaturated fatty acids.

The salmon (40-50 mm in length) that remained in freshwater for a period of time longer than normal contained much lower concentrations and percentages of 20:5, 22:5

TABLE III

Percentages[a] of Eight Major Fatty Acids in Chum Salmon Fry[b]

| Length range | Group | Sample size | Per cent | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | 20:5 | 22:5 | 22:6 |
| 30-40 | FW | 28 | 2.9 | 21.3 | 4.7 | 7.2 | 23.2 | 7.1 | 4.1 | 17.5 |
| 30-40 | SW | 11 | 2.0 | 23.2 | 3.0 | 5.6 | 19.6 | 8.9 | 3.0 | 25.8 |
| 30-40 | FW→SW | 20 | 2.0 | 24.2 | 3.4 | 9.3 | 23.2 | 5.6 | 5.4 | 15.9 |
| 40-50 | FW | 25 | 2.6 | 33.0 | 5.2 | 9.5 | 21.6 | 3.7 | 2.9 | 11.9 |
| 40-50 | SW | 9 | 2.4 | 21.9 | 3.3 | 5.4 | 17.7 | 10.6 | 2.9 | 24.9 |
| 40-50 | FW→SW | 26 | 1.9 | 30.5 | 3.6 | 9.3 | 19.9 | 5.1 | 4.0 | 15.6 |
| 50-60 | SW | 9 | 2.3 | 23.6 | 3.3 | 5.5 | 15.0 | 11.5 | 3.1 | 25.6 |
| 50-60 | FW→SW | 8 | 2.1 | 31.8 | 6.4 | 10.5 | 21.8 | 4.1 | 2.6 | 10.3 |

[a]Relative to total fatty acids.

[b]See Table I.

and 22:6 than the salmon that migrated immediately after emergence (30-40 mm in length). These larger fish also possessed more 16:0, 16:1 and 18:0 than did the latter.

Salmon captured in freshwater and held in circulating sea water on the floating laboratory for 7 days experienced substantial diminutions in total lipids, and in general, declines in the concentrations of all eight major fatty acids. The percentage contributions of 14:0, 20:5, 22:5 and 22:6 to the total fatty acids tended to be lower, while percentages of 16:0, 16:1, 18:0 and 18:1 were higher.

## DISCUSSION

The fatty acids of chum salmon were influenced by length of time spent in freshwater, length of residence in the marine environment, and the combined stresses of handling, anesthesia, confinement and possible starvation. The time spent in the respective environments was also presumed to reflect such influences as growth, diet and general environmental adaptation.

Young chum salmon emerging from the redd must depend mainly on the lipid components absorbed from the yolk. Most fish reside in the stream for only a few hours to a few days and hence would feed minimally during this period. Thus the nutrients available to the fish after emergence from the redd must be sufficient for their migration into sea water, where adjustments to different environmental conditions are necessary.

The salmon (40-50 mm in length) that remained in freshwater for a longer period than usual had lower lipid contents than fish (30-40 mm) that did not. Concentrations of all eight fatty acids were less in these fish than in the smaller fish. The percentage contributions of 14:0, 20:5, 22:5 and 22:6 to the total fatty acids were also lower, whereas the percentage contributions of 16:0, 16:1, 18:0 and 18:1 were greater than in smaller fish. These changes are not felt to reflect starvation since general observations of stomach contents indicated sufficient quantities of food organisms (mostly aquatic insect larvae). The fish appeared in excellent physical condition, although it has been observed that they may experience greater mortality as a result of stress, e.g., capture and anesthesia, compared with the 30-40 mm freshwater chum fry. It is possible that the observed fatty acid changes were an innate physiologic response since Parker and Vanstone (17) observed declines in lipid content in migrating juvenile pink salmon smolts *(O. gorbuscha)* irrespective of diet or size per se. An apparent inverse correlation between lipid content and size was observed in the salmon captured in Hood Canal. These data may in part contradict observations by Vanstone et al. (18) on juvenile chum salmon (0.3-7 g in size), that large fish possessed greater percentages of total body lipids than small fish. However those fish that had completed the migration to sea water contained lower percentages than did premigrants. It is also necessary to consider the effect of a freshwater diet since the chum salmon from Big Beef Creek had stayed in freshwater for 2-3 weeks and grown approximately 10 mm while foraging primarily on drifting aquatic insects. It is possible that these fish may have acquired a fatty acid composition more characteristic of freshwater fish oils, since there was an increase in C-16 and C-18 fatty acids and diminutions in C-20 and C-22 (19) acids.

During migration of the chum salmon into Hood Canal, concentrations of total monounsaturated fatty acids declined, and concentrations of total polyunsaturated fatty acids

TABLE IV

Mean Concentrations of Eight Major Fatty Acids in Chum Salmon Fry[a]

| Length range | Group | Sample size | Milligrams per gram | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | 20:5 | 22:5 | 22:6 |
| 30-40 | FW | 28 | 0.51 | 3.24 | 0.77 | 1.08 | 3.69 | 1.18 | 0.66 | 2.80 |
| 30-40 | SW | 11 | 0.30 | 3.49 | 0.45 | 0.84 | 2.94 | 1.34 | 0.46 | 3.88 |
| 30-40 | FW→SW | 20 | 0.21 | 2.41 | 0.45 | 0.96 | 2.45 | 0.62 | 0.60 | 1.71 |
| 40-50 | FW | 25 | 0.22 | 2.79 | 0.44 | 0.80 | 1.81 | 0.32 | 0.28 | 1.00 |
| 40-50 | SW | 9 | 0.31 | 2.81 | 0.43 | 0.69 | 2.27 | 1.36 | 0.37 | 3.21 |
| 40-50 | FW→SW | 26 | 0.19 | 2.25 | 0.28 | 0.69 | 1.59 | 0.32 | 0.15 | 0.50 |
| 50-60 | SW | 9 | 0.29 | 2.89 | 0.40 | 0.67 | 1.83 | 1.40 | 0.38 | 3.17 |
| 50-60 | FW→SW | 8 | 0.18 | 2.70 | 0.58 | 0.88 | 1.84 | 0.32 | 0.19 | 0.76 |

[a]See Table I.

rose. These alterations may reflect the influences of a marine diet in conjunction with enhanced metabolic requirements arising from migration and general adaptation to the marine environment. The salmon in the 30-40 mm length range from Hood Canal had been in sea water for less than 7 days; hence changes in lipid composition could have occurred only during this period. It is well known that marine zooplankton, the primary food source of the juvenile salmon, are particularly rich in long chain polyenoic acids which they acquire by elongation and desaturation of the C-16 and C-18 fatty acids of phytoplankton (20,21). However certain marine organisms do not follow this pattern. Ackman et al. (22) have shown that some marine diatoms, e.g., *Skeletonema costatum,* have the ability to produce long chain highly unsaturated fatty acids similar to those characteristic of marine fish. Furthermore it has been demonstrated that the planktonic crustaceans *Pandalus borealis* and *Meganyctiphanes norvegica* synthesize high proportions of 20:1 and 22:1, unlike most marine planktonic crustaceans (23). Consumption of zooplankton could partially account for the differences observed in the 30-40 mm Hood Canal salmon. In addition to the impact of the marine diet, exercise and environmental stress in general would be expected to have a contributory, although unascertained, influence on the observed fatty acid responses. Krueger et al. (7) have shown that moderate exercise leads to declines in the proportions of 16:0, 16:1 and 18:1 in juvenile coho salmon. Temporary fasting also results in enhanced catabolism of these same fatty acids. Chum salmon probably do not forage actively during their seaward migration, although the influence of fasting in this instance is considered to be very small.

Chum salmon artificially introduced into sea water and confined for 7 days apparently encountered substantial stress from agents previously mentioned. It is presumed that sufficient food was not available to the fish during holding, although definitive observations of zooplankton abundance in the aquarium and analyses of stomach contents of the fish were not possible. Recent studies on hematological (24) and fatty acid changes (25) in juvenile pink salmon *(O. gorbuscha)* subjected to similar stresses have also documented physiological disturbances arising from these noxious stimuli. As a result there apparently was an increased metabolic requirement in the chum salmon fry from the stressers, which enhanced catabolism of the normally quite stable, tissue-building polyunsaturated fatty acids. Mead et al. (26), who examined the biogenesis of polyunsaturated fatty acids in fish, postulated that fish have the ability to biosynthesize saturated and monounsaturated fatty acids, but must acquire the long chain polyunsaturated fatty acids either from the diet or by elongating 18:2 ($\omega$6) and 18:3 ($\omega$3). Other workers have confirmed the essential nature of the $\omega$3 and $\omega$6 fatty acids and the pathways of biosynthesis from essential precursors (5,27-29).

In conclusion, data are presented which demonstrate the general impact of environment on the fatty acids of juvenile chum salmon. Because of the complex and as yet sparsely investigated effects of environmental factors on the lipids of natural populations of salmon, it is as yet impossible to rank the many environmental variables as to their respective, and perhaps interacting, influences on fatty acid metabolism of these fish. However it seems that diet is a very important factor. Furthermore it is obvious that substantial care is required to minimize the effects of stress on juvenile salmon that are captured in their natural environment and then handled and kept under laboratory conditions.

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# Clinical Evaluation of MK-185: A New Hypolipidemic Drug[1]

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## ABSTRACT

A double-blind crossover study in 11 patients suggests that MK-185 may be a useful drug in lowering plasma triglyceride levels, whereas it has minimal effects on cholesterol levels. A good correlation between the hypouricemic and protein bound iodine lowering effects and plasma level of MK-185 was seen. No correlation with the hypolipidemic effect was noted.

MK-185, 2-acetamidoethyl (*p*-chlorophenyl) (*m*-trifluoromethylphenoxy) acetate (recently assigned the generic name halofenate), exerts hypolipidemic effects in rats, dogs and monkeys. In rats the decrease occurs in both plasma cholesterol and triglycerides. The mechanism of action of MK-185 is not yet completely understood. The drug does not significantly inhibit incorporation of $^{14}$C-acetate into cholesterol by the rat liver and accumulation of desmosterol does not occur (unpublished data, MK-185 Preclinical Evaluation, Merck Sharp and Dohme Research Laboratories).

[1]One of eight papers presented at the symposium "Recent Advances in Drugs Affecting Lipid Metabolism," AOCS Meeting, Houston, May 1971.

[2]Merck International Fellow in Clinical Pharmacology.

The data reported herein are the results of a double-blind trial involving 11 patients, six with type II and five with type IV hyperlipoproteinemia according to Fredrickson et al. (1).

## MATERIALS AND METHODS

Eleven patients, five women and six men, ranging in age from 25-60 were selected. Written consent was obtained following a thorough explanation of the inconveniences and hazards to be expected. They were known to have persistent elevations of serum cholesterol (>250 mg/100 ml) or glyceride glycerol (>15 mg/100 ml) or both. Diabetic patients were not accepted and only one patient had previously received a hypolipidemic drug. The patients had a complete physical examination, including a slit-lamp examination. The latter was repeated periodically throughout the study.

The patients were classified as type II or type IV by lipoprotein electrophoresis (2) on agarose gel in conjunction with determination of plasma cholesterol (3) and glyceride glycerol (4). The electrophoretic strips were scanned with a Beckman Model RD-2 Duostat Densitometer and the counts given by the integrator for each band were computed as percentages of the total (5).

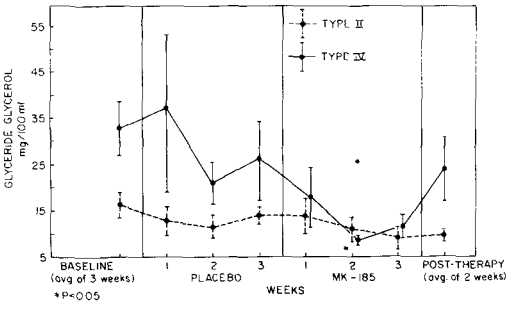


FIG. 1. Changes in serum triglycerides during treatment with placebo and MK-185. The order of administration of the medications was randomized during the trial, although for practical purposes the data has been arbitrarily unified. Each point is mean ± SEM for all patients.

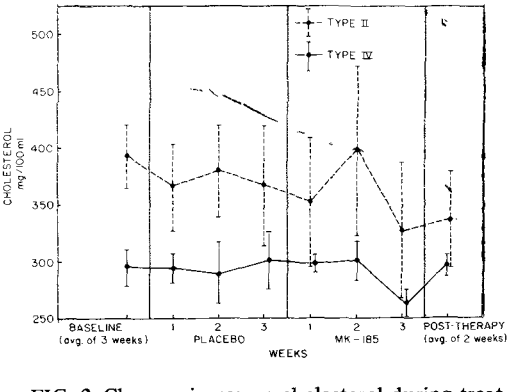


FIG. 2. Changes in serum cholesterol during treatment with placebo and MK-185. The order of administration of the medications was randomized during the trial, although for practical purposes the data has been arbitrarily unified. Each point is mean ± SEM for all patients.

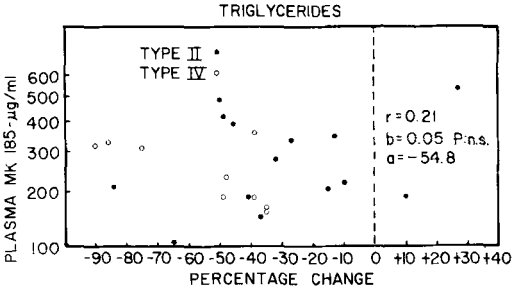


FIG. 3. Correlation between the decreases in triglycerides and the plasma level of MK-185.

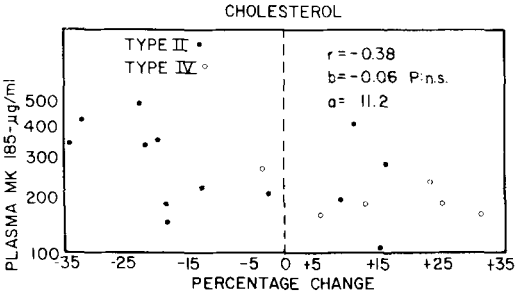


FIG. 4. Correlation between the decreases in cholesterol and the plasma level of MK-185.

The study was divided into four parts of 6 weeks each: pre-therapy control period, treatment period A, treatment period B, post-therapy control period. The patients were seen at 2 week intervals. At the first and last visit of each period, a complete series of toxicity tests was performed (urinalysis, complete blood count, BUN, bilirubin, SGOT, SGPT, alkaline phosphatase, uric acid, fasting blood sugar, creatine phosphokinase [CPK], protein bound iodine [PBI], and serum electrolytes). An EKG and lipoprotein electrophoresis were obtained at the end of each 6 week period. During treatment period A, patients received either MK-185 or a placebo according to a random schedule; during treatment period B, they crossed over the medication they did not have during period A. Placebo and MK-185 (500 mg) were administered in identically-appearing capsules every 12 hr. At each visit an interim physical examination was performed and blood samples for the determination of plasma lipids and MK-185 levels were drawn. No medication was given during either the pre- or post-therapy control periods. The patients did not follow any specific dietary regimen during the study.

The MK-185 serum level was estimated by gas liquid chromatography. Three milliliters serum were added to an equal amount of 0.1 N

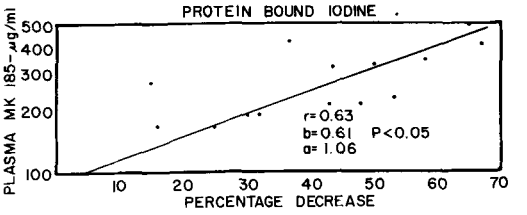


FIG. 5. Correlation between the decreases in uric acid and the plasma levels of MK-185.

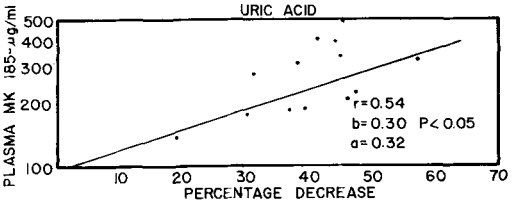


FIG. 6. Correlation between the decreases in protein bound iodine and the plasma levels of MK-185.

HCl and 20 ml chloroform. After centrifugation the bottom chloroform layer was collected and evaporated to dryness in a warm water bath under a stream of air. The dried residue was alkalinized with 5% $K_2CO_3$; 0.2 ml dimethyl sulfate was added, and heated at 70 C for 5-10 min. The tubes were allowed to cool to room temperature while under an air stream. One milliliter acetate buffer (pH 5.6) and 10 ml chloroform were added to each tube. After shaking and centrifuging, the chloroform layer was recovered and evaporated. One $\mu$l samples of the dried residue dissolved in chloroform were analyzed using a 1% QF-1 on 80/100 Gas Chrom Q (Applied Science Labs) column at 165 C. The ethyl ester of MK-185 was used as the internal standard.

Statistical analysis of the data was carried out by Student's "t" test and correlation coefficient "r" (6). P values less than 0.05 were considered significant.

## RESULTS

### Serum Lipids

*Triglycerides:* MK-185 was quite effective in lowering triglycerides in all patients. The decreases (considering as baseline the average of the three values during the pre-therapy control period) ranged between 35-90% in the five patients with type IV abnormality. In the six type II patients, the decrease ranged between 10-65%, although in one patient, at two bi-weekly visits, the triglyceride level was elevated (10-27%). The active drug produced a

TABLE I

Densitometric Percentages of Lipoprotein Electrophoretic Fractions[a]

| Type | β | | pre-β | | α | |
|---|---|---|---|---|---|---|
| | Before MK-185 | After MK-185 | Before MK-185 | After MK-185 | Before MK-185 | After MK-185 |
| II | 51.6 | 61.2 | 19.2 | 15.4 | 31.1 | 24.4 |
| IV | 39.4 | 46.8 | 38.6 | 37.8 | 22.0 | 15.4 |

[a]The values of above are the mean = percentage of the total lipid staining determined densitometrically for each lipoprotein fraction.

significant decrease in triglyceride as compared to the placebo (Fig. 1), in both groups of patients.

*Cholesterol:* In four of the six type II patients, the plasma cholesterol level decreased during MK-185 administration (4-34%); in one there was practically no change, and in another a modest increase. In three of the five type IV patients, the cholesterol plasma level decreased (1-29%), and in the remaining two there was a modest increase (Fig. 2). The cholesterol changes were not significant in either group.

The changes in the blood lipid levels could not be correlated to MK-185 blood levels (Figs. 3,4).

### Other Findings

The drug produced remarkable decreases of the uric acid (UA) and PBI levels. The percentage decrease (15-67%) of both UA and PBI was generally similar in the same patient, the UA decrease being more consistent between patients. Plotting the percentage decrease of UA and PBI against logarithms of the corresponding MK-185 serum levels yielded a significant correlation (Figs. 5,6). MK-185 significantly decreased the total serum bilirubin (from a mean of 0.36 to 0.19 mg/100 ml, $P < 0.05$) and white blood count (from a mean of 6418 to 5449 per $mm^3$, $P < 0.01$); it significantly increased creatine phosphokinase activity (from 29.6 to 47.7 U, $P < 0.05$).

Lipoprotein electrophoresis showed an increased density of the β band after treatment in both groups of patients; the percentage counts for this band were significantly higher after treatment in the type II patients (Table I).

### Side Effects

One type II female patient, after two weeks of treatment, experienced a generalized maculopapular skin rash, and swelling of the face and hands during period A (MK-185). Drug treatment was discontinued and the rash subsided. The medication of period A was restarted and in a few days the rash reappeared. The rash again disappeared when the medication was discontinued and the patient was started on the medication of period B (placebo) without any adverse reactions during the next 6 week period. The patient was given clofibrate at the end of the study, also without reappearance of the rash. Two other type II patients (brothers) complained of dizziness and nausea during coitus. Mild viral infections were prevalent in members of their families at this time. The treatment was not discontinued and the symptoms disappeared within two weeks. None of these reactions was associated with an

unusually high serum level of MK-185.

## DISCUSSION

MK-185 was quite effective in lowering serum triglycerides, both in patients of type IV as well as in type II disease where triglyceride levels were generally within the normal range. The decrease was very striking in some cases (between 80-90%), but could not be correlated with level of the drug in serum. The variations of cholesterolemia were minimal, inconsistent and not statistically significant.

A good correlation was found, on the contrary, between the blood levels of the drug and the decrease in PBI and UA, findings which were not expected from the preclinical animal data. Jain et al. (7) recently showed that MK-185 produces a dose related uricosuria and does not increase the elimination of oxypurines as does allopurinol. This good correlation between the serum level can be used as an index of patient compliance to drug ingestion.

These preliminary studies suggest that MK-185 has an effect comparable to clofibrate in reducing plasma triglyceride levels. The effect on cholesterolemia is not very remarkable with either compound. The hypouricemic effect, if also confirmed in patients with hyperuricemia, may indicate a special rationale for this drug in the not uncommon type IV patient with mild diabetes and hyperuricemia.

The short period of treatment and small number of patients in this study preclude any prediction on the usefulness of this drug in long term treatment of hyperlipoproteinemia. However the good correlation between plasma level of the drug and the decrease in UA and PBI provide evidence that these changes can be used to monitor patient compliance to drug intake. The changes noted may be related to displacement of these substances from sites of plasma protein binding.

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# Effect of Androgens on Serum Lipids and Lipoproteins[1]

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## ABSTRACT

The effects of androgens on lipid transport and metabolism have been reviewed. These effects are probably independent of the androgenic and anabolic activities of the androgens, although the molecular mechanism of action is still not known. Presumably the lowering by androgens of the concentrations of serum lipids and lipoproteins could be the consequence possibly of a primary, inhibitory effect on the synthesis of apolipoprotein A. In addition the role of increased lipolytic activities in plasma and of effects on intermediary metabolism has been considered.

## INTRODUCTION

Androgens are steroids known for their anabolic and masculinizing effects. Androgenicity refers to an anabolic effect on the sex organs and on cell differentiation, while in the common usage of the term an anabolic effect signifies the extragenital stimulation of protein synthesis. Testosterone, the male sex hormone, is one of the most potent anabolic steroids known.

Testosterone is bound to a protein component of the chromatin at the site of active RNA synthesis within the nuclei of accessory sex tissues. This occurs after conversion of testosterone by nuclear 5α-reductase into dihydrotestosterone (5α-androstanolone), which appears to be the active form of testosterone in these tissues (1). However in nontarget tissues, e.g., liver, metabolites other than dihydrotestosterone are formed when testosterone is incubated with nuclei or cytoplasm. These results indicate that the metabolic actions of androgens are mediated possibly through different metabolites in different tissues.

The intermediary metabolism of testosterone proceeds essentially via three main reactions (Fig. 1): (a) 17β-hydroxysteroid dehydrogenase (17-β-hydroxysteroid:NAD/NADP 17-β-oxydoreductase, EC 1.1.1. j/k): oxidation of the 17β-hydroxy group resulting in 17-ketosteroid formation (andostenedione); (b) $\Delta^4$-5α- or $\Delta^4$-5β-reductase: reduction of the 4,5-double bond in ring A (2 isomers); (c) 3α- and 3β-hydroxysteroid dehydrogenase (3-α-hydroxysteroid: NAD(P) oxydoreductase, EC 1.1.1.50, and 3-β-hydroxysteroid:NAD(P) oxydoreductase, EC 1.1.1.51): formation of the 3-hydroxy-5-androstan-17-one metabolites (4 isomers).

These reactions describe two pathways through which the metabolites of testosterone are formed: the "17-oxo" and the "17-hydroxyl" pathways. The androgenicity of the 17-hydroxyl metabolites are significantly greater than that of the corresponding 17-oxo compounds (2). Among the metabolites with ring A saturated, the 5α isomers are the biologically important ones. Furthermore in females and in prepubertal males or in male hypogonadism, testosterone is metabolized predominantly along the "17-oxo" pathway. In males after puberty testosterone metabolism proceeds mainly via the "17-hydroxyl" pathway (3). The substitution of an alkyl group in the 17α-position prevents the oxidation of the 17β-hydroxy group. Methyltestosterone (17α-methyl-17β-hydroxyandrost-4-en-3-one) is unique among 17α-substituted alkyl derivatives in retaining androgenic potency when given orally. Other chemical modifications of testosterone caused significant dissociation of andro-

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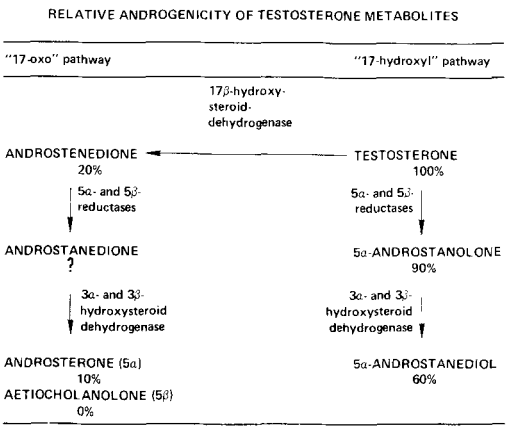


FIG. 1. Pathway of testosterone metabolism. The percentage values indicate the relative androgenicity of testosterone metabolites.

TABLE I

Effect of Androgenic and Anabolic Steroids on Serum Lipid and Lipoprotein Levels[a]

| Steroids | Species | Serum lipids | Serum lipoproteins VLDL (pre-β) | LDL (β) | HDL (α) | Reference |
|---|---|---|---|---|---|---|
| Testosterone | Man | U | | I | D | 13 |
| | | D | | | D | 14 |
| | Dog | D | | | D | 15 |
| | | D | | | | 16 |
| | Rat | U | | | | 17 |
| 17-α-Methyl-testosterone | Man | U | | I | D | 13 |
| | | I | | I | D | 18 |
| | | D | | I | D | 19 |
| | | D | D | I | D | 12 |
| | Dog | D | | | D | 15 |
| | | D | | D | D | 20 |
| | | D | | D | D | 21 |
| | | D | D | D | D | 22 |
| | Rat | D | | D | D | 23 |
| Androsterone | Man | D | | | | 24 |
| | | D | | (I) | D | 25 |
| 19-Nor-testosterone | Man | D | | (I) | D | 26 |
| $\Delta^1$-Testololactone | Man | D | | U | D | 27 |
| 17α-Methyl-androstenediol | Man | D | | | D | 14 |
| 17α-Methyl-5α-androstanediol | Dog | D | | | | 28 |

[a]U=unchanged; D=decreased; I=increased. VLDL = very low density lipoproteins. LDL = low density lipoproteins. HDL = high density lipoproteins.

genic and anabolic activities (4), indicating that these biological effects of the androgens are not inherently related.

## EFFECTS ON LIPID METABOLISM

During puberty the subcutaneous fat decreases in males but not in females, and eunuchs have a tendency to obesity. These developments suggest that androgens affect adipose tissue metabolism. In fact prolonged administration of high doses of testosterone or methylandrostenediol reportedly decrease the total fat content of the body (5,6). Furthermore treatment of fasting female rats or castrated male rats with testosterone causes a marked increase in the plasma concentrations of free fatty acids, possibly reflecting a direct fat-mobilizing activity of the androgens (7,8). Administration of synthetic anabolic steroids, such as 19-nor-androstenolone (Durabolin) and methandrostenolone (Dianabol), reproduced the testosterone effect. However the same authors (8) found that estrogens increased plasma levels of free fatty acids as well, and when estrogens were administered together with androgens they acted synergistically.

Recently it was found (9) that the administration of the synthetic androgen oxandrolone increased the postheparin lipolytic activities in human plasma, with concomittant diminution of the plasma level of triglycerides. However the potent progestin norethindrone, which is only mildly androgenic, had similar effects.

In healthy adult subjects sex differences are evident in the concentrations of serum lipids and lipoproteins: in men more circulating lipid is in the form of lower density lipoproteins than is the case in women, whereas women have more high density lipoproteins than men (10,11). Furthermore administration of gonadal hormones characteristically alters the levels of lipids and patterns of lipoproteins in the serum (12). In general androgens diminish the concentration of high density lipoproteins, both in man and in experimental animals. In man this effect is accompanied by unchanged or increased concentrations of low density lipoproteins, and by decreased concentrations of very low density lipoproteins. In the dog and rat administration of testosterone or methyltestosterone also lowers the concentration of low density lipoproteins. Although serum levels of cholesterol may not change significantly in healthy subjects, the hypocholesterolemic and hypolipemic effect of methyltestosterone and other testosterone anologs has been well documented in lipemic patients. The data clearly indicate that not only methyltestosterone, but analogs of testosterone with significantly lower androgenic activity, exert the same effect on serum lipoproteins. In addition a major fecal metabolite of methyltestosterone, 17α-methyl-5α-androstane-3β, 17β-diol, which is a weaker

androgen than the parent compound, reproduces completely the hypocholesterolemic effect of methyltestosterone in dogs (Table I).

A possible interrelationship between androgen metabolism and serum lipid levels may be illustrated by a consideration of certain pathological states. The association of serum lipid abnormalities with thyroid dysfunction is well known. In thyrotoxicosis low and high density lipoproteins and serum lipid levels are decreased, whereas in hypothyroid subjects low density lipoproteins and lipid levels are increased (29-32). Moreover in myxoedema there is a fall in the excretion of total androgens attributable mainly to a diminution of androsterone component (33). Determination of the androsterone to aetiocholanolone urinary excretion ratio is useful for obtaining information on the relative activities of 5α- and 5β-reductases. A decrease in this ratio indicates the relative or absolute diminution of the biologically active 5α-reduced products of both the "17-oxo" and "17-hydroxyl" pathways of testosterone metabolism, i.e., androsterone and 5α-androstanediol. This is the case in myxoedema (33), after corticosteroid administration (34), in hyperlipemic patients including familial hypercholesterolemia (35), and in myocardial infarction (36), i.e., when serum lipid levels are generally increased. On the other hand, in thyrotoxicosis higher androgenic activity can be expected from the increased ratio (33) which coincides with lower serum lipid levels. The relationship between the kinetics of testosterone metabolism and thyroid function provides further support for the possibility that the effect of the thyroid on lipid metabolism is mediated, at least in part, through androgens. Hyperthyroidism is associated with increased plasma concentration of testosterone, whereas in hypothyroidism the serum testosterone concentration is decreased (37). All these findings agree with the previous observation that in myxoedema the hyperlipemia can be ameliorated by the administration of androsterone (24,25).

Androgens may also influence carbohydrate metabolism and thereby affect lipid metabolism. Low excretion of dehydroepiandrosterone has been reported in diabetic patients with obesity (38). This androgen inhibits glucose-6-phosphate dehydrogenase (39). It was postulated, therefore, that lower level of dehydroepiandrosterone would result in higher enzyme activity and consequently increased synthesis of NADPH. This latter effect could account for an increment in the synthesis of fatty acids and other lipids. Furthermore administration of dehydroepiandrosterone reduces serum lipid levels in man (40) and in rat (41). In hyperlipemic patients the plasma level and the urinary excretion of dehydroepiandrosterone is lower, and a significant reciprocal correlation between plasma phospholipid levels and urinary excretion of this steroid has been established (35).

To explain the hypolipemia and hypocholesterolemia caused by androgens a limited number of investigations have also evaluated the effect of androgens on cholesterol biosynthesis and metabolism. In rats methyltestosterone produces a significant decrease in hepatic synthesis of cholesterol, but this response is compensated by a significant increase in intestinal cholesterol synthesis, and total cholesterol biosynthesis in these tissues remains unaltered (42). The oxidation of cholesterol to biliary and fecal bile acids is not increased significantly in rats treated with methyltestosterone (43). The observation that methyltestosterone decreases the incorporation of acetate into serum cholesterol in the dog (21) may provide an explanation for the changes in cholesterol metabolism, but this in itself is inadequate to account for the marked decrease which has been demonstrated in all three major lipid classes.

## EFFECT ON SERUM APOLIPOPROTEINS

In recent studies in dogs we investigated the possibility that a primary effect on the metabolism of apolipoproteins is the mechanism by which androgens lower the concentrations of serum lipids and lipoproteins. The distribution of apolipoprotein components in canine serum was determined by immunochemical means, and we estimated that the characteristic protein components of the high density lipoproteins, designated as apolipoprotein A, may comprise as much as 90% of total apolipoproteins (44). Lipoproteins of the density range of 1.110-1.250 g/ml contain only apolipoprotein A, and this high density lipoprotein fraction was used to study the effect of methyltestosterone on the metabolism of apolipoprotein A. Methyltestosterone produces an increment in albumin concentration and decrement in $\alpha_1$- and β-globulins, without altering total protein levels in serum (22). At the same time it causes a reduction of the apolipoprotein and lipid concentrations of all density classes of lipoproteins in canine serum. Although no qualitative changes have been demonstrated by immunochemical analyses, marked diminution in the quantity of all high density lipoprotein components is observed. The amino acid composition of apolipoprotein A is not altered appreciably, a finding that suggests that methyltestosterone

produces a quantitative rather than a qualitative change in apolipoprotein A. The diminution in low density lipoproteins comprises a proportionately greater reduction in lipoproteins containing apolipoprotein A than in those containing apolipoprotein B. The metabolic studies, carried out in the same four dogs before and during methyltestosterone treatment, have demonstrated that the relative amount of $C^{14}$-lysine incorporated into the apolipoproteins of high density and low density lipoproteins is reduced, whereas that incorporated into albumin is increased. To evaluate the half-lives and rates of turnover of these proteins, $C^{14}$-albumin and high density lipoproteins containing $C^{14}$-apolipoprotein A were obtained from a donor dog given $C^{14}$-lysine. In whole serum and within the high density lipoproteins methyltestosterone reduces the mean turnover rate and the total exchangeable pool for apolipoprotein A to less than 50% of controls. The half-time for the disappearance of apolipoprotein A from serum is not significantly changed by the treatment. The rate of turnover of albumin increases almost threefold and the total exchangeable pool by almost 50% during methyltestosterone treatment, while the half-life decreased. These findings demonstrate that methyltestosterone decreases synthesis of apolipoprotein A and increases synthesis of albumin (45).

The effects of androgens on the metabolism of individual serum proteins are unknown. However the available data on the changes in concentrations of serum proteins clearly imply that individual proteins are affected differently and that there is no uniform anabolic action of androgens with regard to serum proteins. The finding of the increase in albumin concentration and decrease in apolipoprotein A and $\beta$-globulin concentrations during methyltestosterone treatment in the dog (22) is supported by the recent report on the semiquantitative immunochemical evaluation of the effects of testosterone administration on serum protein concentrations in the domestic fowl (46). Moreover in man androgen treatment increases the concentration of thyroxine-binding pre-albumin (47), and may diminish the concentration of thyroxine-binding $\alpha_1$-globulin (47,48). On the basis of the findings described above, these changes in serum protein concentrations may reflect a differential effect of androgens on the synthesis of serum proteins in the liver. If a primary action on the synthesis of apolipoprotein A is indeed responsible for the hypolipemic effects of androgens, this finding would mean that the anabolic and hypolipemic activities are mediated through different mechanisms.

## MECHANISM OF ACTION

Our present understanding of the mechanism whereby androgens affect serum lipids and lipoproteins is very limited. The possible and in part speculative explanations for the findings of the clinical and experimental animal investigations reviewed above will be offered by discussing the questions below.

I. Are the biological effects of androgens, namely the androgenicity, anabolic activity and hypolipemic effect, mediated through the same mechanism or metabolite, i.e., are these effects inherently related? The data already discussed suggest that almost certainly they are not. This conclusion implies the possibility of finding a particular testosterone metabolite or a synthetic steroid, or both, which will selectively affect lipid metabolism.

II. Which are the possible points of action of androgens on lipid metabolism? (a) Mobilization of free fatty acids from adipose tissue may increase. As a result levels of free fatty acids in plasma would increase, with a possible consequent increased synthesis of triglycerides in the liver. However this action in itself, if indeed operative, would tend to be hyperlipemic rather than hypolipemic. A decrease in the fat content of the body may also be the consequence because of the increased fatty acid utilization. (b) The increase in plasma lipoprotein lipase activity, or postheparin lipolytic activity in general, should increase the breakdown of triglycerides and the utilization of fatty acids. This action could account for an increase in plasma levels of free fatty acids and the decreased plasma level of triglyceride. In addition a possible increase in the turnover of triglycerides might be predicted, especially if an increased mobilization of free fatty acids is also taken into account. (c) Carbohydrate metabolism might be affected, e.g., by an inhibitory action on glucose-6-phosphate dehydrogenase, in such a way that a decrease in lipid synthesis would result. This action could contribute to the decrease of both the fat content of the body and the serum levels of lipids. (d) Inhibition of cholesterol biosynthesis in liver or intestine, or both, could explain the hypocholesterolemic action, but would be insufficient as the initial event to account for the other effects. It may well be the consequence, however, of the actions mentioned under points (c) and (e). (e) Selective inhibition of the synthesis of apolipoprotein A may explain the decreased concentration in the plasma of the high density lipoproteins. It could possibly account also for other effects, as discussed below.

III. Which of the androgen effects on lipid

metabolism might be explained by a selective inhibition of the synthesis of apolipoprotein A? (a) The decrease in the concentration of high density lipoproteins (observed in man, dog and rat) may be the consequence of diminution in the production of these lipoproteins in the liver. The increased ratio of cholesterol to protein in the high density lipoproteins in man (12) may reflect a primary alteration in the protein moiety in the lipoproteins. (b) The increase in the concentration of low density lipoproteins in man may reflect the increased breakdown of very low density lipoprotein triglyceride by increased lipolytic activities or because transport of relatively more lipid in this class may be required as a result of the diminution of apolipoprotein A, or both. (c) The decrease in the concentration of very low density lipoproteins could be explained by an increased lipolytic activity in the plasma. The increase of postheparin lipolytic activities observed in human plasma may be independent of a mechanism involving apolipoprotein A. Recent findings (49-51) indicate that specific apolipoprotein peptides play the role of activators for lipoprotein lipase. The peptides which correspond to the apolipoprotein A, i.e., the characteristic major peptides of high density lipoproteins, are not activators. It is not known whether the concentration of small activator peptides (which may be referred to as parts of apolipoprotein C) might increase during androgen treatment. However it is tempting to speculate that under physiological conditions the enzyme activation may depend not simply on the absolute amount, but on the relative proportion of certain apolipoprotein peptides. Thus the diminution of nonactivating peptides from apolipoprotein A might correlate with increased lipolytic activities. (d) The decrease of cholesterol synthesis observed in dog and in rat may be explained by the recent finding that an apolipoprotein, possibly apolipoprotein A, plays an essential role in the promotion of hepatic cholesterol biosynthesis (52,53). (e) The decrease in the dog of all serum lipid levels, i.e., cholesterol, phospholipid and triglyceride, may be explained by a possibly coordinated production of all components of high density lipoproteins, with a primary role for the protein moiety in this process (54). With regard to apolipoprotein A, the levels of phospholipid and cholesterol would be principally affected by this mechanism. In addition an increased catabolism of triglycerides could explain the lowering of plasma levels of triglycerides. (f) The lack of development of fatty liver in the dog (15), and the protective action against the ethionine-induced fatty liver in rat (55) might also be related to the role apolipoprotein A may play in the regulation of the synthesis of the lipids it transports, i.e., the majority of plasma lipids in these species. In contrast a selective impairment of the synthesis of apolipoprotein B, as in the case of treatment with orotic acid (56), would be expected to cause fatty liver.

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# A Review of Studies on the Mode of Action of Clofibrate and Betabenzalbutyrate[1]

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**ABSTRACT**

Clofibrate and betabenzalbutyrate produce a variety of metabolic alterations in vivo. These include reduction in serum triglyceride and alterations in adipose tissue uptake and release of lipids. Clofibrate displaces thyroxine from albumin binding sites and produces an enlargement of liver and changes in ultrastructure. The biochemical changes produced by clofibrate include reduction in adenyl cyclase activity, inhibition of acetyl CoA carboxylase, inhibition of cholesterol biosynthesis and inhibition of triglyceride formation. Recent studies in this laboratory have shown that the inhibition of hepatic triglyceride formation is an early metabolic consequence of clofibrate administration and precedes the fall in serum triglyceride and several of the other biochemical alterations. Moreover clofibrate and betabenzalbutyrate inhibit the esterification of *sn*-glycerol-3-P by rat liver homogenate and microsomal preparations. The initial step in this pathway, acyl-CoA-*sn*-glycerol-3-P acyltransferase, is inhibited by both drugs, in vitro. It is possible that this in vitro inhibition explains the early hypotriglyceridemic effect of these agents.

## INTRODUCTION

Clofibrate (chlorophenoxyisobutyrate) and betabenzalbutyrate have been shown to lower serum triglyceride levels in experimental animals and in humans (1-5). Both agents also have been reported to lower serum cholesterol in a variable manner (6,7). Although these drugs are used clinically and appear to be relatively safe for long term use in humans, the mode of action remains unclear. An extensive literature has appeared since 1963 on the many metabolic and histological alterations caused by administration of clofibrate to various animal species and to man.

A number of these metabolic alterations have been well-documented and some have been postulated as essential to the hypolipidemic effects of the drug in vivo. In 1962, Thorp described the binding of clofibrate to serum albumin (8). Shortly thereafter it was postulated that clofibrate might displace thyroxine from binding sites on albumin with a resultant increased uptake of thyroxine by liver (9). Since thyroid hormone has a recognized hypocholesterolemic and hypotriglyceridemic effect, this action of clofibrate might produce an alteration of lipid metabolism by such an indirect mechanism. This hypothesis was supported by the findings of Westerfeld et al. (10,11), demonstrating a significant increase in the activity of mitochondrial *sn*-glycerol-3-P dehydrogenase activity. This latter enzyme is specifically increased by thyroxine administration (12). Furthermore the hypolipidemic effect of clofibrate is reduced or abolished in thyroidectomized animals (13), and the thyroxine binding capacity of thyroxine binding globulin is increased by clofibrate administration in man (14,15). Although these metabolic alterations have been clearly demonstrated, they are

[1]One of eight papers presented at the symposium "Recent Advances in Drugs Affecting Lipid Metabolism," AOCS Meeting, Houston, May 1971.

TABLE I

Changes in Serum Triglyceride and Glycerol and in Liver Triglyceride and *sn*-Glycerol-3-P Levels after 14 Days of Clofibrate[a]

| | Serum triglyceride, mg % | Liver *sn*-glycerol-3-P μmoles/g | Liver triglyceride, mg/g |
|---|---|---|---|
| Control | 50.8 ± 6.9 | 6.1 ± 0.23 | 6.5 ± 1.0 |
| Clofibrate | 36.8 ± 3.9 | 3.3 ± 0.15 | 5.9 ± 0.6 |

[a]Results are means of 10 rats ± SEM.

not correlated with lowering of serum triglyceride levels, and no changes in serum free thyroxine levels have been noted (15). Thyroid hormone reportedly increases the degradation of cholesterol and lowers serum cholesterol and phospholipid to a more pronounced degree than serum triglyceride. The reverse seems true of clofibrate. Therefore the relevance of these changes in thyroid hormone metabolism to the hypotriglyceridemic effect of these drugs remains conjectural.

Other studies have shown that adipose tissue uptake of triglycerides may be accelerated by prior clofibrate administration (16) and the relase of fatty acids from adipose tissue inhibited by either in vitro or in vivo administration of this agent (17,18), Perhaps these alterations in adipose tissue metabolism may be attributed to the suppression in adenyl cyclase activity recently reported by Greene et al. (19). Changes in the tissue levels of ATP and 3′,5′ cyclic AMP would be expected to effect both lipolysis and lipoprotein lipase activity. It is well established that serum free fatty acid levels (18,20) and serum glycerol (21) levels fall following the administration of clofibrate, and this supports the physiological importance of these effects on adipose tissue metabolism.

Other studies have demonstrated inhibition of hepatic fatty acid biosynthesis at the acetyl CoA carboxylase step (22,23) and of cholesterol biosynthesis (1,2,24,25) when clofibrate or betabenzalbutyrate is added to various preparations of liver in vitro. Inhibition of hepatic release of lipids in man was suggested by the studies of Bierman et al. (26). Also the turnover rate of low density lipoproteins was found to be lowered by clofibrate treatment (27). These data suggest that suppression of hepatic triglyceride formation or release into serum may be a major factor in lowering serum triglyceride levels in vivo.

Other studies have suggested that administration of clofibrate may alter the formation or release of lipoproteins by liver (28). Thus the inhibition of hepatic triglyceride output could be attributed to suppressed fatty acid synthesis or esterification, decreased lipoprotein formation, or a reduction in release of lipoproteins from liver.

Previous studies of the esterification of fatty acids with *sn*-glycerol-3-P did not suggest an inhibition at this stage in triglyceride synthesis. However more recent studies (21 and Lamb and Fallon, unpublished observations) have demonstrated a reduction in triglyceride synthesis from glycerol in vivo and an inhibition of fatty acid esterification in vitro. These latter studies are briefly reviewed below.

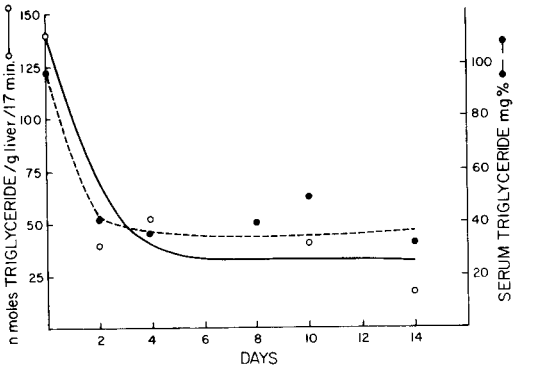


FIG. 1. The inhibition of hepatic triglyceride formation and the fall in serum triglyceride in rats fed 0.25% clofibrate in chow. The maximum decline in both determinations occurred by 2 days and was maintained throughout the 14 day period.

## METHODS

Male adult Sprague-Dawley rats were individually caged and selected to weigh 200-250 g at the start of studies. Clofibrate was administered, mixed with ground chow in a proportion of 0.25 g/100 g chow. Controls were pair fed chow. Weight gain and calorie intake was unaffected by clofibrate. Rats were killed without prior fasting at various time periods, from 6 h to 14 days, after beginning this diet. Rats studied at 6 and 12 h were begun at 8 PM and 2 AM on the chow and clofibrate diet. Serum clofibrate levels were determined (21).

The incorporation of $^{14}$C-glycerol into hepatic and serum triglycerides was measured 17 min after ip or iv administration. This time period was optimum for incorporation if isotope into hepatic triglycerides. The details of methods for the determination of triglyceride biosynthesis by this technique are described elsewhere (21). Serum glycerol was determined as described by Laurell and Tibbling (29) and glycerol radioactivity measured after separation from glucose and other carbohydrate intermediates by thin layer chromatography (21).

The formation of glycerolipids by homogenate preparation of liver was measured by methods described previously (21,30). Microsomes were prepared from rat liver and acyl CoA-*sn*-glycerol-3-P acyltransferase measured as described previously (31,32). Other enzymes of glycerolipid formation were measured by modifications of published methods.

All data were analyzed by the standard "t" test and variance was estimated by the SE of the mean.

## RESULTS

Administration of clofibrate for 14 days

TABLE II

Inhibition of *sn*-Glycerol-3-P Acyltransferase by Clofibrate and Benzalbutyrate[a]

| Drug, mM | Inhibition of MAGP formation, % | |
|---|---|---|
| | CPIB | BBB |
| 0.5 | 11 | 17 |
| 1.0 | 35 | 33 |
| 2.5 | 46 | 59 |
| 5.0 | 66 | 62 |
| 10.0 | 78 | 71 |

[a]The incubation mixture was identical to that described previously (31,32). The drugs were added in the concentration indicated as a sodium salt at pH 6.5. All results are means of at least three determinations.

resulted in a significant fall in serum triglyceride ($p < 0.01$) as noted in Table I. No significant change in liver triglyceride content was observed in the majority of experiments. The liver concentration of *sn*-glycerol-3-P fell as reported previously (10). Serum clofibrate levels varied from 0.5 to 1.5 mM.

The formation of hepatic and serum triglyceride measured by the incorporation of $^{14}$C-glycerol into these lipids was reduced 35-50%. This reduction in triglyceride synthesis in vivo was significant when calculated on the basis of total liver weight and total body weight. A comparable decrease in isotope incorporation into diglyceride and lecithin occurred.

These observations demonstrate that administration of clofibrate for 14 days results in a lowered rate of hepatic and serum triglyceride formation from glycerol and a sustained fall in serum triglyceride and glycerol levels.

The time sequence of these changes during the 14 day period is shown in Figure 1. The fall in serum triglyceride was maximum between the second and fourth day, and the fall in triglyceride formation was maximum by the second day. Other studies show that the fall in hepatic tritlyceride synthesis occurred as early as 6 hr following ingestion of clofibrate and was associated with a decline in serum triglyceride. Both effects were maximum between 12 and 24 hr (21). In these studies no fall in serum glycerol level or in liver *sn*-glycerol-3-P occurred until after the decline in serum triglyceride and in hepatic triglyceride formation was noted (21). Moreover no changes in hepatic triglyceride levels were detected at the earliest time periods. These data provided evidence that inhibition of hepatic triglyceride formation in vivo was one of the earliest metabolic consequences of clofibrate administration. The mechanism of this decrease in triglyceride formation has been studied in vitro.

The incorporation of $^{14}$C-*sn*-glycerol-3-P into diglyceride and triglyceride fraction was inhibited up to 90% (27) by addition of clofibrate in a concentration of 1-10 mM to liver homogenate preparations. The related antilipemic agent, betabenzalbutyrate, also was found to inhibit diglyceride and triglyceride formation in the same preparation. The inhibition was not competitive with respect to *sn*-glycerol-3-P.

Further studies were initiated to determine the mechanism of inhibition by these agents (Lamb and Fallon, unpublished observations). No inhibition of long chain fatty acyl CoA synthetase, phosphatidic acid phosphatase, or diglyceride acyltransferase were found. However marked inhibition of acyl-CoA-*sn*-glycerol-3-P acyltransferase was observed. Inhibition of this reaction was noted in the range of 1-10 mM for both clofibrate and betabenzalbutyrate as noted in Table II. Inhibition was 80-90% at 10 mM. This inhibition was not competitive with respect to *sn*-glycerol-3-P. Because of the protein binding of the other substrate, palmityl CoA, no kinetic analysis was made with respect to this compound.

The reaction mixture used to determine microsomal acyl CoA-*sn*-glycerol-3-P acyltransferase activity contains albumin in a concentration of 3.75 mg/ml. Because of the initial data of Thorp demonstrating binding of clofibrate by albumin, studies of the effect of clofibrate and betabenzalbutyrate on substrate binding by albumin were performed. Clofibrate and betabenzalbutyrate both displace palmityl CoA from albumin binding sites and increase the amount of palmityl CoA bound by microsomes under incubation conditions (33). The binding of palmityl CoA by albumin and microsomes was determined by ultracentrifugation, millipore filtration, and sedimentation by centrifugation (33).

Since increasing concentrations of microsomal bound palmityl CoA were previously shown to inhibit this reaction (31,32), further studies of this process were undertaken. It was demonstrated that a portion of the inhibition by clofibrate in vitro could be attributed to an increase in microsomal bound palmityl CoA without a change in total palmityl CoA concentration in the mixture. However this effect could account for no more than 25% of the inhibition by these agents. Both clofibrate and betabenzalbutyrate inhibited the acylation of *sn*-glycerol-3-P at optimum microsomal bound palmityl CoA concentrations and in the absence of albumin. This provided further evidence for a direct inhibition of this reaction by these agents.

## DISCUSSION

The recent evidence summarized in the preceding section strongly suggests that one early effect of clofibrate in vivo is an inhibition of triglyceride formation in the liver. This change apparently precedes inhibition of adipose tissue lipolysis as measured by a fall in serum glycerol levels. In addition the fall in serum triglyceride level occurred in the absence of a rise in hepatic triglyceride concentration. This would suggest that inhibition of hepatic formation of triglyceride, rather than assembly of lipoprotein complexes or their release, is the initial mechanism by which clofibrate inhibits triglyceride output from liver. However the data cannot exclude an additional effect of this agent. on lipoprotein synthesis, assembly or release which may contribute to or maintain a hypotriglyceridemic effect.

Both the in vivo and in vitro data suggest that reduction in fatty acid esterification accounts for the inhibition of triglyceride formation. Inhibition of fatty acid biosynthesis also may contribute to the hypolipidemic effect by reducing substrate concentrations. Further studies will be necessary to determine the precise interrelation of the various metabolic abnormalities produced by this drug in liver.

The observation that betabenzalbutyrate also inhibits diglyceride and triglyceride formation by reducing the rate of *sn*-glycerol-3-P acylation suggests that this agent also may inhibit hepatic triglyceride biosynthesis in vivo. Studies to demonstrate this are in progress. Since administration of betabenzalbutyrate is not reported to result in hepatomegaly or many of the structural alterations attributed to clofibrate administration (34), it seems likely that these effects are not related to the hypolipemic effects of these compounds.

Final interpretation of the results of the in vitro studies using betabenzalbutyrate and clofibrate must await adequate determinations of hepatic drug levels. Early studies suggest that clofibrate is not concentrated by liver, and in fact very low levels of drug were detected in these studies (8). However the obvious structural changes (35) and metabolic alterations attributed to this agent suggest that hepatic uptake probably occurs. Since clofibrate and betabenzalbutyrate are bound by proteins, their exact intracellular distribution and concentration are not yet known and may be of considerable importance in elucidating the site of biochemical activity of these agents.

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# Progestagens, Anabolic-Androgenic Compounds, Estrogens: Effects on Triglycerides and Postheparin Lipolytic Enzymes[1]

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**ABSTRACT**

Oral contraceptives, estrogens, progestagens and anabolic-androgenic compounds have extensive effects on plasma triglycerides and on triglyceride clearing enzymes. This review will center on recent advances in the understanding of the mode of action of these compounds, both in normals and in patients with hyperlipoproteinemia.

## ORAL CONTRACEPTIVES, ESTROGENS AND TRIGLYCERIDES

Increases in plasma triglyceride levels have been reported in normal women taking a variety of oral contraceptives containing estrogens and progestins (1-5). Triglycerides rose in nine out of ten subjects studied by Hazzard et al (3), and in 16 of 19 reported by Sachs et al. (4). The triglyceride increments on estrogen were, however, primarily within broad normal limits (<150/100 mg). There were accompanying increases in prebeta-lipoproteins (3,4). The rise in triglycerides appeared to be caused by the estrogen component alone (3). Qualitatively similar elevations of triglycerides were noted by Robinson and LeBeau (6) in post menopausal women given equine estrogen in a dose which lowered plasma cholesterol.

The significance of moderate increases in plasma triglycerides observed in women taking oral contraceptives remains unclear. Although no definite causal relationship has been shown between triglyceride increments on oral contraceptives and vascular disease, the incidence of myocardial infarction (7) and cerebral vascular accidents (8) has been reported to be significantly increased as compared to premenopausal women on no oral contraceptives.

Postheparin lipolytic activity (PHLA) has been shown to be depressed in many normal women on mixed oral contraceptives (3,9). The depression in PHLA appears to be owing to the estrogen component (3). Fabian et al. reported depressed PHLA in patients receiving parenteral estradiol therapy (10) and in patients during the last trimester of pregnancy (11). Adipose tissue lipoprotein lipase activity has been studied in pregnant animals and was found to be decreased (12).

The decrements of PHLA and elevation of triglycerides have been shown to be concurrent (3,10,11). The major difficulty in attributing a direct etiological role to estrogen-induced changes in PHLA is the dichotomy between depression of PHLA well below normal range (3,10) and only minimal increases in endogenous lipoprotein and plasma triglyceride levels (3,4).

An estrogen-induced increase in apo-lipoprotein and triglyceride synthesis has also been suggested as a potential mechanism for hypertriglyceridemia produced by oral contraceptives (2).

Triglyceride increments on oral contraceptives may possibly be related to estrogen-induced changes in insulin and glucose metabolism. Oral contraceptives have been reported to cause increased basal immunoreactive insulin levels (3,13). This increase in immunoreactive insulin has been attributed in part to elevated serum growth hormone and resultant tissue insulin resistance in women taking oral contraceptives (14,15). Reaven, Bierman and respective colleagues (16,17) have further suggested that hepatic triglyceride synthesis may be increased by elevated insulin levels. The weak association between basal immunoreactive insulin and triglyceride in patients with endogenous hypertriglyceridemia emphasizes the fact that insulin level is only one of the many factors which contribute to regulation of triglyceride transport (18,19). Recent work by Basso and Havel in diabetic dogs suggests that defects in peripheral triglyceride removal are primary causes of elevated triglyceride levels (20). Havel and coworkers have subsequently shown that efficiency of extrahepatic triglyceride removal in normal and hyperlipemic subjects, rather than increased triglyceride synthesis, must play a primary role in plasma triglyceride levels (21). Further study of effects of estrogen on both extrahepatic triglyceride removal and hepatic triglyceride synthesis needs to be done before estrogen-mediated increments in plasma insulin can be implicated in the concurrent estrogen-induced increments in

[1]One of eight papers presented at the symposium "Recent Advances in Drugs Affecting Lipid Metabolism," AOCS Meeting, Houston, May 1971.

plasma triglycerides.

Oral contraceptives also may diminish glucose tolerance in normal women (22-24). The estrogenic component alone apparently is the cause of decreased intravenous (23) and oral glucose tolerance. Increased cortisol levels during oral contraceptive treatment (25) might also reduce glucose tolerance and increase triglycerides. The diabetogenic action of estrogens may then be further related to increases in triglycerides, since increasing glucose intolerance and increases in triglycerides often occur together (26).

In patients with pre-existing endogenous hyperglyceridemia, estrogen and oral contraceptives can further markedly increase triglyceride levels (27,28). Plasma triglycerides increased from the 250 mg/100 ml range to over 1200 mg/100 ml in two hyperglyceridemic patients treated with mestranol-chlormadinone acetate, and mestranol (27). Plasma PHLA was normal at the height of the hyperglyceridemia in both subjects. It was postulated that the estrogen-induced increase in triglycerides in the two patients was secondary to hyperinsulinism and hyperglycemia, or to increased hepatic synthesis of prebeta-lipoproteins (27).

Exacerbation of mixed endogenous and exogenous hyperglyceridemia, coupled with development of abdominal pain or pancreatitis (29), has also been noted in certain women with familial Type V hyperlipoproteinemia given estrogens (30). In a woman with familial Type V given diethylstilbesterol 3 mg/day (30), triglycerides were elevated to 5200 mg/100 ml and PHLA was depressed to 0.130 μEq FFA/ml/min (normal 0.25-0.55) (31). Two weeks after the estrogen was stopped, and on an isocaloric diet, triglycerides had fallen to 852 mg/100 ml and PHLA rose to 0.460 μEq FFA/ml/min. Levels of both chylomicron and prebeta-lipoprotein triglyceride fell sharply when estrogen was discontinued. Qualitatively similar changes in triglycerides and PHLA have been observed in women with familial Type IV hyperlipoproteinemia (32). Especially in familial Type V, superimposition of an estrogen-related decrease in PHLA and pre-existing decreased triglyceride clearing capacity may severely exacerbate hyperglyceridemia.

If estrogen-related changes in lipid and carbohydrate metabolism in normal and hyperglyceridemic patients are judged to be undesirable, they may in part be side-stepped by use of contraceptives having progestational activity alone, by reducing the dose of estrogen supplementation for post menopausal women, and by exercising caution in administration of estrogen to patients with pre-existing hyperglyceridemia.

## PROGESTAGENS AND TRIGLYCERIDES

In direct contrast to estrogens, progestational compounds lower triglyceride levels in normals and hyperglyceridemic patients, and concurrently increase PHLA (33).

We initially observed a striking fall in plasma triglyceride in a young woman with familial Type V hyperlipoproteinemia given norethindrone actate (17 α ethinyl, 19 nortestosterone) for mestrual irregularity (34). This C-17 alkylated nortestosterone derivative is a steroid derivative with primary and potent progestational activity. Subsequent administration of norethindrone acetate 5 mg/day resulted in marked decrements in plasma triglyceride in four women with familial Type V hyperlipoproteinemia (29). Concurrently PHLA rose in all four patients from low pretreatment levels to the normal or low normal range. Increments in PHLA appeared to be dose-related. Free fatty acids, fasting blood glucose and immunoreactive insulin were not altered by therapy. The fall in triglycerides was also associated with marked diminution in abdominal pain. Triglycerides rose, abdominal discomfort returned, and PHLA fell to pretreatment levels after substitution of placebo or discontinuation of medication (29).

These studies were then extended to include six men with familial Type V hyperlipoproteinemia, and four normal women (33,35). Triglycerides fell in three of the men on norethindrone acetate 5 mg/day and there were concurrent increases in PHLA. Neither triglycerides nor PHLA were changed during therapy in the three other men. In four normal women (35), mean triglyceride fell slightly from 50 to 37 mg/100 ml, accompanied by an increase of mean PHLA from 0.37 off medication to 0.54 μEq FFA/ml/min. Norethindrone then appeared to have similar qualitative effects in normals and patients with familial Type V hyperlipoproteinemia (33).

Norethindrone acetate also effectively lowers triglycerides and elevates PHLA in patients who have endogenous hyperglyceridemia (32,33). On short term therapy (10-12 days) an average 30% decrease in triglyceride was accompanied by an average 28% increase in PHLA in 13 patients with Types III and IV hyperlipoproteinemia (33). Mean triglycerides in seven of these patients (522 mg% before therapy, range 225-1500) fell to 222 mg% (range 100-520) after 6 months of norethindrone.

These studies suggest that synthetic steroids with progestational activity may lower endogenous and exogenous triglycerides in part by increasing the activity of the triglyceride lipases

(29,32,33) and by improving triglyceride removal. The effect appears to be particularly marked in women with Type V hyperlipoproteinemia who have low PHLA prior to treatment (29, 33). In direct contrast to progestagen therapy, administration of estrogens to such patients (32), further depresses PHLA, impairs hydrolysis of triglyceride, and exacerbates Type V hyperlipoproteinemia.

In earlier studies of drugs with progestational activity, little effect on plasma lipids was reported, but these studies centered primarily on changes in cholesterol and phospholipids. Svanborg and Vikrot (36) found no change in free fatty acids, triglycerides, cholesterol and total phospholipids in oophorectomized women given 17 alpha-hydroxy progesterone caproate. Engelberg and Glass (37) observed no changes in cholesterol or phospholipids in normal men and postmenopausal women given 10-30 mg progesterone/day for 3-6 months. Oliver and Boyd (38) found no changes in cholesterol or cholesterol-phospholipid ratios in six hypercholesterolemic men given a short term course of progesterone.

In contrast to previous reports of limited effects of progestational compounds on plasma lipids, there are many studies that document extensive lipid lowering effects of androgenic-anabolic compounds. Many of these androgenic compounds are testosterone derivatives and are structurally similar to progestational compounds.

## ANABOLIC-ANDROGENIC COMPOUNDS AND TRIGLYCERIDES

Norethindrone acetate is a 17 alkylated nortestosterone derivative which has potent progestational activity. Since similar androgenic-anabolic testosterone and nor-testosterone derivatives also lower triglycerides (39-46), one important question to be resolved is whether all of these structurally related compounds alter plasma glycerides and triglyceride clearing enzymes by similar mechanisms. Moreover it is not yet known if the lipid lowering effects can be separated from the anabolic, androgenic or progestational activities of these compounds. Previous work by Furman, Howard and coworkers (39-41), Berkowitz et al. (42), Sachs and Walfman (43), Gherondache (44), Skikantia (45), Cohen (46), and respective colleagues, suggests that many structurally related testosterone derivatives have similar triglyceride lowering effects in normal and hyperglyceridemic subjects. These testosterone and nortestosterone derivatives are primarily anabolic, androgenic, or both, and include the following: Methyltestosterone (41); Oxymetholone (42) (2-hydroxy-methylene-17 αmethyl-17β hydroxy-3-androstanone); Oxandrolone (43,44) (17α methyl-2-oxa-5α androstan-17β ol-3-one); Androstanopyrazole (40) (17β hydroxy-17α methyl-androstano-3, 2-c pyrazole); Methandrostenolone (45); Androsterone (46).

Despite different "hormonal" activity, effects of oxandrolone and norethindrone acetate on postheparin hydrolytic enzymes and triglycerides appear to be similar (32,33,47). In three patients with familial Type V, the mean per cent fall in triglycerides on 10-14 days of oxandrolone (7.5 mg/day) was 39, while the per cent increase in PHLA was 81 (47). In 16 patients with endogenous hyperglyceridemia, (Type IV) on 10-14 days of oxandrolone, the mean per cent fall in triglycerides was 23, while the per cent increase of PHLA was 76. It was postulated that stimulation of postheparin lipolytic activities and increased peripheral triglyceride hydrolysis might, in part, account for the triglyceride lowering effect of oxandrolone.

In seven patients with endogenous hyperglyceridemia on long term oxandrolone (7.5 mg/day, 4-7 months), mean triglycerides fell from 522 to 222 mg% (47). As has previously been reported for androgenic compounds (48), 25% of the patients treated also had improvements in glucose tolerance on oxandrolone (47).

The similarity in action between norethindrone and oxandrolone (drugs which are very dissimilar in "hormonal" activity, but structurally similar) suggests that the triglyceride lowering action may not be directly related to "hormonal" activity. Evaluation of other C-17 and C-19 alkylated testosterone and nortestosterone derivatives (some having no progrestational or anabolic activity) may lead to the development of new and potent triglyceride lowering compounds.

It is now increasingly apparent that synthetic and naturally occurring estrogens, progestagens and anabolic-androgenic compounds have substantial effects on plasma triglycerides in normal and hyperglyceridemic subjects. At least some of these effects appear to be mediated through changes in postheparin lipolytic enzyme activities. Estrogen-induced changes appear to be directly opposite to those induced by progestagens and by anabolic-androgenic compounds. In selected patients with familial hyperglyceridemia (Types III, IV and V hyperlipoproteinemia) norethindrone acetate and oxandrolone represent effective additions to the armamentarium of triglyceride lowering compounds. Coupled with weight reduction to ideal body weight and continued balanced diet, they may well be the treatments of choice in

patients with familial Type V hyperlipoproteinemia.

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# Effects of Ethyl *p*-Chlorophenoxyisobutyrate on Biliary Secretion of Bile Acids, Cholesterol and Phosphatidyl Choline[1]

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## ABSTRACT

The effect of chronic administration of ethyl *p*-chlorophenoxyisobutyrate (CPIB) on the secretion of bile lipids was studied in four dogs with surgically implanted Thomas cannulae for periods of 2-7 months. The concentration of cholesterol, triglycerides and *p*-chlorophenoxyisobutyric acid in serum and of bile acids, cholesterol and phospholipids (phosphatidyl cholines) in bile were measured. Chronic administration of CPIB resulted in a marked increase in the concentration of cholesterol, bile acids and phosphatidyl cholines in the bile of all dogs, and a decrease in serum cholesterol and triglyceride concentration in serum in three of the four dogs. Serum concentrations of *p*-chlorophenoxyisobutyric acid were monitored to insure the presence of the drug in the dogs; however, no correlation between serum levels of *p*-chlorophenoxyisobutyric acid and the concentration of biliary lipids was noted. The bile acids and *p*-chlorophenoxyisobutyric acid were determined by gas chromatographic procedures and the structures were confirmed by gas chromatography-mass spectrometry.

## INTRODUCTION

The current therapeutic use of ethyl *p*-chlorophenoxyisobutyrate (CPIB, Atromid) is based upon its reported action in lowering serum concentrations of cholesterol and triglycerides. Several mechanisms of action have been suggested, including inhibition of cholesterol biosynthesis, but very little is known about the effect of CPIB administration on the excretion of lipids and on the enterohepatic circulation. Grundy et al. (1) observed an increase in neutral fecal steroids for patients during CPIB administration, and concluded that the increase was owing to mobilization and loss of endogenous cholesterol from the body.

The precise determination of fecal lipid output, including bile acids, is difficult, and the evaluation of results is complicated by the reabsorption of cholesterol and bile acids and subsequent biliary reexcretion. We therefore undertook a study of methods for the direct determination of drug effects on the biliary

[1]One of eight papers presented at the symposium "Recent Advances in Drugs Affecting Lipid Metabolism," AOCS Meeting, Houston, May 1971.

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TABLE I

Effects of CPIB[a] Administration Upon Concentrations of Bile Acids, Lecithins, Cholesterol and Triglycerides in Bile of Dogs

| Dog no. | Experiment | Bile acids,[b] mg/ml | Lecithin,[b] mg/ml | Cholesterol,[b] mg/ml | Triglycerides,[b] mg/ml |
|---|---|---|---|---|---|
| 320[c] | Untreated | 24 | 17 | 0.4 | --- |
| | CPIB | 51 | 41 | 1.1 | --- |
| 487[d] | Untreated | 25 | 21 | 0.3 | --- |
| | CPIB | 57 | 40 | 0.8 | --- |
| 530[e] | Untreated | 13 | 7 | 0.7 | 0.3 |
| | CPIB | 51 | 55 | 1.3 | 0.2 |
| 825[f] | Untreated | 30 | 18 | 0.4 | 0.2 |
| | CPIB | 40 | 40 | 1.1 | 0.2 |

[a]*p*-Chlorophenoxyisobutyrate.

[b]Values are averages of three observations, made at intervals of about 10 days, at the beginning of the experiment (untreated) and at the end of the dosage period (CPIB). Typical results observed over the drug course are in Figures 1, 5 and 6.

[c]Treatment period: 56 days, 500 mg/day; two courses.

[d]Treatment period: 210 days, 500 mg/day.

[e]Treatment period: 198 days, 500 mg/day.

[f]Treatment period: 168 days, 1500 mg/day.

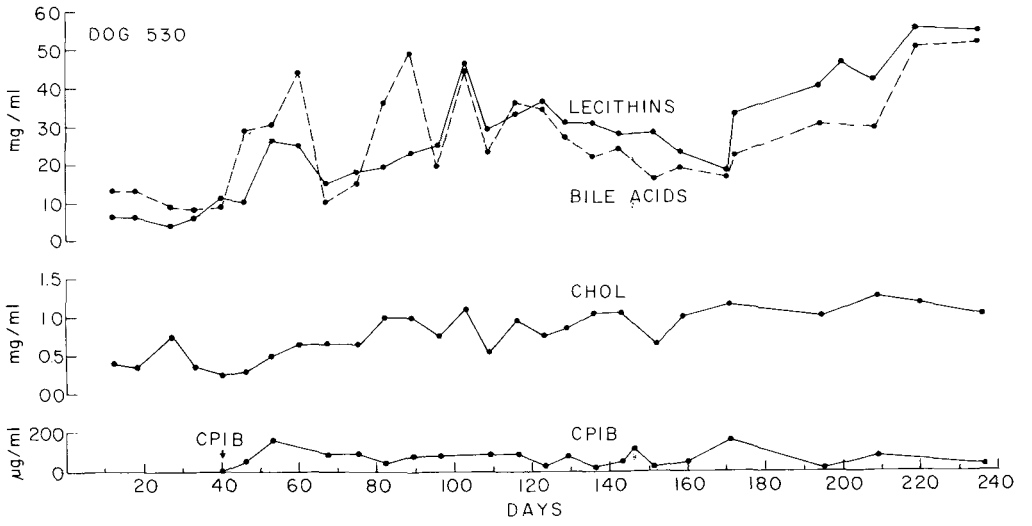


FIG. 1. Effect of chronic administration of ethyl *p*-chlorophenoxyisobutyrate (CPIB) (500 mg/day) on the bile concentration of lecithin (mg/ml), bile acids (mg/ml) and cholesterol (mg/ml). The serum concentration of CPIB acid (µg/ml) was determined at the time of each collection of bile. There was a two-fold increase in the concentration of cholesterol, a four-fold increase in bile acid and a seven-fold increase in lecithin concentration in the bile during the 198 days of drug administration.

secretion of bile acids, cholesterol and lecithin (the phospholipids of bile are almost totally lecithins). The dog was used as an experimental animal. Surgical implantation of a modified Thomas cannula made it possible to collect repetitive bile samples, without undue interruption of normal biliary function, and to carry out long term (1-2 year) studies of drug action on biliary secretion.

The effect of CPIB administration was studied in this way, with dosages comparable to those used in humans. The serum concentrations of cholesterol and triglycerides were also measured during the study. The drug concentration in serum (CPIB is present in blood as the free acid) was also determined. The results obtained with four female dogs receiving CPIB for periods of 2-7 months are summarized in this paper.

## EXPERIMENTAL PROCEDURES

### Animals

After cholecystectomy a modified Thomas cannula was implanted surgically in the second portion of the duodenum, opposite the ampulla of the common bile duct. Bile collections were made on fasting dogs (2 hr collection from 8:30-10:30 AM on appropriate days) by inserting a catheter directly through the ampulla into the common bile duct. Blood samples were obtained by extremity venipuncture during the period of bile collection. Bile and serum were stored at -14 C until analyzed. Triglyceride and cholesterol analyses were usually carried out within 24 hr of collection.

During drug treatment CPIB was administered to each dog once a day by means of a capsule imbedded in a bolus of food. The drug (500 mg/capsule) was obtained from the hospital pharmacy and was the same preparation administered to human patients. The doses employed were 500 (3 dogs) and 1500 (1 dog) mg/day. A 500 mg dose administered to a 20 kg dog is comparable to the dose (25 mg/kg) recommended for human patients. Dogs dislike the taste and odor of the drug, and at times the food-drug combination was rejected. Serum concentrations of *p*-chlorophenoxyisobutryic acid were determined to confirm the presence of the drug at the time of bile collection.

### Bile Analyses

*Bile cholesterol and triglycerides:* The determination of triglycerides and cholesterol concentrations in bile was carried out with 0.5 ml samples of bile. A Technicon Autoanalyzer and Technicon procedures were used; the method was the same as that used for serum analyses.

*Bile acids:* An aliquot of bile, usually 1 ml, was partitioned between chloroform and methanol (2:1) according to the Folch procedure. The chloroform layer contained neutral lipids and phospholipids; conjugated bile acids were in the methanol-water phase.

A 1.0 ml sample of bile was placed in a separatory funnel; 50 ml chloroform-methanol 2:1, and 10 ml distilled water were added. The separatory funnel was shaken vigorously 10

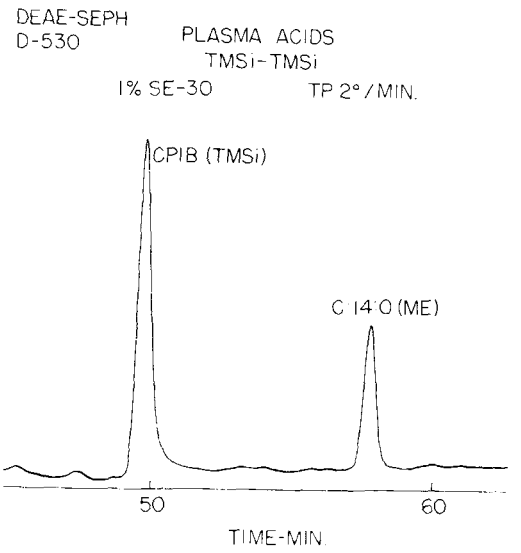


FIG. 2. Gas chromatographic analysis of the acid fraction isolated from plasma of Dog 530. The major metabolite of CPIB, *p*-chlorophenoxyisobutyric acid, was separated as the trimethylsilyl ester derivative; the internal standard was methyl myristate (C:14:0, ME). The separation was carried out with a 12 ft 5% SE-30 column with temperature programming at 2 deg/min from 100 C.

min, and then was placed in a cold room (4 C) overnight in order to complete the separation of the two phases. Difficulties owing to emulsions were not encountered.

The upper (water-methanol) phase containing bile salts was separated from the chloroform layer and concentrated (rotary evaporator) at 40 C to remove most of the methanol; the water was then removed by lyophilization. The residue was dissolved in glass-distilled water and hydrolyzed enzymically with cholylglycine hydrolase (EC 3.5). To an aliquot (.01) of the lyophilized methanol-water phase there was added 0.1 ml of sodium acetate solution (0.1 molar, pH 5.6), 0.2 ml of EDTA solution (1.86% of the disodium salt), 0.1 ml of 0.2M Cleland's reagent and 0.1 ml of enzyme (2). The reaction mixture was incubated at 37 C for 30 min and then acidified with 0.5 ml of glacial acetic acid. The bile acids were extracted four times with 4 ml portions of benzene-acetone 2:1. The combined benzene-acetone extracts were taken to dryness under reduced pressure (rotary evaporator); the residue was dissolved in 0.5 ml methanol. The overall recovery of cholic acid-$C_{14}$ from taurocholic acid (carbonyl-$^{14}C$) added to bile was 90±4%.

The bile acids were converted to methyl ester-trimethylsilyl (ME-TMSi) derivatives for analysis by gas chromatography (GC). An ethereal solution of diazomethane was added to the methanolic solution (0.5 ml) of bile acids until the yellow color persisted. After standing at room temperature 30 min, the excess ether, methanol and diazomethane were removed with a nitrogen stream. The residue of bile acid methyl esters was dissolved in 0.1 ml pyridine and 0.1 ml *bis*-trimethylsilyltrifluoroacetamide (BSTFA) or N-trimethylsilylimidazole (TSIM), and 0.05 ml of trimethylchlorosilane (TMCS) was added. The mixture was allowed to stand overnight at room temperature to insure complete silylation of all hydroxyl groups. An aliquot of the solution was analyzed directly by gas chromatography.

*Bile phospholipids:* After separating the

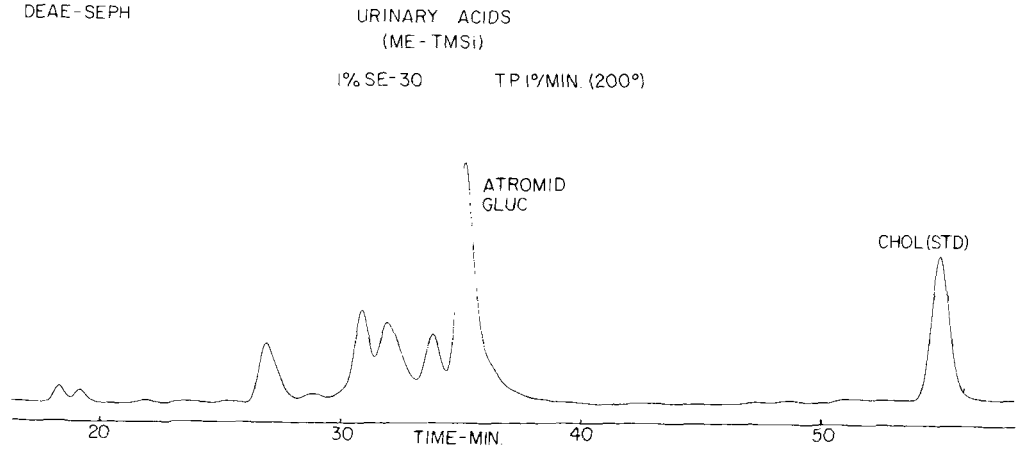


FIG. 3. Gas chromatographic (GC) analysis of urinary acids isolated from the urine of a dog treated with ethyl *p*-chlorophenoxyisobutyrate (CPIB). The acids were separated as the methyl ester-trimethylsilyl ether derivatives. Separation was carried out on a 12 ft 1% SE-30 column by temperature programming at 1 deg/min from 200 C. The ester glucuronide of CPIB acid (ATROMID GLUC) was separated by GC as the intact glucuronide. The structure was confirmed by GC-MS techniques.

TABLE II

Effects of CPIB[a] Administration Upon
Serum Concentrations of Cholesterol and Triglycerides in Dogs

| Dog no. | Experiment | Cholesterol,[b] mg% | Triglycerides,[b] mg% |
|---|---|---|---|
| 320[c] | Untreated | 123 | 55 |
| | CPIB | 131 | 29 |
| 487[d] | Untreated | 192 | 67 |
| | CPIB | 119 | 28 |
| 530[e] | Untreated | 175 | 87 |
| | CPIB | 130 | 27 |
| 825[f] | Untreated | 145 | 36 |
| | CPIB | 120 | 41 |

[a]See Table I.

[b]Values are averages of three observations, made at intervals of about 10 days, at the beginning of the experiment (untreated) and at the end of the dosage period (CPIB). Typical results observed over the drug course are in Figures 1, 5 and 6.

[c]Treatment period: 56 days, 500 mg/day; two courses.

[d]Treatment period: 210 days, 500 mg/day.

[e]Treatment period: 198 days, 500 mg/day.

[f]Treatment period: 168 days, 1500 mg/day.

methanol-water layer, the lower (chloroform) phase containing neutral lipids and phospholipids was evaporated with a rotary evaporator at 40 C. The residue was transferred with chloroform to a calibrated stoppered centrifuge tube, and the solution was reduced to 1 ml with a nitrogen stream. An aliquot (0.1) of the chloroform solution was analyzed by thin layer chromatography (TLC) using glass fiber paper impregnated with silicic acid (ChromAR 500, Mallinckrodt Chemical Co.).

After activating the sheet (20 cm x 20 cm) in an oven at 100 C for 30 min, a 100 µl sample was applied with an applicator in a band. Lecithin and cholesterol were used as markers. The paper was developed first in a solution of chloroform-acetone 60:40 until the solvent front was 16 cm from the origin. After drying in air 3 min, a strip ca. 2 cm wide was cut including the solvent front. This band contains neutral lipids (cholesterol and triglyceride) which can be eluted and analyzed by TLC, GC or colorimetrically. The remainder of the ChromAR sheet (16 cm) was then developed in the same direction using chloroform-methanol 4:1 as the solvent system. Lecithins, the major phospholipids in bile, have an Rf of 0.1-0.2 in this system; cephalins, which are found in small amounts, have an Rf of 0.7. The time required for each thin layer separation was ca. 20 min. After drying 30 min the sheet was sprayed with a 1% solution of Rhodamine 6G in ethanol; the bands were visualized under a UV lamp; usually only a lecithin fraction was present.

The lecithin band was cut into small strips (2 mm x 10 mm) and the strips placed in a chromatographic column (1 cm diam). The lecithin was eluted by allowing solvents (60 ml chloroform followed by 40 ml methanol) to flow slowly through the column. The combined chloroform and methanol extracts were taken to dryness (rotary evaporator), and the residue was transferred immediately with methanol to a graduated stoppered centrifuge tube for phosphorus analysis.

Phosphorus analyses were carried out on duplicate samples according to the Bartlett procedure (3). The phosphorus values were multiplied by a factor of 25 to estimate milligrams of phospholipid.

### Serum Analyses

*Serum cholesterol and triglycerides:* Analyses for cholesterol and triglycerides were carried out with 0.5 ml samples of serum using a Technicon Autoanalyzer. Reference analyses were included in all runs.

*p-Chlorophenoxyisobutyric acid:* The major metabolite of CPIB in blood is the acid, *p*-chlorophenoxyisobutyric acid. The metabolite was identified and quantified by GC analysis as the trimethylsilyl ester.

One ml serum was diluted to 20 ml with glass-distilled water, and after adjusting the pH to 4-4.5 with acetic acid, the sample was transferred to a 10 cm x 1 cm DEAE-Sephadex column (4). Neutral and basic fractions, including proteins, were washed from the column with water, and the acids were eluted with a solution of 150 ml 1.5 M pyridine-acetic acid. After lyophilization the residue was transferred with methanol to a graduated centrifuge tube; the volume of methanol was reduced to approximately 0.2 ml with a nitrogen stream. The

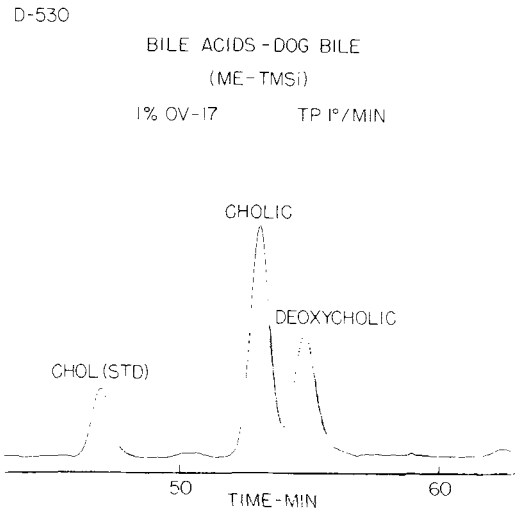


FIG. 4. Bile acid "profile" of the total bile acids isolated from bile (Dog 530) after enzymatic hydrolysis. The bile acids were separated as the methyl ester-trimethylsilyl ether derivatives with a 6 ft 1% OV-17 column with temperature programming at 1 deg/min from 190 C. The major bile acids are cholic and deoxycholic acids. The internal standard was the trimethylsilyl ether derivative of cholesterol (CHOL, STD).

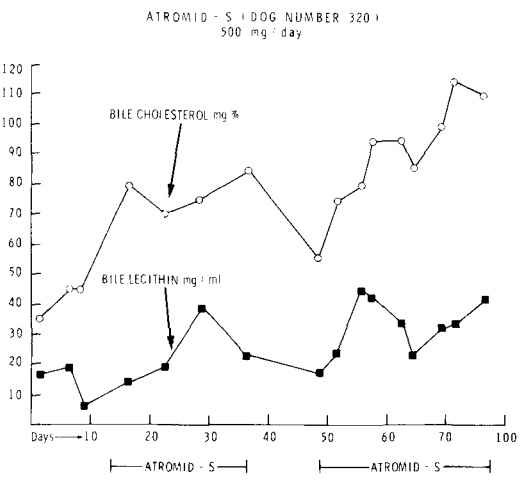


FIG. 5. The effect of administration and withdrawal of ethyl *p*-chlorophenoxyisobutyrate (CPIB) on bile cholesterol and lecithin concentrations. During the two periods of CPIB ingestion there was a rise in both bile cholesterol and bile lecithin. When the drug was withdrawn, the levels of cholesterol and lecithin declined.

solution was transferred quantitatively to a 1 ml vial; 10 $\mu$l pyridine was added, and the solution was evaporated (nitrogen stream). The pyridine salt of *p*-chlorophenoxyisobutyric acid was redissolved in 50 $\mu$l of pyridine, and 100 $\mu$l of *bis*-trimethylsilyltrifluoroacetamide (BSTFA) was added. The contents of the vial were mixed (Vortex mixer) and allowed to stand overnight.

For quantitative work an internal standard (methyl myristate) was added to the sample.

#### Instruments and Separation Conditions

GC separations were carried out with Barber-Colman Model 5000 gas chromatographs with Keithley Model 417 picoammeters and Texas Instruments recorders. Separations of bile acids were carried out by temperature programming from 200 C at 1 deg/min. The columns were 6 ft x 3.4 mm ID glass U-tubes, packed with 1% OV-17 or 1% SE-30 on 100-120 mesh acid-washed and silanized Gas Chrom P, prepared by our usual procedure (5). The separation of trimethylsilyl *p*-chlorophenoxyisobutyrate was carried out by temperature programming from 100 C at 2 deg/min. The column was a 12 ft x 3.4 mm ID glass W-tube, packed with 5% SE-30 on 80-100 mesh acid-washed and silanized Gas Chrom P. The injector temperature was 260 C, and the detector chamber was at 300 C in all separations.

Mass spectrometric analyses were carried out with an LKB Model 9000 gas chromatograph-mass spectrometer. The column was a 9 ft x 3.4 mm ID glass coil with either 1% OV-17 or 1% SE-30 packing on 100-120 mesh acid-washed and silanized Gas Chrom P. The accelerating voltage was 70 eV and the current was 60 $\mu$A. The ion source temperature was 250 C.

## RESULTS

Four dogs, each with a modified Thomas cannula arranged so that bile could be collected, were maintained for periods of 2-7 months with daily administration of CPIB. A stabilization period of 4-6 weeks was allowed after cholecystectomy and insertion of the cannula. In one instance drug administration was interrupted and then resumed.

The effects of CPIB on fasting concentrations of cholesterol, bile acids and lecithin in bile are summarized in Table I. A rise in cholesterol concentration occurred 3-6 days after drug treatment was started, and elevated concentrations persisted as long as the drug was administered. The effect on bile acids and lecithin concentrations was striking; an increase in these components of bile occurred when the drug was administered, and the rise continued, with some interruptions, during the period of drug administration. Rises of up to 8-fold in lecithin and 4-fold in bile acids were observed (Dog 530). During this time and during the stabilization period, the volume of bile collected varied in volume, but no change was observed in the average volume during drug

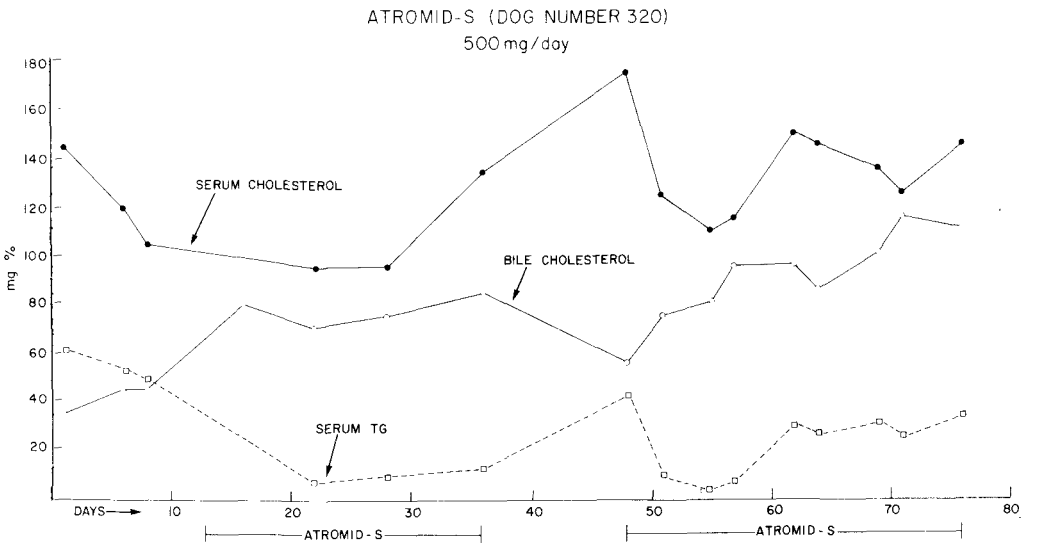


FIG. 6. The effect of ethyl *p*-chlorophenoxyisobutyrate on serum cholesterol and triglycerides and on bile cholesterol, demonstrating an inverse relationship between the serum and bile cholesterol levels. The changes in serum triglycerides paralleled the changes in serum cholesterol. Withdrawal of the drug resulted in a rise in serum cholesterol and triglycerides and a fall in concentration of bile cholesterol. There was a three-fold increase in the bile cholesterol concentration by day 76, but no overall change in the serum cholesterol concentration was observed.

treatment. The drug effect seems therefore to be primarily upon concentration of bile components rather than upon bile volume.

Figure 1 shows typical results obtained for a dog (No. 530) over a period of 198 days with daily administration of 500 mg CPIB. While fluctuations occurred, there was a gradual increase in the observed concentrations of cholesterol, bile acids and lecithin. Similar results were found for all dogs in the study.

The effects of CPIB on fasting serum concentrations of cholesterol and triglycerides are in Table II. A decrease in cholesterol concentration was observed for three dogs treated 168-210 days; for one dog treated 56 days no effect was found. The serum concentration of triglycerides fell for three dogs. Bile usually contains very little triglycerides; no effect was noted for this lipid.

Two metabolites of CPIB were found in the bile: *p*-chlorophenoxyisobutyric acid and the ester glucuronide of the acid. These are also the major urinary metabolites. Serum concentrations of the acid were determined during the experiments. This was done in order to confirm the presence of the drug; dogs dislike CPIB and rejection of the food-drug bolus was observed at times. The gradual increase in biliary secretion of bile acids, lecithin and cholesterol was found to correlate with the presence of the acid in blood, but not with its concentration at any given time. The increase continued with continual administration of the drug, but without concomitant rise in serum concentration of the acid. Concentration data for Dog 530 are in Figure 1. Figures 2 and 3 show the GC separation of the acid and the ester glucuronide from blood and urine, respectively.

No great difference in effects was noted for a dosage of 1500 mg/24 hr over 500 mg/24 hr.

The bile acid concentrations in bile were determined by a GC method, and this permitted an examination of the drug effect on individual bile acids. Figure 4 shows a typical analysis of dog bile acids (Me-TMSi derivatives). The ratio of cholic acid to deoxycholic acid varied during the experiments, but the ratio was never inverted.

## DISCUSSION

The marked increase in the concentration of bile acids and lecithin in bile observed in this study suggests that CPIB administration has a direct effect upon bile composition. The major metabolites of the drug in blood, bile and urine are the free acid and the ester glucuronide; it seems likely that the active agent(s) is one or both of these metabolites, since the drug in ester form is not present in appreciable amount in blood. The effect upon bile acid concentration is particularly marked. For example if a bile volume of 250 ml/24 hr is assumed for Dog 530 (Fig. 1), the increase in bile acid secretion after prolonged drug administration would be from 3.2 g/24 hr to 12.8 g/24 hr. An increase of this magnitude represents either a major

increase in pool size or a major increase in daily biosynthesis, or both, depending upon the pool size-excretion rate relationship. This was not determined in these experiments. The increase in lecithin concentration paralleled the bile acid increase; for example Dog 530 showed an increase in lecithin secretion from 1.8 g/24 hr to 13.7 g/24 hr after prolonged drug treatment (assuming 250 ml/24 hr bile volume). This presumably required an increase in daily biosynthesis. The fatty acids were probably recirculated; the degree of recirculation of the choline moeity was not determined and is not known.

The concentration of cholesterol in bile also increased with drug administration. In experiments with Dog 530, a 2-fold increase was observed; this however represents an increase of only about 150 mg/24 hr in cholesterol secretion. The degree of recirculation was not determined.

There seems to be no doubt that the observed effects were due to CPIB administration. When the drug course was interrupted the concentration of these three bile components fell, and when drug administration was resumed a rise in concentration followed. This effect is shown in Figure 5 (lecithin and cholesterol) for Dog 320. The actual concentration was not dependent upon blood concentration of the acid or its glucuronide ester; prolonged administration of the drug resulted in a slow but prolonged and ultimately marked increase in biliary concentration for these components. These effects suggest that the liver adapted to meet the circumstances induced by the drug. This phenomenon was reversible within the time span of these experiments. The dogs lost weight near the end of their respective experimental periods, however, indicating that additional and undefined metabolic effects were occurring.

The effect of CPIB upon serum or plasma concentrations of cholesterol and triglycerides has been debated on numerous occasions. In these experiments three dogs responded to the drug with a decrease in fasting serum concentration of cholesterol; one dog did not respond. Figure 6 shows an inverse relationship between the blood and bile concentrations of cholesterol for Dog 320, suggesting that the prolonged and marked rise in bile concentration should have resulted in an equally prolonged and marked fall in blood cholesterol concentration. Wide swings in the serum concentration occurred, but at the end of the experiment (80 days) there was little change in the concentration from the start of the experiment. The explanation for this effect is probably that the biosynthetic rate adjusts slowly to the circumstances; a fall while on the drug, and a rise when the drug is removed, may well occur either with or without a long term effect on the blood concentration. The effect on serum triglycerides was somewhat more uniform; decreases in plasma concentration were observed for three of the four dogs. A positive effect is shown in Figure 6 for Dog 320. An inverse relationship with the biliary changes was observed, and the long term effect was a moderate decrease in serum triglycerides.

It was considered possible that the large changes in bile concentration of bile acids caused by CPIB administration would result in an altered ratio of cholic to deoxycholic acid. The GC analytical records showed some variations in acid ratios during the experiments, but no major effect was observed; a typical cholic-deoxycholic acid ratio is shown in Figure 4.

No attempt was made in this study to evaluate CPIB as a therapeutic agent; the objective was to determine if CPIB affected the enterohepatic circulation and biliary secretion. The effect in dogs, with amounts equivalent to those used for humans, is marked and dependent upon continued administration of the drug. The changes are of sufficient magnitude to suggest that lipid metabolism is affected materially, although possibly in complex ways, and also that a determination of effects on biliary secretion should be made for other drugs which might act in the same way. The relationship between dog and human effects also remains to be established; it is known however, from the work of Hunninghake et al. (5) and others that CPIB administration leads to a modest decrease in serum concentrations of cholesterol in some patients, and to a more marked decrease in serum concentrations of triglycerides in most (75% or more) patients. These effects are very nearly the same as those observed in this work with dogs. The biliary effects may well be similar.

ACKNOWLEDGMENTS

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# Inhibition of the Plasma Lecithin-Cholesterol Acyltransferase Reaction by Hydrogen Peroxide and Peroxidized Lecithin[1]

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## ABSTRACT

Inhibition of the plasma lecithin-cholesterol acyltransferase reaction by hydrogen peroxide and peroxidized lipids was studied by two methods: a radiochemical tracer technique and a direct colorimetric assay. Protection of sulfhydryl groups with *p*-chloromercuribenzoate, prior to exposure to peroxide, resulted in restoration of acyltransferase activity on removal of the inhibitors with mercaptoethanol. On the basis of these observations, it is proposed that lipid peroxides inhibit the plasma lecithin-cholesterol acyltransferase reaction by alteration of sulfhydryl groups necessary for enzymatic activity.

## INTRODUCTION

Lipid peroxides have been shown to react with sulfhydryl groups of protein (1,2). This reaction is thought to proceed through a free radical mechanism. Similarly sulfhydryl groups are involved in the activity of the plasma lecithin-cholesterol acyltransferase reaction since activity is abolished with *p*-chloromercuribenzoate (3). Previously it has been shown that the plasma lecithin-cholesterol acyltransferase reaction is inhibited by 0.1 M hydrogen peroxide (4), the same concentration which decreased stability of plasma lipoproteins (5). Furthermore in individuals exposed to pure oxygen atmosphere at 258 mm Hg for 30 days, a significant decrease in the extent of esterification of the plasma lecithin-cholesterol acyltransferase reaction occurred concomitant with a reduction of the plasma cholesteryl ester concentration (6). This paper presents evidence for the alteration of the sulfhydryl groups necessary for activity of the plasma lecithin-cholesterol acyltransferase reaction by hydrogen peroxide and lipid peroxides.

## MATERIALS AND METHODS

Chemicals were obtained from the following sources: (a) L-α-lecithin (egg), chromatographically pure, General Biochemicals (No. 100060), Chagrin Falls, Ohio; (b) cholesterol, reagent grade, Pfanstiehl Chemical Co., Waukegan, Ill.; (c) 4-$^{14}$C-cholesterol, purity greater than 97%, specific activity 57 mc/mmole, New England Nuclear Corp., (NEC-018), Boston, Mass.; (d) hydrogen peroxide, analytical grade, 30% solution, Mallinckrodt Chemical Co., St. Louis, Mo.; (e) *p*-chloromercuribenzoate, sodium salt, Mann Research Laboratories, (No. 6345), New York, N.Y.; (f) 2-mercaptoethanol, Calbiochem, (No. 4449), Los Angeles, Calif.; (g) silicic acid, specially prepared for the chromatography of lipids, Bio Rad Laboratories, Richmond, Calif.; (h) scintillation counting materials: toluene, scintillation grade, Mallinckrodt Chemical Co.; PPO (2,5-diphenyloxazole), scintillation grade, Packard Instrument Co., Downers Grove, Ill.; dimethyl POPOP (1,4-bis-2-[4-methyl-5-phenyloxazolyl]-benzene), scintillation grade, Packard Instrument Co.; (i) chloroform, analytical reagent grade, Mallinckrodt Chemical Co.; (j) methanol, analytical reagent grade, Mallinckrodt Chemical Co., (redistilled before use); (k) benzene, analytical reagent grade, Mallinckrodt Chemical Co., (redistilled before use); (l) Skelly Solve B, Sargent-Welch Chemical Co., Skokie, Ill. (purified with concentrated, reagent grade, sulfuric acid, and redistilled); (m) ethyl ether, absolute, reagent grade, Mallinckrodt Chemical Co.

The extent of esterification during a 24 hr incubation of the plasma lecithin-cholesterol acyltransferase reaction was assayed by incubating, in quadruplicate, 2 ml samples of pooled human sera 24 hr at 37 C with 4-$^{14}$C-cholesterol. All incubations were performed under aseptic conditions to minimize bacterial contamination. All glassware used in handling the serum was previously washed with detergent, dried in a hot oven, acetone-washed and dried under a stream of nitrogen prior to use. Human serum was prepared by drawing 50 ml blood from at least three healthy males, ages 20-47. After centrifugation of clotted blood, serum was removed by Pasteur pipet and pooled. The blood was drawn 1-2 hr prior to

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[1] Presented in the symposium, "Cholesteryl Esters," AOCS Meeting, Houston, May 1971.

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TABLE I

Role of the Sulfhydryl Groups in Hydrogen Peroxide Inhibition of the Plasma Lecithin-Cholesterol Acyltransferase Reaction[a]

| Sample | Colorimetric method | | Radioisotopic method | |
|---|---|---|---|---|
| | Cholesterol esterified, mg % | P | Cholesterol esterified, mg % | P |
| Control | 30.41 ± 0.96 | --- | 20.30 ± 0.82 | --- |
| Hydrogen peroxide 0.1 M | 3.24 ± 1.01 | <0.001 | 2.76 ± 0.14 | <0.001 |
| *p*-Chloromercuribenzoate 0.001 M | 0.84 ± 0.93 | <0.001 | 1.42 ± 0.15 | <0.001 |
| Mercaptoethanol 0.01 M | 25.16 ± 1.08 | <0.01 | 20.02 ± 0.88 | N.S. |
| *p*-Chloromercuribenzoate 0.001 M, mercaptoethanol 0.01 M | 31.84 ± 0.59 | N.S. | 21.36 ± 0.34 | N.S. |
| *p*-Chloromercuribenzoate 0.001 M, hydrogen peroxide 0.1 M | -2.12 ± 0.71 | <0.001 | 1.07 ± 0.13 | <0.001 |
| Hydrogen peroxide 0.1 M, mercaptoethanol 0.01 M | 11.38 ± 2.61 | <0.001 | 10.12 ± 0.65 | <0.001 |
| *p*-Chloromercuribenzoate 0.001 M, hydrogen peroxide 0.1 M, mercaptoethanol 0.01 M | 27.76 ± 0.83 | N.S. | 19.56 ± 0.56 | N.S. |

[a]Each sample was performed at least in quadruplicate on pooled sera from three donors. The results are expressed as the standard error of the mean. The values for *P* represent the difference from the control value. N.S. means not significant statistically, with a value of $P > 0.1$.

use of the pooled serum. One hundred microliters of a solution of 4-$^{14}$C-cholesterol in benzene, containing 0.1 μc ($1.7 \times 10^{-3}$ μmoles or 0.68 μg cholesterol) was added to each incubation vial and evaporated to dryness under a stream of nitrogen. Lipids being studied were then added in appropriate volumes of chloroform solutions and similarly evaporated under nitrogen. Two milliliters of pooled sera was added immediately upon removal of the vial from the nitrogen atmosphere. If hydrogen peroxide, 2-mercaptoethanol or *p*-chloromercuribenzoate (PCMB) was required, the substance was added in appropriate amounts to a 10 ml serum pool to achieve the desired final concentration. Crystals of PCMB were added to pooled sera and dissolved at room temperature, using gentle agitation for approximately 1 hr. Hydrogen peroxide was added by microliter syringe to pooled sera, both treated and untreated, and allowed to mix at room temperature 15 min with gentle agitation. Two milliliter aliquots of the several pooled sera were added to vials containing 4-$^{14}$C-cholesterol coated to the side. Vials were capped promptly and incubated 25 hr at 37 C with continuous shaking.

When peroxidized lecithin was substituted for hydrogen peroxide, the lipid was coated to the side of the vessel by drying under nitrogen, prior to adding previously treated or untreated sera. The sera was kept at room temperature 30 min, with gentle agitation, prior to transfer to incubation vials.

After the incubation was completed, 1 ml serum samples were extracted with 24 volumes of chloroform-methanol 2:1 v/v in a 25 ml volumetric flask. Lipids were chromatographed over silicic acid columns using the Leeder and Clark adaptation (7) of the Hirsch and Ahrens method (8). Radioactivity in each column fraction was measured by evaporating the column effluent in a scintillation vial and adding 8 ml toluene containing 5 g liter PPO and 0.1 g/liter dimethyl POPOP. The cholesterol content of the free and esterified cholesterol fractions was determined on an aliquot of the column fraction, using the method of Rosenthal et al. (9).

Extent of cholesterol esterification during a 24 hr incubation was calculated on the basis of the cholesteryl ester formed, as indicated by two independent sets of data: (a) the free and ester cholesterol levels measured colorimetrically before and after incubation, and (b) the amount of labeled cholesterol found in the cholesteryl ester fraction after incubation. By the first method the cholesteryl ester formed was equal to the difference between the concentrations of cholesteryl esters after and before incubation. By the second (radioisotope) method, the radioactivity in the cholesteryl ester fraction after the incubation was expressed as per cent of the total radioactivity (sum of radioactivity in all fractions). Multiplication of this per cent by the concentration of free cholesterol prior to incubation gave the net

TABLE II

The Effect of Commercially Obtained Egg Lecithin on Plasma Lecithin-Cholesterol Acyltransferase Activity[a]

| Sample | Conversion of 4-$^{14}$C-cholesterol to ester, % | P |
|---|---|---|
| Control | 35.8 ± 2.3 | --- |
| Egg lecithin 100 mg% peroxide number 400 | 2.3 ± 0.6 | <0.0001 |
| Egg lecithin 100 mg% eluted from column peroxide number zero | 42.5 ± 0.7 | <0.05 |

[a]See Table I.

increase in the concentration of 4-$^{14}$C-cholesteryl esters during the incubation.

The absolute values obtained by these two different methods of measuring esterification differed significantly (Tables I and III). Rose (10) has shown that the equilibration of radioisotopic cholesterol with human serum lipoprotein cholesterol is dependent on the particle size of the dispersion, the larger the particle size the less complete the equilibration at 37 C after 20 hr. Exogenous 4-$^{14}$C-cholesterol, coated to the walls of the incubation vessel, may be distributed slowly to sites on serum lipoproteins and to sites that are not primarily associated with enzymatic esterification, such that there is not complete miscibility of the exogenous with endogenous cholesterol. Alternately the labeled cholesterol may contain trace amounts of peroxidized lipid sufficient to cause difference between the two methods. Despite the differences in absolute values obtained by the two methods, the conclusions indicated by the experimental results are the same (Tables I and III). This similarity of results obtained by two independent methods increases confidence in the validity of the findings.

Lecithin was peroxidized by exposing a thin film of lipid to an oxygen atmosphere at 60 C for 1 hr using a rotary evaporator. The products of exposure of lecithin to oxygen were not purified. Peroxide numbers were measured by the Olcott and Dolev modification (11) of the AOCS Official Method (Cd-53) (12).

## RESULTS

Table I summarizes the activities of the plasma lecithin-cholesterol acyltransferase reaction observed in the presence of reagents that provide (2-mercaptoethanol), bind (*p*-chloromercuribenzoate) or oxidize (hydrogen peroxide) sulfhydryl groups. Hydrogen peroxide at concentration of 0.1 M inhibited acyltransferase activity to approximately 10% of the control (line 2 vs. line 1, Table I), 0.001 M PCMB inhibited the activity even more (line 3). Treatment with 0.01 M 2-mercaptoethanol alone produced an inconsequential decrease in esterification by colorimetric method and no decrease by the radioactivity method (line 4), but when added after PCMB it restored acyltransferase activity to control values by both methods (line 5). Likewise when serum was exposed to 0.001 M PCMB prior to addition of 0.1 M hydrogen peroxide, the serum enzyme activity was restored when the serum was exposed to 0.01 M 2-mercaptoethanol after 30 min treatment with peroxide (line 8). If mer-

TABLE III

Role of Sulfhydryl Groups in Peroxidized Lecithin Inhibition of the Plasma Lecithin-Cholesterol Acyltransferase Reaction[a]

| | Colorimetric method | | Radioisotopic method | |
|---|---|---|---|---|
| Sample | Cholesterol esterified, mg % | P | Cholesterol esterified, mg % | P |
| Control | 26.16 ± 0.28 | --- | 13.72 ± 1.85 | --- |
| Peroxidized lecithin (1150) | -1.09 ± 0.80 | <0.001 | 0.39 ± 0.01 | <0.001 |
| *p*-Chloromercuribenzoate, 0.001 M, peroxidized lecithin (1150), mercaptoethanol, 0.01 M | 24.59 ± 0.59 | N.S. | 10.76 ± 1.86 | N.S. |

[a]See Table I.

captoethanol was not added to the incubation mixture, there was virtually complete loss of enzyme activity (line 6). However when the serum was treated first with 0.1 M hydrogen peroxide alone for 30 min, subsequent addition of 0.01 M 2-mercaptoethanol restored acyltransferase activity to only one-third to one-half of normal (line 7). PCMB provided virtually complete protection against the effect of peroxide, whereas mercaptoethanol alone was only partially effective, in the same concentration as used to remove PCMB from enzymes sites and following the same time of reaction with peroxide. It is probable that the difference represents irreversible peroxidative alteration of the enzyme when not protected by PCMB.

Commercially obtained, "chromatographically pure" egg lecithin had a peroxide number of 400 and inhibited the enzyme activity (see Table II). After elution from a 1.0 g silica gel column with methanol, the peroxide number was zero, and stimulation of activity occurred. The purified egg lecithin was peroxidized by exposure to an oxygen atmosphere, resulting in a peroxide number of 1150. A concentration of 100 mg% peroxidized lecithin (approximately 0.001 meq/liter lipid peroxide) markedly inhibited the plasma lecithin-cholesterol acyltransferase reaction and caused appreciable turbidity of the serum as well. Sera made 0.001 M with PCMB prior to exposure to the peroxidized lecithin also became turbid, but had virtually complete recovery of the enzymatic activity on removal of the PCMB from sulfhydryl groups with 0.01 M 2-mercaptoethanol (Table III).

There was a variation in control values as given in Tables I, II and III. This variation may be explained by our use of sera pooled from varying combinations of donors, and by slight variations in the time or temperature of incubation of the experiments. However all the samples for each of the experiments as given in the respective tables were performed on the same pooled sera under identical conditions.

## DISCUSSION

Inhibition of the plasma lecithin-cholesterol acyltransferase reaction results from exposure to peroxidized lecithin as well as exposure to 0.1 M hydrogen peroxide. The mechanism of the inhibition by peroxides involves alteration of sulfhydryl groups necessary for enzyme function. Protection of sulfhydryl groups by reaction with PCMB prior to exposure to hydrogen peroxide or peroxidized lecithin resulted in virtual restoration of activity to control values upon removal of the inhibitors with 2-mercaptoethanol.

The mechanism of attack of hydrogen peroxide and peroxidized lecithin on the enzymatic activity involves sulfhydryl groups, since protection of sulfhydryl groups by reaction with PCMB results in restoration of the enzyme activity on removal of the inhibitors with 2-mercaptoethanol. However 0.001 meq/liter peroxidized lecithin resulted in appreciable turbidity of the sera, regardless of whether or not PCMB was present, in contrast to the effect of 0.1 M hydrogen peroxide treatment which resulted in only bleaching of the sera without turbidity. It is probable that lipid peroxides cause denaturation of plasma proteins by attack on other nucleophiles than solely sulfhydryl groups (13). The inhibition of hydrogen peroxide alone is partially reversed on addition of 2-mercaptoethanol (Table I). In other experiments the inhibition by peroxidized lecithin alone is not reversed appreciably on treatment with 2-mercaptoethanol. It is possible that the lipid peroxide forms a condensation product with the enzyme or cross linking of the protein, which is not reversible upon the addition of free sulfhydryl groups in the form of 2-mercaptoethanol.

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# Control of Lipid Metabolism in Cultured Cells[1]

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## ABSTRACT

Several studies are presented which indicate that composition of cell lipid is regulated by interaction between intracellular metabolism and lipid transport processes. When the fatty acid composition of cells cultured in essential fatty acid deficient conditions was studied, activation of synthesis of unusual polyunsaturated fatty acids was observed for a number of cell lines. In addition cells contained persistent residual amounts of linoleic acid, presumably owing to efficient scavenging mechanisms. The source of cell lipids was studied in both chemically defined and serum-supplemented media. In the absence of exogenous lipid, cells synthesize lipids from simple precursors, a process which is inhibited by adding serum. When serum lipid is present, cells preferentially utilize fatty acids as a source of nonsterol lipid. These are subsequently esterified intracellularly to make glycerides and phospholipids. When triglyceride is utilized as a source of cell lipid, it is first hydrolyzed before being taken up. By use of a nonhydrolyzable cholesterol ester analog, it is confirmed that both free and ester cholesterol are taken up and excreted by cells. Intracellular cholesterol content is thus regulated by rates of uptake, hydrolysis and excretion as well as by biosynthesis.

## INTRODUCTION

Cultured cells provide a promising system for studying the regulation of lipid metabolism. In the first place their rather homogeneous character allows the examination of events separated from the complex physiological interactions encountered in vivo. Moreover nutritional and environmental conditions can be more easily manipulated to facilitate examination of molecular mechanisms. When studying lipid metabolism in cultured cells, several factors must be considered. Lipid composition is affected by the interaction and regulation of the individual pathways for biosynthesis and degradation. In addition cultured cells like cells in vivo grow in an environment containing available lipid in the form of the serum liproteins. The contribution of this to the lipid content of the cell is affected by interaction of extracellular lipid and lipoprotein with cell surface lipoprotein, and upon mechanisms of selective transport and excretion. These latter mechanisms appear to regulate at the level of permeability. In the present paper studies are presented on three areas of lipid metabolism—the contribution of exogenous lipid, the regulation of de novo lipid synthesis and the transport processes for lipids across the cell membrane. In each case our results indicate that lipid composition is regulated by an interaction between intracellular metabolism and trans-membrane permeability processes.

## MATERIALS AND METHODS

Lipid extracts for routine measurement of lipid content were prepared by the chloroform-methanol extraction procedure of Folch et al. (1), and the individual lipid classes were separated by thin layer chromatography by the method of Stahl (2). For experiments in which essential fatty acids (EFA) were to be measured, lipid extracts were prepared overnight by extraction at 4 C with 5 ml or 20 volumes, whichever was greater, of ethanol-diethyl ether 1:1. α-Tocopherol (0.2 mg) in ethanol-ether 1:1 was added to prevent oxidation of polyunsaturated fats. Ethanol-ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness at room temperature in a stream of nitrogen. The lipid residue was redissolved in hexane. It has been shown previously that these relatively mild extraction procedures are necessary for quantitative recovery of polyunsaturated fatty acids (3). Methyl esters of the fatty acids were prepared by transesterification of the lipid extracts in methanol with boron trifluoride (14%) as a catalyst (4), and analyzed by gas liquid chromatography using a 1662 series gas chromatograph (HCL Scientific Inc., Chicago, Ill.) equipped with a hydrogen flame ionization detector. The chromatograph was fitted with glass columns packed with ethylene glycol-succinate polyester (15%) on a 100-120 mesh Gas Chrom Q.

[1]One of 13 papers presented at the symposium "Lipid Metabolism in Cells in Culture," AOCS Meeting, Houston, May 1971.

TABLE I

Distribution of Fatty Acids in Cells as Compared to Their Culture Medium[a]

| Fatty acid | Calf serum[b] | WI-38 cells | WI-38VA13A cells | Mouse blood | Ehrlich Ascites cells | Sarcoma 180 cells |
|---|---|---|---|---|---|---|
| 14:0 | 0.9 ± 0.2 | 1.6 ± 4.2 | 1.8 ± .11 | n.d. | n.d. | n.d. |
| 14:1 | n.d.[c] | Trace | Trace | n.d. | n.d. | n.d. |
| 16:0 | 26.6 ± 2.0 | 22 ± 1.2 | 25 ± 1.6 | 23.0 ± 1.3 | 14.8 ± 0.5 | 14.7 ± 3.0 |
| 16:1 | 3.5 ± 0.3 | .38 ± .06 | Trace | 1.3 ± 0.5 | 1.7 ± 0.1 | 2.2 ± 0.3 |
| 18:0 | 20.8 ± 1.6 | 18 ± 1.3 | 15 ± 1.6 | 10.0 ± 0.6 | 18.2 ± 0.3 | 16.0 ± 1.4 |
| 18:1 | 29.2 ± 1.4 | 32 ± 1.2 | 39 ± 2.1 | 23.7 ± 2.6 | 25.0 ± 0.9 | 22.4 ± 0.8 |
| 18:2 | 12.6 ± 1.6 | 6.6 ± .71 | 10.1 ± 1.8 | 18.4 ± 1.0 | 25.0 ± 0.4 | 20.0 ± 1.2 |
| 18:3 | n.d. | 1.9 ± .20 | Trace | 1.4 ± 0.3 | 2.2 ± 0.2 | 1.6 ± 0.2 |
| 20:2 | n.d. | n.d. | n.d. | 1.5 ± 0.3 | 1.7 ± 0.2 | 1.2 ± 0.2 |
| 20:4 | 5.1 ± 3.5 | 18 ± 1.6 | 8.7 ± 2.1 | 12.8 ± 0.6 | 11.0 ± 0.6 | 17.1 ± 2.5 |
| 22:0 | n.d. | n.d. | n.d. | n.d. | 1.6 ± 0.2 | 1.8 ± 0.3 |

[a]Values are mean % ± S.E. For analysis of the fatty acids of cell cultures, lipid was saponified at 70 C for 4 hr in 4 N Ethanolic NaOH. After removal of nonsaponifiables and acidification, fatty acids were extracted with ethyl ether and converted to methyl esters by reaction with diazomethane (16). For mouse blood and ascites tumor cells, lipids were extracted and fatty acid composition determined by gas liquid chromatography as described in Methods.

[b]Data of Spitzer et al. (24).

[c]n.d. = None detected.

Column temperature was 165 C; injector temperature was 268 C; and the carrier gas (helium) flow rate was 75 ml/min.

Cultures were grown as monolayers using conventional sterile techniques. They were supplemented with serum and an antibiotic mixture of penicillin, streptomycin, mycostatin and achromycin. When chemically-defined media was used, the antibiotics were omitted. Incubation temperature was 37 C, and the gas phase and pH in cultures were adjusted by flushing with filtered 5% $CO_2$ in air before closing the bottles. Solutions for addition to experimental cultures were sterilized by autoclaving or, for thermolabile components, by filtration through Millipore bacteriological filters. After being washed twice with 2 volumes of balanced salt solution, cells were harvested from the glass by trypsinization or by scraping with a rubber policeman.

Chemically defined media and sera were obtained from Microbiological Associates, Bethesda, Md. Human serum was inactivated by heating at 57 C for 1 hr before use to destroy toxic factors. Stock L-2071 strain mouse fibroblast cells adapted to growth on synthetic medium for a number of years were supplied by Virginia Evans' laboratory, National Cancer Institute, and MBIII cells by G.O. Gey, Finney Howell Cancer Research Laboratory, Johns Hopkins Hospital. $^{14}C$-labeled compounds ([2-$^{14}C$] acetate, [2-$^{14}C$] mevalonolactone, [U$^{14}C$] glucose, 1-$^{14}C$ palmitic and oleic acids, tripalmitin and 4-$^{14}C$ cholesterol), were from Nuclear Chicago Inc., and were checked for purity by paper and silicic acid chromatography. Purified lipids for addition to cultures were from California Biochemicals and Nutritional Biochemicals Companies. Silicic acid, "chromatography grade," was from Mallinckrodt Chemicals and was sieved between 100 and 200 mesh screens before use.

Cholesterol-4-$^{14}C$ α-α methyl ethyl caproate was synthesized according to the method of Swell and Treadwell (5), and purified by chromatography on silicic acid. Cholesterol in lipid extracts was precipitated for radioactivity determinations by the digitonin-$AlCl_3$ procedure (6).

Radioactive lipids were added to the medium, dissolved in small amounts of ethanol, and sterilized by filtration. Water soluble radioactive compounds were added as a sterile saline solution. Radioactivity determinations were made by liquid scintillation counting, with appropriate corrections for sample quenching.

## RESULTS

### EFA in Cultured Cells

The fatty acid composition of cells cultured in the presence of normal serum usually reflects the fatty acid composition of the culture medium. Table I shows examples of the similarity between the fatty acids of a number of different kinds of cells and their culture media. The fatty acids in WI-38 and WI-38VA13A cells are similar, and resemble the fatty acids of calf serum which was used in the culture medium; similarly the fatty acids of two strains of ascites tumor cells (the Ehrlich Ascites and an ascitic form of Sarcoma 180) closely paralleled the

TABLE II

Fatty Acid Biosynthesis by L-Strain Mouse Fibroblasts From Various $^{14}C$ Precursors in Lipid Free Synthetic Medium[a]

| | $^{14}C$ Oleic acid | | $^{14}C$ Glucose | | $^{14}C$ Acetate | |
|---|---|---|---|---|---|---|
| Fatty acid | Mass | Isotope | Mass | Isotope | Mass | Isotope |
| 16:0 | 24 | 0 | 26 | 31 | 24 | 39 |
| 16:1 | 11 | 0 | 8 | 6 | 10 | 7 |
| 18:0 | 22 | 0 | 16 | 8 | 21 | 22 |
| 18:1 | 36 | 100 | 38 | 42 | 37 | 20 |
| 18:2 | 6 | 0 | 10 | 0 | 7 | 0 |

[a]Monolayers of L 2071 strain mouse fibroblasts were incubated in serum free culture medium (NCTC 135) containing either 1-$^{14}C$ oleic acid, U-$^{14}C$ glucose or 2-$^{14}C$ acetate, for 96 hr. Lipid was extracted and methyl esters prepared as described in Methods. Extracts were analyzed for both chemical composition (listed under the column heading "Mass" in the table) and for radioactivity in the individual fatty acids (listed as "Isotope") using a 1662 series gas chromatograph fitted with a stream splitter and a radioactivity monitor (H.C.L. Scientific). Note that the small amounts (8-10%) of linoleic acid found in cells cultured in lipid free chemically defined medium are not synthesized de novo from any of the tested precursors. Note that also oleic acid supplied exogenously in the culture medium is incorporated intact into the cellular lipids without apparent interconversion to other fatty acids.

fatty acids in the serum of mice in which they were grown. Further evidence that fatty acid composition is usually regulated in a passive manner by uptake of serum fatty acids without extensive modification or interconversion comes from experiments in which a single labeled fatty acid ($^{14}C$ oleic acid) was added to lipid free synthetic media (Table II). The label is found exclusively in the oleic acid of the cell lipids, and no interconversion of fatty acids by these cells was observed over the 4 day growth periods employed, i.e., under conditions in which the serum lipids are not completely depleted. These results imply therefore that under normal (short term) culture conditions, the fatty acid composition of cells is regulated by nonselective uptake of all available serum fatty acids.

However where there is a deficiency of EFA

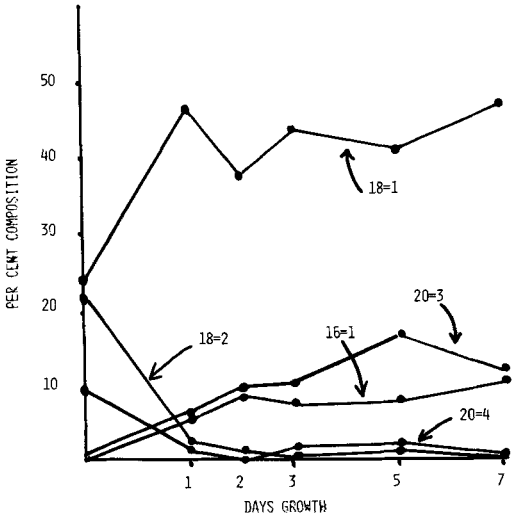


FIG. 1. Changes in fatty acid composition of normal Sarcoma 180 cells grown in EFA deficient mice. Ehrlich Ascites or Sarcoma 180 cells were grown in the peritoneum of CF1 mice fed either normal diet (Purina chow) or fat free diet (Nutritional biochemicals). At the indicated intervals cells were harvested and lipid extracted and fatty acid methyl esters prepared and analyzed as described in Methods.

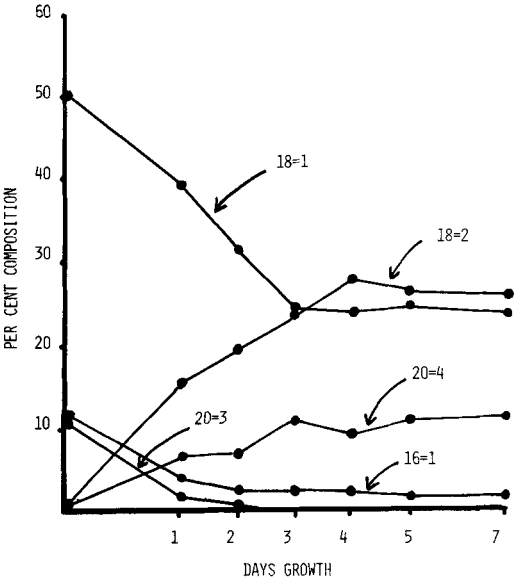


FIG. 2. Changes in fatty acid composition of EFA deficient Ehrlich Ascites cells grown in normal mice. See Figure 1.

TABLE III

Lipid Content of Lipid Free Chemically Defined Media[a]

| Medium | Source | Total lipid, mg/100 ml | Linoleic acid, μg/100 ml |
|---|---|---|---|
| NCTC 135 | Schwartz | 1.12 | 30 |
| NCTC 135 | Microbiological | 1.49 | 39 |
| NCTC 109 | GIBCO | 1.31 | 33 |
| 7C LF | Custom | 0.43 | 10.3 |

[a]100 ml quantities of various commercially available synthetic media were dried by lyophilization and the lipids were extracted and analyzed by gas liquid chromatography as described in Methods. The 7CLF medium was custom prepared (using the formula of Gey et al. (17) but omitting lipids), from components which were individually tested for lipid contamination (and if necessary freed from lipid by cold hexane extraction). The figures for 7CLF, showing a residual contamination of some 10 μg/100 ml of linoleic acid, illustrate the difficulty of obtaining a synthetic medium truly free from essential fatty acids.

in the culture medium, the cells compensate in several ways. When L strain mouse fibroblasts were cultured in lipid free medium, i.e., one that had no EFA supply, the cells synthesized most of the cell lipids from simple precursors in the medium. Under these conditions the content of linoleate drops from 25% of the total fatty acids for cells grown on serum to about 5%, but rarely falls below this level. However this residual level is not the result of biosynthesis, for when $^{14}$C-glucose or acetate was added to the medium no radioactivity was incorporated into the linoleic acid fraction (Table II), although all of the nonessential fatty acids were labeled. This shows that the traces of EFA present in the cells are not synthesized de novo, but represent a very efficient conservation or scavenging mechanism on the part of the cells. We have found that commercially available synthetic media contain appreciable amounts of EFA as contaminants, and EFA are also present in traces in media custom made in the laboratory using stringent precautions to avoid contamination by lipids.

TABLE IV

Fatty Acid Composition of MBIII Cells Grown on Lipid Free Medium[a]

| Fatty acid | Control, 10% serum | Lipid free |
|---|---|---|
| 14:0 | 4.3 ± 0.9 | Trace |
| 16:0 | 23.5 ± 2.7 | 8.9 ± 4.1 |
| 16:1 | 13.8 ± 2.0 | 4.0 ± 2.3 |
| 18:0 | 19.5 ± 4.2 | 5.2 ± 1.4 |
| 18:1 | 28.9 ± 2.3 | 5.2 ± 4.3 |
| 18:2 | 11.2 ± 1.9 | 5.2 ± 3.4 |
| 18:3 | ---[b] | ---[b] |
| 20:3 | ---[b] | 29.0 ± 5.0 |
| 20:4 | ---[b] | Trace |
| 22:3 | ---[b] | 42.6 ± 6.6 |

[a]Cells were adapted to lipid free medium 7CLF over a period of 3-4 weeks by serial passage into decreasing serum concentration. Data was obtained from cultures which had been on lipid free medium for 7 weeks. Note that 20:3 and 22:3, not normally found in cells, comprise over 70% of the fatty acids in cells grown in synthetic lipid free medium.

[b]Did not appear on chromatogram.

The studies of EFA deficient cells show that in addition to scavenging EFA they also adapt, by accumulating large amounts of polyunsaturated fatty acids of a type not usually found in normal cells. Figure 1 shows the fatty acid composition of Ehrlich Ascites cells grown in EFA deficient mice, an experimental situation in which rapid and efficient depletion of EFA can be effected. The content of 18:2 and 20:4 drops in 2-3 days, and there is an appearance of 20:3 which increases from undetectable levels to almost 15% of the total fatty acids. The levels of 20:3 decrease when cells are transplanted back into normal mice, and the essential fatty acid composition rapidly returns to normal (Fig. 2). A similar appearance of new polyunsaturated fatty acids is observed in cells cultured in lipid free chemically-defined medium. This is illustrated by the data in Table IV, which compares the fatty acids of MBIII cells cultured in the presence of serum with those of cells grown in a medium (Gey's 7CS synthetic medium) devoid of fatty acids. Although the content of linoleate remains at ca. 5%, large quantities of 20:3 and 22:3 are synthesized. These polyunsaturates were also observed to accumulate in L cells and Chang liver cells grown in lipid free media. (More recent experiments have failed to confirm this finding for L cells.) These fatty acids presumably can replace the function of EFA at the cellular level, since the growth rate and transplantability of the EFA deficient ascites tumor cells are not significantly different from those of normal cells. The absence of these unusual fatty acids in normal cells implies that the pathways for their biosynthesis are probably repressed when sufficient EFA are present in the medium and are induced only when the

TABLE V

Per Cent of Cellular Lipid Derived From
Biosynthesis in Serum-Supplemented and in Lipid Free Medium[a]

| | $^{14}$C Glucose | | $^{14}$C Acetate | |
|---|---|---|---|---|
| Type of medium | Serum | Lipid free | Serum | Lipid free |
| S.A. precursor, dpm/μg | 300 | 150 | 857 | 1000 |
| S.A. cell lipid | 23.3 | 136 | 5.82 | 82 |
| Biosynthesized,[b] % | 7.8 | 90 | 0.68 | 8.1 |

[a]Cells were cultivated in either Basal Medium (Eagle) containing 10% calf serum or lipid free NCTC 135. Glucose was measured enzymatically (Glucostat) and $^{14}$C acetate was added with known amounts of unlabeled sodium acetate. After 7 days cells were harvested and lipid extracted. For additional details see Methods.

[b]Per cent biosynthesized calculated as ratio specific activity of lipid to that of precursor.

content of EFA is decreased.

### The Source of Cellular Lipid in Cultured Cells

We are also studying the sources of the various components of the complex lipids in cells. When cells are cultured in lipid free chemically defined medium, all components of the lipids are derived by de novo biosynthesis. Table V shows results of experiments in which various $^{14}$C-labeled carbon sources were added to the growth medium for cells cultured in the presence and absence of serum. The data indicate that glucose and acetate are the two main carbon sources for lipid biosynthesis for strain L2071 cells, and provide approximately 90% and 8% of the total lipid respectively. When the medium is supplemented with serum, however, lipid biosynthesis from the simple precursors is inhibited up to 95%. When the specific activity of the cell lipid is compared to that of the particular radioactive precursor, it is found that less than 1% of the lipid is derived from acetate and only about 8% from glucose, when serum is present. This indicates that under the usual culture conditions, when the medium is supplemented with serum the cells derive their lipids from the abundant serum lipoproteins. We then set out to determine whether there is a uniform uptake of all serum lipid or whether certain serum lipids can serve as preferential sources of cellular lipid. Isotopic dilution studies adding $^{14}$C-labeled fatty acid or $^{14}$C-triglyceride to the medium (Table VI) show that under normal conditions of growth, about 85% of the cell lipid is derived from serum free fatty acids and only 2% of the lipid is derived directly from serum triglyceride. These experiments suggest that although there is a far greater amount of triglyceride and phospholipid present in serum, the cells preferentially take up free fatty acid and use it to synthesize glycerides and phospholipids intracellularly. This is supported by experiments that determine the source of glycerol in the cell lipids (Table VII). The data indicate that the glycerol portion of the lipid is derived almost entirely from glucose. $^{14}$C-glycerol added to the medium is utilized only in the case in which there is a large excess available. If serum glycerides or phospholipids were taken up intact, the specific activity of the lipid glycerol would be expected to be much lower than that

TABLE VI

Per Cent of Cellular Lipid Derived From
Serum Triglycerides vs. Free Fatty Acids Present[a]

| Precursor | 1-$^{14}$C Tripalmitin | 1-$^{14}$C Na Palmitate |
|---|---|---|
| S.A. precursor, dpm/μg | 65.8 | 22.7 |
| S.A. cell lipid | 1.49 | 19.4 |
| Derived lipids,[b] % | 2.3 | 85 |

[a]WI-38 cells were grown to a 10-fold increase in cell mass in 10% calf serum to which tracer amounts of radioactive lipid were added. Specific activity of precursors was assayed by extracting total lipid, and isolating free fatty acid and triglyceride by the method of Howard and Kritchevsky (18). Triglyceride was assayed by the method of van Handel and Zilversmit (19), and fatty acid by the method of Duncombe (20). For additional details see Methods.

[b]Per cent of lipids derived from given precursor calculated as ratio S.A. of lipid to that of precursor.

TABLE VII

Source of Glycerol in Cell Lipids[a]

| Precursor | $^{14}$C Glucose | $^{14}$C Glycerol | $^{14}$C Glycerol plus excess glycerol |
|---|---|---|---|
| S.A. precursor, dpm/μg | 66.7 | 2605 | 136 |
| S.A. lipid glycerol | 56.8 | 232 | 24 |
| Derived from given precursor, % | 85 | 8.8 | 17 |

[a]L cells were subcultivated in Minimal Medium (Eagle) containing 10% fetal calf serum and supplemented with $^{14}$C glucose or glycerol. Glucose in the medium was measured enzymatically (Glucostat), and glycerol by the method of Spinella and Mayer (21). Cell lipids were saponified overnight at 60 C in 4 N ethanolic KOH. Glycerol was assayed on the remaining aqueous residue by the method of Bailey (22) after both nonsaponifiables and fatty acids were extracted.

observed experimentally. It should be noted that the derivation of the glycerol portion of the triglyceride molecule from glucose can account for the above-mentioned figure of 8% of the lipid derived from glucose when cells are grown in serum. Further evidence on the differential sources of the precursors of the complex lipids is obtained when phospholipid synthesis is studied. When $^{32}$P and $^{14}$C-acetate incorporation into lipids of these serum-grown cultures are compared (Fig. 3) the rate of $^{14}$C-acetate incorporation is low, indicating that the fatty acid portion is not synthesized de novo. $^{32}$P incorporation is much more rapid, suggesting that phospholipids are assembled intracelluarly rather than being taken up and used intact. Our calculations indicate that the rate of $^{32}$P incorporation into lipids is more than adequate to account for the measured cellular content of phospholipids.

Since the experiments described above were conducted using cultures having a relatively low population density ($10^5$ cells/ml), one obviously wonders what happens in conditions in which the free fatty acid is depleted. Figure 4 shows results of earlier experiments not utilizing radioactive precursors, which indicated that both triglyceride and phospholipid can be utilized in serum supplemented cultures. In these cultures, which were 50-100 times more dense than those of the above experiments (having 5 x $10^6$ cells/ml), 100% of the triglyc-

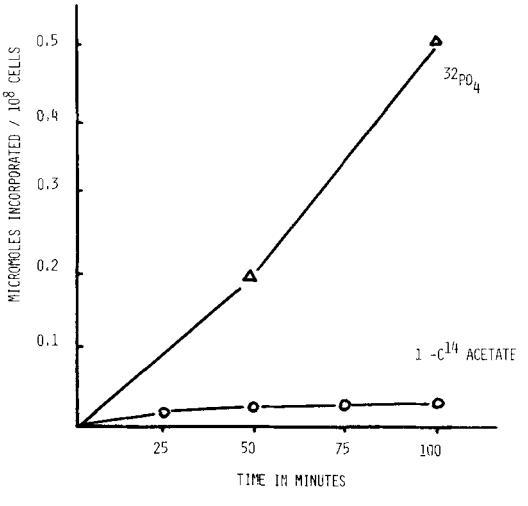


FIG. 3. Incorporation of $^{32}PO_4$ and acetate-1-$^{14}$C into phospholipids of WI-38 cells. WI-38 cells grown in Basal Medium Eagle supplemented with 10% calf serum were harvested and suspended in buffered balanced salt solution ($10^7$/ml) containing the appropriate isotope of known specific activity. The suspension was shaken in a water bath at 37 C and at intervals aliquots were removed and the cells washed in cold balanced salt solution. Phospholipids were isolated by thin layer chromatography, and radioactivity was determined without elution from the silicic acid by the method of Snyder and Stephens (23).

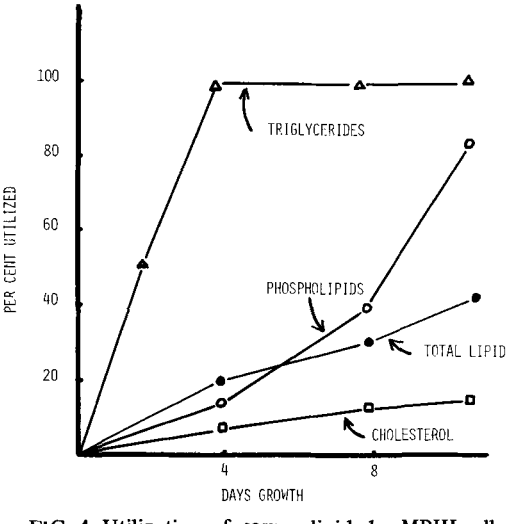


FIG. 4. Utilization of serum lipids by MBIII cells. Depletion of phospholipids and triglycerides was measured in roller tube cultures of MBIII cells in a medium consisting of human placental cord serum diluted with one part of balanced salt solution. Lipids were extracted and assayed as described in Methods. Final population density was 6x$10^6$ cells/ml.

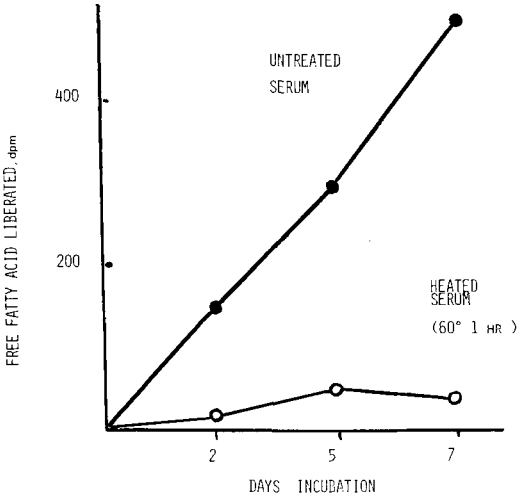


FIG. 5. Hydrolysis of $^{14}$C-triglyceride in culture medium supplemented with fetal calf serum in the absence of cells. Minimal Medium (Eagle) was supplemented with 10% fetal calf serum to which had been added tracer amounts of $^{14}$C carboxyl labeled tripalmitin using the procedure described in Methods. The sterile medium was incubated for 7 days at 37 C. Lipid was extracted from samples of the medium at the indicated intervals, and fatty acid was isolated and the radioactivity determined as described in the footnote to Table 6.

eride was depleted and more than 50% of the phospholipid was also taken up. Recent experiments suggest that the triglyceride and presumably also phospholipid are not taken up intact, however, but are first hydrolyzed to free fatty acids. Fatty acid utilization was assayed suing triglyceride labeled with $^{14}$C fatty acids in cultures having a population density of about $10^6$ cells/ml. Under these conditions 33% of the cell lipid was derived from triglyceride; however triglyceride radioactivity also appears in the free fatty acids of the medium. When the specific activity of the various fractions is measured, it is found that the specific activity of the cell lipids more closely resembles that of the free fatty acids in the medium rather than the triglycerides. Moreover under the same culture conditions, when radioactive free fatty acid was added, a rapid decrease in radioactivity in the medium was observed (Table VIII). Almost all of the original fatty acid as measured by radioactivity was utilized, but the actual free fatty acid content of the medium as measured by chemical analysis remained relatively constant during the 7 day culture period. This implies that the fatty acid used is being replaced from some endogenous source. The source of the nonradioactive free fatty acid is most likely serum triglycerides, although there is evidence suggesting that sterol esters may also

TABLE VIII

Utilization of Serum $^{14}$C Fatty Acids in Dense Cultures[a]

| | Day 0 | Day 7 |
|---|---|---|
| dpm | 49,900 | 992 |
| μg | 220 | 306 |
| Specific activity | 442 | 3.24 |

[a]L strain mouse fibroblasts were cultivated in Basal Medium (Eagle) supplemented with 5% fetal calf serum. $^{14}$C-Palmitic acid was added to the medium in tracer amounts. Specific activity of the fatty acids in the medium was measured at zero time and after growth for 7 days as described in Table VI. Note the marked decrease in radioactivity without a corresponding decrease in the total fatty acid content of the medium.

be a significant source of free fatty acid (see below). The results suggest that when triglyceride is hydrolyzed, the products enter the free fatty acid pool of the medium and are taken up and utilized by the cells in that form.

The mechanism of the hydrolysis of triglyceride is currently being investigated. Preparations of calf and fetal calf sera appear to contain a triglyceride lipase (Fig. 5) which can be detected by addition of $^{14}$C-triglyceride to culture medium in the absence of cells. This hydrolysis of triglyceride is eliminated by preheating the serum at 60 C. The observed rate of hydrolysis is not large enough, however, to account for the total cell free fatty acid requirements in dense cultures. It seems that under these conditions hydrolysis is also mediated by a cellular enzyme. The possible secretion of such a lipase into the medium and conditions which may regulate this are at present under investigation.

### Uptake and Excretion of Sterol Esters by Cultured Cells

Earlier experiments indicated that when cultured in lipid free medium, cells are able to synthesize cholesterol from labeled acetate or glucose added to the medium. This biosynthesis is inhibited up to 95%, however, when serum or cholesterol itself is added to the medium. The studies indicated that when grown in serum, most of the cell cholesterol is derived from the medium. The proportion of ester to free cholesterol in serum, however, is usually ca. 2:1, whereas in cells the free form of cholesterol generally predominates. Work from both our laboratory and that of Rothblat and Kritchevsky (7) indicated that there was a steady flux of both free and esterified cholesterol into and out of the cell, with sterol levels being controlled by the rates of uptake and excretion, plus any

TABLE IX

Uptake of Labeled Cholesterol, Cholesterol Esters and a Synthetic Nonhydrolyzable Ester Analog by MBIII Cells[a]

| | Radioactive test compound added | | |
|---|---|---|---|
| | Cholesterol | Cholesterol oleate | Cholesterol methyl ethyl caproate |
| Total in medium, mg[b] | 13.4 | 19.3 | 3.7 |
| Total dpm in medium[b] | 923,200 | 137,000 | 24,870 |
| Total dpm in cells[b] | 225,000 | 33,620 | 7,120 |
| Uptake of isotope, % | 24.4 | 24.6 | 28.6 |
| In cell particulates, %[c] | 82 | 36 | 36 |
| In cell sap, %[c] | 18 | 64 | 64 |

[a]Each value represents pooled sample of five tissue culture bottles grown for 6 days in medium of 45% calf serum prelabeled with isotope by roller tube procedure. Note that cholesterol methyl ethyl caproate distributes in cell in same manner as normal esters. MBIII cells were cultured in Basal Medium Eagle containing 45% calf serum prelabeled with isotope. After 6 days cells were harvested, homogenized and centrifuged at 150,000 x g. Sterol was isolated from particulate and supernatant fractions by digitonin precipitation and radioactivity was assayed as described in Methods.

[b]Based on 100 ml medium.

[c]Based on chemical identification of isotope by digitonin fractionation.

endogenous synthesis. The uptake of esterified cholesterol by cells could not be easily studied, however, because esters readily undergo both extracellular and intracellular hydrolysis and transesterification. We have therefore utilized a synthetic cholesterol ester, cholesterol 4-$^{14}$C-α, α-methyl ethyl caproate (CMEC), which because of the steric hindrance of the α-Carbon atom is completely resistant to hydrolysis by esterases. The radioactive CMEC was introduced into serum by depositing the compound as a thin film inside roller tubes, and then allowing it to exchange with serum cholesterol esters by incubating the serum in the roller tube for 3 days. Cultures of MBIII mouse lymphoblast cells were then grown on medium containing the labeled serum (Table IX). The data showed that the labeled CMEC was taken up over the 6 day growth period to approximately the same extent as labeled cholesterol and cholesterol esters. Furthermore the radioactive CMEC in cell homogenates distributed between the particulate and supernatant cell fractions to the same proportions as normal cholesterol esters. Excretion was then measured in stationary cultures of prelabeled cells (Table X). In the presence of serum there was a progressive release of radioactivity from all three components—cholesterol, cholesterol ester and CMEC, into the medium; and the rates of excretion did not differ significantly for the three types of compounds. These results showed that esters are not necessarily hydrolyzed to the free form, but may also be taken up and excreted intact.

TABLE X

Excretion of Labeled Cholesterol, Cholesterol Esters and Ester Analog From Cultured MBIII Cells[a]

| Time of incubation | Cell population, millions/ml | Per cent isotope excreted | | |
|---|---|---|---|---|
| | | Cholesterol | Cholesterol oleate | Cholesterol methyl ethyl caproate |
| 2 hr | 4.45 | 9.6 | 10.0 | 3.5 |
| 2 days | 4.60 | 25.8 | 26.4 | 34.4 |
| 5 days | 5.23 | 72.8 | 69.7 | 53.8 |
| 8 days | 4.44 | 73.1 | 70.8 | 56.2 |

[a]Each value is the average of two tissue culture bottles harvested at the indicated times. Population density was chosen so as to maintain cells in steady state. Medium was 45% calf serum in Basal Medium Eagle. Stationary phase cultures of MBIII cells which had accumulated isotope from prelabeled serum were transferred to medium containing fresh unlabeled serum. At various times bottles were harvested and total lipid was extracted. Cholesterol and cholesterol esters were isolated by the digitonin procedure, and radioactivity was determined as described in Methods.

These experiments indicate that cell levels of cholesterol and cholesterol esters thus depend on the rates of uptake and excretion of both forms of the sterol independently, in addition to the previously observed intracellular hydrolysis of the esters (8).

## DISCUSSION

Our studies of EFA deficient cultured cells can be correlated with studies on EFA deficient animals by Mead (9) and Klenk (10). These investigators reported the presence of an elongation and desaturation pathway beginning with either oleic or palmitoleic acids, and resulting in polyunsaturated fatty acids having the structures $\omega$-7 or $\omega$-9. It is most likely that the fatty acids identified as 20:3 and 22:3 observed in our cell cultures under EFA deficient conditions represents induction of these biosynthetic pathways, since there is independent evidence that these pathways are probably not active in cells growing in serum. These enzymes may be the same or different from those normally involved in conversion of linoleic acid to arachidonic. EFA deficient cultured cells afford an opportunity to study the role of EFAs in cell metabolism, and further experiments are in progress to investigate the mechanism of induction of synthesis of unusual polyunsaturates and their role in replacing the essential fatty acids in normal cell functions.

Earlier studies (11) indicated that there is efficient regulation of cellular lipid biosynthesis. The results so far do not indicate unequivocally which enzymes are involved, and whether the regulation is at the level of enzyme activity or if induction and repression of enzyme synthesis is involved. Preliminary studies indicate that significant increases in an acetate-activating enzyme system occur when cells are transferred to serum free medium containing acetate. The changes in rates of lipid biosynthesis in the presence and absence of serum should provide an excellent model system for the study of mechanisms of enzyme regulation in mammalian cells.

Cultured cells also provide an experimental system for the study of cellular lipid uptake and excretion. The observation that free fatty acid is the primary source of cell lipid is consistent with experiments of Mackenzie et al. (12) and Geyer (13) who linked accumulation of intracellular fat droplets with the free fatty acid content of sera. The results from cell cultures also correlate with studies in vivo. Shapiro and others have demonstrated that in the intact animal, plasma free fatty acid, although only a small proportion of the total serum lipid, has a very short half life (14). In addition recent in vivo experiments with radioactive precursors suggest that even liver cells are unable to utilize intact triglycerides (15). We are currently extending our studies on lipid utilization to investigate conditions in which free fatty acid is depleted or absent. It seems that the utilization of serum glycerides and phospholipids under these conditions may be mediated by lipolytic enzymes, similar to those observed in vivo.

In summary, results have been presented which indicate that cultured cells control their lipid composition in a number of different ways. One is by regulation of biosynthesis. Cells were observed to synthesize lipid in lipid free medium, and this synthesis was inhibited in the presence of an external lipid supply. Similarly under conditions of EFA deficiency, quantities of polyunsaturated fatty acids of a type not normally found in large proportions are biosynthesized. This synthesis is repressed during normal EFA supply. However an additional type of regulation, involving transport into and out of the cell seems to be equally important in cell cultures. Cholesterol and cholesterol ester content is a result of both passive uptake and selective excretion. The cells preferentially utilize free fatty acid as a source of nonsterol lipid and esterify it to make glycerides and phospholipids. In addition permeability of essential fatty acids may increase under conditions in which they are depleted. Under favorable conditions all of these mechanisms act in concert toward the end result of a lipid composition which is optimal for the cellular requirements.

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# Lipid Metabolism of Brain Tissue in Culture[1]

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**ABSTRACT**

Tissue explants from frontal lobes of rat brain were used for the study of cerebral fatty acid metabolism. After tissues had been maintained in serum-supplemented medium, a lipid free medium was substituted and metabolic studies were carried out. Under these conditions explants continued to take up lipid precursors for at least 48 hr. Stearic acid 1-$C^{14}$, palmitic acid 1-$C^{14}$ and lignoceric acid 1-$C^{14}$ were bound to cells as the free fatty acids or incorporated into neutral lipids (particularly triglycerides), glycolipids and phospholipids. In the galactolipid fraction, cerebrosides were the principal radioactive lipids. Choline phosphoglycerides, ethanolamine phosphoglycerides, inositol phosphoglycerides and serine phosphoglycerides were the principal radioactive phospholipids. Fatty acids were incorporated into cellular lipids either unchanged or after desaturation, chain elongation, or both. In a patient with a demyelinating disease, precursor uptake was reduced and chain elongation and desaturation of the fatty acid was diminished. In a patient with generalized $G_{M2}$ gangliosidosis, glycolipids other than cerebrosides were labeled to a greater extent than normal. These studies exemplify the usefulness of tissue explants for prolonged metabolic studies in normal and pathological specimens of brain.

In order to study the synthesis and turnover of myelin lipids with long half lives, a system had to be devised which would allow metabolic studies over prolonged periods. Homogenates and subcellular fractions of brain are suitable only for an investigation of cerebral lipids with short half lives, whose principal localization is outside the myelin sheath and which are probably only secondarily involved in the demyelinating disorders.

The development of methods for cultivation of brain tissue in vitro provided a new and potentially useful system for the study of lipid metabolism under normal and pathological conditions. Mammalian cells in tissue culture derive most of their lipids from the serum customarily added to the growth medium. Growth of a number of cell strains can also be supported by chemically defined media which do not contain serum. Under these conditions cell lipids are synthesized from precursors added to the medium. Our initial studies indicated that brain explants remain metabolically active for several days in a chemically defined, serum free medium and that they incorporate radioactive lipid precursors such as mevalonate and fatty acids into a number of lipid fractions (7).

In this report we wish to review the metabolic data obtained on brain explants from immature and mature rats and contrast them with those obtained by surgical biopsy in patients with demyelinating and lipid storage diseases.

## METHODS

### Cell Cultures

For the animal experiments frontal poles from brain of immature rats of Sprague-Dawley strain aged 2-22 days, and mature animals aged 5-6 months were used. After removal from the cranium, tissued was left overnight at 4 C in 50 cc Hanks Salt Solution (Grand Island Biological Company, Berkeley, Calif.), containing 0.5 cc antibiotic-antimycotic solution 524 (Grand Island Biological Company, Berkeley, Calif.). The next day dura and blood vessels were carefully removed under a dissecting microscope, and the remaining tissue was further cut into fragments ca. 1 mm in diameter. These were placed into plastic Falcon flasks (30 ml) (Becton, Dickinson and Co., Oxnard, Calif.). Four flasks were used for frontal lobes of a 3-day-old animal. Medium 199 0.5 ml (Hyland Laboratories, Costa Mesa, Calif.), enriched with 20% v/v calf serum, 10% v/v egg ultrafiltrate and 300 mg glucose per milliliter, was added to all cultures, In addition 1 ml of antibiotic-antimycotic solution 524 was added to each 100 ml of medium. The flasks were placed for 8-12 hr in a 37 C incubator under an atmosphere of 95% air, 5% $CO_2$. This allowed the fragments to adhere to the plastic. Five cubic centimeters more of incubation medium were added, and the tissue was grown for 8-10 days at 37 C in an atmosphere of 95%

[1]One of 13 papers presented at the symposium "Lipid Metabolism in Cells in Culture," AOCS Meeting, Houston, May, 1971.

TABLE I

Distribution of Radioactivity in Lipids Isolated From Brain Tissue Explants After Incubation With Various Lipid Precursors

| Fraction | Radioactivity[a] Sodium acetate -1-$C^{14}$ [5] | Palmitic acid -1-$C^{14}$ [2] | Stearic acid -1-$C^{14}$ [5] | Lignoceric -1-$C^{14}$ [1] |
|---|---|---|---|---|
| Total radioactivity uptake (cpm x $10^{-3}$/mg protein) | 5.4-62 | 104-177 | 67-241 | 5.6 |
| "Neutral lipids" | 80.3 | 54.7 | 76.9 ± 7.4 | 90.7 |
| Triglycerides | 73.7 | 67.5 | 41.8 ± 10.9 | 10.9 |
| Diglycerides | 2.7 | 8.7 | 7.9 ± 2.6 | 3.2 |
| Cholesterol | 1.9 | 2.6 | 3.0 ± 1.3 | 2.0 |
| Free fatty acid | 6.0 | 12.3 | 39.2 ± 10.1 | 79.1 |
| Glycolipids | 0.7 | 0.6 | 1.9 ± 0.7 | 2.3 |
| Cerebrosides | 34.8 | 22.9 | 45.3 ± 8.5 | 58.5 |
| Sulfatides | 0 | 8.1 | 8.1 ± 2.6 | 23.4 |
| Phospholipids[b] | 18.8 | 44.8 | 23.2 ± 7.8 | 6.9 |
| PE | 9.0 | 5.5 | 19.2 ± 1.0 | 5.5 |
| PI + PS | 4.1 | 23.4 | 15.6 ± 2.3 | 4.3 |
| PC | 53.0 | 26.3 | 40.7 ± 6.0 | 65.3 |
| Sphingomyelin | 15.7 | 21.2 | 5.5 ± 0.7 | 8.4 |

[a]Figures represent the percentages of total radioactivity in principal subfractions and per cent of subfraction found in each lipid species. Each value is the sum of 10 flasks. Value for stearic acid and sodium acetate were derived from five sets of experiments and represent means ± S.E.M.; values for palmitic acid are the mean of two sets of experiments. Figures in brackets indicate number of experiments. Experimental conditions are described in the body of the paper.

[b]PE = phosphatidyl ethanolamine; PI = phosphatidylinositol; PS = phosphatidyl serine; PC = phosphatidyl choline.

air to 5% $CO_2$ with the medium being changed after 4 days.

Microscopic examination at that time showed outgrowth of multipolar glial cells from the margin of the explants. The outgrowth was better in explants obtained from 3- and 6-day-old animals than those derived from older rats. At the conclusion of the initial incubation the growth medium was removed and replaced with the experimental medium 5 ml NCTC 109 (Hyland Laboratories), enriched with 300 mg glucose per milliliter and 1 ml of the antibotic-antimycotic solution containing the labeled substrate. Human brain tissue obtained at surgical biopsy of the nondominant frontal lobe was treated in an identical manner.

In experiments designed to measure fatty acid uptake one of the following precursors was used: stearic acid [1-$^{14}$C] (0.2$\mu$Ci), (48.4 mCi/m mole) (Amersham-Searle, Des Plaines, Ill.), palmitic acid [1-$^{14}$C] (0.2$\mu$Ci), (10.0 mCi/m mole) linoleic acid [1-$^{14}$C] (0.2$\mu$Ci), (52.9 mCi/m mole) (Amersham-Searle, Des Plaines, Ill.), lignoceric acid [1-$^{14}$C] (0.2$\mu$Ci) (courtesy of J. Mead) and sodium acetate -1-$^{14}$C (0.10 mCi), (50 mCi/m mole) (Mallinckrodt Chemical Co.). The fatty acids were added in the form of their albumin complexes prepared according to the method of Fillerup et al. (4). Molar ratios of added free fatty acid-albumin of the order of 0.003 resulted in optimal microscopic appearance of explants.

#### Lipid Extraction and Fractionation

At the termination of the experiment, tissue from the four flasks was combined; cells were scraped off with a policeman; the medium was removed by centrifugation, and cells were washed three times with 0.15 M NaCl. The tissue was homogenized in 3 ml chloroform-methanol 2:1 v/v, and aliquots were removed for determination of radioactivity and protein content (5).

The homogenate was filtered through a medium porosity sintered glass funnel; portions were taken for counting and the solution was evaporated to dryness under $N_2$ and placed overnight in a desiccator prior to fractionation on an anhydrous silica column. After suspension in 0.2 ml chloroform containing 0.1 g. of silicic acid the lipids were applied to a 0.5 x 6 cm silicic acid solumn (Sigma Chemical Co., St. Louis, Mo.) and fractionated according to the method of Sun and Horrocks (16). Neutral lipids were eluted with 60 ml chloroform, glycolipids with 50 ml of chloroform-acetone 1:1, v/v, followed by 50 ml of acetone, and phospholipids with 100 ml methanol. Portions of each fraction were taken for determination of radioactivity, the remainder was separated by thin layer chromatography (TLC).

Lipids in each fraction were separated by

TABLE II

Pattern of Labeled Fatty Acids in Tissue Explants Cultured From Frontal Lobes of 3-Day-Old Rats in the Presence of Stearic Acid [1-$^{14}$C], Palmitic Acid [1-$^{14}$C] and Lignoceric Acid [1-$^{14}$C]

| Fatty acid fraction | Radioactivity[a] | | | |
|---|---|---|---|---|
| | Sodium acetate, % | Palmitic acid, % | Stearic acid, % ± SE | Lignoceric, % |
| Nonhydroxy fatty acids, % total fatty acids | 96.8 | 84.4 | 97.7 ± 1.3 | 96.8 |
| Saturated fatty acids, % nonhydroxy fatty acids | 51.9 | 95.9 | 86.9 ± 2.8 | 88.9 |
| 16:0 | | 89.0 | 9.7 | |
| 18:0 | | 8.0 | 87.9 | |
| 20:0 | | 0.5 | 1.0 | |
| 22:0 | | 0.5 | 0.3 | |
| 23:0 | | 0.2 | 0.1 | |
| 24:0 | | 0.4 | 0.1 | |
| Monounsaturated fatty acids, % nonhydroxy fatty acids | 13.0 | 4.7 | 10.2 ± 2.6 | 9.6 |
| 16:1 | | 51.8 | 1.1 | |
| 18:1 | | 43.6 | 89.4 | |
| 20:1 | | 2.4 | 1.7 | |
| Polyunsaturated fatty acids, % nonhydroxy fatty acids | 35.0 | 0.1 | 1.1 ± 0.2 | 1.6 |

[a]Radioactivities of the methyl esters of each fatty acid were measured as described in the text, and are expressed as percentages of the radioactivity in the principal fatty acid fractions. Stearic acid [1-$^{14}$C] (0.3 μCi, 4.1 x $10^{-9}$M) and palmitic acid [1-$^{14}$C] (0.2 μCi, 2.0 x $10^{-8}$M) were added with culture conditions as described in the text; growth was terminated after 48 hr. Lipids from 10 culture flasks were combined for fatty acid analysis. Values for stearic acid are the result of five experiments and represent means ± S.E.M.

TLC on 20 x 20 cm plates. For the separation of neutral lipids Silica Gel G (Merck, AG, Darmstadt, Germany) powder was slurried in 0.01 M $Na_2CO_3$ and the plates were developed in hexane-ether 70:30 v/v, according to the procedure of Sun and Horrocks (16). Glycolipids were separated on Silica Gel G with a solution of Chloroform-acetone-methanol-water 60:20:20:1 by volume. Phospholipids were separated on Silica Gel H with chloroform-methanol-acetic acid-water 20:15:4:2 by volume, according to the method of Skipski et al. (14). Areas containing lipids were located by a brief exposure to iodine vapors and were scraped from the silica gel and transferred to a counting vial, to which 15 ml scintillation mixture (0.5% [w/v] 2, 5-diphenyloxazole [PPO] and 0.03% [w/v] 1,4-bis [2-(5-phenyloxazolyl)]-benzene [POPOP] in toluene) and 0.6 g Cab-O-Sil (15) were added. Uptake of radioactivity was a function of the amount of tissue incubated. Since this varied considerably between experiments all data were expressed in terms of milligrams of tissue protein.

### Identification of Fatty Acids

For identification of the labeled fatty acids the lipid extract was dried under a stream of $N_2$. Fatty acid methyl esters were prepared by heating the dried sample at 100 C for 90 min with 14% w/v boron trifluoride in methanol (10). The methyl esters were extracted into hexane, purified by TLC on Silica Gel G plates developed with hexane-ether 9:1 v/v, and separated according to degree of unsaturation on silver nitrate-impregnated plates developed in chloroform (9). Gas liquid chromatography (TLC) of each fraction was performed on columns of 16% w/v diethyleneglycol succinate on Gas Chrom Z as stationary phase (Applied Science Laboratories, State College, Pa.). Gas chromatography was carried out with a Barber-Coleman model 10 gas chromatograph with argon detector. The column was operated at 175-195 C at 19 lb. argon pressure. Peaks were identified by comparison with standard mixtures of fatty acid esters. Radioactive fatty acid methyl esters were collected from the gas chromatograph by means of a glass U-tube (4 mm in diameter) directly connected to the heated outlet and cooled in a dry-ice mixture. The fatty acid esters were rinsed from the collection tubes into a scintillation vial with 15 ml of scintillation mixture. Approximately 90% of the radioactivity injected into the column was recovered.

## RESULTS

### Incorporation of Fatty Acids Into Tissue Lipids

Even when experimental conditions were

TABLE III
Distribution of Radioactivity in Brain Tissue Explants Incubated in Presence of High Phenylalanine Concentrations

| Fraction | Sodium acetate-1-$C^{14}$[a] | | Palmitic acid-1-$C^{14}$[a] | | Stearic acid-1-$C^{14}$[a] | |
|---|---|---|---|---|---|---|
| | Control, [4] | PA,[b] [4] | Control, [2] | PA, [2] | Control, [4] | PA, [4] |
| Total lipids, cpm/mg protein x $10^{-3}$ | 30.2 ± 20.8 | 32.3 ± 21.8 | 140 | 186 | 124.7 ± 79.1 | 89.2 ± 38.2 |
| Neutral lipids, % total lipid radioactivity | 80.3 | 77.6 | 54.6 | 57.0 | 69.5 ± 13.7 | 69.6 ± 17.0 |
| Glycolipids, % total lipid radioactivity | 0.7 | 0.7 | 0.6 | 1.0 | 2.0 ± 1.3 | 2.5 ± 1.5 |
| Phospholipids, % total lipid radioactivity | 10.8 | 21.7 | 44.8 | 42.0 | 28.4 ± 14.6 | 27.9 ± 17.8 |

[a]Figures represent percentage of total radioactivity in the three principal subfractions. Incubation conditions as in text. Figures in brackets indicate number of experiments.

[b]PA = high phenylalanine medium.

well-standarized the total fatty acid uptake varied considerably between experiments. In tissues derived from a 3-day-old rat incubated with stearic acid-1-$C^{14}$, uptakes ranged from 67,000-240,000 cpm/mg protein. When fatty acids were added in the form of their albumin complexes a considerable proportion of the uptake remained unmetabolized, probably in the form of protein-bound free fatty acids. However the relative amount of label incorporated into each of the lipid classes was nearly constant (Table I). In the neutral lipid fraction triglycerides were the principal labeled lipid, with lesser amounts of radioactivity in diglycerides and cholesterol esters. Only a small amount of label was present in cholesterol. A considerable proportion of fatty acids added to the cultures became rapidly attached to the explant and could not be removed desite repeated washings. (Table I). The proportion of fatty acid so bound was least when acetate was used as lipid precursor, and highest for lignocerate. Cerebrosides were the principal labeled lipids identified in the glycolipid fraction. Fatty acids were incorporated into all major phospholipids, notably choline phosphoglycerides and ethanolamine phosphoglycerides, which together accounted for about 60% of the total phospholipid radioactivity (Table I).

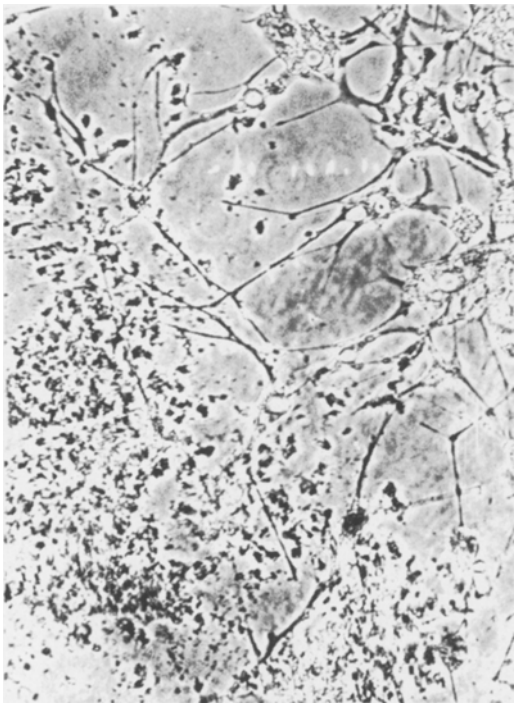

FIG. 1. Explant from 1-day-old rat brain frontal lobe after 8 days incubation in serum-enriched medium. Outgrowths include multipolar glial cells with a cytoplasm filled with refractile bodies. There was no conclusive evidence of neuronal outgrowth. The edge of the original explant is on the lower left. Phase microscopy x 100.

When the uptake of the various fatty acids into the phospholipid fraction was compared, palmitic acid was found to be incorporated to the greatest extent, lignoceric the least.

Under the culture conditions employed, a significant proportion of stearic, palmitic and lignoceric acids underwent desaturation (Table II). Chain elongation of palmitic to stearic acid occurred in the explants, and proceeded more actively than the elongation of stearic arachidic acid (20:0). Labeled acetate was incorporated into all fatty acids, including the polyunsaturated fraction.

### Lipid Metabolism of Explants in Various Disease States

*Metachromatic leukodystrophy:* In this progressive demyelinating disease incorporation of stearic acid-[1-$^{14}$C] was markedly reduced.

TABLE IV

Pattern of Labeled Fatty Acids in Tissue Explants Incubated in Presence of High Phenylalanine

| Fatty acid fraction | Sodium acetate-1-$C^{14}$[a] | | Palmitic acid-1-$C^{14}$[a] | | Stearic acid-1-$C^{14}$[a] | |
|---|---|---|---|---|---|---|
| | Control, [1] | PA[b] [1] | Control, [2] | PA, [1] | Control, [5] | PA, [3] |
| Nonhydroxy fatty acids, % total fatty acids | 96.8 | 88.9 | 89.8 | 79.3 | 98.3 | 93.5 |
| Hydroxy fatty acids, % total fatty acids | 3.2 | 11.1 | 10.2 | 18.6 | 1.3 | 5.5 |
| Saturated fatty acids, % nonhydroxy fatty acids | 55.5 | 54.7 | 91.9 | --- | 89.8 | 89.9 |
| Monounsaturated fatty acids, % nonhydroxy fatty acids | 16.0 | 14.6 | 4.7 | --- | 8.6 | 9.6 |
| Polyunsaturated fatty acids, % nonhydroxy fatty acids | 28.5 | 30.7 | 3.0 | --- | 1.3 | 0.7 |

[a]Figures in brackets indicate number of experiments. Values indicate per cent of radioactivity in terms of incorporation into total fatty acids in nonhydroxy fatty acids. Incubation conditions as given in text.

[b]PA = high phenylalanine medium.

Specific activity of lipids was 320 cpm/mg protein, contrasted with 3681 and 1555 cpm/mg protein for two age-adjusted rat explants, run concurrently. Incorporation of label was reduced for all lipid fractions. Desaturation and chain elongation of stearic acid were impaired in this condition. Oleic acid 18:1 had a relative specific activity of 0.2 compared with a control value of 0.7; arachidic acid (20:0) had a relative specific activity of 0.1 compared with a control value of 1.5.

*Generalized $G_{M2}$ gangliosidosis juvenile variant:* In this variant of Tay-Sachs disease (8) the uptake of stearic acid and palmitic acid was normal (6130 cpm/mg protein and 23,090 cpm/mg protein respecitvely). A significant abnormality was noted in the distribution of labeled in the glycolipid fraction. Only 10.7% and 12.0% of the total radioactivity was found in the cerebroside fraction after incubation with stearic acid and palmitic acids respectively. The major amount of radioactivity was found in lipids whose chromatographic behaviour corresponded to ceramide di- and polyhexosides.

*Lipid biosynthesis in the presence of high phenylalanine levels:* For this purpose a chemically defined incubating medium was prepared, the amino acid profile of which was identical to that of the usual patient with phenylketonuria (12). NCTC 109 medium (Grand Island Biological Company, Berkely, Calif.) was used, but the concentration of L-phenylalanine was raised to 350 mg/liter ($2.1 \times 10^{3}$M) of medium. In order to maintain the total amino acid concentration, the concentration of the other essential amino acids were reduced. For example L-tyrosine levels were 9.86 mg/liter compared with 16.4 mg/liter in the medium, as available commercially, and L-tryptophan was reduced from 17.5 mg/liter to 13.1 mg/liter.

Analysis of the amino acid composition at the beginning and end of a 48 hr incubation failed to show any significant reduction in the phenylalanine content, a finding to be expected in view of the absence from brain tissue of phenylalanine hydroxylase.

The uptake and incorporation of acetate, stearate and palmitate was studied, using concurrent controls and incubating conditions identical with those employed for the other metabolic studies.

As shown in Table III, no significant differences in uptake and distribution of incorporation in the presence of high phenylalanine is evident, even though stearic acid uptake was somewhat higher in the control cultures. When the amount of tissue-bound fatty acid was subtracted from the total uptake, no difference between the two groups of values was noted.

Upon examination of the distribution of the labeled fatty acids, the proportion of label in hydroxy fatty acids was slightly but not significantly increased in the presence of phenylalanine. Incorporation of stearate and acetate into saturated, mono- and polyunsaturated nonhydroxy fatty acids was unaffected by the presence of phenylalanine, and there were no consistent differences in the specific activity of the various fatty acids.

In two experiments sodium phenylpyruvate was added to NCTC 109 to a final concentration of 3.3 mg% ($1.8 \times 10^{-4}$M). This did not effect uptake or distribution of labeled stearate and acetate.

## DISCUSSION

The development of methods for routine cultivation of nervous tissue explants in vitro provides a new approach for the study of cerebral lipid metabolism, particularly for the

slower metabolic reactions relating to the synthesis and turnover of myelin components. Outgrowth from explants at the end of the incubation in enriched serum consisted of multipolar glial cells, principally fibrous astrocytes whose cytoplasm contained refractile bodies. A small number of bipolar fibroblasts, probably derived from the meninges and vasculature included in the explant, was also observed. No neuronal outgrowth was seen (Fig. 1). After subsequent incubation in a chemically defined media there was little change in the appearance of the outgrowths. In interpreting data obtained from this study several points must be emphasized: The cellular environment during the metabolic studies is highly artifical; in the absence of serum the explant cannot be maintained indefinitely and there is considerable biochemical and morphologic evidence that the metabolic processes begin to slow down after ca. 72 hr of incubation, as substrates are depleted and end products accumulate. When the growth environment was enriched by the addition of lipid containing serum, uptake of lipid precursors was reduced considerably (7), a finding consistent with the experience of Bailey (2) who noted that incorporation of [1-$^{14}$C] acetate into lipids of L-strain mouse fibroblasts was almost completely inhibited by the addition of serum to growth medium. In view of the complexity of the cellular environment that is required for maintenance of normal cerebral lipid metabolism, it seems unlikely that the chemically defined medium used in these experiments is adequate for optimal lipid biosynthesis. We have found that adjustments in culture techniques enhanced significantly lipid biosynthesis, particularly in explants taken from brain during periods of active deposition of myelin (E. Yavin and J.H. Menkes, in preparation).

Our work indicates that fatty acids, when added to brain tissue explants in a lipid free incubation medium, are in part incorporated into a variety of lipid fractions, notably triglycerides and phospholipids, and in part remain as tissue-bound free fatty acids. Since triglycerides constitute a minor fraction of brain lipids this finding suggests that the acyl moiety of triglycerides is characterized by a more active uptake of radioactivity than the other lipid fractions, a suggestion consistent with in vivo observations of Sun and Horrocks (16) and implying that in explants at least, triglyceride serves as a readily available source of acyl groups. Much less radioactivity was incorporated into cholesterol esters; apparently in the present cellular environment cholesterol esters are of little importance as a source of fatty acids for phospholipid and glycolipid biosynthesis.

Examination of the fatty acid composition of lipids in the tissue explants indicates that a significant portion of the stearic acid [1-$^{14}$C] and palmitic acid [1-$^{14}$C] utilized by the cells underwent chain elongation or desaturation. This observation is compatable with our previous finding, that brain, particularly at the state of myelination or earlier, is capable of both metabolic processes (1). Our initial studies on pathological material indicate that incorporation of lipid precursors can be severely impaired as in the demyelinating disorder, or can undergo a redistribution as in the lipid storage disease. Our preliminary observations that incorporation of lipid precursors proceeds normally in the presence of high phenylalanine levels and at concentrations of phenylpyruvate comparable to those encountered in the blood of the usual untreated phenylketonuric patients (6), is at variance with the observations of a number of workers.

It would therefore appear that this method will aid in the understanding the various metabolic disorders of the brain for which as yet no enzymatic defect has been found.

Barbato et al. (3) found diminished $^{14}$C acetate uptake into myelin lipids including cerebrosides, in 12-day-old rabbits in the presence of high phenylalanine levels. However phenylalanine concentrations between 68 and 115 mg% were required to demonstrate this effect, and at levels comparable to those established in our experiments no reduction in acetate incorporation was observed.

In the experiments of Weber et al. (17), significant inhibition of glucose-6-$^{3}$H incorporation into lipids was observed at 2.5 x $10^{-3}$M phenylpyruvate, a concentration much higher than serum levels in phenylketonuria.

Shah et al. (13) found inhibition of in vivo incorporation of U-$^{14}$C glucose into rat brain lipids when brain phenylalanine levels were 0.15-0.21 $\mu$M/g tissue. Inhibition was only 8% in 5-day-old animals, with the maximum effect of 26% being observed in 13-day-old rats. However these workers were unable to show any effect of phenylalahine on lipid biosynthesis in brain homogenates. Phenylpyruvic acid also impaired incorporation of glucose into brain lipids, but concentrations of at least 5 x $10^{-3}$M were required to show any significant differences.

The experiments reported at this time are only preliminary, and need to be repeated on explants derived from animals at various stages of brain maturation. However at present it is likely that the deleterious effects of phenylalanine cannot be demonstrated unless the blood

brain barrier remains intact.

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# Phospholipid Metabolism in Cells in Culture[1,2]

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## ABSTRACT

The proportions of the different lecithin fractions have been determined in HeLa and KB tissue culture cells and Ehrlich Ascites tumor. 82.8% of the total phosphatidyl choline phosphorus is found in fraction 3 of HeLa cells. The major phosphatidyl cholines found in KB cells and Ehrlich Ascites tumor are in fractions 3 and 4 and representing 66.6% and 88.7% of the total phosphatidyl choline P, respectively. The incorporation of 1,2-$^{14}$C-choline and 1,2-$^{14}$C-ethanolamine into the various phosphatidyl choline fractions has been assayed to determine their biosynthesis in Ehrlich Ascites tumor. The incorporation of 1,2-$^{14}$C-choline into fractions 3 and 4 is 100 times the 1,2-$^{14}$C-ethanolamine. This evidence indicates that the methylation pathway of phosphatidyl choline synthesis is very low in HeLa, KB and Ehrlich Ascites cells.

## INTRODUCTION

There are a number of indications that the invasiveness of cancer cells may be related to the characteristics of the cell surface membranes (1-3). It is now known that lipids, and in particular phospholipids, are essential components of all cell membranes including both the plasma membrane and the highly specialized membranes of mitochondria, microsomes and nuclei. Phospholipids represent 21-32% of the dry weight of mitochondria and microsomes (4-6). The composition of phosphatidyl choline as per cent of total lipid P of plasma membrane (7,8) is 37.4%, mitochondria (9) 45.0%, and microsomes (9) 48.5%. Phosphatidyl choline can now be fractionated into four different lecithins according to the degree of fatty acid unsaturation by thin layer chromatography on Silica Gel H and $AgNO_3$ (10). Phosphatidyl choline biosynthesis is known to occur by two different major pathways. The Kennedy and Weiss (11) pathway involves cytidine diphosphocholine and $\alpha$-$\beta$-diglycerides to form phosphatidyl cholines. The Bremer and Greenberg pathway (12) involves the methylation of phosphatidyl ethanolamine from adenosylmethionine to form phosphatidyl choline. The specificity of the incorporation of 1,2-$^{14}$C-choline and 1,2-$^{14}$C-ethanolamine into these phosphatidyl choline fractions, as a means of measuring these two biosynthetic pathways of lecithin synthesis, has been determined (13). Phosphatidyl cholines of fractions 1 and 2 (Table I) are chiefly incorporated from 1,2-$^{14}$C-ethanolamine and provide a lecithin rich in polyunsaturated fatty acids (13). The phosphatidyl cholines of fraction 3 and 4 are mainly synthesized by the 1,2-$^{14}$C-choline pathway (13). The composition of these phosphatidyl choline fractions and their biosynthesis has not been determined in cancer cells.

## MATERIALS AND METHODS

Choline chloride-1,2-$^{14}$C (specific activity 2.5 mc/mmole) was purchased from Mallinckrodt Nuclear, St. Louis, Mo. Ethanolamine-1,2-$^{14}$C (specific activity 3.7 mc/mmole) was purchased from the New England Nuclear Corp., Boston, Mass. The isotopic compounds were injected ip (0.83 $\mu$c/animal) into the mice and the Ehrlich Ascites tumor cells harvested 1 and 4 hr later. Protein standard albumin was obtained from Armour Pharmaceutical Company, Chicago, Ill. Silica Gel H was purchased from Brinkmann Instruments, Westbury, N.Y. Two kinds of heteroploid serial cultures were obtained from Microbiological Associates, Inc., Bethesda, Md., HeLa (Gey-human carcinoma of cervix) and KB (Eagle-human carcinoma of nasopharynx). The Ehrlich Ascites tumor was maintained in female mice, strain C3H (Texas Inbred Mice Company, Houston, Tex.). The Ehrlich Ascites tumor cells (9 days after inoculation), HeLa and KB cells were centrifuged at 2000 x g for 10 min, washed twice with 0.9% saline, and recentrifuged with each washing. An aliquot was taken for protein analysis (14). The cells were homogenized with methanol in a Potter-Elvehjem homogenizer with a teflon pestle. The teflon pestle was washed with chloroform. The method of Folch et al. (15) was employed to extract and purify the lipids. The lipids were stored in a dilute chloroform

[1]One of 13 papers presented at the symposium "Lipid Metabolism in Cells in Culture," AOCS Meeting, Houston, May 1971.

[2]Part of a thesis submitted to the Graduate School of the University of North Dakota in partial fulfillment of the Degree of Master of Science.

solution under dry nitrogen at -18 C. The total phospholipid phosphorus (16,17) was determined on an aliquot of the chloroform solution. Phosphatidyl cholines were isolated from the lipid extract by thin layer chromatography by the method of Parker and Peterson (18) using as solvent chloroform-methanol-acetic acid-water, 65:25:4:1.4 by volume. Phospholipids were identified by comparison with purified phospholipid standards. Plates were sprayed with 0.008% rhodamine 6G solution and viewed under UV light to identify and outline the band of gel containing the phosphatidyl cholines. The silica gel containing the phosphatidyl choline was scraped into a flask containing 20 ml chloroform-methanol 2:1 v/v. The phosphatidyl cholines were eluted from the gel by filtration of the chloroform-methanol solution with the aid of a sintered glass funnel (medium porosity). The gel was washed twice with chloroform-methanol-water 200:97:3 and once with methanol. Quantitative recovery of phosphatidyl choline was possible with this extracting procedure. Recovery values represented 94% of the total phosphorus. The filtrate was washed with 0.2 volumes of 0.04% calcium chloride. A dilute solution of the phosphatidyl choline extract in chloroform was stored under dry nitrogen at -18 C. On an aliquot of the chloroform the total phosphatidyl choline phosphorus (16,17) was determined, and the per cent of total lecithin phosphorus per total phospholipid phosphorus was calculated. An aliquot was taken for radioactivity analysis. Fractionation of the phosphatidyl choline fractions was carried out by thin layer chromatography on Silica Gel H impregnated with silver nitrate (10). The phosphatidyl choline fractions were identified by spraying with 0.01% methanolic solution of 2,7-dichlorofluorescein and viewing under UV light (10). The phosphatidyl choline fractions were scraped into tubes containing 15 ml chloroform-methanol 2:1 v/v and 9 ml 0.04% $CaCl_2$ was added. The mixture was shaken and filtered with sintered glass funnel (medium porosity) to remove the silver ion and collect lipid-silica gel. The lipids were extracted from the gel with 15 ml chloroform-methanol-water 200:97:3 v/v/v; 5 ml methanol and chloroform was added to adjust the ratio of 2:1 (chloroform-methanol). The solution was shaken, centrifuged and aspirated to remove the methanol-water phase. Fifteen milliliters methanol and 9 ml 0.04% $CaCl_2$ was added and the washing procedure was repeated. Fifteen milliliters chloroform was added to the lipid extract, cooled in a refrigerator, and the water layer was removed by aspiration. The volume of lipid

TABLE I

Phosphatidyl Choline Concentration in Ehrlich Ascites Tumor Cells, HeLa Cells and KB Cells[a]

| Cells | Number experiments | Total phospholipid-P, μg | Total phospholipid-P/mg protein N, μg/mg | Phosphatidyl choline-P % Total phospholipid P | Phosphatidyl choline fractions, % total phosphatidyl choline-P 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|---|
| Ehrlich Ascites tumor | 6 | 1435 ± 870 | 7.98 ± 0.59 | 40.5 ± 2.7 | 4.2 ± 0.8 | 7.1 ± 1.4 | 42.4 ± 10.1 | 46.3 ± 9.7 |
| HeLa | 5 | 948 ± 413 | 7.62 ± 0.68 | 37.2 ± 3.1 | 1.6 ± 1.5 | 8.2 ± 2.3 | 82.8 ± 3.2 | 7.1 ± 3.4 |
| KB | 3 | 683 ± 195 | 6.54 ± 0.43 | 47.4 ± 2.4 | 10.6 ± 2.6 | 12.4 ± 2.0 | 30.9 ± 5.2 | 46.1 ± 10.7 |

[a]Numbers following ± are standard deviations.

TABLE II

Specific Activity[a] (counts/min/μg P) of 1,2-$^{14}$C-Choline and 1,2-$^{14}$C-Ethanolamine Incorporation into Total Phosphatidyl Choline and Phosphatidyl Choline Fractions of Ehrlich Ascites Tumor Cells

| Sample injected | Time after injection, hr | Specific activity[b] | | | | |
|---|---|---|---|---|---|---|
| | | Total lecithin | Lecithin fractions | | | |
| | | | 1 | 2 | 3 | 4 |
| 1,2-$^{14}$C-Choline | 1 | 49.6 ±20.8 | 17.4 ±7.2 | 26.6 ± 9.4 | 50.1 ±17.1 | 41.8 ±18.2 |
| 1,2-$^{14}$C-Choline | 4 | 56.6 ±11.1 | 17.4 ±7.2 | 38.4 ±11.3 | 62.5 ±20.8 | 50.8 ±15.1 |
| 1,2-$^{14}$C-Ethanolamine | 1 | 0.352 ± 0.086 | 0.106 ± 0.100 | 0.144 ± 0.116 | 0.194 ± 0.079 | 0.168 ± 0.126 |
| 1,2-$^{14}$C-Ethanolamine | 4 | 1.309 ± 0.089 | 1.079 ± 0.236 | 0.238 ± 0.223 | 0.510 ± 0.160 | 0.392 ± 0.031 |

[a]cpm/μg P.
[b]Numbers following ± are standard deviations.

extract was diluted to 50 ml with chloroform. Recovery values represented 90-95% of the phosphorus applied to silica gel impregnated with silver nitrate. Samples were taken for phosphorus (16,17) and radioactivity analysis. All radioactivity measurements were made in a Packard TriCarb liquid scintillation counter operating at 70% efficiency. The scintillation solvent consisted of 0.4% of 2,5-diphenyloxazol (PPO) and 0.005% of 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP) in toluene. Specific activity is expressed as cpm/μg lecithin phosphorus.

## RESULTS AND DISCUSSION

The per cent of total lipid P as phosphatidyl choline of HeLa and KB cells (Table I) is similar to that reported (19). Wood and Snyder (20) have made an extensive study of the fatty acids in total phosphatidyl cholines of Ehrlich Ascites tumor cells. They have shown that the diacylphosphatidyl cholines of Ehrlich Ascites tumor have much less of the polyunsaturated fatty acids, arachidonic and docosahexaenoic than does rat liver (20,21). The phosphatidyl choline fraction 1, which contains the major polyunsaturated fatty acid, is very low in HeLa, KB and Ehrlich Ascites tumor. 82.8% of the total lecithin P is found in fraction of HeLa cells. Fractions 3 and 4, which have been shown to contain arachidonic, oleic and linoleic acids as the major unsaturated fatty acids, constitute the major phosphatidyl cholines of Ehrlich Ascite tumor, HeLa and KB cells. Rytter et al. (13) have shown that the phosphatidyl choline of fractions 3 and 4 are synthesized by the 1,2-$^{14}$C-choline pathway; whereas the phosphatidyl cholines of fraction 1 are chiefly formed in the 1,2-$^{14}$C-ethanolamine pathway. It is apparent from the data of Table I that the major phosphatidyl cholines found in Ehrlich Ascites tumors are in fractions 3 and 4 (88.7% of the total lecithin P). Donisch and Rossiter (22) demonstrated that 1,2-$^{14}$C choline was incorporated 33-16 times greater into the total lecithins of Ehrlich Ascites tumor than was 1,2-$^{14}$C-ethanoline. However the biosynthesis of the various lecithin fractions was not determined. It is apparent from the data of Table II that the uptake of 1,2-$^{14}$C-choline into fractions 3 and 4 represents the major pathway of lecithin synthesis in Ehrlich Ascites tumor cells. There is very little incorporation of 1,2-$^{14}$C ethanolamine into the lecithin fractions. These observations further support the chemical analysis which shows the 86.7% of total lecithin is in fractions 3 and 4. The incorporation of 1,2-$^{14}$C-choline into fractions 3 and 4 is 100 times that of the 1,2-$^{14}$C-ethanolamine. These two observations give evidence that the

CDP-choline-diglyceride pathway of lecithin synthesis provides most of the lecithins found in Ehrlich Ascites tumor. Figard and Greenberg (23) have shown that the enzyme responsible for methylation of phosphatidyl ethanolamine to form lecithin is less active in Ehrlich carcinoma than in mouse liver.

The enzymatic activity of phosphatidylmethyltransferase was shown to be linear with time and concentration of enzyme (24). The average of three analyses for the enzymatic activity of the methylating enzyme (phosphatidylmethyltransferase) with 10 mg of protein and at 10 min of assay gave $6.25 \times 10^{-4}$ $\mu$m lecithin/mg of protein in HeLa cells, and $1.8 \times 10^{-2}$ $\mu$m lecithin/mg protein in rat liver. This observation shows that the system for methylation of phosphatidyl ethanolamine in HeLa cells is much less active than in liver.

The existence of two pathways of phosphatidyl choline biosynthesis provides a basis for different lecithin molecules, and may be a sensitive means of controlling the availability of specific lecithins for normal function and integrity of the membranes of the cell. The decrease in activity of the biosynthesis of lecithin by the methylation pathway may help to provide a phospholipid in the membrane which is different from that in the normal cell, and their invasiveness or malignant change may be owing, in part, to these lipid changes.

## ACKNOWLEDGMENT

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# Regulation of Lipid Metabolism in in Vitro Cultured Minimal Deviation Hepatoma 7288C[1]

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## ABSTRACT

Hepatoma tissue culture (HTC) cells (derived from minimal deviation hepatoma 7288C) were cultivated in a complete medium containing either glucose, fructose or glycerol as the primary carbon source. Growth was rapid on glucose and very low on fructose containing media. Glycerol did not support any growth. Glucose-$^{14}$C (U) and fructose-$^{14}$C (U) are incorporated into the total lipid fraction of HTC cells. However the level of conversion of glucose into lipid is much greater than fructose. Tritiated water is rapidly incorporated into the saponifiable and nonsaponifiable lipid fractions of growing HTC cells. The level of incorporation is greater than that observed with glucose-$^{14}$C (U) and the difference is constant over the experimental period studied. Lipoprotein poor serum (LPPS) isolated from a calf-fetal calf serum mixture (1:1) supported growth at a similar rate as the unfractionated serum combination (DS). However the total lipid and total cholesterol content of the fractionated serum was one sixth and one fiftieth the level found in the whole serum mixture, respectively. The level of incorporation of glucose-$^{14}$C (U), acetate-$^{14}$C (U) and tritiated water into the nonsaponifiable fraction of HTC cells grown on LPPS was 3 to 8-fold greater than that for DS. However mevalonate-2-$^{14}$C incorporation was stimulated only 1.3 to 1.7-fold. In general there was a much smaller response in the level of incorporation of radioactive metabolites into the saponifiable lipids. From these studies and additional data, it was tentatively concluded that HTC cells can respond to nutritional pertubations caused by changes in the exogenous lipid content. It was not determined if the apparent responsiveness in the lipids of the nonsaponifiable fraction is due to "feedback control" or some other regulatory mechanism.

[1]One of 13 papers presented at the symposium "Lipid Metabolism in Cells in Culture," AOCS Meeting, Houston, May 1971.

## INTRODUCTION

The metabolism of lipids is a fundamental biological process which is found in all living systems. As is associated with all fundamental biological processes, there are numerous unanswered questions regarding the nature of endogeneous or exogenous regulatory effectors and their mode of action on the multitude of reactions of lipid biosynthesis. These effectors are responsible for both short and long term control of metabolic pathways (1,2).

Studies on metabolic regulation have progressed significantly from the initial observations of Pasteur effect seen in yeast (3). However the ability to use differentiated, isogenic, single cells, derived from higher eukaroytic organisms such as the rat, had to wait until routine methods were developed for their isolation, maintainance and long term cultivation (4,5). Today there are large numbers of malignant, diploid, highly differentiated, poorly differentiated eukaroytic cells available for cultivation in vitro (6-8).

I have chosen to utilize the experimental advantages of cultured cells to study the regulation of lipid metabolism in mammalian cells.

We are employing cells derived from Morris minimal deviation hepatoma number 7288C (hepatoma tissue culture [HTC] cells) (9,10). These cells exhibit some of the differentiated functions found in normal liver. The enzyme tyrosine amino transferase can be induced in HTC cells by specific glucocorticoid steroid hormones (10,11). Also they show increased cell-cell adhesiveness in the presence of these hormones (12). Considering these differentiated properties of HTC cells and their derivation from an organ which has a known high capacity for lipogenesis (2,14), in addition to availability, I have chosen to use these cells for my studies instead of other widely investigated experimental cell systems (18,21).

## MATERIALS AND METHODS

### Cultivation of Cells

HTC cells were maintained in spinner culture on a modified Swim's 77 medium, containing 16.7 mM glucose and 10% serum (9,10). The different sera (intact or fractionated) were always dialyzed against 0.9% NaCl for at least 24 hr before they were used. The maximum cell density obtained for HTC cells grown in suspen-

sion under normal conditions is $1 \times 10^6$ cells/ml with a generation time of 24-30 hr at 37.5 C.

When different carbon sources were tested for their capacity to support growth, a glucose free Swim 77 medium was used. The concentration of these carbon sources was 4-16 mM.

#### Metabolite Assays

Glucose, fructose and glycerol were measured in the growth medium after removal of the proteins by precipitation with 6% $HClO_4$. The $HClO_4$ supernatant fluid was adjusted to pH 5.8 with a 3*M* $K_2CO_3$, 0.5 *M* Triethanolamine solution and the $KClO_4$ precipitate removed by centrifugation.

Specific enzymatic assays for glucose (44), fructose (44) and glycerol (45) were performed on the clarified perchlorate extracts.

#### Total Lipid Analysis and Saponification

Lipids were extracted from all samples using the methods of Folch et al. (43). We used 0.2 volumes water for the first wash and 0.2 volumes Folch's theoretical upper phase containing NaCl for three additional washes. Centrifugation was used throughout to clarify and separate the solvent phases. The isolated lipids were dissolved in toluene and stored at -10 C until used.

Total lipid was determined by using the $H_2SO_4$-dichromate oxidation method of Amenta (46). A mixture of cholesterol and palmitate 2:1 w/w dissolved in toluene was used as our standard lipid solution.

Isolated lipids, and whole cells were saponified at 90 C for 3 hr after adding 2 ml of 5 *N* NaOH and 1 ml methanol. The nonsaponifiable lipid fraction was extracted from the hydrolysis solution with three 5 ml aliquots of petroleum ether (bp 30-60 C). Saponifiable lipids were removed after acidification of the aqueous with 1.5 ml of concentrated HCl and extraction with petroleum ether as above.

Ether was removed by evaporation in a vacuum oven. The lipid residue was either dissolved in a fixed volume of toluene and saved for lipid analyses or Liquifluor (New England Nuclear, Boston, Mass.) for radioactivity measurements.

#### Radioactivity Measurements

Radioactive samples were counted in either toluene or dioxane based solvent systems as described before (52).

Radioactive lipids, separated by thin layer chromatography (TLC), were removed from the plate after visualization with iodine vapor and comparison with reference compounds. All of the samples were removed by scraping and counted in Liquifluor (New England Nuclear, Boston, Mass.).

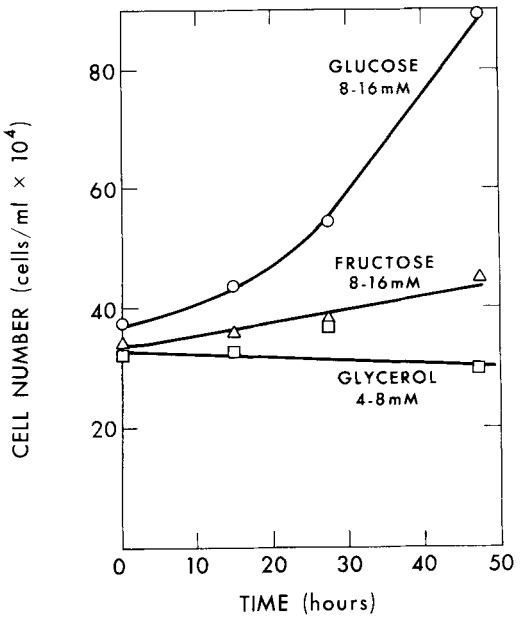


FIG. 1. Growth of HTC cells on different carbon sources.

#### Enzyme Assays

All enzyme activities in HTC cells were performed on extracts prepared from thrice-washed cells, resuspended in 0.25 *M* sucrose 1:3 w/v. This suspension was sonicated 25 sec (15 sec on, 30 sec off, 10 sec on) at 40 C using a Bronwill sonicator set at 20% maximal output and the microtip probe.

The sonicate was centrifuged at 105,000 x g for 35 min. We used the clear infranatant fluid between the pellet and prominent cellular lipid layer for all of the enzyme results presented in this paper.

Citrate cleavage enzyme (47), acetyl CoA carboxylase (48), fatty acid synthetase (49), malic enzyme (53), malate (lactate) dehydrogenase (50), hexokinase (54), acetate thiokinase (47), glucose-6-phosphate dehydrogenase (55) and 6-phosphogluconate dehydrogenase (55) were assayed according to the methods cited. Enzyme activities are expressed as m$\mu$moles of product formed per hour, per milligram protein. Protein was determined by the method of Lowry et al. (51).

## RESULTS AND DISCUSSION

Our initial efforts were directed at obtaining general information about the carbohydrate metabolic capacity of HTC cells grown in a 10% serum (calf-fetal calf 1:1 v/v), modified Swim's

TABLE I

Distribution of Radioactivity in Cellular Neutral Lipids From HTC Cells Grown on Different Carbon Sources[a]

| Lipid class | Carbon source,[b] % counts found | | |
|---|---|---|---|
| | Glucose-$^{14}$C (U.L.) | Fructose-$^{14}$C (U.L.) | Glycerol-$^{14}$C (U.L.) |
| Phospholipid | 66-73 | 75-80 | 86 |
| CH+DG+MG | 6-12 | 9-11 | 7 |
| FFA | 7-9 | 3-7 | 0.1 |
| TG | 16-30 | 6-11 | 6.1 |
| CH-E | 0.5 | 0.0 | 0.5 |
| Saponifiable fraction[c] | 91 | 66 | 50(6 cpm)[d] |
| Nonsaponifiable fraction[c] | 9 | 34 | 50(6 cpm)[d] |

[a]Cellular lipids were extracted by the method of Folch et al. (43) and separated by thin layer chromatography using the developing solvent *n*-hexane-ethyl ether-glacial acetic acid 70:30:1 v/v.

[b]Generally 85-95% of the applied radioactivity was recovered in the respective fractions.

[c]An aliquot of sonicated cellular suspension was saponified at 90 C for 3 hr in 5*N* NaOH plus 33% methanol.

[d]The total of 12 cpm found in hexane soluble fractions represent 1% or less of the total radioactivity in the unsaponified lipid extract.

77 medium (10). We fed the cells glucose, fructose and glycerol as primary carbon sources in the absence hormones. Figure 1 shows typical growth response curves for HTC cells cultivated on the different carbohydrates.

It is apparent that only glucose supports a significient level of cellular proliferation, and fructose facilitates, at best, very little growth. This lack of rapid growth on fructose was observed over a range of concentrations, i.e., 4-16mM. At no time have we observed any cellular proliferation on glycerol.

The inability of HTC cells to grow rapidly on fructose of glycerol is dissappointing in view of the results with whole animals which show that these carbon sources are hyperlipogenic (13-15). There are numerous possible reasons for this lack of growth on glycerol or fructose. We plan to continue these studies in the hope of determining conditions necessary to obtain rapid proliferation of HTC cells on hyperlipogenic substances, i.e., low levels of glucose may have to be added to overcome a minimal metabolic energy requirement or it may be necessary to add a hormone such as insulin to the growth medium, or both. It is also possible that necessary enzymes such as glycerol kinase, α-glycerol phosphate dehydrogenase or fructose kinase are absent from these cells. This is not unlikely because of the numerous reports demonstrating the absence of α-glycerol phosphate dehydrogenase in malignant cells (41,42).

When uniformally labeled $^{14}$C-substrates were used to monitor whether glycerol, glucose or fructose were taken into the cell, radioactivity was found in the TCA precipitable and total lipid fractions. Table 1 shows a summary of the radioactivity distribution pattern of cellular lipids isolated, and separated by TLC from HTC cells grown on the various carbon sources.

There was significant incorporation of glucose into the neutral lipid and phospholipid classes. Also a similar distribution of radioactivity was seen with fructose-$^{14}$C (U), but the absolute level of incorporation was much less than that obtained from glucose-$^{14}$C (U) (3% conversion vs. 30% conversion). However there was a slight increase in the percentage of radioactivity found in the phospholipid fraction of fructose grown cells relative to those grown on glucose. With glycerol-$^{14}$C (U) there was incorporation into the total lipid fraction, but it appeared to be limited to glycerides.

It is also shown in Table I that the relative radioactivity found in the saponifiable and nonsaponifiable fractions derived from the total lipids extracted from HTC cells grown on the different carbon sources is different. When glucose was used most of the hexane soluble counts were found in the saponifiable fraction (91%). The results obtained with fructose are significantly different from those obtained for glucose. It appears that a larger percentage of extractable radioactivity is found in the nonsaponifiable lipid fraction of cells grown on this carbon source. Since there was no indication of an increase in the percentage of radioactivity in the region where cholesterol, hydrocarbons or cholesterol esters migrate, we suggest that these nonsaponifiable counts might be owing to glyceryl ethers—especially when one considers the normal metabolism of fructose (16) and the dihydroxy acetone phosphate dependent glyceryl ether biosynthetic pathway (17). We have not chromatographed the nonsaponifiable frac-

tion from fructose grown cells to determine the number of radioactive components present.

The hexane soluble radioactivity (saponifiable and nonsaponifiable fractions) isolated after saponification of the lipids derived from glycerol-$^{14}$C (U) grown cells were essentially unlabeled. Ca. 80-90% of the radioactivity in the total lipids was found in the aqueous fraction. Since there was very little hexane extractable radioactivity (12 cpm) and the isotope distribution pattern of the lipids found after TLC of a Folch et al. (43) extract of cells exposed to glycerol-$^{14}$C (U), we have concluded that this carbon source is not rapidly converted to two carbon fragments or used for nonglyceride purposes in HTC cells.

Limited growth of HTC cells for 2-2.25 generations on uniformally labeled glucose gives rise to a level of radioactivity in the total cellular lipid fraction equivalent to a 30-35% conversion (uncorrected for lipid present in original innoculum). This is much higher than published data for other eukaroytic cells grown in media containing serum (18,19). Typical values for normal rabbit liver (20) and diploid W138 (21) cells are in the range of 3-10% conversion.

The above results indicate that radioactive glucose carbon atoms can be readily incorporated into the total lipid fraction of HTC cells. Also our TLC data show that this radioactivity is distributed in both neutral and phospholipids. The latter have yet to be resolved and the former appears to show radioactivity in all of the common lipid classes.

It became necessary at this point to determine the kinetics of incorporation of glucose into lipids and to ascertain the level of contribution by nonglucose carbon atoms to the acetyl coenzyme A pool. In order to facilitate these studies we monitored the appearance of radioactivity into the saponifiable and nonsaponifiable fractions of the cellular lipids from rapidly growing HTC cells.

TLC of the saponifiable and nonsaponifiable fractions (and digitonin precipitation) assured us that at least 80% of the radioactivity found in these fractions was owing to fatty acids and 3β-hydroxysterols, respectively (the latter assumed to be cholesterol).

These studies were accomplished by performing double labeled experiments using glucose-$^{14}$C (U) and tritiated water. The $^{14}$C counts represent the carbon atoms which glucose contribute to the isolated lipids, and the input of all lipid precursors in the medium in addition to those synthesized by the cell is represented by the level of tritium incorporated. The results in Figure 2 are from a typical

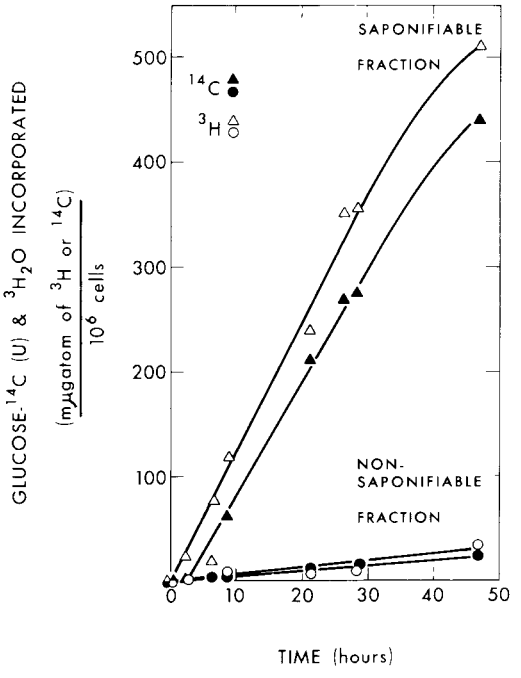


FIG. 2. Kinetics of glucose-$^{14}$C and $^{3}H_2O$ incorporation into saponifiable and nonsaponifiable fractions of growing HTC cells.

experiment for cells grown on 10% serum medium containing both isotopes.

It is apparent that with the specific activity of substrates used and sample size taken, we could detect incorporation of radioactivity into both the nonsaponifiable and saponifiable fractions as early as 2-4 hr after innoculation. This incorporation for both isotopes is linear throughout most of the experimental period, thereby suggesting that fatty acids and sterols in these cells turn over very slowly. Also there appear to be more tritium atoms incorporated per unit time than carbon atoms, thus indicating that carbon sources other than glucose are contributing to the acetyl coenzyme A pool. This is not unexpected in view of the fact that alanine and other amino acids are present in the growth medium (our serum is always dialyzed for 24 hr against 9% NaCl at 4 C in order to reduce the level of potential metabolic precursors).

The value for the ratio of tritium atoms incorporated to carbon atoms is 1.18 for the saponifiable fraction. This is significantly higher than the value of 0.87 found by Jungas for fatty acid synthesis in adipocytes (22). This ratio of 1.18 represents about a 35% contribution of nonglucose carbon atoms to the newly synthesized fatty acids. The ratio found for the nonsaponifiable lipid fraction is essentially 1.0.

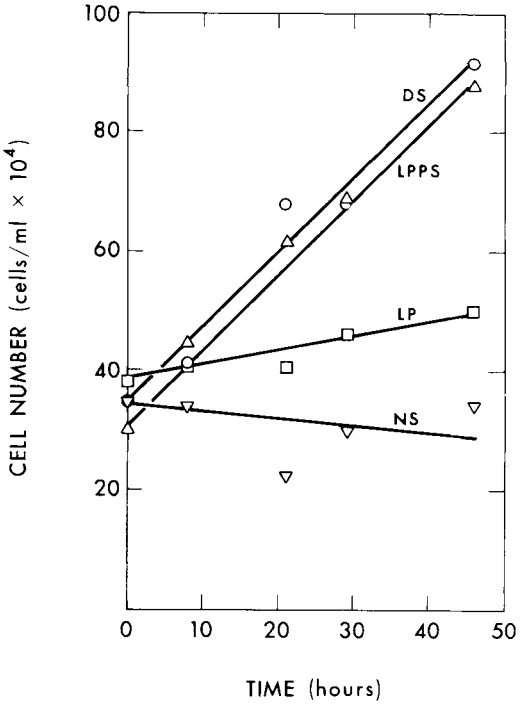


FIG. 3. Growth of HTC cells on intact and fractionated serum components (DS, dialyzed serum; LPPS, lipoprotein poor serum; LP, lipoprotein; and NS; no serum).

At present we have been unable to determine in the literature any value for the ratio of carbon atoms to tritium atoms incorporated.

It should also be noted that the level of incorporation into the saponifiable fraction is usually 10-15 times more than that found in the nonsaponifiable fraction. This is consistant with published data for in vivo incorporations studies in rats (23).

These data clearly indicate that radioactive glucose or tritiated water are adequate monitors of both fatty acid and nonsaponifiable lipid biosynthesis. The use of these precursors is preferred over acetate, because they are physiological and the level of radioactivity incorporated is more than adequate for our kinds of experiments. Also the sustained linear level of incorporation gave us a significant time period to perturb the cells before their immediate precursor pools for fatty acids and nonsaponifiable lipids had apparently reached a constant specific activity.

We were now able to ask the question: can HTC cells respond to nutritional pertubations related to the level of total or specific lipids in the growth medium?

In order to begin to answer this and related questions, we had to reduce our serum's total and specific lipid content. This was accomplished by ultra centrifugation (24). The serums were centrifuged at 120,000 x g for 22-26 hr with KBr at the level of 3.54 g/10 ml of serum. Typical results from a growth experiment using the fractionated serum are shown in Figure 3.

These data show the growth response of HTC cells to our standard dialyzed serum mixture (DS), the lipid protein poor serum (LPPS), the separated total lipoprotein (LP) fraction and no serum (NS).

It is apparent that LPPS supported growth at the same rate and level as DS. LP and NS supported very little, or no growth, respectively, during the 50 hr cultivation. When LP is added back to LPPS there is no apparent effect on the growth rate relative to DS. Thus this simple serum fractionation gives a protein solution with a significantly reduced lipid content but capable of supporting rapid growth.

Limited lipid analyses of DS, LPPS and LP show that LPPS has one sixth to one eighth the total lipid of DS and 10-30 times less total cholesterol. This is not unexpected because the cholesterol rich-lipoproteins are removed from whole serum by our fractionation conditions (24). Also we have found that ca. 80% of the total lipid in our serum mixture is derived from the calf serum. This observation has led us to begin studies using fractionated fetal calf serum as a starting material instead of the usual calf-fetal calf 1:1 mixture.

Since LPPS contsins approximately 1-5% of the total cholesterol found in dialyzed serum and supports rapid growth, studies were initiated to compare the lipogenic capacity of HTC cells grown on this fractionated serum mixture. The results from an experiment designed to ascertain whether we could obtain differences in the rates and levels of radioactive precursor incorporated into the nonsaponifiable lipids are shown in Figure 4.

Tritiated water incorporation was used as our indicator. These data show that the level of tritium incorporation reflects the normal and reduced total lipoprotein nutritional environment employed to cultivate the cells. The NS culture contains no lipid and the highest unit of incorporation of tritium atoms into the nonsaponifiable fraction is obtained under these conditions. However when HTC cells are exposed to a *no* serum environment, there is increased protein degradation (25) and no growth. This makes interpretation of results from cells incubated under these conditions somewhat difficult. On the other hand LPPS facilitates rapid cell growth and contains very little lipid. The level of incorporation of tri-

tiated water into nonsaponifiable lipids is 4- to 6-fold that seen with DS. Although the LP culture in this particular experiment shows a 2-fold increased level of incorporation relative to DS, we normally observe a rate which is below or equivalent to the dialyzed serum cultures. Also HTC cells cannot multiply in a LP medium. Other experiments with HTC cells incubated in different concentrations of LP suggest that the level of isotope incorporation is dependent on the final level of lipoprotein in the growth medium. In this experiment the level was less than that found in dialyzed serum. One obtains similar results to those in Figure 4 when uniformally labeled glucose is used as the radioactive substrate.

The level of radioactivity found in the saponifiable fraction of HTC cells cultivated of LPPS and DS are not markedly different. These results are highly variable. Most of the experiments performed to date show a 50-200% increased level of isotope incorporation by cells grown on LPPS. However we feel that this is owing to the level of free fatty acid in the serum which is variable from serum to serum. Also the LPPS derived from these serums has a high variability owing to the fractionation procedure employed—the levels of denatured protein vary from preparation to preparation and we do not add LPPS to the cultures at a constant level of free fatty acids (constant protein instead).

Table II summarizes data from six double labeled experiments measuring the rates of incorporation of $^{14}$C-glucose and tritiated water into the nonsaponifiable and saponifiable fraction of HTC cells grown under different nutrutional conditions.

These results are a quantitative representation which extends the information given in Figure 4 showing the sensitivity of the lipids found in the nonsaponifiable fraction to the nutritional pertubations used and the minimal response of the saponifiable lipid fraction. Also these data would suggest that either the total lipoprotein fraction removed by ultracentrifugation is responsible for the regulation of the biosynthesis of a lipid or lipids in the nonsaponifiable fraction of HTC cells, or that there are specific lipid or nonlipid components of this fraction which are regulatory effectors. An obvious extrapolation and conclusion from these results would be that the LPPS cholesterol level is so low that "feedback" inhibition (26,27) by the sterol is relieved, thereby causing enhanced cholesterol biosynthesis to meet cellular needs.

We have tested this by determining the effect of increasing LP on isotope incorporation

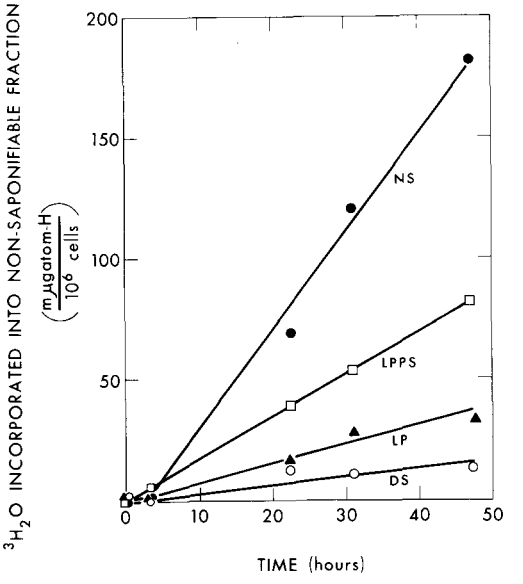


FIG. 4. $^3H_2O$ incorporation into nonsaponifiable lipid fraction of HTC cells grown on intact and fraction serum components (DS, dialyzed serum; LPPS, lipoprotein poor serum; LP, lipoprotein; and NS, no serum).

into the nonsaponifiable fraction of cells grown on LPPS, Table III shows the results obtained. Also the results obtained with a DS control to which LP was added are shown to determine whether isotope incorporation into cells grown on this serum could be suppressed further.

The data given in Table III demonstrate that the readdition of increasing amounts of LP can reduce the level of enhanced isotope incorporation into the lipids of cells grown on LPPS. This includes the saponifiable and nonsaponifiable lipid fractions.

The addition of LP lipid to the level found in DS causes a 33% inhibition of glucose-$^{14}$C (U) and $^3H_2O$ incorporation into the saponifiable and nonsaponifiable lipid fraction of the cells. This would suggest that the basal level of incorporation seem with HTC cells cultured on DS is not the lower limit. We don't know what the relative levels of incorporation would be with increasing DS or LP added to DS. These experiments will have to be performed.

When increasing amounts of LP are added to cultures containing LPPS there is a marked suppression of glucose-$^{14}$C (U) and $^3H_2O$ incorporation into the saponifiable and nonsaponifiable lipid fractions. The inhibition is most pronounced in the nonsaponifiable lipid fraction. There is a 60% inhibition by adding back one fourth the level of total lipid found in DS. Further addition of LP to the concentra-

TABLE II

Rates of Incorporation of Glucose-$^{14}C$ (U.L.) and $^{3}H_2O$ Into the Saponifiable and Nonsaponifiable Fractions of HTC Cells[a]

| Addition to medium | mμg Atoms/hr/$10^6$ cells[b] | | | |
|---|---|---|---|---|
| | Saponifiable | | Nonsaponifiable | |
| | $^{14}C$ | $^{3}H$ | $^{14}C$ | $^{3}H$ |
| DS[c] | 7.5 | 8.2 | 0.41 | 0.36 |
| LPPS | 6.7 | 6.8 | 1.98 | 2.15 |
| NS | 8.8 | 10.0 | 3.60 | 3.20 |

[a]Serial aliquots were removed from the cultures over a 54 hr incubation period. The rates were calculated from the slope of the curve from 10-54 hr (essentially linear over this range).

[b]$10^6$ Cells are equivalent to ca. 0.5 mg protein for cells grown in DS.

[c]The abbreviations used are: DS, dialyzed serum; LPPS, lipoprotein poor serum; and NS, no serum.

tion of total lipid found in DS results in a 80% suppression of the enhanced incorporation found in the LPPS culture lacking added lipoprotein. However this inhibited level of isotope incorporation was still slightly higher than that seen in the DS control culture (lines 1 and 6). Presently we think that this lack of coincidence is owing to (1) denatured lipoproteins (2) altered lipid composition and/or (3) lack of equivalency of LPPS: LP mixture to DS.

The incorporation of radioactivity into the saponifiable fraction from cells grown on LPPS with increasing amounts ot total lipid is inhibited about 30% over the range tested. This is in sharp contrast to the level of inhibition obtained for the nonsaponifiable lipids. Also we have found in numerous experiments that the effect of readdition of lipoprotein to LPPS cultures on isotope incorporation can be minimized under conditions in which inhibition is greater than 50% in the nonsaponifiable lipid fraction, thereby suggesting that the readdition of LP has an effect which is highly specific for the synthesis of nonsaponifiable. lipids rather than fatty acids.

LPPS still contains the bulk of the albumin-bound free fatty acids (24) which can inhibit fatty acid synthesis. Also it contains essentially

TABLE III

Effect of Added Lipoprotein on Glucose-$^{14}C$ (U) and $^{3}H_2O$ Incorporation Into Saponifiable and Nonsaponifiable Fractions[a]

| Condition | cpm/$10^8$ Cells | | | |
|---|---|---|---|---|
| | Saponifiable fraction | | Nonsaponifiable fraction | |
| | $^{14}C$ | $^{3}H$ | $^{14}C$ | $^{3}H$ |
| DS | | | | |
| LP added, ml[b] | | | | |
| 0.0 | 6440 | 10,950 | 710 | 980 |
| 1.0 | 4250 | 7350 | 460 | 720 |
| LPPS | | | | |
| LP added, ml | | | | |
| 0.0 | 13,710 | 23,900 | 4740 | 6100 |
| 0.5 | 9840 | 16,600 | 1500 | 1830 |
| 1.0 | 9050 | 15,100 | 900 | 1350 |
| 2.0[b] | 11,550 | 16,400 | 950 | 1340 |

[a]HTC cells were innoculated into 31 ml cultures containing glucose-$^{14}C$ (U) (15,000 cpm/μmole) and $^{3}H_2O$ (6,600 cpm/μmole) in addition to the amounts of LP indicated above. LPPS contained 360 μg of total lipid per milliliter and 31 μgm total cholesterol per milliliter. LP contained 10.7 mg of total lipid per milliliter, 4,970 μgm total cholesterol per milliliter and 8.8 mg protein per milliliter. All cultures were cultured for 50.5 hr and duplicate aliquots were removed from each culture, saponified and extracted as described in Methods. The abbreviations used are DS, dialyzed serum; LPPS, lipoprotein poor serum; and LP, lipoprotein.

[b]The final concentration of total lipid contributed by the volume of DS used was 690 μgm/ml and 2.0 ml of LP added to the cultures is equivalent to this concentration.

no cholesterol. The readdition of lipoprotein, which is rich in total cholesterol and relatively poor in free fatty acids (assuming no degradation of acyl esters), should have a pronounced inhibitory effect on the biosynthesis of sterols (assuming that HTC cells possess a sterol biosynthesis regulatory system) and a minimal effect on fatty acid biosynthesis. Our results are consistent with this.

It has been suggested for normal liver cells that cholesterol biosynthesis is regulated by a "feedback control" mechanism (28). Increased dietary levels of cholesterol will lead to a decreased conversion of glucose or acetate to this sterol but a minimal rate change in the conversion of mevalonate (27). It has been shown by numerous investigators (29-32). that the primary regulated enzyme in cholesterol biosynthesis is the NAPH dependent, 3-hydroxy, 3-methyl glutaryl coenzyme A reductase (E.C.1.1.1.34). This enzyme gives rise to mevalonic acid which is converted to cellular sterols (33).

However there are numerous reports for Morris minimal deviation hepatomas cultured in vivo which suggest that these cells have lost the capacity for "feedback" control of the cholesterol biosynthetic pathway (27,34-36). Our data would suggest that Morris minimal deviation hepatoma 7288C cultured in vitro has a sterogenic regulatory apparatus which is capable of responding to the environmental pertubations we have employed. Also many of the participants in this symposium have demonstrated with a variety of malignant cell types that some form of metabolic regulation for sterol biosynthesis is expressed by in vitro cultured cells.

The data in Table IV summarize results from four independent experiments we designed to ascertain, in a crude manner, the general location of the apparent metabolic block for isotope incorporation into the nonsaponifiable lipids.

All of the experiments were double labeled with tritiated water as the constant radioactive source. This was used to monitor the total biosynthetic activity and cellular viability. The level of incorporation obtained for cells cultured in dialyzed serum was used as our reference.

When glucose-$^{14}$C (U) acetate-$^{14}$C (U) and tritiated water were used to monitor the effect of the different serums on sterol biosynthesis, it was found that incorporation of these compounds into the nonsaponifiable fraction was stimulated 4- to 5-fold. One would expect these results if the regulated step is beyond acetyl CoA.

TABLE IV

Incorporation of $^{3}H_2O$ and Glucose-$^{14}$C (U) DL-Mevalonate-2-$^{14}$C, or Sodium Acetate-1,2-$^{14}$C Into Nonsaponifiable Fraction of Growing HTC Cells[a]

| Serum and radioactive carbon compound | Nonsaponifiable fraction, cpm/$_{10}$8 cells | |
|---|---|---|
| | $^{14}$C | $^{3}$H |
| DS | | |
| Glucose | 540 | 460 |
| Acetate | 280 | 600 |
| Mevalonate | 630 | 710 |
| LPPS | | |
| Glucose | 4460 | 3920 |
| Acetate | 2670 | 3700 |
| Mevalonate | 980 | 3080 |

[a]HTC cells were innoculated into 30 ml cultures containing $^{3}H_2O$ (8.3 x $10^3$ cpm/μmole) plus 1 mM DL-Na mevalonate, 7.5 mM Na acetate and 16.7 mM glucose, Only one of these carbon substrates was labeled with $^{14}$C in any given culture. The specific activity of each compound was normalized to 5.5 x $10^3$ cpm/μmole. All of the cultures were incubated 48 hr and duplicate aliquots were removed for analysis as described in Methods. The abbreviations used are DS, dialyzed serum; and LPPS, lipoprotein poor serum.

The addition of dl-mevalonic acid -2-$^{14}$C to the culture gave rates of total sterol biosynthesis, as shown by the tritium incorporation data which were reflective of the nutritional pertubations. However the radioactive carbon atoms derived from mevalonic acid did not indicate a strong metabolic response owing to the changes in the nutritional environment. There is only a slight enhancement of radioactivity incorporated in cells grown on LPPS, and this level of stimulation is much smaller than that seen with glucose-$^{14}$C (U) or acetate -$^{14}$C (U).

Mevalonic acid is the product of HMG CoA reductase, and the results presented in Table IV are consistant with a rate limiting step before its formation. Since we observed similar levels of stimulation for both glucose and acetate incorporation and a much smaller level with mevalonic acid, we conclude that the metabolic block is between acetyl coenzyme A and mevalonic acid. This is consistant with observations made for normal liver (28) and L-cells (18,37) in a high and low sterol environment. A more detailed resolution will have to await further in vivo and in vitro studies.

It is recognized that these data were derived from levels of radioactivity incorporation and that specific activity measurements of proper immediate precursors are necessary before one can firmly establish that a net increase in sterol synthesis had occurred. However our tritiated water data are not dependent on the pool sizes

TABLE V

Effect of Nutritional Conditions on Glycolytic and Lipogenic Enzymes in HTC Cells[a]

| Enzyme | Growth condition,[b] mμmoles/hr, mg HSS | | |
|---|---|---|---|
| | DS | LPPS | NS |
| H Kinase | 760 | 763 | 1261 |
| LDH | 184,062 | 68,262 | 49,270 |
| MDH | 126,402 | 61,368 | 51,456 |
| G6PDH | 12,145 | 9852 | 11,445 |
| 6-P, GADH | 7034 | 6885 | 6962 |
| CC Enzyme | 365 | 591 | 595 |
| Malic enzyme | 692 | 642 | 737 |
| Fatty acid synthetase | 146 | 157 | 155 |
| Acetate thiokinase | 198 | --- | --- |
| Acetyl CoA carboxylase | 25 | --- | --- |

[a]All of the enzyme assays were performed with the 105,000 x g avg. supernatant fluid from cells washed and sonicated in 0.25*M* sucrose.

[b]Cells were harvested from cultures 42-48 hr old. The abbreviations used are DS, dialyzed serum; LPPS, lipoprotein poor serum; and NS, no serum.

of metabolic intermediates, and these could be used to argue for true increased rates of sterol biosynthesis. Also Avigan et al. (38) have published similar levels of stimulation of radioactive precursors into the nonsaponifiable fraction of nongrowing HTC cells incubated in a sterol-deficient medium using radioactive acetate or tritiated $^3H_2O$, or both.

We have yet to measure any of the specific enzymes related to sterol biogenesis in HTC cells. However a variety of other lipogenic enzymes in cells grown on NS, LPPS and DS have been assayed. Our results are summarized in Table V.

The level of the lipogenic enzymes for cells grown on dialyzed serum are similar to the values published for livers of normal Buffalo rats fed a chow and high fat diet (34,39). The latter is especially true for acetyl coenzyme A carboxylase and fatty acid synthetase (40).

A comparison of individual enzyme specific activities, assayed in HTC cells grown under the various nutritional conditions, show that very few of the enzymes respond to the metabolic pertubations tested. The most striking change is in the activities of MDH and LDH. These activities are total rates, and no attempt has been made to ascertain whether specific isoenzyme losses were responsible for the observed changes.

We consider the changes in citrate cleavage enzyme activity as the only significant response of an enzyme intimately related to lipogenesis. If one assumes that the acetyl CoA for sterol biosynthesis is provided by the citrate cleavage enzyme pathway (2), then it is not surprising to see an increase in this enzyme's activity in cells grown on LPPS.

All of the enzymic changes seen in cells incubated on NS must be interpreted cautiously, the primary reason being that there is less protein per cell under these conditions and the measured specific activities are probably reflective of differences in half lives for the different enzymes assayed. This can lead to an apparent increased specific activity, i.e., hexokinase and citrate cleavage enzyme.

The studies I have presented represent initial efforts directed toward understanding the regulation of lipid metabolism in cultured cells. We are intensifying our efforts to decipher the apparent regulation of sterol biosynthesis in these minimal deviation hepatoma cells by measuring specific enzymes of cholesterol biosynthesis and the in vitro synthesis of sterols from radioactive precursors.

Also we have begun to utilize methods which fractionate the total lipoprotein fraction into its component lipoprotein classes in the hope of defining their sterol biosynthetic suppressive activity.

## ACKNOWLEDGMENTS

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# SHORT COMMUNICATION

## 24-Methylenecholesterol: Isolation and Identification as an Intermediate in the Conversion of Campesterol to Cholesterol in the Tobacco Hornworm


### ABSTRACT

24-Methylenecholesterol was positively identified as an intermediate in the dealkylation and the conversion of campesterol to cholesterol in the tobacco hornworm, *Manduca sexta* (L.). Biochemical and nutritional studies with $^{3}H$-labeled campesterol and 24-methylenecholesterol indicated that the conversion of campesterol to cholesterol in this insect includes the following metabolic sequence: campesterol, 24-methylenecholesterol, desmosterol, cholesterol.


We recently reported on the identification of fucosterol as a metabolite of β-sitosterol and a probable intermediate in the conversion of this phytosterol to cholesterol in the tobacco hornworm, *Manduca sexta* (L.) (1). If fucosterol, which has a 24-ethylidene group, is a primary intermediate in the conversion of β-sitosterol to cholesterol, then by analogy 24-methylenecholesterol could be an early intermediate in the conversion of campesterol, the 24-methyl homolog of β-sitosterol, to cholesterol in this insect (Fig. 1). We previously showed that the terminal steps in these biochemical pathways are similar in that desmosterol is a common intermediate in the conversion of β-sitosterol, campesterol and certain other phytosterols to cholesterol in the hornworm (2). The present report describes the isolation and positive identification of 24-methylenecholesterol as a constant metabolite of campesterol and presents evidence that it is an intermediate in the conversion of campesterol to cholesterol in this insect.

Hornworms were reared from hatch on an artificial agar-base diet containing highly purified 2,4-$^{3}H$-campesterol (0.026% wet weight). After the total sterols were isolated from prepupae and purified as previously described (3), the sterols were acetylated, and the acetates were chromatographed on a 20% $AgNO_3$-impregnated silicic acid column (4) (Unisil). This was typically a 6 g column (1.2 x 11 cm) eluted with 50 ml of each of the following: 10, 15, 20, 25 and 30% benzene in *n*-hexane, followed by 100% benzene. Monitoring of the column fractions by gas liquid chromatography (GLC) and thin layer chromatography (TLC) on $AgNO_3$-impregnated Silica Gel H indicated that a diunsaturated sterol acetate was present in both the 30% benzene in *n*-hexane and the benzene fractions. These two fractions were combined and rechromatographed on 3 g of 20% $AgNO_3$-impregnated Unisil and eluted with 25 ml of each of the following: 10, 15, 20, 25, 30, 35, 40 and 45% benzene in *n*-hexane and 100% benzene. The latter two fractions, which were shown by GLC and TLC analyses to contain a sterol acetate corresponding to 24-methylenecholesterol acetate, were combined. The major component (77%) of this sample contained about 68% of the radioactivity as determined by trapping from GLC effluent. The minor component was identified as desmosterol acetate and the major sterol acetate had relative retention times (RRTs) on two GLC systems (Table I) corresponding to those of authentic 24-methylenecholesterol acetate. Also, when it was chromatographed on a $AgNO_3$-impregnated Silica Gel H plate developed in benzene-hexane 125:75, the major

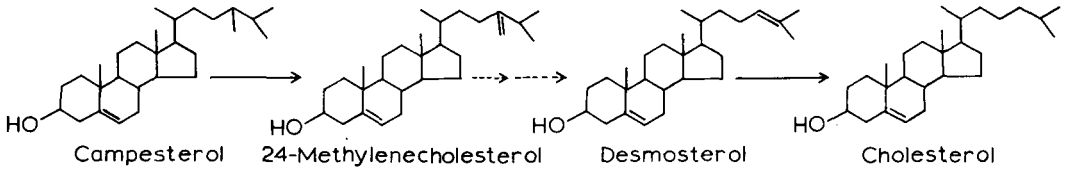


FIG. 1. Conversion of campesterol to cholesterol in the tobacco hornworm.

component had an $R_f$ value identical to that of authentic 24-methylenecholesterol acetate and produced a similar color when sprayed with 50% $H_2SO_4$ and heated to 100 C. Analysis by TLC revealed that 63% of the recovered radioactivity was present in the area corresponding to 24-methylenecholesterol acetate. The IR spectrum of the unknown showed strong bands at 1645 and 885 $cm^{-1}$ typical of a *gem*-disubstituted olefinic group and was identical with the spectrum of authentic 24-methylenecholesterol acetate. The gas chromatography-mass spectrometry (GC-MS) of this sterol acetate and 24-methylenecholesterol acetate were identical: in both spectra, the $M^+$ peak was absent and the highest mass peak appeared at m/e 380 (M-60); also the second most abundant peak in both spectra appearing in the high mass range occurred at m/e 296 (M-60-84), indicating a loss of acetic acid and the expected side chain cleavage of the 22-23 bond accompanied by a hydrogen transfer.

Saponification of these $^3H$-labeled sterol acetates yielded a mixture of sterols in which the major and minor components had RRTs identical to those of 24-methylenecholesterol (Table I) and desmosterol, respectively, on two GLC systems. About 80% of the radioactivity of this preparation was present in the peak corresponding to 24-methylenecholesterol and analysis by GC-MS substantiated that the major component of this sample was 24-methylenecholesterol. Quantitation by GLC indicated that 24-methylenecholesterol comprised approximately 1.5% of the total sterols isolated from these insects.

Tobacco hornworms were also reared on diet containing $^3H$-campesterol in combination with 20,25-diazacholesterol dihydrochloride (both at 0.026% wet weight) to determine whether a significant change in the level of 24-methylenecholesterol might result because of the presence of the sterol inhibitor in the diet, as had previously been observed with the accumulation of desmosterol in a similar test situation (2). After fractionation of the sterols as their acetates on $AgNO_3$-impregnated Unisil, 11.2 and 4.6% of the total radioactivity of the sterol acetates was present in the $^3H$-desmosterol and $^3H$-24-methylenecholesterol acetate fractions, respectively. Although the 24-methylenecholesterol acetate was only 72% pure, as indicated by GLC, analyses of the sterol and its acetate by GC-MS further confirmed that the compound isolated from the azasterol-fed insects was $^3H$-24-methylenecholesterol.

Further proof that 24-methylenecholesterol is an intermediate in the conversion of campesterol to cholesterol was obtained from tests in which 2,4-$^3H$-24-methylenecholesterol was fed in the diet alone and in combination with 20,25-diazacholesterol dihydrochloride at a concentration of 0.026% wet weight. The GLC analysis of the tritiated sterols from the insects fed 24-methylenecholesterol alone showed that 68% of the radioactivity was present in the cholesterol fraction, thus indicating an efficient conversion to cholesterol. In the sterols from insects fed both the dietary sterol and azasterol, only about 7% of the radioactivity was present as cholesterol and 49% was present as desmosterol. These results are in general agreement with those obtained in previous studies with the unlabeled sterol (2).

TABLE I

Gas Liquid Chromatography Analyses of $^3H$-24-Methylenecholesterol Isolated From the Tobacco Hornworm, Its Acetate, and Authentic Standards

| Compound | RRTs[a] 0.75% SE-30 | RRTs[a] 3.0% QF-1 |
|---|---|---|
| Insect sterol | | |
| 24-Methylenecholesterol | 2.32 | 3.52 |
| 24-Methylenecholesterol acetate | 3.21 | 5.66 |
| Authentic standards | | |
| 24-Methylenecholesterol | 2.31 | 3.50 |
| 24-Methylenecholesterol acetate | 3.24 | 5.65 |

[a]Retention time relative to cholestane. Column conditions were as previously described (4).

Dihydrobrassicasterol, the 24-S isomer of campesterol, was also fed to hornworm larvae at a dietary concentration of 0.026% wet weight. Analyses and quantitation of the sterols from these insects by GLC and TLC demonstrated that 24-methylenecholesterol constituted about 1.4% of the total sterols and that cholesterol was the major sterol present. Thus the hornworm apparently uses the same pathway for dealkylation of a 24-methyl substituent, whether a 24-R (campesterol) or 24-S (dihydrobrassicasterol) configuration is involved. Ritter and Wientjens reported the tentative identification, by GLC, of 24-methylenecholesterol as a metabolite of unlabeled dihydrobrassicasterol in the German cockroach, *Blattella germanica* (L.) (5).

We previously suggested the involvement of 24-methylenecholesterol as an intermediate in the dealkylation and conversion of campesterol to cholesterol (2,6); however the present is the first report of conclusive identification of 24-methylenecholesterol as a metabolite of campesterol and a normal intermediate in this pathway. The occurrence of $^3H$-24-methylene-

cholesterol as a constant metabolite of $^3$H-campesterol, the efficient conversion of $^3$H-24-methylenecholesterol to $^3$H-cholesterol, and the fact that desmosterol is a common intermediate in the conversion of both campesterol and 24-methylenecholesterol to cholesterol, all serve to substantiate our premise (2) that 24-methylenecholesterol is an intermediate in the conversion of campesterol to cholesterol in the tobacco hornworm. These findings also tend to support our previous suggestion that the process of C-24-dealkylation of sterols in insects may, at least in part, be the reverse of the C-24-alkylation mechanism in plants (1,2).

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S.R. Dutky of our laboratory provided GC-MS spectra, and P.D. Klein, Argonne National Laboratory, Argonne, Ill., assisted in preparation of 2,4-$^3$H-campesterol and 2,4-$^3$H-24-methylenecholesterol (7); G.D. Searle and Co. supplied a sample of 20,25-diazacholesterol dihydrochloride (SC-12937).

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## ERRATUM

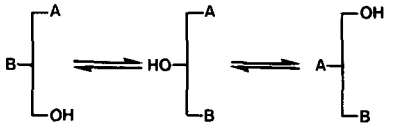


FIG. 3. Isomerization of *sn*-1,2-diacyl glycerol.

Captions under Figures 3 and 4 in "The Structural Analysis of Wheat Flour Glycerolipids" (Lipids 6:768[1971]) were inverted. Figures 3 and 4 with appropriate captions are reprinted on this page.

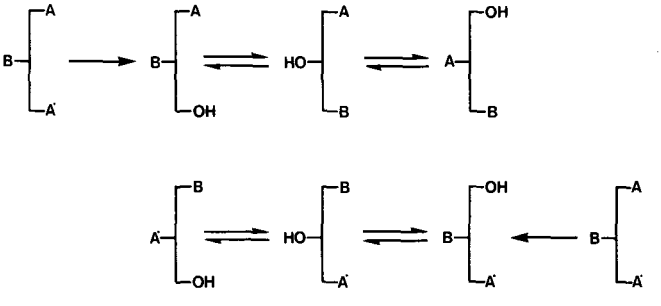


FIG. 4. Isomeric diglycerides after lipolysis of a triglyceride.

cholesterol as a constant metabolite of $^3$H-campesterol, the efficient conversion of $^3$H-24-methylenecholesterol to $^3$H-cholesterol, and the fact that desmosterol is a common intermediate in the conversion of both campesterol and 24-methylenecholesterol to cholesterol, all serve to substantiate our premise (2) that 24-methylenecholesterol is an intermediate in the conversion of campesterol to cholesterol in the tobacco hornworm. These findings also tend to support our previous suggestion that the process of C-24-dealkylation of sterols in insects may, at least in part, be the reverse of the C-24-alkylation mechanism in plants (1,2).

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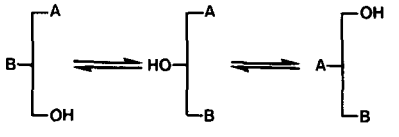


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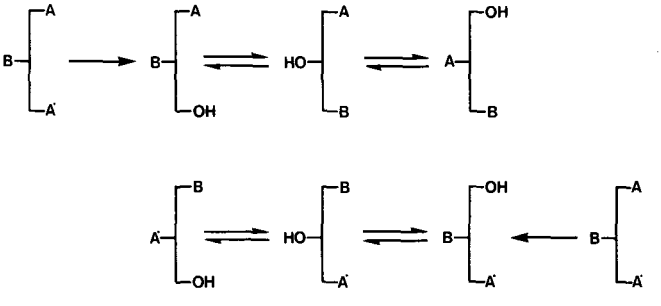


FIG. 4. Isomeric diglycerides after lipolysis of a triglyceride.

# Fat Metabolism in Higher Plants: LIII. Characterization of the Product of the Peanut α-Oxidation System

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## ABSTRACT

The α-oxidation system of germinated peanut cotyledons was purified some 60-fold. Throughout the purification procedure the two activities, namely the formation of 2-hydroxypalmitate and the formation of $CO_2$, remained with a single protein fraction. A hydrogen peroxide generating system was still required by the 60-fold purified system. Unlike the pea leaf system, molecular oxygen was ineffective. D-2-Hydroxypalmitic acid accumulates in the reaction mixture as α-oxidation proceeds while L-2-hydroxypalmitate does not accumulate. Competition studies with unlabeled L-2-hydroxypalmitate would suggest it as the intermediate substrate of further breakdown.

## INTRODUCTION

Newcomb and Stumpf (1) and Martin and Stumpf (2) observed and described the biological degradation of long chain fatty acids in peanut cotyledons via an α-oxidative process. Fatty acids were decarboxylated by a specific peroxidase to yield an aldehyde shorter by one carbon atom. The aldehyde was oxidized by a DPN-specific dehydrogenase to the corresponding acid which, in turn, could be oxidized by the peroxidase and dehydrogenase to the next fatty acid shorter by one carbon. Later, Hitchcock and James (3,4) demonstrated successive α-oxidation by pea leaf tissue with oleic acid and palmitic acid as the initial substrates. Oxygen and not hydrogen peroxide was required for this oxidative sequence, and the α-hydroxy acids were also identified and placed in the degradative scheme.

This report represents a reexamination of the α-oxidative system in peanut cotyledons. 2-Hydroxypalmitic acid accumulated during the oxidation of palmitic acid in the presence of a $H_2O_2$-generating system. Its configuration was shown to be that of the D-isomer.

## MATERIALS AND METHODS

### Materials

The $^{14}C$-labeled palmitic acid (55 mC/mmole) was purchased from Amersham-Searle. Methyl D- and L-2-hydroxypalmitate having specific rotations in chloroform of $[\alpha]_{546.1\ m\mu}^{28°} = -8.02°$ and $+7.99°$ respectively, were from Unilever Research Laboratory, Sharnbrook, Bedford, England. Glucose oxidase was purchased from Worthington Biochemical Corp. and an aqueous solution containing 1 mg/ml was prepared and stored at 4 C for not longer than 4 weeks. DEAE-cellulose (DE-52) was purchased from Whatman and Sepharose 4B and Sephadex from Pharmacia.

### Enzyme

Acetone powders of cotyledons of germinating peanuts (6 days) were prepared, extracted

TABLE I

Identification of 2-Hydroxy[1-$^{14}C$]palmitate by Gas Liquid Radiochromatography and Thin Layer Radiochromatography

| Compounds | GLC FFAP 165 C | GLC OV-210 220 C | Compounds | TLC $R_f$[a] |
|---|---|---|---|---|
| Standards | | | Standards | |
| Methyl palmitate | 3.8[b] | 1.8 | Palmitate | .75 |
| Methyl 2-hydroxypalmitate | 18.0 | 5.0 | 2-Hydroxypalmitate | .20 |
| Experimental | 18.1[c] | 5.1 | Experimental | .19 |

[a]Solvent system described in Materials and Methods.

[b]Retention times (min) of mass peaks.

[c]Retention times (min) of radioactive peaks.

TABLE II

Recrystallization of Biosynthetic 2-Hydroxyl[1-$^{14}$C]palmitate With D- and L-2-Hydroxypalmitate

| | Specific activity, mμ C/mg | |
|---|---|---|
| | D- | L- |
| Initial mixture | 4.06 | 7.60 |
| 1st mother liquor | 4.08 | 8.82 |
| Crystals | 3.85 | 2.87 |
| 2nd mother liquor | 4.18 | |
| 2nd crystals | 4.04 | |

with buffer and fractionated with ammonium sulfate to 50% saturation to obtain an "AS" enzyme fraction according to Martin and Stumpf (2). The "AS" enzyme was used in the initial experiments in which the 2-hydroxy acid was detected.

For enzyme fractionation, phosphate buffer, pH 7.5, was used routinely, all procedures were carried out at 4 C, and protein concentrations were estimated according to Lowry et al. (5). Assay procedure is described in the next section. One unit of activity is defined as that amount catalyzing the formation of 1000 cpm of $^{14}CO_2$ or 2-hydroxy[1-$^{14}$C]palmitate per 15 min. Acetone powder was prepared (2) and extracted with 0.01 M potassium phosphate buffer containing 0.1% Lubrol for 1 hr with stirring (15 ml buffer per g of acetone powder). The mixture was forced through two layers of cheesecloth before being centrifuged at 4000 x g for 5 min. The supernatant solution was treated with 0.01 volume of 1.0 M $MnCl_2$ with the pH being maintained at 7.5 by dropwise addition of 0.1 N KOH. After stirring for 15 min the precipitate was removed by centrifugation at 30,000 x g for 15 min. Solid ammonium sulfate was added to bring the supernatant solution to 20% saturation. The pH was kept at 7.5 with 0.1 N potassium hydroxide during addition of ammonium sulfate and the suspension was stirred gently for 20 min, followed by centrifugation for 15 min at 30,000 x g. The supernatant solution was brought to 40% saturation with solid ammonium sulfate with stirring and pH monitoring as above. Following centrifugation the precipitate was taken up in 0.01 M potassium phosphate buffer and dialyzed for several hours against the same buffer. Dialyzed enzyme solution was concentrated by ultrafiltration and applied to a DEAE-cellulose column (2.5 x 44 cm) previously equilibrated with 0.01 M potassium phosphate buffer, and elution was carried out with a KCl gradient between 1 liter of 0.01 M phosphate buffer containing 0.02 M KCl and 1 liter of the same buffer containing 0.2 M KCl. Fractions were assayed for $^{14}CO_2$ evolution and 2-hydroxy[1-$^{14}$C]palmitate formation. Since both activities peaked together, these fractions were pooled and brought to 60% saturation with solid ammonium sulfate as before. The sedimented fraction was resuspended in buffer, dialyzed, concentrated by ultrafiltration and added to a Sephadex G-150 column (2.5 x 40 cm) previously equilibrated with 0.01 M potassium phosphate buffer. Elution was with the same buffer and both activities peaked in the same tube. This tube and the following one were pooled, concentrated by ultrafiltration and 0.1 ml of this enzyme preparation was added to 4.6 ml of a 5-20% sucrose (w/v) gradient. Centrifugation was performed in a Spinco model L centrifuge equipped with a SW-39 swinging bucket rotor. The run was carried out at 39,000 rpm for 12 hr. The bottom of the tube was punctured and 25 fractions were collected and assayed. Activity for 2-hydroxy[1-$^{14}$C]palmitate formation peaked in fraction 16, $^{14}CO_2$ activity in fraction 17 and protein concentration in fraction 20.

Disc gel electrophoresis was carried out essentially as described by Hedrick and Smith (6). Small bore gel concentrations were 5 and 7% for each sample of protein. Analytical ultracentrifugation was accomplished at 59,780 rpm in a model E analytical ultracentrifuge in a 12 mm cell containing 5 mg protein in 0.01 M phosphate buffer, pH 7.4.

### Enzyme Assay

The $^{14}CO_2$ and 2-hydroxy[1-$^{14}$C]palmitate were recovered from the following reaction mixture: 10 μl [1-$^{14}$C]palmitate (10 nmoles, 150,000 cpm); 50 μl phosphate buffer, 0.01 M, pH 7.5; 30 μl enzyme; 5 μl glucose (0.5 μmole); 5 μl glucose oxidase solution. The assay vessel was a No. 1 hollow polyethylene stopper fitted with a rubber snap cap. A plastic center well (Kontes) was inserted into the cap so that the well was suspended above the reaction mixture. Hyamine hydroxide (0.1 ml) was injected through the cap into the well and the reaction mixture was shaken at 25 C for 15 min. The reaction was terminated by injection of 10μl of approximately 10 N $NH_4OH$ and the center well was placed into Bray's solution (7) and analyzed for $^{14}CO_2$ by counting in a Packard Tri-Carb scintillation spectrometer. A portion of the reaction mixture (30-50 μl) was streaked on a 200 x 50 mm strip of Gelman SG chromatographic medium for the separation of 2-hydroxy[1-$^{14}$C]palmitate from residual [1-$^{14}$C]palmitate substrate. Strips were devel-

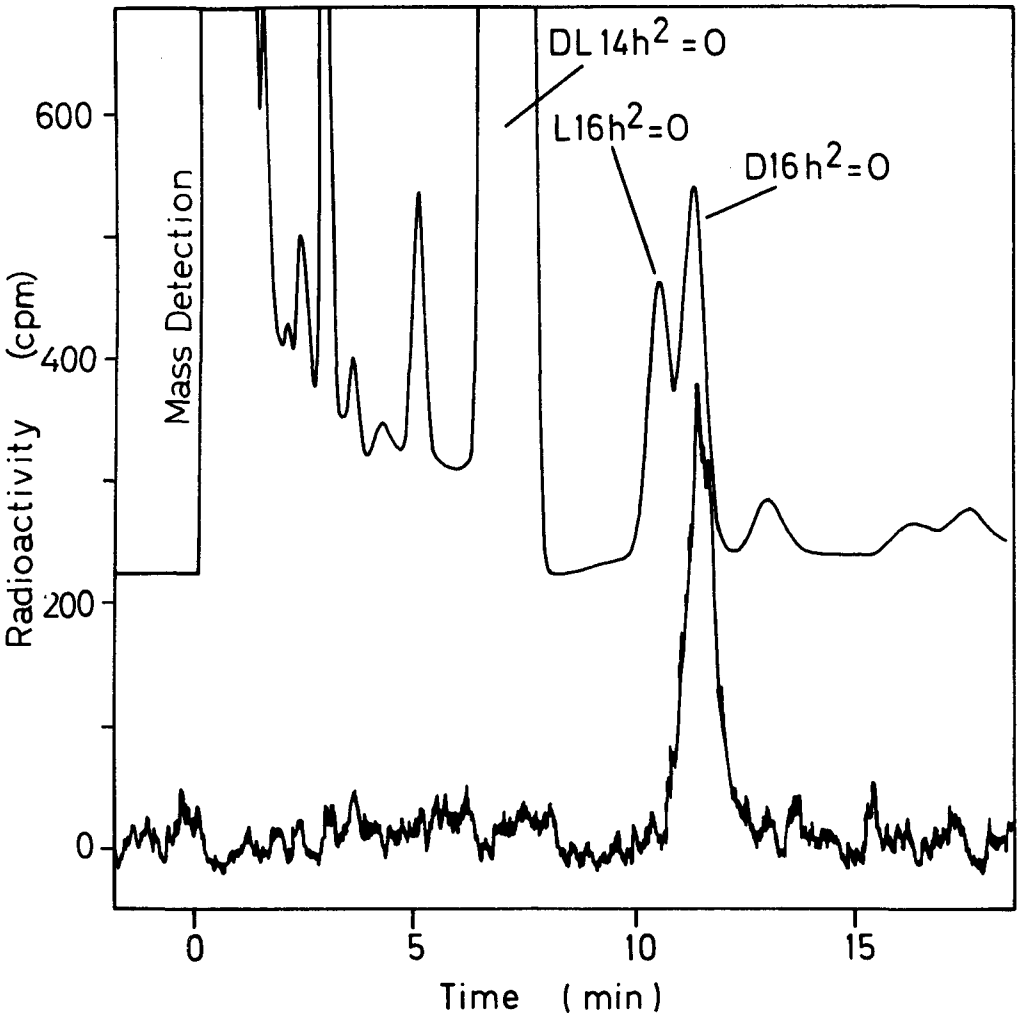


FIG. 1. Gas liquid radiochromatogram of 2-(-)menthyloxycarbonyl[1-$^{14}$C]-Palmitic acid methyl ester. The hydroxy acid was recovered from the peroxidation of [1-$^{14}$C]palmitate as described in Materials and Methods and was converted to the methyl ester and subsequently treated with (-)menthylchloroformate. Authentic (-)menthylchloroformate methyl esters of DL-2-hydroxymyristic and DL-2-hydroxypalmitic acids were added before the analysis. The stationary phase was 1.6% OV-210, the carrier gas argon and the column temperature 210 C. Upper curve: mass detection. Lower curve: radioactivity detection.

oped in hexane-acetic acid-HCl (100:1:0.5 v/v/v) and scanned with a Packard model 7201 radiochromatogram scanning system. Alternatively, the area corresponding to known 2-hydroxypalmitate was cut from the chromatogram and analyzed by scintillation spectrometry.

#### 2-Hydroxy[1-$^{14}$C]palmitate Detection

For gas liquid radiochromatographic detection of the 2-hydroxy acid, enzyme assays were performed as above except that the reaction was terminated by addition of 0.1 ml of 20% $H_2SO_4$. The mixture was extracted three times with ether. The ether extract was taken to dryness, treated with 15% boron trifluoride-methanol at 80 C for 15 min and the extracted methyl esters were analyzed on an Aerograph A-90P (Varian-Aerograph) fitted with a thermal conductivity detector coupled to a Nuclear-Chicago Biospan model 4998 radioactivity detector. Columns used were (a) 2 ft x 0.25 in. stainless steel packed with 5% FFAP on Chromosorb AW/DMCS at 165 C; (b) 6 ft x 0.125 in. stainless steel packed with 1.5% OV-210 on Gas-Chrom Q at 220 C.

TABLE III

Palmitate vs. D- and L-2-Hydroxypalmitate Competition[a]

| Additions | $^{14}CO_2$ mμ C | $^{14}CO_2$ Inhibition, % | 2-Hydroxy[1-$^{14}$C]-palmitate, mμ C |
|---|---|---|---|
| None | 4.30 | 0 | 3.86 |
| D-2-hydroxypalmitate | 3.30 | 24 | 3.36 |
| L-2-hydroxypalmitate | 0.43 | 89 | 0.82 |
| DL-2-hydroxypalmitate | 0.31 | 93 | 0.97 |
| Boiled enzyme | 0.07 | 98 | --- |

[a][1-$^{14}$C]Palmitate (10 nmoles, 18 mμ C) was incubated with a 20-40% ammonium sulfate fraction (0.4 mg protein) and $^{14}CO_2$ evolution and 2-hydroxy[1-$^{14}$C]palmitate formation were determined (see Materials and Methods) in the presence and absence of 20 nmoles of D-, L- or DL-2-hydroxypalmitate.

**Configuration of 2-hydroxy[1-$^{14}$C]palmitate**

(a) By recrystallization with D- and L-isomers–Approximately 15 mg of D- and of L-2-hydroxypalmitate were added to tubes, each containing 25-28 mμ C of the isolated radioactive 2-hydroxy acid which had been recovered from numerous reaction mixtures by preparative thin layer chromatography (TLC). Hexane (0.5 ml) was added, an aliquot was removed for counting and each mixture was recrystallized. The mother liquor was evaporated to dryness and the residue was weighed. The residue was resuspended in hexane and an aliquot was counted. Crystals were dried, weighed and resuspended in hexane and an aliquot was counted. The specific activity was calculated for each step. (b) By gas liquid radiochromatograph–Methyl esters of DL-2-hydroxy myristic, palmitic and stearic acids with C-values of 15.15, 17.15, and 19.15, respectively, from SE-30 analysis were used as reference compounds. Retention times were related to methyl esters of straight chain saturated fatty acids by making a linear plot of the logarithm of the retention times versus carbon atoms and interpolating the logarithm of the retention time of the compound in question. The values obtained were designated as C-values. (-)Menthylformate derivates of the three reference hydroxy acids were prepared with (-)menthylchloroformate (8) and purified on Silica Gel G thin layer chromatograms developed with benzene-dioxane (93:7 v/v). Each compound gave two partially separated peaks by GLC analysis on OV-210. C-values of each pair were 26.26/26.55, 28.25/28.55, and 30.25/30.55, respectively. Gas liquid and gas liquid radiochromatographic analyses were performed using a Barber Colman series 5000 gas chromatograph equipped with a hydrogen flame ionization detector. Columns were U-shaped (195 cm x 6 mm) packed with 1% SE-30 or 1.6% OV-210, both on Gas Chrom Q (100/120 mesh). Derivatives of the experimentally derived 2-hydroxypalmitic acid were prepared as follows: (a) methyl esters were prepared by reflux in methanol-concentrated HCl (75:1 v/v) for 1 hr, followed by purification by TLC (solvent system, ether-hexane 40:60 v/v); (b) (-)menthylformate derivatives were prepared and purified as described above.

## RESULTS

**Detection of 2-Hydroxypalmitate From Palmitate Peroxidation**

"AS" enzyme from acetone powders of cotyledons of germinating peanuts was incubated with [1-$^{14}$C]-palmitate plus the hydrogen peroxide-generating system. Following incubation the acidified reaction mixture was extracted with ether, the extract was methylated and analyzed by gas liquid radiochromatography. Results are shown in Table I. A radioactive peak was detected having a retention time corresponding to methyl-2-hydroxypalmitate. Thin layer radiochromatographic analysis of the ether extract before methylation revealed a radioactive component with an $R_f$ value corresponding to 2-hydroxypalmitate.

**Configuration of 2-hydroxypalmitate**

The 2-hydroxy[1-$^{14}$C]palmitate recovered from reaction mixtures was crystallized from hexane with D- and L-2-hydroxy-palmitate and the results are summarized in Table II. The specific activity of the radioactive acid-D-isomer mixture remained relatively constant throughout the recrystallization. However, an increase in specific activity in the mother liquor coupled with a low specific activity in the crystals was noted when L-hydroxypalmitate served as carrier. These results indicated that the radioactive hydroxy acid accumulating in the reaction mixture was the D-isomer.

Confirmatory evidence for D-2-hydroxy-

TABLE IV

Purification of the α-Oxidation System

| Fraction | Volume, ml | Total protein, mg | Total units | Specific activity units/mg | Recovery, % |
|---|---|---|---|---|---|
| 1. Extract | 80.0 | 2065.0 | 49,600 | 24 | 100.0 |
| 2. $MnCl_2$ supernatant | 75.0 | 1655.0 | 41.400 | 25 | 83.5 |
| 3. $(NH_4)_2SO_4$(20-40% saturation) | 19.6 | 299.0 | 12,540 | 42 | 25.2 |
| 4. DEAE-cellulose | 11.6 | 75.5 | 10,420 | 138 | 21.0 |
| 5. $(NH_4)_2SO_4$(60% saturation | 5.0 | 9.1 | 11,000 | 1200 | 22.1 |
| 6. Sephadex G-150 | 7.7 | 7.7 | 8500 | 1100 | 17.1 |
| 7. Sucrose gradient (5-20%) | 0.15 | 0.2 | 320 | 1600 | 0.7 |

palmitate was obtained by gas liquid radiochromatography. 2-Hydroxy[1-$^{14}$C]palmitate (about 20 μg, 18,000 cpm) was methylated and subjected to TLC. The main radioactive component ($R_f$0.53) comigrated with authentic methyl 2-hydroxypalmitate. Following elution from the plate (yield 9,000 cpm), a portion was diluted with unlabeled methyl DL-2-hydroxypalmitate and analyzed on an SE-30 column. The radioactivity cochromatographed with the reference compound that had been added (C-value 17.15). The remaining material from the thin layer chromatogram was converted to the (-)menthyl-formate derivative and resubjected to TLC. One radioactive component ($R_f$ 0.78) was detected which was eluted from the plate. This, together with 100 μg of the reference compound, was analyzed by gas liquid radiochromatograph on OV-210. The radioactive component had a C-value of 28.55. In another run the same amount of radioactive derivative plus approximately 1 μg of unlabeled authentic methyl DL-2-(-)menthyloxycarbonyl palmitate was similarly analyzed (Fig. 1). It was evident that the radioactive component cochromatographed with the isomer of the internal standard that had the longer retention time. The mass peak of this isomer was correspondingly increased and therefore the biosynthetic acid was concluded to be of the D-configuration (8). The rapid rise of the radioactivity curve further indicated a high degree of optical purity of this acid (> 95% D-2-hydroxy[1-$^{14}$C]palmitate).

#### Isotopic Competition

Competition experiments using synthetic unlabeled D- and L-2-hydroxypalmitate were performed in an attempt to establish the role of the D-isomer, which accumulated in this system, in the α-oxidation of palmitate. A 20-40% ammonium sulfate fraction was used for competition experiments. $^{14}CO_2$ was measured from enzyme plus [1-$^{14}$C]palmitate, and from [1-$^{14}$C]palmitate plus unlabeled D-, L- or DL-2-hydroxypalmitate. Table III lists the results obtained when unlabeled competitors were present at twice the molar concentration of [1-$^{14}$C]palmitate, i.e., 20 nmoles. Similar results were obtained at levels of 5 and 10 nmoles. Addition of the D-isomer to the reaction caused a slight decrease in radioactive $CO_2$. However, a marked decrease in $^{14}CO_2$ evolution was seen when the L-isomer was added. DL-2-hydroxypalmitate was as effective as the L-isomer in decreasing $^{14}CO_2$. Addition of the unlabeled D-isomer had little effect on the formation of radioactive D-hydroxypalmitate from labeled palmitate. In contrast, essential no radioactive D-hydroxypalmitate was formed in the presence of the L-isomer.

#### Fractionation of the α-Oxidation System

Table IV shows the results of preliminary purification. Specific activities given are for $^{14}CO_2$ evolution. Specific activities with reference to 2-hydroxy[1-$^{14}$C]palmitate formation followed the same pattern. In other words, the two activities i.e., peroxidation to form the hydroxy acid and decarboxylation yielding $CO_2$, have not been separated. Also, adsorption to calcium phosphate gel resulted in the simultaneous adsorption and elution of both activities. The final fraction from the purification scheme was checked by disc gel electrophoresis and analytical ultracentrifugation. In each case a major and a minor component (approximately 85 and 15%, respectively) were noted. Correlation of components with enzymatic activity was not done.

#### Effect of Imidazole and Metal Chelating Agents

Imidazole had been shown by Martin and Stumpf (2) to inhibit α-oxidation. Using an "AS" enzyme preparation, imidazole at a con-

centration of 1 mM inhibited $^{14}CO_2$ evolution by 96% with no appreciable 2-hydroxy[1-$^{14}$C] palmitate being detected by gas liquid radiochromatographic analysis. A fraction from pea leaves prepared in the same manner as "AS" enzyme from peanuts was inhibited by 86% with respect to $^{14}CO_2$ production with only a 57% inhibition in 2-hydroxy[1-$^{14}$C]palmitate formation.

Various chelating agents were tested for their effect on α-oxiation. EDTA, *o*-phenanthroline, 8-hydroxyquinoline and α-α'-dipyridyl at a concentration of 1 mM were preincubated for 5 min with the enzyme preparation before addition of [1-$^{14}$C]palmitate and the $H_2O_2$-generating system. EDTA inhibited $^{14}CO_2$ production by 89%, whereas no effect was observed with the other chelating agents.

## DISCUSSION

Formation of the D-isomer of 2-hydroxypalmitate from palmitate was previously reported by Hitchcock et al. (9) to occur with unfractionated acetone powders from pea leaves. Based on isotopic competition experiments, it was suggested by these authors that the D-isomer of 2-hydroxypalmitate accumulated whereas the L-isomer was decarboxylated (10). The interference by nonlabeled L-2-hydroxypalmitate in the conversion of the carbonyl carbon of palmitate to $CO_2$, coupled with the slight effect on $CO_2$ evolution by the D-isomer, was taken to mean that the unlabeled L-isomer was utilized preferentially. Morris and Hitchcock (11) demonstrated that D-2-hydroxypalmitate was formed by replacement of the D-hydrogen by a hydroxyl group with over-all retention of configuration. Further experiments by Morris and Hitchcock (12) showed that D-[2-$^3$H]palmitate was converted to pentadecanal with loss of label, while L-[2-$^3$H]palmitate yielded the aldehyde with retention of $^3$H. The precursor of the aldehyde was concluded therefore to be D-2-hydroxypalmitate and not the L-isomer. To explain these results and those previously obtained by competition experiments it was proposed that α-oxidation of palmitate occurs by two pathways, i.e., through D-2-hydroxypalmitate and pentadecanal which both accumulate, and through L-2-hydroxypalmitate which does not accumulate. The latter pathway, with the aldehyde not being an intermediate, was suggested to be the major pathway.

As indicated above in Results, isotopic competition experiments with peanut cotyledons based only on $^{14}CO_2$ measurements could be interpreted in the same way as the pea leaf system of Hitchcock et al. (10). We have confirmed the results of Hitchcock and James (4) that the pea system requires molecular oxygen rather than hydrogen peroxide as one of the reactants. Of interest, the peanut system is completely inactive under the reaction conditions of the pea system. Thus, in the initial phases of attack, the pea system employs molecular oxygen and the peanut system hydrogen peroxide. While imidazole completely blocks the formation of 2-hydroxypalmitate with the peanut system, only a partial block is observed with the pea system. The two systems accumulate large amounts of D-2-hydroxypalmitate. Competition experiments outlined in Table III would suggest utilization of the L-2-hydroxypalmitate for the formation of $CO_2$ and the $C_n$-1 fatty acid. However, direct experiments with appropriately labeled substrates and with highly purified enzymes are necessary before precise conclusions can be made.

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# The Lipid Composition of Microsomal Preparations From Lactating Bovine Mammary Tissue

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## ABSTRACT

The microsomes isolated from lactating bovine mammary tissue contained 4.3 mg lipid per milligram nitrogen. Phospholipids comprised 83% of the lipids. The neutral lipids were composed of triglycerides (20-30%), diglycerides (5-10%), free fatty acids (15-30%, cholesterol (35-40% and cholesterol esters (10-12%, respectively. Phosphatidylcholine was the predominant phospholipid component (>50%), and the remainder consisted of phosphatidylethanolamine (21-13%), phosphatidylserine (4-6%), phosphatidylinositol (8%), sphingomyelin (9%) and lysophosphatidylcholine (2%) respectively. The composition of the microsomal phospholipids was similar to that of isolated mammary cells and tissue homogenates but quite different from milk and fat globule membrane phospholipids. The triglycerides contained short chain fatty acids but their relative concentrations were lower than in milk triglycerides. The various lipid fractions had a variable proportion of saturated fatty acids, i.e., triglycerides (47.7%), diglycerides (86.7%), free fatty acids (70.6%), phosphatidylcholine (50.6%), phosphatidylethanolamine (50.8%), phosphatidylserine (35.3%), phosphatidylinositol (40.5%) and sphingomyelin (82.3%), respectively. The molecular distribution of fatty acids in the microsomal triglycerides and phosphatidylcholine was similar to that occurring in milk, i.e., the short chain and unsaturated fatty acids were concentrated in the primary positions (*sn*1 and *sn*3) of the triglycerides, and the unsaturated acids were preferentially located in position *sn*2 of the phosphatidylcholine. The compositional data indicate that mammary microsomes are not the direct source of the phospholipids of the milk fat globule.

## INTRODUCTION

Cellular microsomes are the site of glycerolipid synthesis (1,2). Stein and Stein (3) using autoradiographic techniques demonstrated that glyceride synthesis occurred in the endoplasmic reticulum of mouse mammary tissue. Microsomal preparations from lactating bovine mammary tissue actively synthesize glycerides and phospholipids from labeled precursors (4). Despite their role in lipid biosynthesis little is known of the composition of the lipids of mammary microsomes and whether or not they resemble the lipids secreted in milk. Because of this and the fact that lipids are essential for the activity of the enzyme stearyl desaturase (5) which is very active in bovine mammary microsomes (4,6), the composition of the lipids of bovine mammary microsomes has been analyzed and compared with the lipid composition of mammary tissue and milk fractions.

## METHODS

Mammary tissue obtained from lactating cows (four Holsteins, one Guernsey) was sliced, washed and minced. The minced tissue was homogenized in a Waring blender for 30 sec with three times its volume of phosphate-bicarbonate buffer (7mM $KHCO_3$, 85mM $K_2HPO_4$ and 9mM $KH_2PO_4$) pH8.0 at 4 C. The homogenate was centrifuged at 5 C for 10 min at 5000 g, and the supernatant was recentrifuged at 10,000 g for 15 min in a Sorvall RC-2B centrifuge with an SS34 rotor. The microsomes were deposited by centrifuging the latter supernatant at 105,000 g for 70 min in a Beckman model E ultracentrifuge. These microsomes were resuspended in buffer and recentrifuged as above. The pellet thus obtained was referred to as the microsomes. Mammary and liver microsomes from lactating rats were prepared in a similar manner. The microsomes were freeze dried and stored at -30 C until analyzed.

Dispersed mammary cells were prepared using collagenase (Worthington Biochemical, N.J.) as described previously (6,7). The microsomes were isolated from these cells by the procedure described above. The lipids were extracted from the microsomes, tissue, intact cells and milk according to the procedure of Folch et al. (8). Protein was determined by a micro-Kjeldahl method (9). The lipids were fractionated by thin layer chromatography (TLC) (7,10,11). Standard mixtures of phos-

TABLE I

Composition of Phospholipids Isolated From the Microsomes of Lactating Bovine Mammary Tissue

| Lipid class | Holstein | Guernsey |
|---|---|---|
| Lysophosphatidylcholine | 2.2 ± 0.3 | 2.3 ± 0.2 |
| Sphingomyelin | 9.6 ± 0.6 | 9.9 ± 1.2 |
| Phosphatidylcholine | 52.8 ± 4.2 | 48.5 ± 3.7 |
| Phosphatidylserine | 3.8 ± 1.2 | 6.0 ± 2.6 |
| Phosphatidylinositol | 7.9 ± 1.9 | 8.2 ± 1.2 |
| Phosphatidylethanolamine | 21.6 ± 3.2 | 23.6 ± 2.1 |
| Unidentified | 2.0 | 1.5 |

pholipids and neutral lipids (Applied Science, State College, Pa.) were cochromatographed for identification and quantification purposes. The approximate distribution of the neutral lipids (NL) was obtained by densitometry following charring with 50% sulfuric acid (12). Lipid phosphorus was quantified by the method of Rouser et al. (10) following calibration using a standard mixture of phospholipids. Recoveries of lipid phosphorus ranged from 87-104% and appropriate corrections were made in calculating the reported data.

The fatty acid composition of the lipid classes fractionated by TLC was determined by gas liquid chromatography (GLC) of the methyl esters. The lipid components were located with dichloroflourescein, recovered from the thin layer plates and transmethylated using a 14% boron trichloride methanol reagent. The methyl esters were separated using a 6 ft U-shaped glass column packed with 12% diethyleneglycoladipate on gas chrom P (Applied Science, State College, Pa.) programed from 120-220 C at 7.5 deg/min in a Barber-Colman model 500 gas chromatograph (Barber Colman, Rockford, Ill. Recoveries of individual fatty acids were calibrated from standard mixtures of representative fatty acids. Recoveries of short chain fatty acids C4:0-C8:0 averaged 60%. The other major fatty acids were analyzed with a relative error of 5%. Appropriate corrections were included in calculation of the data.

Three samples each of phosphatidylcholine and triglycerides from mammary microsomes were isolated by TLC eluted from the silica gel with an excess of chloroform-methanol 50:50 v/v and diethyl ether, respectively. These were carefully dried under nitrogen. The intramolecular distribution of the major fatty acids in the phosphatidylcholine (Pc) was determined using phospholipase A *(Crotalus adamateus)* (Calbiochem, Los Angeles, Calif.) according to the procedure of Wells and Hanahan (14). The triglycerides were positionally hydrolyzed with Pancreatic lipase using the method of Luddy et al. (15). After extraction the products were separated by TLC (6). The fatty acid methyl esters containing 4-18 carbons were separated by GLC using the equipment described above but programing from 100-195 C at 10 deg/min.

## RESULTS

The mean lipid content from 12 analyses of microsomes from lactating bovine mammary tissue was 4.3± 2.0 mg total lipid per milligram protein nitrogen. The phospholipids (PL) comprised 83% ± 6% of the total lipids. Corresponding values for rat mammary and liver microsomes ranged between 0.9-1.2 and 1.0-2.6 mg PL per milligram protein nitrogen, respectively. The composition of the NL fraction was variable, especially with regard to the glyceride and free fatty acid (FFA) content. The percentage composition ranged as follows: cholesterol (35-40%), cholesterol ester (10-12%), diglycerides (DG) (5-10%), triglycerides (TG) (20-30%) and FFA (15-30%), respecitvely.

Triplicate analyses of the PL from microsomes of mammary tissue of lactating Holstein and Guernsey cows revealed that Pc is the major component in both breeds (Table I). There was no significant difference between the microsomal PL from the two breeds analyzed.

The PL composition of whole tissue, mammary cells, microsomes, milk and milk fractions are tabulated (Table II) to compare similarities and reveal possible relationships between these components in relation to milk synthesis and secretion. Noteworthy is the similarity in composition of PL from mammary tissue, cells and microsomes with the sole exception of cardiolipid. This lipid was absent from the microsomes indicating the absence of mitochondrial contamination from these preparations. There are marked differences between the composition of milk PL and microsomal PL. The Pc concentration is less and the phosphatidylethanolamine (Pe) and sphingomyelin (Sph) is markedly greater in the milk than in the mammary microsomes. Because the PL of milk are located in the milk fat globule membrane

(FGM) and the milk serum in approximate ratio of 64:35, the composition of the PL from these are also included. Though these data are obtained from different analyses they do reveal disparities in the composition, i.e., the milk serum contains a greater concentration of Pc and Pe than the FGM. Furthermore the latter has a PL composition similar to the cell plasma membrane which in turn is markedly different from that of the mammary microsomes.

Short chain fatty acids C4:0-C10:0 occurred in low concentrations in the triglycerides (Table III). Palmitic, stearic and oleic acids were the major components in all classes, with the exception of sphingomyelin. Fatty acids containing more than 18 carbons were absent from the NL. Diglycerides contained the greatest amount of saturated acids. The phosphoglycerides were more unsaturated than the glycerides mainly because of their content of polyunsaturated fatty acids. Arachidonic acid occurred in greatest concentration in Pe. Sphingomyelin contained large quantities of saturated fatty acids of long chain lengths.

The positional distribution of the fatty acids on the Pc as determined by phospholipase A is shown in Table IV. These representative data show that the polyunsaturated fatty acids and the fatty acids of medium chain length C12 and C14 were concentrated on position *sn*2 of the Pc; the saturated acids C16 and C18 were concentrated on position *sn* 1, and the oleic acid was approximately equally distributed between these positions.

In the microsomal TG the reverse situation was obtained, i.e., the saturated fatty acids were located mostly in position *sn*-2 whereas the short chain and unsaturated acids were located preferentially in the primary positions (Table V).

TABLE II

Distribution of the Major Phospholipid Classes in Tissue Microsomes and Secretory Products of Lactating Bovine Mammary Tissue

| Lipid class | Percentage distribution | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Homogenized tissue | Dispersed cells | Cell microsomes | Tissue microsomes | Fresh whole milk | Milk[a] serum | Milk fat[b] globule membrane | Cell[b] plasma membrane |
| Phosphatidylcholine | 51.5 | 50.3 | 52.5 | 52.8 | 33.9 | 37.0 | 31.3 | 30.8 |
| Lysophosphatidylcholine | 0.8 | 2.4 | 2.4 | 2.2 | 1.0 | --- | --- | --- |
| Phosphatidylethanolamine | 23.3 | 22.6 | 25.5 | 23.6 | 35.1 | 32.0 | 27.2 | 21.3 |
| Phosphatidylinositol | 7.6 | 7.8 | 6.7 | 7.1 | 6.0 | 6.0 | 10.0 | 12.3 |
| Phosphatidylserine | 5.0 | 3.8 | 3.8 | 4.9 | 3.0 | 3.0 | 9.1 | 8.5 |
| Phosphatidic acid | --- | trace | 0.2 | trace | --- | --- | --- | --- |
| Cerebrosides | --- | --- | --- | --- | 2.0 | c | --- | c |
| Cardiolipid | 3.0 | 2.1 | --- | --- | t | --- | --- | --- |
| Sphingomyelin | 8.8 | 11.0 | 8.9 | 9.4 | 19.0 | 22.0 | 22.4 | 27.0 |

[a]Data of Patton and Keenan (31).
[b]Data of Keenan et al. (22).
[c]Cerebrosides account for 6, 9, and 17.6% of polar lipids of milk, fat globule membrane and milk serum, respectively (43,47,42).

## DISCUSSION

The microsomes from lactating bovine mammary tissue have a relatively high content of lipids when compared to those from rat liver. Korn (16) reported a range of 1.5-5 mg lipid per milligram microsomal protein. However Glaumann and Dallner (17) showed that the protein content of microsomes was variable depending on preparative treatments, i.e., washing of microsomes removed large amounts of protein. Rouser et al. (18) reported that smooth endoplasmic reticulum (ER) from beef liver had approximately 7 mg lipid per milligram nitrogen whereas the rough endoplasmic had approximately 5 mg. The consistently greater lipid content of smooth ER from rat liver has also been reported by others (1,7,19).

TABLE III

Average Fatty Acid Composition of Lipid Classes Isolated from Microsomes of Lactating Bovine Mammary Tissue, wt %[a]

| Fatty acid | TL | TG | DG | FFA | Pc | Pe | Ps | Pi | Sph |
|---|---|---|---|---|---|---|---|---|---|
| C4:0 | --- | 0.9 | --- | --- | --- | --- | --- | --- | --- |
| C6:0 | --- | 0.2 | --- | --- | --- | --- | --- | --- | --- |
| C8:0 | trace | 0.2 | --- | --- | --- | --- | --- | --- | --- |
| C10:0 | 0.2 | 1.4 | 1.0 | --- | --- | --- | --- | --- | --- |
| C12:0 | 0.8 | 1.3 | 3.0 | 3.0 | 0.3 | --- | --- | --- | --- |
| C14:0 | 3.9 | 7.9 | 13.2 | 10.6 | 4.1 | 2.5 | 2.7 | 1.7 | 0.9 |
| C14:1 | 0.5 | 0.2 | --- | --- | --- | --- | --- | --- | --- |
| C15:0 | 0.6 | 0.4 | --- | 0.6 | 0.5 | --- | 0.8 | --- | --- |
| C16:0 | 25.2 | 30.3 | 49.2 | 40.8 | 26.4 | 16.4 | 13.5 | 17.3 | 26.9 |
| C16:1 | 2.1 | 2.0 | 0.3 | 0.7 | 1.5 | 1.2 | 3.6 | 2.6 | --- |
| C17:0 | 0.5 | 0.3 | --- | --- | 0.7 | --- | 0.8 | --- | --- |
| C18:0 | 17.3 | 14.8 | 20.3 | 15.6 | 17.4 | 19.3 | 16.6 | 20.7 | 16.1 |
| C18:1 | 36.3 | 35.5 | 13.0 | 28.4 | 33.2 | 38.1 | 42.3 | 43.7 | 9.3 |
| C18:2 | 6.7 | 2.6 | trace | 0.3 | 10.6 | 9.2 | 13.0 | 7.6 | 1.2 |
| C18:3 | 2.0 | 2.0 | --- | --- | 2.0 | 1.3 | 2.7 | 2.3 | 1.0 |
| C20:0 | 0.2 | --- | --- | --- | 1.2 | 2.6 | 0.9 | 0.8 | 3.8 |
| C20:4 | 3.7 | --- | --- | --- | 2.1 | 9.4 | 3.1 | 3.3 | 6.2 |
| C22:0 | trace | --- | --- | --- | --- | trace | --- | --- | 14.8 |
| C23:0 | --- | --- | --- | --- | --- | --- | --- | --- | 12.7 |
| C24:0 | --- | --- | --- | --- | --- | --- | --- | --- | 7.1 |
| Saturated % | 48.7 | 57.7 | 86.7 | 70.6 | 50.6 | 40.8 | 35.3 | 40.5 | 82.3 |

[a]Legend: TL, total lipids; TG, triglycerides; DG, diglycerides; FFA, free fatty acids; Pc, Pe, Ps, Pi denote phosphatidyl choline, ethanolamine, serine and inositol respectively; Sph, sphingomyelin.

Conceivably this is attributable to the lower protein content of these membranes. The present data and those of Rouser et al. (18) may indicate that the microsomes from bovine tissues have a greater lipid content compared to rats. However variations in preparative and washing procedures could partly account for this disparity.

Bovine mammary microsomes prepared by Bailie and Morton (25) had a much greater NL content than those analyzed herein. This may be explained by a greater quantity of entrapped glycerides in their preparation which were prepared by different techniques. Microsomes isolated from milk (25) contained less lipid material and had a lipid-protein ratio within the range found in the present study. Bailie and Morton (25) presented evidence to support their suggestion that milk microsomes were derived from mammary microsomes.

The concentration of PL in mammary microsomal lipids was very similar to that reported for rat liver microsomes (20), but less than that reported by Glaumann and Dallner (17). In the review by Rouser et al. (18) the PL content of microsomes from mouse liver and pig heart ranged from 62.7-94% of the TL, respectively. This value may be greatly influenced by the source of the microsomes, i.e., microsomes obtained from tissue actively synthesizing triglycerides might be expected to have relatively lower PL concentration because of entrapped glycerides. This suggestion is supported by our compositional data which showed a relatively high concentration of glycerides and fatty acids in these mammary microsomes. Cholesterol, and perhaps cholesterol esters, are assumed to be structural components of the microsomes (17,18) and accordingly these were the least variable components of the mammary microsomes analyzed.

The relative distribution of the phospholipids is somewhat different from that reported for microsomes from rat liver by Keenan and Morre (20) and by Rouser et al. (18) for beef liver. The mammary microsomes contained more Sph and less Pc than the microsomes from rat and beef liver, respectively.

The distribution of the phospholipid classes in the mammary tissue, isolated cells and the microsomes was very similar indicating that the latter is the predominant pool of PL in the mammary cell. The PL patterns in milk and milk fractions are quite different from those of the microsomes. Thus in milk the concentration of Pc, Ps and Pi is lower, and the Pe, Sph and glycolipids are much higher. This is consistent with the knowledge that microsomes are not the direct source of milk phospholipids (22). Approximately 70% of the PL in freshly secreted milk are located in the fat globule membrane (31). The similarity in PL composition between the FGM and the plasma membrane has been adduced as evidence that the

FGM is derived from the latter, and there is much evidence in support of this (22-27).

Milk serum phospholipids are somewhat closer in composition to the microsomes and conceivably they are composed of a mixture of microsomal and fat globule membrane PL. Several authors have provided biochemical, centrifugal and microscopic evidence for the presence of microsomal particles in milk (25,27-30). Recently Patton and Keenan (31) interpreted their data to support the secretion of discrete lipoproteins into milk. The quantitative and physiological importanct of this source of PL to milk synthesis and secretion has still to be determined.

The puzzling feature of these and other data (32) concerns the source of the relative increase in concentration of Pe in the milk PL. Its intracellular concentration is less than half that of Pc (21,32). It is not accounted for by its concentration in the plasma membrane which evidently is the origin of the FGM and most of the milk PL (22,23,26). Conceivably Pe is preferentially secreted from the plasma membrane or from a specific intracellular pool. However biochemical data from in vivo and in vitro studies have consistently shown that the rate of Pe synthesis is less than Pc synthesis (7,31,33,35,37). The involvement of an ion-catalyzed ethanolamine exchange reaction with diacylglycerolipids may afford one explanation (34). The increased Pe/Pc ration in milk PL compared to the microsomes might also be attributable to the continual conversion of some of the intracellular Pc to diglycerides or other glycerolipids during secretion, as suggested by Patton et al. (13,35).

The presence of short chain fatty acids in the triglycerides indicated the presence of some "milk" glycerides in the microsomal preparations. However their relative concentration was quite low compared to milk triglycerides indicating that there was little contamination of the microsomes with milk fat globules. The absence of short chain fatty acids from the diglycerides is consonant with the knowledge that in their conversion to triglycerides these are predominantly acylated by short chain acids or oleic acid (6,7,13,36,37), or both. The data from the pancreatic hydrolysis of the microsomal triglycerides support this by showing the concentration of short chain and unsaturated acids in the primary position of the triglycerides. This distribution is similar to that of secreted milk triglycerides (38-40).

The fatty acid composition of the Pc from mammary microsomes was quite similar to Pc isolated from isolated mammary cells (7), fat globule membrane (28,41), milk serum (42) and milk (43). The microsomal Pc was quite different from the microsomal DG in fatty acid composition, containing more unsaturated acids. This would tend to preclude the proposition that the DG are the direct precursors of the Pc. However the presence of Pc acyl transferase could conceivably alter the original Pc composition by replacing a portion of the saturated acids with unsaturated acids at position *sn*-2 of the molecule.

TABLE IV
Positional Distribution of Fatty Acids in the Phosphatidylcholine Isolated From Bovine Mammary Microsomes, wt %

| Fatty acid | Total Pc[a] | Position *sn*2 | Position *sn*1 |
|---|---|---|---|
| C12:0 | 0.5 | 2.0 | --- |
| C14:0 | 4.7 | 6.8 | trace |
| C16:0 | 37.5 | 33.4 | 43.8 |
| C16:1 | 2.2 | 2.3 | trace |
| C18:0 | 12.7 | 5.2 | 24.2 |
| C18:1 | 33.0 | 32.4 | 30.4 |
| C18:2 | 7.7 | 14.5 | 1.6 |
| C18:3 | 1.3 | 2.4 | trace |
| Saturated % | 55.8 | 48.4 | 68.0 |

[a]Pc, phosphatidylcholine.

Comparison of the distribution of the fatty acids in the microsomal Pc using phospholipase A showed that the unsaturated acids tended to be concentrated in position *sn*-2. This is similar to milk fat, i.e., myristic acid and the unsaturated acids, particularly linoleic and linolenic acid, are preferentially esterified in the secondary position of Pc (42,43).

The Pe from lactating bovine mammary microsomes had a fatty acid composition quite similar to the Pe of mammary cell plasma membrane, milk fat globule membrane and milk (22,41,43-45), except that the concentration of oleic acid was lower in the microsomal Pe. The microsomal phosphoglycerides, especially Pe, Ps and Pi, had very similar fatty acid patterns and contained more unsaturated fatty acids than the other lipids. The Pe had the highest concentration of arachidonic acid. The fatty acids of microsomal Ps were very similar to those of milk Ps as reported by Morrison et al. (43), but quite different from that reported by others (44,46). The microsomal Ps contained less arachidonic acid than found in the mammary cell plasma membrane (22).

The Sph contained a preponderance of saturated long chain fatty acids which occurred in different proportions than in milk and plasma membrane Sph (43,22). The Sph from the microsomes had a higher content of stearic, oleic and arachidonic acid than that isolated from milk (43).

These data reveal that the distribution of

TABLE V

Positional Distribution of Fatty Acids in Triglycerides Isolated From Bovine Mammary Microsomes, wt %

| Fatty acids | Position TG[a] | Position *sn*2 | Position *sn*1 + *sn*3 |
|---|---|---|---|
| C4:0 | 0.8 | --- | 0.8 |
| C6:0 | 0.2 | --- | 0.2 |
| C8:0 | 0.4 | --- | 0.2 |
| C10:0 | 2.3 | --- | 2.5 |
| C12:0 | 3.4 | 1.8 | 3.0 |
| C14:0 | 12.1 | 18.3 | 8.4 |
| C16:0 | 30.3 | 57.8 | 29.6 |
| C16:1 | 1.7 | 0.8 | 1.5 |
| C18:0 | 16.3 | 15.6 | 20.1 |
| C18:1 | 30.8 | 5.7 | 32.0 |
| C18:2 | 1.7 | trace | 1.7 |
| Saturated % | 65.0 | 78.7 | 64.0 |

[a]TG, triglycerides.

phospholipids in mammary microsomes are quite different from that of the plasma membrane and milk and are consistent with the contention that most of the PL of milk are derived from the mammary cell plasma membrane (22).

The Sph contained a preponderance of saturated long chain fatty acids which occurred in different proportions than in milk and plasma membrane Sph (22,43). The Sph from the microsomes had a higher content of stearic, oleic and arachidonic acid than that isolated from milk (43).

These data reveal that the distribution of phospholipids in mammary microsomes are quite different from that of the plasma membrane and milk and are consistent with the contention that most of the PL of milk are derived from the mammary cell plasma membrane (22,23,26). Confirmation of the suggestion that the microsomes of milk are derived from mammary cell microsomes (25,31) awaits further comparison of the detailed composition of thes two components.

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# Occurrence of Glyceryl Tridocosahexaenoate in the Eye of the Sand Trout *Cynoscion arenarius*

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## ABSTRACT

The pigment epithelium of the eye of the sand trout *(Cynoscion arenarius)* contains a reflecting layer or tapetum lucidum, consisting of lipid spherules approximately 400 nm in diameter. The lipids consist amost exclusively of triglycerides and the fatty acids contain up to 95% docosahexaenoic acid. Thus the lipid of this reflecting layer appears to be nearly pure glyceryl tridocosahexaenoate. The adjacent tissues contain much less docosahexaenoic acid (retina 65%; choroid 9%) and little, if any, tridocosahexaenoin. The possible importance of this nearly pure, highly unsaturated, mono acid triglyceride is briefly discussed.

## INTRODUCTION

Reflecting layers or tapeta lucida are found in the eyes of many fishes and the reflecting material has generally been regarded as guanine (1). A recent survey has shown, however, that many teleosts have a lipid tapetum. This structure is localized in the pigment epithelium of the eye, the cells of which contain a great number of lipid spheres (2). Preliminary studies making use of thin layer chromatography (TLC) have indicated that the tapetal lipids in seatrouts (Sciaenidae) and carp are triglycerides, and analysis by gas liquid chromatography (GLC) has revealed a high proportion of long chain fatty acids (3,4). A functional appraisal of this system presents many interesting problems, not least of which is a determination of its composition. The present paper attempts to achieve this task by an analysis of the tapetum lucidum of the sand trout *(Cynoscion arenarius)*. An account of its histology and ultrastructure is being presented elsewhere (4).

## MATERIALS AND METHODS

### Biological Material

The eyes of sand trouts, like those of other neritic teleosts, execute retinomotor movements and the retina can be separated from the pigment epithelium only in dark-adapted eyes. Previous studies of isolated pigment epithelium and of tapetal reflecting material by TLC have established that the major lipid component behaves as a triglyceride, and that there is very little triglyceride in the retina (4). Therefore to secure substantial amounts of tapetal lipid for analysis, eyes from light-adapted fish were used.

### Material for Identification of Docosahexaenoic Acid in Tapetal Lipids

The retina plus adherent pigment epithelium was removed from the eyes of 50 light-adapted fish and the tissues were rinsed in several changes of teleost Ringer and saline. Tissue and fluid amounting to about 30 cc were collected on ice; the tissue was homogenized in a glass tissue grinder, and was extracted in chloroform-methanol by the method of Folch et al. (5). The chloroform-methanol extract was placed in a separatory funnel, water added and the chloroform layer was withdrawn. The chloroform was removed under vacuum in a rotating evaporator below 50 C, and the lipids were dissolved in hexane.

The extract, in hexane, was chromatographed on a column of silicic acid monitored by TLC on Silica Gel G, and the fraction containing the triglycerides was purified further by preparative TLC. The material was streaked on a plate of Silica Gel F, 2 mm thick, and developed in hexane-ether-acetic acid 90:10:1, v/v. The triglyceride band was visualized by UV, scraped off and eluted with chloroform-ether.

### Material for Identification of Tapetal Lipid as a Triglyceride

Lipids were obtained as already described, from the retina plus pigment epithelium of 22 light-adapted fish. The triglyceride fractions was isolated from the total lipids by preparative TLC, on a 1 mm layer of Silica Gel H. Approximately 250 mg of lipid in hexane was streaked on a 20 x 20 cm plate and developed with 15% ether in Skellysolve B. The lipid zones were located under UV light after spraying with 0.001% aqueous rhodamine 6G. The

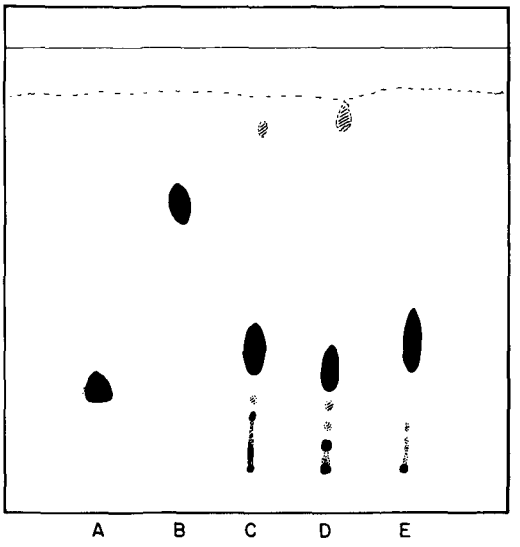


FIG. 1. Thin layer chromatogram of total lipids from the eye of the sand trout. Silica Gel H developed with Skellysolve B-ether-acetic acid 90:10:1; sprayed with $H_2SO_4/K_2CR_2O_7$ and charred. A, free fatty acids; B, methyl stearate; C, corn oil; D, lipids of tapetum lucidum; E, lipids of choroid.

triglyceride band was quickly transferred into a sintered glass filter funnel (JM 5355, Scientific Glass Apparatus Co., Bloomfield, N.J.) containing 5 ml of methanol saturated with nitrogen. The sides of the tube were rinsed with an additional 15 ml of methanol and the silica gel particles stirred with a stream of nitrogen, allowed to settle and filtered under slight vacuum. The adsorbent was further extracted five times with 5 ml portions of chloroform. The combined methanol-chloroform solution was mixed with 75 ml water in a separatory funnel and the chloroform layer was removed. The aqueous fraction was washed once with 5 ml chloroform and the chloroform layers were evaporated under vacuum on a rotary evaporator at 0-4 C. The residue was dissolved in 75 ml freshly distilled ethyl ether, washed twice with 25 ml portions of 5% sodium bicarbonate, dried over anhydrous sodium sulfate, freed of solvent on a rotating evaporator and taken up in a small amount of Skellysolve B. GLC analysis of methyl esters from a small portion of this material showed 94.6% docosahexaenoate.

#### Retinal Lipids

Retinae were removed from several dark-adapted eyes, rinsed in Ringer and homogenized. Lipids were extracted in chloroform-methanol and transferred to hexane as described for tapetal lipids. The triglyceride and phospholipid fractions were isolated by TLC. Frequently a small amount of tapetal tissue adheres to the outer surface of the retina when the two tissues are pulled apart, and retina lipids may be slightly contaminated with tapetal glycerides. Conversely a few outer segments of the visual cells often come away with the pigment epithelium.

#### Choroid Lipids

The outer region of the choroid contains deposits of large fat cells adjacent to the retia mirabilia. The cells give the usual histological tests for fats (staining with sudan dyes and osmic acid). The rete is a vascular system intimately concerned with supplying the retina, and the interesting possibility presented itself that the choroidal fat depots might be the fatty acid pool for the tapetum. Therefore fat deposits of the choroid were collected, extracted and fractionated by TLC as in the case of the tapetal lipids.

#### Analytical Procedures

The methyl esters used to identify docosahexaenoic acid in tapetal lipids were prepared by saponifying the glyceride fraction with 3% methanolic potassium hydroxide under reflux for 1 hr. The fatty acids were isolated, transferred to benzene, and esterified by refluxing with 10% w/w boron trifluoride in methanol for 15 min. The methyl esters were extracted with hexane.

All other methyl esters were obtained by direct transesterification of the lipid with 6% sulfuric acid in methanol at 90 C for 1 hr, followed by extraction with hexane.

Fatty acid composition was determined by GLC of the methyl esters on a 6 ft x 1/8 in. column of 10% EGSS-X on 100-120 mesh Gas Chrom P at 180 C, using an F & M 810 gas chromatograph with a flame ionization detector. The methyl esters were analyzed directly and also after hydrogenation in methanol, with a platinum catalyst at room temperature under 40 psi hydrogen. Identification was by direct comparison of retention values with standards whenever possible, or by comparison of equivalent chain lengths. Peak areas were obtained by triangulation. Composition is expressed as area per cent.

Double bonds were located by ozonolysis in pentane at -50 C and subsequent reduction using the apparatus and method of Beroza and Bierl (6,7). The aldesters were identified by GLC on a 10% EGSS-X column at 80 C and the short chain aldehydes on a Porapak Q column at 200 C.

Ultraviolet spectral absorption was measured

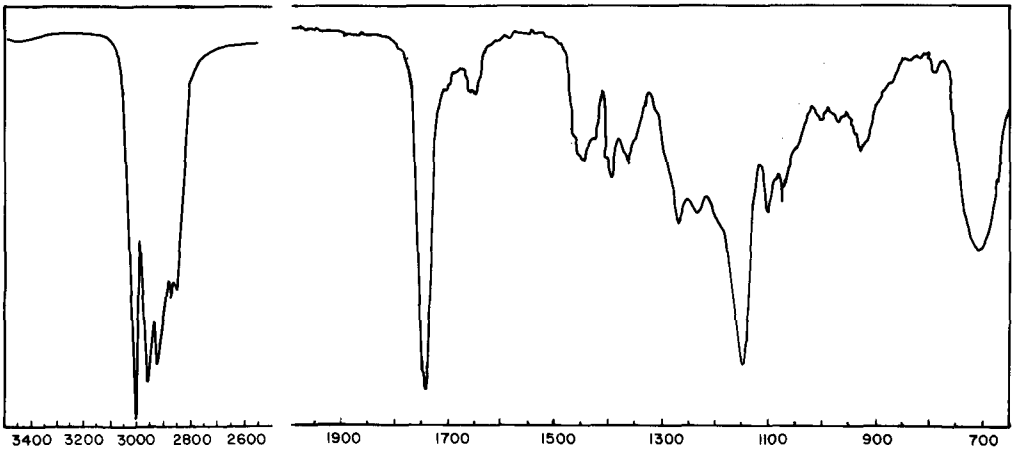


FIG. 2. Infrared spectrum of glyceride fraction of tapetum lucidum of sand trout, 3400-2500 $cm^{-1}$, $CCl_4$ solution with grating instrument. 2000-650 $cm^{-1}$, NaCl prism instrument, $CS_2$ solution except 1650-1400 $cm^{-1}$ $C_2Cl_4$ solution.

with a Beckman DU spectrophotometer and IR spectra were obtained with a Perkin Elmer Model 21 instrument equipped with sodium chloride prism and with a Perkin Elmer Grating IR spectrophotometer model no. 237B.

Mass spectra were obtained with a Hitachi RMU6D mass spectrometer operated at an ionization potential of 70 eV.

The alcohol moiety of the lipid was identified as follows: 1 mg lipid was transesterified with 2 ml of 0.1 N sodium methoxide in methanol for 1 hr at room temperature. The mixture was neutralized with 0.1 N aqueous hydrochloric acid and extracted six times with 3 ml portions of Skellysolve B to remove the methyl esters. The squeous layer was evaporated to dryness and the dry residue was extracted three times with 0.25 ml portions of methanol. Each of the three methanol extracts was evaporated in a stream of nitrogen in the same reaction vial ("Reacti-Vial" 0.3 ml, Pierce Chemical Co., Rockford, Ill.) and the open vial was placed over potassium hydroxide in a vacuum dessicator overnight.

The sample vial and identical vials containing 75 $\mu$g of glycerol, ethylene glycol, 1,4-butanediol or 1,6-hexanediol as reference compounds were capped with teflon-lined screw cap septums. To each vial the following compounds were added through the septum: 50 $\mu$l pyridine, 20 $\mu$l hexamethyldisilazane and 10 $\mu$l trimethylchlorosilane. The samples were mixed after addition of each reagent and the reaction was allowed to proceed at room temperature for 40 min. The reaction products were analyzed directly by GLC at 100 C on a 4 ft x 1/8 in. column of 10% SE-30 on 80-100 mesh Gas Chrom Q.

## RESULTS

Figure 1 shows a typical thin layer chromatogram of the lipids extracted from the tapetum lucidum of the eyes of sand trouts. The lipids consisted largely of a fraction with a retention value similar to that of triglycerides. The major lipid spot, however, was somewhat more polar than the corn oil triglycerides used as a reference material on this plate, suggesting high unsaturation. On another chromatogram the Rf value of the material was found to be virtually identical with that of an authentic sample of glyceryl tridocosahexaenoate.

Ultraviolet spectroscopy showed that the material contained no conjugated unsaturation. The IR spectrum (Fig. 2) is consistent with a highly unsaturated triglyceride. In the C-H stretch region the strongest peak at 3010 $cm^{-1}$ is owing to *cis* hydrogen attached to double bonded carbon atoms. The peaks owing to $CH_2$ (2850 and 2925 $cm^{-1}$) and $CH_3$ (2875 and 2960 $cm^{-1}$), groups, which are the strongest bands exhibited by common fatty acids with up to four double bonds, are correspondingly smaller. The ester carbonyl band appears at its normal position at 1750 $cm^{-1}$, indicating the absence of ethylenic conjugation with the carbonyl, confirmed by appearance of the C=C stretching vibration at the expected 1660 $cm^{-1}$. The absence of significant bands at 1100, 965 and 900 $cm^{-1}$ characteristic of ethers, *trans* unsaturation and terminal double bonds, respectively, indicates that these groupings do not occur in the molecule.

Gas chromatography of the trimethylsilyl ether of the alcohol moiety of the lipid gave only one peak with a retention time identical to

TABLE I

Fatty Acid Composition[a] of Lipids From the Eye of the Sand Trout

| Fatty acid[b] | Tapetum lucidum triglycerides | | Retina | | |
|---|---|---|---|---|---|
| | Original | Hydrogenated | Triglycerides | Triglycerides | Phospholipids |
| 14:0 | --- | --- | 2.85 | 2.04 | 2.85 |
| 14:1 | --- | --- | --- | 1.02 | 1.19 |
| 15:0 | --- | --- | 0.45 | 1.18 | 1.78 |
| 15:1 | --- | --- | --- | 0.58 | 1.13 |
| 16:0 | 1.50 | 1.87 | 30.85 | 10.92 | 20.48 |
| 16:1 | 0.47 | --- | 16.91 | 6.12 | 10.24 |
| 17:0 | --- | --- | 2.05 | 1.21 | 1.31 |
| 17:1 | --- | --- | 1.14 | 0.45 | 0.77 |
| 18:0 | 0.41 | 1.49 | 4.91 | 3.56 | 10.00 |
| 18:1 | 0.88 | --- | 22.28 | 5.80 | 12.50 |
| 18:2 | --- | --- | 1.25 | 0.71 | --- |
| 20:0 | --- | 2.62 | --- | 1.08 | --- |
| 20:1 | --- | --- | 1.48 | --- | --- |
| 20:4 | 0.75 | --- | 2.17 | --- | --- |
| 20:5 | 1.57 | --- | 4.57 | --- | --- |
| 22:0 | --- | 94.00 | --- | --- | --- |
| 22:5 | 1.63 | --- | --- | --- | --- |
| 22:6 | 92.74 | --- | 9.02 | 65.26 | 37.70 |

[a]Expressed as peak area per cent of gas liquid chromatography recording.

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

that of the glycerol derivative, and quite different from the diol ethers examined.

The mass spectra of the normal and hydrogenated lipids were also consistent with the triglyceride structure. The mass spectra of triglycerides are characterized by several peaks at m/e $[M-RCO_2]^+$, $[RCO+74]^+$, $[RCO+115]^+$ and $[RCO+128]^+$ all owing to fragments containing the glyceryl moiety (8,9). For glyceryl tridocosahexaenoate (M = 1022; RCO = 311) peaks at m/e 695, 385, 426 and 439 are expected, and for glyceryl tridocosanoate (M=1058; RCO = 323) the corresponding fragments have m/e of 719, 397, 438 and 451. These groups of peaks were present in the mass spectra of the isolated lipid and of the hydrogenated product, respectively. In all cases the expected peak was more prominent than neighboring peaks in each region.

GLC of the methyl esters from several glyceride fractions, obtained by preparative TLC without further purification, showed that they contained between 90 and 95% docosahexaenoate. Typical results are given in Table I. The hydrogenated material contained only four compounds corresponding to methyl palmitate, stearate, arachidate and behenate. Thus fatty acids with branched chains or with other functional groups that would modify their retention times are absent. The major peak in the original material had the same retention time as that of an authentic sample of methyl docosahexaenoate (Hormel Institute Lipids Preparation Laboratory). The minor polyunsaturated components were identified by comparing their equivalent chain lengths with published values. Excellent agreement existed between the chain length distribution calculated for the original material and that found in the hydrogenated sample.

The absence of ethylenic and carbonyl conjugation and of terminal unsaturation shown by UV and IR spectroscopy data left three possible structures for the docosahexaenoic acid of this lipid. The double bonds could be located at positions 3,6,9,12,15,18-, 4,7,10,13,16,19- or 5,8,11,14,17,20. GLC of the ozonolysis products of the methyl esters showed that succinic aldester and propionaldehyde were the major compounds formed. Acetaldehyde was present also in the ozonolysis products of both the fish eye methyl esters and of several pure polyunsaturated standards. The formation of acetaldehyde in varying amounts during ozonolysis of fatty acids known to contain no double bonds in the *n*-2 position has been reported previously by Berosa and Bierl (7). Therefore this compound is not considered to be indicative of double bond location and its presence has been disregarded. Thus the major fatty acid of this lipid is 4,7,10,13,16,19-docosahexaenoic.

The results show that this particular lipid sample is a triglyceride of fatty acids containing about 93% docosahexaenoic acid. If we assume that the other fatty acids present occur exclusively as the third fatty acid moiety esterified to glyceryl didocosahexaenoate, it can be calculated that the lipid contains at least 76%

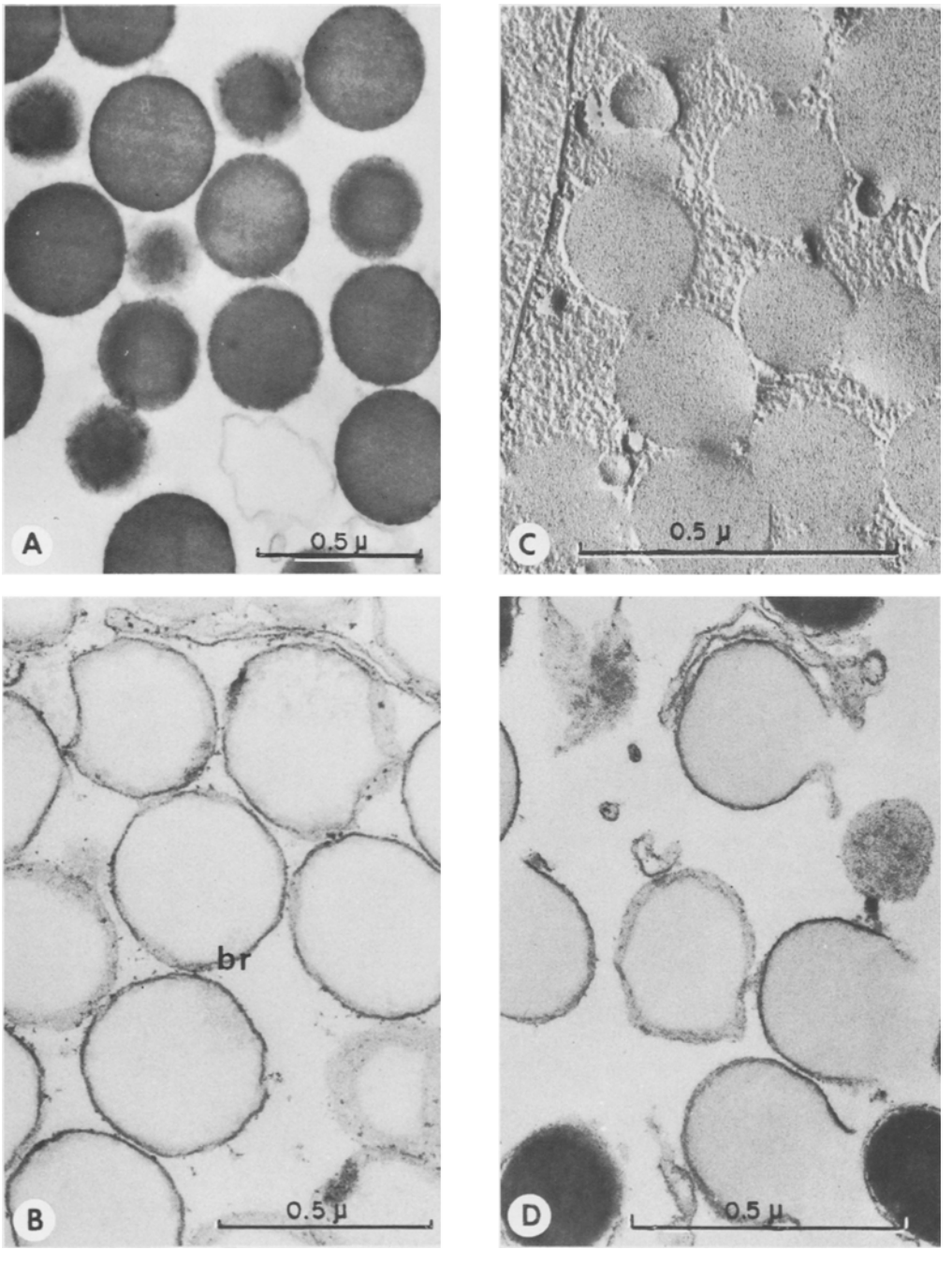


FIG. 3. Electron photomicrographs of tapetal spheres in the pigment epithelium of the sand trout under different conditions of fixation. A, B and D, epoxy sections; C, freeze-etch. Tissues were fixed in glutaraldehyde-acrolein followed by oxmium tetroxide (A,B,D) and glutaraldehyde followed by glycerol (C). br, Boundary membrane.

tridocosahexaenoin. These results, however, were obtained on a triglyceride fraction which was not highly purified. Analytical silver nitrate TLC of this fraction gave a single elongated spot with a leading edge less intense than the rest. When the lower two-thirds of a similar spot obtained from a preparative silver nitrate TLC phase was analyzed, it was found to have

been enriched in docosahexaenoic acid. Thus partial purification of the triglyceride is possible. The lipids of the choroid and of the retina contain a much lower concentration of polyunsaturated fatty acids (Table I) and silver nitrate TLC of these materials showed the presence of large amounts of more saturated triglycerides. In collecting the reflecting layer of the eye, contamination with the adjacent retina is difficult to avoid, and this would decrease the degree of unsaturation of the glyceride fraction. Therefore it is possible that the triglyceride of tapetum lucidum may be nearly pure tridocosahexaenoin.

## DISCUSSION

The pigment epithelium of the sand trout contains an unusually large amount of lipid, much of which is triglyceride. The latter, constituting the reflecting material, is localized in small spheres (Fig. 3) which pack the cytoplasm of the pigment epithelium cells (4). In contrast the triglycerides of a nontapetal pigment epithelium (human) are a minor fraction, amounting to only 3% of total lipids (10).

Marine fish oils contain generally around 10-20% docosahexaenoic acid (11), and in triglycerides it is located preferentially in the β-position (12). Recently docosahexaenoic acid has been reported to account for 22%-37% of the fatty acids in the total lipids of bovine whole retina (13) outer rods and cones (14-16). Most of the lipid was phospholipids, and docosahexaenoic acid was most abundant (29%) in phosphatidyl ethanolamine (13,15). These values and those found in the retina and choroid lipids of the sand trout (Table I) do not suggest the presence of any large amounts of tridocosahexaenoin in these tissues.

Docosahexaenoic acid and its derivatives should be very sensitive to autoxidation. It is not likely that autoxidation is an integral part of the function and metabolism of this lipid in the eye, and therefore mechanisms to prevent autoxidative deterioration in situ must be present. When these mechanisms fail, however, autoxidation may be expected to proceed and result in visual defects.

The lipid of the reflecting layer is localized in small spheres, illustrated in Figure 3. Each sphere is invested by a boundary membrane (br of Fig. 3B), which isolates the lipids of the tapetal sphere from the cytoplasm and other organelles of the pigment epithelium cell. Exchange of vitamin A is known to take place between the pigment epithelium and the retinal rods (17). Carotenoids are destroyed rapidly by cooxidation in presence of autoxidizing polyunsaturated lipids. If autoxidation of the lipids of the pigment epithelium takes place, vitamin A and retinene, the essential visual pigments, might also be destroyed.

The existence of a simple triglyceride, tridocosahexaenoin, in the tapetum suggests some interesting questions regarding its purpose, origin, biosynthesis and metabolism. The refractive index of this lipid, measured in situ, was $n^{23}=1.500$, very close to the value $n_D^{20}=1.497$ reported for methyl docosahexaenoate (18). Electron microscope studies show that the lipid is enclosed in spherules, about 400 nm in diameter (4).

Lipid tapeta lucida have been found, *inter alia*, in drums or sciaenids and grunts or haemulids, which are mostly marine, and in carps, which are fresh water fishes (1). The former inhabit turbid coastal waters, the latter ponds and slow moving streams. In these environments light penetration may be much reduced. When the level of illumination is low, a tapetum lucidum can enhance the amount of light absorbed by the retina. The dark-adapted tapetum forms a good diffusing surface and its ability to backscatter light depends upon a complex of factors, among which the high refractive index of the lipid material is an important property. Photometric studies of the tapetum are now in progress.

## ACKNOWLEDGMENTS

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# Metabolism of 12-Ketooctadecanoic Acid by *Escherichia coli K-12* and *Candida tropicalis*

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**ABSTRACT**

The mode of degradation of long chain keto acids by two microorganisms was investigated. *Escherichia coli K-12* converted 12-ketooctadecanoic acid to 4-ketodecanoic acid, accumulating some amounts of intermediates, 10-ketohexadecanoic, 8-ketotetradecanoic and 6-ketododecanoic acids. In contrast, *Candida tropicalis* completely metabolized the keto acid with transient accumulation of the metabolites mentioned above. The difference between the metabolism by *E. coli* of hydroxy acid and keto acid is that 12-hydroxyoctadecanoic acid is degraded as far as 6-hydroxydodecanoic acid, while 12-ketooctadecanoic acid can be degraded as far as 4-ketodecanoic acid.

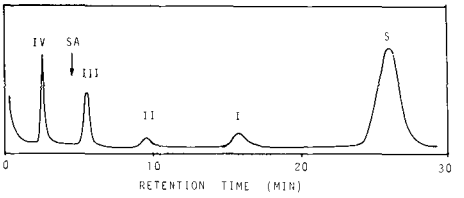


FIG. 1. Gas chromatogram of the metabolites of 12-ketooctadecanoic acid produced by *E. Coli K-12.* Peaks are methyl esters of 12-ketooctadecanoic (S), 10-ketohexadecanoic (I), 8-ketotetradecanoic (II), 6-ketododecanoic (III), stearic (SA) and 4-ketodecanoic (IV) acids.

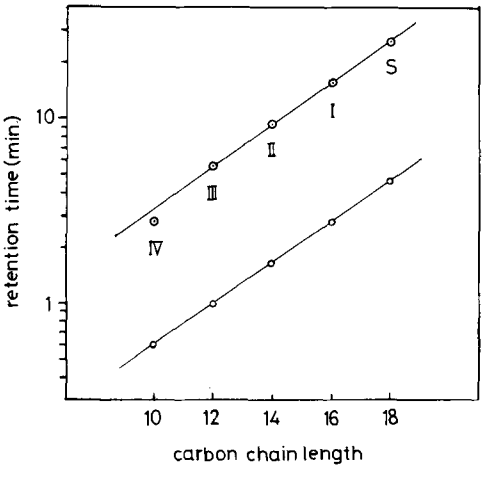


FIG. 2. Relation between carbon chain length and retention times of the metabolites of 12-ketooctadecanoic acid produced by *E. coli K-12.* The abbreviations are defined in Figure 1. —●— = Methyl esters of the metabolites. —○— = Methyl esters of normal saturated fatty acids.

## INTRODUCTION

It is known that carbonyl-containing fatty acid molecules are found in denatured lipids (1,2). Their toxic effect has been reported by investigators in the field of food science (3,4). We have previously investigated the metabolic fate of long chain hydroxy acids by microorganisms in the intestine; it was ascertained that 12-hydroxyoctadecanoic acid was degraded by *Escherichia coli* as far as 6-hydroxydodecanoic acid, accumulating 10-hydroxyhexadecanoic and 8-hydroxytetradecanoic acids (5,6), while the degradation of the hydroxy acid by *Candida* was complete as shown by the presence of intermediates, 4-hydroxydecanoic and 2-hydroxyoctanoic acids (7,8). The administration of long chain hydroxy acid to animals caused the accumulation of several kinds of hydroxy acids in adipose tissue (9,10). Both medium and short chain keto acids will be formed and accumulated in animals when long chain keto acids undergo the same metabolic degradation as that of hydroxy acids. This investigation was performed using two different

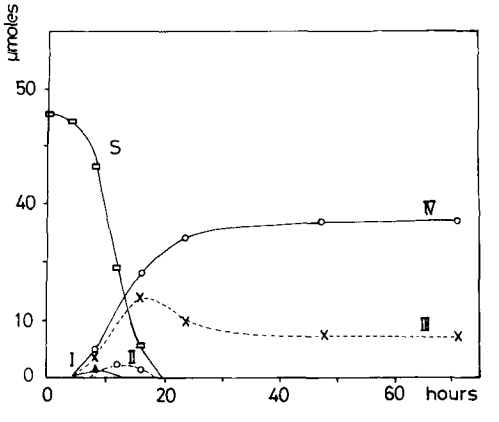


FIG. 3. The conversion of 12-ketooctadecanoic acid in the culture medium at different intervals of incubation of *E. coli K-12.* The abbreviations are defined in Figure 1.

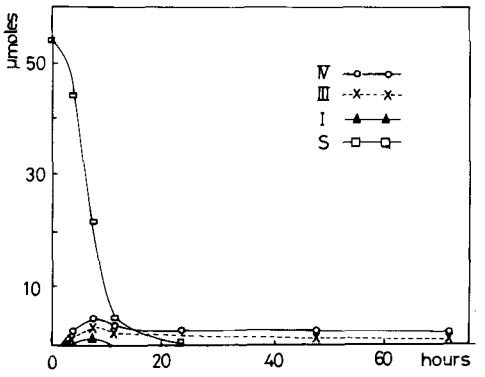


FIG. 4. The conversion of 12-ketooctadecanoic acid in the culture medium at different time intervals of incubation of *Candida tropicalis*. The abbreviations are defined in Figure 1.

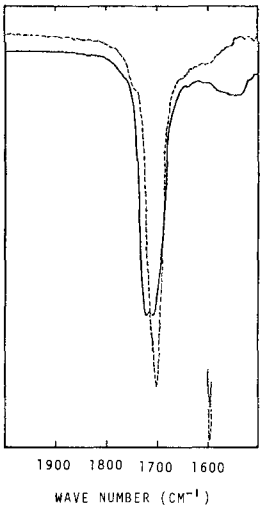


FIG. 5. Infrared absorption spectra of component IV (- - - -) and its methyl ester (———) in chloroform.

microorganisms, to clarify how a long chain keto acid is metabolized and whether its degradation differs from that of a hydroxy acid.

## MATERIALS AND METHODS

12-Ketooctadecanoic acid was prepared by treating ricinoleic acid with $CrO_3$ in acetic acid solution, followed by catalytic reduction of the double bond in the presence of palladium-carbon (11).

The cells of *Esherichia coli K-12* or *Candida tropicalis* were grown in a liquid medium (pH 7.2) containing 2% bovine meat extract (Kyokuto Seiyaku Co., Ltd., Tokyo) and 0.5-0.6 mM concentration substrate. Shaking flasks containing 100 ml medium were used. After sterilization in an autoclave (120 C, 20 min) media were inoculated (10%) with suspensions of cells grown 18 hr in a medium containing 2% bovine meat extract. Incubation was performed with vigorous shaking at 37 C for *E. coli* or 27 C for *C. tropicalis*. Ten milliliter samples of medium were withdrawn at different time intervals of incubation. The samples were acidified with 2N hydrochloric acid and, after addition of 5 μmoles of pure stearic acid as an internal standard, extraction was carried out repeatedly with ether or ethyl acetate. The extract was methylated with diazomethane (12) and analyzed with a Shimadzu GC-1B gas chromatograph equipped with a hydrogen flame ionization detector. A glass column (1.5 m x 4 mm) was packed with 5% diethyleneglycol succinate polyester coated on 60-80 mesh Celite-545 run at 190-195 C (13).

The conversion of substrate during incubation was expressed as the amount in a flask in Figures 3, 4 and 7. Sodium borohydride (Wako Pure Chemical Co., Ltd., Osaka) was used for reduction of a carbonyl group to a hydroxy group. Lactones were reduced to diols with lithium aluminum hydride (Wako Pure Chemical Co., Ltd., Osaka). Mass spectrometry was performed by a direct injection method using a Hitachi RMU-7 instrument.

For the purification of the final metabolite of 12-ketooctadecanoic acid by *E. coli*, a crude extract from culture medium was chromatographed on a silica gel column. The crude extract (170 mg) was dissolved in 2 ml dry carbon tetrachloride and was loaded on a column (1.2 x 28 cm) packed with Wakogel C-200 (silica gel for column chromatography, Wako Pure Chemical Co., Ltd., Osaka) which was previously dried at 115 C for 2 hr. The solvents used for elution were increasing concentrations of ether in hexane (10, 15, 20, 30 and 50%; 100 ml each).

## RESULTS

### Metabolism of 12-Ketooctadecanoic Acid by *E. coli K-12*

Figure 1 shows an example of a gas chromatogram of an ether extract from medium, to which 12-ketooctadecanoic acid (S) was added and metabolized by *E. coli*. Four peaks precede the peak S in this figure. They were denoted as components I, II, III and IV, in the decreasing order of their retention times. There was a linear relationship between the retention times and number of carbon atoms when S, I, II and III were plotted on a semilogarithmic graph (Fig. 2). These lines were parallel to the slope of the retention times of methyl esters of

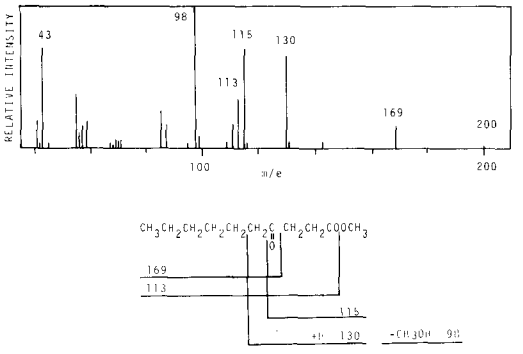


FIG. 6. Mass spectrum (80 ev) of 4-ketodecanoic acid methyl ester.

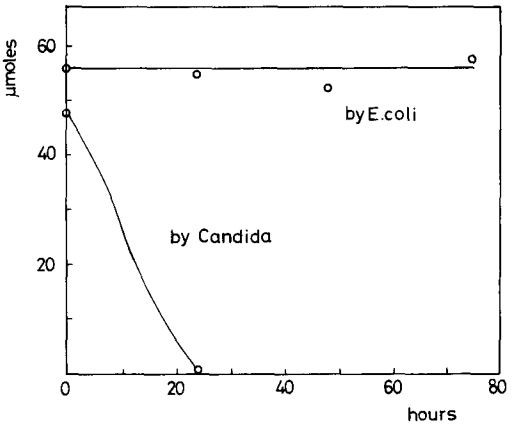


FIG. 7. The degradation of 4-ketodecanoic acid at different time intervals during incubation of *E. coli K-12* and *Candida tropicalis*.

saturated straight chain acids (10:0 to 18:0). The results from these data suggested that I, II and III were 10-ketohexadecanoic, 8-ketotetradecanoic and 6-ketododecanoic acids, respectively. Because the retention time of IV was faster than expected, and it was assumed that IV was 4-ketodecanoic acid, IV was isolated, and a structural analysis was performed.

The results from time studies of the metabolic conversion of the substrate indicated that S was consumed within 24 hr (Fig. 3). Components I and II had maxima in 8 and 12 hr, respectively, with transient and small accumulations. Component III reached its maximum in 16 hr and had a plateau after 24-36 hr (17%). Component IV, the main metabolite, increased in concentration through 24-36 hr incubation and was maintained at a constant level (60%) thereafter.

**Metabolism of 12-Ketooctadecanoic Acid by *C. tropicalis***

It was confirmed by gas chromatographic analysis that S was metabolized, and that the metabolites from I to IV transiently appeared when *C. tropicalis* was cultured under conditions similar to those for *E. coli* (Fig. 4). Component S was rapidly consumed and only small amounts of I and II were detected after incubation for 8-12 hr. Component III gradually accumulated until it reached a maximum in ca. 12 hr and the amount gradually decreased after that time. On the other hand IV reached its maximum in 8 hr but began to decrease thereafter, allowing only an amount equivalent to that of III to be detected. The results from time studies for detection of IV demonstrated that *Candida* would degrade IV further.

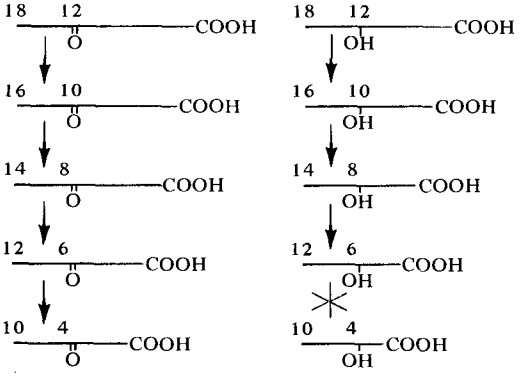


FIG. 8. Comparison of the degradation mode of 12-ketooctadecanoic acid (left) and 12-hydroxyoctadecanoic acid (right) by *E. coli K-12*.

**Isolation and Identification of Component IV**

After 48 hr incubation of *E. coli* the metabolites of 12-ketooctadecanoic acid (ca. 300 mg) were extracted with ether and the extract was added to new medium in which *E. coli* was again cultured. The crude extract (170 mg) from the latter medium was chromatographed on a silica gel column. A fraction eluted with 30% ether in hexane contained IV, which was determined by gas chromatographic analysis to be greater than 98% purity.

The IR absorption spectrum of IV showed a strong absorption at 1708 $cm^{-1}$ and a shoulder at 1715 $cm^{-1}$ (Fig. 5). When IV was treated with diazomethane, the absorption at 1708 $cm^{-1}$ was shifted to near 1730 $cm^{-1}$ but the absorption at 1715 $cm^{-1}$ remained, providing evidence that a carbonyl group was present in IV in addition to a carboxyl group. Component IV had properties like a keto acid when subjected to thin layer chromatography on a Silica Gel G plate using hexane-ether 75:25 as a solvent. Also the product of reduction of IV

with sodium borohydride showed the same retention time on a gas chromatogram with or without the treatment of diazomethane, and agreed with the retention time of authentic 4-decalactone. From these observations it was concluded that IV was 4-ketodecanoic acid.

The mass spectrum of the methyl ester of IV is shown in Figure 6. The molecular ion peak (m/e 200) was observed. Such ion peaks as m/e 130, 115, 113 and 98 indicated that the carbonyl group had existed in the fourth position. The ion m/e 130 was generated by the cleavage between the fifth and sixth positions after a McLafferty rearrangement of a hydrogen atom of the seventh position carbon to the oxygen of the fourth carbonyl group. The ion (m/e 98), formed by splitting of $CH_3OH$ from the ion (m/e 130), became the base peak. The ion peaks m/e 115 and 113 were owing to the so-called α-cleavage. Their cleavages were shown to occur respectively between the fourth and fifth positions, and also between the carbons in the third and fourth positions. The same mass spectrum was obtained from authentic 4-ketodecanoic acid prepared by bromoxidation of authentic 4-decalactone (13). The structure of IV was reconfirmed by mass spectral analysis to be 4-ketodecanoic acid.

### Metabolism of 4-Ketodecanoic Acid (IV) by *E. coli K-12* and *C. tropicalis*

When highly purified 4-ketodecanoic acid (IV) obtained by purification of the *E. coli* metabolites was added to a medium as new substrate, it was ascertained that IV was completely degraded metabolically by *C. tropicalis* within 24 hr, while it was essentially undegraded by *E. coli* (Fig. 7). This was expected from our previous experimental results (Figs. 3 and 4).

## DISCUSSION

It was confirmed that *E. coli* degraded 12-ketooctadecanoic acid (S) to 4-ketodecanoic acid (IV) with the transient accumulation of 10-ketohexadecanoic (I), 8-ketotetradecanoic (II) and 6-ketododecanoic (III) acids. Degradation of S by *C. tropicalis* continued past I, II, III and IV until it was completely degraded. It was inferred that 2-ketooctanoic acid would be formed following the formation of IV when *C. tropicalis* was used, but this compound was not identified by gas chromatography. Even after the metabolic mixture produced from 12-ketooctadecanoic acid by *C. tropicalis* was treated with sodium borohydride to give the corresponding hydroxy acids, the presence of 2-hydroxyoctanoic acid was not proven, whereas the 2-hydroxy acid was detected when 12-hydroxyoctadecanoic acid was added to a culture medium of *C. tropicalis* (7,8). The mechanism of this degradation is under investigation.

A typical difference between the metabolism of a hydroxy acid and of a keto acid by *E. coli* is that 12-hydroxyoctadecanoic acid is degraded as far as 6-hydroxydodecanoic acid (6), while 12-ketooctadecanoic acid when used as a substrate can be degraded further to 4-ketodecanoic acid as illustrated in Figure 8.

In view of the fact that keto acids can be easily absorbed through the intestinal wall (M. Sato, unpublished observation), the fact that short chain keto acids are formed by degradation of long chain keto acids by microorganisms in the intestine suggests that further consideration on the effect of short chain keto acids in vivo is warranted.

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# An Interaction of DDT in the Metabolism of Essential Fatty Acids[1]

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## ABSTRACT

The growth of female rats was depressed further by the incorporation of DDT into a ration deficient in essential fatty acids (EFA). With female rats fed a ration supplemented with EFA, DDT produced a slight stimulation in growth. DDT also produced an increase in the 20:3ω9/20:4ω6 ratio in liver lipids of male rats fed a ration deficient in EFA. These data indicate an effect in EFA nutrition. Substantial changes in the fatty acid composition of liver lipids resulted from the feeding of DDT. The proportion of 16:0 was decreased, while that of 18:0 was increased. With rats on the supplemented rations an increase in the proportion of 20:4ω6 was observed, while in the deficient rats a comparable increase was observed in the proportion of 20:3ω9. These changes in fatty acid composition have been related to the proliferation of hepatic smooth endoplasmic reticulum induced by the DDT, and it is suggested that this effect could increase the demand for EFA by the liver, thus influencing EFA nutrition.


## INTRODUCTION

The growth of female rats depressed by the feeding of rations deficient in essential fatty acids (EFA) was reduced further by the addition of 30 ppm of dieldrin to the diet (1). An interaction between the nutritional and toxic stress was suggested since the dieldrin did not produce a comparable response when fed with rations supplemented with EFA. The dieldrin also produced changes in the fatty acid composition of liver lipids, which in the deficient rats indicated an accentuation of the nutritional stress. The latter changes were observed both in male and female rats. This effect of dieldrin has been related to its ability to induce the proliferation of hepatic smooth endoplasmic reticulum. Since this induction phenomenon is produced by many different substances (2), chemicals other than dieldrin should influence an EFA deficiency if this proposed relationship is valid.

[1]Technical Paper No. 3156, Oregon Agricultural Experiment Station.

TABLE I

Ration Composition

| Component | Per cent by wt |
|---|---|
| Casein | 22.0 |
| Cerelose | 48.0 |
| H.M.W. salts[a] | 4.0 |
| Hydrogenated coconut oil | 25.0 |
| Vitamin mix[b,c] | 1.0 |

[a]Zinc was added at a level of 6 mg/kilo as zinc acetate.

[b]Provided, as mg/100 g diet, the following: Thiamine HCl, 0.4; riboflavin, 0.8; pyridoxine HCl, 0.50; D-calcium pantothenate, 4.0; inositol, 20; menadione, 0.40; folic acid, 0.40; niacin, 4.0; choline dihydrogen citrate, 424; biotin, .03; $B_{12}$, .02.

[c]Vitamins A, D and E were added in ethanol solution to provide per 100 g of diet, 875 IU of vitamin A, 125 units of vitamin D, and 3.64 mg of D-α-tocopherol acetate.

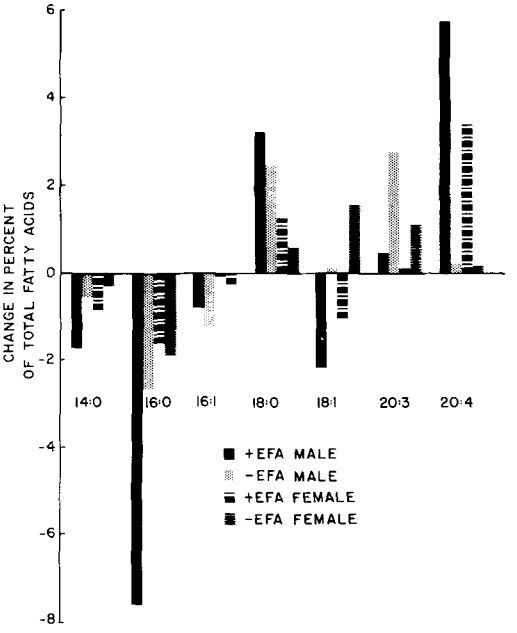


FIG. 1. DDT-induced changes in the proportions of fatty acids in liver lipids. The change in proportion of the fatty acid from animals fed DDT is plotted relative to the proportion observed in animals not fed DDT, the latter being set at zero.

TABLE II

Influence of DDT and EFA[a] Nutrition on Body Weight, Liver Size and Fatty Acid Composition of Liver Lipids

| Subject | Ration | | | |
|---|---|---|---|---|
| | +EFA - DDT | +EFA +DDT | -EFA -DDT | -EFA + DDT |
| Males | | | | |
| 12 week body wt, g | 384 ± 38[b] | 271 ± 12 | 353 ± 37 | 242 ± 9 |
| Liver size, g/100 g body weight | 3.02 ± 0.39 | 4.02 ± 0.20 | 3.96 ± 0.18 | 5.17 ± 0.08 |
| Fatty acid composition | | | | |
| 14:0 | 4.35 ± 0.95 | 2.66 ± 0.40 | 2.62 ± 0.27 | 2.05 ± 0.30 |
| 16:0 | 22.90 ± 2.43 | 16.97 ± 1.19 | 19.70 ± 0.48 | 17.06 ± 0.88 |
| 16:1 | 3.28 ± 0.42 | 2.47 ± 0.37 | 6.64 ± 0.21 | 5.39 ± 0.69 |
| 18:0 | 11.77 ± 0.76 | 14.99 ± 0.49 | 12.42 ± 0.65 | 14.84 ± 1.08 |
| 18:1 | 24.49 ± 1.27 | 22.31 ± 1.32 | 32.44 ± 2.06 | 32.53 ± 0.78 |
| 18:2 | 9.60 ± 2.55 | 10.75 ± 1.58 | 1.41 ± 0.13 | 1.53 ± 0.04 |
| 20:3ω9 | 0.53 ± 0.10 | 1.25 ± 0.35 | 13.16 ± 1.18 | 15.91 ± 1.19 |
| 20:3ω6 | 0.89 ± 0.20 | 1.38 ± 0.18 | 0.83 ± 0.20 | 1.04 ± 0.31 |
| 20:4 | 14.30 ± 1.32 | 20.19 ± 1.20 | 4.04 ± 0.17 | 4.24 ± 0.77 |
| 22:5 | 2.62 ± 0.29 | 2.88 ± 0.55 | 0.93 ± 0.11 | 0.81 ± 0.25 |
| 20:3ω9/20:4 | --- | --- | 3.26 ± 0.27 | 3.85 ± 0.52 |
| Females | | | | |
| 12 week body wt, g | 212 ± 10 | 189 ± 4 | 216 ± 8 | 175 ± 6 |
| Liver size, g/100 g body weight | 3.73 ± 0.19 | 4.22 ± 0.33 | 4.21 ± 0.26 | 4.81 ± 0.19 |
| Fatty acid composition | | | | |
| 14:0 | 3.89 ± 0.80 | 3.01 ± 0.47 | 2.68 ± 0.65 | 2.42 ± 0.56 |
| 16:0 | 17.94 ± 1.38 | 16.27 ± 1.00 | 17.73 ± 1.55 | 15.83 ± 0.73 |
| 16:1 | 2.24 ± 0.26 | 2.29 ± 0.37 | 4.86 ± 0.99 | 4.58 ± 0.77 |
| 18:0 | 15.91 ± 1.02 | 17.16 ± 1.02 | 15.51 ± 1.61 | 16.10 ± 0.98 |
| 18:1 | 20.27 ± 1.11 | 19.20 ± 1.00 | 28.87 ± 1.43 | 30.41 ± 0.48 |
| 18:2 | 8.75 ± 0.64 | 7.84 ± 1.84 | 1.39 ± 0.07 | 1.48 ± 0.13 |
| 20:3ω9 | 0.51 ± 0.05 | 0.57 ± 0.08 | 14.62 ± 1.69 | 15.71 ± 1.09 |
| 20:3ω6 | 1.24 ± 0.23 | 1.13 ± 0.09 | 0.92 ± 0.14 | 1.02 ± 0.29 |
| 20:4 | 18.74 ± 2.05 | 22.11 ± 0.99 | 5.46 ± 1.04 | 5.58 ± 0.55 |
| 22:5 | 4.75 ± 0.33 | 4.87 ± 0.62 | 1.36 ± 0.24 | 0.61 ± 0.32 |
| 20:3ω9/20:4 | --- | --- | 2.72 ± 0.28 | 2.83 ± 0.10 |

[a]EFA = Essential fatty acids.

[b]Mean and standard deviation.

This paper summarizes experiments designed to explore this hypothesis replacing the dieldrin with p,p'-DDT which is also active in inducing the proliferation of smooth endoplasmic reticulum (3).

## EXPERIMENTAL PROCEDURES

The composition of the semipurified ration used in this study is given in Table I. A control ration was obtained by adding 1.7% corn oil, replacing an appropriate amount of the hydrogenated coconut oil to give a level of linoleic acid of 1.5% of total calories. Both the deficient and supplemented rations were fed with and without p,p'-DDT (ESA Pesticide Reference Standard, City Chemical Corp., N.Y.) which was added to the rations dissolved in hydrogenated coconut oil to give a level of 150 ppm.

Litter mate groups of four (males or females) 28-day-old weanling rats from our closed colony of Wistar rats were weaned onto each of the four experimental rations giving a total of eight (four males and four females) on each ration. Rations were fed ad libitum. After a 12 week feeding period the animals were killed and samples of liver tissue were taken for analysis.

Liver lipids were extracted using chloroform-methanol and fatty acid methyl esters were prepared using anhydrous HCl in methanol-ether. Separation of these mixtures of methyl esters was accomplished with a gas chromatograph fitted with a 6 ft x 1/8 in. OD aluminum column of 15% ethylene glycol succinate on AW Chromosorb P (80-100 mesh) at 190 C. A gas flow of 30 ml/min was used. Details of these procedures have been given (4).

## RESULTS AND DISCUSSION

The influence of the experimental variables on growth, liver size and liver lipid fatty acid composition is summarized in Table II. For

TABLE III

Fatty Acid Composition of Total Lipids and Microsomal Lipids of Liver

| | Per cent by wt | | | |
|---|---|---|---|---|
| | EFA-deficient[a] | | EFA-supplemented[b] | |
| Fatty acid | Total lipid | Microsomal lipid | Total lipid | Microsomal lipid |
| 14:0 | 3.2 | 2.1 | 3.0 | 1.6 |
| 16:0 | 21.2 | 16.4 | 21.2 | 15.0 |
| 16:1 | 3.8 | 2.6 | 5.8 | 3.2 |
| 18:0 | 16.7 | 23.6 | 15.2 | 23.4 |
| 18:1 | 26.6 | 18.2 | 24.3 | 16.1 |
| 18:2 | 1.9 | 1.8 | 6.9 | 8.4 |
| 20:3ω9 | 10.5 | 16.4 | 4.5 | 7.5 |
| 20:4 | 5.9 | 7.7 | 9.6 | 13.5 |

[a]Male rat raised on EFA-deficient ration for 6 weeks.

[b]Male rat raised on EFA-deficient ration for 6 weeks followed by a 10 day feeding period on the EFA-supplemented ration.

specific fatty acids the influence of DDT is illustrated in Figure 1. DDT depressed growth in the deficient but not the EFA-supplemented females. This interaction between the nutritional and toxic variables could be demonstrated as early as 5 weeks into the feeding period. No such interaction was observed with male rats. A comparable distinction between males and females had also been observed when dieldrin was used as the toxicant (1). Both stresses produced liver enlargement; however when imposed simultaneously no interaction is indicated.

The proportions of 18:0, 20:3ω9 (This notation specifies the number of carbons in the chain, the number of double bonds, and the number of carbon atoms after the terminal double bond in the molecule.) and 20:4ω6 are increased with corresponding decreases in the proportions of 16:0, 14:0 and 16:1 (Fig. 1). Statistical analysis has demonstrated these effects of DDT to be highly significant ($p<.01$). It should be noted that these changes are more pronounced in male rats. In liver lipids the DDT did not produce a consistent change in the proportion of 18:1.

The ratio of 20:3ω9/20:4ω6 has been used as a biochemical index of the status of essential fatty acid nutrition (5). By this criterion one would conclude that in male rats DDT accentuates an EFA deficiency since it produces a statistically significant ($p<.05$) increase in this ratio (Table II).

These changes in fatty acid composition could well result from the changes in cellular membrane complement produced by the DDT. The smooth endoplasmic reticulum constitutes a major part of the microsomal fraction which has been shown to contain 38% of the total phospholipids in rat liver (6). Thus if the level of smooth endoplasmic reticulum is increased several fold by the DDT, changes in the total complex lipid and fatty acid composition will reflect the changes in membrane content. The fatty acid composition of the total lipids in livers of rats ingesting DDT should tend to become more like that of the lipid of smooth endoplasmic reticulum. That this is so can be seen by comparing the effect of DDT on the fatty acid composition of liver lipids (Table II) with the characteristic fatty acid composition of the lipids of the microsomal fraction (Table III). That the changes in fatty acid composition in liver lipids observed in female rats are not as pronounced as those observed in male rats would be consistent with the fact that the proliferation of the smooth endoplasmic reticulum induced by DDT is not as extensive in female rats (7).

With male rats fed the ration supplemented with EFA, DDT produced a 31% increase in liver size and a 38% increase in the proportion of the linoleic (ω6) series of polyunsaturated fatty acids in liver lipids. The combination of these two factors would mean that the actual amount of this series of fatty acids in the liver would be increased by 80% by the DDT exposure, making the comparison with rats of the same size. In this study DDT has not shown a marked effect on the crude lipid content the liver, another factor which could influence this relationship. The extent to which this increased demand for EFA by the liver would become significant for the animal as a whole would be determined by the comparative contribution of the liver in the overall handling of EFA and the amounts of EFA available to the animal.

The above hypothesis propounded to explain this interaction would be invalid if the EFA deficiency inhibited the induction process. Kaschnitz (8) has reported that in rats deficient in EFA the hepatic levels of aniline hydroxylase and cytochrome P-450 were reduced; however the inductive effect of phenobarbital on these factors was not inhibited. From somewhat similar data Marshall and McClean (9) have concluded that polyunsaturated fatty acids permit the induction process to occur. More definitive studies are required to clarify this relationship.

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# Long Chain Fatty Acid Methyl Ester Hydrolase Activity in Mammalian Cells

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## ABSTRACT

Long chain fatty acid methyl esters were hydrolyzed by cell free homogenates and subcellular fractions prepared from Ehrlich ascites tumor cells. The highest enzyme specific activity was observed in the microsomal fraction. Maximum hydrolase activity occurred in the pH range of 6.5-7.0. The relative activities for the methyl ester substrates that we tested were: methyl palmitate > methyl laurate > methyl oleate > methyl stearate. Butyl palmitate also was hydrolyzed by this enzyme. Enzymatic activity increased when the methyl ester-albumin complex concentration or the methyl ester-albumin molar ratio was raised. In addition to Ehrlich cells, methyl ester hydrolase activity was observed in homogenates of rat heart and liver. The highest enzyme specific activities also occurred in the microsomal fractions prepared from these tissues.

## INTRODUCTION

Long chain fatty acid methyl esters can be taken up by Ehrlich ascites tumor cells, rat heart slices and rat liver slices (1). When fatty acid-labeled methyl esters were employed, radioactivity appeared in tissue phospholipids, glycerides, free fatty acids and $CO_2$. These results suggested that methyl esters may be useful model compounds for studies of esterified fatty acid metabolism by mammalian tissues. Therefore we thought it of interest to investigate the mechanism whereby methyl esters are utilized. Since the above data indicated that hydrolysis probably was the initial reaction in this process, we have examined several tissues for the presence of long chain fatty acid methyl ester hydrolase activity. While our studies were in progress, the presence of a methyl ester hydrolase in human adipose tissue was reported by other workers (2).

## METHODS

Unlabeled methyl esters and methyl esters containing $^{14}C$ in the carboxyl carbon atom of the fatty acid moiety were purchased from Applied Science Laboratories, Inc., State College, Pa. They were dissolved in hexane and extracted with alkaline ethanol to remove any fatty acid impurities (3). Thin layer chromatography revealed that after purification more than 99% of the radioactivity migrated in the methyl ester zone. Radioactivity was measured in a Packard Tri-Carb 3375 B liquid scintillation spectrometer using a scintillator solution containing 18 ml toluene-methanol 7:3 v/v, 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis (2-[4-methyl-5-phenyloxazolyl])- benzene (3). Quenching was monitored with the external

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TABLE I

Distribution of Methyl Ester Hydrolase Activity in Subcellular Fractions of Tissue Homogenates

| Subcellular fraction | Enzymatic activity, nmoles/mg protein[a] | | |
|---|---|---|---|
| | Ehrlich cells | Rat heart | Rat liver |
| Intact homogenate | 32 | 73 | 1740 |
| Nuclear[b] | 30 | 139 | 1510 |
| Mitochondrial[c] | 39 | 338 | 2240 |
| Microsomal[d] | 62 | 725 | 3920 |
| Supernatant[e] | 11 | 126 | 97 |

[a]Methyl palmitate-1-$^{14}C$ was the substrate. Incubation was for 30 min at 37 C.
[b]Sedimented at 600 x g for 10 min at 0 C and washed once.
[c]Sedimented between 600 x g and 12,000 x g for 10 min as above.
[d]Sedimented between 12,000 x g and 100,000 x g for 1 hr as above.
[e] Supernatant solution of 100,000 x g centrifugation.

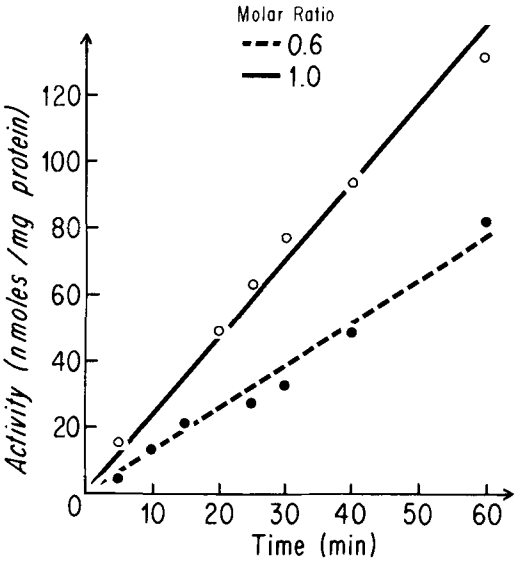


FIG. 1. Time course of methyl palmitate-1-$^{14}$C hydrolysis by the Ehrlich cell microsomal fraction. Incubation was at 37 C. The dashed curve represents an experiment in which the methyl palmitate-albumin molar ratio was 0.6; the solid curve one in which the ratio was 1.0.

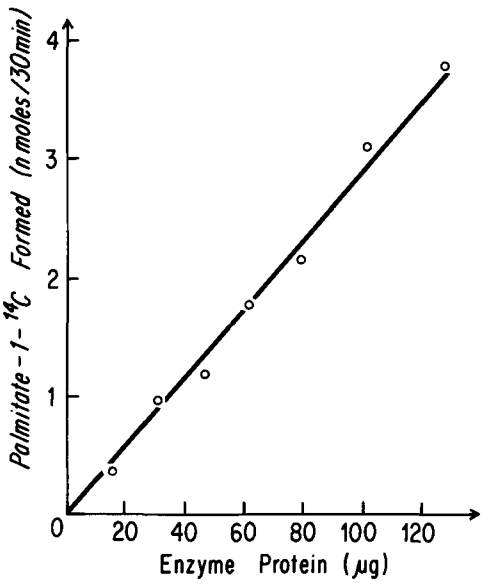


FIG. 2. Effect of enzyme concentration on the hydrolysis of methyl palmitate-1-$^{14}$C. Incubation was for 30 min at 37 C, and a microsomal fraction prepared from Ehrlich cells was the source of the enzyme.

standard. The specific radioactivity of each methyl ester preparation was approximately 0.7 Ci/mole. The methyl esters were added to washed Celite (4), and in most preparations, there was 1 μmole of methyl ester per 100 mg of Celite.

Free fatty acid (FFA)-free Fraction V bovine serum albumin was purchased from Miles Laboratories, Inc., Elkhart, Ind. These albumin preparations contained less than 0.02 μeq/μmole free fatty acid as determined by titration (5). The protein was dissolved in a solution containing 132 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$ and 16 mM $Na_2HPO_4$, pH 7.4. Protein content was measured by the method of Lowry et al. (6). Labeled methyl esters were added to the albumin solutions by incubation with the methyl ester-coated Celite preparations (1,4).

Washed Ehrlich cells were suspended in 250 mM sucrose containing 10 mM $K_2HPO_4$, pH 7.4, and 1 mM EDTA. Homogenates and subcellular fractions were prepared as described previously (7). Homogenates and subcellular fractions of rat hearts and livers were prepared as above except that the sonic irradiation step was omitted. Each of the isolated subcellular fractions was washed once and resuspended in the buffered sucrose solution. The Ehrlich cell microsomal fraction, sedimented between 12,000 and 100,000 g for 1 hr, could be stored in buffered sucrose at -20 C for up to 3 weeks without appreciable reduction in enzymatic activity.

The incubations were initiated by adding 25 μl of the enzyme preparation to 175 μl methyl ester-albumin solution. In some experiments chloroform-methanol 2:1 v/v was added, and the lipid extract was chromatographed on Adsorbosil 5 using petroleum ether-diethyl ether-acetic acid 180:20:2. The enzymatic activity was calculated from the amount of methyl ester radioactivity converted to free fatty acid. In other experiments the liquid-liquid partition system of Belfrage and Vaughan (8) was employed to separate methyl esters from free fatty acids. Radioactivity was measured following addition of 0.5 ml of the upper phase to a dioxane scintillator solution (8). Similar values for enzymatic activity were obtained when duplicate samples were analyzed by either the thin layer chromatography or the liquid-liquid partition method.

## RESULTS

In the initial experiments, the lipid extracts obtained following incubation of fatty acid-labeled methyl esters with subcellular fractions prepared from Ehrlich cells were analyzed by the thin layer chromatography procedure. It was observed that radioactive FFA was pro-

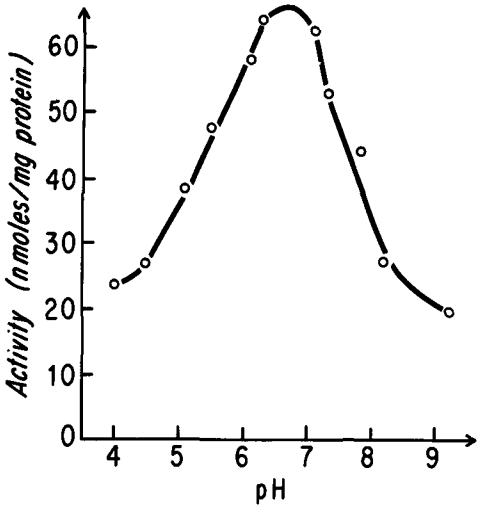


FIG. 3. Enzymatic activity relative to the pH of the incubation medium. The conditions of incubation were the same as those described under Figure 2.

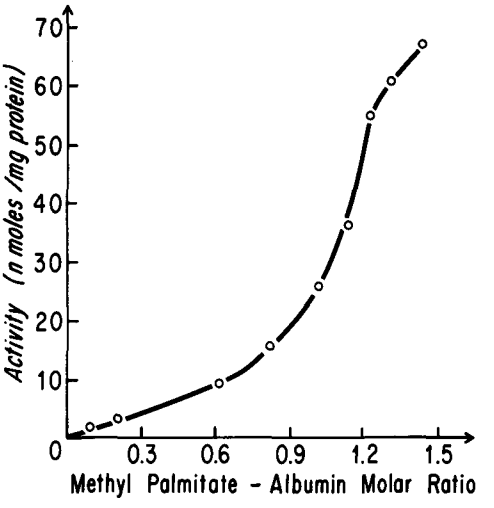


FIG. 4. Effect of methyl palmitate-1-$^{14}$C-albumin molar ratio on enzymatic activity. The albumin concentration was identical in each flask (87.5 nmoles). The conditions of incubation were the same as those described in Figure 2.

duced when the tissue was incubated with the methyl esters. After 1 hr of incubation at 37 C, as much as 77% of the added radioactivity was recovered as FFA. The rest of the radioactivity remained in methyl esters except for 0.1-3.0% which was present in the other zones of the chromatogram. Identical values for methyl ester hydrolysis to fatty acid were obtained when these samples were analyzed by the method of Belfrage and Vaughan (8), and subsequent experiments were performed according to the latter procedure.

The distribution of methyl palmitate hydrolase activity in the tissue homogenates is shown in Table I. The highest enzyme specific activity was obtained with the microsomal fractions. Attempts to solubilize appreciable amounts of the enzyme contained in the mitochondrial or microsomal fractions by either freezing and thawing or sonification were unsuccessful. The highest activities were found in the subcellular fractions of liver, but the heart fractions also contained greater activities than those of the Ehrlich cells. However the Ehrlich cell enzyme was chosen for further study because most of the metabolic studies that were done with methyl ester substrates employed this tissue (1). The Ehrlich cell microsomal fraction, when fortified with the appropriate cofactors (7), did not oxidize palmitate-1-$^{14}$C to $^{14}CO_2$. A vigorous oxidation of palmitate-1-$^{14}$C under these conditions was observed with the mitochondrial fraction. These results suggest that the microsomal fraction was essentially free of contamination by functioning mitochondria.

Figure 1 illustrates the time course of methyl palmitate hydrolysis by the Ehrlich cell microsomal enzyme. Two conditions were tested: a methyl palmitate-albumin molar ratio of 0.6 and a ratio of 1.0. In both cases the reaction was linear during a 1hr incubation at 37 C. The enzymatic activity was 1.8 times greater at the higher molar ratio.

As shown in Figure 2, hydrolytic activity increased linearly with respect to protein concentration when the Ehrlich cell microsomal fraction was incubated with methyl palmitate. Almost no enzymatic activity was observed with the microsomal fraction at 2 C. Between 10 and 37 C the increase in activity was approximately 3-fold for each 10 deg rise in temperature. A small increase in activity occurred between 37 and 49 C.

Figure 3 illustrates the effect of pH on methyl palmitate hydrolysis by the Ehrlich cell microsomal fraction. Maximum activity occurred at pH 6.7, but the pH optimum was rather broad between pH 6.3 and 7.1.

As shown in Figure 4, methyl palmitate hydrolysis by the Ehrlich cell microsomal fraction increased markedly when the molar ratio of methyl palmitate-1-$^{14}$C to albumin was raised. In this experiment increasing amounts of methyl palmitate were added to media containing a fixed quantity of albumin. As seen in Figure 5, hydrolysis decreased when increasing amounts of albumin were added to media containing a fixed amount of methyl palmitate, i.e., hydrolysis decreased as the methyl palmitate-albumin molar ratio was lowered. In

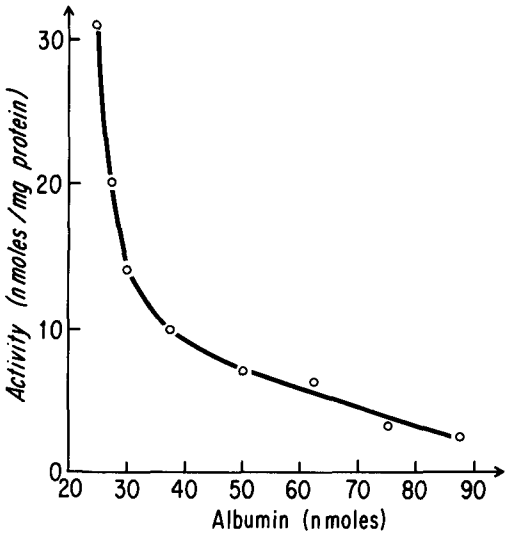


FIG. 5. Effect of medium albumin content on enzymatic activity. Each flask contained 30 nmoles of methyl palmitate-1-$^{14}$C and the amount of albumin shown on the graph. The conditions of incubation were the same as those described under Figure 2.

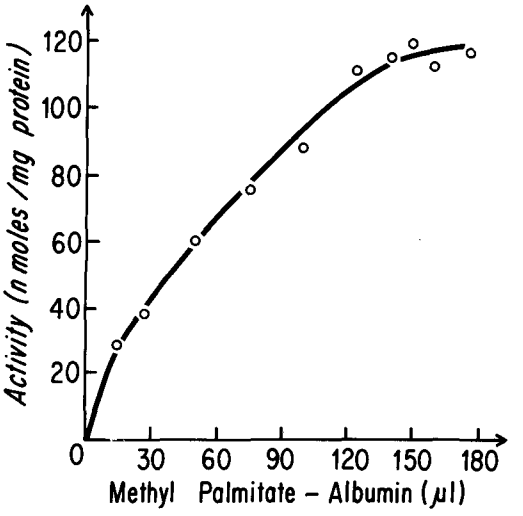


FIG. 6. Enzymatic activity relative to the amount of methyl palmitate-1-$^{14}$C-albumin complex added to the incubation medium. The conditions of incubation were the same as those described under Figure 2.

addition to molar ratio, hydrolysis was also dependent upon the concentration of the methyl palmitate-albumin complex. Figure 6 illustrates that an increased amount of hydrolysis occurred as the concentration of the complex was raised. In this experiment both the methyl palmitate and albumin concentrations were increased concomitantly so that at the start of the incubation the molar ratio of methyl palmitate to albumin was the same in each of the media. Even at the lowest concentration only 20% of the available methyl palmitate was hydrolyzed under the conditions of this experiment. Therefore the differences in enzymatic activity were not simply the result of variations in the methyl palmitate-albumin molar ratio that developed as the incubation progressed.

The effect of methyl ester structure on the rate of hydrolysis by the Ehrlich cell microsomal fraction is presented in Table II. Each experiment was performed with a different enzyme preparation, and considerable variation in absolute activities occurred. However in each of the four experiments the relative activities were: methyl palmitate > methyl laurate > methyl oleate > methyl stearate. In additional studies we found that butyl palmitate was hydrolyzed approximately as rapidly as methyl palmitate by the microsomal enzyme.

## DISCUSSION

Ehrlich ascites tumor cells as well as rat heart and rat liver contain an enzyme that can hydrolyze long chain fatty acid methyl esters. A similar enzymatic activity has been observed in human adipose tissue (2). These findings

TABLE II

Effect of Methyl Ester Structure on Hydrolase Activity[a]

| Substrate | Enzymatic Activity nmoles/mg protein ± SE[b] | | | |
|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 |
| Methyl laurate-1-$^{14}$C | 44 ± 9 | 49 ± 2 | 73 ± 6 | 40 ± 4 |
| Methyl palmitate-1-$^{14}$C | 87 ± 9 | 80 ± 1 | 125 ± 5 | 61 ± 2 |
| Methyl stearate-1-$^{14}$C | 4 ± 0.2 | 7 ± 1 | 8 ± 1 | 4 ± 1 |
| Methyl oleate-1-$^{14}$C | 27 ± 7 | 30 ± 1 | 50 ± 5 | 31 ± 2 |

[a]Incubation was for 30 min at 37 C. The molar ratio of methyl ester to albumin was 0.7 in each case. A microsomal fraction prepared from Ehrlich cells was the source of enzyme, and each experiment was done with a different enzyme preparation.

[b]Each value was the mean of five determinations ± SE.

support the suggestion that hydrolysis is probably the initial step in methyl ester utilization by mammalian tissues (1).

The methyl ester hydrolase of the Ehrlich cell microsomal fraction is similar in several respects to the monoglyceride lipase of myocardium (9), adipose tissue (10) and intestinal mucosa (11,12). Like the monoglyceride lipases (9-12), the methyl ester hydrolase is largely membrane bound. Its pH optimum, 6.7, is almost identical to that of the monoglyceride lipase (9), but it differs considerably from those of the triglyceride and heparin-sensitive lipases (13-15). Ehrlich cells can metabolize fatty acids contained in chylomicron triglycerides (16) and endogenous glycerides (17). Hence these cells almost certainly contain a monoglyceride lipase. In view of this and the similarity between the methyl ester hydrolase and the monoglyceride lipases of other tissues, it is possible that the enzyme which we have investigated may actually function physiologically as a monoglyceride lipase.

At this time it is not possible to interpret definitively the observed kinetics of methyl ester hydrolysis. Because of the very poor solubility of long chain fatty acid methyl esters in aqueous media, some method of "solubilization" had to be developed for these substrates. Albumin binding was chosen as the means of solubilization, for this protein also was required in the assay system in order to bind the fatty acid released during the course of the reaction. Under these conditions the actual substrate could be the albumin bound methyl ester, the very small smount of unbound methyl ester in equilibrium with that bound to albumin, or both. Which of these possibilities actually occurs when protein bound lipids serve as the substrate for an enzymatic reaction has not been determined. Moreover the unbound methyl ester concentration relative to the methyl ester-albumin molar ratio cannot be calculated accurately at present because the binding reaction has been examined only superficially (18,19). Hence the data contained in Figures 4-6 should not be considered true substrate concentration-velocity plots, and the exact significance of the sigmoid-shaped curve in Figure 4 remains open to question. These graphs should be considered only descriptively, for further kinetic interpretations must await elucidation of the above points. Likewise the exact interpretation of the substrate specificities noted in Table II must await comparative measurements of the binding of these methyl esters to albumin.

ACKNOWLEDGMENT

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# Hydroperoxides from Oxidation of Linoleic and Linolenic Acids by Soybean Lipoxygenase: Proof of the *trans*-11 Double Bond

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**ABSTRACT**

Hydroperoxides produced by oxidation of linoleic and linolenic acids with soybean lipoxygenase were analyzed by nuclear magnetic resonance. The isomerized double bond $\alpha,\beta$ to the hydroperoxide group at carbon-13 was determined to be *trans*. The complete structures of the major products proved to be 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid from linoleic acid and 13-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid from linolenic acid. The configuration of the double bonds indicates that oxidation took place through a free radical mechanism as proposed previously by others.

Investigating the oxidation of linoleic acid by soybean lipoxygenase (EC 1.13.1.13), Privett et al. (1) found that the principal products were *cis,trans* conjugated monomeric hydroperoxides. The position of oxidation is primarily at carbon-13 in both linoleic (2-4) and linolenic (3) acids. Largely confirming the work of others, we found that the soybean lipoxygenase (Pierce Chemical Co., Rockford, Ill.) used in this study oxidized linoleic and linolenic acids primarily at carbon-13 with a small amount of oxidation being at carbon-9, as determined by the mass spectral method of Dolev et al. (4). Of the four conjugated isomers possible from linolenic acid (oxidation at carbons-9, -12, -13 and -16), only a trace of oxidation occurred at carbons-12 and -16. These mass spectral data indicated a slight specificity toward oxidation at carbon-9, which could be owing to action of an isoenzyme. Analysis of the methyl hydroxystearates by a thin layer chromatography (TLC) densitometry method (5) established that linoleic acid was 83% oxidized at carbon-13 and 17% at carbon-9 by our sample of soybean lipoxygenase and confirmed an earlier study by one of us (6) using a different method.

The position of the isomerized double bond has been less thoroughly researched. Previously it was assumed that the isomerized double bond of the fatty acid hydroperoxide ($\alpha,\beta$ to the hydroperoxide) became *trans* through a free radical mechanism characteristic of autoxidation. This assumption was proved valid by Hamberg and Samuelsson (3), who showed that *cis*-8,*cis*-11,*cis*-14-eicosatrienoic acid was oxidized to 15-hydroperoxy-*cis*-8,*cis*-11,*trans*-13-eicosatrienoic acid by soybean lipoxygenase; however they did not study the position of the *cis,trans* double bonds of the hydroperoxides from linoleic or linolenic acids. Lipoxygenase

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TABLE I

NMR[a] Chemical Shifts (CS) and Coupling Constants (J) for Linoleic and Linolenic Acid Hydroperoxides Compared With Their Hydroxy Analogs

| Proton (identified by carbon no.) | 13-Hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid | | Methyl coriolate[b] | | 13-Hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid | | 13-Hydroxy *cis*-9,*trans*-11, cis-15-octadecatrienoic acid | |
|---|---|---|---|---|---|---|---|---|
| | CS (δ) | J (Hz) | CS | J | CS | J | CS | J |
| 8,9 | | 7.5 | | 8 | | --- | | --- |
| 9 | 5.45 | | 5.30 | | --- | | --- | |
| 9,10 | | 11 | | 11 | | --- | | --- |
| 10 | 5.98 | | ~5.90 | | 5.95 | | 5.94 | |
| 10,11 | | 10.8 | | 10 | | 11 | | 11 |
| 11 | 6.53 | | ~6.35 | | 6.54 | | 6.49 | |
| 11,12 | | 15 | | 14.4 | | 15 | | 15 |
| 12 | 5.55 | | 5.56 | | 5.54 | | 5.64 | |
| 12,13 | | 8 | | 7 | | 8 | | 7 |
| 13 | 4.37 | | 4.05 | | 4.38 | | 4.20 | |

[a]NMR, nuclear magentic resonance.

[b]Tallent et al. (7)

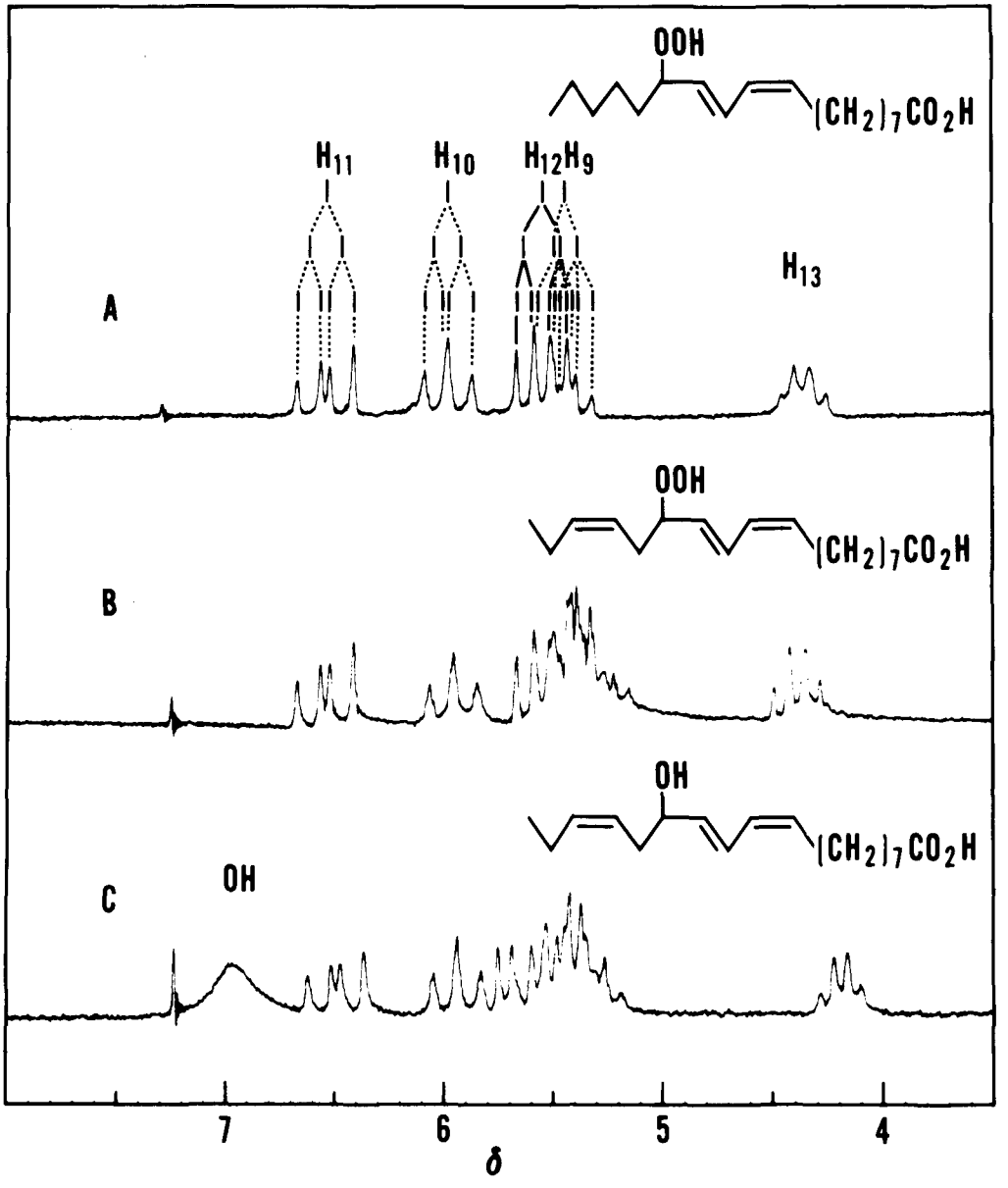


FIG. 1. Nuclear magnetic resonance spectra of hydroperoxydiene, hydroperoxytriene and hydroxytriene moieties of 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (A), 13-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (B) and 13-hydroxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (C).

from corn oxidizes linoleic acid to a hydroperoxide with a *trans* double bond $\alpha,\beta$ to the oxidized carbon (5). However the mechanism of oxidation by corn lipoxygenase may be decidedly different since carbon-9 appears to be oxidized exclusively.

During a study concerned with the reactivity of fatty acid hydroperoxides with $\alpha$-tocopherol, we examined by nuclear magnetic resonance (NMR) linoleic and linolenic acid hydroperoxides generated by soybean lipoxygenase. After examining the coupling constants by double resonance experiments, we established the geometry of the double bonds relative to the hydroperoxide group. The position of the hydroperoxide was ascertained by mass spectrometry.

Either linoleic or linolenic acid was incubated with soybean lipoxygenase by a procedure essentially as described by Gardner (6).

Oxygenation was improved by bubbling $O_2$ through a sintered glass disc, which reduced the amount of byproduct, oxooctadecadienoic acid, to undetectable quantities and simplified subsequent isolation of hydroperoxides by column chromatography as we had described earlier (5). The 100 MHz NMR spectra, recorded by standard methods (6), indicated that the hydroperoxides were highly purified, except the linolenic acid hydroperoxide had a detectable amount of what appeared to be epoxide proton absorptions. The epoxide absorptions were not detected after reduction of the hydroperoxide with $NaBH_4$ and isolation of the hydroxyoctadecatrienoic acid by preparative TLC.

The NMR spectrum of 13-hydroperoxyoctadecadienoic acid (Fig. 1A) is similar to one reported for methyl coriolate, methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate, by Tallent et al. (7). The most striking difference between spectra of the two compounds is the downfield displacement of the C-13 proton (0.32 ppm) in the hydroperoxy compound. The absorptions of the protons on carbons-11, -10 and -9 are slightly downfield compared to similar absorptions in methyl coriolate as shown in Table I. Coupling constants are also listed in Table I.

We applied the double resonance technique by which Tallent et al. (7) determined the position of the *cis* and *trans* double bonds in methyl coriolate to identify the geometry of the hydroperoxydiene moiety. Irradiation of the C-13 proton decoupled $H_{12}$ and the doublet-split-doublet collapsed to a doublet ($J_{11,12}$ = 15 Hz). The magnitude of the coupling constant was indicative of a *trans* proton coupling across a double bond. Irradiation of the C-8 methylene protons decoupled $H_9$ and collapsed the triplet-split-doublet to a doublet ($J_{9,10}$ = 11 Hz). The magnitude of this coupling constant is indicative of a *cis* double bond.

The hydroperoxide of linolenic acid, 13-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid, had an NMR spectrum (Fig. 1B) similar to 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid. The $H_{15}$ and $H_{16}$ resonance centered at $\delta$ 5.40 were superimposed on part of the $H_{12}$ and $H_9$ absorptions. Irradiation of $H_{13}$ decoupled it from $H_{12}$ and allowed the measurement of $J_{11,12}$ = 15 Hz despite the interference of the additional vinyl absorptions. This result was identical to that found for linoleic acid hydroperoxide and established the *trans*-11 double bond.

After $NaBH_4$ reduction of linolenic acid hydroperoxide and subsequent isolation of the hydroxyoctadecatrienoic acid from preparative TLC, an NMR spectrum was obtained of the isolate (Fig. 1C). The $H_{12}$ absorption was displaced to lower field simplifying the decoupling experiment, which gave the same result as described for the previous two compounds.

With the aid of double resonance experiments we confirmed the geometry of the hydroperoxydiene for linoleic and linolenic acid hydroperoxides. We also demonstrated the purity of the *cis,trans* configuration in the products since *trans,trans* absorptions were not evident. Linoleic acid hydroperoxide appeared to be much purer than linolenic acid hydroperoxide as we did have some extraneous epoxide absorptions in the isolate of linolenic acid hydroperoxide.

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# Subfractionation of $S_f$ 4-$10^5$, $S_f$ 4-20 and High Density Lipoproteins[1]

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## ABSTRACT

Subfractionation of the total low density $S_f$ 4-$10^5$, the low density $S_f$ 4-20 and high density plasma (or serum) lipoproteins has been accomplished using a cumulative flotation rate procedure. Fractionation employs nonlinear salt gradients and high performance swinging bucket rotors. Subfractionation of the total low density lipoproteins with minimal contamination allows an extremely accurate lipoprotein mass measurement of $S_f > 400$, total very low density lipoproteins and low density lipoproteins (LDL) by elemental CHN analysis. Physical and chemical data on LDL and high density lipoprotein (HDL) subfractions are in general agreement with earlier data. Lower molecular weight data are obtained for HDL subfractions than reported earlier; however this may be the result of the different fractionation procedures used.

## INTRODUCTION

Plasma (or serum) low density lipoproteins (LDL) of the $S_f$ 0-20 class previously have been subfractionated on density gradients (1-3). These studies have shown that with increasing $S_f$ rate, molecular weight increases and hydrated density decreases. The change in hydrated density appears to be primarily the result of changes in protein content. Similarly fractionation of the high density lipoprotein (HDL) spectra on the basis of density (4) has usually given two fractions, $HDL_2$ ($1.063 < \sigma_2 < 1.125$) and $HDL_3$ ($1.125 < \sigma_3 < 1.20$). Again the content of protein appears to be significantly greater (5,6) in the more dense lower molecular weight (7,8) $HDL_3$ fraction.

Previous methodology usually has involved either equilibrium density gradient procedures or two or more preparative stages in angle head rotors, each requiring density manipulation. The present method is an extension of a cumulative flotation rate procedure described earlier (9,10) for the $S_f > 400$ chylomicron containing fraction and the $S_f$ 20-400 very low density lipoproteins (VLDL). The present availability of high performance long radial path swinging bucket rotors allows extension of this method to the much lower molecular weight LDL and HDL spectra. Also subfractionation of the total $S_f$ 4-$10^5$ lipoproteins conveniently separates all the major low density classes and provides a residue fraction of $\sigma > 1.061$ g/ml containing total HDL and the serum proteins.

## METHODS

### $S_f$ 4-20 (LDL) Subfractionation

Before the LDL subfractionation, $S_f > 20$ lipoproteins must first be removed from the plasma. Two milliliter aliquots of plasma are each mixed with 4 ml of 0.195*m* NaCl (containing 10 mg/100 ml EDTA). Six of these 6 ml mixtures are centrifuged at 40,000 rpm at 18 C for 18 hr in a 40.3 Beckman rotor (Beckman Instruments, Inc., Palo Alto, Calif.). A Beckman L2-65 preparative ultracentrifuge, modified for accurate temperature control (± 0.2 C) and equipped with a variable overspeed safety device, was used in all fractionations (10). This consisted of a model 3324 TXA 100 μA full scale contactless relay meter, calibrated to read rotor speed in thousands of rpm (Simpson Electric Co., Chicago, Ill.). The $S_f > 20$ lipoproteins are removed with a special thin-walled Pasteur capillary pipette with an inside bore of 0.4-0.6 mm (Microchemical Specialties, Berkeley, Calif.) and the bottom 2 ml of each preparative tube pooled. The small molecule background density of this $S_f < 20$ plasma is raised to a density (unless otherwise indicated densities are given at 20 C) 1.118 g/ml by adding 2045 mg NaCl to 12 ml of this bottom fraction. Before 2 ml aliquots of this bottom fraction are pipetted into 9/16 in. OD x 3 1/2 in. heavy-walled preparative tubes, a few drops of 1.118 g/ml NaCl solution are added in the bottom. Then a hemispherical insert is placed in the bottom of the tube as shown in Figure 1. These are made by casting a powdered aluminum-epon mixture (27% Al w/w) directly into the preparative tubes. After hardening, the inserts are machined, removed and lapped to equalize their weights to within 20 mg. A lucite sleeve, also fabricated, is inserted into the

[1]Presented in part at AOCS Meeting, New Orleans, April 1970.

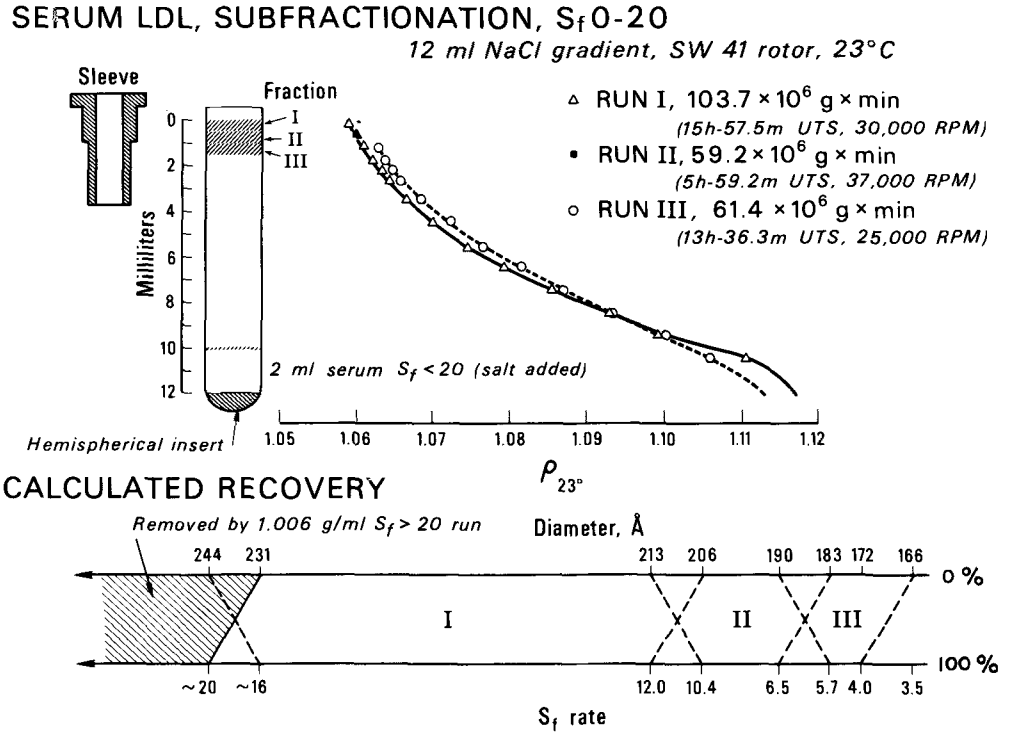


FIG. 1. Sodium chloride density gradient used for plasma (or serum) low density lipoprotein (LDL) subfractionation. The gradient was selected for stability and to allow collection of successive LDL fractions in NaCl background densities appropriate for direct analytic ultracentrifugation, i.e. 1.060-1.063 g/ml. The g x min values listed for each successive centrifugation are equivalent up-to-speed (UTS) time calculated as total UTS time plus one-third (acceleration + deceleration time). On the scales below are given the $S_f$ values and the corresponding lipoprotein diameters which apply to both the threshold and 100% recovery of each fraction. For example fraction I includes 0% (or threshold) concentrations of $S_f$ 20 and $S_f$ 10.4 lipoproteins and 100% recovery of $S_f$ 12-16 lipoproteins. $S_f$ regions included below the right hand diagonal line and between the crossed lines indicate lipoproteins of flotation rates that are partially recovered in adjacent fractions.

preparative tube prior to transfer of the 2 ml $S_f$ < 20 fraction, avoiding contact of the small bore tip pipette with the inside wall of the preparative tube. Contamination on the upper wall of the preparative tube and the occurrence of bubbles in the layered fraction must be scrupulously avoided. If a small bubble is discovered in the layered serum solution it must be removed before overlayering by minimal aspiration with a Pasteur pipette. Optically clear preparative tubes therefore are required. After transfer the tube is rotated to 25° from the horizontal and overlayered with a 10 ml gradient. Solutions are added dropwise in volumes (from highest to lowest density) of 1 ml for the first two solutions and 2 ml for the remainder. Densities are 1.0988, 1.0860, 1.0790, 1.0722, 1.0641 and 1.0588 g/ml, respectively. All salt solutions used in this and the HDL fractionation contain 3 mg EDTA/100 ml. Details of the procedure including gradient preparation and sample collection are as described earlier (10). Fractions of 0.5 ml layers are removed from the top of the tubes after three successive centrifugations in a Beckman SW 41 Ti rotor. It should be emphasized that in all manipulations great care should be exercised to minimize disturbance of the gradients. For example the rotor should be moved slowly and loaded into the centrifuge without jarring. Also the gradients should be set up and the fractions removed as close to the centrifuge as laboratory conditions permit. Running times and calculated $S_f$ recovery of each fraction as well as the gradient and its stability are shown in Figure 1.

The method for calculating centrifugal conditions needed for 100% recovery of lipoproteins of a given $S_f$ rate utilizes interpolated literature values (9,11) for $\sigma$ as a function of $S_f^\circ$ rate. Calculation of the migration rate ($F^\circ$) for any lipoprotein of a given $S_f^\circ$ value in a medium of defined $\rho_j$ and $\eta_j$ is given from the relationship:

$$S_f^\circ = F_j^\circ(\rho_s-\sigma)\eta_j/(\rho_j-\sigma)\eta_s \quad [1]$$

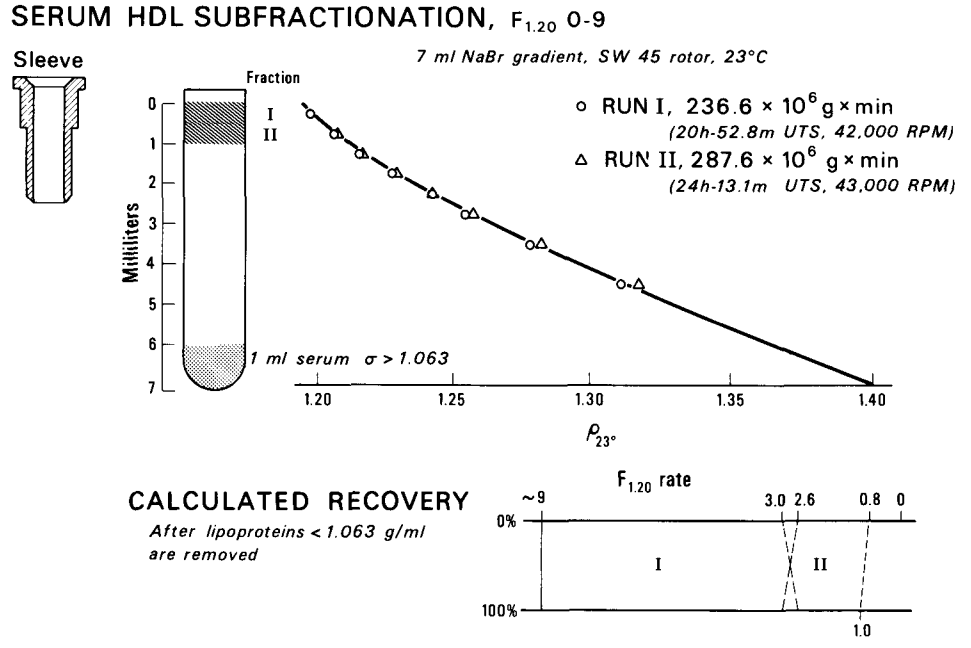


FIG. 2. Sodium bromide density gradient used for plasma (or serum) high density lipoprotein (HDL) subfractionation. All solutions used contain 0.195*m* NaCl and 3 mg/100 EDTA. This gradient was selected to allow collection of HDL subfractions in a NaCl/NaBr medium appropriate for direct analytic ultracentrifugal analysis, i.e., 1.195-1.205 g/ml. See Figure 1 legend for explanations of the recovery calculations (which here refer to those HDL molecules present in the fraction recovered in the 3.5-5.5 ml layer of the preliminary ultracentrifugation).

where standard values at 26 C for 1.744*m* NaCl are $\rho_s$ = 1.0630 g/ml and $\eta_s$ = 1.0260 cp. Since relatively dilute lipoprotein concentrations are involved during fractionation, no $S_f$ vs. concentration dependence corrections are made and the actual F rate is assumed to equal the $F^\circ$ rate at infinite dilution. To further simplify these calculations, each .5 ml volume of the salt gradient is assumed to be a homogeneous region

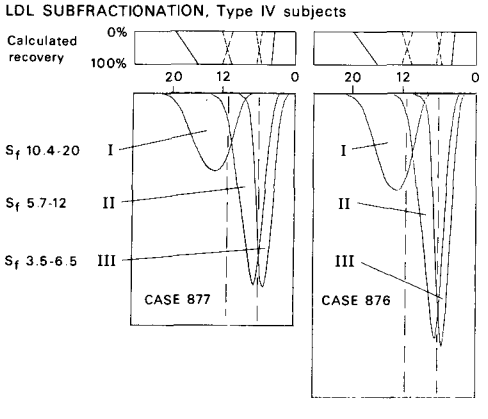


FIG. 3. Corrected schlieren plots of low density lipoprotein (LDL) subfractions for subjects 876 and 877 (linear scale). Calculated recovery is shown above.

(j) of $\rho_j$ and $\eta_j$ whose lower boundary at a radial distance $r_j$ is determined by the rotor and rotor tube. See Figure 3, Reference 12 for a visual presentation of the definitions of threshold (0%) and 100% recovery. For a given g x min run, threshold recovery is defined in terms of the $S_f$ value of a lipoprotein that will migrate from the top of the layered fraction to the lower radial boundary of the .5 ml fraction that is removed. Theoretically no molecules of this $S_f$ value and of lower $S_f$ values float into the recovered fraction. Complete recovery (100%) in the same run is defined in terms of molecules of $S_f$ rate that will migrate from the bottom of the tube to the lower boundary of the recovered fraction. Obviously *all* molecules of this and faster $S_f$ rate are completely recovered. Lipoproteins of $S_f$ value between the threshold and 100% value are partially recovered. For a specified $S_f^\circ$ value equation [1] yields $F_j^\circ$, a close approximation to the actual migration rate $F_j$ through each region (j). Given the centrifugal conditions of rotor rpm and the values for the upper and lower radial boundary of each region j, the time $\Delta t_j$ is determined from:

$$F_j = (\ln r_{j+1} - \ln r_j)/\omega^2 \Delta t_j \qquad [2]$$

TABLE I

Low Density Lipoprotein Subfractionation, Type IV Fasting Subjects

| Case | $S_f$ Range | $\sigma(\rho$ Inter.) g/ml | Peak $S_f^\circ$ rate (svedbergs) | Mol. wt ($10^6$ daltons) | Protein wt % | Phospholipid wt % |
|---|---|---|---|---|---|---|
| | (I) | | | | | |
| 876 | 10.4-20.0 | 1.0079 | 13.5 | 3.04 | 16.9 | 24.8 |
| 877 | | 1.0110 | 13.5 | 3.34 | 17.9 | 22.2 |
| | (II) | | | | | |
| 876 | 5.7-12.0 | 1.0272 | 6.89 | 2.17 | 22.6 | 23.3 |
| 877 | | 1.0281 | 6.87 | 2.25 | 23.8 | 22.6 |
| | (III) | | | | | |
| 876 | 3.5-6.5 | 1.0348 | 4.96 | 1.91 | 25.5 | 22.3 |
| 877 | | 1.0393 | 4.13 | 1.88 | 26.6 | 21.2 |

The total time required for migration through all regions considered is obtained by summing:

$$\omega^2 T = \Sigma_{j=n}^{m} \omega^2 \Delta t_j \quad [3]$$

where T is the total equivalent up-to-speed (UTS) time and n and m are the first and last regions, respectively, appropriate for threshold and 100% recovery. For example in the first LDL run the values for m will be the same in both calculations, whereas the values for n will be 1 and 5 for 100% and 0% recovery, respectively (the 2 ml layered fraction itself constitutes the first four regions in the preparative tube). In a multistage subfractionation, the equivalent UTS time required for each successive run is calculated from the increment of $\omega^2 T$ over the previous state (or stages). Since a 0.5 ml fraction is removed after each run, the last region m is one less than in the preceding run.

Similar calculations are made for the HDL subfractionations utilizing $F_{1.20}^\circ$ vs. $\sigma$ values (Lindgren, unpublished data).

Laboratory equipment, including the rotor, will determine the acceleration and deceleration characteristics to and from full speed. This will allow calculation of the total time at full speed as well as the shut-off time from the start of the run. This latter information is very useful whether or not a Beckman $\omega^2 t$ device is used. We manually accelerate slowly to 1000 rpm, thereafter accelerating at the full rate. We do not use the brake during deceleration.

Centrifugal conditions have all been given here at 23 C; however various laboratory conditions may require fractionations from 20-26 C. An appropriate correction of the total g x min and equivalent UTS times in the amount of plus or minus 2.1% per degree below and above 23 C, respectively, should be made.

The molecular diameters for LDL lipoproteins of certain $S_f^\circ$ values are given assuming Stokes's spheres. They are calculated from the formula:

$$S_f^\circ = d^2(\rho_s - \sigma)/18\eta_s \quad [4]$$

where standard conditions at 26 C in 1.744*m* NaCl are as given above. Values for $\sigma$ appropriate for given $S_f^\circ$ values were interpolated from literature data (9,11).

### $S_f$ 4-$10^5$ Lipoprotein Subfractionation

The above LDL gradient system also may be conveniently used to subfractionate the total low density lipoproteins (TLDL). For example, using whole sera in the above gradient system and centrifuging in three steps, the $S_f > 400$, $S_f$ 20-400 and $S_f$ 4-20 fractions are successively removed as in the LDL fractionation. At 23 C the appropriate centrifugal conditions for each stage are 3.57, 56.5 and 164.2 x $10^6$ g x min, respectively. A reasonable and convenient work cycle is achieved using 1 hr, 14.2 min UTS equivalent at 20,000 rpm; 5 hr, 43.1 min UTS and 16 hr, 36.6 min UTS equivalent at 37,000 rpm, respectively. If a lower $S_f$ rate 100% recovery is desired, for example $S_f 2$, 350.6 x $10^6$ g x min or 35 hr, 31.1 min UTS equivalent at 37,000 rpm is required. The advantage of this procedure is that the three major low density lipoprotein classes of metabolic interest are separated, without density manipulation, in an essentially contamination free medium of defined small molecule compositon.

### HDL Subfractionation

Because of their low molecular weight, subfractionation of HDL is more difficult, requiring a very high performance rotor. Additional complications include the need for long periods of centrifugation at high rotor speeds, suggesting the need for a stable equilibrium type gradient. Since for a given rotor speed the radial path length determines the extent of such an equilibrium gradient as well as the times needed to recover HDL subfractions, reduction in tube path length and increased rotor performance are indicated. A suitable compromise is achieved using a SW 45

TABLE II

Total $S_f$ 4-$10^5$ Low Density Lipoprotein Subfractionation, Fasting Subjects

| Case | $S_f$ Range | Protein wt % | Phospholipid wt % | Serum concentration mg/100 ml |
|---|---|---|---|---|
| 2699 | $S_f > 400$ | --- | --- | 8.5 |
| 2700 | | | | 1.1 |
| 2699 | $S_f$ 20-400 | 9.5 | 20.5 | 468.8 |
| 2700 | | 10.9 | 20.2 | 148.5 |
| 2699 | $S_f$ 4-20 | 24.1 | 24.2 | 342.8 |
| 2700 | | 22.3 | 27.1 | 523.4 |

Ti rotor which can safely run in both the L2-65 and L2-65B machines. This rotor, available from Beckman Instruments, Inc., is equipped with special buckets for .5 x 2.5 in. preparative tubes. To utilize this maximum radial path length, no inserts are used.

An essentially equivalent alternate arrangement can be made using a SW-41 Ti Beckman rotor. On request, the Beckman Company can provide a set of special buckets and an appropriate overspeed rotor decal. This configuration (with the smaller buckets only) can be used in the L2-65B machine and in the L2-65 machine, if the latter is properly equipped with a variable overspeed safety device. We recommend such a safety feature since the HDL fractionation utilizes a maximum preparative tube volume (7 ml) of mean density 1.3 g/ml. Thus the two runs at 42,000 rpm and 43,000 rpm are actually close to the maximum safe rotor speeds. In addition to HDL subfractionation, either of the above rotor-bucket configurations might also be used with smaller serum volumes for both VLDL and LDL subfractionation, with considerable shortening of running times.

Before subfractionation of the HDL spectra the total low density lipoproteins $\sigma < 1.065$ g/ml must be removed first. For this purpose and to achieve concentration of the HDL lipoproteins, three 4 ml aliquots of serum are mixed each with three 2 ml aliquots of a 0.195*m* NaCl and 2.434*m* NaBr solution ($\rho$ = 1.1816 g/ml) which contain 10 mg/100 ml EDTA. After centrifugation for 24 hr at 18 C in a 40.3 rotor, a concentrated fraction containing most of the HDL is removed in the 3.5-5.5 ml layer, measured from the top of the tube. To 6 ml of this fraction 3022 mg of solid NaBr are added bringing the density to approximately 1.395 g/ml. In a manner similar to LDL subfractionation, 1 ml aliquots of this HDL containing fraction are placed in the tubes and overlayered with an approximate equilibrium NaCl/NaBr density gradient. Two 0.5 ml solutions of 1.3622 and 1.3424 g/ml are first added. Then two 1 ml solutions of 1.3161 and 1.2856 g/ml are added, and finally two 1.5 ml solutions of 1.2521 and 1.1973 g/ml complete the 7 ml density gradient. The details of this gradient and calculated recovery are shown in Figure 2.

#### Analytical Ultracentrifugation of Lipoprotein Fractions

Computer analysis of the total low density and high density lipoprotein spectra from the analytic ultracentrifuge schlieren patterns is essentially as described by Ewing et al. (13). Graphical presentation of the schlieren data, including the subfractions, on either a linear or a logarithmic flotation rate scale is as presented by Jensen et al. (14). $S_f^\circ$ (and $F^\circ_{1.20}$) rates, molecular weights and hydrated densities are calculated using $\eta F^\circ$ versus $\rho$ plots, described in detail elsewhere (3). Flotation rates for the low density subfractions were measured at approximately 1.061 and 1.098 g/ml. Flotation rates for the HDL subfractions were measured at approximately 1.200 and 1.286 g/ml. Solid NaCl or NaBr was added to appropriately raise the density of the low and high density subfractions, respectively. In all cases densities and density manipulations were monitored by precision refractometry (15).

#### Elemental CHN and P Analysis

Total lipoprotein mass was determined by CHN elemental analysis (11) and the known elemental composition of the lipoprotein. From a total lipoprotein elemental phosphorus determination (16) and the N/NCH or N/C elemental ratios, protein and phospholipid (PL) content of each lipoprotein fraction was determined (10). All elemental measurements were duplicate analyses and CHN determinations were made using a modified Model 185 CHN analyzer (Hewlett-Packard, Palo Alto, Calif.).

## RESULTS AND DISCUSSION

#### $S_f$ 4-20 (LDL) Subfractionation

LDL subfractionation was performed on

TABLE III

High Density Lipoprotein Subfractionation, Normal Nonfasting Subjects

| Case | $F_{1.20}$ Range | $\sigma(\rho$ Inter.) g/ml | Peak $F^{\circ}_{1.20}$ rate (svedbergs) | Mol. wt (daltons) | Protein wt % | Phospholipid wt % |
|---|---|---|---|---|---|---|
| 879 | (2.7-9.0) | 1.0988 | 4.30 | 265,000 | 43.6 | 33.1 |
| 881 | | 1.0950 | 4.14 | 236,000 | 43.4 | 31.4 |
| 879 | (0.8-3.0) | 1.1437 | 1.73 | 170,000 | 53.9 | 27.0 |
| 881 | | 1.1346 | 1.84 | 148,000 | 55.0 | 25.7 |

plasma from two fasting patients, clinically characterized as type IV hyper-pre-$\beta$-lipoproteinemia (17). Figure 3 shows the corrected schlieren plots of each subfraction from each of the subjects. The observed recoveries agree well with the calculated values. A summary of the physical and chemical data are shown in Table I. These data show that with increasing $S^{\circ}_f$ rate, molecular weight increases and both hydrated density and protein content decrease, the latter confirming earlier data (1). Lee and Alaupovic also have observed consistent decreases in protein content with lower hydrated density among immunochemically heterogeneous LDL subfractions (18). However Mills (19) has reported no correlation between $S^{\circ}_f$ rate of the major component and total LDL protein composition. If protein content increases with decreasing $S^{\circ}_f$ rate of LDL subfractions, the protein content of the total LDL is determined by the lipoprotein profile within intervals of the $S_f$ 0-20 class and not by the $S^{\circ}_f$ rate of the major component. The range of molecular weight from 1.9-3.3 millions for LDL fractions whose major component varied from $S_f$ 4-13 agrees generally with recent physical data on these lipoproteins (3,20,21). However Mauldin and Fisher have reported somewhat higher values (22).

### $S_f$ 4-$10^5$ (TLDL) Subfractionation

Examples of total low density subfractionation from two fasting plasma samples are given in Figure 4. Comparison of the completely recovered total low density spectra and the $S_f$ 20-400 and $S_f$ 4-20 subfractions from the corresponding sera indicate adequate separation at about $S_f$ 20. When the major LDL component is faster than $S_f$ 4, LDL quantification is similar by the two procedures; but where substantial amounts of LDL are below $S_f$ 4 there is incomplete LDL recovery as would be expected. Calculated 0% (or threshold) recovery for this procedure is $S_f$ 3.5, which accounts for the incomplete recovery of $S_f$ 0-4 lipoproteins, particularly in case 2699. Table II presents the elemental data giving the total mass, protein and phospholipid content of the three main low density classes fractionated by this procedure.

### HDL Subfractionation

HDL subfractionation was performed on serum from two normal nonfasting adults. The

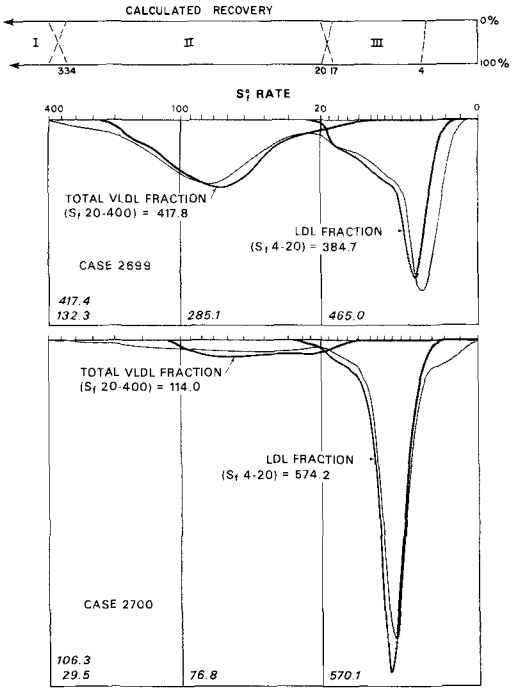


FIG. 4. Comparison of the standard low density spectra (shaded) with the very low density lipoprotein (VLDL) and low density lipoprotein (LDL) subfractions for subjects 2699 and 2700 (logarithmic scale). Calculated recoveries for the subfractions are shown above (see Fig. 1 legend for an explanation of these calculations). Note the favorable comparison between the calculated range of recoveries and the $S_f$ rate distribution actually obtained in the two subfractions. After the last centrifugation and collection of the $S_f$ 4-20 subfraction, most $S_f$ 0-4 lipoproteins are in the 1.5-6 ml region of the gradient, measured from the top. Considering this there is reasonable agreement between the quantitative recovery of the subfractions as compared with the complete lipoprotein recovery obtained in the corresponding standard low density run.

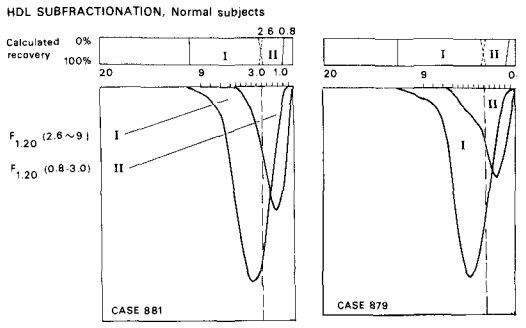


FIG. 5. Corrected schlieren plots of high density lipoprotein (HDL) subfractions for subjects 879 and 881 (linear scale). Calculated recovery is shown above.

corrected schlieren plots for the two subfractions from each subject are shown in Figure 5. Again observed recoveries agree approximately with calculated recoveries. The decision to achieve 100% recovery of $F_{1.20}$ 3.0 molecules was arbitrary. Actually fractionation at $F_{1.20}$ 3.5 or 4.0 would probably yield subfractions somewhat closer in identity to the earlier $HDL_2$ and $HDL_3$ components. It should be observed that the earlier techniques fractionated primarily on the basis of density. In contrast our procedure fractionates essentially on the basis of particle size and therefore the two procedures may be achieving a different type of fractionation within the HDL spectra.

The physical and chemical data are summarized in Table III. These preliminary data show similar hydrated densities but lower molecular weights than earlier data, particularly for the less dense $F_{1.20}$ 3.0-9.0 components (6-8). These discrepancies, in part, may be the result of the different fractionation procedures used. However chemical composition, and in particular protein content (44-55%), is in general agreement with the data based on the older $HDL_2$-$HDL_3$ subfractionation. In order to compare procedures in the future, it will be necessary to determine the density heterogeneity of each subfraction acquired by "cumulative flotation rate" separations.

#### Potential Applications and Comparison of Procedures

The earlier techniques have certain advantages in that the use of angle head rotors simplifies methodology and at the same time permits fractionation on a larger scale. However many of these angle-rotor fractionations, particularly for HDL, have involved serial centrifugations to avoid contamination from albumin and other nonlipoprotein protein (23,24). On the other hand our procedure provides lipoprotein fractions in a medium of defined small molecule composition with minimal contamination from other plasma lipoproteins and proteins. There is also essentially no contamination from the plasma small molecule components. For example serum fractions containing essentially no LDL and no HDL (1.063 and 1.21 g/ml bottom fractions) were subjected to the LDL and HDL gradient procedures, respectively. No contamination by either large or small molecules was detected by elemental CHN analysis of the first three milliliter fractions. Any contamination, if present, was below the error of measurement, approximately 1 mg/100 ml (25). Where contamination is important, as in immunological and peptide studies, such density-gradient fractionation may help define more accurately the characteristics of the protein moiety within subfractions of all the plasma lipoproteins. For example such studies might help determine the fate of apoproteins during catabolism of LDL. Other potential applications include resolving lipoprotein differences between type III and type IV hyperlipoproteinemias. In addition the "cumulative flotation rate" procedure is flexible, allowing the collection of two or more subfractions of any desired flotation rate interval within the important LDL and HDL spectra. Another useful example of this flexibility is in TLDL subfractionation where all three major classes are separated. An analogous fractionation of chylomicra + VLDL and LDL using a zonal rotor has been reported earlier (26). Further, where very large fractions from a single plasma sample are needed, the general techniques described here might be used in a zonal rotor (26), but with reduced resolution.

ACKNOWLEDGMENTS

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# Long Term Human Studies on the Lipid Effects of Oral Calcium

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## ABSTRACT

Ingestion of 2 g of supplemental dietary calcium carbonate daily over a period of one year by eight hyperlipemic men and two hyperlipemic women caused a significant 25% decrease in serum cholesterol, after these subjects had shown stable levels for the previous year, and when compared to a group without therapy. Body weights for both groups remained stable throughout the period of observation. The experimental group also showed a reversal in the cholesterol-phospholipid ratio from a preexperimental ratio of 1.04 to a ratio of 0.92. In addition there was a 113 mg per 100 ml decrease in serum triglycerides and a 48 mg per 100 ml decrease in serum phospholipids, but these were not statistically significant due to the large between individual variations and the limited sample size. Calcium carbonate should be considered as a potential agent for usage in long term studies designed to produce hypolipemia, since it appears to be effective and without significant side effects.

## INTRODUCTION

Previous three-week studies in man (1) demonstrated the short term hypocholesterolemic and hypotriglyceridemic action of supplemental dietary calcium. In experiments with rats it was noted that the calcium appeared to act by increasing fecal bile acids, fecal 3-β-hydroxysterols and fecal free fatty acids (2,3) and not by a redistribution of lipids between the blood and tissue pools. More recently, experiments in rabbits have indicated that increased dietary calcium could significantly decrease the severity of atheroma formation when the rabbits were fed a relatively high fat-high cholesterol diet (4). In view of these findings, the question of whether the hypolipemic effect of supplemental dietary calcium in humans was transient or sustained assumed more importance, since there appeared to be a good therapeutic potential for this agent. This is a report of 12 months' experience with 10 ambulatory volunteers taking supplemental dietary calcium to provide a more prolonged experience.

## METHODS

Both the experimental and control groups were selected from 20 successive patients exhibiting either hypercholesterolemia, hypertriglyceridemia or mixed hyperlipemia. The experimental subjects were eight men and two women ranging in age from 26 to 61 years. The degrees of elevation in serum lipids were determined in accordance with the age adjusted levels recommended by Fredrickson et al. (5). Three subjects had previously documented myocardial infarctions and three others suffered from angina pectoris. Lipoprotein phenotyping indicated that the experimental group consisted of five phenotype II and five pheno-

TABLE I

Effect of Supplemental Dietary Calcium Upon Serum Cholesterol (mg/100 ml)

| Patient | Phenotype | 12 months before study | At start of study | After 6 months' supplemental dietary calcium | Per cent change[a] | After 12 months' supplemental dietary calcium | Per cent change[a] |
|---|---|---|---|---|---|---|---|
| 1 | IV | 328 | 316 | 297 | - 6.0 | 274 | -13.3 |
| 2 | IV | 410 | 435 | 324 | -25.5 | 272 | -46.7 |
| 3 | II | 506 | 525 | 375 | -28.6 | 360 | -31.4 |
| 4 | IV | 374 | 393 | 306 | -22.1 | 293 | -25.4 |
| 5 | IV | 297 | 307 | 257 | -16.3 | 270 | -12.5 |
| 6 | II | 350 | 372 | 255 | -31.5 | 269 | -27.7 |
| 7 | II | 257 | 247 | 213 | -13.8 | 206 | -16.6 |
| 8 | IV | 246 | 244 | 241 | - 1.2 | 224 | - 8.2 |
| 9 | II | 266 | 276 | 184 | -33.4 | 187 | -32.2 |
| 10 | II | 400 | 375 | 324 | -13.6 | 326 | -13.1 |
| Mean ± S.D. | | 343 ± 25.9 | 349 ± 28.3 | 278 ± 18.3 | -20.8 | 262 ± 16.6 | -24.9 |

[a]Compared to values at start of study.

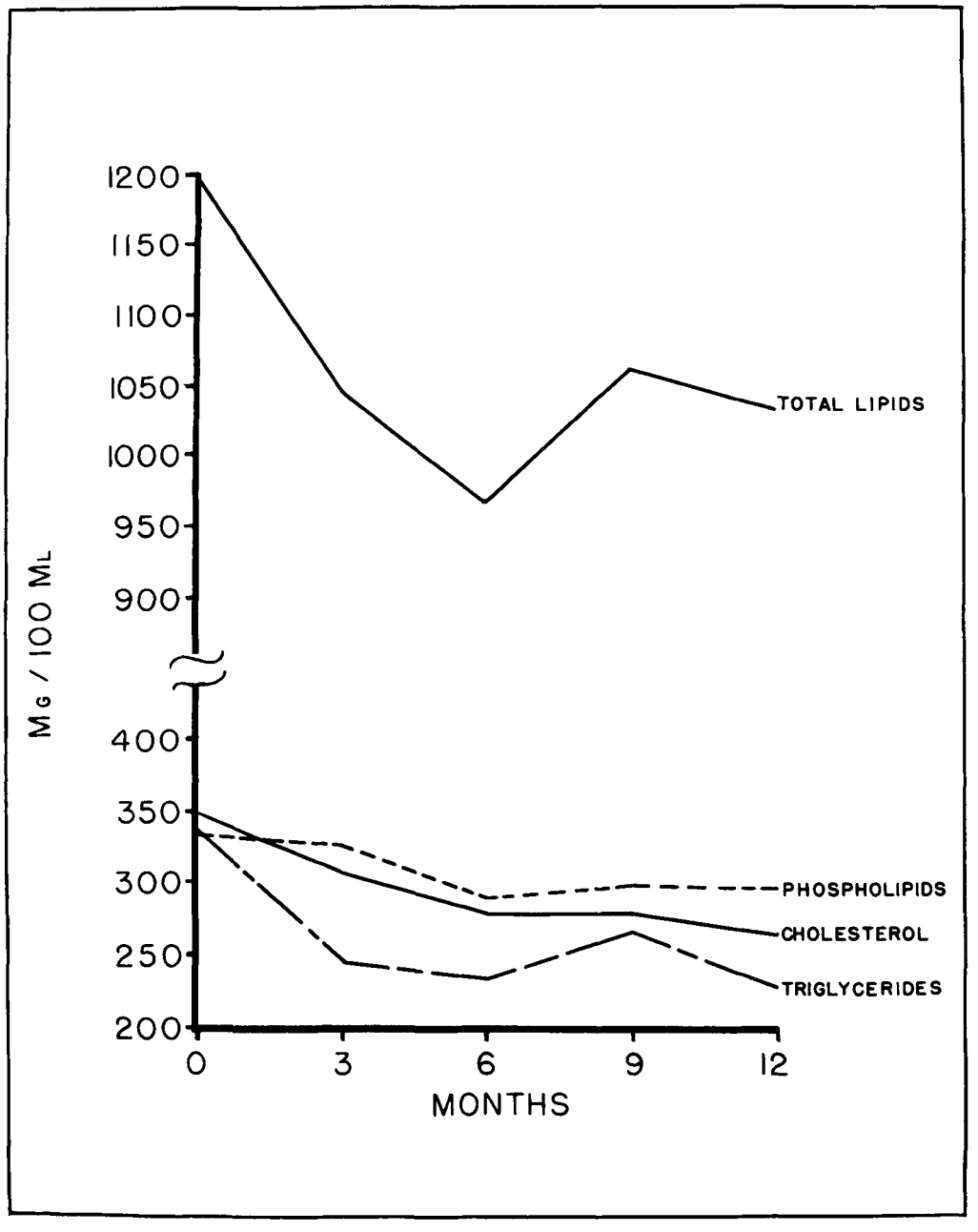


FIG. 1. Effect of supplementary oral calcium upon serum lipids in man.

type IV individuals. The control subjects consisted of eight males and two females ranging in age from 33 to 63 years. Two of this group had previous myocardial infarctions and four suffered from angina pectoris. This group consisted of four phenotype II subjects and six phenotype IV individuals. The alternate assignment of the subjects to either treatment or control group produced an essential balance in lipoprotein abnormalities, but due to the small group size a considerable difference in group cholesterol levels was present at outset.

All subjects consumed the typical American dietary pattern as determined by repetitive seven-day diet histories and dietary recall evaluations. The experimental subjects ingested 2 g

TABLE II

Serum Cholesterol Levels (mg/100 ml) in Comparison Group

| Patient | Phenotype | 12 months before study | At start of study | Per cent change[a] | After 12 months | Per cent change[a] |
|---|---|---|---|---|---|---|
| 11 | IV | 240 | 248 | + 3.3 | 252 | + 5.0 |
| 12 | II | 280 | 280 | 0 | 308 | +10.0 |
| 13 | IV | 253 | 262 | + 3.6 | 242 | - 4.3 |
| 14 | II | 325 | 351 | + 7.7 | 341 | + 4.9 |
| 15 | IV | 244 | 223 | - 8.6 | 235 | - 3.7 |
| 16 | II | 280 | 283 | + 1.1 | 268 | - 4.3 |
| 17 | IV | 310 | 310 | 0 | 315 | + 1.6 |
| 18 | IV | 276 | 280 | + 1.4 | 232 | -15.9 |
| 19 | IV | 250 | 250 | 0 | 252 | + 0.8 |
| 20 | II | 272 | 258 | - 5.1 | 265 | - 2.6 |
| Mean ± S.D. | | 273 ± 8.8 | 275 ± 11.4 | + 0.7 | 271 ± 22.8 | - 0.7 |

[a]Compared to initial value, 12 months before start of study.

calcium carbonate (Eli Lilly and Co.) daily, subdivided into four doses. No other medications with reported effect upon lipid metabolism were administered during this period. Patients with angina pectoris were given nitroglycerine.

Medical examinations and biochemical studies were conducted quarterly for one year, and similar data were available for one year prior to initiation of the study. Blood was sampled after a 14 hr fast; the serum was separated and stored at -20 C for analysis. Analysis was always performed within 72 hr. Serum total lipids, phospholipids, total cholesterol and triglycerides were assayed by previously described procedures (6,7) with standard errors of analysis of 20.0, 2.58, 2.22 and 2.53 mg per 100 ml respectively. Serum glutamic oxalacetic transaminase was determined by the Reitman-Frankel colorimetric method (8). Urine was examined by standard clinical semiquantitative procedures.

## RESULTS

Mean body weights for both experimental and comparison groups are as follows: the study group started at 157.2 lb, after 12 months weighed 157 lb, and after an additional 12 months while on therapy with calcium carbonate showed a mean weight of 156.9 lb; for the comparison group, mean weights at the same intervals were 158.3, 157.2 and 158.8, respectively. There was no significant fluctuation for either group and no significant difference between the two groups.

The effect of supplemental dietary calcium upon serum cholesterol is shown in Table I. The mean preexperimental serum cholesterol level was 349 ± 28.3 mg per 100 ml, showing very little change from the level of 343.0 ± 25.9 mg per 100 ml of one year earlier. After therapy for six months the mean serum cholesterol had decreased (61.4 ± 4.73 mg per 100 ml, $P<0.01$) to 278 ± 18.3 mg per 100 ml. At the end of the

TABLE III

Effect of Supplemental Dietary Calcium Upon Serum Triglycerides (mg/100 ml)

| Patient | Phenotype | 12 months before study | At start of study | After 6 months' supplemental dietary calcium | Per cent change[a] | After 12 months' supplemental dietary calcium | Per cent change[a] |
|---|---|---|---|---|---|---|---|
| 1 | IV | 235 | 228 | 213 | - 6.6 | 180 | -21.1 |
| 2 | IV | 875 | 1500 | 950 | -36.7 | 590 | -60.7 |
| 3 | II | 118 | 69 | 105 | +52.2 | 72 | + 4.3 |
| 4 | IV | 575 | 615 | 323 | -47.5 | 400 | -35.0 |
| 5 | IV | 210 | 202 | 115 | -43.1 | 108 | -46.5 |
| 6 | II | 95 | 88 | 73 | -17.0 | 151 | +71.6 |
| 7 | II | 122 | 112 | 49 | -56.3 | 122 | + 8.9 |
| 8 | IV | 290 | 257 | 343 | +33.5 | 330 | +28.4 |
| 9 | II | 110 | 102 | 90 | -11.8 | 66 | -35.3 |
| 10 | II | 106 | 96 | 128 | +33.3 | 109 | +13.5 |
| Mean ± S.D. | | 273.6 ± 81.12 | 327.0 ± 384.2 | 248.9 ± 85.4 | -23.9 | 212.8 ± 53.6 | -34.9 |

[a]Compared to values at start of study.

TABLE IV

Serum Triglyceride Levels (mg/100 ml) in Comparison Group

| Patient | Phenotype | 12 months before study | At start of study | Per cent change[a] | After 12 months | Per cent change[a] |
|---|---|---|---|---|---|---|
| 11 | IV | 452 | 400 | -11.5 | 412 | - 8.8 |
| 12 | II | 108 | 96 | -11.1 | 113 | + 4.6 |
| 13 | IV | 205 | 226 | +10.2 | 232 | +13.2 |
| 14 | II | 105 | 104 | - 1.0 | 106 | + 1.0 |
| 15 | IV | 224 | 227 | + 1.3 | 350 | +56.3 |
| 16 | II | 197 | 212 | + 7.6 | 244 | +23.9 |
| 17 | IV | 272 | 250 | - 8.1 | 265 | - 2.6 |
| 18 | IV | 470 | 435 | - 7.4 | 410 | -12.8 |
| 19 | IV | 308 | 290 | - 5.8 | 320 | + 3.9 |
| 20 | II | 68 | 73 | + 7.4 | 60 | -11.8 |
| Mean ± S.D. | | 240.9 ± 43.6 | 231.3 ± 38.6 | - 4.2 | 251.2 ± 39.9 | + 4.2 |

[a]Compared to initial value, 12 months before start of study.

year the mean serum cholesterol exhibited a further decrease to 262 ± 16.6 mg per 100 ml, a reduction of 23 mg per 100 ml from the level at six months. The total decrease over the entire year was 84.44 ± 16.6 mg per 100 ml ($P<0.01$). It should be noted that every subject exhibited a decrease in serum cholesterol, with the total over the one-year period being 25%.

The cholesterol changes for the comparison group are shown in Table II. No significant changes were noted during the entire period of observation. In addition, although the study group started at a higher initial level, ingestion of calcium carbonate for 12 months brought the cholesterol level below that of the comparison group.

All the serum lipid data for the study group are graphically depicted in Figure 1. The preexperimental mean serum triglycerides were 327 ± 384 mg per 100 ml (Table III). After six months there was a mean decrease in serum triglycerides of 78 mg per 100 ml to 249 ± 85.4 mg per 100 ml. At the end of a year there was a further mean decrease of 36 mg per 100 ml to 213 ± 53.6 mg per 100 ml. The total decrease for the one year experimental period was 114 mg per 100 ml. This decrease, although highly suggestive of the possible hypotriglyceridemic action of supplemental dietary calcium, did not achieve statistical significance due to the extremely large variability in preexperimental serum triglyceride levels and the variability of the response. The variation in serum triglycerides in the control group appeared to be essentially random (Table IV).

Serum phospholipids decreased from a preexperimental level of 336 ± 17.4 mg per 100 ml to 288 ± 25.5 mg per 100 ml after six months and then were essentially stable for the remainder of the experimental period. This constituted a mean decrease of 48 mg per 100 ml. Again, although the results are highly suggestive, statistical significance was not achieved due to the large variability.

An apparent decrease was noted in serum total lipids from a preexperimental mean level of 1196 mg per 100 ml to a level of 1030 mg per 100 ml after one year. Again this decrease of 166 mg per 100 ml was only suggestive and was not statistically significant due to the large variation in preexperimental total serum lipid values which ranged from 750 to 2150 mg per 100 ml, and the still relatively small number of 10 subjects.

For the comparison group the serum triglycerides, phospholipids and total lipids showed no significant change during the 24 month observation period. This finding added further to the impression of stability of serum lipids without intervention as had been noted for the serum cholesterol level.

An examination of the curves for total serum cholesterol and phospholipids for the study group indicated a rapid reversal in the cholesterol to phospholipid ratio. This reversal occurred within the first three months on supplemental calcium. The preexperimental ratio was 1.04. There was a steady decline to 0.97 at six months and a further decline to 0.94 at the end of the year's observation. The standard deviation of this decline was 0.027 and the decline in the cholesterol-phospholipid ratio was significant at $P<0.01$.

Examination of serum glutamic oxalacetic transaminase for study subjects indicated no significant elevation of level and examination of the urine elicited no case of alkaline urine nor any other abnormality throughout the observation period.

## DISCUSSION

The 25% decrease in serum total cholesterol noted in this study is in agreement with

previously reported findings of Yacowitz et al. (1) in humans in 21-day experiments, and with those of Fleischman et al. (2,3) in experiments lasting as long as six months in rats. When the significant decrease in serum cholesterol is coupled with the highly suggestive decreases in serum phospholipids and serum triglycerides, it would appear that supplemental dietary calcium is an effective and a relatively safe hypolipemic agent suitable for larger scale studies in humans.

Of particular interest is the possible biological meaning of the highly significant reversal of the cholesterol-phospholipid ratio noted in the presence of supplemental dietary calcium. It has been shown (9) that as the density of the serum lipoproteins increases, the ratio of phospholipids to other lipids also increases. Thus, it is reasonable to assume that there was an increase in the relative concentration of the higher density alpha lipoproteins and a decrease in the relative concentration of lower density beta and pre-beta lipoproteins with time on supplemental dietary calcium. Whether the decreases noted in serum lipids and the reversal of the cholesterol-phospholipid ratio could prevent or retard atheroma formation in humans is still unanswered. In rabbits, Yacowitz et al. (4) demonstrated a decrease in severity of atheroma formation with increasing dietary calcium. Epidemiological studies by Schroeder (10), Morris et al. (11), Biörck et al. (12), Crawford and Crawford (13) and Bierenbaum et al. (14), among others, have shown a negative correlation between cardiovascular disease and the mineral content of drinking water, particularly calcium. These epidemiological studies have not definitively demonstrated that the calcium of the water acts through the lipids to decrease cardiovascular disease morbidity and mortality. Thus larger and longer term experiments evaluating the effect of supplemental dietary calcium upon the morbidity and mortality of atherosclerotic heart disease appear to be in order. Initial work in this direction is now being done.

ACKNOWLEDGMENT

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# Lipid Synthesis by Rat Lung in Vitro[1]

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## ABSTRACT

Oxidation and lipogenesis in isolated rat lung tissue were studied in vitro. The minced tissue was incubated in a Krebs-Ringer bicarbonate buffer (pH 7.4) with 1-$^{14}$C-acetate, 2-$^{14}$C-pyruvate, U-$^{14}$C-D-glucose, 1,5-$^{14}$C-citrate, 1-$^{14}$C-laurate, 1-$^{14}$C-palmitate, 1-$^{14}$C-stearate, 1-$^{14}$C-oleate, 1-$^{14}$C-linoleate. The lung tissue readily oxidized all of these substrates to $^{14}CO_2$ and incorporated them into $^{14}$C-lipids with the exception of 1,5-$^{14}$C-citrate, for which there was no significant incorporation into $^{14}$C-lipids. Most of the lipid $^{14}$C was recovered in phospholipids, more specifically phosphatidyl choline. Twenty-eight per cent of glucose carbons was incorporated into the fatty acid moiety of phospholipids, while more than 90% of the carbons of other substrates was found in phospholipid fatty acids. The main fatty acid of the phospholipid fraction synthesized from acetate, pyruvate or glucose was palmitic acid. The oxidation of fatty acids was apparently influenced both by the carbon chain length and number of double bonds. Accumulation of $^{14}$C-fatty acids in the tissue was observed when fatty acids were used as substrates; this finding suggests that the rate limiting step was not in the uptake of fatty acids. Chemical degradation of $^{14}$C-myristic and palmitic acids obtained by hydrolysis of phospholipids biosynthesized from 1-$^{14}$C-laurate indicated that the phospholipid fatty acids were synthesized via the de novo synthesis pathway.

## INTRODUCTION

It is known that the presence of a surface active material (surfactant) in mammalian lung is essential for maintaining aveolar stability (1). Pattle reported that the surfactant is a lipoprotein complex with a high phospholipid (PL) content (2), and the PL is predominately in the form of dipalmityl lecithin (3,4). A number of investigators have demonstrated the synthesis of lipids by mammalian lung tissue from acetate (5-9), glucose (6,9), glycerol (7,10), fatty acids (6,9,10), amino acids and other precursors (9-12). All of these studies indicated that the lung is an active site for lipid metabolism.

The present work was designed to study and compare the synthesis of lipids from various substrates and to determine the possible pathway by which fatty acids, particularly palmitic acid, were synthesized in the lung tissue.

## MATERIALS AND METHODS

### Animals

Normal fed male rats of Sprague-Dawley strain weighing 250-300 g were used. The animals were killed by decapitation. The lung tissues were promptly removed and placed in ice cold Krebs-Ringer bicarbonate buffer (KRB) (13) and minced with scissors.

### Incubation of Tissues

The incubation system consisted of KRB buffer; pH 7.4, 0.3 g minced lung tissue, 4 μmoles $^{14}$C-labeled substrate with a specific activity of 0.25 μCi per μmole, 2 μmoles glucose and 60 mg bovine serum albumin (BSA) to a final volume of 3 ml and placed in a stoppered plastic vial saturated with 95% $O_2$-5% $CO_2$ gas phase. Incubation was carried out in a Dubnoff metabolic shaker at 37 C for 1 hr. Fatty acids used as substrate were complexed with BSA by a modification of the method of Fillerup et al. (14).

### Analysis of Lipids

At the end of incubation, the mixture was centrifuged. The tissue was washed and extracted for lipids by the method of Folch et al. (15). After determination of $^{14}$C activity of total lipids they were separated into neutral lipids (NL) and phospholipids (PL) by silicic acid column chromatography (16). NL and PL were further separated by thin layer chromatography (TLC) (17,18). The lipid spots on TLC plates were scraped directly into glass counting vials for measurement of radioactivity. The recovery of radioactivity in all cases was more than 90%. Free fatty acids (FFA) were separated from NL by TLC. PL were hydrolyzed with 10% ethanolic potassium hydroxide by refluxing at 100 C for 1 hr under an atmosphere of nitrogen using 0.02% hydroquinone as an antioxidant. Fatty acids (FA) were extracted from the acidified hydrolysate and

[1]Presented at the AOCS-ISF World Congress, Chicago, September 1970.

TABLE I

Conversion of Various $^{14}C$-Substrates Into Lipids and $CO_2$ by Minced Rat Lung

| Substrate | $CO_2$ | | Lipids | |
|---|---|---|---|---|
| | μmoles/g/hr[a] (mean±SE) | %[b] | μmoles/g/hr[a] (mean±SE) | %[b] |
| 1-$^{14}C$-Acetate | 1.29±0.15 (3)[d] | 32.3 | 0.28±0.05 (6)[c] | 6.8 |
| U-$^{14}C$-D-Glucose | 1.59±0.23 (3) | 39.4 | 0.25±0.04 (8) | 6.2 |
| 2-$^{14}C$-Pyruvate | 1.23±0.17 (3) | 30.8 | 0.14±0.03 (5) | 3.5 |
| 1-$^{14}C$-Laurate | 0.21±0.02 (3) | 5.5 | 0.20±0.02 (6) | 4.6 |
| 1-$^{14}C$-Palmitate | 0.16±0.03 (3) | 4.1 | 0.80±0.05 (4) | 19.4 |
| 1-$^{14}C$-Stearate | 0.11±0.02 (3) | 2.7 | 0.52±0.07 (4) | 12.8 |
| 1-$^{14}C$-Oleate | 0.17±0.02 (3) | 4.3 | 0.55±0.04 (4) | 13.4 |
| 1-$^{14}C$-Linoleate | 0.18±0.02 (3) | 4.4 | 0.49±0.05 (4) | 12.2 |
| 1,5-$^{14}C$-Citrate | 0.15±0.03 (3) | 3.8 | <0.01 (3) | <0.2 |

[a]μMoles of substrate per gram wet tissue per hour.
[b]Per cent of initial amount of substrate utilized.
[c]Figures in parentheses represent the number of experiments.

methylated by the method of Metcalfe and Schmitz (19). The radioactivity of individual fatty acid methyl esters in a sample was measured directly by continuous monitoring with a radio-gas flow proportional detector (Nuclear Chicago model 4998) connected to the exit port of the thermal conductivity detector of a model 810 F & M gas chromatograph. Degradation of FA was carried out by the method of Dauben and coworkers (20). $CO_2$ production was measured by using a hyamine trap (21) in separate experiments not designed for lipid analysis. Radioactivity was assayed in a Packard liquid scintillation spectrometer operating at 75% efficiency using fluors suggested by Davidson and Feigelson (22) or Bray (23).

1-$^{14}C$-acetate, 2-$^{14}C$-pyruvate, U-$^{14}C$-D-glucose, 1,5-$^{14}C$-citrate, 1-$^{14}C$-palmitic, 1-$^{14}C$-stearic, 1-$^{14}C$-oleic and 1-$^{14}C$-linoleic acid were purchased from New England Nuclear Corp., Boston, Mass. 1-$^{14}C$-lauric acid was purchased from Amersham/Searle Corp., Arlington Heights, Ill. These materials were tested for purity by TLC and gas liquid chromatography (GLC), and were found to be 99% pure or better.

## RESULTS

### $CO_2$ Production and Lipid Synthesis

Table I summarizes the results of $CO_2$ production and lipid synthesis from various substrates in the lung. It can be seen that all substrates were oxidized to $CO_2$. When acetate, glucose and pyruvate were used, more than 30% of the initial substrate was oxidized in 1 hr. The $CO_2$ production ranged from 2.7-5.5% in experiments using fatty acids as substrates. The $CO_2$ production from citrate was also within this range.

With the exception of citrate, all substrates were incorporated into lung lipids. In experiments using long chain fatty acids, especially palmitate, as substrates, there was a greater amount of $^{14}C$ incorporated into complex lipids than in those using acetate, glucose and pyruvate. The incorporation of laurate $^{14}C$ into lung lipids was low compared to that from other fatty acids. Palmitate as a substrate ranked first in the total amount of lipid synthesized; it was followed by oleate, stearate, linoleate, acetate, glucose, laurate and pyruvate. The $CO_2$ to lipid ratio was 5:1, 6.5:1 and 9:1 when acetate, glucose and pyruvate were used as substrates, respectively. The $CO_2$ to lipid ratios were much smaller when fatty acids were used: 1.4:1, 1:3.0, 1:3.2, 1:2.3 and 1:1.5 for laurate, palmitate, stearate, oleate and linoleate, respectively.

### Fractions of Lung Lipids

Table II shows the synthesis of phospholipids and neutral lipids. All substrates were incorporated mainly into PL and to a less extent into NL. When palmitate was used, the NL fraction accounted for only 10% of the $^{14}C$ in the total tissue lipids. The NL fraction had 18-24% of the total tissue lipid $^{14}C$ when other fatty acids were used. The $^{14}C$-PL to $^{14}C$-NL ratios were 5.5:1, 6.8:1 and 8.0:1 when $^{14}C$-acetate, $^{14}C$-glucose and $^{14}C$-pyruvate were used, respectively. This ratio (4.6:1) was greater when $^{14}C$-palmitate was used as a substrate as com-

TABLE II

Incorporation of Various Substrates Into Lung Lipid Fractions[a]

| Substrates | Phospholipids | | Neutral lipids | | Free fatty acids | |
|---|---|---|---|---|---|---|
| | μmoles/gm/hr[b] | %[c] | μmoles/gm/hr[b] | %[c] | μmoles/gm/hr[b] | %[c] |
| 1-$^{14}$C-Acetate | 0.21 | 75 | 0.038 | 13 | 0.032 | 12 |
| U-$^{14}$C-D-Glucose | 0.21 | 84 | 0.031 | 12 | 0.009 | 4 |
| 2-$^{14}$C-Pyruvate | 0.12 | 86 | 0.015 | 8 | 0.009 | 6 |
| 1-$^{14}$C-Laurate | 0.10 | 50 | 0.047 | 24 | 0.052 | 26 |
| 1-$^{14}$C-Palmitate | 0.39 | 50 | 0.084 | 10 | 0.326 | 40 |
| 1-$^{14}$C-Stearate | 0.26 | 50 | 0.093 | 18 | 0.168 | 32 |
| 1-$^{14}$C-Oleate | 0.27 | 50 | 0.116 | 20 | 0.162 | 30 |
| 1-$^{14}$C-Linoleate | 0.24 | 50 | 0.110 | 22 | 0.135 | 28 |

[a]Total lipids were separated by silicic acid column chromatography, neutral lipids were eluted with chloroform and phospholipids with methanol.

[b]μMoles substrate per gram wet tissue per hour. Results are average of four experiments.

[c]Per cent of total lipids calculated from (radioactivity of individual lipid fraction per radioactivity of total lipids) x 100.

pared to those in experiments using other FA as substrates. There was accumulation of FFA in the tissue when fatty acids were used as substrates.

Further analysis of PL and NL by TLC showed the phosphatidyl choline (PC) contained the major amount of $^{14}$C in the PL fraction ranging from 73-80% of the total PL$^{14}$C activity. Seven to twelve per cent of PL label was recovered in the phosphatidyl ethanolamin fraction. Sphingomyelin, Lyso-PC and other PL accounted for the remaining 10-15% of PL $^{14}$C activity. In NL the distribution of $^{14}$C label was mainly found in triglycerides, ranging from 60-80% of the total NL radioactivity. The remaining 20-40% of NL label was distributed in the other NL; in the decreasing order of diglycerides, monoglycerides, cholesterol and cholesterol esters.

### Distribution of Radioactivity in Lung Phospholipid Fatty Acids

Ninety to ninety-five per cent of the PL label was recovered in the FA moiety for all substrates with the exception of U-$^{14}$C-D-glycose, in which the recovery in the FA moiety was 29%. Further analysis of FA methyl esters of lung PL by GLC revealed that acetate, glucose and pyruvate were converted primarily to palmitic acid. Most of the FA substrates were apparently incorporated directly into PL. Ninety-seven per cent of the $^{14}$C in the phospholipid fatty acids was in palmitic acid when palmitate was used. More than 85% of $^{14}$C in the PL fatty acids was in the form of the substrate used in the case of $^{14}$C-stearic, $^{14}$C-oleic and $^{14}$C-linoleic acids. In addition 6.5-13.5% of the activity in PL fatty acids was in palmitic (Table III). Of the $^{14}$C of lung PL fatty acids, 46.5 and 11.8% was in palmitic and myristic acids, respectively, when laurate was used as a substrate; only 37.5% of the $^{14}$C of PL fatty acids was lauric.

### Chemical Degradation of Fatty Acids Biosynthesized From 1-$^{14}$C-Laurate in Rat Lung

Carboxyl carbons of $^{14}$C myristic and palmitic acid biosynthesized from 1-$^{14}$C-laurate were degradated. For both $^{14}$C myristic and palmitic acid the ratio of the specific activity of average FA carbons to that of carboxyl carbon was 1:2.1.

## DISCUSSION

The results of the present study confirm and extend the findings of other investigators (5-10) that rat lung is an active site for oxidation of various substrates to $CO_2$ and incorporation of them into complex lipids, mostly PL. However it is difficult to compare the quantitative relationship in $CO_2$ production and lipid incorporation from acetate, glucose and pyruvate, since these substrates used in this study were labeled at different positions. When 1-$^{14}$C-pyruvate was incubated, it was recovered only as $^{14}CO_2$ but not as $^{14}$C-lipids (Wang and Meng, unpublished results). The $CO_2$ production from acetate, glucose and pyruvate indicated active operation of glycolytic and tricarboxylic acid cycle in the lung tissue.

As expected, $CO_2$ production was much less and incorporation into complex lipids was much greater from FA substrates than those from acetate, glucose and pyruvate. Among all FA tested, oxidation of laurate was the highest

TABLE III

Distribution of $^{14}C$ in Fatty Acids of Phospholipids Synthesized From Various Substrates[a]

| Fatty acids | Composition of normal rat, %[c] | $^{14}C$-Substrate | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Acetate | Glucose | Pyruvate | Laurate | Palmitate | Stearate | Oleate | Linoleate |
| 12:0[b] | 5 | 7.3[d] | 6.3 | 9.1 | 37.5 | Trace | 1.0 | 1.3 | Trace |
| 14:0 | | | | | 11.8 | | | | |
| 16:0 | 42 | 87.5[e] | 90.2 | 86.0 | 46.5 | 97.0 | 6.5 | 13.5 | 10.3 |
| 16:1 | 5 | | | | | | | | |
| 18:0 | 14 | 4.5 | 3.3 | 4.2 | 4.5 | Trace | 91.5 | Trace | 1.2 |
| 18:1 | 15 | --- | --- | --- | --- | --- | --- | 85.5 | --- |
| 18:2 | 8 | --- | --- | --- | --- | --- | --- | --- | 88.3 |
| 18:3 | 2 | --- | --- | --- | --- | --- | --- | --- | --- |
| 20:4 | 10 | --- | --- | --- | --- | --- | --- | --- | --- |

[a]Values are expressed as per cent of total $^{14}C$-fatty acids incorporated into phospholipids (average of two experiments).

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

[c]Fatty acid composition of normal rat lung phospholipids.

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

[e]Combination of 16:0 and 16:1.

followed by linoleate, oleate and palmitate. Incorporation of palmitate into complex lipids was the highest and is significantly greater than those from other fatty acids. Laurate was the poorest substrate for complex lipid synthesis.

In the experiments using U-$^{14}C$-glucose as a substrate, we were unable to confirm the findings reported by Felts (6) who studied the complex lipid synthesis by rabbit lung slices. Felts showed that relatively little $^{14}C$ was recovered in $CO_2$ from U-$^{14}C$-glucose as compared to $^{14}C$-acetate, and that over 90% of PL radioactivity was in the glycerol moiety. Results of the present study showed a slightly higher recovery of $^{14}CO_2$ from U-$^{14}C$-glucose than that from 1-$^{14}C$-acetate, and ca. 25-30% of PL radioactivity was in the FA moiety. The differences could be due to species (rabbit vs. rat) or substrate concentration effect. The poor recovery of FA radioactivity from PL indicates the conversion of glucose carbons to glycerol moiety of PL by providing $\alpha$-glucerophosphate for esterification of FA. However we were not able to demonstrate increased utilization of $^{14}C$-glucose by the addition of palmitate and acetate in our system (Wang and Meng, unpublished data). In contrast to glucose, the carbons of acetate and pyruvate were incorporated into long chain FA, principally palmitic acid of PL (Table III).

The accumulation of FA in the lung tissue using FA as substrates suggests that the rate limiting step for incorporation of complex lipids is not the uptake of FA by the tissue. The finding of the high PL to NL ratio suggests greater synthesis of PL than NL.

It is interesting that 97% of Phospholipid FA-$^{14}C$ was palmitic with only a trace as myristic and stearic, when palmitate was used as a substrate. Decreasing per cent of stearic linoleic and oleic acids with increasing per cent of shorter carbon chain fatty acids including palmitic in phospholipids was observed when linoleate, stearate and oleate were used. The presence of a high per cent of palmitic and myristic acid in phospholipids seems to indicate that fatty acids of long carbon chains are directly incorporated into PL after activation and esterification, and this is true especially for palmitic acid. In addition, palmitic, stearic, linoleic and oleic acids are also degraded to C-2 units from which palmitic acid may be synthesized. However when laurate was used as a substrate, high percentage of PL fatty acids as palmitic and myristic, but relatively low percentage of lauric was observed (Table III). Palmitic acid may be synthesized from FA of short chain length by either addition of acetyl CoA to the preexisting short chain FA (24) or by de novo synthesis from acetyl CoA, which is the product of $\beta$-oxidation (25) of the preexisting FA. Long chain FA may be converted to palmitic acid either by direct cleavage of one or more C-2 units or by de novo synthesis from the product of $\beta$-oxidation, acetyl CoA, via the malonyl CoA pathway (26). Tombropoulos (27) reported that the lung mitochondria-rich fraction was the most active subcellular fraction in synthesizing long chain FA from $^{14}C$-1-acetate. Schiller and Bensch (28) found that the supernatant fraction from rabbit lung homogenates actively synthesizes long chain FA. They

suggested that the lung mitochondrial fraction synthesizes long chain FA via elongation pathway while the supernatant fraction is the de novo FA synthesizing site. Our finding of the degradation of palmitic and myristic acids recovered from the lung tissue in experiments using 1-$^{14}$C-laurate as a substrate indicate that myristic and palmitic acids in PL were synthesized via de novo pathway. It seems reasonable to conclude that 1-$^{14}$C-laurate is degraded by $\beta$-oxidation to acetyl CoA. The resulting acetyl CoA is either oxidized to $CO_2$ or converted to long chain FA.

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# Lipid Composition of Further Purified Bovine Liver Nuclear Membranes[1]


## ABSTRACT

The lipid content, distribution and fatty acid composition of highly purified bovine liver nuclear membranes was determined and compared to those of microsomes prepared in parallel. Contrasted with microsomes, nuclear membranes while containing nearly the same levels of lipid had more cholesterol and total neutral lipid and less phospholipid. Phospholipid and neutral lipid patterns generally were similar for the two types of membranes. The same fatty acids, in similar proportions, were observed in respective total lipid, total polar lipid, phosphatidyl choline and phosphatidyl ethanolamine fractions of the two membrane types. The microsomal lipid fractions contained slightly greater percentages of unsaturated fatty acids. With respect to previous results from preparations contaminated with nonmembranous nuclear material, purified fractions contained more total lipid on a protein basis and more total unsaturated fatty acids. Only minor differences in levels and distribution of phospholipids and neutral lipids were observed between the crude and highly purified fractions.


We recently reported details of the lipid composition of nuclear membranes isolated from bovine liver (1). Kleinig (2) and Franke et al. (3) have investigated the lipid composition of both rat and pig liver nuclear membranes, and Lemarchal and Bornens (4) as well as Kashnig and Kasper (5) have reported observations on lipid distribution in rat liver nuclear membranes. Although our original isolation procedure yielded highly purified nuclear membrane fractions with respect to mitochondrial and microsomal contamination (6), subsequent investigation revealed the preparations to be contaminated with nonmembranous nuclear constituents such as nucleoli (7,8). This procedure has recently been modified to yield a more highly purified membrane fraction, and it appeared worthwhile to examine the lipid composition of this preparation (7,8). These analyses are reported herein along with data on microsomal preparations prepared in parallel.

Crude nuclear membranes were obtained from purified nuclei as described (6). This fraction was purified further by centrifugation in a discontinuous sucrose density gradient (7). Mitochondrial contamination was assessed by assays for succinoxidase and succinate-cytochrome *c* reductase activities, and microsomal contamination was estimated by measuring NADPH-cytochrome *c* reductase activity (7). Contamination of the nuclear membrane fraction by mitochondria was consistently less than 4% on a protein basis and by microsomes was consistently less than 8% (7). Electron microscopic examination revealed the absence of nucleoli fragments (7). Microsomal and mitochondrial fractions were isolated by dif-

[1]Purdue University AES Journal Paper No. 4482.

TABLE I

Composition of Nuclear Membranes and Microsomes Isolated from Bovine Liver

| Constituent | Nuclear membranes[a] | Microsomes[b] |
|---|---|---|
| Total lipid, mg/mg protein | 0.52 ± 0.05 | 0.52 |
| Lipid phosphorus, μg/mg lipid | 26.0 ± 1.26 | 35.2 |
| Total cholesterol, μg/mg lipid | 53.2 ± 2.23 | 34.1 |
| Phospholipid, % of total lipid | 64.9 ± 1.26 | 82.7 |
| Sialic acid, mμM/mg protein | 6.09 | 17.0 |

[a]Values are means ± standard deviations of four preparations, except sialic acid, which was determined with one preparation.

[b]Average values for two preparations.

TABLE II

Lipid Composition of Nuclear Membranes and Microsomes from Bovine Liver

| Component | Per cent of total lipid phosphorous | | Per Cent of total lipid | |
|---|---|---|---|---|
| | Nuclear membranes[a] | Microsomes[b] | Nuclear membranes[c] | Microsomes[c] |
| Sphingomyelin | 3.5 ± 0.82 | 3.9 | | |
| Phosphatidyl choline | 55.6 ± 3.12 | 57.6 | | |
| Phosphatidyl serine | 4.0 ± 0.73 | 2.7 | | |
| Phosphatidyl inositol | 10.0 ± 1.57 | 11.9 | | |
| Phosphatidyl ethanolamine | 21.1 ± 2.94 | 22.5 | | |
| Lysophosphatidyl choline | 2.7 ± 0.42 | 1.5 | | |
| Cardiolipin | 3.1 ± 0.38 | --- | | |
| Diglyceride | | | 0.6 | 0.3 |
| Cholesterol | | | 10.2 | 3.2 |
| Free fatty acid | | | 14.8 | 5.9 |
| Triglyceride | | | 7.7 | 4.7 |
| Methyl ester | | | --- | 2.1 |
| Cholesterol ester | | | 4.7 | 1.0 |

[a]Means ± standard deviations for four preparations.

[b]Averages for two preparations.

[c]Values from a representative preparation.

ferential centrifugation as described previously (6).

Methods of lipid extraction (9), separation by column (10) and thin layer chromatography, and gas chromatographic analysis of fatty acid methyl esters (1) have been described in detail. Methods for phosphorus and protein determination were those used previously (1). Cholesterol was determined according to Stadtman (12). Distribution of neutral lipids was estimated by densitometry (13), using a quantitative reference mixture (Hormel Institute, Austin, Minn.) to correct for deviations from linearity. Sialic acid was determined in the residue remaining after extraction of lipids according to Warren (14).

Nuclear membranes and microsomes contained the same amount of lipid, 0.52 mg/mg protein (Table I). Whereas the amount of lipid recovered from nuclear membrane fractions was consistent, considerable difference was observed between the two microsomal fractions analyzed (0.4 and 0.65 mg lipid per mg protein). Nuclear membranes contained considerably more cholesterol and less phospholipid than did microsomes. Glycolipids were not detected in nuclear membrane preparations by thin layer chromatographic analysis nor by reacting lipid extracts with anthrone reagent (15). Trace amounts of cerebrosides were detected in microsomal lipid extracts, suggesting the presence of low levels of plasma membranes in this fraction (11). Microsomal membranes contained much more sialic acid than did nuclear membranes (Table I). Compared to rat and pig liver nuclear membranes, those from bovine liver contain larger amounts of cholesterol, less phospholipid and more total lipid (3). These purified fractions had lower levels of total lipid than did crude fractions although the per cent of phospholipids was nearly identical (1).

The lipid distribution patterns are given in Table II. With one exception, nearly identical distribution of phospholipids was observed for nuclear membranes and microsomes. Phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol were the major components. Smaller amounts of phosphatidyl serine, sphingomyelin and lysophosphatidyl choline were also detected. Other lysophospholipids and phosphatidic acid were not observed. Nuclear membranes, but not microsomes, contained a component tentatively identified as cardiolipin by virtue of its identical mobility with authentic cardiolipin according to both one- and two-dimensional thin layer chromatography. In addition this component had a fatty acid composition similar to that of cardiolipin from bovine liver mitochondria treated in the same manner as were the nuclear membranes. The maximum contamination of our nuclear membrane fraction by mitochondria is 4%, and thus the level of cardiolipin encountered, 3.1% of the total lipid phosphorus, is too high to be accounted for by mitochondrial contamination (the phospholipid fraction of bovine liver mitochondria contains 18% cardiolipin) (16). Conclusive identification of this component as cardiolipin awaits further work. These phospholipid distribution patterns are similar to those observed with crude bovine liver nuclear mem-

TABLE III

Fatty Acid Composition of Major Lipids from Bovine Liver Nuclear Membranes and Microsomes

| Fatty acid | Total lipid | | Total polar lipid | | Phosphatidyl choline | | Phosphatidyl ethanolamine | |
|---|---|---|---|---|---|---|---|---|
| | NM[a] | M[a] | NM | M | NM | M | NM | M |
| 14:0 | 1.5 | 1.0 | 1.2 | 0.9 | 0.5 | 0.3 | 1.3 | 0.8 |
| 16:0 | 16.5 | 14.2 | 15.4 | 12.1 | 18.3 | 14.3 | 8.1 | 9.6 |
| 16:1 | 1.8 | 1.8 | 1.5 | 1.1 | 1.0 | 1.1 | 0.7 | 0.5 |
| 18:0 | 33.1 | 24.8 | 34.3 | 29.6 | 29.5 | 28.7 | 31.6 | 28.0 |
| 18:1 | 21.1 | 20.9 | 19.7 | 19.3 | 23.7 | 22.0 | 17.4 | 18.7 |
| 18:2 | 8.2 | 10.0 | 8.9 | 10.2 | 10.6 | 10.2 | 8.8 | 11.7 |
| 18:3 | 1.6 | 1.3 | 1.4 | 1.2 | 1.4 | 1.2 | 0.9 | 1.2 |
| 20:3 | 4.4 | 8.8 | 4.7 | 8.8 | 5.2 | 8.7 | 4.1 | 5.2 |
| 20:4 | 6.2 | 8.5 | 7.0 | 8.4 | 4.8 | 5.6 | 14.9 | 12.9 |
| 20:5 | 0.5 | 0.7 | 1.0 | 0.7 | 0.5 | 0.5 | 1.4 | 0.7 |
| 22:4[b] | 1.9 | 3.2 | 1.2 | 3.1 | 1.4 | 2.9 | 4.3 | 4.1 |
| 22:5[b] | 2.8 | 4.4 | 3.1 | 4.2 | 2.7 | 4.1 | 5.8 | 5.9 |
| 22:6 | 0.3 | 0.5 | 0.7 | 0.5 | 0.4 | 0.3 | 0.6 | 0.7 |
| Total unsaturated | 48.8 | 61.0 | 49.1 | 57.4 | 51.7 | 56.7 | 59.0 | 61.6 |

[a]Abbreviations: NM, nuclear membranes; M, microsomes.

[b]Tentative identifications.

branes (1) and with rat and pig liver nuclear membranes (2).

The distribution of neutral lipids in nuclear membranes and microsomes was similar in that cholesterol, free fatty acids and triglycerides were the major components. Both contained low levels of cholesterol esters and diglycerides. Microsomes contained significant quantities and nuclear membranes only trace amounts of a component migrating with methyl esters on thin layer separation. Presumably these were formed from exogenous methanol via the action of an acylase enzyme during the initial stages of lipid extraction.

Data on the fatty acid composition of total lipid, total polar lipid, phosphatidyl choline and phosphatidyl ethanolamine fractions in nuclear membranes and microsomes are presented in Table III. Somewhat higher total amounts of unsaturated acids were present in all microsomal lipid fractions compared to the respective fraction from nuclear membranes. There was a high degree of similarity in that all lipid fractions contained the same fatty acids in roughly equal proportions in the two membranes. Myristic, palmitic and stearic acids were the only saturated fatty acids encountered in appreciable proportions (greater than 0.2% of the total). Oleate, linoleate, dodecatrienoate and arachidonate were the major unsaturated acids encountered. Low but appreciable amounts of acids tentatively identified as 22:4 and 22:5 also were observed. Only trace amounts of eicosahexaenoate were present in the fractions analyzed. Although the same major fatty acids were present in both purified and crude nuclear membrane fractions, the former had higher total percentage of unsaturated acids (1).

Microsomal membranes are derived largely from endoplasmic reticulum, a membrane which shows certain ultrastructural similarities to the nuclear membrane (17). Direct continuity between the outer nuclear membrane and the endoplasmic reticulum has been observed (18), and this continuity along with ultrastructural studies of developing endoplasmic reticulum has given rise to the hypothesis of direct derivation of endoplasmic reticulum from nuclear membrane during early development (19,20). The observed similarities in distribution and fatty acid composition of phospholipids may be a reflection of the structural and biogenetic relationship between these membranes. The membranes do differ, however, in neutral lipid content and this may reflect specializations of the respective membranes. The phospholipid distribution of both membranes resembles that observed with rat and pig liver microsomes and nuclear membranes (2) as well as ribosomes-studded endoplasmic reticulum from rat liver (11). This phospholipid distribution pattern differs from that of the other liver cytomembranes which have been characterized, Golgi apparatus and plasma membranes (11). The major difference is that the latter membranes contain elevated levels of sphingomyelin and lower levels of phosphatidyl choline. The significance of these differences in cytomembrane differentiation has been dis-

cussed elsewhere (11,21).

Although the lipid patterns determined in the present study compare favorably with those determined with our initial crude preparations (1), some differences in fatty acid composition are evident. The major differences are a lower total percentage of unsaturated fatty acids in the total lipid fraction and higher total unsaturated fatty acid percentages in phosphatidyl choline in the purified membrane fraction. Whether these differences represent exclusion of lipids from nonmembranous nuclear material in the purified fraction or simply variation among animals cannot be determined at present.

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# Stereospecific Analysis of Triglycerides From *Monnina emarginata* Seed Oil[1]

**ABSTRACT**

In *Monnina emarginata* seed oil triglycerides, more than two-thirds of the *S*-coriolic (13L-hydroxy-*cis*-9,*trans*-11-octadecadienoic) acid occurs in position 3 (relative to *sn*-glycerol). All but a trace of the remainder is in position 1.

Our previous work has shown that triglycerides of *Monnina emarginata* seed oil incorporate some unusual oxygenated fatty acids (1), the most prominent of which is *S*-coriolic (13L-hydroxy-*cis*-9, *trans*-11-octadecadienoic) acid (30% by weight). Subsequently (2), we established that these oxygenated acids are combined in a complex array of glyceride structures including some with estolide linkages. (The term estolide, as used here, means any compound which may be derived by esterifying the hydroxyl group of a hydroxy acid with any other acid.) Practically all of the *S*-coriolic acid (97%) is bonded at the α-positions in monocoriolins. We now summarize efforts to achieve a Brockerhoff-type stereospecific analysis of these unusual triglycerides.

Brockerhoff's Grignard procedure (3) was not applicable to *M. emarginata* triglycerides because of the constituent estolide linkages and free hydroxyl groups. The Grignard procedure also gave unsatisfactory results when applied to methoxytriglycerides because the derived methoxydiglycerides were only partially separated from the concomitant monoglycerides. Conse-

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

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In *Monnina emarginata* seed oil triglycerides, more than two-thirds of the *S*-coriolic (13L-hydroxy-*cis*-9,*trans*-11-octadecadienoic) acid occurs in position 3 (relative to *sn*-glycerol). All but a trace of the remainder is in position 1.

Our previous work has shown that triglycerides of *Monnina emarginata* seed oil incorporate some unusual oxygenated fatty acids (1), the most prominent of which is *S*-coriolic (13L-hydroxy-*cis*-9, *trans*-11-octadecadienoic) acid (30% by weight). Subsequently (2), we established that these oxygenated acids are combined in a complex array of glyceride structures including some with estolide linkages. (The term estolide, as used here, means any compound which may be derived by esterifying the hydroxyl group of a hydroxy acid with any other acid.) Practically all of the *S*-coriolic acid (97%) is bonded at the α-positions in monocoriolins. We now summarize efforts to achieve a Brockerhoff-type stereospecific analysis of these unusual triglycerides.

Brockerhoff's Grignard procedure (3) was not applicable to *M. emarginata* triglycerides because of the constituent estolide linkages and free hydroxyl groups. The Grignard procedure also gave unsatisfactory results when applied to methoxytriglycerides because the derived methoxydiglycerides were only partially separated from the concomitant monoglycerides. Conse-

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

TABLE I

Stereospecific Analysis of *Monnina emarginata* Triglycerides[a]

| Acid | Weight % attached to various positions of *sn*-glycerol | | | |
|---|---|---|---|---|
| | Position 1 | Positions 2 + 3 | Position 2[b] | Position 3[c] |
| 14:0 | 0.4 | 0.1 | 0.1 | 0.2 |
| 15:0 | 0.2 | --- | --- | --- |
| 16:0 | --- | --- | --- | --- |
| 16:1 | 11.1 | 2.7 | 0.9 | 4.5 |
| 18:0 | 6.2 | 1.8 | 0.4 | 3.1 |
| 18:1 | 33 | 30 | 35 | 25 |
| 18:2 | 31 | 45 | 63 | 27 |
| 18:3 | 2.4 | 0.5 | 1.3 | --- |
| 20:0 | 0.4 | 0.4 | --- | 0.8 |
| 18:3 conjugated[d] | 15.3 | 20 | 0.6 | 39 |
| 18:1 keto | 0.9 | 0.2 | --- | 0.4 |

[a]Carried out after coriolate moieties had been converted to methoxydienoate groups.

[b]These figures represent a weighted average of values for Fractions B-3 and D as given in Table II, Reference 2. They were computed thus: $\frac{(\%\text{ in B-3})\ (0.37) + (\%\text{ in D})\ (0.27)}{0.64}$; the coefficients are from Figure 3, Reference 2.

[c]These values were computed by subtracting position 2 from positions 2 + 3. (Values for positions 2 + 3 are doubled in this computation [7].)

[d]This component is the major product formed by thermolysis of the methoxydiene esters in the injection port of the chromatograph. The percentages are based on gas liquid chromatographic data to which an empirically determined correction factor (1.38 for R-446 column and 2.6 for Apiezon L) has been applied. A sample of methyl esters from another seed oil, known to contain 49% $C_{18}$ conjugated triene, was used to determine the correction factors.

quently use of pancreatic lipase (EC 3.1.1.3) was a more satisfactory method for generating the diglycerides required for stereospecific analysis. Thin layer chromatographic (TLC) separations of reaction products, which are so essential to this overall scheme of analysis, were complicated by the unusual nature of *Monnina* triglycerides. Recoveries were undesirably low, apparently because some unusual components were more strongly adsorbed than those from "ordinary" triglycerides; in addition there was a problem with overlap of components which required repeated TLC separations in certain cases.

In the manner described previously (2), *M. emarginata* seed oil (0.744 g) was treated with a sulfuric acid-methanol-ethyl ether solution to cleave estolide linkages and to etherify the free dienol groupings. The recovered methanolysis product was fractionated on 1 mm layers of silica with petroleum ether-ethyl ether(2:1)as the developing solvent and yielded four fractions: Fraction A, 0.239 g (41%), estolides and common triglycerides in a 1:1 ratio, as judged by IR; Fraction B, 0.284 g (49%), triglycerides whose original coriolate moieties had been converted to methoxydienoate groupings; Fraction C, 0.034 g (6%), a mixture; Fraction D, 0.023 g (4%), diglycerides with hydroxyacyl groups that were nonallylic (hence unreactive in the etherification reaction).

Thin layer chromatographic Fraction B was hydrolyzed with pancreatic lipase by a procedure slightly different from that described by Luddy et al. (4,5). The reaction products were fractionated by TLC under the conditions indicated above. The residual, unreacted triglycerides (50% of recovered products) were hydrolyzed with another portion of pancreatic lipase. The diglycerides, mixed with free fatty acids from the two hydrolyses, were treated with excess ethereal diazomethane, and the diglycerides were freed from the resulting methyl esters by TLC.

The diglycerides derived from Fraction B (68 mg) were phosphorylated with phenylphosphoryl dichloride, and the resulting phosphatides were hydrolyzed with phospholipase A (EC 3.1.1.4) as described previously (6). The reaction products from this hydrolysis were analyzed as in Reference 6. Methyl esters of methoxydienoic acids were thermolyzed to conjugated trienes during gas liquid chromatography and were measured as such. A suitable reference standard was used to provide an estimate of column losses of these trienes, and the operating conditions were similar to those in Reference 6. Data from these analyses are summarized in Table I.

Although experimental difficulties limit the accuracy of the values in Table I, these data provide a basis for certain conclusions. *Monnina*

triglycerides are quite unsymmetrical with respect to the positional distribution of coriolic acid. Disregarding the portion attached via estolide linkages, at least two-thirds of the coriolic acid occurs in position 3 with most of the rest in position 1 and no more than a trace in position 2. There are, necessarily, quantitative differences in distribution of the more conventional acids, but none of them show such a marked preference for a single position. A similar stereospecific analysis carried out on TLC Fraction A likewise revealed some quantitative differences in the positional preferences of the nonoxygenated fatty acids.

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# Effects of Changes in the Major Carbon Source on the Fatty Acids of *Euglena gracilis*

**ABSTRACT**

*Euglena gracilis* was cultured under both heterotrophic and phototrophic growth conditions using ethanol, glucose or $CO_2$ as the major carbon source. Total fatty acid analyses indicated that ethanol produced more highly unsaturated acids than did glucose under both growth conditions. Growth in the light on $CO_2$ yielded a very high content of 18:3, 16:3 and 16:4 (33%), compared to ethanol (11%) or glucose (10%). These two preformed carbon sources enhanced the content of the $C_{20}$ and $C_{22}$ polyenes compared to $CO_2$, and growth in the dark on ethanol caused a further increase in these polyenes. Growth in the dark on glucose caused only a slight increase of the $C_{20}$ and $C_{22}$ polyenes compared to growth in the light on this carbon source. When the fatty acid patterns of the two dark-grown heterotrophs were compared, two observations were quite evident. First, there was a two-fold increase in the saturated acids in the cells grown on glucose. This was largely due to myristic acid. Second, the $C_{20}$ and $C_{22}$ polyenes were almost twice as concentrated in the cells grown on ethanol.

The adaptability to various environments of the phytoflagellate *Euglena gracilis* has been well documented (1), and dramatic changes in the fatty acid patterns have been correlated with environmental conditions. Bloch and his coworkers (2-4) as well as Rosenberg's group (5,6) have been primarily interested in changes brought about by the presence or absence of light. The present communication reports the effects which changes in the major carbon source have on the fatty acid composition of *E. gracilis* grown either in the dark or in the presence of light.

Cultures of *E. gracilis* were obtained either from the culture collection of algae, Indiana University, Bloomington, Indiana, or as a gift from J. Kahn, North Carolina State University, Raleigh, N.C. The cells were cultured on a rotary shaker at 24 C in a modified Hutner's medium (7) using either ethanol, glucose or $CO_2$ as the primary carbon source. Malic and glutamic acids were present in the medium, but their function was mainly to help buffer the pH at 3.3. The dark-grown cultures were carefully shielded from the light, while the light-grown cultures were illuminated as previously described (8). Only the flasks containing the cells grown on $CO_2$ were flushed continuously with a 5% $CO_2$-in-air gas mixture. The cells were

triglycerides are quite unsymmetrical with respect to the positional distribution of coriolic acid. Disregarding the portion attached via estolide linkages, at least two-thirds of the coriolic acid occurs in position 3 with most of the rest in position 1 and no more than a trace in position 2. There are, necessarily, quantitative differences in distribution of the more conventional acids, but none of them show such a marked preference for a single position. A similar stereospecific analysis carried out on TLC Fraction A likewise revealed some quantitative differences in the positional preferences of the nonoxygenated fatty acids.

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**ABSTRACT**

*Euglena gracilis* was cultured under both heterotrophic and phototrophic growth conditions using ethanol, glucose or $CO_2$ as the major carbon source. Total fatty acid analyses indicated that ethanol produced more highly unsaturated acids than did glucose under both growth conditions. Growth in the light on $CO_2$ yielded a very high content of 18:3, 16:3 and 16:4 (33%), compared to ethanol (11%) or glucose (10%). These two preformed carbon sources enhanced the content of the $C_{20}$ and $C_{22}$ polyenes compared to $CO_2$, and growth in the dark on ethanol caused a further increase in these polyenes. Growth in the dark on glucose caused only a slight increase of the $C_{20}$ and $C_{22}$ polyenes compared to growth in the light on this carbon source. When the fatty acid patterns of the two dark-grown heterotrophs were compared, two observations were quite evident. First, there was a two-fold increase in the saturated acids in the cells grown on glucose. This was largely due to myristic acid. Second, the $C_{20}$ and $C_{22}$ polyenes were almost twice as concentrated in the cells grown on ethanol.

The adaptability to various environments of the phytoflagellate *Euglena gracilis* has been well documented (1), and dramatic changes in the fatty acid patterns have been correlated with environmental conditions. Bloch and his coworkers (2-4) as well as Rosenberg's group (5,6) have been primarily interested in changes brought about by the presence or absence of light. The present communication reports the effects which changes in the major carbon source have on the fatty acid composition of *E. gracilis* grown either in the dark or in the presence of light.

Cultures of *E. gracilis* were obtained either from the culture collection of algae, Indiana University, Bloomington, Indiana, or as a gift from J. Kahn, North Carolina State University, Raleigh, N.C. The cells were cultured on a rotary shaker at 24 C in a modified Hutner's medium (7) using either ethanol, glucose or $CO_2$ as the primary carbon source. Malic and glutamic acids were present in the medium, but their function was mainly to help buffer the pH at 3.3. The dark-grown cultures were carefully shielded from the light, while the light-grown cultures were illuminated as previously described (8). Only the flasks containing the cells grown on $CO_2$ were flushed continuously with a 5% $CO_2$-in-air gas mixture. The cells were

TABLE I

The Total Fatty Acid Composition of *Euglena gracilis* Grown in the Light or in the Dark on Different Carbon Sources

| | Light | | | Dark | |
|---|---|---|---|---|---|
| Fatty acid | Ethanol | Glucose | $CO_2$ | Ethanol | Glucose |
| 12:0 | 1[a] | --- | --- | 1 | 2 |
| 13:0 | 3 | 2 | --- | 1 | 3 |
| 14:0 | 10 | 7 | 3 | 8 | 23 |
| 15:0 | 1 | 2 | 2 | tr | 2 |
| 15:1[b] | --- | --- | 6 | --- | --- |
| 16:0 | 9 | 16 | 6 | 9 | 12 |
| 16:1 | 3 | 7 | 5 | 3 | 5 |
| 16:2 | 2 | 2 | 7 | tr | 1 |
| 16:3 | 4 | 3 | 6 | 1 | 1 |
| 16:4 | 1 | 1 | 9 | 1 | tr |
| 18:0 | 1 | 1 | tr[c] | 1 | 2 |
| 18:1 | 8 | 14 | 3 | 8 | 7 |
| 18:2 | 3 | 2 | 7 | 2 | 3 |
| 18:3 | 6 | 6 | 18 | 2 | 2 |
| 20:2 | 5 | 2 | 3 | 8 | 3 |
| 20:3ω6 | 9 | 3 | tr | 6 | 6 |
| 20:4ω6 | 16 | 13 | 8 | 20 | 12 |
| 20:4ω3[b] | 2 | 2 | 1 | 4 | 3 |
| 20:5ω3 | 4 | 5 | 6 | 9 | 4 |
| 22:4ω6 | 4 | 5 | tr | 3 | 4 |
| 22:5ω6 | 6 | 4 | 5 | 9 | 3 |
| 22:5ω3 | 1 | 1 | tr | 1 | 1 |
| 22:6ω3 | 2 | 3 | 4 | 5 | 1 |
| Saturates | 25 | 28 | 11 | 20 | 42 |
| Monoene | 11 | 21 | 14 | 11 | 12 |
| Polyene | 65 | 53 | 75 | 71 | 44 |
| $C_{20}$ | 36 | 26 | 19 | 47 | 28 |
| $C_{22}$ | 13 | 13 | 9 | 18 | 9 |

[a]The fractional percentages were rounded to the next highest. The numbers are weight per cent.

[b]Tentative identification.

[c]tr = trace or under 0.4%.

harvested in late logarithmic phase of growth by centrifugation and washed with distilled water.

The washed cells were sonicated and extracted according to the procedure of Bligh and Dyer (9). When butylated hydroxytoluene was used as an antioxidant, it was removed by thin layer chromatography prior to the gas chromatographic analysis of the fatty acids. Methyl esters were prepared from the total extract by the procedure of Metcalf et al. (10), and the gas chromatographic analysis was performed on a Perkin-Elmer Model 900 gas chromatograph equipped with dual hydrogen flame ionization detectors. The two 12 ft x 0.08 in. ID analytical columns were packed with 12% EGSS-Y coated on 100/120 mesh Gas Chrom Q (Applied Science Labs.). The column temperature was 205 C. Calibration was done using a quantitative fatty acid mixture obtained from The Hormel Institute, Austin, Minn. The various peaks were identified by comparison with known standards and by using plots of the logarithm of the retention time versus the number of carbon atoms in the hydrocarbon chain. Overlap presented some problems in quantitating 16:3, 16:4, 18:0 and 18:1; therefore a 2% OV-17 column, 6 ft x 0.08 in. ID operating at 185 C, was used to separate the $C_{16}$ group from the $C_{18}$ group. The effluent stream was split and each group was collected and reinjected onto the EGSS-Y column to obtain the percentage composition. This calculation for percentage composition was done by the peak height-retention time method of Carroll (11).

A comparison of the $C_{20}$ and $C_{22}$ polyene composition (Table I) indicated that in either the light or in darkness, the more highly oxidized carbon source (ethanol) resulted in a higher total percentage of these unsaturated acids. The cells grown in the light on $CO_2$ contained the lowest percentage (28%) of these fatty acids of any of the cells studied. It should be pointed out that the cells grown on $CO_2$ contained the highest total percentage (75%) of

polyenoic fatty acids, and that this additional 47% consisted mainly of linolenate and the $C_{16}$ triene and tetraene which have been related to photosynthesis (4). However the $C_{16}$ and $C_{18}$ dienes constituted 14% of this total. The most concentrated single fatty acid of the $CO_2$ grown cells was α-linolenate which made up 18% of the total. This is in concert with the findings of others (2-6); however these researchers tended to find a higher percentage of this acid. The next most concentrated acids were 16:4 (9%) and 20:4ω6 (8%).

Two differences were noted when comparing the fatty acid composition of the light-grown heterotrophs. First, the monoenes were twice as concentrated in the cells grown on glucose, and second, the $C_{20}$ polyenes were 1.4 times as concentrated in the cells grown on ethanol. The polyene differences in the ethanol-grown cells were primarily due to 20:2, 20:3ω6 and 20:4ω6, whereas both monoenes (16:1 and 18:1) were elevated to about the same extent in the glucose-grown cells.

When the cells were grown in the dark, a quite different fatty acid pattern resulted from growth on ethanol as compared to glucose. Probably the most striking difference was that the $C_{20}$ and $C_{22}$ polyenes of the cells grown on ethanol were almost double the concentration of those from the cells grown on glucose. This difference was due mainly to 20:2, 20:4ω6, 20:5ω3, 22:5ω6 and 22:6ω3. One other point of interest was that the cells grown on glucose contained 23% myristic acid, and as a result of this, the total saturated fatty acid content was double that of the ethanol-grown cells. The total concentration of the photosynthetically related fatty acids (18:3, 16:3) was quite small, being only 3-4% of the total. The concentration of these fatty acids might serve as an indicator of the distribution of energy metabolism between the "animal type" or mitochondrial and the "plant type" or chloroplast. In these dark-grown cells the concentration of the acids was only 4%, while in the heterotrophic light-grown cells it climbed to 10%, and finally in the cells grown on $CO_2$ it reached 33% of the total. As pointed out earlier, the $CO_2$-grown cells contained malic and glutamic acids, and these organic acids must have supplied some preformed carbon source for mitochondrial function, because Erwin and Bloch (2) found that *Euglena* grown on a completely inorganic medium with only $CO_2$ as a carbon source contained roughly 50% of the total fatty acid concentration as 18:3 and 16:4.

The differences in the fatty acid patterns between the cells grown on glucose and those grown on ethanol may be a result of the differences in the metabolic schemes available for the utilization of these two carbon sources. Barry (12) has indicated that the Embden-Meyerhof-Parnas pathway is the principal route of glucose metabolism and that the pyruvate thus formed is further oxidized via the Krebs cycle (13-15). On the other hand, the glyoxylate pathway is intimately involved in the utilization of ethanol and acetate (16,17) and it seems likely that the Krebs cycle is "shut down" during ethanol utilization (18). Carbon-balance studies (19,20) have indicated that for each mole of ethanol used, 0.97 moles of $O_2$ were consumed and only 0.29 moles of $CO_2$ were produced. This stoichiometry could be accounted for only if considerable amounts of compounds more reduced than carbohydrate were produced. These products could be lipid in nature if a large portion of the ethanol carbon were converted into fatty acid carbon; however there is no data available at the present time to substantiate this. The carbon source also has a marked effect on the respiration of *Euglena.* Two-carbon compounds such as ethanol or acetate result in very high levels of $O_2$ consumption, whereas Krebs cycle intermediates (13) and glucose (21) cause a very negligible stimulation of respiration. Thus the utilization of the Krebs cycle for glucose metabolism compared to the utilization of the glyoxylate cycle for ethanol metabolism might necessitate a need for a different combination of fatty acids within the lipids of the subcellular organelles involved. From this research it is concluded that the carbon source as well as light and darkness have profound effects on the biosynthesis of fatty acids and thus their patterns in *Euglena gracilis.*

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# Distribution and Specific Activities of RNAs in Isolated Adipose Cells With Relation to Exogenous Lipids

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## ABSTRACT

Several observations suggested that the rate of adipose cell formation is influenced by the fatty acid composition of the dietary fat: morphological studies, counting of cells, nuclear and mitochondrial DNA specific activities (SA). Therefore adipose cell RNAs were fractionated in an attempt to localize the site of this increased activity. Weanling rats were fed a diet containing 20% of sunflower oil (SO) or lard (L) for 21 days and fasted for 18 hr. They were then injected with either (6-$^{14}$C) orotic acid or $^{32}$P-phosphate and killed 210 min or 18 hr later. The adipose cell ribosomal and nuclear RNAs were extracted and fractionated by ultracentrifugation in a sucrose gradient. Comparison of the SA of the fractions revealed that: (1) after 210 min of incorporation, ribosomal RNAs were more active in cells from rats fed SO; after 18 hr, nuclear RNAs of these cells were more active; (2) in each extract, 18S RNAs were always more active in SO than in L; (3) in the cells from the rats fed SO, at the nuclear level the synthesis of rapidly labeled heterodisperse RNAs seemed accelerated; at the ribosomal level they seemed to be utilized more rapidly. This is interpreted as indicative of stimulated enzymic induction.


## INTRODUCTION

There is some evidence to suggest that the metabolic activity of the epididymal fat pad is higher in rats fed a dietary fat rich in linoleate than in rats fed a fat containing a high proportion of oleate. For example, lipogenesis and lipolysis (1,2) were more rapid in adipose cells of linoleate-fed rats, and these cells were formed and transformed at a faster rate (3,4).

Because of the possibility that this increased cellular activity was a consequence of enzymic induction in cells containing a high concentration of linoleate, measurements were undertaken to determine whether RNA specific

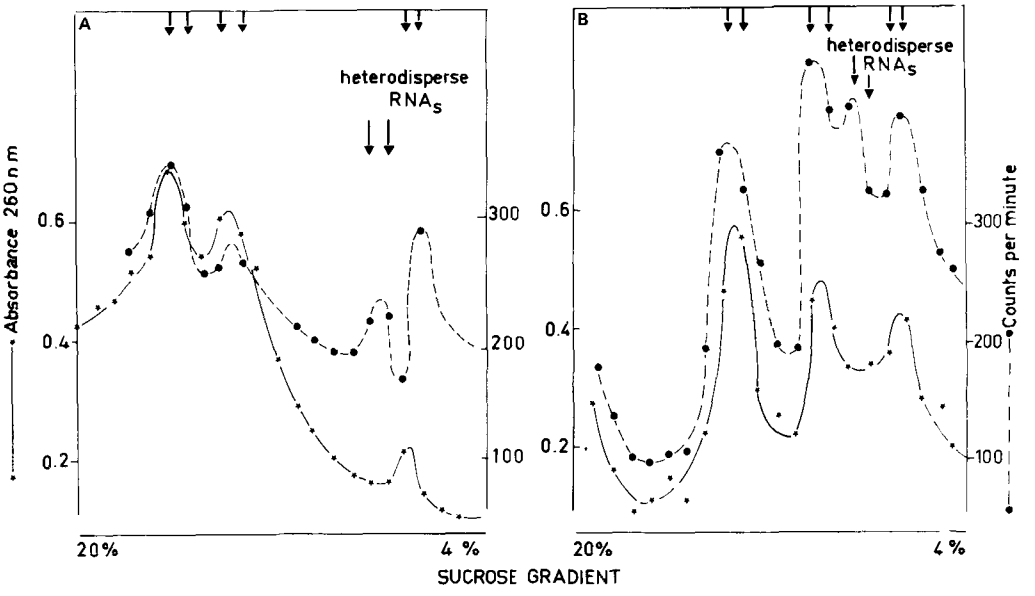


FIG. 1. Sedimentation profiles of adipose cell RNAs (18 hr labeled precursor in vivo incorporation; RNAs extracted at 45 C). A: Lard-fed rats; B: Sunflower oil-fed rats. Specific activities were calculated on samples indicated under arrows.

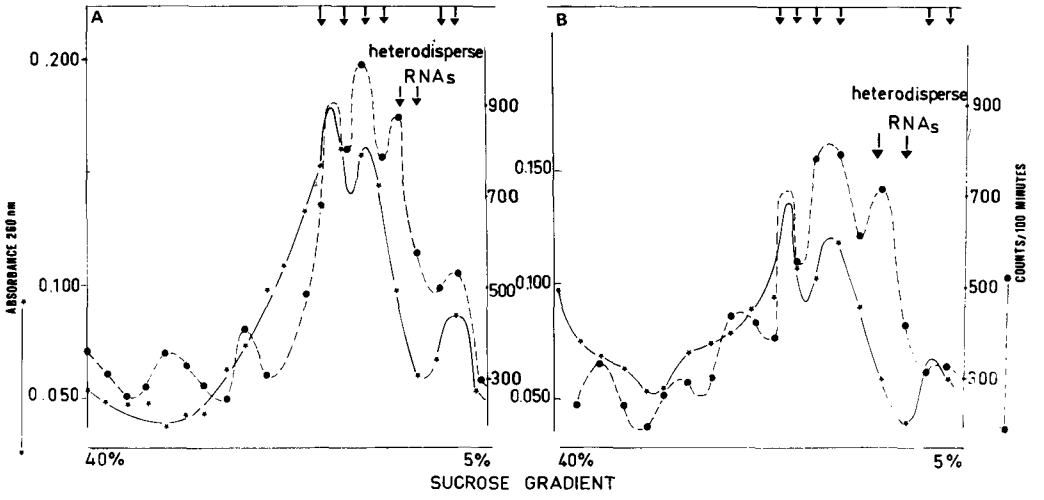


FIG. 2. Sedimentation profiles of adipose cell RNAs (210 min labeled precursor in vivo incorporation; RNAs extracted at 65 C). A: Lard-fed rats; B: Sunflower oil-fed rats. Specific activities were calculated on samples indicated under arrows.

activity would vary with adipose tissue linoleate level in young rats (5).

## METHODS

### In Vivo Labeling of RNAs

Weanling rats were fed a diet containing 20% of either lard (L) or sunflower oil (SO). After 21 days of feeding they were fasted for 18 hr, injected intravenously with either 15 $\mu$C (6-$^{14}$C) orotic acid or 300 $\mu$C $^{32}$P-$PO_4HNa_2$ (in solution in 0.5 ml of 0.9% NaCl) as RNA precursor, and sacrificed 210 min or 18 hr later.

### Extraction of RNAs From Adipose Cells

Epididymal fat pads were dissociated with collagenase (6) in the presence of Macaloid (150 $\mu$g/ml in incubation buffer medium) to inhibit RNA hydrolysis by RNAse. Adipose cells were collected, washed in the same buffer and homogenized in a 0.02 M sodium acetate buffer (pH 5.2) containing 50 $\mu$g/ml of potassium polyvinyl sulfate (PVS). PVS was added at every step of the RNA extraction as an RNAs inhibitor because of its property of precipitating with nucleoproteins. The homogenates were washed two times with ether to remove their lipids.

RNA extraction was carried out in the presence of 0.1% sodium dodecyl sulfate according to Georgiev (7) with modifications described by Kempf and Mandel (8) and Beck et al. (9,10) after phenol treatment at 0 C (Extract 1), 45 C (Extract 2) and 63 C or 65 C (Extract 3). With these procedures it was hoped that the fraction extracted at 0 C would contain mainly ribosomal RNAs; the one extracted at 45 C, a mixture of ribosomal and nuclear

TABLE I

RNA Specific Activities of Adipose Cells in Rats (dpm/mg) 210 Minutes After Intravenous Injection of (6-$^{14}$C) Orotic Acid

| | | RNA extracted at temperature of: | | |
|---|---|---|---|---|
| Dietary treatment[a] | Sedimentation value | 0 C | 45 C | 65 C |
| Lard (L) | 28S | 954 | 900 | 1930 |
| 80 fat pads | 18S | 1193 | 932 | 2380 |
| | 10-12S | --- | --- | 3871 |
| | 4S | 1570 | 824 | 2740 |
| Sunflower oil (SO) | 28S | 1136 | 1009 | 1870 |
| 80 fat pads | 18S | 1350 | 1100 | 2780 |
| | 10-12S | --- | --- | 4476 |
| | 4S | 2160 | 1004 | 2080 |

[a]All rats were given the diet for 21 days from weaning.

TABLE II

RNA Specific Activities of Adipose Cells in Rats
(dpm/mg) 18 Hours After Intravenous Injection of (6-$^{14}$C) Orotic Acid

| Dietary treatment[a] | Sedimentation value | RNA extracted at temperature of: 0 C | 45 C | 65 C |
|---|---|---|---|---|
| Lard (L) | 28S | 18,450 | 17,200 | 7,100 |
| 30 fat pads | 18S | 18,250 | 11,950 | 5,675 |
| | 10-12S | 15,675 | --- | --- |
| | 4S | 8,850 | 12,425 | 5,650 |
| Sunflower oil (SO) | 28S | 7,975 | 20,850 | 10,250 |
| 30 fat pads | 18S | 8,750 | 13,050 | 9,625 |
| | 10-12S | 9,775 | --- | --- |
| | 4S | 8,775 | 15,400 | 8,000 |

[a]All rats were given the diet for 21 days from weaning.

RNAs; and the one extracted at 65 C, nuclear RNAs (phenol was saturated with 0.02 M acetate buffer containing 0.1% 8-hydroxyquinolein). Although one cannot be sure that Extract 1 contained only ribosomal RNAs, it seemed the best way to obtain this fraction as it was not possible to isolate ribosomes from the very tiny ribosomal sediment.

#### Purification

RNAs were precipitated with cold absolute ethanol (24 hr at -20 C) collected by centrifugation and purified twice according to Sevag et al. (11). After solubilization of RNAs in $10^{-3}$M NaCl containing 50 μg/ml of PVS, NaCl was added to obtain the final concentration of $10^{-1}$M. The solution was then shaken with 0.5 volume of chloroform-isopentanol (v/v) and centrifuged 5 min at 2000 g at 4 C. RNAs were precipitated from the aqueous phase with cold absolute ethanol (-20 C overnight). RNAs were solubilized in $10^{-3}$M NaCl for determination of the optical density at 260 nm in a Beckman DB spectrophotometer.

#### Fractionation of RNAs

Fractionation of these RNA extracts was carried out by ultracentrifugation (35,000 rpm for 16 hr in a Beckman Spinco, SW 39 rotor) in 4-20% or 5-40% sucrose density gradients using about 200 μg RNAs on each gradient. RNAs were collected in 10 drop aliquots for determination of radioactivity in relation to the constant of sedimentation (Intertechnique SL 30).

## RESULTS

It was difficult to carry out every step of the adipose cell RNA specific activity (SA) determinations inasmuch as only a few hundred μg of purified RNA were actually obtained despite the very careful extraction carried out on adipose cells from 80 fat pads of young rats.

In these conditions, optical densities (OD) and radioactivities (dpm) were rather low. However, these values seemed sufficiently accurate to be taken for granted. And it is important to emphasize that SA must not be considered as an absolute value but that one must

TABLE III

RNA Specific Activities of Adipose Cells in Rats
(dpm/mg) 18 Hours After Intravenous Injection of $^{32}$P-Phosphate[a]

| Dietary treatment[b] | Sedimentation value | RNA extracted at temperature of: 45 C | 65 C |
|---|---|---|---|
| Lard (L) | 28S | 11,800 | 4987 |
| 30 fat pads | 18S | 11,300 | 1920 |
| | 10-12S | 31,600 | --- |
| | 4S | 27,200 | 3737 |
| Sunflower oil (SO) | 28S | 16,800 | 6400 |
| 30 fat pads | 18S | 23,200 | 9150 |
| | 10-12S | 26,100 | --- |
| | 4S | 22,500 | 6700 |

[a]In this experiment carried out without any SDS, RNAs were not obtained at 0 C.
[b]All rats were given the diet for 21 days from weaning.

recognize which synthesis of RNA could undergo a stimulation under one of our experimental conditions.

When RNAs were extracted from adipose cells of rats fed a lab chow diet (4% of lipids) their constants of sedimentation on a sucrose gradient seemed identical to those of RNAs extracted from liver. When RNAs were extracted from L or SO adipose cells their sedimentation constants were a little lower. Thus, in Figures 1 and 2, no constants are specifically mentioned because these small differences are now under study. However, SA values are given in Tables I-III with conventional sedimentation (S) values (28S, 18S and 4S) in order to simplify interpretation of the results.

It was first noted that, as expected, incorporation of labeled orotic acid (or phosphate) followed the same pattern in these adipose cells as in other mammalian cells (12-14). SA values were much higher after 18 hr than after 210 min of in vivo incorporation (Tables I-III). After 210 min (Table I) nuclear RNAs (65 C) were more radioactive than ribosomal RNAs (0 C and 45 C); after 18 hr of incorporation (Tables II, III) ribosomal RNAs were more radioactive than nuclear RNAs. After 210 min the SA of the ribosomal RNAs had the sequence 4S>18S>28S.

In addition, it was observed that various SA values were influenced by dietary fat. After 210 min of incorporation, 18S RNAs in every extract were more radioactive in the SO adipose cells than in those from animals fed lard (Table I).

After 18 hr of incorporation (Tables II, III) the 18S RNA of the SO cells was more radioactive than in L cells only in the fraction extracted at 65 C. In fact, in the 0 C fractions the opposite was the case; L 18S RNA was more radioactive.

In a sedimentation zone covering messenger RNA, the SA values of a few isolated points were especially high. In the nuclear fractions (65 C), after 210 min of incorporation, the SAs of the heterodisperse RNA were higher in the SO cells than in those from L-fed animals (Table I); in the ribosomal fractions extracted at 0 C after 18 hr of incorporation, the SAs of the L heterodisperse RNAs were higher than in the corresponding fractions from SO cells (Tables II, III).

## DISCUSSION

Although the level of (6-$^{14}$C) orotic acid incorporation in RNAs was not very high after 210 min, the radioactivity of RNAs in general ran parallel to the optical density, indicating that the various sediments had similar SA. One zone of RNA sedimentation was an exception: in the region of messenger RNA (mRNA) the OD was very low—approaching zero—whereas, in every extract, radioactivity profiles showed a sharp peak. In the ribosomal extracts (0 C) this mRNA was more active in the L adipose cells; in the nuclear extracts (65 C) the SO mRNA was more active. These differences were more apparent in the 18 hr extracts where the level of radioactivity was higher.

These observations concerning the zones of rapidly labeled RNA suggest that, in adipose cells rich in linoleate, synthesis of mRNA in the nucleus is enhanced and utilization at the ribosomal level is also rapid. This may be interpreted as indicative of increased enzymic induction in comparison to cells containing high levels of oleate.

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# Composition and Concentration of Lipoproteins in the Serum of Normal Rats and Rats Deficient in Essential Fatty Acids

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## ABSTRACT

A comparison is made of the concentration and chemical composition of serum lipoproteins of normal rats and rats deficient in essential fatty acids. The concentration of very low density lipoproteins (VLDL) and of low density lipoproteins (LDL) in serum of deficient rats is about half that found in normal rats, but the concentration of high density lipoproteins (HDL) is higher than normal and they contain an increased amount of cholesterol esters. The proportion of cholesterol that is esterified is much greater than normal in the serum of deficient rats. The deficiency of essential fatty acids also appears to result in compensating changes occurring in the composition of serum lipoproteins. In both VLDL and LDL of deficient rats the proportion of protein is raised and that of phospholipid lowered compared to normal, while the proportions of triglyceride and cholesterol esters are unchanged.

## INTRODUCTION

Animals reared on a diet deficient in essential fatty acids, either fat free or with saturated fat, develop the well documented symptoms of this deficiency (1-3). Among these symptoms are an alteration in the fatty acid composition of the body lipids and changes in the level of lipids in the serum (1-5). Sinclair and Collins (5) have found that in deficient rats the concentration of serum triglycerides is lowered compared to control animals and that most of this change occurs in the very low density lipoprotein fraction. In order to define this difference in concentration of serum lipids more clearly we have measured the level and composition of very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) in the serum of normal and deficient rats.

## MATERIALS AND METHODS

### Animals

The rats used in these experiments were males of an inbred strain of Sprague-Dawley originating from Holtzman's Rat Farm, Madison, Wis. They were weaned at between 19 and 24 days and then fed either a stock diet of dog cubes (6) or a synthetic fat free diet supplemented with 5% hydrogenated beef fat (5). After 14 weeks the animals on the synthetic diet showed all the symptoms of a deficiency of essential fatty acids (1,5). The rats had continuous access to food and water and they were not fasted prior to slaughter. As both diets had 5% fat other dietary differences were minimal (5).

### Preparation of Lipoproteins

Twenty eight rats and 32 deficient rats were anesthetized with diethyl ether, killed by decapitation and their blood collected, pooled and allowed to clot. After centrifuging, the serum was collected and 5% EDTA (pH 7.0) added so that the final concentration of EDTA was 0.05%. Chylomicrons were removed by centrifuging the serum at 9500 g for 30 min (compare 12,000 g for 20 min [7]). VLDL ($d<1.006$), LDL ($d<1.006$-1.063 and HDL ($d<1.063$-1.202) were then separated by flotation in a No. 40 head of a Spinco model L preparative ultracentrifuge as described by Havel et al. (8). The fraction ($d<1.040$-1.063) which was discarded by Koga et al. (7) in their fractionation of rat plasma lipoproteins was included in the LDL. It is thus possible that this fraction may be contaminated by a small quantity of HDL (7). Each lipoprotein fraction was freed from contamination with heavier fractions by flotation through sodium chloride solutions of the appropriate density, which had been layered above each sample before centrifuging. In addition VLDL was washed once with 0.15M NaCl, containing 0.05% EDTA and concentrated by centrifuging. The LDL and HDL fractions were dialyzed against 0.15M NaCl containing 0.05% EDTA, to remove excess salt. Samples of the lipoprotein fractions were frozen and stored at -20 C until analyzed.

### Chemical Analyses

Total lipids were extracted from freeze-dried samples of lipoproteins using three extractions with chloroform-ethanol (2:1 v/v). The extracts were taken to dryness under vacuum, redis-

TABLE I

Concentration and Composition of Lipoproteins in Serum of Normal and Deficient Rats

| Lipoprotein[a] | mg/100 ml serum Normal | Deficient |
|---|---|---|
| VLDL (d<1.006) | | |
| Protein | 10.1 ( 9.5) | 5.7 (11.5) |
| Phospholipid | 14.5 (13.6) | 5.6 (11.3) |
| Triglyceride | 72.8 (68.1) | 33.8 (68.5) |
| Cholesterol ester | 5.3 ( 4.9) | 2.8 ( 5.6) |
| Free cholesterol | 4.1 ( 3.9) | 1.5 ( 3.1) |
| | 106.8 | 49.4 |
| Total cholesterol | 7.2 ( 6.8) | 3.2 ( 6.5) |
| LDL (d<1.006-1.063) | | |
| Protein | 15.6 (28.0) | 9.9 (37.6) |
| Phospholipid | 10.4 (18.7) | 3.8 (14.5) |
| Triglyceride | 8.0 (14.3) | 4.4 (16.7) |
| Cholesterol ester | 14.6 (26.2) | 6.3 (23.8) |
| Free cholesterol | 7.1 (12.8) | 2.0 ( 7.4) |
| | 55.7 | 26.4 |
| Total cholesterol | 15.8 (28.3) | 5.7 (21.7) |
| HDL (d<1.063-1.202) | | |
| Protein | 146.0 (61.0) | 172.0 (51.8) |
| Phospholipid | 36.2 (15.1) | 48.9 (14.7) |
| Triglyceride | 1.5 ( 0.6) | 2.1 ( 0.6) |
| Cholesterol ester | 42.1 (17.6) | 107.2 (32.3) |
| Free cholesterol | 13.7 ( 5.7) | 1.9 ( 0.6) |
| | 239.5 | 332.1 |
| Total cholesterol | 38.7 (16.2) | 65.5 (19.8) |
| Total serum (by summation) | | |
| Protein | 172 | 188 |
| Phospholipid | 61 | 58 |
| Triglyceride | 82 | 40 |
| Cholesterol ester | 62 | 116 |
| Free cholesterol | 25 | 5 |
| Total cholesterol | 62 | 74 |

[a]Lipoproteins were prepared from pooled serum of 28 normal and 32 deficient rats. The figures in parentheses show percentage composition. (VLDL = very low density lipoproteins; LDL = low density lipoproteins; HDL = high density lipoproteins).

solved in chloroform and filtered. Samples of the filtrate were taken for lipid phosphorus analysis and for thin layer chromatography (TLC). Lipid phosphorus was measured by the method of Itaya and Ui (9) and the phospholipid content of the lipoproteins calculated, taking the molecular weight of phospholipid as 800. Lipids were separated by TLC as described by Sinclair and Collins (5). The triglyceride, cholesterol ester and phospholipid spots were scraped from the plates quantitatively and their fatty acid composition analyzed by gas liquid chromatography (GLC) (10), using a known amount of methyl arachidate as an internal quantitative standard. The following methyl esters were identified by comparison with authentic samples—14:0, 16:0, 16:1, 18:0, 18:1, 18:2 (n-6); 20:3 (n-9); 20:3 (n-6); 20:4 (n-6); 20:5 (n-3) and 22:6 (n-3). Other methyl esters were identified by their relative retention times. From these analyses the ratios of triglyceride and of cholesterol ester to phospholipid were calculated and hence the concentration of triglyceride and cholesterol ester in the lipoprotein preparations.

Protein and total cholesterol content of the lipoproteins were measured before extraction. Protein was measured by a slight modification of the method of Lowry et al. (11). Turbidity caused by high concentrations of lipids was removed by extraction with diethyl ether at room temperature after the color had fully developed (12). Total cholesterol was measured by the method of Searcy and Bergquist (13) and free cholesterol calculated from this by subtracting the cholesterol equivalent of the cholesterol esters.

The sampling errors were minimized by using a pooled sample from 28 normal rats and another pooled sample from 32 deficient rats. The lipid extractions were at least 95% complete and all chemical analyses were performed in duplicate and agreed to within 3%.

## RESULTS AND DISCUSSION

Table I shows the amounts of the three lipoprotein classes found in the serum of normal and deficient rats and also the composition of each of the lipoproteins. Table II gives the fatty acid composition of each of the lipids in Table I.

### Lipoproteins of Normal Rats

There are few analyses published in the literature of lipoproteins of rat serum and these give a range of values for both the amount and composition of the lipoproteins (5,7,14-16). This variation is probably due to several factors; differences in the ages of the rats examined, variations in their diet and in particular to differences in the severity of starvation before slaughter. The latter has varied from no starvation to up to 20 hr, which would lead to large variations in analytical results, since starving rats for 18 hr greatly reduces the level of lipid in their serum (5).

The concentration of LDL and HDL found in the serum of our unstarved normal rats is similar to that reported by Lombardi and Ugazio (14) for rats starved 20 hr, but the concentration of VLD in our rats is considerably higher than theirs. The amount of serum triglyceride we find, and its distribution be-

TABLE II

Fatty Acid Composition of Lipids in the Serum Lipoproteins of Normal and Deficient Rats

| Fatty acid | Normal | | | Deficient | | |
|---|---|---|---|---|---|---|
| | VLD | LD | HD | VLD | LD | HD |
| | | | Phospholipids[a] | | | |
| 16:0 | 17.2 | 14.0 | 15.3 | 17.8 | 13.9 | 13.6 |
| 16:1 | 0.7 | 0.8 | 1.4 | 2.2 | 2.1 | 2.5 |
| 18:0 | 23.8 | 19.3 | 21.9 | 25.0 | 19.3 | 19.7 |
| 18:1 | 11.9 | 12.5 | 18.5 | 25.3 | 21.8 | 23.3 |
| 18:2 (n-6) | 22.8 | 22.6 | 17.0 | 1.3 | 3.1 | 1.9 |
| 20:3 (n-9) | 0.3 | 0.7 | <0.1 | 16.8 | 17.9 | 21.6 |
| 20:4 (n-6) | 12.0 | 11.9 | 3.0 | 2.3 | 3.9 | 3.0 |
| | | | Triglycerides[a] | | | |
| 16:0 | 21.5 | 16.2 | 28.8 | 26.3 | 18.2 | 27.9 |
| 16:1 | 3.4 | 5.6 | 6.0 | 11.9 | 14.1 | 9.8 |
| 18:0 | 6.0 | 6.5 | 8.2 | 5.3 | 14.0 | 7.6 |
| 18:1 | 37.9 | 30.9 | 35.4 | 50.2 | 34.8 | 43.0 |
| 18:2 (n-6) | 19.6 | 24.0 | 7.3 | 0.8 | 3.2 | 1.7 |
| 20:4 (n-6) | 1.7 | 5.8 | 0.6 | 0.1 | 0.6 | 0.4 |
| | | | Cholesterol esters[a] | | | |
| 16:0 | 12.9 | 9.9 | 14.2 | 14.8 | 9.5 | 6.3 |
| 16:1 | 4.6 | 3.1 | 5.3 | 15.9 | 12.3 | 9.1 |
| 18:0 | 10.0 | 2.1 | 1.6 | 7.0 | 2.4 | 0.8 |
| 18:1 | 28.7 | 16.8 | 18.8 | 38.5 | 24.3 | 16.5 |
| 18:2 (n-6) | 16.7 | 23.0 | 23.4 | 3.5 | 6.7 | 9.0 |
| 20:3 (n-9) | 0.7 | <0.1 | 0.5 | 9.5 | 15.9 | 28.0 |
| 20:4 (n-6) | 19.5 | 33.7 | 8.8 | 3.7 | 8.9 | 12.6 |

[a]Per cent of total fatty acids by weight. Fatty acids that occurred in all fractions with an abundance of less than 10% have not been included in the table (Abbreviations as in Table I).

tween VLDL, LDL and HDL, agrees with that reported by Sinclair and Collins (5) for rats fed a control diet. The percentage composition of the lipoproteins is similar to the range of compositions quoted in the literature (7,14-16).

#### Comparison of Deficient Rats with Normal Rats

*Amount of lipoproteins:* The concentration of VLDL and LDL in serum of rats deficient in essential fatty acids is about half that in serum of normal rats but the concentration of the HDL is about 40% higher.

*Total serum lipids:* The level of serum triglycerides in deficient rats is about half that of normal animals. This difference occurs mainly in VLDL which is the chief source of serum triglyceride in rats. The level of LDL triglyceride in deficient rats is also lower than normal, but that of HDL triglyceride is slightly raised. The total amount of serum cholesterol is also a little higher in deficient rats. This is due to a large increase in the amount of their HDL cholesterol esters. In all other fractions deficient rats have less cholesterol. There is also a large increase in the proportion of cholesterol that is esterified, from 60% in normal animals to 93% in deficient animals. This increase occurs in all lipoprotein fractions but is most marked in HDL. The total amount of serum phospholipid is the same in both normal and deficient rats, but the deficient animals have less VLDL and LDL phospholipid and correspondingly more HDL phospholipid. Lipoprotein protein is similarly distributed.

*Composition of lipoproteins:* VLDL from both normal and deficient rats contains the same proportions of triglyceride and of total cholesterol. The proportion of protein plus phospholipid is also the same in VLD from both groups of animals, but the deficient rat has more protein and correspondingly less phospholipid than normal.

Comparison of LDL from normal and deficient rats gives a similar result. Protein content is raised and phospholipid content lowered in deficient animals, while triglyceride and cholesterol ester content is unaltered.

HDL differs from the other two lipoprotein fractions in containing very little triglyceride. Also there is no difference in phospholipid

content between normal and deficient animals and the protein content of the HDL of deficient rats is slightly lower than normal. The most marked difference between HDL of normal and deficient rats is the high content of cholesterol ester and low content of free cholesterol in the deficient animal.

*Fatty acid composition:* Fatty acid analysis of the lipids (Table II) shows the usual marked differences between normal and deficient rats. The linoleic and arachidonic acids of the normal animals are replaced in the deficient animals by palmitoleic, oleic and 5,8,11-eicosatrienoic acids. The proportions of the saturated fatty acids are not altered markedly. These changes occur to a similar extent in all the lipid classes of each of the three lipoprotein fractions. Within the one group of animals there are no marked differences in the fatty acid compositions of the same lipid class in the different lipoproteins, except in the cholesterol esters of HDL. Here in normal animals the content of arachidonic acid was lower and in the deficient animals that of eicosatrienoic acid higher than in the corresponding VLDL or LDL, suggesting that the abnormally large amount of cholesterol esterified in HDL of deficient rats is esterified mainly with this latter fatty acid. Calculation of the absolute concentrations in serum of the various cholesterol esters of HDL shows that while the concentration of cholesterol linoleate is the same in both normal and deficient rats, the concentration of cholesterol palmitoleate, oleate, eicosatrienoate and arachidonate are all increased markedly in the deficient animals, the greatest increase being in the concentration of eicosatrienoate.

These results suggest that in a deficiency of essential fatty acids the alteration in fatty acid composition of the serum lipids may lead to compensating changes in the composition of the serum lipoproteins. It has been suggested that change in fatty acid composition affects the binding of phospholipids to protein (17,18). In the case of serum VLDL and LDL of deficient rats the change in fatty acid composition appears to lower the ratio of phospholipid to protein without changing the over-all proportion of polar components (protein, phospholipid and cholesterol) to nonpolar components (triglyceride and cholesterol esters). This may be related to a need to maintain a constant ratio of hydrophilic surface material to hydrophobic core in these lipoproteins.

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# Effects of pH, Concentration and Aging on the Malonaldehyde Reaction With Proteins[1]

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## ABSTRACT

Malonaldehyde, a major product of unsaturated lipid oxidation, reacts with enzymes to cause inactivation, fluorescence production and crosslinking. The pH dependence for each of these reactions shows a different optimum. Investigation of the stability of malonaldehyde shows that it undergoes decomposition which is dependent on time, reagent concentration and pH. The products resulting from this decomposition are also capable of causing enzyme inactivation and fluorescence production.

## INTRODUCTION

Oxidized unsaturated lipids and their products have been implicated in a variety of deleterious biological phenomena (1-3). The activity of these oxidized materials results in part from their ability to react with proteins, causing crosslinking, modification of critical amino acid residues and loss of functional activities (1). The exact mode of action of the products of lipid oxidation or proteins is still unclear. Malonaldehyde, one of the major products of lipid oxidation, reacts fairly readily with proteins (4). Some of the parameters affecting this reaction have been investigated in more detail in an effort to enhance our understanding of the effect of malonaldehyde on proteins. During the course of this investigation it was found that malonaldehyde itself undergoes a reaction to generate products which are also reactive toward proteins and which may contribute to the effects observed in biological systems and in model studies.

[1]Publication No. 2388 of the Oklahoma Agricultural Experiment Station.

## EXPERIMENTAL PROCEDURES

### Materials and Methods

Malonaldehyde was prepared from 1,1,3,3-tetramethoxypropane (Aldrich Chemical Co.) according to the procedure of Chio and Tappel (5). All enzymes and substrates used were purchased from Sigma. Ribonuclease (RNase) was assayed by the procedure of McDonald (6); papain was assayed by procedures from the Worthington Enzymes Manual (7). Fluorescence was measured with an Amin-co-Bowman spectrofluorometer standardized with quinine sulfate. Free amino groups were

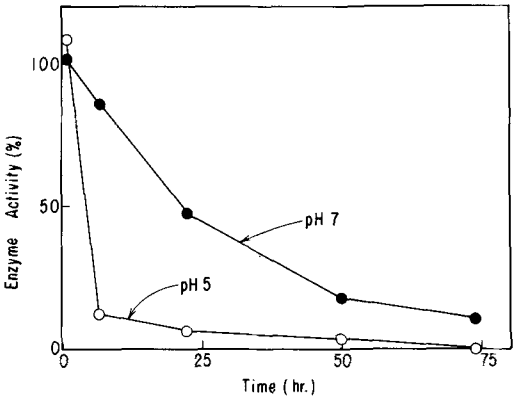


FIG. 1. Effect of malonaldehyde on RNase activity. Enzyme (1 mg/ml) was incubated at 37 C in 0.05 M acetate buffer (pH 5.0) or in 0.05 M phosphate buffer (pH 7.0) with 10 mM malonaldehyde. Activities are expressed as percentage of control which was incubated for same period in the absence of malonaldehyde.

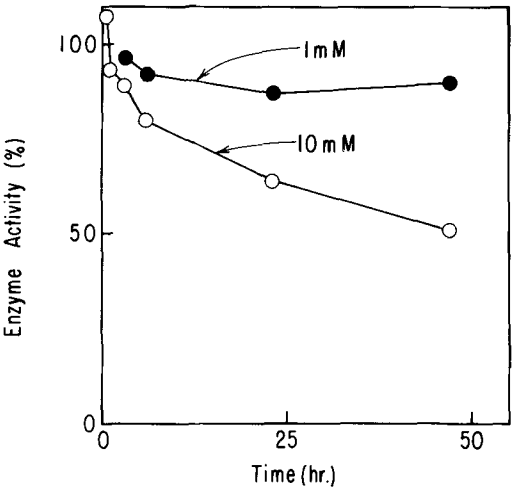


FIG. 2. Effect of malonaldehyde on papain activity. Enzyme (1 mg/ml) was treated with malonaldehyde in 0.05 M phosphate buffer (pH 7.0) at 37 C.

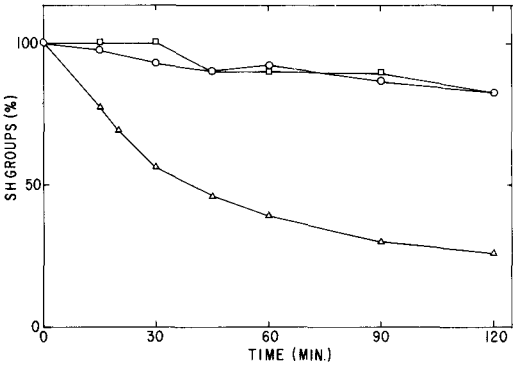


FIG. 3. Effect of malonaldehyde or oxidized linolenic acid on sulfhydryl group of N-acetylcysteine. Reaction mixture contains $10^{-2}$ M malonaldehyde, $10^{-3}$ M N-acetylcysteine and 5 x $10^{-2}$ M $MgCl_2$. The reaction in the absence of $MgCl_2$ showed essentially the same time course. Reaction with oxidized linolenic acid contained $10^{-2}$ M acid which had been oxidized 24 hr under UV light in oxygen. Control with no malonaldehyde (○—○); malonaldehyde-treated (□—□); linolenic acid-treated (△—△).

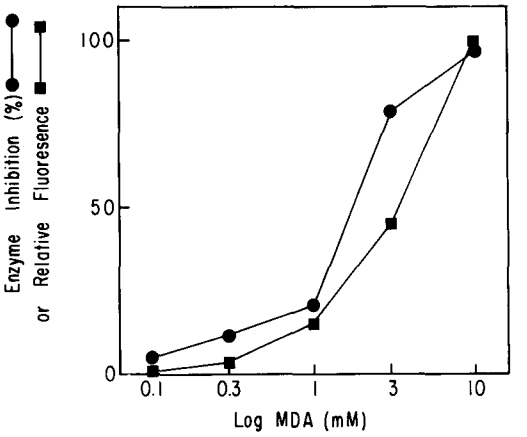


FIG. 5. Effect of malonaldehyde concentration on its reaction with RNase. Enzyme (0.5 mg/ml) was incubated in acetate buffer (pH 5.0) at 37 C with varying concentrations of malonaldehyde. RNase activity and fluorescence intensity were measured at time intervals up to 20 hr. The values shown are for a 20 hr incubation and are typical for the entire period.

determined by the procedure of Moore and Stein (8).

#### Reaction of Enzymes With Malonaldehyde

Enzymes (1 mg/ml) were treated with the appropriate malonaldehyde concentrations at 37 C in 0.05 M acetate buffer (pH 5.0) or 0.05 m phosphate buffer at pH 7-8. Aliquots were taken at timed intervals and assayed for RNase activity, fluorescence or amino groups by The pH dependence of the reaction of malonaldehyde with RNase was studied in a mixed citrate-phosphate buffer (9) from pH 3-7 and in 0.05 M phosphate buffer at pH 7-8. Aliquots were taken at timed intervals and assayed for RNase activity, fluorescence or amino groups by ninhydrin assay (8). No dependence on the buffer type was seen in overlapping points. The concentration dependence of the malonaldehyde reaction with RNase was studied at pH 5.0 in 0.05 M acetate buffer. Aliquots were taken at timed intervals and assayed for RNase activity or fluorescence.

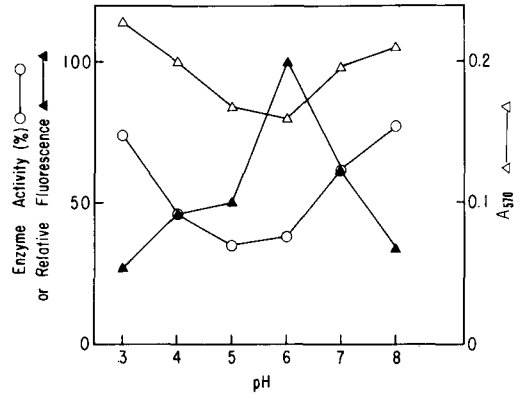


FIG. 4. Effect of pH on malonaldehyde reaction with RNase. Enzyme (1 mg/ml) was incubated at various pH values in citrate-phosphate or phosphate buffer with 4 mM malonaldehyde. Aliquots of incubation mixture were diluted at timed intervals up to 70 hr and assayed for fluorescence (excitation 400 nm, emission 460 nm), amino groups and RNase activity. The values shown were obtained after a 70 hr incubation, but the relative values were similar over the entire period.

#### Polyacrylamide Electrophoresis

Samples for electrophoresis were dissolved in 1.0% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol in 10 mM phosphate at pH 7.0 and incubated at 37 C. Samples were applied directly to the top of 7% polyacrylamide gels containing 0.1% SDS and run for 3.5 hr at 8 ma per gel after prerunning gels for 40 min to remove persulfate. Gels were stained in 0.05% comassie blue in 9% methanol and 7% acetic acid and destained in 7% acetic acid. The gels were scanned using a Gilford 2000 spectrophotometer equipped with a model 2410 linear transport accessory. To estimate the amount of protein on the gel, traces of the gel scans were cut out and weighed. Additional descriptive material on our electrophoretic procedures has been published previously (10).

#### Storage of Malonaldehyde

Malonaldehyde solutions were adjusted to pH 2.5 or 5.0 with sodium hydroxide and

stored at the appropriate concentration or temperature (4 C or 37 C). Aliquots of the malonaldehyde solution were diluted with 20 mM acetate buffer (pH 5.0) for absorbance and fluorescence assays. To determine the effects on RNase activity, aliquots of the aged malonaldehyde solutions were mixed with RNase in acetate buffer at pH 5.0, as previously described, to give the desired concentration of malonaldehyde.

#### Effect of Malonaldehyde on Sulfhydryl Groups

N-Acetylcysteine (1.0 mM) and malonaldehyde (10 mM) were allowed to react at pH 7.5 in 0.1 M phosphate at 37 C in the presence and absence of 0.05 M $MgCl_2$. Aliquots were removed at timed intervals and assayed for sulfhydryl groups with 5,5'-dithiobis-(2-nitrobenzoic acid) according to Ellman et al. (11). A sample of linolenic acid which had been oxidized in the presence of UV irradiation was allowed to react concurrently with N-acetylcysteine to show the loss of sulfhydryl by this method.

## RESULTS AND DISCUSSION

As a preliminary characterization of the reaction of malonaldehyde with proteins, studies were performed on the time course of malonaldehyde inactivation of several enzymes. Figures 1 and 2 show some typical results of these experiments for the enzymes ribonuclease and papain. Both pH and malonaldehyde concentration are important to the rate of inactivation, but the presence of essential sulfhydryl groups in the enzyme does not appear to be significant, since the rate of inactivation of several sulfhydryl enzymes we have studied is not appreciably faster than that for nonsulfhydryl enzymes. This observation is consistent with the low reactivity of malonaldehyde toward sulfhydryl groups as shown in Figure 3.

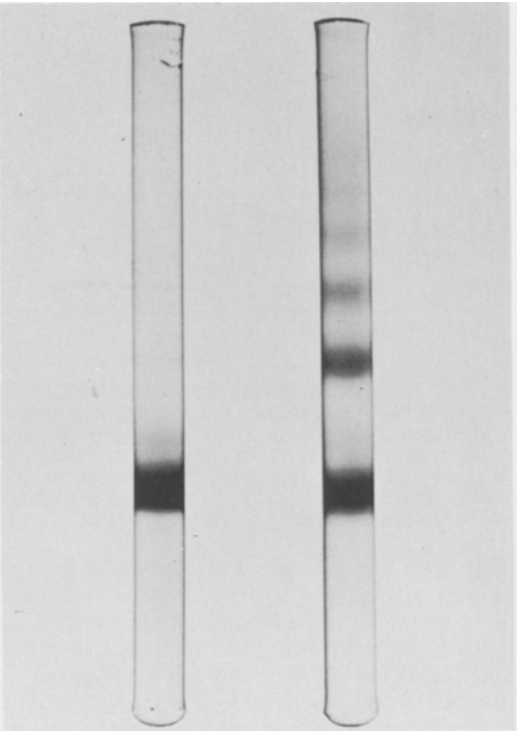

FIG. 6. Acrylamide gel electrophoresis patterns of malonaldehyde-treated RNase. Left gel, untreated RNase.

The loss of the sulfhydryl of N-acetylcysteine in the presence of malonaldehyde was not significantly greater than in the absence of malonaldehyde. The presence or absence of $Mg^{++}$ did not affect the reaction. This result contrasts with work of Buttkus (12), in which cysteine was found to react with malonaldehyde with an apparent loss of sulfhydryl. Cysteine is not a proper model compound for the reactivity of protein-bound sulfhydryl, however, since presence of the amino group

TABLE I

pH Dependence of Crosslinking of RNase by Malonaldehyde

| | RNase amount present, % of total[a] | | | |
|---|---|---|---|---|
| Reaction pH | Monomer | Dimer | Trimer | Tetramer |
| 3 | 97 | 3 | 0 | 0 |
| 4 | 79 | 17 | 3 | 1 |
| 5 | 84 | 12 | 3 | 3 |
| 6 | 94 | 6 | 0 | 0 |
| 7 | 97 | 3 | 0 | 0 |
| 8 | 95 | 5 | 0 | 0 |
| Untreated[b] | 100 | 0 | 0 | 0 |

[a]Estimated from coomassie blue stained gels as described in Experimental Procedures.
[b]Control sample which was not treated with malonaldehyde.

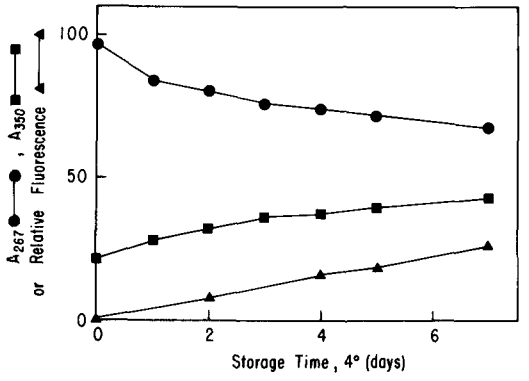


FIG. 7. Effect of storage on malonaldehyde. The reagent was brought to pH 5.0 with sodium hydroxide and stored at 4 C at a concentration of 20 mM. Samples were checked for fluorescence or absorbance at indicated intervals after diluting with 20 mM acetate at pH 5.0. The effect of storage at pH 2.5 was much less pronounced.

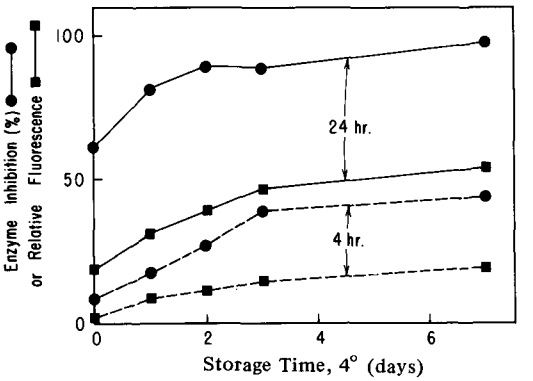


FIG. 9. Effect of aged malonaldehyde on RNase. Reagent (20 mM) was stored at 4 C for timed intervals after adjusting to pH 5.0 with sodium hydroxide. Aged reagent (2 mM) was incubated with RNase (0.5 mg/ml) in 0.05 M acetate buffer (pH 5.0) at 37 C for 0-72 hr. Values for enzyme activity and fluorescence intensity are shown for incubation periods of 4 and 24 hr.

may alter the sulfhydryl group reactivity. This is particularly true for the reaction with compounds such as malonaldehyde which are known to react with amino groups. It seems likely that the amino group may be required for the sulfhydryl reactivity in the case of cysteine, although we have not yet investigated the full range of reaction conditions.

The results obtained with ribonuclease at pH 7 are similar to those obtained by Chio and Tappel (4) under similar conditions. To characterize further the malonaldehyde reaction(s), the effects of pH and malonaldehyde concentration on the reaction with ribonuclease were observed. Loss of amino groups and activity and fluorescence production all show somewhat different characteristics with respect to pH (Fig. 4). Optimal fluorescence production and loss of amino groups occur at pH 6, while loss of activity peaks at pH 5. These differences suggest that different processes may be involved in inactivation and fluorescence production, although the results are not conclusive. The fluorescence production and inactivation also show differences when observed as a function of malonaldehyde concentration (Fig. 5).

Polymerization of proteins by malonaldehyde can be demonstrated simply by electrophoresis on polyacrylamide gels in sodium dodecyl sulfate. Figure 6 shows patterns for control and malonaldehyde-treated ribonuclease. Both ribonuclease and bovine serum albumin (BSA) gave patterns indicative of at least three polymeric forms after treatment with malonaldehyde. The molecular weights of the treated samples were estimated by comparisons with proteins of known molecular weight in the same electrophoresis system. Values of 15,500, 30,000, 43,000 and 56,000 for ribonuclease and 68,000, 145,000 and 185,000 for BSA were estimated for the bands which could be clearly separated and distinguished on the gels. These values correspond qualitatively to a polymeric series with increasing numbers of monomers bound together. The variations of the molecular weight from the theoretical values for untreated proteins probably result from conformational restrictions on the proteins due to intramolecular crosslinking. Use of the SDS acrylamide systems for analysis gave a pH optimum for the crosslinking reaction of ribonuclease between pH 4 and 5 as shown in Table I. Therefore the crosslinking reaction has a lower pH optimum than any other parameters observed.

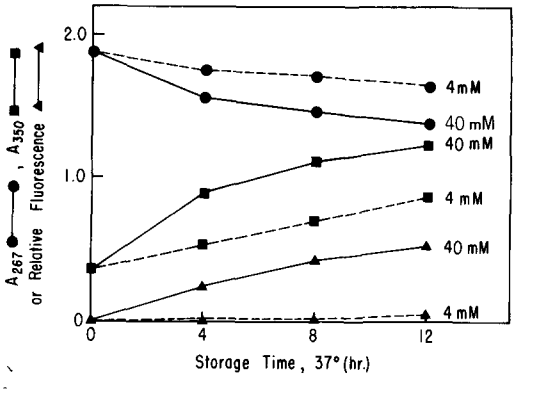


FIG. 8. Effect of incubation at 37 C on malonaldehyde. Reagent was incubated at two different concentrations after adjusting to pH 5.0 with sodium hydroxide.

An adequate characterization of the malonaldehyde reactions must also consider the stability of the reagent. Much of the reactivity of glutaraldehyde, a similar dialdehyde crosslinking agent, can be attributed to its reactions to yield other reactive intermediates (13). Since the effects of malonaldehyde require fairly long incubation periods, reagent stability might be a significant factor. Some typical experiments to determine malonaldehyde stability are shown in Figures 7 and 8. The loss of malonaldehyde was estimated from its absorbance at 267 nm. In addition to the loss of malonaldehyde, the formation of at least two new products can be determined from the absorbance at 350 nm and the fluorescence emission at 550 nm with excitation at 400 nm. Figure 7 shows the changes in these parameters for malonaldehyde solutions stored at pH 5 and 4 C. A slow loss of malonaldehyde occurs with concomitant formation of products. The reactions are much faster at 37 C and are dependent on reagent concentration (Fig. 8). It was also of interest to determine whether the products of malonaldehyde decomposition were responsible to any degree for its enzyme-inactivating and fluorescence-producing effects. Figure 9 shows that both these parameters are affected by the age of malonaldehyde solutions stored at pH 5 and 4 C, indicating that degradation products of malonaldehyde do alter the properties of ribonuclease. The time course of the malonaldehyde degradation indicates that significant quantities of the products could form during the course of protein incubations with malonaldehyde. The degradation reaction is also pH dependent, occurring at a faster rate at pH 5.0 than at pH 2.5. It is not possible at present to determine the relative contributions of the various malonaldehyde products to the reaction with proteins, but it is obvious that the reaction between malonaldehyde and proteins can be quite complex, depending on the particular conditions involved.

The effects of oxidized unsaturated lipids on proteins arise from a very complex set of reactions. The relative roles of these reactions in protein modification is still a debated question (14). The reaction of malonaldehyde with proteins has been shown to yield 1-amino-3-iminopropene derivatives, which can result in crosslinking of protein molecules (5). The present results indicate that the effects of malonaldehyde cannot necessarily be attributed to a single reaction. Although the reaction parameters (pH optima, fluorescence) of the reactions to inactivate enzymes are consistent with 1-amino-3-iminopropene formation, further studies on malonaldehyde indicate that it undergoes additional reactions to yield products which can also react with enzymes to cause inactivation and increase of fluorescence. The nature of these products is unknown, but they probably result from condensation reactions of a type similar to those found with glutaraldehyde (13). The biological significance of these reactions is also difficult to assess. Malonaldehyde is produced in a variety of systems, ranging from dry mixtures (14) to cellular organelles (15). The self-condensation reactions depend on malonaldehyde concentration, the concentrations of other reactive species present, pH, solvent composition and temperature. It appears that the number of variables involved would tend to refute simple solutions to the problem of the reactions of either malonaldehyde or other products of oxidized lipids with proteins.

ACKNOWLEDGMENT

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# The Emulsifying Properties of Egg Yolk Phosphatidylcholine

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## ABSTRACT

The effects of lipid concentration and w/w ratios on the formation of emulsions by some combinations of phosphatidylcholine, cholesterol oleate, cholesterol and triglyceride were studied, with the following results. Phosphatidylcholine emulsified cholesterol oleate and triglyceride, the degree of dispersion and emulsion stability increasing with phosphatidylcholine concentration. Cholesterol reduced the degree of dispersion of phosphatidylcholine-triglyceride emulsions but enhanced the emulsion stability. Triglyceride disrupted the colloidal phase of phosphatidylcholine and cholesterol in high concentrations and did so more effectively when the proportion of cholesterol to phosphatidylcholine was high.

## INTRODUCTION

Phospholipids are often the most effective dispersants for some of the neutral lipids in the body and influence the size of lipoproteins and chylomicrons (1,2).

In this study we have examined the dispersing and emulsifying properties of phosphatidylcholine with respect to cholesterol oleate, the most abundant (3) and the most sclerogenic (4) of the cholesterol esters, and triglyceride. The nature of a cholesterol-phospholipid-triglyceride system in which the triglyceride concentration was progressively increased was also examined, since it is well known that cholesterol can be taken up by both these lipids, and that it can form a colloidal dispersion with phosphatidylcholine alone.

## MATERIALS

### Phosphatidylcholine

Phosphatidylcholine was prepared from hen's egg yolks, tested for purity, and estimated as reported in an earlier paper (5).

### Cholesterol Oleate

Cholesterol oleate (B.D.H. reagent grade, mol wt 651.12) was purified by the method of Barron and Hanahan (6).

Estimation of the ester was carried out by the method of Leffler (7). Purity was assessed by thin layer chromatography (TLC) methods after development in petroleum ether (bp 40-60 C)-diethyl ether-acetic acid 90:10:1 v/v/v (8).

### Triglyceride

Locally purchased lard was rendered down by boiling in distilled water. The neutral fat (triglyceride) was skimmed off the top and the infranatant solution discarded. The process was repeated.

Triglyceride estimation was carried out by the method of Young and Eastman (9). Purity of the triglyceride was assessed by TLC, using the same solvent system as for cholesterol oleate.

### Cholesterol

Cholesterol was purchased from B.D.H., England, purified, and estimated as reported in an earlier paper (5).

## METHODS

### Preparation of Emulsions

Aqueous emulsions of the following con-

TABLE I

The Fatty Acid Composition of Egg Yolk Phosphatidylcholine and of Triglyceride

| Compound | Fatty acids (% of total by weight) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 | 20:4 |
| Phosphatidylcholine | trace | 42.8 | 0.5 | 12.7 | 31.9 | 12.1 | --- | --- |
| Triglyceride | 1.2 | 25.8 | 4.7 | 9.9 | 44.4 | 13.9 | --- | --- |

TABLE II

The Characteristics of Emulsions of Some Combinations of Phosphatidylcholine, Cholesterol, Cholesterol Oleate and Triglyceride

| System | Ratio, w/w | Emulsion dispersion[a] | Globule behavior |
|---|---|---|---|
| Cholesterol oleate | --- | Coarse | Creaming and coalescence within 24 hr |
| Phosphatidylcholine + cholesterol oleate | 4:1 | Fine | No coalescence within 72 hr |
| | 1:1 | Medium (mainly sludge at high concentrations). | Some coalescence within 72 hr |
| Triglyceride | --- | Coarse | Coalescence within 72 hr |
| Phosphatidylcholine + triglyceride | 1:6 | Medium | Creaming and coalescence within 24 hr |
| | 1:1 | Very fine | No coalescence within 72 hr |
| Phosphatidylcholine + cholesterol + triglyceride | 1:1:1 | Medium | Some clumping |
| | 1:1:0.5 | Medium | Some clumping |
| | 1:1:6 | Coarse | Coalescence and clumping within 72 hr; no creaming |

[a]Key: Very fine - below light microscopic visibility; fine - average globule diameters in the range 1-3$\mu$; medium - average globule diameters in the range 4-8$\mu$; coarse - average globule diameters in the range 9-20$\mu$.

tents were prepared: (a) phosphatidylcholine and cholesterol oleate in concentrations ranging from 0.05-0.60 g/100 ml for each lipid at 1:1 w/w ratios and 0.04-0.60 g/100 ml phosphatidylcholine and 0.01-0.15 g/100 ml for cholesterol oleate at 4:1 w/w ratios; (b) cholesterol oleate alone at concentrations of 0.10-0.60 g/100 ml; (c) phosphatidylcholine and triglyceride in concentrations ranging from 0.05-0.60 g/100 ml for each lipid at 1:1 w/w ratios and 0.05-0.10 g/100 ml phosphatidylcholine and 0.30-0.60 g/100 ml triglyceride at 1:6 w/w ratios; (d) triglyceride alone at concentrations of 0.05-0.60 g/100 ml; (e) phosphatidylcholine 0.05 g/100 ml, cholesterol 0.05 g/100 ml and triglyceride 0.30 g/100 ml; phosphatidylcholine 0.10 g/100 ml, cholesterol 0.10 g/100 ml and triglyceride 0.60 g/100 ml at 1:1:6 w/w/w ratios; (f) phosphatidylcholine and cholesterol at fixed concentrations of either 0.15 or 0.30 g/100 ml of each lipid with concentrations of triglyceride varying from 0.40 g/100 ml, i.e., 1:1:0-2.7 w/w/w ratios; and (g) phosphatidylcholine 0.23 and 0.62 g/100 ml and cholesterol 0.10 and 0.30 g/100 ml and concentrations of triglyceride varying from 0-0.60 g/100 ml, i.e., 2:1:0-2 w/w/w ratios.

Ethanolic solutions of phosphatidylcholine were pipetted into small glass screw-top jars (5.5 x 2.4 cm) and the solvent completely evaporated in a warm water-bath under a stream of nitrogen.

Aliquots of cholesterol oleate in chloroform were then pipetted into the jars and the solvent evaporated as before. Ten milliliters of glass-distilled, boiled water were added, and the resultant dispersion, after standing at room temperature for 2 hr, was ultrasonicated as previously described (5).

Triglyceride was weighed into each jar. A small quantity of chloroform was added to dissolve it; aliquots of ethanolic solutions of phosphatidylcholine, with and without cholesterol, were added, and after removal of the organic solvents the dispersions were ultrasonicated.

Some of the ultrasonicated dispersions were examined microscopically at various intervals over a period of several weeks. Others were ultracentrifuged at 35,000 rpm (representing 93,000 g with the rotor used) within 24 hr of preparation in a Beckman Model L preparative ultracentrifuge. After ultracentrifugation chemical estimations of the infranatant solution were carried out.

### Stability of Emulsions

Emulsion stability was estimated with the light microscope. Emulsions were considered stable if no coalescence of globules occurred

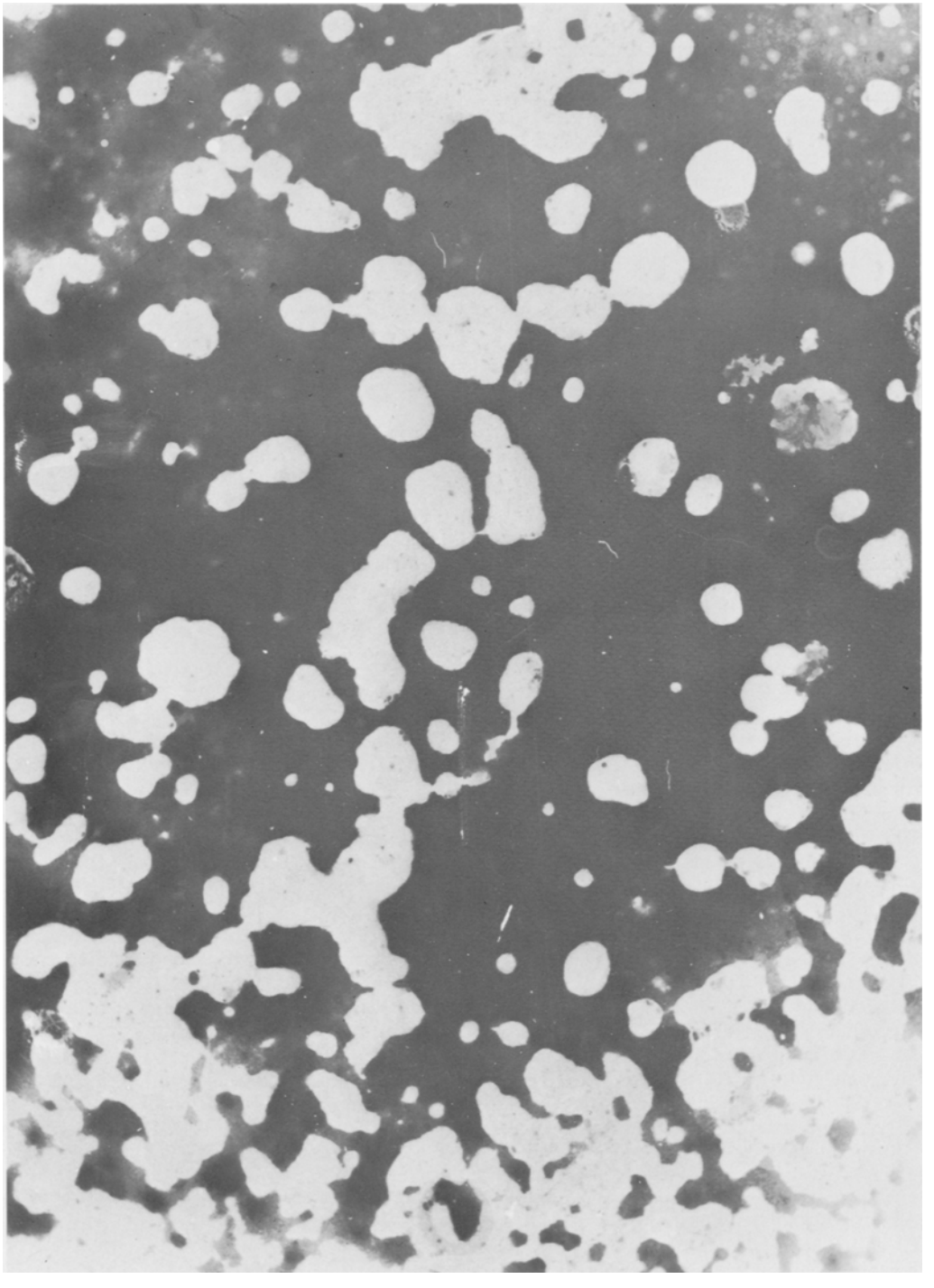

FIG. 1. Electron photomicrograph of phosphatidylcholine and triglyceride in a 1:1 w/w ratio. Magnification: x 56,430.

within 72 hr. Creaming (without coalescence) and clumping were not regarded as evidence of instability. (Clumping is defined as adherence of discrete globules and coalescence as the formation of one globule from two, three or more globules. Creaming is defined as a rise of globules to the surface of the liquid and does not necessarily involve either clumping or coalescence.) Coalescence was assessed by counting the number and size of globules in a diluted sample per unit field in the light microscope using an ocular graticule. An overall increase in size of the globules over a period of 3 days was taken as evidence of emulsion instability.

#### Electron Microscopy

Electron microscopy of a dispersion of phosphatidylcholine and triglyceride in which no particulate matter could be seen with the light microscope was carried out to assess whether solubilization or emulsification had occurred. Negative staining with potassium phosphotungstate, pH 7.4, was carried out and a Formvar carbon-coated copper grid, mesh 400, was used. The preparation was photographed in a Phillips EM 300 electron microscope at 80 KV and instrumental magnifications of 4000 and 34,000.

#### Fatty Acid Analysis

Fatty acid analysis of the triglyceride and phosphatidylcholine was carried out by gas liquid chromatography using standard methods.

Phosphatidylcholine, cholesterol and cholesterol oleate were chromatographically pure. The neutral fat from the lard was found, by TLC, to consist principally of triglycerides with minute traces of mono- and diglycerides. The fatty acid composition of the phosphatidylcholine and triglyceride is given in Table I.

## RESULTS

#### Phosphatidylcholine-Cholesterol Oleate Emulsions

Stable emulsions were produced with w/w ratios of phosphatidylcholine-cholesterol oleate of 4:1. These were finely dispersed emulsions with globule diameters of up to 2$\mu$. In w/w ratios of 1:1, however, unstable emulsions were produced consisting mainly of sludge at all but the lowest concentration, 0.05 g/100 ml (see Table II).

After ultracentrifugation (carried out within 24 hr of ultrasonication) ca. 78% of the phosphatidylcholine and 5% of the cholesterol oleate remained in the infranatant solution in all preparations. Microscopic examination of the intranatant solutions showed that they contained a few emulsion globules, consequently the remaining cholesterol oleate was considered to be a contaminant. Coalescence of globules occurred within a week. Clumping was not observed.

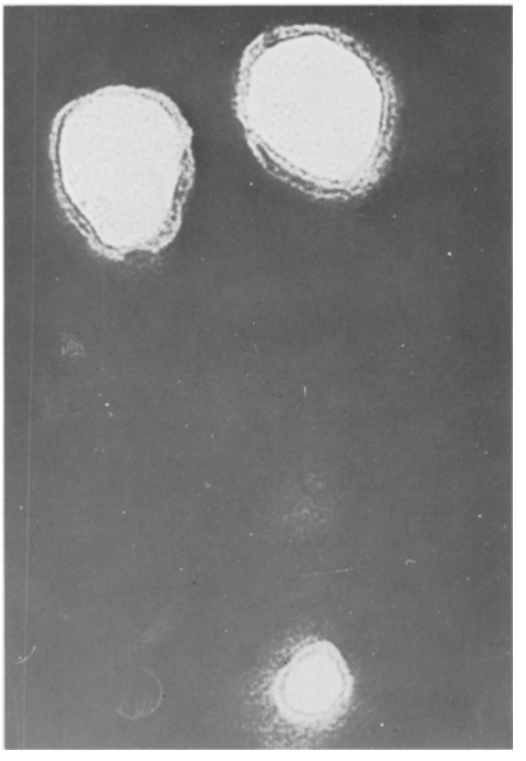

FIG. 2. Electron photomicrograph of lamellae of phosphatidylcholine around globules of triglyceride. Magnification: x 342,000.

Cholesterol oleate ultrasonicated alone in water produced a course emulsion with globule sizes of up to 15$\mu$. Creaming and globule coalescence commenced within 2 days. The globules were birefringent when viewed in polarized light, but the nature of the birefringence was not examined. Ultracentrifugation removed 95% of the oleate, leaving the infranatant solution optically clear.

#### Phosphatidylcholine-Triglyceride Emulsions

Stable emulsions with globule sizes up to 5$\mu$ were produced at a phosphatidylcholine-triglyceride w/w ratio of 1:6. With increasing amounts of phosphatidylcholine more finely dispersed emulsions were produced. At w/w ratios of 1:1 in the concentration range 0.1-0.4 g/100 ml of total lipid, globule size was too small to be seen in the light microscope and the mixes were opalescent (see Table II and Fig. 1). An emulsion of total lipid concentration of 0.4 g/100 ml gave an optical density reading of

0.460 at 500 nm which remained unchanged for a week. Thereafter an increase in optical density occurred. Electron micrographs of this emulsion showed fat particles in the size range 260-3000 Å, many of which were spherical or spheroidal. Some of these globules were surrounded by a lamellar membrane, approximately 60 Å thick (see Fig. 2).

Lamellar spherical micelles of phosphatidylcholine were also seen, possibly representing the "unbound" phosphatidylcholine. After 3 months coalescence had occurred in these emulsions, and globules could be seen in the light microscope.

Ultracentrifugation separated the two components almost completely in all systems. The infranatant dispersion contained approximately 90% of the phosphatidylcholine and only 5% of the triglyceride. This triglyceride was considered as a contaminant since few emulsion globules were seen in the infranatant dispersion by light microscopy.

Triglyceride alone in water produced a coarse emulsion of globule size up to 16$\mu$. Creaming and coalescence commenced within 2 days. Clumping did not occur (see Table II). Ultracentrifugation removed ca. 96% of the triglyceride, leaving the infranatant solution optically clear.

#### Phosphatidylcholine-Cholesterol-Triglyceride Emulsions

Emulsions were produced by ultrasonicating phosphatidylcholine, cholesterol and triglyceride together at all the ratios and concentrations studied.

The presence of cholesterol initially reduced the degree of dispersion of phosphatidylcholine and triglyceride considerably, but as the cholesterol content was raised this degree of dispersion improved. When emulsions were constituted in phosphatidylcholine-cholesterol (w/w) ratios of 2:1, emulsification of the triglyceride was not as effective as when constituted in a w/w ratio of 1:1. In the former the globule size was greater, and slight clumping occurred within 2 days. However stability was not affected. Negligible coalescence had occurred in these emulsions after a period of 3 months (see Table II).

After ultracentrifugation the infranatant solution contained micellar phosphatidylcholine and cholesterol in progressively lower concentrations as the triglyceride concentration increased. Less micellar lipid remained after ultracentrifugation of emulsions constituted in phosphatidylcholine-cholesterol w/w ratios of 1:1 than in those constituted in w/w of 2:1. In these former emulsions almost all the micellar lipid was removed at triglyceride concentrations of 0.15 g/100 ml and above, leaving the infranatant solution almost optically clear.

## DISCUSSION

The rate of coalescence or the separation of the dispersed phase is used as the measure of stability (10). Clumping and creaming are not included in this criterion since the globules remain discrete. The size-distribution counts were a rough estimate of coalescence. For a statistically accurate assessment of dispersion and stability, size-distribution counts of at least 2000 globules must be made on each reading (11).

The most important factor determining coalescence in most emulsions is the nature of the adsorbed film, which forms a mechanical or electric barrier to coalescence. It is considered that this film must form either (a) a coherent, mechanically strong film; (b) an orientated unimolecular layer; or (c) a layer consisting of tightly packed solid particles (10). The surface viscosity or plasticity is considered to be the most important mechanical factor in the absence of a surface charge since the droplets must be deformed during coalescence. Therefore high surface viscosity and low surface plasticity, i.e., rigidity, militate against coalescence (12).

Cholesterol oleate required more phosphatidylcholine than the triglyceride used for a comparable degree of globule dispersion. However since cholesterol oleate has a higher melting point than triglyceride, this effect may have been due to resistance to ultrasonic fragmentation. In contrast to the findings in this study, phosphatidylcholine has been reported to be capable of solubilizing cholesterol esters (with saturated fatty acids ) (13).

The dispersion of triglyceride into colloidal-sized particles in the presence of sufficient phosphatidylcholine illustrates the ability of surfactant to produce a spectrum of emulsion sizes, from coarse to ultramicroscopic, depending on its proportion.

The presence of cholesterol reduced the degree of dispersion, increased the period of emulsion stability, and initiated clumping in emulsions of phosphatidylcholine and triglyceride. This suggests that the cholesterol was located principally in the adsorbed film around the triglyceride globule, causing rigidity and stickiness, with a resultant decrease in the degree of emulsion dispersion. In its absence, the film was less rigid, resulting in an increased degree of globule dispersion.

The stability of colloidal phosphatidylcho-

line and cholesterol seemed to be modified by triglyceride, judging by the synchronous removal of the colloid with the triglyceride during ultracentrifugation. The lamellar membranes seen around the triglyceride globules (Fig. 2) probably consist of phosphatidylcholine, and this system therefore resembles serum chylomicrons (14).

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# The Metabolism of Neutral Lipids in the Spur Dogfish, *Squalus acanthias*

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## ABSTRACT

Cell free studies have shown that liver and intestine are the major sites of synthesis of triacyl glycerols in *Squalus acanthias*. The liver and to a lesser extent the intestine and stomach are major sites of wax ester synthesis. Muscle does not synthesize either triacyl glycerols or wax esters significantly. In vivo studies have shown that intravenously injected ($^3H$) fatty alcohol is massively oxidized to ($^3H$) fatty acid, the bulk of which appears in muscle. Liver appears to export both free fatty acids and triacyl glycerols to serum and thence to muscle. Free fatty acids, triacyl glycerols, wax esters and cholesteryl esters are all turned over within 48 hr in *Squalus* serum. The turnover of triacyl glycerols greatly exceeds the turnover of alkyl diacyl glycerols.

## INTRODUCTION

The livers of many species of sharks are characterized by the presence of large amounts of lipids that are not commonly found in appreciable amounts in the depot fats of land animals. It is certain that liver lipids in sharks are present as metabolic energy reserves but it has also been argued that the more unusual lipids, such as hydrocarbons and alkyl diacyl glycerols, may also be involved in buoyancy regulation (1,2). The spur dogfish conforms to the general pattern in sharks in that its liver contains 50-70% w/w of oil that consists almost entirely of triacyl glycerols and alkyl diacyl glycerols (3-6). The flesh of *Squalus* contains approximately 3-5% w/w of oil (7) that again consists almost entirely of triacyl glycerols and alkyl diacyl glycerols (6).

Traces of wax esters are present in liver oil (6) but considerable amounts of wax esters, as well as triacyl glycerols and alkyl diacyl glycerols, are present in the serum of *Squalus* (8). Cell free preparations of the liver of *Squalus* synthesize both triacyl glycerols and wax esters at an appreciable rate although the synthesis of alkyl diacyl glycerols is very limited (8,9).

The present work is concerned with investigating the roles of the different tissues of *Squalus* in lipid metabolism and the mechanisms whereby lipid metabolism in the different tissues is correlated. Some aspects of the data have already been communicated (10).

## MATERIALS AND METHODS

(1-$^{14}C$) Oleic acid (54 mC/m-mole) and (9,10-$^3H_2$) oleic acid (750 mC/m-mole) were purchased from the Radiochemical Centre, Amersham, England. The latter compound was reduced to (9,10-$^3H_2$) oleyl alcohol with lithium aluminum hydride in anhydrous ether, washed with 1% $NaHCO_3$ and dried.

Lipid standards for thin layer chromatography (TLC) were as described earlier (8). ATP, L-α-glycerophosphate, reduced glutathione and dihydroxyacetone phosphate (dimethyl ketal, cyclohexylammonium salt) were purchased from Sigma Ltd., London. Reduced coenzyme A and NADPH were purchased from Boehringer Corp. Ltd., London.

Adult male specimens of spur dogfish *(Squalus acanthias)* of 1.0-1.5 kilos were caught in Loch Etive, Scotland, and maintained in the laboratory aquarium for about 14 days at 14 C. During this time the fish did not accept food.

To prepare cell free fractions all tissues were homogenized in 0.1 M tris-HCl adjusted to pH 7.4 at 14 C, containing 0.25 M sucrose (medium A). Muscle was first macerated by treating 1 g wet wt with 2.5 vol medium A in the Ultra-Turrax disintegrator (Janke and Kunkel, Staufen i Br., West Germany) for 2 min. The preparation was finally homogenized in a glass-Teflon homogenizer as described earlier (8). Stomach and the intestinal tract were dissected from fish and cut open. No food contents were apparent. The tissues were rinsed thoroughly with sea water, blotted dry and the mucosal layers scraped off using a glass microscope slide. Mucosal scrapings were immediately homogenized in 2.5 vol of medium A. Liver and testis were first chopped finely with scissors and homogenized with 2.5 vol of medium A. All homogenates were centrifuged at 1000 g for 10 min and samples of the supernatant withdrawn for assay taking care not to remove oil which floated to the top of the tube in most cases. All the above procedures except pH adjustment were carried out at 0-2 C.

TABLE I

Synthesis of Lipids by Cell-Free Preparations of Different Tissues of the Dogfish

| Tissue[a] | | Triacylglycerols | | | Alkyl diacylglycerols | | Wax esters | |
|---|---|---|---|---|---|---|---|---|
| | | $^{14}C$ | $^{3}H$ | $^{3}H/^{14}C$ | $^{14}C$ | $^{3}H$ | $^{14}C$ | $^{3}H$ |
| Liver | +ATP | 5510 | 4960 | 0.90 | 320 | 1110 | 4220 | 6540 |
| | -ATP | 860 | 1380 | | 70 | 930 | 660 | 1500 |
| Muscle | +ATP | 444 | 440 | 0.99 | 100 | 470 | 650 | 1090 |
| | -ATP | 1090 | 1200 | | 190 | 1080 | 1590 | 2630 |
| Testis | +ATP | 3890 | 2300 | 0.59 | 270 | 770 | 8400 | 9900 |
| | -ATP | 840 | 1280 | | 170 | 890 | 3020 | 3050 |
| Intestinal | +ATP | 90,960 | 36,000 | 0.40 | 670 | 1610 | 19,720 | 19,350 |
| Mucosa | -ATP | 930 | 1170 | | 120 | 1170 | 4510 | 6180 |
| Stomach | +ATP | 1670 | 1610 | 0.96 | 480 | 1000 | 17,560 | 13,550 |
| Mucosa | -ATP | 870 | 1470 | | 480 | 1580 | 7650 | 7030 |

[a] 1 g wet wt of each tissue was homogenized with 2.5 ml of 0.1 M tris-HCl, pH 7.4, containing 0.25 M sucrose. 0.1 ml of the supernatants obtained after centrifuging the homogenates at 1000 g for 10 min was incubated for 3 hr at 15 C in a total volume of 1 ml with 10 mM ATP, 10 mM $MgCl_2$, 0.1 mM coenzyme A, 2.5 mM glutathione, 100 mM L-α-glycerophosphate, 50 mM dihydroxyacetone phosphate, 200 mM sucrose, 80 mM tris-HCl (pH 7.4) and 0.5 μC each of (1-$^{14}C$) oleic acid (1 mC/m-mole) and (9,10-$^{3}H_2$) oleyl alcohol (1 mC/m-mole). The isotopes were added in 10 μl of 2% Triton X-100. The reaction was terminated by isolating total lipid. Data are expressed as dpm incorporated into a given lipid per 1 ml assay.

Serum was isolated after direct withdrawal of blood from unanesthetized fish by cardiac puncture. The blood was allowed to clot at room temperature for 60 min and serum was collected after centrifuging at 1000 g for 10 min.

For intravenous injection ($^{3}H$) oleyl alcohol was equilibrated for 2 hr at 15 C with serum that had been heated at 60 C for 30 min to inhibit possible acyl transferase activity. The radioactive serum was injected directly into the pectoral vein complex without anesthesia.

In all cases total lipid was isolated by the method of Bligh and Dyer (11). For in vitro assays the reaction media were chilled and the reaction was terminated by applying total lipid extraction. With in vivo experiments the fish were first bled by cardiac puncture to isolate serum, then killed and total lipid was isolated from the various tissues with the homogenizer.

Lipids were separated on thin layers of Silica Gel G (250 μ thick) in petroleum ether-diethyl ether-acetic acid (90:10:1 or 80:20:1) and benzene-hexane-acetic acid (40:60:1). The latter solvent provided slightly better separation of wax esters and cholesteryl esters than the former. Lipid zones were detected by exposure to iodine vapor and scraped directly into scintillation vials for determination of radioactivity (9).

In cell free assays data were expressed as dpm incorporated into a given lipid class per 1 ml assay system (see Table I). For in vivo experiments data were first expressed as dpm incorporated into a given lipid class per 10 mg of total lipid in a particular tissue. The data were then converted into dpm incorporated into a given lipid in 1 g wet wt of tissue using the following experimentally determined values for g of total lipid per 100 g wet wt of tissue: liver 50; serum 0.125; intestinal mucosa 2.0; stomach mucosa 3.0; muscle 5.0. Finally the data were converted to dpm in a given lipid in each entire tissue of the dogfish. For the latter calculation it was determined experimentally that a dogfish weighing 1 kilo contained approximately 750 g muscle tissue, 100 g liver and ca. 5 g each of intestinal and stomach mucosal scrapings. The blood volume of a 1 kilo fish was assumed to be 80 ml (12).

## RESULTS

Previous work was concerned with comparing the rates of synthesis of triacyl glycerols, alkyl diacyl glycerols and wax esters in cell free fractions of the liver of *Squalus* (8,9). In the present work attention was concentrated initially on the abilities of cell free fractions of different tissues to synthesize these lipid classes. Equal wet weights of the different tissues were homogenized in 2.5 vol of buffer, and 0.1 ml of the supernatant obtained after centrifuging the homogenate at 1000 g for 10 min was assayed—that is, equal wet weights of the tissues were assayed. The assays were identical in all cases and ($^{14}C$) oleic acid was used together with ($^{3}H$) oleyl alcohol to moni-

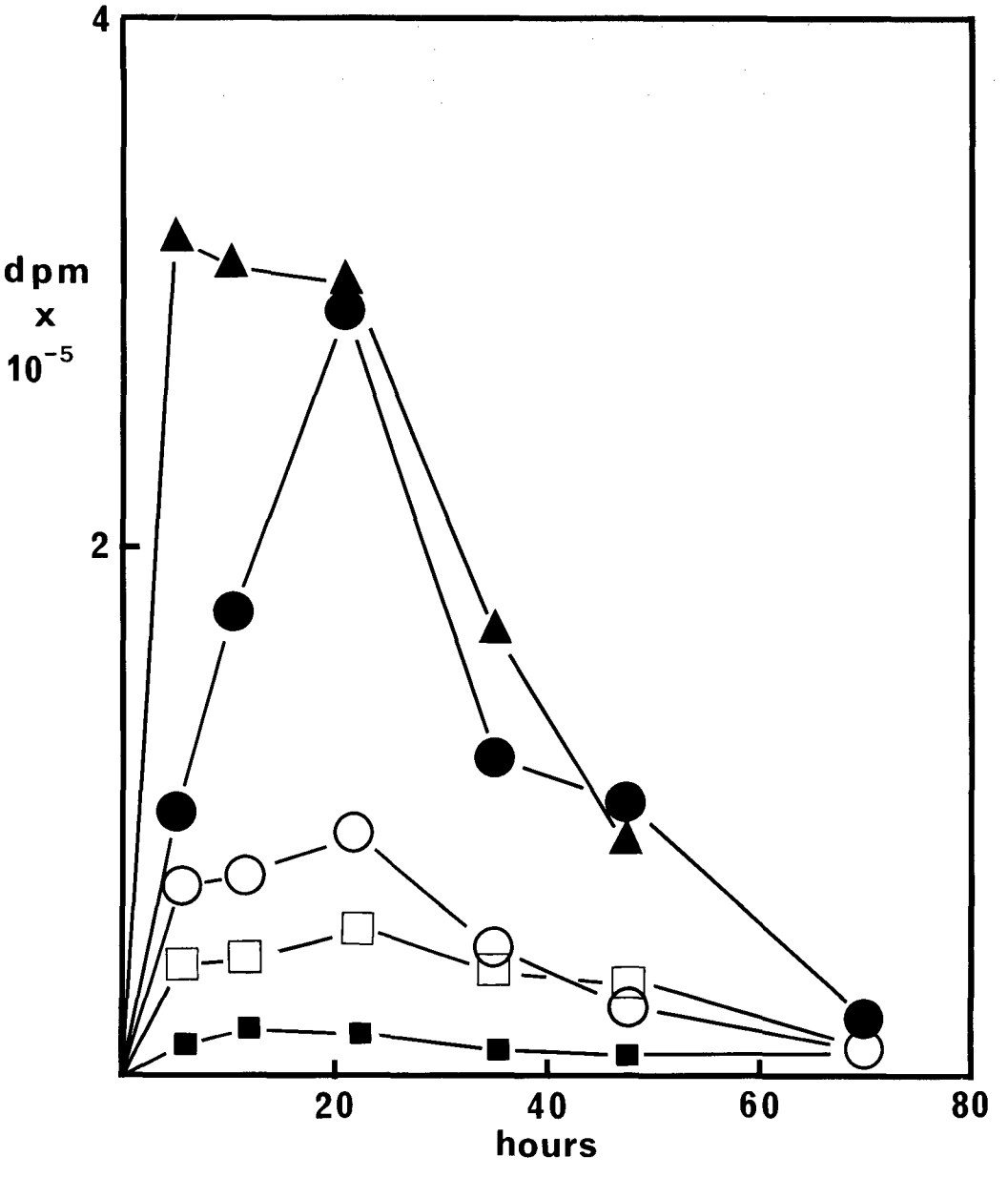


FIG. 1. Incorporation of radioactivity into serum lipids after injecting (9,10-$^3H_2$) oleyl alcohol intravenously into *Squalus*. Each fish received 100 $\mu$C of (9,10-$^3H_2$) oleyl alcohol (750 mC/m-mole) in 0.1 ml of heat treated *Squalus* serum intravenously. At times shown the fish were killed, sera collected and total lipids isolated and analyzed as in Methods. Data are expressed as dpm incorporated into all of a given lipid class in the entire serum of each fish (▲ free fatty acids; ● triacyl glycerols; ○ wax esters; □ cholesteryl esters; ■ alkyl diacyl glycerols).

tor the formation of ester and alkyl bonds respectively. Use of these isotopically labeled compounds also gave a measure of the interconversion of fatty acid and fatty alcohol.

In Table I the ratio of tritium-carbon-14 in triacyl glycerols gives an index of the ability of the various tissues to oxidize fatty alcohol to fatty acid. On this basis it can be seen that liver, muscle and stomach mucosa are more active in oxidizing fatty alcohol to fatty acid than are testis and intestinal mucosa. As in previous work, fatty alcohol recovered from the system contained negligible levels of carbon-14 indicating that conversion of fatty acid to fatty

TABLE II

Incorporation of Radioactivity From (9,10-$^3H_2$) Oleyl Alcohol Into Lipids of Different Tissues of the Dogfish in vivo[a]

| Lipid class | Hours | Liver | Muscle | Serum | Intestinal mucosa | Stomach mucosa |
|---|---|---|---|---|---|---|
| | 12 | 396 | 4930 | 32 | --- | --- |
| Free fatty acids | 23 | 294 | 4288 | 30 | --- | --- |
| | 47 | 219 | 3623 | 9 | --- | --- |
| | 12 | 311 | 289 | 18 | 9 | 5 |
| Triacyl glycerols | 23 | 170 | 321 | 28 | 9 | 3 |
| | 47 | 100 | 98 | 11 | 8 | 4 |
| | 12 | 275 | 97 | 2 | --- | --- |
| Alkyl Diacyl glycerols | 23 | 92 | --- | 2 | --- | --- |
| | 47 | 32 | 147 | 2 | --- | --- |
| | 12 | --- | --- | 8 | --- | --- |
| Wax esters | 23 | 107 | --- | 9 | --- | --- |
| | 47 | 5 | 92 | 4 | --- | --- |
| | 12 | 80 | 71 | 5 | 7 | 2 |
| Cholesteryl esters | 23 | --- | 142 | 6 | 2 | 3 |
| | 47 | 35 | 62 | 3 | 3 | 2 |

[a]Each fish received an intravenous injection of 100 $\mu$C of (9,10-$^3H_2$) oleyl alcohol (750 mC/m-mole) in 0.1 ml of heat-treated *Squalus* serum. At 12, 23 and 47 hours fish were killed, tissues excised and total lipids isolated and separated as detailed in Methods. Data are expressed as dpm x $10^{-4}$ incorporated into all of a given lipid class in an entire tissue. —Signifies 0.5.

alcohol was minimal (8,9). The order of activity of the different tissues in synthesizing triacyl glycerols in Table I is intestinal mucosa $\gg$ liver $>$ testis; muscle and stomach mucosa are essentially inactive. The order of activity in synthesizing wax ester is intestinal mucosa $>$ stomach mucosa $>$ testis $>$ liver; muscle once more is inactive. It may be noted that substrate levels of fatty alcohol are present in these assays so that maximal rates of synthesis of wax ester are probably occurring. Marked dependence on added fatty alcohol is known to occur for wax ester synthesis in liver (8). In all cases in Table I incorporation of either tritium or carbon-14 into alkyl diacyl glycerols was essentially negligible. This situation did not change when the medium was supplemented with NADPH after the first hour of incubation in order to reduce alkyl dihydroxyacetone phosphate formed to alkyl glycerophosphate and so promote the formation of alkyl diacylglycerol (9).

Table II presents data obtained after injecting (9,10-$^3H_2$) fatty alcohol intravenously into a series of fish and determining the radioactivity associated with the different lipid classes in the different tissues. It should be noted that the data in Table II are expressed as the level of radioactivity associated with all of a given lipid class in an entire tissue—that is, the data are concerned with how much radioactivity is present in a given lipid in any one tissue at a given time. On this basis it is immediately obvious that the bulk of radioactivity ends up as free fatty acid in muscle. For example the radioactivity present in free fatty acid in muscle after 12 hr corresponds to 22% of the radioactivity injected as fatty alcohol. Considerable amounts of radioactivity are present in liver and significant amounts are present in serum, free fatty acid being most heavily labeled in these tissues. Second to fatty acids, triacyl glycerols are most heavily labeled, radioactivity being associated mainly with muscle and liver. Alkyl diacyl glycerols are significantly labeled only in liver and to a lesser extent in muscle, with serum containing barely significant radioactivity. Some radioactivity is associated with wax esters and cholesteryl esters in liver. It is noteworthy that both intestinal and stomach mucosa contain negligible levels of radioactivity in free fatty acids, though in both cases small levels of radioactivity are present in both triacyl glycerols and cholesteryl esters.

Figure 1 presents more comprehensive data from the sera of the same fish as in Table II. It is immediately obvious that the most heavily labeled lipids in serum are free fatty acids and triacyl glycerols. The peak of radioactivity occurs in free fatty acids before it occurs in triacyl glycerols. It may be noted (Table II, Fig. 1) that the peaks of radioactivity for free fatty acids and triacyl glycerols in serum tend to coincide in time with the peaks for these lipids in muscle. The peaks for triacyl glycerols in serum and muscle occur after the peak for triacyl glycerols in liver, suggesting a precursor-product relationship between the triacyl glyc-

erols of liver and those of serum and muscle. Of the remaining lipids in serum only wax esters and cholesteryl esters are heavily labeled, with alkyl diacyl glycerols being barely significantly labeled (Fig. 1). Wax esters in serum are interesting since saponification of this lipid class and separation of the resulting fatty acid and fatty alcohol by TLC shows that 88% of the radioactivity is present as fatty alcohol. In contrast 86% of the very small levels of radioactivity in alkyl diacyl glycerols is present as fatty acids, the remainder being associated with glyceryl ether. Negligible amounts of free fatty alcohols could be recovered from serum by TLC and, even at the earliest time point in Figure 1, insignificant amounts of radioactivity were associated with the free fatty alcohol zone. It can be concluded from the data in Figure 1 that all the serum lipids are turned over in *Squalus* serum within 48 hr. Free fatty acids and triacyl glycerols are turned over at a much faster rate than either wax esters or cholesteryl esters. Alkyl diacyl glycerols are turned over at a very much lower rate.

## DISCUSSION

Previous work had shown that cell free fractions of *Squalus* liver synthesized triacyl glycerols and wax esters at essentially the same rate, while alkyl diacyl glycerols were synthesized at a rate which was at best one tenth the rate of triacyl glycerols (8,9). The data in Table I confirm these findings for liver and show additionally that intestinal mucosa is some 20 times more active at synthesizing triacyl glycerols than is liver on a wet weight basis, and some five times more active in synthesizing wax esters. However, in the adult fish used in these experiments (air weight ca. 1 kilo) the liver weighs approximately 100 g and about 5 g of scrapings can be obtained from either the intestinal or stomach mucosa. Consequently in the whole animal the liver and intestine are capable of contributing to approximately equal extents to the over-all levels of triacyl glycerols synthesized. Liver is the major organ of wax ester synthesis, although intestinal and stomach mucosae contribute significantly in this respect. Muscle appears to have little or no ability to synthesize either wax esters or triacyl glycerol and in no tissue was the synthesis of alkyl diacyl glycerols really significant.

The results of the cell free experiments were obtained with a basic assay that was applied equally to all tissues without attempting to optimize conditions for biosynthesis of different lipids that may vary from tissue to tissue. Nonetheless the results from the cell free experiments agree well with those from the in vivo experiment (Table II, Fig. 1) in that both approaches show an extensive oxidation of fatty alcohol to fatty acid, relatively rapid rates of biosynthesis of triacyl glycerols and very low rates of biosynthesis of alkyl diacyl glycerols.

A major aspect of the data in Table II is the finding that massive amounts of radioactivity are associated with free fatty acid in muscle. Since fatty alcohol was injected intravenously into the fish it is likely that the fatty acid in muscle has been derived from two major sources: first, from oxidation of fatty alcohol entering the muscles directly and second, from oxidation of fatty alcohol entering the liver with subsequent export of free fatty acid from the liver. These deductions are supported by the ability of both muscle and liver to oxidize free fatty alcohol (Table I, [8]) and by the presence of radioactivity in free fatty acid in liver (Table II). Fatty acid in serum is heavily labeled and turns over rapidly (Fig. 1); it is highly probable that this turnover reflects transport from liver to muscle rather than vice versa. Fatty acid in muscle could also arise from hydrolysis of triacyl glycerols imported into muscle. The major source of these triacyl glycerols is very likely to be the liver (Tables I, II; Fig. 1) since it is unlikely that intestine is contributing significantly to synthesis of triacyl glycerols in a nonfeeding fish. The latter assumptions are strengthened by the relatively low amounts of radioactive triacyl glycerols in intestine as compared to liver, and by the negligible levels of radioactivity in free fatty acids in intestine (Table II).

The results here may be compared with those of Kayama and Tsuchiya (13) who found that the great bulk of ($^{14}$C) stearic acid administered to carp by intestinal intubation appeared as stearic acid in muscle after 10 hr. Recently it has been found that ($^{14}$C) fatty acid fed to trout is rapidly and substantially incorporated as free fatty acid in serum with triacyl glycerols being labeled to a much lesser extent (J. Mead and J.S. Robinson—personal communication). Malins (14) has convincingly shown that both ($^{14}$C) palmitic acid and ($^{14}$C) chimyl alcohol administered intrahepatically to *Squalus* end up substantially as free fatty acid and triacyl glycerol in *Squalus* liver within 18 hr.

The present data may therefore be interpretated as showing that fatty alcohol injected intravenously enters liver and muscle to a substantial extent. In the liver the alcohol is incorporated to a limited extent into wax ester but is more massively oxidized to fatty acid. The latter is exported substantially as free fatty

acid but is also incorporated to a considerable extent into triacyl glycerols which are also exported. Consequently the liver of *Squalus* may be regarded as being biochemically similar to mammalian liver in that it exports triacyl glycerols, but it is also biochemically similar to mammalian adipose tissue in that it exports fatty acids. This is entirely to be expected in view of the adipose nature of *Squalus* liver. It is virtually certain that fatty acids and triacyl glycerols exported from liver are destined largely to the muscles where they are oxidized to provide metabolic energy. It is well established that the red muscle of *Squalus*, used continuously for cruising, utilizes lipid (7).

A direct conclusion from the present work is that triacyl glycerols are turned over very much more rapidly than alkyl diacyl glycerols in nonfeeding fish (Table I, Fig. 1). A corollary of this is that alkyl diacyl glycerols are relatively conserved. Such a situation may reflect a requirement to conserve alkyl diacyl glycerols with respect to a possible role in buoyancy (2).

Finally, the finding that wax ester is turned over relatively rapidly underlines the metabolic importance of wax ester in the serum of *Squalus*. The enzyme forming wax esters in liver is very much more active than the enzyme forming glyceryl ethers in liver (8,9)—a situation that also applies in the other tissues studied here (Table I). Consequently fatty alcohol is preferentially incorporated into wax esters under the present conditions. Recent work has shown that while the fatty alcohol moieties of wax esters in serum are very largely 18:1 and 16:0 chains, the fatty acid moieties are largely C20 and C22 polyunsaturated acids (Sargent, Gatten, Barone, Robisch and Malins, unpublished data). Furthermore, waxes in serum are known to be transported on serum lipoproteins (Sargent, Gatten and McIntosh, unpublished data). Such data strongly indicate that the striking ability of *Squalus* tissues to synthesize wax may reflect an important role for this lipid class in transporting "essential" fatty acids in serum.

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# The Synthesis of Prostaglandins in Human Platelets

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## ABSTRACT

Human blood platelets were found to possess the whole biological system for synthesis of prostaglandins from $C^{14}$-labeled acetate and inherent derivatives of essential fatty acids. The incorporation of radioactive acetate into prostaglandins, polar lipids and neutral lipids was followed as function of acetate concentration and time. Of the fractions mentioned, ca. 10% of the label was found in PGEs. Among the PGEs, $PGE_1$ and $PGE_2$ account for 48.6 and 43.4% of the radioactive label. The results obtained were related to the theory of the inhibiting function of prostaglandins on the platelet agglutination.

## INTRODUCTION

Platelet aggregation in human, rat and pig plasma is induced by ADP (1) and inhibited by $PGE_1$ (2). Aggregation caused by epinephrine or collagen, the increased adhesiveness of platelets to glass and the reduced electrophoretic mobility of the platelets effected by ADP are also reduced by $PGE_1$ (3,4). Among the other prostaglandins, $PGA_1$ and $PGF_{1\alpha}$ are less inhibitory (2,5). The activities of a number of hormones, whose effects are presumably mediated by cyclic AMP, are inhibited by prostaglandins (6-12).

Since, however, the half-life of the prostaglandins in the blood stream is only about 2 min due to inactivation in the lung tissue (13), the role of prostaglandins as inhibitors of agglutination of the platelets may be of special biological importance if the blood platelets do possess the whole enzymic system for prosta glandin synthesis. The present communication has been designed to elucidate this question.

## MATERIALS AND METHODS

### Preparation of Platelets

This was done by a method modified after Deykin and Desser (14). Blood was collected from normal human donors using trisodium citrate (3.8% adjusted to pH 7.4 with citric acid) as anticoagulant in the ratio of 9:1 v/v. All glassware was siliconized. Platelet-rich plasma (PRP) was obtained by centrifugation at 275 g for 15 min at room temperature. The PRP was pipetted out into another centrifuge tube and centrifuged at 2500 g for 5 min. The platelet-pellet so obtained was suspended into the Krebs-Ringer-Phosphate buffer (KRP) to a final concentration of about $2.0 \times 10^9$ platelets per milliliter. The platelets were counted in a Bürker-Türk's counting chamber. The suspension used was 99.9% pure with practically no admixture of leucocytes and erythrocytes (15).

### Chemicals

The chemicals were of the highest obtainable purity from British Drug Houses, Poole, Dorset, England.

### Preparation of Acetate-1-$^{14}$C

Sodium acetate (The Radiochemical Centre, Amersham, Buckinghamshire, England, specific activity 59 mCi/m mole, total activity 250 $\mu$Ci) was adjusted to 50 $\mu$moles of acetate by adding nonradioactive acetate, and raised to a volume of 1000 $\mu$liters.

### Glutathione

Eleven milligrams glutathione (reduced form) were dissolved in 2 ml redistilled water. stored in a refrigerator and used within a week.

Prostaglandins E were prepared from human seminal plasma as described by Hamberg and Samuelsson (16). Reference prostaglandin $E_1$ was a gift from the Unilever Research Laboratories, Vlaardingen, The Netherlands.

### Metabolic Studies

Incubations were done in siliconized pyrex glass test tubes at 37 C with a continuous passage of oxygen (11 ml/min in water-saturated $O_2$). In order to define the experimental conditions, two kinds of incubations were done: (a) Incubations with varying concentration of acetate: the incubation mixture consisted of 1 ml platelet suspension, 1 ml buffer (KRP), 0.39 $\mu$moles glutathione and an appropriate amount of the radioactive acetate (total volume of incubation mixture 2.2 ml). The incubation was done for 2 hr with continuous supply of oxygen; (b) Incubations with varying time: in these, the incubation mixture contained 1 ml platelet suspension, 1 ml buffer, 0.39 $\mu$moles glutathione and acetate in a concentration of 2.4 mmoles/liter.

The reactions were initiated by adding sodium acetate and terminated by shaking the

incubation mixture with chloroform-methanol 7:3 v/v.

#### Extraction of Lipids

Five hundred microliters clear plasma were added to the incubation mixture. The mixture was extracted with 20 ml chloroform-methanol mixture at 4 C for 16 hr. The clear upper aqueous phase was isolated, and 1 ml of distilled water was added to the extraction mixture in such a way that it did not mix with the organic phase (as judged from no emulsion resulting on addition of water). The water phase was again pipetted off immediately. This procedure was done twice. The organic phase was filtered through a phase separating filter (Whatman No. 1ꝑ) paper soaked previously with the extraction mixture. The filter paper was washed twice with 2 ml of the solvent. The filtrate was concentrated to dryness under $N_2$ at 65 C followed by further drying under vacuum at 80 C. This step was essential because sodium acetate is soluble both in methanol and in methanol-water mixture but is insoluble in chloroform. The dry residue was extracted with 10 ml chloroform and filtered again through a phase separating paper (vide supra). This paper was previously soaked in chloroform prior to filtration. This procedure was made in a desiccator so that water vapor, which might condense due to the cooling produced by the evaporation of chloroform during the filtration, did not dissolve the acetate and thereby transfer it through the paper. The filter paper was washed with 3 ml chloroform three times, and then the filtrate was evaporated to a residue under $N_2$ at 60 C.

Experiments controlling the filtration process were done using 25 μliters of radioactive sodium acetate (specific activity 59 mCi/m mole; total activity 250 μCi in 1000 μliters) and equilibrating this between 2 ml water with 15 ml extraction mixture. The residue obtained by the process described above showed less than $10^{-4}$ μ Ci.

#### Thin Layer Chromatography

Thin layer chromatography (TLC) was performed on Silica Gel G (0.25 mm) (Merck, Darmstadt, W. Germany) using the equipment of Desega Werke (W. Germany) (17). The plates were activated at 110 C for 30 min and stored in a desiccator until use. The PGE mixture (ca. 500 μg) was added as ethereal solution to the residue mentioned above, and the material was applied with a Hamilton syringe, 50 μliters on the TLC plate. The plate was developed with ethylacetate-isooctane-acetic acid-water 110:20:10:100 v/v, equilibrated for 1 hr before using the organic phase. Reference spots of the PGE mixture were placed on the lateral parts of the plate. A total run of 12 cm was maintained in all the experiments. The lipid fractions were shown by iodine vapor.

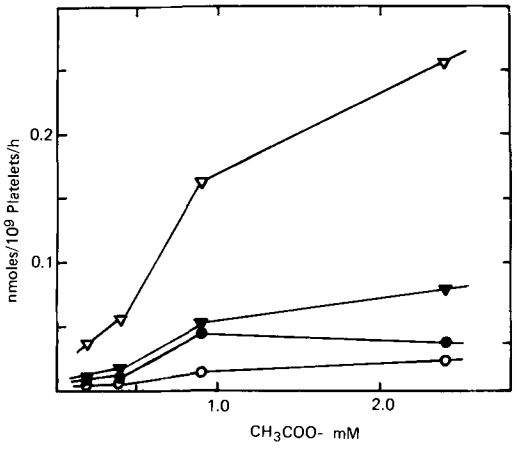


FIG. 1. The incorporation of $C^{14}$-labeled acetate in lipid fractions of human platelets. Conditions of incubation: Krebs-Ringer-Phosphate buffer (pH = 6.9); gluthathione, 55 μg/ml; platelets, 1.0 x $10^9$/ml; incubation time, 120 min. Ordinate: Acetate incorporated (nmoles acetate per $10^9$ cells per h); abscissa: mM acetate. Total acetate incorporated, −▽−▽−▽−; polar lipids, −▼−▼−▼−; prostaglandin E, −○−○−○−; neutral lipids, −●−●−●−.

#### Assay of Radioactivity

The radioactivity of the various zones from the plate was measured in a Beckman liquid scintillation counter (model 230) with a fluor prepared by mixing 8.0 g of 2-(4′-t-Butylphenyl)-5-(4′-biphenyl)-1,3,4-oxdiazole and 0.5 g of 2-(4′-Biphenyl)-6-phenyl-benzoxazole (Beckman Inc., Fullerton, Calif.) in 1 liter of toluene. Counting efficiency was 72%. Corrections for quenching were performed with an external standard. Total acetate incorporated in all the experiments had been calculated on the basis of the sum of all the activity from the entire plate which was divided into five zones as follows: (i) application-line zone, representing an area 0.5 cm above and below the application line, and containing most of the polar lipids; (ii) zone between (i) and (iii) the PGE zone (Rf 0.48-0.49); (iv) between the PGE zone and the solvent front; and (v) the solvent front which contained most of the neutral lipids.

The major part of the radioactivity was present in zones (ii) and (iv), which in the present solvent system might contain other prostaglandins as well.

#### Isolation of Crude PGE Compounds

Thin layer chromatography: 500 μliters clear

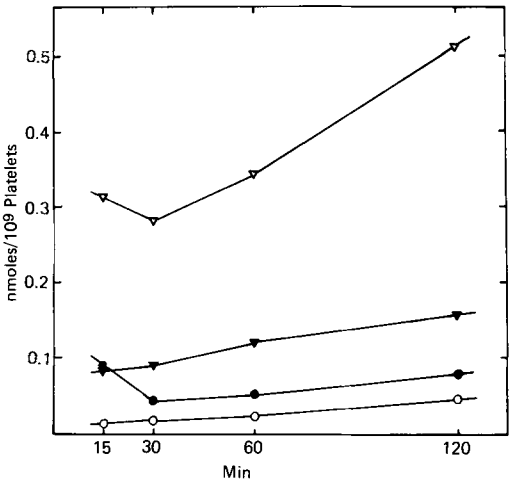


FIG. 2. The incorporation of $C^{14}$-labeled acetate into lipid fraction of human platelets. Conditions of incubation: KRP buffer, pH = 6.9; glutathione, 55 μg/ml; platelets, 1.0 x $10^9$/ml; acetate, 2.4 mmoles/liter or 2.4 μmoles/ml. Ordinate: nmoles acetate incorporated per $10^9$ cells; abscissa: time, min. Polar lipids (stay at the line of application), —▼—▼—▼—; neutral lipids, —●—●—●—; prostaglandin E, —○—○—○—; total acetate incorporated, —▽—▽—▽—.

plasma were added after completion of incubation and prior to extraction of the incubation mixture with $CHCl_3$-$CH_3OH$ 7:3 v/v. The material obtained on extraction of the incubation mixture followed by other treatments described above was taken up in 200-300 μliters chloroform to which were added 500 μg PGE mixture and resolved by TLC. The plate (20 x 20 cm) was coated with a 0.25 mm thick layer of Silica Gel G and activated at 110 C for 30 min. A margin 3 cm wide was marked on either side, while the rest of the plate was divided into two halves. On the margin the mixture of PGE markers was applied. The developing solvent consisted of ethylacetate-2,2,4-trimethylpentane-acetic acid-water 110:20:10:100 v/v, equilibrated for 1 hr before using the organic phase (18). The zones were shown by exposure to iodine vapor. The zone due to PGE compounds (PGEs) (Rf 0.48-0.49) was marked. The polar lipids (phospholipids) remained at the application line, and a zone 0.5 cm above and below the line of application was marked. The solvent front mainly due to neutral lipids was also marked. The plate was kept in the open until such time as the iodine was completely evaporated, as judged by the disappearance of the stain due to it. The marked areas were scraped off, eluted with methanol (vide infra) and counted.

#### Fractionation of PGE Compounds

In another similar experiment the zone due

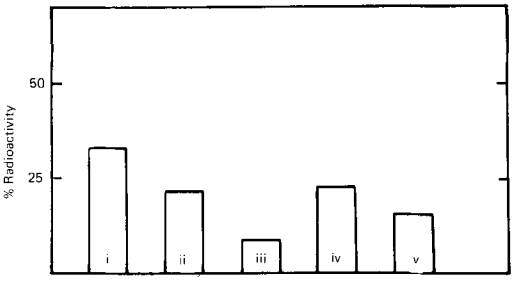


FIG. 3. Distribution of radioactivity among broad lipid classes after incubation with acetate-1-$^{14}$C. The incubation mixture consisted of 1 ml of the platelet suspension (2.0 x $10^9$ cells per ml) 1 ml of KRP buffer, glutathione (55 μg/ml) and acetate (2.4 mmoles/liter) in a total volume of 2.2 ml (see text). Ordinate: per cent of radioactivity on thin layer chromatography plate. (i) Application line zone containing highly polar lipids; (ii) zone between (i) and (iii) containing some of the less polar lipids; (iii) PGE zone; (iv) zone between PGE and solvent front; and (v) solvent front - containing neutral lipids.

to PGE compounds was scraped off and extracted with 3 ml methanol three times. The clear supernatant extracts were pooled together after centrifugation and methanol evaporated under $N_2$ at 60 C. The material thus obtained was taken up in 200 μliters methanol and resolved by TLC on $AgNO_3$-impregnated Silica Gel G 1:25 w/w, 0.25 mm layer activated at 110 C for 30 min (16). The solvent system consisted of ethylacetate-acetic acid-methanol-2,2,4-trimethylpentane-water 110:10:15:10:100 v/v, equilibrated for 1 hr before using the organic layer. The zones due to $PGE_1$, $PGE_2$ and $PGE_3$ were visualized by water spray with their respective Rf values as 0.74, 0.54 and 0.30. The zones were scraped off and counted for radioactivity.

## RESULTS

Figure 1 demonstrates the incorporation of $C^{14}$-labeled acetate into neutral lipids, polar lipids (phospholipids) and prostaglandins as function of acetate concentration. At low acetate concentration (until 0.9 mM of acetate) the labeling of neutral lipids and phospholipids increased parallelwise. Hereafter the phospholipid labeling only increased slightly and the labeling of neutral lipids showed only a slightly lower value (0.039 nmoles acetate per $10^9$ platelets per h) at 2.4 mM acetate than at 0.9 mM, where the incorporation was 0.047 nmoles acetate per $10^9$ cells per h. The incorporation of label into PGEs steadily increased as function of acetate concentration during the whole experimental period. The same was the case with the labeling of the total lipids, of which

only about 60% are accounted for by the lipids mentioned.

Figure 2 demonstrates the labeling as function of time. The labeling of phospholipids and PGEs steadily increased during the period of 2 hr. The total label and the labeling of neutral lipids initially (within the first 30 min) showed a slight fall followed by a steady increase in labeling during the next 90 min. Furthermore, from Figure 1 and 2 it is obvious that only ca. 10% of the total label ends up in the PGEs.

Figure 3 shows the percentage distribution of radioactive label on the TLC plate. It is seen that the highest label is encountered at the application area, where polar lipids are localized (33%). High label (22%) is also in the interzone between application area and PGE zone (9%). This is also the case with the interzone between PGE and the solvent front where the neutral lipids are found (23%). At the moment these intermediate radioactivities have not been identified with known label fractions.

Table I demonstrates the results of separation on TLC of the PGEs. Although $PGE_1$ possesses the highest percentage of label (48.6%), $PGE_2$ has nearly an equal label (43.4%). On the other hand, the $PGE_3$ and a fraction at the application line share 7.2% of the label in equal proportions.

TABLE I

Composition of PGE Compounds Formed in Human Platelets

| PGE Compound | Per cent radioactivity |
|---|---|
| $PGE_1$ | 48.6 |
| $PGE_2$ | 43.4 |
| $PGE_3$ | 7.2[a] |

[a]$PGE_3$ and the line of application share ca. 7.2% of the activity in almost equal proportions.

## DISCUSSION

Previous authors have advanced two major theories for the biological functions of the essential fatty acids (EFA). The first theory relates the function of EFA to their incorporation either directly, or after chain elongation and desaturation into the membrane lipids, hereby influencing the membrane properties (19,29). The second theory on the function of EFA is related to their transformation into prostaglandins (21-23). These components act on the hormone-stimulated cyclic-AMP system, (4,6-12). Our paper may support the latter theory as it shows PGEs to be enzymically formed in platelets. Adrenalin and ADP enhance the agglutination of circulating thrombocytes, probably through the cyclic-AMP system; prostaglandin $E_1$ inhibits this agglutination (9). Perhaps this phenomenon explains the acute formation of thrombi in the arteriosclerotic subjects (9) who have a relatively low supply of linoleic acid, since this acid can be transformed into di-homo-γ-linolenic and arachidonic acids, through chain elongation and desaturation, which in turn give rise to the formation of $PGE_1$ and $PGE_2$ ($PGF_{2\alpha}$) (21-23). These data may explain why dietary experiments have shown that a diet rich in butter or stearic acid strongly predisposes towards ADP and endotoxin-initiated thrombosis. These reactions, however, are reversed by feeding a diet rich in linoleic acid (24,25). The present study shows that blood platelets can be added to the series of "cells," namely the epithelial cells of the vesicular gland and the collecting tubules of the kidney which synthesize prostaglandins (26). The data showing active metabolic incorporation of radioactive acetate of platelets into different lipid fractions confirm previous studies (14,27-29). These show that both acetate and fatty acids are incorporated into the platelet lipids, which in turn suggests that these corpuscular elements in the blood stream play an active metabolic role.

From Figure 2 and Table I, it can be roughly calculated that under the experimental conditions used in the present paper, a net gain of 12 picomoles $PGE_1$ occurs per hour per $10^9$ platelets. Taking into account a mean volume of human platelets as 5 $\mu m^3$ (30), the total volume of $10^9$ platelets is $5 \times 10^{-6}$ liters. This would correspond to a net gain in the molarity of $PGE_1$ to 2.4 $\mu M$. This concentration seems even higher than that used by Kloeze (31), who showed the effect of $PGE_1$ in a concentration range from 11-45 nM in the external medium of thrombocytes. It is therefore tempting to ascribe a biological role of the PG synthesizing system of thrombocytes, especially with regard to the regulation of thrombocyte agglutination.

ACKNOWLEDGMENT

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# Lipid Metabolism of *Agaricus bisporus* (Lange) Sing.: II. Biosynthesis of Sporophore Lipids[1]

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## ABSTRACT

In vivo biosynthesis of lipids of *Agaricus bisporus,* the cultivated mushroom, was studied by examining incorporation of uniformly labeled $^{14}$C-linoleic acid and $^{14}$C-acetic acid added as supplements to mushroom compost. Incorporation of $^{14}$C from both substrates showed similar patterns. Lipids from sporophores were labeled proportionally to their mass with the exception of free sterol which was labeled to a greater proportion in relation to its mass. Quantitative analyses of free sterol was accomplished using pyruvyl chloride 2, 6 dinitrophenyl hydrazone derivatives. Qualitative analysis of free sterol components was made using mass spectrometry.

## INTRODUCTION

Dramatic increases in yield of the cultivated mushroom, *Agaricus bisporus,* have resulted from supplementation of compost with various refined and crude oils (1). Schisler (2) found the greatest stimulatory effect when compost was supplemented at the casing stage of the crop cycle. Recently Schisler and Patton (3) showed that linoleic acid can account for the stimulation of yield by vegetable oil supplements. Ethyl linoleate produced the same effect as vegetable oil containing an equivalent amount of linoleic acid. Addition of sterols did not enhance yield. Also Wardle and Schisler (4) showed that ethyl linoleate and ethyl oleate stimulated growth of mushroom mycelia when added to an artificial basal media.

Analyses of the lipids of mycelia and sporophores, grown under normal production conditions, showed that little or no free sterol was present in the mycelia, whereas free sterol is abundant in sporophores (5). Although both mycelial and sporophore lipids were high in linoleic acid, sporophore lipids contained a greater percentage of linoleic acid than did the mycelial lipids.

Little information is available concerning the biosynthesis of lipids in *Agaricus bisporus.* Wehrli and Rast (6) studied $^{14}CO_2$ incorporation into mushroom tissue homogenates and showed that radioactive lipids had the shortest half-life relative to proteins and carbohydrates.

The aforementioned studies stimulated this study of lipid utilization and biosynthesis in the cultivated mushroom using isotopically labeled compounds.

## MATERIALS AND METHODS

### Isolation of Sporophore Lipids

Strain 310 was obtained from the culture

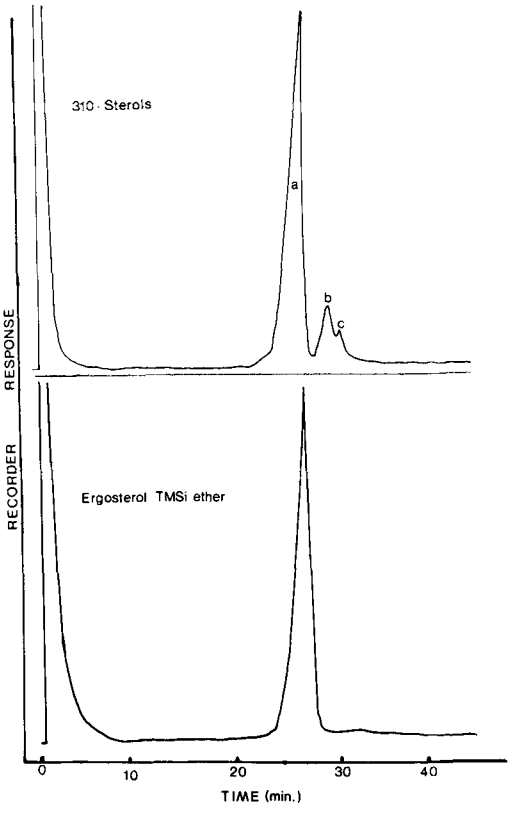


FIG. 1. Above, a gas chromatographic separation of free sterols from mushroom sporophore tissue showing three compounds: a, b, c. Below, a gas chromatogram of a standard trimethylsilyl-ergosterol.

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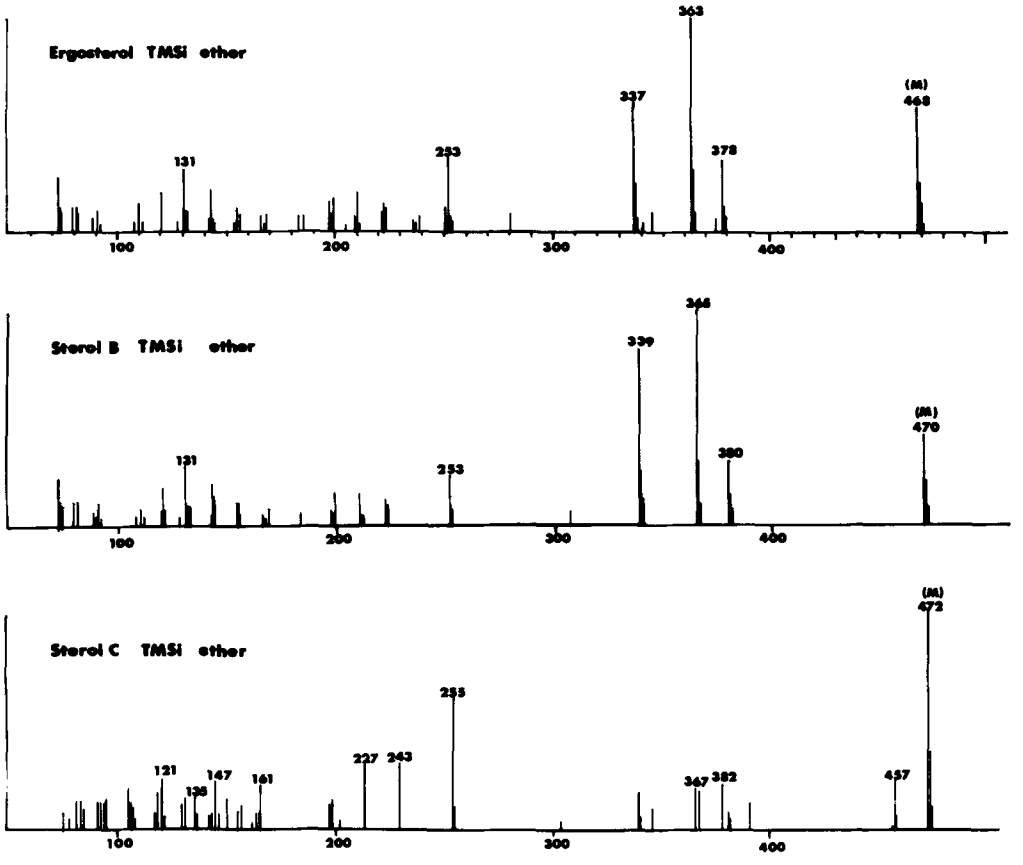


FIG. 2. Mass spectra of mushroom sterols.

collection of The Pennsylvania State University. The general method of mushroom culture and compost preparation as described by Schisler (2) was used in these studies. Glass pots containing approximately 2 kg of compost were used to grow the mushrooms. The pots were spawned (seeded with mycelia) and the mycelia were allowed to grow for 16 days. At that time the tracer was mixed with the compost and the pots were cased (covered with topsoil).

Because linoleic acid has been shown to be a stimulatory agent of *A. bisporus* (3), uniformly labeled $^{14}$C-linoleic acid was added to the mushroom compost at casing. Uniformly labeled $^{14}$C-acetic acid also was added. The mushroom sporophores that developed 16-18 days after casing were analyzed for incorporation of label into the various lipid classes.

Fifty $\mu$Ci. of uniformly labeled $^{14}$C-linoleic acid (Applied Science Laboratories) were diluted in 5 ml linoleic acid for addition to compost. One hundred $\mu$Ci. of uniformly labeled $^{14}$C-Na acetate (New England Nuclear) were diluted in 5 ml distilled water for addition to compost.

Sporophores were harvested from the first break or flush and their lipids were extracted according to the method of Folch et al. (7). Neutral lipids were separated from polar lipids using a silicic acid column as described by Hirsch and Ahrens (8).

### Thin Layer Chromatography

The neutral lipid classes were separated on glass plates coated with Silica Gel G (Merck) in a solvent system of low-boiling petroleum (bp 60-70 C)-diethyl ether-acetic acid 90:10:1 v/v. The polar lipid classes were separated by two-dimensional chromatography on Silica Gel H (Merck) as described by Parsons and Patton (9). A solvent system of chloroform-methanol-water-28% aqueous ammonia 130:70:8:0.5 v/v was used for the first direction and chloroform-acetone-methanol-acetic acid-water 100:40:20:20:10 v/v was used for the second direction. Lipid components were made visible by exposure to iodine vapor. The components were then scraped into scintillation vials and their radioactivity was counted in a liquid scintillation spectrometer.

TABLE I

Per Cent Incorporation of Recovered $^{14}C$ in Neutral and Polar Lipids

| Lipid | U-$^{14}C$-Acetate | U-$^{14}C$-Linoleate |
|---|---|---|
| Neutral | 33.1 | 34.4 |
| Polar | 66.8 | 65.5 |

TABLE II

Per Cent Incorporation of Recovered $^{14}C$ Into Neutral and Polar Lipid Classes

| Lipid | U-$^{14}C$-Acetate | U-$^{14}C$-Linoleate |
|---|---|---|
| Neutral | | |
| Monoglycerides | 8.7 | 14.5 |
| Diglycerides | 3.5 | 2.1 |
| Sterol | 73.6 | 73.8 |
| Free fatty acid | 1.7 | 3.3 |
| Triglycerides | 4.2 | 2.3 |
| Fatty acid esters | 6.8 | 1.2 |
| Sterol esters | 1.4 | 2.4 |
| Polar | | |
| Phosphatidyl inositol | 5.2 | 5.0 |
| Phosphatidyl serine | 2.7 | 4.9 |
| Phosphatidyl choline | 40.6 | 34.0 |
| Phosphatidyl ethanolamine | 41.0 | 43.2 |
| Other polar lipids | 10.3 | 12.4 |

### Gas Liquid Chromatography (GLC)

Fatty acid methyl esters were formed by saponification of the total lipid extract in 0.5% KOH in methanol and refluxing in $BF_3$-methanol (Applied Science Laboratories). Methyl esters were extracted in hexane and chromatographed on a Barber-Coleman 5000 Gas Chromatograph equipped with a 10 ft x .25 in. column packed with 10% DEGS and 2% $H_3PO_4$ on Gas Chrom A (Applied Science Laboratories). The column effluent was trapped from a heated outlet manifold in glass U-tubes filled with silica sand. The fatty acid methyl esters were eluted from the sand traps with scintillation fluid directly into scintillation vials and were then counted in the scintillation spectrometer.

### Quantification of Sterols

Total sterols were quantitatively analyzed as their pyruvyl 2, 6-dinitrophenyl hydrazones (2, 6-DNPH), as described by Schwartz and Brewington (10), and modified by Timmen et al. (11).

Qualitative analyses were accomplished by scraping the sterols from the thin layer plates and eluting them with chloroform-methanol 2:1 v/v. The sterols were dissolved in 150 μliters of tetrahydrofuran and combined with 100 μliters of hexamethyl disilazane and 50 μliters of trimethylchlorosilane to form trimethylsilyl derivatives. These derivatives were then chromatographed on a Hewlett-Packard 5750 Gas Chromatograph using a 12 ft x 1/8 in. 1% SE-30 column maintained at 250 C. Mass spectrometry of sterols was accomplished using an LKB-9000 Gas Chromatograph-Mass Spectrometer equipped with a column of the dimensions and packing described above. Spectra of the trimethylsilyl (TMS) derivatives of sterols were recorded at 70 ev.

## RESULTS

The quantitative analyses of free sterol gave an average of 349.3 μmoles/g total lipid or 135.7 mg (ergosterol)/g total lipid based on five experiments. Figure 1a shows a representative separation of the trimethylsilyl derivatives of the three major mushroom sterols by GLC. One component (a) was the predominant sterol. This component cochromatographs with a standard TMS-ergosterol as shown in Figure 1b. Components (b) and (c) did not cochromatograph with the following standard sterols: cholesterol, campesterol, stigmasterol and B-sitosterol. The mass spectra of the three sterol components are shown in Figure 2. The mass spectrum of component (a) was superimposable with that of a standard TMS-ergosterol analyzed under the same conditions. Component (b) gave a similar mass spectrum to that of ergosterol except that most of the major peaks were shifted two mass units higher than that of ergosterol. Component (c) showed a spectrum with two major diagnostic peaks—one at m/e 255 and the other, the molecular ion, at m/e 472.

The percentages of incorporation expressed are the averages of the two experiments. The incorporation of $^{14}C$ into neutral and polar lipid fractions was similar for both $^{14}C$-linoleic acid and $^{14}C$-acetic acid (Table I). The percentage incorporation of $^{14}C$ into major lipid classes of the neutral and polar lipid fractions is given in Table II. The pattern of incorporation into the lipid components of the neutral and polar fractions was the same for acetate and linoleate. Monoglycerides, sterols, phosphatidyl ethanolamine and phosphatidyl choline, which are the major lipid classes in mushroom tissue are labeled similarly from both acetate and linoleic acid. Incorporation of $^{14}C$ into major fatty acids is shown in Table III. Both acetate and linoleate again show

TABLE III

Per Cent Incorporation of $^{14}C$ Among Fatty Acids of Total Lipids

| Fatty acid | U-$^{14}C$-Acetate | U-$^{14}C$-Linoleate |
|---|---|---|
| C:16 | 8.4 | 6.8 |
| C:18 | 2.6 | 3.8 |
| C:18:1 | 1.6 | 2.0 |
| C:18:2 | 87.4 | 87.6 |

similar labeling patterns. All major mushroom fatty acids, palmitic, stearic, oleic and linoleic are labeled.

## DISCUSSION

Sterol (a) chromatographed with and gave an identical mass spectrum to that of TMS-ergosterol. Sterols (b) and (c) did not cochromatograph with available sterol standards, but did give satisfactory mass spectra. Tentative structural assignments can be made from the interpretation of these spectra.

Figure 2 shows the mass spectra of sterols (b) and (c). The fragmentation pattern of sterol (b) is similar to ergosterol. However the four most abundant peaks are shifted to mass units higher than those in ergosterol suggesting that a single double bond had been saturated. Characteristic peaks at m/e 131, m-131 and m-105 disclose that the structural difference is in the side chain as reported by Brooks et al. (12). They reported this pattern when comparing ergosterol to 7-dehydro cholesterol. These data suggest then that the structure of sterol (b) is identical to ergosterol with the exception of the 22 double bond which is saturated. The tentative identification of sterol (b) is 22-dihydroergosterol ($\Delta$ 5, 7-ergosta-diene-3B-ol).

Sterol (c) gave a mass spectrum with only two major diagnostic peaks: the molecular ion at m/e 472 and a major fragment at m/e 255. A similar spectrum was noted by Brooks et al. (12) for 7-cholestene-3B-ol trimethyl silyl ether. The molecular weight was 458, whereas the molecular weight of sterol (c) is 472. This suggests that sterol (c) has a similar structure to $\Delta$ 7-cholestene-3B-ol with the addition of one methyl group. The authors (12) showed that the m/e 255 fragment was characteristic of the ring cleavage of a $\Delta$ 7-stenol nucleus. By methylation of the 4 position the m/e 255 fragment was shifted 14 mass units to m/e 269. Sterol (c) therefore is a 7-cholestene-3B-ol type with the addition of a methyl group. Sterols of this type are usually substituted at the 24 position (13). If this is the case sterol (c) would have the structure of 7-ergostene-3B-ol or commonly known as fungisterol. The labeled compounds were added to the compost and the casing layer applied. Fifteen days later the sporophores matured and were harvested. The lipids analyzed represent the end products of synthesis during the fruiting process. This is also the time when lipid supplements are added to the compost (2).

Holtz and Schisler (5) reported the weight per cent of neutral and polar lipids in sporophores of strain 310 to be: 32.6% neutral and 67.3% polar. The percentage incorporation of $^{14}C$ from acetate and linoleic acid into these fractions, as seen in Table I, demonstrates that the lipids were labeled in relation to their mass. Generally the major classes of the neutral and polar lipid fractions were labeled in proportion to their mass from both linoleic acid and acetate, with the exception of free sterol. It was calculated, based on previous data that free sterol accounted for 42% of the neutral lipid (5). Free sterol incorporated 73% of the label from both linoleic acid and acetate which shows that free sterol incorporated a greater proportion of the label compared with its mass.

The most probable pathway for incorporation of label from a fatty acid into a free sterol is the oxidation of the fatty acid to acetate units which can then be utilized for sterol synthesis via the mevalonate pathway. The labeling of mushroom free sterols from labeled linoleic acid suggests that linoleic acid is oxidized to acetate before it is incorporated into sterols.

Palmitic, stearic, oleic and linoleic acids were labeled proportionally to their mass from both acetate and linoleic acid. These data suggest that metabolic fatty acids are also synthesized from acetate. If linoleic acid was a direct precursor of palmitic, stearic and oleic acids, it would be unlikely that identical incorporation patterns would be observed from labeled acetate and linoleic acid. Likewise the same labeling pattern of general lipid classes from acetate and linoleic acid supports the concept that linoleic acid is oxidized to acetate before incorporation into lipids. If linoleic acid were incoporated directly onto the glycerol moiety, a different labeling pattern would be expected from labeled linoleic acid than from labeled acetate. It is also suggested that free sterol was synthesized at a greater rate than other lipids, since it was labeled in a greater proportion than its mass from both acetate and linoleate.

The data from oil supplementation of mushroom compost have shown that linoleic acid was a stimulatory lipid (3). The evidence presented in this report suggests that linoleic acid was utilized as a source of acetate units for synthesis of sporophore lipids in *A. bisporus.*

Oil supplementation to compost, therefore, may provide a specific fatty acid substrate for metabolism by the mushroom. Mushroom tissue may either transport linoleic acid preferentially or have oxidative enzymes specific for linoleic acid.

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# Stereochemical Course of Diacylglycerol Formation in Rat Intestine


## ABSTRACT

The biosynthesis of diacylglycerols from 2-monoacylglycerols and free fatty acids was examined in evert sacs of rat intestinal mucosa. By means of alternate labeling of the monoacylglycerols and fatty acids, and conventional stereospecific analysis, it was shown that the main products of synthesis were the *sn*-1,2-diacylglycerols (53-63%), but *sn*-2,3-diacylglycerols (37-47%) were also formed in significant amounts. The total yield and proportions of the isomeric diacylglycerols recovered appeared to vary with the nature of the monoacylglycerol and the complexity of the free fatty acid mixture supplied.


Two pathways of triacylglycerol formation have been demonstrated in the intestinal mucosa. The bulk of the dietary fat is resynthesized into triacylglycerols by acylation of 2-monoacylglycerols (1,2), while any excess of free fatty acids is incorporated into the acylglycerols via the phosphatidic acid pathway (3). The stereochemistry of the intermediate diacylglycerols has not been extensively studied, because it has been widely assumed that the products of the 2-monoacylglycerol pathway would be equal amounts of the *sn*-1,2- and *sn*-2,3-diacylglycerols, and those of the phosphatidic acid pathway exclusively the *sn*-1,2-diacylglycerols (4). Recent work by Johnston et al. (5), however, has shown that the acylglycerol synthetase present in microsomal preparations of hamster intestine yields essentially *sn*-1,2-diacylglycerols also from 2-monoacylglycerols. The present study confirms the preferential formation of *sn*-1,2-diacylglycerols from 2-monoacylglycerols in evert sacs of rat intestine, but notes that *sn*-2,3-diacylglycerols are synthesized in only slightly lower yields.

Labeled 2-(glycerol-2-$H^3$)-monooleoylglycerol and monopalmitoylglycerol were synthesized according to the method of Serdarevich and Carroll (6). The final products were purified twice by thin layer chromatography (TLC) on Silica Gel G impregnated with boric acid using chloroform-acetone-methanol 96:4:0.5 as the solvent system (7). The specific activities were 17.9 dpm/nmole for monopalmitoylglycerol and 117.9 dpm/nmole for monooleoylglycerol. Incubations of evert sacs were carried out in a Krebs-Ringer phosphate buffer (pH 6.3, lacking Ca and Mg), glucose and lipid micelles of fatty acid, monoglycerides and sodium taurocholate in the respective concentrations of 1.2, 0.6 and 8.0 μmoles/ml. The medium was preincubated under $O_2$ atmosphere for 30 min after which the evert sacs were added and incubated for 40 min. At the end of incubation, the sacs were removed and washed with a 1% solution of taurocholate. The mucosal cells were scraped off from the muscle layers, weighed, and then homogenized and extracted with chloroform-methanol (8). The neutral lipids were separated by TLC using heptane-isopropylether-acetic acid 60:40:4. The diacylglycerols were located by UV light after the plate had been sprayed with fluorescein. The silica gel in the corresponding area was removed and extracted with 20% methanol in ether. These extracts were pooled, diluted with petroleum ether, washed with distilled water and dried over anhydrous sodium sulfate. After dilution with cold carrier diacylglycerols (10 mg), which had been prepared by pancreatic lipase hydrolysis of lard triacylglycerols, the 1,3-diacylglycerols were resolved from the X-1,2-diacylglycerols by TLC on the borate plates using chloroform-acetone 96:4 as the solvent. In some experiments the labeled diacylglycerols were resolved into classes differing in degree of unsaturation by argentation TLC. The plates were developed in chloroform-methanol 98:2 and the diglycerides were located and recovered as described above. The X-1,2-diacylglycerols were converted to phosphatidylphenols as described by Brockerhoff (9) using an excess of phenyldichlorophosphate. The phosphatidylphenols were dissolved in 3 ml diethylether and digested with phospholipase $A_2$ *(Crotalus atrox)* in a glass-stoppered Erlenmeyer flask (125 ml) containing 30 ml of 0.05 M aqueous triethylammonium bicarbonate (pH 7.4, prepared by saturating triethylamine in water with carbon dioxide), 0.1 ml $CaCl_2$ (0.1 M) and 10 mg enzyme. The mixture was stirred vigorously with a magnetic stirrer for 4

TABLE I

Distribution of Radioactivity in *sn*-1,2- and *sn*-2,3-Diacylglycerols Synthesized by Incubation of Evert Sacs With Fatty Acids and 2-Monooleoylglycerol

| Diacylglycerol isomer[a] | Lipid precursors,[b] % total X-1,2-isomer | | | |
|---|---|---|---|---|
| | 16:0 + 18:2 + MO | | 16:0 + 18:1 + 18:2 + MO | |
| | A | B | A | B |
| 1,2 | 57.0 | 53.2 | 56.0 | 61.2 |
| 2,3 | 42.9 | 46.8 | 44.0 | 38.8 |

[a]Isomers identified by the esterified hydroxyl groups of the glycerol molecule corresponding to *sn*-glycerol-3-(dihydrogen phosphate).

[b]Lipid precursors were fatty acids (indicated by the total number of carbon atoms and double bonds) and labeled 2-monooleoylglycerol (MO). The concentrations are given in the text. A and B represent separate incubations.

hr at room temperature. At the completion of the digestion, the reaction mixture was extracted with chloroform-methanol (8) and the chloroform layer was taken to dryness. The residue was redissolved in chloroform and applied to a thin layer plate, and the free fatty acids were removed by chromatography in chloroform-methanol-acetic acid 60:40:4. The lysophosphatidylphenols and residual phosphatidylphenols were extracted from the gel with chloroform-methanol 1:1. This extract was rechromatographed on another thin layer plate in chloroform-methanol-3% ammonia 63:30:7. The phosphatidylphenols were recovered from the gel by extraction and the radioactivity assayed by liquid scintillation counting. This technique of stereospecific analysis gave a yield of 50% *sn*-1,2-diacylglycerol isomer for the X-1,2-diacylglycerols derived from corn oil by digestion with pancreatic lipase and 100% *sn*-1,2-diacylglycerol isomer for the neutral products of hydrolysis of egg yolk lecithin by phospholipase C.

The yield of diacylglycerols from a 40 min incubation was 10-30 nmoles/100 mg wet tissue. This represented approximately 12-25% of the total esterified radioactivity. Incubations involving a monoacylglycerol and several fatty acids always gave more diacylglycerol than those containing only one type of fatty acid. Time studies indicated little change in the quantity of diacylglycerols from 10-40 min, while the amount of triacylglycerols continued to increase throughout the incubation period (10). There was no evidence of extensive isomerization or interesterification since the synthesized triacylglycerols contained over 90% and the recovered monoacylglycerols over 95% of the original fatty acid in the 2 position. The X-1,3-diacylglycerols accounted for 15-20% of the total diacylglycerol radioactivity when 2-monopalmitoylglycerol was used and 5-12% with 2-monooleoylglycerol.

Tables I and II show the distribution of radioactivity between the *sn*-1,2- and *sn*-2,3-diacylglycerols. With 2-monooleoylglycerol as the fatty acid acceptor (Table I), 53-63% of the radioactivity was associated with the *sn*-1,2-diacylglycerols, while 37-47% was present in the *sn*-2,3-diacylglycerols. A combination of 2-monopalmitoylglycerol with various fatty acids (Table II) consistently yielded 56-60% of the radioactive X-1,2-diacylglycerols as the *sn*-1,2-diacylisomers. Further digestion of the

TABLE II

Distribution of Radioactivity in *sn*-1,2- and *sn*-2,3-Diacylglycerols Synthesized by Incubation of Evert Sacs With Fatty Acids and 2-Monopalmitoylglycerol

| Diacylglycerol isomer[a] | Lipid precursors,[b] % total X-1,2-isomer | | | | | |
|---|---|---|---|---|---|---|
| | 18:1 + MP | 16:0 + 18:2 + MP | 16:0 + 18:1 + 18:2 + MP | | | |
| | Total | Total | Total | Saturates | Monoenes | Dienes |
| 1,2 | 56.0 | 56.6 | 59.3 | 57.3 | 60.6 | 61.5 |
| 2,3 | 44.0 | 43.4 | 40.7 | 42.7 | 39.4 | 38.5 |

[a]Isomers identified as in Table I.

[b]MP, labeled 2-monopalmitoylglycerol; other lipid precursors as in Table I. Saturates, monoenes and dienes correspond to the chemical classes of diglycerides with 0-2 double bonds isolated by argentation thin layer chromatography. Each fraction was subjected to a separate stereospecific analysis.

residual phosphatidylphenols with phospholipase $A_2$ failed to produce additional radioactive lysophosphatides indicating that the initial digestion had been complete. When the X-1,2-diacylglycerols synthesized from 2-monopalmitoylglycerol and mixed palmitic, oleic and linoleic acids were resolved on the basis of unsaturation by silver nitrate TLC, subsequent stereospecific analysis of the fractions demonstrated that there was preferential synthesis of the *sn*-1,2-diacylisomers in all diacylglycerol classes. A statistically significant positional preference for specific fatty acids, however, could not be shown. Somewhat lesser proportions (25-30%) of the *sn*-2,3-diacylglycerols were identified to result from incubations of unlabeled 2-monoacylglycerols and labeled fatty acids (2 incubations), but in these instances 15-25% of *sn*-1,2-diacylglycerols contained the labeled fatty acid in position 2, which suggested that they represented the contribution of the phosphatidic acid pathway. Synthesis via the phosphatidic acid pathway was also held responsible for the exclusive finding (2 isolations) of *sn*-1,2-diacylglycerols among the neutral lipids in the intestinal mucosa of the fasting rat (10). Thus 13 separate stereospecific analyses of the X-1,2-diacylglycerols recovered from 9 independent incubations demonstrated that both *sn*-1,2- and *sn*-2,3-diacylglycerols are formed in significant amounts from 2-monoacylglycerols in the mucosal cells of the rat intestine during active fat absorption. Although a definite preference for the formation of the *sn*-1,2-diacylisomers is seen, it is not as pronounced as that described by Johnston et al. (5) for the microsomes isolated from the mucosal cells of the hamster. This discrepancy cannot be fully explained on the basis of species difference, since other work (O'Doherty and Kuksis, unpublished results) has shown that the microsomes of the rat intestinal mucosa also form largely *sn*-1,2-diacylglycerols (84%), although *sn*-2,3-diacylglycerols (16%) may be identified.

In order to explain the low and variable yields of the *sn*-2,3-diacylglycerols by the microsomal preparations, it is postulated that the enzyme activity responsible for their synthesis is largely lost during the isolation of the microsomes. In this connection it may be pointed out that various laboratories have reported (11-14) that the major product formed from 2-monoacylglycerol by the microsomal fractions is not triacyl- but diacylglycerol, now identified as the *sn*-1,2-diacylisomer (5). Obviously then, the microsomes as commonly prepared may frequently possess little of the acyl transferase responsible for the acylation of the 3 position of the *sn*-1,2-diacylglycerol. It is possible therefore that the ready formation of the *sn*-2,3-diacylglycerols in the evert sacs is due to this enzyme. In view of the recent work of Schiller et al. (15) and Robins et al. (16), it is unlikely that the site of the synthesis of the 2,3-diacylglycerols or indeed of the bulk of the triacylglycerols (14) would be the brush border, since the membranes of the microvilli were shown to be free of glyceride acylating activity. The acylglycerol synthesis by the intestinal mucosa in vivo could then be attributed to the action of two stereospecific acylglycerol acyl transferases: one concerned with the acylation of the 1 position in 2-monoacyl-, 3-monoacyl- and 2,3-diacylglycerols, and the other with the acylation of the 3 position in the 1-monoacyl-, 2-monoacyl and 1,2-diacylglycerols. This would allow the conversion into triacylglycerols of a large variety of isomeric mono- and diacylglycerols with a minimum number of different enzymes without compromising the stereochemical requirements of the catalytic surfaces involved. All of the above transformations have been shown to take place with a variety of preparations of rat and hamster intestine as well as in vivo (4). For the present purposes it may be claimed that the relative rates of acylation of the different mono- and diacylglycerol isomers by the two transacylases would vary according to the affinity of the enzyme for the available substrate. The occurrence of a triacylglycerol synthetase as an apparent complex only, which actually consists of two stereospecific acyl transferases located at two different microsomal sites, might explain the lack of success with the isolation of a specific synthetase (17,18) as well as some of the species differences encountered (13).

Conceivably a reacylation of the 2-monoacylglycerols via both *sn*-1,2- and *sn*-2,3-diacylglycerols would allow a much more rapid absorption and resynthesis of the dietary fat by the intestinal mucosa, than would be possible if the conversion of the 2-monoacylglycerols into triacylglycerols was confined to only one of the two possible stereochemical routes. Verification of the above postulated mechanisms for the reacylation of 2-monoglycerols will require the separation of the two acyl transferases and a demonstration of acylation of the appropriate mono- and diacylglycerols by each enzyme.

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# Sulfatide Biosynthesis by Intact Microsomes and Triton Extracts of Normal and "Quaking" Mouse Brain

**ABSTRACT**

Microsomes from "Quaking" mice were unable to catalyze the transfer of $^{35}S$ from $PAP^{35}S$ to ceramide galactoside to form sulfatide. However Triton X-100 extracts were found to possess this activity.

The "Quaking" mouse is a mutant characterized histologically by a dimunition of myelin content and genetically by an autosomal recessive mode of inheritance (1). A striking decrease in the content of the characteristic lipids of the myelin sheath, principally cerebrosides and sulfatides, as well as a decrease in the fatty acids usually associated with these compounds, have been reported by several laboratories (2-7). In vivo studies have indicated a reduced capacity for the incorporation of several radioactive lipid precursors into these glycosphingolipids in "Quaking" mouse brain (8,9). In vitro experiments have indicated no reduction in the capacity of the "Quaking" brains for the synthesis of 3-keto-dehydrosphingosine, cera-

TABLE I

Comparison of Cerebroside Sulfotransferase by Intact and Triton X-100 Extracted Microsomes From "Quaking" and Normal Mice

| Microsome | Counts per minute sulfatide[a] per mg protein |
|---|---|
| Normal intact, no additions | 338 |
| Normal intact + galactocerebroside | 490 |
| "Quaking" intact, no additions | 133 |
| "Quaking" intact + galactocerebroside | 197 |
| Normal extract, no additions | 842 |
| Normal extract + galactocerebroside | 1076 |
| "Quaking" extract, no additions | 674 |
| "Quaking" extract + galactocerebroside | 1358 |

[a]Total counts cochromatograming with sulfatide standard on thin layer chromatography. These results represent the data derived from a single typical experiment. The average of four experiments were not provided due to the documented variability of animals (19).

ACKNOWLEDGMENT

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Microsomes from "Quaking" mice were unable to catalyze the transfer of $^{35}S$ from $PAP^{35}S$ to ceramide galactoside to form sulfatide. However Triton X-100 extracts were found to possess this activity.

The "Quaking" mouse is a mutant characterized histologically by a dimunition of myelin content and genetically by an autosomal recessive mode of inheritance (1). A striking decrease in the content of the characteristic lipids of the myelin sheath, principally cerebrosides and sulfatides, as well as a decrease in the fatty acids usually associated with these compounds, have been reported by several laboratories (2-7). In vivo studies have indicated a reduced capacity for the incorporation of several radioactive lipid precursors into these glycosphingolipids in "Quaking" mouse brain (8,9). In vitro experiments have indicated no reduction in the capacity of the "Quaking" brains for the synthesis of 3-keto-dehydrosphingosine, cera-

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[a]Total counts cochromatograming with sulfatide standard on thin layer chromatography. These results represent the data derived from a single typical experiment. The average of four experiments were not provided due to the documented variability of animals (19).

mide, sphingomyelin, nor in the hydrolysis of either ceramide glucoside or ceramide galactoside (10-12). The decrease in the activity of the ceramide-galactosyl transferase and sphingosine-galactosyl transferase has been reported (8,11,13,14). A 50% reduction in the ceramide galactoside-PAPS sulfotransferase activity of intact "Quaking" brain microsomes has been reported (15). The purpose of this report is to further document this deficiency, as well as to present data indicating that the decreased activity is no longer apparent when Triton X-100 extracts of microsomes are assayed for sulfatide synthesis.

## MATERIALS AND METHODS

Phosphoadenosine phosphosulfate-$^{35}$S (PAP$^{35}$S) was prepared according to the procedure of Robbins (16), its purity monitored by paper chromatography (17) and found to be approximately 75-80% pure. Galactocerebroside was prepared from calf brain as previously described (18). Brain microsomes were isolated from 15 to 20-day-old animals according to a published procedure (19) and suspended in 0.25 M sucrose. Aliquots of the microsomal suspensions were sonicated in the presence of 1% Triton X-100 with 2, 30 sec bursts with a semimicro tip on a Branson W 125 Sonifier at step 7. The samples were then centrifuged at 100,000 x g for 1 hr, and the clear supernate was removed and employed as the "Triton-extract". Each incubation tube contained: 50 $\mu$moles imidazole buffer pH 6.5, 1 $\mu$mole ATP, 0.5 $\mu$moles $MnCl_2$; 0.25 $\mu$moles EDTA; PAP$^{35}$S 1.5 x $10^6$ total cpm, enzyme source (.5-2.0 mg protein) in a total volume of 0.45 ml. Galactocerebroside, when present, was added by diluting a solution of 10 mg/ml in 1% Triton X-100 1:10 with distilled water and using 0.1 ml per incubation tube. The samples were incubated at 37 C for 30 min and then processed as previously described (10). An aliquot of the final lower chloroform phase was counted in a liquid scintillation counter. The remainder was chromatogramed on Silica Gel G plates (Analtech Corp.) using chloroform-methanol-water 65:25:4 as solvent. The plates were scanned on a Berthold Thin Layer Radioscanner (Varian Corp.). The areas containing radioactivity cochromatogramming with sulfatide standard (Supelco Corp.) were scraped and counted in Aquasol (New England Nuclear Corp.).

## RESULTS AND DISCUSSION

Experiments employing intact microsomes indicate that the capacity of the particles from the "Quaking" animals to incorporate $^{35}$S into lipid was reduced nearly 50% as compared to that from normal animals. Boiled enzyme controls gave values close to background. Thin layer radiochromatograms revealed the presence of two radioactive products, one of which remained at the origin. The nature of this material is currently unknown. The data presented in Table I, therefore, represent the quantity of radioactivity that cochromatogramed with sulfatide standards. The amount of $^{35}$S present in this area was proportional to the amount of enzyme source added. It is evident that although the intact microsomes from the "Quaking" animal have an apparent reduced sulfotransferase activity, no differences are seen when Triton X-100 extracts are employed. The addition of microsomes from "Quaking" animals to those from normal mice did not result in any inhibition or stimulation. It should be noted that other investigators employing only intact microsomes have reported similar deficiency with "Quaking" (15) or "Jimpy" (20) mice. The finding that the deficiency observed with intact microsomes is not present when a Triton extract is used could suggest that the sulfotransferase is not reduced in the "Quaking" animal. It is possible, therefore, that there is an organizational or structural defect in the machinery responsible for sulfatide biosynthesis in these mutants. Reports have indicated that there is no evidence for a deficiency in the sulfotransferase activity in kidney of "Quaking" and "Jimpy" mice (15,20) nor in spinal cord of "Jimpy" animals (20).

As noted by Farrell and McKhann (19), a large variation in absolute enzyme activity is observed from preparation to preparation, therefore the results presented represent those obtained from a single typical experiment. The presence of enzyme activity in Triton X-100 extracts and the absence in intact microsomes from "Quaking" mouse brain has been seen with three separate preparations. The Triton X-100 extracts and insoluble residue derived from both normal and "Quaking" microsomes were found to be incapable of catalyzing the ceramide:galactosyl transferase reaction (21).

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# Synthesis of 1,2 Dilinoleoyl and 1,2 Dipalmitoyl *sn*-3-Glycerophosphorylcholine[1]

## ABSTRACT

The synthesis of 1,2 dilinoleoyl *sn*-3-glycerophosphorylcholine (1,2-18:2-*sn*-3-GPC) and 1,2 dipalmitoyl *sn*-3-glycerophosphorylcholine (1,2-16:0-*sn*-3-SPC) is described. Synthesis was accomplished by acylating free glycerophosphorylcholine (GPC) with the anhydride and potassium salt of the desired acid. Purification of 1,2-16:0-*sn*-3-GPC was accomplished by crystallization, while purification of 1,2-18:2-*sn*-3-GPC required the use of alumina column chromatography and then crystallization from acetone at -7 C.

## INTRODUCTION

The synthesis of diacylglycerolphosphorylcholine is best accomplished by the procedures described by Cubero Robles and van den Berg (1). In addition to the synthesis of 1,2-di 16:0-*sn*-3-GPC, the method was found to be equally suitable for the synthesis of 1,2 di-18:2-*sn*-3-GPC. Slotboom and Bonsen (2) mentioned that heretofore no data were available on whether lecithins with polyunsaturated acids could be made by the method. Alternate methods of purification are described for these compounds.

## EXPERIMENTAL PROCEDURES

### Glycerophosphorylcholine

Glycerophosphorylcholine (GPC) was obtained as outlined by Chadha (3) utilizing the phospholipid fraction obtained from spray dried egg yolk. The necessity to purify the crude egg phospholipids by column chromatography is eliminated. Free GPC was obtained by removing the water under high vacuum and then drying over $P_2O_5$, rather than by crystallization as described. GPC is obtained as a clear highly viscous material.

### Palmitic and Linoleoic Anhydride

Palmitic and linoleic anhydride were prepared using dicyclohexyldiimide (4). Palmitic anhydride was isolated and purified, while the linoleic anhydride $CCl_4$ solvent-reaction mixture was filtered directly into the reaction

[1]Scientific contribution N. 491, Agricultural Experiment Station, University of Connecticut, Storrs.

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# Synthesis of 1,2 Dilinoleoyl and 1,2 Dipalmitoyl *sn*-3-Glycerophosphorylcholine[1]

## ABSTRACT

The synthesis of 1,2 dilinoleoyl *sn*-3-glycerophosphorylcholine (1,2-18:2-*sn*-3-GPC) and 1,2 dipalmitoyl *sn*-3-glycerophosphorylcholine (1,2-16:0-*sn*-3-SPC) is described. Synthesis was accomplished by acylating free glycerophosphorylcholine (GPC) with the anhydride and potassium salt of the desired acid. Purification of 1,2-16:0-*sn*-3-GPC was accomplished by crystallization, while purification of 1,2-18:2-*sn*-3-GPC required the use of alumina column chromatography and then crystallization from acetone at -7 C.

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### Palmitic and Linoleoic Anhydride

Palmitic and linoleic anhydride were prepared using dicyclohexyldiimide (4). Palmitic anhydride was isolated and purified, while the linoleic anhydride $CCl_4$ solvent-reaction mixture was filtered directly into the reaction

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system. The molar ratio of reactants in both acylation reactions was: GPC/potassium salt/acid anhydride 1:2:2.

**1,2-18:2-*sn*-3-GPC**

Every step of the following reaction was conducted under $N_2$. To 14.7 g (0.957 M) GPC, was added 36.9 g (0.116 M) potassium linoleate and 120 mg of the antioxidant, tert-butylhydroquinone, Eastman Chemical Prod., Inc. After admixture of the linoleoic anhydride-$CCl_4$ solution, the $CCl_4$ was removed under vacuum and the reaction allowed to proceed under vacuum (ca. 8 mm) at 44 C.

After 60 hr, 500 ml dry ethanol-free $CHCl_3$ was added and the mixture was kept at 44 C until the reactants dissolved. The mixture was placed in a freezer at -7 C for 12 hr. A thick, white precipitate rose to the top. The bottom $CHCl_3$ fraction was removed and retained and the above procedure was repeated twice with 500 ml quantities of $CHCl_3$.

The combined $CHCl_3$ fractions were evaporated to determine the approximate volume of reaction mixture. The mixture was diluted again with $CHCl_3$ to a 1:1 ratio to facilitate and standardize further purification by column chromatography.

Alumina grade F-20, 80-200 mesh, Alcoa Chemicals, was activated by heating for 12 hr at 260 C before use (5). It was found that 17 g of alumina per gram of reaction mixture was necessary for adequate purification.

The column, 40 x 2.5 cm, containing 175 g alumina was washed with one bed volume (130 ml) of $CHCl_3$. A 20 ml reaction mixture aliquot was added, followed by successive bed volumes (130 ml each) of $CHCl_3$, $CHCl_3/CH_3OH$ 90:10, and $CHCl_3/CH_3OH$ 3:2. After the first 200 ml of effluent subsequent to the addition of the sample, 30 ml fractions were collected. The second, third and fourth 30 ml fractions contained pure product with subsequent fractions containing in addition to lysolecithin, other more polar contaminants. Collected fractions were monitered by thin layer chromatography (TLC) in $CHCl_3/CH_3OH/H_2O$ 65:25:4 on Silica Gel H, 0.25 mm plates. Purified fractions from each column run were consolidated. After removing the $CHCl_3$ under vacuum, 500 ml redistilled acetone was added, from which the final product was isolated after 12 hr at -7 C. The product was isolated as a slightly opaque grease-like material totaling 29.8 g (66.7% yield).

**1,2-di-16:0-*sn*-3-GPC**

The synthesis of 1,2-di-16:0-*sn*-3-GPC is basically that previously described for 1,2-di-18:2-*sn*-3-GPC. To 3.1 g (0.012 M) GPC was added 7.1 g (0.024 M) potassium palmitate and 11.9 g (0.025 M) palmitic anhydride. The reaction proceeded at 80 C under constant vacuum (ca. 8 mm) for 72 hr.

After this time the solid mixture was broken and washed with 3-250 ml portions of ethyl ether (Mallinckrodt, anhydrous, ethanol-free) and then dissolved in 500 ml $CHCl_3$. With slight cooling below room temperature, the majority of the unwanted reactants crystallized and were then filtered from the $CHCl_3$ solution. Water and $CH_3OH$ were added to the $CHCl_3$ solution to a final ratio of 1:2:4, respectively, without vigorous shaking. The bottom phase was removed, dried through anhydrous $Na_2SO_4$, and evaporated to dryness. The residue, 8.7 g, was twice dissolved and reprecipated from 250 ml of methanol at -7 C. The precipitate was washed and filtered three times from 200 ml acetone-ethyl ether 97:3 at 30 C. The product, 6.6 g, was obtained as white amorphous solid (75.8% yield).

The final products were analyzed by enzymatic degradation with phospholipase A (6). After 10 hr the digestions were complete as evidenced by only the presence of free fatty acid and lysolecithin in a TLC system of $CHCl_3/CH_3OH/H_2O$ 65:25:4 with Silica Gel H plates. Gas liquid chromatograph analysis of the fatty acids of the intact products and the products resulting from phospholipase A digestion indicated the presence of only linoleic and palmitic acid in the respective lecithin.

The observed mp for 1,2-16:0-*sn*-3-GPC was 232 C, reported mp 234-235 (7).

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# The Hypolipidemic Effect of Calcium-Containing Compounds and Vitamin $D_2$ in the Rat


## ABSTRACT

Two per cent dietary calcium carbonate alone (Group 1), in combination with vitamin $D_2$ (Group 2) or oyster shell calcium with vitamin $D_2$ (Group 3), was added to an atherogenic diet containing 20% fat and 2% cholesterol and fed to adult male Holtzman strain rats to evaluate the hypolipidemic effects of these supplements. A control group received the same diet, but the calcium was replaced by sand. After 21 days the serum cholesterol concentrations were lower by 35, 27 and 22%, respectively, in experimental groups 1, 2 and 3, compared to the controls. Serum triglyceride concentrations were lower by 10% in groups 1 and 2, and 17% in group 3. Concomitant with these decreases in serum lipids, there were increases in fecal 3-β-hydroxy-5-ene sterol, total glyceride and free fatty acid excretion. Serum calcium was elevated in the rats fed diets containing calcium when compared with the controls, whereas serum magnesium was depressed in thesy animals. Less calcium was excreted in the feces of rats fed calcium carbonate in combination with vitamin D than in those fed calcium carbonate. The results indicate a possible means of simultaneously treating both calcium deficiency and hyperlipidemia with a single agent.


## INTRODUCTION

Supplementary dietary calcium exhibits significant hypolipidemic effects in man (1) and rats (2-4). Commercially available oyster shell calcium supplemented with vitamin $D_2$ has been safely employed in the treatment of calcium deficiency in humans. Preliminary to an examination of the possible hypolipidemic action of this combination in humans, a study of its effect upon serum and fecal lipids in mature rats when fed in combination with an atherogenic diet containing 20% fat, 2% cholesterol was under taken. The results of this study are presented here.

## EXPERIMENTAL PROCEDURES

Eighty male Holtzman strain albino rats, mean weight 509.6 g (SD ± 8.0 g), were divided into four groups and fed a previously reported corn-soya diet containing 18% added cocoa butter and 2% added cholesterol (2). For the three experimental groups, 2% calcium was added to this basal diet either in the form of (a)

TABLE I

Trace Metal Composition of the Feed and Drinking Water

| Component[a] | Basal diet, μg/g diet | Added by oyster shell calcium, μg/g diet | Water, mg/liter |
|---|---|---|---|
| Cobalt | 0.30 | | |
| Cadmium | 0.20 | | |
| Chromium | 2.00 | | |
| Copper | 7.36 | 0.20 | |
| Iron | 75.00 | 3.50 | 0.06 |
| Manganese | 44.12 | 18.20 | |
| Magnesium | 1000 | 3.40 | |
| Nickel | 2.00 | | |
| Lead | 0.44 | | |
| Zinc | 44.12 | 6.20 | 0.04 |
| Silica | | 7.80 | |
| Total hardness ($CaCO_3$) | | | 152 |
| Total alkalinity (as $CaCO_3$) | | | 115 |
| Sodium | | | 16 |
| Chloride | | | 11 |
| Fluoride | | | 0.5 |
| Nitrate | | | 2 |
| Sulfate | | | 51 |
| pH | | | 7.6 |

[a]Arsenic, chromium, copper and manganese were assayed for in the water, and were *not* detected.

TABLE II

Effect of Calcium Carbonate and OS-CAL on Serum Lipids and Serum Ions in Rats Fed a 20% Fat, 2% Cholesterol Diet

| Component | Controls | 2% $CaCO_3$ | 2% OS-CAL[a] | 2% $CaCO_3$ 25 IU/g vitamin D |
|---|---|---|---|---|
| Phospholipids, mg/100 ml | 176 ±10.3[b] | 138 ±10.0[c] | 151 ±11.0 | 136 ±7.0 |
| Cholesterol mg/100 ml | 138 ±12.4 | 90 ± 4.8[c] | 107 ±9.4[d] | 100 ±3.0[d] |
| Triglycerides, mg/100 ml | 418 ±10.3 | 377 ±19.0[d] | 348 ±14.8[c] | 370 ±19.0[c] |
| Free fatty acids, μEq/l | 117 ±5.9 | 154 ±4.8[c] | 180 ±5.5[c] | --- |
| Calcium, mg/100 ml | 9.36 ±0.03 | 10.08 ±0.05[c] | 10.01 ±0.04[c] | 11.16 ±0.04[c] |
| Magnesium, meq/l | 2.06 ±0.04 | 1.53 ±0.03[c] | 1.61 ±0.03[c] | ---- |
| Phosphorus, mg/100 ml | 4.76 ±0.21 | 4.30 ±0.17 | 4.32 ±0.23 | --- |

[a]Contained 400 U.S.P. Units of vitamin $D_2$ per gram of calcium.

[b]Mean ± SD of mean, 20 rats per dietary group.

[c]$P<0.01$ compared to controls.

[d]$P<0.05$ compared to controls.

calcium carbonate, (b) ground oyster shells containing 400 USP units of vitamin $D_2$ per gram calcium (OS-CAL, Marion Laboratories, Inc., Kansas City, Mo.) or 2% calcium carbonate plus 25 IU of vitamin $D_2$ per gram diet. The fourth group served as controls; 2% sand was added in place of calcium. The rats were fed diet and tap water ad libitum.

Trace metal analyses of the feed, oyster shell and the drinking water are given in Table I. Since all rats received the same drinking water, any effect of trace metals and calcium in the drinking water affected all groups equally.

After 21 days and following an 18 h fast, the rats were anesthetized with 50 mg sodium pentobarbital per kilogram body weight and exsanguinated via the dorsal aorta. Blood was permitted to clot, centrifuged, and immediately assayed for serum cholesterol, triglycerides, phospholipids and free fatty acids, and for calcium, magnesium and phosphorus. Feces were collected by cage for 3 day periods during weeks 1 and 2, and assayed for lipids, ash and calcium. The analytical procedures for serum and fecal lipids have been previously detailed (3,5). Calcium (6), magnesium (7), and phos-

TABLE III

Effect of Calcium Carbonate and OS-CAL Upon Fecal Lipids and Calcium inRats Fed a 20% Fat, 2% Cholesterol Diet

| Component | Control | | 2% $CaCO_3$ | | 2% OS-CAL | | 2% $CaCO_3$ + 25 IU/g Vitamin D |
|---|---|---|---|---|---|---|---|
| | Week 1 | Week 2 | Week 1 | Week 2 | Week 1 | Week 2 | Week 2 |
| Fecal excretion, wet wt g/rat/day | 5.3 ± 0.6[a] | 3.7 ± 0.8 | 10.2 ± 1.0[b] | 5.5 ± 0.3[b] | 9.5 ± 0.2[b] | 5.6 ± 0.2[b] | 8.4 ± 0.4[b] |
| Fecal moisture, % | 29.6 ± 2.0 | 27.1 ± 1.8 | 25.0 ± 2.2 | 19.0 ± 1.3 | 24.2 ± 4.0 | 20.5 ± 1.6 | 29.1 ± 1.8 |
| Ash, mg/rat/day | 1021± 86.1 | 856 ± 61.8 | 1860 ± 132.9 | 1118± 77.4 | 1225± 95.9 | 944± 26.1 | 851 ± 31.1 |
| Calcium, mg/rat/day | 19± 1.7 | 19 ± 1.1 | 659 ± 39.6[b] | 420± 71.0[b] | 358± 27.2[b] | 303± 18.0[b] | 342 ± 13.5[b] |
| Total lipids, mg/rat/day | 481± 34.9 | 474 ± 27.3 | 1810 ± 73.6[b] | 1796± 88.8[b] | 1199± 87.2[b] | 1770± 79.4[b] | 1969 ± 308[b] |
| Lipid phosphorus, mg/rat/day | 1.7 ± 0.2 | 8.3 ± 1.0 | 3.2 ± 0.3 | 10.4 ± 0.5 | 3.0 ± 0.04 | 10.5 ± 0.3 | 8.0 ± 0.12 |
| 3-β-hydroxy-5-ene sterols, mg/rat/day | 188± 12.5 | 121± 9.9 | 425 ± 6.6[b] | 325± 16.8[b] | 327± 10.6[b] | 296± 20.8[b] | 298 ± 21.8[b] |
| Total glycerides as triolein, mg/rat/day | 94± 13.6 | 87± 5.4 | 272 ± 49.9[b] | 110± 13.1 | 247± 20.2[b] | 135± 2.8[b] | --- |
| Free fatty acids, μeq/rat/day | 536± 148.9 | 395± 29.1 | 3294 ± 320.0[b] | 2337± 116.5[b] | 2894± 280.2[b] | 2245± 210.5[b] | --- |

[a]Mean ± SD of mean, 20 rats per dietary group.

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

phorus (8) were assayed by automated methods.

## RESULTS AND DISCUSSION

Weight gain was essentially similar on all diets, 48 ± 3.2 g. The mean feed consumption was 20.9 ± 0.89 g per day on all diets.

Serum phospholipids were significantly lowered only in the calcium carbonate fed groups (Table II). Compared to the control, serum cholesterol decreased 35% in the calcium carbonate fed group, 22% in the OS-CAL fed group, and 27% in the calcium carbonate group with vitamin D. Vitamin D is known to be hypercholesterolemic (9-11); inclusion of vitamin D in the OS-CAL could account for the lesser hypocholesterolemic effect noted in rats fed these diets. In previous work, addition of 25 IU/g feed of vitamin D to the diet used in this study supplemented with 1.2% calcium as calcium carbonate did not significantly influence the hypocholesterolemic effect of exogenous calcium carbonate (4). Similar results are reported here.

Serum triglycerides decreased 10% in the calcium carbonate treated groups with or without inclusion of vitamin D, and 17% in the OS-CAL treated group. Vitamin D has been shown not to affect the hypotriglyceridemic action of exogenous calcium (4). On the other hand various trace metals such as chromium, cobalt, cadmium, copper, manganese and zinc have been reported to affect lipid metabolism (12-15). Inclusion of oyster shell calcium in the diet significantly increases the amounts of manganese and zinc ingested (Table I). Both these metals have been shown to exhibit a hypolipemic effect (15) and this may, in part, explain the greater hypotriglyceridemic effect of OS-CAL compared to calcium carbonate.

Serum free fatty acids were 32% and 55% higher in the calcium carbonate and OS-CAL treated rats compared to the rats on the control diet. The rise on the OS-CAL diet was greater than that on the calcium diet, $P<0.01$. Serum calcium was significantly elevated and serum magnesium was significantly depressed in both the calcium carbonate and OS-CAL treated groups, compared to the control group. No significant differences were noted in serum phosphorus.

A four-fold elevation in fecal total lipids was noted in all experimental groups compared to the control (Table III). Fecal 3-$\beta$-hydroxy-5-ene sterol excretion was significantly higher in all treated groups compared to the control group. Since the rats consumed 20.9 ± 0.89 g feed daily, 418 mg of exogenous cholesterol would be consumed daily. In the calcium carbonate treated groups essentially all of the dietary cholesterol could be accounted for by the fecal 3-$\beta$-hydroxy-5-ene sterol.

Fecal glyceride was significantly increased in the OS-CAL treated groups during both weeks, and in the calcium carbonate treated group during week 1. Fecal free fatty acid excretion increased four-to-six-fold in the calcium carbonate and OS-CAL treated groups, compared to the control during both weeks. This increased excretion could account for the pronounced hypotriglyceridemic effect noted. Although no significant variation was noted in lipid solvent soluble phosphorus excretion among the various groups, excretion during the second week was significantly higher than the first week.

Fecal calcium excretion was significantly higher in all experimental groups compared to the control. The effect of added vitamin $D_2$ can be seen by noting that the calcium carbonate treated group excreted twice as much fecal calcium during the first week and 25% more calcium during the second week than did the OS-CAL treated rats ($P<0.05$). A similar result was obtained during week 2 in the group fed calcium carbonate plus vitamin D. This could indicate that the vitamin $D_2$ in the OS-CAL diet caused significant calcium absorption concomitant with serum lipid lowering and could lead to the deposition of a significant amount of exogenous calcium in the bone.

In those individuals suffering from the dual pathologies of osteroporosis and hyperlipidemia, a means of increasing calcium absorption and concomitantly lowering serum lipids could provide an effective procedure for combating both pathologies. Since OS-CAL has already been shown to be safe and is employed in the treatment of osteroporosis in humans, and since calcium carbonate has been demonstrated to have a hypolipidemic effect in human adults (1), studies on the possible hypolipidemic effect of OS-CAL in humans are indicated.

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# Jimpy Mouse: In Vitro Studies of Brain Sphingolipid Biosynthesis

**ABSTRACT**

Microsomal preparations from brains of Jimpy mice are able to synthesize only 10-20% as much galactosylceramide as preparations from control littermates. Synthesis of glucosylceramide and ketodihydrosphingosine is only slightly impaired.

Mice hemizygous for the sex-linked mutation Jimpy are characterized by severe neurological disturbances and death between 3 and 4 weeks of age (1). Histological (1) and ultrastructural (2) observations demonstrate a lack of myelin and of sudanophilic accumulation in certain white matter tracts. Chemical analysis reveals a paucity of white matter components (3-8). The lipid class most specific to myelin, and most severely depressed in Jimpy, is galactosylceramide (cerebroside). We utilized an in vitro assay system to determine the level of enzymatic activity available for galactosylceramide synthesis in microsomal preparations from the brains of Jimpy mice and controls. Activities for formation of galactosylceramides containing hydroxy fatty acids (HFA) or nonhydroxy fatty acids (NFA) were measured separately. A control enzymatic activity, formation of glucosylceramide, was also measured. Glucosylceramide is not found to any significant extent in myelin; it occurs primarily as precursor to the rapidly turning over gangliosides of gray matter. Biosynthetic pathways for the two compounds, galactosylation of ceramide by UDP-galactose to give galactosylceramide (9-11) or glucosylation of ceramide by UDP-glucose to give glucosylceramide (12), are similar. Formation of ketodihydrosphingosine (13,14), precursor to the long chain base components of both galactosylceramides and glucosylceramides, was also assayed.

The Jimpy mice were offspring of breeding pairs obtained from the Jackson Laboratories, Bar Harbor, Maine. The animals were sacrificed at the indicated ages, microsomes prepared (15), and stored at -50 C until used. The enzyme assays were carried out as reported previously (15); for each assay a radioactive substrate and nonradioactive acceptor were incubated with microsomal fractions and the appropriate cofactors. After incubation the lipids were extracted and separated by thin layer chromatography using the appropriate solvent system. The lipid product being studied was scraped from the plate and was quantitated by scintillation counting. The individual assay conditions are explained below.

HFA-Galactosylceramide: the substrate, 0.25 mg of HFA-ceramide was coated onto 25 mg of Celite by evaporation from chloroform-methanol 2:1 (9,15). The incubation mixture of 0.13 ml contained also 75 $\mu$moles of Tris-HCl at pH 7.4, 0.3 $\mu$moles of neutralized ATP, 0.15 $\mu$moles of dithiothreitol, 0.3 $\mu$moles of $MgCl_2$ and 12.5 nmoles of $^{14}$C-UDP-galactose at a specific activity of 10 $\mu$C/$\mu$mole.

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# Jimpy Mouse: In Vitro Studies of Brain Sphingolipid Biosynthesis

**ABSTRACT**

Microsomal preparations from brains of Jimpy mice are able to synthesize only 10-20% as much galactosylceramide as preparations from control littermates. Synthesis of glucosylceramide and ketodihydrosphingosine is only slightly impaired.

Mice hemizygous for the sex-linked mutation Jimpy are characterized by severe neurological disturbances and death between 3 and 4 weeks of age (1). Histological (1) and ultrastructural (2) observations demonstrate a lack of myelin and of sudanophilic accumulation in certain white matter tracts. Chemical analysis reveals a paucity of white matter components (3-8). The lipid class most specific to myelin, and most severely depressed in Jimpy, is galactosylceramide (cerebroside). We utilized an in vitro assay system to determine the level of enzymatic activity available for galactosylceramide synthesis in microsomal preparations from the brains of Jimpy mice and controls. Activities for formation of galactosylceramides containing hydroxy fatty acids (HFA) or nonhydroxy fatty acids (NFA) were measured separately. A control enzymatic activity, formation of glucosylceramide, was also measured. Glucosylceramide is not found to any significant extent in myelin; it occurs primarily as precursor to the rapidly turning over gangliosides of gray matter. Biosynthetic pathways for the two compounds, galactosylation of ceramide by UDP-galactose to give galactosylceramide (9-11) or glucosylation of ceramide by UDP-glucose to give glucosylceramide (12), are similar. Formation of ketodihydrosphingosine (13,14), precursor to the long chain base components of both galactosylceramides and glucosylceramides, was also assayed.

The Jimpy mice were offspring of breeding pairs obtained from the Jackson Laboratories, Bar Harbor, Maine. The animals were sacrificed at the indicated ages, microsomes prepared (15), and stored at -50 C until used. The enzyme assays were carried out as reported previously (15); for each assay a radioactive substrate and nonradioactive acceptor were incubated with microsomal fractions and the appropriate cofactors. After incubation the lipids were extracted and separated by thin layer chromatography using the appropriate solvent system. The lipid product being studied was scraped from the plate and was quantitated by scintillation counting. The individual assay conditions are explained below.

HFA-Galactosylceramide: the substrate, 0.25 mg of HFA-ceramide was coated onto 25 mg of Celite by evaporation from chloroform-methanol 2:1 (9,15). The incubation mixture of 0.13 ml contained also 75 $\mu$moles of Tris-HCl at pH 7.4, 0.3 $\mu$moles of neutralized ATP, 0.15 $\mu$moles of dithiothreitol, 0.3 $\mu$moles of $MgCl_2$ and 12.5 nmoles of $^{14}$C-UDP-galactose at a specific activity of 10 $\mu$C/$\mu$mole.

TABLE I

Sphingolipid Biosynthesis in Jimpy Mice,[a] nmoles/mg protein/hr

| Enzymatic activity (product) | Mouse phenotype | Age, days | | |
|---|---|---|---|---|
| | | 15 | 19 | 23 |
| HFA-Galactosylceramide | Control | .45 | .56 | .38 |
| | Jimpy | .08 | .06 | .04 |
| NFA-Galactosylceramide | Control | .13 | .20 | .18 |
| | Jimpy | .035 | .025 | .02 |
| Glucosylceramide | Control | 1.25 | 1.05 | 1.05 |
| | Jimpy | 0.95 | 0.8 | 1.00 |
| Ketodihydrosphingosine | Control | 2.10 | 1.85 | 1.80 |
| | Jimpy | 1.60 | 1.50 | 1.55 |

[a]Average of triplicate determinations (in each case the range of values was within ±10% of the average).

Incubation was for 120 min at 34 C with violent agitation.

NFA-Galactosylceramide: as above except with the substitution of NFA-ceramide for HFA-ceramide. The NFA-ceramide was co-evaporated onto the Celite with 0.25 mg of crude lecithin, this additional polar lipid being necessary to optimize formation of NFA-galactosylceramide (10).

Glucosylceramide: as above (NFA-ceramide and lecithin) except that $^{14}$C-UDP-glucose was substituted for $^{14}$C-UDP-galactose (10).

Ketodihydrosphingosine: incubation mixtures (14,15) of 0.13 ml contained 1 mg of microsomal protein, 7.5 μmoles of potassium phosphate buffer at pH 7.5, 25 μmoles of L-serine, and 0.1 μmole of $^{14}$C-palmitoyl-CoA at 5 μC/μmole. Incubation was for 15 min at 34 C with violent agitation.

The results obtained (Table I) indicate that galactosylceramide synthesis from both HFA- and NFA-ceramide is markedly affected; control activity was 5 to 10-fold higher than that of mutant animals. The other enzymatic activities studied, formation of ketodihydrosphingosine and glucosylation of ceramide, were only slightly but consistently depressed by some 5-25%.

The observation that the Jimpy mutant has a severe deficiency in enzymatic activity for galactosylceramide synthesis does not imply that this is the primary or initial effect of the genetic lesion. It is quite possible that, if for any reason myelin cannot be assembled normally, many enzymes involved in myelinogenesis will be repressed. A relevant observation is that other enzymes involved in myelinogenesis, such as hydroxymethyl glutaryl-CoA reductase (16) and activities for biosynthesis of monogalactosyl and digalactosyl diglyceride (17), are severely depressed in Jimpy mice as compared to controls. The depression of HFA-galactosylceramide synthesis in vitro (18) and in vivo (19) has also been reported previously. Our data indicate that related enzymatic activities, biosynthesis of ketodihydrosphingosine and glucosylceramide are only 5-25% reduced. This slight depression may be relatively nonspecific and reflect only the poor clinical state of the animal. This correlates with in vivo studies (20) which indicate a normal synthesis and turnover of gangliosides. Such results, severe depression of enzymatic activities related to myelinogenesis and normal levels of other lipid synthetic enzymes, have been reported for another myelin deficient mouse mutant, Quaking (15,21). We interpret these data from various laboratories as indicating that the synthesis of myelin specific components is coordinately controlled (22).

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# Methanolysis of Cerebrosides With Boron Trifluoride-Methanol

**ABSTRACT**

A time study was carried out to determine conditions under which $BF_3$-methanol could be used reliably for methanolysis of cerebrosides. Results indicate that heating at 100 C in a sealed tube with 14% w/v $BF_3$-methanol for 60 min yields satisfactory cleavage of cerebrosides to fatty acid methyl esters without significant destruction of normal or hydroxy fatty acids.

Various investigators have used different methods to determine the fatty acid composition of cerebrosides. Kishimoto and Radin (1,2) and Kates (3) have described the successful use of methanol-HCl for methanolysis of cerebrosides. Berry and Cevallos (4) and Blass (5) employed a methanol-HCl-dimethoxypropane mixture for this purpose. O'Brien and Rouser (6) used aqueous HCl to produce free fatty acids, which were then esterified with $BF_3$-methanol. More recently Rouser et al. (7) have recommended direct methanolysis of cerebrosides with $BF_3$-methanol.

The methanol-HCl methods described require a number of hours of heating, while the methanol-HCl-dimethoxypropane was found, in this laboratory, to produce too many fast-running artifacts to permit successful analysis of shorter chain fatty acid methyl esters by gas liquid chromatography (GLC). While methanolysis with $BF_3$-methanol appears to offer an attractively simple method for cerebroside fatty acid analysis, insufficient data is available for reliable use of this method. Morrison and Smith (8) demonstrated that $BF_3$-methanol will completely cleave sphingomyelin, but omitted cerebrosides from their study of conditions required for methanolysis of various lipids with this reagent, while Rouser et al. (7) have warned against loss of the more unsaturated fatty acids under the conditions they used. In addition Fulk and Shorb (9) recently reported production of an artifact from oleic acid during methanolysis with $BF_3$-methanol which was not freshly prepared. Because of these uncertainties, a time study was carried out to determine optimum conditions for $BF_3$-methanol methanolysis of cerebrosides.

Fatty acid methyl ester standard mixtures HA, HC and F & OR Mixture No. 6, bovine cerebrosides, Gas Chrom Q (100-120 mesh), and GC Grade GE-SE-30 were purchased from Applied Science Laboratories, Inc. Diethyleneglycol succinate (DEGS) was obtained from Supelco, Inc. Boron trifluoride was purchased from Matheson Gas Products. Nanograde methanol and nanograde hexane, obtained from Mallinckrodt Chemical Works, were both glass-redistilled before use.

All GLC analyses were performed using Hewlett-Packard models 5750 and 7620A, equipped with dual hydrogen flame detectors. Glass columns (.25 in. OD, 6 ft coils, Supelco, Inc.) were silanized with freshly prepared 5% dimethylchlorosilane in toluene v/v, as was all Pyrex wool used for packing. Gas-Chrom Q was resilanized in the same manner before use. Four per cent SE-30 and DEGS column packings were prepared by the filtration method (10).

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TABLE I

Composition of Fatty Acid Methyl Ester Mixtures After Heating With $BF_3$-Methanol Reagent[a]

| Fatty acid methyl ester | Heating time, min: 0 | 30 | 60 | 90 |
|---|---|---|---|---|
| | Per cent | | | |
| 14:0 | 7 | 7 ( 7.5±0.7)[b] | 7 | 6 |
| 16:0 | 25 | 26 (25.6±1.3) | 25 | 26 |
| 18:0 | 18 | 20 (19.7±1.9) | 18 | 19 |
| 16:1 | 2 | 2 ( 2.3±0.3) | 2 | 2 |
| 18:1 | 27 | 25 (25.0±1.0) | 25 | 26 |
| 18:2 | 4 | 5 ( 5.1±0.3) | 4 | 4 |
| 18:3 | 1 | 2 ( 1.7±0.06) | 2 | 2 |
| h14:0 | 5 | 4 ( 4.0±0.3) | 5 | 5 |
| h16:0 | 5 | 4 ( 4.4±0.3) | 5 | 5 |
| h18:0 | 6 | 5 ( 4.7±1.0) | 7 | 5 |

[a]Aliquots of a known mixture of fatty acid methyl ester standards, 2.5 mg/ml in 14% $BF_3$-methanol, heated at 100 C in Teflon-lined screw-cap tubes. Analyses are by gas liquid chromatography of hexane extracts. Values are composites from a 4% SE-30 column, which separated all but the 18-carbon series from one another, and a 4% DEGS column, which clearly separated 18:1, 18:2 and 18:3. Hydroxy fatty acid methyl ester data have been adjusted using a correction factor of 1.1 (see text).

[b]In parentheses are the means ±SD for three replicate GLC analyses. These are included to explain use of integers for all other data, which were obtained from single GLC analyses.

TABLE II

Composition of Fatty Acid Methyl Ester Mixtures Produced by Methanolysis of Beef Brain Cerebrosides With $BF_3$-Methanol[a]

| Fatty acid methyl ester | Heating time, min: 30 | 60 | 90 |
|---|---|---|---|
| | Per cent | | |
| 16:0 | 1 | 1 | 1 |
| 18:0 | 3 | 3 | 3 |
| 22:0 | 3 | 3 | 3 |
| 23:0 | 4 | 3 | 3 |
| 24:0 | 15 | 15 | 15 |
| 24:1 | 5 | 6 | 4 |
| 25:1 | 3 | 3 | 2 |
| 26:1 (estimated) | (2) | (2) | (1) |
| h18:0 | 24 | 18 | 17 |
| h22:0 | 3 | 5 | 5 |
| h23:0 | 4 | 5 | 6 |
| h24:0 | 19 | 22 | 24 |
| h24:1 | 8 | 10 | 10 |
| h25:1 | 3 | 2 | 3 |
| unknown (estimated) | (3) | (2) | (2) |

[a]Aliquots of a mixture of purified beef brain cerebrosides, 5.0 mg/ml in 14% $BF_3$-methanol, heated at 100 C in Teflon-lined screw-cap tubes. Analyses are by gas liquid chromatography of hexane extracts. Components present at less than one per cent are not included and are considered as zero in calculating per cent composition. Hydroxy fatty acid methyl ester data have been adjusted using a correction factor of 1.1 (see text).

The SE-30 column was used at 193 C, and the DEGS column at 115 C. Helium flow rate in both cases was 55 ml/min. Peak areas were determined by triangulation (11). Eight analyses using fatty acid methyl ester standard mixture HC were used to establish that a correction factor of 1.1 applied to hydroxy methyl ester areas served to accurately reproduce the composition distribution, by weight, of the mixture.

Plates for thin layer chromatography (TLC) were prepared by spreading solvent prewashed Silica Gel G (E. Merck) on 20 x 20 cm glass plates. Silica Gel G was prewashed with chloroform-methanol 4:1, and dried overnight at 100 C in an air oven.

$BF_3$-methanol solutions were freshly prepared each day this reagent was to be used. Approximately 14% w/v solutions of $BF_3$-methanol were prepared by slowly bubbling gaseous boron trifluoride through 50 ml methanol in an amber glass screw-cap bottle until 7 g of the gas were taken up. The warm solution was immediately capped loosely with Teflon tape and a screw-cap, which was subsequently tightened as the solution cooled to room temperature.

The mixture of fatty acid methyl esters used for methanolysis was prepared by mixing 1 ml of mixture HA (10 mg/ml in hexane) with 1 ml of F & OR Mixture No. 6 (15 mg/ml in hexane). Commercial bovine cerebrosides were further purified by preparative TLC. After development with chloroform-methanol-water 130:50:8 and air-drying, the clearly visible cerebroside bands were removed by scraping and were eluted from the silica gel by several extractions with chloroform-methanol 2:1. Although elution in this manner produces slightly less than quantitative recovery, gravimetric and TLC estimations indicated nearly 90% recovery of cerebrosides sufficiently representative for the purposes of this study. Portions of cerebroside or methyl ester solution, gravimetrically determined to contain 5 mg of cerebrosides or 2.5 mg of esters, were pipetted into screw-cap glass tubes, and evaporated to dryness under nitrogen. All methanolyses were performed in duplicate.

Before initiating methanolysis, solubility of lipids in the methanolic reagent was enhanced by pipetting 0.3 ml hexane into each tube. One milliliter of the $BF_3$-methanol solution was added to each tube, after which the tube was flushed briefly with nitrogen and quickly sealed with Teflon tape and a Teflon-lined screw-cap. The sealed tubes were placed in a block heater maintained at 100 C, with the exception of the

unheated, "zero-time" controls, which were kept at room temperature not more than several minutes before being subjected to the next step. Heated tubes were removed from the block heater at appropriate times and allowed to cool at room temperature for several minutes. One milliliter saturated aqueous NaCl was added to each tube, followed by 6 ml hexane. After mixing, tubes were centrifuged for 10 min at moderate speed in a clinical centrifuge. The top layer was removed with a pipette, and the bottom layer was extracted twice by the same procedure. Combined hexane extracts were concentrated under nitrogen to approximately 1 ml for GLC analyses, which were performed primarily with the SE-30 column. Since the SE-30 column did not separate the methyl esters of 18:1, 18:2 and 18:3, these components were subsequently determined using the DEGS column. Analyses are reported as composite data for the two columns. One inadequately resolved pair, 26:1 and an unknown minor component, are reported as estimates in Table II.

The aqueous residues from cerebroside methanolyses were examined for completeness of methanolysis. Each residue was freed of hexane under a stream of nitrogen, then extracted with 20 volumes chloroform-methanol 2:1. After this extract was freed of water by repeated concentration in a rotary evaporator, the resulting salt suspension was taken up in 5 ml chloroform-methanol 2:1. The chloroform-methanol supernatant solutions were examined by TLC for the presence of cerebrosides and ceramides, using 10, 50 and 100 μliters of each solution and appropriate amounts of cerebroside standards. After development with chloroform-methanol-water 130:50:8 and air-drying, plates were sprayed with the orcinol-sulfuric acid reagent of Skipski et al. (12) for detection of glycolipids or with 50% aqueous sulfuric acid, followed in each case by heating approximately 20 min at 115 C in an air oven.

A similar TLC analysis was performed on the remainder of the 30 min methanolysis hexane extract used for GLC analysis. The GLC chromatograms were used to roughly estimate that the remaining hexane extract used for this purpose contained approximately 1.5 mg of fatty acid methyl esters.

An additional 60 min methanolysis was performed for the purpose of determining completeness of recovery of methyl esters in the hexane extract. Since analytical TLC revealed the presence of small amounts of sphingosine bases in the extract (indicating some cleavage of the galactosylsphingosine bond), preparative plates were used to obtain pure, total fatty acid methyl esters. Gravimetric analysis was performed on this preparation, using the electrobalance micro-method of Rouser et al. (7).

While TLC analysis of "zero-time" cerebroside methanolysis residues revealed faint cerebroside spots when 10 μliters of chloroform-methanol extracts were chromatographed, only spots corresponding to psychosine were visible after chromatography of 100 μliters of parallel extracts of the 30, 60 and 90 min methanolysates. Absence of cerebroside and ceramide spots at this level indicates that in all cases more than 90% of the cerebrosides were cleaved, and that any ceramides produced were present at a level less than 10% that of the original cerebrosides. Similar analyses demonstrated no significant amount of methyl esters in methanolysis residues, while gravimetric analysis of total methyl esters of a hexane extract revealed a recovery of 44.5% of cerebroside weight as fatty acid methyl ester. This compares well with a theoretical yield of 44.9%, calculated from the fatty acid composition data.

Since some ceramide can apparently be extracted into hexane (1,3), a thin layer chromatogram of hexane extract containing approximately 1.5 mg of fatty acid methyl esters from a 30 min methanolysis was examined for the presence of ceramides. No ceramides were seen after spraying with aqueous sulfuric acid and charring, a procedure which clearly revealed 10 μg of a ceramide standard. Taken together, the above data indicate that no significant amount of fatty acid is left bound as ceramide after 30 min of methanolysis, and that essentially all of the fatty acid is recovered as methyl ester in the hexane extract.

Examination of the GLC data shows that the normal, unsaturated and hydroxy fatty acid methyl esters examined in these experiments were quite stable for 60 min under the conditions used. Although the stability data in Table I were accumulated using a mixture of methyl esters containing no chain lengths longer than 18, the repetitive analyses of standard mixture HC, used to establish a correction factor for hydroxy methyl esters, gave no indication of differential behavior among chain length isomers on the SE-30 column. The data in Table I provide no evidence of any change in the fatty acid methyl ester mixture used, even after methanolysis for 90 min. The significant difference of the 30 min compositional analysis reported in Table II, in contrast to the close agreement between the other two, suggests that methanolysis may not have been quite complete in 30 min. (These data are in broad but

not close agreement with fatty acid compositional analyses of bovine brain cerebrosides reported by O'Brien and Rouser (13). That a difference should exist is not surprising, because nothing is known of such important variables as animal age, tissue origin or selectivity of TLC recovery, regarding the bovine cerebrosides used in these experiments. In addition no attempt was made to detect normal fatty acid methyl esters with carbon chains longer than 26, or hydroxy fatty acid methyl esters with carbon chains longer than 24.) GLC chromatograms also demonstrated that no interfering amounts of artifacts were formed during any of the methanolyses, with the possible exception of the minor, unidentified peak appearing near 26:1 on SE-30 chromatograms.

Consideration of all the results of these experiments leads to the conclusion that 60 minutes of methanolysis with the $BF_3$-methanol reagent under these conditions will yield reasonably complete cleavage of cerebrosides, producing fatty acid methyl esters without significant destruction of the classes of methyl esters examined in this study.

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# Analysis of Commercially Available Ganglioside Standards

**ABSTRACT**

Procedures for the quantitative estimation of sialic acid, sphingosine and fatty acid were employed to assess the purity of commericallly available ganglioside standards. Based on the weight printed on the label, experimental values of mono-, di- and trisialoganglioside standards represented approximately 7, 77 and 25%, respectively, of the expected value. Thin layer chromatography (TLC) revealed that the monosialoganglioside was contaminated with phospholipid, possibly phosphatidyl choline. Trisialoganglioside was resolved into three spots on TLC, two of which were ninhydrin positive and resorcinol negative. These results suggest that caution should be observed when dealing with commercially available standards even though a high degree of purity is guaranteed.

During the course of our work on the nature and distribution of gangliosides in subcellular fractions of mouse brain, it became necessary to search for a source of commercially available pure standards. One source was found which guaranteed their standards to be at least 98% pure as assessed by chromatographic means. However it became clear that these ganglioside standards fell far short of the professed purity. Because of the cost and importance of these standards, the data accumulated in our laboratory on these materials may be of interest to others in the area of ganglioside research.

Monosialo- (MS), disialo- (DS) and trisialo (TS) gangliosides were purchased from a commercial supplier of lipid standards. Each container of standard ganglioside was opened, quantitatively transferred and diluted using the solvent stated on the vial ($CHCl_3$ for MS and

not close agreement with fatty acid compositional analyses of bovine brain cerebrosides reported by O'Brien and Rouser (13). That a difference should exist is not surprising, because nothing is known of such important variables as animal age, tissue origin or selectivity of TLC recovery, regarding the bovine cerebrosides used in these experiments. In addition no attempt was made to detect normal fatty acid methyl esters with carbon chains longer than 26, or hydroxy fatty acid methyl esters with carbon chains longer than 24.) GLC chromatograms also demonstrated that no interfering amounts of artifacts were formed during any of the methanolyses, with the possible exception of the minor, unidentified peak appearing near 26:1 on SE-30 chromatograms.

Consideration of all the results of these experiments leads to the conclusion that 60 minutes of methanolysis with the $BF_3$-methanol reagent under these conditions will yield reasonably complete cleavage of cerebrosides, producing fatty acid methyl esters without significant destruction of the classes of methyl esters examined in this study.

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# Analysis of Commercially Available Ganglioside Standards

**ABSTRACT**

Procedures for the quantitative estimation of sialic acid, sphingosine and fatty acid were employed to assess the purity of commericallly available ganglioside standards. Based on the weight printed on the label, experimental values of mono-, di- and trisialoganglioside standards represented approximately 7, 77 and 25%, respectively, of the expected value. Thin layer chromatography (TLC) revealed that the monosialoganglioside was contaminated with phospholipid, possibly phosphatidyl choline. Trisialoganglioside was resolved into three spots on TLC, two of which were ninhydrin positive and resorcinol negative. These results suggest that caution should be observed when dealing with commercially available standards even though a high degree of purity is guaranteed.

During the course of our work on the nature and distribution of gangliosides in subcellular fractions of mouse brain, it became necessary to search for a source of commercially available pure standards. One source was found which guaranteed their standards to be at least 98% pure as assessed by chromatographic means. However it became clear that these ganglioside standards fell far short of the professed purity. Because of the cost and importance of these standards, the data accumulated in our laboratory on these materials may be of interest to others in the area of ganglioside research.

Monosialo- (MS), disialo- (DS) and trisialo (TS) gangliosides were purchased from a commercial supplier of lipid standards. Each container of standard ganglioside was opened, quantitatively transferred and diluted using the solvent stated on the vial ($CHCl_3$ for MS and

TABLE I

Nana, Sphingosine and Fatty Acid Content of Ganglioside Standards

| | Nana[b] | | Sphingosine[b] | | Fatty acid present as Amide[c] | | Fatty acid present as Ester[c] | |
|---|---|---|---|---|---|---|---|---|
| | μmoles | | | | | | | |
| Standard[a] | Theory[d] | Observed | Theory[d] | Observed | Theory[d] | Observed | Theory[d] | Observed |
| MS | 3.27 | 0.22 | 3.27 | 0.18 | 3.27 | 0.28 | 0.0 | 7.73 |
| DS | 5.53 | 4.35 | 2.77 | 2.15 | 2.77 | 2.10 | 0.0 | 0.0 |
| TS | 7.04 | 1.75 | 2.35 | 0.75 | 2.35 | 0.50 | 0.0 | 0.0 |

[a]MS = monosialoganglioside, DS = disialoganglioside, TS = trisialoganglioside.

[b]Observed values are averages of four analyses.

[c]Amide fatty acids = total fatty acids (based on esterification with $BF_3$-methanol at 80 C) minus ester fatty acids (based on esterification with 0.5 N methanol at room temperature). Observed values are averages of duplicates.

[d]Theory calculated from the weight stated on the vial (5 mg) and assuming an average molecular weight of 1531, 1808 and 2131, respectively, for MS, DS and TS (based on the work of Avrova, Ref. 8).

$CHCl_3$-methanol 2:1 for DS and TS) and aliquots were assayed for N-acetylneuraminic acid (NANA), sphingosine (SPH) and fatty acid (FA) content. The fluorometric procedures of Hess and Rolde (1) and Coles and Gray (2) were used to estimate the contents of NANA and SPH, respectively. Standard SPH was purchased from Supelco and the NANA standard was purchased from Sigma Chemical Co.

An internal standard (IS) procedure (3) employing nonadecanoic acid (19:0) was used to estimate FA content. Total FA, including the IS, was converted to methyl esters using $BF_3$-methanol as described by Morrison and Smith (4), except that esterification was carried out at 80 C for 24 hr. The $BF_3$-methanol reagent, 14% w/v, was prepared in our laboratory and discarded after one month. The resulting methyl esters were purified by thin layer chromatography (TLC) and analyzed by gas liquid chromatography (GLC) using a Hewlett-Packard model 700 unit equipped with dual flame detectors and stainless steel columns (10 ft x 1/8 in) containing 15% DEGS on 70/80 mesh Anakrom ABS (Analabs) or 0.5% Apiezon L coated on Glassport M (Hewlett-Packard). To detect the presence of ester linked fatty acids, duplicate samples were treated with 0.5 N methanol-HCl, at room temperature for 12 hr prior to TLC and GLC. In addition each standard was assayed by TLC using a 1-propanol-water 70:30 v/v solvent system for development and resorcinol (5), ninhydrin (6) and phosphomolybdate (7) spray reagents as visualization aids.

The MS, DS and TS standards yielded approximately 7, 77 and 25%, respectively, of the amount stated on the vial (Table I). In addition there was evidence of extremely large amounts of nonamide linked FA in the MS standard. Greater than 90% of the total FA in the MS standard was esterified under the mild room temperature conditions. In contrast we were able to detect only amide linked FA in the DS and TS samples.

The atypical pattern observed for total FA in the MS standard (Table II) substantiated the presence of nonganglioside FA in this sample.

TABLE II

Fatty Acid Distribution of Standard Gangliosides[a]

| Fatty acid | Monosialoganglioside | | Disialoganglioside | | Trisialoganglioside | |
|---|---|---|---|---|---|---|
| | (1) | (2) | (1) | (2) | (1) | (2) |
| 12:0 | trace | trace | --- | --- | --- | --- |
| 14:0 | trace | trace | --- | --- | --- | --- |
| 16:0 | 41.9 | 43.5 | 12.4 | 11.4 | trace | 4.7 |
| 16:1 | 1.2 | 1.0 | --- | --- | --- | --- |
| 18:0 | 14.6 | 15.8 | 81.9 | 84.8 | 100.0 | 95.3 |
| 18:1 | 41.0 | 38.1 | 3.1 | 1.7 | --- | --- |
| 20:0 | 1.3 | 1.6 | 2.6 | 2.1 | --- | --- |

[a]Values express wt% of total fatty acids for duplicate runs; trace = less than 0.5% of total fatty acids.

TABLE III

Thin Layer Chromatography of Ganglioside Standards[a]

| Ganglioside | Spray reagents | | |
|---|---|---|---|
| | Ninhydrin[b] | Resorcinol[c] | Phospho-Molybdate[d] |
| Monosialoganglioside | - | - | + |
| Disialoganglioside | - | + | - |
| Trisialoganglioside | | | |
| spot 1 | - | + | - |
| 2 | + | - | - |
| 3 | + | - | - |

[a] [a]Support: Silica Gel G, 0.5 mm thickness. Solvent system: 1-propanol-water 70:30 v/v; saturated system. Time for development: 3 hr, 10 min.

[b]Skipski et al., 1962 (Ref. 6).

[c]Svennerholm, 1958, (Ref. 5).

[d]Dittmer and Lester, 1964 (Ref. 7).

Nonganglioside contamination in the MS standard was further established when the standards were analyzed by TLC (Table III). The major component in the MS standard was phosphorous positive and migrated similarly to phosphatidyl choline. No resorcinol positive material was detected in this standard. The TS standard was resolved into three spots, two of which were ninhydrin positive and resorcinol negative. This undoubtedly accounts for the relatively high SPH/FA ratio (Table I) observed in the TS standard. The primary amine of SPH is involved in the complex formation and other amines may interfere with the assay for SPH in the fluorometric method (2) employed.

Of the three ganglioside standards available, only the DS appeared to be free from serious contamination, and even this sample contained less than 80% of what was theoretically expected. In view of our results we caution workers in this area to be extremely dubious when purchasing ganglioside standards, even though a high degree of purity may be guaranteed.

Since the completion of this work, we have obtained monosialoganglioside from a colleague who purchased this standard from the same commercial source as we did. Upon analysis we obtained results essentially identical to those reported in this paper.

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# LETTER TO THE EDITOR

## Formation of an Artifact During Methylation of Conjugated Fatty Acids

Sir: Studies at the Northern Laboratory on the mechanism of hydrogenation involve extensive use of various conjugated isomers of linoleic acid. When methyl esters of alkali-isomerized linoleic(*cis*-9,*trans*-11- and *trans*-10,*cis*-12-octadecadienoic) acid are being prepared with boron trifluoride, sulfuric acid and perchloric acid catalysts, we have observed that an artifact (3-4%) is formed, which has an equivalent chain length of 21.7 compared to 21.4 for *trans,trans* conjugated ester on EGSS-X columns. Fortunately this unknown compound elutes ahead of *trans,trans*-conjugated diene as a separate peak during chromatography on a silver-saturated resin column described by Emken et al. (JAOCS 41:388, 1964). Ultraviolet spectrum of the isolated artifact showed no conjugation. Infrared absorption spectrum showed a strong band for *trans* and a strong methoxy band at 1100 $cm^{-1}$. Catalytic reduction with palladium catalyst formed a new compound with an equivalent chain length of 21.4. Based on these observations we have tentatively identified the artifact as methyl methoxyoctadecenoate. Final confirmation was based on the mass spectrum of the hydrogenated artifact which showed the methoxy group at the 10 and 12 positions (Ryhage and Stenhagen, Ark. Kemi 15:545, 1960).

Methyl methoxyoctadecanoate was also isolated as described below. Alkali-isomerized linoleic acid was converted to methyl esters with boron trifluoride-methanol reagent following the procedure of Metcalfe and Schmitz (Anal. Chem. 33:363, 1961). The esters were hydrogenated with palladium catalyst and most of the stearate was removed from methoxystearate by crystallization from methanol. The remainder was removed by preparative gas liquid chromatography. Mass spectrum of the methoxyoctadecanoate so prepared also showed the methoxy group at the 10 and 12 positions.

Methyl methoxystearate was previously found as a product of methylation of oleic acid with boron trifluoride (Lough, Biochem. J. 90:4C, 1964; Fulk and Shorb, J. Lipid Res. 11:276, 1970). However such artifacts are produced only during severe treatment (longer heating and higher concentration of boron trifluoride than those suggested by Metcalfe and Schmitz or when aged $BF_3$-methanol reagent is used). Oleic, linoleic and linolenic acids did not produce artifacts under the conditions suggested by Metcalfe and Schmitz. Methyl methoxyoctadecenoate also formed from alkali-conjugated linoleic acid even if the $BF_3$-methanol (14% w/v) reagent was freshly prepared. Again methyl methoxyoctadecenoate formed when sulfuric and perchloric acid served as the catalysts during methylation of conjugated fatty acids. This artifact was not observed when diazomethane was employed for the preparation of methyl esters of conjugated fatty acids.

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# Characterization and Metabolism of Free Fatty Alcohols From *Escherichia coli*

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## ABSTRACT

Free fatty acohols have been established as lipid components of *E. coli K-12*. Using combined gas liquid chromatography-mass spectrometry, the major alcohols in aerobically grown cells were identified as 1-tetradecanol (18%), 1-hexadecanol (28%), 1-octadecanol (14%), and 2-pentadecanol (27%). Small amounts of 1-hexadecenol (3%), 2-tridecanol (8%), and 2-tetradecanol (1.5%) were also detected. Analysis of anaerobically grown cells has shown a selective decrease of the secondary alcohols. 2-Pentadecanol was present as only 7% of the total alcohol fraction, and only traces of 2-tridecanol and 2-tetradecanol were found. The major alcohols in anaerobic cells were 1-tetradecanol, 1-pentadecanol, 1-hexadecenol and 1-hexadecanol. The above observations strongly suggest two pathways for the synthesis of fatty alcohols in *E. coli*. One pathway synthesizes the primary alcohols and does not require molecular oxygen, and a separate pathway synthesizes the secondary alcohols and has a requirement for molecular oxygen.

## INTRODUCTION

The chemistry and biochemistry of bacterial fatty alcohols are the subjects of extensive research (1-5). These studies show that bacteria synthesize fatty alcohols by reductive (4) and oxidative pathways (1-3,5) using acyl-CoA and hydrocarbons, respectively, as precursors. It is also established that hydrocarbons are oxidized to both primary (2,3,5) and secondary alcohols (1).

Although considerable work has been done on bacterial fatty alcohol metabolism, this subject has not been investigated in *E. coli*. This report established the presence of free fatty alcohols in *E. coli* and presents information concerning the free fatty acids, fatty aldehydes, waxes and glyceryl ethers. Experiments implicating oxygen as a regulating factor in free fatty alcohol metabolism are also described.

## METHODS

### Bacteria

Stock cultures of *E. coli K-12* were maintained at room temperature on slants of Trypticase Soy Agar. Seed cultures were prepared by inoculating Trypticase Soy Broth with *E. coli* and then incubating the cultures at 37 C with shaking. When the seed cultures attained logarithmic growth, 100 ml was added to 1 liter Trypticase Soy Broth, and these larger cultures were incubated as before. Anaerobic cultures were prepared by filling the flasks to the neck with media and fitting them with a pyragallol plug. Oxygen-free nitrogen was then bubbled through the media. The bacteria were harvested when they reached stationary phase by centrifuging at 10,000 x g for 20 min at 4 C. The cells were washed twice with physiological saline, frozen and stored at -20 C for future use.

### Thin Layer Chromatography (TLC)

Preparation of the chromatoplates and the techniques used for isolation and recovery of lipid have been described previously (6). The

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TABLE I

Results of Lipid Analysis

| Experiment number | Total lipid, μmole/100 mg | | | |
|---|---|---|---|---|
| | Fatty acid | Aldehyde | Alcohol | Wax |
| 1 | 0.820 | 0.126 | 0.152 | 0.040 |
| 2 | 0.636 | 0.163 | 0.136 | n.d.[a] |

[a]Not determined

TABLE II

Relative Retention Times of Acetoxy Alkanes and Free Fatty Aldehydes on OV-101

| Acetoxy alkanes | | | Free fatty aldehydes | | |
|---|---|---|---|---|---|
| Shorthand[a,b] designation | Natural mixture | Standards | Shorthand[a] designation | Natural mixture | Standards |
| 12:0 | 0.070 | 0.070 | 14:0 | 0.155 | 0.160 |
| 13:0 sec. | 0.078 | 0.077 | 15:0 | 0.270 | 0.270 |
| 14:0 | 0.172 | 0.172 | 16:0 | 0.400 | 0.393 |
| 15:0 sec. | 0.190 | 0.187 | 17:1 | 0.549 | 0.554 |
| 15:0 | 0.267 | 0.265 | 17:0 | 0.625 | 0.625 |
| 16:1 | 0.371 | 0.371 | 18:0 | 1.000 | 1.000 |
| 16:0 | 0.414 | 0.414 | | | |
| 18:0 | 1.000 | 1.000 | | | |

[a]For the corresponding lipid—no. of carbons per no. of double bonds; sec. = secondary alcohol with hydroxyl on no. 2 carbon.

[b]Number of carbon atoms refers to alcohol from which acetoxy alkane is derived.

solvent systems used in the development of the chromatograms are defined below and assigned a Roman numeral. This designation will be used throughout the text when referring to these solvent systems.

I. Hexane-Chloroform-Methanol 73:25:2
II. Hexane-Ethyl ether-Acetic acid 30:70:1
III. Hexane-Ethyl ether-Acetic acid 90:10:1
IV. Hexane-Chloroform-Methanol 65:25:10

#### Lipid Extraction and Purification

Lipids were extracted (7), concentrated under vacuum and diluted to a known volume with nitrogen-equilibrated *n*-heptane.

Preliminary lipid separations were achieved on 18 gm, *n*-heptane equilibrated, silicic acid columns (8). Developing solvents were added in the following sequence: 250 ml of 4% ethyl ether in *n*-heptane (free fatty aldehydes, waxes and other more nonpolar lipids); 250 ml of 10% ethyl ether in *n*-heptane (free fatty acids); 300 ml of ethyl ether (remaining neutral lipids); 400 ml of methanol (phospholipid).

Further resolution of these lipids was achieved by TLC. Waxes ($R_f$ 0.8) were separated from free fatty aldehydes ($R_f$ 0.5) with solvent system I. Chromatography with solvent system II resolved the free fatty alcohols ($R_f$ 0.5) and free fatty acids (FFA) ($R_f$ 0.75). The FFA were methylated by heating for 60 min at 70 C with anhydrous methanolic HCl, 2%, and the resulting esters purified using solvent system I. The alcohols were converted to acetoxy alkanes (9) and this derivative was purified by TLC using solvent system III. Secondary alcohols and their acetoxy alkane derivatives had the same chromatographic mobility as the primary isomers in these TLC systems.

#### Lipid Analyses

Free fatty aldehydes and aldehydogenic phospholipids were quantitated as their *p*-nitrophenylhydrazones (10). The waxes, methyl esters and acetoxy alkanes were estimated as hydroxamates (11). Total lipids and phospholipids were reduced with $LiAlH_4$ (12) and the glyceryl ethers isolated by TLC (solvent system IV) and quantitated (13).

#### Gas Liquid Chromatography (GLC) and Mass Spectrometry (MS)

Samples were analyzed on an F & M Model 400 gas chromatograph equipped with a hydrogen flame-ionization detector. Separations were made on a 1/8 in. x 6 ft U-shaped, borosilicate glass column packed with 3% OV-101 on silanized, 100-120 mesh Gas-Chrom Q. The argon pressure was 40 psi and the flow rate 112 ml/min. Free fatty aldehydes were chromatographed at 150 C, and acetoxy alkanes and methyl esters at 160 C.

Conditions used for combined gas chromatography-mass spectrometry (GC-MS) are published (14). During this procedure the gas chromatograph was operated at 140 C to achieve a better separation between the 1 and 2 acetoxy alkanes.

## RESULTS AND DISCUSSION

#### Lipid Composition

The lipid extracted from two 60 g preparations of *E. coli* weighed 1.08 g each. Following column chromatography 92% of the lipid, by weight, was in the phospholipid fraction. Quantitative values for several purified lipid types are given in Table I. The presence of FFA has been previously indicated by IR spectroscopy (15).

Our analysis has confirmed this finding using TLC and GLC, and, further, quantitated and qualitatively identified the FFA. Our findings also establish that free fatty alcohols are constituents of *E. coli* lipids and indicate the presence of free fatty aldehydes and waxes. Glyceryl ethers were not detected in either the total lipid or phospholipid fractions. The latter result is consistent with published results (16).

Identification of the above mentioned compounds was based on several criteria. Natural lipid components that eluted in column fractions known to elute hexadecanal, 1-hexadecanol and hexadecyl octadecanoate also migrated with these standards in two different TLC systems (systems I and II). The lipid chromatographing as hexadecanal migrated as the hydrazone of hexadecanal following treatment with *p*-nitrophenylhydrazine (10), and the lipid migrating with 1-hexadecanol chromatographed as the acetoxy alkane of 1-hexadecanol following acetylation (9). The lipid chromatographing with hexadecyl octadecanoate formed hydroxamates (11), and after saponification yielded two components that migrated as 1-hexadecanol and hexadecanoic acid. During GLC the fatty aldehydes and acetoxy alkanes cochromatographed with the corresponding reference compounds. Table II compares the relative retention times of the *E. coli* free fatty alcohols and aldehydes with the synthetic standards.

To the authors' knowledge separation of acetoxy alkanes of secondary alcohols by gas chromatography has not been investigated before; therefore it was necessary to establish conditions for their separation. A series of 1-acetoxy alkanes and 2-acetoxy alkanes was chromatographed under the conditions stated previously. A semilogarithmic plot of the relative retention times of the acetoxy alkanes versus their carbon number if given in Figure 1. From these data it can be seen that the two homologous series form parallel lines, and the 2-acetoxy alkanes can be separated from the 1-acetoxy alkanes by GLC.

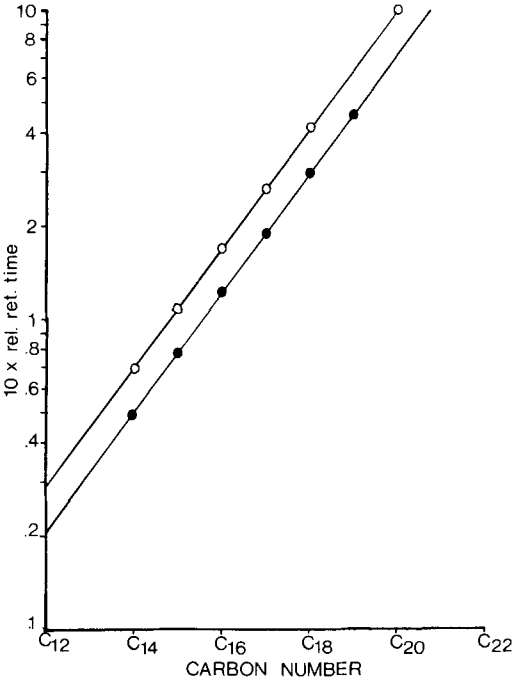



FIG. 1. Semilogarithmic plot ot the relative retention times of acetoxy alkanes versus their carbon number.

#### Mass Spectrometry of Acetoxy Alkanes

The structure of the acetoxy alkanes was further verified by GC-MS. First the fragmentation patterns of 2-acetoxy dodecane through 2-acetoxy heptadecane were compared with the spectra of the corresponding 1-acetoxy alkanes. In general these fragmentation patterns agreed with those reported elsewhere (17-19). Since these references also contain a detailed discussion correlating the mass spectra with molecular structure, this discussion will only deal with the spectral differences that result from isomerism.

1-Acetoxy hexadecane (1-AHD) and 2-acetoxy hexadecane (2-AHD) are typical spectra (Fig. 2) and will be used to illustrate the difference in the fragmentation patterns of the two isomers. The differences are as follows: fragments at m/e 87 and m/e 102 occur in 2-AHD but not in 1-AHD; fragments at m/e 73 and m/e 116 are present in 1-AHD but not 2-AHD; and the intensity of the mass ion, m/e 61, is decreased from 43% in 1-AHD to 8.3% in 2-AHD.

The above spectral differences can be correlated with the structural differences by using principles already deduced (17). The bond between the 1 and 2 carbon atoms of the alkyl moiety is cleaved in acetoxy alkanes.

$$R-CH_2-\underset{\displaystyle X}{\underset{|}{CH}}-O-\overset{\displaystyle O}{\overset{\|}{C}}-CH_3 \quad \text{where X = H or } CH_3$$

When this occurs in 1-AHD (X=H) the resulting fragment is m/e 73, while in 2-AHD ($X=CH_3$), the fragment will appear at m/e 87. It is also noted that this cleavage is more intense in 2-AHD (30%) than in 1-AHD (8%). This is consistent with 2-AHD having a secondary structure, since the fragment ion will have the

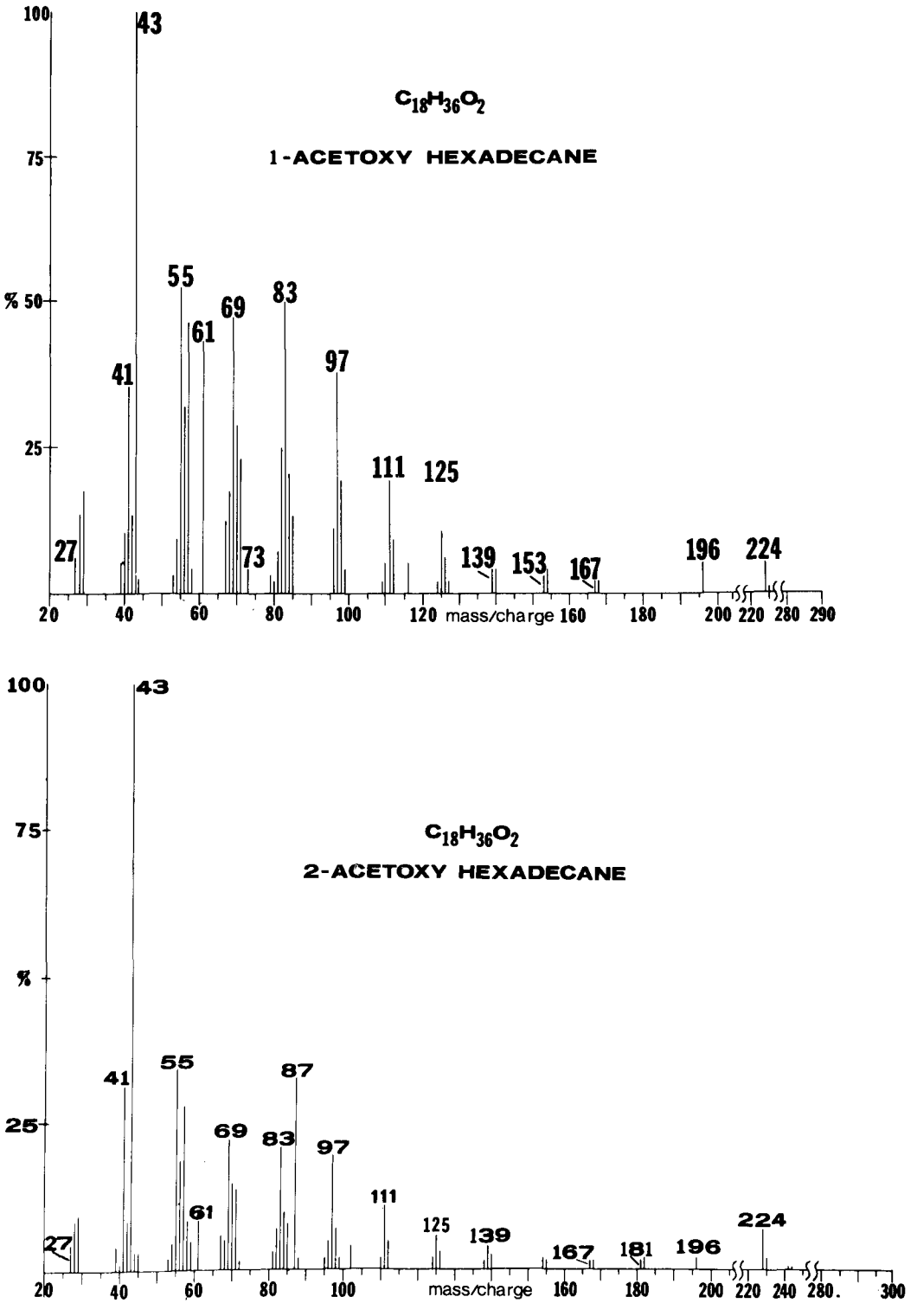



FIG. 2. Mass spectra comparing isomeric acetoxy hexadecanes.

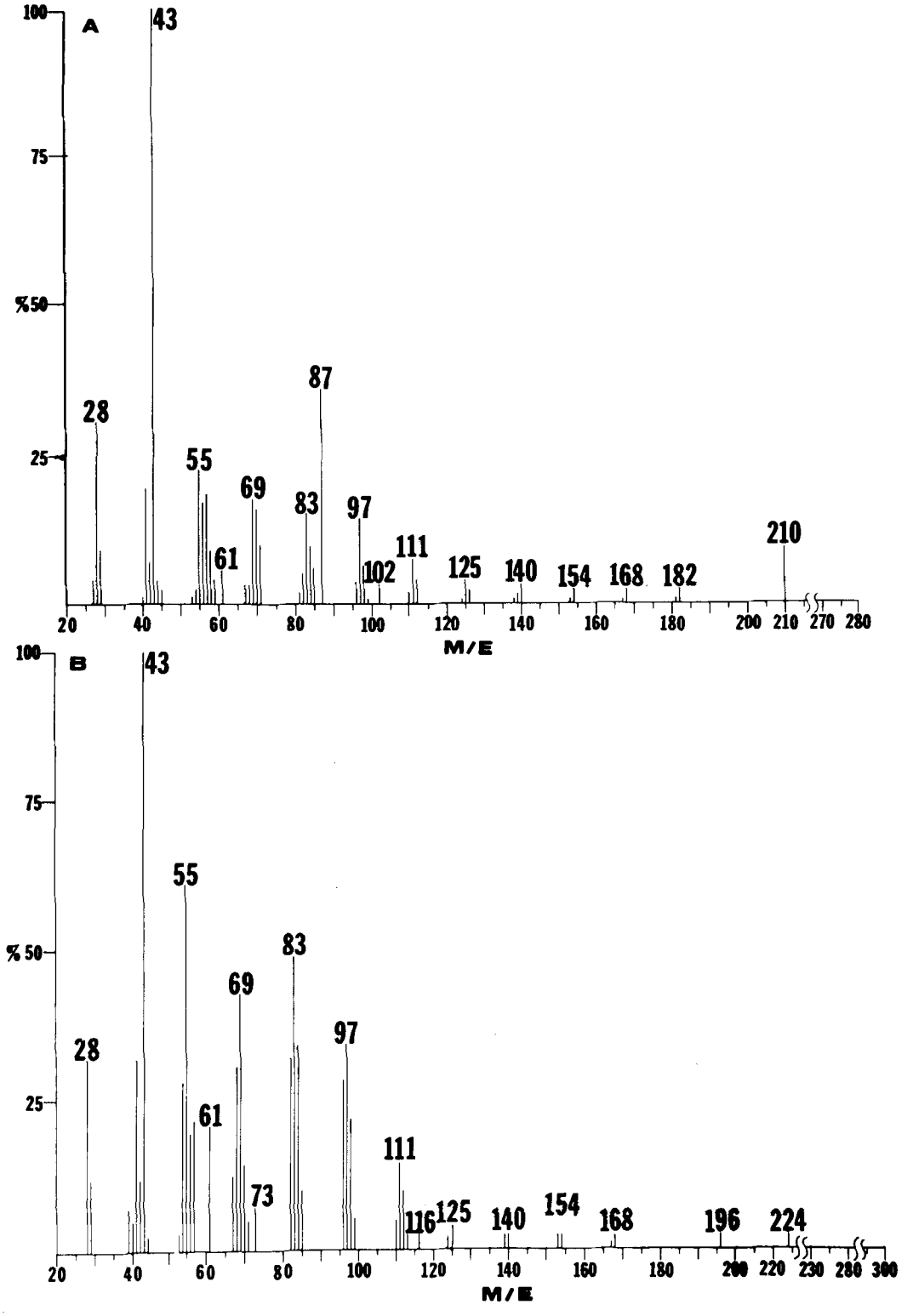



FIG. 3. Mass spectra of acetoxy alkanes derived from *E. coli* free fatty alcohols. A: 2-acetoxy pentadecane; B: 1-acetoxy hexadecane.

TABLE III

Chain Length Distribution

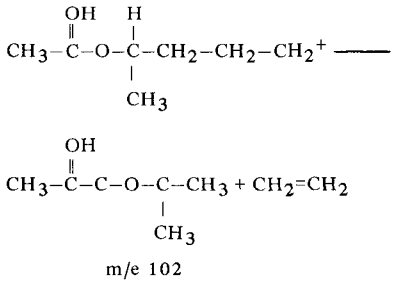
| Shorthand[a] designation | Free fatty acids | | Aldehydes | | Alcohols | |
|---|---|---|---|---|---|---|
| | %[b] | Range | %[b] | Range | %[b] | Range |
| 12:0 | 1.63 | 0-4.9 | --- | --- | 2.76 | 2.5-3.0 |
| 13:0 sec. | --- | --- | --- | --- | 8.41 | 3.5-14.3 |
| 13:0 | 0.66 | 0.2.0 | --- | --- | Trace | Trace |
| 14:0 sec. | --- | --- | --- | --- | 1.50 | 1.2-1.8 |
| 14:0 | 8.17 | 6.5-11.2 | 12.35 | 12.0-12.7 | 18.23 | 14-23.7 |
| 15:0 sec. | --- | --- | --- | --- | 26.73 | 25.3-29 |
| 16:1 | 3.65 | 3.3-3.8 | --- | --- | 2.69 | 1.2-3.5 |
| 16:0 | 43.00 | 39.2-47.6 | 32.5 | 29-36 | 28.60 | 27.2-30 |
| 17:1 | --- | --- | 1.15 | 0.2.3 | Trace | Trace |
| 17:0 | 4.26 | 3-6.7 | 20.9 | 18.3-22.6 | Trace | Trace |
| 18:1 | 24.30 | 15.0-32.6 | --- | --- | Trace | Trace |
| 18:0 | 11.31 | 9.7-12.8 | 17.8 | 16-19.7 | 13.6 | 12.1-15.1 |

[a]See Footnote a in Table II.

[b]Average of three experiments.

positive charge localized on a secondary carbon giving a more stable ion.

In 1-AHD, m/e 116 results from cleavage between the fourth and fifth carbon atoms accompanied by a hydride ion transfer (17). However in 2-AHD this fragmentation proceeds further, and ethylene is lost giving the mass ion at m/e 102.



This occurs because the positive charge will then reside at a secondary carbon which will offer stabilization to the ion. This situation does not occur in 1-acetoxy alkanes.

The decrease in intensity of m/e 61, $CH_3COOH_2^+$, in 2-AHD is also a consequence of the secondary structure of the molecule. It has been shown above that the fragment resulting from cleavage of the carbon-carbon bond between carbon atoms 1 and 2 is more favored in the 2-acetoxy alkanes than in the 1-acetoxy alkanes. This fragmentation is in competition with that resulting in mass ion 61, and therefore when the former becomes more stable, the latter should decrease. This is observed in the fragmentation patterns.

In summary, secondary alcohols can be distinguished from primary alcohols on the basis of the mass spectra of their acetoxy alkane derivatives. Mass spectra of acetoxy alkanes of secondary alcohols exhibit a large ion at m/e 87 (30%) accompanied by a fragment at m/e 102 and a small ion at m/e 61 (8%). Acetoxy alkanes of primary alcohols generate spectra with a small ion at m/e 73, accompanied by a large fragment at m/e 61 (43%) and one at m/e 116. These are the criteria that were used to assign structures to the alcohols.

Figure 3A and B contains typical spectra obtained from the acetoxy alkane derivatives of *E. coli* fatty alcohols. The molecules generating the spectra in Figure 3A and B were assigned structures of 2-acetoxy pentadecane and 1-acetoxy hexadecane. It is quite clear from the spectra that the above criteria are satisfied for these structures. The spectra of the other acetoxy alkanes satisfied the criteria for their assigned structures, and it is concluded from this that the identification from GLC is correct.

### Qualitative Analysis of Free Fatty Acids, Aldehydes and Alcohols

A comparison of the chain length distribution of the free fatty acids, aldehydes and alcohols was made to see if a metabolic interrelationship between these moieties was indicated. In drawing conclusions from this data, the assumption that the individual pools are not compartmentalized is made. This seems a valid assumption in *E. coli*, since all of the bacterial lipids studied so far are localized in the membrane (20). Possible interconversion of the acids, aldehydes and alcohols can be assessed, therefore, by examining the fatty chains.

The qualitative distribution shown in Table III clearly indicates a lack of similarity between

the three lipid classes. Another pathway, not involving direct reduction of a fatty acid, must be postulated to explain the presence of secondary alcohols since there is no acid corresponding to these alcohols. It is possible that alcohols in *E. coli* arise from two separate pathways. The primary alcohols could result from fatty acid reduction, and secondary alcohols could be formed from hydrocarbon oxidation. It is also conceivable that all of the alcohols are formed from hydrocarbon oxidation; the primary alcohols would be further oxidized to acids and the secondary alcohols to ketones.

#### Effects of Anaerobiosis on Alcohol Metabolism

It is known that the oxidative formation of alcohols from hydrocarbons in the organisms previously investigated requires molecular oxygen (21). If this situation occurs in *E. coli*, growing them anaerobically may give some indication as to their origin. Analysis of the free alcohols from anaerobically grown *E. coli* shows that a selective decrease of secondary alcohols occurred in anaerobiosis. The percentage of 2-pentadecanol and 2-tridecanol falls from 27% and 8.5%, respectively, in aerobic cells to 7% and 1% in anaerobic cells. Primary alcohols now become the major alcohols in anaerobes.

That the decrease in the relative proportion of secondary to primary alcohols did, in fact, reflect a decrease in the amount of secondary alcohol and not an increase in primary alcohol is established from quantitative data. The fatty alcohol fraction in the aerobic cells was 0.144 $\mu$moles/100 mg total lipid, while this fraction in anaerobic cells was 30-50% less. An increase in alcohol synthesis therefore did not occur.

Whether this reflects a regulation of the synthesis or utilization of fatty alcohols cannot be determined from this data. If this data does imply a decrease in the synthesis, it would suggest that the secondary alcohols arise from hydrocarbon oxiation. In any case, oxygen is a regulatory factor of secondary alcohol metabolism.

To summarize, the experiments reported here establish that *E. coli* contain free fatty alcohols and indicate the presence of free fatty aldehydes and waxes. Both primary and secondary alcohols were found, with the latter comprising 30% of the total alcohols. Monoenoic fatty alcohols were detected in only small amounts, and polyenoic alcohols were not detected. The media, prior to inoculation, was extracted and analyzed for free or esterified alcohols, but none were found. From this information, the chain length distribution data and the effects of anaerobiosis, it must be concluded that biosynthesis of both primary and secondary fatty alcohols occurs in *E. coli* by two different pathways. One pathway synthesizes the primary alcohols and does not require molecular oxygen, and a separate pathway synthesizes the secondary alcohols and has a requirement for molecular oxygen. Fatty acids or hydrocarbons, or both, may serve as precursors to the alcohols.

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# Is There an Entero-Hepatic Circulation of the Bile Phospholipids?

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**ABSTRACT**

Bile previously labeled with tritiated oleic acid (the main radioactivity was on bile phospholipids) was mixed with pure isolated phospholipids previously labeled with $^{14}C$ oleic acid; this mixture was perfused during 6 or 23 hr into the duodenum of test rats bearing a bile fistula. At the time of decapitation, in the small intestine a large hydrolysis of the $^{14}C$ phospholipids was found. In contrast no bile phospholipid hydrolysis was observed. In the collected bile samples of the test rats, no $^{14}C$ could be detected (this means a very large decrease of the $^{14}C$ fatty acids specific activities by the body fatty acids), and the tritiated fatty acids specific activities were only 2.5-12 times lower than in the perfused bile. These results can be explained, assuming that the bile phospholipids enter in an entero-hepatic circulation and are preserved from the dilution in a large pool of lipids.

Several results of our previous experiments (1,2) allow us to ask this question. Thus, after an introduction into the duodenum of a small volume of bile labeled with tritiated oleic acid (70% of the whole radioactivity was on the phospholipids [PL]) in fat fed rats bearing a lymphatic fistula, the chylomicrons were poorly labeled, but the portal blood PL were significantly labeled (1). These bile PL should have had a special comportment, because it was well established (3,4) that after ingestion of isolated PL labeled on the fatty acids, the main radioactivity was recovered as triglycerides (TG) in the lymphatic chylomicrons. The bile PL should have been hydrolyzed in the intestinal lumen by the pancreatic phospholipase $A_2$ (EC 3.1.1.4) (5), and tritiated oleic acid should have been released since this enzyme hydrolyzes the fatty acids located at the 2 position of 1.2 diacylphosphoglycerides and since in the natural PL the unsaturated fatty acids are usually at this 2 position. But in incubations of bile with pancreatic juice or snake venom, these biliary PL presented a resistance to the phospholipase A hydrolysis; on the contrary the bile PL were completely hydrolyzed when they were isolated from the fresh bile, prior to the incubations, as we have demonstrated in a recent report (2). This fact can explain our previous results concerning the absorption of bile PL: the nonhydrolyzed biliary PL can arrive unchanged at the liver via the portal blood and may perhaps be excreted again in bile. The purpose of this study was to verify this last hypothesis.

In our experiments we have studied the incorporation of the radioactivity in the collected bile of the test rats, which have received in the duodenum a mixture of bile (collected previously on a rat which received 9-10. $^{3}H_2$ oleic acid by iv injection) and isolated PL (labeled with 1 $^{14}C$ oleic acid). The bile taken on the test rats was collected in different samples for analysis. Moreover the lipid radioactivities of the small and large intestines were determined in order to measure the two phos-

TABLE I

Composition and Distribution of the Radioactivity $^{3}H$ (Bile) and $^{14}C$ (Isolated Phospholipids) of the Test Mixture[a]

| | | Bile | | | Isolated Phospholipids | | |
|---|---|---|---|---|---|---|---|
| | | Total fatty acids | | | | | |
| Experiment | Volume, ml | Weight, mg | Radio-activity, dpm $^{3}H$ | Specific activity, dpm $^{3}H$/mg | Weight, mg | Radio-activity, dpm $^{14}C$ | Specific activity, dpm $^{14}C$/mg |
| A | 3.5 | 7.7 | 1.700.000 | 220.500 | 7.2 | 140.000 | 19500 |
| B | 11.3 | 22 | 4.850.000 | 220.500 | 19.8 | 386.000 | 19500 |

[a]Introduced into the duodenum of each rat during 6 hr and 23 hr in experiments A and B, respectively (see text).

TABLE II

Distribution of the Radioactivity and Mass (%) in Lipid Classes and Total Isolated Fatty Acids[a,b]

| | In nonphosphorus lipids | | | | | | In phospholipids | | | | | In total fatty acids | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Compound | Whole | CE | TG | FFA | DG+C | MG | Whole | AP + Cd | Cp | L | S | <16:0 | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | Unsaturated, 20[c] | 20:4 |
| Radioactivity Bile labeled with 9-10 $^3H_2$ oleic acid | 35 | 0 | 4 | 11 | 10 | 10 | 65 | | | | | 7 | 7 | 8 | 2 | 60 | --- | 11 | 5 |
| Isolated hepatic PL labeled with 1-$^{14}$C oleic acid | 5 | | | | | | 95 | 5 | 17 | 63 | 10 | --- | 7 | 2 | 7 | 84 | --- | tr. | tr. |
| Mass Bile labeled with 9-10 $^3H_2$ oleic acid | 12 | | | | | | 88 | | | | | 2 | 40 | 4 | 5 | 10 | 29 | 1.5 | 8.5 |
| Isolated hepatic PL labeled with 1-$^{14}$C oleic acid | 2 | | | | | | 98 | 9 | 30 | 54 | 5 | 0.5 | 22 | 1.5 | 21 | 9 | 16 | --- | 30 |

[a]From bile lipids and isolated phospholipids of the test mixture.

[b]CE = cholesterol esters, TG = triglycerides, FFA = free fatty acids, DG+C = diglycerides + cholesterol, MG = monoglycerides, AP = phosphatidic acids, Cd = cardiolipids, Cp = cephalins, L = lecithins, S = sphingomyelins.

[c]Except 20:4.

TABLE III

Fatty Acid Radioactivities (% of the Test Mixture Radioactivities) Recovered in the Small Intestine (Lumen + Mucosa), the Large Intestine Lumen,[a] and Bile Samples[b,c]

| Experiment | Rat no. | Small intestine | | Lumen of large intestine | | Collected bile | | |
|---|---|---|---|---|---|---|---|---|
| | | $^{14}C$ | $^{3}H$ | $^{14}C$ | $^{3}H$ | Collection time, hr | $^{14}C$ | $^{3}H$ |
| A | 1 | 59.6 | 60.3 | 1.2 | 0.6 | 0- 2 | 0 | 0.93 |
| | | | | | | 2- 4 | trace | 3.53 |
| | | | | | | 4- 6 | trace | 4.26 |
| | | | | | | 0- 6 | trace | 8.70 |
| | 2 | 69.3 | 72.2 | 1.4 | 0.2 | 0- 2 | 0 | 1.32 |
| | | | | | | 2- 4 | trace | 2.72 |
| | | | | | | 4- 6 | trace | 4.40 |
| | | | | | | 0- 6 | trace | 8.40 |
| | 3 | 64.3 | 82.6 | 2.2 | 0.7 | 0- 2 | 0 | 0.70 |
| | | | | | | 2- 4 | trace | 1.80 |
| | | | | | | 4- 6 | trace | 2.80 |
| | | | | | | 0- 6 | trace | 5.30 |
| B | 4 | 31.5 | 45.9 | --- | 18.8 | 0- 6 | trace | 13.30 |
| | | | | | | 6-20 | trace | 8.30 |
| | | | | | | 20-23 | trace | 3.40 |
| | | | | | | 0-23 | trace | 25.00 |
| | 5 | 24.9 | 40.9 | --- | 23.1 | 0- 6 | trace | 1.50 |
| | | | | | | 6-20 | trace | 7.10 |
| | | | | | | 20-23 | trace | 0.70 |
| | | | | | | 0-23 | trace | 9.30 |
| | 6 | 10.1 | 40.3 | --- | 26.9 | 0- 6 | trace | 2.00 |
| | | | | | | 6-20 | trace | 8.40 |
| | | | | | | 0-20 | trace | 10.40 |

[a]Collected at the time of decapitation.

[b]Collected during 6 hr (experiment A) or 23 hr (experiment B) after the perfusion of the test mixture.

[c]Amounts of perfused radioactivity: experiment A = 140,000 dpm $^{14}C$ and 1,700,000 dpm $^{3}H$; experiment B = 386,000 dpm $^{14}C$ and 4,850,000 dpm $^{3}H$.

pholipid substrates which have left the duodenum.

## MATERIALS AND METHODS

### Test Animals

Male albino rats weighing 250 g were of the Wistar strain and had been maintained on commercial diet till the surgical operation.

Bile fistula was near the liver leaving the pancreatic duct intact (6). Bile fistula and duodenal cannula were inserted during ether anesthesia on fed animals. Then they were placed in restraining cages, and immediately upon recovery from anesthesia (ca. 1 hr) the administration of the test mixture into the duodenum began. Two kinds of experiments were carried out (see experiments A and B).

### Preparation of the Test Mixture

The test mixture contained the tritiated bile and the $^{14}C$ PL.

To obtain the tritiated bile, one animal received by iv perfusion during 40 min, 17 mg pure potassium 9-10. $^{3}H_2$ Oleate 0.5 mc (C.E.A., France) bound on 750 mg serum albumin dissolved in 4 ml physiologic buffer. Then 3.5 ml radioactive bile was collected during a 6 hr period after the beginning of the perfusion and was mixed with 40 ml inactive bile (obtained from 15 rats during a 6 hr period after the introduction of the cannula). The final specific activity of the total bile fatty acids was 220,500 dpm/mg.

To obtain the $^{14}C$ PL, an iv perfusion was performed on a rat (as described above) with 70 $\mu$c of 1.$^{14}C$ pure potassium oleate (C.E.A., France). The animal was decapitated 1.5 hr after the beginning of the perfusion, and the liver was removed. The whole PL were isolated from lipid extracts by chromatography on silicic acid supercel column. The specific activ-

TABLE IV

Distribution of the Radioactivity at the Time of Decapitation[a,b]

| Experiment | Rat no. | $^{3}H$ PL | MG | DG+C | FFA | TG | CE | $^{14}C$ PL | MG | DG+C | FFA | TG | CE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 78 | 6 | 3 | 11 | 2 | 0 | 33 | 8 | 6 | 23 | 26 | 4 |
| A | 2 | 56 | 10 | 10 | 9 | 12 | 3 | 28 | 8 | 13 | 20 | 29 | 2 |
| | 3 | 67 | 13 | 5 | 4 | 9 | 2 | 21 | 10 | 12 | 17 | 37 | 3 |
| | 4 | 58 | 9 | 13 | 11 | 6 | 3 | 30 | 3.5 | 15 | 30 | 17.5 | 4 |
| B | 5 | 51 | 11 | 17 | 9 | 10 | 2 | 28 | 3 | 17 | 30 | 21 | 1 |
| | 6 | 50 | 11 | 15 | 12 | 9 | 3 | 19 | 6 | 12 | 31 | 29 | 3 |

[a]In the small intestine lipids (lumen and mucosa) 6 hr (experiment A) or 23 hr (experiment B) after the perfusion of the test mixture.

[b]Abbreviations: see Table II.

ity of PL fatty acids was 19.500 dpm $^{14}C$/mg.

The hepatic PL were dissolved in chloroform-methanol 1:1 and introduced into a flask. The solvents were evaporated to dryness. The tritiated bile was added, and the flask was shaken at 30 C to obtain a homogenous mixture called "test mixture."

#### Experiment A

Each rat received by the duodenal cannula 3.5 ml of the test mixture with a constant rate of 0.6 ml/hr in a 6 hr period, to equal the loss of bile drainage, using a constant rate infusion pump. No drink was given.

#### Experiment B

In a 23 hr period, each rat received by the same procedure 11.3 ml of the test mixture which was mixed with 2.4 g honey and 9 ml physiological buffer. On rat No. 4, 200 mg casein was added.

#### Extraction and Separation of Lipids

After death, the small and large intestine samples were immediately isolated, and their lipids and those of collected bile were extracted with dimethoxymethane-methanol 4:1 v/v. Proteins were removed and solvents evaporated to dryness. Lipids were recovered in pure chloroform, but bile samples, to avoid a loss of PL, were recovered in chloroform-methanol 1:1. Neutral lipids were separated on 250$\mu$ thin layer chromatography plates developed in hexane-ether-acetic acid-methanol 90:20:2:3 v/v/v/v. Different PL were separated with chloroform-methanol-water 65:25:4 v/v/v. Spots were visualized using iodine vapor.

#### Isolation of Total Fatty Acids

Total fatty acids were isolated after saponification or as butyl esters by transesterification, according to Clement and Bezard (7); their weights were determined with an internal standard (c17:0) by gas liquid chromatography (GLC).

#### Measures of Radioactivity

The spots of the thin layer chromatography plates were scraped and introduced into a scintillation vial containing 1 ml methanol; 2 hr later, 15 ml scintillation liquid was added. Radioactivity was counted in a Packard Tri-Carb Model 3324 scintillation spectrometer. The quenching was automatically corrected.

The mass and the radioactivity of each fatty acid was determined after separation by GLC and recovery at the end of the column according to the procedure described by Bezard et al. (8).

## RESULTS

In a previous experiment (2) we checked the position of the oleic acid in the labeled PL isolated from bile or liver on rats having received the labeled oleic acid by an iv injection; 85% of the radioactivity was found at the 2 position. This result agreed with other findings on human and sheep bile PL (9,10).

In Table I, the weights and the $^{3}H$ and $^{14}C$ radioactivities of the test mixture are shown; the distribution of the two radioactivities and mass in the different classes of lipids and in the fatty acids are given in Table II.

In the test mixture the biliary PL mass was very near the isolated PL one; they were 88% (unpublished data) of the bile lipids and they bore 65% of the total tritium; the isolated PL bore 95% of the total $^{14}C$. The mass of $^{3}H$ or $^{14}C$ oleic acid represented 9-10% of the total fatty acid mass. In bile, only 60% of the radioactivity was on the tritiated oleic acid (expressed in per cent of the whole fatty acid radioactivity), compared to 84% on the $^{14}C$ oleic acid in the isolated PL.

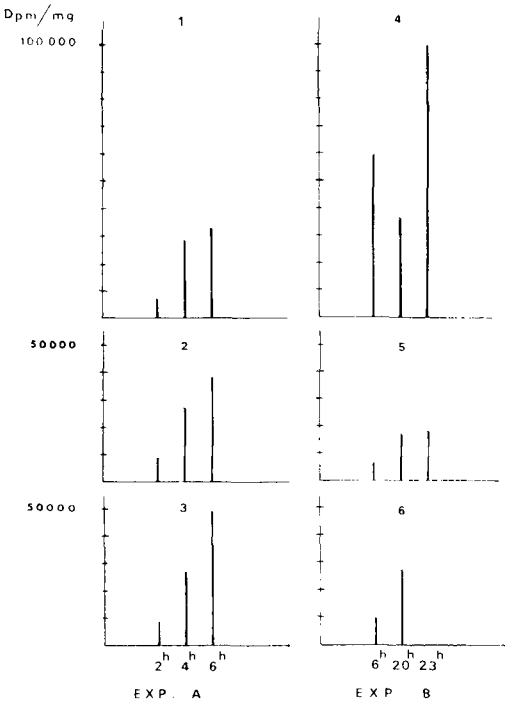


FIG. 1. Specific radioactivities of the tritiated fatty acids in bile of the six test rats (specific activity of the perfused bile fatty acids: 220,500 dpm $^{3}H$/mg).

In experiment A (Table III, rats 1, 2 and 3) at the time of decapitation, there were found in the small intestine ca. 60-80% of the tritiated and $^{14}C$ radioactivity introduced into the duodenum; the large intestine was not labeled (0.2-1.4%). No $^{14}C$, but 5-9% of the test mixture tritiated radioactivity, was present in the bile collected during 6 hr. In experiment B (Table III, rats 4, 5 and 6) at the time of decapitation, the amounts of the radioactivities found in the small and large intestines were smaller than in experiment A, but at this time a large part of the $^{3}H$ radioactivity had not left the intestine; there was no $^{14}C$ in the large intestine. The excreted bile contained only tritiated fatty acids and has incorporated 9.3-25% of the test mixture radioactivity.

The distribution of the tritiated radioactivity in the different lipid classes of the introduced bile (Table II), and in the small intestine, one of the test rats (Table IV), is slightly changed.

In contrast the $^{14}C$ PL (Table IV) bore only 21-33% of the total $^{14}C$ instead of 95% in the test mixture (Table II); the free fatty acids TG and DG appeared adequately labeled (ca. 50% of the total radioactivity).

The tritiated specific activities of the excreted bile fatty acids are given in Figure 1. They increased from the first to the third bile collection samples in experiments A and B, except in rat No. 4, but reached only 40% of the test mixture tritiated fatty acid specific radioactivities. The distribution of $^{3}H$ into the collected bile lipids classes was not always tested, but two analyses showed the following distribution: 85% in PL and 15% in the DG.

Table V gives the tritiated distribution in the collected bile fatty acids of test rats 4 and 5. The oleic acid radioactivity per cent is much smaller than in the test mixture. The unsaturated 20 C fatty acids bore the most of the radioactivity.

## DISCUSSION

The absorption processes were perhaps slowed down and disturbed since the test mixture was introduced into the duodenum 1 hr after the end of the anesthesia. Our experimental procedure was thought good enough because we can follow the two labeled oleic acids which were incorporated in equal quantity into the biliary and into the isolated PL.

The $^{14}C$ PL hydrolysis, by the phospholipase $A_2$, inside the small intestine lumen appeared to a large extent because the $^{14}C$ fatty acids were present in free fatty acid fraction, DG and TG (in lumen + mucosa) (Table IV). A large amount of this $^{14}C$ material will go to the lymphatic chylomicrons triglycerides (3,4). In contrast there is not an important change in the distribution of the tritiated radioactivity between the small intestine and the perfused bile. These results on animals ascertain our previous in vitro experiments (2), since during incubation of bile, the bile PL are preserved in a large part from the pancreatic juice hydrolysis, whereas the isolated PL (isolated from bile or liver) are not.

This fact emphasizes the possible particular way of fatty acid incorporation, since they come from two phospholipidic substrates differing in physicochemical state, but introduced into the duodenum as a homogeneous mixture. Since after a labeled bile perfusion into the duodenum of a rat bearing a lymphatic fistula (1), the lymph TG contained only 10% of the perfused radioactivity and the portal blood PL were labeled, we can assume that the nonphosphorus bile lipids and the small amounts of the fatty acids hydrolyzed from the bile PL can go to the lymph (they represented a maximum level equal to 35% of the perfused bile radioactivity [but we have seen in Table II that this biliary material is small in mass]) as chylomicrons triglycerides. Then they will be much diluted since the lymph reach the blood circulation, but they will not be incorporated extensively in the collected bile, because in

TABLE V

Distribution of $^3H$ Radioactivity (%) in Collected Bile Fatty Acids[a] and Test Mixture Bile Fatty Acids

| Rat no. | Collection time, hr | <16:0 | 16:0 | 16:1 | 18:0 | 18:1 | Unsaturated, 20 |
|---|---|---|---|---|---|---|---|
| 4 | 6-20 | 19 | 6 | 12 | 3 | 19 | 41 |
| | 20-23 | 21 | 15 | 9 | 6 | 15 | 34 |
| 5 | 0- 6 | 15 | 24 | 11 | 3 | 18 | 29 |
| | 6-20 | 18 | 9 | 10 | 3 | 20 | 40 |
| Test mixture | | 7 | 7 | 8 | 2 | 60 | 16 |

[a]*Experiment B, rats no. 4 and 5.*

experiments not described here, the bile samples collected during 6 hr bore only 0.4-1.4% of the radioactive fatty acid introduced in the blood by an iv perfusion of 40 min, as well with a 1.$^{14}C$ oleic acid as with a 9.10$^3H_2$ oleic acid. A similar distribution of the $^3H$ and $^{14}C$ radioactivities was found in the different bile lipid classes (the distribution seen in Table II). These data appear independent of the nature of the labeled molecule $^{14}C$ or $^3H$ oleic acid.

The findings explain that in Table III, we do not have significant amounts of $^{14}C$ fatty acids in the bile samples, because the $^{14}C$ fatty acids released from the $^{14}C$ PL of the test mixture also reach the blood circulation and are much diluted. This important dilution is in agreement with Saunders (11) who measured a very small amount of $^3H$ or $^{14}C$ (0.3-1.1 of $^3H$ and 4.5-4.8 of $^{14}C$) in human bile when a patient receives a dietary labeled lecithin ($^3H$ palmitoyl-oleyl-phosphoglycero-$^{14}C$ choline) or $^3H$ palmitate + $^{14}C$ choline chloride.

If we consider a complete absorption of the perfused radioactive material which has disappeared from the intestine, till the death time (Table III), this means that 75% of the $^{14}C$ but only 30-35% of the tritiated radioactivities of the test mixture would have been absorbed. We would have found more $^{14}C$ than tritiated radioactivity (in per cent of the perfused radioactivity) in the carcass of the test rats. Unfortunately the carcass radioactivity has not been counted.

Furthermore it is possible (although the test mixture was given) that the oleic acid oxidation occurred. If the $^{14}C$ and $^3H$ oleic acids, which are equal in mass, in the two phospholipids in the test mixture had reached the liver together, and if they had been incorporated into the similar molecules, the $^{14}C$ and $^3H$ oxidation per cent would have been the same (taking into account the position of the isotope in the molecule); and we would have obtained as much $^{14}C$ as $^3H$ in bile samples (in per cent of the perfused radioactivities).

But, in bile samples, 8-25% of the tritiated radioactivity perfused in the duodenum were found. We should be able to infer that the PL of the biliary origin, making their way through the portal blood, would have a particular absorption and would return again in bile. This point seems to be true, because if we look at the following estimation, the calculations agree with the experimental determinations, i.e., in experiment B: the perfused bile PL bore 65% of the bile radioactivity (Tables I and II): 4,850,000 x $\frac{65}{100}$ = 3,152,000 dpm $^3H$. One third of these PL (see Table III) would have left the intestine in 23 hr and would have given 1,050,000 dpm $^3H$, which would belong to the biliary lipid radioactivity excreted in a 23 hr period, if we assume a complete return of this material to the liver through the portal blood. We had 1.2, 0.4 and 0.5 x $10^6$ dpm $^3H$, respectively, for rats 4, 5 and 6. No $^3H$ radioactivity would be lost in rat 4 which received 200 mg casein with the test mixture, the honey and the physiological serum (we cannot be sure if casein was essential in these data because only one animal was tested), and we would have a loss of 50% in the two other rats.

If the bile PL enter the portal blood and are excreted again in bile, it is surprising to observe (Table V) a high metabolism of the oleic acid, since it bears only 15-20% of the $^3H$ bile fatty acid radioactivity instead of 60% in the perfused bile. Several authors (12-14) have described a pool of bile lecithin in the liver (this pool would appear different from the liver lecithin one). We can think of a metabolism of the oleic acid to ensure a particular distribution or structure; this is a hypothesis. It would be interesting to experiment with a bile labeled with a $^{14}C$ oleic acid for verification, if we again find this metabolism.

Let us notice again that in our experiments, no $^{14}C$ lipid in the large intestine lumen (these

$^{14}C$ fatty acids have been previously absorbed by the duodenum), but 20% of the perfused tritiated radioactivity, was found (in experiment B). Since in the large intestine the biliary salts are not absorbed and do not return to the entero-hepatic circulation, we can consider these results as more evidence enabling us to speak about the formation of the biliary complex (PL-biliary salts-cholesterol-proteins), one part of which would be excreted and the other which would enter the portal blood and, as we suggest with our experiments, would enter an entero-hepatic cycle.

We wish to proceed with this subject, and think that our results agree with the description of a biliary lecithin pool in the liver (12-14) and with the need for a normal biliary salts rate in bile which appears necessary to obtain a normal bile phospholipid excretion (15,16).

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# Lipids of *Echinococcus granulosus* Protoscolices

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## ABSTRACT

The fatty acid composition of the triglyceride and the total phospholipid fractions of ovine liver *Echinococcus granulosus* protoscolices was determined by gas chromatography and compared with that of the healthy and Echinococcus infected livers. The chain length of the major saturated fatty acids identified in both the host tissues and the parasite ranged from 12-22 carbons. Oleic and linoleic acids were the only detectable unsaturated fatty acids identified in protoscolices and the liver samples. Comparison of the amounts of the fatty acids from the three different sources mentioned above by analysis of variance and the contrast method of Scheffe, revealed a significant decrease in the level of oleic acid in triglyceride fraction of the infected livers compared with normals. Thin layer chromatography of the polar lipid fraction of the protoscolices resulted in tentative identification of lysolecithin, sphingomyelin, lecithin, phosphatidyl inositol, sulfatides, cerebrosides, cephalins and cholesterol.

## INTRODUCTION

Echinococcosis caused by *Echinococcus granulosus* is a widespread disease of man and sheep, involving the liver with an approximate incidence of 70%. Dog is the common host of this tape worm with man and sheep as the intermediate hosts. Infection in the sheep is produced following grazing on pasture contaminated with the feces of the infected dogs; the ova pass through the intestinal mucosa and are transported to the liver. The ova develop into adult cysts in the liver resulting in complications such as intrabiliary and intraperitoneal rupture; the latter results in the development of new cysts. Also, due to the presence of foreign proteins in the fluid, severe anaphylactic shock may occur. Chemotherapy and radiotherapy have not been effective, and in humans one has to resort to surgical removal of the cysts (1,2).

Biochemical studies on *E. granulosus* and their comparison with those of the host tissues may eventually result in the development of

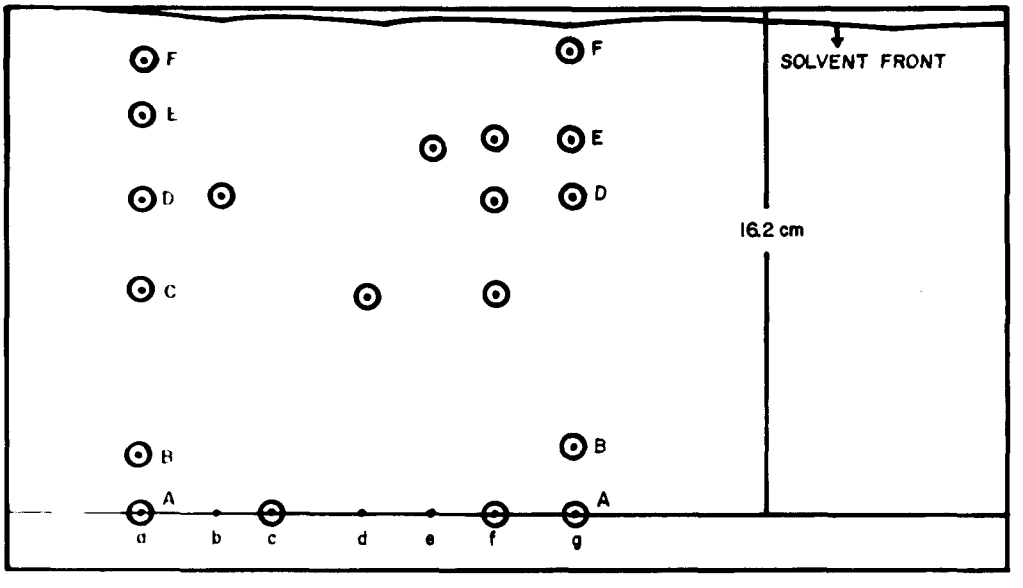


FIG. 1. Schematic representation of thin layer chromatography of neutral lipids. Development and detection methods were the same as described in the text: a, 6.0 μliters of protoscolices neutral lipids; b, c, d and e, 0.10 mg each of stearic acid, phosphatidyl ethanolamine, cholesterol and tripalmitin, respectively; f, a mixture of b, c, d and e (0.05 mg each); g, 10 μliters of neutral lipids of infected ovine liver.

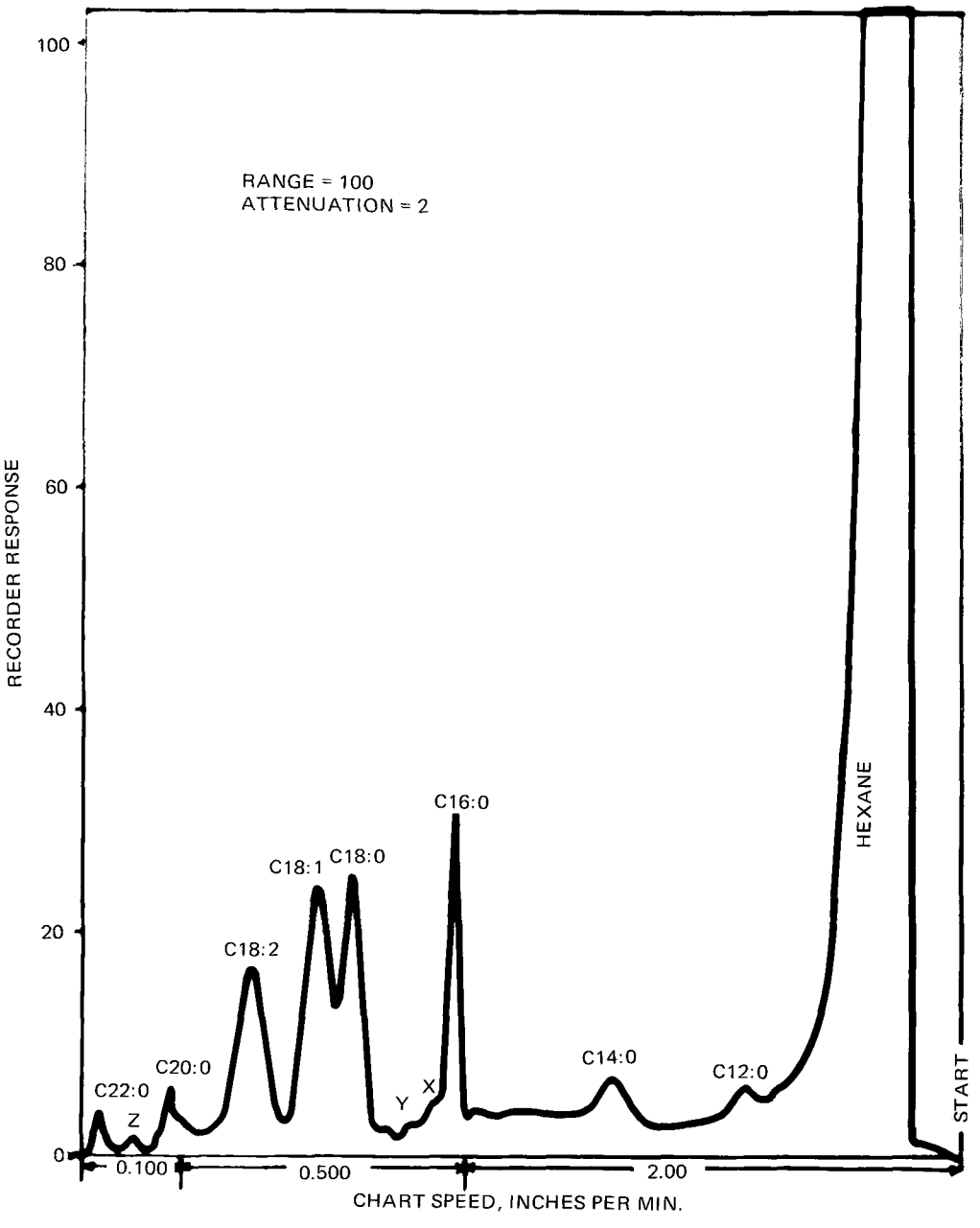


FIG. 2. A gas liquid chromatogram of fatty acid methyl esters obtained from neutral lipid fraction of protoscolices. Experimental conditions are described in the text. Unidentified fatty acid methyl esters are denoted by letters X, Y, Z, etc.

chemotherapeutic measures (3).

Present studies were undertaken in order to compare the lipid components of the parasite with those already established in the host. The information concerning the lipid components of *Echinococcus granulosus* is scant. It is known that approximately 13.6% of the dry substance of the scolices is composed of lipids (4). Among lipid components, cholesterol has been found by Cameron and Fitzpatrick (5) to be present in hydatid cyst fluid, cyst wall and scolices, while the presence of lecithin in scolices has been reported by Kilejian and coworkers (6). The literature concerned with

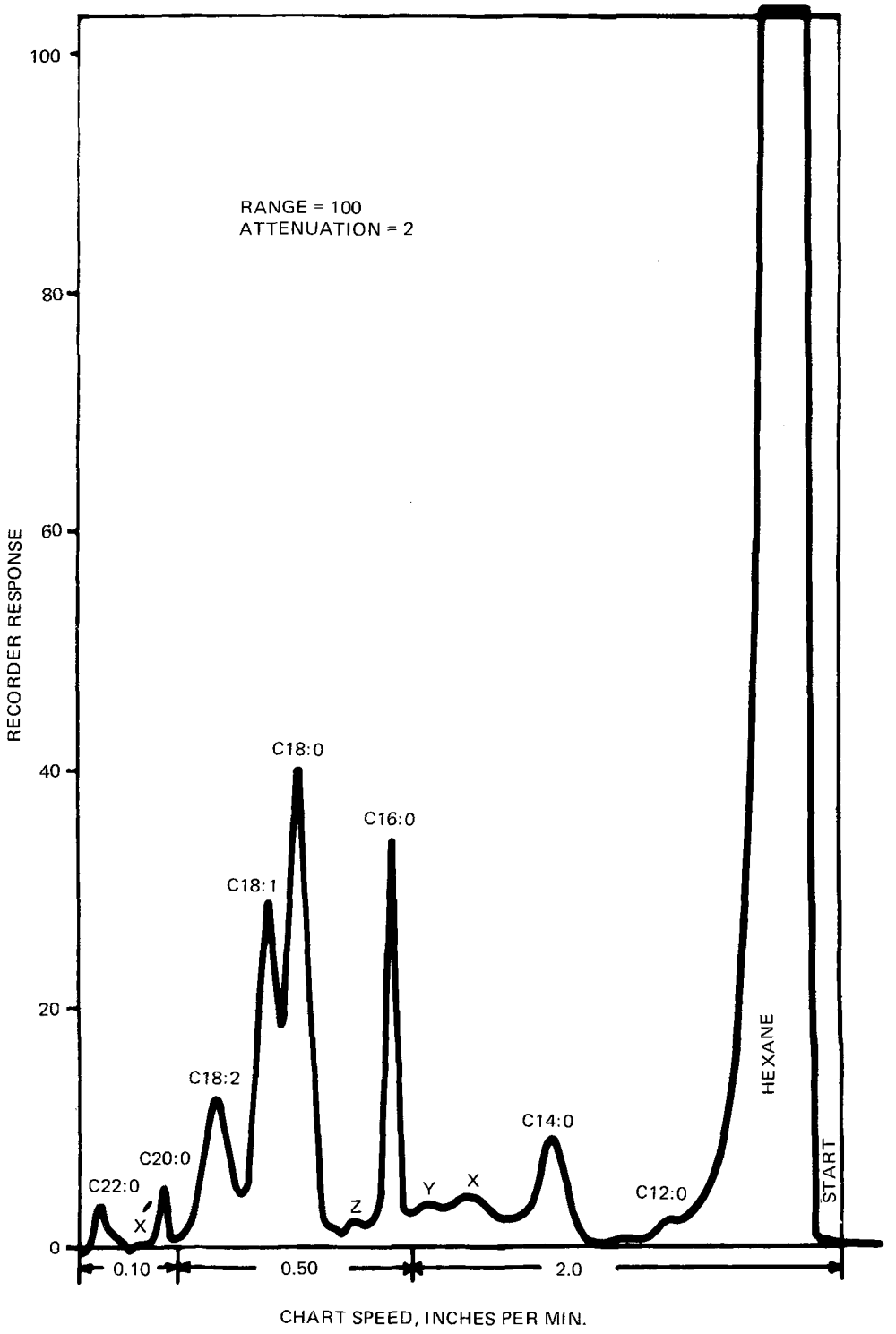


FIG. 3. A gas liquid chromatogram of polar lipid fatty acid methyl esters of protoscolices. Experimental conditions are described in the text. Unidentified fatty acid methyl esters are denoted as X, Y, Z, etc.

TABLE I

Major Fatty Acid Methyl Esters of *E. granulosus* Protoscolices, Healthy and Infected Ovine Livers[a]

| Fatty acids[c] | Area % ± SD[b] | | | | | |
|---|---|---|---|---|---|---|
| | Neutral lipids | | | Phospholipids | | |
| | Protoscolices | Healthy liver | Infected liver | Protoscolices | Healthy liver | Infected liver |
| 12:0 | 2.6 ± 0.8 | --- | 2.8 ± 0.8 | 1.0 ± 0.3 | --- | --- |
| 14:0 | 2.8 ± 0.4[d] | 0.9 ± 0.2[d] | 1.3 ± 0.5[d] | 2.3 ± 0.2[d] | 0.7 ± 0.3[d] | 0.8 ± 0.2[d] |
| 16:0 | 11.7 ± 0.6 | 17.0 ± 2.0 | 13.5 ± 2.4 | 12.9 ± 2.8 | 14.7 ± 1.3 | 14.5 ± 1.1 |
| 18:0 | 21.3 ± 2.5 | 21.8 ± 2.2 | 24.8 ± 3.5 | 29.3 ± 3.2 | 32.3 ± 1.7 | 32.0 ± 1.8 |
| 18:1 | 23.9 ± 2.0 | 29.2 ± 2.5[d] | 16.4 ± 2.8[d] | 23.9 ± 2.5 | 23.2 ± 1.0 | 21.0 ± 2.7 |
| 18:2 | 17.5 ± 3.7[d] | 8.8 ± 1.5[d] | 10.2 ± 1.6 | 9.9 ± 0.6 | 7.9 ± 1.0 | 9.4 ± 0.7 |
| 20:0 | 8.4 ± 1.7[d] | 4.6 ± 0.8 | 3.9 ± 1.0[d] | 5.9 ± 0.6 | 4.9 ± 1.8 | 6.4 ± 1.9 |
| 22:0 | 8.2 ± 1.9 | 3.6 ± 1.0 | 7.6 ± 2.5 | 6.4 ± 1.4 | 9.8 ± 0.7 | 7.8 ± 1.4 |

[a]Minor peaks which were not identified were excluded from this table.

[b]Standard deviations were based on results obtained for six replications of the gas chromatographic procedure.

[c]Numbers before the colon indicate the number of carbon atoms and those after the colon refer to the number of double bonds.

[d]Refer to the text for statistical differences among fatty acid methyl esters.

the lipid composition of this parasite has been reviewed by Agosin (7).

The only data available on the fatty acid composition of the scolices is that of Digenis et al. (8), who demonstrated the presence of saturated and unsaturated fatty acids ranging in chain length from 12-20 carbon atoms in the saponifiable fraction of the neutral lipids of the scolices.

The present investigation deals with the fractionation of the total lipids of protoscolices of ovine liver *E. granulosus* into neutral and polar lipids by solvent extraction. The major fatty acids present in these fractions were identified, estimated and compared with those present in the neutral and polar lipid fractions of the healthy and Echinococcus-infected ovine livers. Also, the major phospholipids and glycolipids present in the polar lipid fraction of the protoscolices were tentatively identified by thin layer chromatography.

## MATERIALS AND METHODS

### Materials

The following reagents were obtained from the commercial sources indicated: silica gel, Chemie-Erzeugnisse and Absorption Technik AG, Müttenz, Schweiz; fatty acid methyl esters, Polyscience Corp., Evanston, Ill.; bovine sphingomyelin, sulfatides, cerebrosides and phosphatidyl serine, plant lecithin, phosphatidyl inositol, and egg lysolecithin from Supelco, Inc., Bellefonte, Pa.

Methanol was purified by refluxing 1.0 liter portions of the solvent with 5 g KOH and 12.5 g zinc dust for 3 hr, followed by collection of the fraction distilled between 59-60 C. Chloroform was redistilled and the fraction boiling between 55-56 C was collected.

### Methods

*Preparation of hydatid cyst protoscolices:* Ovine livers infected with *E. granulosus* were freshly secured from Isfahan and Shiraz slaughter houses and the identity of the parasite as *E. granulosus* was established by the procedure described elsewhere (3).

The infected livers were kept at 4 C and the protoscolices prepared according to Zekavat and Khayat (9), except that ice cold 0.15 M NaCl was substituted for the washing solution recommended. Hydatid cysts with secondary infections were excluded during the preparation of the protoscolices.

The wet weight of protoscolices harvested was 3-5 g per infected liver depending on the progress of the disease.

*Extraction of total lipids:* Wet protoscolices (33 g) were suspended in 104 ml methanol-water 100:4 v/v and disrupted by sonic oscillation in a "Biosonik" sonic oscillator (Bronwill Scientific, Rochester, N.Y.) at the maximum frequency for 10 min at 4 C. Sonication resulted in almost complete rupture of the protoscolices as evidenced by examination under light microscope. The total volume of the sonicate was brought to 130 ml with methanol.

To the sonicate, 100 ml chloroform and 50 ml water were added; the suspension was filtered through Whatman No. 1, and the volume was brought to 280 ml with chloro-

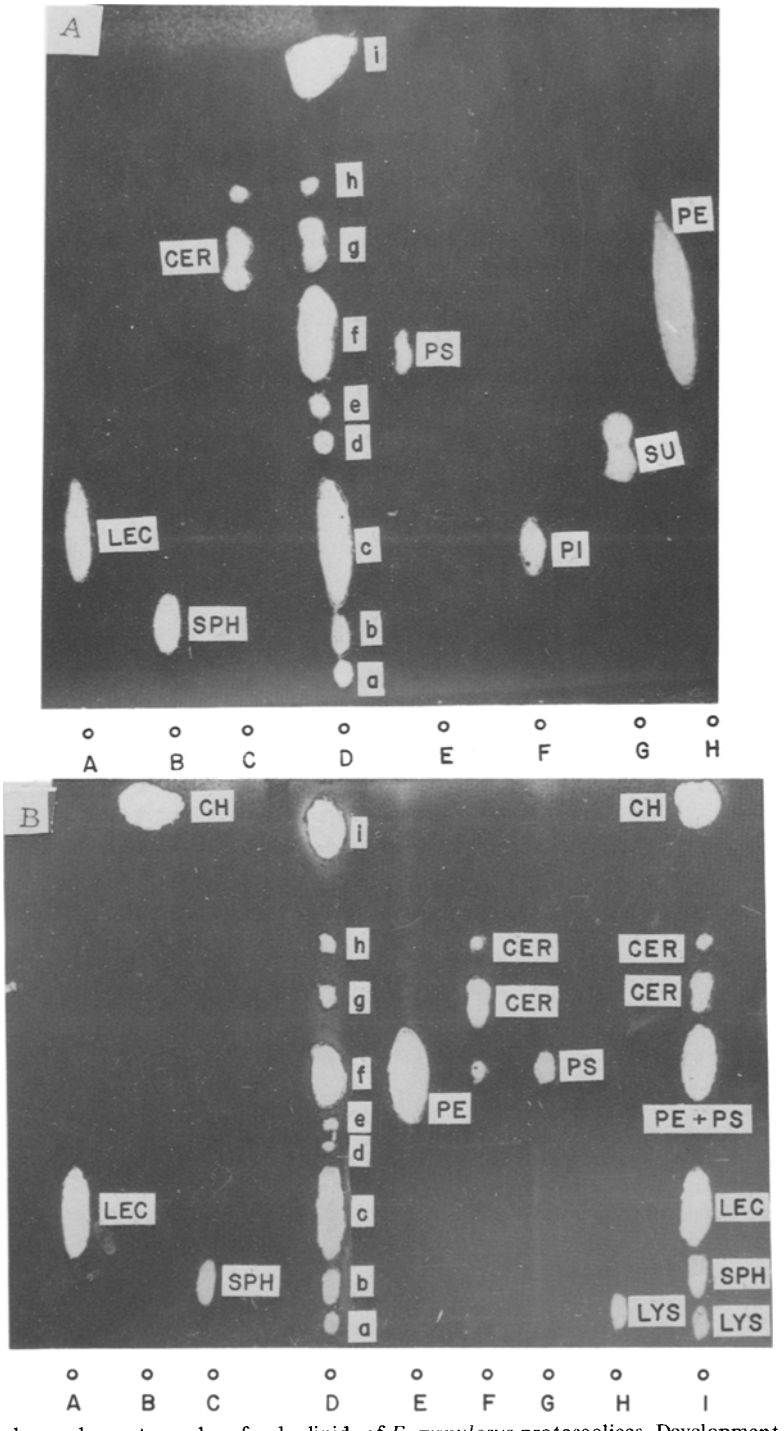


FIG. 4. Thin layer chromatography of polar lipids of *E. granulosus* protoscolices. Development and detection methods were the same as described in the text. Spot D (Fig. 4A and B) was 20 μliters of protoscolices polar lipid fraction. The following abbreviations are used: LEC, lecithin; CER, cerebrosides; PS, phosphatidyl serine; PI, phosphatidyl inositol; SU, sulfatides; PE, phosphatidyl ethanolamine; CH, cholesterol; and LYS, lysolecithin. The amount of lipid standards applied per spot was 0.10 mg, with the exception of PI, 0.03 mg and LEC, 0.05 mg. Spot I in Fig. 4B was a mixture of known standards. All standard phospholipids were dissolved in chloroform-methanol 1:1 v/v except LEC which was dissolved in benzene.

TABLE II

Sprays Used for the Identification of Amino Compounds, Glycolipids and Choline Containing Compounds

| Unknowns | Ninhydrin | Diphenylamine[a] | Chargaff's |
|---|---|---|---|
| Spot A (LEC + PI)[b] | + | + | + |
| Spot B (PE + PS) | + | - | - |

[a]A plus sign denotes positive and minus means negative reaction.

[b]Abbreviations are the same as in Figure 4.

form. The total lipids were extracted by a procedure based on that of Peters and Smith (10). The filtrate was separated into a chloroform and a methanol-water phase in a separatory funnel. To the methanol-water layer, 100 ml chloroform, 100 ml methanol and 65 ml water were added, and the two phases were separated as described. The methanol-water phase was reextracted several times with 150 ml chloroform, and the phases were separated following each extraction. The chloroform phases containing total lipids were combined (600 ml).

*Separation of polar and nonpolar lipids:* Separation of polar from nonpolar lipids was carried out in petroleum ether-87% aqueous ethanol system as described by Galanos and Kapoulas (11). Total lipids in chloroform (150 ml) were dried in vacuo under nitrogen gas. The dried lipids were dissolved in 100 ml ethanol-petroleum ether 3:1 v/v; 12 ml water and 25 ml petroleum ether were added, and the aqueous ethanol and petroleum ether phases were separated. The aqueous ethanol phase was extracted three times with 20 ml portions of petroleum ether followed by the separation of the two layers. The petroleum ether phase was also extracted three times with 17 ml portions of 87% ethanol and the two phases formed were separated. The aqueous ethanol phases containing polar lipids were combined, dried in vacuo under nitrogen gas and dissolved in 4 ml chloroform-methanol 1:1 v/v. The petroleum ether phases containing neutral lipids were also combined, treated as above and dissolved in 3.5 ml chloroform-methanol 1:1 v/v.

For comparison of the host and parasite lipids, 50 g of wet liver from two healthy sheep and 50 g of wet liver from the uninfected portion of two *E. granulosus*-infected livers were taken through the extraction procedure for total lipids. The final volume of healthy ovine liver total lipids in chloroform was 650 ml, and that of infected liver was 850 ml. Half of the total lipid fractions from each of the above sources was used for separation of the neutral from polar lipids according to the procedure described. The polar lipids from each source were dissolved in 25 ml chloroform-methanol 1:1 v/v and the neutral lipids in 5 ml of the solvent mixture mentioned.

*Thin layer chromatography of neutral lipids:* A modification of the procedure of Freeman and West (12) was employed. The adsorbent was prepared by suspending 25 g of silica gel (no binder) in 70 ml water. The plates were 20 x 20 cm in size with an adsorbent thickness of 250 $\mu$. Neutral lipids (6 $\mu$liters) were applied on the plates along with cholesterol, tripalmitin, phosphatidyl ethanolamine and stearic acid standards. The chromatograms were developed first in diethyl ether-benzene-ethanol-acetic acid 40:50:2:0.2 v/v solvent and subsequently with diethyl ether-petroleum ether 6:94 v/v in the same direction (12). The spots were visualized upon spraying the plates with sulfuric acid-potassium dichromate mixture, followed by heating at 100-110 C for 30 min (13).

*Gas chromatography:* (a) Preparation of fatty acid methyl esters: The neutral lipid fraction of the protoscolices was methylated according to the procedure of Morrison and Smith (14) for the methylation of triglycerides and sterol esters using 0.50 ml aliquots of the neutral lipids. The methylating agent used was boron-trifluoride-acetic acid complex (14% in methanol). The methyl esters were recovered in hexane which had been purified in a Silica Gel G column. The volume of the methyl esters of the neutral lipids thus obtained was brought to 0.20 ml with hexane. Two microliter aliquots were used for injection to the gas chromatograph.

The protoscolices polar lipids were also methylated according to a similar procedure described for major phospholipids (14) using 0.5 ml aliquots of the polar lipids. The final volume of methyl esters extracted in hexane was 0.15 ml. Two microliter aliquots were used for gas chromatography.

The neutral lipid fractions of healthy and Echinococcus-infected ovine livers were methylated according to the same procedure described for protoscolices neutral lipids, except that 0.10 ml of the neutral lipids of the infected liver and 0.20 ml of the healthy liver

were used for methylation, and the final volume of methyl esters were brought to 0.20 ml with hexane. Two microliter aliquots of the infected liver and 1.0 μliter aliquots of the healthy liver neutral lipid methyl esters were used for gas chromatography.

The polar lipid fractions of the healthy and infected livers were methylated in the same manner used for the protoscolices, except that the final volume of methyl esters in hexane was 0.20 ml. Two microliter aliquots were used for gas chromatography.

(b) Gas chromatography: The instrument used was a Varian Aerograph Model 600-D with a flame ionization detector using hydrogen at a flow rate of 25 ml/min. The column was a 5 ft x 1/8 in. stainless steel packed with 20% polydiethyleneglycol succinate (DEGS) on Chromosorb W 60-80 mesh. Nitrogen was used as the carrier gas at a flow rate of 55 ml/min as measured by a soap-bubble flow meter at the column outlet. The chromatograph was equipped with a 10 in. Beckman recorder with a variable chart speed. The voltage was maintained at 1 mv throughout the studies.

Specified aliquots of fatty acid methyl esters were injected in the chromatograph using a 10 μliter Hamilton microsyringe. Column temperature was maintained within the range of 196-199 C. Chromatographic separations were performed at a range reading of 100 and an attenuator reading of 2.

The major fatty acid methyl esters were identified by comparing the retention time of the unknown esters with those of the known standard fatty acid methyl esters chromatographed under the same conditions.

The area under each peak was calculated by multiplying the height of the peak by the width at half height (15), and the results were expressed in terms of per cent of the total area.

*Thin layer chromatography of polar lipids:* The polar lipid fractions were chromatographed on Silica Gel G (5% $CaSO_4$) containing ammonium sulfate according to the procedure of Mangold (16). The thickness of the plates used were 500 μ. Standard phospholipids, sulfolipids and glycolipids were chromatographed along with the polar lipid fraction. The developing solvent was chloroform-methanol-water 65:25:4 v/v suggested by Mangold (16), and the spots were visualized by iodine vapor (13). The identity of the spots as phospholipids was confirmed by ammonium molybdate-perchloric acid reagent (13).

## RESULTS AND DISCUSSION

### Thin Layer Chromatography of Neutral Lipids

The results of the thin layer chromatography (TLC) of the neutral lipid fractions of *E. granulosus* protoscolices and the Echinococcus-infected ovine livers are schematically presented in Figure 1. As indicated, free cholesterol, free fatty acid(s) and triglyceride(s) were observed in the neutral lipid fraction of the protoscolices. The spot which behaved like phosphatidyl ethanolamine, i.e., it did not migrate in the solvent system used, was identified according to Freeman and West (12) as a phospholipid (Fig. 1, $a_A$), although it may be an oxidized material yielding a spot upon charring with sulfuric acid-dichromate spray. The two additional spots in the neutral lipid fraction of the protoscolices (Fig. 1, $a_B$ and $a_F$) corresponded to monoglyceride and cholesterol ester, respectively (12). Similar compounds were detected in the infected liver neutral lipids with the exception of free cholesterol (Fig. 1). The latter compound was completely extracted into the polar lipid fraction of the liver as examined by TLC of this fraction (M. Vessal, unpublished data). The difference in the behavior of cholesterol in the protoscolices and the liver tissue may be explained on the basis of the high solubility of cholesterol in bile salts, resulting in the extraction of this compound in the polar lipid fraction of the liver.

### Gas Chromatography of Fatty Acid Methyl Esters

Typical gas chromatograms of fatty acid methyl esters of the neutral and the polar lipid fractions of the protoscolices are shown in Figures 2 and 3, respectively. As seen, the major fatty acids of the neutral and the polar lipid fractions of the protoscolices identified based on their chromatographic behavior were $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:0}$ and $C_{22:0}$.

Table I shows the average per cent area of the different fatty acid methyl esters of the neutral, and the polar lipid fractions of the protoscolices, healthy and Echinococcus-infected ovine livers together with their standard deviations. As noticed, more than 50% of the total fatty acids of the protoscolices is composed of $C_{18}$ fatty acids. This is in accord with the results obtained by Digenis et al. (8) and the results of work done on other cestodes (17).

A comparison of the amounts of the various fatty acids of the neutral and the polar lipid fractions of the parasite, the infected and the healthy livers was made by analysis of variance (18) together with the contrast method of Scheffe (19).

The only statistically significant differences ($P = 0.05$) obtained among the various fatty acids of the three sources were as follows: (a)

$C_{14:0}$ fatty acid of the neutral and the polar lipid fractions of the protoscolices were significantly higher than the corresponding fatty acid in the healthy and the infected host tissue, (b) $C_{18:1}$ fatty acid of the neutral lipid fraction of the healthy liver was significantly higher than the corresponding fatty acid in the livers infected with the parasite, (c) $C_{18:2}$ fatty acid of the neutral lipid fraction of the protoscolices was significantly higher than that of the healthy liver, and (d) $C_{20:0}$ fatty acid of the neutral lipid fraction of the protoscolices was significantly higher than that of the infected liver.

The major point demonstrated by statistical analyses is that the level of oleic acid ($C_{18:1}$) in the neutral lipid fraction is decreased significantly upon invasion of the liver by the parasite. This finding may indicate that this organism requires oleic acid and obtains this fatty acid from its host by hydrolyzing the triglycerides of the host and using oleic acid for its own synthetic purposes.

#### Tentative Identification of Polar Lipids

The results of one-dimensional TLC of *E. granulosus* protoscolices polar lipids are presented in Figure 4. As seen (Fig. 4A and B), the following polar lipids were tentatively identified based on their $R_f$ values: sphingomyelin, cerebrosides, lysolecithin and cholesterol. Lecithin had the same $R_f$ value as phosphatidyl inositol, and phosphatidyl serine had the same $R_f$ value as phosphatidyl ethanolamine. In order to identify the compounds present in these two unknown spots, chromatograms which had been developed in the same manner were sprayed with ninhydrin, diphenylamine and Chargaff's reagents used for amino groups, glycolipids and choline containing compounds, respectively (13). The results of the action of the named sprays on the two unknown spots are summarized in Table II. Spot A gave positive reactions with the three reagents. This is an indication of the presence of a choline containing phospholipid and a sugar containing lipid, and since the $R_f$ value of this spot corresponded to those of phosphatidyl inositol and lecithin, the components present in this spot were tentatively identified as lecithin and phosphatidyl inositol (Fig. 4). In addition to the results mentioned, the work of Kilejian et al. (6) has demonstrated the presence of lecithin in the protoscolices of this parasite.

Spot B gave only a positive reaction with the ninhydrin reagent (Table II) indicating the presence of an amine containing phospholipid(s) without a carbohydrate or a choline moiety; therefore the spot was tentatively identified as one that contains cephalin(s). Whether this spot contains either or both phosphatidyl serine and phosphatidyl ethanolamine requires further investigation.

Comparison of the results on the phospholipids of protoscolices reported heretofore, with those of Peters and Smith (10) on the phospholipid fraction of ovine liver, indicates certain similarities in the types of phospholipids contained in the host and the parasite, except for sulfatides which have not been demonstrated in ovine liver so far.

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# Mass Spectra of Tocopherols[1,2]

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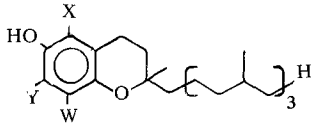
## ABSTRACT

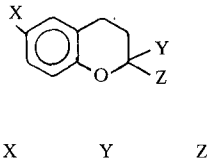
The mass spectra of α-, β- and γ-tocopherols (*I*) are characterized by the absence of numerous fragmentation processes. Ion intensities, but not the reaction pathways, are strongly dependent on temperature. Principal fragment ions are formed (a) by cleavage through the non-aromatic portion of the chromanol ring both with and without hydrogen transfer and (b) via loss of the isoprenoid side chain. For (a) occurring with hydrogen transfer, ion-kinetic energies favor a tropylium ion structure for the daughter ion. Loss of ketene from the molecular ions of α- and β-tocopherol acetates producing the molecular ion of the corresponding tocopherol is the dominant process.

## INTRODUCTION

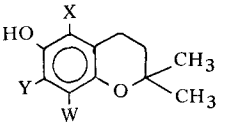
Considerable interest has been focused upon the mass spectra of quinones that function as electron transfer reagents in biochemical systems (1). Related compounds that function as naturally occurring antioxidants are α- (Vitamin E), β- and γ-tocopherols (*I*) (2). Surprisingly no detailed study of the mass spectra of these compounds has been reported. The spectra of α-*I* and its trifluoroacetate have been published (3) and some details of the fragmentation processes of γ-*I* have been commented upon (4). Consequently we report and discuss the mass spectra of α-, β- and γ-*I* and the acetates of α- and β-*I*. Considerations of molecular structure dictate comparison of these results with those obtained for chroman (*II*) (5), 2,2-dimethylchroman (*III*) (5), 6-chromanol (*IV*) (5), and 2,2-dimethyl-6-chromanol (*V*) (6) and various C-5, C-7 and C-8 methylated congeners (tocopherol model compounds) (6).



| | X | Y | W |
|---|---|---|---|
| α-*I* | $CH_3$ | $CH_3$ | $CH_3$ |
| β-*I* | $CH_3$ | H | $CH_3$ |
| γ-*I* | H | $CH_3$ | $CH_3$ |



| | X | Y | Z |
|---|---|---|---|
| *II* | H | HH | HH |
| *III* | H | $CH_3$ | $CH_3$ |
| *IV* | HO | H | H |



| | X | Y | W |
|---|---|---|---|
| *V* | H | H | H |
| α-*VI* | $CH_3$ | $CH_3$ | $CH_3$ |
| β-*VI* | $CH_3$ | H | $CH_3$ |
| γ-*VI* | H | $CH_3$ | $CH_3$ |

TABLE I

Percentage of the Total 70 ev Ion Current ($\%\Sigma_{25}$) for the Principal Ions in the Spectra of Tocopherols[a]

| | Per cent ionization of | | |
|---|---|---|---|
| m/e | α-*I* | β-*I* | γ-*I* |
| 430 | 40.9 | | |
| 416 | | 30.6 | 41.0 |
| 205 | 2.2 | | |
| 165 | 16.0 | | |
| 164 | 5.3 | | |
| 191 | | 3.3 | 3.0 |
| 151 | | 12.0 | 18.5 |
| 150 | | 6.3 | 4.1 |
| 137 | 0.5 | | |
| 123 | | 0.6 | 0.5 |

[a]Per cent total ionization.

[1]Taken in part from the Ph.D. thesis of Charles J. Rudolph, Jr.

[2]Journal article No. 2335 of the Oklahoma Agricultural Experiment Station.

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TABLE II

Ion Intensity Ration, $I(416)/I(151)$ in the Spectrum of γ-Tocopherol as a Function of Instrument Parameters

| Ion source, C | Molecule separator,[a] C | Direct probe, C | Ratio $I(416)/I(151)$[b] |
|---|---|---|---|
| 310 | 250 | | 0.44±0.05 |
| 310 | | 23 | 0.75±0.01 |
| 265 | 250 | | 0.56±0.02 |
| 265 | | 23 | 1.05±0.02 |
| 230 | | 23 | 1.31±0.02 |

[a]Gas liquid chromatography column: 1% OV-1; temperature 200 C; injector 250-270 C. He flow 36 mm/min.; retention time 35 min.

[b]Average of two determinations.

## EXPERIMENTAL PROCEDURES

### Mass Spectra

Samples of α-, β-, γ-tocopherols and the α- and β-tocopherol acetates were obtained from Pierce Chemical Co. Rockford, Ill. α-Tocopherol was from Calbiochem, Los Angeles, Calif., and γ-tocopherol was obtained from Distillation Products Industries Division, Eastman Organic Chemicals, Rochester, N.Y. β-Tocopherol was prepared by hydrolysis of the β-tocopherol acetate.

Low resolution mass spectra were obtained with the LKB 9000 by introducing samples via either the direct probe or gas liquid chromatography (GLC) column (see Table II, footnote a). Spectra were recorded with an electron beam energy of 70 ev and a trap current of 65 μa. The spectra reproduced in Figures 1-3 were recorded with an ion-source temperature of ca. 230 C and at ambient probe temperature. High resolution data were obtained from Purdue University.

Since the shape of the flat-topped metastable peaks in the spectra of the five compounds being studied was observed to depend upon the electron-multiplier focusing, each metastable peak was scanned at several electromultiplier focus settings. Measurements were made at accelerating voltages of 3.5 and 2.5 kv. The kinetic energies were calculated from the widths of the metastable peaks using the published procedure (6).

## RESULTS AND DISCUSSION

Mass spectra of α-, β, and γ-*I* obtained by introducing samples into the ion source via the direct probe and corrected for naturally occurring heavy isotopes of carbon, hydrogen and oxygen (7) are shown in in Figures 1,2 and 3, respectively. Samples were also introduced as an effluent from a GLC column. The percentages of the total 70 ev ion current for the principal ions in the three spectra are listed in Table I. The spectra are characterized by the absence of numerous fragmentation reactions. The molecular ion which constitutes the base peak in each spectrum accounts for ca. 31-41% of the total ion current. Similarly the molecular ion constitutes the base peak in the spectra of chroman (5), 4- and 6-chromanol (5), and the methyl ethers of 6- and 8-chromanols (5). In contrast the molecular ion (a) constitutes 42% of the base peak in the spectrum of 2,2-dimethylchroman (5) and (b) varies in intensity from ca. 50-90% (relative abundance) in the spectra of α-, β-, and γ-*VI* (6).

The present spectrum of α-*I* and the one published by Nair and Luna (3) differs significantly with respect to several ion intensities. First, Nair and Luna report *M*-1 and *M*-2 ion intensities of ca. 36 and 71% of *M*, whereas we observe intensities of only 0.9 and 2.0. Second, in their spectrum the intensity at m/e 165 is ca. 200% of *M* compared to our value of 39.1. Although it is impossible to compare the conditions under which these spectra were recorded, we have observed that instrumental factors are of crucial importance in determining the intensities at m/e 165 or 151 relative to the intensity at *M*. For γ-*I* the data in Table II reveal that the ratio of ion intensities at m/e 416 and m/e 151 (a) increases with decreasing ion-source temperature and (b) for a given ion source temperature is lower when introduced via the GLC column–molecular separators than via the direct probe. At 230 C the ratio is 1.3, which is not in excellent agreement with the value obtained from Figure 3. This difference appears at least in part to reflect the conditions of the ion source, i.e., the source was cleaned prior to recording the spectrum in Figures 1-3 but not in collecting the data in Table II. These results would indicate that thermal excitation influences the extent of fragmentation but not the fragmentation processes.

The appropriate metastable transitions

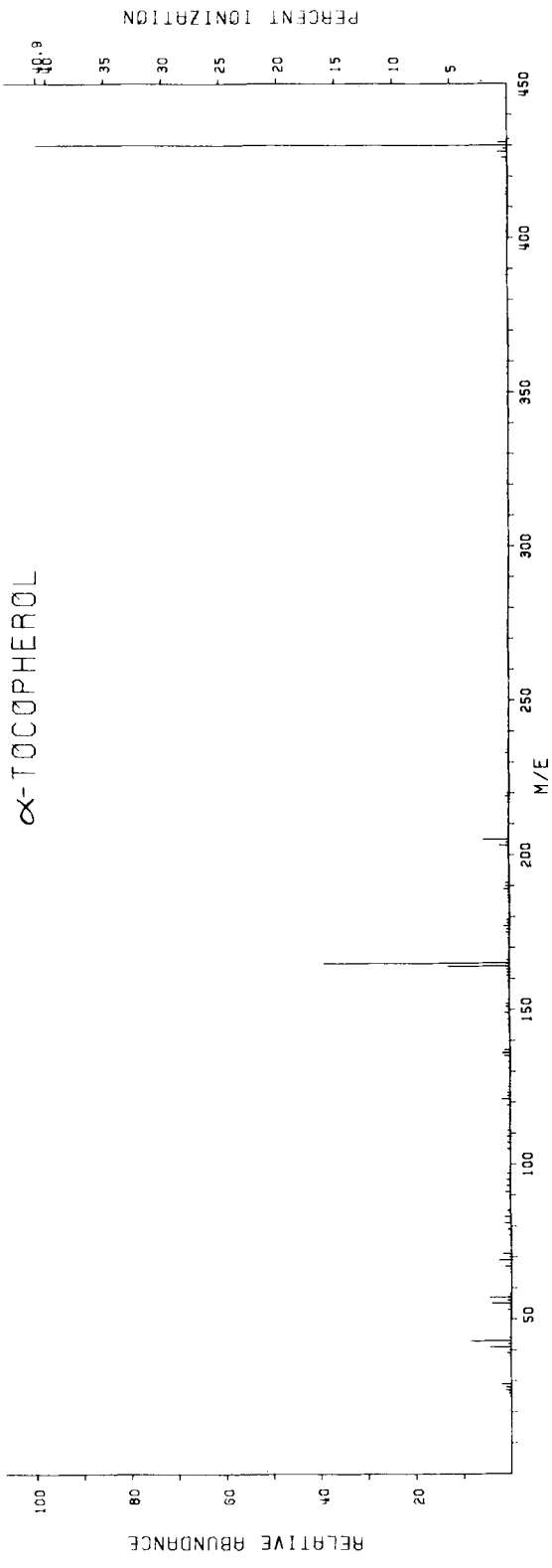



FIG. 1. Mass spectrum (70 ev) of α-tocopherol.

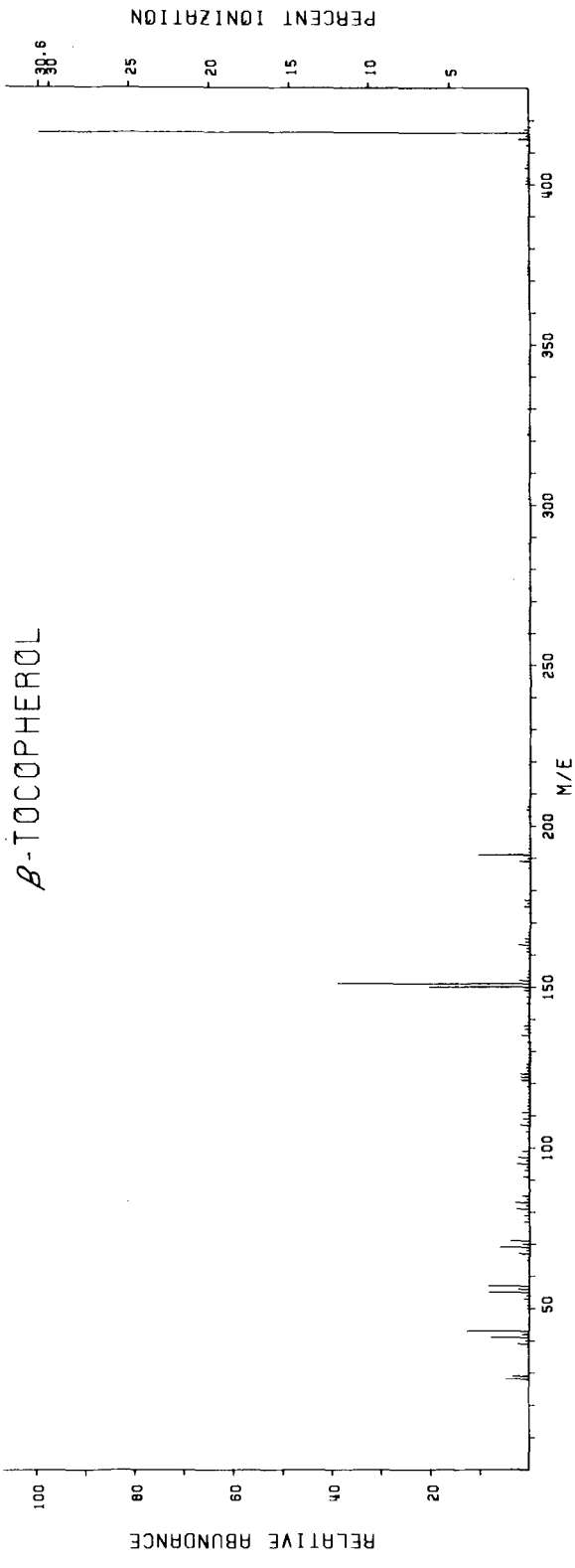



FIG. 2. Mass spectrum (70 ev) of β-tocopherol.

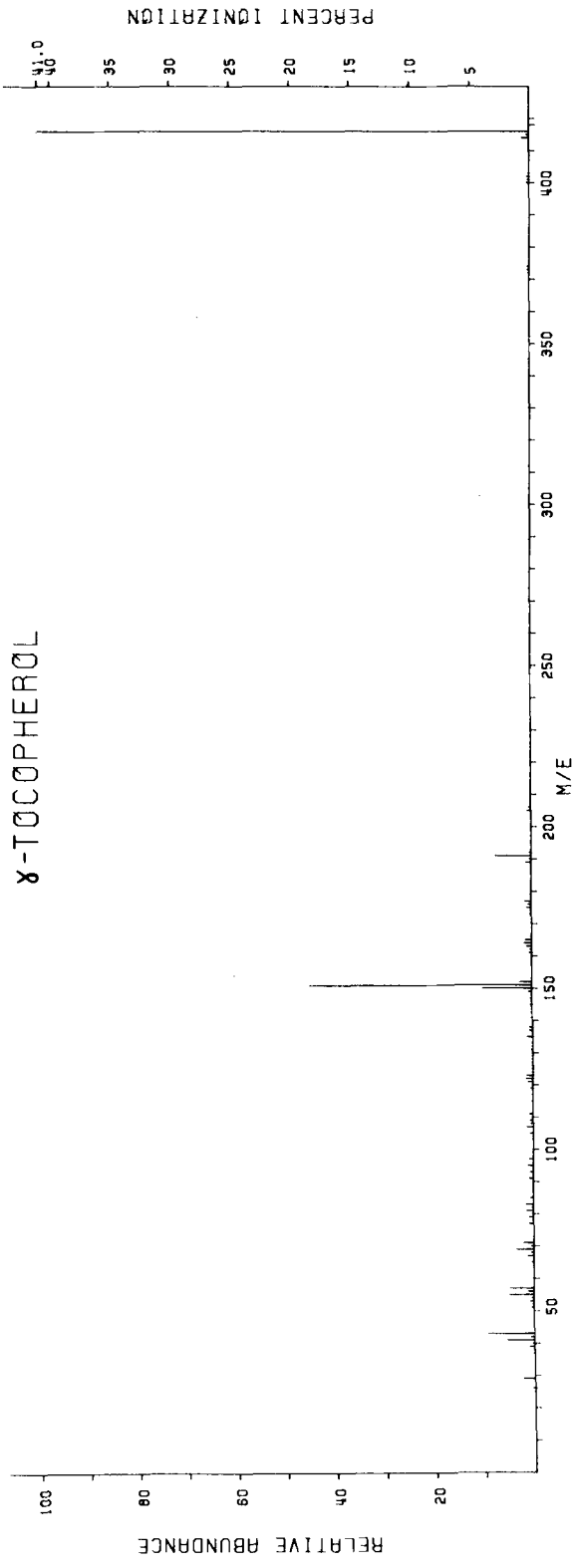


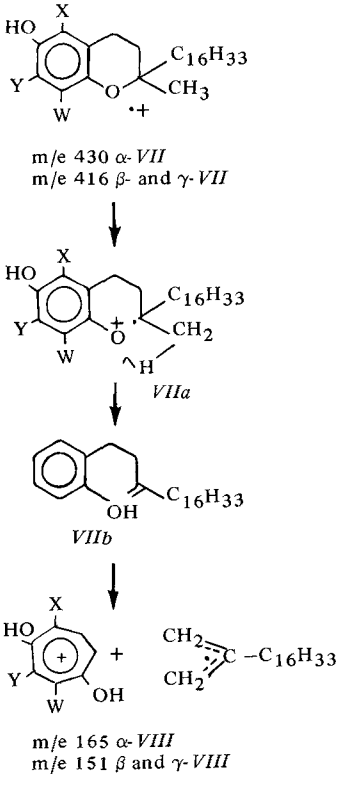

FIG. 3. Mass spectrum (70 ev) of γ-tocopherol.

TABLE III

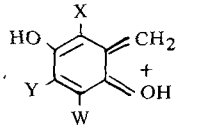
Metastable Transitions in the Mass Spectra of α-, β- and γ-Tocopherols

| Tocopherol | Transition | $M^*$ |
|---|---|---|
| α | $430^+ \rightarrow 205^+ + 225$ | 97.7 |
| | $430^+ \rightarrow 165^+ + 265$ | 63.4 |
| | $430^+ \rightarrow 164^+ + 266$ | 62.6 |
| | $165^+ \rightarrow 137^+ + 28$ | 113.8 |
| β | $416^+ \rightarrow 191^+ + 205$ | 87.7 |
| | $416^+ \rightarrow 151^+ + 265$ | 54.8 |
| | $416^+ \rightarrow 150^+ + 266$ | 54.1 |
| | $151^+ \rightarrow 123^+ + 28$ | 100.2 |
| γ | $416^+ \rightarrow 191^+ + 205$ | 87.7 |
| | $416^+ \rightarrow 151^+ + 265$ | 54.8 |
| | $416^+ \rightarrow 150^+ + 266$ | 54.1 |
| | $151^+ \rightarrow 123^+ + 28$ | 100.2 |

(Table II) and the elemental compositions of the ions at m/e 165 ($C_{10}H_{13}O_2$) and m/e 151 ($C_9H_{11}O_2$) in the spectra of α-, β- and γ-*I* confirm that all molecular ions fragment with loss of a $C_{19}H_{37}$ radical to form m/e 165, 151 and 151, respectively. The percent ionization of the daughter ion in each spectrum, i.e., 16.0, 12.0 and 18.5, confirms this as the principal fragmentation process. This process involves cleavage through the nonaromatic ring of the chromanol moiety. A mechanism is outlined in Scheme 1. Phenomenologically the partial



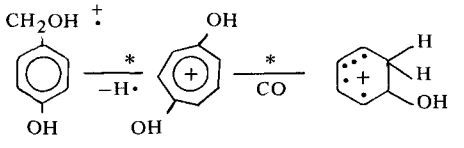

cation radical character of oxygen in the heterocyclic ring of the molecular ions α-, β- and γ-*VII* can be viewed as providing the driving force for rupture of the O–C-1 bond producing α-, β- and γ-*VIIa*. A 1,8-hydrogen transfer from methyl to oxygen leads to the cation radical *VIIa*. Homolytic cleavage of the original C-3–C-4 bond of *VII* in *VIIa* results in formation of the even-electron ions α-(m/e 165), β-(m/e 151) and γ-(m/e 151) *VIII*. Alternatively, the rupture of the O-C bond, transfer of the methyl hydrogen, and dissociation of the C-C bond could be synchronous processes. An alternate structure for the 165 and 151 ions is *VIIIa*. The tropylium ion structure (*VIIIa*) is



*VIIIa*

favored over the *o*-quinonoid one (*VIII*) for the ion formed in the corresponding fragmentation in the spectra of *II* and *III* (5) and *V* and *VI* (6).

Kinetic energies of fragmentation (8) have been utilized in an attempt to determine the structure of the 165 ion and 151 ions. Although not intense processes (see Table I), the appropriate metastable transitions (Table III) and the elemental composition of each daughter ion reveal that m/e 165 and m/e 151 in the spectra of α-*I* and β- and γ-*I* lose CO. Furthermore each metastable peak is flat-topped. Evidence (9) indicates that the *M*-1 ion in the spectrum of *p*-hydroxybenzyl alcohol possesses a tropylium ion structure (Scheme 2).



*IX*, m/e 124 *X*, m/e 23 m/e 95

The metastable peak (9) for loss of CO from m/e 123 (*X*) producing m/e 96 was found to be flat-topped. Ion *X* is structurally related to α-, β- and γ-*VIII*. We reason that equivalent kinetic energies of fragmentation for CO loss from α-, β- and γ-*VIII* and from *X* would indicate the same basic structure for each parent and daughter ion. The measured kinetic energies are listed in Table IV. As a calibration the kinetic energy release for loss of NO from the molecular ion of *o*-nitrophenol (*XI*) was determined. Our average value, 0.77 ev, is in good agreement with the value of 0.76 ev reported by Beynon et al. (8). The agreement between the values of

TABLE IV

Kinetic Energies

| Compound | Transition | Accelerating voltage, kv | Kinetic energy, ev[a] |
|---|---|---|---|
| *XI* | $139^+ \rightarrow 109^+ + 30$ | 3.5 | 0.79±0.02[b] |
| | | 2.5 | 0.75±0.02[c] |
| *α-I* | $165^+ \rightarrow 137^+ + 28$ | 3.5 | 0.22±0.03[d] |
| *β-I* | $151^+ \rightarrow 123^+ + 28$ | 3.5 | 0.21±0.03[c] |
| *γ-I* | $151^+ \rightarrow 123^+ + 28$ | 3.5 | 0.23±0.02[c] |
| | | 2.5 | 0.23±0.02[c] |
| *IX* | $123^+ \rightarrow 95^+ + 28$ | 3.5 | 0.21±0.02[e] |

[a]Deviations are average deviations.
[b]Average of 10 determinations.
[c]Average of four determinations.
[d]Average of 12 determinations.
[e]Average of five determinations.

the kinetic energy release for CO loss from *γ-VIII* measured at accelerating voltages of 3.5 and 2.5 kv is excellent, i.e., 0.23 ± 0.02 and 0.23 ± 0.01. The kinetic energies for CO loss from *α-*, *β-* and *γ-VIII* are seen to be (a) identical within the limits of experimental precision and (b) equivalent to the value for CO loss from *X*. We thus conclude, other factors being equal, that these data provide quantitative evidence favoring the tropylium ion structure (*VIII*) for m/e 165 and 151.

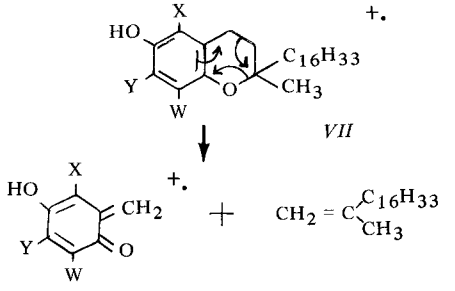
Transfer of a hydrogen from C-1 of the isoprenoid side chain of the various *VII* to oxygen constitutes a reasonable variant to the mechanism illustrated in Scheme 1. The salient requirement of the mechanism is the presence of a alkyl group at C-2 possessing a hydrogen at C-1 of this moiety. The loss of a vinyl radical (5) ($C_2H_3\cdot$) from the molecular ions of *II* and *III*, although a low-intensity process, suggests the existence of a second pathway. Transfer of hydrogen from C-3 concomitant with rupture of the O–C-2 and C-3–C-4 bonds in each *VII* would produce the various homologs of *VIII* and a vinylic radical *XII*. The differences in

$$\cdot CH = \overset{CH_3}{\overset{|}{C}}\text{-}C_{16}H_{33}$$

*XII*

stability between isomeric allylic and vinylic radicals would indicate that the extent of cleavage through the heterocyclic ring (with hydrogen transfer) via this pathway should be minimal compared to that via Scheme 1 or its variant. The dramatic increase in this mode of molecular ion decomposition upon replacement of the C-2 hydrogen atoms in *II* with methyl (5) groups supports this conclusion.

The appropriate metastable transitions and the elemental composition at m/e 164 ($C_{10}H_{12}O_2$) and at m/e 150 ($C_9H_{10}O_2$) confirm that cleavage through the nonaromatic rings of the various homologs of *VII* also proceeds without hydrogen transfer (Scheme 3). The molecular ions of *V* and the tocopherol model compounds (6) fragment analogously with loss of $C_4H_8$. Interestingly this mode of fragmentation is not observed in the spectra of 2,2-dimethylchroman (5), i.e., cleavage of the heteroring proceeds only with hydrogen transfer and loss of the $C_4H_7$ radical. Thus the dynamics for this mode of fragmentation (Scheme 3) may be critically dependent




m/e 164 *α-XIII*
m/e 159 *β-* and *γ-XIII*

upon the presence of a hydroxyl group at C-6.

The absence of the appropriate metastable peaks at least suggest that m/e 164 and 150 are not formed by hydrogen atom loss from m/e 165 and 151, respectively. Such a pathway would require the usually unfavorable formation of an odd-electron ion from an even-electron ion (10).

*α*-Cleavage involving loss of the isoprenoid side chain accounts for 2-3% of the 70 ev ion current in the tocopherol mass spectra (see Scheme 4). The alternate mode of *α*-cleavage is

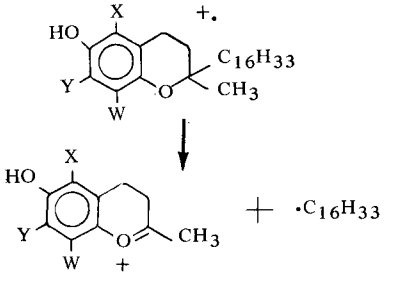
TABLE V

Partial Spectra of Tocopherol Acetates

| | α-Isomer | | β-Isomer | |
|---|---|---|---|---|
| m/e | RA[a] | %Σ[b] | RA[a] | %Σ[b] |
| 472 | 21.9 | 9.5 | | |
| 458 | | | 21.2 | 9.5 |
| 430 | 100.0 | 43.2 | | |
| 416 | | | 100.0 | 44.6 |
| 207 | 7.4 | 3.2 | | |
| 205 | 2.6 | 1.1 | | |
| 193 | | | 5.6 | 2.5 |
| 191 | | | 4.4 | 2.0 |
| 165 | 21.8 | 9.4 | | |
| 164 | 7.9 | 3.4 | | |
| 151 | | | 15.2 | 6.8 |
| 150 | | | 8.6 | 3.9 |

[a]Relative abundance.

[b]Per cent total ionization.




a negligible process, i.e., in the spectra of α-, β- and γ-*I* per cent ionizations for the *M*-15 ions are 0.13, 0.15 and 0.12, respectively. The preferential loss of the $C_{16}H_{33}$ radical follows the normal pattern of α-cleavage (11).

The data in Table V reveal that the loss of ketene from the molecular ion constitutes the dominant fragmentation pathway in the acetate spectra. The similarity of these spectra with those of α- and β-*I* indicates that ketene loss results in formation of α- and β-*VII*. The elemental compositions of the ion at m/e 207 ($C_{12}H_{15}O_3$) and at m/e 193 ($C_{11}H_{13}O_3$) in the spectra of the α- and β-acetates indicates these molecular ions undergo a fragmentation exactly analogous to the one for the free tocopherols given in Scheme 1. Clearly the acetic esters would be suitable derivatives for mass spectral identification of tocopherols.

ACKNOWLEDGMENTS

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# Cholesterol Metabolism in Aorta and in Tissue Culture

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## EDITOR'S INTRODUCTION

As *Lipids* readers know, it is now a policy to consider for publication a limited number of submitted suitable reviews on appropriate subjects while still maintaining this journal's principal function, the publication of papers describing new and original research. When the foregoing policy was adopted, the *Lipids* Advisory Board recommended also that the Editor invite an occasional review from an authority on a timely subject.

To give, insofar as possible, a free hand to the writer of an invited review, he is encouraged to exercise his own judgment in preparing his material in a manner that he believes will be best for the reader. Also, he is given considerable leeway concerning the length of his review, the choice of the portion of a subject matter area to be covered and other points.

The author of the following invited review, Dr. David Kritchevsky, has long been known to lipid scientists, especially for his work and publications concerning cholesterol and its metabolism.

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In the absence of an unequivocal antemortem diagnosis for a myocardial infarction, studies in the field of atherosclerosis research have generally been based upon the exploitation of observed chemical or physiological differences. The ensuing discussion will focus on aortic chemistry and metabolism, an area of research which has received little attention until recently. The official World Health Organization definition of atherosclerosis (1) is: "Atherosclerosis is a variable combination of changes of the intima of arteries (as distinct from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes."

Smith (2) has summarized the differences in mucopolysaccharides (MPS), collagen and lipid between normal human aortas and aortas with different stages of atherosclerosis. As can be seen from Table I, the change of greatest magnitude between the normal and calcified plaque is found in the lipid fraction.

Early studies of the lipids of human plasma and plaques suggested a similarity in composition which seemed to support the Virchow hypothesis that lipid accumulation in the aorta was a result of filtration of serum components by the vascular bed. Table II summarizes the average values obtained in studies carried out 20 years apart (3-6). However studies dating back to Windaus in 1910 (7) had indicated an increase in the cholesteryl ester content of atherosclerotic human aortas. Böttcher (8), in a study of lipids of human aortas, showed that the greatest increase between the ages of 6-56 was in the ester cholesterol fraction. Between the ages of 6-56, the percentage of lipids as dry weight of aorta rose by 344%; among the various lipid classes the phospholipids increased by 145%; free fatty acids fell by 33%; triglycerides rose by 150%, cholesterol by 900% and cholesteryl ester by 5800%. In experimental atherosclerosis, similar changes have been observed. The ratio of free to ester cholesterol in the normal rabbit aorta ranges between 10-40. Aortas from cholesterol-fed rabbits exhibit a free to ester cholesterol ratio ranging from 0.4-1.0 (9-11). Even in atherosclerosis caused by a cholesterol-free diet, the ratio falls to 2 (12). Newman and Zilversmit (13) fed rabbits an atherogenic diet for 120 days and in that time measured aortic levels of phospholipid, triglyceride, free cholesterol and ester choles-

TABLE I

Mucopolysaccharide, Collagen and Lipid in Human Aortas[a]

| Component | Normal, mg | Fatty streak | | Fibrous plaque | | Calcified plaque | |
|---|---|---|---|---|---|---|---|
| | | mg | % Normal | mg | % Normal | mg | % Normal |
| Mucopolysaccharide | 2.6 | 3.0 | 115 | 2.5 | 96 | 1.2 | 46 |
| Collagen | 24.8 | 26.3 | 106 | 41.0 | 166 | 60.7 | 285 |
| Lipid | 9.8 | 30.6 | 312 | 47.3 | 483 | 109.0 | 1112 |

[a]After Smith (2).

TABLE II

Lipids of Human Plasmas and Plaques[a]

| Lipid | Plaques | Plasma |
|---|---|---|
| Free cholesterol | 19.3 | 13.1 |
| Ester cholesterol | 37.6 | 37.2 |
| Total cholesterol | 56.9 | 50.3 |
| Phospholipid | 18.5 | 22.4 |
| Neutral fat | 20.6 | 26.7 |

[a]Percentage of total lipids.

TABLE III

Tissue Reaction to Lipid Implants[a]
(Macrophage, Collagen, Fibroblast Persistence)

| Compound | Score (maximum = 85) |
|---|---|
| Cholesteryl oleate | 85 |
| Cholesteryl *trans-trans* linoleate | 85 |
| Cholesteryl linoleate | 45 |
| Cholesteryl palmitate | 45 |
| Cholesteryl arachidonate | 21 |
| Cholesterol | 70 |
| Linoleic acid | 56 |
| Linolenic acid | 49 |
| Oleic acid | 18 |
| Triolein | 14 |
| Tristearin | 9 |
| Lecithin (saturated) | 2 |
| Lecithin (unsaturated) | 0 |

[a]Abdulla et al. (20).

TABLE IV

Sterol Content of L5178Y Cells

| | Dry wt, μg/mg | | | |
|---|---|---|---|---|
| Sera | Free (C) | Ester (CE) | Total | CE/C |
| Horse | 6.1 | 10.0 | 16.3 | 1.7 |
| Fetal bovine | 6.4 | 5.1 | 11.5 | 0.8 |
| Calf | 5.7 | 15.5 | 21.2 | 2.7 |

terol. By 60 days levels of phospholipid had increased by 300%, triglyceride by 33%, free cholesterol by 1900% and ester cholesterol by 9000%. At 120 days these lipids had risen by 1300%, 83%, 2650% and 10,000%, respectively.

With the advent of gas liquid chromatography, the fatty acid spectra of aortic and serum cholesteryl esters were compared. The major difference lies in the ratio of oleic to linoleic acids. In the serum the ratio of 18:1 to 18:2 is ca. 0.64 and in the plaques it is 0.94 (14). Smith (2), in a study of normal and diseased areas of human arteries, showed that the ratio of free to ester cholesterol was always higher in the normal areas, and the 18:1 to 18:2 ratio was lower no matter how advanced the lesion. Recent studies from several laboratories have shown that there is an increased rate of fatty acid synthesis during atherogenesis and that most of the newly synthesized fatty acid is esterified to cholesterol. It has been estimated that as much as half of the accumulated aortic cholesteryl esters may be attributable to local synthesis (15-19). St. Clair et al. (19) have recently found that, when pigeons are fed an atherogenic diet, an increase in cholesterol esterification can be observed in the aorta before any lesions can be demonstrated. Since the aorta cannot synthesize linoleic acid, the increase in oleic-linoleic acid ratio in the cholesteryl ester fraction of aorta is understandable.

In an effort to learn if any specific lipids were more irritating to normal tissue than others, Abdulla et al. (20) implanted a number of lipids in the abdominal wall of rats and, after a given time interval, assayed macrophage response, collagen response, fibroblastic activity, and persistence. Based on their grading scheme, scores were assigned to the various compounds. The highest possible score, 85, was obtained with cholesteryl oleate, cholesteryl palmitoleate

TABLE V

Hydrolysis of Cholesteryl-4-$^{14}$C Esters by Growing Tissue Culture Cells

| | Cholesteryl ester, % recovered as free cholesterol | | | | | |
|---|---|---|---|---|---|---|
| | L-Cells | | | L5178Y Cells | | |
| | Fetal calf serum, 7.5%[a] | | Horse serum, 7.5%[a] | Fetal calf serum, 5%[a] | Horse serum, 5%[a] | |
| Fraction analyzed | Acetate | Oleate | Oleate | Oleate | Oleate | Acetate |
| Culture supernatant | 1 | 2 | 1 | 2 | 1 | 1 |
| Uninoculated medium | 1 | 2 | 1 | 1 | 1 | 1 |
| Cells | 58.8 (44.0-68.9) | 66.3 (55.3-79.0) | 48.1 (32.5-63.7) | 10.3 (5.0-17.7) | 6.4 (1.8-12.1) | 11.9 (5.7-16.6) |

[a]Heat inactivated, 60 C/30 min.

TABLE VI

Aortic Lipolysis or Esterolysis[a]

| Species | Substrate | |
|---|---|---|
| | Lipemic serum | β-Naphthyl acetate |
| Rat | 1.00 | 1.00 |
| Rabbit | 0.20 | 0.35 |
| Guinea pig | 0.40 | --- |
| Cockerel | 0.40 | 0.50 |

[a]Zemplenyi et al. (22).

and cholesteryl *trans-trans* linoleate. Unsaturated lecithin gave a score of zero. Some of the 29 lipids studied are shown in Table III.

Some years ago we began to study cholesterol metabolism in tissue culture cells in hopes of developing a model system which would help us to understand aortic metabolism. We used a stable line of lymphoblasts derived from a murine leukemia, commonly designated as L5178Y. We observed (21) that the free cholesterol content of the cells remained constant regardless of the source of serum used to prepare the medium, but the cholesteryl ester content rose when more lipemic sera were used (Table IV). A survey of the ratio of ester to free cholesterol of a number of cell lines showed a range between 0 for (MBIII cells) and 0.8 for L5178Y. None of the other cell lines had an ester to free cholesterol ratio above 0.4. We compared cholesteryl ester metabolism in L5178Y and L cells, in which the ratio of ester to free cholesterol ratio ranges from 0.15-0.31. We found that the two cell lines took up about the same amount of steryl ester from rabbit serum (0.50 μg/mg dry wt/hr). The two cell lines synthesized little steryl ester; hence the only possible reason for accumulation of steryl ester in L5178Y would be an inability to hydrolyze ester. As can be seen from Table V, this was indeed the case. The L cell was capable of hydrolyzing more than half the steryl ester which it had incorporated, whereas the L5178Y cells could only hydrolyze ca. 10%.

TABLE VII

Comparison of Cholesteryl Ester Hydrolase and Synthetase Activity

| Species | Specific activity | | |
|---|---|---|---|
| | Hydrolase | Synthetase | Hydrolase-synthetase |
| Rat | 176 | 63 | 2.6 |
| Rabbit | 55 | 99 | 0.6 |

The possibility exists then that certain aortas (species-specific or genetically determined within species) lack the ability to hydrolyze sterol esters. Zemplenyi et al. (22) compared lipolysis and esterolysis by aortic homogenates from several species and found that hydrolytic potential was lower in aortas of susceptible species. Using lipemic serum or β-naphthyl acetate as substrates, they obtained the results given in Table VI.

We have also recently investigated the activity of cholesteryl ester hydrolase present in acetone powder preparations of aortas (23). We found that this preparation had both ester synthesizing (S) and hydrolyzing (H) activity. The preferred substrates were an emulsion for synthesis and a micellar preparation for hydrolysis. In Table VII we show that both rat and rabbit aorta contain each enzymic activity, but the ratio of hydrolysis to synthesis is much higher in the resistant rat than in the susceptible rabbit. Comparison of cholesteryl ester hydrolytic to synthetic activity in the aortas of Show Racer pigeons (resistant) to those of White Carneau pigeons (susceptible) shows a similar difference. The H/S ratio in the Show Racer is 1.37, compared to 0.82 for the White Carneau.

The role which phospholipids may play in cholesterol metabolism should be mentioned in passing. Böttcher (8) found that with increasing

TABLE VIII

Inhibition of Free Cholesterol Uptake by L5178Y Cells: Influence of Phospholipids

| Phospholipid | Free Cholesterol uptake, % | |
|---|---|---|
| | Phospholipid/ml, 5 μg | Phospholipid/ml, 25 μg |
| Lysolecithin | 114.4 | 92.5 |
| Phosphatidyl serine | 93.9 | 84.8 |
| Phosphatidyl ethanolamine | 82.5 | 65.6 |
| Mixed rabbit serum phospholipids | 84.6 | 42.4 |
| Lecithin | 84.2 | 34.1 |
| Sphingomyelin | 59.8 | 17.6 |

[a]Based upon uptake of cholesterol by control system containing no phospholipid.

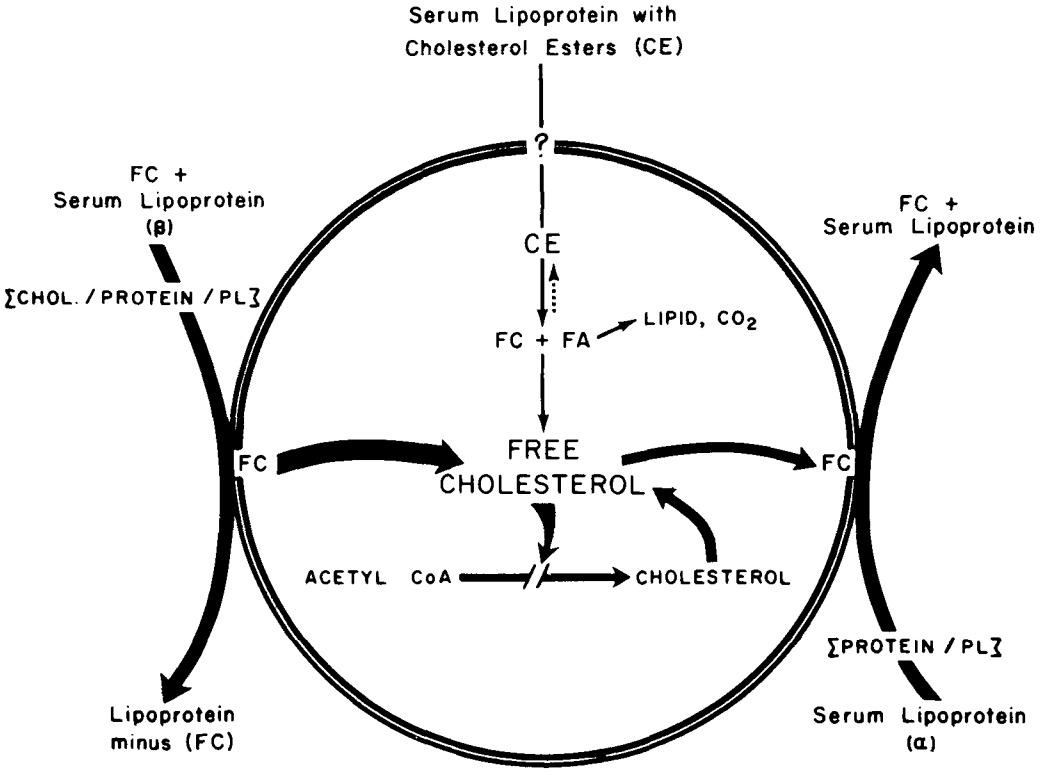


FIG. 1.

severity of human atherosclerosis the percentage of cephalins and lecithins in the aorta fell by 50% and 47%, respectively, whereas sphingomyelin content rose by 80%. In a study of factors which affect uptake of cholesterol by L5178Y cells, we examined the effects of free fatty acids, glycerides and phospholipids (24). The cells were exposed to the test lipid and cholesterol which had been added to delipidized serum.

At a level of 10 μg/ml, all the fatty acids used stimulated uptake of cholesterol: 110% for linoleic, 120% for oleic and 135% for palmitic and stearic acids. At levels of 25 μg/ml, all fatty acids caused cell lysis. At the level of 20 μg/ml, triolein inhibited cholesterol uptake by 10%. The effect of various phospholipids is shown in Table VIII. It is evident that even at 5 μg/ml, sphingomyelin inhibits cholesterol uptake. The role which sphingomyelin plays in cellular metabolism of cholesterol and its esters is not clear.

A scheme for cellular metabolism of cholesterol is shown in Figure 1. This scheme was originally presented in a review (25), but has been refined to its present form by Rothblat (26). How much of this metabolic hypothesis is valid for the L5178Y cell or for the aorta is not yet known, but it can explain a number of published observations. Only further experimentation along several lines—tissue culture cells and aortic metabolism—will tell us if we are on the right track.

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# Lipid Metabolism During Cold-Exposure and During Cold-Acclimation[1,2]

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## ABSTRACT

The lipid-containing tissues are important in cold-exposure (exposure to cold of animals not previously living in the cold) and in cold-acclimation (the adaptive state achieved when animals have lived in the cold for several weeks); these are the white adipose tissue and the brown adipose tissue. The white adipose tissue serves as a store of readily mobilized substrate (free fatty acids [FFA]) for calorigenesis in other tissues during cold-exposure, principally for shivering thermogenesis in muscle. The mobilization of the stored lipid is brought about through activation of the sympathetic nervous system by the cold stress. The brown adipose tissue has two functions in cold-exposure and in cold-adaptation, both quite distinct from the function of the white adipose tissue. These functions are heat production and the maintenance of the adaptation to cold. The triglycerides stored in the brown adipose tissue are mobilized as FFA, also via activation of the sympathetic nervous system, but the FFA are used primarily within the brown adipose tissue itself. The FFA are the agents which switch on the calorigenesis in the brown adipose tissue (via a poorly understood form of "loosening" of the coupling of oxidative phosphorylation); they also serve as the substrate for the calorigenesis. The heat-producing function of the brown adipose tissue occurs in both cold-exposed and in cold-acclimated animals; it is of greater importance in the latter because this tissue normally grows in response to cold. Much of the heat production in cold-acclimated animals (nonshivering thermogenesis) occurs outside the brown adipose tissue itself, most probably in the muscles, and the cold-acclimated animal differs from the cold-exposed animal in being able to switch on nonshivering thermogenesis via activation of the sympathetic nervous system. The maintenance of this adaptation for nonshivering thermogenesis in tissues other than the brown adipose tissue itself depends upon the brown adipose tissue. The adaptation disappears if the brown adipose tissue is removed; the adaptation does not develop if the normal proliferation of mitochondria in the growing brown adipose tissue is inhibited (with oxytetracycline) during acclimation of rats to cold. The mechanism by which the brown adipose tissue exerts this second function is at present unknown. An increased turnover of certain mitochondrial proteins occurs in those tissues (skeletal muscle and brown adipose tissue) in which nonshivering thermogenesis occurs in cold-acclimated rats; no change in turnover of mitochondrial proteins occurs in other tissues (liver and kidney). The relation of this alteration in mitochondrial proteins to the adaptation for nonshivering thermogenesis is at present unknown. However this first demonstration of a biochemical difference between skeletal muscle of cold-acclimated rats and skeletal muscle of warm-acclimated rats opens up a new approach to the study of the nature of both the adaptation for nonshivering thermogenesis and of the role of the brown adipose tissue in the development and maintenance of this adaptation.

## INTRODUCTION

The homeothermic animal exposed to cold is faced with the problem of a rapid loss of heat and a consequent lowering of body temperature. However exposure to cold brings into play a variety of regulatory mechanisms which contribute to the maintenance of a normal body temperature. These regulatory mechanisms may be classified into two main categories: those which reduce heat loss and those which increase heat production. The former category includes piloerection and peripheral vasoconstriction, responses which are both mediated by the activation of the sympathetic nervous system

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produced by the exposure to cold. The latter category of regulatory mechanisms includes the increase in metabolic rate produced by shivering thermogenesis and the increase in metabolic rate produced by means other than shivering or other muscle movements, usually referred to as nonshivering thermogenesis. This review is concerned chiefly with the role of lipid metabolism, and more particularly with the roles of the two lipid-storing tissues, the white adipose tissue and the brown adipose tissue, in the regulatory mechanisms for increasing heat production in the cold. Three distinct roles for these tissues will be described: (a) provision of substrate to support the increased metabolic rate (white adipose tissue and brown adipose tissue); (b) heat production (brown adipose tissue); (c) maintenance of the adaptive state necessary for an animal to be able to use nonshivering thermogenesis (brown adipose tissue). These three roles are not all exercised simultaneously: role (a) is important principally during acute cold-exposure whereas roles (b) and (c) are of greatest importance during long periods of exposure to cold when the animal can be considered to be cold-acclimated.

It is necessary at this point to define some of the terms which will be used in this discussion. Cold-exposure means the fairly brief (hours or up to 2 days) exposure of an animal to a temperature lower than that to which it has been accustomed. For a rat living at usual laboratory temperature (25-28 C) this temperature may be as high as 10 C or as low as -20 C; a temperature of 4 C is commonly used. For a newborn rat living in the nest with its mother a temperature of 30 C may represent cold-exposure. Even a cold-acclimated rat (see below) living at 4 C may be subjected to a more severe cold-exposure such as exposure to a temperature of -20 C. Cold-exposure is, therefore, exposure to a temperature sufficiently low to call forth regulatory mechanisms to maintain body temperature in addition to those already in use. If an animal is exposed to cold (above freezing) for periods of more than 3-4 weeks it adapts to living in the cold and maintaining a high metabolic rate. The principal change in the adapted or cold-acclimated animal is the replacement of shivering thermogenesis by nonshivering thermogenesis. The cold-acclimated animal has undergone various adaptive adjustments during this acclimation period in which the replacement of shivering thermogenesis by nonshivering thermogenesis has occurred; this adaptive process and its resulting adaptive state are referred to as cold-acclimation. The species which adapts most readily in this way is the rat, and this species has been the most extensively studied. For this reason this discussion will consider principally the adaptive changes that occur in the rat during acclimation to cold.

The nature and mechanism of shivering thermogenesis can fairly readily be described; the metabolic processes involved in the increase in heat production due to shivering are the same as those involved in the increase in heat production during exercise. In contrast, the biochemical nature of nonshivering thermogenesis is not understood. In order to follow this discussion of the possible nature of nonshivering thermogenesis there are two characteristics of this process which must be understood. The first of these is the adaptive nature of nonshivering theremogenesis. It occurs in certain species when they are cold-acclimated; it does not occur to any appreciable extent in these same species when they are not cold-acclimated. Nonshivering thermogenesis also occurs in certain newborn species and in hibernating mammals; it does not occur in these same species when they are adult or when they are not hibernating. Thus there exists an adaptation for nonshivering thermogenesis. The second characteristic of nonshivering thermogenesis is of importance in considering what the biochemical nature of this adaptation might be. Nonshivering thermogenesis is a facultative process. It does not occur continuously in the cold-acclimated animal or other animal possessing the necessary adaptation, but it can be switched on and off in accordance with the need for the heat it produces. The switching on and off of nonshivering thermogenesis is controlled by the sympathetic nervous system. Indeed the sympathetic nervous system plays a major role in the overall response of an animal to cold-exposure. It brings into operation the mechanisms for decreasing heat loss, for mobilizing the extra substrate needed to support the increased metabolic rate and, in cold-acclimated animals in particular, for switching on the process of nonshivering thermogenesis. An additional role for the sympathetic nervous system is in the development of the adaptation for nonshivering thermogenesis during acclimation to cold.

This discussion will concentrate first on changes in metabolism, particularly in lipid metabolism, occurring in response to cold-exposure and on the role of the white adipose tissue in shivering thermogenesis. The nature of nonshivering thermogenesis will then be discussed, together with the roles of the brown adipose tissue in this process. A comprehensive review on the effect of cold on the metabolic use of lipid was presented fairly recently by Masoro (1). The older literature will not,

therefore, be reviewed again in detail here.

## SHIVERING THERMOGENESIS

Shivering involves the normal mechanism of muscle contraction, and its effect of raising heat production is much like that of light exercise (2). The rate of oxygen utilization (and substrate utilization) by muscle mitochondria is normally controlled by the supply of ADP, and the utilization of more ATP for the muscle contraction of shivering results in an increased availability of ADP which causes accelerated oxidative phosphorylation and electron transport, increased oxygen uptake and increased utilization of substrates. Shivering itself is under the control of the central nervous sytem (2,3), but the increased supply of substrate which is produced simultaneously with the need for it is under the control of the sympathetic nervous system, just as in the case of exercise. That the sympathetic nervous system is activated by cold-exposure is indicated by the large increase in the excretion in the urine of noradrenaline, adrenaline and their metabolites, normetanephrine, metanephrine, 3-methoxy-4-hydroxyphenylglycol and 3-methoxy-4-hydroxymandelic acid; both sympathetic nerve endings and adrenal medulla are stimulated to release noradrenaline and adrenaline, respectively, by the cold stimulus (4).

The principal, but not the only, substrate utilized during cold-exposure is lipid. The concentration of FFA in the blood rises during cold-exposure (5-10). There is accelerated lipolysis in the white adipose tissue (7,8,11,12) and activation of the white adipose tissue lipase (7). The oxidation of plasma FFA is increased (13), as is also their turnover (13), and the store of triglyceride in the white adipose tissue decreases (14,15). The triglyceride and diglyceride content of skeletal muscle also decreases (16), but the triglyceride and diglyceride content of liver does not change appreciably (16,17) unless the animals are also starved, when it increases markedly (17). The FFA content of the liver increases, whereas that of the muscle does not change (16). The most marked change in plasma lipids, apart from the increase in FFA concentration noted above, is a decrease in the triglyceride content, particularly of the triglyceride of the very low density lipoproteins (18); the decrease occurs rapidly but recovery to normal levels after return to room temperature is slow. This change resembles the rapid and prolonged decrease in plasma triglyceride, particularly in the very low density lipoprotein fraction, that occurs during exercise (19,20). The reason for the reduction in very low density lipoproteins is not clear. An increased utilization seems likely, in view of the increased activity of the lipoprotein lipase observed in the heart during cold-exposure (21) and during exercise (22,23), and the increased lipolytic activity of muscle observed during cold-exposure (24). A decreased production of very low density lipoproteins by the liver is also possible (25).

Lipid is not however the sole substrate used to support the increase in metabolic rate during exposure to cold. The concentration of glucose in the blood also rises during cold-exposure, at least in fed rats (6,9,10,26). An increased oxidation and turnover of glucose occurs in fed rats (26,27) and also in fasting rats (28) in which little or no rise in glucose concentration occurs in response to cold-exposure (28). The proportion of respiratory carbon dioxide derived from glucose oxidation is in fact the same whether the rat is exposed to cold or not (27). Although glycogenolysis in the liver accounts for some of the increased glucose production, there is in addition a marked increase in gluconeogenesis in rats exposed to cold (29,30). Protein and amino acid catabolism are increased, judging from the increase in blood urea concentration (30,31).

Although a large increase in lipid mobilization and utilization does occur to contribute to the support of shivering thermogenesis, such an increase is not actually an essential part of the initial response to cold. Antilipolytic agents (nicotinic acid[31]; 3',5'-dimethylpyrazole [32]) prevent partially or completely the rise in plasma FFA concentration but do not impair maintenance of body temperature or survival in the cold. In fact rats are quite adaptable with regard to the substrate used, since they also survive without the usual mobilization of glucose. Thus adrenodemedullated rats do not become hyperglycemic on cold-exposure (6,9,33) and survive in the cold despite impaired regulation of their blood glucose concentration (33); the mobilization of lipid as FFA is not impaired by adrenodemedullation (6,7,9,34). Procedures which block both the mobilization of lipid and the mobilization of carbohydrate do impair survival in the cold; examples of such procedures are adrenalectomy (34,35) and ganglionic blockade (6,7,9).

The resemblance of the increase in metabolic rate due to shivering to the increase in metabolic rate due to light or moderate exercise is quite striking. The mechanism of the increase in metabolic rate of the muscles is similar in these two states of increased activity, and the two do not summate during exposure to cold, exercise replacing shivering as a mechanism of increasing

heat production (36). During exercise there is increased utilization of both carbohydrate and lipid substrates (37) but, as with shivering, rats are quite adaptable with regard to the substrate used during exercise. Adrenodemedullated rats can exercise despite impaired regulation of their blood glucose concentration (38,39); the mobilization of lipid as FFA is not impaired by this procedure. In man, impairment of lipid mobilization with nicotinic acid does not impair the capacity for exercise (40). Procedures which block mobilization of both lipid and carbohydrate do impair the capacity for exercise; examples of such procedures are adrenalectomy (38) and adrenodemedullation together with treatment with adrenergic neurone blocking agents (38) or together with treatment with a ganglionic blocking agent (39).

In summary, the mobilization of lipid from the white adipose tissue occurs simultaneously with the need for increased utilization of substrate imposed by shivering and supplies that need. The mobilization of lipid is not essential for shivering to occur, but it does normally supply a large part of the required substrate.

## NONSHIVERING THERMOGENESIS

Although there is such a similarity between shivering and exercise in the nature of the process responsible for the increase in metabolic rate and in the mechanism and nature of the increase in substrate supply, there is marked difference between them when they are continued for prolonged periods. The adaptive changes which occur during training on the one hand and during cold-acclimation on the other are entirely different. During repeated exercise a number of adaptive changes occur which permit a greater capacity for exercise; the animal is said to be trained. However during continuous (or repeated) exposure to cold there is no improvement in the capacity for shivering; on the contrary the capacity for shivering is progressively reduced, and the production of the extra heat needed to maintain body temperature is taken over by another mechanism, nonshivering thermogenesis (41-43). The capacity to increase metabolic rate in response to cold is indeed improved in the cold-acclimated animal, not by an improvement in the process involved in the warm-acclimated animals but by a replacement of that process by a different one. The adaptive changes of training, therefore, although they render an animal better able to exercise and even better able to shiver, do not produce the adaptive state necessary for nonshivering thermogenesis to occur (44).

There is still no satifactory biochemical explanation for nonshivering thermogenesis despite intensive investigation of the nature of this process during the last 15 years. There is considerable evidence that the adaptation for nonshivering thermogenesis is associated with a very marked enhancement of the calorigenic response to noradrenaline and to adrenaline (45-49), and that it is the noradrenaline liberated from the sympathetic nerve endings which is responsible for the switching on and off of nonshivering thermogenesis in the cold-acclimated rat (45,50,51).

The increased noradrenaline secretion during acclimation to cold is probably itself responsible for the development of the enhanced response to noradrenaline: treatment of rats with noradrenaline for long periods (3-6 weeks) results in an enhancement of the calorigenic response to noradrenaline in the treated rats (52-54). The nature of the calorigenic response to noradrenaline is itself not understood in any detail, and the mechanism of the remarkable enhancement of the calorigenic response to noradrenaline which occurs in the cold-acclimated rat is not understood at all.

The site of nonshivering thermogenesis would appear to be principally skeletal muscles and brown adipose tissue. Calorigenic responses to cold (55) and to noradrenaline (55,56) have been demonstrated to occur in skeletal muscle of cold-acclimated rats. The evidence that the brown adipose tissue can produce heat during nonshivering thermogenesis is substantial and will not be reviewed in detail here; the reader is referred to reviews by Smith and Horwitz (57) and by Chaffee and Roberts (58) for this topic. The brown adipose tissue undergoes marked changes during acclimation to cold. There is growth of the tissue (57), and not only are there more cells but more mitochondria per cell and more cristae per mitochondrion (59-62). Thus the capacity of the brown adipose tissue to produce heat (due to increase in size and increase in the concentration within the cell of the heat-producing organelles) increases during acclimation to cold. A special function for the heat-producing capacity of the interscapular and associated brown adipose tissue has recently become apparent. Smith and Roberts (63) first drew attention to the special location of this tissue and the direction of its heat specifically to the spinal cord and thoracic organs. Brück and coworkers (64,65) have demonstrated the presence in the spinal cord of receptors that control shivering and have suggested that the warming of this region by the heat coming from the interscapular brown adipose tissue is responsible for the suppression of shivering at the

time when the animal is raising its heat production by nonshivering thermogenesis. The growth of the brown adipose tissue during cold-acclimation parallels the progressive suppression of shivering during this same period. It should be noted here that brown adipose tissue is also capable of increased heat production even in animals that are not cold-acclimated (66-68), but this is presumably not great enough to suppress shivering.

Tissues other than the skeletal muscles and the brown adipose tissues do not appear to be of great importance as sites of nonshivering thermogenesis. That the liver and other viscera are not essential is indicated by the persistence in the functionally eviscerated cold-acclimated rat of the enhanced calorigenic response to cold (69) and of the enhanced calorigenic response to noradrenaline (46).

The substrate mixture mobilized and utilized to support nonshivering thermogenesis appears to be much the same as the mixture used to support shivering thermogenesis. Cold-acclimated rats living in the cold and producing heat by nonshivering thermogenesis have the same pattern of glucose metabolism (26,27) and of FFA metabolizm (13) as do warm-acclimated rats exposed to cold for the first time and producing heat by shivering thermogenesis. The concentration of FFA in their blood is the same (10) or lower (13) than that of warm-acclimated rats at room temperature. The concentration of glucose in their blood is also the same (10,26) or less (70) than that of warm-acclimated rats at room temperature, although the turnover is greater (26). Much of the extra glucose produced can come from gluconeogenesis since a similar increase in glucose turnover and oxidation is seen in fasting cold-acclimated rats in which the contribution of liver glycogen would be minimal (28). Increased gluconeogenesis also occurs in the fed cold-acclimated rat living in the cold (29). There is normally an increased intake of all foodstuffs by the cold-acclimated rat living in the cold, and this also increases the supply of substrates to support the increase in metabolic rate.

The concentration of triglyceride in the very low density lipoprotein fraction of the plasma lipoproteins decreases within one day of exposure to cold and remains at a low level in the cold-acclimated rat (18); this probably represents an increased rate of utilization not quite balanced by an increased rate of production. A decreased transport of triglyceride in the very low density lipoproteins has been reported (25), but since the cold-acclimated rats studied in these experiments were at room temperature they were probably not actually using nonshivering thermogenesis when the measurements were made. There are adaptive changes in the amount of the enzyme responsible for utilizing the triglyceride of the very low density lipoproteins, lipoprotein lipase, which suggest that the triglyceride is directed to different sites of utilization in the cold-acclimated rat. The lipoprotein lipase activity of the white adipose tissue decreases and the lipoprotein lipase activities of brown adipose tissue and cardiac muscle increase within 1 day of exposure to cold (21,71); little or no change occurs in liver (71). The changes persist in the cold-acclimated rat (71), and in addition an increase in the lipolytic activity of skeletal muscle has also been observed in rats intermittently exposed to cold for 2 weeks (24). Thus it seems likely that the triglyceride of the very low density lipoproteins is directed as an energy source to those tissues involved in nonshivering thermogenesis, brown adipose tissue and muscle, and is withheld from the tissue which would normally store the lipid, the white adipose tissue. Similar adaptive changes occur in fasting (23) and are believed to be brought about chiefly by the reduced availability of insulin. Although there is a reduction in plasma insulin concentration in cold-acclimated rats (70), it is probable that this contributes only in part to the changes in lipoprotein lipase activity which occur in the cold-acclimated rat. The administration of insulin to rats exposed to cold for 2 days completely reverses the reduction in white adipose tissue lipoprotein lipase activity, and the increase in heart lipoprotein lipase activity but only slightly inhibits the increase in brown adipose tissue lipoprotein lipase activity (71). Administration of noradrenaline or adrenaline to warm-acclimated rats induces an increase in the lipoprotein lipase activity of heart (71,72) and of brown adipose tissue (71). Thus most probably the changes induced by cold are attributable to reduced availability of insulin in the white adipose tissue, to both reduced availability of insulin and increased availability of noradrenaline in the heart and to increased release of noradrenaline in brown adipose tissue.

There appear to be other adaptive changes in the white adipose tissue of cold-acclimated rats. The lipolytic response to noradrenaline is enhanced in this tissue (15,73-76). Noradrenaline-induced lipolysis represents a complex sequence of events subsequent to the initial stimulation by noradrenaline of both $\alpha$ and $\beta$-receptors in the plasma membrane, a stimulation which results in a change in adenyl cyclase activity ($\alpha$-receptor stimulation decreases and $\beta$-receptor stimulation increases adenyl cyclase activity;

the latter usually predominates over the former) (77). The specific activity of noradrenaline-stimulated adenyl cyclase in white adipose tissue is increased in cold-acclimated rats (78), but since the white adipocytes of these animals appear to be smaller but more numerous (15,79) this presumably represents a greater amount of plasma membrane per unit weight of tissue or of tissue protein, rather than any specific change in the composition of the plasma membrane itself.

Changes in metabolic processes involving lipids also occur in brown adipose tissue of cold-acclimated rats. In intact cold-acclimated rats living in the cold there is a very marked enhancement of the incorporation of glucose carbon into glyceride-glycerol (10,80,81). The enhancement is associated with nonshivering thermogenesis itself rather than the adaptation for nonshivering thermogenesis, because it is not seen in cold-acclimated rats when they are at room temperature (10) nor does it occur to any appreciable extent in warm-acclimated rats when they are exposed to cold (10,82). It has, however, proved impossible to demonstrate this enhancement in vitro. Slices of brown adipose tissue from cold-acclimated rats do incorporate more glucose carbon into glyceride-glycerol in vitro, but the incorporation is not stimulated by noradrenaline (80,83-85) as would be expected from the observations made with the intact animal. It seems likely that brown adipose tissue slices, particularly those from tissue of cold-acclimated rats, may be altered in such a way that they are no longer susceptible to stimulation. High rates of lipolysis occur in slices of brown adipose tissue of cold-acclimated rats and noradrenaline does not stimulate lipolysis in these slices (86). The lack of sensitivity to noradrenaline cannot be attributed to any change in the amount of noradrenaline-sensitive adenyl cyclase system since that is normal (87). It seems more likely that the cells of the brown adipose tissue of cold-acclimated rats are more readily damaged by the isolation and incubation procedures, and that the processes which occur in vitro do not faithfully reproduce those processes which occur in vivo. In keeping with this suggestion is the difficulty experienced in preparing isolated fat cells from brown adipose tissue of cold-acclimated rats. Procedures which produce metabolically intact cells in good yield from brown adipose tissue of warm-acclimated rats are not suitable for isolation of cells from brown adipose tissue of cold-acclimated rats; most cells disintegrate, and the few which are recovered show inhibited respiration and poor response to noradrenaline (Behrens and Himms-Hagen, unpublished observations).

The increased oxidation of FFA in cold-acclimated rats is associated with altered concentrations of carnitine in tissues. Increases in total carnitine occur in liver, skeletal muscle and heart (88)—the increases being due to increases in the acetylcarnitine fraction in heart and muscle and to increases in all three carnitine fractions (free carnitine, acetylcarnitine and acylcarnitine) in liver. An increased turnover and a large increase in body pool size of carnitine in cold-acclimated rats has also been reported (89), but the rats were at room temperature during the 8 days when the turnover was measured, and the relationship of this finding to nonshivering thermogenesis is uncertain; moreover there is some doubt as to the specificity of the methods used for the estimation of the carnitine (88). Brown adipose tissue normally contains a fairly high concentration of carnitine (90,91), but no measurements of the carnitine content of brown adipose tissue of cold-acclimated rats have been reported.

With the idea that accelerated fatty acid oxidation must be an essential part of nonshivering thermogenesis and that an adaptation might involve increased availability of carnitine, Delisle and Radomski (88) studied the effect of treatment of warm-acclimated rats with carnitine on their resistance to cold. The treatment did not alter basal oxygen uptake nor oxygen uptake in the cold and in fact impaired the survival of the rats in extreme cold (-20 C). On the other hand the calorigenic effect of small doses but not of large doses of noradrenaline is enhanced by carnitine in warm-acclimated rats (92).

The suggestion that raised concentrations of FFA in the plasma might be responsible for the effect of noradrenaline to increase the metabolic rate in cold-acclimated rats was originally made ca. 12 years ago by Masoro (93), and this idea is still brought up occasionally (79) despite the now considerable evidence that the plasma FFA cannot be considered to be the agent responsible for the increase in metabolic rate (94,95). Two major pieces of evidence against this idea are: (a) the normal or less than normal concentration of FFA in the plasma of cold-acclimated rats living in the cold (10,13) and (b) the negative correlation between plasma FFA concentration and oxygen uptake in cold-acclimated rats receiving an infusion of noradrenaline at room temperature (96).

The mechanism of nonshivering thermogenesis can at present be described only to a limited extent in biochemical terms. Nonshivering thermogenesis is an exaggerated calorigenic response to noradrenaline, occurring principally in brown adipose tissue and skeletal muscle of

cold-acclimated rats; the mechanism by which the metabolic rate is elevated is still in doubt. Several attempts to find metabolic differences between liver mitochondria of cold-acclimated rats and warm-acclimated rats were made some years ago, and considerable excitement was produced by observations of reduced P/O ratios and partial uncoupling of oxidative phosphorylation in liver mitochondria from cold-acclimated rats (97,98). However it was subsequently found that this uncoupling occurred during the isolation of the mitochondria and was due to another component of the homogenate (99); well-washed mitochondria did not exhibit the uncoupling (99-102). Liver mitochondria from cold-acclimated rats were also observed to be more sensitive to aging (100,103) and to the swelling induced by thyroxine (104). The physiological significance of these observations has never been elucidated, and it is now apparent that there are two criticisms of this older work; first, one would not expect that permanent uncoupling of mitochondria could account for the high metabolic rate in the cold of the cold-acclimated rat, because such permanent uncoupling should produce a permanent increase in metabolic rate and could not, therefore, account for nonshivering thermogenesis which can be rapidly switched on and off; second, the liver is not a major site of nonshivering thermogenesis, and an adaptive change of the mitochondria of liver should not, therefore, be responsible for the adaptation for nonshivering thermogenesis. The obvious tissues in which to look for mitochondrial alterations responsible for nonshivering thermogenesis are the brown adipose tissue and skeletal muscle. Indeed the mitochondria of brown adipose tissue of cold-acclimated guinea pigs do appear to become progressively more loosely coupled during brief periods of adaptation to cold and to revert slowly to normal when the animals are returned to room temperature (105); the loose coupling is characterized by normal P/O ratios and a high rate of respiration that is little influenced by ADP, i.e., the respiratory control ratio is reduced. These mitochondria appear to be substrate-controlled rather than ADP-controlled in the cold-acclimated state. Whether mitochondria from brown adipose tissue and muscle of cold-acclimated rats become more loosely coupled in this way is unknown. Despite intensive investigations into the properties of the mitochondria of brown adipose tissue during the last few years (49,57,106), relatively few studies have involved cold-acclimated rats and in no study have their mitochondria been compared with the mitochondria of warm-acclimated rats. It does seem to be fairly well established that the FFA liberated in the brown adipose tissue by the lipolytic effect of noradrenaline are responsible for inducing the "loosely coupled" state (49,106-108).

In addition to its heat-producing function brown adipose tissue has another different role to play in cold-acclimation in rats. This role, which is that of promoting and maintaining the altered adaptive state in other tissues of cold-acclimated rats, first became apparent in experiments in which the interscapular brown adipose tissue was removed surgically in order to obtain an estimate of the quantitative contribution of this part of the total brown adipose tissue (about one-third of the total brown adipose tissue in the body of the cold-acclimated rat is in the interscapular region) to the increase in oxygen uptake occurring in the intact animal during infusion of noradrenaline or while in the cold. Four days after removal of the interscapular brown adipose tissue the enhancement of the calorigenic response to noradrenaline was indeed reduced sufficiently to suggest that as much as 40% of the enhanced oxygen uptake might be occurring in the interscapular brown adipose tissue (48,49,109). However this appeared to be an impossible estimate when the size of the tissue removed and the size of the reduction in oxygen uptake were compared; in order to consume that amount of oxygen the interscapular brown adipose tissue would have had to have received more than half of the cardiac output (49). It became clear that this was an impossible estimate when it was found that immediately after the removal of the interscapular brown adipose tissue from a cold-acclimated rat, there is actually no change at all in the enhancement of the calorigenic response to noradrenaline (48,49). The loss of the enhanced response occurs progressively during the 4 days following removal of the interscapular brown adipose tissue (48). This progressive nature of the loss of the enhanced response proves that the enhanced consumption of oxygen does not occur in the interscapular brown adipose tissue itself. Why then should the enhanced response disappear after the interscapular brown adipose tissue is removed? The most likely explanation is that the interscapular brown adipose tissue is influencing in some way the capacity of other tissues, presumably skeletal muscle, to respond calorigenically to noradrenaline. This implies a secretion of some factor by the interscapular brown adipose tissue which acts upon other tissues, i.e., an endocrine function of this organ. The observation, that replacement of the interscapular brown adipose tissue fragments in the peritoneal cavity

of the rats from which it was removed partially prevented the loss of the enhanced response to noradrenaline, supported this postulated endocrine function of the brown adipose tissue (48). However it has not been possible to prevent the loss of the enhancement by administration of extracts of the brown adipose tissue. Thus the brown adipose tissue has a role in the maintenance of the adaptation for nonshivering thermogenesis. Does it have a similar role in the development of the adaptation during acclimation to cold? The classical approach of observing the changes which follow removal of an endocrine organ is not possible in the case of the interscapular brown adipose tissue of rats during acclimation to cold, because the hypertrophy of brown adipose tissue elsewhere in the body compensates for the loss of the interscapular brown adipose tissue (110). Another experimental approach was therefore sought which would allow partial or complete suppression of the functioning of the brown adipose tissue during acclimation to cold.

The approach chosen was the treatment of rats with oxytetracycline during acclimation to cold. The rationale behind this approach, chosen to suppress the functioning of the brown adipose tissue in rats undergoing acclimation to cold, was as follows: there is a marked proliferation of mitochondria and of mitochondrial inner membrane in the cells of the brown adipose tissue during acclimation to cold (59-62); mitochondria are known to be able to synthesize some of their own proteins, particularly those of the structural proteins of the inner membrane (111,112); the mammalian mitochondrial protein synthesizing system is like that of bacteria rather than that of the mammalian cell cytoplasm and is inhibited by certain antibiotics which do not affect the cytoplasmic system (113,114). The approach was therefore to inhibit, in the brown adipose tissue of rats undergoing acclimation to cold, the marked proliferation of mitochondria and of mitochondrial inner membrane by treatment with the antibiotic oxytetracycline, a known inhibitor of mammalian mitochondrial protein synthesis (113,114). The administration of oxytetracycline to rats during acclimation to cold for 2 weeks did indeed not only inhibit the proliferation of mitochondria and of mitochondrial inner membrane in the brown adipose tissue, as judged by the lack of the usual increase in specific activity of cytochrome oxidase, a marker for inner membrane, but also inhibited the development of the enhanced calorigenic response to noradrenaline (115). The growth of the rats and the growth of the brown adipose tissue were not affected by the treatment with oxytetracycline, and the metabolic rate of the oxytetracycline-treated rats while they were exposed to cold were at the usual high level observed in control rats, the treated rats apparently using shivering thermogenesis. No changes in specific activity of the cytochrome oxidase of skeletal muscle or of liver were detected in these experiments. Thus it can be concluded that the development of the adaptation for nonshivering thermogenesis during acclimation to cold requires the synthesis of protein by the mitochondria of the brown adipose tissue.

Not only can the development of the adaptation be inhibited by treatment with oxytettracycline, but the maintenance of the adaptation can be prevented by this treatment. Treatment with oxytetracycline of rats already acclimated to cold for 2 weeks reverses the enhanced calorigenic response to noradrenaline and the increased specific activity of cytochrome oxidase in the brown adipose tissue (110). The effects of oxytetracycline are rapidly reversible; when the treatment is stopped the enhancement of the response to noradrenaline and the increase in specific activity of the cytochrome oxidase are rapidly restored to the normal high level. It can be concluded that the synthesis of proteins by the mitochondria of the brown adipose tissue is essential not only for the development but also for the maintenance of the adaptation for nonshivering thermogenesis.

During 4 weeks of treatment with oxytetracycline it is possible to detect a reduction in the specific activity of cytochrome oxidase in muscle and liver; thus oxytetracycline is not a specific inhibitor for the synthesis of proteins by the mitochondria of brown adipose tissue, which would not indeed be expected. However it does appear that its effect is most apparent in the tissue in which the mitochondrial protein synthesis is proceeding most rapidly, namely the brown adipose tissue (110). For this reason this experimental approach does not yield further information about the postulated endocrine role of the interscapular brown adipose tissue, but it does nevertheless open up an entirely new experimental approach to the nature of the adaptation.

Another indication that mitochondrial protein synthesis is accelerated in cold-acclimated rats comes from studies of the half life of mitochondrial proteins. The half lives of various mitochondrial protein of warm-acclimated rats have been measured by a number of different workers (see Table I). Most of the information pertains to the liver mitochondria and relatively little information is available about heart, brain and kidney mitochondria; the only observations

TABLE I

Half Lives (in Days) of Some Mitochondrial Proteins in Warm-Acclimated and Cold-Acclimated Rats

| | | | | Soluble proteins | | | Insoluble proteins | | | "Pure" proteins | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tissue | Rat | Label | Total | Total | $H_2O$ Soluble | Contractile | Total | Structural | Cyto chrome | Cyto chrome[c] | Haem | References |
| Liver | Warm | Arg (guanido) | 5.0 | | | | | | | | | 128 |
| | | | 6.8 | | | | | | | | | 129 |
| | | | 4 - 6 | | 4.2-5.1 | 4.6-5.3 | | 4.3 - 5.3 | 3.9 - 4.8 | 4.5 - 5.0 | | 130 |
| | | | 4.0 | | | | | | | | | 131 |
| | | Arg-U-$^{14}$C | 9.0 | | | | | | | | | 129 |
| | | Lys | | | | | | | | 10.5 | | 132 |
| | | | | | | | | | | 28 | | 133 |
| | | Leu | 7.0 | | | | | | | | | 128 |
| | | | 10.3 | 10.8 | | | 10.1 | | | 9.7 | | 134 |
| | | | 8.4 | | 8.6 | 8.4 | | 8.8 | 8.2 | 8.6 | | 135 |
| | | | | | 8.2 | 6.9 | | 10.2 | | | | 136 |
| | | | 7.0[a] | | 7.5[a] | 6.0[a] | | 6.0[a] | 5.5[a] | 6.0[a] | | 116 |
| | | Acetate | 9.2 | | | | | | | | | 117 |
| | | Acetate | 6.0[a] | | | | | | | | | 118 |
| | | Met-35S ALA[b] | | | | | 9.0 | | | 5.5 | 5.6 | 137 |
| | | | | | | | | | | 6.1 | | 128 |
| | Cold | Leu | 7.8[a] | | 7.5[a] | 6.5[a] | | 6.5[a] | 5.3[a] | 6.0[a] | | 138 |
| | | Acetate | 7.6[a] | | | | | | | | | 116 |
| Kidney | Warm | Lys | | | | | | | | 11.5 | | 118 |
| | | Leu | 8.6 | | 6.0 | 7.6 | | 9.0 | 8.4 | | | 132 |
| | | | 7.5[a] | | 5.5[a] | 11[a] | | 9.5[a] | 10[a] | 7[a] | | 135 |
| | Cold | Leu | 6.8[a] | | 5.3[a] | 9.0[a] | | 8.8[a] | 7.3[a] | 6.8[a] | | 116 |
| Brain | Warm | Leu | 26.3 | | 17.9 | | | 31.4 | | | | 116 |
| | | | | | | | | | | | | 135 |
| Heart | Warm | Arg (guanido) | 6.2 | | | | | | | | | 128 |
| | | | 5.2 | | | | | | | | | 131 |
| | | Lys | | | | | | | | 43 | | 132 |
| | | Leu | 6.1 | | | | | | | | | 128 |
| | | ALA | | | | | | | | 5.8 | 5.9 | 128 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Skeletal muscle | Warm | Lys | | | | | | | 32 | | 132 |
| | | Leu | 22.5[c] | | 15[a] | 21[d] | 31[d] | 21[a] | | | 116 |
| | | Acetate | | | | | | | 13.8[a] | | 119 |
| | Cold | Leu | 11.5[c] | | 11.3[a] | 8.3[d] | 13.5[d] | 17.5[a] | | | 116 |
| | | Acetate | | | | | | | 14.8[a] | | 119 |
| Brown adipose tissue | Warm | Leu | 12[d] | | 12[a] | | 12[c] | 9[a] | | | 116 |
| | Cold | Leu | 7.4[d] | | 8.8[a] | | 7.3[c] | 12[a] | | | 116 |

[a]Difference between warm-acclimated rats and cold-acclimated rats is not significant.
[b]ALA = δ-amino levulinic acid.
[c]Difference between warm-acclimated rats and cold-acclimated rats is significant, $P < 0.02$.
[d]Difference between warm-acclimated rats and cold-acclimated rats is significant, $P < 0.01$.

on the skeletal muscle and brown adipose tissue fractions are our own (116). Cold-acclimation has previously been observed not to alter the half lives of liver mitochondrial proteins (117,118); our findings are in agreement with this (116). In addition we find no change in the half lives of kidney mitochondrial proteins. There is however a decreased half life of some groups of proteins (the "structural proteins" and "contractile proteins") of skeletal muscle and of brown adipose tissue in cold-acclimated rats but no change in other proteins; unchanged turnover of cytochrome *c* in muscle has previously been reported (119). Thus by virtue of its location this decrease in the half lives of some mitochondrial proteins can be associated with the occurrence of nonshivering thermogenesis. Does an increased turnover of mitochondrial proteins actually represent the heat-producing process itself, i.e., an increased utilization of ATP, or does it represent the alterations involved in maintaining the adaptive state? It seems most likely that the latter is correct, since inhibition of mitochondrial protein synthesis with oxytetracycline during the infusion of noradrenaline into a cold-acclimated rat does not alter the enhanced response to noradrenaline (Himms-Hagen, unpublished observations).

It is obvious that an increased turnover of mitochondrial lipids might be expected to occur in a coordinated fashion with the increased turnover of some of the mitochondrial proteins (120); nothing is known about turnover of mitochondrial lipids in cold-acclimated rats.

It is of interest that training also induces a change in the morphology and enzymic composition of the mitochondria of heart and skeletal muscle, tissues which have to sustain prolonged periods of elevated metabolic rats during exercise. The heart acquires more mitochondria with more closely packed cristae in trained rats (121-123). Skeletal muscle acquires larger and longer mitochondria with more densely packed cristae (124). There is an increase in the total amount of certain mitochondrial enzymes (cytochrome oxidase, succinoxidase) which parallels the increase in mitochondrial mass (125,126), but all mitochondrial enzymes do not increase in parallel so that the mitochondria of the trained muscle actually have a different enzymic composition from those of untrained muscles (126,127). This adaptive change in the mitochondria of skeletal muscle during training appears to be quite different from the adaptive change which occurs during acclimation to cold; although there is an adaptive change of some kind, as indicated by the reduced half

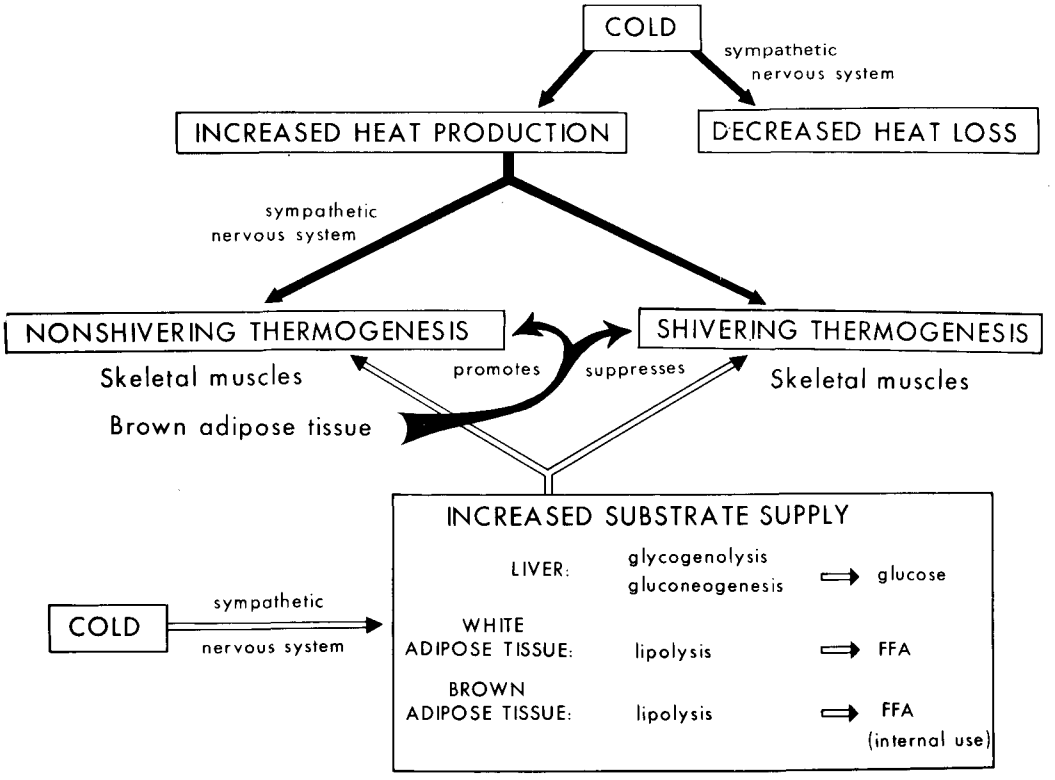


FIG. 1.

lives of some of the mitochondrial proteins, there does not appear to be any change in the mitochondrial mass, as judged by the lack of change in the specific activity of cytochrome oxidase in muscle homogenates of cold-acclimated rats (Himms-Hagen, unpublished observations).

To end this discussion on a note of speculation the following working hypothesis is offered: during acclimation to cold the adaptation responsible for the enhanced calorigenic response to noradrenaline (and for nonshivering thermogenesis) is produced by an altered synthesis of one or several proteins in the mitochondria of two tissues, brown adipose tissue and skeletal muscle, which results in an alternation in the properties of the mitochondria such that they can become "loosely coupled" when stimulated appropriately. This property, already present to some extent in the mitochondria of brown adipose tissue even before acclimation to cold, could be induced by a single stimulus (noradrenaline?) acting upon both tissues simultaneously, or could be induced in skeletal muscle by the postulated factor coming from the brown adipose tissue.

## CONCLUSIONS

The two lipid-storing tissues, white adipose tissue and brown adipose tissue, serve specialized functions during exposure to cold and during acclimation to cold. It is perhaps unfortunate that the names of these two tissues are so similar because their functions are quite different (see Fig. 1).

The principal function of white adipose tissue is to provide at short notice one of the substrates, FFA, needed to support the increased metabolic rate during acute exposure to cold. This function is controlled by the sympathetic nervous system and represents the emergency function of the sympathetic nervous system called forth by the stress of exposure to cold. This aspect of an emergency supply of substrate is less emphasized in the cold-acclimated rat in which there must be a continuously elevated metabolic rate. The white adipose tissue undoubtedly plays an important part in supplying FFA to keep the supply in the plasma at the normal level, but much of the extra substrate required in this new steady state must also come from the extra intake of food.

The brown adipose tissue has two functions, both exercised to the greatest extent during cold-acclimation (see Fig. 1). The first of these functions is the production of heat, a metabolic

process under the control of the sympathetic nervous system. The lipolytic effect of noradrenaline on the brown adipose tissue liberates FFA from the triglyceride stores; these FFA have the dual function of altering the mitochondria so that they become "loosely coupled" and respire at a high rate indenpendently of the supply of ADP, and of providing the substrate to support the elevated metabolic rate in the brown adipose tissue itself. The amount of heat produced by brown adipose tissue is relatively small compared with the total heat production by the cold-acclimated rat living in the cold, but is nevertheless important because of its local distribution, particularly in the thoracic region. The principal function of the heat produced by the brown adipose tissue in this region may be the suppression of shivering via the warming of receptors for shivering in the spinal cord. The heat-producing function of the brown adipose tissue occurs in both cold-exposed and cold-acclimated rats; it is of greater importance in the latter because of the growth of this tissue in response to cold.

The second function of the brown adipose tissue is in the maintenance and probably also in the development of the adaptation that is acquired during acclimation to cold and that permits the enhanced metabolic response to noradrenaline, the basis of nonshivering thermogenesis. It is possible to partially prevent this function of the brown adipose tissue by surgical removal of the interscapular brown adipose tissue, about one-third of the total brown adipose tissue, and to prevent it entirely by inhibiting with the antibiotic oxytetracycline the synthesis of proteins by the mitochondria of this tissue, organelles which normally not only proliferate but also acquire more densely packed cristae during the growth of the tissue induced by prolonged exposure to cold.

Increased turnover of certain proteins of mitochondria normally occurs in both brown adipose tissue and in skeletal muscle of cold-acclimated rats, both tissues in which nonshivering thermogenesis occurs. No change in mitochondrial turnover occurs in liver or kidney of cold-acclimated rats, both tissues in which nonshivering thermogenesis does not occur. It is unknown whether any changes in lipid turnover accompany the changes in protein turnover. The relation of the alteration in mitochondrial proteins to the adaptation for nonshivering thermogenesis is at present unknown. However this first demonstration of a biochemical difference between skeletal muscle of cold-acclimated rats and skeletal muscle of warm-acclimated rats opens up a new approach both to the study of the nature of the adaptation for nonshivering thermogenesis and to the study of the nature of the role of the brown adipose tissue in the development and maintenance of the adaptation.

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# Radical Addition of Linoleic Hydroperoxides to α-Tocopherol or the Analogous Hydroxychroman[1]

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## ABSTRACT

Either linoleic acid hydroperoxide (LOOH) or methyl linoleate hydroperoxide react anaerobically with either α-tocopherol (TOH) or its model compound–2,2,5,7,8-pentamethyl-6-hydroxychroman (COH)–to form principally an addition compound of the two reactants. The reaction can be catalyzed either by 1.28 X $10^{-5}$ M Fe(III) or by proflavin (0.01%) sensitized by visible light. The presence of air in the reaction terminates the addition, and quinones become the major products from TOH or its model compound. The addition compound synthesized from COH and LOOH (a 4.9:1 ratio of 13-hydroperoxy-*cis*-9, *trans*-12-octadecadienoic acid and 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid) was used to solve structural details of the bridging function. Three isomers of the addition compound (methyl esterified) were isolated and identified as methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-12,13-epoxy-*trans*-9-octadecenoate; methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*trans*-12,13-epoxy-*trans*-9-octadecenoate; and methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-9,10-epoxy-*trans*-12-octadecenoate in order of decreasing abundance. The mechanism appears to be free radical addition brought about by the catalytic formation of alkoxy radicals from the hydroperoxide and chromanoxy radicals from TOH or its model.

## INTRODUCTION

That organic peroxides will oxidize α-tocopherol (TOH) has been reported by others; for example, benzoylperoxide oxidizes TOH or its model compound–2,2,5,7,8-pentamethyl-6-hydroxychroman (COH)–to α-tocopherolquinone or the analogous quinone of COH (1), dimer (2) and trimer (3). TOH in the presence of autoxidizing methyl linoleate was oxidized to these products, as well as other more polar products (4). Gruger and Tappel (5) used Fe(III) to catalyze oxidation of TOH by pure lipid hydroperoxides. They obtained data indicating that α-tocopherolquinone was formed through an intermediate.

Aside from oxidation to α-tocopherolquinone, dimer and trimer, TOH is known to form addition products with organic peroxides. Benzoylperoxide adds to the 5-methyl of TOH (2) and COH (1). TOH and linoleic acid react to form an addition compound when adsorbed on silica gel under oxidizing conditions (6). In this addition linoleic acid bridges the 5-methyl and 6-hydroxyl of TOH and forms a new chroman ring. In a similar study (7) methyl linoleate adds under peroxidizing conditions to the 7-methyl of tocored (a TOH oxidation product).

Under anaerobic conditions we observed a radical-initiated reaction between linoleic acid hydroperoxide (LOOH) or methyl linoleate hydroperoxide (MLOOH) and TOH or COH. Homolytic conditions were generated by Fe(III) or proflavin sensitized by visible light. The principal products were isomeric addition compounds. The reaction products between LOOH and COH were used to characterize the details of their structures (Fig.1), which had in common an ether link between the 11-carbon of an epoxyoctadecenoic acid and the 6-carbon of COH. These addition compounds have not been observed in previous research work, possibly because reactions between fatty acid hydroperoxides and TOH or COH in the absence of oxygen have not been reported.

## METHODS

### Hydroperoxides

LOOH was prepared by oxidation of linoleic acid (ca. 0.9 g) with soybean lipoxygenase by a method described previously (8). Oxygenation of the potassium linoleate-lipoxygenase solution was improved by a flow of pure oxygen through fritted glass. Foaming was mitigated by silicone grease. At the completion of the reaction the solution was acidified to pH 5 and then extracted immediately with chloroform-methanol 2:1. The crude LOOH recovered from the extract was purified by column chromatography (9). Traces of oxooctadecadie-

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noic acid, a byproduct which often interfered with a clean separation of LOOH in previous studies, was virtually eliminated by the improved oxygenation of the reaction solution. Nevertheless fractions eluting from the column were assayed for oxooctadecadienoic acid, which absorbs at about 277 nm, so we could be certain that this impurity was not present. Problems associated with autodecomposition of LOOH were minimized by periodic isolations of freshly prepared LOOH. Autodecomposition was never serious enough to affect results significantly.

MLOOH was prepared from crude LOOH with diazomethane. The methyl ester was isolated by thin layer chromatography (TLC).

The authenticity and purity of LOOH and MLOOH were confirmed by IR and UV spectra [$\epsilon_{max}$ = 22,900 to 25,000 at 232 nm in methanol (10)]. Peroxide values (11) of LOOH averaged 6740 meq peroxide per kilogram oil (theoretical, 6400).

Our sample of soybean lipoxygenase oxidized linoleic acid to predominantly 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid as determined by nuclear magnetic resonance (NMR) and mass spectra (MS) (data to be published elsewhere). As indicated by earlier work (8) and by this study, about 17% of the total LOOH was 9-hydroperoxyoctadecadienoic acid with the remainder being the 13-hydroperoxide.

#### Reaction Conditions

The photocatalyzed addition of either LOOH or MLOOH to either TOH (the *d*-isomer, used as received), purchased from Eastman Kodak Co., Rochester, N.Y., or COH, synthesized according to the procedure of Smith et al. (12), was sensitized by 0.01% proflavin, obtained from Aceto Chemical Co., Flushing, N.Y., in methanol. The concentration of the reactants were 3.2 mM of either LOOH or MLOOH, and 6.4 mM of either TOH or COH. Before exposure to light the solution and reaction vessel were thoroughly deaerated with a stream of $N_2$. Deaeration continued throughout the reaction (ca. 6 hr). The apparatus and visible light used for irradiation were described by Grams et al. (13). Aliquots were taken from the reaction at time intervals for assay of the products by TLC.

The Fe(III)-catalyzed addition proceeded in the presence of 1.28 X $10^{-5}$ M $FeCl_3$ (anhydrous) in anhydrous methanol. The concentration of reactants was identical to that used for the proflavin-mediated reaction. The solution was thoroughly purged with $N_2$ before addition of Fe(III), and the purge continued until the reaction was complete (ca. 5 hr). The solution was kept in the dark at room temperature throughout the reaction. At intervals 1 ml samples were assayed for products by TLC after being extracted with 3 ml chloroform and 1 ml aqueous ethylenediaminetetraacetic acid (EDTA) $10^{-2}$ M.

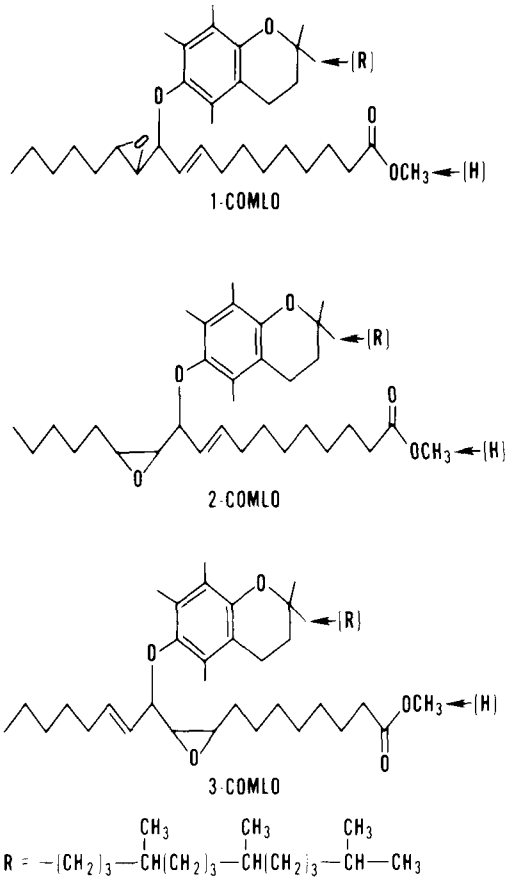



FIG. 1. Structures of methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*trans*-12,13-epoxy-*trans*-9-octadecenoate (1ΣOMLO); methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-12,13-epoxy-*trans*-9-octadecenoate (2-COMLO); and methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-9,10-epoxy-*trans*-12-octadecenoate (3-COMLO). When ($OCH_3$) is replaced with (H), a mixture of these three structures is designated COLO. With (R) replacing ($CH_3$) the mixture is designated TOMLO, and with (H) and (R), TOLO. The isomers of COMLO (1-, 2- and 3-COMLO's) are addition compounds of methyl linoleate hydroperoxide (MLOOH) and 2,2,5,7,8-pentamethyl-6-hydroxychroman (COH). Addition compound(s) (isomers are suspected but not directly demonstrated) of linoleic acid hydroperoxide (LOOH) and COH are designated COLO. TOLO and TOMLO are addition compounds (including possible isomers) of α-tocopherol (TOH) with LOOH and TOH with MLOOH, respectively.

TABLE I

Thin Layer Chromatography of Reactants and Products From Radical Addition Reactions

| | $R_f$ value | | | |
|---|---|---|---|---|
| | Isooctane-ether-acetic acid | | Isooctane-ether | |
| Compounds[a] | 50:50:1 | 60:40:1 | 7:3 | 8:2 |
| COLO | 0.46 | | | |
| LOOH | 0.42 | 0.30 | | |
| COH | 0.61 | | 0.44 | |
| COMLO | | | 0.56[b] | |
| MLOOH | | | 0.37 | 0.27 |
| TOLO | | 0.48[b] | | |
| TOMLO | | | | 0.57[b] |
| TOH | | 0.66 | | 0.44 |

[a]Abbreviations of the compounds are described in Figure 1.

[b]Average $R_f$ value for two to three barely separable spots (isomers).

#### Chromatography

Silicic acid columns were used to isolate LOOH and the addition products. LOOH was eluted from the column by a hexane-ether method (9). The compound COLO formed by addition of LOOH with COH was isolated by the same technique with slight modification of the elution sequence. The mixing chamber was filled with 70 ml hexane, and the replenishing reservoir was filled consecutively with 200 ml 10% ether, 200 ml 20% ether, 250 ml 30% ether, 250 ml 40% ether and 200 ml 50% ether in hexane. COLO methyl esters (COMLO) were separated by an elution sequence of 70 ml hexane in the mixing chamber being replenished from a reservoir filled consecutively with 100 ml hexane, 250 ml 2.5% ether, 250 ml 5% ether, 550 ml 7.5% ether and 750 ml 10% ether in hexane.

Thin layer chromatography served for analytical and preparative separations. Analytical plates were prepared with Silica Gel G 250 $\mu$ thick and activated at 110 C. Solvent systems tried in the separations are listed in Table I. Separated products were visualized by charring on a hot plate after spraying with 50% $H_2SO_4$. Preparative TLC plates (20 x 20 cm) were spread in 250 $\mu$, 0.5 and 1 mm thicknesses. Mixtures weighing 24 mg or less were isolated on plates 250 $\mu$ thick; 24-90 mg, plates 0.5 mm thick; and 90-180 mg, plates 1 mm thick. Recovery of the addition compounds from the plates was aided by their weak yellow fluorescence in mercury vapor UV light. Compounds isolated from plates developed with solvent mixtures containing acetic acid had to be given an additional purification. This step involved filtering the isolate through silicic acid, a few cm thick (Mallinckrodt, 100 mesh), and washing with ether. MLOOH was isolated from preparative plates after development with hexane-ether 6:4.

#### Spectroscopy

Infrared and NMR spectra were recorded as described previously (8,9). Supplemental IR spectra were obtained with 0.1 mm thick NaCl cells containing 10% solutions in $CCl_4$ or $CS_2$.

Mass spectra of methyl hydroxystearates were determined essentially as described by Dolev et al. (14) and that of the addition compounds by a probe set at 152 C with the source being held at 152 C.

#### Derivatives

Epoxides were derivatized on a microscale with picric acid as described by Fioriti et al. (15). The crude picrate was extracted from the reaction mixture with ether, and the ether layer was washed several times with water. The picrate was purified by TLC in an isooctane-ether 7:3 solvent system.

Epoxides were cleaved directly with periodic acid (16). Cleavage products, hexanal or methyl azeleate semialdehyde, were detected by gas liquid chromatography (GLC) and compared with standards as in an earlier study (8). Hexanal was eluted from a 6 ft column (1/8 in. OD) packed with 5% Carbowax 20 M on Fluoroport T (Applied Science Laboratories, State College, Pa.). The temperatue of the column was 50 C, and the column gas flow was 30 cc/min. Methyl azeleate semialdehyde was eluted with a temperature program from 110-200 C at 5 C/min.

Fatty acids were esterified by diazomethane (17). Methyl oxooctadecadienoate was reduced by $NaBH_4$ (9). Hydrogenations were in methanol with a 10% palladium catalyst on charcoal at 1 atm and 25 C.

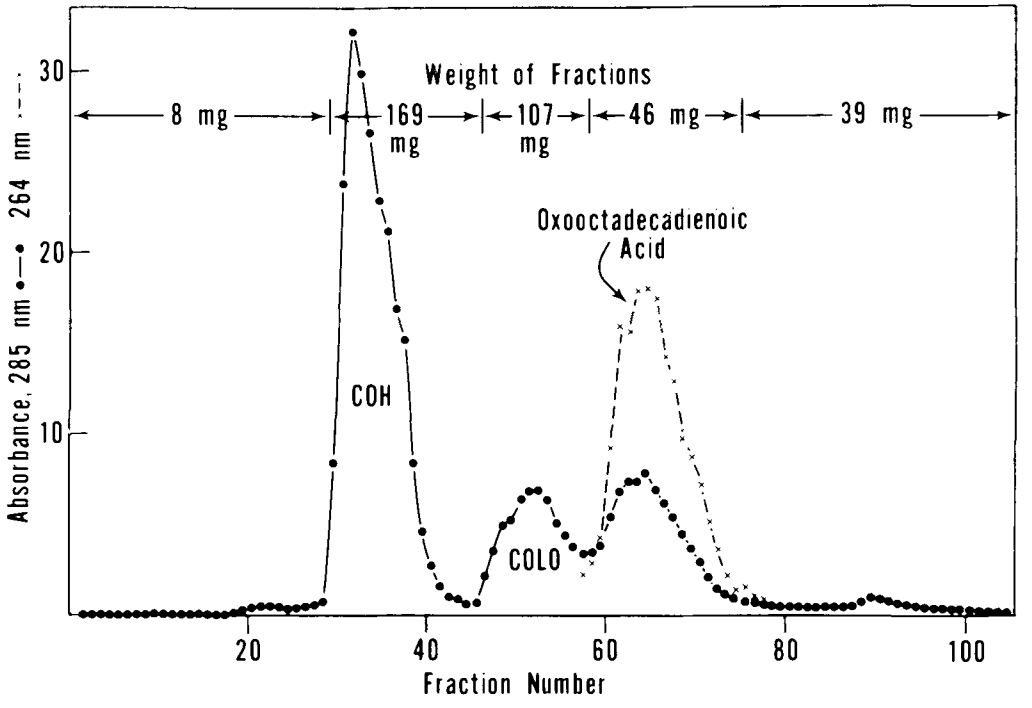



FIG. 2. Column chromatography of products obtained by the reaction of LOOH with COH catalyzed by light-sensitized proflavin. The addition compound between LOOH and COH is designated COLO.

## RESULTS

### Addition Reactions

*LOOH and TOH:* The formation of an addition compound (TOLO) between TOH and LOOH was catalyzed by either Fe(III) or light-sensitized proflavin under anaerobic conditions. TOLO was isolated from both the Fe(III) and photocatalyzed reactions by TLC (Table I). Infrared spectra of TOLO from either source were identical.

Spectral analyses indicated the structure of TOLO. The UV spectrum had a $\lambda_{max}$ at 288.5 nm with a shoulder at 282 nm in hexane or 95% ethanol ($\epsilon_{max}$ = 2460 in hexane). The UV spectra of TOLO compared with the spectra of methyl and allyl ethers of TOH reported by Porter et al. (6). The IR spectra of TOLO had features that appeared to be the additive absorptions of LOOH and TOH with a few exceptions. The following absorptions were absent: hydroperoxide OH at 3550 $cm^{-1}$, phenol OH at 3635 $cm^{-1}$, *cis,trans* conjugated diene at 984 and 948 $cm^{-1}$ and aromatic phenol absorptions at 1213 $cm^{-1}$ (strong) and 1340 $cm^{-1}$ (medium). Absorptions not found in the spectra of TOH or LOOH were detected in the spectrum of TOLO as follows: isolated *trans* at 968 $cm^{-1}$, epoxide at 895-900 $cm^{-1}$ (weak) and aromatic ether at 1250 $cm^{-1}$ (increased in intensity).

*LOOH and COH:* To facilitate structural determination of the addition compound, the model compound, COH, was reacted with LOOH to synthesize COLO. The reaction appeared to proceed in a manner similar to the addition of TOH and LOOH.

Excluding air from the reaction is required if COLO (or TOLO) is to be formed. Air in the proflavin-sensitized photolysis caused COH (or TOH) to disappear rapidly with no apparent effect on LOOH. An IR spectrum of the major product isolated partially pure from the air-reaction by TLC indicated that the compound was primarily α-tocopherolquinone or the analogous quinone of COH. An IR absorption band at 1680 $cm^{-1}$ indicated the presence of some of the epoxide of α-tocopherolquinone as described by Grams et al. (13). α-Tocopherolquinone or the analogous quinone of COH was also among the products when Fe(III) catalyzed the reaction in the presence of air. No COLO was formed regardless of the catalyst selected.

Blank or control experiments were negative. In the absence of Fe(III) or proflavin, LOOH and COH did not react under nitrogen with or without irradiation by visible light. After proflavin was added to a solution of reactants kept

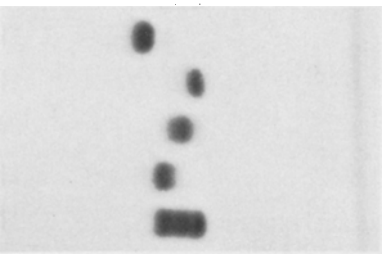



FIG. 3. Thin layer chromatogram of COH and three isomeric addition compounds: 1-, 2- and 3-COMLO. Developing solvent was isooctane-ether 75:25.

in the dark and anaerobic, only a trace of COLO formed. These proflavin-dark experiments were completely negative when 0.5 x $10^{-3}$ M EDTA (disodium salt) was included in the formulation. Evidently trace metal ions catalyze the dark reaction. When the EDTA-treated solution was irradiated, COLO was synthesized, a condition proving the reaction was due to light-sensitized proflavin.

The formation of COLO was not affected by other variables investigated, although their influence on reaction rates was not established. COLO formation occurred either in anhydrous or 99% (aqueous) methanol. No effect was produced when the COH/LOOH ratio was varied from 1 to 4, nor when temperature was varied between 20 and 34 C.

A quantity of COLO was produced via the proflavin-sensitized reaction so that its structure could be investigated. The product mixture was separated by column chromatography (Fig. 2). COLO made up 43% of the total products found. Oxooctadecadienoic acid was another significant product; column fractions 59 to 75 were 18% of the total.

COLO proved to be a mixture of isomers inseparable by our methods. Occasionally two discernible spots were barely separated by TLC. An NMR spectrum of COLO revealed mixed absorptions in the region where epoxide protons absorb.

After methyl esterification of COLO, the isomers could be separated by chromatographic techniques. Column chromatography separated three isomers of COMLO, which are designated 1-COMLO [methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*trans*-12,13-epoxy-*trans*-9-octadecenoate]; 2-COMLO [methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-12,13-epoxy-*trans*-9-octadecenoate]; and 3-COMLO [methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-9,10-epoxy-*trans*-12-octadecenoate]. In fractions 110-116 the 2-COMLO was pure, and 1-COMLO was partially resolved in fractions 92-100. 1-COMLO was mixed with a large amount of COH and traces of 2-COMLO. 3-COMLO was collected from fractions 122-130 mixed with a significant amount of 2-COMLO. Preparative TLC was used as a final purification procedure to obtain highly purified isomers. Figure 3 shows the three isomers separated by TLC as they were finally isolated. The isomers migrated too close to one another to analyze the amount of each isomer present, but an estimate was made from the weights of combined fractions and by TLC separations of the mixtures. The ratio of the isomers was approximately 1-COMLO:2-COMLO:3-COMLO (2:6:1).

Methyl oxooctadecadienoate was obtained impure from the column in fractions 131-150 after the isomers of COMLO had been eluted. Preparative TLC with hexane-ether 7:3 separated this compound in about 95% purity.

*MLOOH with COH or TOH:* MLOOH, being more representative of a model for peroxidized oil, was used in addition reactions with COH or TOH. Both Fe(III) and light-sensitized proflavin systems were tested as catalysts. Addition compounds (COMLO and TOMLO) were produced, which gave IR spectra identical to addition compounds formed from LOOH and subsequently methyl esterified. The carboxylic acid group does not appear to affect the reaction mechanism significantly.

#### Structure Determinations

*1-COMLO and 2-COMLO:* IR spectra of isomeric 1- and 2-COMLO's were nearly identical. The absorption at 895 $cm^{-1}$ of 1-COMLO was slightly more intense than 2-COMLO, but there was no difference in the absorption frequency as might be expected (18). The spectra appeared to show superimposed absorptions of the reactants, COH and MLOOH, and exceptions were clues to their structure. Figure 4 shows IR spectra of the reactants and 2-COMLO. Interpretations from Figure 4 were essentially the same as those described previously when TOLO spectrum was compared with TOH and LOOH spectra. The absorption at 895 $cm^{-1}$ disappeared when the COMLO isomers were treated with an epoxide-cleaving reagent, picric acid. The resultant derivative had all the IR absorptions expected for the picrate of an epoxide (15).

Ultraviolet spectra of 1- and 2-COMLO's were identical with a $\lambda_{max}$ at 288.5 nm and a shoulder at 282 nm ($\epsilon_{max}$ = 2450 in hexane). These spectra were identical to those from TOLO.

Mass spectrometry yielded scanty information, but it did show the molecular ion and a few fragmentations. The major problem ap-

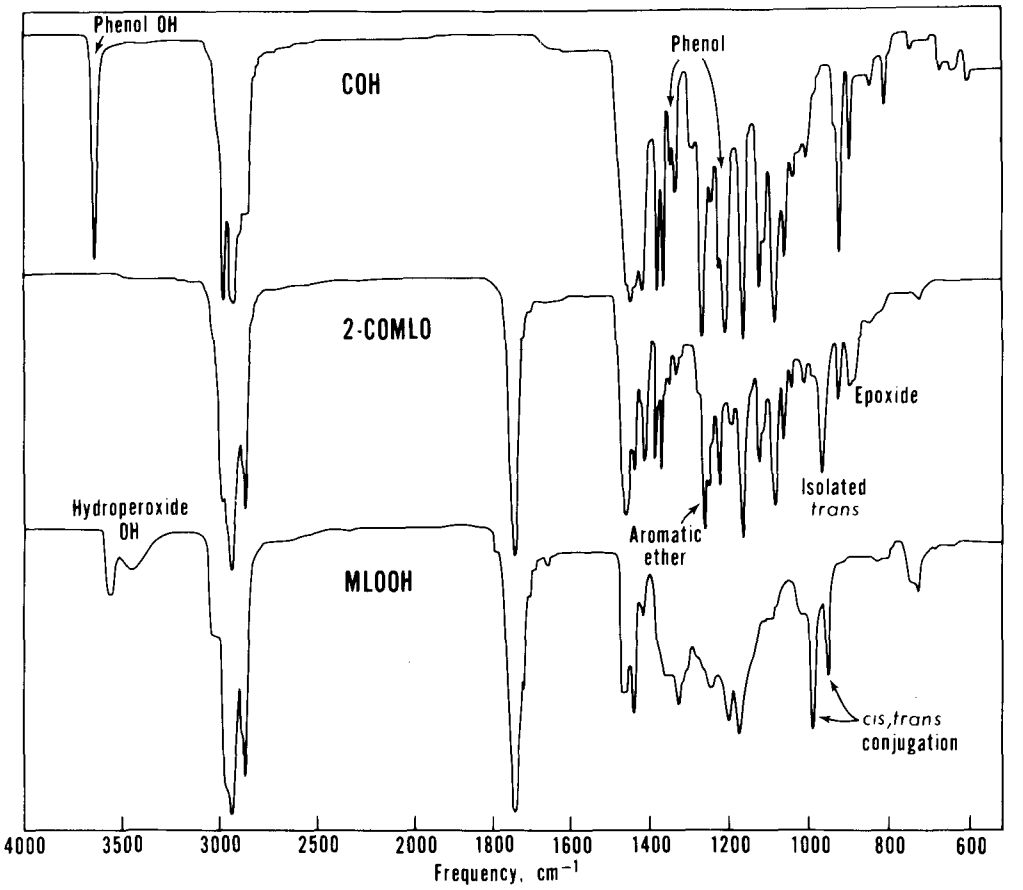


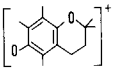
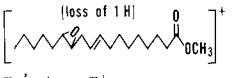
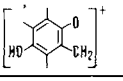

FIG. 4. Infrared spectra of COH, 2-COMLO and MLOOH.

peared to be due to the ease of fragmentation of COH from the addition compound (Table II). Also the table reveals that the spectra of 1- and 2-COMLO's are nearly identical. The most helpful information was confirmation of the molecular weight by an m/e peak at 528 for 1-COMLO. The 2-COMLO molecular ion peak was at M + 1 (529). Catalytic reduction of 2-COMLO gave a product isolated by TLC that had a molecular ion peak at 530; this result is compatible with the reduction of a double bond. An NMR spectrum confirmed that the double bond was reduced and the epoxide was intact. Table II also shows that the methyl epoxyoctadecenoate ion fragment detected at m/e 308 apparently has lost a hydrogen to the COH fragment. Hydrogenated 2-COMLO resulted in a methyl epoxyoctadecanoate fragment, which retained its hydrogen (m/e 311).

NMR spectroscopy (Fig. 5) confirmed that the products were *cis*- and *trans*-epoxide isomers. The 2-COMLO had characteristic proton resonance at 3.00 δ (split triplet, 1 H) and 2.79 δ (multiplet, 1 H) for the epoxy ring protons on C-12 and C-13, respectively. The deshielded proton was assigned the position on C-12 owing to the effect of the pentamethyloxychroman group on C-11. From the spectrum the position of resonance of the epoxy proton on C-13 established the epoxide as *cis* (19). Double resonance experiments showed conclusively that the proton on C-11 (multiplet, 3.80 δ, 1 H) was coupled with the C-12 epoxy proton and the vinylic protons at C-9 and C-10. The split triplet at 3.00 δ collapsed to a doublet and the multiplet at 5.60 δ (2 H, vinylic) simplified upon irradiation at 3.80 δ. 1-COMLO had characteristic proton resonance at 2.90 δ (split doublet, 1 H) and ca 2.6 δ for the epoxy protons on C-12 and C-13, respectively. From the spectrum the epoxide was assigned the *trans* configuration (19). Upon irradiating the C-11 proton at 3.86 δ (multiplet, 1 H) the split doublet at 2.90 δ collapsed to a doublet and the multiplet at 5.48 δ (2 H, vinylic) simplified which effect established the assigned structure.

TABLE II

Mass Spectral Analysis of 1-COMLO and 2-COMLO[a]

| m/e | Relative intensity 2-COMLO | Relative intensity 1-COMLO | Possible ion structure | Possible origin |
|---|---|---|---|---|
| M[b] | --- | 0.9 | | |
| M + 1 | 0.6 | --- | | |
| M - 31 | --- | 0.6 | | Loss of $CH_3O$ |
| M + 1-31 | 0.1 | --- | | |
| 219 | 65 | 63 |  | Cleavage from fatty ester |
| 219 + 1 | 100 | 100 | | |
| M - 220 | 3.1 | 6.0 | | Loss of pentamethyloxychroman + 1 |
| 164 | 40 | 43 |  | Cleavage of the chroman ring |
| 164 + 1 | 37 | 39 |  | |

[a]Two of the three isomers of COMLO (see Fig. 1).

[b]The molecular ion data could be subject to minor counting error due to difficulty of computer-counting m/e peaks that have been separated by large gaps in the spectrum. Replicate spectra of 1- and 2-COMLO gave M - 1 and M + 1 as well as the expected molecular ion (M).

The *cis* and *trans* assignment of the epoxide by NMR was confirmed by the greater mobility of the *trans* isomer relative to the *cis* by TLC (Fig. 3), which is a known property of *cis*- and *trans*-epoxy fatty esters (20).

The position of the epoxide was established at C-12 and C-13 for both 1- and 2-COMLO's by periodic acid oxidation. Both compounds were practically unreactive, especially 1-COMLO; however the expected fragment, hexanal, was detected by GLC.

Periodic acid data were confirmed by additional information obtained from catalytic reductions of 1- and 2-COMLO's. In addition to forming the expected product—methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-12,13-epoxy-octadecanoate—hydrogenations yielded COH and methyl hydroxystearates. Catalytic hydrogenation of epoxystearates under the proper conditions forms hydroxystearates (21). Reduction of the addition compounds yielded pure methyl 13-hydroxystearates as analyzed by MS rather than the expected mixture of 12- and 13-hydroxystearates. Apparently the pentamethyl-6-oxychroman group adjacent to the C-12 had some steric or electronic effect causing the epoxide ring to open toward C-13.

*3-COMLO:* IR, UV and NMR spectra were essentially identical to those reported for 2-COMLO. NMR spectral information established the epoxide as *cis* and placed the pentamethyl-6-oxychroman group between the *trans* double bond and the epoxide as described in the preceding section.

Simply on the basis of its mobility on TLC (Fig. 3), one might expect that 3-COMLO was the 9,10-epoxide as compared with mobilities of various methyl epoxystearates (20). This postulate was proved by fragmenting the epoxide. Periodic acid oxidation yielded methyl azeleate semialdehyde as determined by GLC. Hydrogenation of 3-COMLO gave methyl 9-hydroxystearate as determined by MS.

*Methyl oxooctadecadienoate (trans, trans):* IR and UV spectra of the compound that we have identified as methyl oxooctadecadienoate were virtually identical to the spectra of methyl 9-oxo-*trans,trans*-10,12-octadecadienoate reported by Binder et al. (22).

When reduced with $NaBH_4$, the compound gave IR and UV spectral data comparable to that from methyl dimorphecolate (23) and confirmed that it was a conjugated *trans,trans* methyl hydroxyoctadecadienoate. The position of the hydroxyl was not determined, but probably corresponded to the hydroperoxide from which it was derived (mostly C-13).

## DISCUSSION

Catalyses by both light-sensitized proflavin and Fe(III) appeared to function by a common mechanism, since the products were similar. Light-sensitized proflavin can initiate free radicals through its first-excited triplet state. Fe(II) and Fe(III), known one-electron reducing or oxidizing agents, would also be expected to generate radicals. A mechanism is proposed in Figure 6 that may explain how radical addition

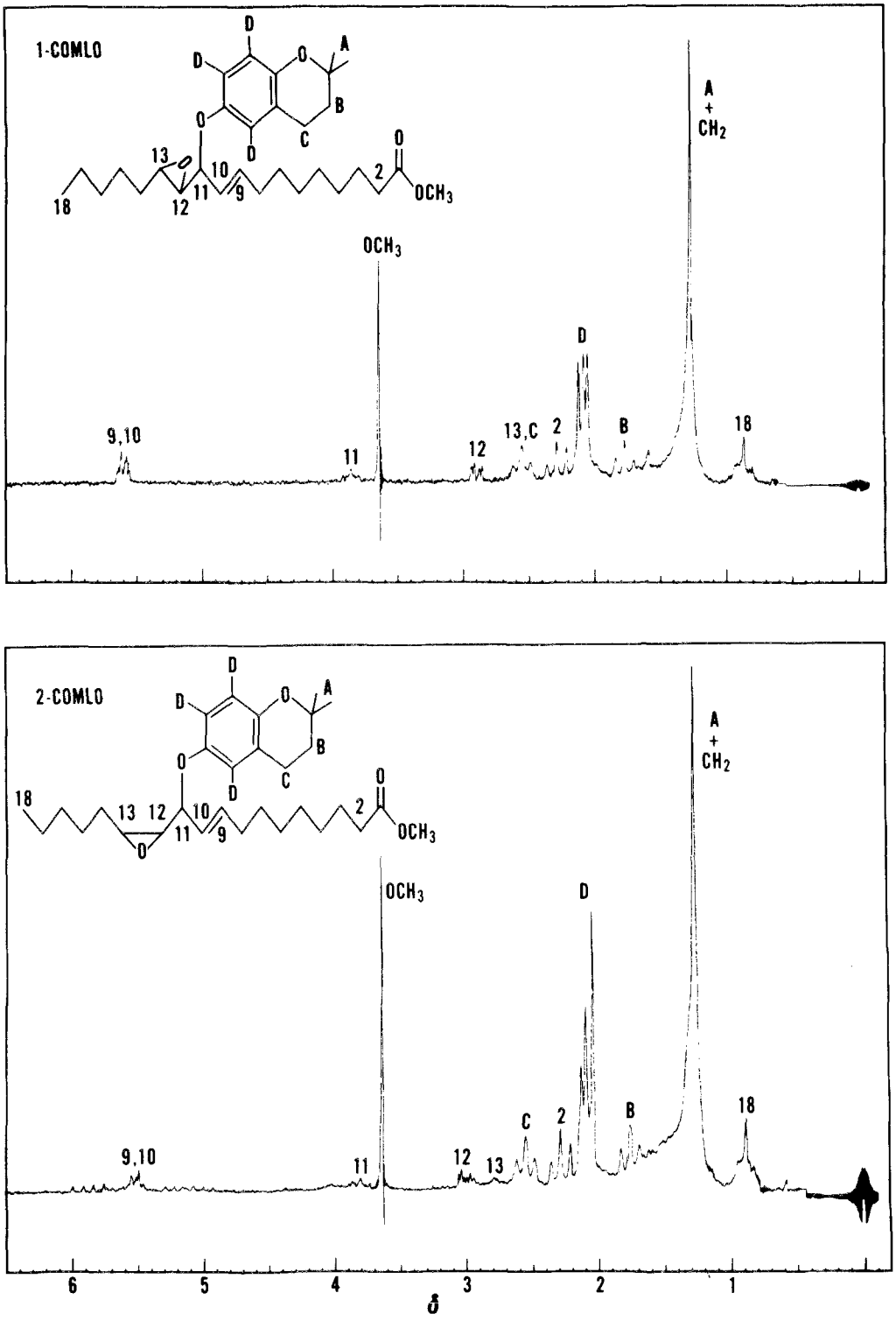



FIG. 5. Nuclear magnetic resonance, spectra of 1- and 2-COMLO.

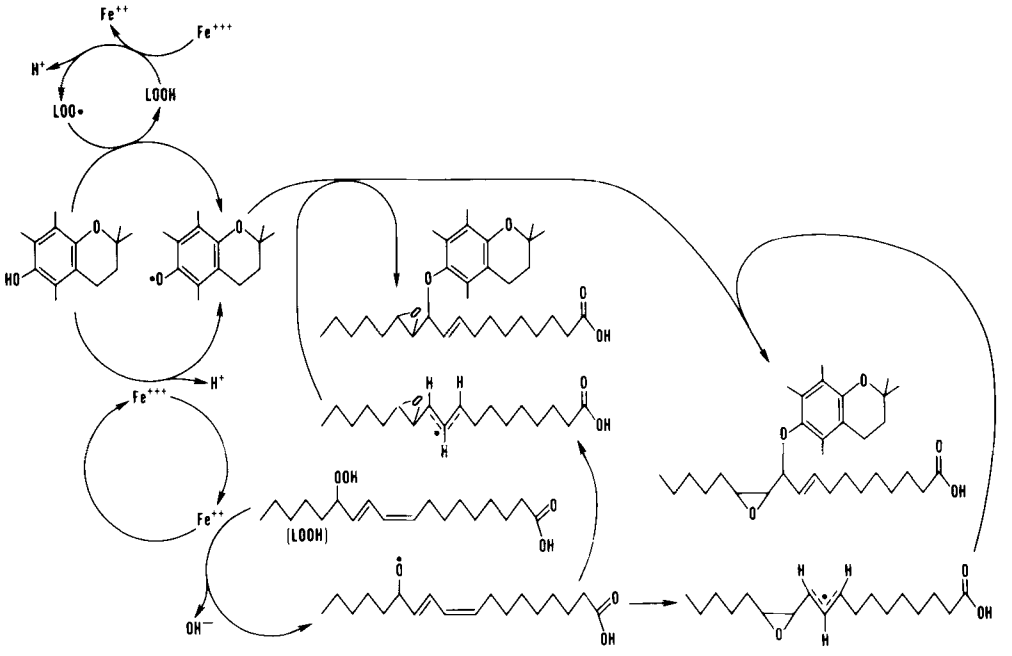



FIG. 6. Proposed mechanism of addition between COH and LOOH catalyzed by Fe (III).

of COH and LOOH can occur through a Fe(II)-Fe(III) cyclic redox system. The initial reaction would form the pentamethyloxychroman radical—a semiquinone—and an alkoxy radical of the fatty acid. The alkoxy radical apparently is transformed into another intermediate, an epoxy-allylic radical, as shown in Figure 6. The products, isomeric COLO and *trans,trans* oxooctadecadienoic acid, were evidence for this intermediate. Presumably oxooctadecadienoic acid was formed by loss of a hydrogen radical from the epoxy-allylic or alkoxy-diene radicals. The conjugated *trans, trans* oxodiene is at the lowest possible thermodynamic energy level that could be achieved after the homolytic loss of hydrogen. Oxidation to the semiquinone probably occurred by the peroxy radical of the fatty acid and Fe(III). The peroxy radical possibly is the most important oxidant as $FeCl_3$ oxidizes TOH or COH slowly. The alkoxy radical of the fatty acid was not likely an important oxidant of TOH or COH, since hydroxyoctadecadienoic acid was not observed among the products.

Although the mechanism outlined in Figure 6 shows how 1- and 2-COMLO's might be formed from 13-hydroperoxyoctadecadienoic acid, the same type of mechanism would apply to the formation of 3-COMLO from 9-hydroperoxyoctadecadienoic acid. In fact the amount of 3-COMLO found relative to 1- and 2-COMLO's was about equivalent to the 9- and 13-hydroperoxide ratio found in the reactants.

No isomers formed with the epoxide conjugated to the double bond, e.g., 9-(2,2,5,7,8-pentamethyl-6-oxychroman)-12,13-epoxy-*trans*-10-octadecenoic acid, although this occurrence would appear to be possible on the basis of intermediates proposed in Figure 6. An analogous compound, 9-hydroxy-*cis*-12,13-epoxy-*trans*-10-octadecenoic acid (a hydroxyl added rather than the semiquinone), was formed in wheat flour extracts (24). This compound arose from lipoxygenase oxidation of linoleic acid most likely through a 13-hydroperoxyoctadecadienoic acid intermediate. Apparently in the reaction we studied, the charge distribution on the epoxy-allylic radical favored addition of the pentamethyloxychroman semiquinone at C-11 rather than C-9. The addition at C-9 may have occurred to much lesser extent that at C-11, in which case detection of minor quantities of this isomer would not be noticed.

Another possible mechanism of epoxidation, the direct attack of alkenes by hydroperoxide oxygen such as those catalyzed by molybdenum or vanadium compounds (25), was eliminated from consideration because of the products obtained. They were mostly *cis*- and *trans*-12,13-epoxides and minor amounts of *cis*-9,10-epoxide in proportion to the 13-and

9-hydroperoxyoctadecadienoic acids used as reactants. Although the *trans*-9,10-epoxide was not observed, it probably was present in amounts too minor to be detected. Direct epoxidation of the double bonds by hydroperoxide or peroxide oxygen would yield mostly *trans*-11,12- and *cis*-9,10-epoxides with minor quantities of *cis*-12,13- and *trans*-10,11-epoxides. Clearly, the mechanism of epoxidation must be cyclization of the alkoxy radical.

Self-dimerization apparently was not significant in the reaction, as self-coupled products were either minor or not present. Dimers of TOH or COH are common oxidation products under proper conditions. Although the presence of COH or TOH dimers was suspected, they were minor components of the reaction mixtures. Similarly, dimers of LOOH or MLOOH were not significant enough to be detected.

A peculiarity of the reaction was the requirement for anaerobic conditions. Products other than the addition compounds were formed in the presence of air regardless of the catalyst selected. We assumed that quinone formation caused by light-sensitized proflavin resulted from oxidation by singlet oxygen, which was generated by proflavin in its excited-state triplet exchanging spin with ground-state triplet oxygen. Grams et al. (13) reported that the photooxidation of TOH by proflavin forms 8a-methoxy-α-tocopherone and 4a, 5-epoxy-8a-methoxy-α-tocopherone in methanol. The photooxidation by proflavin that we observed was undoubtedly similar, since TOH or COH was rapidly oxidized while LOOH or MLOOH appeared to be largely unaffected. Rather than obtaining the tocopherones as found by Grams et al., we obtained the α-tocopherolquinone (and corresponding epoxide) or the analogous quinone and quinone epoxide of COH. This was undoubtedly due to hydrolysis of the 8a-methoxy, which occurred in our work-up procedure as we did not take precautions to exclude water from the reaction products. In the Fe(III)-catalyzed reaction (kept aerobic) α-tocopherolquinone or the analogous quinone of COH was formed, whereas not even traces of the addition compound could be detected. Gruger and Tappel (5) working with Fe(III)-catalyzed reactions between TOH and MLOOH, presumably in the presence of air, also concluded that α-tocopherolquinone was formed. However, under nitrogen and using the same concentration of Fe(III) as Gruger and Tappel, we found the reaction changes to yield the addition product with only traces of α-tocopherolquinone being detected. For the Fe(III) reaction we can conclude from the data that only under nitrogen is the semiquinone stable for a long enough time interval to be trapped by reacting with the alkoxy radical, but when exposed to aerobic conditions the semiquinone apparently reacts immediately with oxygen. Oxygen, a biradical, could conceivably react readily with the semiquinone and form an unstable hydroperoxide para to the 6-oxo. This derivative could exchange with solvent leading to formation of 8a-methoxy-α-tocopherone in anhydrous methanol and finally to opening of the chroman ring during work-up forming the quinone. Fujimaki et al. (26) have already proposed that a tautomer of the pentamethyloxychroman radical may react with molecular oxygen.

## ACKNOWLEDGMENT

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# Ring Position in Cyclopropene Fatty Acids and Stearic Acid Desaturation in Hen Liver

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## ABSTRACT

Four cyclopropene fatty acids, having the double bond of the cyclopropene ring at the 8,9, 9,10, 10,11 and 11,12 positions, respectively, were tested as inhibitors of stearic acid desaturation by the desaturase enzyme system of hen liver. The first three were powerful inhibitors, but the last was not. The cyclopropene acids with the 9,10 and 10,11 double bonds were equally strong inhibitors, while the acid with the 8,9 double bond was less effective. To account for the specificity of those cyclopropene fatty acids in which the C9 or C10 carbon atom is included in the cyclopropene ring, it is suggested that the conformation and structure of the CoA derivatives of these acids is such that they can irreversibly occupy the site on the enzyme responsible for 9,10-desaturation.

## INTRODUCTION

The cyclopropene fatty acids, malvalic (8,9-methyleneheptadec-8-enoic acid) and sterculic (9,10-methyleneoctadec-9-enoic acid), irreversibly inhibit the 9,10-desaturation of the CoA derivatives of fatty acids by the desaturase enzyme system of hen liver (1,2). It has been suggested (2,3) that inhibition is the result of the formation of a carbon-sulfur bond between a carbon atom of the cyclopropene ring and a thiol group at the desaturating site of the enzyme. Desaturation always occurs at the 9,10 position of the fatty acid irrespecitve of chain length (2,4). Sterculic acid is a more effective inhibitor than malvalic acid, and in this respect the former, with the double bond at the 9,10 position, appears to have a more favorable structure for occupying the desaturating site than the latter, which has the double bond at the 8,9 position.

Pande and Mead (5) have suggested that inhibition by the cyclopropene fatty acids is a nonspecific, detergent-type effect of the free fatty acids. We have observed (2) similar nonspecific inhibition of the hen liver desaturase system when high levels of linoleic acid or dihydrosterculic acid were added. Pande and Mead used stearoyl-CoA as substrate and ammonium sterculate as inhibitor in their desaturation system, which may be incapable of forming CoA derivatives. The levels of ammonium sterculate used were considerably in excess of the substrate. Other workers (1-3,6), using systems capable of forming CoA derivatives, have shown that cyclopropene fatty acids act as specific inhibitors of 9,10-desaturation at levels down to .01 that of the substrate. The observation of Pande and Mead that ammonium sterculate at much higher concentrations did not markedly inhibit desaturation suggests that it is the CoA derivatives of the cyclopropene fatty acids which are the active inhibitors. Further evidence for this suggestion is provided by the fact that sterculyl alcohol is a less effective inhibitor of desaturation than either sterculic or malvalic acids (2).

The importance of the position of the cyclopropene ring in relation to the carboxyl group has been examined further. Two higher homologs of sterculic acid were synthesized, namely the $C_{20}$ cyclopropene acid, 10,11-methylenenonadec-10-enoic acid, and the $C_{21}$ cyclopropene acid, 11,12-methylene-eicosa-11-enoic acid. The effectiveness of these acids as inhibitors of 9,10-desaturation was compared with that of malvalic and sterculic acids.

## EXPERIMENTAL PROCEDURES

### Preparation of Cyclopropene Methyl Esters

Methyl malvalate ($C_{18}$ cyclopropene ester) and methyl sterculate ($C_{19}$ cyclopropene ester) were prepared from *Sterculia foetida* seed oil (7,8). The $C_{20}$ cyclopropene methyl ester was synthesized from methyl sterculate by chain elongation as described below. The $C_{21}$ cyclopropene methyl ester was then prepared from the $C_{20}$ ester by a similar chain elongation.

The general procedure for chain elongation involved the following steps. (The antioxidant butylated hydroxytoluene [0.01% by weight] was added to all the cyclopropene intermediates during the preparation.) The cyclopropene methyl ester to be used as starting material was chromatographed on Florisil (Floridin Co., Fla.) (9). The purified ester (usually ca. 2.5 mmoles) was reduced to the corresponding alcohol with lithium aluminum hydride (8) and the product recovered by applying the sodium hydroxide treatment suggested by Mićović and

TABLE I

Desaturation of Stearic Acid (0.1 mmolar) by Desaturase Systems of Hen Liver, in the Presence of Cyclopropene Fatty Acids

| Cyclopropene fatty acid | Experiment 1 | Experiment 2 | Experiment 3 |
|---|---|---|---|
| None (control) | 40.6 (100)[a] | 22.8 (100) | 49.1 (100) |
| $C_{18}$ (0.001 mmolar) | 31.2 (77) | 17.5 (77) | 35.4 (72) |
| $C_{19}$ (0.001 mmolar) | 15.8 (39) | 11.0 (48) | 20.3 (41) |
| $C_{20}$ (0.001 mmolar) | 17.6 (43) | 10.9 (48) | 19.8 (40) |
| $C_{20}$ (0.005 mmolar) | ---[b] | --- | 7.6 (15) |
| $C_{21}$ (0.001 mmolar) | --- | 23.4 (103) | 44.9 (92) |
| $C_{21}$ (0.005 mmolar) | --- | --- | 39.7 (81) |
| $C_{21}$ (0.5 mmolar) | --- | --- | 4.0 (8) |

[a]Figures in parentheses express desaturation as a percentage of the control value (100% conversion).

[b]Dash signifies experiment not performed.

Mihailović (10).

The reduction product was chromatographed on a column of 7% w/w hydrated Florisil (20 g). Elution was commenced with 5% v/v diethyl ether in hexane until unchanged cyclopropene ester had been eluted (ca. 60 ml), then continued with 20% v/v diethyl ether in hexane (ca. 200 ml) to recover the cyclopropene alcohol. The course of the elution was followed by subjecting fractions to thin layer chromatography (TLC) on a Silica Gel G plate developed with hexane-diethyl ether-acetic acid 90:10:1 v/v. After development the plate was sprayed first with a saturated solution of silver nitrate in methanol, which immediately revealed cyclopropene compounds as brown spots (11), then with 2',7'-dichlorofluorescein solution (0.2% w/v in ethanol) to detect byproducts which no longer contained an intact cyclopropene ring. In a typical experiment 2.5 mmoles cyclopropene methyl ester yielded 2.2 mmoles pure cyclopropene alcohol.

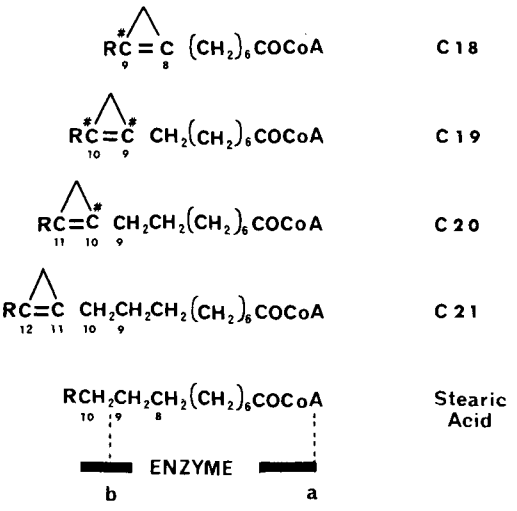


FIG. 1. Positions of the cyclopropene fatty acids and stearic acid in relation to the desaturating enzyme. $C_{18}$, $C_{19}$, $C_{20}$ and $C_{21}$ represent the CoA derivatives of the cyclopropene acids; R is $CH_3(CH_2)_7$-. The site of initial attachment of the substrate or inhibitor to the enzyme is indicated by the point *a*, the site of desaturation of substrate by the point *b*. The configuration of the enzyme surface between *a* and *b* is such that desaturation only occurs at the 9,10 position of the substrate.

The cyclopropene alcohol was converted to the methanesulfonate (mesylate) using the method of Baumann and Mangold (12). The yield of recrystallized mesylate was 75-80%. The mesylate was treated with potassium cyanide in dimethyl sulfoxide (13). The resulting nitrile was hydrolyzed using the alkaline hydrolysis procedure of Gensler et al. (14) to prevent loss of the cyclopropene ring, and the cyclopropene acid thus produced was promptly methylated with diazomethane (14). The ester was purified by chromatography on Florisil (9), and converted to the urea adduct for storage. The yield of ester from the complete elongation sequence was 60-65%.

The purity of each of the $C_{18}$, $C_{19}$, $C_{20}$ and $C_{21}$ cyclopropene methyl esters was checked by IR and NMR spectroscopy, and by TLC and gas liquid chromatography (GLC), and found to be respectively greater than 98%, 99%, 98% and 96%. For GLC analysis the cyclopropene esters were treated with silver nitrate in methanol (15) and the reaction products injected directly into the gas chromatograph (Packard Model 7401, 6 ft glass U-tube, 2-mm ID, 15% diethyleneglycol succinate on Gas-Chrom Q, 180 C). Each cyclopropene methyl ester yielded three products, representing ca. 3%, 85% and 12% of the original ester. The retention times, relative to methyl stearate (1.0), for the smallest peaks were 1.6, 2.15, 2.85 and 3.9, respectively, for

the $C_{18}$, $C_{19}$, $C_{20}$ and $C_{21}$ derivatives. The retention times of the major peaks were 3.0, 4.0, 5.4 and 7.2, respectively, and of the remaining peaks 6.3, 8.4, 11.0 and 14.9, respectively. Schneider et al. (15) have identified the major derivatives of malvalate and sterculate as ethers, and the long-running derivatives as ketones. Plots of log retention time vs. carbon number showed parallel linear relationships for each series of peaks, confirming that a homologous series of cyclopropene esters had been obtained.

#### Effect of Cyclopropene Fatty Acids on the Hen Liver Desaturase System

The influence of the $C_{18}$, $C_{19}$, $C_{20}$ and $C_{21}$ cyclopropene fatty acids on the conversion of stearic acid to oleic acid by the desaturase enzyme system was measured using procedures described previously (1,16), except that argentation TLC was used instead of argentation column chromatography to determine percentage conversion. TLC plates of Silica Gel G containing 10% silver nitrate were employed. The purified methyl esters derived from each incubation were applied in 1 cm lanes scored on each plate, and the plates were developed twice in the same direction with hexane-diethyl ether 92:8 v/v. After development the plate was sprayed with 2′,7′-dichlorofluorescein, and each lane was divided into two zones, one containing the saturated fatty acid methyl esters only, and the other containing all the unsaturated esters. The two zones from each lane were transferred quantitatively to counting vials, scintillation fluid (PPO-POPOP-toluene) was added, and the radioactivity of each zone was determined with a liquid scintillation spectrometer (Packard Tri-Carb, Model 3003). The percentage conversion (desaturation) was calculated from the ratio of the count found in the unsaturated ester zone to the total count of the two zones.

## RESULTS AND DISCUSSION

The results of three separate experiments are shown in Table I. In each experiment three incubations were run as controls, three with the $C_{18}$ cyclopropene acid added, three with the $C_{19}$ cyclopropene acid added, and so on; the stearic acid (substrate) concentration was 0.1 mmolar and the concentration of the cyclopropene acids was usually 0.001 mmolar. Argentation TLC was performed twice on the methyl esters derived from each incubation sample. The reproducibility of the TLC results was extremely high, and the results from each set of three incubations agreed to within 1%. The results from each group were averaged, and the percentage conversions of the groups containing the cyclopropene fatty acids were expressed as percentages of the control value (100% conversion).

Malvalic acid ($C_{18}$ cyclopropene acid) was not as strong an inhibitor as sterculic acid ($C_{19}$), as shown previously (1,2). The $C_{20}$ cyclopropene acid was equally as effective as sterculic acid in inhibiting the conversion.

On the other hand, the $C_{21}$ cyclopropene acid did not inhibit the desaturation of stearic acid at the 0.001 mmolar level, and only slightly at the 0.005 mmolar level. Inhibition was not pronounced until the concentration of the $C_{21}$ acid was greater than that of the substrate stearic acid, an effect which is also obtained with other fatty acids such as linoleic acid at such high concentrations (1).

It has previously been suggested (2,3) that sterculic acid or its CoA derivative irreversibly and completely occupies the site of the enzyme normally occupied by a substrate during desaturation, as a result of the formation of a carbon-sulfur bond between the C9 or C10 of sterculic acid and a thiol group in the desaturating site of the enzyme. Furthermore the above results show that, of those cyclopropene fatty acids tested, only those in which the C9 or C10 carbon atom is present in the ring will inhibit 9,10-desaturation of fatty acids (Fig. 1). Thus the $C_{18}$, $C_{19}$ and $C_{20}$ cyclopropene fatty acids, having the double bond of the cyclopropene ring at respectively the 8,9, the 9,10 and the 10,11 positions, are desaturase inhibitors, while the $C_{21}$ cyclopropene acid (double bond at 11,12) is not.

It would be of interest to obtain the $C_{17}$ cyclopropene fatty acid, 7,8-methylenehexadec-7-enoic acid, to determine if this compound is an inhibitor of stearic acid desaturation. As yet is has not been possible to obtain this acid, although the recent synthetic procedures devised by Gensler et al. (14,17) and by Williams and Sgoutas (18) indicate how such a synthesis may be attempted.

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# Lipids of *Crassostrea virginica*. I. Preliminary Investigations of Aldehyde and Phosphorous Containing Lipids in Oyster Tissue[1]

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## ABSTRACT

Oyster tissue contained 2.4% lipid, 0.14 μmole aldehyde per milligram lipid and at least 10 μg phosphorous per milligram lipid. The neutral lipid represented 58%, the glycolipid 6%, and the polar lipid 36% of the total lipid recovered after silicic acid column chromatography. Aldehydes were found in all fractions, but the presence of plasmalogen was verified in only the neutral and polar lipid fractions. At least 68% of the plasmalogen in oyster lipid was found in the polar lipid fraction. At least 13% of the phosphorous in oyster lipids was present as phosphonolipid. The distribution of phosphate and phosphonate lipids was: diacyl phospholipid 38.1%, plasmalogen phospholipid 21.8%, sphingophosphonolipid 13.5%, glyceryl ether phospholipid 8.3%, sphingophospholipid 6.9%, plasmalogen phosphonolipid 6.4%, diacyl phosphonolipid 2.6%, and glyceryl ether phosphonolipid 2.4%. When the per cent of phosphorous as phosphonolipid within the plasmalogen and glyceryl ether classes was calculated, similar values were obtained. These results support the hypothesis that there is a product precursor relationship between these two classes of lipids.

## INTRODUCTION

The growing interest in marine and estuarine food sources as well as the general lack of information on the lipid composition of oyster tissue prompted us to begin a detailed investigation of the lipids in the Virginia oyster. A number of studies have dealt with the gross composition of oyster tissue, but reports of individual classes of lipids have generally been qualitative in nature. Exceptions are studies on sterol components (1), glycolipids (2), and several contradictory reports on fatty acid composition of oyster tissue (3-5).

Extremely little has been reported on the composition of phospholipids in oyster. Hack et al. (6) reported that oysters were a good source of serine plasmalogen, and Rapport (7) found that gill tissue of oysters contains relatively large amounts of plasmalogens. In view of the current interest in the biosynthesis and function of plasmalogens, we concentrated our initial efforts on examining these compounds in oyster tissue. In this paper we report our observations on the distribution of aldehyde (plasmalogens) among the major fractions of oyster lipid and the distribution of phosphorous among the polar lipids obtained from the tissue of the Virginia oyster.

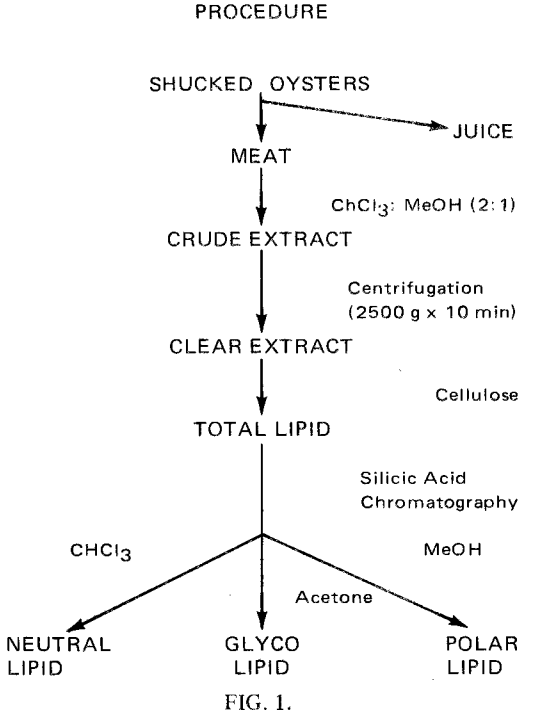


FIG. 1.

[1]Some of the data taken from a thesis to be submitted to the Graduate School, University of Maryland, by Leslye Johnson in partial fulfillment of the requirements for the M.S. degree in biochemistry.

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TABLE I

Lipid, Phosphorous and Aldehyde Content of Total Oyster Extract and Fractions Obtained After Silicic Acid Chromatography

| Fraction | Weight % | Phosphorous content[a] | | Aldehyde content[b] | |
|---|---|---|---|---|---|
| | | μg/mg lipid | %[c] | μmoles/mg lipid | %[c] |
| Total lipid | 2.4 (±0.2)[d] | 10 (±0.5) | --- | 0.139 (±0.001) | --- |
| Neutral | 58 (±2.0)[e] | 0.08 (±0.03) | 0.5 | 0.080 (±0.008) | 28.3 |
| Glyco | 6 (±1.5)[e] | 2.3 (±0.4) | 2.1 | 0.068 (±0.002) | 3.0 |
| Polar | 36 (±1.9)[e] | 24 (±1.8) | 97.4 | 0.272 (±0.022) | 68.7 |

[a]Estimated by the method of Bartlett (10); averages of 10 assays (±SEM).

[b]Estimated by the methods of Rapport and Alonzo (13) and/or Katz and Keeney (14); averages of 10 assays (±SEM).

[c]Per cent of total phosphorous or aldehyde in these fractions after silicic acid chromatography.

[d]Based on wet weight of oyster tissue; average of six extractions (±SEM).

[e]Based on the lipid recovered after chromatography; average of eight separations (±SEM).

## MATERIALS AND METHODS

### Oysters

The oysters used in this study were harvested in February 1970, from the estuary ca. 3 miles off the shore at Hallowing Point, Md. Edible adult oysters (average size 4 x 4 in.–average oyster contained 11 g tissue and 12 g juice) were transported within 2 hr after harvest to the University where they were stored in a cold room at 4 C. Within 2 days after harvest the oysters were shucked and the juice decanted from the oyster flesh. Aliquots of oyster tissue (usually 5-6 oysters) were stored at -10 C until they could be extracted and analyzed.

### Extraction Procedure

With the aid of a Waring blendor, oyster tissue was homogenized in methanol containing butylated hydroxytoluene (BHT). For each 100 mg of expected lipid, 0.1 mg of BHT was added to the methanol. The volume of methanol used in this step was one-half of the total volume necessary to prepare a chloroform-methanol 2:1 v/v extract, according to the method of Folch et al. (8). The homogenate was transferred to an Erlenmeyer flask and the Waring blendor was rinsed with the remaining methanol. The appropriate amount of chloroform was added to the homogenate and the mixture was magnetically stirred at room temperature for 30 min. Insoluble debris was removed by centrifugation at 2,500 x g for 10 min. To insure complete extraction of lipid, the pellet obtained was washed twice by resuspending in chloroform-methanol 2:1 v/v (1/20 of the initial volume of extracting solvent) and centrifuging as above. The supernatants were combined and concentrated under a stream of nitrogen at room temperature. During this concentration step, additional chloroform was added to help remove most of the methanol and water.

Nonlipid contaminants were removed from the clear extract by an initial quick filtration through cellulose powder followed by cellulose column chromatography. For each gram of lipid expected, 15 g Whatman standard grade ashless cellulose powder was prewashed as described by Rouser et al. (9) and layered to a depth of ca. 2 cm over a piece of filter paper in a Buchner funnel. The lipid extract was placed upon the powder and washed through the cellulose using 1 liter of chloroform-methanol 9:1 v/v saturated with water. The eluate was concentrated as above and placed on a column of cellulose prepared essentially as described by Rouser et al. (9). The column consisted of 15 g prewashed cellulose powder packed to a height of 20 cm. The lipid was eluted at a flow rate of 5 ml/min with 20 column volumes (1400 ml) of chloroform-methanol 9:1 v/v saturated with water. The solvent was removed under a stream of nitrogen and the total lipid was stored in chloroform at -10 C until used.

### Silicic Acid Chromatography

The total lipid was separated into three fractions by chromatography on Biosil A (Calbiochem.). The elution scheme used was essentially that described by Rouser et al. (9). For each 150-300 mg of lipid, a column containing 20 g of Biosil A was packed in chloroform to a height of ca. 12 cm. The lipid in 5 ml chloroform was added to the column and chloroform (200 ml) was used to elute the neutral lipids. This was followed by acetone

(400 ml) to collect a glycolipid fraction, and methanol (200 ml) to elute the polar lipids. BHT (0.1 mg/100 mg expected lipid) was added to the glycolipid and polar lipid fractions. The procedure employed to extract and separate the lipid in oyster tissue is summarized in Figure 1.

#### Analytical Procedures

Lipid phosphorous was estimated by the method of Bartlett (10) and Ames (11) as described by Aalbers and Bieber (12). The latter procedure, which utilizes $Mg(NO_3)_2$ in the ashing step, was employed when it was evident that the Bartlett procedure was not accounting for all of the possible lipid phosphorous.

Plasmalogen concentrations were estimated by converting the aldehydogenic lipids into aldehydes, reacting the aldehydes with *p*-nitrophenylhydrazine (13) or 2,4,-dinitrophenylhydrazine (14) and measuring the absorption of the derivatives at 390 or 341 nm, respectively, on a Beckman B spectrophotometer. Hydrogenated samples were analyzed periodically. Also the procedure described by Parks et al. (15) to separate free aldehydes from lipid extracts was employed to check for aldehydes in the neutral lipid fraction.

The sequence of chemical hydrolyses devised by Dawson (16) was employed to estimate the major classes of phospholipid. As a further check on plasmalogen phospholipid, samples were analyzed both before and after hydrogenation.

#### Hydrogenation

Hydrogenations were performed in a 15 ml Parr hydrogenation cup using ca. 50 mg lipid dissolved in 7-10 ml methanol or methanol-methylene chloride 2:1 v/v, 10 mg Pd/C or platinum oxide, and 300 psi hydrogen pressure. The hydrogenation mixture was constantly stirred with the aid of a teflon magnet and a magnetic stirrer. After 20 min the catalyst was removed by filtration.

#### Thin Layer Chromatography (TLC)

Aliquots of total, neutral, glycolipid and polar lipid were spotted on 250$\mu$ layers of Silica Gel G and checked in two solvent systems. Petroleum ether-ethyl ether-acetic acid 90:10:2 v/v/v was used to separate neutral lipids, and chloroform-methanol-acetone-acetic acid-water 55:15:20:5:5 by volume was used to check for the presence of glycolipid or polar lipid. All separations were conducted in nonsaturated developing tanks. Exposure to iodine fumes or spraying with $H_2SO_4/H_2O$ 50:50 v/v followed by heating was employed to visualize the developed TLC plates.

TABLE II

Estimates of Some Phospholipid Classes in Oyster Lipids[a]

| | Phosphorus | | | |
|---|---|---|---|---|
| | Before hydrogenation | | After hydrogenation | |
| Class | $\mu$g | % | $\mu$g | % |
| Diacyl | 127.0 | 55.6 | 112.0 | 57.0 |
| Plasmalogen | 66.6 | 29.4 | 1.5 | 0.8 |
| Sphingolipid | 17.1 | 7.5 | 13.1 | 6.7 |
| Glyceryl ether | 16.8 | 7.4 | 69.6 | 35.5 |

[a]Estimated by the Dawson Hydrolytic Procedure (16) using the method of Bartlett (10) for phosphorous determinations.

## RESULTS AND DISCUSSION

Based on the wet weight, on the average, 2.4% of oyster tissue was lipid (Table I). This value compares favorably with the 2.5% lipid reported in *Crassostrea gigas* (3) and the values for medium and large samples of *Crassostrea virginica* (2.36% and 2.56%, respectively) as reported by Ackman and Cormier (17).

The average values for the per cent of the total lipid recovered in each fraction after chromatography are presented in Table I. The average values for phosphorous and aldehyde contents in these fractions and in the total lipid are also summarized in this table.

On an average the chloroform fraction represented 58% of the total lipid recovered from the column. Similar values for the acetone and methanol fraction were 6% and 36%, respectively. The separation between the acetone and methanol fractions on Biosil A was variable as was the recovery of the total lipid from the column. In general it was found that separations and recoveries were best when the total oyster lipid applied to the column did not exceed 200 mg.

Based on TLC and phosphorous determinations, the chloroform fraction contained all of the neutral lipids with little or no contamination from glycolipid or polar lipid. Sterols, triglycerides and sterol esters were the predominant lipid classes in this fraction. Lesser quantities of material with $R_f$'s between triglycerides and sterol esters were tentatively identified as neutral plasmalogens and glycerol ethers.

The material collected in the acetone fraction gave a positive anthrone test. Also material in this fraction when submitted to TLC ran coincidentally with a galactosyl diglyceride standard. In contrast we were unable to detect

TABLE III

Estimates of Phosphorous Distribution Among Polar Lipids in Oyster Tissue

| Class | Total[a] | | Phosphate[b] | | Phosphonate[c] | | Per cent of each class as phosphonolipid |
|---|---|---|---|---|---|---|---|
| | μg | %[d] | μg | %[d] | μg | %[d] | |
| Diacyl | 122.7 | 40.7 | 114.8 | 38.1 | 8.0 | 2.6 | 6.5 |
| Plasmalogen | 85.0 | 28.2 | 65.6 | 21.8 | 19.4 | 6.4 | 22.8 |
| Sphingolipid | 61.6 | 20.4 | 20.8 | 6.9 | 40.8 | 13.5 | 66.2 |
| Glyceryl ether | 32.1 | 10.6 | 25.0 | 8.3 | 7.1 | 2.4 | 22.1 |

[a]Estimated by the Dawson Hydrolytic Procedure (16) using the digestion conditions recommended by Ames(11 ) followed by color development as described by Bartlett (10) for phosphorous determinations.

[b]Estimated as in (a) except digestion conditions were as described by Bartlett (10).

[c]Estimated by difference (a - b = c).

[d]Per cent of total (phosphate + phosphonate) phosphorous.

glycolipids in the other fractions. In some separations phospholipids were apparently also eluted with acetone, since anywhere from 1-5% of the total recovered phosphorous, as measured by the procedure of Bartlett (10), was found in the glycolipid fraction. Some of this material may have been due to phosphorous-containing glycolipids as these have been reported in oyster tissue (2). Virtually all of the remaining phosphorous was found in the methanol fraction.

When the fractions were assayed for aldehydogenic material, the total lipid contained, on the average, 0.14 μmole aldehyde per milligram lipid. Most of this was recovered in the polar lipid fraction, as the methanol fraction contained 68.7%, the chloroform fraction contained 28.3%, and the acetone fraction contained 3.0% of the total aldehydogenic lipid recovered after silicic acid chromatography. Some of the aldehyde content in the chloroform fraction was due to free aldehydes. However, when the neutral lipids were passed through a celite column impregnated with sodium bisulfite (15) to remove free aldehydes prior to assay for plasmalogen, at least 33% of the aldehyde in this chloroform fraction was still present in the eluant. This lends support to the tentative identification of plasmalogens as one of the minor components in oyster neutral lipids.

When the polar lipids were analyzed for distribution of phospholipid classes (Table II), the presence of plasmalogens in this fraction was verified. Before hydrogenation the plasmalogen phospholipids represented 29.4% of the phosphorous, and after hydrogenation this value dropped to less than 1%. A concomitant increase was found in the glyceryl ether fraction. Thus most, if not all, of the aldehydogenic lipid in the polar lipid fraction was present as the classical vinyl ether-containing plasmalogen.

Since we had obtained values in two different ways for the plasmalogen content in the polar lipid fraction, it was possible to check one assay against the other. The samples used for the Dawson hydrolysis contained 0.34 μmole plasmalogen per milligram polar lipid as measured directly (13). However, based on the results of the Dawson hydrolysis (Table II) and the total phosphorous in this fraction as estimated by the Bartlett procedure (10), these same samples contained only 0.26 μmole of plasmalogen phosphorous per milligram of polar lipid.

At this time it came to our attention that Quin and Shelburne (18) had reported the presence of carbon-bound phosphorous in oyster tissue. If the Bartlett procedure did not recover phosphonate phosphorous, this could explain the discrepancy between the two estimates for plasmalogen that we found in our polar lipids.

With this in mind we compared the release of lipid phosphorous in the polar fraction, using the digestion procedure described by Bartlett (10) and the digestion procedure of Ames as described by Aalbers and Bieber (12). After digestion all samples were treated for color development as described by Bartlett (10).

The Bartlett procedure consistently gave lower values for lipid phosphorous when compared to the procedure recommended by Aalbers and Bieber. The average μmole phosphorous per milligram lipid ± SEM was 26 ± 0.7 compared to 32 ± 0.3 when assayed by the Bartlett and Ames procedures, respectively. Based on these assays anywhere from 13-26% of the total phospholipid in oyster tissue may have been present as phosphonolipid. Analyses of lipid phosphorous based on other methods (16,19) yielded similar values and confirmed

that as much as 25% of the phosphorous-containing lipid in oyster tissue may have a direct phosphorous-carbon bond.

Employing these two methods (10,12) as estimates of total and phospholipid phosphorous, the sequence of Dawson hydrolyses (16) was repeated to obtain an estimate of the distribution of phosphonate and phosphate in the diacyl, plasmalogen, sphingolipid and glyceryl ether fractions from oyster polar lipid. These values are displayed in Table III.

The diacyl phosphate lipid was the largest fraction obtained and represented 38.1% of the total phosphorous in oyster tissue phospholipid. The glyceryl ether and diacyl phosphonate lipid fractions were the smallest obtained, representing 2.4% and 2.6%, respectively, of the total phosphorous content in oyster polar lipid. The highest amount of phosphonate phosphorous was found in the sphingolipid fraction and represented 13.5% of the total phospholipid.

It was clear that the distribution of phosphonolipid among the classes was not random. Based on the assays used (Table III), it was estimated that among the phosphorous-containing oyster lipid, 6.5% of the diacyl class, 22.8% of the plasmalogen class, 66.2% of the sphingolipid class and 22.1% of the glyceryl ether class were present as phosphonate.

A number of studies have been concerned with the biosynthesis of lipids containing ether bonds (20). Based on labeling studies with the terrestrial slug, *Arion ater,* Thompson (21) has proposed that the alkyl ethers are precursors to the alk-1-enyl ethers. The similar percentages of phosphonolipid in the plasmalogen and glyceryl ether fractions in oyster lipid (see above) supports the current hypothesis that there is a product precursor relationship between these two lipid classes.

The values shown in Table III represent estimates from a single analysis. When the analysis was repeated similar values were obtained. However it should be pointed out that the Bartlett procedure apparently can hydrolyze some phosphonate bonds. In this respect the amount of $H_2O_2$ used in the digestion step has been reported to be crucial (12). Although we were unable to observe any consistent effect of $H_2O_2$, the values for phosphonolipid in oyster tissue may actually be higher than those observed in this work. Conversely, the amounts of phosphate phospholipid may be slightly lower.

ACKNOWLEDGMENT

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# Formation of Lymph Chylomicron Phosphatidylcholines in the Rat During the Absorption of Safflower Oil or Triolein

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## ABSTRACT

The chylomicron phosphatidylcholines from rats fed safflower oil or triolein were isolated and separated into four different fractions according to the degree of unsaturation. Fraction 1, which was rich in palmitic, stearic and oleic acid, was a minor fraction (7.6-11.6 mole%) during the absorption of safflower oil, but was quantitatively important (27-51 mole%) after triolein feeding when significant amounts of dioleoyl-phosphatidylcholine were present. Fraction 2, which was a major fraction in all the experiments, contained linoleic acid in combination with a saturated or monounsaturated fatty acid. The third fraction contained mainly linoleic acid and was present only after safflower oil feeding. This indicates that dilinoleoyl-phosphatidylcholine is formed in the intestine after ingestion of linoleic acid. Fraction 4, which was rich in arachidonic acid and saturated fatty acids, accounted for 15-20 mole% of the chylomicron phosphatidylcholines with both kinds of fat meals. Incorporation of $^{3}$H-choline indicated that the dilinoleoyl- and dioleoyl-phosphatidylcholines were formed by synthesis de novo while the majority of the remaining phosphatidylcholines originated partly from acylated biliary lysolecithins and partly from the existing pool of mucosal phospholipids not formed during active fat absorption.


## INTRODUCTION

A significant proportion of the ester bound fatty acids transported by the lymph chylomicrons is of endogenous origin. Although bile is the most important source of these endogenous fatty acids during the absorption of a fat-free diet (1,2), it is doubtful if this is true during active fat absorption (3). Endogenous fatty acids are present in all chylomicron ester fractions but are relatively most predominant in the phosphatidylcholines, where endogenous palmitic and linoleic acids are the major fatty acids in the 1 and 2 positions, respectively, independently of the diet fed (4). In the present study phosphatidylcholines from rat chylomicrons obtained after feeding safflower oil or triolein were separated into four fractions of differing degrees of unsaturation, and the incorporation of intravenously injected, labeled choline was determined early after injection. By comparing the specific activities of the different phosphatidylcholine fractions, information

TABLE I

Relative Amounts of Different Phosphatidylcholines in Rat Chylomicrons Isolated From Thoracic Duct Lymph During Fat Absorption[a]

| Treatment of rats | Total phosphatidyl-cholines, % total lipids | Phosphatidylcholine fraction, % total phosphatidylcholines | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| Safflower oil, | 4.8 | 9.6 | 67.0 | 7.9 | 15.5 |
| no bile fistula | 3.8-5.8 | 8.8-10.0 | 64.6-68.3 | 6.8-9.3 | 15.0-16.0 |
| Triolein, | 5.1 | 29.2 | 51.8 | --- | 19.1 |
| no bile fistula | 5.3 | 30.2 | 51.1 | --- | 18.7 |
| Hydrolyzed safflower oil, | 2.5 | 12.5 | 60.0 | 20.6 | 6.9 |
| bile fistula | 2.2 | 78.9[b] | | 10.1 | 11.0 |
| Oleic acid, | 3.0 | 50.0 | 38.8 | --- | 11.2 |
| bile fistula | 2.8 | 38.6 | 49.5 | --- | 11.9 |

[a]The figures shown are individual data for two rats in each group except for the safflower oil fed rats without a bile fistula where mean values and range for three rats are given.

[b]Fractions 1 and 2 were analyzed together due to scarcity of material.

TABLE II

Fatty Acid Composition of the Isolated Phosphatidylcholine Fractions[a]

| Treatment of rats | Phosphatidyl-choline fraction | Fatty acid,[b] mole % | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 20:4 | 22:6 |
| Safflower oil, no bile fistula | 1 | 38.3 | 2.5 | 14.0 | 45.1 | --- | --- | --- |
| | 2 | 25.9 | 0.4 | 17.0 | 7.3 | 49.7 | --- | --- |
| | 3 | 3.7 | 0.8 | 2.1 | 4.1 | 89.4 | --- | --- |
| | 4 | 24.4 | 0.4 | 20.0 | 4.2 | 3.2 | 42.2 | 5.5 |
| Triolein, no bile fistula | 1 | 17.4 | 7.8 | 6.2 | 68.6 | --- | --- | --- |
| | 2 | 23.6 | 1.2 | 12.7 | 14.5 | 48.2 | --- | --- |
| | 4 | 26.9 | 1.0 | 15.2 | 9.6 | 7.5 | 36.4 | 3.2 |
| Hydrolyzed safflower oil, bile fistula | 1 | 23.5 | 7.5 | 5.9 | 63.0 | --- | --- | --- |
| | 2 | 20.2 | 0.9 | 15.9 | 14.3 | 48.8 | --- | --- |
| | 3 | 6.3 | 0.7 | 2.1 | 3.6 | 87.1 | --- | --- |
| | 4 | 23.0 | 0.5 | 17.1 | 8.6 | 4.0 | 35.6 | 11.0 |
| Oleic acid, bile fistula | 1 | 17.7 | 8.4 | 5.0 | 68.7 | --- | --- | --- |
| | 2 | 17.6 | 2.0 | 13.6 | 20.4 | 46.4 | --- | --- |
| | 4 | 20.5 | 1.3 | 14.6 | 12.3 | 19.2 | 24.5 | 7.5 |

[a]Individual data for one animal of each group are shown.

[b]Designated by number of carbon atoms per number of double bonds.

could be gained about the role of mucosal de novo synthesis in the formation of various chylomicron phosphatidylcholines.

## METHODS

### Animals

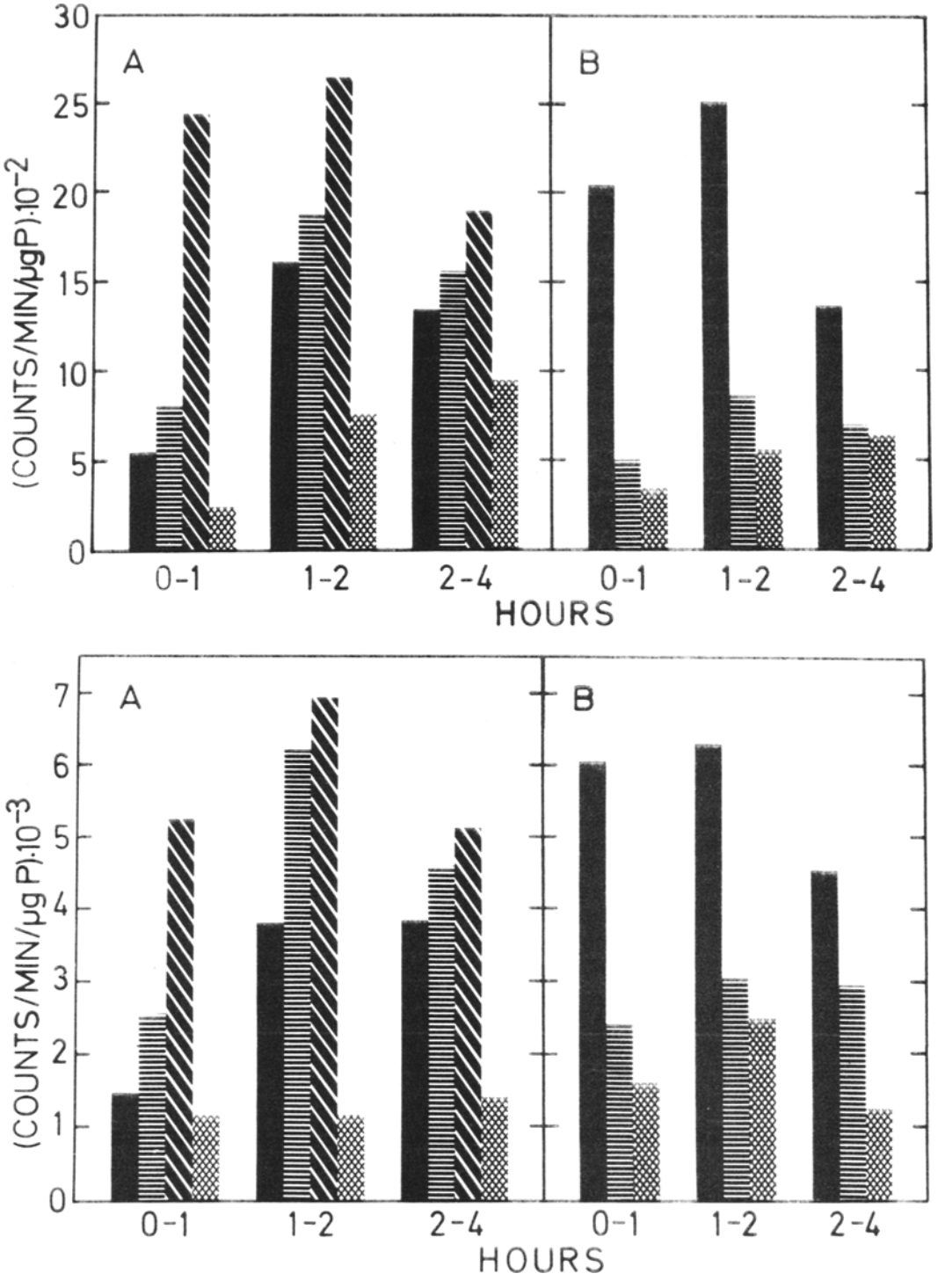
Male white Sprague-Dawley rats were purchased from Anticimex, Stockholm, Sweden. They were kept on a standard pellet diet and weighed 250 g at the beginning of the experiment. The animals had been fasting for ca. 24 hr when the lymph ducts were cannulated under ether anesthesia according to Bollman et al. (5). About 24 hr later three animals received 0.8 ml safflower oil (Fatty acid composition [wt%]: palmitic acid 7.6, stearic acid 2.7, oleic acid 14.2 and linoleic acid 75.6. The oil contained no detectable phospholipids.), and two animals, 0.8 ml triolein. The fat was administered through a stomach fistula. One hour after the administration of fat when the lymph had a milky appearance indicative of active fat absorption, 200 $\mu$c of [Me-$^3$H]choline (200 mc/mmole) obtained from the Radiochemical Centre, Amersham, England, was injected into the jugular vein under ether anesthesia. The animals were then returned to the restraining cages, and lymph was collected. In four other rats with cannulated lymph ducts the bile was drained off through a polyethylene cannula inserted into the bile duct. Via a gastric incision a polyvinyl tubing was inserted into the duodenum for infusion of fatty acid emulsions. The infusion was started 24 hr after the operation. Two rats received an emulsion of oleic acid and the other two rats an emulsion of hydrolyzed safflower oil. The fatty acids were emulsified in a phosphate buffer, pH 7, containing 10 mg sodium taurocholate per milliliter. The rate of infusion was 3 ml/hr corresponding to 100 mg fatty acid per hour. After 4 hr of continuous infusion each rat received 200 $\mu$c of [Me-$^3$H]choline by injection into the jugular vein.

### Isolation of Chylomicrons

In each animal three samples of thoracic duct lymph were analyzed. The samples were collected during the time intervals 0-1, 1-2 and 2-4 hr after choline injection. Chylomicrons were isolated as the creamy supernatant obtained by centrifugation of the lymph at 25,000 g for 30 min in an angle head rotor.

### Analytical Procedures

The chylomicron lipids were extracted with chloroform-methanol 2:1 v/v and washed (6). The lipids, in chloroform solution, were dried over anhydrous sodium sulphate and weighed after evaporation of the chloroform in vacuo. The phosphatidylcholines were isolated by preparative thin layer chromatography (7) and then fractionated according to the degree of unsaturation on the same type of $AgNO_3$-impregnated plates as used before (7). Three fractions were obtained from each sample of the triolein fed rats and four from the safflower oil fed rats. The fractions were eluted from the adsorbent and analyzed for phosphorus and radioactivity as described previously (7). Aliquots of chromatographically identical frac-




FIGS. 1 and 2. Specific radioactivities of phosphatidylcholine fractions 1 (■), 2 (▬), 3 (◣) and 4 (◎) during three consecutive time intervals after injection of [Me-$^3$H]choline. Individual data are shown for each of two rats without a bile fistula in Figure 1 and for two rats with biliary drainage in Figure 2. The fat being absorbed during the experiment was safflower oil in Figure 1A, triolein in 1B, hydrolyzed safflower oil in 2A and oleic acid in 2B.

tions from different time intervals of lymph collection were pooled separately for each rat, and the fatty acid composition was determined (7).

## RESULTS

The mass distribution among the phosphatidylcholine fractions did not change significantly during the collection of lymph. Representative data for one time interval (1-2 hr after the injection of choline) are shown in Table I. The fatty acid composition of the isolated fractions (Table II) was similar in animals belonging to the same experimental group. In the safflower oil fed rats the fastest migrating fraction on the $AgNO_3$-impregnated plates (fraction 1) contained only saturated and monounsaturated fatty acids, whereas phosphatidylcholines containing linoleic acid in combination with a monounsaturated or a saturated fatty acid were collected in fraction 2. Fraction 3 contained the dilinoleoyl-phosphatidylcholine, and fraction 4, phosphatidylcholines of higher degree of unsaturation. Chylomicrons produced by the triolein fed animals contained three phosphatidylcholine fractions, namely 1, 2 and 4, but no measurable amounts of dilinoleoyl-phosphatidylcholine (Tables I and II). Instead some dioleoyl-phosphatidylcholine was present in the chylomicrons from these animals, since fraction 1 contained 68-69 mole% oleic acid.

These data indicated that when large doses of fat, which is rich in linoleic and oleic acid, respectively, is fed, formation of some dilinoleoyl- and dioleoyl-phosphatidylcholine occurs in the intestinal mucosa. Still the extent to which the fatty acid composition of the chylomicron phosphatidylcholines can be changed by feeding different fatty acids is limited. In all rats without a bile fistula the second phosphatidylcholine fraction accounted for 50-70% of the total phosphatidylcholine mass. The fatty acids of this fraction contained 48-50 mole% linoleic acid independent of the type of fat given. In the safflower oil fed rats palmitic and stearic acid accounted for 43-47 mole% of the fatty acids in fraction 2, indicating that in these rats ca. 90 mole% of the phosphatidylcholines in fraction 2 contained linoleic acid in combination with a saturated fatty acid. In the triolein fed rats only 35.8-36.3 mole% of the fatty acids in fraction 2 consisted of saturated fatty acids, while monounsaturated fatty acids accounted for 14.5-15.7 mole%. It can therefore be concluded that in these rats ca. 30 mole% of the phosphatidylcholines in fraction 2 contained linoleic acid in combination with a monounsaturated fatty acid, mainly oleic acid.

Absorption and conversion of biliary lysolecithin to chylomicron-phosphatidylcholines is known to occur (8,9) and could be an important pathway for the formation of phosphatidylcholine fractions 2 and 4, which remained unaffected by changes in the dietary fat. The effect of biliary drainage on the composition of the chylomicron phosphatidylcholines was therefore investigated. Although fractions 2 and 4 were somewhat decreased in these animals, and fractions 1 and 3 concomitantly increased, the changes were not drastic (Table I). Fraction 2 was still the largest fraction, except in one of the oleic acid fed animals in which fraction 1 dominated.

In order to study the relative importance of de novo synthesis in the formation of the various chylomicron phosphatidylcholines, the incorporation of radioactively labeled choline was determined during the period of active fat absorption. Both in the safflower oil and in the triolein fed animals the specific activities of the various phosphatidylcholine fractions differed considerably from each other (Figs. 1 and 2). Different animals belonging to the same experimental group exhibited the same pattern of labeling. The highest specific activities occurred in fraction 3 in the safflower oil experiment and in fraction 1 in the triolein experiment, i.e., those fractions that contained dilinoleoyl- and dioleoyl-phosphatidylcholines, respectively. These phosphatidylcholines were synthesized specifically, depending on the type of fat given, and were probably not diluted to any appreciable extent by preformed phosphatidylcholines of the same specific fatty acid structure. It would then be possible to estimate the role of de novo synthesis for the other, quantitatively dominating fractions by dividing their specific activities at the earliest time interval with that of fraction 3 in the safflower oil experiment and that of fraction 1 in the triolein experiment. According to such calculations for the rats without a bile fistula, only 30% of the total chylomicron phosphatidylcholines originated from synthesis de novo in the safflower oil fed animals and 45% in the rats given triolein. In the rats with a bile fistula the figures varied between 55-90%. However these figures should be regarded only as rather crude estimates, and they are probably too high for the following reasons. Firstly some incorporation into the chylomicron phosphatidylcholines of labeled blood phospholipids, formed by other organs, cannot be excluded even during the earliest time interval after the injection of labeled choline. Secondly in the triolein experiments, the true specific radioactivity of the dioleoyl-phosphatidylcholine in fraction 1

might well have been higher than that of the entire fraction 1 which also contained appreciable quantities of other phosphatidylcholines.

## DISCUSSION

The present study confirms the earlier finding of Whyte et al. (4) that the fatty acid composition of rat chylomicron phosphatidylcholines can be significantly changed by altering the fatty acid composition of the ingested fat, but only within certain limits. The two phosphatidylcholine fractions which contain linoleic acid (fraction 2) or arachidonic acid (fraction 4) in combination with a saturated or unsaturated fatty acid were quantitatively important chylomicron constituents after feeding either triolein or safflower oil. However 20-30% of the chylomicron phosphatidylcholine was different in the triolein fed and in the safflower oil fed animals. The appearance of dilinoleoyl-phosphatidylcholine after the ingestion of safflower oil is of particular interest, since this fraction has not earlier been found in significant amounts in mammalian tissues (10). This fraction had a very high specific activity after the $^{3}$H-choline injection. It is therefore most likely that it was formed mainly from the reaction between the proper diglyceride precursor and CDP-choline. The presence in rat intestinal mucosa of the enzymes necessary for this conversion is well established (11). In vitro studies of this enzyme system in rat liver have not revealed any specificity towards certain diglycerides (12). In vivo, the intestinal enzyme system seems to share this property since the incorporation of radioactive choline into the various chylomicron phosphatidylcholines was very much dependent on the fatty acid composition of the absorbed fat. It is therefore unlikely that the quantitative dominance of fractions 2 and 4 is due to a selective utilization of specific diglycerides by the intestinal mucosa in synthesis de novo of chylomicron phosphatidylcholines. Incorporation of biliary lysolecithin into chylomicron phosphatidylcholines is known to occur and to increase during active fat absorption (8,9). However this pathway and synthesis de novo cannot fully account for the dominance of fractions 2 and 4, since these fractions were quantitatively important and had a low specific radioactivity also in rats with biliary drainage. Nor is it likely that the phosphatidylcholines in fractions 2 and 4 were formed by methylation of phosphatidylethanolamines, since this pathway for phosphatidylcholine biosynthesis is of minor importance in the intestine (13,14). It therefore seems probable that a significant portion of the chylomicron phosphatidylcholines was not formed during the short period of active fat absorption but originated from the existing pool of mucosal phospholipids. A recent work by Yurkowski and Walker (15) showed that phosphatidylcholine species corresponding to our fractions 2 and 4 are normally present in rat intestinal mucosa.

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# Stearyl CoA as a Precursor of Oleic Acid and Glycerolipids in Mammary Microsomes From Lactating Bovine: Possible Regulatory Step in Milk Triglyceride Synthesis

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**ABSTRACT**

The stearyl desaturase of lactating bovine mammary tissue is located in the microsomes and requires activated fatty acid and NADH for activity. Other enzymes, acyl-transferase(s) and deacylase which apparently compete with the desaturase for substrate are also present. Both the substrate 1-$^{14}$C-stearyl CoA and the oleic acid produced by desaturase are esterified into the various lipid classes. The oleic acid is preferentially acylated into position *sn*-3 of the triglycerides and *sn*-2 of the phosphatidylcholine. Experimental conditions causing reduced desaturase activity depressed triglyceride synthesis, and stimulation of desaturation by NADH, L$^{\alpha}$ GP, acidic pH, 5.6, was accompanied by increased incorporation of radioactive fatty acids into the triglycerides. These data indicated that desaturase and glyceride acyl transferase were located contiguously within the microsomal membranes. The possibility that desaturase activity might control triglyceride synthesis in vivo is discussed. It was observed that mammary tissue from nonlactating cows 1-2 weeks and 2 days prior to calving lacked or possessed very low stearyl desaturase activity.

Oleic acid is the principal unsaturated acid in bovine milk, and its concentration and intramolecular distribution may be very significant in reducing the melting point of bovine milk fat and maintaining it in a fluid condition at physiological temperatures (1). The quantity of *cis* octadecenoic acid in milk is variable ranging from 50-70% of the total octadecenoic acid present (2). It is estimated that 30-40% of the *cis* isomer, i.e., oleic acid, is synthesized in the mammary tissue by the action of the anzyme stearyl desaturase on absorbed exogenous stearic acid (1-5). The possible importance of exogenous stearic acid and its metabolism in lactating bovine has been alluded to by some authors (6-8,34) in that it may directly or indirectly regulate triglyceride synthesis. The possible essential role of oleic acid in milk fat synthesis has been discussed (1). In the present paper the finding that stearyl desaturase activity affects triglyceride synthesis by mammary microsomes in vitro corroborates the above intimations.

## MATERIALS AND METHODS

Mammary tissue was obtained from lactating (30/40 lb. milk per day) Holstein cows following slaughter. The tissue was minced in a meat grinder, diluted 1:2 in 66 mmoles potassium phosphate buffer pH 7.4, and homogenized for 30 sec in Waring blendor. The homogenate was rehomogenized for 30 sec in a small mill (Polyscience Corp., Ill.) and filtered through cheese cloth. Temperature was kept at 4 C. The filtrate was sequentially centrifuged at 5000 g for 10 min, 10,000 g for 15 min, and 100,000 g for 60 min, to remove cell debris and mitochondria and other material and to recover microsomes in the final step (31). The microsomal pellet was washed with buffer and then freeze-dried and stored at -30 C.

The standard assay system consisted of 66 mmoles potassium phosphate buffer pH 7.4, 150 nmoles NADH, 50 nmoles 1-$^{14}$C-stearyl CoA and approximately 5 mg microsomal protein in 2 ml. Incubations were carried out at 37 C in a Rotamix shaker using capped culture tubes. The reaction was stopped after 15 min by extraction of lipids by the procedure of Folch et al. (9).

Nonlactating mammary tissue obtained by biopsy from pregnant cows, just prior to parturition, was minced in two volumes of tissue culture media 199 (Grand Island Biological Co., N.Y.) under aseptic conditions. Two milliliter aliquots of this homogenate were incubated with 50 nmoles Na-1-$^{14}$C-stearate (20 $\mu$c/$\mu$mole) for 2 hr after which the lipids were extracted (9).

The lipids were separated by thin layer chromatography (TLC) into the respective neutral lipid and phospholipid (PL) classes (1,10,11). The radioactivity in specific classes was determined by liquid scintillation counting (Packard Tri Carb). Radioactivity in the fatty acids was quantified by radio gas chromatography (1). Desaturase activity was measured by

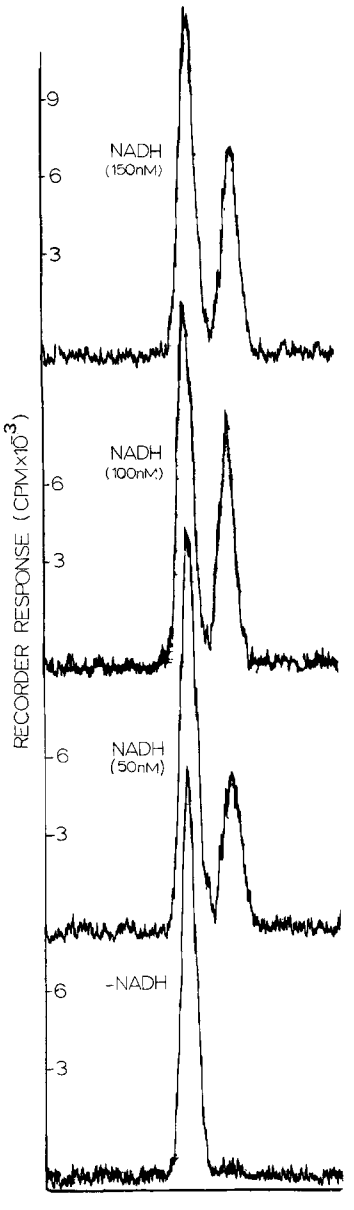


FIG. 1. Radiogas chromatograms of the methyl esters prepared from total lipid extracts of bovine mammary microsomes showing the effect of NADH on the desaturation of 1-$^{14}$C stearyl CoA to $^{14}$C-oleic acid.

determining the quantity of the substrate 1-$^{14}$C-stearyl CoA (C18CoA) converted to labeled oleic acid (1), hereinafter referred to as endogenous oleic acid.

To determine the distribution of the labeled oleic acid (C18:1), the triglycerides (TG) and the phosphatidylcholine (Pc) isolated by TLC were enzymatically hydrolyzed by the methods of Luddy et al. (12) and Wells and Hanahan (13), respectively, and the products were analyzed as previously described (1). Protein was quantified by micro-Kjeldahl (14) or Lowry (15) method.

Prepared radiochemicals were purchased from New England Nuclear (Boston, Mass.) and reliable NADH was obtained from Sigma (Sigma Chemicals, St. Louis, Mo.).

## RESULTS

Of the various subcellular components tested only the particulate fraction sedimented at 100,000 g, i.e., microsomal fraction, possessed stearyl desaturase activity. In the standard incubations used throughout this study desaturase activity varied among four different cows, but a mean value of 31±7% desaturation of substrate stearyl-CoA was obtained (20 incubations). In preliminary experiments when 1-C$^{14}$-stearyl CoA was replaced by 50 nmole Na-1-$^{14}$C-stearate and appropriate amounts of cofactors (ATP, CoASH, NADH Mg $Cl_2$) desaturation was only 7%, indicating low thiokinase activity in these microsomal preparations.

Chemically pure NADH was essential for stearyl desaturase (Fig. 1). The concentrations cited in Figure 1 are approximate. Under optimum standard conditions a maximum 38% of the substrate 1-$^{14}$C-stearyl CoA was desaturated to oleic acid.

Analyses of the microsomal lipids revealed that both the substrate $^{14}$C stearyl CoA and the endogenous labeled oleic acid were mostly esterified. The TG and PL contained most of the radioactivity (Tables I and II). The data (Table I) indicate that the microsomes contained active acyl transferases and deacylase. Because of their apparent propinquity and concurrent activity both the desaturase and acyl transferase(s) enzymes were monitored, and the experimental results are tabulated together.

The distribution of radioactive stearic and oleic acid in various lipids from a representative standard incubation is shown in the radiogas chromatograms (Fig. 2) The observation that the TG and Pc contained the preponderance of the endogenous oleic acid is noteworthy. The 1,2 diglycerides and phosphatidylethanolamine (Pe) had low levels of labeled oleic acid. The ratio of stearic to oleic acid in the free fatty acids reflects that in the total lipid extract.

A number of experimental variables influenced the production of oleic acid and its incorporation into specific glycerolipids (Table II). The total extent of desaturation and acylation in the standard incubations were quite

TABLE I

The Distribution of Radioactivity in the Lipid Classes of Bovine Mammary Microsomes[a]

| Lipids | Percentage distribution | |
|---|---|---|
| | Standard | Standard minus NADH |
| Phospholipids | 48 ± 8.6 | 67.4 |
| Diglycerides | 4.6 ± 2.0 | 5.2 |
| Free fatty acids | 15 ± 3.1 | 11.0 |
| Triglycerides | 29 ± 8.2 | 16.3 |
| Cholesterol ester | 0.1 ± 0.1 | 0.1 |
| Amount of substrate esterified, % | 85 | 89 |

[a]Following incubation with 1-C$^{14}$-stearyl CoA under standard conditions with and without NADH.

consistent with exception of experiment IV. Replacement of 0.5 ml of buffer with an equal volume of supernatant caused a ca. 33% decrease in TG synthesis and an equal depression in extent of desaturation within the triglycerides. There was a concomitant increase in amount of radioactivity in the phospholipids though desaturation therein decreased slightly.

Omission of NADH, which eliminated desaturase activity (experiment II and Fig. 1), markedly reduced triglyceride labeling, whereas the labeling in the phospholipids was increased by 35%. L-α-Glycerolphosphate (20 nmole) stimulated both triglyceride synthesis and desaturase activity and enhanced acylation of endogenous oleic acid into the triglycerides, whereas PL synthesis was depressed.

Acidic incubation conditions reduced transacylase activity, especially with respect to the phospholipids. Desaturation was not impaired, and the quantity of endogenous oleic acid incorporated into the triglycerides actually increased slightly. Alkaline conditions decreased the activities of both desaturase and acyl transferase(s).

These data (experiments I-IV) revealed that the incorporation of radioactivity into the triglycerides was stimulated when desaturase activity was enhanced, whereas diminution or elimination of desaturase activity enhanced incorporation of label into the phospholipids.

Because the preponderance of the endogenous $^{14}$C-oleic acid in the PL was associated with the Pc, the distribution of radioactivity in the PL in absence of endogenous oleic acid was examined (Table III). Significantly even though there was a marked increase in PL acylation (Table II), there was a diminution in Pc labeling and a marked increase of radioactive stearic acid in Pe and Pi. There two classes normally had low levels of endogenous oleic acid as shown (Fig. 2). These data may indicate that the availability of endogenous oleic acid may also influence the synthesis of Pc in mammary

TABLE II

The Effects of Experimental Factors on the Extent of Acylation and Desaturation of 1-$^{14}$C-Stearyl CoA[a]

| Experimental conditions | Amount esterified, nmoles | | Amount desaturated, nmoles | |
|---|---|---|---|---|
| | Triglycerides | Phospholipids | Triglycerides | Phospholipids |
| Experiment I | | | | |
| Standard | 15.0 | 19.5 | 8.7 | 7.4 |
| +Supernatant | 10.5 | 23.5 | 5.5 | 7.0 |
| Experiment II | | | | |
| Standard | 14.1 | 24.0 | 8.1 | 6.9 |
| -NADH | 8.1 | 33.7 | trace | 0 |
| Experiment III | | | | |
| Standard | 16.0 | 23.0 | 8.8 | 6.3 |
| +L-α-Glycerolphosphate | 21.5 | 16.1 | 12.9 | 5.3 |
| Experiment IV | | | | |
| Standard (pH 7.2) | 12.5 | 18.0 | 7.2 | 7.4 |
| pH 5.6 | 9.6 | 9.0 | 8.5 | 6.0 |
| pH 8.3 | 5.0 | 10.0 | 3.3 | 3.4 |

[a]By bovine mammary microsomes incubated under standard conditions (see Methods).

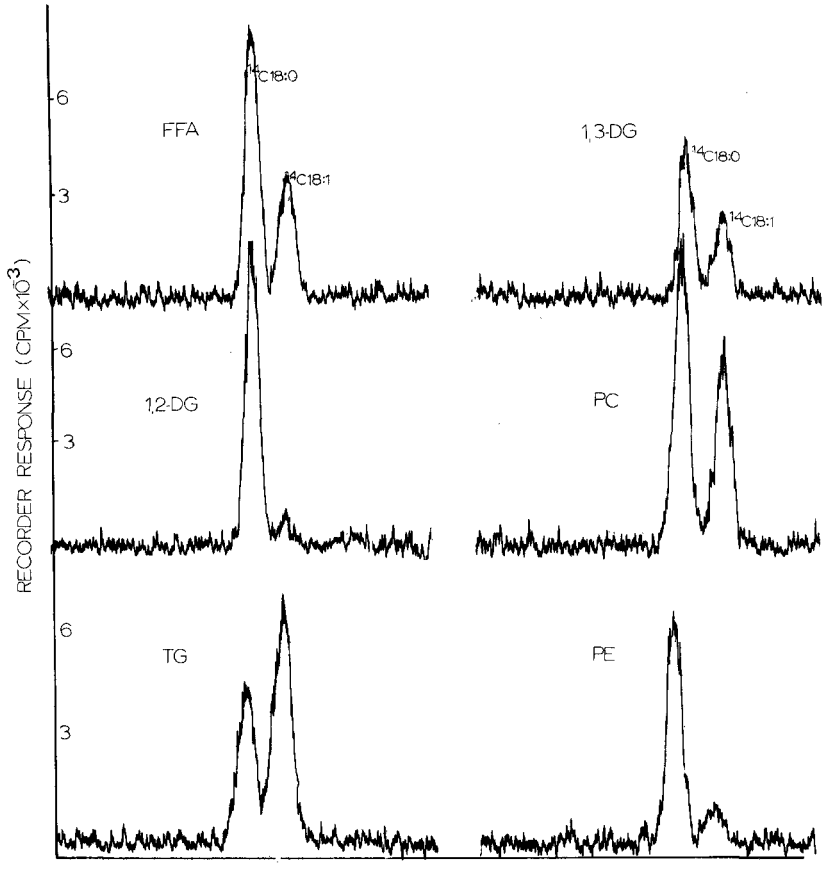


FIG. 2. Representative radiogas chromatograms showing the location and relative concentration of radioactivity in the various lipid classes, isolated following the incubation of bovine mammary microsomes with 1-$^{14}$C-stearyl CoA under standard conditions. The proportion of endogenous radioactive 1-$^{14}$C-oleic acid in these classes varied, i.e., free fatty acids (FFA) 32; 1,2 diglycerides (1,2DG) 13; 1,3 diglycerides 1,3DG) 27; triglycerides (TG) 60; phosphatidylcholine (Pc) 43; phosphatidylethanolamine (Pe) 16 and phosphatidylinositol (not shown) 5%, respectively.

tissue.

The intromolecular distribution of endogenous oleic acid was revealed following lipolysis (Fig 3). In the TG most of the $^{14}$C-oleic acid was in the primary positions of the glyceride glycerol. Assuming that these triglycerides were synthesized from the 1,2 DG pool (Fig. 2), then conceivably most of the labeled oleic acid was on position *sn*-3 of the triglycerides. Endogenous oleic acid was concentrated in position *sn*-2 of the Pc.

Because stearyl desaturase, which is a mixed function oxidase (16), is inducible, it may be influenced by the endocrinological status of the cow. Hence we compared the desaturase activity in mammary tissue from pregnant nonlactating and lactating cows which were on similar diets. Because of the small amounts of tissue procured by biopsy, homogenates rather than microsomes were incubated with Na-1$^{14}$C stearate (Fig. 4). The homogenate from the lactating tissue possessed much more active desaturase and acyl transferase(s) than the tissues from the nonlactating cows. The mammary homogenates from the cow 1-2 weeks prepartum lacked desaturase activity completely.

## DISCUSSION

These data demonstrate that the stearyl desaturase is located in the microsomes of lactating bovine mammary tissue, and its cofactor requirements are quite similar to the desaturase of rat and hen liver (17-23), rat adipose (24), goat mammary (2,5), fungal microsomes (25), plants and bacteria (16). These mammary microsomes also possessed active acyl trans-

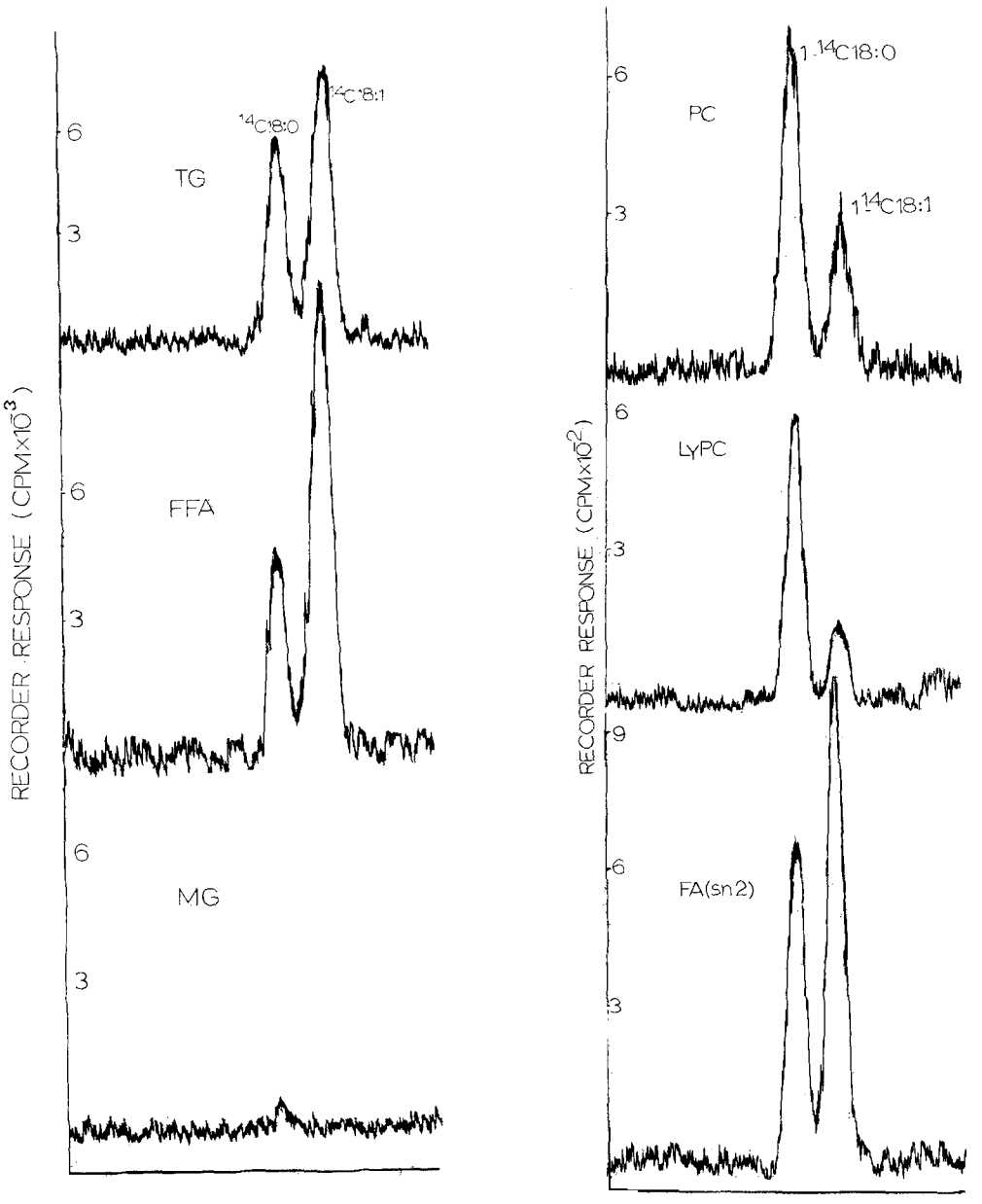


FIG. 3. Radio gas chromatograms showing the relative concentrations and positional distribution of radioactive stearic and oleic acid in triglycerides (TG) and phosphatidylcholine (Pc) isolated following the incubation of bovine mammary microsomes with 1-$^{14}$C-stearyl CoA. The original lipids, i.e., triglycerides (TG) and phosphatidyl-choline (Pc) were hydrolyzed with lipases (see methods) and the radioactive methyl esters of the products, separated by thin layer chromotography, were analyzed by radio gas chromatography.

ferases and deacylase. Probably these enzymes competed for available substrate as indicated by the consistent acylation and deacylation of stearyl CoA even when the acyl desaturase was inactivated. The apparent affinity of the transacylase enzymes for activated stearic and oleic acid differed with the glyceride acyl transferase preferring the endogenous oleic acid, whereas the phosphatide acyl transferase apparently preferred stearic acid.

The selectivity may be attributed to chemical specificity or to the juxtaposition of these enzymes in the microsomal membranes. Baker and Lynen (25) suggested some models

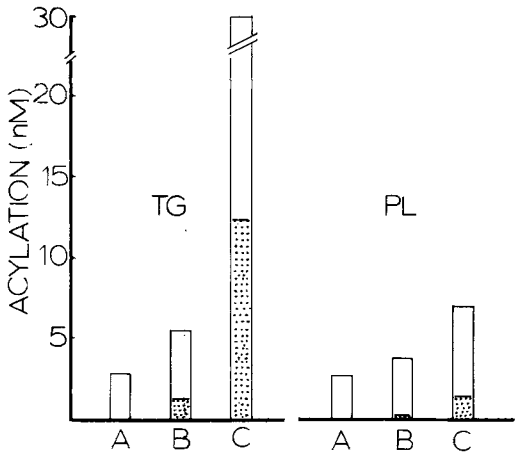


FIG. 4. Histogram showing the extent of esterification and desaturation (stippled areas) of Na-1-$^{14}$C-stearate by homogenates of bovine mammary tissue from non-lactating cows ca. 1-2 weeks (A) and 2 days (B) weeks prior to calving, and lacting cow (C) ca. 3 months after calving. TG and PL denote the triglycerides and phospholipids recovered from the incubated tissue.

pertaining to this with respect to fungal microsomal desaturase. In the latter the endogenous oleic acid is placed preponderantly in the PL, whereas in the bovine microsomes it is esterified into TG and PL and when more oleic acid is produced more TG is made. In the present context the negligible effect of acidic pH on desaturase and triglyceride acylation may be construed to suggest that the desaturase and glyceride acyl transferase enzymes are located close together in the interior of the mammary microsomal membranes, where because of its hydrophobicity the effects of H ion concentration on functional charged groups are minimized (26). Experimental evidence showing that lipids are necessary for desaturase activity (22) would support the above suggestion. Conceivably, as might be rationalized from surface chemistry requirements, the phosphatide acyl transferases are situated in the surface region of the membranes, and consequently they utilize the substrate stearyl CoA more effectively for PL synthesis. The closely parallel effects of various incubation conditions on desaturase activity and triglyceride synthesis in the present study are consistent with this suggestion. Attempts to isolate the individual enzymes from this complex are in progress to determine if this proposition is valid.

The depression of desaturation and glyceride synthesis by supernatant fractions was also observed in goat mammary microsomes (2). This could be explained by several factors in

TABLE III

Distribution of Radioactivity in the Phospholipids[a]

| Lipid class | Percentage distribution of radioactivity: Standard incubation +NADH | Standard incubation -NADH |
|---|---|---|
| Lysophosphatidylcholine | 1.1 | 1.2 |
| Sphingomyelin | 2.0 | 1.3 |
| Phosphatidylcholine | 46.5 | 31.1 |
| Phosphatidylserine | 4.2 | 3.6 |
| Phosphatidylethanolamine | 26.7 | 39.3 |
| Phosphatidylinositol | 19.5 | 23.5 |
| Per cent of substrate esterified[b] | 46 | 67 |

[a]Following the incubation of bovine mammary microsomes with 1-$^{14}$C-stearyl CoA in presence and absence of NADH.

[b]In phospholipids

the supernatant, i.e., added free fatty acids which inhibited the desaturase (2), additional PL-acyl transferase activity, and/or the progress of reactions which consumed the available NADH.

The finding that both desaturase activity and triglyceride labeling varied in a corresponding manner may be significant and help to explain some observations made with regard to milk glyceride synthesis in vivo (6,7). The relatively consistent structure of milk fat and nonrandom pattern of fatty acid acylation reflects an orderly assembly of milk glycerides in presence of the proper concentration and ratios of fatty acids. On the basis of experimental data a number of authors have concluded that mammary triglyceride synthesis is somehow controlled by the particular types and concentrations of long chain fatty acids available to the synthetic enzymes, and in the absence of these conditions triglyceride synthesis is depressed (6,7,34). Thus in cows on special concentrate rich diets, changes in the availability of stearic acid have been related to depressed milk fat production (7). Because in the present study triglyceride synthesis is reduced when desaturase activity is depressed, it is possible that in vivo the availability of a specific quantity of endogenous oleic acid may be the critical factor in facilitating or limiting triglyceride production. Thus any factor which influences the activity of desaturase, i.e., availability of activated precursor stearic acid, local NADH levels, and the inherent enzyme concentration may theoretically regulate triglyceride synthesis.

Steele et al. (27) have reported that feeding stearic acid to cows increased the secretion of oleic acid and milk fat yield, and Askew et al. (8) showed that stearic acid stimulated esterifi-

cation of fatty acids by homogenates of lactating mammary tissue. Both of these effects may have been mediated via the action of the stearyl desaturase.

The intramammary levels of NADH could act as a potent regulator of desaturase activity and thereby influence milk glyceride production. The enhanced incorporation of stearic acid into PL in absence of NADH (and hence desaturase activity) is consistent with the possible competition of the stearyl desaturase and PL acyl-transferase for the substrate. The concomitant depression in TG synthesis may be relevant to observations of changes in fat synthesis in certain in vivo situations. Conceivably under dietary conditions, e.g., high concentrate diets, and/or specific physiological conditions, e.g., ketosis, glucose and acetate concentrations in mammary tissue may be reduced with a subsequent drop in production of reducing equivalents (NADH, NADPH) (28,34). This would impair desaturase function and thereby limit milk triglyceride production.

Dietary and physiological factors, i.e. fasting and diabetes, may directly affect the level of desaturase in mammary tissue as occurs in the rat (24,29,30).

The effect of LαGP in stimulating desaturation and glyceride synthesis in the bovine microsomes may reflect the availability of extra acyl acceptors with consequent reduction of desaturase inhibition by the free fatty acids present in the microsomes (8). Goat mammary microsomes showed a similar response (2). The effect of LαGP on microsomal desaturase in vitro may obtain in vivo in mammary tissue of cows on high concentrate diets, though in these tissues LαGP concentration is quite high (34).

The intramolecular distribution of endogenous oleic acid in TG and Pc is similar to that obtained using mammary cells in vitro and that found in milk, and further indicates that mammary microsomes possess the enzymes required for milk fat synthesis (1,31,32).

The apparent absence of the stearyl desaturase in nonlactating tissue may reflect the inducible nature of this particular enzyme or of some of its components (24,29,22). The fact that stearyl desaturase of rat liver responds to insulin may indicate that the desaturase of bovine mammary tissue requires the lactogenic hormones for activity. However the difficulty in interpreting this type of data especially in bovine mammary tissue has been discussed by Baldwin (33).

ACKNOWLEDGMENTS

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# Cuticular Lipids of Insects: V. Cuticular Wax Esters of Secondary Alcohols From the Grasshoppers *Melanoplus packardii* and *Melanoplus sanguinipes*[1]

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## ABSTRACT

Wax esters of secondary alcohols constitute 18-20% of the cuticular lipid extract of *Melanoplus packardii* and 26-31% of the cuticular lipids of *Melanoplus sanguinipes*. The total number of carbons in the wax esters range from 37-54 with 41 predominating in both species. The fatty acids of *M. packardii* wax esters are 16:0, 18:0, 14:0, 20:0 and 12:0 in decreasing quantity. The fatty acids of *M. sanguinipes* wax esters are 18:0, 20:0, 16:0, 22:0, 14:0, 19:0 and 17:0 in decreasing quantity. The secondary alcohols from the wax esters of *M. packardii* are $C_{25}$, $C_{23}$ and $C_{27}$ in decreasing quantity, and the secondary alcohols of the *M. sanguinipes* are $C_{23}$, $C_{25}$, $C_{21}$, $C_{27}$, $C_{24}$, $C_{22}$ and $C_{26}$ in decreasing quantity. Each secondary alcohol consists of two to four isomers with the hydroxyl group located near the center of the chain.

## INTRODUCTION

A material with a unique $R_f$ value on a silica gel thin layer plate was found in the outer surface lipids of *Melanoplus sanguinipes* defectus (Walker) (1). They found here the material chromatographed with an $R_f$ value that was greater than that of a normal wax ester but less than the $R_f$ value of paraffin hydrocarbons. Infrared spectrophotometry showed the material to have ester linkages, and gas chromatography showed it to consist of four major compounds. Mild bromination did not alter the gas chromatogram, indicating that the esters were not unsaturated. Saponification gave acid and alcohol moieties which were confirmed by IR spectrophotometry. A combination of thin layer chromatography (TLC) and gas liquid chromatography (GLC) showed the acid moiety to be mainly octadecanoic acid, but small amounts of hexadecanoic, heptadecanoic and eicosanoic acids were also present. The IR spectra and TLC data indicate that the alcohol moieties were not primary alcohols and therefore might be secondary or teriary alcohols. Likewise, the branching from the secondary or tertiary alcohols may interfere with the adsorption of the esters on the silica gel thin layer plate and thereby display greater $R_f$ values than normal wax esters.

We now report the investigation of the cuticular lipid wax esters of *Melanoplus packardii* and *Melanoplus sanguinipes*. The wax esters were hydrolyzed, and the structures of the acids and the secondary alcohols with a hydroxyl group near the middle of the chain are reported here. To our knowledge this type of wax ester or secondary alcohol has not been previously characterized from insect cuticular lipids.

## EXPERIMENTAL PROCEDURES

*Melanoplus packardii* and *Melanoplus sanguinipes* were collected from wild populations in southwestern Montana. The insects were sacrificed at the time of collection by freezing with dry ice and stored at −29 C until needed.

Only intact specimens were used, thereby lessening possible contamination from internal lipids. Time studies indicated that slurrying the insects for up to 15 min in hexane provided a good yield of surface lipids without removing internal lipids.

The surface lipids were extracted by slurrying for 15 min in hexane. The extract was filtered through glass wool and then evaporated to dryness and weighed. The surface lipids (200-700 mg) were then chromatographed on a 2.5 x 18 cm column filled with silicic acid (Biosil A). An eight-fraction stepwise elution solvent system consisted of: (a) 100 ml hexane; (b) 100 ml hexane; (c) hexane-chloroform 95:5 ml; (d) hexane-chloroform 90:10 ml; (e) hexane-chloroform 90:20 ml; (f) hexane-chloroform 80:40 ml; (g) 100 ml chloroform; and (h) chloroform-methanol 67:33 ml. Fraction four contained the unique wax esters.

Purity of the wax esters was vertified on analytical TLC plates developed in chloroform-hexane 50:50 ml. Analytical GLC of the unique wax esters was carried out on a 2% SE-54 on Gas Chrom Q column 5 ft x 1/8 in. programed

[1]Montana Agriculture Experiment Station, Journal Series No. 332.

TABLE I

Composition of Cuticular Wax Esters of Secondary Alcohols

| Number of carbons | Melanoplus packardii, area % | Melanoplus sanguinipes, area % |
|---|---|---|
| Wax esters | | |
| 37 | 1 | Trace[b] |
| 39 | 16 | 18 |
| 40 | Trace | Trace |
| 41 | 68 | 38 |
| 42 | Trace | Trace |
| 43 | 15 | 27 |
| 44 | ---[a] | Trace |
| 45 | Trace | 11 |
| 47-54 | --- | Trace |
| Resulting fatty acids | | |
| 12:0 | 1 | --- |
| 14:0 | 11 | 4 |
| 15:0 | 2 | --- |
| 16:0 | 63 | 11 |
| 17:0 | Trace | 1 |
| 18:0 | 14 | 48 |
| 19:0 | --- | 1 |
| 20:0 | 9 | 28 |
| 21:0 | --- | 1 |
| 22:0 | --- | 5 |
| Resulting secondary alcohols | | |
| 21 | Trace | 12 |
| 22 | --- | 2 |
| 23 | 15 | 59 |
| 24 | --- | 2 |
| 25 | 82 | 20 |
| 26 | --- | 1 |
| 27 | 2 | 3 |
| β-Amyrin | --- | 2 |

[a]Indicates not detectable at the 0.1% level.

[b]Trace equals less than 1% but greater than 0.1%.

from 225-300 C. Area per cent was obtained by disc integration. Retention times were compared with those of a standard normal wax ester mixture.

The unique esters were reduced with lithium aluminum hydride (LAH) (2), and saponified with 5% KOH in ethanol mixed with hexane 2:1. The products of both reactions were separated by preparative TLC in chloroform-hexane 50:50 ml.

Analytical GLC of the alcohol fractions was carried out on a 4 ft x 1/8 in. 1% SE-30 column programed from 150-250 C.

The alcohol fraction which migrated higher in the TLC system than normal alcohols was oxidized with Jones (3) reagent. This alcohol fraction was separated and collected by using preparative GLC with a stream splitter on a 6 ft x 1/8 in 1% SE-30 column step programed from 140-180 C.

Synthesis of 12-tricosanone was carried out by pyrolysis of the barium salts of dodecanoic acid (4).

Mass spectra were obtained with a Varian CH5 instrument operating at ionizing voltage of 70 eV, current 100 μA. The samples were inserted into the ion source from a gold crucible.

The fatty acids from the ethanolic KOH saponification were methylated by the method of Schlenk and Gellerman (5), and gas chromatographed on a 6 ft x 1/4 in. 15% EGS (ethylene glycol succinate) column at 165 C. The retention times were compared to authentic standards. Integration was obtained by a disc integration.

## RESULTS

The hexane-extracted cuticular lipids from both *M. packardii* and *M. sanguinipes* amounted to ca. 0.5 mg per insect. Of this the wax esters of secondary alcohols amounted to 18-20% on *M. packardii* and 26-31% on *M. sanguinipes*. The wax esters of secondary alcohols are simpler from *M. packardii* than from *M. sanguinipes*. The gas chromatogram from *M. packardii* shows one major peak at 41 carbons, lesser peaks at 39 and 43 carbons and only trace quantities at 37, 40, 42 and 45 carbons for a range of 37-45 carbons (Table I). The wax esters from *M. sanguinipes* range from 37-54

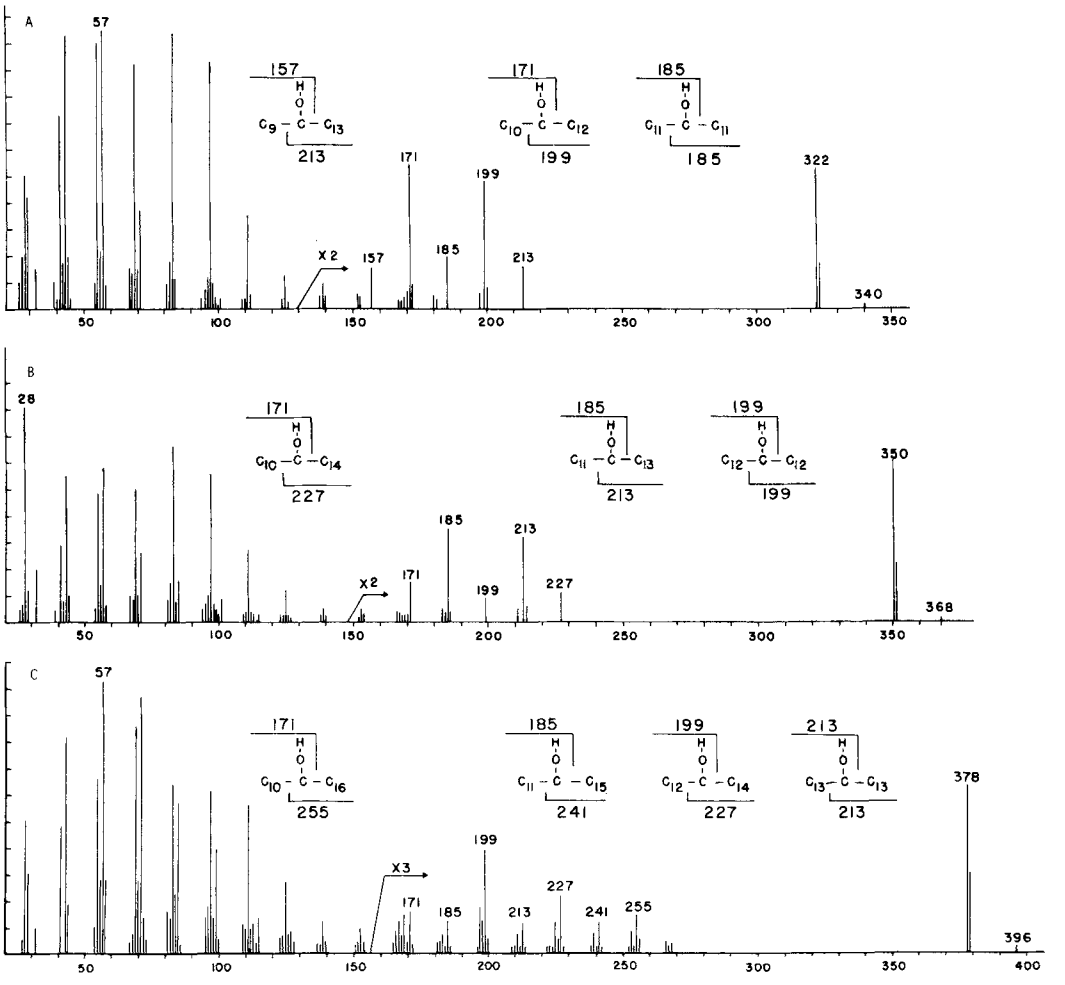


FIG. 1. Mass spectra of secondary alcohols from the secondary alcohol wax esters of *Melanoplus packardii*.

carbons (Table I), again with the major peak at 41 carbons, lesser peaks at 39, 43, and 45 carbons and trace quantities at 37, 40, 42, 44 and 47-54 carbons. The wax esters in the 47-54 carbon range are very likely esters of normal secondary alcohols and long chain fatty acids, as well as esters of $\beta$-amyrin found in the secondary alcohols of *M. sanguinipes* (Table I); because of this mixture, the GLC peaks are not well defined and were therefore not accurately measurable.

Saponification of both secondary alcohol wax ester samples yielded only saturated fatty acids (Table I). The fatty acid compositions of the two insects were different. The major fatty acid from *M. packardii* is palmitic acid 16:0 with 77% of the fatty acids being palmitic acid or shorter; however the major fatty acid from *M. sanguinipes* is stearic acid 18:0 with 83% of the fatty acids being stearic acid or longer. The results from the GLC of the methylated fatty acids from saponification and from the GLC of normal alcohols from LAH reduction gave similar quantitation for the fatty acid composition.

The alcohol portion of the wax esters under consideration was found to be a series of aliphatic secondary alcohols, most of which have an odd number of carbons (Table I). Jones oxidation (3) of this fraction gave aliphatic ketones, some of which were subjected to mass spectrometry to substantiate the structures of the secondary alcohols. The GLC retention times of these secondary alcohols are quite different from normal alcohols, with the secondary alcohol retention time being almost one carbon unit shorter than a normal alcohol under the same conditions.

The mass spectrum of each secondary alcohol peak shows that the sample is a mixture of

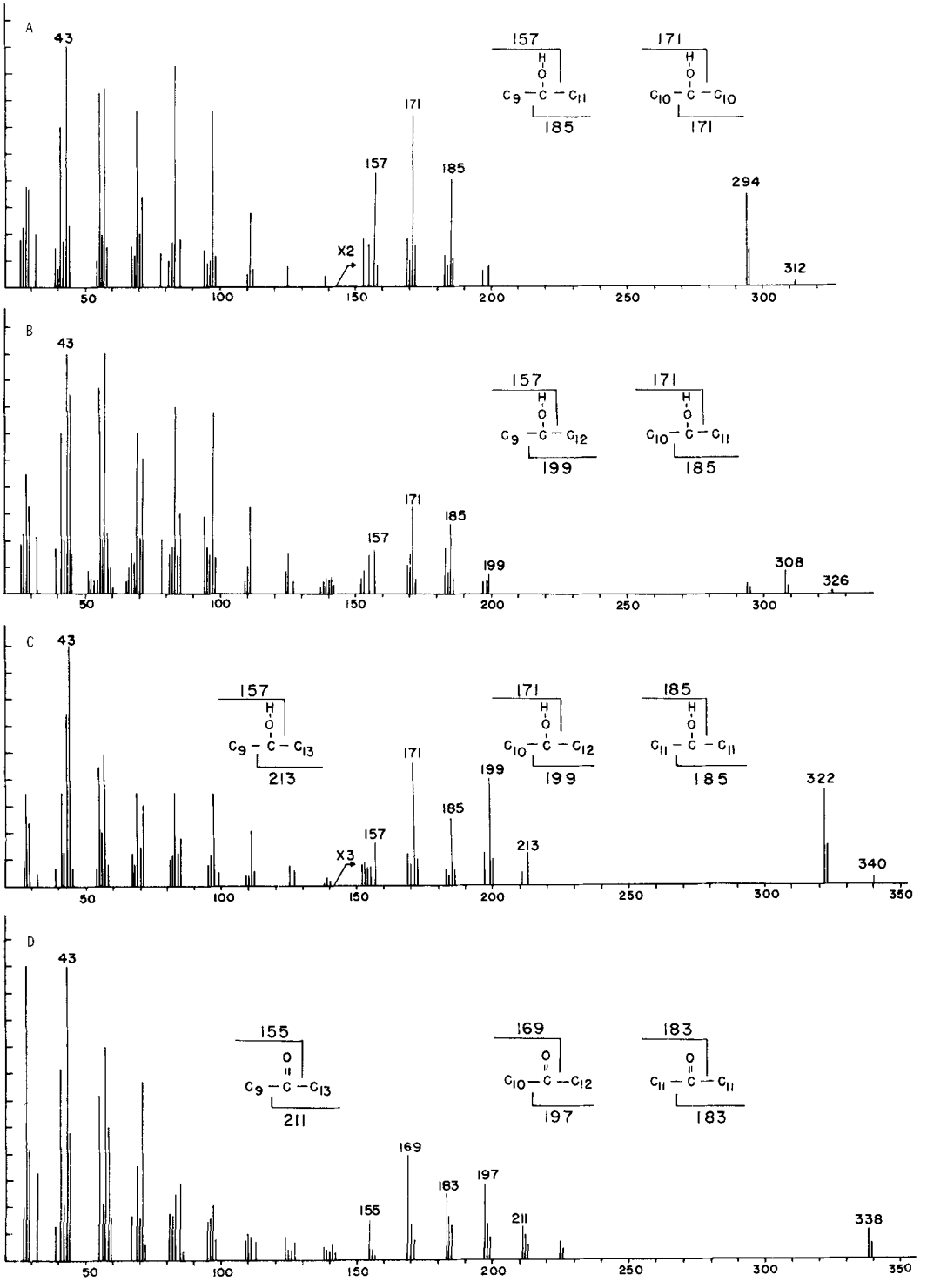


FIG. 2. Mass spectra of secondary alcohols from the secondary alcohol wax esters of *Melanoplus sanguinipes*. Spectra D is of the ketones resulting from oxidation of the secondary alcohols in spectra C. Sequence continues on following page.

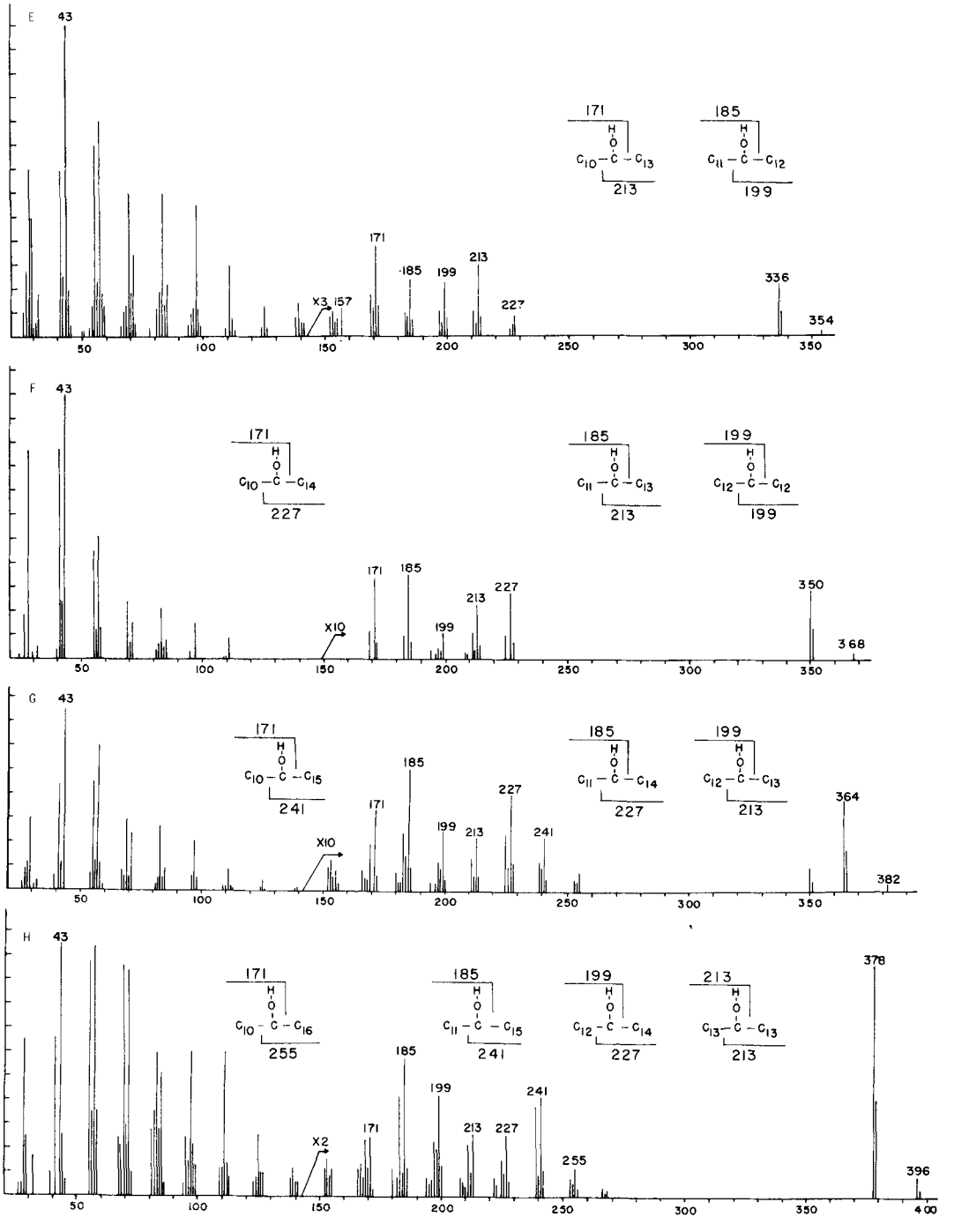


FIG. 2. Sequence continued from previous page.

two to four isomers with the hydroxyl group being located near the middle of the chain. In all cases the parent peak is very small, while the usual M-18 peak observed in alcohols is predominant.

**Secondary Alcohols of *M. packardii***

The $C_{23}$ alcohol fraction (Fig. 1A) contains three isomers consisting mostly of 11-tricosanol followed by 10-tricosanol and then 12-trico-

sanol. The mass spectrum shows major peaks of interest at 322, 213, 199, 185, 171 and 157. The peak at 322 is the result of M-18. The peaks at 199 and 171 arise from cleavage at either side of carbon eleven giving ions $(C_{13}H_{27}O)^+$ and $(C_{11}H_{23}O)^+$. The 171 peak is slightly larger than the 185 peak as a result of preferential cleavage leaving the larger alkyl group as a neutral fragment. This type of cleavage is also noticed in all the nonsymmetrical patterns in internal methyl-branched hydrocarbons (6). The peaks at 213 and 157 result from cleavage at either side of carbon ten of 10-tricosanol. The peak at 185 arises from 12-tricosanol, with cleavage at either side of the symmetrical molecule resulting in an ion of $(C_{11}H_{23}O)^+$.

The $C_{25}$ alcohol fraction (Fig. 1B) consists mostly of 12-pentacosanol, with two other isomers, 11-pentacosanol and 13-pentacosanol. The major peaks of interest are 350, 227, 213, 199, 185 and 171 with the M-18 peak being 350. Cleavage at either side of $C_{12}$ in 12-pentacosanol results in the 185 and 213 peaks, while cleavage around $C_{11}$ in 11-pentacosanol produces the peaks at 171 and 227. Cleavage on either side of $C_{13}$ in the symmetrical 13-pentacosanol results in a peak at 199.

The $C_{27}$ alcohol fraction (Fig. 1C) contains four isomers. The major isomer is 13-heptacosanol, while the remaining three isomers are 11-, 12- and 14-heptacosanol. The M-18 peak is seen at 378, leaving significant peaks at 171, 185, 199, 213, 227, 241 and 225. Cleavage at either side of the $C_{13}$ of 13-heptacosanol results in fragmentations with masses at 199 and 227. Cleavage of the 11-heptacosanol produces the 171 and 255 peaks. The peaks at 185 and 241 come from the 12-heptacosanol, and the peak at 213 is a result of the cleavage of the 14-heptacosanol.

#### Secondary Alcohols of *M. sanguinipes*

The $C_{21}$ alcohol consists of two isomers, with about equal amounts of 10-heneicosanol and 11-heneicosanol. The mass spectrum (Fig. 2A) shows major peaks of interest at 294, 185, 171 and 157. The 294 peak is the M-18 peak. Peaks at 185 and 157 arise from cleavage at either side of carbon 10 giving ions $(C_{12}H_{25}O)^+$ and $(C_{10}H_{21}O)^+$ The 157 peak is slightly larger than the 185 peak arising from preferential cleavage of the larger group as a neutral fragment. The peak at 171 arises from the 11-heneicosanol, with cleavage at either side of the symmetrical molecule resulting in an ion of $(C_{11}H_{23}O)^+$.

The $C_{22}$ alcohol consists of two isomers (Fig. 2B) with about twice as much 11-docosanol as 10-docosanol. The peaks at 171 and 185 result from cleavage at either side of carbon eleven, giving ion fragment of $(C_{11}H_{23}O)^+$ and $(C_{12}H_{25}O)^+$. These peaks are about twice as large as the 157 and 199 peaks, which arise due to the cleavage at either side of the carbon 10. Again, as noted earlier, the cleavage of the larger alkyl group as a neutral fragment is preferred, as the 171 and 157 peaks are larger than the 189 and 199, respectively. The peak at 308 is the M-18 peak.

The $C_{23}$ peak consists primarily of 11-tricosanol (Fig. 2C) with smaller amounts of 12-tricosanol and 10-tricosanol. The peaks at 171 and 199 arise from cleavage at either side of carbon eleven, giving ions of $(C_{11}H_{23}O)^+$ and $(C_{13}H_{27}O)^+$. The peaks at 157 and 213 arise from cleavage at either side of carbon ten. The cleavage at either side of the center carbon of the symmetrical 12-tricosanol gives rise to the peak at 185. The 322 peak is the M-18 peak. A mass spectrum of the $C_{23}$ alcohol oxidized to ketone is shown in Fig. 2D. The peaks at 169 and 197 arise from cleavage at either side of the carbon eleven of the 11-tricosanone. $\beta$-Cleavage (7) of the same isomer gives rise to peaks at 184, 185 and part of 183, 212, 213 and part of 211. The cleavage of symmetric 12-tricosanone gives rise to peaks at 183 and through $\beta$-cleavage at 198, 199 and part of the 197 peak. The mass spectrum of the synthesized 12-tricosanone gives similar results.

The $C_{24}$ peak consists primarily of the 11-tetracosanol and 12-tetracosanol (Fig. 2E). The peaks at 171 and 213 arise from cleavage at either side of carbon 11, and the peaks at 185 and 199 arise from cleavage at either side of carbon 12. The M-18 peak is at 336.

The $C_{25}$ secondary alcohol is comprised of three isomers (Fig. 2F) with a hydroxyl group at the 11, 12 or 13 position. The peaks at 171 and 227 arise from cleavage at either side of carbon eleven, peaks at 185 and 213 from cleavage at either side of carbon eleven, peaks at 185 and 213 from cleavage at either side of carbon twelve, and a peak at 199 from cleavage at either side of the central carbon of the symmetrical 12-pentacosanol. The M-18 peak is at 350.

The $C_{26}$ peak consists of three isomers with a hydroxyl group at the 11, 12 or 13 position (Fig. 2G). The peaks at 171 and 241 arise from cleavage at either side of carbon 11, peaks at 185 and 227 from cleavage at either side of carbon 12, and peaks at 199 and 213 from cleavage at either side of carbon 13. The M-18 peak is at 364.

The $C_{27}$ peak contains four isomers (Fig. 2H), with a hydroxyl group at the 11, 12, 13 or

14 position. Peaks at 171 and 255 result from cleavage at either side of carbon 11, peaks at 185 and 241 from cleavage at either side of carbon 12, peaks at 199 and 227 from cleavage at either side of carbon 13, and the peak at 213 from cleavage at either side of the central carbon of the symmetrical 14-heptacosanol. The peak at 378 is the M-18 peak. The peaks at 169, 183, 197, 211, 225, 239 and 253 are thought to arise from dehydrogenation at high temperatures (8).

In addition to the aliphatic secondary alcohols, *M. sanguinipes* wax esters after either LAH reduction or saponification yield an alcohol migrating near the normal alcohols on TLC which is indicated by GLC have ca. 30 carbons as compared to a normal alcohol standard. After subsequent isolation of the compound it was found to cochromatograph with authentic $\beta$-amyrin and the mass spectrum was similar to that of authentic $\beta$-amyrin. $\beta$-Amyrin constitutes ca. 2% of the alcohol fraction of the secondary alcohols from *M. sanguinipes*, but was not present in the secondary alcohols of *M. packardii*.

## DISCUSSION

Wax esters of insects generally have one or more of the long chain, *n*-series primary alcohols and acids (9). To our knowledge wax esters of secondary alcohols have not previously been reported in insect cuticular lipids.

Secondary alcohols reported in insects include a pentacosane-8,9-diol in the larva of *Tenebrio molitar* L. (Coleoptera) (10), and a diol in bees wax which consisted of one primary hydroxyl group and one at the penultimate position (reviewed by Tulloch 11). To our knowledge mono-hydroxyl secondary alcohols with the hydroxyl group located near the center of the chain have not previously been reported in insects. They are however common constitutents of plants (7, 12-14). Wollrab (13) points out that in plants, secondary alcohols are not esterified, in contrast to the situation found among the primary alcohols.

The location of the hydroxyl group has usually been determined by first oxidizing the secondary alcohol to a ketone (13, 14). We found that less ambiguous results were secured by obtaining the mass spectrum of the alcohol directly. This is especially helpful when more than one isomer exists, as it eliminates the $\beta$-cleavages found in mass spectra of ketones. Similar comparisons of mass spectra of ketones and secondary alcohols were shown by Friedel et al. (15, 16).

The origin of the secondary alcohols as well as their wax esters in insect cuticular lipids is unknown. It is possible that the insects obtain the secondary alcohols from their diet and then esterify them. Further studies are being carried out to determine the biosynthesis of the secondary alcohols and their wax esters.

## ACKNOWLEDGMENTS

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# The Activating Effect of Dietary Protein on Linoleic Acid Desaturation

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## ABSTRACT

The desaturation of $^{14}$C-1-linoleic acid to γ-linolenic acid and their incorporation into the microsomal lipids of rats fed on a balanced diet and a protein diet were measured in vitro. It was shown that a protein diet does not change significantly the distribution of the radioactivity among the different lipidic fractions compared to the animals fed on a balanced diet. However the microsomal desaturation of linoleic acid to γ-linolenic acid increased in the rats maintained on a protein diet. Besides, the amount and composition of the free fatty acids present in the microsomes of the animals fed on both diets were similar enough to discard the hypothesis that they may modify the desaturation of linoleic acid produced by the diet. The enzymic activity of the linoleyl desaturase of liver microsomes of animals fed on a protein diet, measured in substrate saturating conditions, is greater than in animals with balanced diet. Consequently the results support the hypothesis that a protein diet increases specifically the desaturating activity of the microsomes.

## INTRODUCTION

Studies on the biosynthesis of polyenoic fatty acids in liver microsomes from normal rats have demonstrated that a protein diet produces an increase in the desaturation of linoleic acid to γ-linolenic acid (1). Considering that this increase produced by the diet is maintained during 24 hr but is not evoked when Actinomycin D, Puromycin or Cycloheximide are simultaneously injected, it was thought that the protein diet probably increased the linoleic acid desaturation by induction of the 6-desaturase (1). Nevertheless, such an effect could be produced by another mechanism.

One of the characteristics of liver microsomes is that they contain a large amount of phospholipids, especially phosphatidylcholine, and a smaller amount of triglycerides, cholesterol, cholesterol esters and free fatty acids (2). Besides, the enzymes that intervene in the synthesis and degradation of the different lipidic fractions are present in the microsomes. Brenner et al. (3) have demonstrated that liver microsomes not only convert linoleic acid into linoleyl-CoA and desaturate it into γ-linolenyl-CoA, but also incorporate both acids into phospholipids. The same authors have found evidence of the competition of the synthesis of phospholipids with the desaturation reaction for the fatty acids in the microsomes (4). Therefore the effect of the proteins could be produced through a modification of phospholipid synthesis and also by a change in the distribution of the microsomal lipidic fractions. This change would produce a variation in the total proportion of linoleic acid and γ-linolenic acid. For this reason the incorporation of linoleic acid in the microsomal lipids and the desaturation to γ-linolenic acid were studied in animals fed on a balanced diet and a protein diet.

On the other hand, the desaturation of linoleic acid to γ-linolenic acid measured in vitro depends on the presence and concentration of other free fatty acids in the medium, as it has been demonstrated before (5). Therefore we also considered it of importance to investigate the amount and composition of the free fatty acids of the microsomes before and after the incubation, of animals fed on a balanced or protein diet.

## MATERIAL AND METHODS

### Animals

Adult female Wistar rats weighing 250-300 g and maintained on standard Purina chow were used.

### Treatment of Animals

The animals were divided into two groups of five animals each. One group was fed a Purina chow diet and water ad libitum, and was used as a control. The other group was force-fed a 20% suspension of casein (Casenolin, Glaxo-Argentina) for 48 hr. These animals received isocaloric diet (25 kcal/100 g body wt), and the total daily food intake was administered accordingly at 4 hr intervals. Water was given ad libitum.

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TABLE I

Effect of Dietary Protein on the Per Cent Distribution of Labeled Fatty Acids in the Microsomal Lipids After Incubation[a]

| Thin layer chromatography zones | Incubation time | | | |
|---|---|---|---|---|
| | 5 min | | 20 min | |
| | Balanced diet | Protein diet | Balanced diet | Protein diet |
| Origin[b] | 0.8[c]± 0.13 | 0.7 ± 0.1 | 1.5 ± 0.3 | 1.5 ± 0.3 |
| Lisophosphatides | 0.7 ± 0.07 | 0.7 ± 0.1 | 1.5 ± 0.2 | 1.6 ± 0.2 |
| Choline phosphatides | 12.0 ± 2.7 | 11.1 ± 2.3 | 29.8 ± 6.3 | 32.3 ± 3.7 |
| Ethanolamine phosphatides | 13.3 ± 2.0 | 9.1 ± 1.9 | 8.4 ± 0.5 | 6.4 ± 0.8 |
| Free fatty acids | 25.4 ± 4.8 | 31.1 ± 4.9 | 13.1 ± 0.8 | 11.8 ± 2.0 |
| Neutral lipids | 47.8 ± 3.7 | 47.3 ± 4.6 | 45.7 ± 6.4 | 46.4 ± 4.2 |

[a]10 nmoles of $^{14}$C-1-linoleic acid were incubated with 2 mg microsomal protein at 25 C with the cofactors detailed in the Materials and Methods.

[b]Lipids were separated in choloroform-methanol-water 65:25:3 v/v/v.

[c]Mean values of five observations ± one standard error of the mean (SEM).

### Isolation of Microsomes and Assay Procedure

The rats were killed by decapitation without anesthesia. The liver was rapidly excised, and placed immediately in ice cold homogenizing medium. The liver was then homogenized in a cold solution (3:1 v/w) consisting of 0.15 M KCl, 0.005 M $MgCl_2$, 0.004 M EDTA, 0.004 M N-acetyl-cysteine, 0.05 M phosphate buffer (pH 7), and 0.25 M sucrose. Cell debris and mitochondria were removed by sedimentation at 12,000 x g for 10 min at 0 C. The microsomes were isolated in the cold by differential centrifugation at 140,000 xg for 60 min in a Spinco Model L2 centrifuge as described previously (3,6).

The desaturation of linoleic acid to γ-linolenic acid by liver microsomal preparation was measured by estimation of the conversion per cent of $^{14}$C-1-linoleic acid (53.0 mci/mmole; 98% Radiochemically pure, Radiochemical Centre, Amersham, England) to γ-linolenic acid. Ten nmoles of labeled linoleic acid were incubated with 2 mg of microsomal protein in a Dubnoff Shaker at 25 C for 5 or 20 min in a total volume of 3 ml of a 0.15 M KCl, 0.25 M sucrose solution containing 4 μmoles ATP, 0.2 μmoles CoA, 2.5 μmoles NADH, 15 μmoles $MgCl_2$, 4.5 μmoles glutathione, 125 μmoles NaF, 1 μmoles nicotinamide and 125 μmoles phosphate buffer (pH 7). The distribution of radioactivity between substrate and product was measured by gas liquid radiochromatography of the methyl esters in a Pye apparatus with a proportional counter under the conditions described in a previous work (1).

TABLE II

Specific Radioactivity of Thin Layer Chromatography Fractions, cpm/μmole[a]

| Fraction | Balanced diet | Protein diet |
|---|---|---|
| Choline phosphatides | 7130[b]± 1700 | 13150 ±5200 |
| Ethanolamine phosphatides | 13720 ± 3400 | 17790 ± 4400 |
| Neutral lipids | 9555 ± 2300 | 13140 ± 3200 |

[a]Experimental conditions as in Table I. Microsomes incubated for 20 min.

[b]Mean values ± 1 SEM.

To measure the specific activity of linoleic acid desaturase, the effect of time, substrate concentration and amount of enzyme were specially tested and fixed. The incubation medium contained: 4 μmoles ATP, 0.2 μmoles CoA, 2.5 μmoles NADH, 7.5 μmoles $MgCl_2$, 2.25 μmoles glutathione, 62.5 μmoles NaF, 0.5 μmoles nicotinamide and 62.5 μmoles phosphate buffer (pH 7) in a total volume of 1.5 ml 0.15 M KCl and 0.25 M sucrose solution. The incubation procedure was the same as before.

The effect of time of incubation on the linoleic acid converted to γ-linolenic acid is shown in Figure 1. Up to 30 min the conversion increased linearly with time. Therefore 30 min of incubation were chosen to get the highest possible yield and increase the accuracy of the measurement. Besides, the linoleic acid desaturation was proportional to the amount of enzyme up to 5 mg of microsomal protein. For this reason the desaturating specific activity of the microsomes was measured in the presence of 5 mg microsomal protein during 30 min and with increasing concentrations of $^{14}$C-1-linoleic acid from 3.3 x $10^{-2}$ mM to 13.2 x $10^{-2}$ mM (diluted with unlabeled fatty acid to maintain 200,000 cpm in each tube).

### Lipid Fractionation

The lipids of the incubation system were

TABLE III

Desaturation Per Cent of Linoleic to γ-Linolenic Acid in Lipidic Fractions[a]

| Thin layer chromatography zones | Incubation time | | | |
|---|---|---|---|---|
| | 5 min | | 20 min | |
| | Balanced diet | Protein diet | Balanced diet | Protein diet |
| Origin | 9.4 | 14.1 | 30.1 | 42.0 |
| Lisophosphatides | 9.1 | 11.3 | 28.6 | 33.5 |
| Choline phosphatides | 3.9 | 7.4 | 12.1 | 17.6 |
| Ethanolamine phosphatides | 4.1 | 11.0 | 9.4 | 24.0 |
| Free fatty acids | 3.0 | 3.5 | 18.8 | 32.9 |
| Neutral lipids | 5.0 | 10.3 | 10.1 | 16.7 |
| Total lipids | 6.4 | 9.2 | 18.7 | 26.1 |

[a]Experimental conditions as in Table II.

extracted with chloroform-methanol 2:1 v/v. The lipid extract obtained was freed of nonlipid impurities by the procedure of Folch et al. (7), concentrated in a rotary evaporator at room temperature, made up in chloroform-methanol 2:1 v/v, and stored at 4 C. Aliquots of this stock solution were evaporated under nitrogen and analyzed by thin layer chromatography (TLC) on plates of Silica Gel G (Merck). The lipids were separated with chloroform-methanol-water 65:25:3 v/v/v. The spots were compared to standards run at the same time. They were developed with iodine vapor. The corresponding fractions of the samples were scraped off and either phosphorous determined according to Doizaki and Zieve (8) or fatty acid ester groups following Rapport and Alonzo (9). Aliquots were counted in a Mark 1, Nuclear Chicago scintillation counter with scintillation solution prepared according to Bray (10). Other aliquots were heated with methanolic 3 M HCl for 3 hr at 68 C and label distribution in fatty acid methyl esters analyzed by gas liquid radiochromatography as formerly described (1).

### Free Fatty Acids

The free fatty acid content of the microsomes was determined by TLC on plates of Silica Gel G (Merck). They were separated with redistilled petroleum ether-ethyl ether-glacial acetic acid 70:30:1.2 v/v/v. Standards of pure linoleic acid (10, 20 and 40 nmoles) were run at the same time. Lipid components were located by aqueous sulfuric acid (1:1 v/v) spray and the plates were heated for 30 min at 180 C. The amount of fatty acids was measured with a Zeiss PMQ II spectrophotometer for chromatograms. The light absorbed by the spots of the fatty acids was measured and compared with the standards.

The composition of the free fatty acids separated by TLC was determined by analyzing their methyl esters by gas liquid chromatography in a Pye apparatus with an argon ionization detector. Six foot columns heated to 180 C and packed with 10% polyethyleneglycol succinate chromosorb W (80-100 mesh) were used. The main peaks were identified by comparison of the adjusted retention times relative to stearate with the corresponding standards. Fatty acid composition was calculated measuring the surface of the peaks by triangulation.

TABLE IV

Free Fatty Acids in Microsomes, nmoles/mg protein

| Diet | Before incubation | After incubation |
|---|---|---|
| Balanced | 34.8[a]± 3.3 | 20.5 ± 1.3 |
| | P>0.05 | |
| Protein | 48.1 ± 7.0 | 19.5 ± 3.4 |
| | P<0.001 | |

[a]Mean values ± 1 SEM.

## RESULTS AND DISCUSSION

In our experimental conditions, the desaturation of linoleic acid to γ-linolenic acid is performed in the microsomes through a series of reactions. The linoleic acid is first converted

TABLE V

Composition of Microsomal Free Fatty Acids of Animals Fed on Balanced Diet and Protein Diet

| Fatty acids | Balanced | Protein |
|---|---|---|
| C 16 | 36.0 | 37.8 |
| C 16:1 | 4.2 | 6.7 |
| C 18 | 24.3 | 25.3 |
| C 18:1 | 13.4 | 13.4 |
| C 18:2 | 2.5 | 2.2 |
| C 20:4 | 10.7 | 10.4 |
| Others | 8.9 | 4.3 |

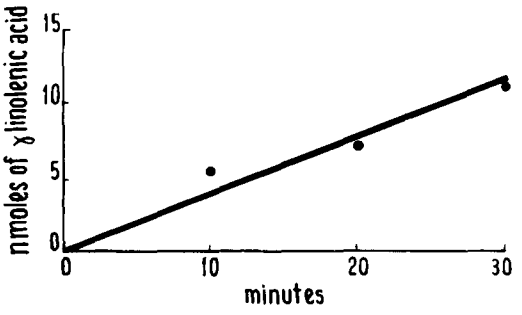


FIG. 1. Effect of time on the speed of linoleic acid desaturation to γ-linolenic acid. Five milligrams microsomal protein incubated with 100 nmoles of labeled linoleic acid.

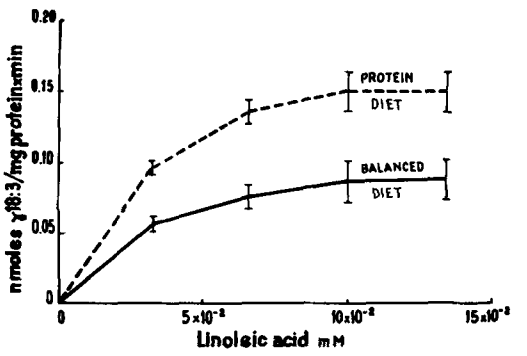


FIG. 2. Effect of a protein diet on the substrate saturation curve of linoleic acid desaturation.

into linoleyl-CoA and then at least two types of reactions are performed: the desaturation into γ-linolenyl-CoA on one hand, and the esterification to synthesize principally phospholipids and triglycerides on the other. The esterification of linoleyl-CoA must be considered as a reaction parallel to the desaturation, and if it decreases under certain conditions, it may consequently favor the desaturation. Because of these reasons, liver microsomes were incubated with $^{14}$C-1-linoleic acid in desaturating conditions, studying the distribution of radioactivity among the different microsomal lipidic fractions (Table I).

The results in Table I show the per cent of labeling of the different lipidic fractions of the microsomes after 5 and 20 min of incubation. They show a rapid labeling in all the lipids, including a remarkable incorporation in the neutral lipids. The radioactivity in the free acids decreases with the increase of the time of incubation. From 5 to 20 min this decrease is correlated with an increase of the per cent of radioactivity in the phosphatidylcholine fraction. After 20 min of incubation it is twice as high as that corresponding to the 5 min. During this interval the incorporation into neutral lipids remains constant. Consequently the labeled acids would be incorporated principally by the lecithin during this period. It may be pointed out that the fact that incorporation of the labeled acid among the fractions of neutral lipids remains constant for 15 min, after a rapid incorporation during the first 5 min, by no means implies a static process, but the existence of a turnover, as is demonstrated by the increase in the levels of γ-linolenic acid between the 5th and 20th min of incubation (Table III).

The data in Table I also show that a protein diet does not change significantly the distribution of the radioactivity among the free acids and the different lipidic fractions when compared to the animals fed on a balanced diet.

The measuring of the specific activity of the phosphatidylcholine, phosphatidylethanolamine and triglyceride fractions (Table II) also shows similar values for the normal rats and the rats maintained on a protein diet.

Table III shows the per cent of distribution of radioactivity among linoleic acid and its conversion product, γ-linolenic acid, in each of the lipidic fractions. The microsomes were incubated 5 and 20 min. The data indicate that there is an increase in the conversion per cent of linoleic acid to γ-linolenic acid along with the time of incubation, and that this increase is shown in all the lipidic fractions when the rats are maintained either on a normal or a protein diet. Besides, the protein diet increases the microsomal desaturation of linoleic acid to γ-linolenic acid, and this increase is distributed in a similar way among all the lipidic fractions.

Therefore, and considering that in our experimental conditions the incorporation of labeled acids into the different lipidic fractions of the microsomes is not substantially modified when a protein diet is administered, and that on the other hand the desaturation is increased in all the same fractions, we may suppose that the increase of desaturation cannot be attributed to a modification of lipid synthesis or to a change in the incorporation of substrate or product, or both, in the lipids. Therefore the protein diet apparently produces the increase of linoleic acid desaturation by means of a different mechanism.

In previous experiments we have demonstrated that the microsomal conversion of linoleic acid to γ-linolenic acid, measured in vitro, depends on the presence and concentration of other fatty acids in the incubation medium (3,5,11,12). Considering that the increase of desaturation observed in the liver microsomes caused by a protein diet could depend on a modification in the concentration of free fatty acids in the microsomes, we

measured the amount of free fatty acids present in them, before and after the incubation period, and for animals fed on both diets. The results are summarized in Table IV. As can be observed although there are significant differences in the fatty acid content of the microsomes before and after incubation ($P<0.001$), there are not significant differences between the control group and the group with protein diet either before or after the incubation. So we can apparently reject the possibility of a modification in the microsomal free fatty acid content being responsible for the increase in the desaturation produced by the protein diet in our experimental conditions.

Nevertheless, considering that the microsomal desaturation of linoleic acid to γ-linolenic acid is modified in different ways by different fatty acids (3,5), it was necessary to check the composition of the free acids of the liver microsomes. The results appear in Table V and they show, as expected, that the composition does not have comparatively important variations between the control animals and the ones maintained on a protein diet.

Therefore the increase of the desaturation of linoleic acid produced by the protein diet cannot be attributed to a change of the composition of the microsomal free fatty acids induced by the protein diet.

Finally, Figure 2 compares the effect of a protein diet and a balanced one in the microsomal desaturation velocity of linoleic acid to γ-linolenic acid for different concentrations of substrate. In these experimental conditions, substrate saturation curves are found, and the effect of collateral reactions on linoleic acid desaturation are undoubtedly minimized when rising the high concentrations of substrate. Therefore, in these conditions and at the plateau, they may measure with rather good reliability the specific desaturating activity of the microsomes for linoleic acid. It can be observed that in these experimental conditions the enzymic activity of liver microsomes is greater in the rats fed a protein diet than in the animals with normal balanced diet. Consequently the data collected in this series of experiments would add considerable evidence to the hypothesis that a protein diet increases specifically the desaturating activity of the microsomes. Its importance and its mechanism of activation are still to be further investigated.

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# The Metabolism of 1-$^{14}$C-Palmitic Acid in the Testis of the Rat

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## ABSTRACT

The metabolism of 1-$^{14}$C-palmitic acid was studied in rat testis at various intervals after intratesticular injection and after a 2 hr incubation period. Significant catabolism occurred as evidenced by production of $^{14}CO_2$ in both in vivo and in vitro experiments. Of the $^{14}$C activity retained in the injected testis, less than 15% remained as free fatty acid after 2 hr and less than 5% by the end of 2 weeks. Activity of $^{14}$C appeared in phosphatides faster than in the triglyceride fraction, and with time (1-2 weeks) the activity in phosphatides decreased relative to that in triglycerides. In phosphatides of these experiments ca. 80% of the $^{14}$C was in the palmitic acid fraction and the balance predominantly (98%) in palmitate with the balance in stearate and oleate. By the first and second weeks almost half of the $^{14}$C-palmitate present in the testes had been newly synthesized from $^{14}$C-acetyl CoA resulting from oxidation of the administered 1-$^{14}$C-palmitate. In the incubated samples the only fatty acid with $^{14}$C activity in any lipid fraction was palmitate. In these experiments ca 90% of the $^{14}$C was in the free fatty acid fraction, 7% in the phosphatides and 2% in the triglycerides.

## INTRODUCTION

Fatty acid metabolism in reproductive tissue has become a topic of increased interest in the last few years. In reports from this and other laboratories it has been shown that processes of de novo synthesis, of elongation and of conversion of polyenoic acids to longer chain, more highly unsaturated derivatives are all active in the rat testis (1-5). Palmitic acid is a major fatty acid of rat testicular tissue (6), and its concentration changes with concomitant changes in other fatty acids of this organ in degenerative processes (7). Therefore it was thought desirable to investigate further the metabolism of palmitic acid in this organ. This paper reports results of such investigations using $^{14}$C-palmitic acid injected intratesticularly or incubated with sections of testicular tissue.

## EXPERIMENTAL PROCEDURES

Sprague Dawley rats of ca. 1 year of age and maintained on laboratory chow were used. A minimum of three rats was used per time period reported. For the intratesticular injections 1-$^{14}$C-palmitic acid (> 99% pure by gas radiochromatography; obtained from New England Nuclear Corp., Boston, Mass.) was complexed with albumin, and 50 $\mu$l containing 4 $\mu$c and < 0.1 mg were injected into one testicle of each rat. In the experiments in which expired $^{14}CO_2$ was collected the rats were kept in a metabo-

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TABLE I

Amount of $^{14}$C in Expired Air and in Organs of Rats Injected Intratesticularly With 1-$^{14}$C-Palmitate

| Time after injection | Per cent of injected dose | | | |
|---|---|---|---|---|
| | In expired air | In injected testis | In noninjected testis | In liver |
| .5 hr | 1.91[a] (1.58-2.12)[b] | 64.2 (53.0-86.2) | Nil | 3.52 (2.28-5.40) |
| 2 hr | 18.6 (14.9-25.0) | 47.3 (38.5-58.4) | Nil | 2.58 (1.51-3.43) |
| 4 hr | 44.7 (35.7-50.5) | 41.4 (29.0-56.2) | 0.04 (0.02-0.05) | 1.76 (1.24-2.91) |
| 1 week | Not done | 16.1 (12.3-20.3) | 0.16 (0.14-0.17) | 0.63 (0.60-0.69) |
| 2 weeks | Not done | 6.4 ( 3.1-11.9) | 0.30 (0.28-0.32) | 0.26 (0.17-0.32) |

[a]Average of data obtained in three animals.
[b]Range of values.

TABLE II

Distribution of $^{14}C$ in Testes of Rats Injected Intratesticularly With 1-$^{14}C$-Palmitate

| Lipid class | Per cent of $^{14}C$ recovered from thin layer chromatography | | | | |
|---|---|---|---|---|---|
| | .5 hr | 2 hr | 4 hr | 1 week | 2 weeks |
| Phospholipid | 26.9[a] (14.8-48.7)[b] | 54.1 (52.1-61.6) | 65.9 (59.2-76.8) | 32.3 (28.4-37.7) | 35.8 (33.0-38.6) |
| Cholesterol | 4.5 ( 2.3-6.0 ) | 4.5 ( 2.4-5.3 ) | 4.8 ( 3.4-5.3 ) | 0.5 ( 0.4-0.5)) | 0.5 ( 0.3-0.6 ) |
| Free fatty acid | 47.1 (30.9-62.8) | 15.0 (14.9-15.2) | 8.5 ( 4.5-14.9) | 8.2 ( 6.2-10.5) | 3.6 ( 2.2-6.4 ) |
| Triglyceride[c] | 16.1 (12.5-19.4) | 22.6 (18.9-28.7) | 21.4 (12.7-31.3) | 52.2 (43.3-59.6) | 49.6 (36.2-60.8) |
| Cholesterol ester | 1.1 ( 0.7-1.9 ) | 1.1 ( 1.0-1.2 ) | 0.5 ( 0.4-0.8 ) | 2.6 ( 2.0-3.1 ) | 4.4 ( 4.3-4.6 ) |

[a]Average of data obtained on three rats.

[b]Range of values.

[c]Includes glyceryl ether diesters.

lism cage designed according to Roth et al. (8), and the expired $^{14}CO_2$ was trapped in a solution of ethanolamine-ethylene glycol monomethyl ether 1:2 v/v. $^{14}C$ activity of these samples was determined using a toluene-ethylene glycol monomethyl ether solution 2:1 v/v), containing 5.5 g/liter of 2,5 diphenyloxazole. $^{14}C$ content of all other samples (lipids) was determined in toluene containing diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl)-benzene. The counts of the various samples were corrected, when necessary, to the same efficiency. A Packard Tri-Carb liquid scintillation spectrometer was used.

For the incubation experiments the tunica albuginea was removed and each testis, cut into four approximately equal parts, was incubated in a medium composed of 1 ml phosphate buffer (1 M, pH 7.4), 0.1 mg (4 $\mu$c) 1-$^{14}C$-palmitic acid (complexed with albumin) and 1 ml of 10mM glucose. Tissues were incubated with shaking for 2 hr at 37 C in an atmosphere of 95% $O_2$, 5% $CO_2$. At the end of the incubation period the flasks were chilled in ice, the contents centrifuged, and the tissue extracted with Folch mixture. Methods used for lipid extractions, thin layer chromatography (TLC), gas chromatography and gas radiochromatography have been described previously (4,9). Recovery of $^{14}C$ from TLC plates was at least 90% of the activity applied to the plates.

Chemical degradation of testicular palmitic acid isolated pure by gas chromatography was by the procedure of Dauben et al. (10).

## RESULTS

Radioactivity was observed in the expired air as early as 30 min after intratesticular injection of $^{14}C$-palmitate (earliest sample obtained) (Table I). The amount increased to ca. 45% of the injected dose by the end of the fourth hour after injection (the latest sample done). In two incubation experiments 0.6 and 0.8% of the 1-$^{14}C$-palmitate substrate was oxidized to $^{14}CO_2$ by each testis. The amount of $^{14}C$ activity retained by the injected testis decreased rapidly as seen in Table I. At 4 hr after injection only ca. 40% remained, and after two weeks only ca. 6% of the injected dose remained in the injected testis. Not until the 4 hr sample was any amount of $^{14}C$ detected in the noninjected testis (ca. 0.04% of the injected dose). After 2 weeks only ca. 0.3% of the injected dose was present in the noninjected testis. The amount of $^{14}C$ found in liver was highest in the earliest sample (30 min) and decreased rapidly to ca. 0.3% of the injected dose at 2 weeks.

The labeled palmitate was rapidly esterified in the injected testis (Table II). Only ca. 14% of the $^{14}C$ recovered in lipids of the testis 2 hr after the injection was present as free fatty acid and this decreased to ca. 4% by the end of 2 weeks. During the intervals immediately after injection the $^{14}C$ appeared in the phospholipid fraction faster than it did in triglycerides. At the end of the 1 and 2 week periods the activity had decreased in phospholipid relative to that found in triglyceride. A small amount of activity was present in the cholesterol and cholesterol ester fractions.

At the end of 1 week the $^{14}C$-palmitate present in the testes was composed partly of the injected $^{14}C$-palmitate and partly of $^{14}C$-palmitate biosynthesized from $^{14}C$-acetate originating from the beta oxidation of injected

TABLE III

Incorporation of Radioactivity Into Testicular Fatty Acids of Phospholipid Fraction After Intratesticular Injection of 1-$^{14}$C-Palmitate

| Fatty acid | Per cent of Total $^{14}$C recovered | | | | |
|---|---|---|---|---|---|
| | .5 hr | 2 hr | 4 hr | 1 week | 2 weeks |
| <16:0 | 1.3[a] ( 1.0-1.4 )[b] | 1.2 ( 0.4-2.8 ) | Trace | Trace | Trace |
| 16:0, 16:1[c] | 80.1 (77.5-82.3) | 79.4 (77.3-80.1) | 80.9 (75.5-87.2) | 78.5 (76.3-82.3) | 72.2 (70.6-73.2) |
| 18:0 | 7.8 ( 4.2-11.4) | 11.1 ( 9.0-12.8) | 11.8 ( 9.1-13.6) | 13.1 (11.9-14.8) | 12.0 ( 9.5-13.9) |
| 18:1 | 2.5 ( 1.1-4.2 ) | 1.9 ( 1.1-2.2 ) | 2.4 ( 1.6-3.9 ) | 7.6 ( 6.0-9.3 ) | 14.9 (13.7-15.7) |

[a]Average of data obtained on three rats.

[b]Range of values.

[c]Only a minor fraction of the $^{14}$C was in 16:1.

1-$^{14}$C-palmitate. This was evidenced by the change in ratio of specific activity of carboxyl carbon to average fatty acid carbon of 16:1 in the injected material (carboxyl-labeled) to a ratio of 9.5:1 in two 1 week samples. Samples analyzed from testes of rats killed at the end of 2 weeks had ratios quite similar to the 1 week animals.

Reliable data describing the distribution of $^{14}$C in lipid classes of liver were obtained only for the .5 hr and 2 hr experiments, and results were similar for these two time periods. About half of the $^{14}$C was in phosphatides and the balance in triglycerides. Minor quantities only were found in the other lipid fractions. The amount of $^{14}$C in liver lipids isolated at other time periods was too small to permit fractionation.

In the incubation experiments most of the $^{14}$C activity recovered from the TLC plates (ca. 90%) was in the free fatty acid fraction, ca. 7% in the phosphatide fraction, and ca. 2% in the triglyceride fraction. Minor amounts were found in other lipid fractions.

The distribution of $^{14}$C in the phospholipid fatty acids of the injected testis is shown in Table III. Most of the $^{14}$C was in the palmitic acid fraction at all time periods, although with time there was an increase in the activity of 18:0 and 18:1 relative to that of 16:0. In some samples there was $^{14}$C activity in 20-carbon compounds, but the amount was small and the results were not consistent.

In the triglyceride fraction of these testes at all time periods, all but ca. 2% of the $^{14}$C was in the palmitic acid fraction. The balance was distributed in 18:0 and 18:1. In the free fatty acid fraction all the $^{14}$C activity was in the palmitic acid fraction. In the incubated samples the only fatty acid which had $^{14}$C in either phospholipid or triglyceride fraction was palmitic.

## DISCUSSION

The rapid disappearance of $^{14}$C from the injected testis and the rapid appearance of activity in $^{14}CO_2$ indicates that palmitic acid, as other fatty acids reported previously (9,11), is actively catabolyzed or transported out of the organ, or both. At least some of the $^{14}$C in the expired air was due to oxidation of the $^{14}$C-palmitate in the testis as evidenced by the oxidation to $^{14}CO_2$ of $^{14}$C-palmitate incubated with testicular tissue. The amount of $^{14}$C activity present in the injected testis (as per cent of injected dose) 4 hr after injection was slightly greater than reported for 18:2, 20:4 and 22:5 (9,11). At the end of 2 weeks similar amounts were present: with $^{14}$C 18:2, 6%; with $^{14}$C 20:4, 10%; and with $^{14}$C 16:0, 6%.

Rapid esterification of the intratesticularly injected labeled palmitate occurred as it had also been observed previously with other fatty acids. As with the experiments using labeled linoleate and arachidonate (9,11), the $^{14}$C in the testis 4 hr after injection with $^{14}$C-palmitate was largely in the phospholipid fatty acids. These may be contrasted with the lower amount found at the same time period when $^{14}$C 22:5 was used (11). The relative amounts in the triglycerides at this time period were: with $^{14}$C 18:2, 10%; with $^{14}$C 20:4, 18%; with $^{14}$C 22:5, 31%; and with $^{14}$C 16:0, 21%. Thus the pattern for palmitate follows most closely that of arachidonate.

There was little conversion of palmitate to other fatty acids in the injected testis and this was largely to the 18-carbon saturated and monoenoic fraction. However some activity was obtained in 20-carbon compounds in some of

the experiments, particularly 20-carbon polyenes. This was probably the result of elongation of endogenous fatty acids by $^{14}$C-acetyl coenzyme A obtained during the oxidation of the injected palmitate. That some of the resulting $^{14}$C-acetyl CoA was used for re-synthesis of palmitic acid is shown by the change in ratio of specific activities of fatty acid carboxyl carbon to average fatty acid carbon from that of the injected material (carboxyl labeled) to near half the ratio by 1 week. By this time apparently no $^{14}$C-acetyl CoA of significant specific activity was being produced since the ratio found in animals which lived 2 weeks post injection was similar to that of the 1 week animals. Less conversion was noted in the triglyceride fraction than in the phosphatide, and no conversion was noted in the incubated sample in 2 hr.

No significant change in concentration of palmitic acid in testis occurs with development from 4 weeks to 29 weeks (6). However there is a significant decrease in the concentration of this fatty acid in the cryptorchid testis or in the testis of the rat treated with cadmium chloride. In both of these instances there is degeneration of the germinal epithelium of the testis.

The fact that in the testis this fatty acid can be synthesized by de novo synthesis (1), can be elongated to stearic acid (3) and is rapidly catabolyzed and incorporated into important lipid fractions is suggestive evidence of the important role that saturated fatty acids have in the structure and function of reproductive tissue.

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# The Absolute Optical Configurations of the Isomeric 9,10-Epoxystearic, 9,10-Dihydroxystearic and 9,10,12-Trihydroxystearic Acids

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## ABSTRACT

The absolute optical configuration of (-)-*cis*-9,10-epoxystearic acid has been verified as being L. Here (-)-*erythro*-9,10-dihydroxystearic acid, isolated from castor oil, was converted by stereospecific reactions to (+)-*cis*-9,10-epoxystearic acid and was thereby proved to be D-9,D-10-dihydroxystearic acid. Removal of the D-12-hydroxy group from the higher melting *erythro*-9,10,12-trihydroxystearate derived from ricinoleic acid, after protection of the glycol group, gave the L-9,L-10-dihydroxystearate derivative. This proved the high melting diastereoisomer to be L-9,L-10,D-12-trihydroxystearate and directly verified the supposition that the higher melting, arsenite-complexing diastereoisomer of such oxidation pairs has the *trans*-10,12-diol grouping. On this basis, the higher melting *threo*-trihydroxystearate from ricinoleate must be D-9,L-10,D-12-trihydroxystearate and removal of the 12-hydroxy group must give D-9,L-10-dihydroxystearate which proved to be the levorotatory enantiomer. The dextrorotatory L-9,D-10-dihydroxystearate was transformed by stereospecific reactions to (+)-*trans*-9,10-epoxystearic acid, thereby defining the absolute configurations of *trans*-9,10-epoxystearic acids. On the basis of these results conclusions may be drawn as to the stereospecificity and site of action of enzymes which hydrate 9,10-epoxystearic acids.

## INTRODUCTION

The absolute optical configurations of the enantiomers of *cis*-12,13-epoxyoleic (vernolic) acid and of *erythro*- and *threo*-12,13-dihydroxyoleic acids have been established (1,2). Much less is known, however, of the stereochemistry of the corresponding 9,10-substituted acids. Powell et al. (3) described the occurrence of *cis*-9,10-epoxystearic acid and *cis*-9,10-epoxy-*cis*-12-octadecenoic (coronaric) acid in *Xeranthemum annuum* seed oil along with vernolic acid. They verified that (+)-vernolic acid has the D-configuration and established that the two *cis*-9,10-epoxy acids, whose methyl esters are both dextrorotatory, are of the L-configuration.

By a similar approach, namely determination of the rotation of the mixture of 9- plus 10-hydroxystearate isomers obtained by reductive cleavage of the epoxy group, the L-configuration of (+)-methyl *cis*-9,10-epoxystearate, derived in this instance from the uredospore lipids of a wheat stem rust (4), has now been confirmed.

The absolute configuration of the *erythro*-9,10-dihydroxystearic acid from castor oil (5) was established by its conversion, by the stereospecific reactions summarized in Figure 1(a), to a *cis*-9,10-epoxystearic acid and its methyl ester whose rotations then defined the configuration of the *erythro*-dihydroxy acid.

The determination of the absolute configuration of (+)- or (-)-*threo*-9,10-dihydroxystearic acid was not so straightforward. An attempt to elucidate this by comparing the two products of partial tosylation with the two products of cleavage of L-*cis*-9,10-epoxystearic acid with toluene-p-sulphonic acid, in an approach similar to that used to establish the configurations of (+)- and (-)-*threo*-12,13-dihydroxyoleic acids, failed because of the impossibility of separating the isomeric hydroxy, tosyloxystearate products in the absence of any adjacent double bond, as foreshadowed by that earlier paper (2). However, an alternative approach was possible. We had previously suggested (1), on the basis of their melting points, that the higher melting *threo*-9,10,12-trihydroxy derivative from ricinoleate had a *trans*-disposition of the 10- and 12-hydroxy groups and therefore had the D-9,L-10,D-12 configuration. Wood et al. (6) assigned configurations to all four 9,10,12-trihydroxystearic acids, largely on the basis of arguments as to the steric effect of the 12-hydroxy group on the ratio of the individual isomers of each pair of products formed on hydroxylation of the 9,10-double bond but also on the basis of their thin layer chromatography (TLC) and gas liquid chromatography (GLC)

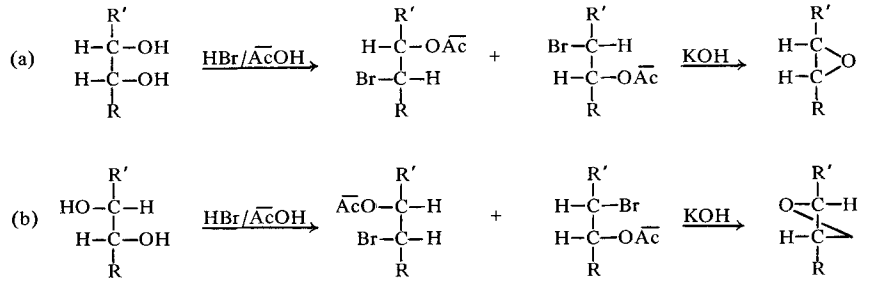

FIG. 1. Summary of reactions used in the stereospecific conversion of (a)(-)-*erythro*-dihydroxystearic acid to (+)-*cis*-epoxystearic acid (R=$CH_3(CH_2)_7$- ; R'= -$(CH_2)_7CO_2H$) and (b)(+)-*threo*-9,10-dihydroxystearic acid to (+)-*trans*-9,10-epoxystearic acid (R=$CH_3(CH_2)_7$- ; R' = -$(CH_2)_7CO_2H$); (+)-*threo*-12,13-dihydroxyoleic acid to (+)-*trans*-12,13-epoxyoleic acid (R=$CH_3(CH_2)_4$- ; R' = -$CH_2CH{=}CH(CH_2)_7CO_2H$); and (+)-*threo*-12,13-dihydroxystearic acid to (+)-*trans*-12,13-epoxystearic acid (R=$CH_3(CH_2)_4$- ; R' = -$(CH_2)_{10}CO_2H$). Ac = -$COCH_3$.

characteristics and their melting points. By similar considerations these authors also assigned configurations to the eight diastereoisomeric 9,10,12,13-tetrahydroxystearic acids. There is only one consistent structural difference between the two diastereoisomers comprising each of the six "oxidation pairs" represented by these 12 compounds, namely that one diastereomer must have the hydroxyl groups in the 10- and 12- positions *cis* (or *erythro*, in the terminology of Wood et al. [6]) relative to each other while the other isomer must have these 10- and 12-hydroxyls in a *trans* (or *threo*) relative configuration. One particularly consistent relationship between the properties of the individual diastereoisomers in each of these six oxidation pairs is that the higher melting isomer always migrates slightly less rapidly on TLC on normal silica gel but much more rapidly on TLC on sodium arsenite impregnated silica gel (6,7). These properties are clearly dependent on the spatial relationship and interactions of the hydroxy groups and this consistency in properties between the individual compounds in the six oxidation pairs must derive from the one consistent structural relationship available. Wood et al. (6) and we conclude that the higher melting isomer of each oxidation pair of these 9,10,12-trihydroxy, and 9,10,12,13-tetrahydroxystearic acids has a *trans* or *threo* disposition of the 10- and 12-hydroxyls.

If these conclusions were correct and the higher melting *threo*-9,10,12-trihydroxystearate were indeed the D-9,L-10,D-12-isomer, then if the 12-hydroxyl were removed we would have either (+)- or (-)-*threo*-9,10-dihydroxystearate of known configuration, namely D-9,L-10. Before this was done, the validity of assigning the *trans*-10,12-configuration to the higher melting isomer of an oxidation pair could be tested with the *erythro*-9,10,12-trihydroxystearates because the absolute configurations of the 12-hydroxyl and the *erythro*-9,10-diol groups were now separately known. The same series of reactions were used in each case for removal of the 12-hydroxyl without affecting the 9,10-diol system and, using the higher melting *erythro*-9,10,12-trihydroxystearate as the example, these are summarized in Figure 2.

Having defined the stereochemistry of a *threo*-9,10-dihydroxystearic acid, it could then be converted stereospecifically to an optically active *trans*-9,10-epoxystearic acid, as summarized in Figure 1(b), thereby establishing the configuration of a *trans*-epoxy fatty acid for the first time. (The opportunity was also taken to prepare similarly *trans*-12,13-epoxystearic and -oleic acids of defined configuration and thereby complete the stereochemical definition of the 12,13-substituted fatty acid series.)

Once the absolute configurations of these 9,10-epoxy acids and 9,10-dihydroxy acids were known, the stereochemistry and site of attack of enzymic hydration of (-)-*cis*-9,10-epoxystearic acid to (+)-*threo*-9,10-dihydroxystearic acid in the spores of various plant stem rusts, (e.g., 8), would become apparent. Similarly, although the hydrating enzyme derived from a *Pseudomonas* species, which specifically attacks only one enantiomer of *cis*- or of *trans*-9,10-epoxystearic acid to give optically active *threo*- or *erythro*-9,10-dihydroxystearic acid, has been proved to attack the 10-position in each case (9,10), the stereochemistry of neither substrates nor products was known but would also now be defined.

## EXPERIMENTAL PROCEDURES

### Materials

D-*cis*-12,13-Epoxyoleic acid was isolated from *Vernonia anthelmintica* seed oil (1,2) and D-12-hydroxystearic acid was derived from

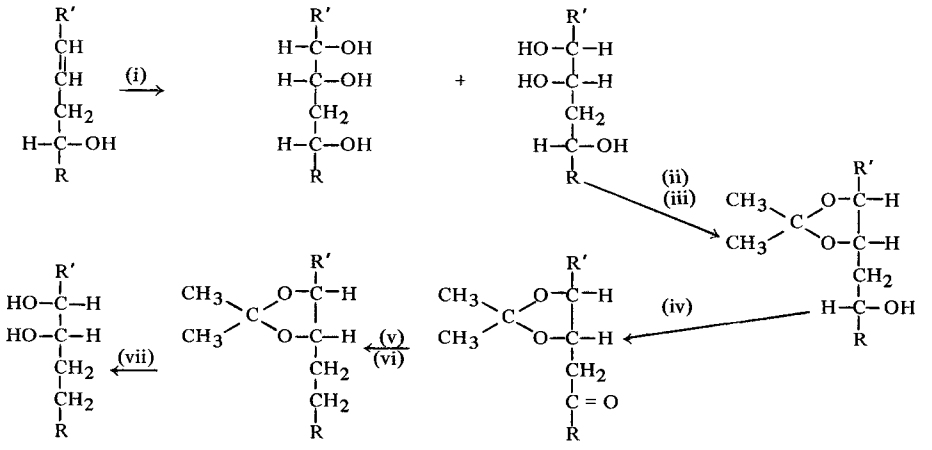

FIG. 2. Summary of the reactions used in the determination of the relative configuration of the 10- and 12-hydroxyl groups of the higher melting isomer of *erythro*-9,10,D12-trihydroxystearate (R= $CH_3(CH_2)_5$- ; R' = $-(CH_2)_7CO_2CH_3$). (A similar sequence was used in the determination of the absolute configuration of *threo*-9,10-dihydroxystearate.) Reactions are: (i) hydroxylation with alkaline $KMnO_4$ (or performic acid); (ii) separation of diastereoisomers on arsenite impregnated TLC layers; (iii) isopropylidene formation with acetone/$HClO_4$; (iv) oxidation with $CrO_3$/AcOH; (v) tosylhydrazone formation with toluene-p-sulphonylhydrazide; (vi) hydrogenolysis with $NaBH_4$ in MeOH; (vii) regeneration of free glycol group with boric acid in 2-methoxyethanol.

ricinoleic acid. D-9-Hydroxystearic acid was obtained by catalytic hydrogenation of (+)-9-hydroxy-*cis*-12-octadecenoic acid isolated from *Strophanthus kombe* seed oil (cf. [11]). D-10-Hydroxystearic acid was prepared by hydration of oleic acid by a *Pseudomonas* species (12) and (-)-*cis*-9,10-epoxystearic acid was isolated from the uredospores of the wheat stem rust *Puccinia graminis* (4).

The (-)-*erythro*-9,10-dihydroxystearic acid was isolated from the mixed fatty acids of castor oil by crystallization at 0 C from 10 volumes of ethyl acetate and (+)-*threo*-9,10-dihydroxystearic acid was isolated from the sclerotial lipids of the fungus *Claviceps sulcata* (13). The (+)-*threo*-12,13-dihydroxyoleic acid was obtained by incubation of ground *V. anthelmintica* seeds in the presence of water, as described previously (2) and (+)-*threo*-12,13-dihydroxystearic acid was obtained from it by catalytic hydrogenation.

The *erythro*- and *threo*-9,10,12-trihydroxystearic acids were derived from ricinoleic acid by hydroxylation with dilute alkaline permanganate and with performic acid, respectively (14). The two diastereoisomers of each oxidation pair were separated and purified by preparative TLC of the esters of silica gel impregnated with sodium arsenite as described previously (1,7).

Methyl esters of all these acids were prepared by reaction with diazomethane in diethylether-methanol solution.

#### Physical Methods

Optical rotations were measured at 546.1 nm in a 2.0 cm cell with an ETL/NPL automatic polarimeter (type 143A), as solutions in methanol, ethanol or chloroform (concentrations 0.1-9%).

Mass spectra were obtained on an AEI MS12 instrument and were interpreted on the basis of published work (e.g., 9,15) and by comparison with the mass spectra of pure authentic samples.

#### Reduction of Epoxy Esters

Methyl D-*cis*-12,13-epoxyoleate and methyl (+)-*cis*-9,10-epoxystearate were each hydrogenated at atmospheric pressure in ethyl acetate-acetic acid (1:1) solution over Adam's platinum oxide catalyst to yield 12- plus 13-hydroxystearate and 9- plus 10-hydroxystearate mixtures, respectively. These mixtures were freed from other minor products by preparative TLC.

#### Conversion of Dihydroxy Acids to Epoxy Acids

As shown in Figure 1, (-)-*erythro*- and (+)-*threo*-9,10-dihydroxystearic acids and (+)-*threo*-12,13-dihydroxystearic and -oleic acids (100-200 mg) were each reacted overnight at room temperature with a 15% solution of anhydrous HBr in acetic acid (10 ml) according to Julietti et al. (16). The resulting mixtures of bromo, acetoxy acids were each isolated and heated with 5% methanolic KOH and the epoxy acid products were esterified with diazomethane and purified by preparative TLC. Yields of pure epoxy ester varied from ca. 40% for *trans*-12,13-epoxyoleate to 80% for *cis*-9,10-

TABLE I

Specific Rotations of Dihydroxy Esters and of Their Epoxy Acid and Ester Products

| Dihydroxyester (source) | [α] 546.1 nm (c; solvent) | Epoxy product | [α] 546.1 nm of acid (c; solvent) | [α] 546.1 nm of ester (c; solvent) |
|---|---|---|---|---|
| (i) *erythro*-9,10-diOH-18:0 (castor oil) | -0.12° (1.1; MeOH) -0.04° (9.0; EtOH) | *cis*-9,10-epoxy-18:0 | +0.21° (2.45; MeOH) | -0.27° (2.75; MeOH) -0.22° (2.8; $CHCl_3$) -0.35° (2.75; hexane) |
| (ii) *threo*-9,10-diOH-18:0 *(Claviceps sulcata)* | +22.5° (1.2; MeOH) | *trans*-9,10-epoxy-18:0 | +10.0° (1.1; MeOH) +9.5° (1.0; $CHCl_3$) | +15.0° (1.3; MeOH) +14.5° (1.4; $CHCl_3$) |
| (iii) *threo*-12,13-diOH-18:1 *(Vernonia* seed) | +20.3° (1.0; EtOH) | *trans*-12,13-epoxy-18:1 | +12.9° (0.7; MeOH) +12.7° (0.5; $CHCl_3$) | +14.6° (0.7; MeOH) |
| (iv) *threo*-12,13-diOH-18:0 (*Vernonia* + $H_2$) | +23.7° (1.35; MeOH) | *trans*-12,13-epoxy-18:0 | +21.4° (1.3; MeOH) | +19.5° (4.3; MeOH) |

epoxystearate.

#### Preparation of Isopropylidene Derivatives

The methyl esters of (-)-*erythro*- and (+)-*threo*-9,10-dihydroxystearic acids and of the higher melting diastereoisomers of *erythro*- and *threo*-9,10,12-trihydroxystearic acids were treated for 15 min at room temperature with ca. 100 volumes of a 0.2% solution of 60% perchloric acid in acetone (17). Almost quantitative yields of isopropylidene derivatives were obtained which were purified by preparative TLC. The *erythro*-trihydroxy ester product contained up to ca. 30% of a second component slightly more mobile than the major 12-hydroxy,9,10-isopropylidene product on TLC. This minor product was removed by preparative TLC and proved to be the 9-hydroxy,10,12-isopropylidene isomer by mass spectrometry.

#### Conversion of 9,10,12-Trihydroxystearates to 9,10-Dihydroxystearates

As shown in Figure 2, the 9,10-isopropylidene derivatives of the higher melting diastereoisomer of *erythro*- and of *threo*-9,10,12-trihydroxystearate (ca. 250 mg) were each oxidized with a 5% solution of $CrO_3$ in glacial acetic acid (25 ml) for 15 min at room temperature. The 9,10-isopropylidene,12-ketostearate products were isolated by ether extraction and thorough washing with water and were purified by preparative TLC to give ca. 50% yield.

The tosylhydrazones of these two 12-keto, isopropylidene compounds (ca. 100 mg) were prepared by their reaction with toluene-p-sulphonylhydrazide (18) (50 mg) in boiling methanol (5 ml) for 6 hr. Each product was extracted into ether, washed with dilute $H_2SO_4$ and then with water till neutral. The tosylhydrazone derivatives (ca. 70% yield) were purified by preparative TLC.

Hydrogenolysis of the tosylhydrazone groups was accomplished by refluxing these derivatives (ca. 70 mg) overnight with $NaBH_4$ (200 mg) in methanol (4 ml) (19). Each reaction mixture was diluted with water, acidified, extracted with ether and the extract thoroughly washed with water. The products were recovered and the isopropylidene derivatives of *erythro*- and *threo*-9,10-dihydroxystearates were isolated (ca. 50% yield) by preparative TLC. The purity of each of these products and the 9,10-position of the isopropylidene grouping were verified by GLC and by mass spectrometry (cf. [15]).

The isopropylidene blocking group was released from each of these products (ca. 20 mg) by heating them at 100 C for 2 hr in a 25% solution of boric acid in 2-methoxyethanol (20). The *erythro*- and *threo*-9,10-dihydroxystearate products were again purified by preparative TLC in almost quantitative yield. The over-all yields from the 9,10,12-trihydroxystearic acids were approximately 12% and 17%, respectively of *erythro*-9,10-dihydroxystearate and *threo*-9,10-dihydroxystearate. TLC on arsenite impregnated silica (14) and mass spectrometry (9) again verified the purity and the positional and structural identity of each product.

TABLE II

Specific Rotations of Natural 9,10-Dihydroxystearates and Their Isopropylidene Derivatives and the Corresponding Products From 9,10,12-Trihydroxystearates

| Compound | [α] 546.1 nm (c; solvent) | Product | [α] 546.1 nm (c; solvent) |
|---|---|---|---|
| *erythro*-9,10,D-12-triOH-18:0 (higher melting isomer) | | *erythro*-9,10-diOH-18:0-isopropylidene derivative | +0.65° (6.5; EtOH) |
| D-9,D-10-diOH-18:0 (castor oil) | -0.04° (9.0; EtOH) | D-9,D-10-diOH-18:0-isopropylidene derivative | -0.50° (6.0; EtOH) |
| *threo*-9,10,D-12-triOH-18:0 (higher melting isomer; i.e., D-9,L-10,D-12) | | D-9,L-10-diOH-18:0-isopropylidene derivative | -29.0° (0.15; EtOH) |
| | | D-9,L-10-diOH-18:0 | -20.6° (0.15; EtOH) |
| *threo*-9,10-diOH-18:0 *(Claviceps sulcata)* | +22.5° (1.2; MeOH) | *threo*-9,10-diOH-18:0-isopropylidene derivative | +26.1° (2.15; EtOH) |

## RESULTS

Catalytic reduction of methyl D-(+)-*cis*-12,13-epoxyoleate gave a mixture of 12-hydroxystearate plus 13-hydroxystearate whose specific rotation (-0.43° in EtOH) was of similar magnitude and the same sign as authentic methyl D-12-hydroxystearate (-0.66° in EtOH). This verified that the reductive cleavage of the epoxy group had proceeded, as expected, with retention of configuration of the oxygenated function and with little racemization. Similar reductive cleavage of methyl *cis*-9,10-epoxystearate (+0.3° in MeOH), the ester of (-)-*cis*-9,10-epoxystearic acid from *P. graminis* spores (4), gave a dextrorotatory mixture of 9-hydroxystearate and 10-hydroxystearate (+0.16° in EtOH, + 0.3° in $CHCl_3$) whereas D-9-hydroxystearate derived from strophanthic acid and D-10-hydroxystearate from the *Pseudomonas* species, whose absolute configurations are now satisfactorily established (21), were both levorotatory (-0.32° and -0.28°, respectively, in $CHCl_3$). Thus the (-)-*cis*-9,10-epoxystearic acid derived from wheat stem rust uredospores was proved to be of the L-configuration (i.e., 9-*R*,10-*S* in the Cahn-Ingold-Prelog system [22]). This confirms the conclusions reached by Powell et al. (3) by a similar procedure applied to the (+)-9,10-epoxy esters derived from *X. annum* seed oil.

Methyl *erythro*-9,10-dihydroxystearate derived from castor oil, when converted stereospecifically to the *cis*-epoxide by the sequence outlined in Figure 1(a), gave a product that was clearly the D-enantiomer of the one characterized above, from the rotations summarized in Table I(i). Therefore (-)-*erythro*-9,10-dihydroxystearic acid was proved to have the D-9,D-10 configuration (i.e., 9-*S*,10-*R*). This, of course merely confirms the elegant and long-standing work of King (5) who effected a similar transformation with the same result but was unable, at that time, to draw the final stereochemical conclusion.

The specific rotations of natural 9,10-dihydroxystearates and their isopropylidene derivatives and the corresponding compounds derived from the higher melting diastereoisomeric forms of *erythro*- and *threo*-9,10,12-trihydroxystearates are summarized in Table II.

The higher melting diastereoisomer of methyl *erythro*-9,10,12-trihydroxystearate, when subjected to the sequence of reactions depicted in Figure 2, gave an *erythro*-9,10-dihydroxystearate isopropylidene derivative that was dextrorotatory. The isopropylidene derivative of the *erythro*-9,10-dihydroxystearate from castor oil, however, which had now been proved to be the D-9,D-10-diol, was levorotatory so that this product from the trihydroxy ester was the L-9,L-10-enantiomer. As the 12-hydroxyl group of the original trihydroxy ester was known to be D, the configuration of ricinoleic acid from which it was derived, this higher melting *erythro*-trihydroxy ester was thereby proved to be L-9,L-10,D-12-trihydroxystearate (i.e., 9-*R*10-*S*,12-*R*).

This result established directly that the higher melting isomer of the *erythro*-9,10,12-trihydroxy oxidation pair from ricinoleate had the 10- and 12-hydroxyl groups in a *trans* or *threo* relative disposition, i.e., L-10,D-12. This agrees with the conclusions of Wood et al. (6) and, on the basis of the considerations outlined in the Introduction, it is therefore considered virtually certain that, in all such oxidation pairs having a 1,3-diol system at positions 10 and 12, the higher melting isomer would have a *trans*- and the lower melting isomer a *cis*-10,12-diol configuration.

On this basis the higher melting *threo*-9,10,12-trihydroxy product from ricinoleate

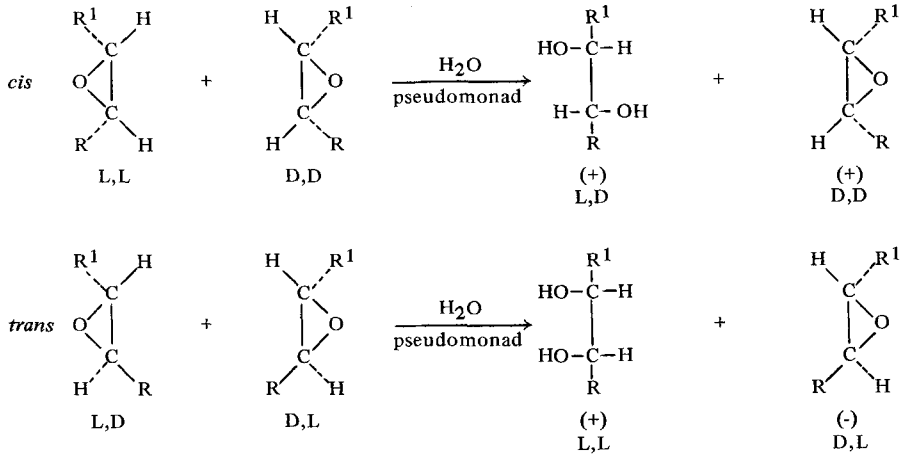


FIG. 3. Summary of the stereochemistry of the action of the pseudomonad hydratase enzyme on racemic cis- and *trans*-9,10-epoxystearic acids. (R = $CH_3(CH_2)_7$- ; R' = -$(CH_2)_7CO_2H$).

must be D-9,L-10,D-12-trihydroxystearate (i.e., 9-*S*,10-*S*,12-*R*). Removal of the D-12-hydroxy group by the reaction sequence of Figure 2, therefore, gave the isopropylidene derivative of D-9,L-10-dihydroxystearate and, on removal of the isopropylidene group, gave free D-9,L-10-dihydroxystearate. Both these products were strongly levorotatory whereas, the *threo*-9,10-dihydroxystearate from *C. sulcata* (13) and its isopropylidene derivative were strongly dextrorotatory and therefore of the L-9,D-10-configuration (i.e., 9-*R*,10-*R*).

The slight residue of doubt that might remain because this configurational conclusion has not been reached directly, but relies on the more general conclusion discussed above that in all oxidation pairs having a 1,3-diol system at positions 10 and 12 the higher melting isomer has a *trans*- 10,12- diol configuration, may be dispelled by the following considerations. The *threo*-9,10-dihydroxystearic acid now assigned the L-9,D-10 configuration has the same sign and similar magnitude of rotation (+22.5°) as the *threo*-12,13-dihydroxystearic acid (+23.8°) derived by hydrogenation from (+)-*threo*-12,13-dihydroxyoleic acid, which we have unequivocally shown to have the L-12,D-13 configuration; i.e., the configuration of the glycol group relative to the chain of the molecule is the same in each case for the same sign of rotation. This relationship between configuration and rotation holds for the corresponding positional isomers of all other substituted stearic acids so far characterized stereochemically. Thus, 9-,10-,12- and 13-hydroxystearic acids of the D-configuration are all levorotatory (3,20), D-9,D-10- and D-12,D-13- dihydroxystearic acids are both levorotatory (5,2), and D-*cis*-9,10-epoxystearic acid and D-*cis*-12,13-epoxystearic acid are both dextrorotatory, their methyl esters both being levorotatory ([3] and this paper). These correlations must clearly be more than fortuitous and, together with the arguments adduced previously as to the configurational relationships within and between the various oxidation pairs of 9,10,12-trihydroxystearates and 9,10,12,13-tetrahydroxystearates, confirm the validity of this conclusion that L-9,D-10-dihydroxystearic acid is the dextrorotatory isomer.

Having thus defined the absolute configuration of (+)-*threo*-9,10-dihydroxystearic acid it was readily possible to transform it stereospecifically, by the reaction sequence of Figure 1(b), to the *trans*-epoxy acid, L-9,D-10-epoxystearic acid (i.e., 9-*R*,10-*R*). This product and its ester were both dextrorotatory as summarized in Table I(ii).

The opportunity was also taken to complete our stereochemical definition of the 12,13-substituted family of acids (1,2) by similar conversion of L-12,D-13-dihydroxyoleic and -stearic acids to the corresponding *trans*-epoxy acids, L-12,D-13-epoxyoleic and -stearic acids (i.e., 12-*R*,13-*R*). These products and their esters were also found to be the dextrorotatory enantiomers, Table I(iii) and (iv), thus maintaining the correlation discussed above between configuration and rotation for 9,10- and 12,13-positional isomers.

## DISCUSSION

The work herein reported has confirmed that (-)-*cis*-9,10-epoxystearic acid has the L-configuration (9-*R*,10-*S*) and that (-)-*erythro*-9,10-dihydroxystearic acid has the D-9,D-10-configuration (9-*S*,10-*R*). It has been estab-

lished that (+)-*threo*-9,10-dihydroxystearic acid has the L-9,D-10-configuration (9-*R*,10-*R*). The stereochemistry of *trans*-epoxy fatty acids has also been defined for the first time, the dextrorotatory enantiomers being L-9,D-10-epoxystearic acid (9-*R*,10-*R*), L-12,D-13-epoxystearic acid and L-12,D-13-epoxyoleic acid (both 12-*R*,13-*R*). In addition, the absolute configurations of the four 9,10,12-trihydroxystearic acid diastereoisomers derived by hydroxylation of ricinoleic acid have been determined, the results confirming the ealier conclusions of ourselves (1) and of Wood et al. (6).

Tulloch (8,23) has shown that in the spores of *P. graminis* and of a number of other plant rusts that contain (-)-*cis*-9,10-epoxystearic acid there is an enzyme system which, on incubation of the spores in water, hydrates the epoxy acid to (+)-*threo*-9,10-dihydroxystearic acid. This enzyme evidently becomes activated in the early stages of germination of such plant rust spores (24,25). The epoxy acid and the dihydroxy acid of these spores have now been proved to have the L-9,L-10- and L-9,D-10-configurations, respectively. Therefore, assuming a normal mechanism for epoxide ring opening, the enzymic hydration must proceed by hydroxyl attack at the 10-position of the epoxide, resulting in inversion at that position. This has now been verified directly by incubating *Puccinia* spores in $H_2{}^{18}O$ enriched water and finding that the $^{18}O$ isotope is enriched exclusively in the 10-hydroxyl group of the product (26,27).

A somewhat different state of affairs exists with respect to the hydrative enzyme isolated from a *Pseudomonas* species, which hydrates oleic acid to D-10-hydroxystearic acid (9,10,28). The same enzyme preparation was recently shown (9,10) also to hydrate *cis*- and *trans*-9,10-epoxystearic acids to *threo*- and *erythro*-9,10-dihydroxstearic acids, respectively, with apparently complete substrate and product stereospecificity. In each case the site of hydroxyl attack was proved to be at the 10-position, by incorporation of $^{18}O$ from $H_2{}^{18}O$ exclusively at that position (9,10). However, the absolute configurations of neither epoxy acid substrates nor of dihydroxy acid products were known so that the detailed stereochemistry of these reactions could not be defined. This is now possible on the basis of the configurational determinations reported herein and is illustrated in Figure 3. The configurations of substrates and products are fully in accord with the results of the isotope studies of Schroepfer and his colleagues (9,21,28) and have enabled a fairly detailed postulate of the relevant features of the hydratase enzyme and its mechanism of action to be made (26,27).

In addition to these conclusions from the results of the present work, the absolute configurations of the optically active trihydroxy acids in *Chamaepeuce* seed oils (29) are also now clear; *threo*-9,10-18-trihydroxystearic acid ($[\alpha]_D$ of methyl ester = +22.3° in EtOH) and *threo*-9,10-18-trihydroxy-*cis*-12-octadecenoic acid ($[\alpha]_D$ of ester - +18.2° in EtOH) from these seed oils must correspond to (+)-*threo*-9,10-dihydroxystearic acid ($[\alpha]_D$ of ester = +22.5° in MeOH) and have the L-9,D-10-configuration (9-*R*,10-*R*). Whether these acids are derived in the seed from *cis*-9,10-epoxy acid precursors is not known.

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# Release of Chylomicrons by Isolated Cells of Rat Intestinal Mucosa[1]

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## ABSTRACT

Fat-laden mucosal cells were isolated by flotation from fed male rats after digesting scrapings of washed jejunum with collagenase in bicarbonate buffer. About 50-60 million cells were obtained per preparation, which were 95-100% viable as assessed by Trypan Blue. The isolated cells were capable of effective incorporation of labeled fatty acids and glucose into triglycerides and phospholipids, and of labeled leucine and glucosamine into the protein envelope of the released chylomicrons. The secretion of the labeled protein paralleled the release of the labeled fat, both of which were linear with the concentration of the albumin in the incubation mixture. About 80% of the total fat of the cell was released as chylomicrons within 30 min when incubated in the presence of albumin-bicarbonate buffer. Injection of puromycin 24 hr prior to harvesting of cells led to a complete inhibition of chylomicron release. Addition of puromycin to the incubation medium gave 50-80% inhibition of release. No inhibition of release of chylomicrons resulted from a treatment with ethionine. The released chylomicrons were separated from the cells by Millipore filtration.


## INTRODUCTION

It has been well established that after hydrolysis in the lumen the dietary fat is absorbed by the mucosal cells of the small intestine, where it is resynthesized into triglycerides and released in the lymph as chylomicrons. While the main biochemical steps of resynthesis of the hydrolysis products of dietary triglycerides have been elucidated (1-3), the process of assembly and extrusion of the chylomicrons has not. In fact, the constancy of composition of the lipoprotein coating of these particles has been questioned (4), and the essential role of protein synthesis in the formation and release of chylomicrons (5) has been disputed (6,7). Much of this uncertainty is due to the complex organization of the intestinal mucosa with the accompanying blood, lymph and nerve supply and its integrated response to outside stimuli. We have therefore used isolated mucosal cells for the study of chylomicron biosynthesis and secretion in vitro. By modifying previously described methods (8,9) we have obtained preparations of mucosal cells which are specifically involved in the resynthesis of dietary fat.

## METHODS

### Isolation of Mucosal Cells

Male Wistar rats, weighing 200-300 g, were maintained on laboratory chow prior to experiments. After a 24 hr fast each animal received under ether anesthesia 1.5 ml corn oil by stomach tube. One hour later the abdomen was opened under ether anesthesia and the upper one-third of the small intestine was excised, care being taken to remove most of the mesenteric fat. The intestine was immediately flushed with ice cold Krebs bicarbonate buffer, pH 7.4, containing 6 μmoles glucose per ml. Suitable lengths (10-15 cm) of the intestine were everted over a stainless steel probe and the mucosal side washed free of the lumen contents. The section of the intestine was tied off at one end and the everted sacs blotted lightly on filter paper. The mucosal cells were scraped off by passing the everted sac between a pair of smooth flattened forceps proceeding from the open end of the sac. During these manipulations (4-5 min) the segments of the intestine remained submerged in ice cold buffer.

The mucosal scrapings were incubated at 37 C with 5 ml of the bicarbonate buffer containing 10 mg collagenase (Type III, *Clostridium histolyticum*, Sigma Chemical Co., St. Louis, Mo.) for 5-60 min with 2 sec mechanical agitation every 15 min. The resulting cell suspensions were centrifuged in polyethylene tubes for 4 min at 400 x g. The fat-laden mucosal cells floated to the surface while other cells were sedimented and could be removed by aspiration. The fat cells were washed thrice by

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suspending them in 10 ml warm (37 C) bicarbonate buffer and recentrifuging for 1 min at 400 x g. The cells were morphologically normal when stained with hemotoxylin or eosin and were 95-100% viable as assessed by the Trypan Blue penetration test. The yield was 50-60 million cells per preparation. In subsequent experiments the cells were used in batches of 5 million, which represented ca. 1.2 mg of triglyceride.

#### Measurement of Chylomicron Release

The release of chylomicrons was assessed by incubating the cells at 37 C in 5 ml bicarbonate buffer containing various concentrations of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). The released chylomicrons were separated from the cells by filtration through a 0.85 or a 1.2 $\mu$ Millipore filter (Millipore Filter Co., Bedford, Mass.). The chylomicrons were recovered from the filtrate by centrifugation at 10,000 x g for 30 min and were washed with bicarbonate buffer and recentrifuged three more times. The triglyceride content of the chylomicrons was determined by direct gas chromatography of the total lipid extracts (10) in the presence of tridecanoin as an internal standard. The chylomicrons were shown to remain intact during Millipore filtration by means of electron microscopy.

#### Electron Microscopy

The isolated cells were fixed in 3% glutaraldehyde in 0.15 M phosphate buffer, pH 7.2, centrifuged, rinsed with buffer and then postfixed in 2% osmium tetroxide in 0.15 M phosphate buffer. The cells were dehydrated in alcohol and propylene oxide, embedded in Epon araldite, polymerized and cut. Before photography the preparations were stained with lead hydroxide and counter-stained with uranyl acetate.

The chylomicrons were examined by suspending a small drop of the filtrate on a Formvar-coated grid which was then exposed for 20 min to osmium tetroxide vapor generated from a 2% solution. The grid was allowed to dry at room temperature and then an examination made in the microscope. Alternatively the chylomicrons were fixed in buffered osmium tetroxide, dehydrated by standard methods, embedded in Epon 812, polymerized, cut and stained as described for the cells. The micrographs were made with an RCA 3G Electron Microscope.

#### Assessment of Metabolic Activity

The metabolic activity of the cells was measured by determining their response to the administration of selected metabolites and known inhibitors of specific metabolic transformations in the intestinal mucosa. The general metabolic activity of the isolated cells was determined by comparing the glycolytic activity of the isolated cells with that of the everted sacs of the jejunum. For this purpose measurements were made of the radioactivity recovered in the esterified glycerol following addition of glucose-U-$C^{14}$ (0.1 $\mu$c/3 $\mu$moles) to the incubation medium. The comparisons were based on equal numbers of cells.

The biosynthesis of protein by the mucosal cells was determined after feeding of leucine-U-$C^{14}$ to the animal along with the corn oil (0.1 $\mu$c/0.1 mg) and after addition of the amino acid to the cell suspension (1.0 $\mu$c/0.002 mg). The radioactivity in the chylomicron envelope from glucosamine-1-$C^{14}$ was determined following addition of 1 $\mu$c/0.02 mg of the amino sugar to the incubation mixture. Uptake and release of lipids was estimated by feeding to the animals 1-$C^{14}$-palmitic acid (5 $\mu$c/0.5 mg) and by adding the palmitic acid (0.1 $\mu$c/0.01 mg) to the cell suspension. The radioactivity of the lipids was measured in the triglycerides of the released chylomicrons.

The effect of inhibitors on the formation and release of chylomicrons by isolated cells of the mucosa was assessed by injecting puromycin (Nutritional Biochemicals, Cleveland, Ohio) 24 hr prior to the oil feeding (5 mg in 1 ml phosphate buffer every 8 hr [5]) and by adding 1 mg puromycin to the incubation medium. The effect of DL-ethionine (Nutritional Biochemicals, Cleveland, Ohio) was determined by adding 10 mg of the dissolved substance to the incubation medium.

TABLE I

Release of Mucosal Cells by Collagenase *(Cl. histolyticum)*[a]

| Time of incubation, min | Released number of cells x $10^6$ (mean ± standard error) |
|---|---|
| 5 | 9.3 ± 2.1 |
| 10 | 18.8 ± 1.9 |
| 15 | 27.2 ± 2.7 |
| 20 | 39.5 ± 2.1 |
| 30 | 50.2 ± 2.7 |
| 60 | 53.2 ± 1.8 |

[a]Data from six incubations of mucosal scrapings from the upper one-third of jejunum of fed male rats.

## RESULTS AND DISCUSSION

#### Release of Fat-Laden Cells

Suspensions of intestinal epithelial cells

were first prepared by Harrer et al. (8), who used trypsin and pancreatin for the digestion of the connective tissue, and disrupted the resulting cell clumps by forcing them through fine wire mesh. Other workers have since employed lysozyme (11) and hyaluronidase (9) or purely mechanical means (12-14) for the preparation of suspensions of intestinal cells. In all cases the cells were obtained from fasting animals and could be collected as a pellet by centrifugations. In the present work the isolation of the fragile fat-laden cells of the intestinal mucosa was achieved by digestion with collagenase. This enzyme had previously given excellent dispersions of the fat cells of adipose tissue (15) and of liver (16). Table I gives the yields of the mucosal cells obtained by digestion of the mucosal scrapings of the upper one-third of rat jejunum with collagenase for various periods of time. Under the experimental conditions nearly complete release of the cells was obtained within 30 min of the start of the incubation. The yield of 50-60 x $10^6$ cells for the upper one-third of the small intestine of the rat obtained in the present study compares favorably with the average yield of 176 x $10^6$ cells for the entire small intestine reported by Harrer et al. (8). The viability of our cells, however, was much higher (95-100% vs. 76%). This was probably due to an effective removal of any broken cells, which would have lost their fat, from the fatty layer during centrifugation. Assuming a volume of 6740 $\mu^3$ per mucosal cell, it may be calculated that the total volume occupied by these cells would be ca. 0.34 ml, which is about one-third of the volume obtained by Perris (9) for the whole intestine of the rat by digestion with hyaluronidase.

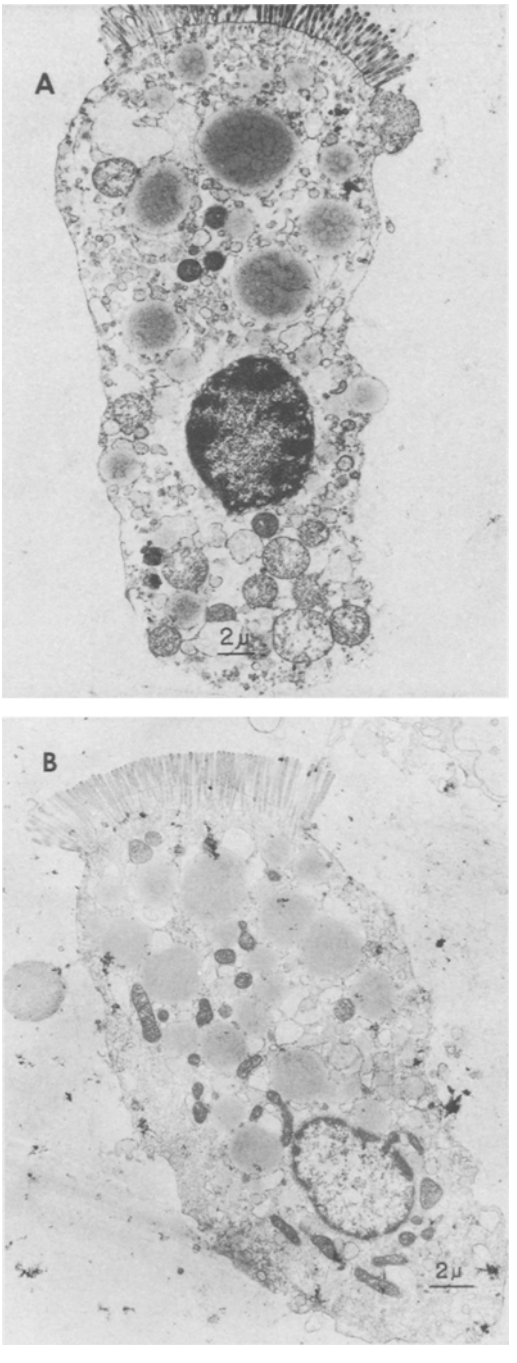


FIG. 1. Electron micrographs of epithelial cells of rat intestinal mucosa. A, a villous cell isolated by collagenase digestion 2 hr after feeding corn oil to a control animal. X9,100; B, a villous cell isolated from a corn oil fed animal pretreated with puromycin. X13,000.

In contrast to previous preparations, the intestinal epithelial cells isolated in this study were free of lymphocytes, granulocytes and immature crypt cells, as well as from bare nuclei and membrane fragments. In the procedure of Harrison and Webster (13) the mucosal cells also are separated from the villi and crypt cells, but due to the exposure to high frequency vibration, damaged membranes remain a possibility (14). The major advantage of the present method of isolation rests on the selectivity which it exhibits for the cells involved in the actual uptake and resynthesis of the dietary fat.

Figure 1 shows electron micrographs of representative intestinal absorptive cells from fed normal and puromycin-treated rats. In general appearance these sections are similar to those commonly observed for individual cells in sections of mucosal tissue. Unlike the individual cells isolated by Iemhoff et al. (14), the present preparation possessed normal endoplasmic reticulum and mitochondria, as well as intact plasma and nuclear membranes. Other sections

TABLE II

Effect of Albumin on the Release of Chylomicrons From Isolated Mucosal Cells[a]

| Treatment | Amount of chylomicron released as fat, mg | | | | |
|---|---|---|---|---|---|
| | 30 min | | 60 min | | 120 min |
| Bicarbonate alone | 51 ± 9 | Not sign. | 64 ± 7 | Not sign. | 72 ± 12 |
| Bicarbonate + 0.01 mg albumin | 113 ± 23 | $P < 0.05$ | 203 ± 17 | $P < 0.1$ | 309 ± 28 |
| Bicarbonate + 0.02 mg albumin | 410 ± 12 | $P < 0.001$ | 604 ± 26 | $P < 0.01$ | 736 ± 22 |
| Bicarbonate + 0.04 mg albumin | 711 ± 11 | $P < 0.001$ | 914 ± 16 | Not sign. | 885 ± 19 |
| Bicarbonate + 0.04 mg albumin + puromycin | 47 ± 10 | Not sign. | 42 ± 6 | Not sign. | 49 ± 4 |

[a]Data from four replicate treatments. The total amount of fat in these cell preparations was 1176 ± 55 mg. The puromycin-treated cells were obtained from animals injected with 15 mg of the antibiotic 24 hr prior to the start of the experiment. The statistical comparisons refer to differences between 30 and 60, and 60 and 120 min time intervals, not between different treatments.

through the clumped cell suspension showed pairs and triplets of cells remaining together. It is seen that both cells contain, in addition to numerous fat droplets surrounded by smooth endoplasmic reticulum, also other much larger fatty drops which are devoid of any membrane and possibly represent the prechylomicrons of Redgrave (17). The smaller droplets surrounded by the membranes are of about the same size as the chylomicrons.

The isolated cells were shown to take up glucose-U-$C^{14}$ at the same rate as everted sacs of the intestinal mucosa and incorporated it into glycerides and phosphoglycerides at corresponding rates. On the basis of these micrographs it could be said that the cells treated with puromycin possessed more of the membrane-free lipid droplets than normal cells, as claimed recently by Friedman and Cardell (18). An examination of numerous electron micrographs of isolated cells and mucosal tissue, however, failed to permit an absolute differentiation between treated and untreated cells, although there might have been a difference in the total cell membrane content. As originally suggested by Sabesin and Isselbacher (5) such a difference would have been anticipated, but it is seen to be only quantitative and not qualitative. A more effective basis for differentiation between normal and puromycin-treated cells was the ability to release chylomicrons.

### Validity of Assay of Chylomicron Release

Although the average chylomicron possesses a diameter which is nearly 20 times smaller than that of the intestinal absorptive cell, it cannot be separated from a fat-laden cell because of closely similar densities. It occurred to us that such a separation could be effected by Millipore filtration in dilute solution. For this purpose we selected filters with average pore sizes ranging from 0.85-2.50 microns. It remained to be demonstrated that the filtration process was not accompanied by cell breakage or oiling out of the chylomicrons, or both. The chylomicrons recovered from the filtrate under the above conditions were shown to have normal appearance in the electron microscope. The micrographs were identical to those reported by Salpeter and Zilversmit (19) for washed chylomicrons from a dog fed corn oil. There was no evidence of structure in the chylomicron core and only a single electron-dense line, which does not resemble plasma membrane, was found. The chylomicrons appearing in the Millipore filtrate were free of contamination with simple fat globules, or subcellular fragments. Furthermore, essentially all of the triglyceride released from the fat cells during incubation with albumin was recovered in the form of chylomicrons in the Millipore filtrate. The rat chylomicrons, however, were smaller than those of the dog, as already pointed out by Zilversmit (4).

### Effect of Albumin

An effective recovery of the fat-laden cells following digestion of the mucosal scrapings with collagenase indicated that the chylomicrons present in the cells did not become

TABLE III

Appearance of Radioactivity From Leucine-U-C14, Glucosamine-U-C14 and 1-C14-Palmitic Acid in Chylomicrons Released by Isolated Mucosal Cells[a]

| Time, min | Release of chylomicrons, mg TG | | Release of radioactivity, cpm | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Leucine | | Glucosamine | | Palmitic acid | |
| 5 | 722 ± 18 | | 7,793 ± 414 | | 15,398 ± 452 | | 8,522 ± 201 | |
| | | Not sign. | | Not sign. | | Not sign. | | Not sign. |
| 15 | 761 ± 13 | | 8,028 ± 358 | | 16,001 ± 432 | | 9,374 ± 223 | |
| | | $P < 0.001$ | | $P < 0.001$ | | $P < 0.001$ | | $P < 0.001$ |
| 30 | 961 ± 28 | | 10,396 ± 366 | | 21,361 ± 403 | | 11,620 ± 303 | |
| | | $P < 0.02$ | | $P < 0.001$ | | $P < 0.001$ | | $P < 0.001$ |
| 60 | 1119 ± 31 | | 14,126 ± 290 | | 24,833 ± 337 | | 14,204 ± 158 | |

[a]Data from four replicate treatments. Radioactive precursors were added to cell suspensions under optimal conditions for chylomicron release. Statistical differences refer to comparisons between time intervals. The correlation coefficients (r) between the appearance of triglyceride mass and radioactivity in the chylomicrons were: triglyceride vs. leucine, 0.9617; triglycerides vs. glucosamine, 0.9777; and triglycerides vs. palmitic acid, 0.9678. The number of degrees of freedom was 30.

released during incubation and subsequent washing of the cells with the bicarbonate buffer. Table II shows that there was little secretion of chylomicrons also during the subsequent incubation with the bicarbonate buffer alone. Addition of albumin, which had been previously reported (20) to compete with the liver cell membrane for chylomicron binding, however, resulted in their rapid release. This discharge of the chylomicrons was approximately linear with increasing concentration of the albumin in the range 0.01-0.04 mg/ml buffer. There was a lesser increase in chylomicron secretion with time of incubation at each of the concentrations. At the optimal concentration of albumin (0.04 mg/ml buffer) ca. 80% of the maximal release was obtained in 30 min. Estimates of the chylomicron release at shorter time intervals showed that up to 50% of the total secretion could be obtained by the end of 5 min of incubation. The maximum amount of triglyceride secreted in the form of chylomicrons was 75-90% of the total triglyceride content of these cells. In other studies (9) with isolated mucosal cells, it has been shown that the presence of 1% albumin greatly enhances the oxygen uptake. An increase in the albumin concentration to 2% had no further stimulatory effect. The addition of up to 0.5% albumin during the release of the cells as a result of hyaluronidase digestion, led to a reduced yield of the cells (9).

Table II also demonstrates that little or no release of chylomicrons takes place from cells poisoned with puromycin 24 hr prior to isolation, even in the presence of optimal albumin concentration. These results would appear to indicate that the impairment in chylomicron secretion resulting from puromycin poisoning is related to some abnormality in function of the mucosal cell as claimed by Sabesin and Isselbacher (5), and not to a decline in lymph flow or to changes in gastric emptying as suggested subsequently by Redgrave and Zilversmit (6) and by Redgrave (7).

### Biosynthesis of Chylomicrons

Table III shows the incorporation with time of radioactive palmitate, leucine and glucosamine into the released chylomicrons. The chylomicron release was quantitated by the amount of triglyceride recovered in the Millipore filtrate. In all cases the release of the radioactivity paralleled closely that of the mass of the chylomicrons. There was a rapid initial release with leveling off after the first 5 min of incubation. The release was resumed at ca. 15 min and progressed rather rapidly until ca. 30 min, by which time most of the chylomicron

TABLE IV

Effect of Ethionine and Puromycin Upon the Appearance of Radioactive Leucine and Glucosamine in the Chylomicrons Released by Isolated Mucosal Cells[a]

| Time, min | Yield of chylomicrons, mg TG | | Release of radioactivity, cpm | | | |
|---|---|---|---|---|---|---|
| | | | Leucine | | Glucosamine | |
| | E | P | E | P | E | P |
| 5 | 737 ± 7 | 536 ± 17 | 8,576 ± 252 | 339 ± 23 | 15,722 ± 547 | 655 ± 21 |
| 15 | 788 ± 6 | 557 ± 10 | 9,613 ± 228 | 390 ± 10 | 16,899 ± 589 | 864 ± 32 |
| 30 | 972 ± 13 | 557 ± 13 | 11,639 ± 256 | 412 ± 27 | 22,340 ± 458 | 939 ± 24 |
| 60 | 1176 ± 47 | 540 ± 13 | 13,988 ± 550 | 356 ± 29 | 25,178 ± 590 | 790 ± 121 |

[a]Data from four replicate treatments. Inhibitors and radioactive precursors were added to the cell suspensions under optimal conditions for release of chylomicrons. TG, triglyceride; E, ethionine; P, puromycin.

had been secreted and the bulk of triglyceride removed. There were only small amounts of chylomicrons released during the final 30 min of incubation. On the basis of electron microscopy it could be suggested that the rapid initial release was probably due to the discharge of preformed chylomicrons present in the cell at the start of the incubation.

In view of the parallel incorporation of the labeled tracers (Table III), it is obvious that the prechylomicrons remain in effective metabolic contact with the cell structure until the moment of their expulsion. In other experiments a comparable relationship was shown between the incorporation of radioactive glucose into the chylomicron lipids and the release of chylomicrons. The incorporation of the glucosamine into the chylomicrons ($d < 1.006$) is related to the presence of a carbohydrate moiety (21) in addition to triglyceride, phospholipid, cholesterol and protein (1). From previous studies it is known that the triglycerides, cholesterol and phospholipids are incorporated into the chylomicrons in the smooth endoplasmic reticulum. The protein component is probably synthesized in the rough endoplasmic reticulum and complexed with the lipid globule either in smooth endoplasmic reticulum (1) or in the Golgi complex (22). The site of carbohydrate addition has been suggested to be the Golgi apparatus. Thus the synthesis and secretion of the chylomicrons appears to require the passage of lipid through the tubules of the endoplasmic reticulum and the Golgi apparatus prior to its release from the cell. It would also require the proper functioning of the entire assembly system, not just a synthesis of protein and lipid.

A rapid incorporation of glucosamine into the glycoprotein of the low density microsomal fraction of rat intestinal mucosa has recently been reported by Forstner (23).

**Effect of Puromycin and Ethionine**

From Table II it was seen that puromycin effectively inhibited the release of chylomicrons from individual mucosal cells isolated from puromycin-poisoned animals. Table IV shows the results obtained by adding puromycin or ethionine to suspensions of mucosal cells isolated from normal animals. While the addition of ethionine had no adverse effect upon the chylomicron secretion and the incorporation of radioactive leucine and glucosamine, inclusion of puromycin in the incubation mixture resulted in an extensive suppression of the secretion of labeled chylomicrons. There was, however, an initial release of chylomicrons, varying from 20-50% of the maximal release observed in control systems. This was attributed to a release of preformed chylomicrons, because they possessed much lower specific activity than those released initially by normal or ethionine-treated cells. The small amounts of radioactivity incorporated initially by the puromycin-treated cells may possibly represent a limited amount of synthesis prior to the realization of the full effect of puromycin.

The lack of effect of ethionine upon the incorporation of radioactive leucine and the secretion of chylomicrons by the isolated cells coincides with the findings of Schlunk et al. (24). These investigators were unable to confirm the earlier claim of Hyams et al. (25) that ethionine inhibited protein synthesis in the intestinal mucosa in vivo and in vitro and interfered with the intestinal transport of triglycerides containing long chain fatty acids. Kessler et al. (26), however, have been able to repeat the work of Hyams et al. (25) using female rats dosed with DL-ethionine 24 hr prior to feeding of 1-$C^{14}$-palmitic acid. They attributed the decreased fat transport observed under these conditions to an impairment of chylomi-

cron completion due to inhibition of either the synthesis of chylomicron apoprotein or the association of preformed triglyceride with the protein moiety of chylomicrons. Since the mechanisms of inhibition of protein synthesis by puromycin and ethionine are unlikely to be the same, and may require different doses and timing for observation, the present results based on addition of ethionine to cell suspensions may not be considered valid evidence in favor of one or the other of the hypotheses.

The results with puromycin appear to support the original claim of Sabesin and Isselbacher (5) that protein synthesis is obligatory for chylomicron secretion. Friedman and Cardell (18) have recently reported the effects of puromycin on the structure of rat intestinal mucosa during fat absorption and have concluded that treated tissue contains much less rough endoplasmic reticulum and Golgi membranes. In another study from our laboratory (27) we have observed that puromycin poisoning leads to a significant suppression of synthesis of phospholipids in addition to proteins while triglyceride formation remains unaffected. Since both protein and phospholipid biosynthesis would be expected to be necessary for membrane assembly, these data support the conclusions of Friedman and Cardell (18) which were based exclusively on electronmicrographic examination of the tissue. Whether the primary defect in puromycin poisoning resides in the interference of the antibiotic with membrane assembly or specific protein and phospholipid synthesis cannot be decided at the present time, if indeed such a distinction can be made at all. In any case additional work with more specific metabolic inhibitors and other precursors of the chylomicron proteins and phospholipids is necessary for a complete understanding of the mechanisms involved in chylomicron formation and release.

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# Biosynthesis of Protein Relative to the Accumulation of Fat in the Liver of EFA Deficient Rats

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## ABSTRACT

Effect of an essential fatty acid (EFA) deficiency in the rat on the incorporation of leucine-$^{14}$C and glucosamine-$^{14}$C into serum and liver protein are reported. Weanling male rats of the Sprague-Dawley strain were raised on a fat-free diet for 10-12 weeks and then switched to diets supplemented with 10% corn oil or 10% hydrogenated coconut oil. Leucine-$^{14}$C or glucosamine-$^{14}$C was injected into the tail veins of the animals of each group. At selected intervals up to 120 min after the injections, the animals were sacrificed and the radioactivity of the liver and serum proteins was measured. The levels of triglyceride (TG) in the serum and the liver were also determined. Less radioactivity was incorporated into the serum $\beta$-lipoprotein ($\beta$-LP) fraction of the hydrogenated coconut oil than the corn oil fed animals injected with leucine-$^{14}$C, but no differences were observed in the incorporation of radioactivity into the liver protein and both albumin and globulin fractions of the serum of these groups of animals. In the similar experiments with glucosamine-$^{14}$C less radioactivity was incorporated into the $\beta$-LP fraction of the serum and into the smooth endoplasmic reticulum of the liver in the hydrogenated coconut oil (EFA deficient) than the corn oil fed animals. Time course studies also indicated that less radioactivity was incorporated into the $\beta$-LP fraction than into the albumin and globulin fractions of the serum of the hydrogenated coconut oil group. These findings suggest that an EFA deficiency results in an impairment of the synthesis or release of lipoprotein.

## INTRODUCTION

In contrast to other experimentally produced fatty livers, the mechanism of the accumulation of fat in the livers of essential fatty acid (EFA) deficient rats is not clear. In recent studies by Fukazawa et al. (1,2) it was shown that the capacity of the liver to secrete triglycerides and phospholipids was impaired by an EFA deficiency. These in vitro studies also indicate that EFA is required for normal lipid transport and synthesis of lipoproteins. The accumulation of fat in the livers of EFA deficient rats could be caused by a combination of factors, particularly by an increase in fatty acid synthesis in the liver (3) and elevated levels of plasma-free fatty acids (4). However the underlying mechanism may involve a defect in the secretion or synthesis of lipoproteins as observed in experimentally produced fatty livers (5). The present investigation was undertaken to obtain information on this aspect of the effect of an EFA deficiency in rats.

## MATERIALS AND METHODS

### Animals

Weanling male rats of the Sprague-Dawley strain (obtained from Dan Rolfsmeyer Co., Madison, Wis.) were raised on a fat-free diet for 10-12 weeks. The rats were then divided into two groups and fed either a diet containing 10% corn oil or 10% coconut oil for 4-6 weeks. Composition of the diets is reported elsewhere (1,2). In order to avoid the influence of fasting on protein metabolism (6), as well as the influence of the fat supplementation on the composition of the blood lipids, rats were fed a fat-free diet overnight prior to their use in an experiment.

### Experiments Using Leucine-$^{14}$C

DL-Leucine-$^{14}$C (specific activity 6.4 mc/mM, obtained from New England Nuclear Corp., Boston, Mass.) dissolved in physiological saline was injected (5 $\mu$Ci/400 g body wt) into the tail vein of animals of each group under a light ether anesthesia. Blood was obtained from the aortas, and the livers were excised 90 or 120 min after the injections. In several experiments 2-3 ml blood was obtained from the retroocular plexus 60 min after injection; blood was withdrawn from the aorta and the liver was excised 120 min after injection. Since the results on blood at 120 min after injection showed no significant difference between the animals with and without bleeding at 60 min, they were combined.

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TABLE I

Animals Injected With Leucine-$^{14}$C

| Group (fat supplement) | Body wt | Liver wt | Liver triglyceride | Serum triglyceride |
|---|---|---|---|---|
| | (g) | (g) | (mg/g liver) | (mg/ml serum) |
| Corn oil | 387 ± 14[a] (11)[b] | 12.5 ± 0.3 (11) | 10.8 ± 0.9 (8) | 65.8 ± 7.8 (8) |
| Coconut oil[c] | 360 ± 10 (12) | 12.5 ± 0.3 (12) | 22.0 ± 2.5[d] (9) | 49.7 ± 7.9 (9) |

[a]Mean ± standard error.
[b]Number of animals.
[c]Hydrogenated coconut oil.
[d]$P < 0.05$ to corn oil group.

The liver was blotted on filter paper, weighed, and a portion of the liver was homogenized in 20 volumes physiological saline with a Potter-Elvehjem homogenizer. Homogenates consisting of 0.05 g original liver were washed three times with 5% trichloroacetic acid (TCA) solution containing 0.5% DL-leucine. After the second TCA washing, liver protein was boiled for 30 min to separate RNA-bound leucine. The TCA insoluble fraction was then delipidized by extractions with ethanol-ether 1:1 v/v and with acetone. After evaporation of the organic solvents in a water bath, the protein residue was dissolved in 1 ml 1N NaOH. Protein was determined by the method of Lowry et al. (7). Radioactivity measurements were made on solutions containing less than 2 mg protein per vial with a Packard Tri-Carb scintillation spectrometer using a scintillator containing toluene-dioxane-triton (8). Activities were expressed as dpm. Corrections of cpm were made by the channel ratio method, using a series of standard vials containing the same scintillation fluid, protein and various amounts of chloroform as a quencher.

Serum β-lipoprotein (β-LP) was determined by the method of Burstein and Samaille (9). This method precipitates both low density lipoprotein (LDL) and very low density lipoprotein (VLDL). However the change in serum β-LP in experimentally induced fatty livers is attributed primarily to the change in VLDL protein (5), and in several experiments no differences were observed in the specific activity of VLDL and β-LP. Therefore the change in serum β-LP in the present study most likely also represents primarily the change in VLDL. Serum β-LP and total serum protein was washed with 5% TCA solution containing 0.5% DL-leucine, as described above, without boiling the protein at the second washing with TCA. The albumin fraction of the serum was separated from TCA-insoluble total serum protein by acidic ethanol (10). The acidic ethanol insoluble fraction of TCA-insoluble total serum protein was expressed as globulin. This fraction contained only trace amounts of albumin as determined by electrophoresis.

Lipids of liver and serum were extracted by chloroform-methanol 2:1 v/v (11) and triglyceride (TG) content measured by the method of Van Handel and Zilversmit (12).

### Experiments Using Glucosamine-$^{14}$C

Experiments with glucosamine-$^{14}$C were performed essentially the same as those with leucine-$^{14}$C. After 12 weeks on a fat-free diet, rats were fed a diet supplemented with 10% corn oil or coconut oil for 4-5 weeks. Rats, fasted overnight, were injected into the tail vein with D-glucosamine-$^{14}$C dissolved in physiological saline (5 μCi/400 g body weight, specific activity 9.74 mc/mM, obtained from New England Nuclear Corp., Boston, Mass.). Blood and liver were obtained 1 hr after injection. The liver and serum proteins were fractionated and purified as described in the leucine experiments, except 0.5% D-glucosamine was used instead of DL-leucine in the TCA washing procedure. A part of the livers was subfractionated as described by Lawford and Schachter (13). Briefly, the liver was homogenized in 0.25 M sucrose and centrifuged for 20 and 15 min at 20,000 x g. Subfractionation of the smooth and rough endoplasmic reticulum was carried out by ultracentrifugation at 144,000 x g from the postmitochondrial fraction overlayered on a solution of 1.3 M sucrose and 15 mM CsCl. Specific activity of the protein of each fraction was measured as described above.

In another series of experiments with rats raised under the same condition as in the previous experiment, the animals were placed under light ether anesthesia 1 hr after injection, and 2-3 ml of blood was obtained from the

retroocular plexus. Finally, blood was withdrawn from the aorta 2 hr after injection. Specific activity of the protein of several serum fractions was measured as described above.

Results were expressed as the mean ± the standard error. The Student's *t* test, or a modified formula to take into account large differences in variance (14), was used for statistical analysis.

## RESULTS

### Experiments Using Leucine-$^{14}$C

The two different diets did not produce significant differences between groups in body or liver weights during the 4-6 week experiment, but the amount of TG in the liver was significantly higher in the animals of the coconut oil group (Table I). Although the differences were not significant, the mean value of serum TG was lower in the coconut oil group in contradistinction to the TG content of the liver.

The amounts of protein of liver and major fraction of serum were not significantly different between the two groups (Table II). The mean values of specific activity of liver protein, serum albumin or serum globulin fractions were higher in the corn oil group at both 90 min or 120 min (Table II) after injection, but the differences between the two groups were not statistically significant.

The amount of protein in the $\beta$-LP was slightly higher in the corn oil than the coconut oil group, but the difference was not significant at 90 or 120 min after injection as in the case of serum TG level (Table III).

The peak in the specific activities of the $\beta$-LP fraction came at 90 min in accordance with observations of Buckley et al. (15) as illustrated in Figure 1. The time course of the incorporation of the radioactivity into the lipoprotein fraction followed the same pattern in the two groups of animals. The specific activities of the fractions obtained from the coconut oil fed animals were generally lower than those of the corn oil fed animals and were significantly different at the 2 hr period. The specific activities of the VLDL at the 90 min period were 2.65 ± 0.40 [3] and 1.77 ± 0.49 [3] dpm per $\mu$g protein for the corn oil and coconut oil groups of animals, respectively (numbers in brackets denote the number of animals used in these determinations); these values were also significantly different.

### Experiments Using Glucosamine-$^{14}$C

Rats fed the corn or coconut oil diet were sacrificed 1 hr after injection. The amount of

TABLE II

Incorporation of Leucine-$^{14}$C Into Protein

| Group | | dpm[a] | | Protein[b] | | Specific activity[c] | |
|---|---|---|---|---|---|---|---|
| | | Time after injection, min | | | | | |
| | | 90 | 120 | 90 | 120 | 90 | 120 |
| Liver | Corn[d] | 50.9 ± 3.9 | 50.2 ± 2.9 | 121 ± 3 | 134 ± 6 | 428 ± 38 | 379 ± 19 |
| | Coconut[e] | 45.6 ± 3.8 | 52.3 ± 5.1 | 129 ± 4 | 144 ± 6 | 355 ± 29 | 360 ± 29 |
| Total serum | Corn | 20.5 ± 1.7 | 25.2 ± 2.0 | 58.3 ± 2.5 | 50.9 ± 1.7 | 351 ± 20 | 495 ± 33 |
| | Coconut | 18.5 ± 1.4 | 23.4 ± 1.6 | 51.5 ± 1.5 | 47.2 ± 1.4 | 358 ± 25 | 496 ± 37 |
| Albumin | Corn | 7.16 ± 0.31 | 9.26 ± 0.41 | 21.4 ± 1.9 | 19.0 ± 0.4 | 346 ± 40 | 486 ± 20 |
| | Coconut | 6.49 ± 0.47 | 8.62 ± 0.63 | 22.7 ± 2.0 | 20.5 ± 0.9 | 294 ± 27 | 427 ± 40 |
| Globulin | Corn | 12.7 ± 0.9 | 16.2 ± 1.0 | 31.6 ± 0.5 | 26.3 ± 1.5 | 403 ± 31 | 617 ± 28 |
| | Coconut | 11.5 ± 0.8 | 15.1 ± 1.8 | 29.3 ± 1.3 | 25.7 ± 1.0 | 397 ± 35 | 588 ± 44 |

[a]dpm x $10^{-3}$ per g liver or ml serum.
[b]mg Protein per g liver or ml serum.
[c]dpm per mg protein.
[d]Five rats for corn oil group and six rats for coconut oil group.
[e]Hydrogenated coconut oil.

TABLE III

Incorporation of Leucine-$^{14}$C Into β-Lipoprotein

| Time, min | Group | dpm[a] | Protein[b] | Specific activity[c] |
|---|---|---|---|---|
| 60 | Corn (3)[d] | 532.±44 | 372 ± 21 | 1.37 ± 0.13 |
| | Coconut[f] (3) | 298 ± 9[e] | 252 ± 9[e] | 1.18 ± 0.04 |
| 90 | Corn (5) | 642 ± 81 | 292 ± 14 | 2.17 ± 0.21 |
| | Coconut[f] (5) | 400 ± 58[e] | 258 ± 25 | 1.53 ± 0.24 |
| 120 | Corn (6) | 504 ± 89 | 293 ± 45 | 1.71 ± 0.09 |
| | Coconut[f] (6) | 389 ± 54 | 286 ± 25 | 1.35 ± 0.14[e] |

[a]dpm per ml serum.
[b]μg Protein per ml serum.
[c]dpm x $10^{-3}$ per mg protein.
[d]Number of rats used for experiments.
[e]$P < 0.05$ to the corn oil group.
[f]Hydrogenated coconut oil.

protein in the serum and the liver fractions was not significantly different between the two groups (Table IV).

As in the previous experiment, specific activities of serum albumin and globulin or total liver protein in the hydrogenated coconut oil group were not significantly different from that of the corn oil groups, but the specific activity of the β-LP fraction showed a significant difference between the two groups. Furthermore the specific activity of the smooth endoplasmic reticulum also decreased significantly in the hydrogenated coconut oil group. The specific activity of β-LP and smooth endoplasmic reticulum was higher than the other serum or liver protein fractions.

In another series of experiments, blood samples were obtained at 60 or 120 min after glucosamine-$^{14}$C injection in order to follow the time course of the specific activity of serum protein. The time course of the incorporation of radioactivity into the albumin, globulin and β-LP fractions of the corn oil and coconut oil groups is shown in Figure 2. These results show that, in general, the specific activities of the serum protein fractions of the hydrogenated coconut oil group are lower than those of the corn oil group. Although the values of the albumin or globulin fraction of the coconut oil group were not significantly different from those of the corn oil group, incorporation of radioactivity into the β-LP fraction was significantly lower in the hydrogenated coconut group than the corn oil group, as in the previous experiment.

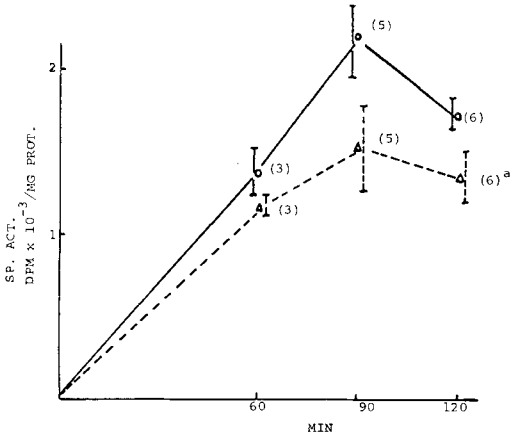


FIG. 1. Incorporation of leucine-$^{14}$C into β-LP. Corn oil group, ○—○; coconut oil group, △– – –△. Vertical bars show the standard error; number in parenthesis = number of animals; a = $P < 0.05$ to corn oil group.

## DISCUSSION

In general, the amount of TG was lower in the liver and higher in the serum of the animals fed corn oil, compared to those fed hydrogenated coconut oil as the sole source of fat, in accordance with the previous study (16).

Normally the amount of TG or VLDL in the serum of fasting animals depends mainly on the balance between the release of lipid from the liver and the hydrolysis of serum lipid in peripheral tissue. The hydrolysis of serum lipid in peripheral tissue has been reported to increase by feeding polyunsaturated fatty acids (17-19). However postheparin lipase activity measured by the method of Kern et al. (20) was found to be increased by an EFA deficiency (unpublished data, T. Fukazawa and O.S. Privett) in accordance with observations by F.D. Collins (personal communication). An increase in lipase activity would increase the hydrolysis of serum lipid and have a tendency to lower the

TABLE IV

Incorporation of Glucosamine-$^{14}$C Into Protein
(60 Min After Injection)

| | Group | dpm[a] | Protein[b] | Specific activity[c] |
|---|---|---|---|---|
| Serum albumin | Corn[d] | 4.66 ± 0.97 | 21.3 ± 0.8 | 221 ± 49 |
| | Coconut[g] | 3.98 ± 0.45 | 22.7 ± 0.7 | 174 ± 16 |
| Globulin | Corn | 55.3 ± 4.9 | 37.4 ± 1.5 | 1.49 ± 0.09 |
| | Coconut | 46.0 ± 3.1 | 33.4 ± 1.0 | 1.39 ± 0.09 |
| β-Lipoprotein | Corn | 2.94 ± 0.31 | 449 ± 53[e] | 6.33 ± 0.50 |
| | Coconut | 1.59 ± 0.20[f] | 370 ± 52 | 4.41 ± 0.28[f] |
| Liver total | Corn | 110 ± 10 | 166 ± 8 | 666 ± 65 |
| | Coconut | 97 ± 7 | 171 ± 5 | 565 ± 31 |
| Smooth endoplasmic reticulum | Corn | 7.49 ± 0.30 | 2.70 ± 0.24 | 2.84 ± 0.22 |
| | Coconut | 5.61 ± 0.76 | 2.67 ± 0.27 | 2.80 ± 0.13[f] |
| Rough endoplasmic reticulum | Corn | 7.91 ± 0.40 | 5.79 ± 0.67 | 1.42 ± 0.13 |
| | Coconut | 6.48 ± 0.12[f] | 6.82 ± 0.72 | 1.11 ± 0.09 |

[a]dpm x $10^{-3}$ per ml serum or g liver.
[b]mg Protein per ml serum or g liver.
[c]dpm per mg protein.
[d]Five rats for each group.
[e]μg Protein per ml serum.
[f]$P < 0.05$ to the corn oil group.
[g]Hydrogenated coconut oil.

serum TG level. Thus conceivably serum triglycerides in the EFA deficient animal could be lowered partially by an increase in lipase activity. If the release of TG from the liver is normal or increased in EFA deficient animals and the level of serum TG is lowered only by the effect of increased lipase activity, then the specific activity of VLDL or β-LP should be normal or higher in the EFA deficient animals. The fact that the specific activity of the β-LP was decreased by an EFA deficiency indicates that the release of TG from the liver was impaired in these animals.

In normal animals, increased FFA supply to the liver (21,22), increased fatty acid synthesis in the liver (23), or increased level of TG in the liver (24), increase (or accelerate) the release of lipid from the liver. Increased secretion of TG from the liver involves an increase in the synthesis and secretion of lipoprotein (25). If the mechanism of TG secretion from the liver is intact in the EFA deficient rat, the same chain of events may be expected to occur.

In EFA deficient rats, high levels of FFA in the blood (5), increased fatty acid synthesis in the liver (4,26), or increased level of TG in the liver (16,27,28) have also been reported. The effect of an EFA deficiency thereby would be to increase the specific activity of the serum β-LP in the present experiments. The fact that the specific activity of the β-LP or VLDL fraction is lower in the EFA deficient animals indicated further an impairment in the synthesis or secretion of lipoprotein from the liver, or both, in accordance with previous in vitro, as well as in vivo studies (2,3).

In several types of experimental fatty liver, the principal cause of the accumulation of fat in the liver is believed to be due to impairment of the synthesis of lipoprotein (5). The present study indicates that there is a decreased synthesis of lipoprotein in the EFA deficient rat. The decreased synthesis of lipoprotein could be an accompanying phenomenon of the accumulation of fat in the liver. However, because of the slight but general effect on protein synthesis by an EFA deficiency, it appears that one of the causes of the accumulation of fat in the liver of EFA deficient rats is an impairment of the synthesis of apolipoprotein. Pertinent also is the fact that the synthesis of apoprotein of VLDL is more sensitive to such agents as actinomycin D (29) or ethionine (30) than the other moieties of serum protein. Furthermore the accumulation of lipid in the liver does not necessarily accompany impairment of radioactive leucine incorporation into plasma proteins and lipoproteins, as observed in ethanol-induced fatty livers (31,32).

Impairment in the synthesis of glycoprotein is reported in the pathogenesis of fatty livers caused by a choline deficiency (31). That the impairment in the synthesis of glycoprotein may also be important in the accumulation of fat in the liver of EFA deficient rats is suggested by the fact that the incorporation of

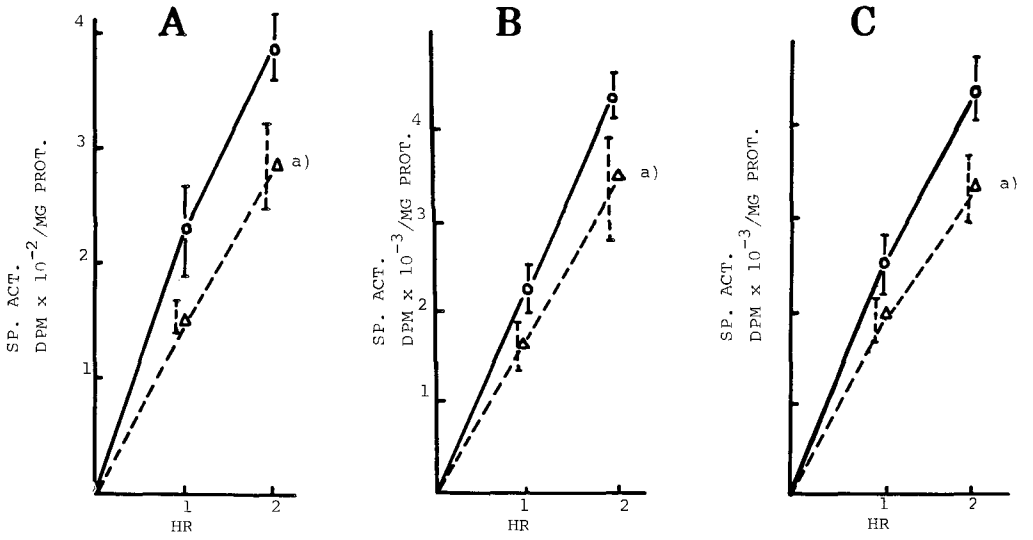


FIG. 2. Incorporation of glucosamine-$^{14}$C into serum protein. Corn oil group, ○—○ (five animals), coconut oil group, △– – –△ (four animals). Vertical bars show the standard error; a = $P < 0.05$ to corn oil group. A = albumin; B = globulin; C = β-lipoprotein.

glucosamine-$^{14}$C is more sensitive to an EFA deficiency than that of leucine-$^{14}$C. The difference could be due to the relative rate of turnover of these compounds as protein precursors. However the difference in the incorporation of radioactivity in the smooth endoplasmic reticulum of the animals that were injected with glucosamine-$^{14}$C suggests that disturbance of a specific function of the smooth microsomes, especially glycoprotein synthesis, may be the critical mechanism involved in the impairment of lipoprotein synthesis. The amount of smooth endoplasmic reticulum in the two groups was not evaluated with the electron microscope; however there was no significant difference in the protein content of smooth endoplasmic reticulum as indicated by Table IV. Smithson observed by electron microscopy a marked reduction of smooth endoplasmic reticulum and an unchanged amount of rough endoplasmic reticulum in the liver of EFA deficient animals (34). This observation, together with our finding of reduced specific activity of incorporated glucosamine-$^{14}$C, suggests that the smooth endoplasmic reticulum and glycoprotein synthesis may be involved in the pathogenesis of lipid accumulation in the liver of EFA deficient animals.

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# The Effect of Cholesterol Supplementation on Glutamate-Induced Hypocholesterolemia in the Mongolian Gerbil

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## ABSTRACT

The effect of cholesterol supplementation on the hypocholesterolemia induced by glutamic acid feeding has been studied in the Mongolian gerbil. Serum triglycerides and phospholipids and hepatic cholesterol and triglyceride contents have also been studied during the control and glutamate feeding. Cholesterol supplementation (0.05% and 1.0%) did not diminish the hypocholesterolemic response (20-24% decrease in serum cholesterol). The glutamate amino acid formula diet caused no change in serum triglyceride, serum phospholipid, hepatic cholesterol and hepatic triglyceride content when compared to the control formula diet. No lesions of the hypothalamic or paraventricular areas of the brain were noted on histological preparation in adult gerbils given large amounts of glutamic acid orally.

## INTRODUCTION

A marked decrease in the serum cholesterol concentration of human subjects fed chemically defined, isocaloric, isonitrogenous amino acid formulas containing glutamic acid (AAFG) as the sole source of nonessential nitrogen was described by Olson et al. (1). Subsequently, Bazzano and Olson (2,3) found the degree of hypocholesterolemia to be roughly proportional to the measured decrease in excretion of fecal steroids and the calculated increase in half-life of endogenous sterols. To better elucidate the mechanism of glutamate-induced hypocholesterolemia, it became necessary to find a suitable animal model. Bazzano (4) described a marked hypocholesterolemic response to AAFG in the Mongolian gerbil, whereas the diet was only mildly effective in the chick, without effect in the rabbit, and hypercholesterolemic in the rat. More recent

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TABLE I

Serum Cholesterol Concentration in Adult, Male Mongolian Gerbils on Purina Chow Food Control Diet (FCD) Supplemented with 0.05% and 1% Cholesterol[a]

| Diet | Number of animals | Weight, g | Serum cholesterol,[b] mg% | |
|---|---|---|---|---|
| FCD + 0.05% Cholesterol | 8 | 69 | 195 ± 13.1 | |
| | | | | $p^c < .001$ |
| FCD + 1% Cholesterol | 8 | 69 | 312 ± 15.2 | |
| | | | | $p < .025$ |
| FCD + 0.05% Cholesterol | 8 | 70 | 205 ± 14.8 | |
| | | | | $p < .25$ |
| FCD + 0.05% Cholesterol | 8 | 67 | 178 ± 15.3 | |
| | | | | $p < .25$ |
| FCD + 0.05% Cholesterol | 8 | 66 | 179 ± 18.6 | |

[a]Weekly measurements with diet changed as shown.
[b]Results expressed as mean ± standard error.
[c]Probability (p) determined by student's *t* test (paired).

TABLE II

Body Weight and Serum Cholesterol Concentration of Adult, Male Mongolian Gerbils on Food Control Diet (FCD) and Casein Diets

| Diet[a] | Number of animals | Weight, g | Serum cholesterol,[b] mg% | |
|---|---|---|---|---|
| FCD | 13 | 76 | 176 ± 15.6 | $p^c < .20$ |
| Casein, 30% | 13 | 75 | 184 ± 13.6 | $p < .50$ |
| Casein 30% | 11 | 75 | 209 ± 27.0 | $p < .001$ |
| Casein 10% | 10 | 74 | 128 ± 13.8 | $p < .20$ |
| Casein 10% | 10 | 71 | 118 ± 9.4 | |

[a]Weekly measurements with diet changes as indicated (0.05% cholesterol supplement).

[b]Results expressed as mean ± standard error.

[c]Probability (p) determined by student's *t* test.

work has indicated that the mechanism of this hypocholesterolemic effect may be, in part, related to a block in cholesterol synthesis between acetate and mevalonate in glutamate-fed gerbils (5). The gerbil accumulates supplemented cholesterol in its blood and tissues (6) to a degree intermediate between that of the mouse and rat, which retain almost no excess, and that of the hamster, which accumulates very large amounts of fed sterol (7). It was therefore necessary to test the AAFG effect during various levels of cholesterol supplementation.

The serum triglyceride and phospholipid, and hepatic cholesterol and triglyceride content were also measured during the various dietary regimens.

During the course of this work, Olney (8), and Olney and Sharpe (9) described hypothalamic lesions in infant mice and a premature newborn monkey given monosodium glutamate subcutaneously in large doses. Included in our study, therefore, are brain histological sections of our adult animals fed ad libitum to determine possible changes in the areas as described by these authors.

## EXPERIMENTAL PROCEDURES

Mature, male Mongolian gerbils (40-90 g) were obtained from Beaumanor Farms, Cleveland, Ohio. The composition of the amino acid formulas has been previously reported (4). The control amino acid formula (AAF) contained equimolar amounts of glycine and ammonium citrate as the nonessential nitrogen source. The AAFG was similar to AAF, except that it contained glutamic acid (220 g/kg) as the sole source of nonessential nitrogen. Other diets utilized in sequence studies included Purina Chow for small laboratory animals, referred to as the food control diet (FCD), and casein control diet in which vitamin-free casein (General Biochemicals, Chagrin Falls, Ohio) constituted the entire nitrogen supplement; while the carbohydrate, lipid, vitamin and mineral supplements remained similar to those in AAF. Cholesterol supplementation was either 0.05% or 1.0%. Dietary periods lasting a week in various sequences utilized each animal as his own control. One week periods had previously been found to be sufficiently long to demonstrate the effects of our amino acid formula diets in the gerbil. Weighing and bleeding by tail

TABLE III

Serum Cholesterol Concentration of Adult, Male Mongolian Gerbils on Formula Diets Supplemented with Cholesterol

| Diet[a] | Number of animals | Serum cholesterol,[b] mg% | |
|---|---|---|---|
| Cholesterol, 0.05% | | | |
| AAF | 28 | 238 ± 16.7 | $p^c < .02$ |
| AAFG | 28 | 182 ± 10.7 | |
| Cholesterol, 1.0% | | | |
| AAF | 28 | 236 ± 16.2 | $p < .05$ |
| AAFG | 28 | 188 ± 16.4 | |

[a]Composite Table of the means of weekly measurements regardless of the sequence of the diets fed. Abbreviations: AAF = amino acid formula; AAFG = amino acid formula containing glutamic acid.

[b]Results expressed as mean ± standard error.

[c]Probability (p) determined by student's *t* test.

TABLE IV

Serum Triglyceride and Phospholipid Concentration of Adult, Male Mongolian Gerbils on Designated Diets

| Diet[a] | Weight, g | Number of animals | Serum triglyceride, mg% | Serum phospholipids, mg% |
|---|---|---|---|---|
| FCD | 71 | 11 | 94 ± 9.0[b] | 253 ± 2.0 |
| | | | p[c] < .6 | p < .01 |
| AAF | 62 | 6 | 102 ± 4.4 | 290 ± 4.2 |
| | | | p < .1 | p < .8 |
| AAFG | 62 | 6 | 86 ± 5.3 | 286 ± 8.0 |

[a]Weekly measurements with diet changed as shown; supplemented with 0.05% cholesterol. For abbreviations see Table III.

[b]Results expressed as mean ± standard error.

[c]Probability (p) determined by student's *t* test (paired).

incision were performed at the end of each week.

Serum cholesterol was measured by the method of Abell et al. (10), serum triglycerides by the method of Van Handel (11), and serum phospholipid phosphorus according to Fiske and Subarrow (12). Pooled serum for triglyceride and phospholipid analysis was extracted by the method of Dole (13). Animals were sacrificed by decapitation. Frozen liver samples were thawed and homogenized with water in a Potter-Elvehjem type Teflon homogenizer. The aqueous homogenates were extracted with isopropanol. Hepatic cholesterol content was measured by the direct addition of Libermann-Burchard reagent to a dried aliquot of the isopropanol extract. Hepatic triglycerides were determined by resuspending an aliquot of the evaporated isopropanol extract in chloroform and applying it to zeolite as in the method of Van Handel (11). Hepatic phospholipid phosphorus was performed by acid digestion of an aliquot of the dried isopropanol extract (12). Histological sections of formalinized brain tissue were performed from paraffin sections in hematoxylin and eosin stain.

## RESULTS

Table I shows the changes in the serum cholesterol concentration during the indicated dietary regimen. An increase of 117 mg% in serum cholesterol concentration was observed when 0.05% cholesterol-supplemented FCD was changed to 1.0%. Following reinstitution of the 0.05% supplement, serum cholesterol concentrations fell to baseline levels after 1 week. This length of time was henceforth selected as the standard period in all subsequent experiments.

Feeding of a 10% casein diet produced a slight weight loss and a significant fall in serum cholesterol (-91 mg% at the end of the second week) as seen in Table II. Thirty per cent casein caused no change in weight and a mild increase in serum cholesterol concentration (+33 mg% after 2 weeks).

Weekly measurements testing glutamic acid in the presence of 0.05% and 1.0% cholesterol, during feeding experiments of a week's duration in all possible combinations, reproduced the familiar hypocholesterolemic effect. In this group of experiments an initial sharp increase in serum cholesterol was observed during the first

TABLE V

Hepatic Cholesterol and Triglyceride Content of Adult, Male Mongolian Gerbils

| Diet | Number of animals[a] | Cholesterol[b] | Triglycerides[b] |
|---|---|---|---|
| FCD | 9 | 496 ± 34.6 | 992 ± 54.9 |
| | | p[c] < .1 | p < .005 |
| AAF | 9 | 416 ± 22.7 | 1672 ± 185.9 |
| | | p < .2 | p < .2 |
| AAFG | 10 | 505 ± 42.7 | 1333 ± 92.6 |

[a]Animals sacrificed after 1 week on the indicated regimen. All diets supplemented with 0.05% cholesterol. For abbreviations see Table III.

[b]Results expressed as mean mg/100 g wet tissue ± standard error.

[c]Probability (p) calculated from student's *t* test.

to second week of feeding either diet, followed by a reduction to a plateau of approximately equal level, independently from the dietary supplementation. Table III shows that both the concentration of serum cholesterol and its relative decrease during the AAFG were approximately equal for the two levels of dietary cholesterol (-56% and -48%, respectively).

Although the amino acid formulas caused a significant elevation in serum phospholipid concentration when compared to the FCD diet, as demonstrated in Table IV, glutamic acid addition to amino acid formulas appeared to have no effect. Serum triglyceride concentration was not significantly altered by either the amino acid formulas or glutamic acid.

No significant differences were found in the hepatic cholesterol, while triglycerides appeared to be increased significantly in the formula-fed animals. Hepatic triglycerides were lower in the AAFG-fed animals, although not significantly, as can be seen from Table V. Histological sections of formalin-fixed brain tissue revealed no differences in the paraventricular structures studied from FCD, AAF or AAFG-fed gerbils. Specifically, no signs of discrete zones of necrosis were seen in animals taking as much as 15-30 g of glutamic acid per day per kilogram body weight.

## DISCUSSION

The Mongolian gerbil demonstrates the same hypocholesterolemic response to glutamic acid previously described in human subjects. The present study was designed to test the relative potency of this glutamate effect during large dietary cholesterol supplementation, since the tendency to accumulate fed sterol is well documented in this laboratory animal. Through a larger pool size and a faster synthetic rate for bile acids, the rat and mouse, respectively, can maintain a tighter control over serum and tissue cholesterol levels (7). Our results show that, given a 20-fold increase in cholesterol supplementations (0.05-1.0% w/w), the gerbil demonstrates serum elevations amounting to 50% of baseline within the first week. This initial sharp increase observed in the FCD sequence is followed by a reduction to a steady plateau at the end of the second week. Henceforth serum cholesterol values appear to be stabilized. This phenomenon, present only in gerbils fed the high cholesterol diets, appears to be peculiar to this animal and has been previously reported by others (14). Even in this setting, however, AAFG still produced a 20-25% fall in serum cholesterol concentration.

Serum triglyceride and phospholipid levels were not altered by the AAFG regimen. Human subjects on similar dietary regimens had revealed decreased levels of phospholipids and $S_f$ 0-12 $\beta$-lipoproteins, but no change in serum triglycerides, during AAFG. It was therefore important to know whether AAFG alters the serum triglyceride or phospholipid content of gerbils. Hepatic content of cholesterol and triglycerides was not altered significantly by the glutamic acid-containing diets. In this setting AAF did produce a slight elevation of serum triglycerides and a significant increase in liver triglycerides. This may have been a response to the refusal to eat and subsequent weight loss frequently noted when the animals were switched from FCD to the amino acid formulas (15).

The absence of brain lesions in adult animals on ad libitum diets containing large amounts of glutamic acid reflects both normal liver function and a mature blood-brain barrier. This is in agreement with previous studies from our laboratory (16), indicating that glutamic acid supplementation is not harmful in the mature animal. The suckling mouse or premature monkey may have neither the hepatic capacity for metabolism of increased amounts of glutamic acid nor the membrane transport discrimination needed to exclude a high concentration of rapidly injected substrated.

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# Ring Location in Cyclopropane Fatty Acid Esters by Boron Trifluoride-Catalyzed Methoxylation Followed by Mass Spectroscopy

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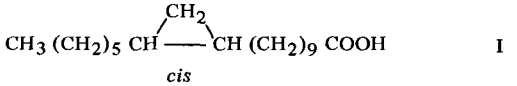
## ABSTRACT

Methyl esters of methyl *cis*- and *trans*-9,10-methyleneoctadecanoic acids react with 50% boron trifluoride-methanol to produce unsaturated and methoxy-esters; both products are shown by gas chromatography to be a mixture of several isomers. Mass spectra of the methoxylated esters are characterized by intense peaks due to cleavage adjacent to methoxy-functions which allow the position of the ring in the original cyclopropane ester to be easily assigned. Methyl oleate is also partially attacked by 50% $BF_3$-MeOH to produce a mixture of methyl 9- and 10-methoxyoctadecanoates. 14% $BF_3$-MeOH does not react with cyclopropane and olefinic esters under the reaction conditions employed.

## INTRODUCTION

Cyclopropane fatty acids, occurring in nature, may be broadly divided into two major groups. The first group comprise those such as lactobacillic acid (I) having *cis*-cyclopropane rings which are derived from unsaturated fatty acids of conventional structure and chain length, i.e., *cis*-octadecenoates (1). The second group include several acids of the mycolic type which are β-hydroxy acids of high molecular weight isolated from Mycobacteria (2). These latter acids may contain either isolated *cis*-cyclopropane rings or *trans*-cyclopropane rings with adjacent methyl branches; examples of these are two mycolic acids (II, III, respectively, for the main components) from *Mycobacterium tuberculosis var hominis* (3).



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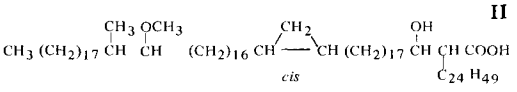

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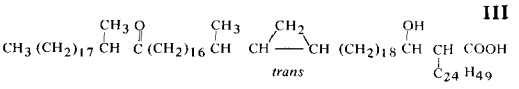
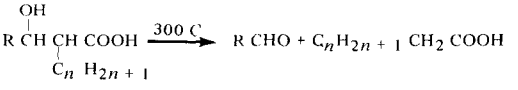


III

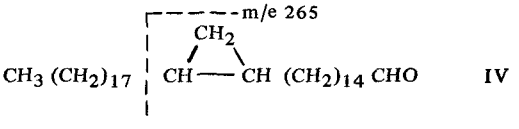
The problem of locating the position of the ring in cyclopropane fatty acids has been the subject of continuing interest. The original studies on lactobacillic acid involved oxidation of a large quantity of material and chemical identification of the degradation products (1). A further development was oxidation of the methyl-branched fatty acids obtained by hydrogenation of the cyclopropane acid (4). With the advent of mass spectrometry, attempts were made to apply this technique to cyclopropane esters. It was discovered that both geometrical and positional isomers of cyclopropane esters gave practically identical mass spectra (5-7). Attention was therefore turned to the possibility of chemical modification of cyclopropane esters in order that derivatives suitable for mass spectrometry might be obtained.

Hydrogenation of cyclopropane esters, to yield a mixture of isomeric methyl-branched esters and a straight chain ester, is well established. The suitability of mass spectrometry for the determination of structure of methyl-branched long chain esters is also well proven (8). Analysis of the methyl-branched esters produced by hydrogenation by coupling a mass spectrometer to the outlet of a gas chromatograph therefore allows a sophisticated method for determination of the structure of simple cyclopropane esters (9,10).

The location of the position of the cyclopropane rings in the mycolic acids is more difficult. The mycolic acids may be degraded by pyrolysis to produce a straight chain acid and a long chain aldehyde (meroaldehyde).



Mass spectrometry of long chain aldehydes similar to meroaldehydes apparently can give some information regarding the position of cyclopropane rings. For example, the mass spectrum of a long chain aldehyde (IV) shows



IV

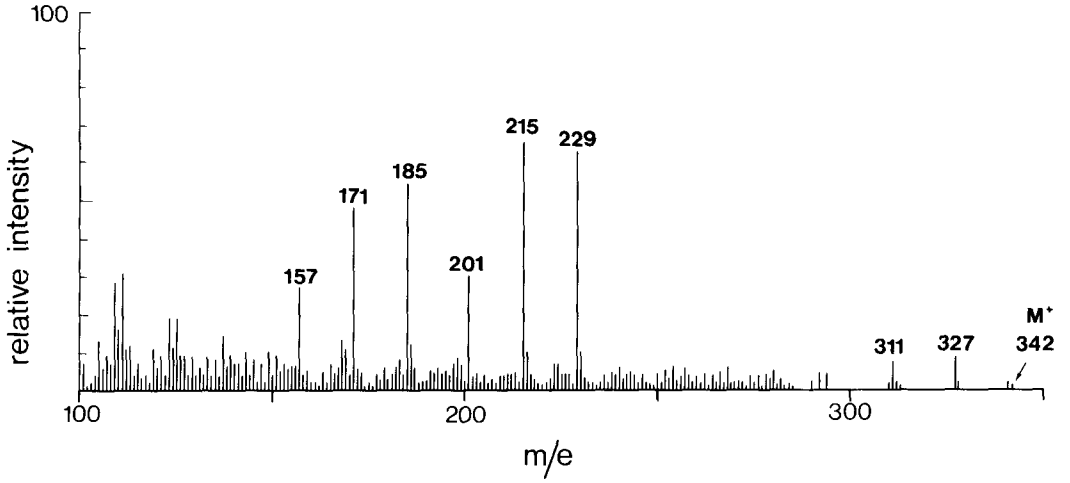



FIG. 1. Partial mass spectrum of methoxy-esters derived from methyl *cis*-9,10-methyleneoctadecanoate (base peak m/e 57).

an intense peak at m/e 265 which is attributable to cleavage at the point indicated (11). Such cleavages, however, are unreliable since they appear to depend on the position of the ring in the chain, and when more than one cyclopropane ring is present, predictable fragmentations are not observed for both rings (12). Hydrogenation of the methyl meromycolates derived from meroaldehydes would produce a mixture of methyl-branched esters which, because of their size ($C_{50}$-$C_{60}$), are not easily separated by conventional gas chromatographic procedures. The meromycolates also usually contain several homologs, and it would therefore be difficult to positively identify the relatively weak mass spectral cleavages due to methyl branches in such mixtures.

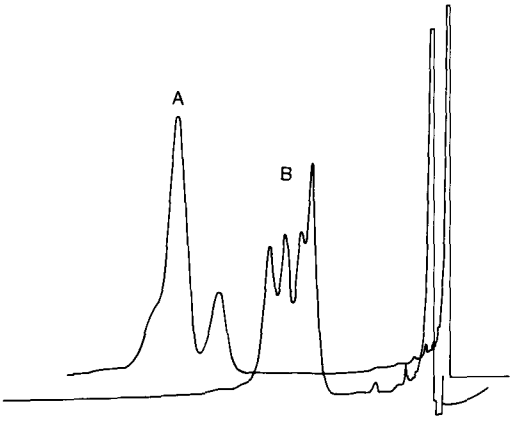



FIG. 2. Gas Chromatograms of methoxy-(A) and unsaturated esters (B) derived from methyl *cis*-9,10-methyleneoctadecanoate (SE 30).

The logical approach to determination of the position of cyclopropane rings is to attack the ring with chemical reagents and produce a derivative which will give intense peaks on mass spectrometry. This has been realized by two approaches. Cyclopropane esters and hydrocarbons derived from mycolic acids have been methoxylated by reaction with boron trifluoride-methanol reagent (3,12,13) and the derivatives found to be suitable for mass spectrometry. Oxidation of long chain cyclopropane compounds with chromium trioxide produces cyclopropyl ketones which are amenable to mass spectral analysis (14,15). This paper describes details of the application of the former procedure to model cyclopropane esters. The latter method with be discussed in light of the results presented here.

## EXPERIMENTAL PROCEDURE

### Thin Layer Chromatography (TLC)

Merck Silica Gel PF 254 + 366 was used for analytical (0.4 mm layers) and preparative (1 mm layers) separations. An irrigation mixture of hexane-diethyl ether 90:10 was employed in all cases.

### Gas Liquid Chromatography

A Perkin-Elmer F11 chromatograph was employed for gas chromatography; nitrogen was used as carrier gas. Stainless steel columns (1.5 m x 3.2 mm OD) were packed separately with methyl silicone (SE 30, 1.5%), fluorosilicone oil (QF 1, 10%) and polyethylene glycol succinate (PEGS, 10%) coated on acid-washed celite (85-100 mesh).

### Mass Spectrometry

A.E.I. MS9 instruments were used for determination of mass spectra at an ionizing potential of 70 ev.

### Synthesis of Cyclopropane Esters

Oleic and elaidic acids (British Drug Houses, Poole, England) were esterified with ethereal diazomethane. Cyclopropane esters were prepared by Simmons-Smith reaction on the above esters according to the procedure of Le Goff (16). The cyclopropane esters were separated from traces of unchanged unsaturated esters by preparative TLC on layers impregnated with silver nitrate (10%). Final purification was achieved by bulb-tube distillation (bp 170-190 C/2-3 mm) and the identity of methyl *cis*- and *trans*-9,10 methyleneoctadecanoates confirmed by elemental analysis, mass spectrometry and proton magnetic resonance spectroscopy (17).

### Reaction of Cyclopropane Esters With 50% Boron Trifluoride-Methanol

Boron trifluoride-methanol (50% $BF_3$), purchased initially from B.D.H., Poole, England, was subsequently prepared from $BF_3$ gas (B.D.H.) and dry methanol. Methyl *cis*-9,10-methyleneoctadecanoate (35 mg) was dissolved in dichloromethane (3 ml) and treated at room temperature with 50% $BF_3$-MeOH reagent (1 ml). The reaction followed by TLC on silver nitrate-impregnated plates was complete after 3 hr when no material which migrated with unreacted cyclopropane ester remained. The reaction mixture was poured into water and the dichloromethane layer separated, washed with water and dried ($Na_2SO_4$). Preparative TLC of the residue, after evaporation of the solvent, gave methoxylated esters (16 mg) and olefinic esters (11 mg).

Methyl *trans*-9,10 methyleneoctadecanoate (10 mg) subjected to the same procedure yielded methoxylated esters (6 mg) and olefinic esters (3 mg).

### Reaction of Unsaturated Esters With 50% $BF_3$-MeOH

Methyl oleate and elaidate (10 mg each) were treated with 50% $BF_3$-MeOH under the same conditions as described for the cyclopropane esters. TLC showed that approximately half the unsaturated esters had been converted to more polar methoxylated esters which were isolated by preparative TLC.

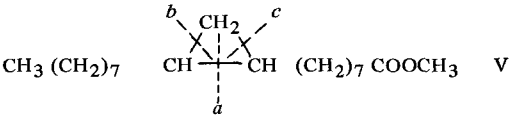
### Attempted Reaction of Unsaturated and Cyclopropane Esters With 14% $BF_3$-MeOH

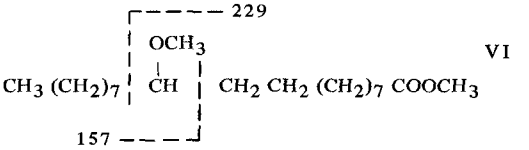
The unsaturated and cyclopropane esters used in the above reactions were treated with 14% $BF_3$-MeOH (B.D.H.) under the same conditions and under reflux for 2 hr both with and without dichloromethane. TLC and gas chromatography of the reaction products showed that these esters were recovered unchanged.

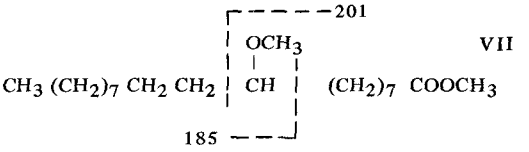
## RESULTS

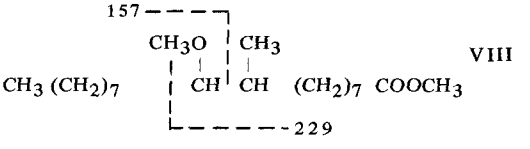
### Mass Spectrometry

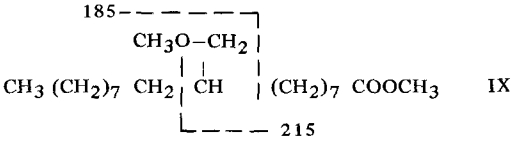
The possible isomeric structures which may be formed from a cyclopropane ester (V) on treatment with $BF_3$-MeOH reagent are shown in formulas VI-XI. Cleavage of the cyclopro-

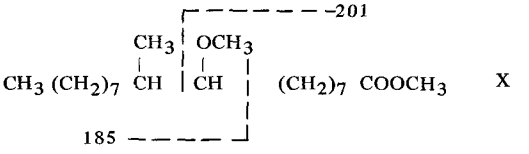


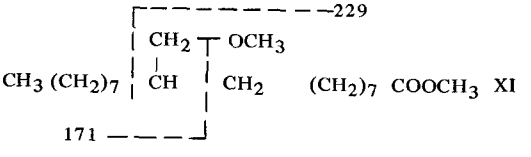



pane ester (V) at position *a* may produce isomers VI and VII; at position *b*, VIII and IX; and at position *c*, X and XI are predicted. The m/e values expected to be prominent on mass spectrometry of the methoxylated esters are indicated in formulas VI-XI.

The mass spectrum of the mixture of methoxylated esters derived from methyl *cis*-9,10-methyleneoctadecanoate is shown in Figure 1. Major peaks due to fragmentations promoted by the methoxy-groups are observed and correspond to the anticipated m/e values shown in formulas VI-XI. The mass spectrum of the methoxylated esters from methyl *trans*-9,10-methyleneoctadecanoate was similar to that shown in Figure 1. The relative intensities (% of base peak) of the diagnostic ions at m/e 157, 171, 185, 201, 215 and 229 in this latter spectrum were 42, 92, 92, 45, 84 and 100, respectively.

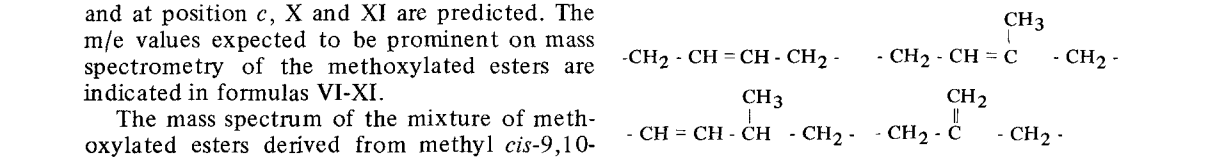
The mass spectrum of the methoxylated esters produced by reaction of 50% $BF_3$-MeOH reagent with methyl oleate was essentially identical to that of the mixture of 9- and 10-methoxyoctadecanoates derived from methyl oleate by methoxymercuration-demercuration; dominant peaks being observed at m/e 157, 171, 201 and 215 (18).

Mass spectrometry of the mixtures of unsaturated esters produced by the reaction of the cyclopropane esters with 50% $BF_3$-MeOH reagent showed them to have molecular weights identical to those of the original cyclopropane esters.

#### Gas Liquid Chromatography

The cyclopropane esters and the methoxylated and olefinic esters derived from them by reaction with 50% $BF_3$-MeOH were subjected to gas chromatography on polar (PEGS), semi-polar (QF 1) and nonpolar (SE 30) stationary phases. Figure 2 shows the gas chromatographic results for the products derived from methyl *cis*-9,10-methyleneoctadecanoate on SE30 stationary phase; similar but quantitatively different traces are shown by the products from the *trans*-isomer. Two main peaks are observed on SE30 for the methoxylated esters with a small shoulder on the main peak indicating the presence of a third component. On QF-1 this third minor component is more clearly resolved, but on PEGS stationary phase it is obscured by the other peaks. The structures VI-XI proposed for the methoxylated esters show that there are three main structural types which might be separated by gas chromatography.

Elimination of methanol from the methoxylated esters VI-XI can give rise to four series of unsaturated esters containing the following structural units of undefined stereochemistry:



Four main peaks (Fig. 2), perhaps corresponding to these structural types, are in fact observed on gas chromagography (all three stationary phases) of the unsaturated esters derived from both *cis*- and *trans*-cyclopropane esters.

The methoxylated ester produced by reaction of 50% $BF_3$-MeOH with methyl oleate cochromatographed with a sample of the methoxylated octadecanoate prepared by methoxymercuration-demercuration of methyl oleate.

## DISCUSSION

The results outlined in the preceding section demonstrate that simple cyclopropane esters may be partially methoxylated by reaction with 50% $BF_3$-MeOH, and that mass spectrometry of these methoxy-esters enables the position of the ring in the original cyclopropane ester to be assigned.

The mass spectra (Fig. 1) of the methoxylated derivatives are dominated by six intense peaks which can be attributed to fragmentations promoted by methoxy-functions. No large secondary peaks derived by loss of methanol are encountered in these spectra, although such further fragmentation is observed in the spectra of methoxylated derivatives from cyclopropane compounds of higher molecular weight (3,12,13). The molecular ions are not intense, but characteristic peaks at m/e 327 (M-15) and m/e 311 (M-31) enable the molecular weight to be determined.

The procedure described here compares well with the other main methods for ring location of cyclopropane esters by mass spectrometry. The method of McCloskey and Law (10) involves hydrogenation followed by gas chromatograph-mass spectrometry of the resulting mixture of methyl-branched and straight chain esters. From an individual cyclopropane ester four significant mass spectral peaks are observed; while this is a simpler fragmentation pattern the intensities of the peaks are much smaller. This method is unsuitable for cyclopropane compounds of more complex structure and higher molecular weight such as the mycolic acids.

The method of Promé (14), which involves

mass spectral analysis of cyclopropyl ketones derived from long chain cyclopropane compounds by chromium trioxide oxidation, may be applied to both simple and complex cyclopropane compounds. In certain cases the two possible cyclopropyl ketones produced from a cyclopropane ester may be separated by TLC. For example methyl *cis*-9,10 methylene-8- and 10-oxo-octadecanoates may be isolated from the products of oxidation of methyl *cis*-9,10-methyleneoctadecanoate and their mass spectra determined separately; other isomeric cyclopropyl ketones, especially those derived from esters of higher molecular weight, are more difficult to separate. The mass spectra of such cyclopropyl ketones are more complex than those of the methoxylated derivatives produced by reaction of cyclopropane esters with $BF_3$-MeOH. In cases where the cyclopropane function is assymmetrically substituted, for example having a methyl branch on one adjacent carbon atom (see formula III), the chromium trioxide oxidation procedure might be advantageous since it would be expected that the keto-function would be assymmetrically located with respect to the cyclopropane ring. The two procedures under discussion are therefore somewhat complementary. The $BF_3$-MeOH method gives the location of a cyclopropane ring in a chain and is independent of the environment, at least when the substituents are alkyl chains. Oxidation with chromium trioxide is more sensitive to environment of the cyclopropane ring (15), and mass spectral analysis of the resultant cyclopropyl ketones gives more information about the nature of the alkyl substituents.

It is notable that in the experiments described above simple cyclopropane and unsaturated esters are not attacked by 14% $BF_3$-MeOH. A recent report (19), however, has demonstrated that cyclopropane esters can be extensively attacked by 14% $BF_3$-MeOH; no detailed structural investigation of the products was made, but the gas chromatographic results were in general agreement with those presented here. Artifacts have also been observed when unsaturated fatty acids and esters are treated with $BF_3$-MeOH reagent. Incompletely characterized methoxy-substitued fatty acids were detected when unsaturated acids were treated with 50% $BF_3$-MeOH (20) and when flour lipids were treated with dilute (12.5%) $BF_3$-MeOH and the reaction mixture concentrated by distillation of methanol (21). The former case of artifact formation was attributed to the high concentration of $BF_3$ and it was concluded that 14% $BF_3$-MeOH was a satisfactory and generally applicable methanolysis reagent (22). Subsequent studies reinforced the suitability of dilute (12.5% or less) $BF_3$-MeOH as a general methanolysis reagent (23), and indeed such a procedure was recommended for fatty acids, e.g., cyclopropene and conjugated acids, which were partially destroyed by conventional mineral acid catalysis (24). A recent study (25), however, demonstrated that artifacts can be produced when unsaturated fatty acids are treated with 14% $BF_3$-MeOH; the ability of the reagent to produce artifacts was suggested to be dependant on its age and history. More recently (26) it was demonstrated that under harsh reaction conditions $BF_3$-MeOH is capable of producing artifacts from unsaturated acids, but the age of the reagent was thought to be unimportant; boron trichloride in methanol was much less prone to such side reactions. It is therefore apparent that while a dilute solution of boron trifluoride in methanol is a useful reagent for the methanolysis of lipids, each batch of reagent should be checked by reaction with a fatty acid mixture of suitable composition before being applied to valuable lipid samples. It may be pointed out that, as described above, analysis by mass spectrometry of methoxylated esters produced by reaction of monounsaturated fatty acids with 50% $BF_3$-MeOH reagent affords a quite efficient procedure for location of the position of the double bond in such esters. It is also notable that unsaturated carbohydrates have been found to add methanol in the presence of boron trifluoride etherate (27).

## ACKNOWLEDGMENTS

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# Properties of Ultrasonically Irradiated Human Serum Lipoproteins

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## ABSTRACT

Human serum low density lipoprotein (LDL) and human serum high density lipoprotein (HDL) were treated with ultrasonic irradiation. The immunochemical properties, spectrophotometrical analysis and thiobarbituric acid test (TBA) value of ultrasonically irradiated lipoproteins were examined. The agar gel precipitin reaction of sonicated LDL disappeared as the irradiation time increased. The effects of ultrasonic irradiation upon human serum lipoproteins resulted in a loss of lipids from the sonicated lipoproteins and are increased in TBA value. TBA value of LDL increased in two steps.

## INTRODUCTION

The antigenic properties of the lipid-containing C-reactive protein (C-reactive protein is an abnormal protein in human serum.) and the lipid-free C-reactive protein have not been clearly differentiated (1). In the case of human serum low density lipoprotein (LDL), other antigenic properties not apparent in native LDL appeared after delipidation of LDL, enzymatic digestion of LDL, or treatment of LDL with surfactants (2). According to Searcy and Bergquist (3), the immunocrit value of LDL in human serum is inactivated by ultrasonic irradiation with release of LDL lipid components.

In this paper, we report the effect of ultrasonic irradiation on human serum lipoproteins and linoleate-bovine serum albumin (BSA) preparations. The latter is used as a model for evaluating immunochemical, spectrophotometric and thiobarbituric acid (TBA) value changes.

## MATERIALS AND METHODS

Human nonfasting serum first was centrifuged at 12,000 x g for 30 min at 4 C to remove chylomicrons in the top layer. HDL and delipidated LDL were prepared from this serum as described in an earlier paper (2). High density lipoprotein (HDL) of density fraction 1.125-1.195 g/cm$^3$ was isolated from serum after LDL had been removed by the procedure of Scanu (4). Disodium ethylenediamine tetraacetic acid (EDTA) (1 mM) was added to all solutions used in lipoprotein preparation. Lipoprotein fractions were dialyzed overnight against 0.14 M NaCl-lmM EDTA at 4 C, and the purity of each fraction was tested immunologically, using agar gel precipitin reaction with antiserum against human serum.

Linoleic acid, pure by gas chromatography, was provided by T. Akiya (Food Research Institute, Ministry of Agriculture and Foresty, Tokyo, Japan). BSA was fraction V from bovine plasma (Armour Co., Kankakee, Ill., U.S.), treated with activated charcoal to remove bound free fatty acids by Chen's procedure (5). Preparation of the linoleate-BSA mixture was carried out as described by Haase and Dunkley (6).

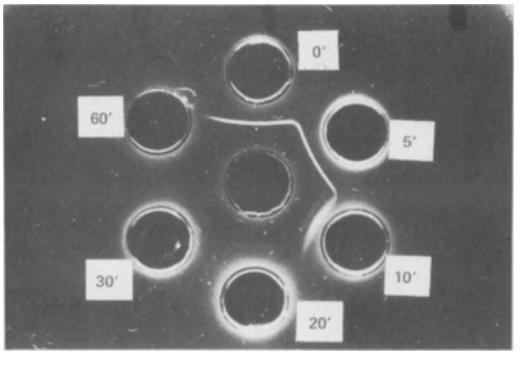


FIG. 1. Agarose gel precipitin reaction of sonicated human serum low density lipoprotein (LDL). Center well contains anti-LDL rabbit serum; surrounding wells contain ultrasonically irradiated LDL for the indicated times.

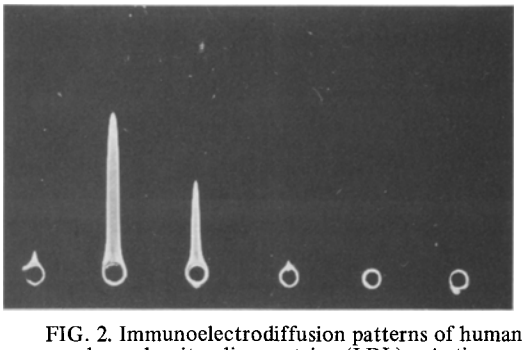

FIG. 2. Immunoelectrodiffusion patterns of human serum low density lipoprotein (LDL). Antiserum against LDL diluted at 1/10. Applied volume of sample solution is 20 μl. Electrophoresis at 30 mA, 70 V for 5 hr at 20 C. From left side to right the material in wells are as follows: Human serum, native LDL, and LDL after sonication for 4 min, 8 min, 12 min and 16 min.

TABLE I

The Hemaglutination Inhibition Reaction of Sonicated Human Serum Low Density Lipoprotein (LDL)

| Irradiation | Dilution of sonicated LDL solution | | | | | |
|---|---|---|---|---|---|---|
| Time, min | x 2 | x 4 | x 8 | x 16 | x 32 | x 64 |
| 0 | --- | --- | --- | --- | ± | + |
| 5 | --- | --- | --- | --- | ± | + |
| 10 | --- | --- | --- | --- | ± | + |
| 15 | --- | --- | --- | --- | + | + |
| 20 | --- | --- | --- | ± | + | + |

The ultrasonic irradiation (20 kc, 150 Watts) was performed in an ice cooled test tube exposed to air using a model 150 T sonicator (Ohtake Workes Co., Tokyo, Japan).

Gel filtration utilized Sepharose 4B (Pharmacia, Uppsala, Sweden) applied to a 22 x 430 mm column. Fractions were eluted using a solution of 0.01 M phosphate buffer, pH 7.2. The protein concentration of each collected effluent volume (3 ml) was determined by spectrophotometric absorbancy at 280 nm, using a model 139 Hitachi Perkin-Elmer UV-VIS Spectrophotometer (Hitachi Co., Tokyo, Japan).

TBA values were determined by the procedure of Wilbur et al. (7). A recording spectrophotometer, model EPS-3T (Hitachi Co., Tokyo, Japan) was used for the spectrophotometric analysis.

The agarose gel electrophoresis was performed in 1.2% agarose gel in veronal buffer solution at pH 8.6, I=0.025, for 50 min at 3 mA/cm (current-constant), using a model PAV-50 electrophoresis apparatus (Johkoh-Sangyo Co., Tokyo, Japan). Protein staining was by Kohn's method (8), using Ponceau 3R instead of Ponceau S dye (Tokushu Chemical Co., Tokyo, Japan).

Serological analyses utilized antisera against LDL, HDL and human serum prepared from common adult albino rabbits. Agarose gel precipitin reaction was carried out in 0.85% NaCl solution containing 1.2% agarose. The hemagglutination inhibition reaction was performed using sensitized red cells according to a standard procedure (9). The Beta-L test kit (Hyland Inc., Los Angeles, Calif., U.S.) was used for the immunocrit of LDL. Immunoelectrodiffusion was performed according to the method of Laurell (10).

## RESULTS

In sonicated LDL, the precipitin lines of the agarose gel precipitin reaction changed in proportion to the irradiation time (Fig. 1), but HDL was not affected by ultrasonic irradiation. Altered antigenic properties of sonicated LDL, not detectable in native LDL, were identified with newly appearing antigenicity of delipidated LDL.

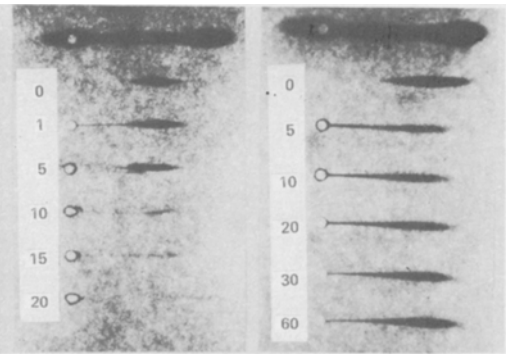


FIG. 3. Agarose gel electrophoresis of sonicated lipoproteins. The left figure is human serum low density lipoprotein (LDL) and the right figure is human serum high density lipoprotein (HDL). The top pattern is human serum and the numbered patterns below indicate the time (min) of sonication of each lipoprotein fraction. Right side is the anode.

The immunoelectrodiffusion patterns of sonicated LDL are shown in Figure 2. In this method the distance of migration of the precipitin line correlates with the quantity of the antigen. Although immunocrit values correlate with the hemagglutination inhibition titers in the ultrasonicated human serum, the hemagglutination inhibition titer of sonicated LDL does not decrease as does the immunoelectrodiffusion assay (see Table I). The electrophoretic pattern of sonicated LDL shows tailing, and the mobility of LDL decreases as the irradiation time increases (Fig. 3).

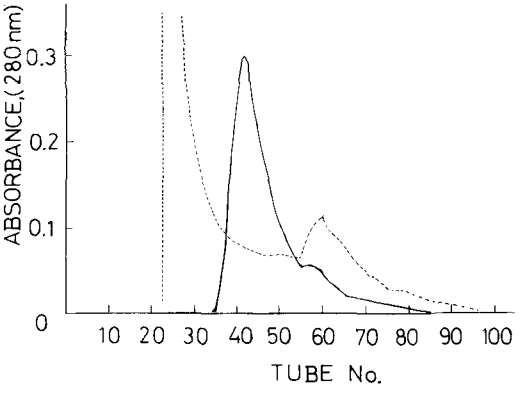


FIG. 4. Gel filtration patterns with Sepharose 4B. The solid line is native human serum low density lipoprotein (LDL) and the dotted line is sonicated LDL after 10 min irradiation.

TABLE II

The Effect of Ultrasonication on the UV Absorption Spectra Maxima and Minima From Human Serum Lipoproteins. Their Derivatives and Bovine Serum Albumin[a]

| Preparation[b] | Absorption[c] | |
|---|---|---|
| | Minimum (nm) | Maximum (nm) |
| LDL | 265 | 278 |
| Protein moiety from delipidated LDL | 260 | 280 |
| HDL | 259 (262) | 280 (274) |
| Bovine serum albumin (BSA) | 252 (256) | 279 (277) |
| Linoleate-BSA mixture | 256 (256) | 279 (277) |

[a]Values in parenthesis are for 10 min ultrasonication.

[b]LDL = human serum low density lipoprotein; HDL = human serum high density lipoprotein.

[c]This value could not be measured as the result of turbidity.

The Sepharose 4B gel filtration patterns of native LDL and sonicated LDL are shown in Figure 4. Sonicated LDL separated into two peaks, the first fraction corresponding to the void volume, and the second to the bed volume.

The absorption maximum and minimum in the UV spectrum of HDL approached each other with ultrasonic irradiation. In the case of sonicated LDL, both the absorption maximum and its minimum disappear. On the other hand, after delipidation with organic solvents, the absorption maximum shifts to a longer wavelength, and the absorption minimum shifts to a shorter wavelength as indicated in Table II.

An analysis of differential UV spectra for LDL, HDL and linoleate show differential maxima at 233 nm, but these maxima shift to 236 nm with BSA and the linoleate-BSA mixture. The visible spectrum of sonicated LDL did not show a shift of the absorption maximum but the absorption minimum in the UV spectrum did. On the other hand, according to Turian (11), the absorption maxima of gamma-carotene at 430 nm and 480 nm disappeared after conversion of gamma-carotene into oxicarotenoid. Similarly, in the case of sonicated LDL, the absorption maxima at 430 nm and

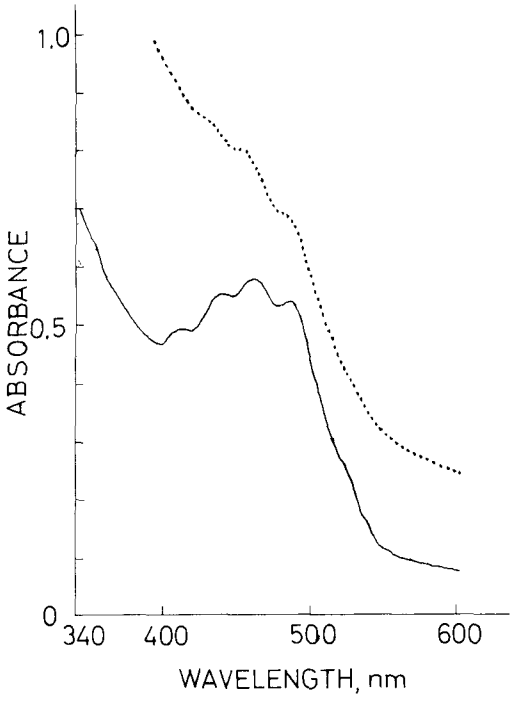


FIG. 5. Comparison of the visible spectra of native human serum low density lipoprotein (LDL) solution (solid) and sonicated (90 sec) LDL solution (dotted line).

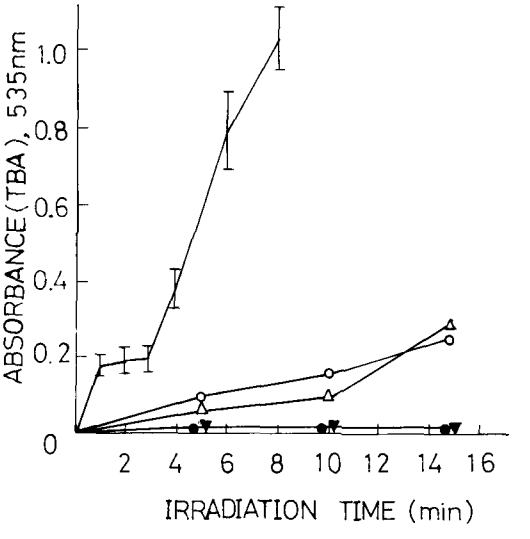


FIG. 6. Comparison of thiobarbituric acid test values of various ultrasonically irradiated preparations. Human serum low density lipoprotein (LDL), 20 mg/ml —┼—, 0.014 M bovine serum albumin (BSA) —●—, 0.02 M linoleate —△—, 0.02 M cholesterol —▼—, 0.02 M linoleate - 0.014 M BSA mixture —⊖—.

TABLE III

Comparison of TBA Values of Various Preparations Before and After Ultrasonic Irradiation (4 sec) showing the Effect of Riboflavin, Ascorbic Acid or Alpha-Tocopherol[a]

| Preparation | TBA value (O D 535 nm) | | |
|---|---|---|---|
| | Before | After | Difference |
| LDL (20 mg/ml) | 0.064 | 0.121 | 0.057 |
| LDL (20 mg/ml) + riboflaving (2 mg/ml) | 0.058 | 0.095 | 0.037 |
| LDL (20 mg/ml) + ascorbic acid (2 mg/ml) | 0.057 | 0.057 | 0.000 |
| LDL (20 mg/ml) + α-tocopherol (2 mg/ml) | 0.077 | 0.083 | 0.006 |
| HDL (20 mg/ml) | 0.196 | 0.289 | 0.093 |
| HDL (20 mg/ml) + riboflavin (2 mg/ml) | 0.138 | 0.165 | 0.027 |

[a]Abbreviations: TBA = thiobarkituric acid test; LDL = human serum low density lipoprotein; HDL = human serum high density lipoprotein.

480 nm in the visible spectra tend to disappear, as shown in Figure 5.

Figure 6 shows the comparison of TBA values with increasing time of ultrasonic irradiation. A two step pattern is seen in the ascending curve of TBA as a result of the ultrasonic irradiation upon LDL. On the other hand, the increased TBA value of sonicated LDL is suppressed by riboflavin, ascorbic acid and alpha-tocopherol (Table III).

## DISCUSSION

From Figures 1 and 2, the agarose gel precipitin reaction of sonicated LDL is suppressed as the ultrasonic irradiation time increased. An explanation for this phenomenon might be as follows: (a) the antigenicity of sonicated LDL decreased because of changes at the active antigenic site as a result of the ultrasonic irradiation; (b) the LDL molecules do not diffuse into the agar gel because of aggregation, and thus the antigenic reactivity diminishes.

According to Searcy and Bergquist (3), the active antigenic sites of LDL disappear on ultrasonic irradiation because of some structural changes in the protein moiety. The hemagglutination inhibition reaction shows that sonicated LDL may possess some of the same antigenic potency as native LDL as shown in Table I. The observations with the gel filtration of Sepharose 4B indicate that sonicated LDL also may be associated to form aggregated LDL. Thus suppression of reactivity of the agarose gel precipitin reaction of LDL can probably be attributed to aggregation of LDL.

The ultrasonic irradiation of LDL may have the same effect on LDL as delipidation because the precipitin lines of sonicated LDL fuse to those of delipidated LDL. Also the ultrasonic irradiation gives the same result as oxidation. According to Haase and Dunkley, the absorption at 233 nm was increased by both oxidation of linoleic acid and by oxidation of LDL.

Robison and Nelson (12) stated that on oxidation of lipoproteins the absorption maximum at 280 nm shifted to 275 nm and that a new absorption appeared at 320 nm. The absorption maximum of the ultrasonically irradiated lipoproteins also shifts to a shorter wavelength, as shown in Table II. An analysis of the differential spectra with LDL, HDL and linoleate shows an increase at 233 nm, increasing with the time of ultrasonic irradiation. The disappearance of the absorption at 410-500 nm in the visible spectrum of sonicated LDL is similar to the results of the oxidation of gamma-carotene by Turian (11).

The oxidizing action by ultrasonic irradiation of lipoproteins exposed to air is also supported by TBA values of sonicated lipoproteins, with or without antioxidant, as shown in Table III. The changes seen in lipoproteins, namely increased TBA value and absorption at 233 nm, are probably localized in the fatty acid portion of the lipoproteins (Fig. 6).

There is some indication that the absorption maximum of LDL at 278 nm shifts to a longer wavelength as a result of the delipidation treatment, as shown in Table II. This is in the reverse direction from the shift resulting from oxidation (12), and may be accounted for as a difference in the scattering backgrounds of LDL and its apoprotein.

## ACKNOWLEDGEMENT

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# Composition, Particle Size and Role in Dietary Fat Transport of Two Different Lipoproteins of the Intestinal Lymph

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## ABSTRACT

Rat intestinal lymph very low density lipoproteins have been separated by cellulose acetate electrophoresis. After administration of a meal including natural fats and labeled fatty acids, two labeled bands were detected on the electrophoregraphs. Further separations of these two kinds of particles were performed by density gradient zonal centrifugation. The isolated lipoproteins were analyzed for chemical composition and investigated by electron microscopy and electrophoresis. The moving particles had a higher protein and cholesterol content and a smaller diameter than the particles which remained at the origin. It has also been shown that the major part of the labeled lymph lipids were transported by the smaller particles.

## INTRODUCTION

In a previous study (1), two of us have shown that cellulose acetate electrophoresis of intestinal lymph (or chylomicrons isolated from this lymph and washed three times) obtained from rats fed a diet containing $^{14}C$ or $^{3}H$ fatty acids, exhibited two labeled bands: the first remained at the origin, whereas the second moved in the region of $\alpha_1$-globulins; generally this second band was the most radioactive. A single radioactive band was found at the origin when this lymph was subjected to electrophoresis on filter paper. These two labeled bands on cellulose acetate electrophoresis may represent one of the following possibilities: either chylomicrons of variable size, which differ significantly in their electrophoretic behavior, or chylomicrons which remain at the origin and very low density lipoproteins (VLDL) showing an electrophoretic mobility. It is known that lipoproteins (LP) have different electrophoretic mobilities according to the support (2,3) and that this property depends also on the product studied; it has been shown by Ockner et al. (4) that plasma VLDL move to the $\alpha_1$ globulin region and lymph VLDL to the $\alpha_2$ region.

A search of the literature reveals that cellulose acetate electrophoresis has been applied only to unlabeled plasma LP of fasting or fed humans or animals. Colfs and Verheyden (5) have obtained two bands: at the origin and in the $\alpha_2$ globulin region. These workers suggest that all the chylomicrons must move in the $\alpha_2$ region and that the saturation of the pores of the medium can explain the material remaining at the origin.

Identical results are reported by Chin and Blankenhorn (6), but their explanation differs significantly because they conclude that chylomicrons do not move and that only the lipids of altered chylomicrons migrate to the $\alpha_2$ region. From a survey of these very different conclusions, it appeared that new experiments should be undertaken using partially the methods previously described (1). Labeled fatty acids allow easy localization of LP that have a role in the transport of exogenous lipids. Under these conditions cellulose acetate electrophoresis separates two labeled fractions.

In the present investigation, the relative proportion of labeling of each fraction is given. Samples of radioactive intestinal lymph were then submitted to zonal centrifugation in a density gradient in order to obtain appreciable amounts of isolated fractions for subsequent analysis (cellulose acetate electrophoresis, elec-

TABLE I

Percentage of Radioactivity in the Two Labeled Bands After Electrophoresis on Cellulose Acetate of Rat Intestinal Lymph After Feeding Labeled Fats

| Number of rats | Diet fats | 1st Band[a] | 2nd Band |
|---|---|---|---|
| 8 | Corn oil + 18:1 $^{3}H$ | 30 (11-44) | 70 (56-89) |
| 2 | Corn oil + 16:0 $^{3}H$ | 17 (15-19) | 83 (81-85) |
| 3 | Butter + 18:1 $^{3}H$ | 30 (15-41) | 70 (59-85) |
| 1 | Margarine + 18:1 $^{3}H$ | 17 | 83 |

[a]Average values (range is indicated in parentheses).

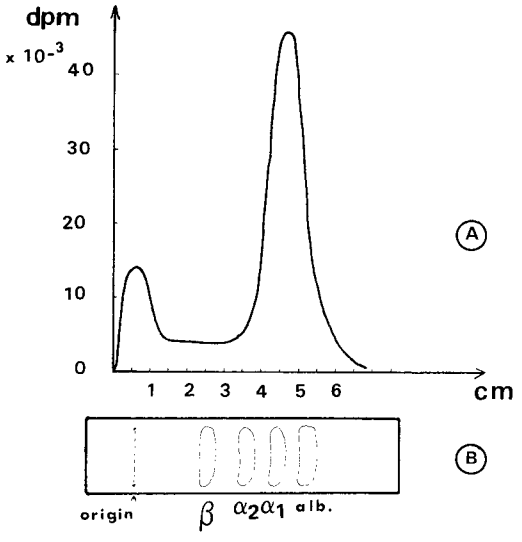


FIG. 1. Radiogram (A) and cellulose acetate electrophoresis pattern (B) of lymph of a rat fed corn oil labeled with 0.15, mCi 16:0 9-10$^3$H. (Staining = amidoschwartz.)

tron microscopy, chemical composition). Some additional experiments have been performed on rat plasma to support the conclusions drawn from our results.

## MATERIALS AND METHODS

### Collection of Lymph and Blood

Adult male Wistar rats (Mignaloux-Beauvoir, France), 180-200 g body weight, fasted overnight, were used in all the experiments. Cannulation of the main mesenteric lymph duct was performed. Twelve hours later the animals received a diet containing 300 mg casein, 500 mg sucrose, 80 mg of natural fats (corn oil or butter or margarine) with 0.15 mCi of $^3$H fatty acids added: palmitic acid 9-10 $^3$H (50 Ci/mM) or oleic acid 9-10 $^3$H (10 Ci/mM) purchased from C.E.A., Saclay, France. Silica gel thin layer chromatography and gas liquid chromatography revealed a radiopurity of 99%. Lymph was collected during 24 hr in an ice bath and immediately analyzed without addition of anticoagulant. During the lymph collection the animal was allowed to drink ad libitum a solution of 5% sucrose, 0.7% NaCl, 0.2% KCl, and to eat some pieces of bread. In some experiments aortic blood was collected in 3.8% sodium citrate from intact rats that 2 hr before had received the meal previously described. Plasma was obtained by centrifugation for 10 min at low speed.

### Cellulose Acetate Electrophoresis. Radioactivity Measurements.

Samples of plasma, lymph or fractions isolated from lymph by zonal centrifugation (see below) were subjected to electrophoresis on strips of cellulose acetate (Cellogel-Chemetron-Milano) according to the procedure described by Colfs and Verheyden (7). In each case two strips were developed: one for staining (amidoschwarz 1%) to allow visual location of LP, the second for radioactivity determination. Strips were cut in 5 mm width segments and placed in counting vials containing 1 ml methanol. Twenty hours later, 15 ml scintillation mixture (4 g of 2,5 diphenyloxazole, 0.1 g of 1,4 bis,

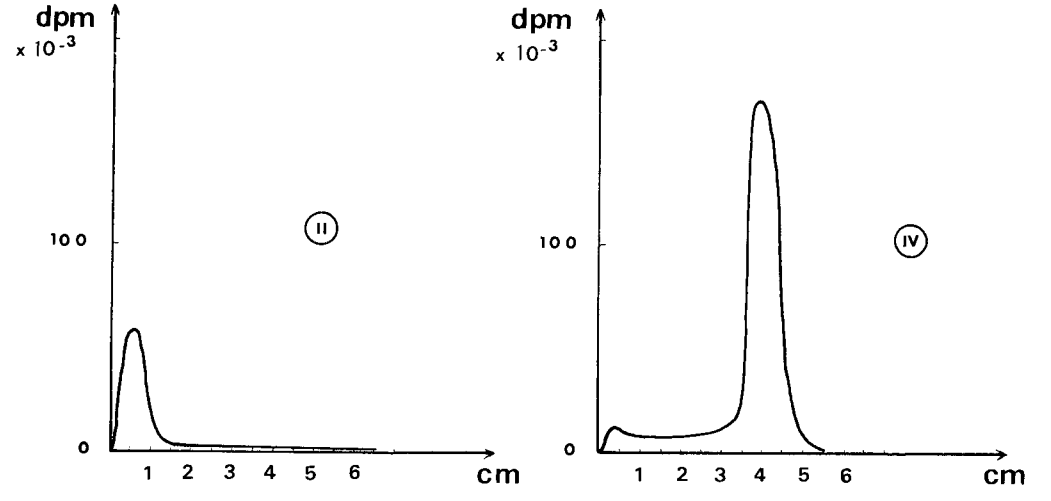


FIG. 2. Radiograms of fractions II and IV obtained after zonal centrifugation in a gradient of sucrose (30-40% w/v) and cellulose acetate electrophoresis.

TABLE II

Weight Percentage of the Lipid Classes in Fractions II and IV Separated by Zonal Centrifugation in a Sucrose Gradient Density. Distribution of the Radioactivity in Triglycerides (TG) and the Other Classes of Lipids[a]

| Diet fats | Isolated fractions | Weight, % | | | | Radioactivity, % | |
|---|---|---|---|---|---|---|---|
| | | Total cholesterol | Proteins | TG | Other classes of lipids[a] | TG | Other classes of lipids[a] |
| Corn oil + 18:1 $^3$H | II | 2 | 7 | 66 | 25 | 93 | 7 |
| | IV | 3 | 20 | 65 | 12 | 94 | 6 |
| Corn oil + 16:0 $^3$H | II | 2 | 10 | 77 | 11 | 93 | 7 |
| | IV | 4 | 27 | 55 | 14 | 91 | 9 |

[a]Monoglycerides + diglycerides + phospholipids + free fatty acids.

2,4 dimethyl-5-phenyloxazole and toluene to a final volume of 1 liter) was added.

Radioactivity was measured in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer. Quenching was monitored by the automatic external standard device (Packard AAA Model 544).

#### Zonal Centrifugations

After defibrination on gauze, the lymph was gently homogenized with a glass rod during 4 min and subjected to zonal centrifugation in two types of density gradient. All centrifugations were carried out in a Spinco Model L2 65B Ultracentrifuge which had been adapted to accept a Ti 14 rotor with removal seals. During the interval when the rotor was loaded or unloaded, its velocity was maintained at 3000 rpm. The rotor volume was filled with the appropriate gradient solution at the rate of 30 ml/min, using a Beckman Model 141 high capacity gradient pump.

a) *KCl-sucrose gradient* according to Wilcox and Heimberg (8): Fifty ml distilled water was placed in the rotor which was then accelerated to 3000 rpm. The gradient materials used were water ($d$=1) and an aqueous solution of sucrose-KBr (400 g, 200 g, water to a final volume of 1 liter [$d$=1.28]). When the rotor was almost completely filled, the lymph (to which had been added Sucrose-KBr 2:1 w/w until $d$=1.28) was injected with a syringe at the periphery of the rotor followed by ca. 10 ml high density solution to ensure that the entire sample was within the rotor. Centrifugation (7 C) was carried out at 47,000 rpm (172,000 x g [All number times gravity are maximum centrifugal forces.]) for 24 hr. The rotor was then decelerated to 3000 rpm and the content of the rotor was displaced by pumping a solution of high density into the periphery of the rotor and collecting through the core. The solution was then divided into fractions of various volumes. During the displacing, this effluent was monitored by measurement of absorption at 280 mμ in a Beckman spectrophotometer equipped with a quartz flow-through cell (1 cm light path).

b) *Sucrose density gradient:* This procedure stems in part from the method of Zilversmit et al. (9). The gradient materials used were aqueous solutions of sucrose (30% and 40% w/v). Sucrose, 40% w/v, was added to the lymph, and the mixture was injected into the rotor at its periphery followed by 10 ml of high density solution.

Centrifugation was performed at various and successive rotation speeds: 1 hr at 8000 rpm (4800 x g); 1 hr at 16,000 rpm (19,000 x g); 1 hr at 24,000 rpm (43,000 x g); 1 hr at 40,000 rpm (119,000 x g). After each centrifugation the rotor was decelerated to 3000 rpm and the opalescent fraction near the core of the rotor was removed by displacing with high density solution injected at the periphery. In this way fractions I (8000 rpm), II (16,000 rpm), III (24,000 rpm), and IV (40,000 rpm) were collected. Then they were diluted by 0.9% NaCl and centrifuged for an hour at 40,000 rpm (105,000 x g) in a 50 Ti Rotor. The surface layer was removed by skimming with a hypodermic syringe; chemical and electrophoretic analyses were performed on these samples.

#### Extraction. Lipid, Protein and Cholesterol Analysis

Total lipids were extracted from lymph or plasma isolated fractions with 15 volumes dimethoxymethane-methanol 4:1 v/v for each volume of biological material according to the procedure of Delsal (10). After protein filtration the larger part of the solvent was removed, a large volume of water was added and lipids were extracted by three washings with diethylether. This extract was taken to dryness under reduced pressure and the residue was suspended in chloroform and filtered.

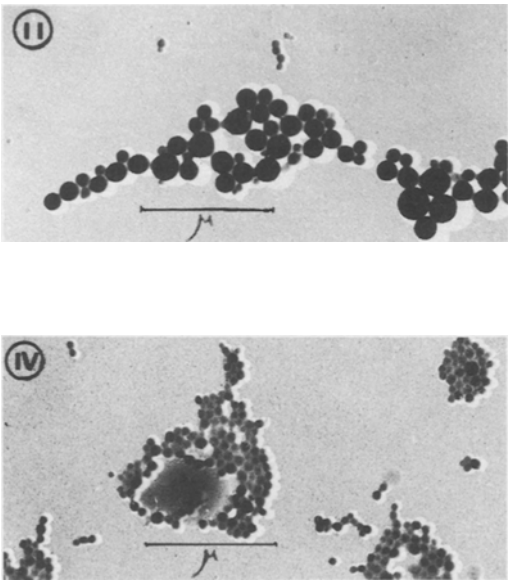


FIG. 3. Electron micrographs of the lipid particles contained in fractions II and IV of the intestinal lymph of a rat fed corn oil. These two fractions fixed with 2% osmium tetroxide were placed on grids with collodium membrane and shadowed with Au/Pd at an angle of 30 degrees. Random fields were photographed in a HU 11E Hitachi electron microscope at an instrumental magnification of 11.000.

Separation of lipids was carried out by thin layer chromatography on gel plates (Kieselgel "G" Merck) (11). Chromatograms were developed with hexane-diethyl ether-acetic acid 90:30:1 v/v/v. Lipids were made visible by exposure to iodine vapors.

Fatty acids were analyzed as butyl esters following the method of Clement and Bezard (12) by gas liquid chromatography for molar composition and for weight determination after the addition of heptadecanoic acid as internal standard.

Total cholesterol was determined according to the method of Delsal (13) and the protein by the method of Lowry et al. (14).

## RESULTS

First, let us notice that the label recovery during the 24 hr collection of the intestinal lymph was 50-70% of the administered dose.

### Distribution of Radioactivity Between the Two Lipoprotein Bands Obtained by Cellulose Acetate Electrophoresis of Intestinal Lymph

As can be seen (Fig. 1 and Table I) there are two very distinct labeled zones as previously shown (1). In each case the lipid material which showed an electrophoretic mobility was more radioactive than the material remaining at the origin. This distribution of radioactivity seems to be not dependent on the nature of dietary fat (corn oil-butter-margarine) but additional experiments should be necessary to clarify this point.

### Electrophoresis, Chemical Composition and Electron Microscopic Studies of Fractions Isolated From Intestinal Lymph by Zonal Centrifugation

Ten 1 ml fractions ($1.00 \leqslant d < 1.01$) were isolated in a density gradient of KBr and sucrose and subjected to cellulose acetate electrophoresis. As with total lymph (Fig. 1), the two labeled regions were already present in each of these fractions, indicating that these two radioactive compounds had a density less than 1.01. Under these conditions LDL, HDL and albumins were not removed from the rotor, so it can be assumed that the electrophoretic mobility of the fraction of very low density material was not due to their presence.

Among the four fractions that had been isolated by means of zonal centrifugation in sucrose gradient (see Methods), only fractions II, III and IV were labeled. Electrophoresis of these three fractions showed (Fig. 2) that they differed significantly in their mobilities. Only one labeled zone was detected at the origin in fraction II and in the $\alpha_1$ globulins region in Fraction IV, while fraction III showed the two labeled zones. Therefore in the following discussion the results relating only to fractions II and IV will be given. Table II shows the composition and the distribution of radioactivity among lipid constituents. Triglycerides are the main constituents in weight as well as in radioactivity in both fractions. Protein and cholesterol are more important in fraction IV than in fraction II. Two additional experiments (results not indicated in Table II) have been performed in order to test the incorporation of the exogenous cholesterol. Two rats were fed a diet as described in Materials and Methods but with 10 mg 4-$^{16}$C cholesterol (0.05 mCi, C.E.A., Saclay, France) added. The isotopic ratio oleic acid $^{3}$H/cholesterol $^{14}$C of the diet had been set at 1. In fractions II the isotopic ratios were 10 and 17, approximately three times higher than in fractions IV (3 and 6, respectively). These results show that the incorporation of exogenous cholesterol compared to $^{3}$H oleic acid was greater in fraction IV than in fraction II. In Figure 3 are shown typical electron micrographs of particles contained in fractions II and IV. The particles of fraction II have a larger diameter (mean value 0.15 $\mu$) than those of fraction IV (mean value 0.05 $\mu$). It should be noted that these diameters are in the

same order as those published by Ockner and Jones (15); but in our experiments these two kinds of particles were present in the sample of lymph collected from a rat fed with corn oil, while Ockner and Jones found significant differences related to the degree of saturation of the fed fatty acids.

## DISCUSSION

The results reported here concerning electrophoresis of radioactive lymph are in agreement with our earlier paper (1); at that time rats were fed corn oil containing labeled free fatty acids or doubly labeled triglycerides (glycerol and fatty acids). In this present study, only the results obtained from lymphs collected during 24 hr are given. But cellulose acetate electrophoresis of labeled lymphs collected at different times after the radioactive meal (2 hr, 4 hr, 6 hr . . .) has shown that the two radioactive peaks are already present with electrophoretic mobilities identical to those observed in the case of lymph collected during 24 hr. We have shown that the two radioactive fractions (obtained by zonal centrifugation) exhibit different electrophoretic mobility and seem to consist of spherical unaltered particles. The mobility of particles contained in fraction IV was not consistent with the conclusions of Colfs and Verheyden (5) who have claimed that the material remaining at the origin was due to the saturation of the pores of the medium. Our observation is also in disagreement with Chin and Blankenhorn (6), because the particles of fraction IV (examined by electron microscopy) seem to be unaltered.

Using agarose gel, Ockner et al. (4) have described different electrophoretic mobilities of lymph VLDL and plasma VLDL. In contrast, we have shown that the electrophoretic moving fraction was always located in the $\alpha_1$ globulin region of each sample (total lymph, fractions of density $< 1.01$ obtained in sucrose–KBr gradient or fraction IV either alone or added with an equal volume of serum collected from fasting rats).

In addition, experiments on the plasma of rats were performed. The animals had been fed, 2 hr previously, a diet containing corn oil and a tracer dose of oleic acid 9-10$^3$H. The plasma was subjected to zonal centrifugation according to Wilcox and Heimberg (8) (see Fig. 4). The results obtained were identical to those of Windmueller et al. (16) and Wilcox and Heimberg (8) in showing the presence of albumin, HDL, LDL and VLDL in small amounts. We have examined all these plasma fractions for radioactivity and found that only VLDL (mainly in TG) and albumin (in free fatty acids) were labeled.

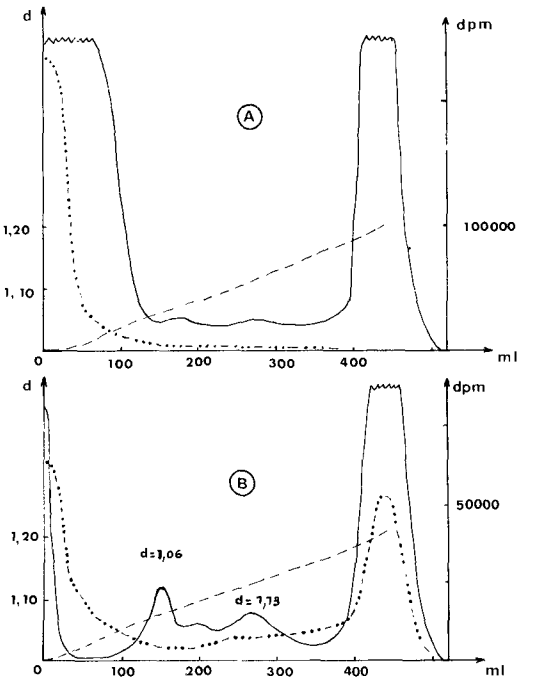


FIG. 4. Resolution with Ti 14 Rotor at 47,000 rpm during 20 hr in a sucrose-KBr gradient. A, Rat intestinal lymph; B, rat plasma; ———— optical absorbance at 280 mμ; – – – – density (a); ●●●–●●● radioactivity.

In contrast, the lymph subjected to centrifugation under the same conditions is richer in LP of very low density than the plasma. Albumins were also present, but there were negligible amounts of HDL and LDL, in agreement with Windmueller et al. (16). In this case the fraction of very low density is labeled in the triglycerides (Table II).

The results concerning the lipoprotein pattern in plasma give us confidence that there was no technical error. By comparison with lymph results, there is evidence that capillary filtration is very efficient, because LP patterns are somewhat different. Apparently the fraction that remains at the origin on electrophoretic strips could be similar to chylomicrons, as defined by Zilversmit (17), taking account of size and relative triglyceride proportion. The protein concentration is higher than commonly considered for these particles, but it is known (18) that separation of very low density particles from the other lymph proteins is very difficult. This contamination could be important in our experiments, because purification by additional "washes" was not performed to avoid label and particle loss.

It is more difficult to come to a conclusion on the nature of electrophoretic moving parti-

cles (small size chylomicrons or VLDL). Indeed their high percentage of triglycerides in weight as well as in radioactivity suggest that they are small size chylomicrons, but in this hypothesis a "tailing" should be exhibited between the origin and the $\alpha_1$ globulin region. In fact the labeled zones are quite distinct. This could be explained if it is assumed that cellulose acetate gel has a behavior somewhat similar to a sieve which prevents the movement of the larger particles.

Several authors suggest that the distinction between chylomicrons and VLDL is difficult, because they are two ends of a single spectrum of particle size and density.

Their chemical composition did not offer sufficient evidence to say anything explicit concerning this question. In fraction IV the greater amounts of protein and cholesterol could be explained by the diameter of the particles, because the smaller particles (fraction IV) have a larger surface to volume ratio than the larger ones, and it is now assumed that cholesterol and protein are present in the polar surface coat (19).

Ockner et al. (4) have demonstrated in the fasting state the importance of lymph electrophoretic moving particles (which they called VLDL) in the transport of endogenous lipids and cholesterol derived from bile and the intestinal wall. In other studies (20) it has been observed that these kinds of particles may contribute to the transport of exogenous lipids. Our findings are in agreement, but the use of labeled fatty acids gave evidence that the small particles are of the greatest importance in this transport process.

Fatty acid composition of fractions II and IV (unpublished observations) were not similar, but these fatty acid patterns were affected by the nature of diet fats (corn oil, butter, margarine). We have seen that this factor did not significantly influence the relative proportion of labeling present in the two kinds of particles. This observation is inconsistent with the findings of Ockner et al. (20), who have shown quantitative differences between chylomicrons and VLDL according to the nature of exogenous fatty acids.

It is known that plasma VLDL concentrations arose from a rich carbohydrate diet. In all the experiments described in this paper a high proportion of sucrose was present, so in some additional experiments rats received a carbohydrate-free diet; we have not seen any differences in the distribution of radioactivity among the two classes of lipoproteins.

Our results have not enabled us to establish definitely if this electrophoretic moving fraction consists of chylomicrons or VLDL; it is even possible that the study of apoproteins would not give any definite solution either because, according to Ockner et al. (21), the apoproteins of the chylomicrons and the VLDL seem to have a similar composition.

However this study (using the method of zonal centrifugation in a sucrose gradient density) enabled us to separate from labeled intestinal lymph two fractions which present a different electrophoretic mobility.

All the papers in this field of study have shown the importance of chylomicrons in this transport. These experiments allow us to state precisely that particles smaller than chylomicrons and moving to the $\alpha_1$-globulins region have also an important role in the transport of exogenous lipids.

## ACKNOWLEDGMENTS

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# Comparison of Dialysis, Thin Layer and Silicic Acid Column Chromatography for Prostaglandin Isolation From Biological Material

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**ABSTRACT**

Chemical and biochemical methods assaying the total content of neutral lipids during some conventional procedures for purification and isolation of prostaglandins from biological fluids revealed all fractions to be contaminated with fatty acids, cholesterol, cholesterol esters, tri-, di- and/or mono-glycerides. It was demonstrated that it was not possible to make pure prostaglandin fractions by simple dialysis. On the basis of these findings a new preparative schedule involving either dialysis or column chromatography on silicic acid, combined with preparative thin layer chromatography, made it possible to isolate substantially pure $PGE_2$ from a synthetic biological system involving arachidonic acid as a precursor and the microsomal synthesizing system from sheep vesicular glands.

Sheep vesicular glands have been used for biosynthesis of prostaglandins (PG) (1). For the study of the metabolism of various prostaglandins, labeled $PGE_1$ has been prepared from 8, 11, 14-eicosatrienoic acid labeled at various positions (2). Recently a method employing arachidonic acid and sheep vesicular glands for the formation of $PGE_2$ has been patented. The method claims a high purity (98%) with quick recovery of $PGE_2$ from the incubation mixture. The essential feature of the method is the use of a dialysis step which is said to be superior to the time-consuming chromatographic procedures (3). In our recent studies on the metabolism of essential fatty acids, we have found it impossible to prepare chemically pure prostaglandins by the above mentioned methods, since cholesterol, cholesterol esters, fatty acids and glycerides were found to contaminate the final products. The present paper therefore gives some data on these contaminants, and on this basis a new approach to the isolation of chemically pure prostaglandin

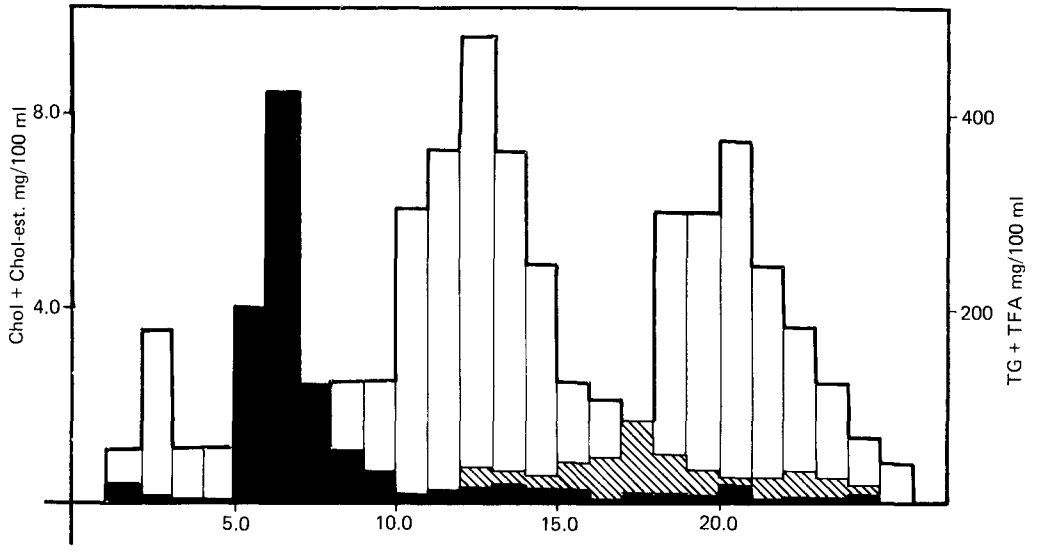


FIG. 1. Elution of lipids from the incubation mixture used for biological synthesis of prostaglandins. The incubation mixture was acidified to pH 3.0, extracted twice with 250 ml ether, washed with water until neutral pH, evaporated and dissolved in 4 ml benzene. The benzene solution was applied to the silicic acid column, and the lipids were eluted with benzene. Twenty-five fractions of 30 ml were collected and assayed. Black squares: triglycerides, ordinate to the right; hatch squares: free fatty acids, ordinate to the right; clear squares: cholesterol esters (greatest peak) and free cholesterol (latest peak) ordinate to the left; ordinate: concentration of neutral lipids (mg/100 ml); abscissa: fraction number.

($PGE_2$) has been developed.

## MATERIALS AND METHODS

### Biological Material

Swine and sheep vesicular glands were obtained fresh from the slaughter house (Roskilde, Denmark). The glands, together with epididyma part, were cut out immediately after slaughter; blood was eliminated by washing in 0.16M NaCl; and the glands were stored until use, sealed in plastic bags in a deep freezer at -20 C.

### Reference Prostaglandins

These were obtained by the fractionation of human seminal plasma (4,5) and finally purified as described in the present communication. The seminal plasma was kindly supplied by the Copenhagen Health Insurance Laboratory and was stored at 4 C in a nitrogen atmosphere in sealed glass ampules. Chemically synthesized $PGE_1$ was kindly supplied by Unilever Research Institute, Vlaardingen, Holland.

### Chemicals

When not otherwise stated chemicals were of highest obtainable purity from British Drug Houses, Dorset, England. Standard chromatographic markers (99% pure), i.e., tri-, di- and monopalmitate, cholesterol, cholesterol palmitate and palmitic acid, were obtained from the Hormel Institute (Austin, Minn.).

### Thin Layer Chromatography (TLC)

TLC was done on Silica Gel G (Merck, Darmstadt, Germany) applied on glass plates (19 x 19 cm). Prior to use the silica gel was washed twice with hot (45 C) methanol followed by drying at 40 C for 12 hr and then for 2 hr at 110 C. The silica gel was spread in a homogenous 0.4 mm layer by means of the spreader recommended by Stahl (6). The plates were activated by heating to 110 C for 30 min. They were stored in a desiccator until used (maximally 4 hr). The samples were applied with a 50 $\mu$l syringe 1 cm from the lower edge of the TLC plate as a 5-10 cm long zone. The development of the plates was performed in all-glass tanks (20 x 20 x 8 cm) in one of three systems (A, B and C) mentioned below. The individual fractions were visualized in the lateral parts of the TLC plate by exposure to iodine vapors; the major part of the TLC plate was covered with a steel plate, the four borders of which had uniform resting edges (10 mm) leaving a 1 cm zone free to iodine exposure. The metal plate was fixed to the TLC plate with the help of cellotape. The plate was then exposed to iodine in a sealed glass tank for 2 min. The areas corresponding to the lateral spots were scraped off and studied chemically (vide infra).

### Column Chromatography

Column chromatography (4,5) was done on a column (40 x 2 cm) of 20 cm silicic acid (100 mesh) (Mallinckrodt Chemical Works, St. Louis, Mo.) suspended in benzene. The samples applied were eluted as described below with a hydrostatic pressure equivalent to 20 cm eluant above the silicic acid surface. Twenty-five fractions, each of 30 ml, were collected automatically in a LKB fraction collector (Ultrorac No 7000, LKB Ltd., Stockholm, Sweden) equipped with a siphon stand or a drop counter.

All solvent compositions used for chromatographic purposes are expressed as volume/volume.

### Assay of Neutral Lipids in Various Fractions From Silicic Acid Chromatography

(a) Neutral fat (tri-, di- and monoglycerides0 was estimated in the individual fractions by the enzymic method of Eggstein (7). (b) Total cholesterol was estimated by the method of Abell et al. (8). (c) Total fatty acids were indirectly estimated after formation of copper-soaps by assay of free $Cu^{++}$ with the dithiocarbamate reaction (9). Prior to the enzymic assay (7), neutral fat was demonstrated by TLC to be either mono-, di- or triglycerides in the solvent system (A) mentioned below, using standard markers to locate these lipids.

### Assay of $PGE_2$

Assay of $PGE_2$ was made by treatment of the samples with 0.5 M NaOH, yielding the $\alpha$-$\beta$ unsaturated ketone ($PGA_2$) which then rearranges itself to the doubly conjugated ketone, $PGB_2$. This is estimated by measuring, at 278 nm, the increase in optical density (or extinction) which occurs with alkali treatment.

### Assay of PGA and B

Assay of PGA and B was made by direct photometric estimation of extinction at 217 nm and 278 nm (10), and indirectly the PGA was assayed as the extinction difference prior to, and after, the chromatographic fractions (2 ml, vide infra) were mixed with 0.5 M NaOH, thereby transforming the A into the B derivative.

### Microsomal System for Biosynthesis of Prostaglandins

About 225 g of the thawed minced glands were mixed in a ratio of 1:2 w/v with 0.1M

$NH_4Cl$ solution, pH 8.5 containing 1 g glutathione per 500 ml. The mixture was homogenized in a Waring blendor for 4 min at 4 C. The suspension was centrifuged at 10,000 x g (max.) for 15 min at 0 C (Sorvall RC-2 superspeed centrifuge). The supernatant was isolated and the particulate residue mixed with 40 ml $NH_4Cl$ solution, homogenized with a Thomas homogenizer (Thomas, Philadelphia, No. C23347) at 4 C (50 rpm) and centrifuged as described above. The two supernatants containing the enzyme which catalyzes prostaglandin synthesis from unsaturated fatty acids were combined and adjusted to pH 8.0 with 0.1 M NaOH. A solution of prostaglandin-precursor fatty acid was prepared from 250 mg arachidonic acid (The Hormel Institute, Austin, Minn. Lot No. C420A; 90% pure as determined by gas chromatographic [11] analysis) suspended in 500 ml of 0.1 M $NH_4Cl$ (pH 8.5) containing 1 g glutathione.

The supernatant containing the enzyme which catalyzed prostaglandin synthesis was mixed with the suspension and the mixture incubated at 37 C for 60 min with constant stirring and a continuous supply of oxygen (11 ml/min water-saturated $0_2$) (1-3). The reaction mixture was then freeze-dried, the lyophilized powder was dissolved in 200 ml water and the pH adjusted to 7.5. The mixture was dialyzed (Visking dialysis membrane pore size 24 Å, width 3.2 cm at normal atmospheric pressure) against distilled water twice, first with 4 liters and then with 3 liters. The dialysates were combined and adjusted to pH 3.0 with 0.5 N sulphuric acid.

## RESULTS

The prostaglandins synthesized in vitro by the microsomal system were extracted from the combined dialysates with 200 ml portions of ether in three aliquots. The ethereal extracts were combined, washed with distilled water until free from mineral acid, dried with anhydrous sodium sulphate, filtered and finally concentrated under reduced pressure. Total solids weighed ca. 25 mg which, when assayed for $PGE_2$ with 0.5 M NaOH, indicated its yield to be 3.5% (calculated on a basis of a 100% conversion of arachidonic acid into $PGE_2$), and a total content of 10.2 mg $PGE_2$ indicating 14.8 mg of other hydrophilic, non-$PGE_2$ components. Thus the crude dialysate contained only 41% $PGE_2$.

Quantitative assay for lipid components in the crude dialysate was made after isolation of the individual components by preparative TLC developed with system A: benzene-ether-

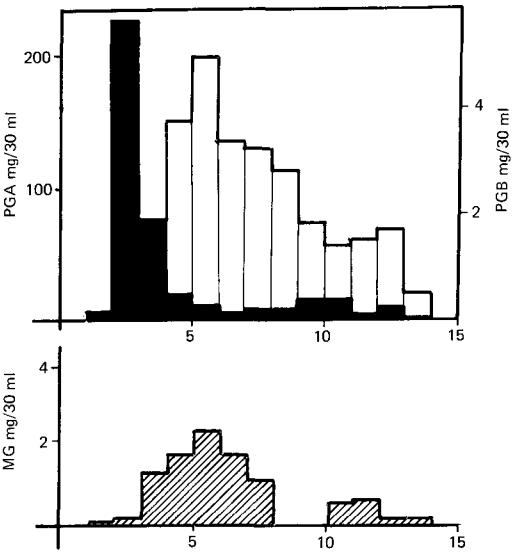


FIG. 2. The elution diagram of the column mentioned in Figure 1 after elution with benzene and benzene-ether 95:5 v/v. The elution hereafter presented in the figure was made with ethyl-acetate-benzene 3:7 v/v. Fractions (30 ml) were collected. Black squares: represent PGB and unknown impurities; clear squares: represent PGA; hatch squares: monoglycerides; ordinate (left side): concentration of PGA (mg/ 30 ml x $10^{-3}$); ordinate (right side): concentration of PGB and impurities (mg/30 ml); abscissa: fraction number.

ethylacetate-glacial acetic acid 80:10:10:0.2. Solvent A is used specifically for the resolution of neutral lipids. This solvent does not resolve the prostaglandins and phospholipids. The neutral lipids which accompanied the $PGE_2$ were triglycerides, and free cholesterol along with trace amounts of monoglycerides. After development the corresponding application bands were scraped off from the plates and pooled together. The silica gel was extracted with methanol three times and filtered through Whatman 40 ashless filter paper which was moistened with methanol prior to filtration. The solution was evaporated under $N_2$, followed by dilution with 5 ml water. This was then acidified with 0.5 N HCl and extracted three times with equal volumes of ether. The ethereal extract was washed with water until neutral. The volume of water used for this purpose was kept to a minimum. The extract was then dried in vacuum. The crude dialysate containing 10.2 mg ($PGE_2$) also contained 6.2. mg triglyceride, 1.4 mg monoglyceride, 0.7 mg total cholesterol and 1.9 mg fatty acids. Minor impurities of phospholipids remained on the TLC plate at the origin.

In order to purify the prostaglandin in the material isolated by the dialysis method as

TABLE I

Biosynthesis of $PGE_2$ From Arachidonic Acid[a]

| Method | Yield, % | Purity |
|---|---|---|
| Dialysis method | 3.5 | Admixed with: 59% (w/w) cholesterol (+ esters), fatty acids and glycerides. |
| Dialysis followed by thin layer chromatography (TLC) | 2.25 | Pure |
| Column chromatography on silica gel combined with preparative TLC | 1.7 | Pure |

[a]Yields obtained by means of different methods of isolation (for details see text).

described above, it was essential to separate it from the impurities by preparative TLC of this material. This was done on Silica Gel G using solvents B: ethyl acetate-iso-octane-acetic acid-water 110:20:10:100 (13) and C: benzene-ethyl-acetate-acetic acid-methanol-water 52:48:10:60. The lipids were separated as sharp narrow zones in both systems even when the maximum loading of 8 mg total lipid was applied to the TLC plate. These double phase systems B and C were equilibrated for 1 hr before using the lipophilic nonaqueous phase. Since a part of the material applied to the TLC plate did not migrate but remained at the point of application, with solvents B and C, it was concluded that there were more polar components, vis., polar lipids. Zones corresponding to Rf 0.49 and 0.25 (respectively with B and C) were scraped off, extracted thrice with methanol and filtered. The filtrate was concentrated under $N_2$, followed by dilution with 5 ml water. This was acidified with 0.5N HCl and extracted three times with equal volumes of ether. The combined ethereal extracts were washed with water until neutral, dried with anhydrous sodium sulphate and finally concentrated under $N_2$. As arachidonic acid is the precursor of $PGE_2$ and accepting that only trace amounts of other PGE components might be present in the microsomal fraction used for $PGE_2$ formation, the yield of $PGE_2$ was assayed to be 2.25% (6.5 mg).

### Silicic Acid Column Chromatography

In another experiment an attempt was made to isolate and purify $PGE_2$ from the incubation mixture without resorting to dialysis. The incubation mixture was adjusted to pH 3.0 by addition of 0.5 N $H_2SO_4$, drop by drop, with continuous stirring. Ether (250 ml) was added twice for extraction. After 1 hr the ether layer was separated, washed with a minimum quantity of water to free it from the mineral acid, dried with sodium sulphate and filtered. The filtrate was concentrated as described before. The residue, which appeared to be a brown mass, was dissolved in a small volume of benzene and applied to a column of silicic acid prepared in benzene. A brown layer stayed at the top during elution with benzene. Twenty-five fractions (each 30 ml) were collected and examined by TLC using solvent A (vide supra). Four neutral lipid components appeared to be eluted in order of their increasing polarity. They were cholesterol esters, triglycerides, free cholesterol and fatty acids (Fig. 1). Quantitative assays for these components in the collected fractions revealed a small cholesterol peak (fractions 1-4 in Fig. 1) appearing before the triglyceride peak (fractions 4-8), then cholesterol esters (fractions 9-15), fatty acids (fractions 16-18) and finally free cholesterol (fractions 18-24) (Fig. 1). Since even the last fraction contained fatty acids, these acids were retained on the column, but they could be quantitatively eluted with benzene-ether 95:5 v/v. The next elution was started with ethyl-acetate-benzene 3:7 v/v. A little portion of the unidentified brown ring slowly proceeded down the column as a sharp band. Elution was continued until it had left the column. In all, 18 fractions (each 30 ml) were collected. All the fractions were examined by TLC (solvent A) and by the chemical methods. No cholesterol, triglycerides, diglycerides or free fatty acids were found. However significant amounts of monoglycerides appeared as two peaks (Fig. 2). Assays of PGA (and PGB) (Fig. 2) on 2 ml aliquots of every fraction (except fractions 2 and 3 of which 200 $\mu$l were used) revealed the PGA peak to be located in fraction 5, and the direct absorption prior to alkaline treatment at 278 nm (PGB) was distributed in two peaks (at fractions 2 and 9), both of which overlapped the PGA fraction. The initial absorbance at 217 and 278 nm prior to alkali treatment was due not only to the $\alpha,\beta$-unsaturated ketone (PGA and PGB) (10) but also to unidentified components. The unidentified brown zone on the column emerged in fraction 18. Fractions 4-17 were combined, concentrated to a small volume under reduced pressure and finally evaporated under $N_2$ at 65 C to a residue. The residue was examined by TLC with solvent B.

Three spots were obtained, of which one corresponded to that for PGA + PGB (0.78). The presence of PGB and PGA was again demonstrated by the increase in absorbance at 278 nm following treatment with alkali. The 18th fraction was found to be a mixture of three components, none of which, however, could be identified chemically. Finally elution was done with ethyl acetate-benzene 6:4. No glyceride, fatty acid or cholesterol could be traced. A total volume of 800 ml was eluted and concentrated as described earlier. The residue was purified by preparative TLC with solvent C. The yield was 1.7% (Table I).

## DISCUSSION

The data of the present communication showed that although prostaglandins are more hydrophilic than cholesterol, fatty acids and triglycerides, it is not possible by a simple procedure (3) to separate these on the basis of their water-solubility. The low yields of prostaglandin in the combined dialysates may be due either to the presence in the microsomal fraction of a specific prostaglandin-binding protein or to biological inactivation during the incubation period used, or to both. As hydrophilic components, monoglycerides are seen in several fractions of the column chromatographic separation together with the prostaglandins. This phenomenon may be explained by their tendency to micelle formation.

Ethyl acetate-benzene (3:7, 6:4, 8:2) solvent systems have been used for the separation of individual groups of prostaglandins (PGA+B, PGE and PGF, respectively) from the seminal plasma (4,5). The material eluted with the 6:4 solvent has been further purified by reversed phase partition chromatography (stationary phase supported on hydrophobic cellulose). In various incubation experiments wherein authors have tried to prepare PGE, attempts have been made to isolate it on silicic acid columns using 6:4 solvent system (2), followed by further purification as described above by the reversed-phase partition chromatography. Hamberg and Samuelsson (1) have purified PGE from such an incubation mixture by eluting unchanged linoleic acid from the column with ether hexane 5:95. More polar compounds were eluted with ether-hexane 20:80. Andersen (15) purified diastereoisomers of PGE contaminated with PGA on silicic acid column by eluting successively with an ethyl acetate-cyclohexane mixture in the ratios of 3:2 and 1:2. He has also recommended SilicAR CC-4 (100-200 mesh) as column adsorbent using the following solvent systems of cyclohexane-ethylacetate: (a) the system (2:1) for the elution of PGA + PGB; (b) the system (1:1) for the diastereoisomers of PGE; and (c) ethyl acetate for PGF diastereoisomers.

The present paper makes it possible to elute PGE from a mixture of other PGs and neutral lipids by successive elution with the following solvents: (a) benzene, (b) benzene-ether 95:5, (c) ethyl acetate-benzene 3:7 and (d) ethyl acetate-benzene 6:4 for gradually removing most of the neutral lipids with solvent (a), the remainder of the free fatty acid (chiefly arachidonic acid used as a substrate) with (b), PGA+PGB+monoglycerides with (c) and finally $PGE_2$ with (d).

While calculating the quantity of $PGE_2$ by measuring the chromophore (conjugated dienone), it has been assumed that the yield of the chromophore for $PGE_2$ is 90%. Our demonstration of the presence of PGA and PGB could be due to either of the two possibilities: (a) they might be present in the microsomal fraction used, or (b) they have been formed during the incubation or have been generated during the various chromatographic steps. However it has been shown that a small part of PGA compound is formed non-enzymatically during the isolation procedure (14). The extinction at 278 nm prior to addition of alkali may not be necessarily due to PGB components, as it has been shown that other components with varying degrees of polarity show an absorption at 278 nm (4).

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# Determination of Specific Activity of Isotopic Materials by Thin Layer Chromatography[1]

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**ABSTRACT**

A modified method for determination of specific activity by thin layer chromatography (TLC) is described. Based on in situ spectrodensitometry after separation by TLC, the method is rapid and requires amounts of reference material in the microgram range.

A problem of analysis of radioactive material isolated from biological sources lies in identification of trace amounts of substances. The concentration method for indication of the purity of isolated material seems to be recrystallization to constant specific activity. However even recrystallization is not always proof for purity of compounds, especially if the compounds are very similar in their chemical structures (1). Methods have been published on determination of specific radioactivity using thin layer chromatograph (TLC) scraping, counting and quantitation by colorimetry (2,3). These methods are either very complicated or not in general use.

A search was made for a faster and more simple method for determination of specific activity. This was provided by in situ spectro-

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

[2]Ford Foundation Fellow in Reproductive Biology.

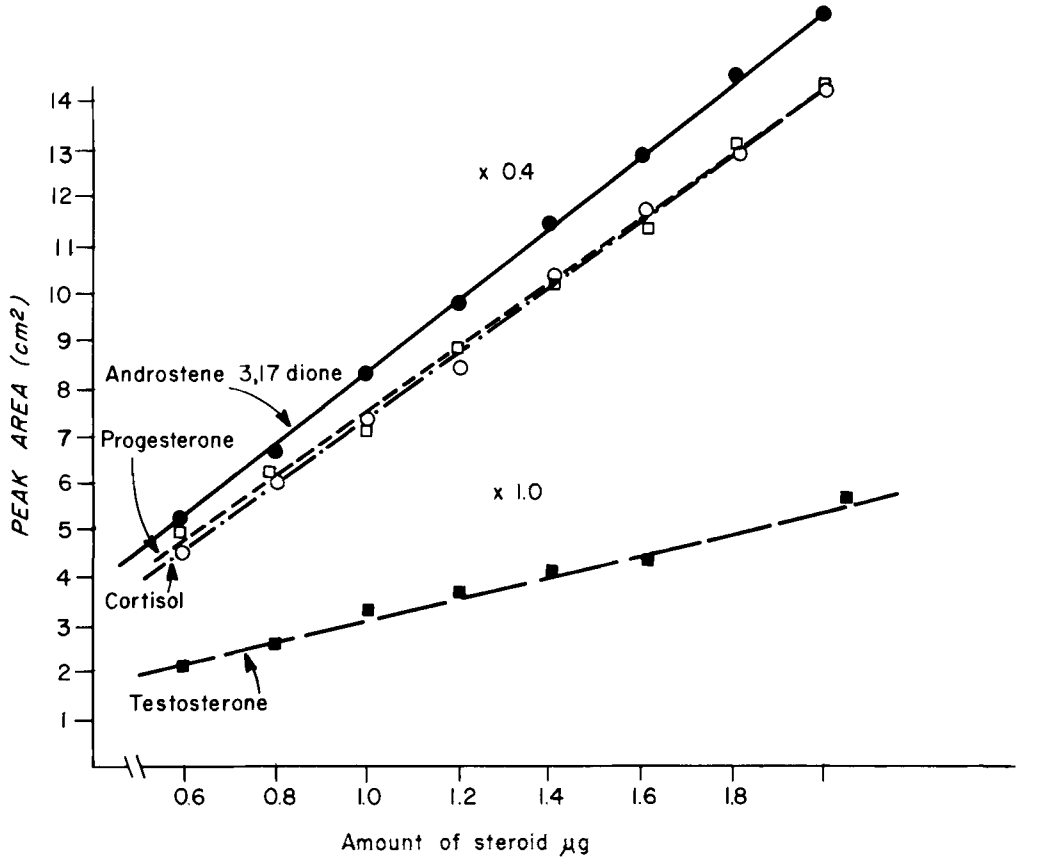


FIG. 1. Calibration curve for serial amounts of steroids determined by fluorescence quenching. The designation x 0.4 refers to instrument attenuation which would be 2.5 times as sensitive as x 1.0.

TABLE I

Solvent Systems

| System | GFW plates for free steroids | System | PMA plates for estrogen acetates |
|---|---|---|---|
| A | Chloroform-methanol 93:7 | E | Benzene-ethanol 99:2 |
| B | Methylene chloride-methanol 95:5 | F | Benzene-chloroform 90:10 |
| C | Benzene-ethanol 95:5 | G | Methylene chloride-isopropyl ether 5:95 |
| D | Benzene-ethanol 93:7 | | |

densitometry of thin layer chromatograms (4), followed by scintillation of the eluted material. Only microgram quantities of reference substances are required. Quantitation is made directly from the plate by spectrodensitometry. Procedures using the technique of fluorescence quenching and absorbance with different types of TLC plates are described. Values for recovery from these plates are presented. Different plates are necessary to visualize both the abosorbing and nonabsorbing compounds.

## MATERIALS

Three different reagent-impregnated silica gel plates, 20 x 20 cm and 250 $\mu$ thick were used. (a) Silica Gel GFW (fluorescent white), obtained from commercial sources; (b) silica gel impregnated with phosphomolybdic acid (PMA) (5); (c) silica gel impregnated with ammonium bisulfate ($NH_4HSO_4$) (6).

All solvents for chromatography were of reagent grade and were redistilled before use. The steroids used in this work were obtained from Mann Research Laboratories, New York, N.Y. The labeled steroids were obtained from New England Nuclear, Boston, Mass, and from biological material isolated during metabolism studies in this laboratory.

## EQUIPMENT AND APPLICATION

A Schoeffel Model 3000 spectrodensitometer was used for in situ quantitation directly on the TLC plate. This instrument was equipped with an analogic computer which converted photomultiplier response to optical density units that appeared on the recorder as peaked curves. The 20 x 20 cm silica gel plates were scored into lanes 10 mm wide to fit the scanning beam of the instrument. The substances were spotted on alternate lanes. On the fluorescence plate the compounds were measured by fluorescence quenching using a wave-

TABLE II

Proof of Purity of Testosterone Using Determination of Constant Specific Activity

| | Counts, dpm | Corrected peak area, $mm^2$ | Steroid, $\mu g$ | Specific activity, dpm/$\mu g$ | Recovery of isotopes, % |
|---|---|---|---|---|---|
| Starting amount | 24,625 | --- | 2.0 | 12,312 | --- |
| 1st TLC | 20,685 | 475 | 1.70 | 12,167 | 84 |
| 2nd TLC | 17,942 | 415 | 1.44 | 12.459 | 87 |
| 3rd TLC | 14,742 | 365 | 1.22 | 12,083 | 82 |
| 4th TLC | 12,914 | 305 | 1.07 | 12,069 | 88 |

[a]Thin layer chromatography.

TABLE III

Proof of Purity of Progesterone Using Determination of Constant Specific Activity

| | Counts, dpm | Corrected peak area, $mm^2$ | Steroid, $\mu g$ | Specific activity, dpm/$\mu g$ | Recovery of isotopes, % |
|---|---|---|---|---|---|
| Starting amount | 25,641 | --- | 2.0 | 12,820 | --- |
| 1st TLC[a] | 20,000 | 1135 | 1.58 | 12,658 | 78 |
| 2nd TLC | 15,885 | 920 | 1.27 | 12,507 | 79 |
| 3rd TLC | 13,028 | 740 | 1.05 | 12,407 | 82 |
| 4th TLC | 10.742 | 620 | 0.83 | 12,942 | 82 |

[a]Thin layer chromatography.

TABLE IV

Proof of Purity of Androstenedione Using Determination of Constant Specific Activity

| | Counts, dpm | Corrected, peak area $mm^2$ | Steroid, μg | Specific activity, dpm/μg | Recovery of isotopes, % |
|---|---|---|---|---|---|
| Starting amount | 18,536 | --- | 2.0 | 9268 | --- |
| 1st TLC[a] | 15,200 | 1335 | 1.66 | 9156 | 82 |
| 2nd TLC | 13,485 | 1165 | 1.46 | 9236 | 89 |
| 3rd TLC | 10,171 | 900 | 1.08 | 9417 | 75 |
| 4th TLC | 9257 | 820 | 0.99 | 9350 | 91 |

[a]Thin layer chromatography

TABLE V

Proof of Purity of Cortisol Using Determination of Constant Specific Activity

| | Counts, dpm | Corrected, peak area $mm^2$ | Steroid, μg | Specific activity, dpm/μg | Recovery of isotopes, % |
|---|---|---|---|---|---|
| Starting amount | 61,987 | --- | 2.0 | 30,993 | --- |
| 1st TLC[a] | 57,038 | 1345 | 1.88 | 30,339 | 92 |
| 2nd TLC | 55,428 | 1300 | 1.82 | 30,454 | 97 |
| 3rd TLC | 53,142 | 1265 | 1.77 | 30,023 | 96 |
| 4th TLC | 49,942 | 1230 | 1.67 | 29,905 | 94 |

[a]Thin layer chromatography.

TABLE VI

Specific Activity of Estrogen Acetates From Liver Perfusates Using PMA Plates

| Estrogen acetate | Counts, dpm | Corrected, peak area $mm^2$ | Steroid, μg | Specific activity, dpm/μg | Recovery of isotopes, % |
|---|---|---|---|---|---|
| Estrone acetate | | | | | |
| Starting amount | 37,247 | --- | 6.0 | 6207 | --- |
| 1st TLC[a] | 23,386 | 501 | 3.94 | 5935 | 63 |
| 2nd TLC | 14,021 | 302 | 2.38 | 5891 | 60 |
| 3rd TLC | 8343 | 175 | 1.38 | 6045 | 60 |
| Estradiol acetate | | | | | |
| Starting amount | 15,393 | --- | 6.0 | 2565 | --- |
| 1st TLC | 10,586 | 766 | 4.30 | 2461 | 69 |
| 2nd TLC | 7422 | 539 | 3.03 | 2449 | 70 |
| 3rd TLC | 4889 | 350 | 1.97 | 2481 | 66 |
| Estriol acetate | | | | | |
| Starting amount | 47,054 | --- | 6.0 | 7842 | --- |
| 1st TLC | 35,239 | 947 | 4.62 | 7627 | 75 |
| 2nd TLC | 27,518 | 748 | 3.65 | 7539 | 78 |
| 3rd TLC | 21,985 | 604 | 2.95 | 7452 | 80 |

[a]Thin layer chromatography.

TABLE VII

Change of Specific Activity During Four Purification Steps on GFW Plates

| Steroid | Specific activity, average of four TLC[a] plus original, dpm/μg | Maximum deviation from starting value, % |
|---|---|---|
| Androstenedione | 9285 | 1.61 |
| Cortisol | 30,342 | 3.51 |
| Testosterone | 12,218 | 1.97 |
| Progesterone | 12,666 | 3.22 |
| Summary of 16 determinations | (average deviation from starting value) | 1.79 |

[a]Thin layer chromatography.

TABLE VIII

Change of Specific Activity During Three Purification Steps on PMA Plates

| Steroid | Specific activity, average of four TLC[a] plus original, dpm/μg | Maximum deviation from starting value, % |
|---|---|---|
| Estrone acetate | 6019 | 5.09 |
| Estradiol acetate | 2489 | 4.52 |
| Estriol acetate | 7615 | 4.97 |
| Summary of 9 determinations | (average deviation from starting value) | 3.94 |

[a]Thin layer chromatography.

length of 250 nm and the transmission mode (4). Impregnated plates were scanned in the transmission mode with a wavelength of 265 nm (6) or 560 nm (5).

A Packard Tricarb liquid scintillation spectrometer (Model 3375) was used for determination of the radioactivity of the eluted material. The eluted and dried samples were dissolved in 4.0 ml methanol and 0.1 ml aliquot taken for scintillation. Sufficient counting time was allowed to obtain a relative standard error less than 2% for each sample.

## METHODS

For the labeled steroids cortisol (1, $2^3$H) progesterone ($7^3$H), androstenedione (1, $2^3$H), and testosterone ($7^3$H), TLC in System A (Table I) served to assure purity.

a) Use of GFW plate: 20,000-40,000 dpm of each of the radioactive substances were spotted on a scored GFW plate, and 2 μg of each corresponding unlabeled carrier were spotted on the same spot. On alternate lanes, 2 μg of each reference compound as internal standard was placed. The compounds were separated on silica gel plates in a series of different solvent systems (Table I). After each development, quantitation by spectrodensitometry was carried out; the zones on the TLC plate (located by UV light) were scraped from the plate, inactivated with some drops of water, and extracted three times with 5 ml dichloromethane-methanol 9:1 and ethanol, and dried at room temperature. An aliquot (1:40) was counted in the Tricarb-Scintillation counter while the remainder was subjected to the next chromatographic step. For determination of quantity a standard curve of each compound (0.6-2.0 μg), as seen in Figure 1, was prepared. The counts after the first TLC plate and the amount of unlabeled carrier determined on the second plate served for the calculation of specific activity.

b) The compounds which did not absorb light or did not absorb the wavelength of activation of the phosphor were measured on the silica gel modified with indicator. One μg of each compound, serving as internal standard, was spotted beside the radioactive compound. On impregnated plates, in addition, a third standard was placed to serve for localization purposes. After development in different systems (Table I), the reference part of the plate was broken away and heated. After localization of the separated substances and the standard, they were eluted. An aliquot of the radioactive substances was counted in the liquid scintillation counter. The eluted standard, serving for determination of recovery, was spotted on the next plate along with a reference standard. The counts after the first plate and the amount of recovered standard on the second plate served for determination of specific activity. The eluted standards were dried and taken up in a minimum of solvent for transfer to the next plate to determine recovery.

Some requirements for working with PMA or $NH_4HSO_4$ plates are as follows:

i) To obtain a good recovery immediate scraping from the plate, and neutralization and inactivation of the silica gel with some drops of water is necessary. However some compounds, i.e., estrogens, give a better recovery from PMA plates after neutralizing the gel with 8% $NaHCO_3$.

ii) After elution from PMA or $NH_4HSO_4$ plates the eluate must be dried at room temperature. Increases of temperature lead to reaction between PMA or $NH_4HSO_4$ and the compound and thus to loss of material.

iii) The part of the plate containing the standard for quantitation or localization on non-UV-absorbing compounds must be heated for 5 min at 110 C for PMA and for 10 min at 140 C with $NH_4HSO_4$. Longer heating can cause darkening of the silica gel. The purified radioactive steroid should never be heated on the plate.

iv) For good separation a solvent system, low to mid-polar, must be chosen to inhibit

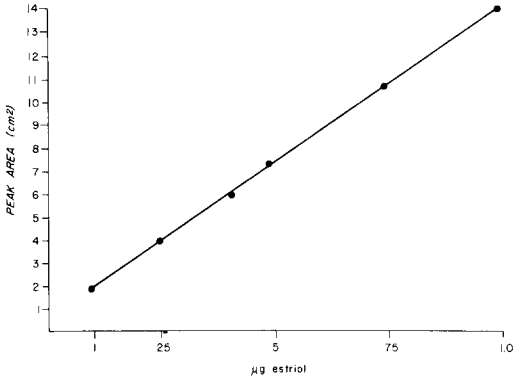


FIG. 2. Calibration curve for serial amounts of estriol separated on silica gel layers impregnated with phosphomolybdic acid.

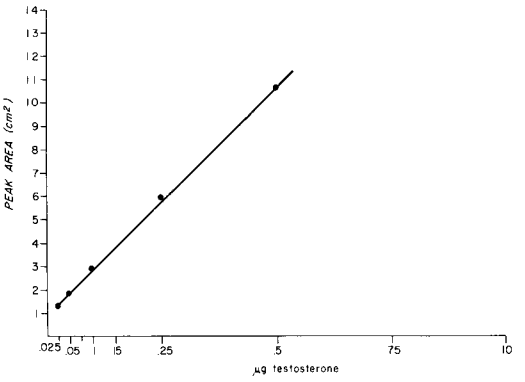


FIG. 3. Calibration curve for serial amounts of testosterone separated on silica gel layers impregnated with ammonium bisulfate.

washout of the impregnated silica gel. Suitable solvent systems for steroids are benzene-acetone 85:15 or benzene-ethanol 90:10. Steroids of the same polarity on the impregnated plates move slightly faster than on normal silica gel. Therefore polar compounds will move with less polar systems.

The determination of quantity was done by triangulation of the peak areas and after correction, interpolation on the standard curves. From results of densitometry of the internal standard, a correction was provided in the formula: corrected peak area = $P_1$ $(P_2/P_3)$ where $P_1$ is the area of the sample curve, $P_2$ is the corresponding area from the standard curve, and $P_3$ is the related area of the internal standard.

## RESULTS

The results of scintillation after each TLC are shown in Table II-V for GFW plates and in Table VI for PMA paltes. The relationship dpm/μg (specific activity) of compounds varied in a range of 5% in the successive separations (Tables VII-VIII). The residual recovery decreased after each step, as expected, but did not affect the specific activity. Too large a change of the specific activity indicated impurities in the material being analyzed. This figure is, of course, dependent on instrumentation and methods used, as well as on stability of the compounds tested. Each chromatographic step employing a different solvent system increases purification as long as there is some impurity. The maximal deviation in specific activity for each compound during the four purification steps on GFW plates was between 1.6 and 3.6% (Table VII); on PMA plates it was between 4.5 and 5.1% (Table VIII). These results indicate that a change in specific activity less than 5% may be considered proof of purity of a compound on any one of the three plates used. Thus two different purification steps within these limits should be considered significant for indication of the purity of a compound.

Silica gel impregnated with PMA or $NH_4HSO_4$ showed a larger variation in recovery than normal G or GFW plates used in TLC. Recovery experiments were done with different steroids of the $C_{18}$ and $C_{19}$ class. Recovery appeared to be dependent on the nature of the compound. With PMA plates the recovery was between 40 and 95%. A recovery between 60 and 90% was obtained with $NH_4HSO_4$ plates. These rates of recovery are sufficient for determination of specific activity where the relation between μg standard and dpm is important.

## DISCUSSION

The most important factor in the present work is in situ quantitation directly on the TLC plate. Having the proper plate for the compound at hand, i.e., a fluorescent plate for compounds absorbing UV light and plates with reagent incorporated for nonabsorbing compounds, leads to a more accurate quantitation by the densitometer. It is necessary to have a standard curve for every compound. The internal standard is required. Figure 2 (estriol on a PMA plate) and Figure 3 (testosterone on a $NH_4HSO_4$ plate) show some of the curves obtained with representative compounds using impregnated silica gel for TLC. All curves show linearity which indicates direct relation between amount of standard and area of the peaks. The sensitivity for detection varies with the steroid and the plate used. On PMA plates 50 ng can be measured.

Purification by recrystallization and purifica-

tion by TLC of two similar steroids, estriol and epiestriol, were compared. After six recrystallizations there was still epiestriol, as well as estriol, in the crystals as determined by TLC. On TLC the separation of both compounds was excellent. On normal silica gel in the system ethanol-benzene 15:85 the Rf value for estriol was 0.35, while for epiestriol it was 0.47 (7). This points out further than recrystallization to constant specific activity is not always a valid method when chemically very similar compounds are involved.

The amount of reference material required for the present method is low, 0.5-3.0 $\mu$g in one development is sufficient. This is an advantage when the reference substance is rare and hard to obtain. During crystallization much larger amounts are required, and recovery is sometimes low.

Using the present technique of quantitation by densitometry fewer steps are required, and on conventional 20 x 20 cm plates scores into 20 lanes of 10 mm width, a number of different compounds can be investigated. Each compound has its reference on a separate lane serving as an internal standard or standard for recovery, or both. The whole procedure should not take longer than 3 hr. The chromatogram of six compounds might be finished in 45-60 min, reading on the densitometer in 15 min, elution and centrifugation in 1 hr, drying and preparing for liquid scintillation ca. 45 min. So it is possible to analyze several compounds in successive TLC separations during one day.

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# On the Rate of Cholesterol Esterification in Cord Blood Serum

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## ABSTRACT

Cholesterol esterification was studied in adult and cord serum by measuring the initial rate of lecithin-cholesterol acyl transferase (LCAT) activity. Cord serum had about one-third as much free and esterified cholesterol and about one-half as much LCAT as adult serum. When the adult LCAT activities are plotted against the individual's serum free cholesterol levels a straight line relationship results (0.101 ± .005% cholesterol esterified per min). Cord serum LCAT activities (.135 ± .0407% cholesterol esterified per min) in the main fall above the adult line. Our results show that cord serum can esterify cholesterol at a rate equal to or higher than adult serum when the LCAT activity is related to the amount of serum free cholesterol present.

## INTRODUCTION

Lecithin-cholesterol acyl transferase (LCAT) is the enzyme reported to esterify cholesterol in human serum (1). LCAT prefers high density lipoproteins (HDL) as substrates while transferring fatty acids from the beta position of lecithin to free cholesterol (2).

Due to the lack of stable homogeneous enzyme preparations (3,4), LCAT activity has been studied mainly in partially purified preparations or in whole serum (5,6). At least two reports have appeared in the literature (7,8) claiming that LCAT activity was significantly lower in cord serum than in adult serum. The measurements of enzyme activity in these experiments, however, involved extended incubation periods (7,8) precluding the study of initial rates and the effect of native endogenous substrates on the reaction.

Stokke and Norum (9) recently developed a new technique that allows the measurement of initial rates of LCAT activity using endogenous lipoproteins during the assay. We have taken advantage of this method to compare LCAT activity in cord serum to that of the adult.

## EXPERIMENTAL PROCEDURE

### Materials

5,5′ Dithiobis-2-nitrobenzoic acid (DTNB) was purchased from Aldrich Chemical Co. (Rochester, N.Y.), Crystalline Serum Albumin (human) was the gift of Cutter Laboratories (Berkeley, Calif.); beta mercaptoethanol, 2,5-Diphenyl oxazole (PPO) and 2,2-P-Phenylenebis-(4-methyl-5-phenyloxazole) (Dimethyl POPOP) were the products of Eastman Kodak Co. (Rochester, N.Y.). Powdered bilirubin was supplied by Fisher Scientific Co. (Pittsburgh, Pa.). All chemicals were of reagent grade and were used without further purification.

$^{3}$H-Cholesterol (generally labeled) was purchased from Schwarz/Mann (Orangeburg, N.Y.) and was found to be 95% radiochemically pure when tested by thin layer chromatography (TLC). The majority of the radioactive contaminants invariably stayed at the origin during chromatography and thus did not interfere with the radioassay. Cholesterol-4-C$^{14}$-oleate was purchased from Applied Science Laboratories (University Park, Pa.).

### Methods

Blood samples were collected from fasting healthy men directly into glass tubes, after an overnight fast and abstinence from smoking for more than 12 hr. Cord blood samples were obtained according to the method described by Glueck et al. (10). All hemolyzed specimens were discarded. The collected blood samples were placed immediately in ice, allowed to clot, and transported at once to the laboratory

TABLE I

Cholesterol Levels and Rate of Esterification of Cholesterol in Adult and Cord Sera

| Sample | No. of subjects | Total cholesterol, mg/dl | Free cholesterol, mg/dl | Per cent esterified/min | Cholesterol esterified mμmoles/min/ml |
|---|---|---|---|---|---|
| Cord sera | 8 | 70 ± 18.7 | 20 ± 5.3 | .135 ± .0407 | .70 ± .183 |
| Adult sera | 9 | 208 ± 46 | 58 ± 16 | .101 ± .005 | 1.51 ± 0.45 |

where they were centrifuged without delay at 3000 g for 15 min at 4 C and the serum used for subsequent studies described below.

Serum total and free cholesterol concentrations were determined using the method of Zak et al. (11).

The assay used for LCAT activity was a slightly modified version of the method described by Stokke and Norum (9). Human serum, 0.5 ml, is incubated with 0.1 ml DTNB for 30 min at 37 C to inactivate the enzyme temporarily. Then 0.15 ml of 5% human serum albumin solution containing ca. 0.5 $\mu$Ci of $^3$H-cholesterol (9) is added and allowed to incubate with the serum for 4 hr at 37 C to facilitate the transfer of $^3$H-cholesterol from the albumin to the serum lipoproteins.

The transesterification is initiated by the addition of 0.1 ml of 0.1 M beta mercapto ethanol which reverses the inhibition caused by DTNB. Then 0.1 ml aliquots are removed from the assay mix into 2 ml chloroform-methanol 2:1 at 0, 10, 20, 30, 40 min and 0, 5, 10, 15, 20 min, respectively, for adult and cord serum assays. The extract is heated for 15 min at 70 C, or left overnight at room temperature to facilitate a thorough extraction of serum lipids by the solvents and then filtered on a sintered glass funnel (medium or coarse grade) using suction. The residue is washed twice with 2 ml chloroform-methanol 2:1, and the extract is combined with the wash and evaporated in vacuo or under a stream of nitrogen. The dry residue is dissolved in a small amount of chloroform and dried down again. The lipids are finally dissolved in a minimum amount of heptane for thin layer chromatography (TLC). Upon spotting the samples on plastic-based silica gel sheets (Eastman Chromagram 6061), TLC was carried out in petroleum ether-ether-acetic acid 70:10:1, and staining was accomplished by exposing the air-dried plate to iodine vapors. After evaporation of the excess iodine, discs containing cholesterol and cholesterol esters are cut out of the plates and placed in scintillation vials for counting. The scintillation fluid contains 400 g naphthalene, 25 g PPO, 0.25 g dimethyl POPOP dissolved in 1925 ml xylene 1925 ml dioxane, and 1150 ml absolute ethanol, as described by Stokke and Norum (9). The radioactive zones containing cholesterol and cholesterol esters were counted in the Packard Tricarb 3375 scintillation counter, and the present esterification established for each time interval. The rate of esterification (m$\mu$ moles/ml/min) is the product of the per cent esterification per min and the free cholesterol concentration present in the sample.

In an attempt to determine the relative importance of substrate concentration and enzyme levels on the rate of initial esterification, we added active cord and adult sera to serum "substrates" which had previously been inactivated by heating for 1 hr at 60 C. One part of labeled cholesterol in a serum albumin emulsion was added to nine parts of heated serum, and 1 ml aliquots were preincubated for 30 min at 37 C. At the end of the preincubation period 100 $\mu$liters of active serum was added to commence the assay. Blank tubes received 100 $\mu$liters of buffer (0.01 M Tris. HCl, 0.001 M EDTA, 0.14 M NaCl pH 7.4). Esterification was allowed to proceed for the times indicated when 2 ml of chloroform-methanol 2:1 was added to stop the reaction. Lipid extraction, TLC and scintillation counting were carried out as described.

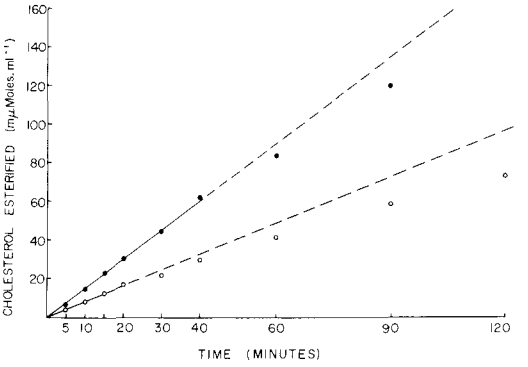


FIG. 1. Progress of cholesterol esterification with time in adult serum (•) and cord serum (o). See text for experimental details.

## RESULTS

The data in Table I show that both the mean cholesterol levels and the LCAT activity are substantially lower in cord serum than in adult serum.

The rates of cholesterol esterification in representative samples of cord and normal serum are shown in Figure 1. In normal adult serum esterification proceeds linearly until ca. 40 min, while in cord serum linearity does not prevail beyond 20 min.

When the initial rates of esterification are plotted against the respective serum free cholesterol concentrations, the pattern indicated on Figure 2 is obtained. There is an apparent linear relationship between the initial rates of esterification and adult serum free cholesterol levels. Cord serum LCAT activities fall into the lower range in keeping with their cholesterol content, but the majority of points fall above the adult line. The decreased LCAT activity is

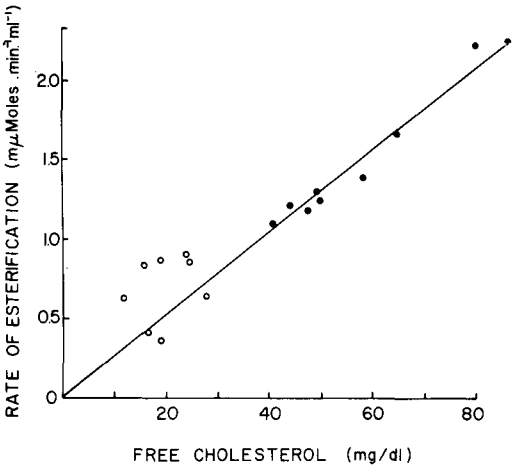


FIG. 2. Initial rate of cholesterol esterification as a function of serum free cholesterol concentration. Adult sera (•), cord sera (○). See text for experimental details.

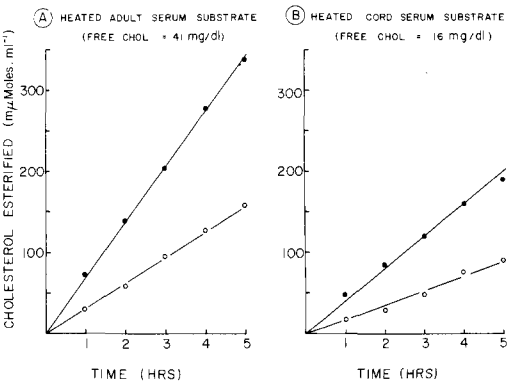


FIG. 3. Cholesterol esterification by adult serum (•) and cord serum (○) using heat inactivated serum substrates. See text for experimental details.

not due to inhibition by excess bilirubin, since normal serum esterification was not affected by concentrations up to 8 mg/dl (about four times the level found in cord serum). Cholesterol esterase was exclused by the failure of cord serum to release free cholesterol from heat-inactivated serum previously labeled with cholesterol-4-$C^{14}$-oleate.

Additional experiments were carried out using heat-inactivated cord and adult sera as substrates in an attempt to specify the cause of the diminished rate of cholesterol esterification in cord serum. The data in Figure 3 show that the LCAT levels in cord serum are about half of that found in adult serum. The rates of esterification of both cord and adult sera are diminished when heated cord serum is substituted for heated adult serum as substrate.

## DISCUSSION

Our data agree with those of Fujita (7) and Cooper and Gulbrandsen (8) who reported low levels of LCAT in cord serum. However Fujita's experiments (7) used incubation of serum samples for 9 hr during which esterification appeared to be linear when tested by chemical and radioactive methods. Such results are difficult to evaluate because they differ substantially from the findings of Stokke and Norum (9) and our own (Fig. 1). Stokke and Norum (9) reported that a linear rate of esterification persists only for ca. 1 hr in normal adult serum, and we found that linearity in adult serum persists only for 45 min. Furthermore our experiments with cord serum show linearity only up to 20 min. It would appear that the maximum time at which linear rates can be measured is not more than 1 hr and is likely to be related to the levels of cholesterol and other substrates in the serum.

Cooper and Gulbrandsen (8) measured net esterification over a 6 hr period rather than the initial rate of esterification, using a 1:1 mixture of active serum and heat-inactivated normal adult serum. At the end of incubation at 37 C, cord serum was found to be 38% as efficient as adult serum in carrying out esterification of cholesterol. We found 45% and 49%, respectively, when heat-inactivated adult and cord sera were used as substrates (Fig. 3A and B). Our results show a linear rate of esterification up to 5 hr because the active serum samples were diluted 1:11 by the substrate in the assay mix. Cooper and Gulbrandsen's lower results (8) could be due to the lower substrate efficiency of cord serum that comprised half of their substrate and to their long (6 hr) incubation period. Our data show that LCAT activities of cord and adult sera are each increased ca. 80% when heat-inactivated adult serum is used as substrate in the place of heated cord serum (Fig. 3A and B).

We have previously shown (12) that there is a good linear correlation between the initial rate of esterification and free cholesterol in normal adult serum. Monger and Nestel (13) have reported similar results using each subject's heat-inactivated plasma as substrates. Such a relationship suggests that the factors that control serum LCAT activity are carefully balanced. We have also found that LCAT activity increased in healthy adults proportionally to the individual's serum-free cholesterol levels when such sera were assayed against a common heat-inactivated substrate.

It is therefore essential that meaningful

comparative studies include the measurement of the physiologically significant initial rate of esterification related to the free cholesterol levels of each subject's serum. Such analysis combined with the use of heat-inactivated serum substrates reveals that the lower rate of cholesterol esterification of cord serum is due to at least two factors, namely, lower enzyme levels and diminished substrate concentration.

Our results also indicate that the initial rate of cholesterol esterification in cord serum falls above the regression line of healthy adult LCAT activity (Fig. 2). This higher relative LCAT activity of cord serum is the result of a higher percentage of cholesterol esterification per hour (Table I) and is probably due to the relatively high amounts of LCAT present. Cord serum contains about one-half of the enzyme but only about one-third of the free cholesterol of healthy adult serum. The LCAT activity in cord serum could also be influenced by the net effects of (a) a higher proportion of lecithin (14) and HDL (15) which enhance LCAT activity, and (b) a relative deficiency of linoleic acid in cord serum lecithin (14) which would tend to impair the rate of cholesterol esterification (16).

In any event our data indicate that cord serum is able to carry out cholesterol esterification rates equal to or higher than adult serum when LCAT activities are expressed as a function of free cholesterol concentration in the respective sera.

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# Total, Polar and Neutral Lipids of *Rhizopus arrhizus* Fischer

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## ABSTRACT

The changes in total lipid content, neutral and polar lipids, total fatty acids, and free fatty acids were investigated over a 4 day period in the zygomycete, *Rhizopus arrhizus* Fischer. The highest concentration of lipids occurred at the 72 hr period. The degree of unsaturation in the total fatty acid fraction increased during the growth period, whereas the degree of unsaturation decreased in the free fatty acid fraction during the same time period. The ratios of neutral to polar lipids over the 4 day period were: 0.75, 0.22, 1.94 and 0.94. The major components of polar lipids were phosphatidyl ethanolamine, lecithin, lysolecithin, and fatty acids. The fatty acids in the mono- and diglycerides were predominately saturated (67-96%). The fatty acids in the triglycerides shifted from a predominately unsaturated (69%, 24 hr) to a more saturated pattern (62%, 96 hr).

## INTRODUCTION

In a previous study we reported the relative distribution of lipids of *Rhizopus arrhizus* Fischer grown in shake culture and harvested at a single time period (7). In order to obtain a more complete picture of what was occurring to the lipids of *R. arrhizus,* we decided to investigate the changes in the lipid components during the growth period of the mycelium. The present study reports the changes in total lipids, neutral lipids, polar lipids and their components, total fatty acids, and free fatty acids at 24 hr intervals in *Rhizopus arrhizus.*

## MATERIALS AND METHODS

The culture of *Rhizopus arrhizus* Fischer used in this investigation was originally isolated from infected peaches in California. Stock cultures were maintained on potato dextrose agar (PDA). The cultures were grown in 250 ml flasks containing 98 ml Fothergill's medium (3) consisting of the following substances: 4% glucose; 0.048 M $K_2HPO_4$; 0.012 M $MgSO_4 \cdot 7H_2O$; 0.025 M $(NH_4)_2 SO_4$; and 2 ppm of Zn, Fe and Mn. A uniform inoculum of 2 ml of mycelial fragments was used in order to obtain uniform mycelial cultures. The mycelial fragments were prepared by homogenizing 1 g of mycelium with 2 ml of sterile water under aseptic conditions. Cultures were incubated at 25 C on a rotary shaker. The mycelial mats were harvested at 24, 48, 72 and 96 hr after inoculation. The mycelium was collected in a Büchner funnel and washed with distilled water. The moist mycelium was weighed, and a weighed fraction of the mycelium was used to determine the moisture content.

### Extraction of Lipids

Lipids were extracted from the mycelial mats by the method of Folch et al. (2) using chloroform-methanol 2:1 v/v followed by several washings with distilled water. The solvents were evaporated under vacuum at 40 C. Total lipid extracted was determined by weighing the dried extracts.

### Separation and Analysis of Lipid Fractions

Various classes of lipids such as polar lipids, monoglycerides, diglycerides, triglycerides, free fatty acids, sterols, sterol esters and hydrocarbons were separated by thin layer chromatography (TLC) on silica gel (Absorbosil-5) with petroleum ether-diethyl ether-acetic acid 65:35:1.5 v/v. The fractions obtained were identified by comparative chromatography with known standards. The standards were visualized by exposure to iodine vapor or by observing

TABLE I

Changes in Total Lipid Content of *Rhizopus arrhizus* During the Growth of the Mycelial Culture

| Time interval, hr | Growth, dry wt of tissue, g | Total lipid wt, mg | Lipid, mg/g tissue |
|---|---|---|---|
| 24 | 2.25 | 14.86 | 6.62 |
| 48 | 2.67 | 38.30 | 14.34 |
| 72 | 3.24 | 159.56 | 49.20 |
| 96 | 2.92 | 62.69 | 21.46 |

TABLE II

Changes in Neutral and Polar Lipids of *Rhizopus arrhizus* During Growth of Mycelial Cultures

| Time interval, hr | Neutral lipids, % | Polar lipids, % | Neutral/Polar |
|---|---|---|---|
| 24 | 43.0 | 57.0 | 0.75 |
| 48 | 18.0 | 82.0 | 0.22 |
| 72 | 66.0 | 34.0 | 1.94 |
| 96 | 48.4 | 51.6 | 0.94 |

with UV light. Each lipid band was removed from the thin layer plate by scraping and the lipid material eluted from the silica gel with water-saturated diethyl ether, or in the case of the polar lipids, with methanol. The extracts were evaporated to dryness at 40 C under nitrogen and the amount of lipid in each fraction determined by weighing.

The polar lipids were further analyzed by two dimensional chromatography on Absorbosil-5 (1). A solvent system of chloroform-methanol-$NH_4OH$ 60:35:5 v/v was used in the first dimension and 35:60:5 v/v in the second dimension. The spots were made visible by exposure to iodine vapor. Individual polar lipids were identified by comparative chromatography with reference standards.

The methyl esters of fatty acids were prepared by hydrolyzing the total lipids, polar lipids, mono-, di- and triglycerides with 0.5 M KOH in methanol and the isolated fatty acids esterified with $BF_3$-methanol (5). The methyl esters were extracted in petroleum ether (boiling point 20-40 C) and then the solvent evaporated. The dried samples were dissolved in carbon disulfide and separated by gas chromatography (GC) using a Packard Model 7400 equipped with Infotronics integrator. The methyl esters were separated on a stainless steel column (10 ft x 1/8 in.) packed with 10% DEGS on chromosorb Q. The gas chromatograph was operated isothermally at 165 C with a flow rate of 60 ml/min. The retention times of methyl esters of fatty acids from the fractions were compared with authentic standards. The free fatty acids fraction obtained by separation with TLC was methylated by the method of Morrison and Smith (5). The methyl esters of fatty acids were characterized with a Varian Mat III GC-mass spectrometer using the DEGS column. Hydrocarbons and sterols were analyzed with a 100 ft capillary column coated with OV-17.

## RESULTS

The maximum lipid content (mg/g) of the mycelium occurred at the 72 hr period and was eight times greater than the lipid content of the mycelium at the 24 hr period (Table I). The per

TABLE III

Total Fatty Acid Composition of *Rhizopus arrhizus* During Growth of Mycelial Cultures

| | Per cent | | | |
|---|---|---|---|---|
| Fatty acids | 24 hr | 48 hr | 72 hr | 96 hr |
| Unknown A | 4.0 | 2.3 | 3.2 | 2.1 |
| Unknown B | 0.8 | 1.5 | 1.8 | 2.0 |
| Unknown C | 7.9 | 6.8 | 0.9 | 1.0 |
| 14:0 | 18.6 | 10.5 | 6.7 | 5.8 |
| 16:0 | 17.7 | 21.1 | 15.3 | 17.2 |
| 16:1 | 1.3 | 2.5 | 0.6 | 0.9 |
| 18:0 | 5.9 | 9.9 | 9.8 | 8.5 |
| 18:1 | 22.1 | 29.7 | 38.7 | 36.9 |
| 18:2 | 9.6 | 7.9 | 11.6 | 12.5 |
| 18:3 | 11.9 | 7.7 | 11.3 | 13.1 |
| 20:0 | *t*[a] | *t* | *t* | --- |
| Degree of saturation | | | | |
| Saturation | 42.2 | 41.5 | 31.7 | 31.5 |
| Unsaturated | 44.9 | 47.9 | 62.3 | 63.3 |
| Unknown | 12.8 | 10.5 | 5.9 | 5.2 |

[a]*t* = Trace (less than 0.1%).

TABLE IV

Free Fatty Acid Composition of *Rhizopus arrhizus* During Growth of Mycelial Cultures

| Fatty acids | Per cent | | | |
|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 96 hr |
| Unknown A | 23.0 | 2.3 | 10.5 | 9.6 |
| Unknown B | 2.4 | 4.1 | 5.3 | 7.7 |
| Unknown C | 5.9 | 10.5 | 0.8 | 0.7 |
| 14:0 | 3.5 | 17.9 | *t* | *t* |
| 16:0 | 17.8 | 27.3 | 49.6 | 41.9 |
| 16:1 | 2.7 | 4.3 | *t* | 1.2 |
| 18:0 | 8.0 | 4.0 | 9.6 | 8.6 |
| 18:1 | 26.1 | 17.8 | 12.8 | 23.7 |
| 18:2 | *t*[a] | *t* | 1.4 | 1.4 |
| 18:3 | 10.6 | 11.9 | 10.0 | 5.3 |
| Degree of saturation | | | | |
| Saturated | 29.2 | 49.2 | 59.1 | 50.5 |
| Unsaturated | 39.4 | 33.9 | 24.3 | 31.6 |
| Unknown | 31.4 | 16.8 | 16.7 | 17.9 |

[a]*t* = Trace (less than 0.1%).

cent of polar lipids was higher than that of the neutral lipids at all periods, except the 72 hr period (Table II). The ratio of neutral to polar lipids over the 4 day period was: 0.75, 0.22, 1.94 and 0.94. The total fatty acids fraction contained more unsaturated than saturated fatty acids at each of the harvest periods. In contrast, the free fatty acids fraction contained predominately saturated fatty acids. Palmitic acid ($C_{16}$) was the predominant free fatty acid (Table IV) and represented almost 50% of the free fatty acids at the 72 hr harvest. The major fatty acid of the total fatty acids fraction was oleic acid ($C_{18:1}$), which represented 39% of the fraction at the 72 hr period, whereas palmitic acid represented only 15% (Table III).

Three unidentified compounds were detected in the fatty acid fractions and their mass spectra obtained. However the interpretation of the spectra was not clear and the identity of these compounds remains unknown.

The results of the analyses of the polar lipids are shown in Tables V and VI. The fatty acids in the polar lipids were about equal in the amount of saturation and unsaturation at the 24 and 72 hr period (4). The unknowns (unknown A, B, C) observed in the free fatty acids fraction were also found in the total fatty acids fraction and in the polar lipids in each interval, but at a lower concentration. A comparison of various compounds detected in the polar fraction by two dimensional thin layer chromatography is shown in Table VI. Phosphatidyl ethanolamine was the major component (50%) followed by lecithin regardless of the time interval. Two unknowns were detected in the

TABLE V

Fatty Acid Composition of Polar Lipids of *Rhizopus arrhizus* During the Growth of Mycelial Culture

| Fatty acids | Per cent | | | |
|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 96 hr |
| Unknown A | --- | *t* | *t* | --- |
| Unknown B | *t*[a] | *t* | *t* | --- |
| Unknown C | 2.0 | 2.9 | *t* | 3.2 |
| 14:0 | 1.9 | 12.1 | 1.4 | 8.4 |
| 16:0 | 41.8 | 10.6 | 36.2 | 26.8 |
| 16:1 | 1.3 | 5.6 | 2.2 | 0.5 |
| 18:0 | 5.1 | 5.1 | 13.8 | 4.6 |
| 18:1 | 37.1 | 25.1 | 17.3 | 20.4 |
| 18:2 | 4.3 | 18.0 | 10.2 | 9.8 |
| 18:3 | 6.5 | 20.8 | 18.9 | 26.4 |
| 20:0 | *t* | *t* | *t* | --- |
| Total saturated | 48.8 | 27.7 | 51.4 | 34.5 |
| Total unsaturated | 49.2 | 69.4 | 48.6 | 57.1 |

[a]*t* = Trace (less than 0.1%).

TABLE VI

Composition of Polar Lipids of *Rhizopus arrhizus* During the Growth of Mycelial Culture

| Various fractions | Per cent | | | |
|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 96 hr |
| Phosphatidyl ethanolamine | 48.2 | 44.7 | 47.6 | 44.2 |
| Lecithin | 26.4 | 31.1 | 28.9 | 30.7 |
| Unknown 1 | 6.2 | 7.9 | 6.9 | 7.1 |
| Unknown 2 | 1.4 | 1.2 | 0.9 | *t*[a] |
| Lysolecithin | 17.9 | 15.1 | 15.7 | 18.1 |

[a] *t* = Trace (less than 0.1%).

polar lipids of *Rhizopus arrhizus.* Unknown 1 amounted to ca. 7% of the fraction at each of the harvest periods. The quantity of unknown 2 (1.4%) decreased in the later time periods. The nature of these unknowns is being investigated further.

The hydrocarbons and sterols did not change significantly during the four harvest periods. We previously reported the presence of the hydrocarbon squalene (7). The results of this investigation indicated that squalene content was low and did not change during the growth period. Minor peaks were detected in the sterol fraction but the results will not be included. Personal communication from Laseter and Weete, who have analyzed the sterol fraction of the same isolate (R-80) of *Rhizopus arrhizus,* established that these minor peaks were the following sterols: ergosterol, fungosterol and 22-dehydroergosterol.

The analysis of the fatty acids in the monoglycerides of the neutral fractions is shown in Table VII. Palmitic, stearic and oleic acids were the predominant fatty acids present in the monoglycerides. The fatty acids were mostly saturated.

The results of the analysis of the fatty acids of the diglycerides are shown in Table VIII. The degree of saturation of fatty acids was higher (96%) in diglycerides, as compared to monoglycerides (70%).

The fatty acids in the triglycerides were mainly unsaturated at the 24 and 48 hr periods and then shifted to a more saturated pattern during the 72 and 96 hr periods (Table IX). The predominant fatty acids in the triglycerides were $C_{16}$, $C_{18:0}$ and $C_{18:2}$. Arachidic acid was detected only in triglycerides of the 48 and 72 hr harvests.

## DISCUSSION

The results of the lipid analyses indicated that the highest concentration of lipids in the mycelium of *R. arrhizus* occurred at the 72 hr period. The lipids at the 72 hr period were predominately neutral lipids. The shift that occurred from the 48 hr period to the 72 hr period is reflected in the ratio change of 0.22 to 1.94 (neutral lipids to polar lipids). This change could be due to changes in the utilization of neutral lipids, an increase in the rate of synthesis of neutral lipids, or the decrease in synthesis of polar lipids from the 48 hr period to the 72 hr period.

The unsaturated fatty acids were major in polar lipids from all harvests except the 24 hr period (Table V). The mono-, di- and triglycerides contained mainly saturated fatty acids,

TABLE VII

Fatty Acid Composition of Monoglycerides of *Rhizopus arrhizus* During Growth of Mycelial Culture

| Fatty acids | Per cent | | | |
|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 96 hr |
| 14:0 | --- | 12.8 | 17.4 | --- |
| 16:0 | --- | 28.2 | 22.0 | --- |
| 16:1 | *t*[a] | *t* | 1.0 | --- |
| 18:0 | --- | 26.9 | 34.8 | *t* |
| 18:1 | *t* | 32.1 | 23.2 | *t* |
| 18:2 | --- | --- | 1.5 | --- |
| Total saturated | --- | 68.0 | 74.2 | --- |
| Total unsaturated | --- | 32.1 | 25.7 | --- |

[a] *t* = Trace (less than 0.1%).

TABLE VIII

Fatty Acid Composition of Diglycerides of *Rhizopus arrhizus* During Growth of Mycelial Culture

| | Per cent | | | |
|---|---|---|---|---|
| Fatty acids | 24 hr | 48 hr | 72 hr | 96 hr |
| Unknown C | --- | *t* | --- | --- |
| 14:0 | 27.3 | 33.0 | 26.4 | 38.6 |
| 16:0 | 62.0 | 54.4 | 69.0 | 49.0 |
| 16:1 | 5.4 | 3.0 | 2.1 | 8.8 |
| 18:0 | 5.2 | 6.1 | 0.8 | 1.0 |
| 18:1 | *t*[a] | 2.0 | 1.3 | 1.8 |
| 18:2 | *t* | 1.5 | 0.4 | 0.8 |
| Total saturated | 94.6 | 93.5 | 96.2 | 88.6 |
| Total unsaturated | 5.4 | 6.5 | 3.8 | 11.4 |

[a]*t* = Trace (less than 0.1%).

TABLE IX

Fatty Acid Composition of Triglycerides of *Rhizopus arrhizus* During Growth of Mycelial Culture

| | Per cent | | | |
|---|---|---|---|---|
| Fatty acids | 24 hr | 48 hr | 72 hr | 96 hr |
| 14:0 | 8.1 | 1.2 | 9.9 | 8.2 |
| 16:0 | 7.3 | 14.5 | 29.2 | 24.9 |
| 16:1 | 7.2 | 2.3 | 0.3 | *t* |
| 18:0 | 14.9 | 27.0 | 30.6 | 31.3 |
| 18:1 | 33.5 | 24.5 | 27.6 | 16.4 |
| 18:2 | 29.1 | 25.5 | 1.4 | 13.2 |
| 18:3 | *t*[a] | 5.1 | 1.0 | 8.0 |
| 20:0 | --- | *t* | *t* | --- |
| Total saturated | 30.2 | 42.6 | 69.7 | 62.4 |
| Total unsaturated | 69.8 | 57.4 | 30.4 | 37.6 |

[a]*t* = Trace (less than 0.1%).

with the exception of the triglycerides, in which the unsaturated fatty acids were predominant in the first two harvests (Tables VII-IX). The analysis of the total fatty acids indicated that unsaturated fatty acids were predominant (Table III). In contrast, the free fatty acid pool was mainly saturated (Table IV). The relationship of polar lipid synthesis and neutral lipids appears consistent with the changes in saturation in the pool of free fatty acids. The naturally occurring fatty acid esters reported previously (4,7) were predominately saturated fatty acids; however they represent a small fraction of the total lipid content. Nevertheless the possibility exists that these esters may be a factor in the control of the synthesis of neutral lipids. Paik and Kim (6) recently postulated that the methylation of proteins was an important factor in the control of synthesis of proteins.

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# The Abundance of *cis*-5-Octadecenoic Acid in *Dioscoreophyllum cumminsii* Seed Oil


## ABSTRACT

Oil from *Dioscoreophyllum cumminsii* (Stapf) Diels contains *cis*-5-octadecenoic acid as 84% of its total fatty acids. The unsaturated acids normally found in seed oils are present in small amounts (oleic, 1%, linoleic, 5.2%; and linolenic, 0.3%). Other unusual acids present, in minor amounts, are *cis*-5-hexadecenoic (0.6%), 11-octadecenoic (0.9%) and two polyenoic acids which are probably unsaturated at the 5 position.


*Dioscoreophyllum cumminsii*, a member of the Menispermaceae, is a woody, twining vine of tropical distribution in Africa (1). Its fruit contains an intensely sweet material reportedly 800-3000 times as sweet as sucrose (1-3). Analysis of the oil from seed collected in Nigeria is given here.

Oil (26% db) was extracted from the ground seed and endocarp (40.0 g/1000 units) with petroleum ether (bp 30-60 C). This oil and the esters prepared from it (4) were analyzed by gas liquid and thin layer chromatography (GLC and TLC) (5). The esters were fractionated according to degrees of unsaturation by preparative TLC on silver nitrate-impregnated layers of Silica Gel G (5), and the fractions were analyzed by GLC and IR spectrometry (5,6). Double bond positions were established by ozonolysis (7) and, in the monoenoic esters, by gas chromatography-mass spectrometry (GC/MS) of methoxy derivatives (5,8).

Direct GLC of the oil showed 98% (area) of the eluted components in the triglyceride region with a major peak corresponding to $C_{54}$. All unsaturation presumably has *cis* geometry since no *trans* absorption (10.4 $\mu$m) was observed in IR spectra of the oil, esters and fractions. A weak absorption band at 7.7 $\mu$m appeared in the oil spectrum; it is atypical of seed oil spectra, and we have not related it to a specific structure.

The total fatty acid composition of *D. cumminsii* seed oil is given in Table I. Identical iodine values, 86, were found by the Wijs method and by calculation from the fatty acid composition. The relative proportions of $\Delta 5$ and $\Delta 9$ 16:1 esters were arrived at by: (a) triangulation of the partially resolved peaks due to these esters in GLC analysis on a polar column (LAC-2-R 446); and (b) comparison of the intensities of the ions obtained from the methoxy derivatives during GC/MS analysis. Both these methods indicated that the ratio of $\Delta 5$:$\Delta 9$ is ca. 2:1. The 18:1 isomers, however, were quantitated by ozonolysis-GLC (7).

Among the polyunsaturated esters (also identified by ozonolysis-GLC), linoleate was the principal diene and linolenate the principal triene. Small peaks from GLC analysis of the diene and triene fractions on LAC-2-R 446 had respective ECL's (9) of 18.8 and 19.3, which are comparable with ECL's of esters known to have $\Delta 5$ unsaturation (10). The monoenes with chain lengths greater than 18 carbon atoms were not investigated.

Although *cis*-5-octadecenoic acid has been found to be present in seed oils (11,12; R. Kleiman, unpublished), *D. cumminsii* is the richest source so far discovered.

TABLE I

Fatty Acid Composition of *Dioscoreophyllum cumminsii* Seed Oil

| Component | Area % by gas liquid chromatography (as methyl esters) |
|---|---|
| 14:0 | 0.1 |
| 15:0 | Trace |
| 16:0 | 1.4 |
| $16:1^{5}$ | 0.6 |
| $16:1^{9}$ | 0.3 |
| 17:0 | Trace |
| 17:1 | 0.1 |
| 18:0 | 4.6 |
| $18:1^{5}$ | 84.5 |
| $18:1^{9}$ | 1.1 |
| $18:1^{11}$ | 0.9 |
| $18:1^{9,12}$ | 5.2 |
| $18:2^{5,?}$ | 0.1 |
| $18:3^{9,12,15}$ | 0.3 |
| $18:3^{5,?,?}$ | Trace |
| 20:0 | 0.2 |
| 20:1 | 0.2 |
| 22:0 | Trace |
| 22:1 | 0.2 |
| 24:0 | Trace |

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# The Effect of Methyl Sterculate on Oleic Acid Synthesis in the Hen

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## ABSTRACT

The enzymic desaturation of saturated fatty acids to monoenes in animals and plants is inhibited by cyclopropene fatty acids, such as sterculic acid. Labeled acetic and stearic acids were administered to laying hens which had received methyl sterculate in the diet for long periods, and the incorporation of the label in the eggs and liver was studied. The egg was used as a "biological trap" to study the metabolism of the hen in relation to its diet. Maximum incorporation of label was observed in the third or fourth egg laid after administration of the labeled compound. Dietary methyl sterculate reduced the incorporation of stearic acid into egg yolk lipids, but the incorporation of acetate was not affected. The formation of oleic acid was inhibited by methyl sterculate irrespective of whether the precursor was acetate or stearate acid. In laying hens receiving methyl sterculate for long periods, no evidence could be obtained for the biosynthesis of oleic acid by an alternative pathway which did not involve the desaturation of stearic acid. The validity of comparing the ratio of the specific activities of stearic acid and oleic acid in control birds with the corresponding ratio obtained for birds receiving methyl sterculate is questioned, because the sizes of the metabolic pools of the product and precursors may be quite different for the two groups of birds.

## INTRODUCTION

It has long been accepted that, in animals, the monounsaturated fatty acids such as oleic and palmitoleic acids are formed predominantly by enzymic desaturation of the corresponding saturated fatty acids. This desaturation process is inhibited by cyclopropene fatty acids such as sterculic acid (1-3). In 1963, Dupuis and Favarger (4) suggested that an alternative pathway to oleic acid may also be present in animals. Reiser and Raju (5,6) and Donaldson (7,8) produced support for this suggestion by using *Sterculia foetida* oil, which contains sterculic acid, to block the normal desaturation pathway in rats and chickens. They interpreted the results of these experiments as evidence

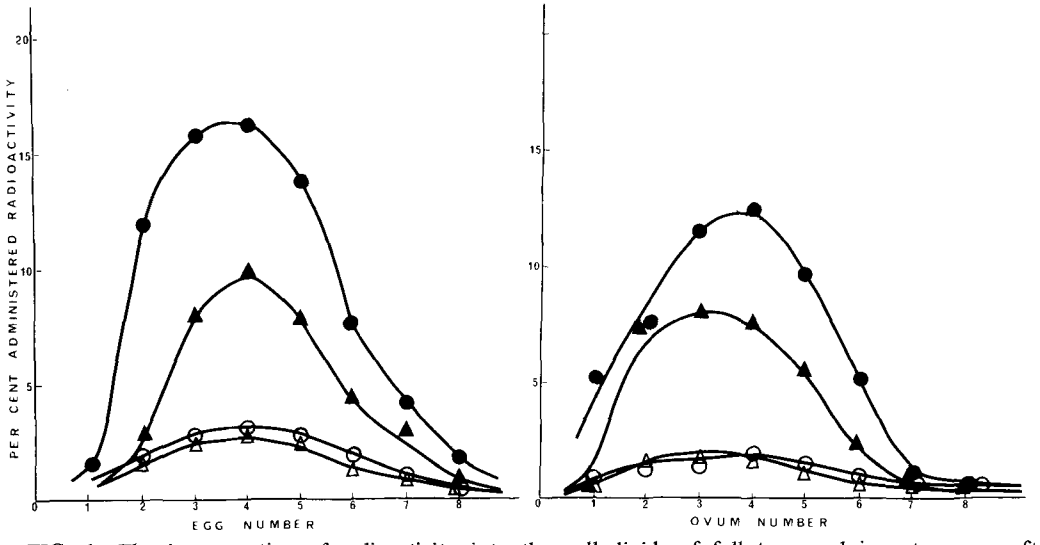


FIG. 1. The incorporation of radioactivity into the yolk lipids of full term and immature eggs after administration of: labeled sodium acetate to control birds ○—○; labeled sodium acetate to sterculate-treated birds △—△; labeled stearic acid to control birds ●—●; labeled stearic acid to sterculate-treated birds ▲—▲. All results corrected to a 400 μc dose basis.

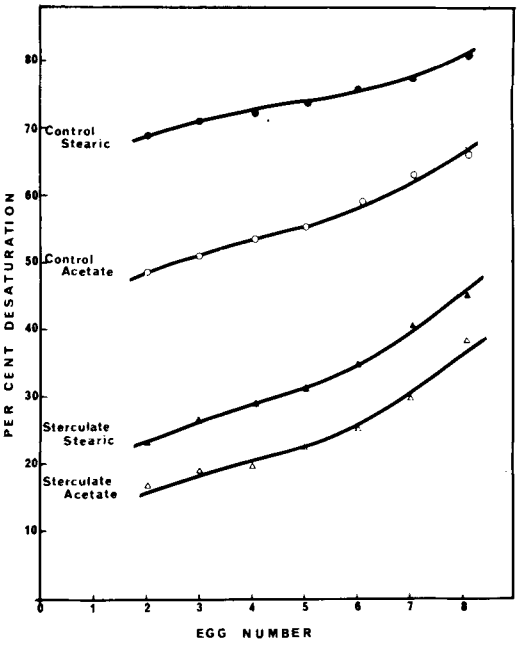


FIG. 2. Per cent desaturation values of yolk lipid after administration of labeled acetate or stearic acid to control and to sterculate-treated hens.

that a saturate-independent, alternative pathway for the biosynthesis of monounsaturated fatty acids was present or had been induced as a result of treatment with *Sterculia foetida* oil. Raju and Reiser (6) have suggested that in rats lauric acid may be utilized for oleic acid synthesis, probably by $\beta,\gamma$-desaturation followed by elongation.

This paper described experiments in which labeled acetate or stearic acid was administered to laying hens, some of which had received pure methyl sterculate in the diet for long periods. The fatty acid composition of the lipid in the liver, developing ova, and mature eggs was studied with particular reference to the distribution of the radioactivity and the specific activites of the $C_{16}$ and $C_{18}$ fatty acids formed.

## EXPERIMENTAL PROCEDURES

A number of laying White Leghorn hens, maintained on a commercial laying ration, were used for these experiments. Some of the hens had received a daily dose of methyl sterculate (12 mg/kg body wt) by capsule for periods ranging from 3 months to 1 year. At the start of each experiment, individual hens were given a single dose, either of sodium acetate-$^{14}$C(U) (400 $\mu$Ci), or of stearic acid-1-$^{14}$C (66 $\mu$Ci or 226 $\mu$Ci) administered orally. Alternatively, for double-label experiments, each hen received sodium acetate-$^{14}$C(U) (400 $\mu$Ci) and stearic acid-9,10-$^{3}H_2$($n$) (0.5 or 1.0 mCi) simultaneously. The labeled compounds were obtained from the Radiochemical Centre, Amersham, England; the specific activities of the [$^{14}$C]-labeled compounds and the [$^{3}$H]-labeled acid were 50 c/mole and 500 c/mole, respectively.

Eggs laid by the hens were collected and numbered in sequence for 14 days after giving the radioactive dose. Hens receiving methyl sterculate were given the usual daily capsule during this period. In some cases the hens were killed 22 hr after receiving the radioactive dose, and the ovary, with developing ova, and the liver were excised.

The radioactivity in the shell, white and yolk of each egg was determined by oxygen-flask combustion of suitable aliquots of dry shell, freeze-dried white and freeze-dried yolk, followed by liquid scintillation counting of the $^{14}CO_2$ or $^{3}H_2O$, or both, produced (9).

Yolk and liver lipid was obtained from each egg by a Bligh and Dyer (10) extraction procedure. Lipid samples were interesterified with boron trifluoride in methanol-benzene (11), and the resulting methyl esters were purified by chromatography on Florisil (Floridin Co., Fla.) containing 7% w/w water (12). The composition of the methyl esters was determined by gas liquid chromatography (GLC) (Packard Model 7508, columns 180 cm x 3 mm ID of coiled glass, stationary phase 25% ethylene glycol succinate (EGS) plus 2% phosphoric acid on Gas-Chrom P).

Argentation column chromatography was used to separate the methyl esters into saturated (S) and unsaturated (US) fractions in order to determine a "per cent desaturation" value, (counts in US x 100) per (counts in US + counts in S) (3). The distribution of radioactivity in the separated fractions was determined by preparative GLC followed by counting of the separated components; a Pye Model 105/15 chromatograph with a 9.1 m x 7 mm ID coiled glass column containing 15% EGS on Gas-Chrom Q was used for the separations. During desaturation of stearic acid-9,10-$^{3}H_2$ to oleic acid, part of the tritum is released, and allowance was made for this when the formation of oleic acid was measured (13). In all cases similar results were obtained when either stearic acid-1-$^{14}$C or stearic acid-9,10-$^{3}H_2$ was used as the precursor.

In the studies with developing ova, the ova were weighed and graded according to size, the largest being number 1. Lipid was extracted from each of the first eight ova, and methyl esters were prepared and analyzed as already

TABLE I

Incorporation of Label into $C_{16}$ and $C_{18}$ Saturated and Monounsaturated Fatty Acids of Hen Lipids After Administration of Radioactive Acetate or Stearic Acid

| Labeled precursor | Diet | Lipid[a] site | Per cent of total methyl ester counts C16:0 | C16:1 | C18:0 | C18:1 |
|---|---|---|---|---|---|---|
| Acetate | Control | Egg 2 | 41.6 | 7.6 | 8.5 | 40.1 |
| | | Egg 6 | 30.8 | 5.7 | 9.5 | 51.0 |
| | | Liver | 29.5 | 3.7 | 16.0 | 47.8 |
| | Sterculate | Egg 2 | 48.3 | 1.9 | 32.5 | 12.1 |
| | | Egg 6 | 35.7 | 1.3 | 40.8 | 18.4 |
| | | Liver | 47.1 | 1.2 | 31.1 | 16.1 |
| Stearic acid | Control | Egg 2 | 3.4 | 0.4 | 28.3 | 63.2 |
| | | Egg 6 | 4.0 | 0.7 | 23.5 | 65.0 |
| | | Liver | 7.1 | 4.8 | 22.8 | 63.2 |
| | Sterculate | Egg 2 | 2.2 | 0.2 | 73.2 | 19.1 |
| | | Egg 6 | 4.0 | 0.3 | 56.4 | 27.9 |
| | | Liver | 12.1 | 1.7 | 58.2 | 25.9 |

[a]*Total yolk lipid of full term eggs. Liver lipid taken from birds killed 22 hr after administration of radioactive precursor.*

described. The data reported below are averages of results obtained from at least two birds. In experiments using full term eggs the birds were used first as controls, then treated with sterculate for a second experiment, and finally sacrificed for studies of liver and ova. In this way each bird served as its own control.

## RESULTS AND DISCUSSION

Combustion analysis showed that in the eggs of both control and sterculate-fed hens the acetate label was incorporated mainly in the yolk lipids and to a small extent in egg shell and white; stearic acid label appeared almost exclusively in the yolk lipids. Maximum incorporation of radioactivity from acetate or stearic acid appeared in the yolk lipid of the third or fourth egg laid after dosing (Fig. 1). Deposition of maximum radioactivity in this egg is apparently determined within a few hours of administering the label, because within 22 hr the third or fourth largest developing ovum already shows a greater incorporation of radioactivity than the other ova. In the intact hen this most active ovum maintains its maximum activity to maturity, so that the third (or fourth) egg laid after dosing contains the most radioactivity.

Hens receiving methyl sterculate incorporated about the same amount of radioactivity from labeled acetate into egg yolk lipid as control hens. However they did not incorporate stearic acid label to the same extent as the controls (Fig. 1).

The proportion of radioactivity associated with the unsaturated fatty acids of egg yolk lipid, expressed in terms of "per cent desaturation," is shown in Figure 2. Similar curves were

TABLE II

Content of $C_{16}$ and $C_{18}$ Saturated and Monounsaturated Fatty Acids in Hen Lipids

| Diet | Lipid[a] site | Per cent total methyl esters C16:0 | C16:1 | C18:0 | C18:1 |
|---|---|---|---|---|---|
| Control | Eggs[b] | 24.0 | 3.3 | 7.2 | 54.1 |
| | Liver | 27.8 | 4.0 | 11.7 | 49.3 |
| Sterculate | Eggs[b] | 34.8 | 1.2 | 30.3 | 23.5 |
| | Liver | 29.8 | 1.5 | 21.8 | 28.6 |

[a]See footnote, Table I.

[b]Average of all eggs.

TABLE III

Ratios of Specific Activities of $C_{16}$ and $C_{18}$ Saturated and Monounsaturated Fatty Acids From Hen Lipids After Administration of Radioactive Acetate and Stearic Acid

| Labeled precursor | Lipid[a] site | Diet | $\frac{C_{16:0}\ dpm/mg}{C_{16:1}\ dpm/mg}$ | $\frac{C_{18:0}\ dpm/mg}{C_{18:1}\ dpm/mg}$ |
|---|---|---|---|---|
| Acetate | Eggs[b] | Control | 0.7 | 1.9 |
| | | Sterculate | 0.9 | 1.8 |
| | Liver | Control | 1.2 | 1.3 |
| | | Sterculate | 1.2 | 3.0 |
| Stearic acid | Eggs[b] | Control | 0.6 | 4.0 |
| | | Sterculate | 0.4 | 3.0 |
| | Liver | Control | 0.2 | 2.2 |
| | | Sterculate | 0.2 | 2.4 |

[a]See footnote, Table I.

[b]Average of all eggs.

obtained when the lipids of developing ova were studied. The per cent desaturation values for yolk fatty acids in the eggs from sterculate-fed hens were considerably lower than the control values, irrespective of whether the fatty acids were derived from labeled acetate or labeled stearic acid, showing that methyl sterculate in the diet had strongly inhibited the formation of unsaturated fatty acids from both sources. In all cases the extent of desaturation appeared to increase after the administration of the precursor. However this is an artificial increase and reflects the fact that the radioactivity was given as a single dose, and that the amount of yolk constituents obtained from the blood and deposited on the developing yolk is related to the size of the yolk. Desaturation is not instantaneous, and shortly after administration of the dose of the labeled precursor a greater amount of the saturated acid will be deposited on the larger ova than on the smaller ova. As desaturation proceeds, a greater proportion of labeled unsaturated fatty acid becomes available for deposition.

The incorporation of label from acetate or stearic acid into the $C_{16}$ and $C_{18}$ saturated and monounsaturated fatty acids of egg yolk and liver lipids is shown in Table I, while the proportions of these acids present in the lipids is given in Table II. It is evident from both these tables that sterculate inhibits the formation of monounsaturated fatty acids from both acetate and stearic acid, as both the level and rate of synthesis of these acids is reduced.

The label from acetate, in both control and sterculate-fed birds, is about equally distributed between the $C_{16}$ and the $C_{18}$ fatty acids in the early eggs, but in later eggs the activity of the $C_{16}$ acids falls while the activity of the $C_{18}$ acids rises, indicating that the $C_{18}$ acids are metabolized via the $C_{16}$ fatty acids. On the other hand, the label derived from stearic acid is largely confined to the $C_{18}$ fatty acids with only a small proportion of label appearing in the $C_{16}$ acids.

The ratios of the specific radioactivities of palmitate and palmitoleate and the ratios of specific activities of stearate and oleate are shown in Table III. With both labeled acetate and stearic acid precursors the ratios found for sterculate-fed birds were of the same order as the corresponding ratios in control birds.

Reiser and Raju (5,6), using rats, and Donaldson (7,8), using chicks, have used this type of specific activity ratio to express the results of their experiments in which labeled acetate and stearic acid were given to control and sterculate-affected animals. When stearic or palmitic acid was the precursor there was an increase in the ratios in sterculate-treated animals, but no increase in the ratio when acetate (or lauric acid [6] ) was the precursor. If inhibition of desaturation was the only factor involved, then this result could be explained by postulating an alternative pathway for the synthesis of oleic acid from acetate. However the specific activities of stearic and oleic acids are dependent not only on the rates of desaturation, but also on the sizes of the metabolic pools of these acids. In sterculate-fed animals there is an increase in the level of stearic acid (Table II), and presumably in its metabolic pool, and a drop in the level of oleic acid. The specific activity ratio will therefore be influenced by the changes in the levels of stearic and oleic acids and will depend upon the amount of sterculate fed to the animal and the length of time over which it is administered. Thus conclu-

sions based solely on specific activity ratios may not be valid. This may account for the differences in the results obtained in this laboratory and those of other workers. Coleman and Friedman (14) have demonstrated a continued increase in the mass ratio of palmitate-palmitoleate and of stearate-oleate in rats receiving sterculate for 34 weeks, and on this basis have questioned the existence of a significant, saturate-independent, alternative pathway for the biosynthesis of oleic acid in this species. It has been shown recently that there is no alternative pathway for oleic acid biosynthesis in the goat mammary gland (15). The results presented in the present paper provide no evidence for the existence of an alternative pathway for oleic acid biosynthesis in the laying hen.

ACKNOWLEDGMENT

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# Sensitized Photooxidation of α-Tocopherol and of 2,2,5,7,8-Pentamethyl-6-Chromanol in Ethyl Acetate

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## ABSTRACT

The major product of each photooxidation was an equimolar mixture of quinone oxide and quinone. The yield of this mixture was 64% and 67% when the substrate was α-tocopherol and 2,2,5,7,8-pentamethyl-6-chromanol, respectively. Neither spirodienone dimer nor trimer was present in the product mixture. Evidently the reaction intermediate is an adduct of tocopherol and singlet oxygen. Tocopherol may protect biological lipids from singlet oxygen degradation.

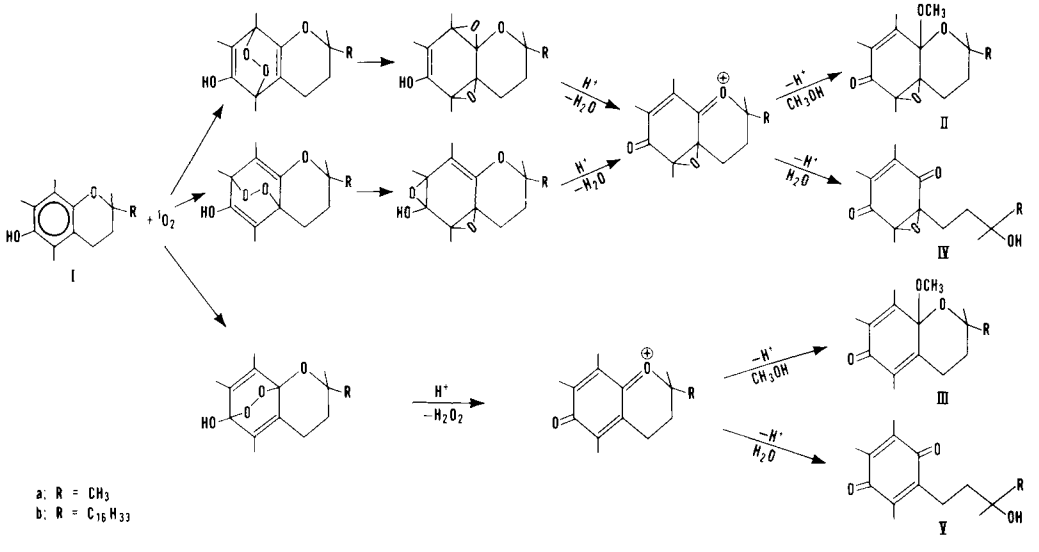

## INTRODUCTION

Recently (1) we proposed that the products (IIb-Vb) of dye-sensitized photooxidation of α-tocopherol (Ib) in methanol result from the solvolysis of an endoperoxide typical of singlet oxygen oxidation reactions (Scheme I). Evidence that singlet oxygen is the oxidizing agent in this reaction has been substantiated by the following findings: (a) the characteristic epoxy ketones were obtained when singlet oxygen was generated either photochemically with a sensitizer (1) or chemically from the $Ca(OCl)_2H_2O_2$ reaction in methanolic solution (2); (b) the kinetics of the methylene blue sensitized photooxidation of tocopherols were characteristic of a singlet oxygen reaction and the singlet oxygen reactivity of tocopherols correlates with vitamin E activity (3).

Csallany et al. (4) have shown that the major oxidation products of α-tocopherol formed during autoxidation of methyl linoleate are identical to compounds produced by mild oxidation of α-tocopherol with alkaline $K_3Fe(CN)_6$ and those isolated from rat liver. The major products of these oxidations (Scheme II) are the spirodienone dimer (VIb) and trimer (VIIb) of Ib. It has been fairly well established that free radical oxidation of I leads to characteristic products from an intermediate believed to be the *o*-quinone methide (VIII). If a suitable dienophile is not present, VI and VII would be expected to be major products of these reactions. We chose to photooxidize Ia and Ib in ethyl acetate under conditions that would lead to dimeric or trimeric products if the photooxidation proceeds by a pathway similar to the autoxidation of tocopherols (5).

## RESULTS AND DISCUSSION

Rose bengal-sensitized photooxidation of Ia




Scheme I.

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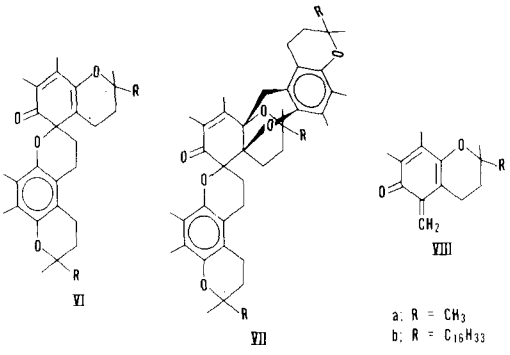
[2]N. Market. Nutr. Res. Div., ARS, USDA.

gave as major products quinone oxide, IVa, and quinone, Va. The ratio of IV to V was 1:1 when either Ia or Ib was the substrate. No spirodienone dimer, VI, or trimer, VII, was present in the product mixture. These results differ significantly from those of Fujimaki et al. (5), who reported trimer VIIa was the major product of the autoxidation of Ia after 40 days in oxygen-saturated heptane at 90 C. The photooxidation of tocopherols in the presence of a sensitizer must proceed by a mechanism different than that of autoxidation. An intermediate must be involved which contains both tocopherol and oxygen to obtain the observed products. We propose that intermediate is the endoperoxide illustrated in Scheme I.




Scheme II.

Chan (6) reported that soybean lipoxidase and horseradish peroxidase can convert singlet oxygen acceptors into characteristic products of singlet oxygen reactions. From his studies it seems likely singlet oxygen or singlet-oxygen-like oxidants occur in nature. Foote and coworkers (7) found that carotenoids may function as singlet oxygen quenchers. The ability of various carotenoids to quench singlet oxygen correlates with their biopotency. β-Carotene was shown to quench singlet oxygen at a diffusion controlled rate. A study in our laboratory (2) has shown tocopherols have the ability to function as singlet oxygen scavengers. A significant correlation was demonstrated between singlet oxygen reactivity and Vitamin E activity of tocopherols. α-Tocopherol reacts with singlet oxygen with a rate approaching that of a diffusion controlled reaction and is one of the most reactive naturally occurring singlet oxygen acceptor.

In 1971 McCay et al. (8) reported rapid metabolism of α-tocopherol by a membrane-bound enzyme system (TPNH oxidase). Their tracer studies showed that the oxidation products of α-tocopherol appear not to be the spirodienone dimer or trimer but rather a compound or compounds more polar than α-tocoquinone. The function of tocopherol as proposed by McCay is to protect the microsomal membrane from oxidative degradation and alteration of structure. Our model appears to be more closely related to the function of tocopherol as proposed by McCay et al. (8) than the general antioxidant hypothesis supported by Csallany et al. (4) and other research groups.

The possibility that α-tocopherol is an efficient scavenger of singlet oxygen in an environment similar to one which might be expected in nature is important. Conceivably on the basis of these results, α-tocopherol can protect biological lipids and membranes from the effects of singlet oxygen.

## EXPERIMENTAL PROCEDURES

### Photooxidation

Ia (234 mg, 1.06 mmoles) was dissolved in ethyl acetate (250 ml) containing rose bengal (10 mg). This solution was photolyzed under a Sylvania FBD 500W-120V iodine vapor lamp operating at 60 v in an Ace Glass Co. photoreactor fitted with a pyrex filter, an oxygen bubbler and a Haake circulating water bath. Photolysis completely converted starting materials to oxidation products in 3 hr at 28 C. The reaction products were extracted with 5% aqueous $NaHCO_3$ (3 x 50 ml) and water (50 ml) containing ethanol (5 ml). The extract was dried over anhydrous $Na_2SO_4$ and concentrated below 40 C to an oily residue. After the residue was dissolved in a small amount of benzene, the mixture was placed on a silica gel column and separated into its components with an ether-benzene gradient. The major fraction (168 mg) consisted of an equimolar mixture of pentamethyl chromanolquinone oxide (IVa) and quinone (Va). Because these compounds were inseparable by silica and reverse phase chromatography and were unstable on alumina, IVa was purified by chromatography on silica after selective reduction of Va to the chromanol (Ia) by the procedure outlined previously for the purification of α-tocoquinone oxide (2).

The yields of IVa and Va (as Ia) were 33% and 31%, respectively. Several minor products observed were not characterized. The total recovery of materials after chromatography was 88%. No spirodienone dimer or trimer was found upon examination of the product mixture by thin layer chromatography.

After column chromatography, recovery of Ia from reactions carried out with no sensitizer, no oxygen and no light was 97%, 99% and 98%,

respectively.

### 2,2,5,7,8-Pentamethyl-6-Chromanolquinone-4a, 5-Oxide, IVa

Nuclear magnetic resonance ($CDCl_3$) - $\delta$ 1.23 *s* (6H, $2CH_3$), 160 m (8H, $2CH_2$, -OH, $CH_3$), 1.93 *s* (6H, $2CH_3$); UV ($C_6H_{12}$), $\lambda_{max}$ 268 nm, $\epsilon_{max}$ 4.0 x $10^3$; IR (neat), 3500, 2990, 2950, 2900, 1680, 1635(w), 1460, 1380 $cm^{-1}$; mass spectrum (70 eV) *m/e* 237 ($C_{13}H_{17}O_4$, 16%), 234 (10%), 219 (5%), 209 ($C_{12}H_{17}O_3$, 46%), 206 ($C_{13}H_{18}O_2$, 31%), 194 (27%), 191 (29%), 167 ($C_9H_{11}O_3$, 31%), 166 (22%), 165 ($C_9H_9O_3$, 44%), 153 (41%), 152 (53%), 151 (54%), 139 (31%), 138 (31%), 137 ($C_8H_9O_2$, 71%), 126 (20%), 109 ($C_7H_9O$, 13%), 99 ($C_5H_7O_2$, 70%), 59 (100%), 43 (50%).

Analysis calculated for $C_{14}H_{20}O_3$: C, 66.64; H, 7.99. Found: C, 66.35; H, 8.19.

### α-Tocopherol Photooxidation

When this same procedure was followed, the reaction gave in 67% yield an equimolar mixture of α-tocoquinone and α-tocoquinone oxide, identical in all respects with the compounds prepared as previously described (1,2).

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# Synthesis of Conjugated Trienoic Fatty Acids by a Cell-Free Preparation of Tung Endosperm

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**ABSTRACT**

An acetone-insoluble fraction that catalyzes the synthesis of conjugated trienoic fatty acid was prepared from maturing tung nuts. Greatest synthesis with the enzymic fraction occurred in the presence of CoASH, NADH and either ATP or ADP.

Consecutive desaturation of long chain fatty acids forms polyunsaturated fatty acids in maturing plant seeds (1). Eleostearic acid (9,11,13-octadecatrienoic acid) is the principal fatty acid elaborated by tung seeds (*Aleurites fordii*, Hemsl). We are investigating the biosynthesis of eleostearic acid in developing endosperm of tung to obtain information needed to isolate a stable enzyme that catalyzes this synthesis and to determine the requirements for catalysis in vitro. In this communication we report the simple preparation and the requirements of an acetone-insoluble fraction of maturing tung nuts that catalyzes the synthesis of eleostearic acid.

Developing tung nuts were collected before, during and after the period of active oil

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TABLE I

Synthesis of Conjugated Trienoic Fatty Acid

| Components of reaction mixture[a] | Relative activity[b] |
|---|---|
| Complete | 1.00 |
| Without CoASH | 0.40 |
| Without NADH | 0.38 |
| Without NADH but with NAD | 0.77 |
| Without NADH but with NADPH | 0.32 |
| Without ATP | 0.05 |
| Without ATP but with ADP | 1.00 |
| Without ATP but with phytic acid | 0.00 |
| Without ricinoleic acid | 0.00 |
| With purified ricinoleic acid | 0.00 |
| Without ricinoleic acid but with stearic, oleic, linoleic, linolenic or alkali isomerized linoleic acid | 0.00 |
| With boiled enzyme | 0.00 |

[a]The complete reaction mixture (2 ml) contained 5 μmoles CoASH, 11 μmoles NADH, 10 μmoles ATP, 1.2 μmoles $MgSO_4$, 230 μmoles ricinoleic acid (containing 0.4% unidentified, conjugated dienoic acid), sufficient 0.5 N KOH to neutralize the above mixture, and enzyme preparation containing 100 μmoles tris-HCl buffer, pH 7.2, 4.0 μmoles dithiothreitol and 15-35 mg protein. Substitutions of another reagent for a component in the complete mixture were quantitatively of the same magnitude. Conditions of enzymic incubation and analyses of reaction product are described in the text.

[b]Change in absorbance at 268 mμ after 45 min of incubation of complete reaction mixtures that were run as controls ranged from 0.08-0.40 units corresponding to 6.2-31.0 mμmoles of conjugated trienoic fatty acid produced.

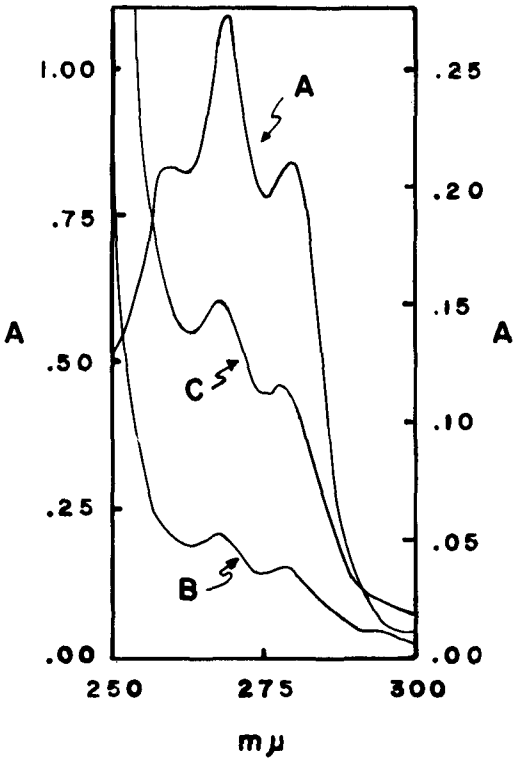


FIG. 1. Spectra of eleostearic acid and hexane extracts of reaction mixtures after 3 and 48 min of incubation. A: 7.1 x $10^{-6}$M eleostearic acid; B: reaction mixture after 3 min of incubation; C: reaction mixture after 48 min of incubation, corresponding to a production of 31 mμ moles of eleostearic acid during 45 min. Conditions of enzymic incubation are given in text and in Table I. Right ordinate is absorbance for curve A; left ordinate is for Curves B and C.

accumulation in the endosperm (2). Thinly sliced tissue was macerated in liquid nitrogen with a mortar and pestle. The small, frozen pieces were added to an equal weight of sand and homogenized at -78 C with sufficient acetone to ensure fluidity of the homogenate. The insoluble residue was washed by vacuum filtration with acetone at -78 C until the filtrate appeared colorless and then with sufficient ether to displace residual acetone. Ether was removed by immediately fluffing the powder at room temperature in an exhaust hood. The powder was further dried over $P_2O_5$ in a vacuum of 0.05 torr and then stored in sealed ampules at -20 C.

Solutions of the enzyme were prepared by triturating the powder in 0.1 M tris-HCl buffer (pH 7.2), filtering the suspension through glass wool, and centrifuging the filtrate at 1100 g for 10 min. Dithiothreitol was added to the supernatant to a concentration of $4 \times 10^{-3}$M. This enzyme preparation, containing 15-35 mg of protein per milliliter (mostly storage protein of the seed), was homogenized with other components of the reaction mixture and the mixture was allowed to incubate at 30 C with continuous homogenization. At various time intervals portions of 0.6 ml were removed and added to 0.4 ml of 0.5 N HCl. Fatty acids were extracted from each acidified reaction mixture into 3.0 ml of hexane (spectrophotometric grade), and the content of conjugated trienoic fatty acid was determined spectrophotometrically (3,4). The production of unsaturation was linear with time and proportional to enzyme concentration when enzyme was rate limiting.

Curve A of Figure 1 shows the absorbance spectrum of $7.1 \times 10^{-6}$M eleostearic acid in hexane (for comparison see Reference 3). Both the $\alpha$ and $\beta$ isomers were present in this preparation. Curves B and C show spectra of hexane extracts of enzymic reaction mixtures after 3 min and 48 min of incubation, respectively. Increase in absorbance at 268 m$\mu$ during incubation indicates production of conjugated trienoic fatty acid. Absorbance in the lower UV portions of the spectra was from conjugated dienes in the sample of ricinoleic acid used as the substrate.

In Table I are summarized the results of experiments to determine conditions for the synthesis of conjugated trienoic fatty acid. Greatest synthesis was obtained with CoASH, NADH and either ATP or ADP in the enzymic reaction mixture. NAD, but not NADPH, replaced NADH to some extent.

Various long chain fatty acids were tested as substrates in our system (Table I). Although ricinoleic acid appeared to be converted to conjugated trienoic fatty acid, this sample of ricinoleic acid contained 0.4% unidentified, conjugated dienoic fatty acid. When purified ricinoleic acid was tested as a substrate, conjugated trienoic unsaturation was not produced. This indicates that the fatty acid precursor was a conjugated dienoic acid in the sample of ricinoleic acid. However conjugated trienoic fatty acid was not formed when a mixture of synthetic conjugated dienoic acids, produced by alkali-isomerization of linoleic acid (4), was used as a substrate (Table I). Perhaps the mixture of synthetic dienes did not include the conjugated dienoic acid associated with ricinoleic acid from castor, or one of the several isomeric dienes in the mixture inhibited the enzyme system. The conjugated dienoic acid of castor oil merits characterization. It is of interest that castor, which is the source of ricinoleic acid, and tung are in the same family *(Euphorbiaceae)* and that conjugated trienoic and dienoic acids are present in small amounts among the fatty acids of castor oil (5).

Only acetone powders of maturing tung nuts that were rapidly accumulating oil (2) were enzymically active. Neither immature nuts that contained small amounts of endosperm nor fully matured, quiescent nuts provided active powders. Apparently enzymes catalyzing the biosynthesis of certain unsaturated fatty acids exist in tung, as in castor (6), for a restricted period in plant ontogeny.

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# Fatty Acid Composition of Carotenoid Esters in Soybean and Rapeseed Oils

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## ABSTRACT

The amounts of the different carotenoids (lutein, lutein monoesters and diesters) in soybean and rapeseed oil were determined through a combination of column chromatography and UV spectrometry. The lutein diesters in the oils have been isolated by a combination of column and thin layer chromatography. Identification and determination of the amount of the various fatty acids of the lutein diesters have been carried out by means of gas chromatography after transesterification of the fatty acids to their methyl esters. Comparison of the fatty acids of the lutein diesters with those of the triglycerides of the oils revealed a striking difference. First, the fatty acids of the lutein diesters have shorter chains than the triglycerides acids. Secondly, the lutein fatty acids are more saturated than the fatty acids of the triglycerides of the corresponding oils. However the amount of linoleic acid in the case of the fatty acids of the lutein diesters in rapeseed oil is greater than that in the fatty acids of the triglycerides in rapeseed oil.

## INTRODUCTION

On the fatty acid composition of carotenoid esters of vegetable origin there exists only a small quantity of data. The fatty acids of these esters, found in various fruits and flowers (2-5), generally appear to have saturated chains, e.g., lauric, myristic, palmitic and stearic acid. In wheat flour (6) and autumn leaves (7) the fatty acids of these esters also consist of unsaturated acids such as oleic, linoleic and linolenic acid.

When comparing the fatty acid composition of the carotenoid esters in paprika (4) and green algae (8) with the fatty acid composition of the accompanying triglycerides, they appear to differ.

In a previous publication from this laboratory (1) it was reported that lutein (3,3′-dihydroxy-α-carotene) occurs as the main carotenoid in soybean, rapeseed and linseed oils. From the chromatographic behavior of the oil pigment before and after saponification it was concluded that part of the lutein was esterified. However no data are known about the composition of carotenoid esters from oleaginous seeds.

In this publication the isolation and fatty acid analysis of lutein esters from soybean and rapeseed oil are described. The fatty acid compositon of the esters is subsequently compared with that of the glycerides of the oils.

## EXPERIMENTAL PROCEDURES

### Materials

Soybean oil was isolated from American soybeans. The beans (2.3 kg) were crushed and extracted twice at room temperature with petroleum ether (bp 40-65 C). The oil solution was concentrated at room temperature in a rotary evaporator. Four hundred grams of oil were obtained. Rapeseed oil was pressed from Canadian rapeseed.

The soybeans and the rapeseed oil were samples taken at random obtained from Verenigde Oliefabrieken, Zwijndrecht, The Netherlands.

### Carotenoid Analysis

The carotenoids in the oil were separated by column chromatography according to Lepage and Sims (6). Five grams of oil were chromatographed on a silica gel column (fein gepulvert, Merck), dimensions 20 x 1.5 cm. Lutein diesters were eluted successively with 100 ml hexane-ether 9:1, lutein monoesters (only detected in the soybean oil) with 100 ml hexane-ether 1:1 and lutein with 75 ml chloroform-methanol 9:1. The carotenoid fractions were concentrated and analyzed spectrophotometrically with a Cary 14 spectrophotometer, using hexane-ethanol 93:7 as solvent.

TABLE I

Carotenoid Analysis in Soybean and Rapeseed Oils (ppm)

| Oil | Lutein | Lutein monoesters | Lutein diesters |
|---|---|---|---|
| Soybean | 19.7 | 1.7 | 2.2 |
| Rapeseed | 39.5 | — | 4.7 |

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TABLE II

Soybean Oil: Fatty Acid Composition of Lutein Diesters and Triglycerides

| Fatty acid | Common name | Carbon number | Calibration factor | Percentage of the different fatty acids: In lutein diesters | Percentage of the different fatty acids: In triglycerides |
|---|---|---|---|---|---|
| | | | | % | % |
| C12:0 | Lauric acid | 11.9 | 1.00[a] | 9.9 | Trace |
| C14:0 | Myristic acid | 14.0 | 1.00[a] | 11.0 | Trace |
| C16:0 | Palmitic acid | 16.0 | 0.92 | 12.5 | 11.0 |
| C18:0 | Stearic acid | 18.0 | 0.96 | 3.7 | 3.4 |
| C20:0 | Arachidic acid | 20.0 | 1.01 | 0.4 | --- |
| C16:1 | Palmitoleic acid | 16.4 | 0.96 | 0.4 | --- |
| C18:1 | Oleic acid | 18.4 | 0.99 | 35.9 | 21.9 |
| C18:2 | Linoleic acid | 19.0 | 1.15 | 18.0 | 56.9 |
| C18:3 | Linolenic acid | 19.8 | 1.01 | 5.7 | 6.8 |
| Unidentified[b] | | | | 2.5 | |

[a]Arbitrary.

[b]Four unidentified different compounds 0.1, 0.1, 0.9 and 1.4%, respectively.

**Isolation of the Lutein Diesters**

The lutein diesters were isolated from the bulk of the oils by a combination of column and thin layer chromatography (TLC). The oil was chromatographed in 25-40 g portions on silica gel columns ($SiO_2$, fein gepulvert, Merck, 20 x 7 cm). In total 300 g of soybean oil and 400 g of rapeseed oil were used, corresponding to ca. 1 mg of lutein diesters in the oil.

The lutein diester band was eluted before the triglycerides with 500 ml hexane-ether 9:1. The remaining triglycerides in the combined lutein diester fractions were largely removed by repeated chromatography on a smaller silica gel column (20 x 1.5 cm) with hexane-ether 9:1.

The last traces of triglycerides which accompanied the lutein diesters were completely removed by means of preparative TLC. The TLC separation was carried out on twelve 20 x 20 cm plates, coated with a 1 mm layer of silica gel G-HR (Macherey, Nagel und Co.). The hexane solution of lutein diesters was brought on the plates as a thin strip, using an "Autoliner" apparatus (Desaga). Development with hexane-ether 9:1 gave a good separation between triglycerides (Rf = 0,30) and lutein diesters (Rf = 0,50). The triglyceride band was located by spraying with a 1% aqueous solution of Ultraphor WT (BASF; an optical bleaching agent) and viewing under UV light.

The lutein diester band was scraped off the plate and extracted with acetone. The combined extracts of the twelve separations were concentrated and rechromatographed with TLC

TABLE III

Rapeseed Oil: Fatty Acid Composition of Lutein Diesters and Triglycerides

| Fatty acid | Common name | Carbon number | Calibration factor | Percentage of the different fatty acids: In lutein diesters | Percentage of the different fatty acids: In triglycerides |
|---|---|---|---|---|---|
| | | | | % | % |
| C12:0 | Lauric acid | 11.7 | 1.00[a] | --- | 0.2 |
| C14:0 | Myristic acid | 13.9 | 1.00[a] | 3.4 | 0.1 |
| C16:0 | Palmitic acid | 16.0 | 0.95 | 9.0 | 3.1 |
| C18:0 | Stearic acid | 18.0 | 1.00 | 1.1 | 1.0 |
| C20:0 | Arachidic acid | 20.0 | 1.00 | 0.4 | --- |
| C16:1 | Palmitoleic acid | 16.4 | 0.91 | 0.5 | 0.2 |
| C18:1 | Oleic acid | 18.4 | 1.00 | 21.6 | 32.1 |
| C20:1 | — | 20.4 | 1.00[a] | 3.8 | 10.8 |
| C22:1 | Erucic acid | 22.3 | 1.00[a] | 9.3 | 20.6 |
| C18:2 | Linoleic acid | 19.0 | 1.14 | 48.8 | 22.1 |
| C20:2 | — | 20.7 | 1.00[a] | --- | 0.5 |
| C18:3 | Linolenic acid | 19.8 | 1.04 | 2.1 | 9.4 |

[a]Arbitrary.

on two plates in the same way to ascertain the absence of triglycerides. The combined acetone extracts were dried ($MgSO_4$) and the acetone was removed in a rotary evaporator (in an $N_2$ atmosphere). The fatty acids from the resulting pure lutein diesters were converted to their methyl esters (vide infra). In the same manner as in the case of the lutein diesters a sample of the triglyceride bands (≈ 100 mg) of the first TLC separation was isolated.

To protect the lipids from autooxidation during the isolation, the TLC-apparature was used in darkness in a nitrogen atmosphere. Black paper was wrapped round the silica gel columns. The solutions of the lutein esters were maintained under nitrogen, and preservation took place at 0 C between the different actions.

The preparative isolation of the observed lutein monoesters in soybean oil has not yet been carried out, because it will take a very long time to separate the monoesters from the great amount of triglycerides by means of repeated chromatography.

#### Fatty Acid Analyses

The fatty acids of the lutein diesters and the triglycerides were analyzed as their methyl esters by using gas chromatography (GLC). Transesterification was carried out as described by Van Wijngaarden (9). The GLC separation of the methyl esters was carried out on a Pye 104 apparatus, equipped with a flame ionization detector. Columns of steel (6 ft x 1/8 in.) were filled with 10% polyethyleneglycol adipate on Gaschrom Q. Carrier gas was nitrogen (35 ml/min) and the column temperature was 185 C (isothermal).

The fatty acids were identified by comparing the retention times with those of known fatty acid methyl esters. For the identification were also used the carbon numbers calculated with known fatty acid methyl esters (10). The peak areas were measured with a planimeter. For most of the compounds the specific sensitivity of the detector was determined using calibration mixtures of known composition. With the calibration factors found the fatty acid composition of the oils as well as of the lutein diesters was calculated, expressed in mole percentages.

## RESULTS AND DISCUSSION

The carotenoids were separated into three fractions by column chromatography. Amounts of the lutein diesters, the lutein monoesters, and lutein, respectively, were determined by UV spectrophotometry. Maximum absorptions were found at 424, 445 and 477 nm. Concentrations (Table I) were calculated using the reported value of $E_{1\,cm}^{1\%}$ = 2377 (at 444 nm) for lutein (6) and the assigned values of $E_{1\,cm}^{1\%}$ = 1286 and 1671 nm for lutein diesters and monoesters, respectively (calculation based on the di- and monoesters of palmitic acid).

The lutein diesters in the soybean and rapeseed oils were isolated by the described combination of column and thin layer chromatography. The fatty acids of the lutein diesters were determined with GLC after interesterification to their methyl esters. The fatty acids of the triglycerides in the rapeseed and soybean oils were analyzed in the same manner to compare the fatty acid composition of the lutein diesters with those of the triglycerides in the corresponding oils. The fatty acid composition of the last amount of triglycerides separated of the lutein diesters by TLC was also investigated. This was done to determine if then the fatty acids were different from those of the starting oils. However no differences were observed.

The results of the GLC analyses are given in Tables II and III. It should be noted that the fatty acid compositions of lutein diesters and triglycerides in both oils are different.

First, the fatty acids from the lutein diesters have shorter chains than the triglycerides acids. Secondly, they differ in degree of unsaturation. The lutein diester fatty acids from the soybean oil are more saturated than the fatty acids of the corresponding triglycerides (number of double bonds per molecule of fatty acid: 0.89 and 1.56, respectively). Most of the fatty acids of the lutein diesters in the rapeseed oil are also more saturated than the fatty acids of the corresponding triglycerides; however there is a greater amount of linoleic acid.

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# Effect of Dietary Linolenic Acid and Docosahexaenoic Acid on Growth and Fatty Acid Composition of Rainbow Trout *(Salmo gairdneri)*[1,2]

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**ABSTRACT**

Methyl linolenate 18:3ω3 and docosahexaenoate 22:6ω3 were incorporated in semipurified diets at several levels and fed to trout previously maintained on a fat-free diet. After 14 weeks, the weight gain and feed conversion of the fish on each diet were determined. The fatty acid composition of the lipid from each group of fish was analyzed by gas liquid chromatography. Both 18:3ω3 and 22:6ω3 fed at the 1% level supported maximum growth of the fish. The control group, which were fed no ω3 fatty acids, exhibited a shock syndrome, poor appetite and a very slow growth rate. Tissue fatty acid analysis revealed eicosatrienoic acid 20:3ω9 accumulated in the phospholipid fraction of this group. The 20:3ω9 level was lowered when either 18:3ω3 or 22:6ω3 was included in the diet. Analysis showed that the dietary 18:3ω3 was rapidly converted by the fish into 22:6ω3 with a high concentration in the phospholipid. However 22:6ω3 fed to the fish remained unchanged and little or no retroconversion of this fatty acid was observed.

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

[2]Technical paper no. 3247, Oregon Agricultural Experiment Station.

TABLE I

Composition of Trout Diets

| Component | Weight % |
|---|---|
| Casein | 53.9 |
| Gelatin | 9.7 |
| Dextrin | 17.1 |
| Lipid[a] | 2.0 |
| Mineral mix[b] | 4.0 |
| Carboxymethylcellulose | 1.4 |
| Alpha-cellulose (Alphacel-NBC) | 8.7 |
| Vitamin mix[c] | 2.0 |
| Choline chloride | 1.0 |
| Vitamin E concentrate (330 IU/g as dl-α-tocopheryl acetate) | 0.2 |

[a]Lipid composition listed in Table II.

[b]Bernhart-Tomerelli Salt Mix, modified by adding NaF and $CaCl_2$ at 0.002 and 0.02%, respectively.

[c]Supplied vitamins at following levels: (mg/kg) thiamine, 64; riboflavin, 144; niacinamide, 512; biotin, 1.6; Ca D-pantothenate, 288; pyridoxine, 48; folic acid, 19.2; menadione, 16; cobalamine, 0.159; i-inositol (meso-), 2500; ascorbic, 1200; and *p*-aminobenzoic acid, 400. Vitamins A and D were added at 25,000 and 4000 IU/kg, respectively.

## INTRODUCTION

Recent studies have demonstrated that fatty acids of the linolenic family (ω3) are essential for rainbow trout, whereas acids of the linoleic family (ω6) do not seem to be required. Lee and coworkers (1) reported that corn oil in a semipurified diet led to poor growth and high mortality of rainbow trout. Replacing the corn oil with fish oil greatly stimulated growth and prevented further mortalities. Castell and coworkers (2,3) demonstrated that trout grew poorly on a diet containing 1% linoleic acid as

TABLE II

Effect of Dietary Lipids on Feed Efficiency and Mortality of Rainbow Trout

| Diet no. | Lipid composition, % 12:0 | 18:3ω3 | 22:6ω3 | Feed efficiency,[a] gain/feed | Accumulated mortality |
|---|---|---|---|---|---|
| 1 | 2.00 | 0 | 0 | 0.32 | 17 |
| 2 | 1.75 | 0 | 0.25 | 0.80 | 0 |
| 3 | 1.50 | 0 | 0.50 | 0.88 | 1 |
| 4 | 1.00 | 0 | 1.00 | 0.91 | 1 |
| 5 | 1.50 | 0.50 | 0 | 0.84 | 3 |
| 6 | 1.00 | 1.00 | 0 | 0.90 | 1 |

[a]Feed efficiency is defined as units of weight gained per unit of dry feed consumed.

the only unsaturated fatty acid. The fish developed a shock syndrome, excessive liver mitochondrial swelling and other symptoms. On the other hand, Castell reported excellent growth of the fish with a diet containing 1% linolenic acid and recommended this level be included in diets for rainbow trout. Their results supported the findings of Richardson et al. (4), Brockerhoff and Hoyle (5), Brenner et al. (6) and Higashi et al. (7) that linolenic acid was nutritionally more important for fish than linoleic acid.

So far only linolenic acid has been used to study the $\omega 3$ fatty acid requirements of fish. The objective of this experiment was to isolate the long chain highly unsaturated docosahexaenoic acid 22:6$\omega 3$ from fish oil and to compare the effect of this fatty acid with that of linolenic acid on growth and fatty acid composition of rainbow trout.

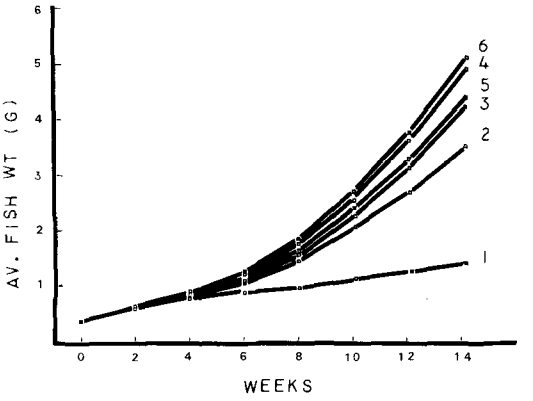


FIG. 1. Effect of dietary fatty acids on growth rate of rainbow trout. Curve 1, control trout diet containing no $\omega 3$ fatty acid. Curves 2, 3 and 4, diets containing 0.25, 0.5 and 1.0% 22:6$\omega 3$, respectively. Curves 5 and 6, diets containing 0.5 and 1.0% linolenic acid, respectively.

## MATERIALS AND METHODS

Methyl linolenate 18:3$\omega 3$ and methyl laurate 12:0, both 99% pure, were obtained from the Hormel Institute. The source of docosahexaenoic acid 22:6$\omega 3$ was Pacific Coho salmon roe which had been freeze dried and the oil extracted with chloroform-methanol 2:1. The egg oil was converted to fatty acid methyl esters by use of sodium methoxide (8). The resulting esters were dissolved in acetone and subjected to low temperature crystallization at -80 C. After removal of the precipitated esters, the 22:6$\omega 3$ content increased from 21.0% to 30.6% as estimated by gas liquid chromatography (GLC). The 22:6$\omega 3$ was isolated from the ester mixture by argentation chromatography using a column packed with silicic acid containing 20% $AgNO_3$ (9). The elution solvents were similar to those described by Stein and Slawson (10) with the addition of equal parts of the antioxidants, butylated hydroxyanisole and butylated hydroxytoluene (0.001%) to prevent autoxidation of the unsaturated esters. The saturated and monounsaturated esters were eluted with diethyl ether-pentane 1:1 and more highly unsaturated esters were eluted with diethyl ether-pentane-cyclohexane 5:3:2. Finally the ester of 22:6$\omega 3$ was eluted with diethyl ether-cyclohexane 1:1. Fractions containing high percentages of 22:6$\omega 3$ were pooled. The final product, as analyzed by GLC, contained 97.0% 22:6$\omega 3$; 2.5% 20:5$\omega 3$, and was free from 18:3$\omega 3$ and $\omega 6$ fatty acids. The isolated 22:6$\omega 3$ was finally put through a silicic acid column to remove the antioxidants (11).

Rainbow trout *(Salmo gairdneri)* was used as the experimental animal. Eggs from the brood stock of this laboratory were hatched and the fry fed a fat-free diet for one month and then randomly divided into lots of 50 fish. Each experimental diet was fed to duplicate lots of fish. The composition of the diets is shown in Table I. The casein, gelatin, dextrin and cellulose used in the diet had been extracted with warm isopropanol to remove trace lipids. The

TABLE III

Effect of Dietary Lipids on Growth of Rainbow Trout

| | Average fish wt (g) in weeks[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Diet | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 |
| 1 | 0.53 | 0.76 | 0.97 | 0.97 | 1.03 | 1.21 | 1.35 | 1.56 |
| 2 | 0.53 | 0.85 | 0.90 | 1.23 | 1.56 | 2.15 | 2.73 | 3.58 |
| 3 | 0.50 | 0.78 | 0.92 | 1.19 | 1.63 | 2.39 | 3.23 | 4.32 |
| 4 | 0.51 | 0.76 | 0.98 | 1.37 | 1.84 | 2.62 | 3.70 | 5.00 |
| 5 | 0.53 | 0.79 | 0.94 | 1.28 | 1.75 | 2.51 | 3.37 | 4.47 |
| 6 | 0.52 | 0.84 | 1.00 | 1.39 | 1.93 | 2.84 | 3.88 | 5.20 |

[a]Average of duplicate tanks.

TABLE IV

Percentage Fatty Acid Composition of Phospholipids[a]

| Fatty acids | Diet | | | | | | |
|---|---|---|---|---|---|---|---|
| | Fat-free, 1 month | 1 | 2 | 3 | 4 | 5 | 6 |
| 12:0 | — | 0.5 | 0.5 | 0.6 | 0.4 | 0.6 | 0.5 |
| 14:0 | 0.9 | 2.4 | 2.2 | 1.8 | 1.7 | 2.2 | 2.0 |
| 16:0 | 14.2 | 16.1 | 15.9 | 16.8 | 18.1 | 11.7 | 16.6 |
| 16:1ω7 | 9.7 | 14.1 | 12.7 | 11.9 | 10.5 | 12.1 | 10.5 |
| 18:0 | 7.2 | 5.1 | 4.6 | 5.3 | 5.7 | 4.8 | 5.0 |
| 18:1ω9 | 30.8 | 35.8 | 32.7 | 29.1 | 27.2 | 31.9 | 29.2 |
| 18:2ω6[b] | 3.2 | 4.3 | 3.2 | 2.7 | 2.3 | 2.8 | 2.1 |
| 18:3ω3 | — | — | — | — | — | 1.6 | 3.8 |
| 20:1ω11[b] | 3.5 | 2.9 | 2.6 | 2.5 | 1.8 | 2.3 | 2.0 |
| 18:4ω3 | — | — | — | — | — | 1.1 | 2.3 |
| 20:2ω9 | 2.4 | 2.5 | 2.5 | 2.3 | 2.1 | 2.4 | 1.4 |
| 20:3ω9 | 4.2 | 8.6 | 5.9 | 4.8 | 3.0 | 3.6 | 1.5 |
| 20:4ω6 | 1.1 | 1.2 | 0.6 | 0.5 | Trace | 0.8 | 0.6 |
| 20:4ω3 | — | — | — | — | — | — | 0.6 |
| 20:5ω3 | Trace | — | Trace | 0.9 | 0.8 | 1.7 | 1.5 |
| 22:5ω6 | Trace | 0.6 | — | — | — | — | — |
| 22:5ω3 | Trace | — | — | — | — | Trace | Trace |
| 22:6ω3 | 23.0 | 5.7 | 16.8 | 21.0 | 26.7 | 16.1 | 20.9 |

[a]Average of analysis of duplicate tanks.
[b]Other isomers may be present.

quantity of the lipids in the diets was adjusted to 2% by varying the amount of the added 12:0 and all diets were isocaloric. The lipids added to each diet are listed in Table II.

The fish were held in 75 liter fiberglass tanks. The water temperature was 11.5 C, and flow rate was ca. 8 liters/min. The fish were fed three times daily. Food was offered only as long as the fish continued to feed.

Feed consumption and mortality were recorded. The fish were weighed every 2 weeks and the experiment was terminated at the end of 14 weeks.

The fatty acid composition of the pooled samples of each lot was determined. The lipid was extracted from the fish by the method of Folch et al. (12) and further separated into phospholipid and neutral lipid fractions as described previously (1). Methyl esters were prepared from these lipids by transesterification with boron-trifluoride in anhydrous methanol (13). Separation and identification of the component fatty acids was carried out as described by Lee et al. (1).

## RESULTS AND DISCUSSION

The growth rate was extremely slow for the group of fish receiving diet 1 (see Table I) which was devoid of ω3 fatty acids. The fish also exhibited poor appetite and a shock syndrome as described by Sinnhuber (14). Addition of ω3 fatty acids, even at a low level (diet 2), to the diet vastly improved the condition of the fish and increased the growth rate (Fig. 1). None of the fish on supplemental diets exhibited the shock syndrome.

The accumulated mortality was high in the group of fish on diet 1, without ω3 acids. Incorporation of ω3 fatty acids in the diets effectively lowered the mortality (Table II). The feed efficiency also increased with increasing levels of ω3 fatty acids as shown in Table II. These results indicated that trout responded equally well to dietary 18:3ω3 and 22:6ω3.

The growth of fish on diet 6, containing 1.0% 18:3ω3, was similar to that reported by Castell et al. (2,3). Their results showed that the growth rate of trout approached a maximum when 1% 18:3ω3 was added in the diet. The growth rate was considerably lower if only 0.5% of either 22:6 or 18:3 was incorporated in the diet (Table III).

The fatty acid composition of phospholipids and of neutral lipids is shown in Tables IV and V. The lipid extracted from the whole fish, after receiving a fat-free diet for one month was 1.14% and showed a high percentage of 22:6ω3 (23.0%) in the phospholipid fraction. The neutral lipid fraction contained ca. 10.0% 22:6ω3. These values represent the carry-over from the egg. Calculation of the total amount of 22:6ω3 remaining in the fish after 14 weeks on diet 1 showed that fish had conserved ca. 70% of the original 22:6ω3.

TABLE V

Percentage Fatty Acid Composition of Neutral Lipids[a]

| Fatty acids | Diet | | | | | | |
|---|---|---|---|---|---|---|---|
| | Fat-free, 1 month | 1 | 2 | 3 | 4 | 5 | 6 |
| 12:0 | — | 4.9 | 9.4 | 9.1 | 5.2 | 7.5 | 5.5 |
| 14:0 | 2.6 | 4.8 | 4.1 | 3.3 | 2.9 | 3.4 | 3.0 |
| 16:0 | 15.8 | 14.7 | 14.6 | 15.9 | 16.5 | 16.0 | 17.5 |
| 16:1ω7 | 11.5 | 13.6 | 12.7 | 13.2 | 14.5 | 13.4 | 12.6 |
| 18:0 | 4.6 | 4.9 | 4.6 | 6.3 | 4.2 | 5.0 | 4.2 |
| 18:1ω9 | 42.6 | 48.6 | 43.5 | 38.0 | 41.0 | 40.7 | 37.4 |
| 18:2ω6[b] | 3.6 | 2.1 | 1.8 | 2.9 | 2.3 | 1.8 | 1.6 |
| 18:3ω3 | — | — | — | — | Trace | 2.5 | 8.5 |
| 20:1ω11[b] | 3.3 | 3.8 | 3.8 | 3.3 | 2.5 | 2.9 | 2.8 |
| 18:4ω3 | — | — | — | — | — | 0.7 | 1.5 |
| 20:2ω9 | 1.2 | 1.8 | 1.8 | 2.0 | 2.1 | 1.5 | 1.4 |
| 20:3ω9 | 1.8 | 1.5 | 1.2 | 1.2 | 1.0 | 0.8 | 1.2 |
| 20:4ω6 | 1.4 | — | — | — | — | — | — |
| 20:5ω3 | 1.9 | — | — | Trace | 1.0 | — | — |
| 22:5ω3 | Trace | — | — | — | — | — | — |
| 22:6ω3 | 10.0 | — | 2.9 | 5.2 | 7.1 | 4.0 | 3.9 |

[a]Average of analysis of duplicate tanks.
[b]Other isomers may be present.

The acid 20:3ω9 was biosynthesized and found at high concentration in the phospholipids of fish which received only lauric acid (diet 1). The concentration of 20:3ω9 was lowered when dietary 18:3ω3 or 22:6ω3 increased (Table IV). Linolenic acid appeared to be somewhat more efficient than 22:6ω3 in reducing the level of 20:3ω9. This is in agreement with the experiment of Brenner and Jose (15) which showed that the 20:3ω9 in liver and heart tissues of the fat deficient rats was lowered when 18:3ω3 or 22:6ω3 was included in their diet. They suggested that 22:6ω3 inhibited the incorporation of 20:3ω9 into the B position of the glycero phospholipids. As a result, further formation of the 20:3ω9 was inhibited. 18:3ω3 competitively inhibited the enzymatic desaturation and chain elongation reaction of 18:1ω9.

The formation of 18:4ω3, 20:4ω3, 20:5ω3, 22:5ω3 and 22:6ω3 was observed in the phospholipids of the fish receiving dietary 18:3ω3 (diets 5 and 6). The concentration of 22:6ω3 was especially high. These results are quite similar to the conversion of linolenic acid into docosahexaenoic acid in kelp bass as reported by Kayama et al. (16). In the groups of fish placed on diets containing 22:6ω3 (diets 2-4), there was a very high concentration of 22:6ω3 in the phospholipids; while only a low concentration of 20:5ω3 was observed.

We believe the origin of the 20:5ω3 is from the small amount (2.5%) which is present in the 22:6ω3 supplement and is not the result of retroconversion. A positive answer will require further experimentation. No formation of 20:4ω3, 18:4ω3 and 18:3ω3 was detected in the lipids of the fish. Rat experiments conducted by Verdino and coworkers (17) and Schlenk et al. (18) showed that a large quantity of 20:4ω6 (essential to rats) was formed by retroconversion by fat deficient animals when placed on diets containing 22:5ω6. Only a trace of 18:2ω6 was formed. Furthermore the conversion of arachidonic acid to 18:2ω6 in rats was insignificant. It seems that 22:6ω3 in trout has a behavior similar to 20:4ω6 in rats.

Fish oils are generally low in 18:3ω3 but rich in other ω3 fatty acids: namely, 18:4ω3, 20:5ω3, 22:5ω3 and 22:6ω3. The total ω3 fatty acid content in fish oil is ca. 20-30%. This experiment reaffirmed the ability of fish to synthesize 22:6ω3 from lower ω3 fatty acid and the capability of both 18:3ω3 and 22:6ω3 to alleviate essential fatty acid deficiency symptoms, increase feed efficiency and promote rapid growth.

This experiment stressed the importance of ω3 fatty acids in trout nutrition but did not rule out a possible requirement for ω6 fatty acids. A long term feeding experiment is in progress to determine whether trout can survive and reproduce without dietary ω6 fatty acids.

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# The Intravenous Use of Fat for the Total Parenteral Nutrition of the Infant

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## ABSTRACT

A new program for the total iv feeding of the postoperative neonate has been described and evaluated. The program is based on a crystalline 1-amino acid solution as the nitrogen source and a soybean fat emulsion as the major source of calories. This program has three main advantages over ones based on hypertonic glucose as the main calorie source: (a) no central venous catheter is needed, (b) the complication of osmotic diuresis is eliminated, and (c) the high caloric value of fat allows small volumes of fluid to be infused into the infant. The present regimen has been shown to sustain nitrogen and potassium retention in amounts similar to those found in normal newborns and in other parenteral feeding programs. In the initial phase of this study, 32 newborns were treated with this program and 26 survived, following surgery for life-threatening congenital anomalies. Since this first evaluation, over 75 infants have been placed on this iv feeding protocol.


## INTRODUCTION

The total iv feeding of infants requires the administration of adequate amounts of nitrogen along with a calorie source. Sufficient calories must be given so that the infused nitrogen (in the form of a crystalline amino acid solution or a protein hydrolysate) can be utilized for the synthesis of a new protein. Since the newborn requires 75-100 cal/kg body wt/24 hr for normal growth and development (1-6), this large amount of calories can only be given in the form of hypertonic glucose or as fat. The disadvantages inherent in the use of a hypertonic glucose solution are as follows: (a) the high osmotic load (2500 milliosmoles/kg in the preparations presently used in the U.S. [7,8]) requires that the solution be administered into an area of rapid mixing such as the large central veins or right atrium. This demands the insertion of a central venous catheter, with all its attendant difficulties and complications (7); (b) the high osmolality of the solution can lead to a significant osmotic diuresis, especially in the newborn with immature kidneys, which can cause dehydration and possible cardiovascular collapse; and (c) because of the relatively low calorie value of carbohydrate (4 cal/gm), relatively large volumes of fluid have to be given in order to supply the appropriate number of calories. On the other hand, the above three disadvantages are obviated by using fat as the major calorie source. Intralipid, (Vitrum Co., Stockholm, Sweden) the solution used in this study, is isotonic, and thereby eliminates the risk of osmotic diuresis and the need for a central venous catheter (it can be infused into a peripheral vein). Its high caloric value (10 cal/gm) allows the administration of relatively small volumes.

Because of the advantages mentioned above, the following study was undertaken to evaluate the use of a fat emulsion for the total iv feeding of the postoperative neonate.

## MATERIALS AND METHODS

During the period January 1, 1969-June 30, 1969, 32 newborns, who had undergone major surgery in the Pediatric Surgical Department of the Rikshospitalet, Oslo, Norway, were placed on the parenteral feeding program described below. The infants were placed on this program for periods ranging from 3-40 days postoperatively. All patients were carefully evaluated clinically and metabolically during the period that they were fed intravenously. Four babies from this group were selected for detailed

TABLE I

Parenteral Feeding Program for Surgical Neonates

| Preparation | Volume, ml/kg/24 hr | Calories |
|---|---|---|
| Crystalline amino acid[a] solution | 30 | 24 |
| Soybean oil emulsion[b] | 15-20 | 30-40 |
| 10% Glucose | 80-90 | 32-36 |
| $KH_2PO_4$, 1 mmole/ml | 1-2 | |
| Calcium, 0.5 meq $Ca^{++}$/ml | 2 | |
| Multivitamin solution | 0.2 | |
| Total | 125-140 | 86-100 |

[a]Aminofusin, L-Forte, J. Pfimmer Co., Erlangen, Germany.

[b]20% Intralipid, Vitrum Co., Stockholm, Sweden.

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TABLE II

Comparison between Human Milk and the Parenteral Feeding Program(9), Volume of 130 ml

| Component | Human milk | Parenteral feeding program |
|---|---|---|
| Calories | 98 | 96 |
| Nitrogen | 0.43 gm | 0.53 gm |
| Fat | 5.4 gm | 4.0 gm |
| Carbohydrate | 9.6 gm | 11.0 gm |
| K+ | 2.3 meq | 1.75-2.75 meq |
| Mg++ | 0.18 meq | 0.15 meq |
| Phosphate | 21 mg P | 36-48 mg P |
| Ca++ | 1.7 meq | 1 meq |
| Na+ | 2.4 meq | 1.05 meq |

metabolic balance studies.

The parenteral feeding program employed is outlined in Table I. A comparison between this program and human milk is presented in Table II. The solutions listed in Table I, except Intralipid, were mixed together each morning and infused over the next 24 hr at a constant rate. Simultaneously, the Intralipid was administered through a separate infusion set, and the two infusion sets were connected to a single needle through a Y-connector. The fluids were administered through a needle placed into a peripheral vein (usually a scalp vein). No central venous catheters were used; in five instances, peripheral venous catheters were utilized for periods of 4-6 days. Heparin was added to the bottle containing the amino acids in a dose of 150 to 300 IU/kg/24 hr to accelerate the triglyceride clearance(10).

Plastic bags were applied to the infants's perineum, and accurate 24 hr urine collections were made. Leakage around the collecting bags was rare and the volumes lost were quite small. The urine was analyzed daily for nitrogen, sodium, potassium, phosphorus, calcium and magnesium. Gastric aspirates were collected daily from a nasogastric or gastrostomy tube; the volumes were measured, and then the entire sample was pooled prior to the analyses already mentioned above.

Sodium and potassium were measured by flame photometry. Calcium and magnesium were analyzed by atomic absorption spectrophotometry. Nitrogen was determined by the micro Kjeldahl method and phosphorus was analyzed by the standard colorimetric technique.

## RESULTS

The weight changes and balance data in the four study infants are shown in Table III. Each newborn showed a weight gain during the period of iv feeding in spite of the fact that this treatment period occurred during the maximal catabolic phase after surgery. In addition, the other 28 patients studied during this period also demonstrated either stable weight or a weight gain during parenteral nutrition. Likewise, the cumulative nitrogen and potassium balances in these infants were positive. In fact, except for patient K.O., the nitrogen and potassium balances were positive during each day of the study period. K.O. was in very minimal negative nitrogen balance for three days, probably secondary to severe peritonitis and intestinal obstruction.

No complications directly related to the parenteral feeding regimen were noted in any of the 32 infants. No episode of thrombophlebitis was noted during any of the infusions. A single peripheral vein usually tolerated the infusion ca. 4 days and then had to be changed because of infiltration. No cases of pyrexia or septicemia that could be directly traced to the infusions were noted. Likewise, no clinical evidence of abnormal blood clotting was noted.

Table IV shows the potassium-nitrogen retention ratios and compares them with the ratios one would see during breast and bottle feeding. The K/N ratio seen in muscle tissue is also listed.

Patients E.F. and K.O. died during the study. Autopsy of these two patients did not reveal any abnormal accumulation of fat in any of their tissues, especially the liver and the reticuloendothelial system.

## DISCUSSION

Normal breast-fed newborns accumulate nitrogen at a rate of between 150 and 250 mg N/kg body weight(3,11). Bottle feeding increases nitrogen retention by about 100 mg(3). Our program supports positive nitrogen balances of the same magnitude as those seen during bottle feeding, even during severe illness and during the immediate postoperative period.

The balance data, described in more detail in a previous communication, support the contention that 3 gm protein/kg body wt/24 hr will support a normal rate of protein synthesis when accompanied by an adequate caloric intake (90-100 cal/kg/24 hr)(9). The weight gains and the nitrogen balances seen in the present study are quite similar to those reported in other parenteral feeding studies in newborns, in which the major calorie source was hypertonic glucose, which was administered through a central venous catheter(7,8).

The balance data for potassium show that between 50% and 80% of the administered dose was retained. In the cases of E.F., R.K. and O.J., where the daily doses were between 2.0 and 2.5 meq K/kg, the ensuing K/N retention ratios were considerably in excess of the K/N ratio in muscle tissue (Table IV). This has also been seen during normal oral feeding, as shown in Table IV. In the case of K.O., the retention of potassium was relatively low, the K/N retention ratio being only 1.7 meq K/gm nitrogen. The potassium supply to this patient was limited to ca. 1 meq/kg daily, and this was probably too small a dose for adequate positive balances. The optimal dose of potassium appears to be ca. 2 meq/kg body wt, provided calories and protein are administered simultaneously in sufficient quantity to allow normal nitrogen retention, and provided the clinical state of the patient is such that the body cells are able to absorb and retain the potassium they need. Element-nitrogen retention ratios and recommended optimal doses for phosphorus, calcium magnesium, and sodium have been described in a previous communication(9).

Previous work in newborn dogs and humans has shown that small doses of heparin (doses that will not affect normal blood clotting), given along with the Intralipid (but not in the same bottle), will enhance the clearance of triglycerides from the blood stream and the appearance of free fatty acids in the blood(10). This has allowed us to increase the amount of fat administered to the surgical neonates from 2 gm/kg body wt/24 hr, the usual recommended dose, to 4 gm/kg/24 hr. No adverse clinical or metabolic affects from this higher dosage were seen, and no abnormal fat accumulations were noted in any of the autopsies done. This increased dosage has allowed us to significantly increase the number of calories given, while the increase in the volume of fluid infused has been insignificant.

The feasibility of administering total iv nutrition to infants for up to 40 days through the sole utilization of peripheral veins is the major finding of the present study and will help

TABLE III

Balance Data

| Patient | Age at operation days | Diagnosis | Operation | Duration of total parenteral feeding, days | Weight during parenteral feeding, gm | | Cumulative nitrogen balance, mg | Cumulative K+ balance, meq |
|---|---|---|---|---|---|---|---|---|
| | | | | | Initial | Final | | |
| K.O. | 3 | Aganglionosis of the small intestine | Ileostomy | 7 | 1600 | 1800 | +1,830 | +3.3 |
| E.F. | 1 | Gastroschisis | Staged repair | 26 | 2350 | 2400 | +16,400 | +71.0 |
| R.K. | 1 | Omphalocele | Repair | 8 | 2765 | 3000 | +3,850 | +30.5 |
| O.J. | 1 | Diaphragmatic hernia | Repair | 3 | 3850 | 4000 | +4,700 | +20.0 |

TABLE IV

K/N Retention Ratios

| Ratio | Muscle tissue | Breast feeding | Bottle feeding | Parenteral Feeding-Present Material | | | |
|---|---|---|---|---|---|---|---|
| | | | | K.O. | E.F. | R.K. | O.J. |
| K/N meq/gm | 2.8 | 5.6 | 6.7 | 1.7 | 5.5 | 7.8 | 4.0 |

broaden the spectrum of parenteral nutrition in newborns.

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# *cis* and *trans* Analysis of Fatty Esters by Gas Chromatography: Octadecenoate and Octadecadienoate Isomers[1]

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## ABSTRACT

A gas chromatographic method has been developed for quantitative determination of the *cis* and *trans* percentages in octadecenoate and octadecadienoate esters. To separate *cis*- and *trans*-monoene and diene isomers on a packed GC column, the fatty esters were stereospecifically epoxidized with peracetic acid. A simple and quantitative epoxidation procedure allows the *cis*- and *trans*-epoxyoctadecanoates to be analyzed without prior isolation from the reaction mixture. No positional or geometric isomerization of the double bond occurred during epoxidation. Synthetic mixtures containing *cis*- and *trans*-6, -9 and -12 octadecenoate isomers were completely separated into *cis* and *trans* fractions. *trans*-15-Octadecenoate was the only isomer investigated that partially interfered in the analysis. Diene mixtures containing *trans,trans*-, *cis,trans*- and *cis,cis*-9,12-octadecadienoates were also successfully analyzed by gas liquid chromatography (GLC) after epoxidation with peracetic acid. Each diene isomer formed two pairs of diepoxy diastereomers, some of which could be separated. One *cis,cis*-diepoxyoctadecanoate diastereomer peak overlapped the *cis,trans*-diepoxyoctadecanoate peaks. The total *cis,cis*-diepoxyoctadecanoate percentages were calculated by using the ratio of the two *cis,cis*-diepoxyoctadecanoate diastereomers. Other positional octadecadienoate isomers were also epoxidized and analyzed by GLC. The large number of possible octadecadienoate isomers requires that some preliminary fractionation be made before GC analysis is practical for diene isomers.

## INTRODUCTION

Determination of *cis* and *trans* by gas liquid chromatography (GLC) has been effectively used as an analytical method in petroleum research for several years. A comparable method has not been available to the lipid chemist because the high column temperatures required in lipid gas chromatographic (GC) work cause the liquid phase to decompose or bleed-off the column. Most available GC methods for separating *cis* and *trans* olefins are limited to compounds that elute at low column temperatures. Silver nitrate in ethylene or propylene glycol is an effective liquid phase only when column temperatures are lower than 65 C (1). Thallium nitrate GC columns have a higher maximum operating temperature, but the upper limit is still only 130 C (2). Neither of these columns is suitable for *cis-trans* analysis of lipid methyl esters.

*cis* and *trans* Analysis by GLC has several inherent advantages over IR analysis. The most important is that small amounts of many common fatty esters, such as palmitate, stearate, linolenate and conjugated octadecadienoates and octadecatrienoates, can be present as impurities in the monoene sample without affecting GC analysis. The small sample requirements (0.1 mg) allow samples obtained by preparative TLC or by preparative GLC to be analyzed. Analysis of partially deuterated monoenes is routine with GLC, but essentially

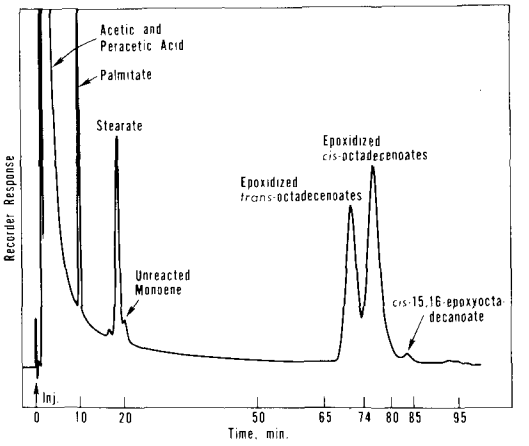



FIG. 1. Separation of epoxidized methyl octadecenoate isomers isolated from hydrogenated vegetable oil by an 11 ft x 4 mm 10% EGSS-X on Gas Chrom P. Gas chromatographic conditions: column temperature, 198 C; He flow rate, 38 ml/min; injection temperature, 205 C; detector temperature, 210 C.

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

[2]N. Market. Nutr. Res. Div., ARS, USDA.

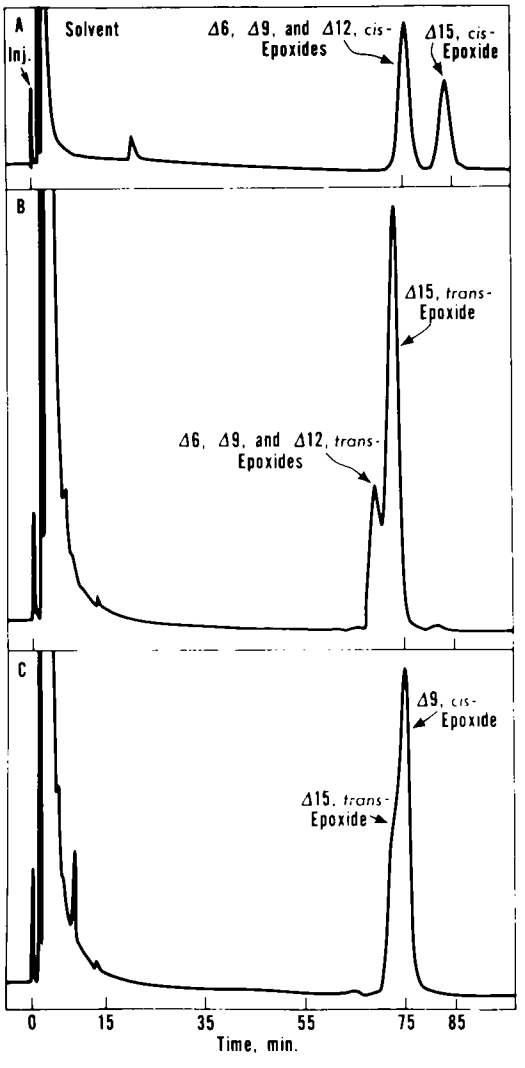



FIG. 2. Separation of epoxidized methyl octadecenoate positional isomers by an 11 ft 10% EGSS-X column. Gas chromatographic conditions same as in Figure 1.

impossible by IR owing to the difference in the carbon-hydrogen and carbon-deuterium force constants (3).

Capillary GLC is the only high temperature GC method that will separate *cis* and *trans* $C_{18}$-methyl esters. Unfortunately *cis* and *trans* analysis of octadecenoates by capillary GC is not possible when the samples contain certain positional isomers. When Δ12 and higher *cis* and *trans* positional octadecenoate isomers are present some of the *trans* positional isomers overlap with certain *cis* positional isomers which prevents reliable *trans* analysis (4).

Recently short chain, $C_7$ to $C_{10}$, *cis*- and *trans*-epoxides have been separated on ordinary packed columns at temperatures of 210 C or less (5). More recently *cis*- and *trans*-epoxyoctadecanoates and mixtures of geometric diepoxyoctadecanoate isomers have been successfully separated on a conventional column packed with 10% EGSS-X (6). The octadecenoate and octadecadienoate isomers were stereospecifically epoxidized with peracetic acid, and the resulting epoxyoctadecanoates and diepoxyoctadecanoates were quantatively analyzed by GLC.

TABLE I

Analysis of Standard Oleate-Elaidate Mixtures

| Per cent elaidate | | | | |
|---|---|---|---|---|
| | | Gas liquid chromatography[b] | | |
| Weight | IR[a] | Run 1 | Run 2 | Average |
| 2.04 | — | 2.2 | 2.0 | 2.1 |
| 4.30 | — | 4.0 | 4.3 | 4.15 |
| 11.07 | 11.7 | 11.5 | 12.3 | 11.9 |
| 50.62 | 51.2 | 51.3 | 52.1 | 51.7 |
| 77.73 | 79.9 | 77.5 | 76.1 | 76.8 |
| 91.06 | — | 92.0 | 90.5 | 91.2 |

[a]Average of three runs.

[b]Standard deviation based on 12 gas chromatographic analyses = 0.8.

This paper further explores the use of conventionally packed GC columns for analysis of epoxidized geometric octadecenoate and octadecadienoate isomers. Various positional octadecenoate isomers were studied in order to detect possible peak overlap between mixtures of *cis* and *trans* positional octadecenoate isomers. The accuracy, reproducibility and limitations of the procedure are demonstrated. A convenient experimental procedure for epoxidizing the samples is described in full detail, and results obtained with epoxidizing reagents other than peracetic acid are evaluated.

## EXPERIMENTAL PROCEDURES

### Methyl Octadecenoate Standards and Mixtures

Pure samples of methyl oleate and methyl elaidate were purchased from The Hormel Institute. Various standard oleate-elaidate mixtures were prepared by mixing weighed portions of these pure standards.

Octadecenoate mixtures containing *cis* and *trans* isomers having the double bonds scattered from the 6 to 17 position were obtained from copper-chromite hydrogenated methyl linolenate, soybean methyl esters and linseed methyl esters (7). Percentage *trans* in the monoene fraction of these samples was determined by IR

analysis (8). The double bond positions were determined by ozonolysis (9).

#### Methyl Octadecadienoate Standards

Methyl *trans*-9,*trans*-12-octadecadienoate and methyl *cis*-9,*trans*-12-octadecadienoate were prepared by countercurrent distribution (10) and further purified by silver resin chromatography (11). The methyl *cis*-9,*cis*-15-octadecadienoate and methyl *cis*-12,*cis*-15-octadecadienoate were prepared as previously described (10,12).

#### *Peracetic Acid*

Peracetic acid (~5%) was prepared by mixing 5 ml reagent grade acetic anhydride with 1 ml 30% hydrogen peroxide, stirring the mixture for ca. 3 hr at room temperature and allowing it to stand at 3 C for 16 hr (13). The peracetic acid was stored at -25 C and was stable for a minimum of 3 weeks.

Commercial 40% peracetic acid was diluted with glacial acetic acid to make a 6% peracetic acid solution; however this diluted peracetic acid gave several unknown side products and lower yields of the desired epoxides.

#### Epoxidation Procedures

*Monoenes:* The monoenes were epoxidized by mixing 50 μliters peracetic acid with 1 μliter monoene and allowing the mixture to stand at room temperature for 2-6 hr (13). *cis*-Monoenes are completely epoxidized after ca. 3 hr, but *trans*-monoenes require up to 6 hr for complete epoxidation. The samples should be analyzed as soon as possible to minimize side products, but samples can be frozen at -25 C for up to 24 hr without seriously affecting the analysis. Previous work has shown that side products are formed at a rate of less than 1% per hr at 25 C (13). The formation of the side products, hydroxy-acetoxy octadecanoate, can be followed by periodically sampling the reaction mixture and chromatographing at 210 C on a 4 ft x 1/4 in. column packed with QF-1. The hydroxy-acetoxy octadecanoate is well separated from the epoxide, and the rate of appearance of this side product was confirmed to be ca. 1%/hr. The QF-1 column can also be used to quickly determine when over 95% epoxidation has been achieved. Significant amounts of hydroxylated and acetylated side products are formed during prolonged storage at -25 C. If it is necessary to store the epoxides for a long time, they should be extracted with petroleum ether (PE) and the extract washed several times with water to remove the acetic acid.

*Dienes:* The dienes were epoxidized by mixing 100 μliters peracetic acid with 1 μliter diene and allowing the mixture to stand at room temperature for 6-8 hr (14). The reaction mixture can then be analyzed directly by GLC, or it can be extracted by mixing with 0.5 ml of PE and 5 ml of $H_2O$. The PE extract should be washed with water to remove any acetic acid.

TABLE II

Analysis of Monoenes from Copper Hydrogenated Fats

| Monoene source | Double bond positions[a] | Per cent *trans* IR | Per cent *trans* GLC[b] |
|---|---|---|---|
| HSBO[c] | 7 to 16 | 31.9 | 34.7 |
| HSBO | 6 to 17 | 63.4 | 68.0 |
| HLSO[d] | 6 to 17 | 40.4 | 43.9 |

[a]Double bond positions determined by reductive ozonolysis.

[b]Gas liquid chromatography.

[c]HSBO = hydrogenated soybean methyl esters.

[d]HLSO = hydrogenated linseed methyl esters.

#### GC Analysis

A Packard 7400 series gas chromatograph equipped with a flame ionization detector was used for most of the GC analyses. An Aerograph gas chromatograph model 1520 also equipped with a flame ionization detector was used occasionally. The Packard gas chromatograph was equipped with coiled 1/8 in. glass columns packed with prestabilized or preconditioned 10% EGSS-X on Gas Chrom P (Applied Science), and the Aerograph instrument used a 1/8 in. copper column. Epoxidized *cis*- and *trans*-monoenes were separated on an 11 ft column, and epoxidized dienes were separated on a 4 ft column.

The packed columns were conditioned overnight at 200 C, cooled to 150 C and then silylated by injecting several 10 μliter portions of *N-O*-bis(trimethylsilyl)-acetamide (BSA) or *N-O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA). Silylation appeared to prevent small extraneous peaks, which occasionally were noted when the columns were not silylated.

GC operating conditions were: injector temperature, 205 C; detector temperature, 210 C; flow rate, 38 ml/min; column temperatures for epoxidized monoenes, 198 C, and for epoxidized dienes, 195 C.

The epoxidized monoenes were analyzed by directly injecting the reaction mixture without prior isolation of the epoxystearates. The epoxidized diene reaction mixtures could be directly analyzed, but the dienes were normally extracted with PE before GC analysis. In every sample analyzed the *trans* epoxides had shorter

TABLE III

Analysis of Epoxidized Synthetic Diene Mixtures

| Diene | Mixture 1 | | Mixture 2 | |
|---|---|---|---|---|
| | Wt % | GLC, %[a] | Wt % | GLC, %[a] |
| *trans*-9,*trans*-12 | 42.67 | 42.2 | 26.38 | 24.9 |
| *cis*-9,*trans*-12 | 14.65 | 15.7 | 56.18 | 57.0 |
| *cis*-9,*cis*-12 | 42.67 | 42.1 | 17.44 | 18.0 |

[a]Gas liquid chromatography percentages are an average of two analyses.

retention times than the *cis* epoxides.

### IR Analysis

Samples of epoxidized monoenes and dienes were collected from an Aerograph autoprep model A-700. The IR spectra of these samples were identical to the spectra previously reported (15). Discrete absorption bands at 11.2 $\mu$ for the *trans*-epoxyoctadecanoate and 12.0 $\mu$ for the *cis*-epoxyoctadecanoate were noted.

Standard *cis*- and *trans*-monoene mixtures were analyzed before epoxidation by the Official AOCS Tentative Method (8).

## RESULTS

### Monoenes

Previous work has shown that *cis*-9- and *trans*-9-epoxyoctadecanoate isomers could be separated by GLC using conventional packed columns (6).

The GC curve in Figure 1 extends this separation to an epoxidized monoene fraction from hydrogenated vegetable oil which contained $\Delta 7$ to $\Delta 15$ *cis* and *trans* positional isomers. The GC curve in Figure 1 indicates that quantitative *cis* and *trans* analysis of samples containing a mixture of positional octadecenoate isomers is possible. The sample analyzed in Figure 1 contained 40.4% *trans* positional isomers by IR analysis after correction for palmitate and stearate impurities. The GC analysis indicated 43.9% *trans* and was not hampered by traces of solvent, palmitate, stearate or other impurities in the monoene fractions, and therefore did not require any corrections. The sample contained several positional *cis* and *trans* octadecenoate isomers, and it will be shown later that the *trans*-15-octadecenoate isomer is responsible for the less than base line separation in Figure 1. The small peak which follows the two larger peaks in Figure 1 cochromatographed with an authentic sample of *cis*-15, 16-epoxyoctadecanoate.

A series of oleate-elaidate mixtures was epoxidized to demonstrate the stereospecificity

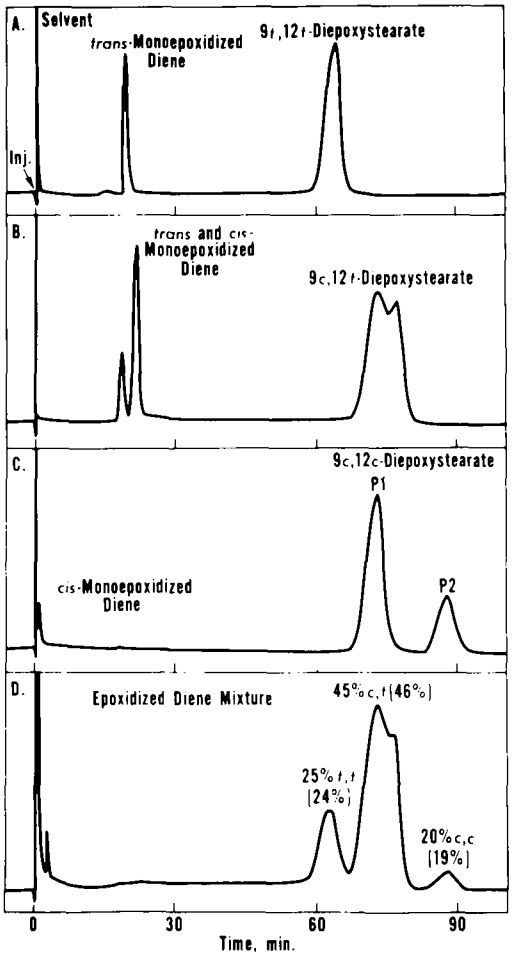



FIG. 3. Epoxidized methyl 9,12-octadecadienoate geometric isomers. Gas chromatographic conditions: column, 4 ft x 4 mm 10% EGSS-X on Gas Chrom P; column temperature, 194 C; He flow rate, 38 ml/min; injector temperature, 205 C; detector temperature, 210 C. Values in parentheses are weight percentages.

of the epoxidation reaction and its applicability to quantitative procedures involving a wide range of *trans* values. GC analyses of standard epoxidized mixtures are listed in Table I and compared with both the actual weight per cent and IR analyses. The response of the flame ionization detector to a weighed methyl vernolate (*cis*-9,10-epoxyoctadecanoate) sample was checked and found to correspond closely to a weighed methyl stearate sample. All samples were analyzed periodically during epoxidation. GC analysis of the mixtures before epoxidation was complete gave lower *trans* percentages, which indicated a slightly slower rate of epoxidation for methyl elaidate (16,17). Since the reaction rate for the *trans* bond is

slower than for the *cis* isomer, care must be taken to epoxidize at least 95% of the total sample for reasonably accurate quantitative work.

At room temperature, epoxyoctadecanoate reacts slowly with acetic acid to form acetoxy-hydroxyoctadecanoate. GC analysis of the reaction mixture using a QF-1 column indicated 2-4% of these side products were formed during normal reaction times. This confirmed previous work which reported this side reaction occurs at a rate of ca. 1%/hr (13). Samples should therefore be analyzed as quickly as possible after epoxidation is complete, and those that must be stored before being analyzed should be frozen at -25 C or extracted with PE to minimize side product formation.

The standard deviation for the *trans* percentages determined by GC analysis of the mixtures in Table I was ±0.8%. This value was based on 12 analyses and is significantly better than the standard deviation of ±1.1%, which was calculated for a series of 12 IR determinations.

The data in Table I demonstrate the accuracy of *trans* analysis by GLC for *cis*-9,10- and *trans*-9,10-epoxyoctadecanoate mixtures. The influence that positional isomers have on the accuracy of the *cis* and *trans* analysis was also investigated. Separation of the *cis*- and *trans*-epoxyoctadecanoates depends somewhat on which positional isomers are available (Fig. 2). The *cis*-6,7-, -9,10- and -12,13-epoxyoctadecanoates have similar retention times. The *cis*-15,16-epoxyoctadecanoate has a longer retention time and separates from the other *cis* epoxides as shown in Figure 2A. The *cis*-16,17- and *cis*-17,18-epoxyoctadecanoates would also be expected to have retention times as long as or longer than the *cis*-15,16-epoxyoctadecanoate.

The *trans*-epoxides behave similarly. The 6,7-, 9,10- and 12,13-*trans*-epoxyoctadecanoates have identical retention, but the *trans*-15,16-epoxyoctadecanoate is partially separated from the other *trans* epoxides and, at the same time, partially overlaps the first *cis*-epoxyoctadecanoate peak. In samples containing *trans*-15,16-epoxyoctadecanoate the resolution between the *cis*- and *trans*-epoxide peaks is partially reduced, and GC analysis for *trans* is slightly low owing to the *trans*-15,16-epoxyoctadecanoate peaks overlapping the *cis*-epoxyoctadecanoate peak. Fortunately, Δ15, Δ16 and Δ17 *trans*-octadecenoate isomers rarely compose a major portion of natural or hydrogenated samples, and thus contribute only a small error to the analysis. The accuracy of the GC method tested on samples containing substantial amounts of Δ15, Δ16 and Δ17 *trans*-octadecenoates is demonstrated in Table II.

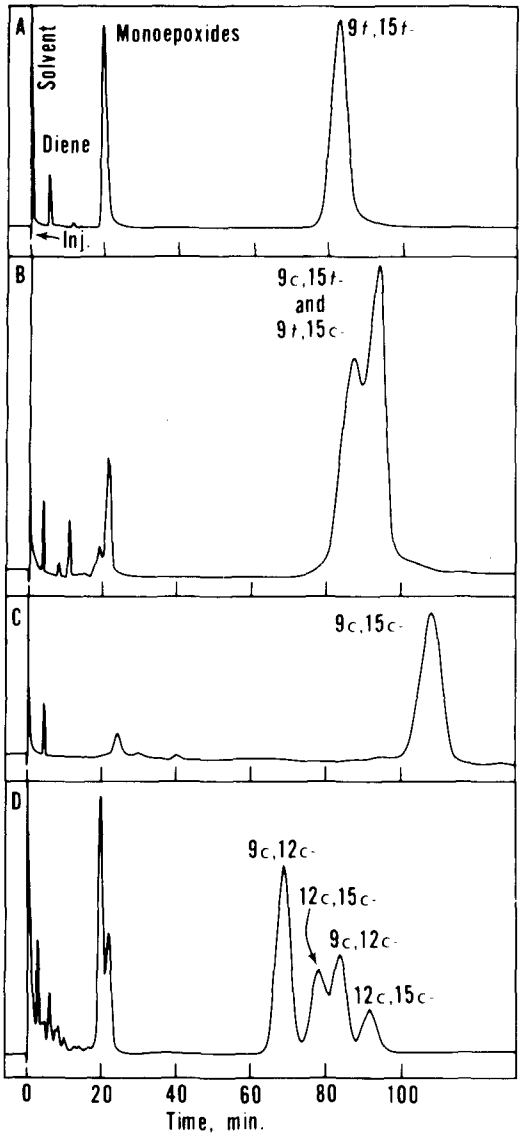



FIG. 4. (A) Epoxidized *trans*-9,*trans*-15-octadecadienoate; (B) mixture of epoxidized *cis*-9,*trans*-15- and *trans*-9,*cis*-15-octadecadienoate; (C) epoxidized *cis*-9,*cis*-15-octadecadienoate; (D) mixture of epoxidized *cis*-9,*cis*-12- and *cis*-12,*cis*-15-octadecadienoate. Gas chromatographic conditions are same as in Figure 3.

The samples analyzed in Table II were from copper-chromite hydrogenated vegetable fats and methyl linolenate (7). These samples contained from 3-10% Δ15, Δ16 and Δ17 *trans*-octadecenoate isomers. When GC analyses were compared with IR analyses, *trans* values were 2-4% higher, although the GC analyses are known to be slightly lower than the true *trans*

percentage. The lower *trans* percentages obtained by IR analysis are caused by the lower molar absorptivity constants for some of the various positional *trans* isomers (C.R. Scholfield, unpublished).

Initially, such aryl peracids as perbenzoic acid were used to epoxidize monoenes, but aryl acids had long retention times and interfered with the unepoxidized monoene peak. Using a peracetic acid-acetic acid mixture eliminated interference by the epoxidizing agent and allowed the entire reaction mixture to be analyzed without prior isolation of the epoxides. Other alkyl peracids, such as performic or trifluoroperacetic, cannot be used because they cause extensive destruction of the epoxide ring by hydrolysis and acylation.

**Dienes**

Various diene isomers and synthetic mixtures were epoxidized with peracetic acid in an attempt to determine if the geometric and positional diepoxyoctadecanoate isomers derived from various octadecadienoate isomers were capable of being resolved by GLC. The curves in Figure 3 show retention times for epoxidized *trans,trans*-, *cis,trans*- and *cis,cis*-9,12-octadecadienoates. Figure 3D is the GC curve for an epoxidized synthetic diene mixture, which contained weighed amounts of three 9,12-octadecadienoate geometric isomers. The epoxidized *trans*-9,*trans*-12-diene isomer has a shorter retention time than either the epoxidized *cis*-9,*trans*-12- or *cis*-9,*cis*-12-diene isomers.

Epoxidized *cis*-9,*trans*-12-octadecadienoate (Fig. 3B) shows two partially separated peaks. These two peaks are not due to a *cis*-9,*trans*-12-, *trans*-9,*cis*-12-diene mixture as the sample was entirely *cis*-9,*trans*-12-octadecadienoate. The best explanation for the two *cis*-9,*trans*-12-diepoxyoctadecanoate peaks is that they originate from two diepoxyoctadecanoate diastereomers (19). The *cis*-9,*cis*-12-diepoxyoctadecanoate sample (Fig. 3C) is also separated into two well resolved peaks (P1 and P2). These two peaks undoubtedly represent two *cis*-9,*cis*-12-diepoxyoctadecanoate diastereomers (19). The larger peak (P1) is eluted between the two *cis*-9,*trans*-12-diepoxyoctadecanoate peaks. Consequently, a mixture of epoxidized *cis*-9,*trans*-12- and *cis*-9,*cis*-12-dienes gives a single broad peak (Fig. 3D) which is actually a composite of three peaks. Fortunately the second *cis,cis* peak (P2) can be used to calculate the integral for the first *cis,cis* peak (P1) by assuming the ratio of P1/P2 remains constant.

The percentages for the diene mixture shown in Figure 3D were calculated as follows:

$I_{total}$ = Total integral

$$\%\ trans,trans = (I_{t,t} \div I_{total}) \times 100 \quad [1]$$

$$\%\ cis,cis = ([I_{P1(c,c)} + I_{P2(c,c)}] \div I_{total}) \times 100 \quad [2]$$

$$\text{Where } I_{P1(c,c)} = I_{P2(c,c)} \times \frac{P1}{P2} \quad [3]$$

$$\%\ cis,trans = 100 - (\%\ trans,trans + \%\ cis,cis) \quad [4]$$

The *trans,trans*-diene percentage is found by equation 1 in the usual manner simply by dividing the *trans,trans* integral ($I_{t,t}$) by the total integral ($I_{total}$). The *cis,cis*-diene percentage is determined by calculating the integral for the first *cis,cis*-diene peak [$I_{P1(c,c)}$] using equation 3. The $I_{P1(c,c)}$ integral is added to the $I_{P2(c,c)}$ integral and divided by $I_{total}$ to obtain the *cis,cis*-diene percentage (equation 2).

The data in Table III compare the percentages obtained by GC analysis of known synthetic diene mixtures. The lower accuracy of the diene determinations compared to the monoene analyses is the result of basing the diene calculations on the $P2_{(c,c)}$ peak and the $P1_{(c,c)}/P2_{(c,c)}$ ratio. The $P1_{(c,c)}/P2_{(c,c)}$ ratio varies depending on reaction conditions, and is a potential source of error. Nevertheless good results can be expected if a linoleate standard is epoxidized and the $P1_{(c,c)}/P2_{(c,c)}$ ratio is determined under the same reaction conditions as used for the diene unknown.

When the pure diene standards were periodically analyzed during epoxidation, the following characteristics of the epoxidation reaction were noted. The dienes were generally ca. 75% monoepoxidized before detectable amounts of diepoxyoctadecanoate were formed. Reaction rates for the diene isomers were in the following order: *cis,cis* > *cis,trans* > *trans,trans*. The differences in reaction rates between diene isomers are greater than those observed between monoene isomers. This difference in reaction rates requires that at least 95% of the diene mixture be epoxidized before quantitative analysis is attempted. If the sample is analyzed too soon, the diepoxide mixture will contain proportionately too much of the epoxidized *cis,cis* isomer. The second double bond in the diene was more difficult to epoxidize than the first double bond, and the total reaction times required to epoxidize a diene mixture completely were 7.5-8 hr at room temperature.

The GC curves in Figure 4 show the effect of double bond position on retention times for various epoxidized octadecadienoate isomers. Retention times for epoxidized 9,15-octadeca-

dienoate isomers are longer than for the same corresponding 9,12-octadecadienoate geometric isomers. The *cis*-9,10:*cis*-15,16-diepoxyoctadecanoate diastereomers (Fig. 4C) were not separated as were the *cis*-9,10:*cis*-12,13-diepoxyoctadecanoate diastereomers (Fig. 3D). The GC curve in Figure 4D is a mixture of epoxidized *cis*-9,*cis*-12- and *cis*-12,*cis*-15-octadecadienoates. The *cis*-12,13:*cis*-15,16-diepoxyoctadecanoate diastereomers are separated from each other and also from the *cis*-9,10:*cis*-12,13-octadecanoate diastereomers.

The sample of *cis,trans*-9,15-octadecadienoate was a mixture of *cis*-9,*trans*-15- and *trans*-9,*cis*-15-diene isomers. The two peaks in Figure 4B are assumed to correspond to these two isomers rather than to separate diastereomers. This assumption is made because the epoxidized *cis*-9,*cis*-15-diene gave a single peak. The epoxidized *trans*-9,*trans*-15-octadecadienoate sample also gave a single peak, which was expected since the *trans*-9,10:*trans*-12,13-diepoxy octadecadienoate diastereomers were not resolved.

The GC curves in Figures 3 and 4 show the retention times of only a few of the many possible geometric and positional diepoxyoctadecanoate isomers. A comparison of retention times demonstrates that epoxidized diene mixtures containing these geometrical and positional isomers would not be completely separated. However if the diene mixture was partially fractionated by other methods, then GC analysis of the epoxidized diene fractions could be quite useful.

The presence of small amounts of other saturated and unsaturated fatty esters does not interfere with diene analysis. The saturated and epoxidized monoene $C_{18}$-esters have much shorter retention times than do the epoxidized dienes. The $C_{18}$-trienes and conjugated dienes are apparently epoxidized, but reaction products do not elute at a column temperature of 200 C.

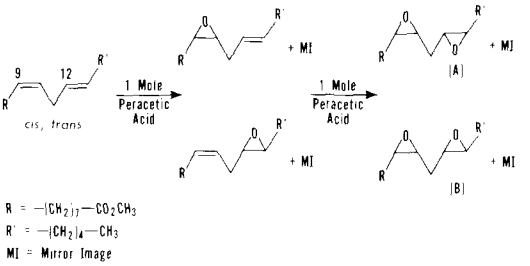
## DISCUSSION

In every example investigated, the epoxidized *trans* isomer had a shorter retention time than the epoxidized *cis* isomer. Stereochemical molecular models of the *cis* and *trans* epoxy isomers show the *trans* epoxide to be a relatively "straight" isomer compared to the "bent" *cis*-epoxide isomer, and which is probably more polar than the *trans*-epoxide isomers.

Experimentally the *trans*-epoxide appears to be the less polar of the two isomers, since it has a shorter retention time on the very polar EGSS-X column. The importance of polarity on the separation of the epoxide isomers is apparent when nonpolar columns are used. Columns packed with nonpolar liquid phases, such as OV-17, SE 30, QF-1 and OV-101, did not separate the *cis* and *trans* epoxy isomers. As more polar columns are used, such as Carbowax-20M, partial separation is achieved. Finally with the very polar EGSS-X column total resolution of the epoxide isomers is possible. These data can be explained by assuming that the differences in the stereochemical geometry of the epoxide isomers are reflected in their polarity.

Factors such as boiling point differences, molecular configuration or orientation as applied to the steric bulk of the molecule are not sufficient to permit separation by nonpolar GC columns, since separation on these columns is based mainly on differences in volatility.

As mentioned under Results, the two partially separated *cis*-9,10:*trans*-12,13-diepoxyoctadecanoate peaks in Figure 3B are not due to a mixture of *cis,trans*- and *trans,cis*-diene isomers. These two peaks are actually diastereomers, which are formed when the diene is epoxidized from opposite sides. The following reaction shows the formation of these diastereomers:




Inspection of stereochemical models of the diepoxyoctadecanoate diastereomers shown in the reaction indicates that isomer A and its mirror image have a spatially straighter orientation than isomer B and its mirror image. Based on previous *cis* and *trans* data, which indicate the straighter *trans* isomer has a shorter retention time, it appears that diastereomer A would have a shorter retention time than diastereomer B. As was observed with the *cis* and *trans* epoxides, these diastereomers could not be separated by nonpolar columns.

The *trans,trans*- and *cis,cis*-octadecadienoates can each form two pairs of diastereomers in a manner similar to the *cis,trans*-octadecadienoate example. For *trans,trans*-octadecadienoate, molecular models show that all the possible epoxide diastereomers have essentially identical spatial configurations. These

diastereomers were not separated under the GC conditions used.

The *cis,cis*-diepoxyoctadecanoate isomers from linoleate have been well studied, and two enantiomer pairs of diastereomers have been separated and their melting points determined (18). The solid isomer has a mp of 32-33 C, and the liquid isomer melts at 7.5 C. The physical constants for these isomers indicate a substantial difference in their physical configuration which would allow their separation by GC. The two enantiomer pairs of *cis,cis*-diepoxyoctadecanoate diastereomers are not formed in equal amounts (18). The ratio of the two diastereomer pairs (P1/P2), as shown in Figure 3C, varies and appears to depend on reaction conditions. The P1/P2 ratio probably varies because of a difference in the rate of formation of the two pairs of enantiomers rather than any type of equilibrium involving interconversion of the isomers.

## ACKNOWLEDGMENTS

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# Egg Plant Lipoxygenase: Isolation and Partial Characterization

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## ABSTRACT

Egg plant lipoxygenase EC 1.13.1.13 when purified on Ecteola cellulose was resolved into two active fractions with most of the activity in the first fraction (A). This fraction when further purified on Sephadex G200-120 had 20 times the specific activity of the crude material. It proved to be a single substance by electrophoresis and immunological technique. The pH optimum was 6.5. Its activity was specific for the *cis,cis*-1,4 pentadiene structure. There was no inhibition by cyanide, azide, EDTA or fluoride. Nordihydroguaretic acid, on the other hand, exhibited strong inhibition at 3 x $10^{-3}$M concentration. The specific antibody caused 50% inhibition.

Lipoxygenase (EC 1.13.1.13) catalyzes the oxidation of fatty acids such as linoleic acid, containing the *cis,cis*-methylene interrupted diene system, forming the conjugated *cis,trans*-diene hydroperoxide. This enzyme is known to occur in legume seeds, notably soybeans, and in some cereal grains and oil seeds (1). Its presence in various other plant tissue, including leaves, has also been reported (2-7). Purified preparations have been obtained from the soybean (8,9) and other legume seeds (10). To our knowledge, aside from a partially purified preparation obtained from potatoes (11), no purified enzyme has been obtained from non-legume sources. The work described below deals with the isolation and partial characterization of egg plant (solanum melongena) lipoxygenase, where the raw material is the whole fruit and not only the seed.

## MATERIALS AND METHODS

### Preparation of Egg Plant Homogenate

Five hundred grams egg plant cut into small pieces were homogenized in a Waring blendor, in 1 liter of cold 0.005 M phosphate buffer pH 6.5 containing 1% Triton X-100, which was added because Pinsky et al. (6) showed that the Triton more than doubled the lipoxygenase activity of the extract. The resulting homogenate was filtered through cheesecloth and was then centrifuged for 10 min at 13,000 rpm (20,000 g). The supernatant which contained all of the enzyme activity was again filtered through cheesecloth. The filtrate is the crude egg plant extract used in further experiments.

### Purification Procedure

Ecteola (Epichlorohydrin Triethanolamine) cellulose (Serva, Heidelberg, Germany) was rinsed with 0.2 M NaOH for 30 min. It was then rinsed with distilled water until the pH of the effluent was in the neutral range. The ecteola cellulose was then brought to pH 6.5 with the aid of 0.005 M $NaH_2PO_4$. After reaching this pH, the ecteola cellulose was again rinsed with 0.005 M phosphate buffer pH 6.5, and then introduced into a column 2 x 10 cm. For chromatography of the crude egg plant lipoxygenase, 50 ml of the egg plant homogenate prepared as described above was dialyzed for 12 hr against 0.005 M phosphate buffer pH 6.5 at 4 C (three changes of 4 liters each). The dialysate was applied to an ecteola cellulose column prepared as described above. Elution with 0.005 M phosphate buffer pH 6.5 (1-2 liters until the absorption of the eluant at 280 nm was zero) removed the detergent and inactive proteins. The column was then placed on a fraction collector. Increasing concentrations of phosphate buffer, pH 6.5, were then

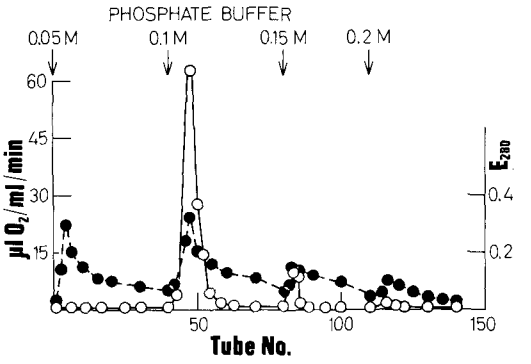


FIG. 1. Column chromatography of a triton extract of egg plant on ecteola cellulose. Fifty milliliters of the extract were adsorbed on an ecteola cellulose column 10 x 2 cm. The column was rinsed with 0.005 M phosphate buffer pH 6.5 to remove the detergent and inactive protein (no lipoxygenase), and elution was then by stepwise increase of buffer concentrations from 0.05 M to 0.1 M, to 0.15 M and finally 0.2 M of the above buffer. Fraction A eluted at 0.1 M buffer, B eluted at 0.15 M buffer. •—•, Protein E 280; ○—○, linoleate oxidation (polarographic method).

TABLE I

The Specific Lipoxygenase Activities and Yields of Egg Plant Extracts and Fractions Purified by Chromatography

| Fractions | Specific activity, μliters absorbed per min per mg protein | Total activity, % recovered |
|---|---|---|
| Crude extract ecteola cellulose eluant | 20 | 100 |
| Fraction A | 203 | 86 |
| Fraction B | 72 | 10 |
| Fraction A after sephadex G 200 | 400 | 86 |

applied, and a number of protein peaks were obtained with 6 ml fractions collected in each test tube (Fig. 1).

Fraction A was precipitated by 60% ammonium sulfate solution saturation. The precipitate, which was dissolved in 10 ml, 0.05 M phosphate buffer, pH 6.5, was placed on a Sephadex G-200-120 (Sigma, St. Louis, Mo.) column (4 x 80 cm) and was then eluted by the above buffer with 6 ml collected in each test tube (Fig. 2).

#### Assay of Enzyme Activity

Lipoxygenase activity of the egg plant fractions on linoleate was determined by three methods: (a) polarographic according to Grossman et al. (12); (b) spectrophotometric according to Ben-Aziz et al. (13); and (c) colorimetric, by measuring the formation of peroxides by the thyocyanate reaction according to Koch et al. (14). In all cases the linoleate was dissolved with the aid of Tween 20 according to Grossman et al. (12).

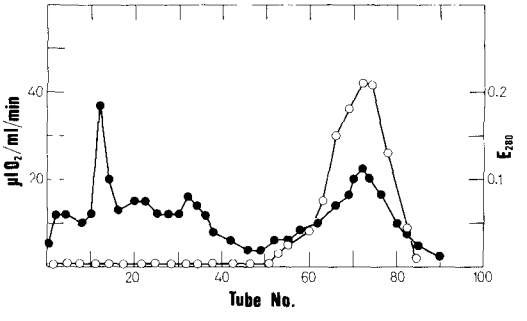


FIG. 2. Chromatography of fraction A from the ecteola cellulose eluate of egg plant lipoxygenase on sephadex G-200-120. The above fraction A was precipitated in 60% ammonium sulfate and then dissolved in 10 ml phosphate buffer 0.05 M pH 6.5. This solution was applied to the sephadex column 4 x 80 cm. Elution was with the above buffer. •—•, Protein E 280; ○—○, linoleate oxidation (polarographic method).

#### Protein Determination

Protein determination of the homogenates was according to Lowry et al. (15). The protein content of the purified fractions was based on UV absorption at 280 nm, the calculation being that an OD of 1.0 is equivalent to a protein concentration of 1 mg/ml.

#### Electrophoresis

Disc electrophoresis was carried with 7.5% polyacrylamide gel in Tris-glycine buffer at pH 8.6, as described by Davis (16). Then 150 μg samples of the ecteola cellulose purified fraction or of the sephadex purified enzyme were applied to each tube, and electrophoresis was allowed to run at room temperature for 2 hr, at 250 v and 5 mA per tube. The gels were stained with coumassie blue.

The active lipoxygenase fractions were identified by cellulose acetate gel electrophoresis in 0.3 M tris-glycine buffer pH 8.6 or in 0.3 M phosphate buffer pH 6.5, and stained according to Grossman et al. (17).

#### Immunization Procedure

Specific antibodies were obtained by hyperimmunization of rabbits with crude homogenate preparation of egg plant. Rabbits (3 months old) were immunized for 8 weeks by weekly injections into the leg muscles of 1 ml quantities of an emulsion of Freund and Bonanto's complete adjuvant (18) containing 2 mg of crude homogenate protein. At the 10th day after the last injection they were bled, and their sera were tested by immunodiffusion for the presence of antibodies against crude homogenate antigens and against the purified enzymatically active fraction. Sera were pooled and divided into 1 ml portions and stored at -20 C.

#### Isolation of γ-G-Globulin

The γ-G-globulin fraction of immune and normal rabbit serum was prepared as described

by Stelos (19). The globulin solutions were brought to the original serum volume by adding 0.15 NaCl and stored at -20 C.

#### Immunodiffusion

Double diffusion was carried out with 1% w/v agarose gel in 0.9% NaCl layered thinly (2 mm) on glass slides (7 x 7 cm). After development of the precipitin arcs, the slides were photographed.

#### Inhibitory Effect of Antibodies on Enzyme Activity

The antibodies were previously tested by the precipitin reaction so that the equivalence zone was known. Quantities of 150 μg purified enzyme were preincubated at room temperature for 1 hr, with increasing amounts of the corresponding antibodies in the antibody excess zone. The resulting antigen antibody complexes were tested for their ability to oxidize linoleate. Normal rabbit serum γ-globulin served as control.

#### Enzyme Insolubilization

Ten milliliters of ethylene diamine were reacted with 20 g agarose which had been treated with cyanogen bromide as described by Porath et al. (20). After stirring for 13 hr at 4 C the gel was washed by large quantity of 0.3 M sodium carbonate and then with distilled water. The gel was then freeze-dried. A 1.2 g sample was stirred with 20 ml sodium carbonate buffer pH 7.2 for 20 min at room temperature, and then 1.7 ml glutaraldehyde was added and the stirring continued for 3 hr. The gel was then centrifuged and washed three times with 40 ml aliquots of the above carbonate buffer pH 7.2. It was stirred for 4 hr at 4 C with 20 ml of the above buffer containing 100 mg of purified fraction A from egg plant. The gel was then centrifuged and washed three times with 40 ml aliquots of the above buffer.

## RESULTS

#### Purification of Egg Plant Lipoxygenase

A typical purification procedure is summarized in Figures 1 and 2 and Table I. Two peaks (A, 0.1 M and B, 0.15 M) exhibiting linoleate oxidizing activity were found by ecteola cellulose chromatography. Figure 1 shows that most of the linoleate oxidizing activity was in peak A. The yields and specific activities of the ecteola column eluates and of fraction A after molecular filtration are given in Table I.

It may be seen that the specific activity of fraction A was 10-fold that of the crude extract. This specific activity (fraction A) was three times that of fraction B. The total yield of both peaks was ca. 96% of the original crude extract. Further purification of fraction A on sephadex G-200-120 is shown in Figure 2. It may be seen that inactive protein was first eluted, and then the active lipoxygenase was

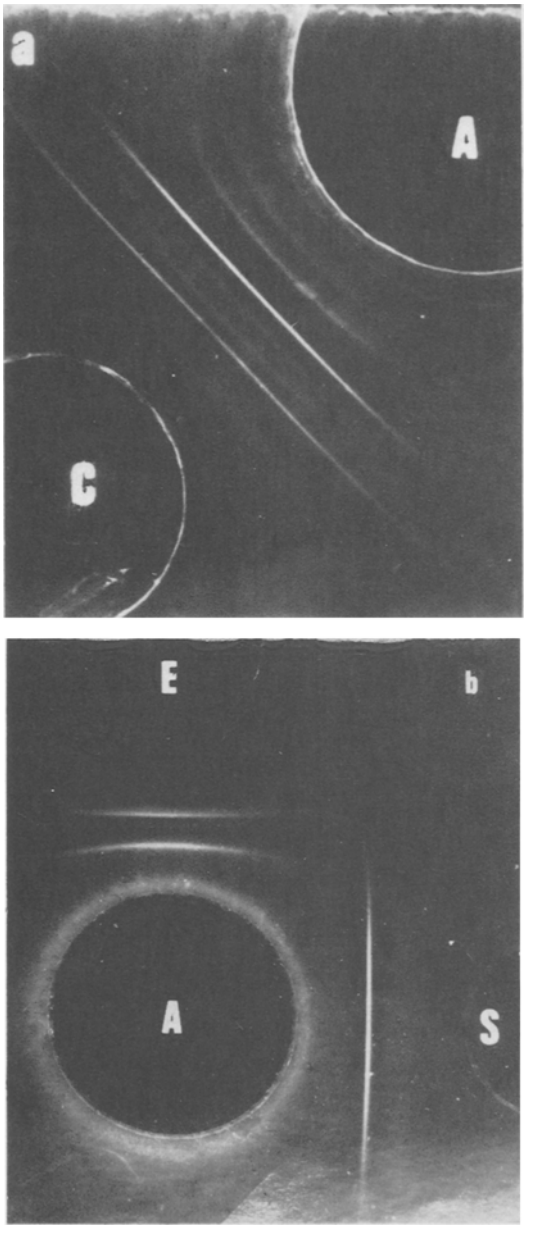


FIG. 3. Immunological identification of the purity of the egg plant lipoxygenase after the various stages of purification. (a) A – Egg plant antibody; C – crude extract. (b) A – Egg plant antibody; E – ecteola cellulose eluate (fraction A); S – sephadex eluate (fraction A from ecteola cellulose).

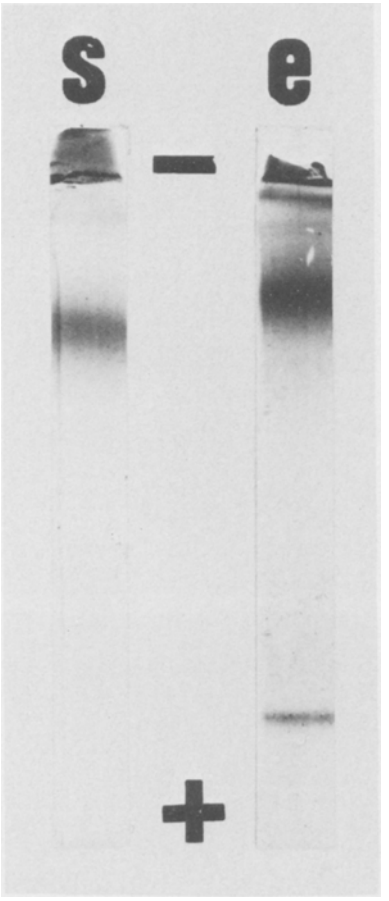


FIG. 4. Protein staining of egg plant lipoxygenase fraction A after acrylamide gel electrophoresis as described in methods. *e* – After ecteola cellulose chromatography; *s* – after sephadex G-200-120 molecular filtration.

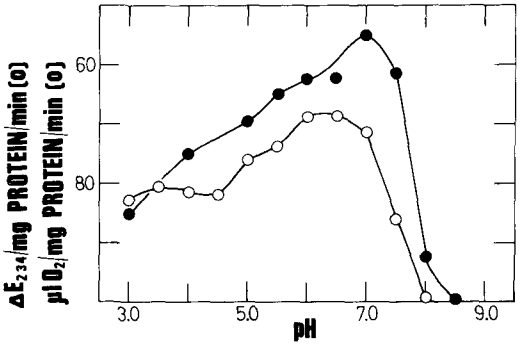


FIG. 5. The effect of pH on egg plant lipoxygenase activity. Purified fraction A of the ecteola cellulose eluate was used. The buffer was 0.2 M citrate phosphate. •—•, Spectrophotometric determination E 234; o—o, polarographic determination.

TABLE II

Oxygen Consumption of Egg Plant Lipoxygenase on Various Substrates

| Substrate[a] | μLiters $O_2$ absorbed per mg enzyme per min |
|---|---|
| Oleic acid | 0 |
| Linoleic acid | 216 |
| Linolenic acid | 220 |
| Linoleic acid methyl ester | 45 |
| Linoleic acid ethyl ester | 18 |
| Dilinolein | 48 |
| Trilinolein | 36 |

[a]The substrates used were purchased from Fluka and were 98% pure.

eluted giving a peak approximately parallel to the protein peak. The peak fraction had a specific activity of 400 μliters $O_2$ per mg protein per min (20-fold enrichment).

#### Antigenic Properties

At least six precipitin lines, which indicate the existence of corresponding allied antigens, could be discerned when egg plant homogenate was reacted against its antibodies (Fig. 3A). After the ecteola purification stage the precipitin lines reduced to essentially two lines with a few allied impurities. After the sephadex stage a clear major line appeared (Fig. 3B).

#### Electrophoresis

Polyacrylamide gel electrophoresis at pH 8.6 of fraction A after ecteola cellulose chromatography gave three protein bands which reduced to one major band with a minor contaminant after sephadex gel filtration (Fig. 4). Cellulose acetate gel electrophoresis at pH 6.5 and at pH 8.6 gave similar results. The broad band contained the active enzyme.

#### Enzyme Stability

The purified enzyme in 0.2 M buffer pH 6.5 was stable for 1 week at 4 C. Freezing caused a pronounced reduction in activity, the reduction being 40% after 1 day and 65% after 5 days. When the enzyme was precipitated by 80% saturated ammonium sulfate and the precipitate was dried by evaporation in a stream of cold air, the resulting powder was stable at 4 C for at least 1 month. It did not lose any activity at room temperature for 24 hr (during the experiments performed).

#### Effect of pH

Figure 5 shows the effect of pH on the linoleate oxidizing activity of the purified fraction. A broad maximum between pH 5.5 and 6.5 was obtained when the enzymic activ-

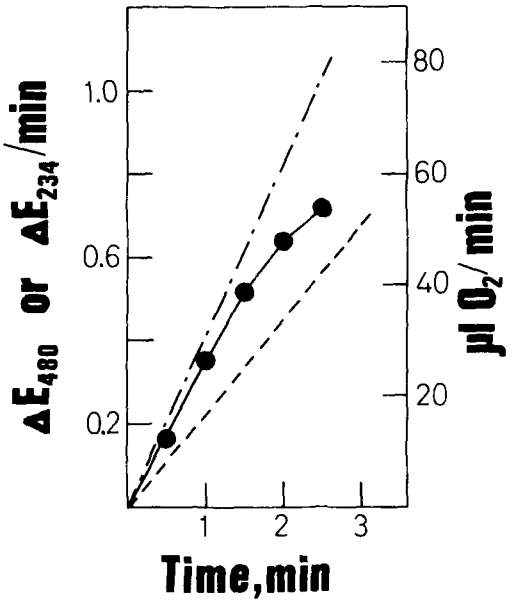


FIG. 6. The oxidation of linoleate by egg plant lipoxygenase in 0.2 M phosphate buffer pH 6.5. –•–•–, Diene production measured at 234 nm; •—•, hydroperoxide production measured by the thiocyanate method at 480 nm; – – –, oxygen consumption; 5 μg enzyme was used for the first two and 50 μg for the final method.

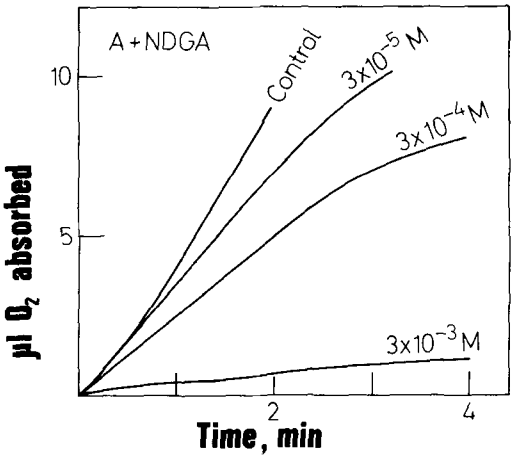


FIG. 7. The oxidation of linoleate by egg plant lipoxygenase in the presence of NDGA. The determination was by the polarographic method with the reaction in 0.2 M phosphate buffer pH 6.5. The NDGA was dissolved in ethanol such that the final concentration of the ethanol in the reaction medium was 4% for 20 μg of enzyme.

ity was measured by oxygen absorption. The determination of conjugated dienes at 234 nm gave a similar pH activity curve, but with a pH optimum ca. 7. Each point represents the mean of at least five determinations where the variations were negligible.

#### Substrate Specificity

The activity of the purified lipoxygenase (fraction A) on a variety of substrates is given in Table II. Of the substrates tested, the greatest activity was on linoleic and linolenic acid, containing the *cis,cis*-1,4 double bond. Much less activity was noted for linoleic acid esters (less than 25%), methyl, ethyl or glyceryl. It may be noted that there was no activity on oleic acid which does not contain the *cis,cis*-1,4 pentadiene system.

Figure 6 shows that each of the three methods described above (polarographic, spectrophotometric or colorimetric) could be used to follow the oxidation of linoleate by the purified enzyme of egg plant.

#### Inhibition Studies

No significant lipoxygenase inhibition was noted when $NaN_3$, NaF, EDTA or NaCN at a final concentration of $10^{-3}$M was added.

Figure 7 shows that an alcoholic solution of nordihydroguaretic acid (NDGA) at 3 x $10^{-3}$M or a higher concentration was a strong inhibitor of the purified enzyme. The per cent inhibition diminished progressively with decrease in the NDGA concentration below 3 x $10^{-3}$M.

Ascorbic acid, on the other hand, showed no lipoxygenase inhibition at 2 x $10^{-3}$M as determined by $O_2$ uptake.

#### Inhibitory Effect of Antibodies

It was found that specific antibodies when present in excess after incubation with the purified enzyme, inhibited only 50% of the linoleate oxidation, as compared to similar concentrations of antibodies from normal rabbit serum which did not influence enzyme activity.

#### Effect of Enzyme and Substrate Concentrations

A linear relationship between the concentration of purified fraction A and linoleate oxidation was found as can be seen from Figure 8. The activity was determined polarographically as described in methods.

A plot of $\frac{1}{V}$ against $\frac{1}{S}$ according to Lineweaver and Burk gave a Km of 7.6 x $10^{-4}$M for this enzyme (Fig. 9).

#### Activity of the Insoluble Enzyme

The eluant, when linoleic acid was percolated through a column of egg plant lipoxygenase insolubilized on agarose as described in methods, showed a UV absorption curve with a peak of 235 nm and a minor peak at 280 nm.

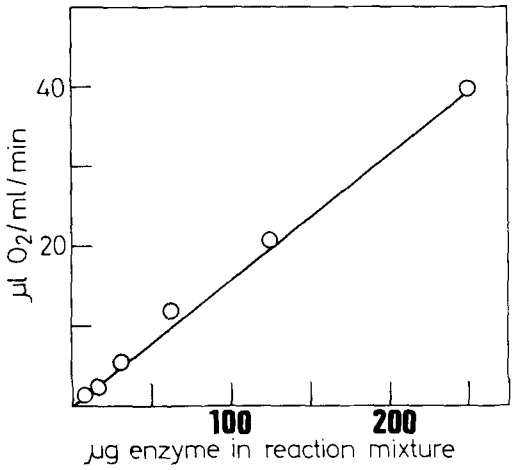


FIG. 8. The effect of the purified egg plant lipoxygenase concentration on linoleic acid oxidation as measured polarographically; the method as described.

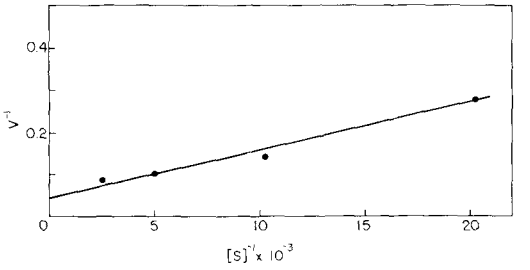


FIG. 9. Lineweaver Burk plot for the kinetics of the purified egg plant lipoxygenase activity. $s$ – linoleic acid concentration; $v$ – μliters $O_2$ per ml enzyme per min.

## DISCUSSION

The results described above show that egg plant contains two fractions A and B, which can oxidize linoleic acid. Most of the activity was in fraction A. The purity of this fraction was shown by immunologic and electrophoretic techniques. Similar to the soybean lipoxygenase isoenzymes, it was not inhibited by relatively high concentrations of NaCN or $NaN_3$, indicating that it does not contain any metal groups as is the case for the hemeproteins. This experiment provides useful information for distinguishing between the linoleate oxidizing activity of heme proteins and lipoxygenase. Whereas lipoxygenase activity is not inhibited by the above reagents, hemeproteins, like cytochrome c, peroxidase or catalase were inhibited (21). Similar findings were observed for the lipoxygenase from alfalfa (22), leaves (4) and potato (7). Egg plant lipoxygenase showed no activity towards oleic acid resembling soybean lipoxygenase isoenzymes in this respect.

The egg plant enzyme (fraction A) resembles soybean isoenzyme 1 (23) in its greater activity towards the free acids as compared to the esters.

The high specific activity of the egg plant lipoxygenase (fraction A) on linoleate, resembles that of the soybean (450 μliters $O_2$ per mg enzyme per min for purified Fluka commercial lipoxygenase determined in this laboratory) and the alfalfa enzymes (300 μliters $O_2$ per mg enzyme per min for fraction B and 651 μliters $O_2$ per mg enzyme per min for fraction C [12]). This specific activity was considerably reduced when hemeproteins were tested as oxidizing agents, especially when the formation of conjugated dienes was measured (13). Moreover, whereas the linoleate oxidizing activity of hemeproteins was markedly inhibited by comparatively high concentration of the same hemeprotein (24), such inhibition was not observed when the egg plant lipoxygenase concentration was raised in the same manner (Fig. 8).

The results showed a linear relationship between linoleate oxidation and concentration of fraction A. Tappel (1) noted that this straight line relationship is characteristic of lipoxygenase, whereas the curve obtained when hemeproteins are used is a straight line only when the oxidation of linoleate is plotted against the square root of the heme protein concentration. Our previous work has shown the same relationship both for lipoxygenase and for heme protein (22).

NDGA at 3 x $10^{-3}$M greatly inhibits linoleate oxidation by egg plant lipoxygenase. This effect is similar to that noted for soybean lipoxygenase. However we must add that this antioxidant inhibits heme protein as well.

The pH optimum for the oxidation of the linoleate by the purified enzyme is in the range of 5.5-6.5. This optimum is similar to that found for lipoxygenase from leaves, potatoes and alfalfa. The egg plant lipoxygenase differs from the others in its relatively high activity at acid pH's down to 3.0.

It appears that the optimum pH for the formation of conjugated dienes is 7.0 for this enzyme. This optimum differs from the optimum determined by oxygen consumption. The dependence of the pH optimum on the assay method was pointed out for soy lipoxygenase (1).

Blain et al. (25) has cast doubts on the presence of lipoxygenase in some plant tissues. Our work has shown that the oxidation of linoleic acid by the enzyme described by us could not have been due to hemeprotein, as emphasized in the above discussion.

That lipoxygenase activity of this fraction was not completely inhibited by its antibodies even in excess could indicate that the antigenic sites differ from catalytic sites and are relatively distant from it.

ACKNOWLEDGMENT

Z. Goldweitz gave technical assistance.

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# Fatty Acid Composition of the Sterol Ester Fraction of Human Adrenal Cortex in Cushing's Syndrome and After Treatment with Aminoglutethimide[1]

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## ABSTRACT

The lipid that accumulated in the adrenal cortex of a patient who had been treated with aminoglutethimide has been compared with the lipid in normal human adrenal cortex and identified as esters of cholesterol with fatty acids. While the concentration of free cholesterol was normal, that of esterified cholesterol was three times greater than that in normal controls. Sterols were analyzed by gas chromatography and found to consist almost wholly of cholesterol. The fatty acid composition of the cholesterol esters in adrenal cortex from patients with abnormal steroid secretion rates was determined. An increased proportion of cholesteryl arachidonate (cholesteryl-3β-[all *cis*]-eicosa-5,8,11,14-tetraenoate) was found in adrenal cortex from patients with decreased steroid secretion rates and a decreased proportion in adrenal cortex from patients with steroid secretion rates raised sufficiently to cause Cushing's Syndrome.

## INTRODUCTION

Aminoglutethimide is an inhibitor of corticosteroid biosynthesis in vivo (1-4) and in vitro (1,5,6); evidence has been presented that it acts by inhibiting the conversion of cholesterol to

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TABLE I

Separation and Recovery of Cholesterol and Cholesterol Esters by Thin Layer Chromatography on Silica Gel

| | $R_f$ of area recovered | $R_f$ of pure compound[a] | Principal components | $^3H$, dpm | Recovery, % | $^{14}C$, dpm | Recovery, % |
|---|---|---|---|---|---|---|---|
| Chromatography in benzene-chloroform 4:1[b] | | | | | | | |
| A | 0-0.11 | 0.054 (cholesterol) | Cholesterol, phospholipids | 209800 | 97.2 | 11 | — |
| B | 0.11-0.45 | 0.39 (tripalmitin) | Triglycerides | 96 | — | 20 | — |
| C | 0.45-0.75 | 0.69 (cholesteryl stearate and oleate) | Cholesterol esters | 107 | — | 144300 | 99.0 |
| D | 0.75-1.0 | — | — | 0 | — | 13 | — |
| Rechromatography of extract of section A in ethyl acetate-hexane 3:7[c] | | | | | | | |
| A′ | 0-0.20 | — | Phospholipids | 215 | — | 179 | — |
| B′ | 0.20-0.45 | 0.36 (cholesterol) | Cholesterol | 210400 | 97.5 | 57 | — |
| C′ | 0.45-10. | — | — | 7 | — | 21 | — |

[a]Marker compounds were detected by spraying with sulfuric acid-ethanol 1:1 and heating at 100 C for 5 min.

[b]Cholesteryl [1-$^{14}$] stearate (145700 dpm) and [7-$^3$H] cholesterol (215800 dpm) were added to the lipid extract from 0.51 g normal human adrenal cortex and chromatographed on thin layers of silica in the system benzene-chloroform 4:1. Sections of the chromatogram were recovered and extracted.

[c]The extract of the $R_f$ 0-0.11 section (A) from the first chromatogram was rechromatographed on silica in the system ethyl-acetate-hexane 3:7.

TABLE II

Columns and Conditions for Gas Liquid Chromatography[a]

| No. | Length m | Internal Diameter mm | Stationary Phase[b] | % Coating by Weight | Support[b] | Mesh of Support | Operating Temp.[c] | Carrier & Flow ml/min | Gas Rate |
|---|---|---|---|---|---|---|---|---|---|
| 1)[d] | 1;5 | 4 | OV1 | 3 | Gas Chrom Q | 100/120 | 250 | $N_2$ | 40 |
| 2)[e] | 1.35 | 5 | EGSSX | 10 | Gas Chrom P | 100/120 | 180 | $N_2$ | 40 |
| 3)[e] | 1.35 | 5 | EGSPZ | 3 | Gas Chrom Q | 100/120 | 180 | $N_2$ | 40 |
| 4)[e] | 2.85 | 3.5 | EGSPZ | 0.5 | Corning GLC 110[f] | 120/140 | 170 | He | 20 |

[a]*Use:* 1) *trimethylsilyl* ethers of cholesterol and related sterols; 2) fatty acid methyl esters with up to 20 carbon atoms; 3) fatty acid methyl esters with 20 or more carbon atoms; and 4) fatty acid methyl esters in the gas chromatograph-mass spectrometer.

[b]Stationary phases and supports were obtained from Applied Science.

[c]In all cases the detector temperature was 300 C. The separator of the gas chromatograph-mass spectrometer was at 250 C. The injection temperature was 50 C above the column operating temperature.

[d]Column 1 was conditioned by heating from 100-300 C at 1 C/min and maintained at 300 C for 15 hr.

[e]Columns 2-4 were conditioned by heating from 100-220 C at 1 C/min, at 220 C for 15 hr and then at 225 C for 15 hr. The 15 cm of the column at the detector end was then filled with 3% OV1 on 100/120 mesh Gas Chrom Q which had been conditioned separately at 250 C. This procedure produced columns of low background (bleed) suitable for use in the gas chromatograph-mass spectrometer.

[f]Siliconized etched glass beads.

pregnenolone (1,5,7). After its administration both to man and to other animals there is observed histologically an accumulation of lipid in the adrenal cortex similar to that described by Prader (8) as "congenital lipoid hyperplasia of the adrenal cortex" (1,3,5). A similar effect has been observed in the ovary (9,10). The lipid substance has been referred to as cholesterol (1,5,9,10) and its accumulation has been adduced as evidence that aminoglutethimide inhibits the first step in the conversion of cholesterol to steroid hormones. It is the purpose of this report to identify this lipid and to compare it with the lipids of normal adrenal cortex.

In comparison with other tissues, the fatty acids esterified with cholesterol in rat adrenal glands (11,12) and testes (13,14) contain unusually high proportions of long chain polyunsaturated acids. Their proportions are affected by hypophysectomy (14) and by stress (12), suggesting that they are involved in some way with steroidogenesis. Therefore the fatty acid composition of the adrenocortical cholesterol ester fraction was determined using adrenal glands from a number of patients with normal and abnormal steroid secretion rates.

The following nonstandard trivial names are used: aminoglutethimide, for 2-ethyl,2-(*p*-aminophenyl)glutarimide; glutethimide, for 2-ethyl,2-phenyl glutarimide; pregnenolone, for 3β-hydroxypregn-5-en-20-one; 20α-hydroxycholesterol, for cholest-5-ene-3β,20α-diol;20α,22R-dihydroxycholesterol, for 22R-cholest-5-ene-3β, 20α,22-triol; arachidonic acid, for *all cis*-eicosa-5,8,11,14-tetraenoic acid; linoleic acid, for *cis*-octadeca-9,12-dienoic acid; oleic acid, for *cis*-octadec-9-enoic acid; palmitic acid, for hexadecanoic acid; palmitoleic acid, for *cis*-hexadec-9-enoic acid; stearic acid, for octadecanoic acid; and ACTH, for adrenocorticotropic hormone.

## MATERIALS

### Adrenal glands

Adrenal #1: This gland was removed surgically from a patient with Cushing's Syndrome due to a pancreatic tumor that secreted an ACTH-like substance. She had been treated with aminoglutethimide for over 6 weeks before adrenalectomy, for the final 3 weeks at a dose of 2 g per day. This resulted in marked clinical improvement and reduction of cortisol secretion rate to only slightly above normal; however plasma ACTH concentration as measured by radioimmunoassay (15) remained extremely high. The adrenal gland was grossly hyperplastic and weighed 15 g. It was yellow and fatty in appearance, and histological examination showed that the cortex consisted of predominantly clear, lipid-laden cells with a few areas of compact cells consistent with previous reports of the effects of aminoglutethimide (3).

Adrenal #2: This adrenal gland was obtained post mortem from a patient who had been treated with large doses of glutethimide and who had suffered from acute porphyria. Histologically this gland appeared normal.

Adrenals #3 and 4: These glands, which were essentially similar, were obtained surgically from two patients with Cushing's Syndrome; histological examination showed nodular hyperplasia of the adrenal cortex (16).

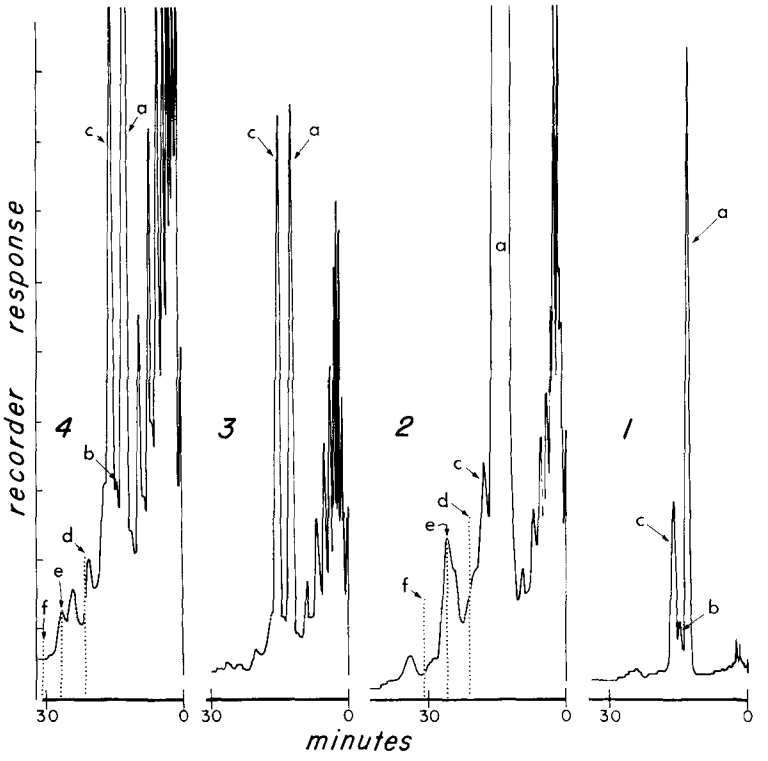


FIG. 1. Gas chromatographs of sterol fractions of human adrenal cortex as trimethylsilyl ethers: 1) trimethylsilyl ethers of sterol fraction of adrenal cortex #1 from a patient who had been treated with aminoglutethimide (+ *n*-dotriacontane); 2) the same as 1) but a larger sample; 3) trimethylsilyl ethers of sterol fraction of adrenal cortex #6 (normal) (+ *n*-dotriacontane); 4) the same as 3) but a larger sample. Details of the adrenal glands are given in the Materials section. *n*-Dotriacontane was added as an internal standard. Key to peaks: (a) cholesterol 3β-trimethylsilyl ether; (b) unidentified peak; (c) *n*-dotriacontane (internal standard). Expected retention time for: (d) 20α-hydroxycholesterol 3β,20α-bis(trimethylsilyl ether); (e) 20α,22R-dihydroxycholesterol 3β,22R-bis(trimethylsilyl ether); (f) 20α,22R-dihydroxycholesterol 3β,20α,22R-tris (trimethylsilyl ether).

Adrenal #5: This gland was obtained surgically from a patient with a functioning adrenal carcinoma and who had elevated corticosteroid secretion rates but no other symptoms of Cushing's Syndrome. The nontumorous part of the adrenal cortex used in these experiments was atrophic.

Adrenals #6-9: These glands were obtained surgically and were histologically normal.

Adrenal glands are only removed from patients who have some serious medical problem and a strictly "normal" adult human adrenal that is not "stressed" cannot be obtained at operation. Thus the use of "normal" for the controls in these experiments only implies that there was no known abnormality of steroid biosynthesis and that the histology was normal.

### Chemicals

Pure fatty acid methyl esters were obtained from Applied Science and from the Hormel Institute (University of Minnesota). [7-$^{3}$H] Cholesterol and cholesteryl [1-$^{14}$C] stearate were obtained from the Radiochemical Center and from New England Nuclear and were purified by thin layer chromatography before use. 20α-Hydroxycholesterol was prepared by condensation of pregnenolone tetrahydropyranyl ether (17) and 4-methylpentyl magnesium bromide by a method similar to that described by Petrow and Stewart-Webb (18). 20α,22R-Dihydroxycholesterol was a gift of the Syntex Corporation. Merck Silica Gel H (without binder) was used for thin layer chromatography.

## METHODS

The adrenal glands were dissected free of adipose tissue and the cortex was separated from the medulla and the capsule. The cortical tissue was weighed, chopped and homogenized with water (5 volumes) and extracted twice with chloroform-methanol 2:1 v/v (10 volumes)

TABLE III

Cholesterol and Cholesterol Ester Content of Normal and Pathological Adrenal Cortex

| Adrenal glands | Free cholesterol, mg/g tissue | Esterified cholesterol, mg/g tissue | Total cholesterol, mg/g tissue | Per cent free |
|---|---|---|---|---|
| Controls[a] | | | | |
| #6 | 5.99 | 32.12 | 38.11 | 15.7 |
| #7 | 4.71 | 40.26 | 44.97 | 10.4 |
| #8 | 6.30 | 26.45 | 32.75 | 19.2 |
| #9 | 4.36 | 34.49 | 38.85 | 11.2 |
| Mean for controls | 5.34 | 33.33 | 38.67 | 14.1 |
| Abnormal[a] | | | | |
| #1 (Aminoglutethimide treated) | 7.49 | 97.85 | 105.34 | 7.1 |
| #2 (Glutethimide treated) | 8.31 | 40.08 | 48.39 | 17.2 |

[a]Details of the adrenal glands are given in the Materials section.

and twice with ethyl acetate (10 volumes). The combined extracts were washed with 10 ml water, evaporated to dryness and stored under nitrogen at -15 C.

Portions of the lipid extract were separated by thin layer chromatography, and after location of marker compounds the plate was divided into segments. Cholesterol, cholesterol esters and triglyceride fractions were recovered quantitatively by extraction successively with chloroform, methanol and ether. The cholesterol fraction was rechromatographed. Details of chromatography are given in Table I.

The cholesterol ester fractions were hydrolyzed with 0.5N alcoholic KOH (0.5 ml) at 50 C in the dark under nitrogen for 24 hr with addition of a few drops of benzene to aid dissolution. After extraction the fatty acid fraction was esterified by adding a freshly prepared and distilled solution of diazomethane in ether. The cholesterol fraction was rechromatographed (Table I). Cholesterol was estimated spectrophotometrically by the method of Mann (19) using cholesterol (Sigma 99%) as standard.

For examination of the sterols present the tissue extract was saponified and then chromatographed as before. The zones corresponding in $R_f$ to cholesterol, 20$\alpha$-hydroxycholesterol and 20$\alpha$,22R-dihydroxycholesterol were recovered and extracted; but, owing to the large amount of cholesterol present, the latter two zones were contaminated with cholesterol. The dried extracts were treated with a mixture of dry pyridine: N,N-bis(trimethylsilyl)acetamide: trimethylchlorosilane 4:4:1 (reagents from Eastman-Kodak and redistilled) at 75 C overnight and then chromatographed on thin layers of silica in the system ethyl acetate-*n*-hexane 1:4 v/v in order to separate the trimethylsilyl ethers from the reactants (20). The products were recovered by extraction of the appropriate areas with dichloromethane, deposited on stainless steel gauzes and were exposed to the vapors of N,N-bis(trimethylsilyl)acetamide and pyridine overnight at 35 C in order to ensure complete silylation (21).

Gas chromatography was performed on a Pye model 104 and a Barber-Colman model A7579 gas chromatograph, both equipped with hydrogen flame ionization detectors. Combined gas chromatography-mass spectrometry was performed on an LKB Model 9000. All instruments were modified for solid sample injection (21). The columns and conditions used for gas chromatography are shown in Table II.

Mass spectra were recorded with the ion source at 270 C and ionizing energy 70 eV. Fatty acid methyl esters all gave very similar mass spectra; the m/e of the molecular ion gives the chain length and degree of unsaturation with some ambiguities which can be resolved by examination of retention times. The achievement of a gas chromatographic column using a polyester as stationary phase but with a sufficiently low rate of bleed (background) to permit use directly coupled to a mass spectrometer is noteworthy. Details are given in Table II.

Quantitation of fatty acid methyl esters using flame ionization detection was achieved by measuring peak areas by triangulation. Using the solid sample injection technique with weighed amounts of pure (>99%) standard fatty acid methyl esters the relation between the peak area and mass was linear and constant for fatty acid methyl esters containing 14-24

TABLE IV

Fatty Acid Composition of Cholesterol Ester Fraction of Human Adrenal Cortex Determined by Gas Liquid Chromatography of the Methyl Esters

| Fatty acid | $RR_t$[a] of standard | Observed $RR_t$ | Adrenal gland number[b] (mole %[c]) 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12.0 | 0.11 | 0.11 | 1.1 | 0.4 | 1.0 | 0.6 | 0.4 | 0.5 | 0.3 | 0.3 | 0.2 |
| 14.0 | 0.20 | 1.20 | 4.6 | 3.6 | 5.3 | 3.7 | 6.0 | 3.9 | 4.2 | 3.9 | 2.8 |
| 14:1(ω5) | 0.25 | 0.25 | 0.6 | 0.7 | 0.7 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.4 |
| 15:0 | 0.27 | 0.27 | 0.5 | 0.6 | 0.4 | 0.3 | 0.8 | 0.3 | 0.2 | 0.5 | 0.7 |
| | | 0.32 | 0.2 | 0.2 | 0.2 | 0.1 | 0.3 | 0.1 | 0.1 | 0.2 | 0.3 |
| 16:0 | 0.38 | 0.38 | 26.4 | 27.0 | 26.1 | 28.1 | 33.5 | 27.8 | 22.6 | 27.8 | 34.5 |
| 16:1(ω7) | 0.44 | 0.44 | 5.2 | 6.9 | 6.3 | 4.7 | 4.5 | 6.7 | 7.0 | 3.3 | 3.1 |
| | | 0.47 | 0.2 | 0.3 | 0 | 0 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| 17:0 | 0.52 | 0.52 | 0.5 | 0.7 | 0.4 | 0 | 0.6 | 0.3 | 0.4 | 0.6 | 1.0 |
| | | 0.59 | 0.5 | 0.6 | 0.4 | 0.4 | 0.5 | 0.4 | 0.4 | 0.3 | 0.4 |
| 18:0 | 0.71 | 0.71 | 6.8 | 7.7 | 6.9 | 6.1 | 7.4 | 6.8 | 5.8 | 11.1 | 11.6 |
| 18:1(ω9) | 0.81 | 0.81 | 40.1 | 43.7 | 41.3 | 46.1 | 33.9 | 40.9 | 47.5 | 39.3 | 30.0 |
| 18:2(ω6) | 1 | 1 | 4.3 | 1.7 | 6.2 | 4.9 | 3.9 | 3.5 | 3.4 | 3.8 | 3.5 |
| | | 1.14 | 0.1 | 0.3 | 0 | 0 | 0.2 | 0.3 | 0.2 | 0.2 | 0.3 |
| | | 1.19 | 0.1 | 0 | 0.2 | 0.1 | 0 | 0 | 0 | 0.1 | 0 |
| | | 1.28 | 0.2 | 0.1 | 0.2 | 0.4 | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 |
| 20:0 | 1.38 | 1.38 | 0.2 | 0.3 | 0.3 | 0.1 | 0.2 | 0.3 | 0.5 | 0.6 | 0.3 |
| 20:1(ω9) | 1.45 | 1.45 | 1.3 | 1.7 | 1.6 | 1.6 | 1.1 | 2.6 | 3.0 | 2.5 | 2.2 |
| | | 1.66 | 0.1 | 0.1 | 0 | 0 | 0.3 | 0.1 | 0.1 | 0.2 | 0.6 |
| 20:2(ω9) | 1.74 | 1.73 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.5 |
| | | 1.91 | 1.8 | 0.4 | 0.2 | 0.2 | 1.5 | 0.5 | 0.2 | 0.6 | 1.8 |
| 20:4(ω6) | 2.02 | 2.02 | 2.9 | 0.4 | 0.2 | 0.4 | 2.1 | 1.8 | 0.7 | 0.6 | 0.5 |
| | | 2.28 | 0 | 0 | 0 | 0.1 | 0 | 0.1 | 0 | 0.1 | 0.3 |
| 22:0 | 2.44 | 2.47 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 | 0 | 0.2 | 0.2 | 0.2 |
| 22:1(ω9) | 2.66 | 2.68 | 0.5 | 0.6 | 1.1 | 0.3 | 0.4 | 0.7 | 1.2 | 1.3 | 0.4 |
| | | 2.99 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0 | 0 |
| | | 3.18 | 0 | 0.1 | 0 | 0 | 0.1 | 0 | 0 | 0 | 0 |
| | | 3.43 | 0 | 0.1 | 0 | 0 | 0.1 | 0 | 0.1 | 0 | 0 |
| | | 3.72 | 0.5 | 0 | 0 | 0.2 | 0.4 | 0.6 | 0.2 | 0.3 | 0.9 |
| 24:0 | 4.40 | 4.47 | 0.4 | 0 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 |
| 24:1(ω9) | 4.79 | 4.81 | 0.4 | 0.4 | 0.2 | 0 | 0.3 | 0.4 | 0.4 | 0.5 | 0.5 |
| | Total | | 99.7 | 99.0 | 99.6 | 99.5 | 99.6 | 99.7 | 99.7 | 99.3 | 98.8 |

[a] $RR_t$ = retention time relative to methyl linoleate.
[b] Details of the adrenal glands are given in the Materials section.
[c] Fatty acids found in quantities less than 0.1 mole % are listed as absent since the amounts were too small to permit definitive identification by gas chromatography-mass spectrometry.

carbon atoms and 0-4 double bonds. Methyl esters of fatty acids having fewer than 14 carbon atoms are too volatile to measure reproducibly by this method.

## RESULTS

The chromatograms of the sterols from the abnormal adrenal glands were qualitatively similar to those of the normal controls (Fig. 1); however there was a very large amount of cholesterol in the gland from the patient who had been treated with aminoglutethimide. The principal sterol present in the normal and abnormal adrenal cortices was cholesterol, as was shown by its mobility on thin layer chromatography and the relative retention time of its trimethylsilyl ether on gas liquid chromatography; 20α-hydroxycholesterol was undetectable. At the retention time of 20α-hydroxycholesterol bis (trimethylsilyl ether) only a "shoulder" was seen (*d*, Fig. 1); and if there was any 20α-hydroxycholesterol present in this shoulder it was estimated to be not more than 5μg/g wet weight of tissue, whereas the amount of cholesterol present was ca. 10,000 times greater (see below).

A small peak of the same relative retention time as 20α, 22R-dihydroxycholesterol 3,22-bis-(trimethylsilyl ether) amounting to about 30μg/g tissue was observed (*e*, Fig. 1). However no peak corresponding to its tris-(trimethylsilyl ether) was detected (*f*, Fig. 1), although both the bis- and the tris-trimethylsilyl ethers were usually formed from 20α,22R-dihydroxycholesterol. Thus its identification is uncertain. There were some other small, unidentified peaks present with longer retention times than that of 20α-hydroxycholesterol bis (trimethylsilyl ether); each of these corresponded to less than 20μg/g tissue assuming the detector re-

sponse to them was similar in magnitude to the response to the sterol trimethylsilyl ethers. There was also an unidentified peak (*b*, Fig. 1) of slightly longer retention time than that of cholesterol trimethyilsilyl ether which amounted to ca. 40μg/g tissue. It was also present in the extract of the normal adrenals.

It was concluded that the major sterol present in the adrenal cortex after aminoglutethimide treatment is cholesterol and not 20α-hydroxycholesterol, 20α,22R-dihydroxycholesterol or other hydroxylated derivative of cholesterol.

The amounts of cholesterol in free and esterified form are shown in Table III. The adrenal cortex from the patient who had been treated with aminoglutethimide contained three times the normal amount of esterified cholesterol, while the concentration of free cholesterol was only slightly greater than that in normal controls. The adrenal cortex from the patient who had been treated with glutethimide contained normal amounts of esterified cholesterol; the concentration of free cholesterol was slightly elevated compared with normal controls.

The fatty acid methyl esters obtained from the cholesterol ester fractions were analyzed by gas liquid chromatography. Over 30 fatty acids having more than 14 carbon atoms and present to the extent of at least 0.1% were detected (Table IV). Palmitate, palmitoleate, stearate, oleate and linoleate together made up over 80% of the total.

Although there were some small variations in percentages of particular fatty acids in the various adrenal glands, all showed the same general pattern. However there was a significantly larger proportion of arachidonic acid in the two adrenal glands from patients whose corticosteroid secretion rate was depressed, in one case by aminoglutethimide treatment (#1) and in the other because of the presence of a functioning adrenal carcinoma (#5). In glands #3 and #4 from patients whose corticosteroid secretion rate was elevated because of nodular hyperplasia (Cushing's Syndrome) there seemed to be relatively less arachidonic acid (Table V). Similarly the gland from the patient who had been treated with glutethimide (#2) had a lower proportion of arachidonic acid.

All the abnormal adrenal glands also showed a decreased proportion of eicos-9-enoic acid (Table VI) compared to the normal controls.

## DISCUSSION

The ability of aminoglutethimide to inhibit adrenal steroidogenesis in vivo and in vitro and at the same time to cause accumulation of a lipid substance in the adrenal cortex is well documented (1-6). Its site of action in the rat was shown by Dexter et al. (1) to be before pregnenolone in the biosynthetic pathway by its failure to inhibit the conversion of pregnenolone to corticosteroids in rat adrenal quarters. The accumulation of lipid, which was referred to by them as cholesterol, was taken as evidence that the site of action of aminoglutethimide is after cholesterol in the biosynthetic sequence. Cohen (5) incubated 20α-hydroxycholesterol with acetone powders of rat adrenal and found that enzymic cleavage of the side chain was stimulated, not inhibited, by aminoglutethimide whereas when cholesterol was the substrate cleavage was inhibited. She concluded that aminoglutethimide acts by inhibition of 20α-hydroxylase which has been assumed to be the first step in conversion of cholesterol to pregnenolone. Recent evidence, however, casts doubt on this hypothesis (22-24).

The failure in this study to detect a large amount of any hydroxylated cholesterol derivative in normal adrenals is in agreement with the findings of other workers (25,26). In the case of the adrenal gland from the patient treated with high doses of aminoglutethimide, failure to detect a large amount of any hydroxylated cholesterol derivative shows that the drug does not act between successive hydroxylations of the cholesterol side chain to produce accumulations of the hydroxylated derivatives thought to be intermediates in steroid hormone biosynthesis. Hall and Koritz (27) have suggested that intermediates between cholesterol and pregnenolone cannot be detected because the pathway operates in a concerted way such that no intermediates are released from the enzyme. In view of the uncertain status of the various hydroxycholesterols in the side chain cleavage sequence (22-25), the most that can be said is that aminoglutethimide produces an accumulation of cholesterol ester in the adrenal cortex.

The adrenal cortex from the patient treated with aminoglutethimide had a normal or slightly elevated concentration of free cholesterol, but the concentration of cholesterol esters was some three times greater than normal. The total weight of cholesterol and cholesterol esters made up ca. 20% by weight of the adrenal cortical tissue. These results are in general agreement with the data obtained by Dexter et al. (1) in rats.

That cholesterol fatty acyl esters have a role in steroidogenesis has been suggested by a number of authors (28-31). Enzymes that esterify cholesterol and hydrolyze cholesterol esters are found in adrenal cortex (32,33).

TABLE V

Relations between Adrenal Function and Proportion of Arachidonic Acid in Fatty Acids of Adrenal-Cortical Cholesterol Ester Fraction

| Adrenal gland number[a] | Arachidonic acid, mole % | | |
|---|---|---|---|
| | Mean, SE | No. of determinations | Significance[b] |
| Controls | | | |
| 6 | 1.82 ± .057 | 8 | |
| 7 | 0.65 ± .024 | 4 | |
| 8 | 0.60 ± .039 | 5 | Mean 1.14 |
| 9 | 1.51 ± .038 | 4 | |
| Cortex from adrenal glands with decreased steroid synthesis | | | |
| 1 | 2.85 ± 0.78 | 5 | $p < .01$ |
| 5 | 2.10 ± .049 | 4 | $p < .05$ |
| Cortex from adrenal glands with increased steroid systhesis | | | |
| 3 | 0.25 ± .011 | 5 | $p < .05$ |
| 4 | 0.42 ± .025 | 8 | $p < .05$ |
| Cortex from adrenal gland from patient with acute porphyria treated with glutethimide | | | |
| 2 | 0.37 ± .021 | 4 | $p < .05$ |

[a]Details of the adrenals are given in the Materials section.
[b]Significance determined by Student's *t*-test.

However the cholesterol ester content of adrenal cortex varies greatly with diet (30) and, in mice, between particular strains (34). The situation is further complicated by the existence of different pools of cholesterol and cholesterol esters within adrenal cells, especially as pregnenolone systhesis is mitochondrial, while cholesterol esters are largely stored in lipid droplets (35). It is clear, however, that aminoglutethimide affects the quantitative relationship between free and esterified cholesterol concentrations and cholesterol side chain cleavage in human and animal adrenal glands by an unknown mechanism that links increased storage of cholesterol esters and decreased cholesterol untilization for steroidogenesis. It is likely that this mechanism is normally controlled by ACTH which has effects opposite to those of aminoglutethimide, causing depletion of cholesterol esters in the adrenal cortex and at the same time stimulation of steroidogenesis (29,31).

Glutethimide, which differs from aminoglutethimide only by the absence of the amino group on the aromatic ring, seems to be without effect on the concentration of cholesterol esters or total cholesterol in the adrenal cortex (Table III). This is in agreement with the findings of Cohen (5) that it was 100 times less effective than aminoglutethimide as an inhibitor of side chain cleavage of cholesterol in vitro. The patient from whom this adrenal was obtained postmortem had apparently been treated with large doses of glutethimide as a sedative, but no details of dosage or steroid secretion rates could be obtained.

The proportions of various fatty acids esterified with cholesterol in all the glands were similar to those described by Riley (28), who also used glands from British patients, but different from the results of Takayasu et al. (36). The latter, using postmortem adrenal glands from Japanese patients, found very much lower proportions of cholesteryl palmitate (7% instead of 30%), rather higher proportions of esters of various polyunsaturated fatty acids, and only 10.6% of esters of saturated fatty acids compared with ca. 40% in the glands from the British patients described here and 60-75% in those described by Riley (28). It seems likely that these large differences can be ascribed to the differences in diets of the Japanese and British patients (30). There were considerable differences between the proportions of fatty acids found and those reported for rat adrenal glands (11,12).

As is shown in Table V, the two adrenal glands in which corticosteroid sysnthesis was decreased, in one case because of administration of aminoglutethimide and in the other because of the presence of a corticosteroid-producing carcinoma, showed an increase in the proportion of cholesteryl arachidonate compared with the normal controls. The two glands in which corticosteroid synthesis was high enough to cause Cushing's Syndrome showed a slight decrease in the proportion of cholesteryl arachidonate. The latter agrees with the results of Gidez and Feller (12) who showed a decrease in the proportion of cholesteryl arachidonate in

TABLE VI

Proportion of *cis*-Eicos-9-enoic Acid in Fatty Acids of Adrenal Cortical Cholesterol Ester Fraction

| Adrenal gland number[a] | Eicos-9-enoic acid, Mole % Mean, SE | No. of determinations | | Significance[b] |
|---|---|---|---|---|
| Controls | | | | |
| 6 | 2.56 ± .068 | 8 | | |
| 7 | 3.00 ± .039 | 4 | Mean 2.55 | |
| 8 | 2.46 ± .025 | 5 | | |
| 9 | 2.19 ± .012 | 4 | | |
| Abnormal | | | | |
| 1 | 1.23 ± .061 | 5 | | $p < .0025$ |
| 2 | 1.71 ± .096 | 4 | | $p < .01$ |
| 3 | 1.58 ± .028 | 5 | | $p < .01$ |
| 4 | 1.59 ± .034 | 8 | | $p < .01$ |
| 5 | 1.08 ± .054 | 4 | | $p < .0025$ |

[a]Details of the adrenals are given in the Materials section.
[b]Significance determined by Student's *t*-test.

the remaining gland of unilaterally adrenalectomized rats which would have been synthesizing steroids at an increased rate.

In rats, Walker and Carney (37) have found that cold stress causes a decrease in the concentration of adrenal cholesteryl arachidonate, compared with other esters, at the same time as increasing the plasma corticosterone concentration. In rats deficient in essential fatty acids, whose adrenal glands contained much less cholesterol arachidonate to begin with, the proportional decrease of this ester was greater, and the increase in plasma corticosterone was smaller, than in the normal controls. Thus it would seem that in rats and in humans, increased adrenal corticosteroid output is associated with preferential depletion of adrenal cholesteryl arachidonate.

The concentration of adrenalcortical cholesteryl arachidonate did not seem to be *directly* related to the concentration of ACTH in plasma. Adrenal #1, from a patient who had been treated with aminoglutethimide to inhibit corticosteroid biosynthesis and which had an increased proportion of cholesteryl arachidonate, had also been exposed to very high levels of a substance with ACTH activity secreted by the patient's pancreatic tumor. Adrenal #2, which also had decreased corticosteroid biosynthesis and increased cholesteryl arachidonate, was from a patient with a functioning adrenal carcinoma and thus had presumably been exposed to very low levels of ACTH due to the operation of the pituitary feedback system by the steroids secreted by the tumor.

Cholesteryl arachidonate may be involved in prostaglandin synthesis. Arachidonic acid can be converted to prostaglandins (38); their synthesis in rat adrenal homogenates is stimulated by ACTH (39), while superfusion of rat adrenal glands with prostaglandin $E_2$ causes an increase in corticosterone production (40). Links between the action of prostaglandins, tropic hormones and cholesterol ester concentration have also been demonstrated in ovary (41). Thus it may be postulated that prostaglandin biosynthesis and steroid biosynthesis in human adrenal cortex are linked by arachidonic acid and perhaps by other long chain polyunsaturated fatty acids.

The other clear trend in the fatty acid compositions of the cholesterol esters from the adrenal glands examined was the finding that the proportion of *cis*-eicosa-9-enoate was significantly higher in the "normal" controls than in the adrenal glands with various disorders of steroid biosynthesis (Table VI), but there was no correlation with the rate of steroidogenesis. Walker and Carney (37) have shown that this ester is preferentially depleted in the adrenal glands of rats exposed to cold stress.

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# Studies of Tocopherol Dimers from Soybean Oil by Reaction Gas Chromatography

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## ABSTRACT

Reaction gas chromatography was found to be helpful in elucidating structures of tocopherol dimers. By this method $\gamma$- and $\delta$-tocopherols were determined as monomers derived from tocopherol dimers, after isolation of the latter compounds from soybean oil. It was shown that gas chromatographic determination of tocopherols, as performed by injection of total unsaponifiables from soybean oil, will give results too high; the eluted tocopherols will account for both tocopherol monomers and dimers.


## INTRODUCTION

Several research workers (1-7) investigated tocopherol dimers in vegetable oils. Various tocopherol dimers were isolated from tung oil (1,2), cottonseed oil deodorizer scum (3), cottonseed oil (4), soybean oil (5) and soybean oil deodorizer scum (6). Nilson (7) provided thorough and valuable information on the structure and the mechanism of formation of dimers and trimers from tocopherols and from related chromanols. Komoda and Harada (5) showed the influence of moisture in raw soybeans on the tocopherol-dimers content of the oil.

The dimers investigated by Nilson and Daves (4) and by Komoda and Harada (5) were shown to be oxidation products of various tocopherols, and it was observed that some of them reacted positively with the Emmerie-Engel reagent. Structures of tocopherol dimers were established by using UV, IR, mass and NMR spectra (2,3,5,7).

The present work differs from the previous ones, in utilization of reaction gas chromatography (8,9) to investigate the structure of tocoph-

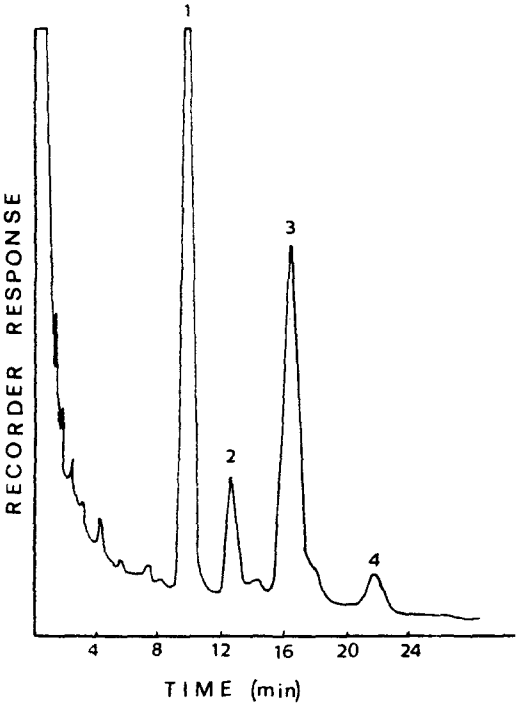


FIG. 1. Gas chromatogram of tocopherol dimer A. Chromatography conditions are given in Experimental Procedures, Gas Liquid Chromatography. 1, squalene; 2, $\delta$-tocopherol; 3, $\gamma$-tocopherol; and 4, unknown.

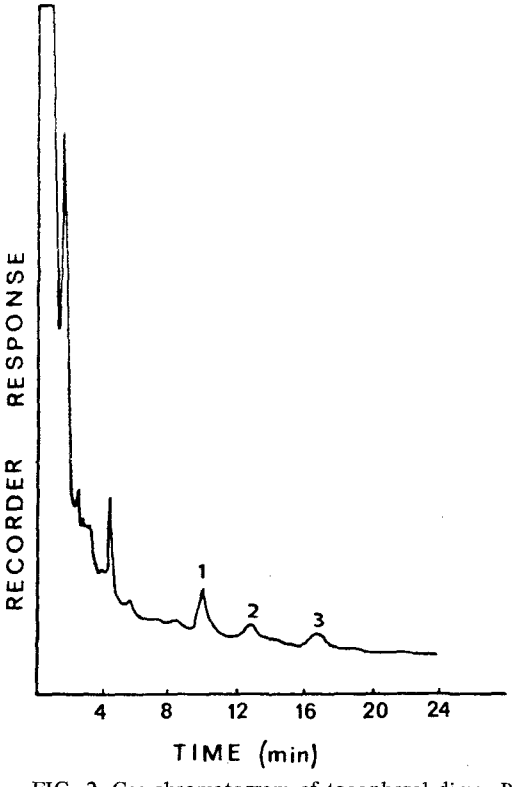


FIG. 2. Gas chromatogram of tocopherol dimer B (see Fig. 1 for explanation).

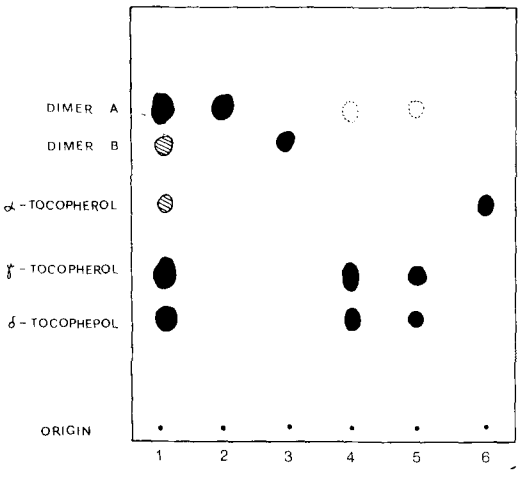


FIG. 3. TLC of pyrolysis products from dimers A and B. Solvent, chloroform; detecting reagent, Emmerie-Engel Spray. 1, unsaponifiable matter from soybean oil; 2 and 3, dimers A and B, respectively, before reaction gas chromatography; 4 and 5, dimer A and B, respectively, after reaction gas chromatography; and 6, α-tocopherol.

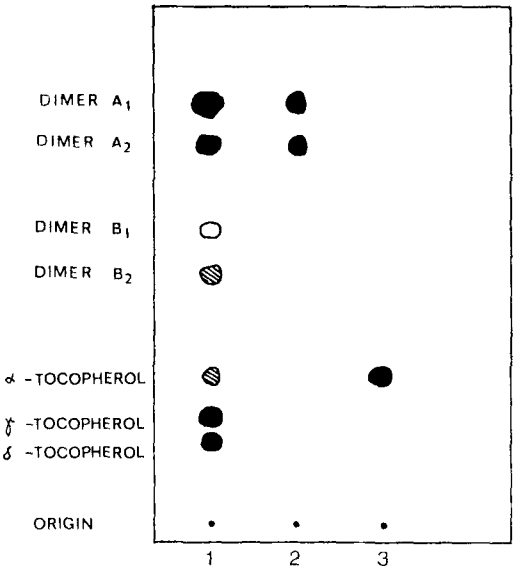


FIG. 4. Rechromatography of dimer A. Solvent, hexane-ethyl ether 90:10; detecting reagent, Emmerie-Engel Spray. 1, unsaponifiable matter from soybean oil; 2, dimer A (obtained after isolation by TLC using benzene as developing solvent) and 3, α-tocopherol.

erol dimers. The authors were concerned mainly with identity of the major tocopherol dimers, the presence of which in the unsaponifiable matter of refined soybean oil was earlier observed by Komoda and Harada (5).

## EXPERIMENTAL PROCEDURES

### Reagents

All solvents and reagents were of analytical grade and were used as received, except ethyl ether, which was purified on a column of basic alumina to remove peroxides. α-Tocopherol was purchased from Fluka AG. Standards of γ- and δ-tocopherols were obtained from soybean oil by thin layer chromatography (TLC).

### Isolation of Unsaponifiables

Refined soybean oil was saponified according to Ames (10). Most of the solvent was removed at 50 C from the extract of unsaponifiables on a rotary vacuum evaporator and then transferred to a small vial. The residual solvent was removed with a stream of nitrogen also at 50 C. The unsaponifiables were subsequently dissolved in a minimum amount of chloroform, for further separation by TLC.

### Thin Layer Chromatography

Preliminary TLC separation was conducted on a 20 x 20 cm plates of Silica Gel G (Merk, catalog no. 7731), 0.5 mm thick. Four different solvent systems were used: no. 1, chloroform; no. 2, benzene; no. 3, hexane-ethyl ether 90:10; and no. 4, petrol ether (bp 60-80 C)-ethyl ether-acetic acid 50:50:1. In each case the solvent front was allowed to advance 17 cm. The developing chamber was lined with filter paper. The detecting reagent was Emmerie-Engel Spray (11). The tocopherols were identified by comparison with standards.

Tocopherol dimers were subsequently separated from the unsaponifiable matter by preparative TLC. The same technique as described above was used, but the Silica Gel G plates contained sodium fluorescein (12). After separation and drying, the plates were observed under the light of 366 nm of a UV lamp. The stripes of tocopherol dimers and monomers were dark purple under the UV light. For confirming, the edges of the stripes were sprayed with the Emmerie-Engel reagent. The stripes, which have been later identified as tocopherol dimers, were scraped off and eluted with several portions of ethanol. The ethanol was evaporated at 50 C with a stream of nitrogen. For further gas liquid chromatography (GLC) separations, the dimers were dissolved in a minimum amount of chloroform.

### Gas Liquid Chromatography

The appartus was a Packard Gas Chromatograph, model no. 7821 with a dual column system and dual no. 811 hydrogen flame ionization detector. Gas chromatography analy-

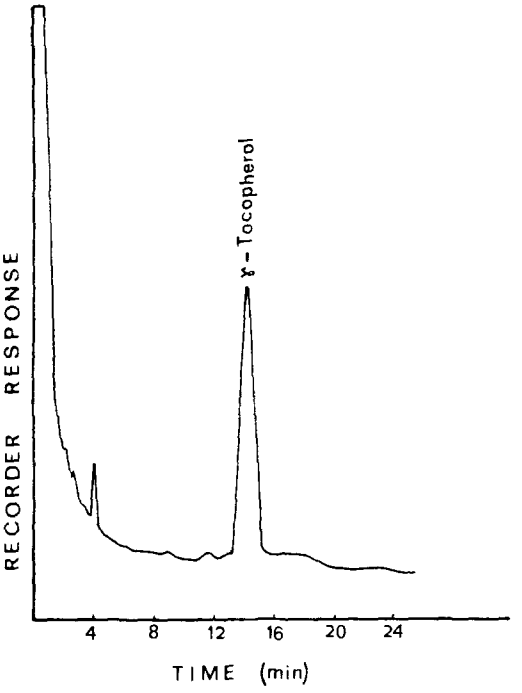


FIG. 5. Gas chromatogram of dimer $A_1$. Chromatography conditions are given in Experimental Procedures, Gas Liquid Chromatography.

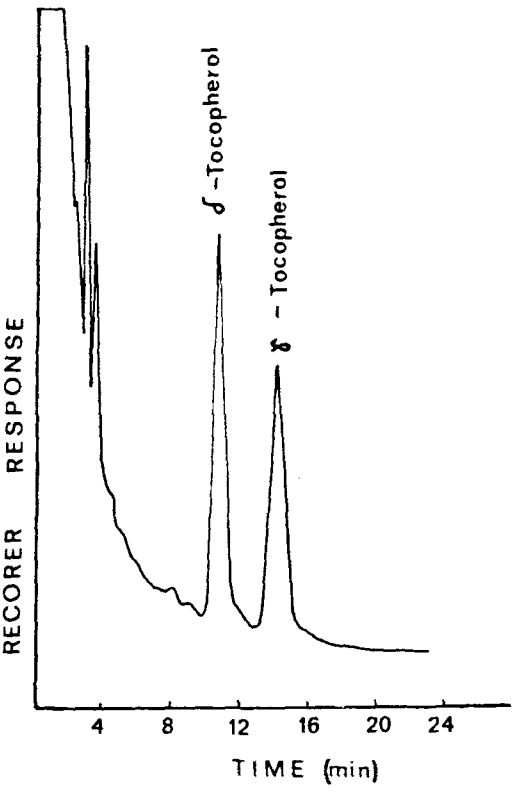


FIG. 6. Gas chromatogram of dimer $A_2$. Chromatography conditions are given in Experimental Procedures, Gas Liquid Chromatography.

ses were conducted on a 6 ft x .25 in. (OD) coiled glass column, packed with 3% SE-30 on Gas Chrom Q 80/100 (Applied Science Laboratories). Column temperature was 232 C. The temperatures of the injection point, detector and outlet were 275 C each. Carrier gas was Argon at a flow rate of 30 ml/min. Identification of the peaks was carried out by comparison of their retention times with those of the tocopherol standards. The relative content of the components in the injected mixture was calculated by triangulation of peaks' areas.

To convert the apparatus for the purposes of preparative reaction gas chromatography, the detector was disconnected and a teflon tube was attached to the outlet of the column. The outlet of the attached tube (20 cm long) was kept at room temperature.

Portions of 10 $\mu$liters of the dimers solution in chloroform were injected on column with 701 N Hamilton syringe every 20 min, during a period of several hours. The tocopherol monomers condensed in that part of the tube which was kept at the room temperature; they were subsequently eluted from the tube with chloroform.

## RESULTS AND DISCUSSION

Separation of the unsaponifiable matter from soybean oil by TLC using either chloroform or benzene as the developing solvent, resulted in five spots of components which reacted positively with the Emmerie-Engel reagent (see no. 1 separation in Figs. 3 and 7). Three of the spots were identified as $\alpha$-, $\gamma$- and $\delta$-tocopherols. The two other spots were identified by us as tocopherol dimers by the method of reaction gas chromatography and also by TLC according to the evidence from the literature (5). Of the two dimers, the component with the higher Rf (0.77 in chloroform and 0.72 in benzene) was named dimer A, and that of the lower Rf (0.68 in chloroform and 0.52 in benzene) – dimer B. The Rf of the dimer A in the TLC separation (when chloroform was used as solvent) was identical to that of 5 - ($\gamma$-tocopheryloxy)-$\gamma$-tocopherol, as reported by Komoda and Harada (5).

Gas chromatograms of the dimers A and B are shown in Figures 1 and 2, respectively. The no. 1 peak is that of squalene: in the first run of the TLC separations (using chloroform), the very big squalene spot interfered with the spots of dimers A and B. The other GLC peaks (nos. 2 and 3) were identified as $\gamma$- and $\delta$-tocopherols

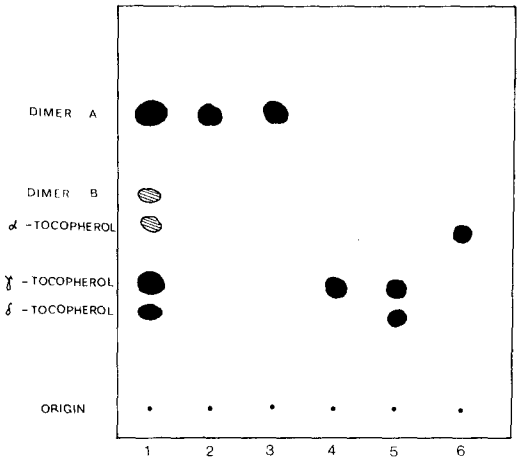


FIG. 7. Thin layer chromatography of pyrolysis products of dimers $A_1$ and $A_2$. Solvent, benzene, detecting reagent, Emmerie-Engel Spray. 1, unsaponifiable matter of soybean oil; 2 and 3 dimers $A_1$ and $A_2$, respectively, before reaction gas chromatography; 4 and 5 dimers $A_1$ and $A_2$, respectively, after reaction gas chromatography and 6, α-tocopherol.

(see Experimental Procedures). The very small no. 4 peak in the chromatogram of dimer A (Fig. 1) was not identified.

The resolution into tocopherols of both the tocopherol dimers A and B was made possible through thermal scission of the dimers, followed by gas chromatographic separation of the monomers. Gas chromatography of the dimer A resulted in pyrolysis of that dimer to its monomers γ- and δ-tocopherols. The separations showed good reproducibility; the standard deviation for δ-tocopherol (calculated from six separation) was 1.8%. The ratio between the amounts of γ- and δ-tocopherol was ca. 4:1 (Fig. 1). It can be deduced, therefore, that the dimer A spot consisted of at least two dimers and probably of not more than three dimers of the same polarity (γ-γ, δ-γ and δ-δ).

The effluents which contained the thermal degradation products of the tocopherol dimers A and B have been subjected to a TLC separation (separation Nos. 4 and 5, Fig. 3). Two spots, of γ- and of δ-tocopherol have been obtained in those separations, thus confirming the previous identification by gas chromatography (Figs. 1 and 2). The TLC separation also showed that the amount of γ-tocopherol was higher than that of δ-tocopherol.

In the TLC separations of the pyrolysis products from the dimers A and B (Fig. 3), a very faint spot was observed at the Rf characteristic for dimer A. According to our observation, the appearance of this spot resulted when the effluent from the gas chromatography of

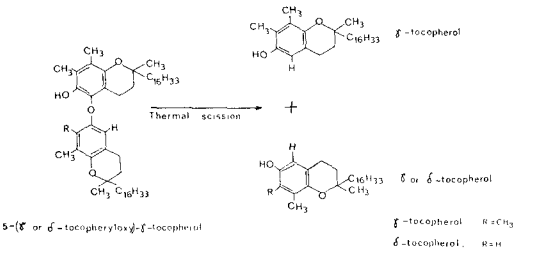


FIG. 8. Pyrolysis of tocopherol dimers $A_1$ and $A_2$ to their monomers.

the dimers A or B was subjected to the separation after some delay. It seems therefore that the small amounts of dimer A resulted from recombination of the γ- and δ-monomers in the eluate from gas chromatography.

An attempt was made to resolve the dimer A spot (after pyrolysis, γ- and δ-tocopherols were obtained in the ratio of 4:1, see Fig. 1). The dimer A was subjected to rechromatography on freshly activated Silica Gel G using hexane-ethyl ether 90:10. In the rechromatography dimer A separated into two components, dimer $A_1$ (Rf = 0.76) and dimer $A_2$ (Rf = 0.68) (Fig. 4).

Gas chromatography of dimer $A_1$ resulted in one peak only, that of γ-tocopherol (Fig. 5); dimer $A_1$ is therefore a γ-γ-component, presumably of the 5-(γ-tocopheryloxy)-γ-tocopherol (5). Gas reaction chromatography of dimer $A_2$ resulted in two peaks, of γ-tocopherol and of δ-tocopherol (Fig. 6). The ratio of those peaks was 1:1.2; we assume therefore that the dimer $A_2$ was an unresolved mixture of δ-γ (main component) and of δ-δ, the structures of which were similar to that of the γ-γ tocopherol dimer $A_1$. As all the $A_1$ and $A_2$ dimers were of the same polarity, it seems feasible that also the δ-γ and δ-δ dimers were of similar structure to the 5-(γ-tocopheryloxy)-γ-tocopherol of the γ-γ dimer.

In order to confirm the gas chromatographic results (Figs. 5 and 6), the pyrolysis products from dimers $A_1$ and $A_2$ were subjected to a TLC separation (Fig. 7). Eluate from dimer $A_1$ gave only one spot (γ-tocopherol), and the eluate from dimer $A_2$ gave two spots of about equal size (γ- and δ-tocopherols).

No such detailed work has been done with the dimer B. However it may be seen from Figure 4 (separation no. 1) that by using a more suitable solvent system the dimer B could also be resolved into two spots.

It is worth mentioning here the results of Seino et al. (6) who isolated three dimers from soybean oil deodorizer distillate and the results of Komoda and Harade (5) who obtained two

spots of dimers in a single layer separation of unsaponifiables from soybean oil. Komoda and Harada (5) did not try to further resolve the dimer spots by TLC, but have proved (with the aid of IR techniques) that the major spot consisted of γ-γ-tocopherol dimer of the structure 5-(γ-tocopheryloxy )-γ-tocopherol. (An evidence of small amounts of a δ-compound has been also produced after reduction of the tocopherol dimer with $LiAlH_4$).

The results of reaction gas chromatography (Figs. 5 and 6) indicate that a scission of the dimer A (5-[γ- or δ-tocopheryloxy] )-γ-tocopherol (5) occurs at the weak oxygen link between the two parts of the molecule (Fig. 8). According to Willmott (13) the mechanism of pyrolysis might involve a free radical reaction, thus in our case the free radical yielding the monomer.

Beroza and Coad (8) believe that for compounds that undergo thermal degradation under 300 C, the injection port acts as pyrolysis chamber. In our work the injections have been made on glass column at 275 C.

As indicated above, the pyrolysis products of the injected dimers have been identified as tocopherols (monomers), according to GLC and TLC procedures. No dimers were eluted during the gas chromatographic determinations, and the same amount of tocopherol monomers were obtained from injections of same volumes of the dimers' solution in chloroform. It appears, therfore, that in the specified conditions of reaction gas chromatography (see Experimental Procedures), thermal degradation of the dimers is complete.

Reaction gas chromatography is advantageous in structure elucidation, and it can be carried out even with small samples and with samples that are not altogether pure. The last statement is true as long as minor contaminants do not have the same retention time as tocopherol monomers, i.e., the presence of squalene together with the dimer A did not interfere with the peaks of γ- and δ-tocopherol (see Fig. 1).

Reaction gas chromatography should therefore be considered a very helpful tool in structure determination of tocopherol dimers. The case is especially true for tocopherol dimers which are not stable enough and may oxidize when purified by repeated TLC and crystallization for further analytical determinations by other techniques, e.g., IR, mass spectra, etc.

According to our observations (Fig. 9), it will be erroneous to determine directly, by GLC the amount of tocopherols in the unsaponifiable matter from those oils which contain also appreciable amounts of tocopherol dimers. To obtain accurate results, the tocopherols should be separated from their dimers by TLC, in a preliminary procedure. This recommendation of ours differs from the procedures recommended by Nelson and Milun (14) for determination of tocopherols.

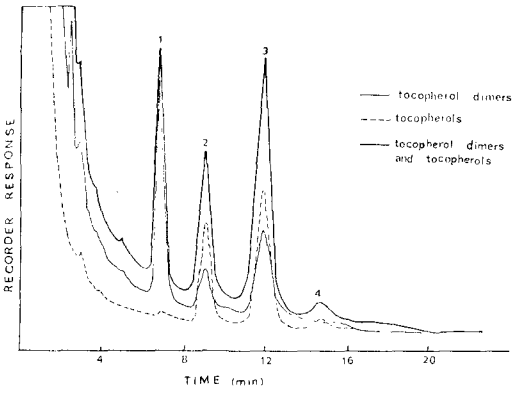


FIG. 9. Gas chromatograms of tocopherols, tocopherol dimers and mixture of tocopherol monomers and dimers. For conditions see Experimental Procedures, Gas Liquid Chromatography. Tocopherols and tocopherol dimers were isolated by TLC using petrol ether(bp 60-80 C)-ethyl ether-acetic acid 50:50:1. The injected amounts of either tocopherols or tocopherol dimers or of their mixture were related to the same amount of soybean oil. 1, squalene; 2, δ-tocopherol; 3, γ-tocopherol and 4, α-tocopherol.

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# SHORT COMMUNICATIONS

## Estimate of Fatty Acid Turnover in Porcine Adipose Tissue


### ABSTRACT

Fatty acid turnover in the domestic pig was estimated by measuring the half-life of linolenic acid depletion in adipose tissue depots which had been made abnormally high in linolenic acid by feeding large quantities of linseed oil. The measured half-life of linolenate in 8- to 12-month-old pigs was 300 days. The apparent half-life of linolenate in muscle lipids was less than that of subcutaneous backfat.


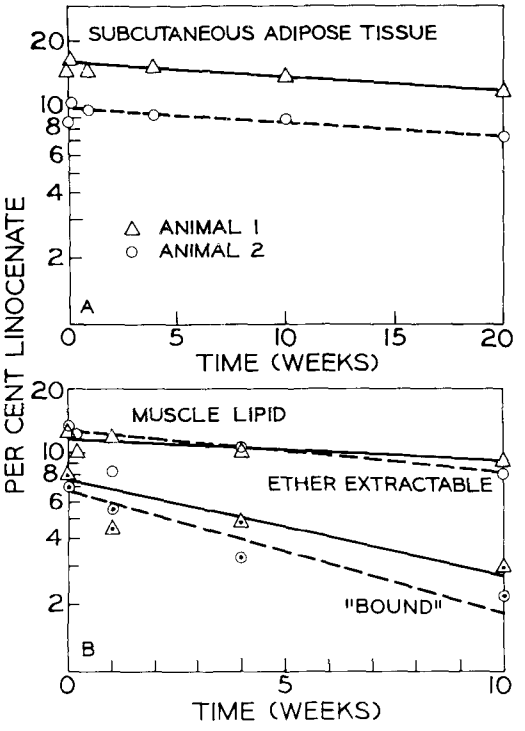


FIG. 1. Semilog plot of adjusted linolenate concentration in subcutaneous adipose tissue (A) and muscle lipid fractions (B) after removal from the high linseed oil diet. Control values; which are the average of previously reported values (10-12), were subtracted from experimentally determined values before plotting. Control values for linolenate used were: subcutaneous lipid, 1.0%; ether extractable muscle lipid, 2.1%; and chloroform-methanol extractable lipids from ether extract residue ("bound" lipids), 1.0%. Initial concentration, $A_O$, and final concentration, A, at time, $t$, were determined from the graph to calculate fractional turnover rate, $k$. $kt = 2.3 \log (A_O/A)$.

Schoenheimer and Rittenberg in their classic experiments with deuterium-labeled fatty acids were the first to demonstrate the dynamic nature of adipose tissue (1). Subsequently a number of researchers have measured the half-life of carcass fat in the rat and reported values ranging from 16-141 days (2-5). Hirsch et al. and Fleischman et al., who measured fatty acid composition changes of humans fed diets high in polyunsaturated fats, have estimated the half-life of fatty acids in human adipose tissue to be between 350 and 700 days (6,7).

Since little information was available on the turnover of fatty acids in porcine adipose tissue, an experiment was conducted to estimate fatty acid half-life in the pig. The fatty acid composition of pig fat can be altered significantly by the fatty acid content of the diet (8,9). Therefore two 6-month-old male castrate pigs were fed ad libitum a diet of 20% w/w linseed oil for two months in order to increase the depot fat concentration of linolenic acid (C-18:3) from the normal 1% to ca. 15%. After 2 months, linseed oil was removed from the diet, and the decline in linolenate concentration back to normal depot fat levels was measured by maintaining the animals at a constant weight and taking biopsy samples of longissimus muscle and backfat for periods of 10 and 20 weeks, respectively. The linolenate concentration of the extracted adipose tissue and muscle lipids was measured by gas liquid chromatography using the procedure of Sink et al. (10). Special care was taken during the biopsy procedure to separate and analyze individually inner and outer layers of backfat, since fatty acid composition differences have been reported for the two layers (10). The basal diet was 12% protein and 4% fat formulated from corn, soybean meal and meat scraps with mineral and vitamin supplementation.

The decline in the linolenate concentration in the diethyl ether extractable lipids of subcutaneous adipose tissue (average of outer and middle layers of backfat) is shown as a semilog plot in Figure 1A. During the 20 week period,

the linolenate concentration decreased from ca. 17.5-13% in the more obese animal (animal 1) and from 11-8.5% in the more muscular animal (animal 2). Muscle lipids were separated into two fractions according to the procedure of Giam and Dugan (11): (a) ether extractable lipids from the freeze-dried muscle; and (b) chloroform-methanol extractable lipids from the ether extract residue. The second fraction, defined as "bound" lipids by Giam and Dugan, is composed largely of membrane lipids which were bound as lipoproteins and were not extracted by ether, but were released by the dehydrating effect of methanol. Throughout the experiment both animals had similar levels of linolenate in the ether extractable and "bound" muscle lipids. The linolenate concentration in the ether extractable muscle lipids (Fig. 1B) decreased from ca. 14-11% in 10 weeks while that in the "bound" muscle lipid fraction decreased from 8.5-3.5%.

The method used for determination of linolenate half-life was essentially that which had been used by Freeland to estimate half-lives of adaptive enzymes (13). Normal linolenate levels which have been previously reported (10-12) were subtracted from the experimentally determined values before plotting. The depletion of linolenate was assumed to be exponential; therefore the results were plotted on a semilog graph to determine half-life values from the slopes. From the initial concentration, $A_o$, and the final concentration, A, at time, $t$, the fractional turnover rate, $k$, was calculated ($kt$ = 2.3 log ($A_o$/A)). Half-life, $t$ 0.5, = .693/$k$. The average half-life of the linolenate in subcutaneous adipose tissue was 300 days; in the ether extractable muscle lipids, 175 days; and in the "bound" muscle lipids, 47 days.

The apparent turnover rate is quite different for each of the three lipid pools measured, suggesting that linolenate does not equilibrate freely throughout the various pools. The half-life of the subcutaneous pool is the most meaningful value, however, since this tissue represents the largest pool in the pig, making up ca. 70% of the total lipid stores in the body; whereas that in all of the muscles accounts for only ca. 5% of total body lipids (14). Even though the turnover of muscle lipids is considerably faster, it is not as important to the total fatty acid turnover in the animal.

The half-life of linolenate as measured in this experiment is probably an underestimate of the half-lives of the more common saturated fatty acids in porcine depot fats for two reasons. Previous work has shown that the turnover of saturated fatty acids (1,2), and in addition the maintenance of constant body weight in this experiment probably decreased the fatty acid pool size somewhat, since it is likely that other tissues (mainly muscle) would continue to increase in size. Consequently a decrease in the pool size would result in a reduction in the measured half-life, assuming constant flux through the pool. Hence the measured half-life of 300 days is probably an underestimate of the true turnover of porcine fatty acids, and thus it would appear that the turnover of fatty acids in the 8 to 12-month-old porcine animal, as estimated by the half-life of linolenic acid in subcutaneous adipose tissue, is a relatively slow process.

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# Biological Implications of Cadmium-Phospholipid Monolayers


## ABSTRACT

The potential interaction of air-borne metal pollutants with pulmonary lung surfactant may be simulated using monolayers of dipalmitoyl phosphatidyl choline (lecithin) spread on aqueous subphases containing different metal ions. Preliminary surface pressure and surface potential results indicate that cadmium interacts with dipalmitoyl phosphatidyl choline, such that the surface characteristics of the phospholipid are altered.


While heavy metals, along with other air-borne contaminants, pose problems in terms of potentially harmful effects on respiration, few studies have been undertaken to demonstrate the effect of such materials on the surface activity and functionality of pulmonary lung surfactant or dipalmitoyl phosphatidyl choline (DPC), its major component. Cadmium, one of the more toxic metals, has a high potential for interaction with DPC at the alveolar interface in the lungs, since the two species are known to react in bulk systems to form a cadmium-DPC adduct (1). Were a similar interaction to occur at the alveolar interface, precipitation of DPC and loss of respiration functionality could occur. In order to assess this possibility, the interaction of cadmium in aqueous subphases with monolayers of DPC was studied. The effect of zinc was also investigated, in order to establish the specificity, if any, of the cadmium-DPC interaction.

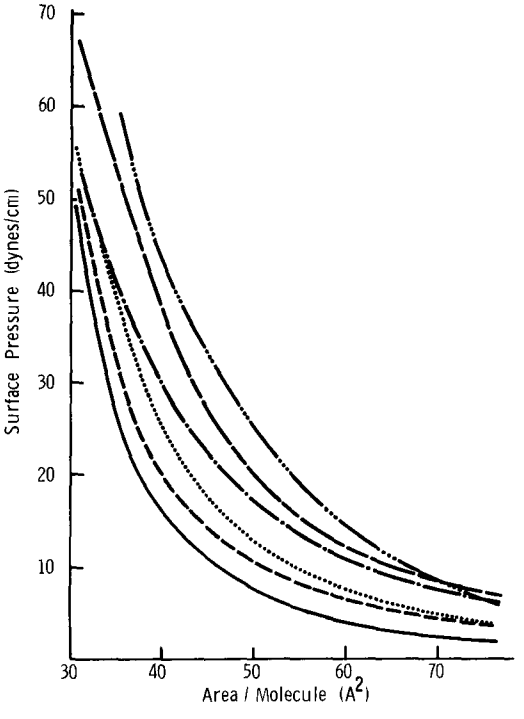


FIG. 1. Compression isotherms of dipalmitoyl phosphatidyl choline and adduct on various aqueous subphases.——— = Water; – – – – – = 0.1 M Zn; ..... = 0.1 M Cd;—.—. = 1.0 M Zn;—.. . = 1.0 M Cd; and—— —= adduct on water, saline.

The interfacial interactions were followed by monitoring changes in surface pressure ($\pi$) and surface potential ($\Delta V$) as a function of area per molecule. The equipment and procedure used have been described elsewhere (2,3). Double distilled water ($K \not> 1.5 \times 10^{-6}$ mhos/cm) was used throughout and other materials were of reagent grade quality. The *sn*-3-DPC, synthesized according to the method of Gordon and Jensen (4), was spread from a chloroform solution. The cadmium-DPC adduct, prepared immediately prior to use, was spread from a 2:1:1 solution of methanol-ethanol-chloroform. Solvent alone, run as a control on the film balance, confirmed the absence of a $\pi$ or $\Delta V$ effect arising from the solvents themselves. The $\pi$ -A isotherms presented represent the average of three separate determinations, the variation being less than $\pm$ 1 dyne/cm at any given area per molecule. Simultaneously recorded $\Delta V$ measurements varied no more than $\pm$ 15 mv.

Figure 1 shows the $\pi$'s generated by DPC spread on water and various aqueous subphases containing $Cd^{++}$ and $Zn^{++}$ compressed at the rate of 2.54 cm/min following a 10 min equilibration period. Also shown is the isotherm for the cadmium-DPC adduct on water and saline. Table I shows the $\Delta V$ changes for systems represented in Figure 1. The extrapolated areas per molecule of 38.9Å² and 40.8Å² on water and saline, respectively, are similar to literature values (5-7). Penetration studies were attempted in which $CdCl_2$ was injected into the subphase below monolayers of DPC compressed to $\pi$'s of 5, 10 and 40 dynes/cm. Final $Cd^{++}$ concentration in these studies was 0.015 M. While no change in $\pi$ was observed over a 30 min period after injection, i.e., no penetration into the monolayer occurred, $\Delta V$ changes indicated an interaction immediately below the surface. Equilibrium $\Delta V$ values were established within 10 and 30 min, respectively, with the saline and water subphases. Values reflected a net negative change in $\Delta V$ independent of initial $\pi$. A progressive expansion of DPC

TABLE I

Surface Potential Data

| Systems | + ΔV (mv) at area per molecule of | | | |
|---|---|---|---|---|
| | 40 Å$^2$ | 50 Å$^2$ | 60 Å$^2$ | 70 Å$^2$ |
| DPC[a] on water | 470 | 390 | 320 | 280 |
| DPC on saline | 515 | 450 | 385 | 335 |
| DPC on 0.1 M Zn | 455 | 415 | 345 | 290 |
| DPC on 1.0 M Zn | 150 | 135 | 125 | 110 |
| DPC on 0.1 M Cd | 385 | 340 | 300 | 260 |
| DPC on 1.0 M Cd | 410 | 400 | 380 | 350 |
| Adduct on water | 535 | 470 | 400 | 340 |
| Adduct on saline | 600 | 555 | 495 | 445 |

[a]DPC = dipalmitoyl phosphatidyl choline.

monolayers was observed on cadmium-containing subphases (Fig. 1), the expansion being related to the metal ion concentration. No change in compressibility was observed at low areas per molecule. Surface potential increased when the cadmium concentration was raised from 0.1 M to 1.0 M. In contrast, ΔV decreased when DPC monolayers were spread on subphases containing 1.0 $ZnCl_2$ when compared to 0.1 M $ZnCl_2$. The compression isotherms (Fig. 1) on zinc-containing subphases also indicate a difference in the type of interaction occurring between, on the one hand, $Zn^{++}$ and DPC, and $Cd^{++}$ and DPC on the other. Thus the expanding effect was not as great in the presence of $ZnCl_2$ as that observed in the presence of $CdCl_2$. Furthermore the isotherm on 1.0 M $ZnCl_2$ showed a condensing effect at higher film pressures, in contrast to the uniform expanding effect noted on $CdCl_2$ subphases. The cadmium-DPC adduct isotherms were similar, irrespective of whether the subphase was water or saline. The extrapolated area per molecule is 54.4 Å$^2$, close to that for DPC on 1.0 M $CdCl_2$, namely 53.2 Å$^2$. The significance of this agreement and the observed differences in surface potential following injection of $CdCl_2$ into the subphase must await the results of additional work currently in progress.

The data indicate the presence of an interfacial reaction between cadmium and DPC. The reaction appears to be specific for cadmium, since zinc, a metal ion of the same group, behaves differently. Additionally, the data suggest that a different interaction occurs depending on whether DPC molecules are compressed to low areas per molecule or allowed to occupy larger areas per molecule.

It would appear that ΔV is an important parameter in differentiating various interactions between DPC in the monolayer and cadmium in the subphase. Assuming that ΔV changes are the result of changes in the vertical dipole component of the lecithin molecule, two interaction mechanisms may be postulated: (a) In films where movement of the molecules is limited, i.e., at low areas per molecule and high surface pressures, cadmium ions are able to react only with an electronegative induced portion of the nitrogen center. This would be expected to lower the surface dipole and hence reduce ΔV. (b) With DPC at larger areas per molecule, the cadmium ions interact at the phosphorus-oxygen region. Such a reaction would add to the surface dipole, thereby increasing ΔV.

The observed interfacial behavior of the cadmium-DPC adduct would indicate a phosphate oxygen-cadmium interaction. This is supported by X-ray diffraction studies on the adduct, which show that in the three dimensional solid state, the phosphate oxygens are bonded to cadmium (8). However there is a difference in electrical behavior, depending on whether the adduct is spread, or whether cadmium and DPC are allowed to react in situ at the interface. This implies that the adduct found in bulk systems may not form at the air-water interface, possibly due to steric factors.

These preliminary investigations point to an interfacial interaction between DPC and both cadmium and zinc, the effect being dependent on the ion involved. Assuming that DPC spread at the air-water interface is a valid model for the lung interface, indications are that the functionality of lung surfactant may be altered by the presence of such air-borne contaminants as cadmium.

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# Surface Tension Studies of Phosphatidyl Glycerol Isolated from the Lungs of Beagle Dogs

**ABSTRACT**

Phosphatidyl glycerol, isolated from Beagle dog pulmonary surfactant, was found to have surface tension properties similar to phosphatidyl choline (isolated from the same source and chemically synthesized). The results show that another phospholipid in addition to dipalmitoyl-glyceryl-phosphoryl-choline contributes to the characteristic surface tension behavior of pulmonary surfactant.

Mammalian lungs contain a specialized acellular lining material with surface active properties that stabilize the pulmonary alveoli against collapse (1). A film of this surfactant material has characteristic surface properties in which the dynamic surface tension decreases sharply as the surface area is decreased and increases sharply as the surface area is increased, producing a hysterisis loop on a surface tension-area diagram (1). The largest single constituent of this surfactant material has been found to be dipalmitoyl lecithin (DPL) (2). This phospholipid in pure form exhibits much the same surface active characteristics as the total surfactant material from lavage fluid, leading other investigators to conclude that it was the actual surface active component of pulmonary surfactant. This report shows that phosphatidyl glycerol (PG), another component of lung lavage material from Beagle dogs, has surface tension properties similar to dipalmitoyl lecithin.

The isolation and identification of PG from Beagle dog pulmonary surfactant material obtained by lavage (3) and a preliminary indication of the surface activity of PG have been reported (2,4). This compound accounted for 10% of the phospholipid phosphorus from Beagle dog pulmonary surfactant (2). The fatty acids from surfactant phosphatidyl glycerol were 72% saturated and contained 58% palmitic acid. The compound was isolated and purified by thin layer chromatography and identified by color reactions, elemental analysis, thin layer chromatography of hydrolyzate products and their derivatives, and IR spectral analyses (2,4).

A modified Wilhelmy balance (Cahn Instrument Co.) was used to measure the surface tension of the phospholipid films using the methods of Harkins and Anderson (5). The films were layered over a 0.9% saline solution and were compressed and expanded in a cyclic fashion by teflon wiper blades. Continuous measurements of surface tension and of surface film area were recorded on an XY recorder. The dynamic surface tension was recorded in dynes/cm on the Y axis, and the area of the film in the trough was recorded in $cm^2$ on the X axis. Cycle time was 4 min, and the temperature was 24 C. Maximum film area was 51 $cm^2$, and the minimum area was 9 $cm^2$. To insure the wettability of the platinum sensor, the sensor was first roughened with sandpaper, then washed in a cleaning mixture (Alconox) and rinsed exhaustively in deionized water followed by an acetone rinse. Finally the sensor was heated in a flame just before use. Before the phospholipid film was layered on the saline, a test cycle was made with only the saline subphase present. The surface tension remained constant at 73 dynes/cm, and no hysteresis was observed. The individual phospholipids were

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always layered in a solution of hexane-ethanol 4:1 v/v. The hexane evaporated rapidly, and the ethanol did not affect the surface tension in a detectable manner (the volume of ethanol was never more than 10 $\mu$l in the ca. 70 ml of saline subphase). The balance and recorder were calibrated so that each cm$^2$ of area on the chart (not to be confused with the film area) of the XY recorder represented 6.33 dyne cm (area of film (cm$^2$) X surface tension [dyne/cm] = dyne cm = ergs). The area recorded on the chart between the surface tension of the saline subphase (73 dynes/cm) and the dynamic surface tension of the phospholipid film during the compression phase represents a summation of the surface work of the film during this compression. This energy parameter was found to be linearly related to the quantity of the phospholipid layered in the film in the range of 4-13 nmoles. A larger trough would extend the permissible upper end of the range. In the range employed, the minimum and maximum surface tensions are constant. The results are reproducible upon recycling (±4%) if precautions are taken to prevent leaking of the film around the teflon blades. The results are constant for cycling speeds of 1, 2 and 4 min. Thus the surface energy per mole of phospholipid is a relative measure of the potential effectiveness of the phospholipid as a surfactant material in the lung. While this calculation cannot be said to represent the exact energy which such a phospholipid film would be able to contribute toward stabilizing the structure of the alveoli during expiration (1,6,7) (in vivo conditions would not be the same as those in vitro), the calculation is a useful parameter to assess the increase in surface energy which becomes available upon compression of a phospholipid film. The energy parameter is utilized to compare various phospholipids as to their potential effectiveness as tensioactive substances in pulmonary surfactant.

The results of the surface tension measurements on PG isolated from Beagle dog pulmonary surfactant material (2) can be seen in Figure 1A. Upon compression of the film containing 7.0 x $10^{15}$ molecules (1.16 x $10^{-8}$ moles) of PG, a minimum surface tension of 5 dynes/cm was reached at a film surface area of 26.5 cm$^2$ or 38 Å$^2$ per molecule of PG (based on a molecular weight of 738 from the fatty acid composition (2). The energy parameter described above for the PG film was 2.2 x $10^3$ ergs and was calculated from the area on the chart between the surface tension of the subphase and the dynamic surface tension of the PG during compression (345 cm$^2$ x 6.3 $\frac{\text{dyne cm}}{\text{cm}^2}$).

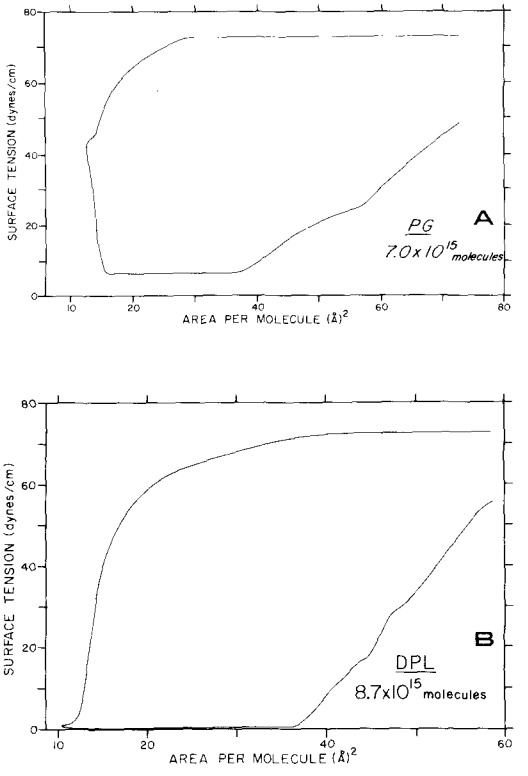


FIG. 1. Dynamic surface tension measurements of a film of phosphatidyl glycerol (PG) and a film of dipalmitoyl lecithin (DPL). Phosphatidyl glycerol isolated from lung washes of Beagle dogs and synthetically prepared L-α-dipalmitoyl lecithin were dissolved in hexane-ethanol 4:1 v/v and layered over a 0.9% NaCl solution. The area of the film was varied from a maximum of 51 cm$^2$ (73 Å$^2$ per molecule of PG and 59 Å$^2$ per molecule of DPL) to 9 cm$^2$ (13 Å$^2$ per molecule of PG and 10 Å$^2$ per molecule of DPL) and back again by teflon wiper blades. The compression phase is represented by the lower part of the curve. The time to complete the cycle was 4 min, and the temperature was 24 C. Surface tension was measured by the pull on a vertically hanging platinum plate attached to an electrobalance.

Thus the energy per mole was 1.9 x $10^{11}$ ergs/mole of PG. This can be compared to the data obtained for phosphatidyl choline (PC) isolated from Beagle dog pulmonary surfactant. A minimum surface tension of one dyne/cm was reached at a surface area of 37 Å$^2$ per molecule of PC with the energy parameter for PC equal to 1.8 x $10^{11}$ ergs/mole (based on a molecular weight of 743 from the fatty acid composition (2).

The dynamic surface tension of pure L-α-dipalmitoyl lecithin (General Biochemicals, Chagrin Falls, Ohio) was also measured (Fig. 1B). Upon compression of a film containing 8.7 x $10^{15}$ molecules of the saturated lecithin, a

minimum surface tension of one dyne/cm was reached at 31.5 $cm^2$ of film corresponding to a molecular area of 36 $Å^2$ per molecule. The energy parameter for DPL was found to be 1.7 x $10^{11}$ ergs/mole.

The results of these experiments indicate that another phospholipid in addition to lecithin contributes to the characteristic surface active properties of pulmonary surfactant. Further investigation involving the isolation of pure dipalmitoyl phosphatidyl glycerol and the study of its surface active properties is planned.

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# *cis*-5-Monoenoic Fatty Acids in Some Chenopodiaceae Seed Oils

## ABSTRACT

Methyl esters from seed oils of four Chenopodiaceae species are unusual in that they contain methyl *cis*-5-hexadecenoate (4.6-12%) and methyl 5-octadecenoate (1.1-1.2%). There are indications of small amounts of $18:2^{5,9}$ and $18:3^{5,9,12}$ along with unsaturated acids commonly found in seed oils—oleic (14-21%), linoleic (53-57%) and linolenic (3.5-7.8%). Fatty acid composition of the oils was determined by gas chromatography, and positions of the double bonds were established by application of gas chromatography-mass spectrometry to the methoxylated methyl esters.

## INTRODUCTION

Seeds from the family Chenopodiaceae are not generally rich in oil (1,2) and have not been chemically characterized extensively. The fatty acid compositons of the oil from a familiar member of this family, spinach (*Spinacea oleracea*), and from two lesser known members, summercypress (*Kochia scoparia*) and Russian-thistle (*Salsola pestifer*), have been reported in a general manner (3,4).

We now report the fatty acid composition (Table I) of four species of the Chenopodiaceae, all with *cis*-5-hexadecenoic and 5-octadecenoic acids, and of one species, *Chenopodium quinoa*, that has no appreciable amount of either. Although both these acids have been identified previously in seed oils (5-7), the amount of the $C_{16}$ homolog is the largest (12%) yet found in seed oils.

## EXPERIMENTAL PROCEDURES

Oils were extracted from ground seed with petroleum ether (30-60 bp). Methyl esters were prepared from the seed oils with methanol-$BF_3$ (8) and were analyzed by gas liquid chromatography (GLC) using both a Packard Model 7401 and a Hewlett-Packard Model 5750. The Packard 7401 was equipped with a 12 ft x 1/4 in. glass column packed with 5% LAC-2-R 446 and a 4 ft x 1/8 in. glass column packed with 5%

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TABLE I

Seed Oil Composition of Five Chenopodiaceae, Per Cent by Gas Liquid Chromatography

| Component | *Bassia hyssopifolia* | *Kochia prostrata* | *Kochia scoparia* | *Suaeda setigera* | *Chenopodium quinoa* |
|---|---|---|---|---|---|
| 16:0 | 10 | 4.0 | 9.4 | 8.5 | 11 |
| $16:1^{5}$ | 5.2 | 12 | 4.9 | 4.6 | 0.2 |
| $16:1^{9}$ | 0.2 | Trace | 0.1 | 0.2 | Trace |
| 18:0 | 2.2 | 1.1 | 2.2 | 2.7 | 1.0 |
| $18:1^{5}$ | 1.2 | 1.2 | 1.1 | 1.2 | — |
| $18:1^{9}$ | 17 | 14 | 17 | 21 | 31 |
| $18:2^{5,9}$[a] | 0.5 | Trace | 0.3 | 0.5 | — |
| $18:2^{9,12}$ | 57 | 54 | 55 | 53 | 45 |
| $18:3^{5,9,12}$[a] | 0.4 | 1.3 | 1.3 | 0.9 | — |
| $18:3^{9,12,15}$[a] | 4.5 | 7.8 | 5.1 | 3.5 | 2.7 |
| Others | 1.8 | 4.6 | 3.6 | 3.9 | 9.3 |

[a]Tentative identification based on equivalent chain length.

Apiezon L. The columns were held at 195 C, and the inlet pressure of the carrier gas (helium) was 40 psig. A 20 ft x 1/8 in. column was used with the Hewlett-Packard 5750. This column was packed with 5% LAC-2-R 446 and was run at 165 C with helium at 100 psig.

Unsaturated esters were converted to methoxy derivatives at the olefinic site by mercuric acetate in methanol and subsequent reduction with sodium borohydride as described by Abley et al. (9). The resulting methoxy esters were recovered with ether and examined by gas chromatography-mass spectrometry (GC-MS) in order to locate original double bond positions. The MS equipment, Du Pont (CEC) 21-492-1, was run in tandem with a Packard 7401 GC equipped with a 6 ft glass column packed with 3% OV-1 and operated isothermally at 200 C.

## RESULTS AND DISCUSSION

Reduction of the methanolic mercuric acetate product from each monoenoic ester provided two methoxy derivatives for analysis by GC-MS (9). Mass spectra were taken at the apex of peaks with equivalent chain lengths (ECL) (10) (OV-1 column) of 17.3 (methoxyhexadecanoates from 16:1), 19.3 (methoxyoctadecanoates from 18:1) and 20.5 (dimethoxyoctadecanoates from 18:2). Major peaks at m/e 145, 159, 185 and 199 in the spectra from the methoxyhexadecanoates show that these esters are primarily methyl 5- and 6-methoxyhexadecanoates. In addition, peaks sometimes observed at m/e 129, 143, 201 and 215 indicate small amounts of 9- and 10-methoxyhexadecanoates. Since no *trans* unsaturation was detected by IR, the 16:1 component is primarily methyl *cis*-5-hexadecenoate with a small amount of methyl *cis*-9-hexadecenoate. GLC of the underivatized esters on a 20 ft LAC-2-R 446 column confirmed the presence of two 16:1 components with ECL's of 16.2 (Δ5) and 16.4 (Δ9) and provided the quantitation shown in Table I. The methoxy component with an ECL of 19.3 (OV-1 column) showed the major group of ions at m/e 157, 171, 201 and 215 and smaller peaks at m/e 145, 159, 213 and 227. These spectra show the major components to be derivatives from methyl oleate and the smaller from 5-octadecenoate. The geometry of the small amount of 5-octadecenoic acid was not established. The GLC analysis of the underivatized esters on the 20 ft column confirmed the presence of two monoenic $C_{18}$ esters with peaks at ECL of 18.2 (Δ5) and 18.3 (Δ9). The methoxylated derivatives with an ECL of 20.5 (OV-1) gave a spectrum consistent with that of the mixture of dimethoxy esters prepared from methyl linoleate. Among the bond-locating ions were m/e 201, 215 (Δ9) and 115, 129 (Δ12). The remaining components listed in Table I were tentatively identified on the basis of the ECL's of their methyl esters. The established presence of two Δ5 components contributes to the conclusion that the peak with an ECL of 18.5 represents $18:2^{5,9}$ and the one with an ECL of 19.1, $18:3^{5,9,12}$. The components listed under "others" in Table I are mostly 20:0, 20:1, 22:0 and 22:1.

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# Uptake of Blood Triglyceride by Various Tissues[1]

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**ABSTRACT**

Triglycerides are transported in the blood in chylomicrons and very low density lipoproteins. Electron microscopic studies indicate that these particles, which range in diameter from 0.03-0.6 $\mu$, cannot cross the capillary endothelium in most tissues. There is now considerable evidence that the triglycerides are hydrolyzed to free fatty acids (FFA) during uptake and that this process is catalyzed by lipoprotein lipase. The enzyme is found in nearly all tissues that utilize circulating triglyceride, and the level of activity, in individual tissues, varies with nutritional and physiological states that affect triglyceride uptake, such as fasting, diabetes and pregnancy. Studies in perfused adipose tissue with doubly labeled chylomicrons showed that hydrolysis occurs outside of the blood stream. Two-thirds of the fatty acids are incorporated into tissue triglyceride and the rest are released as FFA, with glycerol, to the blood. Infusion of heparin causes immediate release of lipoprotein lipase activity to the blood and decreases the amount of chylomicron-triglyceride hydrolyzed by the tissue. Electron microscopic cytochemical studies showed that hydrolysis of blood glycerides by lipoprotein lipase in adipose tissue occurs within the capillary endothelial cells and in the subendothelial space near the pericytes, but not in the capillary lumen or near the fat cells. The results indicate that the fatty acids of chylomicrons cross the capillary endothelium as glycerides and FFA, within a membrane-bounded system, and cross the extravascular space to the fat cells as FFA.

Long chain fatty acids are important as a form of energy that can be readily utilized by cells or stored in adipose tissue until needed. They are also essential constituents of lipids necessary for the normal structure and function of cells. Fatty acids are derived from the diet and from synthesis de novo, mostly in liver and adipose tissue (1-4). Fatty acids are transported in the blood stream as triglyceride, in chylomicrons and very low density lipoporteins (1-4), and as free fatty acids (FFA), bound to albumin (1,2,5).

Chylomicrons are formed in the intestines from dietary lipid. They usually range in diameter from 0.05-0.6 $\mu$ (1-4,6) and consist of a large central core surrounded by a thin surface coat (7,8). The core is mostly triglyceride, with traces of cholesterol ester, and the surface coat consists of phospholipid, free cholesterol and protein (9,10). Very low density (pre-$\beta$) lipoproteins are formed in the liver and usually range in diameter from 0.03-0.08 $\mu$ (11,12). However they too may be particulate, that is, larger than 0.1 $\mu$ in diameter, if the blood triglyceride concentration is high (1,3,13).

The purpose of the symposium in which this paper was presented ("Biochemical and Clinical Aspects of the Use of Fat Emulsions in Parental Nutrition," AOCS Meeting, Atlantic City, October 1971) was to consider different aspects of the use of iv infusion of triglyceride emulsions to meet energy needs of patients with inadequate oral alimentation. The lipid particles in the emulsions, which range from 0.2-0.5 $\mu$ in median diameter (14), are similar in structure to chylomicrons (7,8) and large very low density lipoproteins (15), in that they too consist of a core of triglyceride enclosed by a stabilizing film (8). This paper discusses some of the problems involved in the uptake of blood triglyceride by different tissues and the role of lipoprotein lipase in this process (3,4,16).

Fatty acids in chylomicrons and very low density lipoproteins are rapidly cleared from the blood when injected intravenously. The rate of clearance and fate of the fatty acids, however, depends on the nutritional and physiological state of the animal (17-20). In fed rats injected intravenously with chylomicrons containing [$^{14}$C] palmitate, ca. 32% of the fatty acids removed in 10 min were found in adipose tissue, 32% in skeletal muscle, 18% in liver and ca. 2% in heart, kidney, lung and spleen; 18% were immediately oxidized to $CO_2$ (17). In fasting animals, however, 57% of the fatty acids removed in 10 min were immediately oxidized to $CO_2$, and 25% were found in liver, 10% in skeletal muscle and only 5% in adipose tissue

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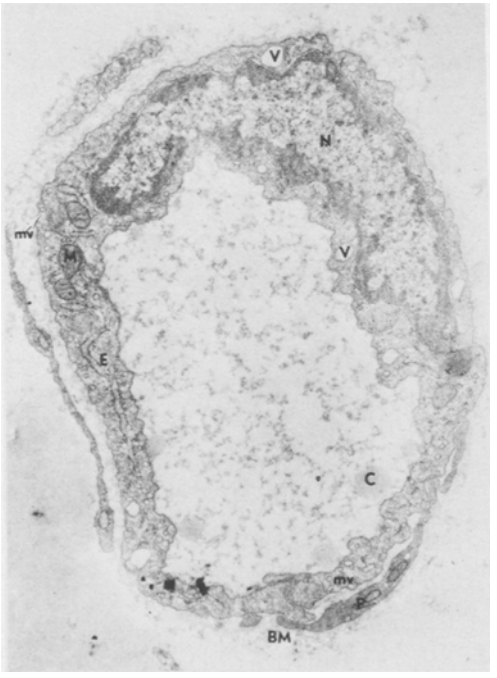


FIG. 1. Electron micrograph of a capillary in rat parametrial adipose tissue perfused 10 min with blood containing chylomicrons. Chylomicrons (C) are present in the lumen near the endothelium (E). Note numberous microvesicles (mv) and large vacuoles (V) in the endothelial cells, the interendothelial junction, and pericytes (P), enveloped by basement membrane (BM), surrounding the endothelium. Mitochondria (M) and a nucleus (N) are also seen inside the endothelial cell. X 10,000 (From Blanchette-Mackie and Scow [40].)

(17). It is well known that excess food intake causes increased deposition of lipid in adipose tissue. In lactating animals, however, food intake is increased two- to three- fold without increasing body fat stores (21,22); the lipid, instead, is diverted to mammary tissue for formation of milk (23).

Nearly all substances removed from the blood stream must cross a capillary wall (24-28). Several electron microscopic studies have shown that capillaries in liver have large fenestrations or openings in the endothelium and no basement membrane (24,26,27). Consequently, it was thought that chylomicrons and other particles could cross through these gaps, which ranged up to 0.5 $\mu$ in width, to the subendothelial space of Disse and come in direct contact with the surface of liver parenchymal cells (24,26,27). Recent studies (29), however, indicate that these gaps may be artifacts of fixation since they were not seen in tissues fixed by perfusion. Instead, the endothelial cells in liver perfused with glutaraldehyde contained multiple fenestrated sieve plates $< 0.1$ $\mu$ wide. Although the role of sieve plates in the transfer of particles across hepatic capillary endothelia has not been studied, the plates may prevent entry of large particles, $> 0.1$ $\mu$, into the space of Disse (29). Various metabolic and radioautographic studies of liver have suggested that plasma triglyceride may be taken up intact by hepatic parenchymal cells (12,18,19,30-32) or hydrolyzed at the parenchymal cell surface during uptake (33). More recent studies (34-38), however, provide strong evidence that plasma triglyceride is not utilized directly by the parenchymal cells, but, instead, is hydrolyzed in peripheral tissues to FFA and glycerol which are then released to the circulation and taken up by the parenchymal cells.

The capillaries in adipose tissue, in contrast to those in liver, consist of a continuous endothelium surrounded by a continuous basement membrane (Fig. 1) (27,39,40). The capillary endothelium is also surrounded by pericytes, which are enclosed within the capillary basement membrane (40). The endothelial cells, which sometimes interdigitate, are separated by a gap 0.01-0.02 $\mu$ in width (26,40). The endothelial cells contain microvesicles, 0.06-0.08 $\mu$ in diameter, located mostly along the luminal and basal surfaces (Fig. 1). The microvesicles are thought to be involved in transport of substances across the endothelium (26). Vacuoles, up to 0.25 $\mu$ in diameter, are also present in endothelial cells (Fig. 1). The capillaries in heart, skeletal muscle and mammary tissue are similar to those in adipose tissue (24-27,41).

Electron microscopic studies of adipose tissue and heart in normal animals have shown chylomicrons and other lipid particles attached to the luminal surface of the capillary endothelium and sometimes partially enclosed by the capillary endothelium but none inside the cells or in extracellular space (26-28,40,42,43). Capillaries of lactating mammary tissue have been studied in mice, rats and guinea pigs injected intravenously with chylomicrons or artifical triglyceride emulsion (Intralipid, Vitrum, Stockholm) (41). Many lipid particles were found in these capillaries, attached to the luminal surface and partially enveloped by the endothelium. Particles were not seen in the extravascular space.

It is evident from the above that intact chylomicrons, and perhaps other lipid particles, do not cross the capillary endothelium in most tissues. There is now considerable evidence that plasma triglycerides are hydrolyzed to FFA during their removal from blood and that this process is catalyzed by lipoprotein lipase

(4,16). Lipoprotein lipase is found in most tissues that utilize plasma triglyceride, and its level of activity usually reflects the capacity of the tissue to remove triglyceride from the blood stream (4,44). The enzyme is quickly released to the blood stream when heparin is injected intravascularly, suggesting that the enzyme is present in or near the vascular wall (4). The level of activity in the tissues is affected by various nutritional and hormonal factors (4). The activity in adipose tissue, for example, is decreased by fasting and diabetes, and increased by refeeding and insulin (44-46).

Lipoprotein lipase activity and uptake of blood triglyceride in mammary tissue are both greatly increased by lactation (47-50). Most of the long chain fatty acids secreted in milk are of dietary origin and thus are transported to mammary tissue as chylomicrons (51-54). Mammary tissue removes up to 50% of the blood triglyceride that enters the gland (47) and accounts for a major part of the additional lipid ingested during lactation (55). There is no appreciable gain in body fat stores in the rat during lactation (21,22), and, accordingly, lipoprotein lipase activity in adipose tissue was found low during the first 3 days of lactation (56). Hypertriglyceridemia develops during pregnancy in the rat but disappears a few days prior to delivery (56,57).

The relationship between lipoprotein lipase activity in adipose and mammary tissue and the plasma concentration of triglyceride during pregnancy and lactation was recently studied in rats in our laboratory (Fig. 2). Lipoprotein lipase activity in adipose tissue increased during the first 19 days of pregnancy, decreased to very low levels several days before parturition and remained low during lactation. The enzyme activity in mammary tissue was low during the first 20 days of pregnancy, increased several days before parturition, decreased sharply at parturition, and increased again immediately postpartum; it remained high throughout lactation.

Plasma triglyceride concentration increased from 0.9 to 3.3 mM between the 12th and 20th days of pregnancy and then decreased 50% during the next 2 days (Fig. 2). It increased again at parturition, and then fell sharply and remained $< 1.0$ mM throughout lactation. Hypertriglyceridemia developed during pregnancy while adipose tissue lipoprotein lipase activity was high, suggesting that plasma triglyceride concentration was independent of the level of enzyme activity in adipose tissue. Later in pregnancy and during lactation, when the enzyme activity was low in adipose tissue, plasma triglyceride concentration was inversely related to lipoprotein lipase activity in mammary tissue.

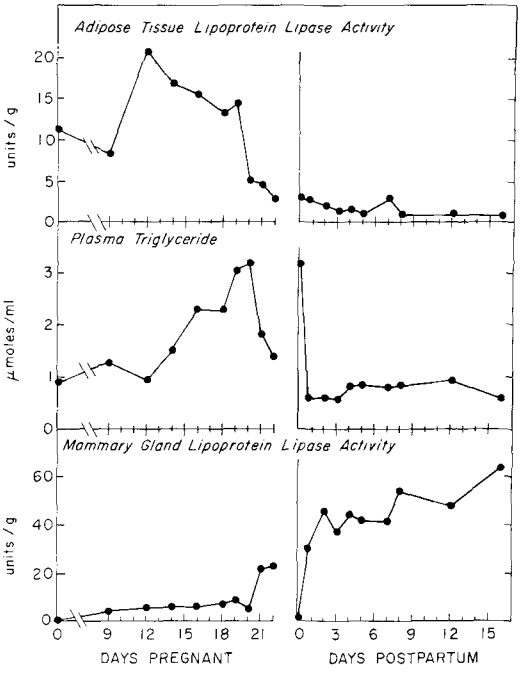


FIG. 2. Effect of pregnancy and lactation on plasma triglyceride and lipoprotein lipase activity of adipose and mammary tissue in rats. Parturition occurred on the 22nd or 23rd day of pregnancy. All rats were suckled after parturition. (After Hamosh et al. [23].)

Nonsuckling caused a marked fall in lipoprotein lipase activity of mammary tissue in lactating guinea pigs (49) and rats (23). It also increased plasma triglyceride concentration and lipoprotein lipase activity in adipose tissue of rats (23). Nonsuckling for 9 hr in rats (23) lowered mammary tissue lipolytic activity 70%, increased plasma triglyceride three-fold (to 3.1 mM) and increased slightly adipose tissue activity. Nonsuckling for another 9 hr completely inhibited lipoprotein lipase of mammary tissue and increased activity of adipose tissue to 55% of that in nonlactating rats; it had no further effect on plasma triglyceride concentration.

The inverse relationship between mammary tissue lipoprotein lipase activity and plasma triglyceride concentration in lactating rats suggests that lipoprotein lipase is involved in the uptake of blood triglyceride by mammary tissue. The relationship between lipoprotein lipase activity in adipose tissue and that in mammary tissue suggests that the hormonal factors necessary for milk secretion divert dietary fatty acids to the mammary gland by suppressing lipoprotein lipase activity in adi-

TABLE I

Hydrolysis of Chylomicron-Triglyceride by Perfused Adipose Tissue[a,b]

| Time, min | Blood triglyceride, mM | Released to blood stream, nmoles/min | | |
|---|---|---|---|---|
| | | Glycerol | FFA[c] | FFA/glycerol |
| 0-15 | .7 | 1.8 | 2.5 | 1.3 |
| 15-30 | .8 | 2.0 | 3.1 | 1.5 |
| 30-45 | .9 | 1.9 | 2.8 | 1.4 |
| 45-60 | 1.0 | 1.8 | 2.2 | 1.2 |
| 60-65 | .5 | 1.5 | 1.8 | 1.1 |
| 65-70 | .02 | .8 | .3 | .3 |

[a]After Evans et al. (62).

[b]Doubly labeled chylomicrons were infused for 60 min. The tissues weighed 400 mg at the end of the perfusion. Chemical analyses of the tissues are given in Table II. The values are means of four experiments.

[c]FFA = Free fatty acids.

pose tissue and stimulating the lipase activity in mammary tissue (23).

Transfer of plasma triglyceride-fatty acids across the capillary wall and the role of lipoprotein lipase in this process have been studied in perfused adipose tissue in our laboratory (58-60). The rat parametrial fat body, which was used for these studies, is a bilateral structure that consists of a fatty appendage that extends laterally from the uterine horn and fatty tissue located along the uterine blood vessels (61). The tissue is isolated by ligating and cutting the uterine vessels near the ovary and the vesical vessels, and by electrocauterizing the small vessels to the uterus. The uterine blood vessels are then cut near their juncture with the common iliac vessels; the fat pad is transferred to the tissue chamber, and the uterine artery and vein are immediately cannulated.

The perfusion apparatus, described elsewhere (39,61), consisted of a reservoir-oxygenator, pulsating pump, filter, manometer, injection site and tissue chamber. The perfusing fluid was composed of defibrinated rat blood diluted 1:10 with Tyrode's solution containing 4% albumin, 0.05% glucose and antibiotics. Radioactive chylomicrons and other substances were injected intraarterially through a needle inserted into the arterial tubing between the manometer and tissue chamber (39,61). Chylomicrons were prepared from chyle collected for 6 hr from the thoracic duct of rats fed corn oil containing [$^{14}$C] palmitic acid and triolein labeled with [$^{3}$H] glycerol (23). Since rat adipose tissue contains very little glycerokinase, the amount of radioactive glycerol released from chylomicrons by the tissue was used as a measure of chylomicron-triglyceride hydrolyzed to glycerol and FFA (58-60).

Earlier studies showed that triglyceride in chylomicrons and artificial emulsions was taken up by perfused adipose tissue (58,59). About half of the triglyceride taken up was hydrolyzed and the fatty acids were reesterified, whereas the other half was retained intact in

TABLE II

Retention of Chylomicron-Fatty Acids in Perfused Adipose Tissue[a,b]

| Portion of tissue analyzed | Wet wt mg | Per cent of fatty acids retained as | | |
|---|---|---|---|---|
| | | Unhydrolyzed chylomicron triglyceride | New triglyceride | Free fatty acids |
| Whole | 400 | 42 ± 11 | 58 ± 11 | <1 |
| Proximal | 300 | 55 ± 8 | 45 ± 8 | <1 |
| Distal | 100 | 9 ± 2 | 91 ± 2 | <1 |

[a]After Evans et. al (62).

[b]The tissues were perfused 60 min with blood containing doubly labeled chylomicrons and flushed 10 min with chylomicron-free blood (see Table I). The proximal portion contained adipose tissue, uterine vessels and uterine suspensory ligament, whereas the distal portion contained only adipose tissue. The values are means ± SE of four experiments.

TABLE III

Hydrolysis of Chylomicron-Triglyceride by Perfused Adipose Tissue[a,b]

| Time min | Blood triglyceride mM | Released to blood stream, nmoles/min | | |
|---|---|---|---|---|
| | | FFA[c] | Glycerol | FFA/glycerol |
| 0-0.5 | 0 | 0 | 0 | 0 |
| 0.5-1 | .1 | .1 | 0 | --- |
| 1-1.5 | 1.4 | 4.1 | 1.0 | 5.3[d] |
| 1.5-2 | 2.8 | 7.7 | 5.1 | 1.5 |
| 2-2.5 | 3.6 | 10.7 | 7.6 | 1.4 |
| 2.5-3 | 4.2 | 13.5 | 9.8 | 1.3 |
| 3-4 | 4.0 | 13.0 | 9.8 | 1.3 |
| 4-5 | 4.2 | 12.4 | 9.0 | 1.4 |

[a]After Evans et al. (62).
[b]Infusion of doubly labeled chylomicrons was begun at 0 time. Values are means of three experiments.
[c]FFA = Free fatty acids.
[d]Significantly higher than other values, $p < .05$.

the tissue. The continued release of glycerol to the blood stream after stopping the infusion of chylomicrons suggested that triglyceride was hydrolyzed after it was removed from the blood stream (58,59).

We have recently studied in more detail the uptake of chylomicron-triglyceride by perfused adipose tissue of fed rats (62). Release of glycerol and FFA to the blood stream during and after infusion of doubly labeled chylomicrons, and incorporation of chylomicron-fatty acids into tissue triglyceride were measured. The blood triglyceride concentration during the infusion, which lasted 60 min, ranged from 0.7-1.0 mM (Table I). The fat pads, weighing 400 mg, released to venous blood 1.9 nmoles glycerol and 2.6 nmoles FFA per minute during the infusion of chylomicrons. After the infusion was stopped for 5 min, the blood triglyceride concentration was decreased 98% and FFA release was decreased 90%, whereas glycerol release was decreased only 55%. This finding supports our earlier conclusion (58,59). that hydrolysis occurs after triglyceride is removed from the blood. The molar ratio of FFA to glycerol in blood was 1.2-1.5 except during the last two collection periods, indicating that about two-thirds of the FFA produced were retained in the tissue. The fat pads hydrolyzed ca. 1% of the infused chylomicron-triglyceride to glycerol and FFA.

Analyses of fat pads at the end of the perfusion showed that 42% of the chylomicron-fatty acids retained in the whole tissue were present in unhydrolyzed chylomicron-triglyceride, 58% were in new triglyceride, and less than 1% were in FFA (Table II), confirming our earlier finding (58,59). that half of the triglyceride taken up was retained intact in perfused tissue. Studies made by others in vivo (63), however, showed that most of the triglyceride taken up from blood by adipose tissue was immediately hydrolyzed to FFA and glycerol. In order to localize the unhydrolyzed triglyceride in perfused adipose tissue, the fat pads were divided into two portions: a distal portion consisting of the tip of the fatty appendage, and a proximal portion consisting of the rest of the fat pad, the large uterine blood vessels, and the small vessels and suspensory tissue that had been separated from the uterus with an electrocautery. The distribution of the fatty acids retained in the proximal segment was similar to that in the whole tissue, 55% in unhydrolyzed chylomicrons and 45% in new triglyceride (Table II). In the distal segment, however, only 9% of the retained fatty acids were present in unhydrolyzed chylomicrons and the rest, 91%, were in new triglyceride. Since edema usually developed during the perfusion near the uterine vessels and in the uterine suspensory tissue, it is possible that abnormal sequestration of chylomicrons occurred in the edematous fluid in the extravascular space, and that this accounts for the large retention of unhydrolyzed blood triglyceride in the proximal portion. The distribution of fatty acids retained in the whole fat body reflects the distribution in the proximal portion because the latter contained 70% of the fatty acids that were retained. The very small amount of unhydrolyzed blood triglyceride found in the distal portion, which was not edematous, suggests that most of the triglyceride taken up by intact adipose tissue is completely hydrolyzed, confirming the findings made in vivo

TABLE IV

Effect of Heparin on Hydrolysis of Chylomicron-Triglyceride and on Release of Lipoprotein Lipase Activity by Perfused Adipose Tissue[a,b]

| Time, min | Released to blood stream | | | |
|---|---|---|---|---|
| | Glycerol, nmoles/min | | Lipoprotein lipase activity, U/min | |
| | Control | Heparin | Control | Heparin |
| 0-2 | 0 | 0 | .03 | .03 |
| 2-5 | 3.3 | 3.4 | .03 | .60[c] |
| 5-10 | 4.0 | 1.5 | .03 | .11[c] |
| 10-15 | 5.2 | 1.3[c] | .03 | .11[c] |
| 15-30 | 4.3 | 1.2[c] | .01 | .07[c] |
| 30-45 | 3.8 | .9[cc] | .007 | .04 |
| 45-60 | 3.4 | 1.0[c] | .015 | .02 |

[a]After Evans et al. (62).

[b]Infusion of doubly labeled chylomicrons and heparin was begun at 0 time, 10 min after the start of the perfusion. Blood triglyceride concentration averaged 0.8 mM in the control group and 0.9 mM in the heparin group, and blood heparin concentration averaged 30 μg/ml in the heparin group. Lipoprotein lipase activity is expressed in units (U) with 1 unit = 1 μmole of triglyceride hydrolyzed per hr (23). There were five tissues in each group.

[c]Difference between control and heparin groups was statistically significant, $p < .01$.

(63).

The rates of release of FFA and glycerol to blood during the first minutes of infusion of chylomicrons are shown in Table III. The time lag of $> 1$ min before chylomicrons appeared in venous blood was due to the volume of tubing between the injection site and the venous blood collecting tube, 0.3 ml, and the rate of blood flow through the tissue, ca. 0.2 ml/min (39). The triglyceride concentration in venous blood began to increase between 1-1.5 min after starting the infusion of chylomicrons, and reached a plateau after 2 min. The molar ratio of FFA to glycerol was 5.3 during the third collection period (1-1.5 min), when the blood triglyceride concentration was first increased, 1.5 during the fourth period (1.5-2 min), and less than 1.5 thereafter (Table III). These findings suggest that blood triglycerides are hydrolyzed first to partial glycerides and FFA, with immediate release of FFA to blood, and hydrolyzed ca. 30 sec later to glycerol and FFA, with release of glycerol to blood and incorporation of the rest of the FFA into tissue triglyceride.

Lipoprotein lipase activity is present in both fat cells and vascular-stromal cells of adipose tissue (4,59,64). Infusion of heparin causes the immediate release of lipoprotein lipase activity to the blood stream, presumably from within or near the capillary wall (59,65). In order to determine the effect of loss of this enzyme activity on the uptake of blood triglyceride by perfused adipose tissue, chylomicrons were infused with and without heparin. Control tissues released less than 0.04 unit of lipoprotein lipase activity per minute and hydrolyzed ca. 4 nmoles of triglyceride per minute (Table IV). Infusion of heparin at a blood concentration of 30 μg/ml immediately increased the release of enzyme activity to 0.6 unit/min and 3 min later decreased the rate of hydrolysis of chylomicrons by the tissue to $<1.5$ nmoles of triglyceride per minute. Although enzyme release was negligible after 45 min of infusion of heparin, the rate of hydrolysis of chylomicrons was still 70% less than that by control tissues. The rapid release of enzyme activity and the subsequent decrease in hydrolysis of chylomicrons when heparin was infused suggest that lipoprotein lipase activity located in or near the capillary wall is involved in the hydrolysis of blood triglyceride.

In order to determine where chylomicron-triglyceride is hydrolyzed in adipose tissue and how triglyceride-fatty acids cross the capillary wall, rat adipose tissue perfused with chylomicrons was studied with the electron microscope. Sites of lipoprotein lipase activity were demonstrated in the tissue with a cytochemical procedure (40,41) based on Gomori's histochemical method for lipase. Tissue specimens, after being fixed with glutaraldehyde, were incubated at 38 C and pH 8.3 in a medium containing $Ca^{++}$. The fatty acids released from chylomicrons by lipoprotein lipase activity formed insoluble fatty acid soaps with calcium at the sites of hydrolysis. Since these precipitates were not visible with the electron microscope, the specimens were treated with $Pb^{++}$ to transform the fatty acid soaps to electron-opaque precipitates. The latter indicated sites of hydrolysis of chylomicron-triglyceride by lipoprotein lipase (40).

A capillary in unincubated perfused adipose tissue is shown in part in Figure 3A. An intact chylomicron is present in the capillary lumen, and another is partially enveloped by the endothelium. A large vacuole and many microvesicles are seen inside the endothelial cell. Capillaries in incubated perfused adipose tissue are shown in Figures 3B and 3C. The endothelial cell in Figure 3B contains two large electron-opaque precipitates, a laminar precipitate in a vacuole and a granular precipitate outlining a circular profile in the cell. An intact chylomicron is present in the capillary lumen near the endothelium. The capillary shown in Figure 3C has a laminar precipitate in the endothelial cell and a similar precipitate in the subendothelial space, between the endothelium and a pericyte. Electron-opaque precipitates were also found in microvesicles of endothelial cells and in chylomicrons closely attached to or enveloped by the endothelial cells (40), but not in unattached chylomicrons (Fig. 3B).

The above cytochemical findings indicate that glycerides are hydrolyzed by lipoprotein lipase to FFA in vacuoles and microvesicles of the capillary endothelium and in the subendothelial space of adipose tissue. The findings also demonstrate that glycerides cross the endothelial cells within a membrane-bounded system. The chemical nature of the glyceride in the endothelium and subendothelial space prior to incubation is not known. The findings presented in Table III would suggest they may be partial glycerides. Hydrolysis also occurred in chylomicrons attached to or partially enveloped by endothelial cells, but not in "free-flowing" chylomicrons (Fig. 3B). The latter finding is supported by the perfusion studies described above showing that practically no lipoprotein lipase activity was released to the blood stream unless heparin was infused (Table IV).

In conclusion, a schema of how chylomicron-fatty acids may cross the capillary wall in adipose tissue is presented in Figure 4. It is based primarily on our biochemical and cytochemical findings in perfused adipose tissue. When a chylomicron becomes attached and partially enveloped by the endothelial cell, triglyceride (depicted by three dots in a cross-hatched area) within the chylomicron is hydrolyzed by lipoprotein lipase to FFA and diglyceride. The FFA (a single dot) is released to the blood stream and the diglyceride (two dots in a cross-hatched area) is taken into a vacuole or microvesicle. Hydrolysis of the diglyceride to FFA and monoglyceride (one dot in a cross-hatched area) occurs during transit across the endothelium and the products are released to the subendothelial space, between the endothelium and pericyte. The FFA is taken up and esterified by the fat cell, and the monoglyceride is hydrolyzed in the subendothelial space to FFA and glycerol. The resultant FFA is taken up and esterified by the fat cell, and the glycerol (cross-hatched area) is released to the blood stream. The chylomicron remnant (37,38) (depicted by the stippled concave area) and most of the FFA and glycerol released to the blood are taken up and utilized by the liver.

This schema also shows how lipoprotein lipase activity within the endothelial cell could

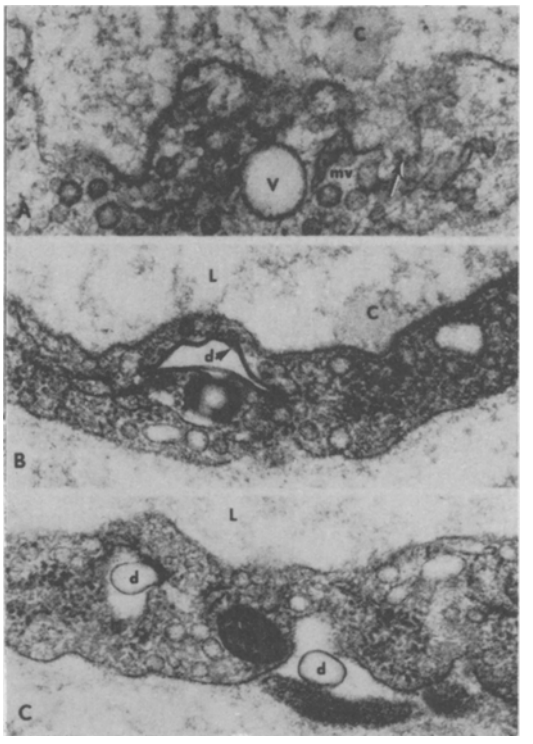


FIG. 3. Electron micrograph of capillary endothelium in rat parametrial adipose tissue perfused 5 min with chylomicrons. A: Unincubated tissue. Note the chylomicron (C) in the capillary lumen (L) and floculent material at the invaginations of the plasma membrane (arrow). Microvesicles (mv) and a large vacuole (V) are present in the endothelium and appear to be continuous with invaginations of the plasma membrane. X 42,500. B: Tissue incubated 1 hr in $CaCl_2$-Tris medium (pH 8.4) and treated with $Pb^{++}$. Note the laminar deposit (d) within a vacuole, and the adjacent granular precipitate outlining a circular profile within the cell. An intact chylomicron (C) is present in the capillary lumen (L). X 38,000. C: Tissue incubated 1 hr in $CaCl_2$-Tris medium (pH 8.4) and treated with $Pb^{++}$. Note the circular profiles (d) within the endothelial cell and in the subendothelial space. These profiles indicate that glyceride was hydrolyzed within the endothelium and in the space between the endothelium and pericyte (P). X 38,000. (From Blanchette-Mackie and Scow [40].)

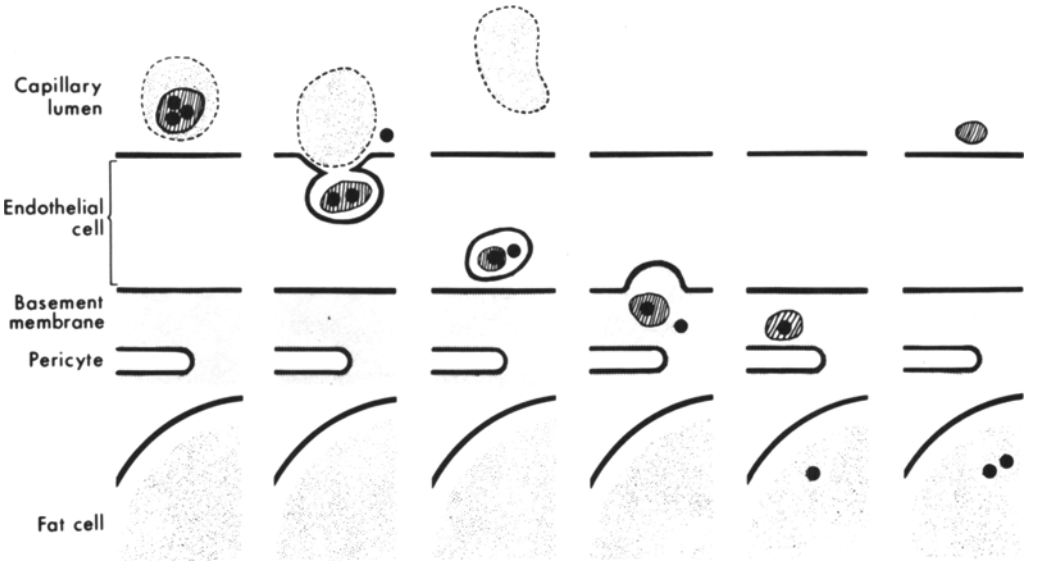


FIG. 4. Model for the transport of chylomicron-fatty acids across the capillary wall in adipose tissue. Triglyceride is depicted by three dots in a cross-hatched area, diglyceride by two dots in a cross-hatched area, monoglyceride by one dot in a cross-hatched area, FFA by single dots, glycerol by the cross-hatched area, and the chylomicron and chylomicron remnant by the stippled area in the capillary lumen.

regulate uptake of chylomicron-triglyceride by adipose tissue and how hydrolysis of partial glycerides within the endothelium and subendothelium could direct two-thirds of the glyceride-fatty acids across the capillary wall to the parenchymal cell, the fat cell. This schema probably applies to the other tissues that have the type of capillary found in adipose tissue, such as mammary gland, skeletal muscle and heart. Where lipoprotein lipase is synthesized, what regulates its activity and how lipid particles are attached to the capillary endothelium are some of the problems awaiting further study.

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# The Fatty Acids of Wax Esters and Sterol Esters from Vernix Caseosa and from Human Skin Surface Lipid

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## ABSTRACT

Separation of sterol esters from wax esters in the lipids of vernix caseosa and adult human skin surface was accomplished by column chromatography on MgO. The fatty acids of the sterol esters and wax esters of both samples were separated into saturates and monoenes, and examined in detail by gas liquid chromatography (GLC). The saturated fatty acids of the wax esters of vernix caseosa and of adult human skin surface were remarkably similar. They ranged in chain length from at least $C_{11}$ to $C_{30}$, six skeletal types being present: straight even, straight odd, iso, anteiso, other monomethyl branched and dimethyl branched. A large number of patterns of monoenes were observed, each pattern consisting of desaturation of a specific chain at Δ6 or Δ9 plus its extension or degradation products. The mole per cent of the total Δ6 and Δ9 patterns of wax ester fatty acid monoenes of vernix caseosa were 87% and 12%, respectively, and 98% and 1%, respectively, for adult human skin surface lipid. The sterol ester fatty acids of vernix caseosa were much different from those of adult human skin surface: vernix caseosa saturates were largely branched and of lengths greater than $C_{18}$, whereas the saturates of adult human surface lipid resembled the wax ester fatty acids. Of the vernix caseosa monoene patterns, the mole per cent was 30% Δ6 and 70% Δ9, whereas of the adult human skin surface sterol ester fatty acids 89% were Δ6 and 11% Δ9. Chain extension was particularly pronounced in the sterol ester fatty acid monoenes of vernix caseosa amounting to 7-8 $C_2$ units in some cases. The fatty acids of the sterol esters of both vernix caseosa and adult human skin surface appear to be derived from the sebaceous gland and from the keratinizing epidermis, but those of the wax esters are from the sebaceous glands only.

## INTRODUCTION

Sterol esters of skin lipids have evoked considerable interest since Rothman reported that psoriatics have a lower amount than normal (1). Their origin and role are still unresolved problems.

Knowledge of skin sterol esters has been hampered by the difficulty in separating them from the wax esters, for in most chromatographic systems the two ester types migrate as a group. Sterol esters can now be separated from wax esters conveniently by chromatography on magnesium oxide (2).

On the assumption that the fatty acid moieties of each of these ester types would give clues as to the origin and role of these lipids, we used the above technique to separate the sterol esters from the wax esters of the lipids of vernix caseosa and of adult human skin surface. A plausible explanation as to how sterol esters are formed, which would fit all of our results and other relevant data, is that sterols, which are products of epidermis, become esterified primarily (but not entirely) with sebum fatty acids in late phases of keratinization or after keratinization is complete, both in the adult human skin and in vernix caseosa. Some unusual fatty acid unsaturation patterns were also observed, as were some unusual extension patterns of both saturated and unsaturated fatty acids.

## EXPERIMENTAL PROCEDURES

Human skin surface lipid was collected daily from a 26-year-old man by the ether scalp soaking technique (3). An aliquot (817.1 mg) from four soaks was chromatographed on a column (205 mm x 44 mm ID) of 134 g silicic acid (Unisil, 100 mesh, Clarkson Chemical Co., Williamsport, Pa.). Fifty milliliter fractions were collected and assayed by thin layer chromatography (TLC). (This was carried out on 250μ layers of silica gel plus magnesium silicate 9:1 developed in hexane-ether 95:5 v/v.) Hexane (550 ml) and 5% benzene in hexane (520 ml) eluted 4.0 mg saturated hydrocarbons and 75.3 mg squalene, respectively. Then 600 ml 20% benzene in hexane eluted 185.1 mg of a mixture of sterol esters plus wax esters, and an additional 550 ml eluted a mixture of 11.80 mg wax esters plus about an equal amount of more polar material. The latter fraction was not included in further work-up.

A 173.7 mg aliquot of sterol esters plus wax esters was chromatographed on a column (92 mm x 44 mm ID) of 85 g MgO (Matheson

TABLE I

Relative Amounts of Wax Esters and Sterol Esters and Their Fatty Acid Moieties in Vernix Caseosa and Adult Human Skin Surface Lipid

| Esters | Vernix caseosa lipid | | | | Adult human skin surface lipid | | | |
|---|---|---|---|---|---|---|---|---|
| | Fatty acid moieties, 100% | | | | | | | |
| | Saturates | Monoenes | Dienes | Polar[a] | Saturates | Monoenes | Dienes | Polar[a] |
| Wax esters, % of total lipid | | 15.9% | | | | 19.6% | | |
| Straight even | 8.5 | 46.8 | | | 16.5 | 40.7 | | |
| Straight odd | 1.8 | 8.1 | | | 2.7 | 4.7 | | |
| Iso | 6.6 | 6.6 | | | 3.5 | 15.0 | | |
| Anteiso | 6.1 | 5.0 | | | 1.8 | 4.0 | | |
| Monomethyl branched[b] | 1.9 | tr[c] | | | 2.2 | tr | | |
| Dimethyl branched[d] | 0.1 | tr | | | 0.2 | tr | | |
| Totals | 25.0 | 66.5 | 6.0 | 2.0 | 26.9 | 64.4 | 4.0 | 4.7 |
| Sterol esters, % of total lipid | | 25.4% | | | | 2.81% | | |
| Straight even | 6.8 | 23.6 | | | 22.5 | 40.5 | | |
| Straight odd | 0.9 | 1.3 | | | 4.4 | 4.1 | | |
| Iso | 38.1 | 3.8 | | | 5.2 | 7.4 | | |
| Anteiso | 19.6 | 0.7 | | | 3.3 | 2.0 | | |
| Monomethyl branched[b] | tr | tr | | | 2.3 | tr | | |
| Totals | 65.4 | 29.4 | 3.7 | 1.5 | 37.7 | 54.0 | 8.3 | tr |

[a]Polar unidentified material.

[b]Equivalent chain lengths ranged from 10.5-20.5 (see Table II).

[c]tr = Trace.

[d]Equivalent chain lengths ranged from 11.2 to at least 17.2 (Table II).

TABLE II

Fatty Acids of Wax Esters and Sterol Esters from Vernix Caseosa and from Human Skin Surface Lipid[a]

| | Wax esters | | | | Sterol esters | | | |
|---|---|---|---|---|---|---|---|---|
| | Saturates | | Hydrogenated monoenes | | Saturates | | Hydrogenated monoenes | |
| ECL[c] | VC[b] % | HSL % | VC % | HSL % | VC % | HSL % | VC % | HSL % |
| 11.22 | .02 | tr | | | | | | |
| 11.50 | .43 | | | | | tr | | |
| 11.65 | tr | .17 | | | tr | .21 | | |
| 12 | .77 | .96 | tr | tr | tr | .64 | | |
| 12.12 | tr | .30 | | | | tr | | |
| 12.48 | 2.01 | 1.24 | | | | .54 | | |
| 12.68 | .87 | .12 | | | tr | tr | | |
| 13 | .76 | .37 | .04 | .04 | tr | .37 | | |
| 13.15 | tr | tr | | | | tr | | |
| 13.20 | .44 | .24 | | | tr | | | |
| 13.3 | | | | | | tr | | |
| 13.48 | .15 | tr | | | tr | .06 | | |
| 13.66 | 9.28 | 5.99 | .03 | tr | 2.25 | 3.18 | tr | tr |
| 14 | 8.13 | 13.04 | 7.62 | 6.76 | 1.25 | 13.69 | .60 | 5.25 |
| 14.2 | tr | tr | Some? | tr? | Some? | Some? | | |
| 14.48 | 2.85 | 2.78 | | | tr | 2.49 | | |
| 14.69 | 16.60 | 5.32 | .63 | 2.23 | 6.41 | 4.75 | tr | 1.05 |
| 15 | 5.22 | 6.85 | 6.86 | 5.06 | 1.02 | 7.12 | .70 | 3.54 |
| 15.2 | tr | tr | Some? | | | Some? | | |
| 15.49 | tr | .07 | | | tr | .20 | | |
| 15.65 | 14.63 | 5.04 | 8.78 | 21.56 | 15.24 | 4.62 | 2.02 | 10.09 |
| 16 | 20.50 | 40.13 | 48.39 | 47.01 | 5.02 | 32.25 | 11.61 | 45.83 |
| 16.2 | tr | tr | Some | Some? | | Some? | | |
| 16.50 | 2.17 | 3.99 | Some | Some? | tr | 3.09 | tr | |
| 16.72 | 5.76 | .75 | 6.92 | 3.84 | 3.45 | 1.31 | 1.21 | 2.22 |
| 17 | .96 | 2.02 | 5.13 | 2.07 | .24 | 2.32 | 1.93 | 3.34 |
| 17.22 | .08 | tr | Some? | | .05 | .07 | | |
| 17.52 | tr | .04 | | Some | | .07 | | |
| 17.65 | .84 | .38 | .98 | 1.49 | 3.65 | .60 | 3.32 | 2.49 |
| 18 | 3.78 | 4.83 | 13.18 | 8.60 | 1.29 | 6.05 | 32.24 | 20.76 |
| 18.2 | tr | tr | Some? | | | .05 | | |
| 18.49 | tr | tr | .02 | | .15 | .05 | | |
| 18.6 | | | | | | .07 | | |
| 18.72 | .69 | tr | .04 | .14 | 1.32 | .21 | .40 | .22 |
| 19 | .07 | .26 | .19 | .21 | .06 | .58 | .66 | .58 |
| 19.66 | .76 | .24 | .09 | .09 | 12.90 | 1.23 | 3.38 | .52 |
| 20 | .25 | .70 | .64 | .53 | .84 | 1.68 | 9.55 | 1.53 |
| 20.4 | | tr | | | .60 | .01 | | |
| 20.5 | tr | tr | | | | .04 | tr? | |
| 20.72 | .25 | .09 | .02 | .01 | 6.80 | .55 | .39 | .06 |
| 21 | tr | .05 | .03 | .01 | tr | .32 | .49 | .06 |
| 21.47 | | | .02 | .03 | Some | | | |
| 21.65 | .30 | .21 | .03 | .01 | 12.43 | 1.25 | 1.59 | .14 |
| 22 | .12 | .47 | .11 | .07 | .64 | 1.23 | 12.47 | .06 |
| 22.2 | | | .02 | | | Some? | | |
| 22.5 | | | | | .60 | .20 | | |
| 22.74 | .15 | .15 | .01 | tr | 4.69 | .49 | .18 | .05 |
| 23 | .02 | .16 | .02 | .01 | .20 | .26 | .35 | .06 |
| 23.2 | | tr | | | | | | |
| 23.3 | tr | | | | | | | |
| 23.4 | tr | | tr | | | tr | | |
| 23.5 | | | | tr | Some | | | |
| 23.64 | .35 | .52 | .01 | .04 | 7.76 | 1.57 | 1.88 | .27 |
| 24 | .30 | 1.11 | .07 | .05 | .95 | 2.90 | 9.04 | .75 |
| 24.2 | | | | | | .07 | | |
| 24.5 | tr | Some? | .01 | | .60 | .05 | | |
| 24.75 | .25 | .43 | .01 | tr? | 3.96 | 1.00 | .16 | .07 |
| 25 | .02 | .28 | .01 | tr | tr | .54 | .14 | .07 |
| 25.4 | | | | | Some? | | | |
| 25.6 | .15 | .33 | .01 | .04 | 3.38 | .88 | .64 | .26 |
| 26 | .03 | .37 | .04 | .04 | .40 | .94 | 1.43 | .19 |

(Continued on following page)

TABLE II
(Continued from preceding page)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 26.4 | | | | | | .03 | | |
| 26.7 | .02 | tr | tr | .02 | 1.11 | .07 | tr | tr |
| 27 | tr | tr | tr | .02 | tr | .04 | tr | tr |
| 27.6 | .02 | tr | .01 | .01 | .60 | .03 | tr | tr |
| 28 | tr | tr | .02 | .01 | tr | .03 | .50 | tr |
| 28.4 | | | | | tr | | | |
| 28.7 | | | | | .14 | | | tr |
| 29 | | | tr | | | .03 | tr | tr |
| 29.5 | | | | | tr | | | |
| 29.6 | | | | | | | | tr |
| 30 | | tr | .02 | | | | .21 | |
| | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |

[a]VC = vernix caseosa, HSL = human surface lipid, tr = trace. Percentage figures are given to two decimal places simply to round out the data and show relative amounts of the minor components. Accuracy is estimated at ± 5%.

[b]Traces of material to ECL's of 10.50 were seen for this sample.

[c]ECL = equivalent chain length (7). The ECL's listed are an average of all eight samples. Where only one decimal is listed the error is estimated at ± .05 for all samples: where two decimals are given maximum error for all samples is ± .04 and usually ± .02 or less.

Coleman and Bell MX-66 catalytic grade 200 mesh adsorptive powder) and eluted with 20 ml hexane and 190 ml 1% acetone in hexane before anything emerged. An additional 80 ml, 140 ml and 550 ml of 1% acetone in hexane eluted 107.25 mg wax esters, 1.14 mg of a mixture of wax esters plus sterol esters and 27.05 mg of a mixture of sterol ester plus cholesterol and wax alcohol respectively. Finally an additional 450 ml 1% acetone in hexane plus 350 ml 10% acetone in hexane eluted 20.23 mg wax alcohol plus sterol. The 27.05 mg fraction was then rechromatographed on 12 g of silicic acid on a column (120 x 16 mm ID) and sterol esters (21.79 mg) easily separated from the free alcohols plus sterols (5.96 mg). This gave a final recovery of 107.25 mg wax esters, 1.14 mg of an equal mixture of sterol esters and wax esters, 21.79 mg sterol esters and 26.19 mg of a mixture of free alcohol and free sterol in proportion of ca. 5:1 (assayed by TLC), making a total of 156.37 mg recovered. The unrecovered 17.3 mg from the original 173.7 mg was presumably held on the column as fatty acid salts of magnesium. Subsequently it was shown that when fines were removed from the magnesium oxide (which was not done in this chromatogram) less than 5% hydrolysis occurred (2). However the proportion of alcohol to sterol recovered indicated that no preferential hydrolysis of either ester class occurred.

An aliquot (26.9 mg) of the wax ester fraction and the entire sterol ester fraction were then individually saponified (10% KOH in 90% ethanol refluxed 3 hr under nitrogen); the saponification mixture was diluted with water, then acidified with 6N $H_2SO_4$ and extracted with hexane. The unsaponifiables were then separated from the fatty acids quantitatively by column chromatography on 4.6 g Florisil (Floridin Co., Tallahasee, Fla.) after a batch of the adsorbent had been washed extensively with distilled water, activated at 120 C for 16 hr, then 7% water added (4). Column dimensions were 105 x 50 mm ID. For the sterol ester separation of saponified products, after hexane had eluted a tiny trace of material, 60 ml of $CHCl_3$ eluted 12.32 mg of sterols and 35 ml chloroform-methanol-formic acid (88%) in the proportion 90:5:5 v/v/v eluted 8.31 mg of fatty acids. The wax esters yielded 14.83 mg of wax alcohols and 13.57 mg of fatty acids in a similar chromatogram. The fatty acids from each group of esters were then esterified with methanolic $BF_3$ and separated into saturates, monoenes, dienes and "polar" material on $AgNO_3/SiO_2$ columns by techniques already reported (5). The saturates were analyzed by GLC and the monoenes were collected by preparative GLC and analyzed by hydrogenation, GLC and ozonolysis by procedures also previously reported (5). Nothing further was done with the polar material or dienes (except as indicated below).

Vernix caseosa (11.82 g) was obtained from a Caucasian male to yield 1.370 g lipid. This was analyzed by a very similar procedure, and details have been reported (6).

## RESULTS AND DISCUSSION

The wax ester fatty acids of both vernix caseosa and adult human skin surface show a remarkable similarity. Not only do these esters make up nearly the same percentage of their corresponding lipid samples (Table I), but their fatty acid moieties show a similar composition

TABLE III

Positions Isomers of Fatty Acid Monoenes in Wax Esters of Vernix Caseosa and Adult Human Skin Surface Lipid

| Monoene carbon skeleton[a] | Mole % of total monoenes | Mole % at Δ positions indicated[b] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Δ4 | Δ5 | Δ6 | Δ7 | Δ8 | Δ9 | Δ10 | Δ11 | Δ12 | Δ13 | Δ14 | Δ15 |
| Vernix caseosa | | | | | | | | | | | | | |
| n-14 | 8.59 | 1 | tr | 96 | 1 | 1 | 1 | tr | | | | | |
| n-16 | 48.88 | | tr | 88 | tr | 5 | 6 | tr | 1 | tr | tr | | |
| n-18 | 12.04 | nd | nd | 14 | tr | 26 | 35 | 1 | 21 | nd | 3 | | |
| n-20 | 0.54 | | tr | 4 | 3 | 2 | 2 | 33 | 9 | 3 | 44 | | |
| n-22 | 0.08 | NA | | | | | | | | | | | |
| n-24 to 30 | 0.10 | NA | | | | | | | | | | | |
| n-13 | .06 | NA | | | | | | | | | | | |
| n-15 | 7.31 | tr | tr | 99 | tr | tr | 1 | nd | | | | | |
| n-17 | 4.91 | | tr | 76 | 4 | 7 | 7 | tr | 6 | tr | | | |
| n-19 | .17 | NA | | | | | | | | | | | |
| n-21 to 27 | .04 | NA | | | | | | | | | | | |
| i-14 | .04 | NA | | | | | | | | | | | |
| i-16 | 8.84 | tr | 1 | 98 | 1 | tr | tr | tr | tr | | | | |
| i-18 | .90 | | 2 | 64 | 5 | 23 | 6 | tr | | | | | |
| i-20 to 28 | .13 | NA | | | | | | | | | | | |
| ai-15[c] | .67 | | 3 | 97 | tr | tr | tr | tr | | | | | |
| ai-17[c] | 6.62 | | tr | 100 | tr | tr | | | | | | | |
| ai-19 to 27 | .08 | NA | | | | | | | | | | | |
| Adult human skin surface lipid | | | | | | | | | | | | | |
| n-14 | 7.57 | nd | nd | 100 | nd | nd | nd | nd | nd | nd | | | |
| n-16 | 47.17 | nd | tr | 95 | tr | 4 | 1 | tr | tr | tr | tr | tr | tr |
| n-18 | 7.81 | nd | tr | 38 | 2 | 52 | 5 | 3 | tr | tr | tr | tr | |
| n-20 | .44 | | nd | nd | 11 | 9 | 3 | 61 | 6 | 10 | tr | tr | |
| n-22 to 28 | .12 | NA | | | | | | | | | | | |
| n-13 | .05 | NA | | | | | | | | | | | |
| n-15 | 5.36 | | nd | 100 | nd | | | | | | | | |
| n-17 | 1.97 | | tr | 75 | 7 | 17 | 1 | tr | nd | | | | |
| n-19 | .18 | | nd | 55 | 13 | 38 | 8 | 34 | 2 | | | | |
| n-21 to 27 | .03 | NA | | | | | | | | | | | |
| i-16 | 21.63 | | | 100 | | | | | | | | | |
| i-18 | 1.35 | | 2 | 39 | 2 | 57 | | | | | | | |
| i-20 | .08 | | 6 | 7 | 1 | 24 | 4 | 59 | tr | tr | | | |
| i-22 to 28 | .08 | NA | | | | | | | | | | | |
| ai-15 | 2.36 | | 1 | 97 | 1 | 1 | | | | | | | |
| ai-17 | 3.66 | nd | nd | 100 | nd | | | | | | | | |
| ai-19[c] | .12 | | | 47 | 9 | 44 | | | | | | | |
| ai-21 to 27 | .02 | NA | | | | | | | | | | | |

[a]n- = normal, i- = iso, ai- = anteiso.

[b]tr = Trace, nd = not detected, NA = not analyzed. All structures were determined by analysis of the aldehydes and aldehyde esters formed on reductive ozonolysis (5).

[c]Structure determined by analysis of aldesters only.

both with regard to the amount of saturates and unsaturates (Table I) and to the types of chains (Table II). The chains range from an equivalent chain length (ECL) (7) of 10.50-30.00, the chain types being straight even, straight odd, iso, anteiso, other monomethyl branched and dimethyl branched. These chain types have all been identified by mass spectrometry (6). The similarity in composition of such a diverse group of acids for these two entirely different samples argues against any significant contamination of either sample. However some differences in distribution (Table I) as well as in double bond patterns (to be discussed) does exist.

The sterol esters of vernix caseosa, on the other hand, make up not only a significantly greater percentage of total lipid than do the sterol esters of adult human skin surface lipid, i.e., 25.4% vs. 2.8%, but the fatty acid moieties of the two samples are considerably different. In this regard the sterol ester fatty acids of adult human skin surface resemble more closely those of the wax esters than they do those of

the sterol esters of vernix caseosa. In vernix caseosa sterol esters the per cent saturated fatty acids is exceedingly high, i.e., 65% (Table I). Most of these saturated acids are branched, iso making up 38% and anteiso nearly 20%. This high amount of saturates is in marked contrast to the other samples which have much more straight even monoenes (∿40%). The sterol ester fatty acids of vernix caseosa differ in another respect from those of the other samples in that they have a higher proportion of the longer chain lengths. For example, 60% of the saturates and 43% of the monoenes are above $C_{18}$ for vernix caseosa, whereas only 16% of the saturates and 5% of the monoenes are above $C_{18}$ for adult human skin surface. The fatty acids of the wax esters on the other hand, have only ca. 5% saturates and 1% monoenes above $C_{18}$ for both samples.

Both vernix caseosa lipid and adult human skin surface lipid have methyl branched acids other than iso and anteiso for both wax esters and sterol esters. The location of these methyl branches is predominantly at position 4, but it also occurs on even C-atoms greater than 4 (6).

Our data show a rough correspondence in chain length distribution with those of Kärkkäinen et al. (8); however identifications of the exact structures are at considerable variance. What they have frequently stated as "iso" we have tabulated as ECL's of 0.45-0.52 which were found to be mixtures of monomethyl branched acids. The basis for their identification as "iso" or "anteiso" must have been solely from GLC retention data, and the hazards of that have been discussed (9).

The molar per cents of the double bond position isomers for each chain length monoene of the wax ester fatty acids of vernix caseosa and adult human skin surface are given in Table III; those for the sterol esters of both samples are given in Table IV. A probable manner in which these position isomers could be biosynthesized can be given if we make four reasonable assumptions: (a) There are two desaturase systems, one placing a double bond between the sixth and seventh C-atoms from the carboxyl group (designated as Δ6), and the other between the ninth and tenth C-atoms (designated as Δ9). (b) Both desaturase systems can desaturate all the major skeletal chain types of the fatty acids found, i.e., straight even, straight odd, iso and anteiso. We have also found that the other mono and dimethyl branched chain types show unsaturation (unpublished), but their structures have not yet been elucidated, and they are not included in this discussion of position isomer data. (c) Chain lengths primarily of $C_{14}$ to $C_{20}$ (usually those occurring in greatest amounts) are the substrates upon which the desaturase systems work. (d) After desaturation has occurred the chains can be further extended by $C_2$ units or degraded by $C_2$ units, the chain extension process predominating. Each initial substrate plus its extension or degradation products constitute what we are here calling a "pattern." The total moles of a pattern can be computed from data given in Tables III and IV. Thus the mole per cent of the 14Δ6 pattern would include, for example, not only the moles of the initial substrate, $C_{14:\Delta 6}$, but also all its extension and degradation products, i.e., $C_{16:\Delta 8}$, $C_{18:\Delta 10}$, $C_{20:\Delta 12}$, $C_{12:\Delta 4}$, etc., if these were present. For the various patterns occurring in the wax ester fatty acids of vernix caseosa and of adult human skin surface lipid, Table V gives the mole per cent of the total monoenes in the pattern as well as the number of molecular species in each pattern. Table VI gives the same data for the sterol ester fatty acids of both samples.

It is not implied that the biosynthetic scheme proposed here is the only one which can explain the data. Other desaturase systems (for example, desaturating between the seventh and eighth C-atoms) or decarboxylating enzymes may be present, which could also explain some of the products. The proposed scheme, however, does have the merit of simplicity and comprehensiveness, and postulates biochemical processes known to exist. We are not aware of any evidence which would necessitate mechanisms additional to those postulated here.

The large number of position isomers present (Tables III and IV) is truly a striking feature. For the wax esters of adult human skin surface lipid, nearly all the monoenes (98%) are of the Δ6 type (Tables II and V). Vernix caseosa wax ester fatty acids also show a preponderance of the Δ6 patterns (87%), but Δ9 patterns are appreciable (12%) (Table V). Of the latter the 16Δ9 and the 18Δ9 make up the largest proportion (5.78 and 4.26 mole %, respectively). The Δ9 patterns are far more prevalent among the sterol ester fatty acids of vernix caseosa, making up ∿70%; but for adult human skin surface, only 11% of the sterol ester fatty acids are Δ9 (Table VI).

It is highly probable that vernix caseosa sterol ester fatty acids originate from both keratinizing epidermis and sebum. Much evidence supports this conclusion: sterol esters are major components of epidermis (10-12); the major fatty acid monoene of epidermis is oleic acid, $C_{18:\Delta 9}$ (13); acids of the Δ6 type (from sebum only) and the Δ9 type (from both sebum and epidermis) occur in total vernix

TABLE IV

Position Isomers of Fatty Acid Monoenes in Sterol Esters of Vernix Caseosa and Adult Human Skin Surface Lipids

| Monoene carbon skeleton[a] | Mole % of total monoenes | Mole % at Δ positions indicated[b] | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Δ4 | Δ5 | Δ6 | Δ7 | Δ8 | Δ9 | Δ10 | Δ11 | Δ12 | Δ13 | Δ14 | Δ15 | Δ16 | Δ17 | Δ18 | Δ19 | Δ20 | Δ21 | Δ22 | Δ23 |
| Vernix caseosa | | | | | | | | | | | | | | | | | | | | | |
| n-14 | 0.78 | | | 100 | | | | | | | | | | | | | | | | | |
| n-16 | 13.50 | | | 62 | 2 | 2 | 31 | nd | 3 | | | | | | | | | | | | |
| n-18 | 37.15 | | nd | 2 | nd | 10 | 64 | 1 | 22 | tr | 1 | | | | | | | | | | |
| n-20 | 9.20 | | | nd | nd | 1 | 1 | 5 | 12 | 1 | 67 | nd | 13 | nd | nd | | | | | | |
| n-22 | 11.04 | | | nd | nd | nd | nd | nd | nd | 2 | 13 | 1 | 65 | tr | 19 | | | | | | |
| n-24 | 7.42 | | | | | | | | nd | tr | tr | 7 | 17 | tr | 73 | nd | 3 | | | | |
| n-26[d] | 1.09 | | | | | | | | | | | | nd | 9 | 11 | nd | 74 | nd | 6 | | |
| n-28[d] | .34 | | | | | | | | | | | | | | | 11 | 5 | nd | 75 | 7 | 2 |
| n-30[d] | .15 | | | | | | | | | | | | | | | | | | pres | pres | maj |
| n-13 | tr | | NA | | | | | | | | | | | | | | | | | | |
| n-15 | .87 | | 3 | 84 | 8 | 1 | 4 | nd | | | | | | | | | | | | | |
| n-17 | 2.12 | | | 25 | 3 | 14 | 45 | 3 | 10 | | | | | | | | | | | | |
| n-19 | .66 | | tr | 2 | 2 | 22 | 35 | 13 | 19 | 1 | 6 | tr | | | | | | | | | |
| n-21 | .44 | | | | | nd | 2 | 4 | 3 | 3 | 41 | 8 | 42 | | | | | | | | |
| n-23[d] | .31 | | | | | | | | | nd | 14 | 10 | 25 | 4 | 48 | | | | | | |
| n-25[d] | .12 | | | | | | | | | | | nd | 16 | 10 | 27 | 9 | 38 | | | | |
| n-27 | tr | | | | | | | | | | | | | | | | | | | | |
| i-14 | tr | | NA | | | | | | | | | | | | | | | | | | |
| i-16 | 2.34 | | tr | 100 | tr | | | | | | | | | | | | | | | | |
| i-18 | 3.48 | | nd | 25 | 4 | 64 | 7 | | | | | | | | | | | | | | |
| i-20 | 3.24 | | tr | 3 | 1 | 29 | 32 | 34 | 1 | | | | | | | | | | | | |
| i-22 | 1.40 | | | | nd | 1 | 1 | 19 | 18 | 55 | 6 | | | | | | | | | | |
| i-24[d] | 1.53 | | | | | | | | nd | 8 | 11 | 79 | 5 | | | | | | | | |
| i-26[d] | .50 | | | | | | | | | | | nd | 14 | 80 | 6 | | | | | | |
| i-28 | tr | | NA | | | | | | | | | | | | | | | | | | |
| ai-15 | tr | | NA | | | | | | | | | | | | | | | | | | |
| ai-17 | 1.25 | | nd | 100 | tr | tr | tr | | | | | | | | | | | | | | |
| ai-19 | .41 | | nd | 6 | 4 | 61 | 20 | 3 | 4 | | | | | | | | | | | | |
| ai-21 | .37 | | | | nd | 5 | 10 | 85 | tr | | | | | | | | | | | | |
| ai-23[d] | .16 | | | | | | | nd | 5 | 90 | 5 | | | | | | | | | | |
| ai-25[d] | .13 | | | | | | | | | | | maj | | | | | | | | | |
| Adult human skin surface lipid | | | | | | | | | | | | | | | | | | | | | |
| n-14 | 6.07 | 3 | tr | 97 | tr | | | | | | | | | | | | | | | | |
| n-16 | 47.11 | nd | 1 | 95 | 1 | 2 | 1 | nd | | | | | | | | | | | | | |
| n-18 | 19.34 | | 3 | 14 | 2 | 46 | 33 | 2 | tr | tr | nd | | | | | | | | | | |
| n-20 | 1.30 | | tr | 4 | 5 | 7 | 2 | 45 | 21 | 7 | 9 | nd | nd | | | | | | | | |
| n-22 | .47 | | | 2 | 4 | 3 | 7 | 4 | tr | 30 | 41 | 6 | 3 | tr | tr | | | | | | |
| n-24 | .55 | | | | 4 | tr | 3 | 3 | 3 | 2 | tr | 42 | 40 | 3 | tr | nd | | | | | |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| n-26 | .14 | | | | | | tr | 1 | 6 | nd | 2 | 2 | 4 | 49 | 37 | tr | nd |
| n-28 | tr | NA | | | | | | | | | | | | | | | |
| n-15 | 3.83 | | tr | 100 | tr | nd | nd | nd | | | | | | | | | |
| n-17 | 3.26 | | tr | 60 | 6 | 25 | 9 | tr | tr | | | | | | | | |
| n-19 | .52 | | | 4 | 3 | 33 | 27 | 22 | 11 | nd | | | | | | | |
| n-21 | .06 | | | | | | tr | 25 | 13 | 28 | 34 | tr | | | | | |
| n-23 | .06 | | | | | | | | | 17 | 14 | 41 | 28 | | | | |
| n-25 | .06 | | | | | | | | | | | 22 | 15 | 31 | 32 | | |
| i-16 | 10.37 | tr | 1 | 98 | 1 | tr | nd | | | | | | | | | | |
| i-18 | 2.30 | | tr | 30 | 2 | 68 | tr | | | | | | | | | | |
| i-20 | .44 | | nd | tr | 2 | 43 | 2 | 48 | tr | tr | | | | | | | |
| i-22[d] | .11 | | *nd* | tr | tr | *tr* | *tr* | 22 | tr | 78 | tr | | | | | | |
| i-24 | .19 | | | | | | tr | 2 | tr | 11 | tr | 87 | | | | | |
| i-26 | .17 | | | | | | nd | 5 | 14 | 1 | 1 | 13 | 2 | 66 | tr | | |
| ai-15 | 1.13 | *nd* | 2 | 97 | 1 | nd | | | | | | | | | | | |
| ai-17 | 2.18 | | tr | 98 | tr | 2 | tr | | | | | | | | | | |
| ai-19 | .19 | | | | pres | maj | pres | | | | | | | | | | |
| ai-21 | .06 | NA | | | | | | | | | | | | | | | |
| ai-23 | .03 | NA | | | | | | | | | | | | | | | |
| ai-25[c] | .06 | | | | | | | nd | 3 | 2 | 4 | 79 | 3 | 6 | 3 | | |

[a]See Table III.

[b]See Table III.

[c]See Table III.

[d]Structure determined by analysis of aldehydes only.

TABLE V

Double Bond Patterns of Wax Ester Fatty Acids in Vernix Caseosa Lipid and in Adult Human Skin Surface Lipid

| | Vernix caseosa lipid patterns,[a] mole % of monoenes (no. molec. species[c]) | | | | Adult human skin surface lipid patterns[a] mole % of monoenes (no. molec. species[c]) | | | |
|---|---|---|---|---|---|---|---|---|
| Pattern initiator[b] | Straight even | Straight odd | Iso | Anteiso | Straight even | Straight odd | Iso | Anteiso |
| 12Δ6 | .09(2) | | | | | | | |
| 13Δ6 | | tr(2) | | | | | | .02(1) |
| 14Δ6 | 10.81(4) | | | | 9.73(4) | | | |
| 15Δ6 | | 7.58(2) | | .65(2) | | 5.75(3) | | 2.30(1) |
| 16Δ6 | 46.42(4) | | 8.87(2) | | 49.14(3) | | 22.45(3) | |
| 17Δ6 | | 3.74(2) | | 6.62(1) | | 1.55(2) | | 3.71(2) |
| 18Δ6 | 1.70(2) | | .57(2) | | 3.01(2) | | .54(2) | |
| 19Δ6 | | | | | | .01(1) | | .06(1) |
| 20Δ6 | .02(1) | | | | | | .01(1) | |
| Total | 59.04 | 11.32 | 9.44 | 7.27 | 61.98 | 7.31 | 23.00 | 6.09 |
| | Total Δ6 = 87.07 mole %[d] | | | | Total Δ6 = 98.28 mole %[d] | | | |
| 14Δ9 | .94(3) | | | | | | | |
| 15Δ9 | | .36(2) | | | | | | |
| 16Δ9 | 5.78(4) | | | | .47(3) | | | |
| 17Δ9 | | .34(2) | | | | .03(2) | | .02(1) |
| 18Δ9 | 4.26(4) | | .14(2) | | .42(3) | | | |
| 19Δ9 | | .20(2) | | .02(2) | | .15(2) | | .02(1) |
| 20Δ9 | .01(3) | | .14(2) | | .17(3) | | .03(2) | |
| 21Δ9 | | | | | | .02(2) | | .01(1) |
| 22Δ9 | .02(1) | | .02(1) | | .05(2) | | .03(2) | |
| Total | 11.01 | .90 | .30 | .02 | 1.11 | .20 | .06 | .05 |
| | Total Δ9 = 12.23 mole %[d] | | | | Total Δ9 = 1.42 mole %[d] | | | |

[a]A "pattern" is here defined as an initial fatty chain substrate plus its extension or degradation products by $C_2$ units (see text).

[b]Chain length and double bond position of the substrate initiating the pattern (see text).

[c]The figures in ( ) are the number of molecular species in the pattern (see text).

[d]The difference between the sum of Δ6 + Δ9 patterns and 100.00% is the moles not analyzed.

caseosa lipid (14); and fetal sebum contains the 16Δ9 pattern (15). In the comedo, too, much evidence supports the conclusion that the sterol esters are derived from sebum as well as from epidermal fatty acids (16). If all the oleic acid present in the sterol ester fatty acids of vernix caseosa is derived from keratinizing epidermis, there still remains a very large portion (~46 mole %) of other Δ9 acids, and many of these, especially those of the 16Δ9 and 14Δ9 patterns as well as the iso and anteiso monoenoic acids, are most likely of sebum origin. It is noteworthy that rat surface lipid, also primarily of sebaceous gland origin, shows mainly the 16Δ9 pattern (5).

It is apparent from Tables III-VI that the straight even fatty acids of vernix caseosa sterol esters have been extended the maximal number of moles compared to the fatty acids of any of the other samples. Of these straight even acids, six times as many are extended in the Δ9 series as in the Δ6 series (Table VII). In both series the $C_{16}$ substrate is maximally extended. These extensions are undoubtedly occurring in the sebaceous gland. A large amount of substrate $C_{18:\Delta 9}$ (23.80 mole %) is present, compared to the moles extended (3.94 mole %); but this is not so for the 16Δ9 series, i.e., 4.19 moles of substrate compared to 28.13 moles extension products. This implies that some oleic acid is in a pool not extended as are the other Δ9 chains. This pool is very likely the sterol esters of the keratinizing epidermis as discussed above. Chain extension to as many as 7 or 8 $C_2$ units is by no means common among lipids of internal tissues.

Wilkinson has published data on the double bond position of the fatty acids of sterol esters of adult human skin surface lipid (17). For the major chain lengths that he found, our data are in general agreement. We have, however, found a good many more components, both of a given chain length and for branched and other skeletal types occurring in small amounts. Downing and Green have published double bond position isomer data for all the fatty acids of vernix caseosa obtained by saponification of the entire lipid sample (14). They have remarked on the uniqueness of the 16Δ9 pattern, and in general

TABLE VI

Double Bond Patterns of Sterol Ester Fatty Acids in Vernix Caseosa Lipid and in Adult Human Skin Surface Lipid

| | Vernix caseosa lipid patterns[a] mole % of monoenes (no. molec. species[c]) | | | | Adult human skin surface lipid patterns[a] mole % of monoenes (no. molec. species[c]) | | | |
|---|---|---|---|---|---|---|---|---|
| Pattern initiator[b] | Straight even | Straight odd | Iso | Anteiso | Straight even | Straight odd | Iso | Anteiso |
| 12Δ6 | .02(3) | | | | | | | |
| 13Δ6 | | .13(6) | | | | | | |
| 14Δ6 | 1.62(6) | | | | 7.36(7) | | | |
| 15Δ6 | | 1.17(6) | | .01(2) | | 4.83(6) | | 1.15(3) |
| 16Δ6 | 13.42(8) | | 8.01(6) | | 54.85(7) | | 12.30(6) | |
| 17Δ6 | | .70(3) | | 2.09(5) | | 2.15(5) | | 2.38(3) |
| 18Δ6 | .82(3) | | 2.20(4) | | 2.83(5) | | .94(6) | |
| 19Δ6 | | .01(1) | | .04(2) | | .02(1) | | tr (1) |
| 20Δ6 | | | .11(2) | | .08(3) | | .01(4) | |
| 22Δ6 | | | | | .01(3) | | .01(1) | |
| Total | 15.88 | 2.01 | 10.32 | 2.14 | 65.13 | 7.00 | 13.26 | 3.53 |
| | Total Δ6 = 30.35 mole % | | | | Total Δ6 = 88.92 mole %[d] | | | |
| 14Δ9 | 4.37(7) | | | | | | | |
| 15Δ9 | | .66(6) | | | | | | |
| 16Δ9 | 32.32(8) | | | | .60(6) | | | |
| 17Δ9 | | 1.44(6) | | .02(2) | | .40(6) | | .01(3) |
| 18Δ9 | 28.01(8) | | .46(6) | | 7.58(7) | | .10(5) | |
| 19Δ9 | | .39(6) | | .09(4) | | .37(6) | | .02(4) |
| 20Δ9 | .09(2) | | 1.67(5) | | .90(6) | | .18(6) | |
| 21Δ9 | | .02(2) | | .05(3) | | .02(3) | | tr (3) |
| 22Δ9 | | | .04(2) | | .70(5) | | .02(5) | |
| 23Δ9 | | | | | | | | tr (1) |
| 24Δ9 | | | | | .05(4) | | .02(3) | |
| 26Δ9 | | | | | .02(2) | | | |
| | 64.79 | 2.51 | 2.17 | .18 | 9.85 | .79 | .32 | .03 |
| | Total Δ9 = 69.65 mole % | | | | Total Δ9 = 10.99 mole %[d] | | | |

[a]See Table V.
[b]See Table V.
[c]See Table V.
[d]See Table V.

their results are consistent with ours. Ansari et al. (15,5) found independently that the 16Δ9 pattern occurred in the monoenoic fatty acids of the alkane diol diesters of vernix caseosa. Since these diesters are sebum components they concluded that the 16Δ9 desaturase system was a sebaceous gland activity. Since Downing and Greene's sample included epidermal as well as sebaceous gland lipids, they were unable to determine the origin of the 16Δ9 pattern. Double bond positions of the wax ester fatty acids of either vernix caseosa or of adult human surface have hitherto not been reported, nor have they been reported for the sterol esters of vernix caseosa.

It is obvious from the foregoing that the sterol esters of vernix caseosa, which constitute ca. 25% of the lipid, are a very complex fraction. This complexity characterizes not only the fatty acid moieties but also the sterols themselves. For instance, Miettienen and Lukkäinen showed that vernix caseosa lipid contains at least eight additional sterols besides cholesterol (18). Of this group the esters of lanostenol (which constitutes less than 2% of the total) would be expected to migrate with the wax esters in our separation scheme, because models of this sterol show that it cannot present much flat surface to the absorbent, MgO, the basis of the separation (2). Although the Liebermann-Burchard test of the wax esters of vernix caseosa did give the yellow color characteristic of lanosterol, GLC of the total wax ester fraction showed extremely little material in the region of lanosterol palmitate or stearate (unpublished) so that contamination of the wax esters by lanosterol esters must be very slight.

The same authors (18) also examined the sterols of amniotic fluid, meconium, placenta and maternal serum, and showed that the sterols of vernix caseosa, amniotic fluid and meconium bore a much closer similarity to each other than they did to those of the placenta or

TABLE VII

Sterol Ester Fatty Acid Chain Extension Pattern of the Straight Even Acids of Vernix Caseosa

| Carbon chain length | Substrate $C_{14}$:Δ6 | Δ6 Extension patterns | | | | | | Δ9 Extension patterns | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Extension | | Substrate $C_{16}$:Δ6 | Extension | | Substrate $C_{18}$:Δ6 | Extension | | Substrate $C_{14}$:Δ9 | Extension | | Substrate $C_{16}$:Δ9 | Extension | | Substrate $C_{18}$:Δ9 | Extension | |
| | | $C_2$ units | mole % | | $C_2$ units | mole % | | $C_2$ units | mole % | | $C_2$ units | mole % | | $C_2$ units | mole % | | $C_2$ units | mole % |
| 14 | .78 | | | | | | | | | not detected | 1 | .40 | 4.19 | | | | -1 | .27 |
| 16 | | 1 | .27 | 8.37 | | | | | | | 2 | .37 | | 1 | 8.17 | 23.80 | | |
| 18 | | 2 | .37 | | 1 | 3.71 | .73 | | | | 3 | 1.21 | | 2 | 6.16 | | 1 | 1.10 |
| 20 | | 3 | .09 | | 2 | .46 | | 1 | .09 | | 4 | 2.10 | | 3 | 7.17 | | 2 | 1.44 |
| 22 | | 4 | .11 | | 3 | .22 | | | | | 5 | .22 | | 4 | 5.42 | | 3 | 1.26 |
| 24 | | | | | 4 | .52 | | 3 | tr | | 6 | .06 | | 5 | .81 | | 4 | .12 |
| 26 | | | | | 5 | .10 | | | | | 7 | .01 | | 6 | .25 | | 5 | .02 |
| 28 | | | | | 6 | .04 | | | | | 8 | tr | | 7 | .15 | | 6 | tr |
| 30 | | | | | 7 | tr | | | | | | | | | | | | |
| Total | | | .84 | | | 5.05 | | | .09 | | | 4.37 | | | 28.13 | | | 3.94 |
| | | Total mole % Δ6 extended = 6.00[a] | | | | | | | | | Total mole % Δ9 extended = 36.44 | | | | | | | |

[a]Includes .02 mole % of $C_{12}$Δ6 pattern not tabulated.

maternal serum. Thus the similarity of the sterol patterns found in vernix caseosa, amniotic fluid and meconium could result from the fact that during intrauterine life the fetus drinks amniotic fluid into which skin cells and surface lipids have been released, but that these substances are not absorbed. However the authors also point out that the intestinal mucosa, being a tissue of ectodermal origin, may synthesize sterols that resemble those of the skin.

Sterol esters of high molecular weight fatty acids are also not well absorbed. This might be a reason as to why they are made, or, if others are made too, why only esters of high molecular weight acids remain in vernix caseosa. The high carbon content of the sterol esters undoubtedly helps to provide a waxy film of low water solubility (along with other sebum components). This prevents excessive wetting of the fetal skin. Esterification of the sterols of vernix caseosa might also provide a means for removing most of the free cholesterol resulting from epidermal lipid. This would help regulate the amount of free cholesterol that gets into the amniotic fluid.

In the case of adult human skin surface lipid, the sterol esters seem to be simply the product of a residual esterase activity set free by the lysosomal enzymes of dying, keratinizing epidermal cells. This implies that the sterol esters of both the adult human skin surface lipid or of vernix caseosa are secondary products. Free sterols are built up by the living epidermis and are then esterified primarily with sebum acids but also with some acids released from the epidermis in late stages of keratinization or after keratinization is complete.

Yardley (19) has suggested that sterol esters of essential fatty acids are necessary compounds for keratinization. We examined the dienoic fatty acids of adult human skin surface sterol esters briefly and found that, of all the acids esterified to sterols, the $C_{18}$ dienes make up at most 1%. If linoleic acid constitutes the same proportion of the dienes of sterol esters as it does of all the dienes of adult human surface lipid (20), then it must make up only ca. 0.25%. Arachidonic acid, if present, would have appeared in the polar fraction of the acids of Table I. Since only a trace of total polar material was recovered in the sterol esters of adult human skin surface lipid, if any, only minute amounts of arachidonic acid must be present. Thus, if sterol esters of essential fatty acids are to be important for keratinization, they would have to be used in extremely small amounts. We are not aware of any evidence that this is so.

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# An Enzymatic Protective Mechanism against Lipid Peroxidation Damage to Lungs of Ozone-Exposed Rats

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## ABSTRACT

The effects of whole animal exposure to ozone and of dietary α-tocopherol on the occurrence in rat lung of lipid peroxidation and alteration of the activity of enzymes important in detoxification of lipid peroxides were studied. Exposure to 0.7 and 0.8 ppm ozone continuously for 5 and 7 days, respectively, significantly elevated the concentration of TBA reactants, primarily malonaldehyde, produced by lipid peroxidation, as well as the activities of glutathione (GSH) peroxidase, GSH reductase and glucose-6-phosphate (G-6-P) dehydrogenase. As a logarithmic function of dietary α-tocopherol (0, 10.5, 45, 150 and 1500 mg/kg), the increase in formation of malonaldehyde and the increase in activities of GSH peroxidase and G-6-P dehydrogenase were partially inhibited. The activity of GSH reductase was not affected by dietary α-tocopherol. The concentration of malonaldehyde and the activity of GSH peroxidase in lung were linearly correlated ($p < 0.001$). This study confirmed the occurrence of lipid peroxidation in the lung during ozone exposure and revealed an enzymatic mechanism against damage. An apparent compensation mechanism is that with increased lipid peroxides there is increased activity of GSH peroxidase, which in turn increases lipid peroxide catabolism. The increased activities of GSH reductase and G-6-P dehydrogenase also function in the protective chain by providing increased levels of GSH and NADPH, respectively.

## INTRODUCTION

Malonaldehyde is derived from lipid peroxides and has been detected in tissue extracts (1-4). However the failure to find increasing amounts of lipid peroxides in the tissue of vitamin E-deficient or other nutritionally stressed animals has initiated a controversy as to whether lipid peroxides are formed in vivo (5). Nevertheless present knowledge favors the conclusion that small amounts of lipid peroxides are formed in vivo.

In a study of rat liver peroxidase, Little and O'Brien (6) demonstrated an intracellular glutathione (GSH) peroxidase (glutathione-hydrogen peroxide oxidoreductase, E.C. 1.11.1.9) that utilized lipid peroxide substrates. Christophersen (7,8) reported that the oxidation of glutathione by linoleate and linolenate hydroperoxides is enzymatically catalyzed in rat liver, and the products formed from the lipid peroxides are their corresponding monohydroxy polyenoic fatty acids. Both reports suggest that the enzymic reaction is probably responsible for the decomposition of most of the lipid peroxides in the liver cell and thus protects the cellular components from the deleterious effects of lipid peroxides.

Lipid peroxides are highly toxic and damaging to biological systems (9-13); however intravenously injected lipid peroxides are rapidly metabolized (14). Animals develop a tolerance to oxidants following prior exposure to sublethal dosages (15-17), and α-tocopherol and other antioxidants show limited effectiveness in the inhibition of lipid peroxidation damage in vivo (18-20). These facts indicate that animal tissue may have other mechanisms for protection against the toxic effect of lipid peroxides through their increased catabolism.

The damaging effects of nitrogen dioxide and ozone are related to their oxidative reactions, including initiation of lipid peroxidation, which can be partially prevented by α-tocopherol and other antioxidants (18-20). To understand how animals respond biochemically under ozone stress, these studies investigated alterations of lung tissue enzyme activities that may be important in the detoxification of lipid peroxides. An enzymatic protective mechanism against lipid peroxidation damage was found as a result of these studies.

## EXPERIMENTAL PROCEDURES

### Experiment I

Five groups of 1-month-old male Sprague-Dawley rats were fed a basal tocopherol-deficient diet (21) containing 15% stripped

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corn oil and 0, 10.5, 45, 150 and 1500 mg dl-α-tocopherol acetate per kilogram of diet, respectively. After 30 days, nine animals from the first dietary group and six from each of the other groups were exposed to 0.70 ± 0.15 ppm ozone continuously for 5 days, except for ca. 15 min daily during which time the chambers were cleaned and the diets replaced. Ozone was admitted to the chambers by a controlled flow device with 10 volume changes per hour. Ozone was monitored continuously by an ozone meter and at intervals by the neutral buffered KI method (22). This research is part of a program study at the University of California, Davis, on pulmonary effects of environmental oxidants. The ozone chambers are operated for this program research. Four rats from each dietary group served as controls. Control animals were placed in ambient air chambers where the highest 1 hr ambient oxidant level was 0.12 ppm. The animals of exposed and control groups were then sacrificed by withdrawal of blood via heart puncture following anesthetization by peritoneal injection of Beuthansia Special (Burns Pharmaceuticals). The lungs were removed, rinsed with 0.15 M KCl, blotted and finely minced within 2 hr after exposure. About 250-300 mg of the minced tissue from each animal was homogenized in a Potter-Elvehjem homogenizer with 6 ml of isotonic potassium phosphate buffer, pH 7.0, and 0.1 ml of 0.6% α-tocopherol in ethanol. The homogenate was filtered by suction through two layers of cheesecloth, and the filtrate was immediately assayed by the thiobarbituric acid (TBA) test. Protein was determined by the method of Miller (23).

The remaining lung minces from two to three animals of the same dietary group were pooled, and a 10% homogenate was made in 0.25 M sucrose containing 1 mM EDTA. After centrifugation for 10 min at 750 x g, the supernatant was centrifuged at 105,000 x g for 60 min in a Beckman Model L ultracentrifuge. The soluble fraction was used for measurement of GSH peroxidase, GSH reductase (reduced-NAD(P):oxidized glutathione oxidoreductase, E.C. 1.6.4.2) (24), glucose-6-phosphate (G-6-P) dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, E.C. 1.1.1.49) (25) and protein.

#### Thiobarbituric Acid Test

One and one-half milliliters of the filtrate and 1.5 ml of 0.8% 2-thiobarbituric acid (Sigma) in 10% trichloroacetic acid were thoroughly mixed in test tubes and then placed in boiling water for 10 min and allowed to cool. After centrifugation, the absorbance of the

TABLE I

Effects of $O_3$ Exposure and Dietary α-Tocopherol on Malonaldehyde Formation and Activities of GSH Peroxidase, GSH Reductase and G-6-P Dehydrogenase (Experiment 1)[a]

| Dietary α-tocopherol, mg/kg diet | TBA reactants, nmoles malonaldehyde/g protein | | | GSH peroxidase, nmoles GSH oxidized/min/mg protein | | | GSH reductase, nmoles NADPH oxidized/min/mg protein | | | G-6-P dehydrogenase, nmoles NADPH formed/min/mg protein | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ |
| 0 | 162 ± 19[b] | 82 ± 18 | <0.001 | 50 ± 4 | 30 ± 0 | <0.01 | 118 ± 9 | 87 ± 7 | <0.05 | 236 ± 7 | 154 ± 11 | <0.01 |
| 10.5 | 134 ± 15 | 70 ± 9 | <0.001 | 39 ± 5 | 26 ± 3 | <0.05 | 114 ± 9 | 107 ± 13 | >0.1 | 216 ± 29 | 158 ± 7 | <0.1 |
| 45 | 125 ± 10 | 54 ± 14 | <0.001 | 39 ± 4 | 24 ± 5 | <0.05 | 116 ± 9 | 98 ± 6 | <0.1 | 210 ± 17 | 156 ± 9 | <0.05 |
| 150 | 122 ± 22 | 54 ± 18 | <0.001 | 37 ± 1 | 24 ± 3 | <0.01 | 125 ± 8 | 105 ± 15 | <0.1 | 209 ± 17 | 159 ± 9 | <0.05 |
| 1500 | 106 ± 11 | 40 ± 0 | <0.001 | 27 ± 8 | 20 ± 4 | <0.1 | 118 ± 4 | 107 ± 15 | >0.1 | 103 ± 12 | 165 ± 2 | <0.1 |
| $\bar{X}$[c] | 113 ± 25 | 63 ± 19 | <0.001 | 38 ± 8 | 25 ± 4 | <0.001 | 118 ± 8 | 101 ± 11 | <0.001 | 213 ± 21 | 159 ± 7 | <0.01 |

[a]GSH = Glutathione; G-6-P = glucose-6-phosphate.

[b]*Mean ± standard deviation.*

[c]Mean ± standard deviation for all dietary groups.

TABLE II

Effects of $O_3$ Exposure and Dietary α-Tocopherol on Malonaldehyde Formation and Activities of GSH Peroxidase, GSH Reductase and G-6-P Dehydrogenase (Experiment II)[a]

| Dietary α-tocopherol, mg/kg diet | TBA reactants, nmoles malonaldehyde/g protein | | | GSH peroxidase, nmoles GSH oxidized/min/mg protein | | | GSH reductase, nmoles NADPH oxidized/min/mg protein | | | G-6-P dehydrogenase, nmoles NADPH formed/min/mg protein | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ |
| 0 | 105 ±15[b] | 80 ± 7 | <0.001 | 32 ±4 | 19 ±2 | <0.001 | 94 ±10 | 81 ±7 | <0.001 | 173 ±27 | 121 ±11 | <0.001 |
| 45 | 90 ±13 | 78 ± 10 | <0.01 | 28 ±4 | 19 ±2 | <0.001 | 96 ± 6 | 83 ±7 | <0.001 | 147 ±17 | 121 ±10 | <0.001 |
| $p$[c] | <0.02 | >0.1 | | <0.01 | >0.1 | | >0.1 | >0.1 | | <0.01 | >0.1 | |
| $\bar{X}$[d] | 98 ±16 | 79 ± 9 | <0.001 | 30 ±4 | 19 ±2 | <0.001 | 95 ± 8 | 82 ±7 | <0.001 | 160 ±26 | 121 ±10 | <0.001 |

[a]See Table I.
[b]Mean ± standard deviation.
[c]Probability value of 0 vs. 45 mg α-tocopherol/kg diet.
[d]Mean ± standard deviation of all dietary groups.

clear supernatant was measured at 532 nm, and the amount of TBA reactants was calculated based on the molar extinction coefficient of malonaldehyde ($1.56 \times 10^5$).

### Preparation of Ethyl Linolenate Hydroperoxide

The hydroperoxide was prepared by oxidation of ethyl linolenate in hexane at room temperature according to a modification of the procedure of Banks et al. (26). Crude ethyl linolenate hydroperoxide, obtained by stripping the solution with 87% ethanol, was purified by thin layer chromatography on Silica Gel G using petroleum ether-ether-acetic acid 8:7:0.5 v/v, or 1% methanol in benzene, or both, as solvent system. The peroxide concentration was determined spectrophotometrically in ethanol at 232 nm, and calculations were made using the molar extinction coefficient, $2.5 \times 10^4$ (27).

### Glutathione Peroxidase Assay

The peroxidase activity was assayed at 25C with glutathione as hydrogen donor using a system containing 0.1 M Tris-HCl buffer, pH 7.6, 200 μM EDTA, 650 μM GSH, 350 μM ethyl linolenate hydroperoxide and ca. 1 mg of enzyme protein in a final volume of 1.0 ml. The reaction was started by addition of the peroxide and was stopped by denaturing the enzyme with 4 ml of 6.3% trichloroacetic acid. The reduced glutathione remaining was measured according to a modification of the procedure of Sedlack and Lindsay (28). The colorimetric determination was carried out by mixing 2 ml of the clear supernatant with 2 ml of 0.4 M Tris-HCl buffer, pH 8.9 and 0.1 ml of 10 mM, 5,5-dithiobis-(2-nitrobenzoic acid) in methanol. The absorbance was read within 5 min after the reaction. Control samples without addition of the peroxide or enzyme, or both, were also determined. A molar extinction coefficient of $1.36 \times 10^4$ for GSH (29) was used for calculations.

### Experiment II

Thirty and twenty-nine 1-month-old male Sprague-Dawley rats (chronic respiratory disease-free) were maintained on a basal diet containing 0 and 45 mg dl-α-tocopherol acetate per kilogram, respectively. These diets were the same as diets 1 and 3 of Experiment I. After 35 days, 15 animals from each group were exposed to 0.79 ± 0.14 ppm ozone continuously for 7 days. The exposed and control animals were then sacrificed. The lung samples were prepared and assayed as described in Experiment I, except samples were not pooled.

## RESULTS

### Occurrence of Lipid Peroxidation Following Ozone Exposure

Tables I and II show the results of Experiments I and II, respectively. The concentration of malonaldehyde, as measured by TBA reactants, in the filtered lung homogenates of all dietary groups was significantly increased by whole animal exposure to ozone. The increases averaged 80% and 24% for Experiments I and II, respectively. The increase of malonaldehyde was partially inhibited by dietary α-tocopherol. Figure 1A shows that in Experiment I the concentration of malonaldehyde decreased linearly as a function of the logarithm of dietary α-tocopherol, with $r$ values of ca. -0.8. Relatively little effect of α-tocopherol in the control group of Experiment II was observed.

The sensitivity of the measurement of malonaldehyde was enhanced by using a large sample volume (up to 100 mg of homogenized tissue in a 3 ml reaction volume). The formation of artifacts due to in vitro lipid peroxidation was minimized by analyzing the α-tocopherol-protected homogenates immediately after their preparation.

### Effect of Ozone and α-Tocopherol on the Activity of GSH Peroxidase

Tables I and II show that the activity of GSH peroxidase in the soluble fraction of lung was significantly elevated by whole animal exposure to ozone in all dietary groups. These increases averaged 52% and 58% for Experiments I and II, respectively. Alteration of the enzymic activity was also modified by dietary α-tocopherol. Figure 1B shows the relationship of GSH peroxidase activity to α-tocopherol concentration in Experiment I. The data indicate that as a logarithmic function of concentration α-tocopherol partially inhibited the elevation of the enzymic activity due to ozone treatment. For the ozone exposed groups $r$ = -0.888 and $p < 0.001$; for the control groups $r$ = -0.809 and $p < 0.01$.

### Effect of Ozone and α-Tocopherol on the Activity of GSH Reductase

The activity of GSH reductase in the soluble fraction of lung (Tables I and II) was also significantly elevated in all dietary groups following ozone exposure. These increases averaged 16% and 17% for Experiments I and II, respectively. The activity of the enzyme was not altered by dietary α-tocopherol.

### Effect of Ozone and α-Tocopherol on the Activity of G-6-P Dehydrogenase

Tables I and II also show that exposure of animals to ozone markedly increased the activity of lung G-6-P dehydrogenase in all dietary groups. The increases averaged 33% and 32% for Experiment I and II, respectively. In Experiment I, dietary α-tocopherol partially, but linearly ($r$ = -0.681, $p$ = <0.01), retarded the alteration of the enzymic activity due to ozone exposure. There was no effect of α-tocopherol on G-6-P dehydrogenase activity in the lung of control animals. Similar results were observed in Experiment II.

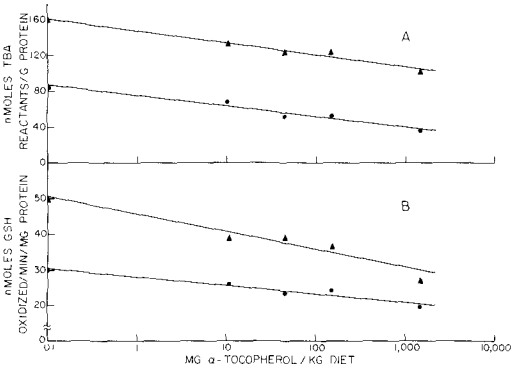


FIG. 1. Linear relationships of logarithm of α-tocopherol concentration with (A) malonaldehyde concentration (ozone exposed animals $r$ = -0.787 and $p < 0.001$; control animals $r$ = -0.773 and $p < 0.001$) and (B) glutathione peroxidase activity (ozone exposed animals $r$ = -0.888 and $p < 0.001$; control animals $r$ = -0.809 and $p < 0.001$). (▲), ozone exposed; (●), control.

### Relationship of Malonaldehyde Formation with GSH Peroxidase Activity

Figure 2 (Experiment I) and Figure 3 (Experiment II) are plots of concentration of malonaldehyde vs. the activity of GSH peroxidase in lung tissue. The linear regression coefficient of the malonaldehyde content and the activity of GSH peroxidase ($r$ = 0.848 and 0.661 for Experiments I and II, respectively) of lung indicate that these two measurements are highly correlated ($p < 0.001$ for both experiments).

## DISCUSSION

Whole animal exposure to ozone elevated the concentration of the lipid peroxidation product malonaldehyde as well as GSH peroxidase activity in the lung tissue, and dietary α-tocopherol partially reversed the elevation of each. The finding that α-tocopherol is partially effective in the inhibition of the formation of lipid peroxidation products confirms that it functions as a biological antioxidant and that lipid peroxidation occurred in vivo during

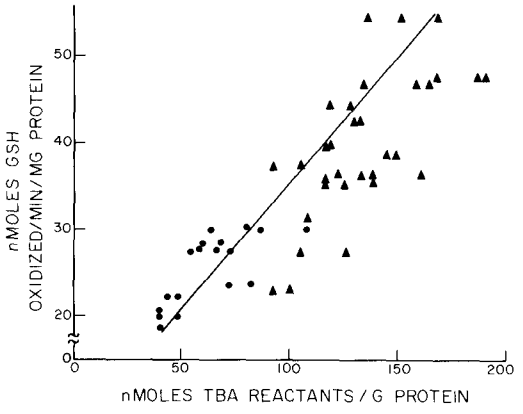


FIG. 2. Correlation between malonaldehyde concentration and glutathione peroxidase activity for Experiment I in all ozone exposed (▲) and all control (●) animals; $r = 0.848$, $p < 0.001$.

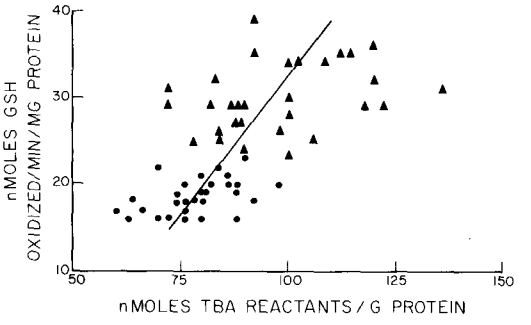


FIG. 3. Correlation between malonaldehyde concentration and glutathione peroxidase activity for Experiment II in all ozone exposed (▲) and all control (●) animals; $r = 0.661$, $p < 0.001$.

ozone exposure. The high correlation between the concentration of malonaldehyde and the activity of GSH peroxidase suggests that the enzyme activity is probably affected by lipid peroxide formation. Whether the increase of GSH peroxidase activity is due to an altered rate of synthesis or to other changes remains to be shown.

The observed elevated activity of GSH reductase following exposure of animals to ozone may be attributable to the increased demand for reduced glutathione as a result of the increased activity of GSH peroxidase. Since dietary α-tocopherol did not modify the GSH reductase activity and since there was a lack of correlation between the activity of this enzyme and that of GSH peroxidase, direct oxidation of GSH by the oxidant may also have contributed to alteration of the enzyme activity. α-Tocopherol does not protect sulfhydryl compounds from direct oxidation.

The increase of G-6-P dehydrogenase activity during ozone exposure reflects the increased oxidation of NADPH due to elevated activity of GSH reductase and perhaps in part the direct oxidation of NADPH. The reported antioxigenic action of GSH and other sulfhydryl compounds (30,31), therefore, could be attributable to their ability to become proton donors for GSH peroxidase. Dietary α-tocopherol partially inhibited the increase of the G-6-P dehydrogenase activity, suggesting that the vitamin might have some relation to this system.

Pinto and Bartley (32) observed that GSH peroxidase activity in liver of male rats increased steadily from foetal stage up to ca. 55 days and then remained unchanged, while GSH reductase activity went up and down during the weaning period and reached a plateau at ca. 45 days. Increased G-6-P dehydrogenase activity has been observed in rat red blood cells during hyperoxia (33) and in rat liver upon starvation and refeeding with a carbohydrate diet containing adequate protein (34,35).

The results reveal that rat lung has an enzymic pathway for reducing lipid peroxides (Fig. 4) in vivo. The ability of animals to respond to an increase of lipid peroxide by increasing the activity of GSH peroxidase, which results in increased peroxide catabolism, is the key feature of the mechanism. Harmless hydroxy fatty acids formed by reduction of lipid peroxides (7,8) can be metabolized via the β-oxidation pathway. The increases in the activity of GSH reductase and G-6-P dehydrogenase are also parts of the protective chain by providing the increased requirement for GSH and NADPH, respectively.

Beutler (36) summarized a mechanism in human erythrocytes for detoxification of hydrogen peroxide formed by oxidative drug metabolism involving GSH peroxidase, GSH reductase and G-6-P dehydrogenase in the maintenance of reduced GSH and the decomposition of $H_2O_2$. When the cells genetically lack one of these enzymes (37-39) or enzymes involved in the maintenance of GSH levels, such as GSH synthetase (40,41), they become susceptible to drug-induced hemolytic anemia. The lack of specificity of rat liver GSH peroxidase using a variety of hydroperoxides as substrate (6) suggests that this is probably a type of protective mechanism similar to that observed in the lung. Genetic deficiency of one of these enzymes would be expected to cause increased susceptibility to lipid peroxidation damage. The findings suggest that the protective mechanism against lipid peroxidation damage is not confined to the lung tissue or to any particular species. Therefore the presence of the suggested

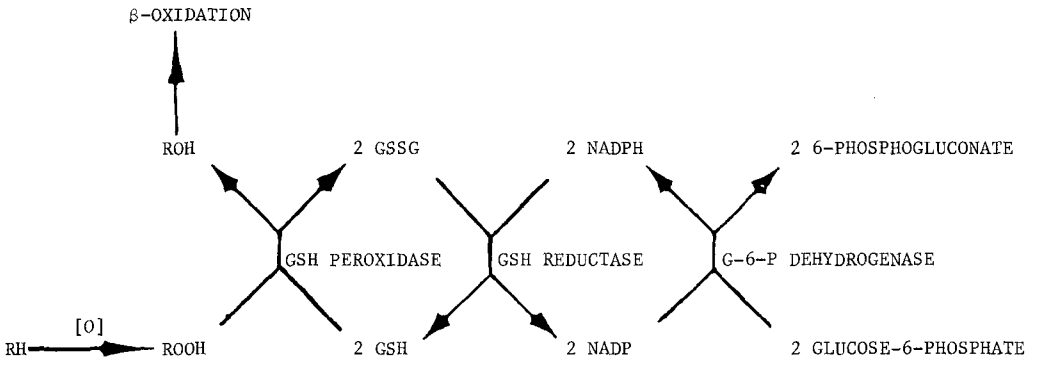


FIG. 4. Scheme of lipid peroxide metabolism. RH, polyunsaturated fatty acid; ROOH, fatty acid hydroperoxide; ROH, hydroxy fatty acid; GSH, reduced glutathione; GSSG, oxidized glutathione.

enzymatic protective mechanism against lipid peroxidation damage may explain the following observations: (a) failure to detect any or to detect increasing amounts of lipid peroxide in nutritionally stressed animals (5); (b) development of tolerance by animals to oxidants, e.g., ozone and $NO_2$, following prior sublethal exposure (15-17); (c) insignificant decrease of SH groups following ozone exposure (Chow and Tappel, unpublished); and (d) rapid metabolism of injected lipid peroxide by the animal (14). The same type of protective mechanism may also apply to lipid peroxidation induced by $NO_2$ exposure (19,20) and hyperoxia (42), as well as $CCl_4$ (43), ethanol (44) and other free radical inducing agents. While the glutathione peroxidase pathway appears to be a major protective mechanism, other types of protective mechanisms would work in parallel.

Alteration in tissue enzyme activity is a more sensitive and specific indicator of tissue damage than other known biochemical measurements. Hence the alteration of the activity of protective enzymes, GSH peroxidase in particular, should be useful as an index for monitoring oxidant damage to lung and the occurrence of lipid peroxidation in vivo.

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# Enzymatic Synthesis of Glucosylsphingosine by Rat Brain Microsomes

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## ABSTRACT

Labeled glucosylceramide was formed when rat brain microsomes were incubated with $^{14}$C-UDP-glucose. When sphingosine was added to the incubation mixture, labeled glucosylsphingosine was synthesized, and the formation of glucosylceramide increased.

## INTRODUCTION

To our knowledge, the synthesis of glucosylsphingosine by an enzymatic system has not been reported. Basu et al. (1) found labeled hexosylsphingosine after incubation of $^{14}$C-UDP-glucose with sphingosine in the presence of a chicken brain preparation, but they did not attempt any separation of the hexosylsphingosines that could have been originated in their incubation system, that is, glucosyl or galactosylsphingosine or both. Kanfer (2), in a similar type of experiment but using a rat brain preparation, failed to find glucosylsphingosine as a reaction product. The present report deals with the separation of labeled glucosylsphingosine after incubation of rat brain microsomes with $^{14}$C-UDP-glucose and sphingosine.

## MATERIALS AND METHODS

DL-erythro-sphingosine and galactosyl-DL-sphingosine were purchased from Miles; Tween 20 and Sephadex G-25 (particle size 10-40 $\mu$) were from Sigma. Dowex 1-X8 (200-400 mesh) and Dowex 50W-X8 (200-400 mesh) were purchased from Baker; U-$^{14}$C-UDP-glucose (specific activity 227 $\mu$c/$\mu$mole) was from New England Nuclear. Glucosylceramide was prepared from the spleen of a patient with Gaucher disease by the method of Radin and Brown (3) up to the first precipitation step and then precipitated twice from glacial acetic acid (4). The method of Cumar et al. (5) was used to prepare glucosylsphingosine from glucosylceramide.

### Chromatographic Methods

Thin layer chromatography (TLC) was carried out on Silica Gel G (Merck). The borate-impregnated plates were prepared with saturated solution of $Na_4B_2O_7$. 10H20-water 1:2 v/v (6). System A: borate-impregnated Silica Gel G plates developed with chloroform-methanol-2 M $NH_4OH$ 84:21:2 v/v/v (2). System B: borate-impregnated Silica Gel G plates developed with chloroform-methanol-water 66:30:4 v/v/v. System C: Silica Gel G plates developed with chloroform-methanol-water 66:30:4 v/v/v. System D: Silica Gel G plates developed with chloroform-methanol-concentrated ammonia 54:36:9 v/v/v. Rf values for glucosylsphingosine and galactosylsphingosine were, respectively, 0.12 and 0.05 in system A and 0.28 and 0.20 in system B. With systems C and D no separation of these glycolipids was obtained; the Rf values for both of them were 0.38 in system C and 0.73 in system D.

To separate glucosylceramide from galactosylceramide, TLC on borate-impregnated Silica Gel G plates developed with chloroform-methanol-water 65:25:4 v/v/v (6) (system E) was used.

### Enzyme Preparation

Microsomes were obtained from brain of 15 to 16-day-old rats, according to Cleland and Kennedy (7), but collecting them by centrifugation at 100,000 x g for 60 min. The microsomes from 5 g of brain were suspended in 1.2 ml of 0.02 M Tris-HCl buffer pH 8.0, and water was added to complete 2.5 ml.

### Incubation System

The reaction mixture contained, in a final volume of 0.12 ml, 1.4 nmole U-$^{14}$C-UDP-glucose; 12 $\mu$moles Tris-HCl buffer pH 8.0; 0.75 $\mu$mole $MgCl_2$; 0.54 $\mu$mole sphingosine (added to the mixture in 25 $\mu$liters 1.2% w/v solution of Tween 20); and 60 $\mu$liters microsome suspension. The incubation time was 15 min and the temperature 37 C.

### Extraction and Fractionation of Labeled Lipid Products

The reaction was stopped by adding 1.5 ml methanol containing 0.2 $\mu$mole glucosylsphingosine as carrier; after heating 10 min at 55 C the mixture was centrifuged and the insoluble material extracted again with 3 ml methanol. The combined methanol extracts were passed through a column of Dowex 1 (Cl'form) equilibrated with methanol-water 97:3 v/v. To the effluent, 9 $\mu$moles of glucose were added, and it was passed through a column of Dowex 50


FIG. 1. Radioautogram obtained from the basic lipid fraction run on thin layer chromatography with system B. The arrow shows the spot that appeared as a finger printing of the glucosylsphingosine carrier revealed with iodine vapor. O, origin; F, front.

(H'form) (7) which had been previously washed with a solution of 0.5 M glucose and then equilibrated with methanol-water 97:3 v/v. After the radioactive solution was passed, the column was washed with the methanol-water solvent until the radioactivity of the eluate was less than 25 cpm/ml. The radioactive fraction obtained with methanol-water will be referred to as the neutral lipid fraction. After this fraction was obtained, 0.4 N HCl in methanol-water 97:3 v/v was passed through the column of Dowex 50 in order to obtain the fraction which will be referred to as the basic lipid fraction.

#### Radioactivity Measurements

Samples were evaporated to dryness in vials and counted in a Beckman LS-200 liquid scintillation counter after addition of 0.25 ml ethanol and 5 ml of a scintillator mixture containing 2 g of PPO, 100 g of naphthalene and dioxane to complete a liter.

## RESULTS AND DISCUSSION

The basic lipid fraction was evaporated to dryness in vacuo and analyzed by TLC. The plates were developed with systems A, B, C and D and then exposed successively to X-ray films and to iodine vapor. In each of the four TLC systems used, a radioactive spot appeared which had absolute coincidence (finger printing) with the iodine spot of carrier glucosylsphingosine. No iodine spot appeared in the area of the plates corresponding to glucosylsphingosine if no carrier glucosylsphingosine had been added. Other radioactive compounds appearing in the radioautograms (Fig. 1) were not identified. They were eliminated by passing the fraction dissolved in 1 ml chloroform-methanol-water 60:30:4.5 v/v/v through a column of Sephadex G-25 (0.8 g) equilibrated with the same solvent mixture (8-10) and then washed with two 1 ml portions of the chloroform-methanol-water solvent; consequently, determinations of glucosylsphingosine synthesized were carried out in the material passed through a column of Sephadex G-25 (Table I). After glucosylceramide (0.12 $\mu$mole) was added as carrier to the neutral lipid fraction, the mixture was evaporated to dryness in vacuo, redissolved in chloroform-methanol-water 60:30:4.5 v/v/v and saponified with 0.05 N NaOH at 40 C for 40 min (11). The saponified material was partitioned with 0.2 volumes of water, and the lower phase was washed once with theoretical upper phase (12). The lower phase was evaporated and then subjected to TLC with system E, exposed to X-ray film and subsequently to iodine vapor.

TABLE I

Glucosylsphingosine and Glucosylceramide Synthesis by Rat Brain Microsomes[a]

| Modification to the incubation system | $^{14}$C-glucose incorporated, pmoles | |
|---|---|---|
| | Glucosylsphingosine | Glucosylceramide |
| None | 2.33 | 2.18 |
| Sphingosine omitted | 0.05 | 1.34 |
| Zero time | 0.13 | 0.02 |

[a]The incubation system contained, in a final volume of 0.12 ml, 1.4 nmole U-$^{14}$C-UDP-glucose; 12 μmoles Tris-HCl buffer pH 8.0; 0.75 μmole $MgCl_2$; 0.54 μmole sphingosine; 0.3 mg Tween 20; and 60 μliters microsome suspension. The incubation time was 15 min and the temperature 37 C.

Two radioactive spots were seen, one of them appearing as a finger printing of the iodine spot of carrier glucosylceramide. The other spot, which appeared only after incubation with sphingosine, was not identified. For the purpose of counting the radioactivity (Table I) the silica from the glucosylceramide area was scraped from the plate and eluted in a column with chloroform-methanol-water 30:25:4 v/v/v, followed by chloroform-methanol 2:1 v/v.

Table I shows that after incubation of rat brain microsomes with $^{14}$C-UDP-glucose and sphingosine, glucosylsphingosine was synthesized. When sphingosine was omitted, labeled glucosylceramide but no glucosylsphingosine was found. Braun et al. (13) did not find free sphingosine in the brain of 15-day-old rats; our failure to synthesize glucosylsphingosine when sphingosine was not added also indicates the lack of free endogenous sphingosine in rat brain microsomes. As it has been previously reported (2,14) an increase in the labeling of glucosylceramide when sphingosine was added was also observed. We did not investigate whether glucosylsphingosine was also synthesized from L-sphingosine. This possibility arises from the presence of DL-sphingosine in our incubation mixture. At present we have no evidence to favor or contradict this possibility. The unidentified radioactive compounds moving slower than glucosylsphingosine on TLC (Fig. 1) are probably nonlipidic substances since they were retained by the Sephadex columns; in control experiments, 95 and 100%, respectively, of $^{14}$C-glucosylsphingosine and galactosylsphingosine, were recovered from these columns in the 3 ml total effluent.

Since Morell et al. (15) have provided proofs of the glucosylation of exogenous ceramide it appears that the pathway of the synthesis of gangliosides through ceramide has been ascertained. Whether or not there is another pathway through glucosylsphingosine cannot be discarded at present since the acylation of glucosylsphingosine to form glucosylceramide has not been investigated. A similar reaction, the synthesis of galactosylceramide by acylation of galactosylsphingosine, has been described (16,17).

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# Glyceryl Ethers in Insects: Biosynthesis of Ethanolamine Phosphoglycerides Containing Alkyl and Alk-1-Enyl Glyceryl Ether Linkages[1,2]

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## ABSTRACT

When $^{14}C$-labeled acetate, fatty acids or fatty alcohols were injected into or fed to the tobacco budworm, acyl, alkyl and alk-1-enyl moieties of the phospholipids incorporated radioactivity. Fatty acids were the principal precursor in acyl bond formation and fatty alcohols in the synthesis of alkyl and alk-1-enyl glyceryl ethers. Detailed analysis of the ether-linked phosphoglycerides revealed that most of the radioactivity was in the ethanolamine phosphoglycerides, and very little $^{14}C$ was found in the choline phosphoglycerides. In experiments of a short duration, the alkyl glyceryl ethers incorporated more radioactivity than the alk-1-enyl glyceryl ethers. The reverse was found with long term experiments, when the alk-1-enyl ethers had higher radioactivity. In addition to demonstrating the synthesis of ether-linked ethanolamine phosphoglycerides, the data suggested that fatty alcohols and acids were interconverted by insects and that the alk-1-enyl ethers were derived from the alkyl ethers.

## INTRODUCTION

Until recently the biosynthetic pathway of ether-bonded lipids was unknown. Several investigators have since established (with cell-free systems from mammals) that dihydroxyacetone phosphate and fatty alcohol are the principal precursors yielding alkyl glyceryl ethers (1,2). The plasmalogens are derived from the alkyl ethers by dehydrogenation involving several possible intermediate reactions (3,4).

In previous studies from this laboratory (5,6) it was shown that labeled acetate, fatty acids and fatty alcohols were incorporated into acyl, alkyl and alk-1-enyl moieties of total phospholipids during various developmental stages of the tobacco budworm (*Heliothis virescens* [F]; Lepidoptera-Noctuidae). Although labeling of acyl moieties occurred readily in both the ethanolamine phosphoglyceride and choline phosphoglyceride fractions, it appeared from preliminary experiments that the ether-bonded components were associated primarily with the PE fraction. The present experiments were done to explore further the relative incorporation of fatty acids and fatty alcohols into PE vs. PC ethers, and to determine by long-duration feeding experiments the possible origin of plasmalogens of insects.

## MATERIALS AND METHODS

### Insect Handling Procedures

The tobacco budworm, *Heliothis virescens*, was used for all experiments. They were reared as previously described (6) and selected for injection of labeled acetate, fatty acids or alcohols at desired stages of development. The fatty acids were converted to the K soap and were complexed with bovine serum albumin fraction V (7). The fatty alcohols were made up as a stable emulsion in 1.5% aqueous Tween 80 with an ultrasonic vibrator (8). Prior to injection the insects were immobilized at 4 C for ca. 15 min, then injected and held at room temperature for varying intervals. Each insect received exactly 2 $\mu$liters of injected material delivering 2 $\mu$Ci of acetate or 0.2 $\mu$Ci of fattty acid or fatty alcohol. The insects were then killed by dropping them in chloroform-methanol 2:1 at -20 C.

Some groups of insects were reared on diets containing labeled substrates and were taken for analysis as pupae 1-3 days old. Depending upon size and lipid content, each experimental replicate consisted of 10-40 insects, and most experiments were replicated several times.

### Chemicals and Standards

The following labeled materials were used: 1-$^{14}C$-acetate (2.0 mCi/mM), 1-$^{14}C$-hexadecanoic acid (12.0 mCi/mM), 1-$^{14}C$-octadeca-

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

[2]The following abbreviations and terminology will be used: PE, PC, PI and PS for the generic terms ethanolamine, choline, inositol and serine phosphoglycerides, respectfully. Alkyl glyceryl ether for 1-alkyl-2-acyl-*sn*-glycerol-3-phosphoryl-, and alk-1-enyl glyceryl ether for 1-alk-1′-enyl-2-acyl-*sn*-glycerol-3-phosphoryl- (commonly called plasmalogen). These are adapted from the tentative rules published in *J. Lipid Res.* 8: 522-528(1967).

noic acid (20.0 mCi/mM), 1-$^{14}$C-hexadecanol (11.4 mCi/mM and 52.0 mCi/mM) and 1-$^{14}$C-octadecanol (21.8 mCi/mM). These were obtained from New England Nuclear (Boston, Mass.), Amersham/Searle (Des Plaines, Ill.) and International Chemical and Nuclear (Irvine, Calif.). The labeled fatty alcohols for injection were further purified by preparative thin layer chromatography (TLC), and all materials were checked by zonal analysis and radioassay and were >99% pure. The labeled hexadecanol was also analyzed by gas liquid chromatography (GLC) by the manufacturer with a stated radiochemical purity of 97-99%. The larger amounts of labeled fatty alcohol needed for preparing diets were synthesized by $LiAlH_4$ reduction of the corresponding 1-$^{14}$C-fatty acid, followed by purification to >99% by preparative TLC. These were also checked by zonal TLC analysis.

The alkyl glyceryl ether standard for photodensitometry consisted of 1-octadecylglycerol (Analabs, Inc., North Haven, Conn.). It was carefully purified by preparative TLC before use. The alk-1-enyl standard was prepared by $LiAlH_4$ reduction of ethanolamine plasmalogen, obtained from white brain matter (Supelco, Bellefonte, Pa.), and was further partially purified by preparative TLC. All other chemicals and standards were as previously described (6).

#### Isolation of PE and PC from Total Phospholipids

Total lipids were extracted, separated into neutral (NL) and phospholipid (PL) fractions by silicic acid chromatography (9) and the PL repurified by preparative TLC to remove traces of NL. The PL were then rechromatographed to separate PE and PC on TLC plates of Silica Gel HR, 300 $\mu$, containing 0.001 M $Na_2CO_3$, activated 1 hr at 110 C. The solvent system consisted of chloroform-methanol-glacial acetic acid-saline 50:25:8:4 v/v/v/v. The PL classes were identified by comparison with authentic purified PL and further characterized with various spray reagents (6). Weighed amounts up to 5 mg of PE and PC were taken for analysis of glyceryl ethers as described by Wood and Snyder (9). The TLC solvent system consisted of diethyl ether-water 400:1 (10) instead of diethyl ether-ammonia as used previously (5,6,9). The water-saturated ether solvent system gave much better resolution of the alkyl from the alk-1-enyl ethers on the Silica Gel G plates.

#### Quantitative Analysis of Glyceryl Ethers by Photodensitometry

Aliquots of *lipids* for zonal analysis and

TABLE I

Specific Activity (dpm/$\mu$g) and Per Cent Distribution of $^{14}$C in Ethanolamine and Choline Phosphoglycerides and Their Constituent Glyceryl Ethers from Larvae and Pupae of *H. virescens* Reared on a $^{14}$C-1-Acetate Diet

| Lipid source | Phosphoglyceride class | Specific activity, dpm/$\mu$g | Per cent $^{14}$C | | Ether lipid type | Specific activity, dpm/$\mu$g | Per cent $^{14}$C |
|---|---|---|---|---|---|---|---|
| | | | Acyl | Ether | | | |
| Mature larva | PE | 25.2 | 80.2 | 12.6 | Alkyl | 442.4 | 22.9 |
| | | | | | Alk-1-enyl | 428.4 | 77.1 |
| | PC | 80.1 | 90.9 | 2.6 | Alkyl | 1787.7 | 30.4 |
| | | | | | Alk-1-enyl | Trace[a] | 69.6 |
| Pharate pupa | PE | 24.1 | 89.4 | 3.7 | Alkyl | 2088.2 | 64.2 |
| | | | | | Alk-1-enyl | 338.3 | 35.7 |
| | PC | 9.2 | --[b] | --- | --- | --- | --- |
| Three-day-old pupa | PE | 26.0 | 89.6 | 4.1 | Alkyl | 1600.0 | 63.3 |
| | | | | | Alk-1-enyl | 501.0 | 36.6 |
| | PC | 17.6 | 94.7 | 1.7 | Alkyl | 5175.0 | 69.2 |
| | | | | | Alk-1-enyl | Trace[a] | 30.8 |

[a]Trace detected; too little for quantitative analysis.
[b]= insufficient material or radioactivity for meaningful quantitative analysis.

TABLE II

Specific Activity of Ethanolamine and Choline Phosphoglycerides and of the Ether-Bonded Ethanolamine Phosphoglycerides in Pupae of *H. virescens* Synthesized from $^{14}$C-Labeled Fatty Acids Administered by Injection

| Substrate and incubation time | Phosphoglyceride class | Specific activity, dpm/μg | Per cent $^{14}$C | | Ether lipid type | Specific activity, dpm/μg | Per cent $^{14}$C |
|---|---|---|---|---|---|---|---|
| | | | Acyl | Ether | | | |
| Hexadecanoic acid, 4 hr | PE | 10.6 | 88.1 | 1.7 | Alkyl | 162.4 | 58.6 |
| | | | | | Alk-1-enyl | 166.7 | 41.4 |
| | PC | 15.2 | --- | --- | --- | --- | --- |
| Hexadecanoic acid, 48 hr | PE | 11.5 | 90.8 | 1.2 | Alkyl | 420.0 | 41.0 |
| | | | | | Alk-1-enyl | 514.9 | 59.0 |
| | PC | 17.0 | --- | --- | --- | --- | --- |
| Octadecanoic acid, 12 hr | PE | 33.0 | 96.8 | 0.3 | Alkyl | 156.0 | 73.6 |
| | | | | | Alk-1-enyl | 35.9 | 26.4 |
| | PC | 25.2 | --- | --- | --- | --- | --- |
| Octadecanoic acid, 55 hr | PE | 26.8 | 92.2 | 0.8 | Alkyl | 240.0 | 64.4 |
| | | | | | Alk-1-enyl | 151.4 | 35.6 |
| | PC | 12.9 | --- | --- | --- | --- | --- |

[a] - = Insufficient material or radioactivity for meaningful quantitative analysis.

radioassay were spotted in duplicate on 20 x 20 cm Silica Gel G plates and developed in the diethyl ether-$H_2O$ solvent system. Quantitative photodensitometry of the alkyl and alk-1-enyl ethers and of triplicates (on each plate) of the standard 1-octadecylglycerol were made by the procedures of Privett et al. (11). Concentrated $H_2SO_4$ was used as the charring reagent. A permanent record of all plates was made by Polaroid photography.

### Radioassay Procedures

Zonal analysis of the $LiAlH_4$-reduction products of the PE and PC fractions (12) and all other radioassay procedures have been described in detail elsewhere (6) and were used essentially unchanged here. All samples were counted with a Beckman LS-250 or DPM-100 instrument for sufficient time to obtain no greater than a ±5% 2σ error.

## RESULTS

Two groups of phospholipids were obtained and purified by preparative TLC, PE and PC, and their alkyl and alk-1-enyl glyceryl ether content was analyzed. The PE fraction contained prominent components for both types of glyceryl ether, whereas only traces of alkyl ether and no alk-1-enyl ether could be detected in the PC. The glyceryl ether components were characterized and further identified by TLC analysis of their hydrogenated and isopropylidene derivatives, and by GLC analysis of isopropylidene derivatives of alkyl ethers, or aldehydes derived from alk-1-enyl ethers before and after hydrogenation. It was also determined by TLC zonal analysis of isopropylidene derivatives of several representative samples that the $^{14}$C in the products reported in the following sections was genuinely associated with the ethers. No radioactivity was found in the TLC chromatographic position of the alkyl-1,2-diols after derivative formation. The free diols have an Rf very close to the alkyl glyceryl ethers (13).

### Biosynthesis of Glyceryl Ethers from Labeled Acetate Diet

The specific radioactivities of PE and PC for three developmental stages of *H. virescens* reared on a larval diet containing 1-$^{14}$C-acetate were determined by zonal analysis and LS assay and are summarized in Table I. The per cent distribution of $^{14}$C in acyl and ether moieties is also given, and for each phosphoglyceride type for which sufficient material was available, the percentage distribution of $^{14}$C and specific radioactivities of alkyl and alk-1-enyl ethers are

TABLE III

Specific Activity of Ethanolamine and Choline Phosphoglycerides and of the Ether-Bonded Ethanolamine Phosphoglycerides in Pupae of *H. virescens* Synthesized from $^{14}C$-Labeled Fatty Alcohols Administered by Injection or in the Larval Diet

| Substrate and incubation time | Phosphoglyceride class | Specific activity, dpm/μg | Per cent $^{14}C$[a] | | Ether lipid type | Specific activity, dpm/μg | Per cent[a] $^{14}C$ |
|---|---|---|---|---|---|---|---|
| | | | Acyl | Ether | | | |
| Hexadecanol, 12 hr | PE | 26.4 | 85.5 | 8.2 | Alkyl | 1249.4 | 78.6 |
| | | | | | Alk-1-enyl | 391.4 | 21.3 |
| | PC | 19.2 | --- | --- | --- | --- | --- |
| Hexadecanol, 48 hr | PE | 17.2 | 81.3 | 9.3 | Alkyl | 852.1 | 62.4 |
| | | | | | Alk-1-enyl | 793.5 | 37.6 |
| | PC | 13.2 | --- | --- | --- | --- | --- |
| Octadecanol, 12 hr | PE | 42.8 | 81.6 | 9.2 | Alkyl | 7547.6 | 81.8 |
| | | | | | Alk-1-enyl | 1470.8 | 18.2 |
| | PC | 25.9 | 94.8 | 0.9 | --- | --- | --- |
| Octadecanol, 72 hr | PE | 65.4 | 91.8 | 7.2 | Alkyl | 1455.0 | 68.1[a] |
| | | | | | Alk-1-enyl | --- | 31.9 |
| | PC | 28.2 | 91.3 | 1.2 | --- | --- | --- |
| Hexadecanol, diet | PE | 48.4 | 92.8 | 3.4 | Alkyl | 674.1 | 32.1 |
| | | | | | Alk-1-enyl | 727.4 | 67.9 |
| | PC | 34.8 | 95.4 | 1.1 | --- | --- | --- |
| Octadecanol, diet | PE | 31.3 | 86.0 | 7.8 | Alkyl | 461.4 | 26.2 |
| | | | | | Alk-1-enyl | 1489.7 | 73.8 |
| | PC | 19.4 | 93.0 | 0.5 | --- | --- | --- |

[a]Per cent $^{14}C$ calculated from dpm.
[b] - = Insufficient material or radioactivity for meaningful quantitative analysis.

summarized. In Table I, and as noted previously for insects (6), formation of the acyl bond predominated in all stages over synthesis of ether-bonded lipid. The highest percentage of labeling of the ethers was found in the PE of mature larvae and the lowest in PC obtained from pupae. The reverse was found for labeling of acyl moieties.

In comparing the ether content of the PE vs. PC lipids, it was clear that the ethers are associated primarily with the PE fraction. In the pharate pupa, extremely low $^{14}C$ was found in the PC ether fraction, making significiant measurements of the alkyl and alk-1-enyl moieties impractical. In larvae, about five times more $^{14}C$ occurred in PE ethers than in PC ethers, and about two and one-half times more occurred in PE ethers of 3-day-old pupae. Subsequent analysis of the PE ethers showed nearly identical specific activities for alkyl and alk-1-enyl glyceryl ethers in mature larvae, with 77% of the radioactivity being found in the alk-1-enyl form. The specific activity of PE alkyl ethers in pharate pupae rose five-fold over that of larvae and dropped slightly in the pupal stage.

The per cent distribution of $^{14}C$ to alkyl and alk-1-enyl PE ethers of pharate pupae and pupae (in Table I) was the reverse of that noted for mature larvae. In these latter two developmental stages, the alkyl ethers incorporated a larger per cent of $^{14}C$ than the alk-1-enyl and had a specific activity of three to six times higher.

### Biosynthesis of Glyceryl Ethers from Fatty Acids and Fatty Alcohols

Biosynthesis of alkyl and alk-1-enyl glyceryl ethers in the PE and PC fractions from pupae reared from diets containing labeled fatty acids or injected with fatty acids are summarized in Table II. With fatty acid as precursor, labeling of PE and PC occurred principally in the acyl moiety, although up to 1.7% of the radioactivity was in the ether-bonded fraction. As noted in Table I for acetate, nearly all of the radioactivity associated with the glyceryl ethers was found in the PE fraction, and so little of the $^{14}C$ was in the PC ethers that meaningful further analysis was impractical in most instances. Both hexadecanoic acid and octadecanoic acid were readily incorporated into acyl bonds and proportionately less into ethers. With fatty acids as the precursor, the highest ether specific activity occurred in the alk-1-enyl fraction with hexadecanoic acid in pupae held for 48 hr after injection.

With fatty alcohols as precursor, the trend was for much higher per cent incorporation and higher specific activities to occur in the ethers (Table III) than had been found with fatty acids. Once again, most of the radioactivity in the glyceryl ethers was associated with the PE fraction, although with octadecanol at 12 and 72 hr, and in the diet, small but sufficient enough PC labeling occurred to provide material for further analysis. These latter analyses showed that almost all of the PC ether radioactivity was in the alkyl glyceryl ether fraction. Occasionally traces of $^{14}C$ could be found in the choline plasmalogens; however, no meaningful calculations of specific radioactivity could be made because of the very small amounts of lipid actually present.

Significant labeling of the acyl groups occurred when fatty alcohols were the precursor (Table III) as noted in Table II for fatty acids. Therefore each precursor was utilized in the synthesis of both acyl and ether bonds. These observations strongly suggested that the two precursors were interconverted by this insect. With 16:0 fatty acid, labeling of PC predominated over PE. The reverse was found with the 18:0 fatty acid precursor (Table II). With fatty alcohols, PE always had higher specific activity than PC for all substrates even though the per cent $^{14}C$ in PC generally predominated slightly over PE (Table III). Judged by the isotope incorporation data, the fatty alcohols were the preferred precursor in ether bond formation.

## DISCUSSION

From the present results it is clear that insects are capable of synthesizing ether-bonded lipids from acetate, fatty acids and fatty alcohols. It also appears that they accomplish ether synthesis by the same pathways recently established for higher organisms. For example several investigators have previously shown that a variety of animals synthesize alkyl glyceryl ethers from fatty acids, fatty aldehydes, fatty alcohols, glycerol and acetate (14). Except for the present series of experiments (5,6), this had not been established for insects, although it had been demonstrated with several other invertebrates. More recently it has been shown conclusively that fatty alcohol is the only substrate that can be utilized in the synthesis of alkyl and alk-1-enyl glyceryl ethers. It is necessary for fatty alcohol to be present with the proper cofactors (2,15) and a glycerol precursor (1) for glyceryl ether synthesis to occur. The present data further substantiate that *H. virescens* has the metabolic capacity to interconvert fatty acids and fatty alcohols, because both precursors readily served in acyl and ether bond synthesis. Enzymic interconversion of these

precursors has recently been shown to occur in unicellular organisms (16), fish (17) and mammals (18).

The present findings also support earlier quantitative data from this laboratory showing that the glyceryl ethers were associated primarily with the PE fraction of the tobacco budworm (6). Although no data on glyceryl ether biosynthesis by other insects are available for comparison, several investigators (3,4,19,20) have reported that the principal ether-bonded component synthesized from labeled hexadecanol is associated with the ethanolamine phosphoglycerides.

The specific radioactivities and percentage distribution of $^{14}C$ between alkyl and alk-1-enyl ethers in the three developmental stages reared on labeled acetate diet (Table I) revelaed a time-dependent relationship between the two ether types. These three stages represented two different experimental situations. First, the time spent during larval feeding was relatively long. By contrast the physiologically different pharate pupal and pupal stages were experimental conditions of a short duration. The present findings show that in short duration experiments, the alkyl ethers had the higher per cent $^{14}C$, whereas in longer experiments the alk-1-enyl ethers had the larger per cent $^{14}C$. These observations strongly suggested that the alkyl ethers were synthesized first and were later desaturated to the alk-1-enyl form. The direct precursor-product relationship between alkyl and alk-1-enyl ethers has been noted in experiments of varying incubation times with the slug *Arion ater* (21). Several other investigators have reported that the alkyl ethers of higher organisms serve by one or more metabolic pathways as precursors of the alk-1-enyl glyceryl ethers (3,22,23).

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# Lipid Binding in Mitochondria from Testes of Normal and Essential Fatty Acid Deficient Rats[1]

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## ABSTRACT

Essential fatty acid (EFA) deficiency in rat causes severe degeneration of spermatogenic tissue. Previously it was shown that the distribution of lipid classes changes very little during tissue degeneration. However it is well known that the fatty acid spectrum in lipids from testicular tissue is altered drastically during EFA deficiency. The molecular binding of lipids in membrane structures might be altered when a larger amount of ω9-acids is present in the various lipid classes in testes of EFA-deficient rats. In the present studies comparison was made of the binding of lipids in testicular mitochondrial membranes from rats fed a fat-free diet or a diet containing 6% peanut oil for 26 weeks. Isolated mitochondria were coated on glass beads, then dried and packed into a column, whereafter the membrane lipids were eluted with solvents with increasing dielectric constants. The differences between the binding of lipid classes in supplemented and EFA-deficient rats were not pronounced, but a tendency to a weaker binding in the EFA-deficient rats was observed. However for both groups the various extracts showed marked differences in the distribution of lipid classes concurrent with the change of the eluent. This indicates a different kind of binding in the membrane, not only for different lipid classes, but also within a special lipid class. Thus both phosphatidylcholines (PC) and phosphatidylethanolamines (PE) were found in extracts with quite different dielectric constants. The fatty acid composition of PC and PE in the major fractions eluted with chloroform and ethanol, respectively, was essentially the same. This indicates that the successive release of phospholipids (PL) in these two fractions was not based on fatty acid solubility properties but on variable binding in the membrane structure. The introduction of ω9-polyenoic fatty acids instead of ω6-polyenoic fatty acids in the PL of mitochondria membranes from EFA-deficient rats seems to be the only deviation in the lipid pattern of EFA-supplemented and EFA-deficient animals, and might therefore be responsible for the symptons of EFA deficiency.

## INTRODUCTION

Essential fatty acid (EFA) deficiency in rats causes severe degeneration of the spermatogenic tissue (1). The distribution of lipid classes in whole testes changes very little during the development of these tissue degenerations (2). However the fatty acid compositon of the lipids is altered drastically during EFA deficiency. Degenerative alterations in the testes of animals on EFA-deficient diets might be a consequence of improper molecular binding of lipids in membrane structures.

Since preliminary studies have shown a close correlation between the phospholipid (PL) composition of total testis and of testis mitochondria, the latter were chosen as representative subcellular membranous particles for this investigation. Furthermore the fatty acid composition in PL is similar when the rats are given the same diet.

The various kinds of interactions between lipids and between lipids and proteins have been reviewed by Chapman (3). Among these, hydrophobic interactions including van der Waals forces and electrostatic forces play the major role in lipid binding in membranes (4,5).

Salem (6) has studied the interaction between two parallel hydrocarbon chains and suggests that the forces are reduced significantly with increasing distance. The introduction of double bonds in fatty acids of a phospholipid causes folding of the hydrocarbon chain and results in a diminished van der Waals force between fatty acids. However folding may allow interactions between PL and hydrophobic regions of membrane bound protein.

Electrostatic binding is dependent on the dielectric constant ($\epsilon$) of the medium, and increasing values of $\epsilon$ (higher solvent polarity) decrease the attraction forces.

In the present study we report on the binding of lipid in mitochondria membranes based on the extractability of lipid classes by solvents with increasing dielectric constants.

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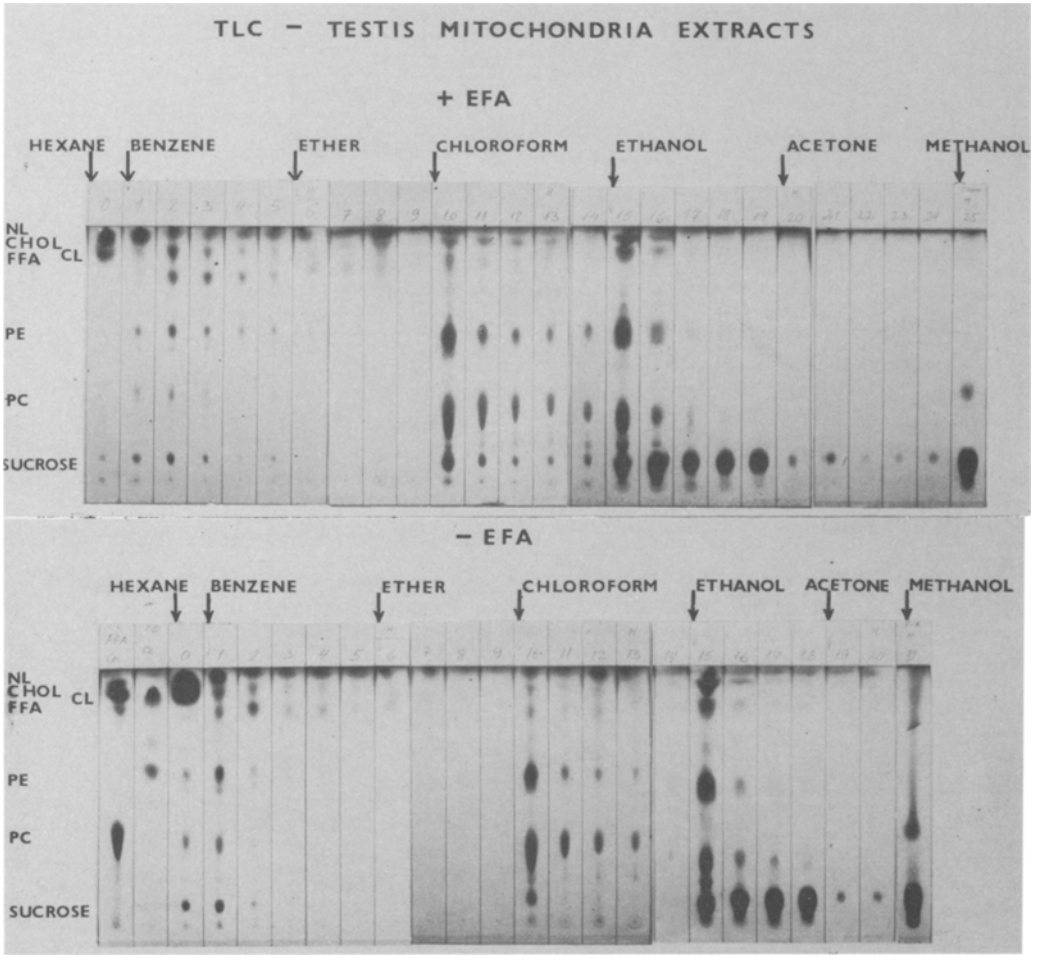


FIG. 1. Thin layer chromatography of 3 ml fractions from successive extractions of testis mitochondria membranes showing the stepwise elution of lipids according to the increasing dielectric constants of the solvents. The arrows indicate changes of solvents. Abbreviations: NL, neutral lipids; CHOL, cholesterol; CL, cardiolipin; FFA, free fatty acids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; and SPH, spingomyelin.

## EXPERIMENTAL PROCEDURES

### Animal Experiments

Weanling rats were reared either on a fat-free diet or the same diet supplemented with 6% peanut oil (1), which provided adequate amounts of essential fatty acids.

The diets and water were given ad libitum to experimental animals for a period of at least 26 weeks. After this time the animals on the fat-free diet showed severe testicular degenerations (1). Gross symptoms in EFA-deficient animals, such as scaliness of feet and tail, kidney lesions followed by hematuria, as well as depigmentation of the skin and abnormally pale livers were also observed. The animals were killed using chloroform anesthesia or by decapitation. The testes were removed for mitochondria preparation. Analytical differences between these two killing methods were not detectable.

### Preparation of Mitochondria

After killing the rats, the testes were immediately excised and immersed in ice cold 0.25 M sucrose in 1 mM EDTA, pH 7.4. After cooling, the testes were punctured, the contents squeezed into a Potter-Elvehjem glass homogenizer equipped with a Teflon pestle, and homogenized in 0.25 M sucrose in 1 mM EDTA, pH 7.4 (4.5 ml/g tissue). Six normal testes or 12 testes from EFA-deficient rats were pooled, corresponding to 8-10 g of tissue.

The homogenate was centrifuged for 10 min in a Sorvall centrifuge (Rotor SS 34) at 0-4 C and 900 x $g_{max}$ to remove cell nuclei and

TABLE I

Total Lipids, Phospholipids and Cholesterol in Rat Testes Mitochondria

| Diet characteristics | EFA-supplemented[a] (6% peanut oil) | EFA-deficient[b] (fat-free) |
|---|---|---|
| Total lipid, mg/mg protein | 0.33±0.02[c] | 0.30±0.01[c] |
| Total PL, mg/mg protein | 0.14±0.01 | 0.14±0.00 |
| Cholesterol, μg/mg protein | 3.0±0.1 | 3.7±0.2 |

[a]Fat-free basal diet supplemented with 6% peanut oil (normal).
[b]Fat-free basal diet (deficient).
[c]SE of the mean of two determinations on mitochondria from pools of six normal testes or twelve deficient testes.

debris. The supernatant fluid containing the mitochondria was then centrifuged at 9000 x $g_{max}$ for 10 min at 0-4 C. A light brown pellet of mitochondria was layered in the bottom of the tube, and the supernatant fluid was decanted off and discarded. The pellet was first resuspended in a small amount of the buffered sucrose, then diluted with additional solution to the original volume of the supernatant fluid. The mitochondria were sedimented at 9000 $g_{max}$ for 10 min, the supernatant solution discarded, and the sediment resuspended in a small amount of the buffered sucrose (ca. 25 mg protein per milliliter). An aliquot was taken for protein determination by the microbiuret method according to Goa (7).

### Total Lipids

Extraction of total mitochondrial lipids was made with chloroform-methanol 2:1 v/v, using a slightly modified procedure of Folch et al. (8). To avoid emulsions the mitochondria sus-

TABLE II

Lipid Distribution in Fractions from Column Extraction of Rat Testicular Mitochondria[a]

| Diet characteristics | Fraction no. (15 ml total) | Solvent | ε | Total lipid, mg | Total lipid, % | PL[b] | Total PL, % |
|---|---|---|---|---|---|---|---|
| EFA-supplemented[c] (6% peanut oil) | N1 | Hexane | 1.9 | 0.76 | 6.0 | 0.04 | 0.8 |
| | N2 | Benzene | 2.3 | 1.43 | 11.4 | 0.15 | 2.8 |
| | N3 | Diethylether | 4.3 | 0.29 | 2.3 | 0.03 | 0.5 |
| | N4 | Chloroform | 4.8 | 2.16 | 17.3 | 1.55 | 29.4 |
| | N5 | Acetone | 20.7 | 1.36 | 10.9 | 0.08 | 1.5 |
| | N6 | Ethanol | 24.3 | 5.82 | 46.5 | 3.31 | 63.0 |
| | N7 | Methanol | 32.6 | 0.70 | 5.6 | 0.11 | 2.1 |
| | | Total | | 12.52 | 100.0 | 5.27 | 100.0 |
| | | Folch extraction | | 12.54 | | | |
| EFA-deficient[d] (fat-free) | F1 | Hexane | 1.9 | 0.74 | 6.5 | 0.02 | 0.5 |
| | F2 | Benzene | 2.3 | 0.56 | 4.4 | 0.08 | 1.6 |
| | F3 | Diethylether | 4.3 | 0.29 | 2.5 | 0.03 | 0.7 |
| | F4 | Chloroform | 4.8 | 2.17 | 19.0 | 1.46 | 28.0 |
| | F5 | Acetone | 20.7 | 1.46 | 12.8 | 0.09 | 1.7 |
| | F6 | Ethanol | 24.3 | 5.53 | 48.3 | 3.41 | 65.5 |
| | F7 | Methanol | 32.6 | 0.75 | 6.5 | 0.10 | 2.0 |
| | | Total | | 11.44 | 100.0 | 5.19 | 100.0 |
| | | Folch extraction | | 11.50 | | | |

[a]Column loading: mitochondria corresponding to 38 mg of protein (testes from six normal animals or from 12 deficient animals were pooled).
[b]Calculated from phosphorus determinations using a factor 26.1 corresponding to an average molecular weight of 808 for phospholipids.
[c]Fat-free basal diet supplemented with 6% peanut oil (normal).
[d]Fat-free basal diet (deficient).

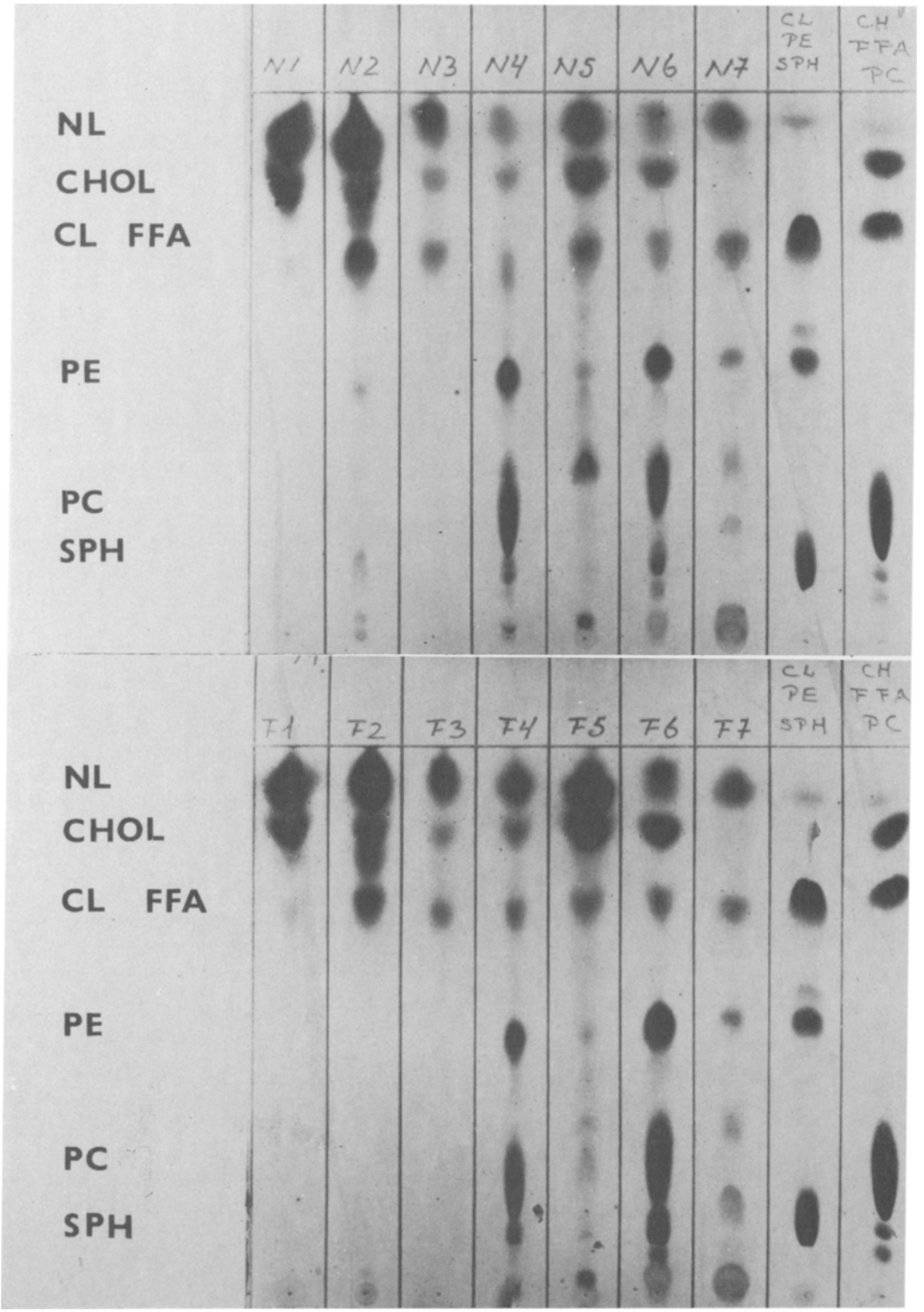


FIG. 2. Distribution of lipids in thin layer chromatography of total fractions (15 ml) from column extraction of testis mitochondria. N1, N2. . . . , and F1, F2. . . . correspond to the fractions in Table II. Equal amounts, ca. 200 μg, were applied except for fractions 3 and 7, where only half this amount was available. Standards are run on the right hand side of the plate. For abbreviations, see Figure 1.

TABLE III

Distribution of Phospholipids in Major Fractions from Column Extraction of Testis Mitochondria. Weight Per Cent of Phosphorus

| Diet characteristics | EFA-supplemented[a] (6% peanut oil) | | | EFA-deficient[b] (fat-free) | | |
|---|---|---|---|---|---|---|
| P-lipid, % of total PL | 2.8 | 29.4 | 63.0 | 1.6 | 28.0 | 65.5 |
| PC(+PS)[c] | 49.4±1.0[f] | 61.5±1.3 | 51.2±2.6 | Major | 44.9±3.5 | 48.2±4.1 |
| PE | 44.3±2.4 | 32.9±1.0 | 21.1±0.2 | Major | 38.8±4.2 | 25.0±1.0 |
| SPH | 4.6±1.0 | 4.1±0.4 | 18.1±4.8 | Minor | 9.0±1.7 | 16.4±1.7 |
| CL | 0.7±0.2 | 1.5±0.3 | 5.7±2.8 | Minor | 5.1±1.5 | 5.5±0.2 |
| PGP[d] | 0.7±0.4 | | 0.2±0.2 | Trace | --- | --- |
| PI[e] | 0.2±0.1 | | 0.7±0.5 | Trace | 2.2±0.6 | 0.5±0.3 |
| Lyso PC | --- | --- | 3.0±1.0 | --- | --- | 4.4±1.3 |

[a]Fat-free basal diet supplemented with 6% peanut oil (normal).
[b]Fat-free basal diet (deficient).
[c]PS = Minor component.
[d]PGP = Polyglycerophosphatidic acid.
[e]PI = Phosphatidylinositol.
[f]SE of the mean of three determinations.

pension to be extracted (30-50 mg protein) was first treated with 5 ml methanol. This resulted in flocculation, presumably of mitochondrial protein. Further addition (10 ml) of chloroform was made before extraction. Two subsequent extractions with chloroform-methanol 2:1 v/v assured removal of total lipids. The combined extracts were washed according to Folch et al. with sodium chloride solution and "upper phase."

#### Extraction of Mitochondria with Solvents of Increasing Dielectric Constants

Extraction of mitochondria was performed in a column (13 x 100 mm) fitted at the bottom with a filter disc (No. G3) and packed with glass beads (Ballottini Beads No. 16, 100 mesh) in three successive layers. First a layer of uncoated beads was packed into the column to a height of 15 mm, secondly 4 g of beads coated with mitochondria (see below), and finally a 2 mm layer of uncoated beads was poured into the column to prevent disturbance of the sample zone during extraction. The glass beads, as well as all other glassware, were rinsed thoroughly with extraction solvents prior to use.

To coat the beads with mitochondria, 1 ml aqueous mitochondria suspension, corresponding to 25-30 mg mitochondrial protein, was mixed with 4 g of glass beads. The solvent was removed in a Büchi Rotavapor operated at 10-15 mm Hg at ambient temperature. After the solvent had evaporated the coated beads were dried over $P_2O_5$ in vacuo at 4 C overnight.

The columns were eluted stepwise using 15 ml portions of an eluotropic series (9) ("$\epsilon$" is the designation of the dielectric constant of a solvent.): (a) hexane, $\epsilon$ = 1.9; (b) benzene, $\epsilon$ = 2.3; (c) diethylether, $\epsilon$ = 4.3; (d) chloroform, $\epsilon$ = 4.8; (e) acetone, $\epsilon$ = 20.7; (f) ethanol, $\epsilon$ = 24.3; and (9) methanol, $\epsilon$ = 32.6. For qualitative analysis, five 3 ml fractions were collected.

#### Quantitation of Lipid Classes

Total PL and the distribution of PL classes after preparative thin layer chromatography (TLC) were quantitated according to the method described by Bartlett (10). Digestion with 10 N $H_2SO_4$ and the subsequent color development were made directly on TLC scrapings. The silica gel was sedimented by centrifugation at ca. 1500 g in a clinical centrifuge before measurements. These were made in a Beckman DB-G spectrophotometer with a flow-through cell connected to an automatic sampler (Samplomat, Struers, Copenhagen), which allowed sampling without interference from the sedimented silica gel.

Total cholesterol content was determined by the method of Herrmann (11).

#### Thin Layer Chromatography

Fractions collected in column extraction were further characterized by TLC. The separation of the PL classes was performed in a solvent system of chloroform-methanol-water 65:25:4 v/v/v on Silica Gel H (Merck, Darmstadt) suspended in 1 mM $Na_2CO_3$ (12). Various visualizing reagents were used for identifying phospholipids and glycolipids (13,14); thus ninhydrin for lipids containing amino groups, Dragendorff reagent for choline-phospholipids and molybdate-spray for all phospholipids (13). Glycolipids were identified by $\alpha$-naphthol followed by sulfuric acid (96%)

TABLE IV

Fatty Acids of Phosphatidylcholines in Major Fractions from Column Extractions of Rat Testes Mitochondria (% of total methyl esters)[a,b]

| Diet characteristics | EFA-supplemented[c] (6% peanut oil) | | EFA-deficient[d] (fat-free diet) | | Total testes from rats on fat-free diet |
|---|---|---|---|---|---|
| Fatty acid | Fraction N4 | Fraction N6 | Fraction F4 | Fraction F6 | |
| 14:0 | 1.3 | 1.5 | 1.3 | 0.4 | 0.8 |
| 16:0 | 39.7 | 43.0 | 39.7 | 46.6 | 41.6 |
| 18:0 | 4.7 | 5.8 | 9.1 | 8.5 | 5.8 |
| 18:1 ω9 | 16.8 | 17.5 | 24.3 | 26.3 | 24.3 |
| 18:2 ω6 | 3.2 | 3.2 | 2.6 | 0.8 | 0.7 |
| 20:3 ω9 | — | — | 12.2 | 11.7 | 14.0 |
| 20:4 ω6 | 14.6 | 15.3 | 2.2 | 1.0 | 4.6 |
| 22:3 ω9 | — | — | Trace | Trace | 0.6 |
| 22:5 ω6 | 11.3 | 10.0 | 2.2 | 1.3 | 3.1 |
| Σ saturated | 46.5 | 50.3 | 48.7 | 55.8 | 48.2 |
| Σ monoenoic | 20.6 | 20.4 | 29.7 | 28.6 | 27.5 |
| Σ ω6-polyenoic | 30.7 | 29.4 | 7.0 | 3.1 | 8.4 |
| Σ ω9-polyenoic | — | — | 12.2 | 11.7 | 15.7 |

[a]Minor amounts of C 15:0, C 16:0 ald., C 16:1 ω7, C 18:0 ald., C 18:3 ω3, C 20:1, C 20:2, C 20:3 ω6, C 22:1, C 22:4 ω6 were also detected. (ald. = Alk-1-enyl moieties in PC plasmalogens determined as dimethylacetals.)

[b]Experimental Period: 28 weeks.

[c]Fat-free basal diet supplemented with 6% peanut oil (normal).

[d]Fat-free basal diet (deficient).

(14). Charrring with 50% $H_2SO_4$ or chromic-sulfuric acid was used for nonspecific identification of the lipids.

### Gas Liquid Chromatography (GLC)

Fatty acid distribution in some of the major lipid classes was determined by preparative TLC followed by GLC.

Lipids were methylated according to Stoffel et al. (15) with 5% dry hydrochloric acid in dry methanol directly on scrapings from TLC plates.

TABLE V

Fatty Acids of Phosphatidylethanolamines in Major Fractions from Column Extraction of Rat Testes Mitochondria (% of total methyl esters)[a,b]

| Diet characteristics | EFA-supplemented[c] (6% peanut oil) | | EFA-deficient[d] (fat-free) | | Total testes from rats on fat-free diet |
|---|---|---|---|---|---|
| Fatty acid | Fraction N4 | Fraction N6 | Fraction F4 | Fraction F6 | |
| 14:0 | 6.1 | 5.7 | 1.6 | Trace | 0.5 |
| 16:0 ald.[a] | — | — | 0.9 | 0.8 | 6.6 |
| 16:0 | 27.6 | 29.5 | 23.0 | 19.2 | 24.5 |
| 18:0 | 9.6 | 8.3 | 13.6 | 11.9 | 12.3 |
| 18:1 ω9 | 7.5 | 7.4 | 16.6 | 17.6 | 13.9 |
| 18:2 ω6 | 1.9 | 3.1 | 0.8 | 2.7 | 0.4 |
| 20:3 ω9 | — | — | 23.5 | 23.9 | 17.1 |
| 20:4 ω6 | 20.9 | 19.3 | 3.9 | 4.2 | 4.8 |
| 22:3 ω9 | — | — | 2.4 | 4.2 | 3.8 |
| 22:5 ω6 | 15.7 | 13.5 | 6.2 | 2.0 | 6.9 |
| Σ saturated | 46.3 | 46.0 | 39.5 | 33.3 | 37.3 |
| Σ monoenoic | 15.0 | 11.3 | 20.5 | 20.4 | 16.0 |
| Σ ω6-polyenoic | 38.5 | 39.6 | 10.9 | 11.8 | 12.5 |
| Σ ω9-polyenoic | — | — | 25.9 | 28.9 | 25.6 |

[a]Minor amounts of C 15:0, C 16:1 ω7, C 18:3 ω3, C 20:1, C 20:2, C 20:3ω6, C 22:1, C 22:4 ω6, C 22:4 ω9 and C 18:0 ald. were also detected. (ald. = Alk-1-enyl moieties in PE plasmalogens determined as dimethylacetals.)

[b]Experimental Period: 28 weeks.

[c]Fat-free basal diet supplemented with 6% peanut oil (normal).

[d]Fat-free basal diet (deficient).

GLC was performed in a Beckman GC 4 using a column 1/8 in. od, 6 ft long, filled with 15% diethylene glycol succinate (DEGS) on chromosorb W (AW) (Applied Science Laboratories, Inc., State College, Pasadena, Calif.). The column temperature was 175 C, and the detector and inlet temperatures were ca. 230 C and 240 C, respectively. Helium was used as a carrier gas at a flow rate of 40 ml/min. The flame ionization detector was operated with a flow of 40 ml $H_2$ per minute and 250 ml air per minute. Quantitation was made either by means of a Disc integrator or by the "retention x peak height" approximation.

#### Materials

All reagents were analytical grade, except some solvents for TLC, which were redistilled before use.

## RESULTS

#### Qualitative Examination of Fractions from Mitochondria Extractions

The lipids from mitochondria membranes could be extracted selectively by varying the dielectric constant of the extraction solvent. Figure 1 shows the different lipid classes separated by TLC of five successive 3 ml fractions of the extracts. Fraction O represents the total hexane fraction. The largest amounts of extracts were obtained with chloroform and ethanol for both groups. Most notably, the first or the second, or both, of the 3 ml fractions contained the majority of the material, and thereafter the extractable amounts decreased appreciably. Consequently cross contamination between fractions was considered insignificant. The difference between fat-free and fat-supplemented animals was small.

The data demonstrate binding of lipids in mitochondrial membranes to be different and the release of lipid to be highly dependent on the capability of the eluting solvent to break interactions between lipids or between lipid and protein. As the important bindings in membranes are of either electrostatic or hydrophobic nature (3), variation in dielectric constants of the surroundings would influence the lipid-binding in different ways, as previously mentioned. A nonpolar solvent with a low dielectric constant would favor extraction of lipids bound by pure hydrophobic interactions as the London van der Waal interactions between hydrocarbon chains.

For both groups of animals a large part of neutral lipids (NL), including cholesterol, was eluted in hexane (fraction O). However later changes in solvent polarity released more cholesterol; therefore some cholesterol could have been unaccessible in structures held together by other types of binding. These data are in contrast to results reported by Parpart and Ballentine (16), who showed, for erythrocytes, that all cholesterol was eluted with dry ethyl ether. However, in agreement with the results presented here, Haahti et al. (17) found a successive elution of cholesterol from erythrocytes.

Small differences between solvent dielectric constants (hexane, $\epsilon = 1.9$, to benzene, $\epsilon = 2.3$) resulted in a different distribution of lipid classes. Benzene gave small amounts of PL, which were mainly PE, some free fatty acids and other NL, among which was cholesterol.

Diethyl ether ($\epsilon = 4.3$) removed only small amounts of lipids from the membranes. The dielectric constant for this solvent was undoubtedly not great enough to disrupt hydrophobic interaction between PL and proteins.

Surprisingly a relatively small change in $\epsilon$, i.e., 4.3 for diethyl ether to 4.8 for chloroform, resulted in a massive release of lipid material from mitochondria membranes, which was primarily PL. The major component in this fraction was PC, but PE was also found in considerable amounts. Some glycolipid was found to cochromatograph with PE in the TLC procedure. A spot which migrated slightly lower than PC also gave a positive reaction for glycolipid.

Fleischer et al. (18) have reported glycolipids to be absent in mitochondria. Their data have been confirmed for liver mitochondria in this laboratory, but in testis mitochondria glycolipids were present in small amounts, probably cerebrosides and cerebroside sulfate. There was no difference between supplemented and deficient animals with respect to the occurrence of glycolipids.

The next change of solvent involved a great alteration in dielectric constant, i.e., from 4.8 for chloroform to 24.3 for ethanol.

In preliminary experiments columns were eluted with ethanol ($\epsilon = 24.3$) prior to acetone ($\epsilon = 20.7$), which resulted in release of lipid in the ethanol fraction, corresponding to the lipid extracted with successive use of acetone and ethanol. These data further support the assumption that the association of different lipids to membranes is a function of the dielectric constant of the environment at the membrane surface.

Increasing the dielectric constant of the solvent from that of chloroform to that of ethanol (a change in $\epsilon$ of ca. 20) caused a large amount of lipid to be released from the mitochondria. These data were interpreted as

the influence of a solvent with high dielectric constant on the electrostatic interactions between lipids and mitochondrial proteins.

The amounts of PC and PE in the ethanol fractions appeared nearly equal in contrast to the chloroform extractable lipid in which PC was more predominant than PE. Sphingomyelin, as well as cardiolipin, was present in this fraction. However cardiolipin, which is a *major* component of mitochondria from other organs, was detectable only in small amounts. Glycolipids were also present in this fraction.

The last fractions were taken with methanol ($\epsilon$ = 32.6) as eluent. Apparently all the PL had already been removed as only two spots were detected; one was identified as sucrose, and the other was not identified. Both components were water-soluble and thus were not lipids. In subsequent experiments these components were removed by washing with sodium chloride according to Folch et al. (8).

These data demonstrate that successive elution of mitochondria with a stepwise increase in dielectric constant of the solvents released lipids of different composition. The differences between EFA-deficient and EFA-supplemented animals was small, but a tendency to looser binding of lipids was observed for mitochondria from deficient animals in the first fractions. The membrane architecture of testis mitochondria for normal and EFA-deficient animals was therefore considered essentially the same as far as the lipid class composition was concerned.

#### Quantitative Analysis of Mitochondria Extracts

Table I shows the total lipids, total PL and total cholesterol contents of mitochondria from testes of rats fed on a fat-free basal diet with and without supplements of 6% peanut oil. The total lipid and total PL contents were nearly the same for deficient and supplemented rats.

Total lipids and total PL in fractions from column extractions are shown in Table II and Figure 2.

The content of total lipid in the ethanol fractions (N6, F6) was much larger than in any other fraction, but large amounts of lipid were also eluted in chloroform. For the animals on supplemented diets, benzene and acetone each removed ca. 10% of the total lipids from the mitochondria. The benzene fraction of mitochondria from rats on fat-free diets contained less lipid. The lipid content of the ether fraction was small in both cases. In general there were only small differences between the EFA-supplemented and the deficient animals.

Quantitative analysis of total extracts made according to Folch et al. (8) was in close agreement with the total amounts of lipids extracted with the different solvents.

The chloroform and ethanol fractions (N4, F4 and N6, F6) contained practically all the PL, and nearly two-thirds of the total PL were present in the ethanol fraction. Again there was little difference between the EFA-supplemented and deficient groups.

For the first three fractions (N1, N2, N3 and F1, F2 and F3) taken with hexane, benzene and ether, respectively, only very small amounts of PL were present, and NL were predominant (Fig. 2). The NL may have been held within the membrane structure only by London van der Waal interactions, which were believed essential for the binding of cholesterol and hydrocarbon chains.

When chloroform was used as the eluent (N4 and F4), significant amounts of PL were released, suggesting hydrophobic interactions between phospholipids and proteins to be disrupted with this solvent. A great change in dielectric constant ($\epsilon$) from 4.8 for chloroform to 20.7 for acetone did not seem to release additional PL (N5 and F5), whereas significant amounts of NL were extracted from the membranes.

As acetone extracts contained predominantly NL, it was reasonable to speculate that they were bound by hydrophobic interactions, as the PL in the preceeding fractions N4 and F4. The retardation of the elution of NL in N5 and F5 was considered a consequence of the removal of surface bound PL-layer before additional NL were extractable. A moderate change in $\epsilon$ from 20.7 for acetone to 24.3 for ethanol (N6 and F6) resulted in a very significant removal of lipid from the membranes. With this solvent, strong electrostatic forces would also be expected to be broken, as well as possible hydrogen bondings. Relatively large amounts of cholesterol were still eluted with ethanol.

There were no major differences between EFA-supplemented and deficient animals.

#### Distribution of Phospholipid Classes in Solvent Fractions

The distribution of PL in the major fractions is given in Table III. Phosphatidylcholines (PC) and phosphatidylethanolamines (PE) were the major components among the PL. In the chloroform fractions (N4 and F4), PC represented from two-thirds to one-half of the total PL. Sphingomyelins (SPH) and cardiolipins (CL) were minor fractions and had slightly higher values for the EFA-deficient rats. Fractions F6 and N6 contained PC as their major component and virtually all the SPH. CL, which was differentiated from free fatty acids (FFA) by a specific PL-reagent, was a minor

constituent of these fractions as well as of the total lipid extract from mitochondria.

### Fatty Acids of Phosphatidylcholines (PC) and Phosphatidylethanolamines (PE) of Rat Testis Mitochondria

The overall composition of PC in fractions 4 and 6 (Table IV and V) was essentially identical for both supplemented and deficient animals. The supplemented groups showed high contents of ω6-acids for both fractions, and approximately half of the fatty acids were saturated. The molecular species may therefore all have one saturated acid, the other one being either a monoenoic acid or an ω6-polyenoic acid. In the deficient rats the content of saturated acids in phosphatidylcholine (PC) was similar to that of supplemented animals, but the amounts of the ω6-polyenoic acids were greatly reduced and partially substituted with ω9-polyenoic acids and monoenoic acids. For comparison, results obtained with whole testes of fat-free animals are also listed in Tables IV and V.

In PE there was also a close agreement between the fatty acid composition of fractions 4 and 6, originating from the same mitochondria. Significant differences in the amounts of ω9- and ω6-acids were detected in PE as the consequence of EFA deficiency. Furthermore the content of saturated acids was lower in PE than in PC, increasing the possible number of molecular species, especially for the fat-free group. Compared to whole testes, only traces of PE-plasmalogens have been found in mitochondria (Table V), whereas they have always been found in whole testes, both in deficient and supplemented groups.

These analyses demonstrate that the subsequent release of PL represented by fractions 4 and 6 was not based on fatty acid solubility properties, but on variable binding in the membrane structure.

## DISCUSSION

The differences in lipid class composition of extracts of mitochondria from rat testes of EFA-supplemented and EFA-deficient groups were small. The subsequent release of the lipids with solvents of increasing dielectric constants were affected in the same way, possibly with a tendency to weaker binding of some of the neutral lipids in the deficient group. The occurrence of successive fractions according to the variation in the dielectric constant of the eluting solvent may have been due to different kinds of interactions between the extracted lipids and other lipids or proteins in the mitochondrial membrane. These interactions are supposed to range from the relatively weak London van der Waal interactions between hydrocarbon chains, almost independent of the dielectric constant of the medium, to the stronger hydrophobic interactions between lipids and proteins and to the strongest electrostatic forces that depend highly on the surroundings.

The lipid composition of membranes in mitochondria from EFA-deficient and EFA-supplemented rats was nearly identical as far as the lipid classes and the binding to the mitochondrial structure were concerned. The only difference was in the fatty acid composition of the PL, where ω9-polyenoic acids predominated, as it typical in EFA-deficiency.

The introduction of ω9-polyenoic acids instead of ω6-polyenoic acids in the 2 position of PL affects the three dimensional structure of the mitochondrial membranes. This deviation might be responsible for the swelling and fragility of liver mitochondria which Houtsmüller et al. (19) have used as a measure of EFA deficiency, and the difference in swelling pattern of mitochondria under various conditions reported by Hayashida and Portman (20). However the role of structural proteins in membranes should also be taken into account. Investigations along this line are now in progress.

## ACKNOWLEDGMENTS

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# Polyunsaturated Fatty Acids of Skin; Identification and $^{14}$C-Acetate Incorporation

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**ABSTRACT**

Following exposure of the tissue to $^{14}$C-acetate, polyunsaturated fatty acids were separated from the total fatty acids of newborn human preputial and mouse skin using argentative thin layer and gas liquid chromatographic procedures. They were characterized by conventional methods including oxidative cleavage and gas liquid chromatography of fission fragments. Members of the linoleic and linolenic acid classes were present in labeled form including the parent acids. Linoleic and arachidonic acids predominated, all others occurring in relatively small amounts. Patterns for the two types of skin were similar in distribution of both mass and radioactivity of individual acids.

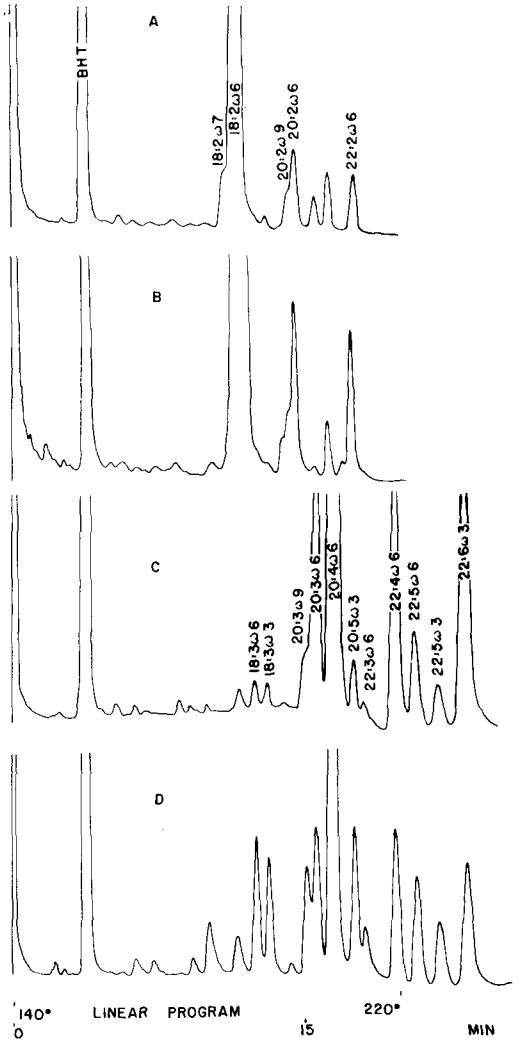


FIG. 1. Gas liquid chromatography traces of dienes (A and B) and polyenes (C and D) from preputial and mouse skin, respectively.

Apart from diene fatty acids of human skin surface lipids (sebum, Ref. 1), little information is available about the polyunsaturated fatty acids (PUFA) of skin. Recently 18:2 and 20:4 were identified in lipids of human preputial and abdominal skin (2), but other PUFA remained undetected.

This report describes the characterization of PUFA in skin and their radioactivity levels following exposure of the tissue to $^{14}$C-acetate in vitro. In an earlier communication, study of the incorporation of acetate into octadecadienoic acids in the lipids of epidermal cells released from human skin by trypsin revealed that labeling was associated unexpectedly almost exclusively with the ω6 isomer, linoleic acid (3). As trypsinization might have modified the cells' enzymatic potential (4), labeling of 18:2 was then studied with intact skin, and the results are reported here.

The present study utilized newborn human and mouse skin because desaturation enzymes show maximal activity in very young tissues (5). However, among the $^{14}$C- labeled fatty acids of skin from young mice, Brooks et al. found that linoleic acid remained unlabeled (6). It was necessary to determine whether or not this represented a difference in PUFA metabolism between mouse and human skin.

## MATERIALS AND METHODS

### Skin

Preputial skin speciments were from the nursery; connective tissue was trimmed off. Newborn mice, strain BALB/c, 1-2 days old were sacrificed, and their skin removed. All tissue was rinsed with saline, blotted dry and weighed.

TABLE I

Occurrence and Radioactivity Distribution among PUFA

| Fatty acid or class | Preputial skin | | | Newborn mouse skin | | |
|---|---|---|---|---|---|---|
| | Occurrence, % of total PUFA[a] | Radioactivity, % of total of PUFA[a] | Specific activity cpm/μg | Occurrence, % of total PUFA[a] | Radioactivity, % of total of PUFA[a] | Specific activity, cpm/μg |
| Saturates | —[b] | 57750[c] | | — | 19593[c] | |
| Monoenes | — | 6875[c] | | — | 3211[c] | |
| Dienes | 43.4[d] | 3592[c] | 83 | 415.8[d] | 1530[c] | 3.7 |
| Polyenes | 76.3[d] | 2899[c] | 38 | 305.8[d] | 2479[c] | 8.1 |
| 18:2ω6 + 18:2ω7 | 31.7 | 40.2 | 60 | 50.7 | 22.5 | 2.3 |
| 20:2ω6 + 20:2ω9 | 1.5 | 13.5 | 430 | 3.2 | 10.1 | 16.3 |
| 22:2ω6 | 0.5 | 1.5 | 148 | 1.9 | 1.2 | 3.2 |
| 18:3ω6 | 0.6 | 3.3 | 268 | 2.0 | 7.7 | 20.1 |
| 18:3ω3 | 0.3 | 1.1 | 124 | 2.1 | 4.1 | 10.6 |
| 20:3ω9 | 0.7 | — | — | 2.4 | — | — |
| 20:3ω6 | 4.4 | 21.1 | 227 | 2.8 | 15.9 | 30.6 |
| 20:4ω6 | 38.4 | 2.6 | 3 | 20.5 | 2.1 | 0.5 |
| 20:5ω3 | 1.1 | 0.4 | 14 | 2.2 | 0.3 | 0.7 |
| 22:3ω6 | 0.4 | 0.6 | 60 | 1.1 | 1.1 | 5.2 |
| 22:4ω6 | 5.3 | 10.7 | 96 | 2.6 | 11.6 | 25.0 |
| 22:5ω6 | 1.8 | 2.2 | 58 | 1.9 | 2.5 | 6.8 |
| 22:5ω3 | 0.9 | 2.1 | 111 | 0.9 | 2.7 | 14.0 |
| 22:6ω3 | 5.1 | 1.2 | 11 | 1.6 | 4.5 | 14.7 |
| Others | 6.6 | 3.3 | | 4.8 | 2.9 | |

[a]Figures are per cent of total amounts for PUFA (dienes + polyenes) except where specified otherwise.
[b]Mass of saturated and monoene classes not determined.
[c]In cpm/100 mg skin.
[d]In μg/100 mg skin.

#### Radioactive Materials

Sodium 1-$^{14}$C-acetate, 54 mCi/mM, and linoleic acid, 10 mCi/mM, methylated with diazomethane, were used.

#### Labeling

Each preputial or mouse skin was minced with scissors and incubated at 37 C in air in a vial containing 2 ml Pucks Saline G, pH 7.4, with 5 μCi of sodium 1-$^{14}$C-acetate and antibiotics (7) for 4 hr, using a shaking incubator.

#### Isolation of Methyl Esters

After labeling, tissue was homogenized in $CHCl_3/CH_3OH$ 2:1. The homogenate was filtered through fritted glass and the solvents removed in a rotary evaporator at 50 C. Ten preputial and 15 mouse skins were labeled and extracted in this manner, and the residues after solvent removal were pooled to obtain two large lipid samples. Methylation and purification of methyl esters by thin layer chromatography (TLC) on silica gel has been described before (7), except that benzene was used here to develop the plates in one step. Purified esters were resolved by argentative TLC. Plates were developed with 0.75% $CH_3OH$ in $CHCl_3$ (8) and ester bands visualized with 0.2% ethanolic 2,7-dichlorofluorescein. This process separated saturated, monoene and diene bands from a fourth band which consisted of the remaining, poorly resolved polyenes. These four bands were scraped from plates, extracted with $CH_2Cl_2/CH_3OH$ 4:1, silica gel filtered off, the filtrate evaporated and the residue extracted with pentane. Bands from each plate were pooled. The fourth fractions (pooled) were reapplied to a single plate, developed with hexane-ethyl ether 4:1 and resolved into tri-, tetra-, penta- and hexaene fractions which were recovered as before. Samples of pure methyl esters of known unsaturation were run alongside.

Each sample of PUFA of defined unsaturation was resolved into its component methyl esters by small scale preparative gas liquid chromatography (GLC) as described before (7). Samples of 1 mg or less were injected. The column was operated either at 182 C or linearly programed from 140-220 C. Emerging peaks were collected in glass tubes as before (7) and recovered by rinsing the tubes with hexane. An aliquot of each hexane solution was evaporated and redissolved in scintillation fluid for radio-

activity assay.

Saturated and monoene fractions were not subjected to GLC, but extracted from the $AgNO_3$/silica gel, dissolved in scintillation fluid and counted.

#### Hydrogenation

Aliquot samples of the hexane solutions of individual methyl esters or classes of esters were evaporated, and the residue was hydrogenated as before (9). Products were analyzed on the same GLC column as above.

#### Double Bond Location

Individual PUFA were oxidized by $NaIO_4$/$KMmO_4$ as before (3), or by reductive ozonolysis (10), or by both methods. Solutions containing oxidation fragments (after diazomethane methylation of carboxyl groups when applicable) were concentrated to 25 $\mu$liters and injected into a GLC column similar to the one used above (10% EGSS-X on Gas Chrom P, 100/120 mesh). Injections were made at 130 C column temperature (injector 230 C, detector 220 C, helium flow 38 ml/min) which was programed to 200 C at 4 C/min. Peaks were collected in glass U-tubes, 3 mm ID cooled in acetone-$CO_2$. Finally each tube was rinsed with scintillation fluid into vials for counting.

#### Reagents

Solvents were reagent grade, distilled before use and with BHT added (0.001%). Fatty acid samples were kept under nitrogen as much as possible. Ozone was obtained from a corona generator with oxygen flow of 2-3 $ft^3$/hr. Scintillation fluid has been described (7), and counting was performed in a Beckmann LS-100 instrument, with 90% efficiency. Samples of PUFA were obtained from Schwarz-Mann, Sigma and Nu Chek Prep, Elysian, Minn. 56028.

## RESULTS AND DISCUSSION

In skin lipids, saturated and monoenoic fatty acids predominate, and the bulk of radioactivity incorporated from $^{14}C$-acetate by skin is associated with these fractions (2). Except for 18:2 and 20:4, PUFA are present in small amounts, and therefore tend to be obscured in GLC analysis of a total fatty acid sample by more abundant and highly labeled components.

In this study, resolution of total samples into groups according to degree of unsaturation was accomplished by small scale preparative $AgNO_3$/TLC. Development of these TLC plates with $CHCl_3/CH_3OH$ (8) produced satisfactory separation of saturates, mono- and dienes from the remaining polyenes. Resolution of the complete PUFA mixture (dienes plus polyenes) by preparative GLC was not satisfactory due mainly to the obscuring of 18:3 peaks by the very large 18:2 peak. However separate GLC treatment of dienes and remaining polyenes provided good resolution of most peaks (Fig. 1). For complete characterization of individual peaks the polyene fraction was separated on a second $AgNO_3$/TLC plate. Morris has described a two-stage separation of saturated and unsaturated (mono- through hexaenes) methyl esters on the same plate (11), but in our hands his method proved less satisfactory.

The efficiency of condensation of methyl 1-$^{14}C$-linoleate in 3 mm glass tubing (95%) was similar to that of methyl stearate (7) over a 5-200 $\mu$g range. Efficiency was lowered by use of wider tubes or higher flow rates, e.g., when use of wider columns was attempted.

Individual PUFA methyl esters were subjected to oxidative degradation using $NaIO_4$/$KMnO_4$, and in some cases by ozonolytic fission with reductive workup (10) to confirm results obtained with the former. In previous studies on monoenes the use of mixed GLC phases (7% silicone DC-560 + 1% polyester EGSP-Z) permitted the resolution of mono- and dicarboxylic methyl esters (3). However oxidation of PUFA produces monocarboxylic (or aldehydic) fragments of only short chain length. Thus the difunctional (dicarboxylic esters or aldehyde-esters) fragments alone were used in structure determination and were conveniently separated on an EGSS-X column, a portion of each being collected as described. The efficiency of collection of each and the loss incurred in evaporation of pentane solutions to volumes suitable for injection were studied with a mixture of ca. 100 $\mu$g of dicarboxylic esters from $C_5$ (glutarate) through $C_{12}$. From the mass traces before and after evaporative and collection procedures it was possible to estimate the overall loss of individual members, such as $C_5$ (74%), $C_6$ (43%) and $C_7$ (25%). Longer chain compounds were recovered almost quantitatively.

The extensive information available on the separation of PUFA on polyester columns was used to predict the identity of many PUFA in this study (12,13). Further confirmation was obtained by conventional methods including information from patterns of degradation fragments.

The GLC patterns of PUFA from preputial and mouse skin were quite similar (Fig. 1) and resembled patterns recorded for other mammalian tissues (13). This similarity was somewhat surprising in view of the ability of mouse skin alone to synthesize monounsaturated fatty acids with up to 35 carbon atoms (9). However

more highly unsaturated chains of this length have not been demonstrated. PUFA belonging to the $\omega 3$, $\omega 6$ and $\omega 9$ series were observed.

Data on the occurrence and label distribution among component PUFA are given in Table I. Hydrogenation showed only traces of odd-numbered or branched chains present. On the basis of cpm per unit weight of tissue, preputial skin incorporated more label into its fatty acids, especially saturates, than did mouse skin. In terms of $\mu$g per unit weight of tissue, however, mouse skin far exceeded preputial skin in amounts of PUFA present. (Determination of mass of saturates and monoenes were not performed in this study; however, from TLC patterns, preputial skin was judged to contain about the same relative amounts of saturates, monoenes and PUFA as mouse skin.)

For both types of skin, oxidation showed that ca. 92% of the 18:2 peak was linoleic acid (18:2$\omega$6), with ca. 95% of its radioactivity associated with the derived $C_9$ fragment (azaleic acid) and the remainder with a $C_8$ fragment, which was probably an oxidation by-product. This confirmed patterns observed earlier with epidermal cells (3). Schmidt degradation of hydrogenated 18:2 (as free acid) revealed that radioactivity was located in the carboxyl carbon. In the absence of evidence to the contrary, carboxyl labeling of 18:2$\omega$6 (and 18:3$\omega$3) is usually attributed to exchange with $^{14}$C-acetate (14). A possible $C_{16}$ precursor of linoleic acid (16:2$\omega$6) was not identified in this study, although its existence is a subject of continuing research.

Linoleic and arachidonic (20:4$\omega$6) acids formed ca. 70% of skin PUFA. Of the total PUFA radioactivity, linoleic acid carried the largest share (40-50%), followed by 20:3$\omega$6 (15-21%), 20:2$\omega$6 (10-13%) and 22:4$\omega$6 (10-11%). Obviously the $\omega$6 pathway of PUFA formation predominated, although conversion of 20:3$\omega$6 to arachidonic acid (lightly labeled) was not very significant.

ACKNOWLEDGMENT

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# Cardiac Lipids in Rats and Gerbils Fed Oils Containing $C_{22}$ Fatty Acids[1]

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## ABSTRACT

Docosenoic acid from rapeseed oil or herring oil in the diet of the young rat promoted an accumulation of cardiac lipid. The triglyceride fraction accounted for most of the deposited fat and contained a high concentration of the docosenoic acid. Liquid rapeseed oil, partially hydrogenated rapeseed oil or partially hydrogenated herring oil increased the amount of cardiac fatty acids at 1 week and led to the development of degenerative lesions at 16 weeks. Whale or seal oils low in $C_{22}$ fatty acids produced little effect on the amount of lipids in the heart of rats or gerbils. The latter species receiving 20% rapeseed oil in the diet showed a peak in cardiac lipid deposition at 4 days with similar levels of total fatty acids to that of rats, but with a lower concentration of erucic acid. Oil from *Limnanthes douglasii* and hydrogenated herring oil also increased the amount of cardiac fatty acids in gerbils. A high intake of docosenoic acid was common to the animals displaying the cardiac alterations.

## INTRODUCTION

There are several reports of myocardial alteration in experimental animals fed rapeseed oil containing an appreciable quantity of erucic acid (1-5). During the first few days of receiving rapeseed oil, rats accumulated fat within the muscle fiber (3,5) and, after a prolonged period, developed necrotic lesions (1-3). The following experiments deal with the early or late cardiac changes of rats or gerbils fed sources of docosenoic acid.

## METHODS

Male weanling rats, COBS® were obtained from Charles River Breeding Labs., Mass., and weanling gerbils from the Canadian Communicable Disease Centre, Ottawa. The animals were fed a diet containing 20% w/w casein, 30% cornstarch, 20% sucrose, 1% vitamin mixture (Each kilogram of diet provided 10 mg thiamine hydrochloride, 10 mg riboflavin, 10 mg

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

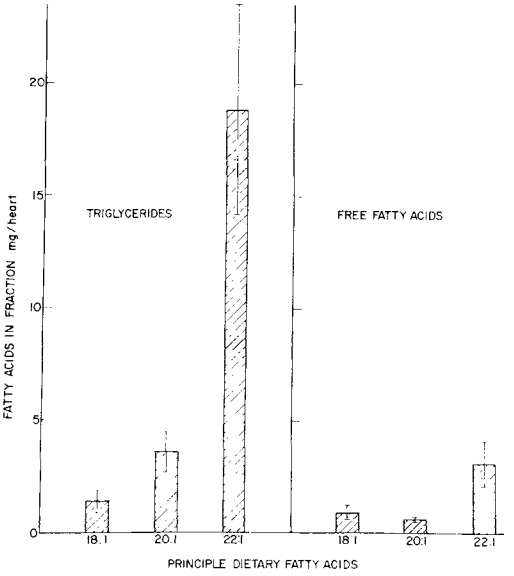


FIG. 1. Cardiac triglycerides and free fatty acids in rats fed for 1 week a synthesized oil containing 72% 18:1 or 66% 20:1 or 73% 22:1 (complete composition of dietary oils given in Reference 6).

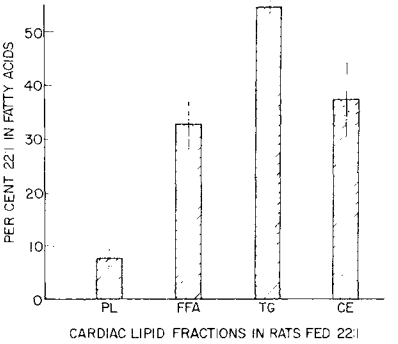


FIG. 2. Proportion of erucic acid in phospholipids (PL), free fatty acids (FFA), triglycerides (TG) and cholesterol esters (CE) of rats fed the synthesized oil containing 73% 22:1.

TABLE I

Cardiac Fatty Acids and Histopathological Scores of Rats Fed 20% Fat Diet[a]

| Dietary oil type | % | 1 Week | | | 16 Weeks | | |
|---|---|---|---|---|---|---|---|
| | | Cardiac fatty acids | | Hearts with +fat stains | Cardiac fatty acids | | Hearts with degenerative lesions[b] |
| | | mg/g | % 22:1 | | mg/g | % 22:1 | |
| LRSO[c] | 0 | 17.3 ± 3.1 (5)[d] | 0 | 0 (10) | 14.5 ± 1.2 (6) | 0 | 0 (12) |
| | 2.5 | 15.7 ± 0.8 (5) | 0.6 ± 0.3 | 0 (10) | 14.3 ± 0.8 (6) | 0.4 ± 0.1 | 0 (12) |
| | 5 | 18.1 ± 0.9 (5) | 3.2 ± 0.4 | 0 (10) | 17.3 ± 0.9 (6) | 1.1 ± 0.3 | 3 (12) |
| | 10 | 24.5 ± 2.7 (5) | 7.7 ± 1.4 | 5 (10) | 17.0 ± 1.0 (6) | 1.8 ± 0.3 | 3 (12) |
| | 15 | 40.6 ± 7.9 (5) | 18.5 ± 0.5 | 9 (10) | 18.2 ± 1.6 (6) | 2.5 ± 0.1 | 6 (12) |
| | 20 | 55.1 ± 7.4 (5) | 26.3 ± 0.5 | 10 (10) | 19.4 ± 0.4 (6) | 4.7 ± 0.3 | 9 (11) |
| HRSO[c] | 0 | 13.5 ± 0.5 (5) | 0 | 0 (10) | | | |
| | 2.5 | 15.7 ± 0.7 (5) | 1.6 ± 0.2 | 0 (10) | 13.3 ± 1.4 (6) | 0.5 ± 0.2 | 0 (12) |
| | 5 | 16.3 ± 1.2 (5) | 2.3 ± 0.2 | 1 (10) | 15.3 ± 0.4 (6) | 0.7 ± 0.1 | 1 (11) |
| | 10 | 17.9 ± 1.5 (5) | 7.7 ± 1.0 | 5 (10) | 17.1 ± 0.5 (6) | 1.7 ± 0.2 | 1 (12) |
| | 15 | 25.0 ± 3.6 (5) | 17.9 ± 2.5 | 10 (10) | 17.1 ± 0.8 (6) | 2.1 ± 0.3 | 7 (12) |
| | 10+5[e] | | | | 17.7 ± 1.2 (6) | 2.5 ± 0.4 | 6 (12) |
| HHO[c] | 0 | 14.3 ± 0.6 (4) | 0 | 0 (8) | 16.2 ± 2.3 (5) | 0 | 0 (10) |
| | 2.5 | 15.5 ± 0.3 (4) | 0.7 ± 0.2 | 0 (8) | 16.7 ± 0.8 (5) | 0.4 ± 0.1 | 3 (10) |
| | 5 | 15.5 ± 0.4 (4) | 1.9 ± 0.3 | 1 (8) | 21.0 ± 1.0 (5) | 1.0 ± 0.1 | 2 (10) |
| | 10 | 17.0 ± 0.9 (4) | 4.3 ± 0.5 | 8 (8) | 19.8 ± 2.7 (5) | 1.6 ± 0.3 | 4 (10) |
| | 15 | 21.9 ± 1.3 (4) | 9.2 ± 0.9 | 8 (8) | 19.2 ± 0.9 (5) | 2.6 ± 0.3 | 6 (10) |

[a]Containing various levels of liquid rapeseed oil (LRSO), partially hydrogenated rapeseed oil (HRSO) or partially hydrogenated herring oil (HHO) for 1 or 16 weeks.

[b]Histological staining with hematoxylin-phloxine-saffron.

[c]LRSO contained 38.1% 22:1; HRSO, 35.2% 22:1; HHO, 31.3% 22:1.

[d]Mean ± standard error of the mean. Number of animals in brackets.

[e]10% HRSO + 5% LRSO.

pyridoxine hydrochloride, 30 mg calcium pantothenate, 500 mg inositol, 50 mg niacin, 100 mg para-aminobenzoic acid, 0.2 mg biotin, 0.02 mg vitamin $B_{12}$, 2 g choline bitartrate, 1.5 mg vitamin A, 0.025 mg vitamin $D_2$, 91 mg dl-α-tocopherol, 5 mg menadione and 2 mg folic acid.), 4% U.S.P. XIV salt mixture (Basal diet contained 12.2 ppm zinc.), 5% alphacel and 20% of one of the following fats or oils: synthesized oils containing a high concentration of oleic, eicosenoic or erucic acid (6); a mixture of lard and corn oil (3:1) to which was added increasing amounts of liquid rapeseed oil, partially hydrogenated rapeseed oil (iodine value 77.9) or partially hydrogenated herring oil (iodine value 76.0); partially hydrogenated whale oil (iodine value 78.0); partially hydrogenated seal oils (iodine value 84.0 or 78.0); *Limnanthes douglasii* oil (7,8).

From each group of animals, some hearts were analyzed for fatty acids and others were examined histologically as previously described (6). Lipid classes were separated by thin layer chromatography employing glass plates coated with 0.5 mm Silica Gel G containing $H_3PO_4$ (9) ; hexane-diethyl ether 70:30 v/v for development, and a spray of 2',7'-dichlorofluorescein for detection. Lipid fractions, quickly located under UV light, were scraped into vials (Reactivials, Pierce Chemical Co.) and methylated with 2 ml 14% $BF_3$ in methanol in an oven at 110 C for 90 min (10). To each cooled vial were added 2 ml methanol and 1 ml water, and the contents were extracted with hexane in a side-arm flask (11). The fatty acids of each fraction were quantitated by the use of methyl lignocerate or methyl cerotate as an internal standard in gas liquid chromatography (6).

## RESULTS AND DISCUSSION

The sequence of early deposition and regression of fat in the heart was previously studied (6). Cardiac fat accumulation in rats fed liquid rapeseed oil reached a peak at 1 week and thereafter decreased, but at 4 weeks still exceeded that of the control rats. Partially hydrogenated rapeseed oil and, to a somewhat lesser extent, partially hydrogenated herring oil promoted an appreciable deposition of cardiac fatty acids. The proportion of $C_{22:1}$ in these fatty acids in rats fed the different sources of docosenoic acid followed the pattern of the deposition of total fatty acids. Further evidence that the intake of docosenoic acid was responsible for the observed fat deposition was obtained by feeding large amounts of erucic acid

TABLE II

Cardiac Fatty Acids and Histopathology of Rats Fed the Control Fat or Partially Hydrogenated Whale or Seal Oils for 1 Week

| Dietary oil | | Cardiac fatty acids | | |
|---|---|---|---|---|
| Type | %22:1 | mg/g | %22:1 | Hearts with + fat[a] |
| Lard-corn oil 3:1 | 0 | 17.9 ± 1.3[b] (5) | 0 | 0 (10) |
| Whale oil (78)[c] | 7.9 | 17.9 ± 0.3 (5) | 2.5 ± 0.5 | $6^{tr}$ (10)[d] |
| Seal oil (84)[c] | 5.4 | 16.2 ± 1.2 (5) | 1.9 ± 0.3 | $4^{tr}$,$6^{tr}$ (10) |
| Seal oil (78)[c] | 6.1 | 20.3 ± 1.6 (5) | 1.5 ± 0.5 | $2^{tr}$,$7^{tr}$ (10) |

[a]Histological staining with Oil Red O.

[b]Mean ± standard error of the mean. Number of rats in brackets.

[c]Iodine number.

[d]$_{tr}$ = trace.

or its positional isomer, cetoleic acid, in the form of synthesized oils (7). Again fatty hearts contained a high content of docosenoic acid derived from the diet. Any effect due to the position of the double bond (*n*-9 or *n*-11) was small compared to that of the chain length of the monoenoic acid.

A high intake of eicosenoic acid (20:1 *n*-9) elevated the triglyceride content of the rat heart, but a more striking increase in this lipid resulted from a high intake of erucic acid (Fig. 1). This docosenoic acid in the diet also increased the free fatty acids of the heart. As seen in Figure 2, the proportion of erucic acid in the fatty acids of the phospholipids was low; that of the free fatty acids and cholesterol esters was higher, and that of the triglycerides highest. Since the triglyceride fraction was dominant, it contained most of the cardiac erucic acid.

The deposition of cardiac fatty acids and the histopathological scores for the fat stains at 1 week and for the degenerative lesions at 16 weeks are shown in Table I which is a summary of results from experiments on each oil. Liquid rapeseed oil, partially hydrogenated rapeseed oil or partially hydrogenated herring oil at a level of 10% or more by weight in the diet was associated at 1 week with an increased amount of cardiac lipid. The accumulation of fatty acids appeared to be related to the concentration of docosenoic acid. At 16 weeks the deposition of cardiac fatty acids was relatively low with some incidence of degenerative lesions in the groups that at 1 week had exhibited fat accumulation. Pronounced necrosis or fibrosis occurred at about the 15% level of these test oils. Thus, under these conditions, oils containing 31-38% docosenoic acid promoted both the early fat deposition and the later degenerative lesions.

Whale oil and seal oils, hydrogenated to different iodine values, contained about half as much $C_{22}$ fatty acids as did the herring oil, and still less of the $C_{22}$ fraction was monounsaturated. The isomers of these oils have been studied by Conacher and Page (12). As seen in Table II, the total amount of cardiac fatty acids did not appear to increase with the marine oils, although trace amounts were observed histologically with the whale oil and definite fat droplets with the seal oils. Cardiac lipid deposi-

TABLE III

Cardiac Fatty Acids of Gerbils Fed 20% Liquid Rapeseed Oil (LRSO) or Partially Hydrogenated Seal Oils for 1 Week

| Dietary oil | Cardiac fatty acids | | |
|---|---|---|---|
| | mg/g | % 20:1 | % 22:1 |
| LRSO | 54.6 ± 4.8[a] (6) | 11.6 ± 0.6 | 8.6 ± 0.7 |
| Seal (84)[b] | 24.6 ± 2.2 (6) | 4.0 ± 0.3 | 0.5 ± 0.3 |
| Seal (78)[b] | 24.5 ± 2.3 (6) | 4.3 ± 0.6 | 0.7 ± 0.3 |

[a]Mean ± standard error of the mean. Number of gerbils in brackets.

[b]Iodine number.

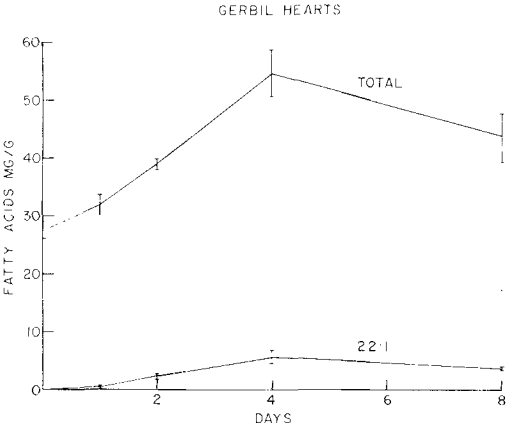


FIG. 3. Deposition of cardiac fatty acid and erucic acid in gerbils fed 20% liquid rapeseed oil for 0, 1, 2, 4 and 8 days.

TABLE IV

Cardiac Fatty Acids in Gerbils Fed 20% Control Fat, *Limnanthes douglasii* Oil or Partially Hydrogenated Herring Oil (HHO) for 4 Days

| Dietary oil | Cardiac fatty acids | | | |
|---|---|---|---|---|
| | mg/g | % 22:1 | % 22:1 | % 22:2 |
| Lard-corn oil 3:1 | 26.8 ± 1.4[a] (4) | 0.2 ± 0.1 | 0 | 0 |
| *L. douglasii*[b] | 78.8 ± 8.9 (4) | 37.8 ± 2.3 | 9.6 ± 0.6 | 8.7 ± 0.5 |
| HHO (78)[c] | 65.1 ± 8.7 (4) | 18.2 ± 0.6 | 12.0 ± 0.3 | 0 |

[a]Mean ± standard error of mean.

[b]Oil contained 61.3% 20:1, 15.4% 22:1, 10.4% 22:2.

[c]Iodine number.

tion was unaffected by a change in the degree of hydrogenation of the seal oil.

In Table III is shown a comparison of the effects on gerbil hearts of 20% liquid rapeseed oil and 20% hydrogenated seal oils fed for 1 week. As observed in the rats, the cardiac fatty acids were high with rapeseed oil but not with the marine oils which contained substantially less docosenoic acids. The proportion of erucic acid in the total cardiac fatty acids was lower in the gerbils than in the rats under similar dietary conditions. Also the gerbils differed from the rats in accumulating relatively large amounts of eicosenoic acid relative to docosenoic acid from a diet rich in the latter. Even so, in both species fed rapeseed oil, there was a build-up of fatty acids in the heart.

Since the gerbils consumed about a third as much food as did the rats of the same age, they appeared advantageous for testing sources of long chain fatty acids in short supply. In addition, the peak accumulation of cardiac lipid occurred at 4 days (Fig. 3). This time interval was used to examine the cardiac fatty acid of gerbils fed oil from *L. douglasii*. As shown in Table IV, both the vegetable oil containing 61% eicosenoic, 15% docosenoic and 10% docosadienoic acids, and the partially hydrogenated herring oil increased the cardiac fatty acids. The high eicosenoic acid from the *L. douglasii* oil was reflected in the fatty acid composition of the cardiac lipids, but, according to previous results (6), rats fed synthesized oils high in this fatty acid exhibited little increase in total cardiac fat compared to that produced by docosenoic acid. The docosadienoic acid (mostly $\Delta^{5,13}$) from the diet appeared in the gerbil hearts. Also $C_{22}$ polyenoic acids which remained after the partial hydrogenation of the whale and seal oils were detected in trace amounts in the hearts of the rats and gerbils studied. Whether these polyenoic acids without $C_{22}$ moneoic acid would increase cardiac fat deposition is still unknown.

Species differences in response to rapeseed oil have been observed previously. Ducklings developed a hydropericardium (4). Miniature pigs and regular baby pigs deposited little docosenoic acid in the heart but did exhibit myocardial fat droplets (13). Unlike rats, squirrel monkeys fed rapeseed oil increased the amount of docosenoic acid in the heart between 1 and 10 weeks (13). So far no species given a high intake of docosenoic acid has been found to be free from cardiac alteration.

The investigations with rapeseed oil, and especially those with erucic acid, are relatively recent compared to those with marine oil. In 1930, Agduhr and Stenström (14-16) described heart lesions and abnormal electrocardiograms of pigs, calves, dogs, rabbits and rats fed cod liver oil. Then 3 years later Madsen et al. (17) reported heart failure in herbivores given cod liver oil and demonstrated that the unsaponifiable fraction was not responsible. From recent work it is evident that docosenoic acid can promote an early deposition of cardiac fatty acids in the form of triglycerides. The occurrence and etiology of the later degenerative lesions in the heart of rats fed processed rapeseed or herring oils requires further investigation.

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# SHORT COMMUNICATIONS

## Azasteroids: Potent Inhibitors of Insect Molting and Metamorphosis

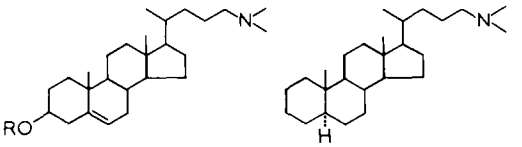

### ABSTRACT

Based on previous structure-activity studies, four new azasteroids were synthesized and tested in several species of insects. These compounds, which are the most potent azasteroid inhibitors of insect growth and development tested to date, affect the hormone-regulated processes of molting and metamorphosis.

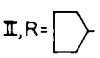

Since our initial report that 22,25-diazacholesterol and triparanol (MER-29) inhibit the $\Delta^{24}$-sterol reductase enzyme system and also disrupt normal growth and development of larvae of the tobacco hornworm, *Manduca sexta* (Linnaeus) (1), we have carried out studies on the structure-activity relationship of more than 20 azasteroids (2). The information obtained from testing these compounds has permitted us to design and synthesize a number of new azasteroids with considerably enhanced activity, certain of which are approximately a thousand times more inhibitory in tobacco hornworm larvae than the 22,25-diazacholesterol originally used in our studies. We now wish to present data obtained from tests with four of these new azasteroids and discuss the significance of these findings. We have extended our research to include comparative studies with several species of insects and have also obtained experimental results that suggest that these steroids may exert their effects by interfering with the endogenous biosynthesis or metabolism of the insect steroid molting hormones the ecdysones.

Larvae of the tobacco hornworm and fall armyworm, *Spodoptera frugiperda* (J.E. Smith), were reared on an artificial diet as previously described (3). β-Sitosterol, the sole added dietary sterol, was coated on the dry components to achieve a concentration of 0.026% wet wt (0.2% dry wt) in the larval diet. German cockroach nymphs, *Blattella germanica* (Linnaeus), were reared on diets (4) containing 0.2% sterol (dry wt) for a test period of 6 weeks. Azasteroids (as the free base) were coated on the diets at appropriate concentrations in the manner used for coating the dietary β-sitosterol. The larval test systems for the yellow fever mosquito, *Aedes aegypti* (Linnaeus), the confused flour beetle, *Tribolium confusum* Jacquelin duVal, and the house fly, *Musca domestica* Linnaeus, were those previously used to assess the inhibitive effects of ecdysones and synthetic analogs on growth and metamorphosis (5).

In the previous studies on the relationship of structure to biological activity, 25-azacholesterol (3β-hydroxy-chol-5-en-24-dimethylamine, I, Fig. 1) was the most potent azasteroid inhibitor tested (2). Using this compound as a point of departure, a number of new 25-azasteroids have been synthesized and tested and four of these, shown in Figure 1, were found to be potent inhibitors of insect development, molting and metamorphosis: 25-azacholesteryl cyclopentyl ether (3β-cyclopentoxy-chol-5-en-24-dimethylamine, II), 25-azacholesteryl methyl ether (3β-methoxy-chol-5-en-24-dimethylamine, III), 25-azacholestane (5α-cholan-24-dimethylamine, IV), and 25-azacoprostane (5β-cholan-24-dimethylamine, V). The cyclopentyl ether and the methyl ether intermediates were prepared by heating the 3β-tosylate of chol-5-en-24-oic acid methyl ester with excess cyclopentanol at 80-85 C for 4 hr and with methanol at reflux temperature for 4 hr, respectively. The esters were then saponified with 5% methanolic-KOH for 3 hr, and upon acidification yielded the cyclopentyl and methyl ethers of chol-5-en-24-oic acid. The 5β-cholanic acid was readily prepared via oxidation




I, R=H

II, R= 

III, R=$CH_3$

IV

V, 5β-H

FIG. 1. Azasteroid structures.

TABLE I

Physical Properties of the New Azasteroids

| Compound | Melting point, C | NMR[a] Methyl resonances, δ 18-H | 19-H | 21-H | | 26- and 27-H | Methoxy | Olefinic proton | Molecular ion[b] |
|---|---|---|---|---|---|---|---|---|---|
| II | 133-134 | 0.684 | 1.00 | 0.891 , | 0.965 | 2.22 | | 5.37 | 455 |
| III | 71-73 | 0.684 | 1.01 | 0.891 , | 0.965 | 2.22 | 3.35 | 5.37 | 401 |
| IV | 86-87 | 0.645 | 0.775 | 0.875 , | 0.967 | 2.21 | | | 373 |
| V | 61-63 | 0.657 | 0.935 | 0.875 , | 0.967 | 2.22 | | | 373 |

[a]Recorded at 60 Mc with a Varian A-60A spectrometer; solvent-deuterated chloroform.

[b]Obtained with an LKB model 9000 mass spectrometer, and sample was introduced directly into the ionization chamber.

of cholic acid methyl ester to the triketone, dehydrocholic acid methyl ester, and its subsequent reduction and saponification by the Wolff-Kishner reaction. The 5α-cholanic acid was prepared by the lithium aluminum hydride reduction of the 3β-tosylate of 5α-cholanic acid methyl ester, followed by oxidation of the resultant 5α-cholan-24-ol to the 5α-cholanic acid. The new azasteroids were all prepared according to the general method of synthesis via reaction of the appropriate 24-carbon steroidal acid with thionyl chloride to give the steroidal acid chloride, and its reaction with dimethylamine to give the amide and its subsequent reduction to the amine (6). The structures of intermediates and final products were confirmed by IR, NMR and mass spectroscopy and the purity of the final products as determined by gas liquid chromatography (GLC) and thin layer chromatography (TLC) was $>99\%$. Certain of the physcial properties of the azasteroids are listed in Table I. Mass spectra of all four azasteroids had a base peak at m/e 58, indicative of cleavage of the 23-24 bond; the $M^+$ peak was the next largest peak and the M-15 was also a prominant peak in all spectra.

Since the 3β-cyclopentyl ether of 20,25-diazacholesterol had previously been shown to be approximately five times more active than the parent diazasterol (2), the 25-azacholesteryl cyclopentyl ether (II) was the first compound prepared and tested. This azasteroid was equal to compound I in its inhibitory activity in the fall armyworm, but was a more potent inhibitor than I in all the other test systems (Table II), including the German cockroach. The 3β-methoxy derivative of I, 25-azacholesteryl methyl ether (III), was as active or more active than compound II in all the test insects; and in three species, the fall armyworm, confused flour beetle, and house fly, it was the most active inhibitor of the four new azasteroids. In the house fly the range of concentrations of III required to cause 75% inhibition or mortality was 350-400 ppm.

The structurally more simple 25-azacholestane (IV), which lacks a C-5 double bond and an oxygen function at C-3, also proved to be a very active inhibitor. Although this compound was only about one-half as active as III in the hornworm, the fall armyworm and the confused flour beetle, it was equally active in the mosquito larva and was the most active of the azasteroid inhibitors for the German cockroach (Table II). The high activity observed for compound IV, with a hydrocarbon steroid nucleus and the 5α-hydrogen (A/B ring *trans*), prompted us to synthesize and test its 5β-isomer, 25-azacoprostane (V), which has the A/B ring *cis* configuration characteristic of the insect molting hormones (ecdysones). This modification resulted in a compound that was less active than IV in the German cockroach and flour beetle, about equally active in the fall armyworm and the mosquito larvae, but which was the most active of the four new azasteroids in the tobacco hornworm (Table II). Based on its inhibitory effects on larval development, molting and metamorphosis in the hornworm, compound V is 16 times more active than I and ca. 1000 times more active than the 22,25-diazacholesterol that was used in our initial tests with this insect.

GLC analysis of sterols isolated from inhibited insects indicated that all of the new azasteroids are potent $\Delta^{24}$-sterol reductase inhibitors that block the conversion of $C_{28}$ and $C_{29}$ plant sterols to cholesterol in the insects and bring about an accumulation of desmosterol and unchanged dietary sterol as previously found with other azasteroids (1,2,9). Four of the test insects—the hornworm, armyworm, cockroach, and flour beetle—must derive their essential cholesterol through the conver-

TABLE II

Range of Concentrations of 25-Azasteroids in the Larval Diet or Medium Required to Kill or Inhibit Development in 75% of the Test Insects

| | Compound | | | | |
|---|---|---|---|---|---|
| | I | II | III | IV | V |
| Insect | Concentration, ppm | | | | |
| Tobacco hornworm | 2.00-4.00 | 0.75-1.00 | 0.25-0.50 | 0.50-0.75 | 0.10-0.25 |
| Fall armyworm | 4.00-8.00 | 4.00-8.00 | 0.75-1.00 | 1.00-2.00 | 1.00-2.00 |
| German cockroach | >1,000 | >1,000 | >1,000 | 250-500 | >1,000 |
| Confused flour beetle | >1,000 | 100-250 | 50-100 | 250-500 | >1,000 |
| Yellow fever mosquito | 2.50-5.00 | 0.50-1.00 | 0.50-1.00 | 0.50-1.00 | 0.50-1.00 |

sion of the plant sterols present in the test diets to cholesterol, but we have previously shown that a severe reduction in cholesterol production is not in itself sufficient to disrupt larval development (2). This indicates that these azasteroid inhibitors interfere with steroid metabolic pathways other than those involved in cholesterol production—possibly those pathways related to molting hormone biosynthesis or metabolism. This is substantiated by our results from tests with the new azasteroids with the yellow fever mosquito and house fly larvae since the test diets of these two insects contain sufficient cholesterol to support normal growth and development. (The house fly larval diet contains 0.2% (dry wt) cholesterol. Cholesterol comprises 22% of the total sterols present in the pulverized dog food that is used as the yellow fever mosquito larval diet, and 62.4% of the total sterols from fourth stage mosquito larvae is cholesterol.) In the latter insect the inhibitive effect of the azasteroids could in no way be due to interference with the conversion of plant sterols to cholesterol, since the house fly lacks this biochemical mechanism (7,8).

Further evidence for the above premise was the characteristic inhibitive effects observed for the azasteroids with each species. As had previously been found for the hornworm (2), these effects involved the processes of molting and metamorphosis (Table III), and higher concentrations usually resulted in inhibition or mortality during earlier stages of development.

In an attempt to determine whether or not the azasteroids do interfere with the pathways of ecdysone biosynthesis and metabolism, various concentrations of compound III were added in combination with the inhibitory ecdysone analog, 22,25-bisdeoxyecdysone (5) to the larval diets of *Tribolium* and the house fly. In *Tribolium* larvae, the ecdysone analog slightly enhanced the activity of the azasteroid, suggesting that an interaction may occur. However the response of house fly larvae was quite different; here a concentration (150 ppm) of compound III that is inactive gives a near complete reversal of the effect of the ecdysone analog at a concentration (75 ppm) that inhibits development to the adult in 96% of the test insects (5). Thus in the house fly this azasteroid severely decreases the inhibitive activity of the ecdysone analog—perhaps by blocking its conversion to a more active compound(s). Additional evidence for such an interaction was obtained by the joint administration in the *Tribolium* larval diet of α-ecdysone and azasteroid III. When α-ecdysone is added to the diet at a 5:1 or 10:1 ratio with compound III at 100 ppm, which is sufficient azasteroid to cause complete mortality or inhibition of development, this insect ecdysone brings about approximately a 50% reversal of the inhibitive effect.

TABLE III

Characteristic Effects of Minimum Inhibitive Concentration of the Azasteroid Inhibitors in the Larval Diet or Medium

| Insects | Susceptible stages | Effects |
|---|---|---|
| Tobacco hornworm | 4th Instar | Precocious prepupa formation-abnormal "4th instar prepupa" |
| Yellow fever mosquito | lst and 2nd Instars | Blocks molting, no further development |
| Confused flour beetle | Last stage larva | Blocks larval to pupal molt |
| House fly | Puparium and pupa | Blocks pupation or adult emergence |

Although the above cited results strongly suggest that an interaction does occur between certain of the azasteroids and ecdysteroids, proof for this must await detailed biochemical studies. If our previous experience with the azasteroids as experimental tools for charting the pathways of conversion (dealkylation) of $C_{28}$ and $C_{29}$ plant sterols to cholesterol in insects can be taken as an indicator (9,10), then these compounds could serve us well in studies on the biosynthesis and metabolism of the ecdysones. Our results with the azasteroid inhibitors, however, also conclusively demonstrate the feasibility of disrupting the hormone-mediated processes of insects with simple nonhormonal compounds—possibly by interfering with hormone biosynthesis and metabolism.

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# The Effect of Two Isomeric Octadecenoic Acids on Alkyl Diacyl Glycerides and Neutral Glycosphingolipids of Novikoff Hepatoma Cells

**ABSTRACT**

The addition of 150 μg of *cis*-6-octadecenoic acid (6-18:1) per milliliter to the growth medium of Novikoff hepatoma cells reduced the rate of multiplication of these cells, whereas the same level of *cis*-9-octadecenoic acid (9-18:1) was without effect. The quantity of alkyl diacyl glycerols in cells grown in medium containing 100 μg/ml of either 6-18:1 or 9-18:1 was reduced to about 10% of that observed in cells grown in unsupplemented media. The presence of 6-18:1 acid led to an increase in the concentration of the dihexosyl ceramide and a corresponding decrease in that of the monohexosyl ceramides of the cells; whereas the presence of 9-18:1 resulted

TABLE I

Concentration and Fatty Alkyl Composition of Alkyl Diacyl Glycerides (ADG) of Novikoff Hepatoma Cells

| Fatty alkyl | Cells grown in: Control medium | Medium + 6-18:1 | Medium + 9-18:1 |
|---|---|---|---|
| 16:0 | 30.1[a] | 35.3 | 22.8 |
| 16:1 | 4.0 | 5.1 | 4.0 |
| 18:0 | 58.9 | 16.4 | 13.5 |
| 18:1 | 7.0 | 43.2 | 59.7 |
| ADG | 8.4[b] | 0.8 | 1.1 |

[a]Percentage by weight.

[b]Percentage by weight of total neutral lipids.

TABLE II

Percentage Composition of Fatty Acids of Alkyl Diacyl Glycerides from Novikoff Hepatoma Cells

| Fatty acid | Cells grown in: Control medium | Medium + 6-18:1 | Medium + 9-18:1 |
|---|---|---|---|
| 14:0 | 6.9[a] | 4.9 | 3.6 |
| 16:0 | 36.4 | 24.2 | 26.4 |
| 16:1 | 4.4 | 4.2 | 3.2 |
| 18:0 | 18.9 | 13.2 | 10.3 |
| 18:1 | 30.2 | 53.4 | 56.0 |
| 18:2 | 2.8 | Trace | Trace |

[a]Percentage by weight.

Although the above cited results strongly suggest that an interaction does occur between certain of the azasteroids and ecdysteroids, proof for this must await detailed biochemical studies. If our previous experience with the azasteroids as experimental tools for charting the pathways of conversion (dealkylation) of $C_{28}$ and $C_{29}$ plant sterols to cholesterol in insects can be taken as an indicator (9,10), then these compounds could serve us well in studies on the biosynthesis and metabolism of the ecdysones. Our results with the azasteroid inhibitors, however, also conclusively demonstrate the feasibility of disrupting the hormone-mediated processes of insects with simple nonhormonal compounds—possibly by interfering with hormone biosynthesis and metabolism.

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|---|---|---|---|
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| 16:1 | 4.0 | 5.1 | 4.0 |
| 18:0 | 58.9 | 16.4 | 13.5 |
| 18:1 | 7.0 | 43.2 | 59.7 |
| ADG | 8.4[b] | 0.8 | 1.1 |

[a]Percentage by weight.

[b]Percentage by weight of total neutral lipids.

TABLE II

Percentage Composition of Fatty Acids of Alkyl Diacyl Glycerides from Novikoff Hepatoma Cells

| Fatty acid | Cells grown in: Control medium | Medium + 6-18:1 | Medium + 9-18:1 |
|---|---|---|---|
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| 16:0 | 36.4 | 24.2 | 26.4 |
| 16:1 | 4.4 | 4.2 | 3.2 |
| 18:0 | 18.9 | 13.2 | 10.3 |
| 18:1 | 30.2 | 53.4 | 56.0 |
| 18:2 | 2.8 | Trace | Trace |

[a]Percentage by weight.

TABLE III

Fatty Acid Composition of Triglycerides of Novikoff Hepatoma Cells

| Fatty acid | Cells grown in: Control medium | Medium + 6-18:1 | Medium + 9-18:1 |
|---|---|---|---|
| 14:0 | 4.0[a] | 2.5 | 1.9 |
| 16:0 | 17.0 | 10.3 | 8.6 |
| 16:1 | 3.3 | 5.2 | 2.2 |
| 18:0 | 25.6 | 6.2 | 6.8 |
| 18:1 | 37.5 | 64.5 | 75.8 |
| 18:2 | 5.6 | 2.4 | 2.3 |
| 18:3 | 2.7 | Trace | Trace |
| 20:0 | 1.0 | Trace | Trace |
| 20:1 | Trace | 5.6 | 1.8 |

[a]Percentage by weight of major fatty acids.

TABLE IV

Percentage Composition of Neutral Glycolipids of Novikoff Hepatoma Cells

| Glycolipid | Cells grown in: Control medium | Medium + 6-18:1 | Medium + 9-18:1 |
|---|---|---|---|
| Monohexosyl ceramides | 24.5 | 15.3 | 18.6 |
| Dihexosyl ceramides | 25.5 | 30.6 | 18.6 |
| Tetraglycosyl ceramides | 50.0 | 54.0 | 62.7 |

in an increase in the relative amount of tetraglycosyl ceramide and corresponding reductions in those of mono- and dihexosyl ceramide. Possible causes for these changes are discussed.

Differential rates of growth of the bacterium, *Leptospira* serotype *patoc*, can be obtained by alteration of the double bond position of octadecenoic acid of the growth medium (1). Makino and Jenkin (2) have shown that the growth of Japanese Encephalitis Virus (JEV) in baby hamster kidney cells is inhibited by *cis*-6-octadecenoic acid (6-18:1), but the host cell is unaffected by the presence of this acid in the growth medium. Novikoff hepatoma cells which had been infected with JEV were grown in medium containing *cis*-6-18:1 or *cis*-9-18:1 acid. It was found that the presence of 6-18:1, but not 9-18:1, reduced both the rate of growth of cells as well as the number of infectious viral particles being released into the medium (unpublished data). The maximum numbers of cells and viruses observed in the medium containing 6-18:1 were ca. 50% and 1%, respectively, of those in the medium containing 9-18:1. This observation suggests that the 6-18:1 isomer was slowing down the metabolic rate of the cells more than the rate of multiplication. Therefore the effects of the presence of these two isomers on the growth rate and on certain lipid components of Novikoff hepatoma cells were further investigated.

## MATERIALS AND METHODS

Novikoff hepatoma cells (N1S1-67) were obtained from P. Plagemann, University of Minnesota. The growth medium was Swims 67G (Grand Island Biological Co., Grand Island, N.Y.). The 6-18:1 and 9-18:1 acids were obtained from the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn. The alkyl diacyl glycerol and alkyl glycerol ether standards were a gift from W.J. Baumann of

TABLE V

Fatty Acid Composition of Di- and Tetraglycosyl Ceramides of Novikoff Hepatoma Cells

| Fatty Acid | Cells grown in: Control medium Di | Control medium Tetra | Medium + 6-18:1 Di | Medium + 6-18:1 Tetra | Medium + 9-18:1 Di | Medium + 9-18:1 Tetra |
|---|---|---|---|---|---|---|
| 14:0 | 7.3[a] | 2.0 | 6.8 | 1.5 | 8.1 | 2.4 |
| 16:0 | 23.2 | 9.0 | 22.1 | 9.5 | 21.5 | 11.4 |
| 16:1 | 9.6 | 2.4 | 9.4 | 2.2 | 2.3 | 3.8 |
| 18:0 | 9.2 | 6.3 | 10.2 | 6.9 | 11.2 | 7.4 |
| 18:1 | 14.7 | 7.9 | 18.6 | 10.3 | 15.0 | 9.4 |
| 20:0 | 2.1 | 3.8 | 0.3 | 4.3 | 1.3 | 4.6 |
| 20:1 | 4.4 | 0.9 | 8.2 | 8.4 | 0.1 | 0.9 |
| 22:0 | 3.2 | 11.2 | 2.0 | 15.8 | 4.4 | 9.0 |
| 22:1 | 0.2 | 1.1 | 0.2 | 5.8 | 0.2 | 0.1 |
| 24:0 | 14.9 | 36.9 | 7.2 | 17.7 | 11.0 | 19.0 |
| 24:1 | 0.3 | 9.2 | 0.1 | 6.3 | 9.3 | 23.7 |

[a]Percentage by weight of major fatty acids.

The Hormel Institute.

The sodium salts of the fatty acids were prepared and sterilized by autoclaving the acids with a 10% (molar) excess of 0.1 N NaOH at 121 C for 15 min. The sodium salts of the acids were dispersed in the medium using 2 mg/ml of bovine albumin as carrier. (Fraction V, fatty acid-free, Pentax Biochemicals, Kankakee, Ill.). Incubations were carried out in screw-cap Erlenmeyer flasks at 37 C in a water bath shaker.

Cell numbers were determined with the aid of a haemocytomer (3). Cells were harvested by centrifugation and the lipids isolated by the method of Bligh and Dyer (4). Further separations and characterizations of the lipids were performed by the standard methods of column (5), thin layer (6,7) and gas liquid chromatography (8).

Cells were grown in the presence of either 6-18:1 or 9-18:1 from 0-150 μg/ml medium. Cells for lipid analysis were harvested from flasks containing either 0 or 100 μg/ml of 6-18:1, or 9-18:1 after 48 hr incubation.

## RESULTS AND DISCUSSION

When 6-18:1 was included in the culture medium in amounts of up to 100 μg/ml, or 9-18:1 in amounts of up to 150 μg/ml, there were no large differences in the rate of multiplication of the cells. Maximum numbers of cells were reached at 60 hr. However, when the medium contained 150 μg/ml of 6-18:1, maximum numbers of cells were reached at 96 hr, and even then the total numbers of cells found were only 30% of that observed at 60 hr in the unsupplemented medium.

The values presented in Table I show that when the cells were grown in the control medium, the alkyl diacyl glyceride (ADG) fraction made up 8.4% of the neutral lipids. High levels of this fraction have been found in a number of cancer cells and tissues (9). However, when either the 6- or 9-18:1 isomer was added to the growth medium, ADG became a minor component (see Table I). It is accepted now that long chain fatty alcohols are efficiently converted to the alkyl chains of glycerol ethers (10) and that fatty acids can be reduced to the corresponding alcohols (11). A possible explanation for the observed reduction of the amount of ADG in the cells when 18:1 was added to the growth medium can be arrived at by comparing the fatty acid composition of either the ADG or TG (Tables II and III) with the fatty alkyl composition of the ADG (Table I). From the values given in these tables, it can be seen that the fatty alkyl chains are less unsaturated than the fatty acyl chains. This appears to be due to the fact that saturated fatty acids are more readily reduced to alcohols than are unsaturated acids (12). Thus an increased supply of unsaturated fatty acids (as when 18:1 was added to the medium) would lead to a slower rate of alcohol production and would therefore reduce the total amount of ADG being synthesized.

The data given in Table IV show that when the cells were grown in the presence of 6-18:1, the concentration of the dihexosyl ceramide was slightly increased and that of the monohexosyl ceramide fraction was slightly decreased; whereas the presence of 9-18:1 in the medium resulted in an increased amount of tetraglycosyl ceramide at the expense of the other two fractions.

Since the fatty acid compositions of the mono- and dihexosyl ceramide fractions were essentially the same, only the compositions of the latter, as well as that of the tetraglycosyl ceramides, are given in Table V. Comparison of the fatty acid composition of the glycosphingolipids (Table V) shows that when 6-18:1 was present in the medium, there was an increase in the amount of 20:1, whereas in the presence of 9-18:1, there was an increase in 24:1. The results presented in Table V also show that the longest chain fatty acids are associated with those glycolipids which have the greater number of hexose moieties. Thus it is to be expected that addition of the 9-18:1 would tend to enhance the levels of the tetraglycosyl ceramides, whereas 6-18:1 would favor production of those glycosyl ceramides with fewer hexose moieties. Hakomori and Murakami (13) have shown that in certain virally transformed cells, there is an increase in the concentration of lactosyl ceramides at the expense of sialolactosyl ceramides. The results presented in Table IV suggest that the effects observed by Hakomori et al. (13) can be reversed.

Thus, by the addition of octadecenoic acids to the growth medium of Novikoff hepatoma cells, it is possible to alter three characteristics of this tumor cell: (a) slow down its growth rate; (b) reduce the amount of ADG to normal levels; (c) increase those neutral glycosyl ceramides which have the greatest number of hexose residues and thereby possibly change the immunological characteristics of the cells.

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[Received June 9, 1972]

# A Comparison of the Absorption of Oleic Acid in Vitro as Measured Isotopically and by Titration

**ABSTRACT**

Uptake of oleic acid by the everted sac of rat intestine has been shown to be net absorption and not merely exchange. Sodium fluoride, 10 mM, has been shown to inhibit absorption of oleic acid.

The everted sac of small intestine has been used extensively in the study of the luminal events of fatty acid absorption (1), and absorption has usually been measured isotopically. These studies were undertaken to test the validity of isotopic measurements of absorption, since by exchange the tissue could conceivably accumulate isotope without net absorption. During the study another possible artifact was observed. It appeared that monoglyceride was hydrolyzed intracellularly, and thus contributed to the chemically measured fatty acid absorption.

Sacs weighing ca. 500 mg were prepared as previously described (2) with the exception that tissue was weighed prior to sac preparation. The oxygenated incubation media were also prepared as previously described with pure bile salts, sodium taurocholate, sodium taurodeoxycholate in a 4:1 molar ratio to give a final concentration of 10 mM. The lipid was 1 mM 1-$^{14}$C-oleic acid with 1 mM 1-mono-olein. Incubation was for 15, 30 or 60 min at 35 C and pH 6.4. After incubation the sacs were washed in saline, the mucosa scraped and the lipid extracted in chloroform methanol 2:1 v/v. One portion was used for titration of fatty acid, another for isotope counting, and a third for thin layer chromatography.

For titration 1 ml distilled water was added to 5 ml of the lipid extract. This was shaken, then centrifuged. The upper phase was discarded and replaced with 2 ml water-methanol 1:1 v/v which was again shaken, centrifuged, and the upper phase discarded. The lower phase was taken to dryness under a stream of $N_2$; then 2 ml of 0.002% bromthymol blue in ethanol was added. This was titrated to a blue end point with NaOH 0.02 N. Thin layer chromatography was performed on a 0.25 mm layer of Silica Gel G in the solvent system hexane-diethyl ether-glacial acetic acid 80:20:2 v/v/v. The lipid was eluted in three fractions by the method of Goldrick and Hirsch (3) with the solvent, chloroform-methanol 2:1 v/v. The fractions comprised (a) phospholipids, monoglyceride, diglyceride and cholesterol; (b) free fatty acids; and (c) triglyceride. Liquid scintillation counting was performed with a Nuclear Chicago Mark I counter with 10 ml of the scintillant-PPO, 4 gm PoPoP, 0.05 gm/liter of toluene. Quench correction was by the channels ratio technique (4). Saponification of the lipid extract prior to estimation of fatty acid produced very erratic results which were presumed to be due to the unwanted inclusion of small

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TABLE I

Free Fatty Acid Increment in Tissue Following Incubation[a]

| Time, min | Standard incubation | | Incubation with NaF, 10 mM | |
|---|---|---|---|---|
| | Isotopic | Titration | Isotopic | Titration |
| 15 | 0.3 | 0.9 | 0.9 | 1.0 |
| 30 | 0.7 | 1.4 | 1.4 | 1.2 |
| 60 | 1.2 | 1.7 | 2.2 | 2.4 |

[a]The incubation media comprised 1-$^{14}$C-oleic acid 1 mM, monoolein 1 mM in a bile salt mixture sodium taurocholate, sodium taurodeoxycholate (4:1 on molar basis) to give a total concentration of 10 mM, pH 6.4 and an incubation temperature of 35 C. NaF, 10 mM, was added to media as indicated. The free fatty acid increment as calculated is indicated in text. The units of measurement are μmoles of free fatty acid per gram of tissue. Each point is calculated from the mean of duplicate sacs for each condition for four rats.

amounts of mesenteric fat. Only the free fatty acid content of the mucosa could be estimated reliably. Since this increment was small, NaF was added to some incubation media to inhibit esterification in order to increase that increment and thereby facilitate measurement. Thus each experiment consisted of two pieces of tissue which were not incubated—two sacs incubated without NaF and two incubated with NaF. The unincubated pieces of tissue were the most proximal and most distal portions of the section of intestine used. The mucosa was scraped off and its lipid content extracted, serving as the control lipid content for the mucosa of that rat. The isotopic uptake of free fatty acid could be estimated from the total isotopic uptake of oleic acid, since the percentage of this which remained as free fatty acid had been estimated by thin layer chromatography. The experiment was designed to allow analysis by the statistical test of Student's *t* value for paired data (5). Duplicate titration values agreed to within 10% and usually within 5%. The coefficient of variation was 3.6%.

The fatty acid content of both test tissues was shown to be significantly greater than control tissues ($p<0.05$) for all incubation tissue. Thus the mean mass uptake of free fatty acid could be calculated by subtraction of the mean control value from each of the test values at each time. The mass and isotopic free fatty acid increments due to incubation are shown in Table I. It can be seen that the mass and isotopic values agree well at all times for the tissue incubated in the presence of NaF. The agreement is not good after incubation in absence of NaF.

It has been shown (6) that NaF at a concentration of 1 mM will inhibit intracellular

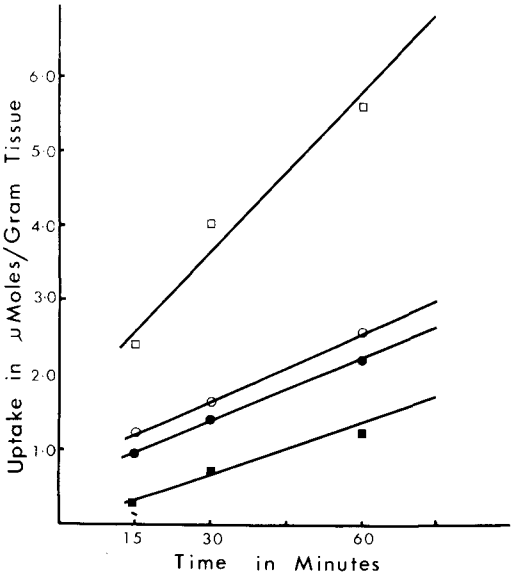


FIG. 1. The effect of NaF on oleic acid uptake and esterification by sacs. The incubation conditions are as described in Table I. Total uptake in μmoles/g wet tissue: □ control sacs; ○ NaF, 10 mM. Free oleic acid, μmoles/g wet tissue: ■ control sacs; ● NaF, 10 mM.

monoglyceride lipase. Thus in the absence of NaF, 1-mono-olein could be hydrolyzed, increasing the apparent mass increment of free fatty acid in the sac due to incubation, but not the isotopic increment. Therefore sacs were incubated in a medium containing oleic acid as the sole micellar solute, and there was reasonable agreement between mass and isotopic measures of free fatty acid increment for incubation with or without NaF. An increment of 0.4 μmoles of fatty acid was estimated isotopically and 0.3 μmoles/g tissue by titration for standard incubation, and 1.5 and 2.0 μmoles/g tissue, respectively, in the presence of fluoride.

These experiments suggest that isotopic uptake measures net absorption of lipid by the everted sac and not merely isotopic exchange. Further, in Figure 1, in contrast to previous studies (7,8) it can be seen that a metabolic inhibitor inhibited total oleic acid uptake by sacs.

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# Ceramide, Triglyceride, and Sterol Ester in Normal Human Whole Brain at Different Ages

**ABSTRACT**

Separation of ceramides from other lipids by two dimensional thin layer chromatography, characterization of ceramide in human brain lipid extracts, and the levels of ceramide, triglyceride, and sterol ester in human brain at different ages (25 week fetus and postnatal ages of 1 day, 3 weeks, 6, 8 and 22 months, and 6, 8.5, 10, 18, 33, 55 and 98 years) are described.

Ceramide, triglyceride and sterol ester are minor components of brain (1). In this report are presented procedures for separation, identification and determination of ceramide in normal human brain and quantitative values for the ceramide, triglyceride and sterol ester levels at different ages.

Brain samples were obtained as soon as possible after death (never more than 18 hr). The whole brain (including cerebellum and brain stem) was cut in half longitudinally, ground to a uniform paste in a meat grinder, and a sample removed for extraction. Samples were extracted and nonlipid contaminants removed by column chromatography on Sephadex as previously described (2,3).

Ceramides are readily separated from other lipid classes by two dimensional thin layer chromatography (TLC). That ceramides containing hydroxy and normal fatty acids are widely separated (Fig. 1) was established with authentic preparations (Supelco Inc., Bellefonte, Pa.). A spot for ceramide with normal fatty acid was readily detected by two dimensional TLC after application of 1 mg total lipid. No spot was observed in the position for ceramide

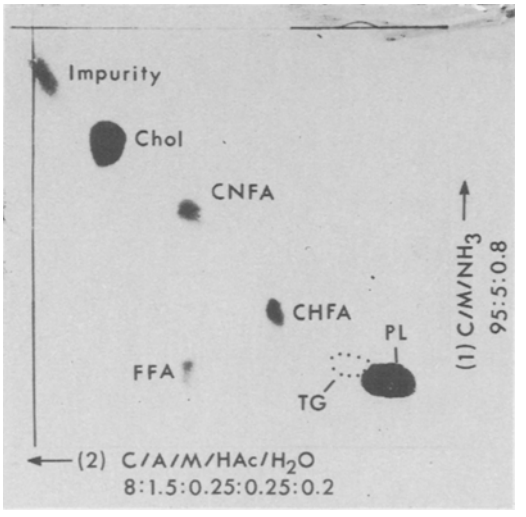


FIG. 1. Separation of ceramides from other lipid classes by two dimensional thin layer chromatography (20 μg each of ceramides with normal and hydroxy fatty acids added to a normal human brain lipid extract). Chloroform-methanol-28% by weight aqueous ammonia 95:5:0.8 was used as the first solvent and followed by chloroform-acetone-methanol-acetic acid-water 8:1.5:0.25:0.25:0.2. The adsorbent was Silica Gel H (Merck)-magnesium silicate 9:1 w/w spread as a slurry (20 g/65 ml) in 0.01N potassium hydroxide. Spots located by charring at 180 C for 30 min after spraying with a mixture of 3 volumes of 37% formaldehyde solution and 97 volumes of 98% sulfuric acid. Abbreviations: Chol, cholesterol; CHFA, ceramide with hydroxy fatty acids; CNFA, cerebroside with normal fatty acids; FFA, free fatty acid; TG, trace glycolipid; PL, phospholipid.

ACKNOWLEDGMENTS

W.J. Simmonds gave advice and guidance. V. Yeoh provided technical assistance. This work was supported by grants from the National Health and Medical Research Council of Australia and from the Medical School Research Grants of the University of Western Australia. The author was a N.H. and M.R.C. postgraduate medical scholar.

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Ceramides are readily separated from other lipid classes by two dimensional thin layer chromatography (TLC). That ceramides containing hydroxy and normal fatty acids are widely separated (Fig. 1) was established with authentic preparations (Supelco Inc., Bellefonte, Pa.). A spot for ceramide with normal fatty acid was readily detected by two dimensional TLC after application of 1 mg total lipid. No spot was observed in the position for ceramide

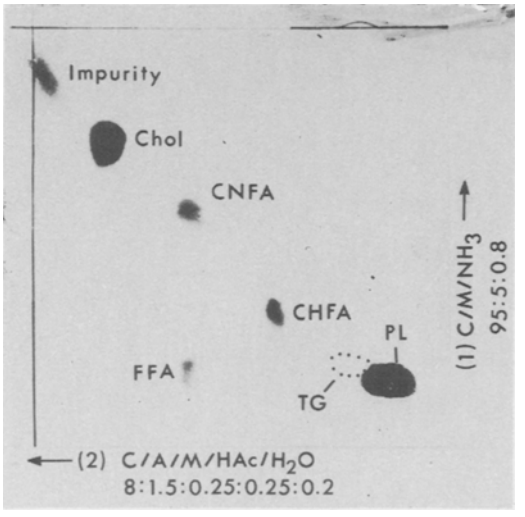


FIG. 1. Separation of ceramides from other lipid classes by two dimensional thin layer chromatography (20 μg each of ceramides with normal and hydroxy fatty acids added to a normal human brain lipid extract). Chloroform-methanol-28% by weight aqueous ammonia 95:5:0.8 was used as the first solvent and followed by chloroform-acetone-methanol-acetic acid-water 8:1.5:0.25:0.25:0.2. The adsorbent was Silica Gel H (Merck)-magnesium silicate 9:1 w/w spread as a slurry (20 g/65 ml) in 0.01N potassium hydroxide. Spots located by charring at 180 C for 30 min after spraying with a mixture of 3 volumes of 37% formaldehyde solution and 97 volumes of 98% sulfuric acid. Abbreviations: Chol, cholesterol; CHFA, ceramide with hydroxy fatty acids; CNFA, cerebroside with normal fatty acids; FFA, free fatty acid; TG, trace glycolipid; PL, phospholipid.

TABLE I

Ceramide Content of Human Brain at Different Ages[a]

| Component | Fetus | 1 D | 3 W | 6 M | 8 M | 22 M | 6 Y | 8.5 Y | 10 Y | 18 Y | 33 Y | 55 Y | 98 Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Per cent of total lipid,[b] | 0.99 ±0.02 | 0.89 ±0.03 | 0.88 ±0.03 | 1.31 ±0.03 | 0.70 ±0.01 | 0.41 ±0.02 | 0.41 ±0.02 | 0.44 ±0.02 | 0.39 ±0.04 | 0.38 ±0.03 | 0.52 ±0.03 | 0.31 ±0.01 | 0.39 ±0.02 |
| Per cent of dry weight, | 0.25 | 0.25 | 0.27 | 0.47 | 0.26 | 0.17 | 0.18 | 0.21 | 0.17 | 0.19 | 0.26 | 0.15 | 0.17 |
| mM/100 g, Fresh weight, | 0.038 | 0.042 | 0.051 | 0.120 | 0.069 | 0.047 | 0.053 | 0.068 | 0.061 | 0.068 | 0.092 | 0.050 | 0.055 |

[a]Fetus 25 week gestation. Abbreviations: D, W, M, Y: days, weeks, months, years of age.
[b]Value ± standard deviation.

TABLE II

Cholesterol Ester and Triglyceride Content of Normal Human Whole Brain at Different Ages[a]

| Component | Fetus | 1 D | 3 W | 6 M | 8 M | 22 M | 6 Y | 8.5 Y | 10 Y | 18 Y | 33 Y | 55 Y | 98 Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Per cent of cholesterol ester | | | | | | | | | | | | | |
| Total lipid[b] | 0.23 ±0.02 | 0.61 ±0.02 | 1.19 ±0.08 | 0.23 ±0.03 | 0.18 ±0.02 | 0.09 | 0.08 | 0.06 | 0.06 | 0.09 | 0.04 | 0.15 ±0.03 | 0.19 ±0.02 |
| Per cent of dry weight, | 0.06 | 0.17 | 0.36 | 0.08 | 0.07 | 0.04 | 0.04 | 0.03 | 0.03 | 0.04 | 0.02 | 0.07 | 0.08 |
| mM/100 g Fresh weight, | 0.009 | 0.030 | 0.071 | 0.021 | 0.018 | 0.010 | 0.011 | 0.010 | 0.010 | 0.016 | 0.007 | 0.025 | 0.027 |
| Triglycerides | | | | | | | | | | | | | |
| Per cent of total lipid,[b] | 0.37 ±0.01 | 0.24 ±0.01 | 0.31 ±0.01 | 0.20 ±0.01 | 0.20 ±0.01 | 0.27 ±0.03 | 0.21 ±0.01 | 0.45 ±0.01 | 0.16 ±0.01 | 0.15 ±0.01 | 0.18 ±0.02 | 0.18 ±0.02 | 0.61 ±0.02 |
| Per cent of dry weight, | 0.09 | 0.07 | 0.09 | 0.07 | 0.07 | 0.11 | 0.09 | 0.21 | 0.07 | 0.07 | 0.09 | 0.09 | 0.27 |
| mM/100 g, Fresh weight, | 0.010 | 0.008 | 0.013 | 0.013 | 0.014 | 0.023 | 0.020 | 0.051 | 0.019 | 0.019 | 0.024 | 0.021 | 0.063 |

[a]D, W, M, Y: days, weeks, months, years of age. Fetus after 25 weeks gestation.
[b]± Standard deviation.

with hydroxy fatty acid. Both types of ceramide are eluted from a diethylaminoethyl cellulose column with chloroform which also elutes cholesterol, cholesterol ester and triglyceride. Ceramide was separated from the other lipids in the ion exchange cellulose column fraction by one dimensional TLC with either of the two solvents shown in Figure 1, located by spraying with water and isolated by scraping from the plate, followed by elution from the wet adsorbent with chloroform-methanol 2:1.

The chromatographic identification of ceramide in human brain was confirmed as follows. The IR spectrum was similar to that described previously (1) and to that of a commercial standard. After acid hydrolysis, sphingosine and fatty acid were detected by TLC with chloroform-methanol-water 65:25:4. Carbohydrate was not detected by anthrone or α-naphthol procedures (4). A molar ratio of long chain base-fatty acid of 0.98 was obtained by determination of long chain base with trinitrobenzene sulfonic acid (5) and fatty acid after conversion to methyl esters by the hydroxamic acid procedure (4).

Triglyceride and sterol ester were separated by TLC with hexane-ether 9:1 as solvent. Spots were located under UV light after spraying with 0.05% methanolic Rhodamine 6G and then scraped into culture tubes with Teflon-lined screw caps. Sterol esters and triglycerides were determined by the hydroxamic acid procedure (4) after conversion to fatty acid methyl esters (6% by volume methanolic sulfuric acid at 105 C for 20 min) and extraction into hexane.

The molar amount of ceramide (Table I) appears to increase somewhat to ca. 6 months of age after which the level first falls and then remains relatively constant. Sterol ester appears to reach a peak at ca. 3 weeks and then to decline and remain relatively constant, whereas the amount of triglyceride appears to be the same at all ages (Table II).

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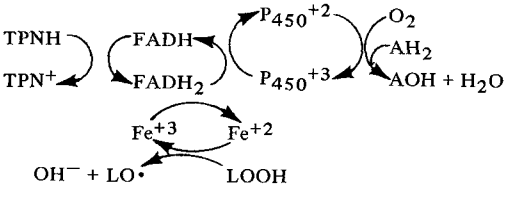
# LETTER TO THE EDITOR

## A Proposed Mechanism for the TPNH Enzymatic Lipid Peroxidizing System of Rat Liver Microsomes

*Sir:* A TPNH lipid peroxidation system was found in rat liver microsomes and first described by Hochstein and Ernster (CIBA Cellular Injury 1964:123). More recent publications from the laboratories of Wills (Biochem. J. 113:315, 325, 333 [1969]) and McCay (May and McCay, J. Biol. Chem. 243:2288, 2296 [1968]; Tam and McCay, Ibid. 245:2295 [1970]; Poyer and McCay, Ibid. 246:263 [1971]; Pfeifer and McCay, Ibid. 246:6401 [1971]; and McCay et al., Lipids 6:297 [1971]) have confirmed the presence of a TPNH oxidase (cytochrome *c* reductase) and have described extensively the requirements necessary for it to be involved in lipid peroxidation. These requirements include the presence of TPNH, iron ($Fe^{+2}$ or $Fe^{+3}$) and oxygen. In addition, this TPNH oxidase is involved in the initial steps of the cytochrome $P_{450}$ hydroxylating system (Wills, Biochem. J. 113:333 [1969]; Cohen and Estrabrook, Arch. Bioch. Biophys. 143:54 [1971]). However the mechanism by which the oxidase functions in lipid peroxidation has not been explained.

A study of the evidence available leads us to propose that the TPNH oxidase is catalyzing the reduction of the iron ($Fe^{+3}$ to $Fe^{+2}$) required in the reaction mixture. The ferrous form of the iron would then cause cleavage of any hydroperoxides (LOOH) present and initiate further peroxidation via the alkoxy radical (LO•).




Metal catalysis has frequently been found to initiate lipid peroxidation. Ingold (in "Lipids and Their Oxidation," Edited by Schultz, Day and Sinnhuber, Avi Publishing Co., 1962, p. 105) has described this catalysis by metals as a cleavage of the hydroperoxides resulting in the oxidation of the metal and the formation of free radicals.

$$LOOH + M^{(n+1)} \rightarrow LO\cdot + HO^- + M^{(n+1)+}$$

$$LOOH + M^{(n+1)+} \rightarrow LOO\cdot + H^+ + M^{(n+1)}$$

The importance of retaining the reduced state of the metal has been discussed by Pryor (Chem. Eng. News, June 7, 1971); the $Fe^{+2}$ state of the iron is $10^8$ times more rapid than the $Fe^{+3}$ state in cleaving hydrogen peroxide. Thus, when iron and ascorbate are used to propagate lipid peroxidation, the ascorbate is acting as a reducing agent maintaining the $Fe^{+2}$ state of the iron (Wills, Biochem. Biophys. Acta 98:238 [1965]).

As indicated in the above figure, the TPNH-lipid peroxidation system interacts with the hydroxylating system, showing an inverse relationship to the presence of hydroxylating substrates (Wills, Biochem. J. 113:333 [1969]). Also, the peroxidation system requires the presence of inorganic (nonheme) iron to be active (Wills, Biochem. J. 113:325 [1969]; Orrenius et al., Biochem. Biophys. Res. Commun. 14:329 [1964]). From the data of Wills (Ibid.), it appears that the iron is bound to the microsomal system, but it has not been clearly established how the iron might be coordinated.

Since the TPNH oxidase appears to be capable of reducing many different iron-containing compounds, including cytochrome *c*, hemoglobin, hematin and cytochrome $P_{450}$ (Wills, Biochem. J. 113:315 [1969]; Cohen and Estrabrook, Arch. Biochem. Biophys. 143:54 [1971]), in the absence of these substrates the TPNH oxidase may nonspecifically reduce the nonheme iron present in the reaction mixture.

When a comparison of the TPNH-enzymatic and the nonenzymatic ascorbate systems is made from the information in the literature cited above, no critical differences can be found (Table I). The similarities between the two systems include: a specific requirement for iron to be present, as noted by the inhibition in the

TABLE I

Comparison of the TPNH and Ascorbate Peroxidation Systems

| | TPNH | Ascorbate | Reference |
|---|---|---|---|
| Metal chelators (EDTA) | Inhibits | Inhibits | Wills, Biochem. J. 113:325 (1969) |
| *Heavy metals present* | | | Wills, Biochem. J. 113:325 (1969) |
| $Fe^{+2}$, $Fe^{+3}$ | Activates | Activates | |
| $Mg^{+2}$ | +/- | +/- | |
| $Ca^{+2}$, $Co^{+2}$, $Zn^{+2}$ | Inhibits | Inhibits | |
| Free radical trapping agents | | | Pfeifer and McCay, J. Biol. Chem. 246:6401 (1971) |
| Diphenylamine | Inhibits | Inhibits | |
| Diphenyl-*p*-phenylenediamine | Inhibits | Inhibits | |
| N-Methylamine | Inhibits | Inhibits | |
| α-Tocopherol | Inhibits | Inhibits | |
| Propyl gallate | Inhibits | Inhibits | |
| High ascorbate (.5mM) | Inhibits | Inhibits | Wills, Biochem. J. 113:315 (1969) |
| Reducing agent required | TPNH | Ascorbate | |
| Polyunsaturated fatty acid | * | # | *May and McCay, J. Biol. Chem. 243:2296 (1968) |
| 22:6 | ++++ | | |
| 20:4 | +++ | ++++ | |
| 18:2 | ++ | +++ | #Victoria and Barber, Lipids 4:582 (1969) |
| 18:1 | ± | + | |
| Moles $O_2$/moles PUFA/moles MA[a] | 24:6:1 | | May and McCay, J. Biol. Chem. 243:2296 (1968) |

[a]Malonaldehyde.

presence of a chelator or other metal ions; the presence of free radical intermediates, which are indicated by the inhibition in the presence of antioxidants and other free radical trapping agents; a requirement for reducing power; and similar damage to the polyunsaturated fatty acids in the membrane present. This is indicative of similar modes of action.

Thus knowledge of the mechanism involved seems to be crucial to understanding the TPNH-enzymatic lipid peroxidation system.

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# Response of Docosapentaenoic Acids of Rat Heart Phospholipids to Dietary Fat

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## ABSTRACT

Groups of male Holtzman strain rats were fed from weanling one of the following diets: 20% hydrogenated soybean fat (20% HF), and 20% HF plus 2%, 3% and 4% corn oil, respectively, for 20 weeks. The animals were killed, and the heart phospholipid fractions isolated by chromatographic procedures. The levels and distribution of the docosapolyenoic acids, especially 22:5ω3, were compared among the animals fed the corn oil supplemented and nonsupplemented diets. Although dietary linolenate (18:3ω3) level was very low in the nonsupplemented diet, 22:5ω3 accounted for 8.4% of the total fatty acids of heart total phospholipids when this diet was fed—half the level of total eicosatetraenoic acids. The amounts of 22:5ω3 were decreased by corn oil supplementation of the diet and got down to the "normal" range of 2.0-2.5% at corn oil supplementation levels greater than 2%. The docosapolyenoic acids were confined largely to the phosphatidylcholine and phosphatidylethanolamine classes of phospholipids. These findings are discussed from the standpoint of the structural role of the phospholipids in the heart subcellular fractions.

## INTRODUCTION

Previous work from this laboratory has shown that dietary partially hydrogenated soybean fat (HF) induced the accumulation of some unusual eicosadienoic acids in the rat liver phospholipids and docosadienoic acids in the adrenal cholesteryl esters and phospholipids (1,2). More recent studies along this line examined the consequences of this and other diets containing increasing amounts of corn oil (58.5% linoleate) on the long chain fatty acids of several rat tissues (3). In a parallel study we have observed a striking elevation in the level of docosapentaenoic acid (22:5ω3) of the heart phospholipids and also a defined response of the level of this fatty acid to the amount of linoleate (as corn oil) in the diet. These

TABLE I

Fatty Acid Composition of Total Heart Phospholipids after Feeding 20% HF and 20% HF + 2% Corn Oil Diets[a]

| Fatty acid | Area % of total fatty acids | | |
|---|---|---|---|
| | ECL[b] | HF | HF + 2% CO |
| 16:0 Aldehyde | 15.6 | 1.2 | 0.6 |
| 16:0 | 16.0 | 5.3 | 4.5 |
| 16:1ω7 | 16.5 | 0.6 | Trace |
| 18:0 Aldehyde | 17.6 | 0.7 | 0.4 |
| 18:0 | 18.0 | 18.9 | 17.8 |
| 18:1ω9 | 18.5 | 28.1 | 16.1 |
| 18:2ω6 | 19.0 | 18.4[c] | 17.1 |
| 18:3ω3 | 20.2 | 0.2 | 0.6 |
| 20:1ω9 | 20.4 | Trace | Trace |
| 20:3ω9 | 21.3 | 0.8 | Trace |
| 20:3ω6 | 21.6 | — | 0.5 |
| 20:4ω6 + ω7 | 22.2 | 15.8 | 35.0 |
| 22:4ω6 | 22.8 | Trace | 0.8 |
| 22:5ω6 | 24.9 | 0.8 | 1.3 |
| Unidentified | 25.1 | Trace | 0.6 |
| 22:5ω3 | 25.3 | 8.4 | 5.1 |
| 22:6ω6 | 25.4 | Trace | Trace |

[a]Results are the average of triplicate gas liquid chromatography analyses on the pooled organs per dietary group; HF = hydrogenated soybean fat.

[b]Equivalent chain length.

[c]Mixture of octadecadienoates (1).

TABLE II

After Feeding 20% HF + 3% and 20% HF + 4% Corn Oil Diets[a]

| Fatty acid | ECL[b] | Area % of total fatty acids | |
|---|---|---|---|
| | | 20% HF + 3% CO | 20% HF + 4% CO |
| 16:0 Aldehyde | 15.6 | 0.3 | Trace |
| 16:0 | 16.0 | 10.0 | 1.8 |
| 16:1ω7 | 16.5 | Trace | Trace |
| 18:0 Aldehyde | 17.6 | 0.2 | --- |
| 18:0 | | 17.7 | 25.4 |
| 18:1ω9 | 18.5 | 14.4 | 27.2 |
| 18:2ω6 | 19.0 | 18.4[c] | 17.8 |
| 18:3ω3 | 20.2 | Trace | --- |
| 20:1ω9 | 20.4 | --- | --- |
| 20:3ω9 | 21.3 | --- | --- |
| 20:3ω6 | 21.6 | 0.5 | --- |
| 20:4ω6 | 22.2 | 33.0 | 24.6 |
| 22:4ω6 | 22.8 | 1.3 | 0.2 |
| 22:5ω6 | 24.9 | 0.9 | 0.3 |
| Unidentified | 25.1 | 0.6 | 0.1 |
| 22:5ω3 | 25.3 | 2.2 | 2.5 |
| 22:6 | 25.9 | Trace | Trace |

[a]Results are the average of triplicate gas liquid chromatography analyses on the pooled organs per dietary group; HF = hydrogenated soybean fat.

[b]Equivalent chain length.

[c]Mixture of octadecadienoates (1).

observations are the subject of this communication.

## MATERIALS AND METHODS

Male Holtzman strain rats, 21 days old, were fed, per group of 4 animals, one of the following diets for 20 weeks: 20% HF, and the 20% HF diet supplemented with 2%, 3% and 4% corn oil. The full fatty composition of the fats, as well as the source and composition of the basic fat-free diet, have been described previously (1). Food and water were provided ad libitum.

The animals were sacrificed by decapitation at the end of the feeding period, at which time the average body weights among the animals receiving the three corn oil supplemented diets were comparable (258 g, 256 g, 260 g) and did not differ significantly from the average body

TABLE III

Influence of Dietary Fat on the Concentration and Distribution of Docosapolyenoic Acids of Rat Heart Phospholipids[a,b]

| Diet | $C_{22}$ Polyene | ECL | Area % of total fatty acids | | | | |
|---|---|---|---|---|---|---|---|
| | | | TL | PC | PE | PS | CL |
| HF | 22:4ω6 | 22.8 | --- | 2.4 | --- | --- | 0.4 |
| | 22:4ω9 | 24.1 | --- | 3.7 | | | 0.5 |
| | 22:5ω3 | 25.3 | 8.4 | --- | 9.6 | --- | --- |
| HF + 2% CO | 22:3ω9 | 23.3 | --- | 0.8 | --- | --- | --- |
| | 22:5ω3 | 25.3 | 5.1 | 4.5 | 1.0 | | |
| HF + 3% CO | 22:3ω6 | 23.8 | --- | --- | 6.0 | --- | --- |
| | 22:5ω3 | 25.4 | 2.2 | 3.6 | 0.0 | --- | --- |
| HF + 4% CO | 22:3ω9 | 23.2 | --- | | 1.1 | --- | --- |
| | 22:4ω9 | 24.0 | 0.3 | | 0.5 | --- | --- |
| | 22:4ω6 | 24.5 | 0.8 | 9.0 | --- | --- | --- |
| | 22:5ω6 | 24.9 | 1.0 | --- | 1.2 | --- | --- |
| | 22:5ω3 | 25.3 | 2.5 | | 3.2 | | |

[a]Results are the average of triplicate gas liquid chromatography analyses on the pooled organs per dietary group.

[b]Abbreviations: HF = hydrogenated soybean fat; ECL = equivalent chain length; TL = total lipids; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PS = phosphatidyl serine; CL = cardiolipin.

TABLE IV

Influence of Dietary Fat on the Concentration and Distribution of Docosapolyenoic Acids of Rat Heart Phospholipids[a]

| Diet | $C_{22}$ Polyene | ECL[b] | Area % of total fatty acids | | | | |
|---|---|---|---|---|---|---|---|
| | | | TL | PC | PE | PS | CL |
| Purina Chow | 22:4ω6 | 24.5 | 0.2 | --- | 7.3 | --- | --- |
| | 2222:5ω6 | 24.9 | --- | --- | 2.1 | --- | --- |
| | 22:5ω3 | 25.3 | 2.1 | 3.7 | --- | --- | --- |
| | 22:6ω6 | 25.9 | 9.9 | 2.0 | 20.9 | --- | --- |
| | 22:3ω9 | 23.2 | --- | --- | 1.0 | --- | --- |
| | 22:4ω6 | 24.5 | 11.1 | 9.3 | 0.8 | --- | --- |
| Fat-free | 22:5ω6 | 24.9 | 1.0 | 9.3 | --- | --- | 2.7 |
| | 22:5ω3 | 25.4 | 5.2 | --- | 1.8 | 5.8 | 1.6 |

[a]Results are the average of triplicate gas liquid chromatography analyses on the pooled organs per dietary group.

[b]Abbreviations: ECL = equivalent chain length; TL = total lipids; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PS = phosphatidyl serine; CL = cardiolipin.

weight of the animals receiving the unsupplemented HF diet (255 g). The hearts of the animals within each dietary group were pooled, weighed and washed in saline at 0 C. Total lipids were extracted from the hearts by the Folch procedure (4) using chloroform-methanol 2:1 v/v followed by the entire work-up procedure of the lipids up to the gravimetric determination of the total lipid extracts (1). Phospholipids were separtated from known aliquots of each sample either by thin lyaer chromatography (TLC), solvent system, high boiling petroleum ether-diethyl ether-glacial acetic acid 70:30:1 v/v, or by silicic acid (Unisil, Clarkson Chemical Co. Inc., Williamsport, Pa.) column chromatography, the nonpolar lipids being eluted first with chloroform followed by the elution of the phospholipids with methanol. Total phospholipids were fractionated into classes by TLC (solvent system, chloroform-methanol-glacial acetic acid-water 25:15:4:2 v/v). The methyl esters from the total phospholipids or the individual phospholipid classes were routinely prepared both by transesterification using boron triflouride-methanol reagent (5) and by methanolysis with sodium methoxide (6).

Gas liquid chromatography (GLC) was done isothermally at 180 C on: (a) a U-shaped glass column, 1.8 m long, id 3.2 mm packed with 15% ethylene glycol succinate on Chromasorb WAW, using a Barber Colman gas chromatograph; (b) a 61 cm steel column, id 3.2 mm, packed with Apiezon L 15% on 60-80 mesh Gas Chrom Q at 210 C, using a Beckmann GC-5 instrument. The details of these analyses have been described elsewhere (1,2). Identification of the peaks was done by the equivalent chain length (ECL) technique coupled with the use of the relation: $ECL_{EGS} - ECL_{Ap.}$ $L$ divided by that difference for a monoene (18:1), equals the number of double bonds in the individual polyenoic acid (1,7). Where possible, the retention times of the unknowns were compared to those of known pure standards (Hormel Institute, Austin, Minn., and Applied Science Laboratories, State College, Pa.) run under identical conditions. Identification of the polyenoic acids was also confirmed by comparison of the $r_{18}$ (retention time relative to $C_{18:0}$) and separation factor values (types I-III) with those reported by Ackman (8) and by Geer et al. (9) for an EGS column.

## RESULTS AND DISCUSSION

Tables I and II show the fatty acid composition of heart total phospholipids following the feeding of the different designated diets. The fatty acids relevant to this discussion are underlined in the tables. It is seen from the tables that rats fed the unsupplemented hydrogenated fat diet for 20 weeks had a comparatively elevated level of docosapentaenoic acid of the linolenate family (22:5ω3) in the total phospholipid fraction of the heart muscle lipids, even though the linolenate content of the dietary fat itself was very low (0.4% of total fatty acids, see also Reference 1). The value was 8.4% of the total phospholipid fatty acids, which was approximately one-half of the level of the total eicosatetraenoic acids (20:4ω6 + 20:4ω7) of the phospholipids. The level of this $C_{22:5}$ acid decreased with corn oil supplementation of the diet. Thus the HF diet supplemented with 2%, 3% and 4% corn oil resulted, respectively, in the values of 5.1%, 2.2% and 2.5% of the total phospholipid fatty acids.

TABLE V

Concentration and Distribution Pattern of Docosapentaenoic Acid (22:5ω3) in Heart Phospholipids of Rats Fed Nonsupplemented and Supplemented Hydrogenated Saoybean Fat[c]

| Diet | Area % of total fatty acids | | | | |
|---|---|---|---|---|---|
| | PL | PC | PE | PS | C |
| 20% HF | 8.4 (100)[b] | — | 9.6 (100) | — | — |
| 20% HF + 2% OO | 5.1 (100) | 4.5 (84.9) | 1.0 (100) | — | — |
| 20% HF + 3% CO | 2.2 (100) | 3.6 (100) | — | — | — |
| 20% HF + 4$ CO | 2.5 (54.3) | — | 3.2 (53.3) | — | — |
| Purina Chow | 2.1 (17.2) | 3.7 (64.9) | — | — | — |
| Fat-free | 5.2 (30.0) | — | 1.8 (50.0) | — | 1.6 (37.2) |

[a]Abbreviations: HF = hydrogeanted soybean fat; PL = phospholipids; PC, PE and PS = phosphatidyl-choline, -ethanolamine and -serine, respectively; C = cardiolipin.

[b]Figures in parentheses are percentages of total $C_{22}$ polyenoic acids of the lipid fraction.

Concomitantly the linolenate levels in the phospholipid fractions, for all the dietary groups (Tables I and II), were found to be very low. For purpose of comparison we point out that similar analyses involving the heart phospholipids from animals fed Purina Chow diet or the fat-free diet described previously (1) gave the values of 2.1% and 5.2%, respectively, for 22:5ω3. The response elicited by the fat-free diet was comparable to that seen with the 20% HF + 2% corn oil diet and tended more closely towards that due to the unsupplemented HF diet. This trend has been noted in another connection altogether (3). It seems also that corn oil supplementation of the diets at levels greater than 2% decreased the amount of 22:5ω3 to normal levels (relative to the value obtained by feeding the Purina Chow diet). It must be pointed out that these differences were not the result of differences in the weights of the hearts themselves. The weights, like the average body weights of the different groups, were really very similar: in grams per heart, 1.75 (20% HF diet); 2.00 (20% HF + 2% corn oil); 1.92 (20% HF + 3% corn oil); 1.85 (20% + 4% corn oil).

Pudelkewicz and coworkers (10), in their studies on the requirements of rats for linolenic acid, did find accumulations of long chain metabolites of linolenate (20:5ω3, 22:5ω3 and 22:6ω3) in the total lipids of both rat heart and liver, but this finding was dependent on an adequate dietary source of linolenate (at 0.3-0.5% of total calories). For the male rat the provision of dietary linolenate within this range of 0.3-0.5% of total calories was reported to be enough to suppress the level of eicosatrienoic acid (20:3ω9) in the total lipid fraction of the heart. However the dietary linolenate level had to be much higher (2.0% of total calories) in order to maintain the triene-pentaene ratio at the low level of 0.40. It is obvious from the linolenate (18:3ω3) content of the dietary fats used in this study (1) that linolenate contribution to the total calories was actually below the minimum requirement level of 0.3-0.5% of total calories. Consequently the finding, that comparatively 22:5ω3 accumulated in the phospholipids, is of interest. This possibly represents an attempt by the heart to conserve this long chain polyene in the phospholipid fraction in the face of limited amounts of $C_{22}$ polyenes of the ω6 family.

Our results suggest that in assessing the requirement for linolenate with respect to the heart, the parameters of Pudelkewicz et al. (10), arrived at on the basis of the total lipids, may not apply for the phospholipids as a class. This is probably due to the usually specific fatty acid compositional pattern of the phospholipids from several rat tissues (1,3), most likely associated with their widely believed structural role in tissues. The suppression of the level of this $C_{22}$ pentaene with supplementation of the diet with corn oil underscores the well known preference for the polyenes of the linolenate (ω6) family in the phospholipids of most rat tissues, under normal conditions (4).

A final observation of significance was the specific location of most of the $C_{22:5}$ acids, irrespective of their family of origin, essentially only in phsophatidylcholine or phosphatidyl-

ethanolamine (Tables III and IV). These two phospholipid types are usually the most predominant phospholipids in rat heart lipids (11). The data (Table IV), obtained from two other groups of rats fed either Purina Chow or a fat-free diet, were included for purposes of comparison. It was only in the fat-free group that relatively significant levels of a $C_{22}$ pentaene ($22:5\omega3$) distributed among the phospholipid classes, being absent only in phosphatidyl choline. At the same time $22:5\omega6$ was also incorporated into phosphatidylserine and cardiolipin. This departure from the otherwise general observation may be related to some metabolic derangement consequent on fat-free feeding. It must be noted that, just as in many mammalian heart phospholipids (12,13), other phospholipid classes (cardiolipin, phosphatidylserine, phosphatidylinositol, sphingomyelin) also occur in varying amounts in rat heart phospholipids (11). Furthermore heart phospholipids are known to be distributed in some pattern between the mitochondrial and microsomal fractions (11-13). If one accepts fat-free feeding as an abnormal stress condition on the animal as noted above, then the specific confinement of these $C_{22}$ polyenes, under fairly normal feeding conditions, to the two usually most predominant mitochondrial phospholipid types (11) may be related to the structural role of these phospholipid types (11) at the subcellular level in the rat heart muscle. The extent to which $22:5\omega3$ accounted for the $C_{22}$ polyenes of each major phospholipid class is shown in Table V. With the unsupplemented HF diet and up to 3% level of corn oil supplementation, $22:5\omega3$ by and large accounted for all the docosapolyenoic acids of the phosphatidylcholine and phosphatidylethanolamine fractions. With the HF + 4% corn oil diet, as well as the Purina Chow and fat-free diets, $22:5\omega3$ no longer occurred at the exclusion of the other $C_{22}$ polyenoic acids, whose identities were presented in Tables III and IV.

The relatively high level of $22:5\omega3$, a metabolite of linolenate which, like linoleate, was very low in the dietary fat whereas the level of oleate was high (28.1%) – was most probably due to the relative ease of the chain elongation-desaturation reactions of these three fatty acid families. The order (14) was found to be: $18:3\omega3>18:2\omega6>18:1\omega9$. That the level of $22:5\omega3$ was highest when the dietary $18:3\omega3$ was lowest may at least in part be due to this substrate preference on the part of the enzymes involved. The observed decrease in the level of this acid, with increasing corn oil (linoleate) supplementation of the diet, could be a reflection of the mutual inhibitions reported for these three major fatty acid families (14,15).

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# Formation of Diglycerides of Long Turnover Time from Labeled Acetate and Glucose in Rat Tissues

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## ABSTRACT

Rat adipose tissue pieces were incubated with acetate-2-$^{14}$C and glucose-$^{14}$C(U), respectively, and liver slices with acetate-2-$^{14}$C. The labeled tissues were then reincubated in inactive medium, and the changes of radioactivity in the different lipid classes were determined. In all three experiments a significant amount of radioactivity was incorporated in the diglycerides. During 1 hr of reincubation in inactive medium the radioactivity of diglycerides decreased from 35 to 26% of the total lipid activity in the adipose tissue labeled with acetate. In the adipose tissue labeled with glucose radioactivity fell from 25 to 19%. In liver slices 11% of the labeled acetate was incorporated in the diglycerides, and during the 2 hr of reincubation this value fell to its half. The radioactivity of the uniformly labeled glucose was distributed equally in the fatty acids and the glycerol. The distribution of radioactive glycerol between diglycerides and triglycerides was similar to that of the labeled fatty acids. Triglyceride synthesis seems to always be accompanied by the formation of diglycerides with a lasting turnover time.


In triglyceride synthesis phosphatidic acid is being formed by acylation of α-glycerophosphate. It is then converted into diglyceride, which is then completed by a third fatty acid to triglyceride (1). Several data show that, in mitochondrial preparates, homogenates and slices of different tissues besides triglycerides, a significant quantity of labeled fatty acids was also incorporated in the diglycerides (2-5).

By incubating slices of different rat tissues for a short time with labeled palmitic acid, then reincubating them in an inactive medium, the radioactivity of diglycerides was found to fall to its half, only after 1-2 hr reincubation (6,7). This half-life seemed too long for an intermediate, the triglyceride synthesis being very rapid.

In the present experiments, instead of labeled fatty acids, labeled acetate and glucose were added to the incubation medium. Incorporation of both compounds in the fatty acids enabled determination of the distribution of endogenous fatty acids between di- and triglycerides. From glucose not only fatty acids but also the glycerol moiety of glycerides were formed, and in this way it was possible to compare the life time of both compounds in the diglyceride molecule.

## MATERIALS AND METHODS

In our experiments we used male Wistar rats weighing 200 g and fed ad libitum on the standard pellet (Phylaxia, Budapest).

The incubation medium was a Krebs-Ringer phosphate buffer (pH 7.4) containing 5% bovine albumin and 0.09% inactive glucose. This solution was used partly in this form as "inactive medium," and was partly completed by labeled acetate or glucose.

The specific activity of the acetate-2-$^{14}$C (Reanal, Budapest) was 4.14 mCi/mmole, and that of the uniformly labeled D-glucose-$^{14}$C (UVVR, Prague), 100 mCi/mmole. The labeled medium contained 2 μCi/ml of either acetate or glucose. The specific activity of glucose was reduced by the unlabeled glucose content of the medium to 0.4 mCi/mmole. Then 150-200 mg pieces of adipose tissue were incubated in 5 ml medium, and 500-800 mg liver slices in 10 ml medium at 37 C by gentle shaking.

TABLE I

The Total Radioactivity of Tissue Lipids (mμCi per Gram Tissue) Before and After Reincubation in the Inactive Medium[a]

| Lipid | No. of animals | Time of reincubation, hr | Before reincubation | After reincubation |
|---|---|---|---|---|
| Acetate-2-$^{14}$C labeled adipose tissue | 3 | 1 | 1010.6 ± 63.5 | 1061.1 ± 44.4 |
| Acetate-2-$^{14}$C labeled liver slices | 3 | 2 | 17.4 ± 2.8 | 20.7 ± 7.3 |
| Glucose-$^{14}$C(U) labeled adipose tissue | 5 | 1 | 117.2 ± 43.6 | 104.8 ± 33.0 |

[a]Mean values ± SEM.

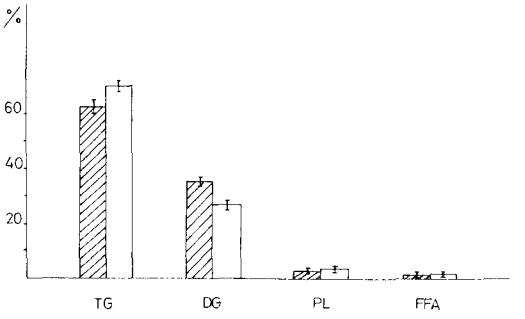


FIG. 1. The distribution of radioactivity in the lipid classes of acetate-2-$^{14}$C labeled adipose tissues before and after 1 hr reincubation in inactive medium. TG = Triglycerides, DG = diglycerides, PL = phospholipids, and FFA = free fatty acids. Hatched bars represent the distribution prior to reincubation, open bars after reincubation. The values reported are means of three animals ± SEM.

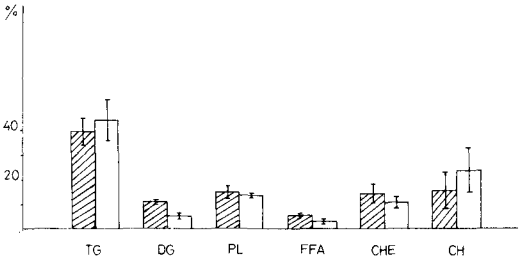


FIG. 2. The distribution of radioactivity in the lipid classes of acetate-2-$^{14}$C labeled liver slices before and after 2 hr reincubation in inactive medium. TG = Triglycerides, DG = diglycerides, PL = phospholipids, FFA = free fatty acids, ChE = cholesterolesters, and Ch = free cholesterol. Hatched bars represent the distribution prior to reincubation, open bars after reincubation. The values reported are means of three animals ± SEM.

All tissues were incubated first for 10 min in the labeled medium, then rinsed with physiological saline and reincubated in inactive medium. This reincubation lasted for the adipose tissue taken from one side of each animal for 10 min and for the contralateral tissues 1 hr longer, i.e., 70 min. In the case of liver one group of the slices made from the same liver was reincubated for 10 min, and the other group for 2 hr longer, i.e., 130 min.

After reincubation the tissues were rinsed again with physiological saline and homogenized in chloroform-methanol 2:1. Lipids were extracted according to Folch et al. (8). The lower phase was washed five times with pure upper phase in order to remove all nonlipidic contaminants.

The different lipid classes were separated by thin layer chromatography and eluted from the silica gel as described earlier (7). Since the free cholesterol and the diglycerides were not separated satisfactorily by this technique, a part of the fraction containing these two lipids was saponified with 0.5 N KOH for 2 hr in $N_2$ atmosphere. The radioactivity of the unsaponifiable part corresponded to the cholesterol, and that of the saponifiable one to the diglycerides. In the adipose tissues the free cholesterol had no measurable radioactivity.

In the adipose tissue incubated with glucose the distribution of radioactivity between the glycerol and the fatty acid parts of the glycerides was to be determined. For this purpose the total radioactivity of diglycerides and triglycerides was determined from a known part of these lipids, while in another aliquot part the fatty acids were transmethylated and the radioactivity of methyl esters measured. In this way the radioactivity of the fatty acid moiety was determined separately. The total radioactivity of glycerides minus this value supplies the radioactivity of the glycerol moiety. For *trans*-methylation 1 ml hexane and 5 ml HCl-methanol (5% concentrated HCl in absolute methanol) were added to maximum 40 mg lipid; the samples were sealed under $CO_2$ and kept at 80 C for 4 hr.

After cooling, 5 ml water was added, and the samples were vigorously shaken three times with 5 ml petroleum ether. The upper phases containing the methyl esters were pooled and washed with distilled water. Control experiments with lipids, labeled only in the fatty acid moiety, showed recoveries above 95% by this technique. The relation of radioactivity of the fatty acids to that of the total lipid was also

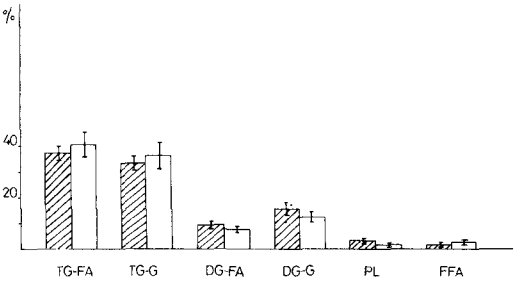


FIG. 3. The distribution of radioactivity in the lipid classes and in the fatty acid and glycerol moiety of the glycerides in the glucose-$^{14}$C(U) labeled adipose tissues before and after 1 hr reincubation in inactive medium. TG-FA = Fatty acid moiety of triglycerides, TG-G = glycerol moiety of triglycerides, PL = phospholipids, DG-FA = fatty acid moiety of diglycerides, DG-G = glycerol moiety of diglycerides, and FFA = free fatty acids. Hatched bars represent the distribution before reincubation, open bars after reincubation. The values reported are means of five animals ± SEM.

determined by saponification. The results obtained by the two techniques were in accord within a 5% limit.

The lipid samples were dissolved in 10 ml toluene containing 0.4% diphenyloxazole and 0.01% 1.4-di-(2-[5-phenyloxazolyl])-benzene. The radioactivity was measured by Packard Tri-Carb liquid scintillation spectrometer.

## RESULTS

During the reincubation in inactive medium, the radioactivity of the total lipids did not change significantly (Table I).

In the adipose tissue incubated with labeled acetate (Fig. 1) the distribution of radioactivity in the different lipid classes was similar to that observed after incubation with labeled palmitic acid (6,7). The permanent radioactivity of diglycerides was also apparent here; as during the 1 hr in the inactive medium, their radioactivity fell only from 35 to 26% of the total activity. This decrease was even slower than in the experiment with labeled palmitic acid, but it seems uncertain whether this difference was due to the origin of fatty acids, for these were two separate experiments carried out at different times.

Also in the case of liver slices incubated with acetate-2-$^{14}$C (Fig. 2), the distribution of radioactivity was similar to that observed after incubation with labeled palmitic acid. Differences here are also caused by acetate incorporation into the cholesterol, thus leading to the labeling of free cholesterol and to the increased radioactivity of the cholesterol ester fraction. Accordingly only 11% of the radioactivity was incorporated into diglycerides, and this value fell to exactly its half during the 2 hr reincubation in the inactive medium. The half-life of diglycerides in liver slices proved to be 2 hr when labeled palmitic acid was used (7).

In adipose tissue incubated with glucose-$^{14}$C (Fig. 3) the fatty acid and glycerol moiety of glycerides had similar radiocarbon content. In triglycerides the fatty acid per glycerol radioactivity ratio was higher than in diglycerides, corresponding to the fact that in triglycerides glycerol is combined with three fatty acids, while in diglycerides only with two. Taking this into account it appears that the radioactive glycerol had the same distribution between triglycerides and diglycerides as the fatty acids synthesized from glucose or acetate. In addition the radioactivity of glycerol diminished in the diglycerides during the reincubation to the same extent as that of the fatty acids.

## DISCUSSION

The above results have shown that the intracellularly synthesized fatty acids are also incorporated in diglycerides, which retain their radioactivity for a long time.

If only the distribution of fatty acids were known, it could be supposed that there is only a single diglyceride pool of the long turnover time, and the rapid appearance of radioactivity in triglycerides is effected by the labeled fatty acids, completing these preexisting diglycerides to triglycerides. In experiments with glucose, however, the glycerol moiety became labeled too, proving rapid de novo synthesis of both glycerides. If the rapid de novo triglyceride synthesis proceeds through diglycerides, then at least two different diglyceride pools must exist, one with a very short and one with the long turnover time here demonstrated.

The existence of these two distinct pools may be associated with the acylation of α-glycerophosphate in both the mitochondria and the microsomes (9), or with the recently discovered alternate route of triglyceride synthesis, starting with the acylation of dihydroxyacetonephosphate instead of α-glycerophosphate (10,11).

It also seems possible that all diglycerides are synthesized at the same site and in the same way; then one part of them is immediately completed to triglycerides, while the other part is passed on to the second diglyceride pool of long turnover time. In this way small lipid droplets might be formed in the mitochondria and in microsomes, consisting of some two-thirds triglycerides and one-third diglycerides. One might speculate whether the diglycerides, being of a more polar character, could play a role in the intracellular transport of these droplets.

Anyway the similar ratio of the radioactivity of fatty acids to that of the glycerol in diglycerides and triglycerides indicates a similar origin.

The relation of radioactivities of the fatty acid and glycerol parts in the diglycerides remained unchanged during the reincubation, demonstrating that the fatty acids were not exchanged, but the molecule is metabolized as a whole. It may be completed to triglyceride or completely hydrolyzed, its constituents reentering the process of triglyceride synthesis.

In one gram adipose tissue some μmole fatty acids are esterified in 1 hr (8,12). As we have seen above, about one-third of these fatty acids is incorporated into diglycerides which have a half-life of ca. 2 hr.

In 1 g adipose tissue 28 μmole diglyceride was found (13). This amount is very low, compared to that of triglycerides, but is sufficient enough to permit -even despite the long half-life–a turnover rate, corresponding to one-

third of the total fatty acid esterification.

In earlier experiments is was demonstrated that in the adipose tissue, diaphragm and liver slices incubated in vitro, but also in the adipose tissue incubated in vivo, a major part of the endogenous fatty acids were incorporated into diglycerides, which remain stable for a long time (6,7).

The present experiments show the same phenomenon in the case of intracellularly synthesized fatty acids and glycerol. It is therefore likely that the formation of diglycerides of long life time is a general concomitant of the triglyceride synthesis.

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# Dietary Lipid, Fatty Acid Synthesis and Cholesterol Metabolism in Aging Rats[1,2]

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## ABSTRACT

Male and female rats were fed diets containing 2% of calories as corn oil or that plus 40% of calories as beef tallow or corn oil. After 3, 6, 12 and 18 months groups were given 4-$^{14}$C-cholesterol ip, and feces were collected for 9 days. Just prior to necropsy $^{3}$H-acetate was administered ip. Samples of serum, liver, heart and carcass were obtained for analysis. Concentrations of fatty acids and cholesterol, synthesis of those and recovery of ring-labeled steroid are reported. Mortality from acute respiratory disease was very high in male rats fed beef tallow or low fat diets and very low in those fed the corn oil diet. In females, only beef tallow diet resulted in a high mortality rate, and this was lower and at a later age than in males. The most notable effects of age were in relation to fatty acid synthesis and presence of $^{14}$C-acidic steroid in the carcass. In 3-month-old rats both fats depressed fatty acid synthesis in comparison to the low fat diet. At later ages beef fat ceased to depress fatty acid synthesis in both sexes. Corn oil continued to depress fatty acid synthesis up to 12 months in males and 18 months in females. The presence of $^{14}$C-acidic steroid in carcass was substantial in 6-month-old rats and constituted ca. 40% of recovered $^{14}$C in 18-month-old rats. The possibility that the increase in acetate incorporation into fatty acids with age in fat feeding is related to chain elongation rather than de novo synthesis is discussed. Both the presence and amount of acidic steroid in the carcass are notable and may be of importance in constructing models of cholesterol turnover.

## INTRODUCTION

Numerous studies have been conducted to determine differential effects of saturated and polyunsaturated dietary lipid on cholesterol metabolism in rats and other experimental animals as well as man. Polyunsaturated fatty acids (PUFA) in the diet reduce serum cholesterol (1) and increase acetate incorporation into cholesterol (2) in comparison to saturated fatty acids (SFA). These effects have been considered desirable for man (1), and a number of studies suggest that PUFA may reduce the incidence of atherosclerosis in man (reviewed in Reference 1).

The mechanisms by which fatty acids affect cholesterol metabolism are not fully understood. One difference in metabolism between PUFA and SFA is more rapid oxidation of PUFA (3). The rapid oxidation to $CO_2$ does not provide, however, excess acetyl-CoA for cholesterol synthesis in vivo (3).

Cholesterol balance studies have failed to discover significant differences when PUFA or SFA was fed. Grundy and Ahrens (4) reported no difference in steroid excretion by humans when butter oil or corn oil was fed. They suggested considerable loss of ring-labeled sterol from the gut by means other than absorption or excretion (5). Wood and Hatoff (6) reported that there is no detectable loss of ring-labeled sterol from incubated feces, which suggests that deterioration of the ring does not occur in the gut.

Most of the studies conducted have been of short duration and considered only one aspect of lipid metabolism in any one study. The following experiment was designed to study cholesterol biosynthesis from acetate, cholesterol retention and excretion, and fatty acid synthesis from acetate in rats fed high PUFA (42% of calories as corn oil), high SFA (40% of calories as beef fat) or low fat diets from weaning to 3, 6, 12 or 18 months of age. Both sexes were used. The location of ring-labeled and newly synthesized cholesterol was determined for the entire animal with distribution among feces, serum, liver, gut and carcass.

## MATERIALS AND METHODS

### Animal Management Procedures

*Animals:* Strain CFE rats of both sexes were obtained from Carworth, Inc., at weaning. Those to be held to 12 and 18 months of age were received at the same time. Nine months

[1]Presented in part at the AOCS Sterol Symposium, April 1970, and the Federation of American Societies for Experimental Biology, April 1971.

[2]Scientific Series Paper No. 1536, Colorado Agricultural Experiment Station.

later those to be held for 3 months were obtained and 3 and 12 month groups were killed simultaneously. The rats to be held 6 months were obtained after the 3 and 12 month groups were killed, and the 6 and 18 month groups were killed simultaneously. The small amount of observed variability among body weights of all groups at 3 and 6 months suggests that the animals were from near identical populations. During the entire experiment the animals were held in a single room maintained at 22 ± 1 C and 50 ± 10% humidity, except for one 3 day period when the first rats were 8 months old. During that period the humidity fluctuated between 10 and 30%. The rats were housed in individual stainless steel wire bottom cages and were randomly located as to sex and diet. The rats held to old age were not placed on the same racks as those started later, but were held in the same room. Old animals (more than 6 months) were selected for termination by lottery. Throughout the experiment, all animals had access to food and deionized water, ad libitum. Where possible six rats were in each group. The 18 month groups did not all contain six animals because of mortality. Some of the means reported contain less than six observations because of loss of samples.

*Diets:* The diet formulation (Table I) was intended to conform to the approximate gross composition of the average diet consumed in the U.S. (7), with variations on the fat content. The beef fat diet (BF) contained 40% of calories as beef fat and 2% as corn oil, providing a polyunsaturated to saturated ratio (P/S) of ca. 0.06 and sufficient essential fatty acids according to current recommendations. Variations were substitutions of the beef fat by carbohydrate (LF) or corn oil (CO). Carbohydrate was supplied as half sucrose and half cornstarch. Fatty acid composition and calorie density were calculated (8). The calorie densities were LF-3.8 cal/g, BT-4.7, CO-4.7. Food intake was determined for individual animals during the 6 days prior to necropsy procedures.

During two periods sulfamerazine was included in the diets of all rats at the concentration of 0.25 mg/kg to control outbreaks of chronic respiratory disease (9). The periods were 3 weeks during the 2nd and 11th months of the rats killed at 3 and 12 months, respectively, and 2 weeks during the 5th and 17th months of those killed at 6 or 18 months, respectively. It was discontinued 3 weeks prior to necropsy procedures.

An error was made in calculating the vitamin A content of the vitamin mix. Consequently the rats maintained during the 1st year of the study received only a small fraction of the recommended dietary allowance (Table I). This affected rats killed at 3 and 12 months. Supplementation with the correct amount of vitamin A began when the 6 month rats arrived. They received adequate vitamin A throughout their lives. The 18 month rats were supplemented from 12 to 18 months with the correct amount of vitamin A. Growth rates of all rats from weaning to 3 and 6 months were very similar, so it is assumed that vitamin A was not limiting in this experiment; however interactions of low vitamin A with other factors cannot be assessed.

*Pathology:* Rats began to exhibit symptoms of chronic respiratory disease (CRD) after the 9th month of age. Mortality records were kept on the original group of rats through 18 months. The number killed was subtracted from the remaining number at 12, 15 and 18 months for statistical computations.

TABLE I

Composition of Diets, Weight Per Cent

| Ingredient | Low fat | Beef tallow | Corn oil |
|---|---|---|---|
| Lactalbumin[a] | 15.0 | 19.0 | 19.0 |
| Cornstarch[b] | 39.9 | 25.3 | 25.3 |
| Sucrose[c] | 36.5 | 25.0 | 25.0 |
| Cellophane spangles[a] | 2.0 | 2.5 | 2.5 |
| Corn oil[d] | 1.0 | 1.2 | 21.2 |
| Beef tallow[e] | — | 20.0 | — |
| Salt mix[a,f] | 4.0 | 5.0 | 5.0 |
| Vitamin mix[g] | 1.6 | 2.0 | 2.0 |

[a]General Biochemicals, Inc., Chagrin Falls, Ohio.

[b]Staley Mfg. Co., Decatur, Ill., or Amaizo, American Maize Products Co., New York.

[c]Beet sugar, Great Western United.

[d]Mazola, obtained on local retail market throughout the experiment.

[e]Fat rendered from beef kidney knobs obtained from the Animal Science Dept., C.S.U.

[f]Williams, M.A., and G.M. Briggs, Fed. Proc. (Abstr.) 22:601 (1963).

[g]Contains per 2 kg of vitamin mixture made up with cornstarch: vitamin A acetate, 1,163 or 400,000 IU (see methods); vitamin $D_2$, 40,000 IU; (in grams) α-tocopheryl acetate, 20; Ca-panthothenate, 2; choline chloride, 400; *p*-aminobenzoic acid, 20; inositol, 20; niacin, 3; ascorbic acid, 10; (in mg) menadione, 100; biotin, 50; $B_{12}$, 10; folic acid, 50; pyridoxine-HCl, 500; riboflavin, 500; and thiamine-HCl, 500.

### Lipid Analyses

*Cholesterol excretion:* Ten days prior to termination procedures each rat was injected ip with 2.5 or 5 μCi of cholesterol-4-$^{14}$C (New England Nuclear Corp., 57 mc/mM) suspended in 0.5 ml 10% ethanol in saline. Data were calculated upon the basis of a dosage of 50

TABLE II

Death from Apparent Chronic Respiratory Disease, Per Cent of Survivors and Number[a]

| Period | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | 3-12 Months | 12-15 Months | Cumulative | 3-12 Months | 12-15 Months | Cumulative |
| LF[b] | 16% | 13% | 29% | 0 | 0 | 0 |
| | 4/25 | 2/15 | | 0/23 | 0/17 | |
| BT[b] | 12% | 37.5% | 49.5% | 0 | 23% | 23% |
| | 3/25 | 6/16 | | 0/23 | 4/17 | |
| CO[b] | 4% | 5.5% | 9.5% | 4% | 0 | 4% |
| | 1/24 | 1/18 | | 1/24 | 0/18 | |

[a]Chi square test of significant difference from the grand mean of 9.68% mortality showed males greater than females, all ages ($P<.05$); diet effect in males at both ages ($P<.01$) and females at 12-15 months ($P<.01$); and sex-diet interactions at both ages ($P<.01$).

[b]LF = Low fat (2% of calories as corn oil); BT = beef tallow (2% corn oil plus 40% beef fat), CO = corn oil (42% of calories as corn oil); see Table I.

$\mu$Ci/100 g body wt. Feces were collected at 3 day intervals for 9 days. Fecal collections were stored at -20 C for $^{14}C$ determination. The whole sample was homogenized in water with a Virtis Homogenizer and aliquots placed in 2 in. concentric ring aluminum planchets, dried and weighed. Radioactivity was determined by counting in a Nuclear-Chicago low background counter equipped with a thin window. Efficiency, background and self-absorption corrections were made using appropriate standards.

*Cholesterol and fatty acid synthesis:* On the evening prior to necropsy food was removed from the cages. Then 8-12 hr later food was replaced for 1 hr. This procedure insured that all rats consumed food at the same time prior to lipid metabolism measurements. At the time the food was removed following this meal, acetate-$^{3}H$ (50 $\mu$Ci in 0.5 ml saline, Amersham-Searle, 500 mc/mM) was injected ip. The rat was decapitated 2.5 hr later, and head, lungs, heart and portions of the liver were removed for other studies. The "carcass," therefore, was the body minus head, gastrointestinal tract, liver, heart and lungs. The serum, remainder of the liver, gastrointestinal tracts and "carcass" were stored at -20 C until analyzed. Data were calculated on the basis of a standard dose of 50 $\mu$Ci/100 g body weight.

Serum and liver were extracted with chloroform-methanol 2:1, incorporating one synthetic upper phase (containing 0.025% $CaCl_2$/liter) wash (10), and liver total lipid was determined gravimetrically as previously described (3). Total lipid $^{3}H$ and $^{14}C$ were determined by differential counting on a Nuclear-Chicago Unilux II liquid scintillation system equipped with external standardization. The scintillation solution consisted of 4 g 2,5-diphenyloxazole (PPO) and 50 mg 4-di-2-(5-phenyloxazolyl)-benzene (POPOP) in 1 liter toluene. An aliquot of the C-M solution was saponified, and nonsaponifiable material counted the same as total lipid. Cholesterol was quantitated as previously reported, and the digitonides $^{3}H$ and $^{14}C$ were determined by dissolving in 1 ml methanol and counting. The scintillation solution consisted of 6 g PPO and 100 mg POPOP in 1 liter toluene. The radioactivity data for fatty acids of serum and liver were calculated by subtracting nonsaponifiable counts from total lipid counts.

"Carcasses" were hydrolyzed in 10% ethanolic KOH, and nonsaponifiable material was extracted three times with 1 volume of petroleum ether each time (2). Samples of this were counted, used for quantitation of cholesterol and for precipitation of cholesterol digitonide. Counting of the fractions was the same as for those of liver and serum. The saponified residue was acidified with HCl and extracted with petroleum ether. The material was quantitated gravimetrically to assess "carcass" fatty acid content, and $^{14}C$ and $^{3}H$ were counted to determine acid sterol metabolism and incorporation rate of acetate into fatty acid, respectively.

*Cholesterol retention:* Cholesterol-4-$^{14}C$ remaining in serum, liver and "carcass" were determined in all the lipids as described above. The $^{14}C$ found in the saponifiable fraction was considered to be acidic steroids. By the time it was apparent that a significant amount of $^{14}C$ had been recovered in the saponifiable petroleum ether extractable fraction of the "carcass," samples had been discarded. Subsequently, however, additional work in this laboratory has shown that petroleum ether soluble, saponifiable material derived from 4-$^{14}C$-cholesterol may be recovered from the serum muscle and adipose tissue of minipigs

TABLE III

Growth, Food Intake and Liver Lipids of Male and Female Rats[a]

| | | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Age, months | Diet | Body wt, g | Food intake, g/day | Liver wt, g | Liver fat, % | Liver cholesterol,[b] mg total | Body wt, g | Food intake, g/day | Liver wt, g | Liver fat, % | Liver cholesterol,[a] mg total |
| 3 | LF[c] | 282 ± 20[d] | 17.8 ± 2.3 | 10.0 ± 0.5 | 4.1 ± 0.1 | 21.8 ± 2.9 | 192 ± 4 | 13.6 ± 1.5 | 5.9 ± 0.2 | 4.7 ± 0.3 | 12.3 ± 0.9 |
| | BT | 306 ± 9 | 16.9 ± 0.7 | 9.7 ± 0.3 | 4.4 ± 0.2 | 20.4 ± 1.6 | 196 ± 4 | 11.8 ± 2.4 | 6.0 ± 0.3 | 4.6 ± 0.5 | 12.7 ± 1.1 |
| | CO | 304 ± 12 | 14.7 ± 0.5 | 9.8 ± 0.4 | 5.1 ± 0.2 | 32.2 ± 3.9 | 196 ± 4 | 11.0 ± 0.3 | 5.9 ± 0.1 | 4.8 ± 0.3 | 18.6 ± 2.4 |
| 6 | LF | 391.± 24 | 20.0 ± 0.9 | 13.0 ± 0.5 | 4.2 ± 0.1 | 34.5 ± 3.5 | 258 ± 6 | 14.0 ± 0.2 | 7.1 ± 0.2 | 4.3 ± 0.2 | 16.8 ± 1.5 |
| | BT | 450 ± 9 | 18.1 ± 0.4 | 13.3 ± 0.5 | 4.4 ± 0.1 | 32.5 ± 2.5 | 280 ± 13 | 13.0 ± 0.6 | 7.5 ± 0.2 | 4.6 ± 0.2 | 17.1 ± 0.9 |
| | CO | 456 ± 8 | 15.8 ± 0.5 | 12.8 ± 0.3 | 5.1 ± 0.1 | 43.7 ± 3.5 | 266 ± 12 | 12.0 ± 0.7 | 6.8 ± 0.3 | 4.8 ± 0.2 | 18.4 ± 1.4 |
| 12 | LF | 447 ± 15 | 16.2 ± 0.8 | 11.9 ± 0.4 | 4.6 ± 0.1 | 37.1 ± 5.8 | 301 ± 15 | 12.6 ± 1.2 | 8.0 ± 0.4 | 4.5 ± 0.1 | 17.8 ± 1.4 |
| | BT | 530 ± 16 | 14.6 ± 0.8 | 12.5 ± 0.9 | 5.2 ± 0.3 | 34.9 ± 2.2 | 308 ± 19 | 9.9 ± 1.3 | 8.1 ± 0.4 | 4.8 ± 0.2 | 17.5 ± 1.3 |
| | CO | 480 ± 22 | 12.4 ± 0.3 | 11.2 ± 0.6 | 6.3 ± 0.3 | 49.3 ± 7.5 | 295 ± 20 | 10.2 ± 1.1 | 7.5 ± 0.5 | 5.2 ± 0.3 | 24.4 ± 1.0 |
| 18 | LF | 490 ± 24 | 17.3 ± 1.7 | 13.6 ± 0.4 | 5.0 ± 0.3 | 38.8 ± 2.7 | 361 ± 12 | 15.5 ± 0.6 | 9.6 ± 0.6 | 5.1 ± 0.2 | 23.4 ± 1.8 |
| | BT | 612 ± 10 | 20.2 ± 0.4 | 15.5 ± 0.9 | 5.0 ± 0.1 | 48.1 ± 8.9 | 416 ± 45 | 14.0 ± 1.2 | 9.8 ± 1.0 | 5.0 ± 0.2 | 20.3 ± 1.5 |
| | CO | 573 ± 16 | 15.5 ± 0.3 | 13.3 ± 0.7 | 5.9 ± 0.1 | 50.0 ± 3.7 | 330 ± 12 | 10.7 ± 0.6 | 7.9 ± 0.5 | 5.0 ± 0.3 | 23.7 ± 2.9 |

[a]Rats 3-18 months of age, fed low fat, high beef fat or high corn oil diets.

[b]Liver cholesterol, mg per liver.

[c]LF = Low fat; BT = beef tallow; CO = corn oil; see Table I.

[d]Mean ± standard error of the mean for four to six rats. Statistical analysis discussed in results.

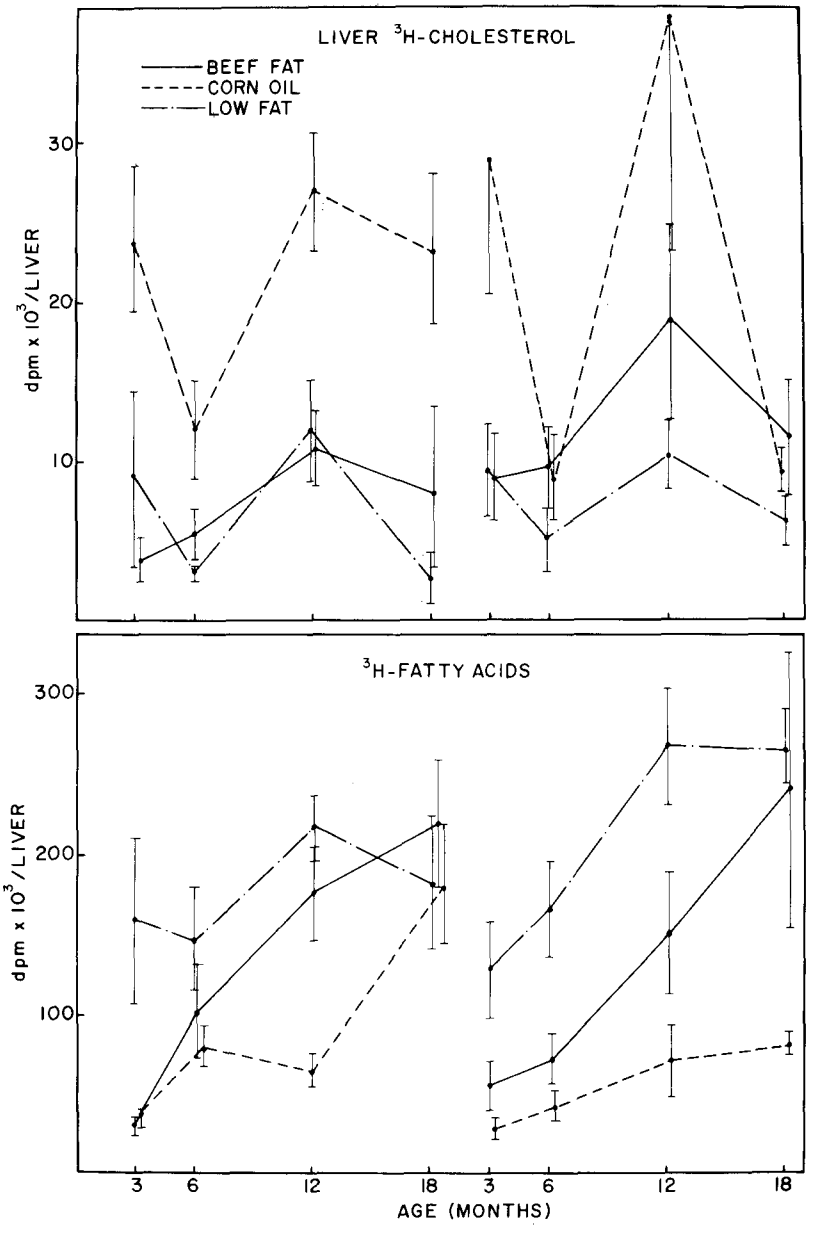


FIG. 1. In vivo incorporation of $^3$H-acetate into liver cholesterol and fatty acids of male and female rats fed low fat, high beef tallow or high corn oil diets (see Table I) from weaning to 3, 6, 12 or 18 months of age. Points indicate mean ± the standard error of the mean for four to six animals. Statistical analysis discussed in Results and shown in Figure 2.

after administration of the tracer. Whether there is only one compound is not yet known, and identification has not been completed. The gastrointestinal tract plus contents were homogenized with water to a consistency enabling plating in a thin layer in 2 in. concentric ring planchets. Radioactivity was determined the same as for feces.

*Statistical analysis:* Mortality data were analyzed by a Chi Square method using the data shown in Table II. Computations of data on radioactivity were made with an Olivetti Programma 101 computer that was on line with the liquid scintillation system. Means and standard errors of the means were determined on unadjusted data and are so reported. Differ-

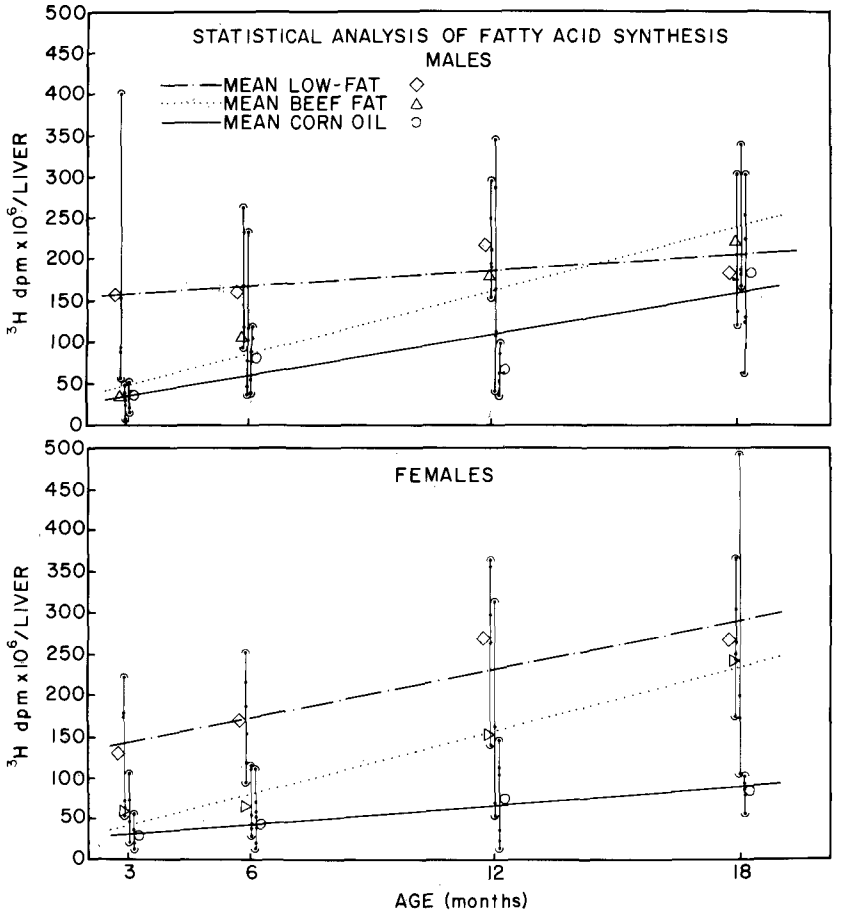


FIG. 2. Linear effect of age on $^3$H-acetate incorporation into liver fatty acids. See Table I for diet composition. In males low fat $r = 0.19$ (n.s.); in females low fat $r = 0.62$ ($P<.05$); beef fat $r = 0.64$ ($P<.05$); corn oil $r = 0.58$ ($P<.05$). Diet effects were different as discussed in Results.

ences due to age, sex and diet effects and those due to interaction effects among age, sex and diet were assessed by a least squares analysis of variance procedure. Unequal numbers of observations were taken into account. Computations were performed on a CDC 6400 computer.

## RESULTS

### Mortality

By the time the rats were 1-year-old there was a significant incidence of death with chronic respiratory disease symptoms in males, but not in females $P<.05$ (Table II). There was a significant dietary difference, with LF and BT diet groups suffering greater mortality than those fed CO ($P<.01$). Between 12 and 15 months the BT males suffered a 37.5% death rate which was significantly greater than the rate for LF or CO groups ($P<.01$). Females fed BT also had a higher death rate than CO or LF during this period (23% vs. 0, $P<.01$). The females, in general, did not suffer as great a death rate during the 12 to 15 month period as the males ($P<.05$). The significant interaction between sex and diet at both age periods ($P<.01$) indicates that diet affected the sexes differently.

Examination of the lungs of rats killed at random at 15 months of age indicated no differences among groups in prevalence of chronic respiratory disease (CRD) infections. The rats that died with apparent acute respiratory disease symptoms had severe infections in the lungs. The details of lung pathology will be reported elsewhere. There was no apparent myocardial infarction nor any significant coronary artery involvement of the hearts, as seen by light microscopy, regardless of sex, diet or age.

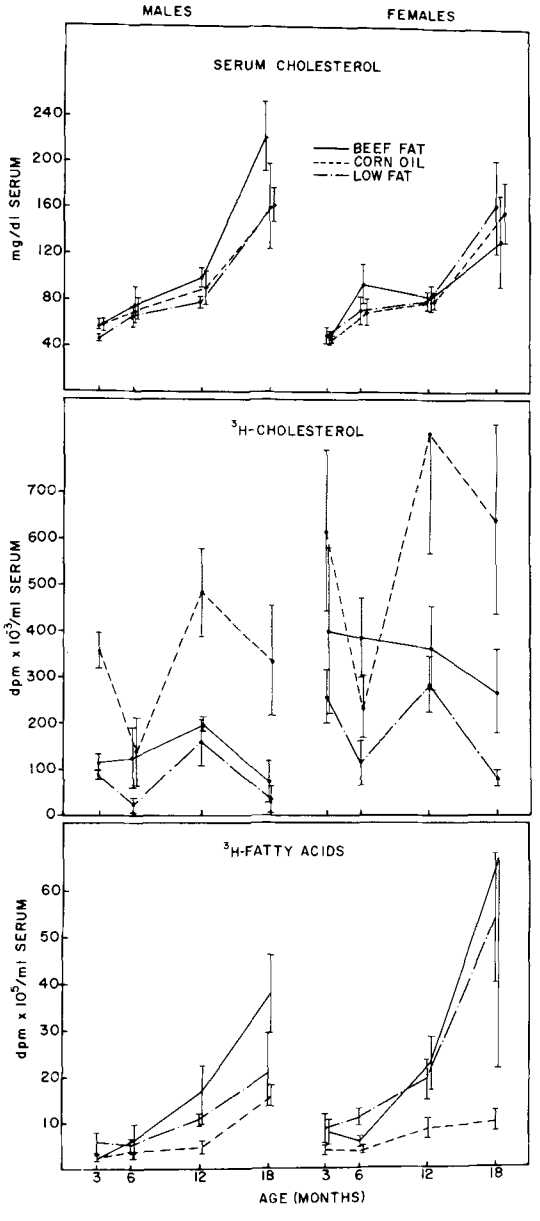


FIG. 3. Serum cholesterol concentrations and $^3$H-cholesterol, and $^3$H-fatty acids from in vivo administered $^3$H-acctate of rats fed low fat, high beef tallow or high corn oil diets for 3, 6, 12 or 18 months. Points indicate mean ± standard error of the mean for four to six animals. Statistical analysis is discussed in Results.

#### Growth, Food Intake and Liver Lipid Composition

The data in Table III show final mean body weights of all the groups. Food intake data are mean daily consumption determined over a 6 day period immediately prior to termination. Body weights increased with age throughout the experiment, but food intake did not vary with age in a consistent manner. Body weight differences were significant ($P<.01$) for age, sex and diet. Calculation of calorie intake per 100 g body wt (not in Table) shows a decline from 3 to 6 and 6 to 12 months, but no change at 12 to 18 months in both sexes. The sex difference in food consumption can be attributed to difference in body weight, although there was a slightly greater cal/100 g body wt in females than males for all ages and diets.

The males fed BT ate more cal/100 g body wt at every age than those fed LF or CO which consumed similar calories. The difference was from 8 to 21% at different ages. There was no such difference in females. This indicates a greater food efficiency for LF and CO than BT diet.

Liver weights were proportional to body weights. Differences were significant for age, sex ($P<.01$) and diet ($P<.05$). Total lipid in liver was proportional to liver size. The values for per cent of lipid were similar and indicated normal liver lipid concentrations. Both total cholesterol per liver and per gram of liver differed significantly for age, sex and diet. There was a general increase with age in both sexes which was greater than increase in liver size. The rats fed CO had higher liver cholesterol than BT or LF in males at 3, 6 and 12 months, but BT equalled CO at 18 months. Females fed CO had higher liver cholesterol than LF or BT at 3 months and 12 months but not at 6 or 18 months. BT values were generally similar to LF for both sexes.

#### Cholesterol Synthesis

Incorporation of ip administered $^3$H-acetate into liver cholesterol is shown in Figure 1. There was no significant sex difference in total liver cholesterol synthesis, but the specific activity (dpm/mg cholesterol) was significantly greater in females than males ($P<.05$). This is due to lower total amounts of cholesterol in conjunction with similar values for acetate incorporation. There was a significant difference due to diet ($P<.05$). This is shown in Figure 1 by the comparison of CO to LF and BT in males of all ages. There was also a significant effect of age, probably due to the great fluctuations shown by CO females. A possible explanation for the general decreases between 3 and 6 months may be continuing developmental changes. Sullivan et al. (11) have shown decreasing synthesis of fatty acids in female rats from 120 to 300 g. The 300 g rats in their experiment would be comparable to the 6-month-old rats in this experiment. The subsequent increase in cholesterogenesis between 6 and 12 months followed by a decrease from 12

TABLE IV

"Carcass" Cholesterol and Fatty Acid Concentration and Labeling from $^3$H-Acetate in Rats 6 and 18 Months of Age[a]

| Age, months | Diet | Fatty acids | | | | Cholesterol | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Total, g | Per cent of carcass | $^3$H Total, dpm, X10-7 | $^3$H Per g fatty acid, X10-3 | Total, mg | Mg per 100 g carcass | Per g fatty acid | $^3$H Total, dpm, X10-6 | $^3$H per g cholesterol |
| Males | | | | | | | | | | |
| 6 | LF[b] | 62.6 ± 8.5[c] | 17.8 ± 1.7 | 153 ± 13 | 274 ± 48 | 315 ± 21 | 91 ± 5 | 5.4 ± 0.7 | 53 ± 17 | 170 ± 56 |
| | BT | 72.2 ± 5.4 | 19.4 ± 1.1 | 77 ± 32 | 96 ± 34 | 299 ± 23 | 81 ± 6 | 4.3 ± 0.5 | 70 ± 17 | 250 ± 69 |
| | CO | 77.2 ± 5.6 | 20.2 ± 1.1 | 31 ± 8 | 40 ± 10 | 304 ± 26 | 80 ± 5 | 4.0 ± 0.3 | 66 ± 20 | 208 ± 54 |
| 18 | LF | 97.8 ± 13.1 | 23.9 ± 2.0 | 260 ± 82 | 303 ± 12 | 316 ± 32 | 78 ± 7 | 3.4 ± 0.4 | 44 ± 10 | 143 ± 34 |
| | BT | 195.1 ± 4.3 | 38.0 ± 1.2 | 77 ± 12 | 39 ± 7 | 317 ± 25 | 61 ± 4 | 1.6 ± 0.1 | 52 ± 13 | 170 ± 48 |
| | CO | 166.4 ± 10.4 | 34.4 ± 1.2 | 67 ± 15 | 46 ± 12 | 342 ± 37 | 71 ± 7 | 2.1 ± 0.2 | 98 ± 19 | 312 ± 74 |
| Females | | | | | | | | | | |
| 6 | LF | 37.4 ± 2.0 | 18.0 ± 0.7 | 174 ± 16 | 465 ± 32 | 181 ± 14 | 89 ± 10 | 5.0 ± 0.7 | 12 ± 2 | 70 ± 14 |
| | BT | 55.6 ± 8.7 | 23.7 ± 2.4 | 27 ± 6 | 49 ± 8 | 170 ± 13 | 76 ± 7 | 3.5 ± 0.6 | 23 ± 4 | 149 ± 32 |
| | CO | 49.2 ± 6.5 | 22.4 ± 1.7 | 26 ± 4 | 53 ± 5 | 191 ± 14 | 90 ± 8 | 4.1 ± 0.5 | 18 ± 2 | 98 ± 13 |
| 18 | LF | 107.3 ± 7.9 | 36.0 ± 1.5 | 245 ± 24 | 227 ± 11 | 189 ± 15 | 65 ± 7 | 1.8 ± 0.3 | 16 ± 4 | 88 ± 20 |
| | BT | 139.0 ± 22.9 | 39.1 ± 1.8 | 95 ± 22 | 76 ± 24 | 188 ± 14 | 56 ± 8 | 1.5 ± 0.3 | 37 ± 15 | 208 ± 99 |
| | CO | 95.6 ± 7.7 | 35.1 ± 1.8 | 25 ± 18 | 27 ± 2 | 196 ± 16 | 73 ± 6 | 2.1 ± 0.2 | 20 ± 4 | 105 ± 23 |

[a]See Methods for definition of "carcass."

[b]LF = Low fat; BT = beef tallow; CO = corn oil; see Table I.

[c]Mean ± standard error of the mean of four to six animals. Statistical analysis discussed in results.

to 18 months is not clearly understood. While variations among animals was great, it seems likely that the age fluctuations are real in view of the repetition for sex and diet groups.

**Fatty Acid Synthesis**

$^3$H-Acetate incorporation into liver fatty acids is shown in Figure 1. The apparent increase with age was much greater than either the increase in liver size or in liver fat (Table III). Analysis of variance suggested significant differences among age, sex and dietary effects.

Separate statistical analyses of the male and female data reveals statistically significant linear differences in fatty acid synthesis with respect to age (3,6, 12 and 18 months; $P<.001$ for males and $P<.001$ for females, Fig. 2). Analysis also indicates that the linear effect of fatty acid synthesis with respect to age cannot be assumed to be the same for all three diets in each of the male and female groups (linear age by diet interaction significance: $P = .0461$ for males and $P = .0493$ for females). Because of the heterogeneous observed variation at different ages it would be inappropriate to use the observed straight line relationship as a means of predicting fatty acid synthesis with age.

Statistical analysis also points out that the diet effects cannot be assumed to be the same for the male and female groups. In view of the importance of the linear (fatty acid synthesis on age) by diet interaction, attention is directed to diet effects at each of the 3, 6, 12 and 18 month age groups (Fig. 1). In males mean responses for LF and CO are statistically different ($P<.05$) at age 3 months and 12 months. LF and BT means are statistically different at 3 months if the highest observation (401) is deleted from the LF data. In females diet means for LF and CO are statistically different ($P = .05$) at ages 3, 6, 12 and 18 months. The mean of LF is different from BT at 6 months. (Note: For the 18 month females, means of BT and CO were not declared statistically different mainly due to the wide range of observation variability occurring in the CO group.)

These data can be summarized to indicate that fatty acid synthesis is less when fat is consumed than when carbohydrate is the major energy source in young rats. As the rats age the response to dietary beef fat is lost in males by 12 months and in females by 18 months. Corn oil has an effect different from beef fat. Males do not lose the response to corn oil feeding until 18 months, and females still respond at 18 months.

**Serum Lipids**

Serum cholesterol concentrations and $^3$H-cholesterol and fatty acids in serum are described in Figure 3. The most striking observation is the age-related increase in serum cholesterol (significant $P<.01$). Between 12 and 18 months of age, rats of both sexes and on all diets doubled their serum cholesterol concentration. The overall analysis of variance indicates no significant sex or diet differences. The BT fed male rats, however, were consistently higher than LF or CO. The large individual variation at 18 months probably precluded statistical significance. The CO group had somewhat lower serum cholesterol than BT at 18 months, but LF was not different from either fat-fed group. Diet was clearly not significant as a factor in the females. Despite the lack of statistical significance, the females tended to have lower serum cholesterol concentrations than males, at all ages except 6 months.

Cholesterol-$^3$H in serum is indicative of transport of newly synthesized cholesterol from liver and possibly gut. The pattern shown in Figure 3 for serum is in most respects similar to that seen in liver. Sex and diet differences were significant ($P<.01$) but age was not. The BT females' serum $^3$H-cholesterol pattern differed from their liver pattern in that there was an apparent increase with age in the liver values but a decrease with age in serum values. This was probably due to cholesterol concentration because specific activity (dpm/mg) of serum $^3$H-cholesterol was significantly affected by age, sex and diet. ($P<.01$, data not shown.)

The data for $^3$H-fatty acids in serum are shown in Figure 3. They indicate transport of newly synthesized fatty acids from liver and possibly other tissues. Age is the most significant ($P<.01$) factor in the amount of $^3$H-fatty acids found in serum 2.5 hr after $^3$H-acetate injection. Diet and sex also have significant effects ($P<.05$). It is clear that CO diet, which depressed acetate incorporation into fatty acids in the liver, resulted in a lower concentration of newly synthesized fatty acids in the serum. The diet effect was more pronounced in females than males, but not sufficiently different to result in a significant statistical diet by sex interaction effect.

Cholesterol-$^3$H and fatty acid-$^3$H in serum are inversely related in CO rats of both sexes. The LF and BT groups are quite similar after 6 months of age, except for 18 month females.

**"Carcass" Lipids**

The "carcass" data are for that portion of the rat's body left after removal of the head, blood, liver, heart and gastrointestinal tract. Weights of the "carcasses" were proportional to body weights shown in Table III. Age, sex and

TABLE V

Location of $^{14}C$ Derived from 4-$^{14}C$-Cholesterol
9 Days After Intraperitoneal Administration to Rats 6 and 18 Months of Age

| | | dpm x $10^{-6}$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | "Carcass" | | |
| Age | Diet | Serum | Liver | Gut | Neutral | Acidic | Feces |
| Males | | | | | | | |
| 6 | LF[a] | 1.2 ± 0.3[b] | 63.7 ± 10.8 | 73.0 ± 6.4 | 389.8 ± 114.7 | 126.1 ± 24.5 | 103.8 ± 19.4 |
| | BT | 1.5 ± 0.3 | 62.8 ± 10.7 | 81.9 ± 7.8 | 465.4 ± 63.0 | 255.8 ± 76.0 | 96.5 ± 16.2 |
| | CO | 1.6 ± 0.3 | 84.8 ± 10.0 | 78.2 ± 10.7 | 453.0 ± 57.4 | 255.7 ± 71.7 | 125.8 ± 17.9 |
| 18 | LF | 3.1 ± 0.9 | 86.1 ± 17.3 | 109.4 ± 16.0 | 460.8 ± 88.8 | 428.6 ± 118.6 | 139.5 ± 38.7 |
| | BT | 3.8 ± 0.3 | 97.0 ± 9.6 | 120.2 ± 16.5 | 656.6 ± 128.0 | 716.8 ± 46.2 | 125.8 ± 28.8 |
| | CO | 2.7 ± 0.4 | 90.1 ± 14.6 | 87.2 ± 23.0 | 552.0 ± 36.5 | 574.2 ± 134.2 | 113.1 ± 7.0 |
| Females | | | | | | | |
| 6 | LF | 1.3 ± 0.3 | 33.0 ± 5.2 | 58.4 ± 9.3 | 250.1 ± 14.2 | 95.0 ± 15.4 | 79.5 ± 21.4 |
| | BT | 0.9 ± 0.2 | 16.4 ± 3.8 | 40.6 ± 7.7 | 175.5 ± 36.2 | 133.6 ± 51.0 | 88.2 ± 13.9 |
| | CO | 0.8 ± 0.2 | 26.4 ± 4.1 | 48.6 ± 7.2 | 234.2 ± 28.5 | 105.0 ± 17.6 | 93.1 ± 13.6 |
| 18 | LF | 2.5 ± 0.6 | 45.8 ± 6.4 | 53.0 ± 6.6 | 322.6 ± 52.6 | 320.5 ± 83.5 | 80.2 ± 23.2 |
| | BT | 2.5 ± 1.0 | 45.6 ± 13.6 | 82.2 ± 13.8 | 300.8 ± 42.2 | 410.7 ± 137.4 | 108.3 ± 15.2 |
| | CO | 1.8 ± 0.3 | 23.7 ± 2.2 | 54.1 ± 4.3 | 278.2 ± 24.8 | 216.5 ± 35.5 | 97.1 ± 18.9 |

[a]LF = Low fat; BT = beef tallow; CO = corn oil; see Table I.

[b]Mean ± standard error of the mean for four to six animals. Statistical analysis discussed in results.

diet all caused significant differences ($P<.001$). Within males there was a significant interaction between diet and age ($P = .034$) caused by the fact that LF diet caused no difference in "carcass" weight between 6 and 18 months, whereas both CO and BT groups "carcass" weights were significantly different ($P<.05$) between 6 and 18 months of age. In females age resulted in significantly different "carcass" weights ($P<.001$), but there was no diet by age interaction. The only significant dietary effect was that the BT group was heavier at 18 months than CO or LF groups ($P = .015$).

Total fatty acids in the "carcass" (Table IV) varied significantly with age, sex ($P<.001$) and diet ($P<.05$), both in quantity and as per cent of carcass. Analysis of male and female data separately revealed that dietary differences were much greater for males ($P<.001$) than for females ($P = .022$ for total fat and $P = .065$ for per cent fat). There was a significant age by diet interaction for males ($P<.001$) but not for females. The interaction resulted from the fact that LF males did not have significantly greater total fat or per cent fat at 18 than at 6 months, while CO and BT groups did have greater values for both factors ($P<.05$). The CO and BT groups did not differ from each other. Females fed LF increased in total and per cent body fat between 6 and 18 months and were not different in this respect from CO or BT groups. The BT group had significantly more body fat than LF or CO, but the percentage was not significantly different.

Newly synthesized fatty acids were estimated by presence of $^3H$ from $^3H$-acetate administration. There were significant sex differences, and the data were analyzed separately for each sex. The data shown (Table IV) are total activity for the whole carcass and specific activity per gram of fatty acid. Activity per gram of carcass weight closely paralleled total activity with similar statistical results in most instances, so those data are not shown. In males there was no significant effect of age, but diet had an effect ($P<.001$). The diet effect was that the LF group at 18 months was higher ($P<.05$) than all other groups at either age and all others were similar to each other.

In females the differences were more complex. Both age and diet had significant effects ($P<.001$) on total $^3H$-fatty acids. Expressed as $^3H$-fatty acids per gram of body weight, age was not significant, but diet was ($P<.001$). The differences between diets in $^3H$-fatty acids per gram body weight were LF at both ages were higher ($P<.001$) than BT and CO at both ages, and the latter did not differ from each other. Total $^3H$-fatty acids were affected by diet similarly to effect as per gram of body weight, except that the BT group at 18 months was not different from the LF group at 6 months. While this suggests that BT and CO were different at 18 months, the difference does not reach significance, probably due to the small number of observations on BT rats. When $^3H$ per gram fatty acid is observed, LF is significantly different from BT and CO which do not differ from each other. There is also a difference between 6 and 18 months of age within the LF diet, with the older group having a significantly lower specific activity than the younger group.

Total "carcass" cholesterol differed between sexes. The entire difference appears to be due to body size, because there is no age or diet difference. In view of differences in "carcass" fat and other parameters, the similarity of total "carcass" cholesterol within sex groups is remarkable. The only observed difference is the BT group had lower ($P<.05$) cholesterol per 100 g "carcass" weight than LF or CO in females. The concentration of carcass cholesterol was not proportional to fatty acid concentration. Since lean body mass was not determined in this study, it can be assumed only that cholesterol concentration may be associated with lean body mass, and changing fat content with age or diet does not affect the cholesterol to lean body mass ratio. The bovine apparently has the greatest concentration of cholesterol and cholesterol esters in serum, followed by intramuscular lipids, with adipose containing very small amounts (12).

The presence of newly synthesized cholesterol in the carcass is shown by $^3H$-cholesterol derived from $^3H$-acetate. There was a significant sex difference in this measurement. The only other difference is a significant ($P = .03$) diet effect in females that appears to be due to higher values for BT than LF or CO groups. In general the differences in $^3H$-cholesterol parallel the differences in total cholesterol, except for the BT groups of females which vary inversely.

#### Recovery of 4-$^{14}$C-Cholesterol

Feces and tissues were analyzed for $^{14}C$ derived from 4-$^{14}$C-cholesterol administered ip 9 days prior to termination. The assumption was made that none of the $^{14}C$ could be lost from steroid ring compounds. Feces and gastrointestinal tracts plus contents were analyzed without any extraction procedures. Livers and sera were analyzed in a manner yielding only neutral sterol data. "Carcasses" were analyzed in a manner which revealed that a substantial portion of the $^{14}C$ was present in an acidic form. The data in Table V indicate, therefore,

total recovery of ring labeled steroid, except for that possibly lost from liver and serum because it was acidic.

Significant sex differences were apparent in every parameter. There were no other differences in feces data whether total for 9 days or rate of excretion were analyzed. There were no diet-related differences in any of the parameters. There were significant age differences in all parameters except feces.

Sex and age effects may be due to body size, but it is difficult to conclude that other effects did not exist as well. The most notable observation is the large increase in acidic steroid ($P<.001$) in the carcass with advancing age. Even though neutral sterol increased, the proportion of neutral to acidic decreased.

## DISCUSSION

This study was designed to explore metabolic differences between rats fed high carbohydrate or high saturated or polyunsaturated fat diets for long periods. The differences in mortality among the groups appears to be related to polyunsaturation of fatty acids in the males. Both beef fat and carbohydrate which yields saturated and monounsaturated fatty acids for storage were deleterious compared to corn oil. The females had low death rates during the length of this study, with beef fat appearing to exert a deleterious effect during the later months.

Observations on fatty acid metabolism differed among groups. The increase in acetate incorporation with age in males fed high fat diets is significant. Fat feeding has been reported to decrease acetate incorporation into fatty acids in comparison to carbohydrate (2,13). The rats used in those studies were young adults. Similar results were obtained in this study in rats of both sexes at 3 months of age. Older rats, however, did not respond to fat feeding in the same manner as those 3 months old. There was a gradual increase in acetate incorporation into liver fatty acids in rats of both sexes fed beef tallow. The rise began earlier in males than females. Corn oil had an effect different from beef tallow at ages greater than 3 months. Not until males were 18 months old did corn oil cease to depress acetate incorporation. In females corn oil depressed acetate incorporation into fatty acids at all ages. These age and sex differences may explain some of the discrepancies in observed effects of different fatty acids on rats as reviewed by Abraham (13).

In assessing these results, the question arises as to whether acetate incorporation into liver fatty acids represents de novo synthesis or chain elongation. Filipovic and Buddecke (14) have reported increased fatty acid synthesis from acetate in arterial tissue of calves when the tissue was incubated under $N_2$ rather than $O_2$. They degraded the fatty acids and found that chain elongation was the predominant mode of incorporation of acetate. Fatty acid degradation was not performed in our experiment. A study of fatty acid oxidation was conducted on tissues of these rats, and the results will be reported elsewhere. Comparison was made between oxidation rates of carboxyl and uniformly labeled fatty acids in liver and heart tissue in vitro. In all cases carboxyl-labeled fatty acids were oxidized ca. 160% as rapidly as uniformly labeled fatty acids. These results and reports by Mounts et al. (15) and Huxtable and Wakil (16), showing differences in metabolism of carboxyl and uniformly labeled fatty acids, suggest that exchange of acetyl units was occurring in addition to complete oxidation of the fatty acids. Acetate incorporation into fatty acids of the carcass did not follow the same pattern as in liver and serum. Both fats depressed it at both 6 and 18 months in both sexes. This suggests that the $^{3}H$-fatty acids in carcass were synthesized at the site. If loss of depressive effect of fat feeding on fat synthesis in liver is related to an increased rate of chain elongation, then this appears not to have occurred in extrahepatic tissue. These lines of evidence suggest that aging rats fed beef fat may have exhibited increased rates of chain elongation by the liver due to the presence of excess fatty acids and acetyl groups and possibly mild hypoxic conditions.

Cholesterol metabolism in aging rats has been studied by a few investigators (17-20). The several studies cited differ in ways which cannot be evaluated because of inadequate descriptions of conditions of the experiments. Acetate incorporation into cholesterol and fecal excretion of $^{14}C$-cholesterol have been reported to decrease with age (20), but no data were available for rats between 4 and 18 months of age. The data obtained in our study indicate that there was no reduction of cholesterol synthesis from acetate from 3 to 18 months. An oscillating effect was observed with peaks at 3 and 12 months and low points at 6 and 18 months. This may have been observed by Trout et al. (19). Excretion of ring-labeled cholesterol over 9 days did not decrease between 6 and 18 months of age.

In order to evaluate the relative amounts of 4-$^{14}C$-cholesterol excreted and retained in different tissues, the summary shown in Figure 4 was prepared. The total $^{14}C$-cholesterol recov-

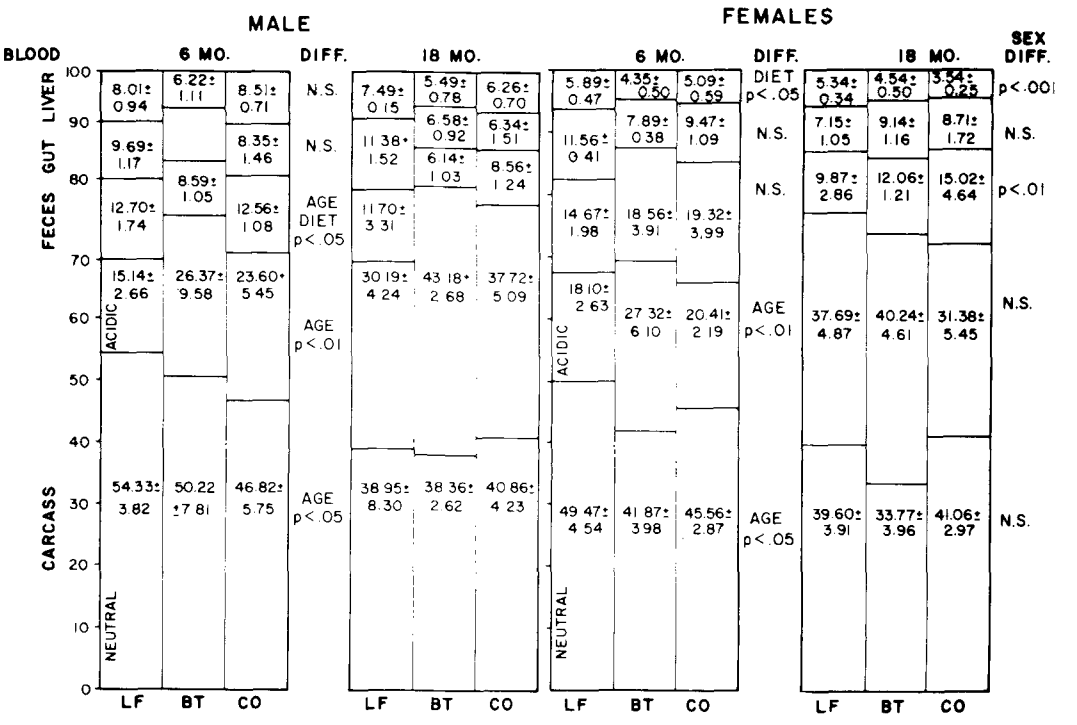


FIG. 4. Proportional distribution of 4-$^{14}$C-cholesterol recovered after 9 days in "carcass," feces, gut, liver and blood of male and female rats fed low fat (LF), beef tallow (BT) or corn oil (CO) diets (Table I) from weaning to 3, 6, 12 or 18 months of age. Figures in bars indicate mean ± standard error of the mean of three to six rats. See Methods for definition of "carcass." Statistical analysis is discussed in Discussion.

ered was considered 100%, and portions thereof in various tissues were calculated. Only 6- and 18-month-old rats were included because of lack of carcass data for the other age groups. By this interpretation of the data, age, diet and sex all affected fecal excretion of $^{14}$C-cholesterol significantly. Less was excreted by older rats than younger, males than females and BT than CO or LF in males.

The presence of acidic steroid in the carcass is notable. This fraction constituted 15-43% of the total recovered $^{14}$C. Age was the major factor affecting the proportion of acidic steroid found, with the older rats having much more than younger rats. The question of whether the acidic fraction originated in extrahepatic tissues or was transported from the liver or gut cannot be answered from these data. It is unlikely that the large proportion found in the carcass originated in liver and was transported to extrahepatic tissues. With only one time point (9 days) represented in this study, it is not possible to draw conclusions regarding rates of metabolism of cholesterol. If one assumes that cholesterol turnover involves neutral cholesterol → acidic steroid → fecal steroids (Fig. 4), then the older rats clearly had slower turnover of cholesterol than the younger rats. Acidic to fecal transfer would appear to be the slower of those steps.

Sex and diet significantly affected fecal and liver but not carcass $^{14}$C recovery. Sex and diet also affected acetate incorporation into liver cholesterol. Age more strongly affected carcass sterol metabolism. Studies conducted with humans have measured only serum and fecal sterols, and bile acids (4,21). The methods of analysis of neutral sterol in serum have precluded finding evidence of acidic steroid. In this study and one other study where retention of ring-labeled cholesterol was determined after 8 days (22), substantial concentrations of acidic steroid were found in extrahepatic tissue. It is possible that the loss of ring-labeled sterol reported by Grundy et al. (5) was due to transformation to acidic steroid.

The measurements of lipid metabolism reported in this study do not account for the mortality differences among groups of rats. The findings in relation to control of fatty acid synthesis and to acidic steroid production in extrahepatic tissue suggest new aspects of lipid metabolism in relation to aging and dietary fat which will be valuable to explore further.

Identification of the acidic steroid(s) is in process.

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# Comparative Effect of a Protein Diet on the Desaturation, Elongation and Simultaneous Desaturation and Elongation of Linoleic Acid

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## ABSTRACT

The effect of a protein diet on the biosynthesis of polyunsaturated fatty acids of the linoleic acid family was studied by incubation of rat liver microsomes with labeled linoleic acid. The incubation was performed in desaturating, elongating and desaturating-elongating conditions. In desaturating conditions, linoleic acid was converted to γ-linolenic acid, whereas in elongating conditions it was converted to 20:2, 22:2 and 24:2. In desaturating-elongating conditions, labeling was found in γ 18:3, 20:2, 20:3, 20:4 and 22:2. A protein diet increased the oxidative desaturation of linoleic acid to γ-linolenic and arachidonic acid biosynthesis, whereas the elongating reaction was not enhanced in the experimental conditions tested. It is suggested that the main controllable step in the linoleic acid family is the oxidative desaturation of linoleic acid to γ-linolenic acid.

## INTRODUCTION

Liver microsomes are able to elongate and desaturate unsaturated fatty acids synthesizing higher members of the different fatty acid series (1-6). Linoleic acid can be converted to arachidonic and docosa-4,7,10,13,16-pentaenoic acid. Moreover different sequences of reactions may lead to the same final products. Stoffel and Ach (6) have shown that linoleic acid may be either desaturated to γ-linolenic acid or elongated to eicosa-11,14-dienoic acid. However Marcel et al. (7) have found that the preferential pathway for arachidonic acid biosynthesis is via γ-linolenic. This acid is subsequently elongated to eicosa-8,11,14-trienoic acid and then desaturated to arachidonic acid. Besides, Ullman and Sprecher (8) have recently found that eicosa-11,14-dienoic acid may be desaturated by rat liver microsomes in a reduced extent. However the product is eicosa-5,11,14-trienoic acid. This acid would not be further desaturated to arachidonic acid.

Among all these reactions that lead to the biosynthesis of polyunsaturated acids, the oxidative desaturation of linoleic acid to γ-linolenic acid is highly dependent on the diet (9). The enhancing effect of a protein diet on the desaturation of linoleic acid has been very well studied and established (10,11). The purpose of the present work is to learn to what extent a protein diet may also modify the elongation reactions and therefore to what extent they may play a regulatory function in the cell. For this reason the effect of a protein diet was studied on the microsomal desaturation, elongation and simultaneous desaturation and elon-

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TABLE I

Chain Lengthening and Oxidative Desaturation of $^{14}$C-1-Linoleic Acid[a]

| | Fatty acids | Distribution of radioactivity % | |
|---|---|---|---|
| | | Normal diet | Protein diet |
| Elongation | 18:2 | 92.3[b]±0.5 | 93.6±0.4 |
| | 20:2 | 2.4 ±0.1 | 2.3±0.1 |
| | 22:2 | 4.3 ±0.3 | 3.2±0.3 |
| | 24:2 | 1.0 ±0.2 | 0.9±0.1 |
| Desaturation | 18:2 | 79.5 ±2.5 | 66.9±2.2 |
| | 18:3 | 20:5 ±1.2[c] | 33.1±1.6[c] |

[a]Ten nmoles of $^{14}$C-1-linoleic acid were incubated with 2 mg microsomal protein at 25 C during 20 min with the cofactors detailed in Materials and Methods.
[b]Means ± one standard error of the mean of duplicate samples from three animals.
[c]$P < 0.01$.

TABLE II

Simultaneous Chain Lengthening and Desaturation of $^{14}$C-1-Linoleic Acid[a]

| Fatty acids | Distribution of radioactivity % | | P |
|---|---|---|---|
| | Normal diet | Protein diet | |
| 18:2 | 76.2[b]±0.6 | 62.4±2.7 | <0.01 |
| 18:3 | 7.0 ±1.5 | 22.1±1.9 | <0.01 |
| x | 3.2 ±0.6 | 2.2±0.7 | NS[c] |
| 20:2 | 5.1 ±0.6 | 1.8±0.2 | <0.01 |
| 20:3 | 1.8 ±0.2 | 2.2±0.2 | NS |
| 20:4 | 1.8 ±0.2 | 7.1±0.9 | <0.01 |
| 22:2 | 4.9 ±0.3 | 2.2±0.2 | <0.01 |

[a]Ten nmoles $^{14}$C-1-linoleic acid were incubated with 2 mg microsomal protein at 25 C during 20 min with the cofactors detailed in Materials and Methods.

[b]Each value is the average in duplicate from three animals ± one standard error of the mean.

[c]Not significant.

gation of linoleic acid.

## MATERIAL AND METHODS

### Animals

Adult female Wistar rats weighing 250-300 g and maintained on standard Purina chow were used.

The animals were divided into two groups of three animals each. One group was fed a Purina chow diet and water ad libitum and was used as a control. The other group was force fed a 20% suspension of casein (Casenolin, Glaxo-Argentina) for 48 hr. These animals received isocaloric diet (25 kcal/100 g body wt), and the total daily food intake was administered accordingly at 4 hr intervals. Water was given ad libitum.

### Isolation of Microsomes

The rats were killed by decapitation without anesthesia. The liver was rapidly excised and placed immediately in ice cold homogenizing medium. After homogenization the microsomes were separated by differential centrifugation by the procedure described in a previous work (9).

### Incubation Procedure for Chain Elongation

The elongation of linoleic acid by liver microsomal preparations was measured by estimating the conversion of $^{14}$C-1-linoleic acid (53.0 mCi/mmole; 98% radiochemically pure, Radiochemical Centre, Amersham, England) to higher homologs. The basic incubation medium contained 4 μmoles ATP, 2.5 μmoles NADH, 0.2 μmoles CoA, 15 μmoles $MgCl_2$, 4.5 μmoles glutathione, 125 μmoles NaF, 1 μmole nicotinamide, 125 μmoles phosphate buffer (pH 7), 3 μmoles KCN, and 0.1 μmoles malonyl-CoA in a total volume of 3.0 ml of 0.15 M KCl and 0.25 M sucrose solution. Then 10 nmoles labeled linoleic acid were incubated anaerobically with 2 mg microsomal protein in a Dubnoff shaker at 25 C for 20 min under argon atmosphere.

### Incubation Procedure for Oxidative Desaturation

The desaturation of linoleic acid to γ-linolenic acid by microsomes was measured by estimation of the per cent conversion of 10 nmoles $^{14}$C-1-linoleic acid to γ-linolenic acid according to the procedure described in a previous work (9). Then 2 mg microsomal protein were incubated during 20 min at 25 C in air.

### Incubation Procedure for Simultaneous Elongation and Desaturation

The microsomes were incubated in the same medium and conditions used for desaturation with the addition of 3 μmoles of malonyl-CoA.

### Analysis of the Fatty Acids and Identification of Incubation Products

At the end of the incubation the fatty acids were recovered by saponification of the incubation mixture. The acids were esterified with methanolic 3 M HCl (3 hr at 68 C), and the distribution of the radioactivity between linoleic acid and γ-linolenic acid or the elongation products, or both, was determined by gas liquid radiochromatography in a Pye apparatus with a proportional counter (12). The methyl fatty acids were identified by equivalent chain length determination and comparison with authentic standards. The structure of the products of linoleic acid desaturation had already been proved by ozonolysis and reduction (12).

## RESULTS AND DISCUSSION

As shown in Table I, rat liver microsomes are

able to elongate linoleic acid to 20:2, 22:2 and 24:2 when incubated in strictly elongating conditions. The same microsomes are also able to desaturate the same acid to γ-linolenic acid when incubated in strictly desaturating conditions. However Table I also shows that both reactions are not equally affected by administration of casein to the rats during 48 hr. Whereas linoleic acid conversion to γ-linolenic acid is enhanced very significantly by this diet, the elongation to either 20:2, 22:2 or 24:2 is not changed. Therefore these results show that the 6-desaturation of linoleic acid is a step that may be regulated by the protein of the diet, whereas the elongation is insensitive to the same control.

These results suggest that a protein diet may also alter the biosynthesis of arachidonic acid when a microsomal system able to desaturate and elongate linoleic acid is tested, and that this modification may be evoked by the desaturation reaction and not by the elongation. Table II confirms this prediction and not only shows that the incubation of labeled linoleic acid in the presence of malonyl-CoA, NADH and $O_2$ synthesized γ-18:3, 20:3, 20:4, 20:2 and 22:2, but also that a protein diet increases significantly the labeling of γ-linolenic and arachidonic acid at the partial expense of a decreased labeling of 20:2 and 22:2. Therefore the synthesis of polyunsaturated fatty acids of the linoleic family may be enhanced by a protein diet through a specific effect on the oxidative desaturation of linoleic acid to γ-linolenic acid and not by an increase of the elongation.

The fact that a protein diet increases the labeling of 20:4, whereas the radioactivity of 20:3 is not changed, suggests that the 5-desaturation of 20:3 to arachidonic acid is also enhanced by the protein diet. This conclusion is coincident with the increase of the 5-desaturation of eicosa-8,11-dienoic acid to eicosa-5,8,11-trienoic acid proposed by Castuma et al. (13) by administration of a casein diet to rats. Therefore the 5-desaturation of 20:3 to arachidonic acid would be a secondary regulating step in the total biosynthesis of the polyunsaturated fatty acids of the linoleic acid series; similarly, 5-desaturation of eicosa-8,11-dienoic acid to eicosa-5,8,11-trienoic acid would be a secondary regulating step in the biosynthesis of fatty acids of the oleic family.

In this experiment, chain lengthening and desaturation of fatty acids are shown to be controlled by quite different mechanisms. In this respect, Lyman et al. (13) have also shown that ehtionine inhibits rather specifically the acyl desaturases but not the elongation reactions. This inhibition has been attributed to a specific effect of ethionine on protein synthesis and would be in agreement with the results obtained in the present experiment.

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# Lysolecithin Acyltransferase in Hamster Intestinal Mucosa

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## ABSTRACT

1-Acyl-lysolecithin acyltransferase has been demonstrated in the microsomal fraction of hamster intestinal mucosa. The characteristics of the enzyme, with respect to substrate concentration, time of incubation and protein concentration, were studied. $Ca^{++}$ was found to severely inhibit enzymatic activity. More modest inhibitors were found to be $Mg^{++}$ and $F^{-}$; EDTA and albumin had no effect. Enzyme activity was reduced when palmityl CoA was substituted for oleoyl CoA as substrate. The specific activity of intestinal microsomes was modestly greater than liver microsomes.

## INTRODUCTION

Dietary lecithin (3-*sn*-phosphatidylcholine) is rapidly hydrolyzed by pancreatic phospholipase A (E.C. 3.1.1.4) within the upper intestinal lumen (1). The resultant 1-acyl-lysolecithin (1-acyl-*sn*-glycerol-3-phosphorylcholine) has been demonstrated by a multiple labeling technique (2) to be absorbed intact and reacylated to lecithin within the intestinal mucosa. Although the presence of 1-acyl-lysolecithin acyltransferase (acyl-CoA: 1-acyl-*sn*-glycerol-3-phosphorylcholine acyltransferase), which catalyzes this reaction, was well established in the liver of a variety of species (3), a direct demonstration of activity in intestinal mucosa at first failed (4). Subsequently a brush border free preparation of rat intestine was found to have 1-acyl-lysolecithin acyltransferase activity (5). This report confirms the presence of this enzyme in intestinal mucosa of the hamster and describes several of its properties.

## MATERIALS AND METHODS

### Substrate Preparations

Crude lecithin was prepared from egg yolks and further purified by chromatography on a neutral aluminum oxide column (BioRad Lab.) as suggested by Hanahan et al. (6). The lecithin was then hydrolyzed by phospholipase A (*Crotalus adamanteus* venom, Sigma Chemical Co.) to form 1-acyl-lysolecithin (7). The isolated product was purified by chromatography on a silicic acid column (BioRad Labs., -325 mesh) with chloroform-methanol 80:20 v/v as the eluent as described by Lea et al. (8). After lecithin had been eluted from the column, lysolecithin appeared in the column effluent. The fractions containing lysolecithin were combined and concentrated under reduced pressure in a $N_2$ atmosphere on a rotary evaporator (Rinco Instrument Co.) at 40 C. The final product migrated as a homogenous band on analysis by thin layer chromatography (TLC) with an $R_f$ of .18 which was equal to that of a sample of commercial lysolecithin (Sigma Chemical Co.) and had a percentage composition of phosphorus (9) of 5.7 (theoretical 5.9). The lysolecithin was dissolved in chloroform and stored at -15 C.

(1-$^{14}$C) Oleoyl-CoA was prepared as described for palmityl CoA (10) from (1-$^{14}$C) oleic acid (ICN Corp.) and Coenzyme A (Calbiochem). The final product was dissolved in

TABLE I

The Effect of Various Additions on Lysolecithin-Acyltransferase Activity[a]

| Addition | (1-$^{14}$C) Fatty acid incorporated into lecithin, mμmoles/min/mg protein |
|---|---|
| None | 9.08 |
| EDTA, 10 μmoles | 9.36 |
| NaF, 20 μmoles | 7.40 |
| $CaCl_2$, 10 μmoles | 2.14 |
| $MgCl_2$, 10μmoles | 5.60 |
| Albumin, 0.5 mg | 9.16 |
| Palmityl CoA, 40 mμmoles | 5.48 |

[a]The incubation conditions were similar to those described in the text, except that additions to the incubation mixture were made as shown. When palmityl CoA was included in the incubation, oleoyl CoA was deleted.

water (final pH 5.5) and assayed at 234 mμ and 260 mμ. The specific activity was 581,548 cpm/μm. (1-$^{14}$C) Palmityl CoA was obtained from ICN Corp. and palmityl CoA from Schwarz-Mann.

All solvents were glass distilled.

#### Enzyme Preparation

Male golden Syrian hamsters weighing ca. 180 g were fasted overnight, sacrificed by dislocation of the cervical vertebrae, and the small intestine was quickly removed. The intestinal mucosa was exposed, cleansed by flushing with cold (2 C) 0.15M NaCl, blotted with tissue paper and removed by careful scraping with a glass microscope slide. The scrapings were collected in a beaker containing 2 ml 0.278 M mannitol in 0.01 M Tris-HCl (pH 7.0), transferred to a Thomas tissue grinder of 10 ml capacity, and the beaker was rinsed with an additional 3 ml of the mannitol Tris solution. The tissue was homogenized with 15 strokes of a blunt Teflon pestle. The homogenate was then poured through two layers of cheese cloth and the microsomal fraction isolated as described by Senior and Isselbacher (11). The microsomes were transferred to a Ten Broeck all glass homogenizer and suspended in 1 ml 0.01 M Tris-HCl (pH 7.0). All operations were carried out at 2 C. An aliquot of 0.05 ml was taken for protein determination.

#### Enzyme Assays and Lipid Extraction

The complete incubation mixture contained in a total volume of 0.5 ml was: 200 mμmoles 1-acyl-lysolecithin (pipetted in chloroform and dried under $N_2$); 100 mμmoles Tris-HCl (pH 7.0) and 40 mμmoles (1-$^{14}$C) oleoyl CoA. Each incubation was performed in duplicate. After incubation at 28 C in a shaker water bath (Research Specialties Co.) at 24 oscillations per minute, the reaction was terminated by the addition of 3 ml 95% ethanol. The lipids were extracted as described by Young and Lynen (10), dried under a stream of $N_2$, redissolved in 0.5 ml chloroform-methanol 2:1 v/v and stored at -15 C. The residual protein from this extraction procedure had less radioactivity than the protein precipitate following lipid extraction by the method of Folch et al. (12) (200 vs. 600 cpm).

#### Analytical Procedures

The phospholipids were separated by TLC using chloroform-methanol-acetic acid-water 150:90:24:12 v/v (13) and identified by exposure to iodine vapors. The gel was allowed to lose its iodine stain, and the lecithin spots were carefully transferred to counting vials. The radioactivity was determined in a Packard Tri-Carb spectrometer (Model 314 EX) using toluene-Cab-O-Sil as the solvent and 2,5 diphenyloxazole and 1,4-bis (2-5-phenyloxazolyl)-benzene as the scintillators. Self quenching with the method has been shown previously to be essentially identical in each sample (14). This was verified by counting 25 samples in a Packard Tri-Carb Model 3375 spectrometer. The external standard ratio was 0.844 ± 0.00806 (SD).

Protein was determined by the method of Lowry et al. (15) using 0.01-0.04 mg crystallized bovine serum albumin (Sigma Chemical Co.) as standards.

## RESULTS AND DISCUSSION

When incubated in the presence of optimal concentrations of both lysolecithin and oleoyl-CoA, enzyme acitivity was directly proportional to the concentration of microsomal protein up to 1.0 mg. Preliminary experiments performed at 37 C showed that enzymatic activity was directly proportional to time for 60 sec; therefore experiments were performed routinely at 28 C. Under these conditions enzyme activity was directly proportional to time for 2 min. In the absence of 1-acyl-lysolecithin, minimal but definite incorporation of oleoyl-CoA was observed indicating the utilization of lysolecithin present in the microsomal particles. As the concentration of 1-acyl-lysolecithin was increased, a maximum reaction rate was reached at a concentration of 400 μM, indicating saturation of the enzyme with respect to this substrate. These results suggest that radiolabeled lecithin formed under these experimental conditions was not due to simple isotopic exchange. When increasing concentrations of oleoyl-CoA were added to the incubation mixture, a maximum reaction rate was reached at a concentration of 80 μM. These characteristics of the enzyme are similar to those reported previously for liver (16,17). In agreement with the work of Van den Bosh et al. (16), the pH optimum was found to be pH 7.0, and in agreement with Lands (17) the microsomal fraction was found to have the highest specific activity.

As shown in Table I, EDTA and albumin did not affect the incorporation of oleoyl-CoA into lecithin, whereas incorporation was greatly inhibited by the addition of $Ca^{++}$. The addition of $Mg^{++}$ and $F^-$ were also found to be inhibitory, although to a lesser extent than $Ca^{++}$. When (1-$^{14}$C) palmityl CoA was substituted in equimolar amounts for (1-$^{14}$C) oleoyl CoA, the acylation of lysolecithin was reduced by 40%. This is consistent with previous work in liver which has shown that the more unsaturated

acyl CoA (oleate) is the preferred substrate over the more saturated acyl CoA (palmitate) in the acylation of 1-acyl lysolecithin (18). However this observation might merely reflect differences in the solubility of the 2 acyl-CoA's used.

Microsomes were isolated from liver and enzyme activity assayed in a manner similar to that used for gut microsomes. Liver microsomes incorporated 5.76 and 5.16 mμmoles of oleoyl-CoA into lecithin per minute per milligram protein in two experiments, whereas gut microsomes from the same animals incorporated 7.72 and 7.36 mμmoles.

The gut acyltransferase pathway of lecithin synthesis described in this report might play an important role in intestinal lecithin synthesis and absorption. Previous studies have shown the conversion of dietary lecithin to 1-acyl-lysolecithin within the intestinal lumen (1) and its incorporation, in toto, into lecithin (2).

ACKNOWLEDGMENT

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# Relationship between Embryonic and Tumor Lipids: I. Changes in the Neutral Lipids of the Developing Chick Brain, Heart and Liver

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## ABSTRACT

Organ dry weight, per cent total lipid, per cent neutral lipid, per cent phospholipid, and neutral lipid class composition of embryonic and mature brain, heart and liver were determined at 10, 13, 16, 19, 22, 27 and 53 days after incubation was initiated. All three tissues showed an increase in total lipids from the 10th day to hatching (21st day). The 10th day brain showed relatively high levels of sterol esters which decreased with increased development while free sterol levels increased. Heart free sterol and sterol ester percentages decreased with increased time, while triglyceride levels increased dramatically after the 16th day. Liver showed a massive accumulation of neutral lipid after the 17th day. The neutral lipid was not triglyceride, as might have been expected, but sterol ester. Liver sterol, sterol ester and triglyceride levels were approximately equal at the 10th and 13th days, after which time sterol ester rose rapidly to more than 90% of the total neutral lipids by the 19th day. The neutral lipid class distributions were characteristically different for each tissue throughout embryonic development. The relative high sterol ester levels in each of the tissues early in development suggests that the high level of sterol esters in neoplastic tissue may be related to the growth process of increasing cell numbers. On the other hand, the absence or the presence of only trace amounts of glyceryl ether diesters in any of the embryonic tissues suggests that the elevated levels of this lipid class in most tumors may be related to the neoplastic process or to conditions resulting from neoplasia.

## INTRODUCTION

Over the last few years I have studied the structure and metabolism of lipids of neoplastic cells, primarily Ehrlich ascites carcinoma cells, to ascertain whether differences exist between normal and neoplastic tissue. The data have indicated a number of basic differences in lipid structure and metabolism between the two tissues (1-5). Numerous other investigators have also observed differences between the lipid metabolism of normal and neoplastic tissue (6-10). Errors in the lipid metabolism of neoplastic cells could affect membrane structure, lipid dependent enzyme systems and other biological processes involving lipids. However not all differences observed between normal and neoplastic tissue can be attributed to errors in lipid metabolism. In addition to any differences in lipid metabolism that may be associated with neoplasia, there are differences associated with rapid cell growth or proliferation. Despite the lack of research in this area, there is adequate evidence that demonstrates the difference in lipid metabolism between the developing embryo and the adult organism (11-13).

There is an accumulation of data that demonstrates the similarities between embryonic tissue and neoplastic tissue. Abelev in 1963 (14) found that hepatomas synthesized and secreted into blood a specific embryonic $\alpha$-globulin which has been confirmed in primary liver tumors of rats, mice and humans (15-16). Isoenzymes of numerous enzymes that predominate in fetal tissue, but are absent or present at very low levels in normal tissue, have been shown to be present at relatively high levels in neoplastic tissue (17-19). The architecture of the surface membranes of embryonic and neoplastic cells exhibit similarities in that they are readily agglutinated by certain carbohydrate-binding proteins, whereas normal adult cells are unaffected (20-22). It has also been shown that the amino acid sequence of the C-terminal portion of glycine-arginine-rich histones isolated from bovine lymphosarcoma, Novikoff hepatoma, fetal calf thymus and pea seedlings are identical (23). Although space does not permit an exhaustive review of the similarities between embryonic and neoplastic tissues, the examples cited are sufficient to demonstrate that many similarities exist.

Comparative lipid data, both metabolic and structural, from neoplastic and embryonic tissue is very limited. However, despite the scarcity of data, some similarities between neoplastic and embryonic tissue lipid metabolism

have been noted. Desmosterol, a sterol first reported in the developing chick brain by Stokes et al. (24) and in rat brain by Kritchevsky and Holmes (25), has been found to occur also in fibroblasts and undifferentiated brain tumors (26,27), but is not a detectable component in the adult brain. The rate of liver cholesterol biosynthesis is decreased by exogenous dietary cholesterol in the adult (28); however all hepatomas examined thus far (6,7) fail to respond to dietary cholesterol, as has recently been found with fetal rat liver (29).

This is the first in a series of papers on the structure and metabolism of lipids of embryonic tissues. It is hoped that the comparison of the lipid structure and metabolism data from embryonic tissues, normal mature tissue and neoplastic tissue will enable us to determine whether the differences observed between the lipid metabolism of normal and neoplastic tissue are associated with growth or neoplasia. These studies will also broaden our knowledge about the involvement and physiological role of lipids during embryonic development. This report compares the neutral lipid class composition of brain, heart and liver at various stages of the chick's development.

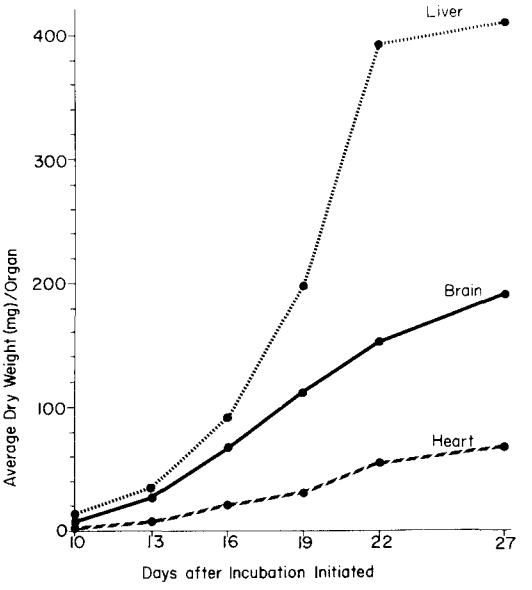


FIG. 1. Average dry weight of different organs as a function of the chick embryo development and maturity.

## MATERIALS AND METHODS

Twenty-eight dozen Standard Northwest Arkansas Broiler strain eggs were selected at random and placed in a large atmospherically controlled incubator. Brains, hearts and livers were excised from embryos and chicks 10, 13, 16, 19, 22, 27 and 53 days after incubation was initiated. Chicks were fed a standard maize base starter ration. The 53 day group was placed on a standard broiler diet ca. 10 days after hatching. Excised organs were held in isotonic saline in an ice bath during collection.

The pooled tissue of each time period was lyophilized, and the total lipids were extracted by the Bligh and Dyer procedure (30). A second extraction was performed by readjusting chloroform and methanol concentrations to again give a single phase. Neutral lipids were separated from the phospholipid fraction by silicic acid chromatography (31). Percentages of neutral lipid and phospholipid fractions were determined gravimetrically. The neutral lipid fraction was separated into classes by thin layer chromatography (TLC) on adsorbent layers of Silica Gel G developed in a solvent system of hexane-diethyl ether-acetic acid 80:20:1 v/v. TLC plates used for qualitative and quantitative estimations were sprayed with sulfuric acid, charred, and documented by photography. Preparative TLC used to isolate each class for quantitation and further analysis was visualized by spraying with Rhodamine 6G and viewing under UV light. The purity and identity of each class were checked by TLC in other solvent systems and by gas liquid chromatography (GLC) of the intact compounds and their hydrolysis products.

Quantitation was achieved by gas liquid chromatography (GLC) analysis of intact classes and their hydrolysis products containing a known quantity of an appropriate internal standard. Each lipid class was eluted quantitatively from the adsorbent, scraped from a developed chromatoplate (5 x 20 cm) that had been streaked with a known quantity (ca. 2.0 mg) of a neutral lipid fraction. One-half of the sterol ester and triglyceride fraction and all of the free fatty acid fraction were converted to methyl esters; a known quantity of methyl arachidate was added, and each analyzed before and after hydrogenation. Cholestane was added to the free sterol fractions and also analyzed by GLC. From the responses of the standards relative to the quantities added, the quantities of fatty acids and cholesterol present were calculated. The remaining 50% of the sterol ester and triglyceride fractions were hydrogenated; then a known quantity of cholesteryl heptadecanoate and dihexadecanoyl hexadecyl glyceryl ether was added to the respective fractions, and they were analyzed intact by high temperature GLC. The quantity of triglyceride

TABLE I

The Percentage Total Lipid, Neutral Lipid and Phospholipid of Brain, Heart and Liver at Various Stages of Embryonic Development

| Days after incubation initiated | Brain | | | | Heart | | | | Liver | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of embryos | Total lipid,%[a] | Neutral lipid,% | Phospholipid,% | No. of embryos | Total lipid,% | Neutral lipid,% | Phospholipid,% | No. of embryos | Total lipid,% | Neutral lipid,% | Phospholipid,% |
| 10 | 141 | 14.4 | 32 | 68 | 141 | 8.0 | 37 | 63 | 141 | 9.2 | 45 | 55 |
| 13 | 60 | 15.0 | 32 | 68 | 84 | 11.3 | 31 | 69 | 84 | 11.4 | 46 | 54 |
| 16 | 30 | 15.4 | 32 | 68 | 30 | 8.0 | 39 | 61 | 30 | 17.6 | 69 | 31 |
| 19 | 17 | 18.6 | 33 | 67 | 17 | 13.5 | 33 | 67 | 17 | 35.3 | 88 | 12 |
| 22 | 8 | 21.6 | 29 | 71 | 8 | 15.3 | 39 | 61 | 8 | 43.5 | 89 | 11 |
| 27 | 8 | 22.2 | 30 | 70 | 8 | 15.8 | 56 | 44 | 8 | 32.6 | 83 | 17 |
| 53 | 6 | 33.5 | 38 | 62 | 6 | 14.4 | 22 | 78 | 6 | 13.5 | 34 | 66 |

[a]Total lipid percentages are based on dry weight.

and cholesteryl esters determined in this manner served as a check on the percentages calculated from the methyl ester data. Duplicate samples usually agreed within ±5% for major components and ±10% for minor components by either method. Agreement between percentages calculated from hydrolysis products and intact lipid classes was ±10%. Analyses of standard mixtures of varying concentrations agreed within ±5% for high levels and ±10% for low concentrations of compounds. Since the attributes of GLC as an analytical tool are well known, the only drawbacks of this approach are limited to technical skill of the operator to make quantitative manipulations and the time and error involved in measuring chromatographic peak areas. The latter is virtually eliminated by the use of a digital integrator.

Gas liquid chromatographic analyses were carried out with an Aerograph Model 2100 instrument. Methyl ester analyses were carried out using a 180 cm x 2 mm (ID) pyrex column (packed with 10% EGSS-X coated on 100-120 mesh Gas Chrom-P) temperature programed from 140-200 C at 4 C/min. Free sterols were quantified using a 180 cm x 2 mm (ID) pyrex column (packed 1% OV-17 coated on 100-120 mesh Gas Chrom-Q) operating at an isothermal temperature of 215 C. Analysis of intact triglycerides and sterol esters was performed on a 75 cm x 2.5 mm (ID) pyrex column packed with 1% OV-17 coated on 100-120 mesh Gas Chrom-Q. Column temperatures were programed from 200-300 and 350 C at 4 C/min for analysis of sterol esters and triglycerides, respectively.

Methyl esters were prepared by the time-honored 2% sulfuric acid-anhydrous methanol procedure. Esterification was carried out either by refluxing for 2 hr with a large excess of reagent or heating in a boiling waterbath in sealed screw cap culture tubes. Neutral lipid standards (purity 99%) were purchased from Nu-Chek-Prep, Inc., Elysian, Minn. All solvents were glass-distilled and obtained from Burdick and Jackson Lab., Muskegon, Mich. Other chemicals and reagents used were reagent grade or better.

## RESULTS

The increased dry weight of each tissue with increased development time is shown in Figure 1. Brain and heart showed a gradual and sustained increase over the measured time period. Liver exhibited a pronounced accumulation of dry weight between the 17th and 22nd days after incubation was initiated. Hatching occurred on the 21st day, and it is assumed that

the increased liver weight occurred before that time, which indicates a ca. 300% increase in liver weight over a 3-4 day period.

**Total Phospholipid and Neutral Lipid**

The number of embryos and chicks, percentage total lipid and percentage neutral lipid and phospholipid for each tissue at each time period is given in Table I. All three tissues showed an increased accumulation of total lipids from the 10th day of incubation to hatching. Total lipid (relative to dry weight) of brain continued to increase from 14 to 33% while the neutral lipid and phospholipid fractions remained constant at approximately one-third and two-thirds, respectively. Heart total lipids increased from 8% at 10 days to 15% at hatching, and, like brain the phospholipid to neutral lipid ratio was approximately two for the same time period. At 27 days after incubation was initiated the heart phospholipid to neutral lipid ratio was down to one, but was back to three at the 53 days. Liver total lipids showed a 400 to 500% increase from the 10th day to hatching, which paralleled the increase in liver mass (Fig. 1). Neutral lipids and phospholipid percentages were approximately equal at the 10th day, but at hatching there was eight times more neutral lipid than phospholipid. After hatching, liver neutral lipids decreased, and at 53 days the phospholipid to neutral lipid ratio was two. Egg neutral lipid to phospholipid ratio was four.

A clearer picture of the lipid changes that occur during development is seen for each tissue in Figure 2, which shows the micrograms of both lipid fractions per milligram of dry weight with time. Except for liver neutral lipid and brain phospholipid fractions, the values for each fraction were bunched in a narrow region between ca. 25 and 75 μg/mg dry weight for all time periods. The liver neutral lipid fraction also fell within this range on the 10th, 13th and 53rd days. Figure 2 shows that the tremendous accumulation of liver lipids was due to neutral lipid fraction and not phospholipid fraction, which remained relatively constant. It can also be seen that the accumulation of total lipids in the brain with development represents primarily an increase in phospholipids.

**Brain**

Sterol esters, triglycerides, free fatty acids and sterols were present at all time periods. The quantitative data shown in Table II indicate a marked decrease in the sterol esters with increased development. Sterols, the major neutral lipid class, showed a small percentage but a definite increase until hatching. Free fatty acids and triglycerides decreased with increased time (Table II). A trace component between the

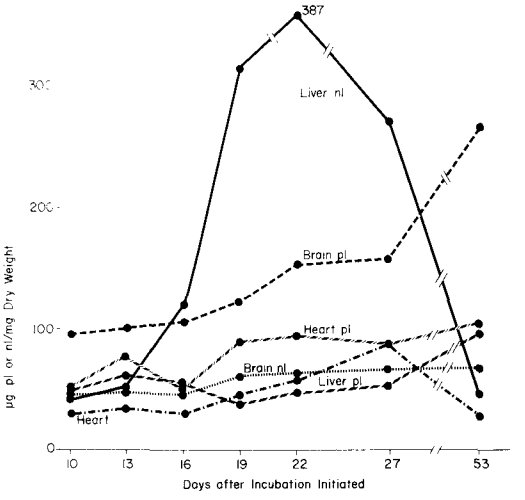


FIG. 2. Micrograms of phospholipid (pl) and neutral lipids (nl) per milligram of total dry weight for brain, liver and heart at various stages of development and maturity of the chick.

sterols and free fatty acids, most prevelant at the earlier time periods, was not characterized. The calculated recovery percentage shown in Table II indicates less than 5% trace components.

**Heart**

The neutral lipid class distributions of heart at various times is shown in Figure 3. Sterol esters, triglycerides and sterols represent the major neutral lipid classes. Triglycerides represent about one-third of the heart neutral lipid at the 10th day, which decreases to half this level by the 16th day and then increases to 75% by the 27th day. Sterol esters showed the reverse picture, increasing when triglyceride levels fell and decreasing when triglyceride levels rose. Cholesterol, the major sterol, represented ca. 50% of the heart neutral lipids through the 16th day and then decreased to ca. 15% by the 53rd day. Free fatty acids were present only as a trace component and was not quantified. A component above the triglyceride band at day 53 was identified as a glyceryl ether diester. A spot below the triglyceride band at the same time period was not characterized.

**Liver**

The most dramatic changes occurred in the neutral lipid classes of the developing liver, as shown in Figures 4 and 5. Cholesterol, triglycerides and sterol esters each represented approximately one-third of the liver neutral lipids at days 10 and 13. However after the 13th day

TABLE II

Percentage of Neutral Lipid Classes at Various Stages of Development in the Chick Embryo Brain[a]

| Days after incubation initiated | Per Cent of total neutral lipids | | | | |
|---|---|---|---|---|---|
| | Sterols | Free fatty acids | Triglycerides | Sterol esters | Recovery |
| 10 | 83.3 | 2.6 | 3.1 | 10.0 | 99 |
| 13 | 83.8 | 2.3 | 1.6 | 7.1 | 95 |
| 16 | 85.6 | 1.4 | 1.6 | 6.3 | 95 |
| 19 | 90.6 | 1.8 | 2.1 | 2.8 | 97 |
| 22 | 91.1 | 1.9 | 1.4 | 3.1 | 97 |
| 27 | 91.3 | 0.7 | 0.9 | 2.3 | 95 |
| 53 | 91.2 | 1.0[b] | 1.0[b] | 2.0[b] | 95 |

[a]Percentages represent the mean of duplicate analysis.

[b]As determined by thin layer chromatography.

cholesterol and triglyceride levels decreased rapidly to a low level which persisted until after the 27th day. The decreased cholesterol and triglyceride levels were offset by an accumulation of cholesterol esters that reached 90% of the neutral lipids by the 19th day and remained at the level until after the 27th day. After maturity (53 days after incubation was initiated) liver triglyceride values represent ca. 75% of the neutral lipids, and cholesterol ester values were down below 5%.

### Egg

Triglycerides represent more than 90% of the egg neutral lipids (Fig. 5). Cholesterol and cholesterol esters were the only other egg lipid classes present in amounts greater than 1% of the neutral lipid fraction.

## DISCUSSION

The literature on the lipids of the developing chick embryo through 1967 has been covered by Romanoff (32). A review of the work covered by Romanoff and a search of the literature since revealed that a quantitative determination of all the neutral lipid classes had not been determined at various developmental stages for the chick heart or brain. The neutral lipid classes of liver, the most extensively examined chick embryo tissue, have been quantitated for various developmental stages; but most of the studies were carried out using column chromatography and gravimetric techniques, which have since been replaced by more accurate methods. The present study, in addition to adding to our knowledge where data are lacking, allows comparisons of changes in the neutral lipid classes within the same study, of neural, muscular and digestive tissue, each of which exhibits disproportionate growth rates occurring at different stages of development.

### Brain

The chick embryo brain has been used as a model by numerous investigators to study cholesterol metabolism. Aside from cholesterol, the other brain neutral lipids have gone almost unstudied, particularly in early stages of development. The data (Table II) show that free fatty acids, triglycerides and sterol esters are present in substantial amounts, in addition to free sterols at 10 days of incubation. All three lipid classes decreased with increased incubation time, and free sterol values increased. The levels and changes in the free fatty acid and triglyceride values at various developmental times of the chick brain do not appear to have been reported previously. Indirect evidence for the presence of cholesterol esters in the developing chick brain was reported by Mandel et al. (33) in 1949. Adams and Davison (34) reported the presence of esterified cholesterol in the developing human nervous system and the chick, but their data on the chick brain was inconsistent and inconclusive. Wells and Dittmer (35) reported significant levels of sterol esters in the three-day-old rat that decreased to undetectable levels after six days.

Examination of the hydrolysis products and the intact sterol esters by GLC has established the identity and presence of this neutral lipid class in the brain beyond any reasonable doubt. The decrease in sterol ester content of the brain with increased maturity suggests it may play a role in cell division or maturation. If the level of sterol esters is related to rate of brain development, sterol ester levels around the 5th day of incubation (when brain development is most rapid [32]) should be still higher than the 10th day values reported here.

### Heart

The lipids of the developing chick heart

apparently have not been reported previously. Total lipids of heart were lower than either brain or liver, and unlike the other two tissues (which showed an increase in total lipids each successive time period) heart decreased from the 13th to the 16th day of incubation (Table I). The decreased total lipid level at this time period corresponds to one of the two self-inhibiting growth retardation periods that are known to occur on the 9th and 16th days of incubation (32). During the growth retardation period at the 16th day, which is the most pronounced, metabolic processes such as adsorption, storage and oxidation of organic matter also show cyclic changes (32). Heart triglyceride levels reached a minimum, and cholesterol ester values reached a maximum, also at this time (Fig. 3). It should also be pointed out that this was the time period when liver sterol ester levels started to increase dramatically (Fig. 2) and when brain sterol esters showed the largest decrease to the next time period (Table II). All of this information seems to show a very active metabolism of sterol esters at this time and suggests further that the observed decrease in total neutral lipids of the heart at the 16th day of incubation, relative to the 13th and 19th days, is a real phenomenon and is not due to technical error.

Heart and liver triglyceride percentages at 10, 13 and 16 days of incubation were similar, but after the 16th day liver triglyceride levels continued to fall while heart triglyceride levels began to rise. At present, triglycerides from all three tissues are being examined in detail to determine whether de novo synthesis or accumulation of preformed yolk triglyceride occurs in these tissues.

#### Liver

The rapid increase of liver dry matter after the 16th day of incubation (Fig. 1) has been observed previously by a number of investigators, and these data have been analyzed from several viewpoints by Romanoff (32). The high percentage lipid of the dry liver weight (Table I) is in good agreement with previously reported values (36,37). Accumulation of cholesterol esters in the neutral lipids (Figs. 4 and 5) was somewhat surprising, since the egg neutral lipids contained more than 90% triglycerides and less than 1% cholesterol esters (Fig. 5). Tsuji et al. (38) first observed the increased cholesterol ester of whole embryos with increased incubation time. Moore and Doran (37), the first to examine the neutral lipids of the developing embryo in detail, found that the cholesterol esters accumulated in the liver, which has since been confirmed by Noble and

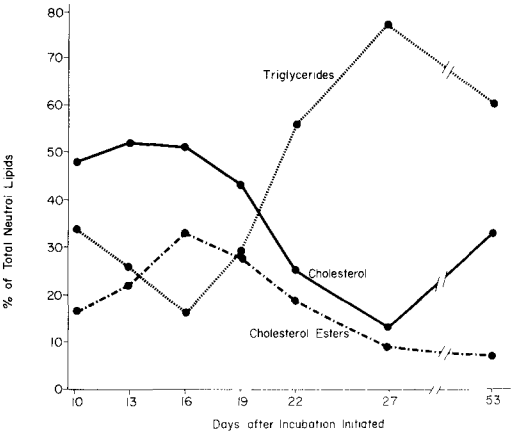


FIG. 3. Quantitative distribution of the neutral lipid classes as function of development and maturity of the chick heart.

Moore (36), Feldman and Grantham (39) and the present study. Recalculation of previous data expressed in terms of wet weight (39), few time periods (37) and incomplete class analysis (36) made comparison of present class percentages with those reported previously difficult. However in general the comparisons agreed reasonably well. The present study, which examined both earlier and later developmental periods than previous studies (36,37,39), shows that at 10 days of incubation cholesterol, cholesterol ester and triglyceride values are approximately equal, and that when the liver rapidly accumulates large amounts of cholesterol esters before hatching, the level remains high at least 6 days after hatching. The eventual fall in cholesterol ester values is accompanied by a concomitant rise in triglyceride level. The storage of lipids in the chick liver in the form of cholesterol esters as opposed to triglycerides (which are most abundant in the egg) poses some most interesting questions about biosynthesis, transport and storage of lipids during development.

#### Embryonic vs. Tumor Lipids

A prime objective in initiating this series of studies on the developing embryonic tissue lipids was to determine whether observed abnormalities in the lipid metabolism of tumors are associated with neoplasia or rapid cell growth and proliferation. The determination of the neutral lipid class composition, a basic but necessary operation, was not expected to provide any insight into this question. However the high level of sterol esters observed early in all three developing tissues at least opens the door to speculation.

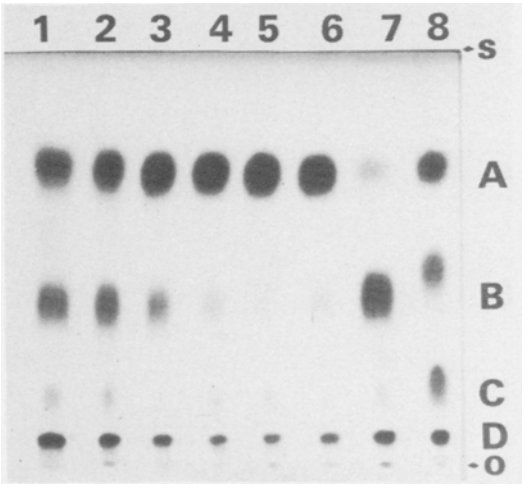


FIG. 4. Thin layer chromatoplate depicting the distribution of sterol esters (A), triglycerides (B), free fatty acids (C) and free sterols (D) of the embryonic chick liver at 10, 13, 16, 19, 22, 27 and 53 days after incubation was initiated (arranged chronologically in lanes 1-7). Lane 8 represents a standard mixture, and the origin and solvent front are indicated by 0 and S, respectively. Chromatography was carried out on adsorbent layers of Silica Gel G developed in a hexane-diethyl ether-acetic acid 80:20:1 v/v solvent system. The standard triglyceride (triarachidin) moved just ahead of the sample triglycerides because of the higher molecular weight.

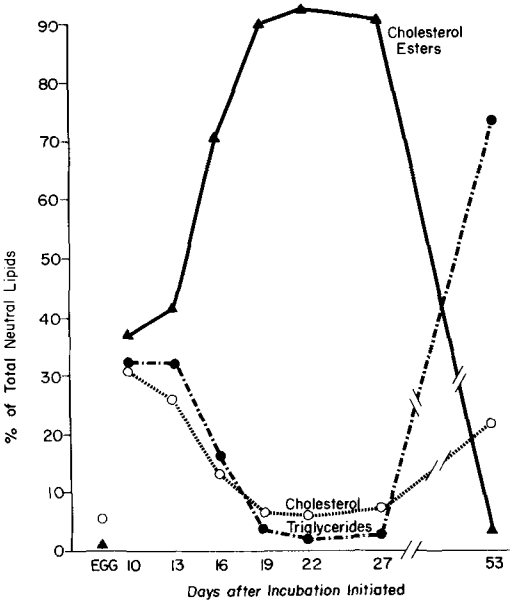


FIG. 5. Quantitative distribution of the neutral lipid classes of egg and of chick liver at different stages of development and maturity.

Sterol ester levels of neoplasms are generally higher than most normal tissue. Boyd et al. (40) reported higher sterol ester levels in Walker 256 carcinosarcoma than any of five normal host tissues examined. Elevated levels of sterol esters have been reported for five transplantable mouse tumors (41), Ehrlich ascites cells (43) and in the blood of patients with cancer (43). In minimal deviation hepatomas, where a valid comparison can be made with normal liver, the sterol ester level has been found to be significantly higher (44). However not all rapidly proliferating cells contain high levels of cholesteryl esters, as data from a number of "normal" cells grown in culture have indicated (45). This observation does not, however, rule out the possible involvement of sterol esters in cell proliferation, because most "normal" cells grown in culture require for growth serum which contains sterol esters. Rothblat et al. (45) have shown that lymphoblast cells (L5178Y) grown on media containing serum incorporate exogenous serum cholesteryl esters. Bailey (46) has shown that a strain of non-tumorgenic lymphoblast cells (MB III) grown in culture do not contain significant levels of cholestryl esters but do not replicate on media without serum. Whether serum sterol esters are required for MB III cell replication remains to be determined. It has been suggested that the levels of sterol esters in cultured cells may be regulated by rates of uptake and hydrolysis of exogenous cholesteryl esters (45). In this light, elevated levels of sterol esters in embryonic tissue may represent a lack of hydrolysis. However high sterol ester levels in the developing liver, heart and even brain, relative to the adult values, may suggest that the elevated sterol ester levels in neoplasms are related to the growth process. If sterol esters are involved in cell proliferation as a structural component, a regulator or even a source of energy, their controlled biosynthesis and transport in a neoplasm could have significant therapeutic value. However the full significance of the observation will have to await more detailed metabolic studies.

The first characterization and identification of glyceryl ether diesters from neoplastic tissue was carried out on lipids isolated from Ehrlich ascites cells (1), and the presence of glyceryl ether diesters in a large number of transplantable rat and mouse tumors has been established more recently (47). Glyceryl ether diesters are not characteristic of neoplastic tissue. They have been isolated from human perinephric fat (48) and beef heart lipids (49), and indirect evidence suggests that they occur in most mammalian tissues (50,51); but based upon indirect quantitative data (52,53) it appears

that generally neoplastic tissue contains elevated levels of glyceryl ether diesters.

The absence or presence of only trace quantities of glyceryl ether diesters in all three embryonic tissues at all time periods, except 53 day heart, suggests that the elevated levels of glyceryl ether diesters found in neoplasms are not due to the growth process, but in fact may be related to the neoplastic process or condition resulting from neoplasia. Recently Howard and Bailey (54) reported a correlation between the elevated glyceryl ether content and decreased activity of the enzyme α-glycerol phosphate dehydrogenase for both hepatomas and cultured cells. These findings suggest that the elevated levels of glyceryl ether diesters may be due to altered enzymatic activities which result in build-up of precursors for their biosynthesis.

ACKNOWLEDGMENTS

N. Holman of the Poultry Husbandry Dept. carried out the egg incubations, and J. Falch gave technical assistance.

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# Health Physics Practices in Laboratories Using $^3H$- and $^{14}C$-Labeled Tracers[1]

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## ABSTRACT

Tritium and $^{14}C$ are often considered relatively safe radionuclides to handle because their permissible body-burdens are orders of magnitude greater than those for most radionuclides. This is reflected in the U.S. Atomic Energy Commission regulations that do not require a license for the use of amounts up to 1000 $\mu$Ci of $^3H$ and 100 $\mu$Ci of $^{14}C$. Although many tracer studies are performed with much smaller quantities, poor control of even these small amounts can result in contamination, causing an internal radiation hazard or the loss of valuable experimental data. This paper discusses the methods of handling radioactive materials that minimize the risks of contamination, and it emphasizes the needs for proper storage, secondary containers, monitoring air and surfaces, and analysis of bioassay samples for measuring body burdens. The user's responsibilities for licensing, waste disposal and transportation are also included. The authors' experience at a laboratory handling both tracer and curie amounts of $^{14}C$ and $^3H$ required for labeling and synthesizing compounds and methods of accident prevention and decontamination are described.

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[2]Under contract with the U.S. Atomic Energy Commission.

## INTRODUCTION

When discussing the hazards of $^3H$ and $^{14}C$, one usually points out that these low energy beta emitters are seldom used in quantities sufficient to create an external radiation hazard. Even the internal radiation hazard is relatively slight. Of the 236 radionuclides listed by the International Commission on Radiological Protection, only $^3H$ and two others have maximum permissible body-burdens (MPBB) as high as 1000 $\mu$Ci (1). Carbon-14 with a permissible body burden of 300 $\mu$Ci is only slightly more toxic.

However to conclude that precautions are unnecessary in handling $^3H$ and $^{14}C$ would be wrong, because these nuclides under certain conditions can cause significant exposure to laboratory personnel and to the public. In addition improper control can result in laboratory contamination and lead to erroneous experimental results.

The low energy beta emission that makes these isotopes relatively nonhazardous also makes them difficult to monitor. This frequently gives a false sense of security which prompts investigators to become careless in the control of the radionuclides. Even a relatively nontoxic radionuclide can become a hazard if handled improperly.

## PHYSICAL AND BIOLOGICAL PROPERTIES

The basic physical properties of $^3H$ and $^{14}C$ are summarized in Table I. Biologically these radionuclides behave essentially the same as their stable isotopes. The uptake of a radionu-

TABLE I

Physical Properties

| Decay scheme | $T_{1/2}$ | Maximum $\beta$ energy | Average $\beta$ energy | Range in air | Range in tissue |
|---|---|---|---|---|---|
| $^3H \xrightarrow{\beta^-} {}^3He$ | 12.3 y | 18 keV | 6 keV | 0.5 cm | 6 $\mu$m |
| $^{14}C \xrightarrow{\beta^-} {}^{14}N$ | 5735 y | 156 keV | 49 keV | 22 cm | 280 $\mu$m |

TABLE II

Radiation Protection Guides

| Type of exposure | Permissible concentration, μCi/cc | |
|---|---|---|
| | $^{3}H$[a] | $^{14}C$ |
| Occupational[b] | | |
| Air | $5 \times 10^{-6}$ | $4 \times 10^{-6}$ |
| Water | $1 \times 10^{-1}$ | $2 \times 10^{-2}$ |
| Nonoccupational | | |
| Air | $2 \times 10^{-7}$ | $1 \times 10^{-7}$ |
| Water | $3 \times 10^{-3}$ | $8 \times 10^{-4}$ |

[a]As $^{3}H_2O$.

[b]Based on permissible body burden of 1000 μCi of $^{3}H$ or 300 μCi of $^{14}C$.

clide, its distribution and residence time in the body, and its physical properties are used to calculate its maximum permissible body-burden and the permissible air and water concentrations. Table II gives these values for tritiated water and readily oxidized $^{14}C$ compounds that have effective half-times in the body of approximately 12 days (2). The body burden and permissible concentrations of $^{3}H$- and $^{14}C$-labeled compounds that have an uptake or retention time different from water and readily oxidized organic compounds will need to be determined by means of the equations given in Reference 1.

## LICENSING REQUIREMENTS

The U.S. Atomic Energy Commission and in some cases the state health departments have the responsibility for the control and safe use of radioactive materials. The radiation protection regulations (2) are based on recommendations of national and international experts (3,4). To obtain a license the user must prove that his training, experience and facility are adequate for the safe handling of the radionuclides. A license will specify the type and maximum amount of activity that may be possessed. Unless authorized by the license, the user may not administer radioactive material to humans. The licensee is inspected periodically by the licensing agency to ensure compliance.

Certain quantitites of radionuclides are license-exempt: 1000 μCi of $^{3}H$ and 100 μCi of $^{14}C$. Even though a license may not be required, the user must still comply with radiation protection standards.

## LABORATORY DESIGN

The International Atomic Energy Agency (5) and the American Standards Association (6) have issued design guides for laboratories handling radioactive materials. The requirements are based on a classification of the radionuclides according to relative radiotoxicity: very high, high, moderate and slight. Carbon-14 and tritium are both considered to be of slight toxicity, and with reasonable care up to 10 mCi may be handled in a good chemical laboratory. If other than standard chemical operations are to be performed, the amount permitted is either reduced or increased according to a modifying factor (Table III). For example only 0.1 mCi of a radioactive gas should be used in such a laboratory. Because most tracer studies require less than 10 mCi in solution or 0.2 mCi as a gas, a good chemical laboratory is generally adequate. If greater quantities of a radionuclide are to be handled, a laboratory of a higher classification is required.

Laboratory design standards recommend a minimum of 150-200 $ft^2$ of floor space per worker (7). Our own experience has shown that spills are more frequent and more rapidly spread in a crowded laboratory. The multiple use of work areas, poor housekeeping and excessive laboratory traffic contribute to the contamination potential. Ideally each radioisotope user should be restricted to a specified laboratory area.

To simplify control of the radionuclide, laboratories handling radioactive materials should be separate from offices, lunch rooms and conference areas. A suitable layout is shown in Figure 1. This arrangement limits the flow of traffic through areas having the greatest potential for a spill or release.

To facilitate decontamination, all laboratory surfaces should be smooth, nonporous and chemically nonreactive. Unsealed and concrete surfaces should be avoided. A single-piece floor covering is considered preferable to tile; however both are suitable provided they are protected by wax. A polyurethane floor covering has proved quite satisfactory. The polyurethane is poured and spread to form a continuous

TABLE III

Type of Operation and Corresponding Modifying Factors for Laboratory Design

| Operation | Modifying factor |
|---|---|
| Storage | x 100 |
| Very simple wet operation | x 10 |
| Normal chemical operation | x 1 |
| Complex wet operations with risk of spills | x 0.1 |
| Simple dry operations | x 0.1 |
| Dry and dusty operations and those where isotopes are evolved as gases | x 0.01 |

crack-free surface, which after curing is resistant to practically all the solvents and chemicals routinely handled. Since the smooth, nonporous surface maintains its sheen, waxing is not required.

Bench tops, sinks and surfaces on which radioactive materials are handled must also be easy to clean. Unsealed or rough surfaces such as soapstone that have become etched are practically impossible to decontaminate.

Each laboratory should be equipped with exhaust ventilation for the control of aerosols, gases and fumes (8). A glove box may be used for those operations requiring strict control. Fume hoods with an average face velocity of 125-200 fpm may be used when less control is required. Only necessary pieces of equipment should be placed in a hood, since such obstructions create turbulence and reduce the degree of control the hood provides. The hood should be located where drafts from windows, doors, air conditioners or personnel traffic will not disrupt the smooth flow of air into the hood.

## ADMINISTRATIVE CONTROLS

The license will specify the maximum amount of radioactivity that may be kept in the laboratory; however, from a radiation safety standpoint, the quantity should be restricted to the minimum required. Additional radionuclide simply increases the risk. Labeled compounds are especially troublesome, since they tend to decompose and may result in an unsuspected release of radioactivity into the laboratory.

Ordering and shipment errors can also create health physics problems (9,10). When each radioisotope is received, the package should be checked carefully. As a minimum the shipping label, assay slip and container label should be examined to determine whether the radionuclide, amount and compound are in agreement with the order.

Each shipment should be checked for damage or contamination, and when possible the identity of the nuclide and the compound should be verified. If contaminated, the entire package should be placed in a sealed container to prevent further release of activity. The vendor should be notified of any irregularities so that he may improve his own operation.

A few years ago, a supplier filled our order for a few *millicuries* of a labeled $^{3}H$ compound with *curie* amounts. Although the package was marked properly, the person opening the container failed to recognize that the potential for hazard had increased 1000 fold. Because the compound was highly volatile, the entire laboratory became contaminated, and a month of valuable research time was lost during cleanup operations.

## STORAGE

Radioactive materials should be stored only in areas designed and designated especially for their safekeeping, and the storage area should be checked frequently to be sure the containers have remained intact. Periodic cleanout is necessary because old samples are often pushed back and lose their identification, only to be rediscovered after their contents have been spilled. If refrigeration is required the unit should be suitable for flammable substances, since many labeled compounds are in flammable solvents. The caps of containers should be checked a few hours after refrigeration, since cooling is apt to loosen the caps and permit vapors to escape. To prevent the rupture of containers, space should be allowed for expansion of the contents resulting from changing temperatures.

## HANDLING

Regardless of how well a laboratory is designed, effective training and attention to detail will still be required to minimize the risk. Good laboratory practice prohibits eating, drinking, smoking and application of cosmetics in a radioactive-materials area since these activities may result in radioactive material entering the body. Operations that require placing laboratory items into the mouth should also be prohibited. Oral pipetting should be replaced

with the use of automatic or manual pipetting devices.

Everyone in a radioisotope laboratory should wear lab coats. Plastic or rubber gloves worn to prevent skin contamination should be changed frequently, because many labeled compounds diffuse slowly through these materials (11). Each person should remove protective clothing and wash his hands thoroughly before leaving the area.

Because of the difficulty in monitoring for low energy beta contamination, disposable supplies such as pipettes and beakers should be used whenever possible. Nondisposable equipment should be marked as potentially contaminated and used only for radioactive material. To prevent cross contamination, the items should be identified according to isotope and the possible level of contamination.

Secondary containers should be provided at all work spaces to limit further spread of spilled liquids. Blotting paper, usually plastic-backed, is adequate if only a few drops of solution are apt to be spilled. If large volumes of liquid are being handled, a tray large enough to contain the entire volume should be used.

When large quantities of radioactive material must be handled, the prevention and consequences of an accidental release are important considerations. One way to anticipate possible problems is to perform the entire experiment without the radioactivity and to look for flaws in the experimental setup, such as vacuum systems that exhaust directly into the room or glassware that may be broken easily. Even though precautions are taken, an accidental release may still occur, and plans should allow for rapidly evacuating the area and limiting the spread of contamination. The consequence of an accidental release should be evaluated carefully in deciding where to conduct high level experiments. Ideally the work should not be performed in an area for low level work, because a release could seriously hamper future operations.

## SURVEYING

Since beta particles emitted by $^{3}H$ and $^{14}C$ have very little energy, they do not penetrate the walls of most radiation detectors. Specially designed thin window or windowless detectors (Fig. 2) are required, but even they cannot detect $^{3}H$ and $^{14}C$ through surface films of dirt or oil. Most of the instruments used for measuring surface contamination are proportional counters with relatively high sensitivities for low energy beta particles; however they also have high backgrounds that limit the amount of contamination that may be detected.

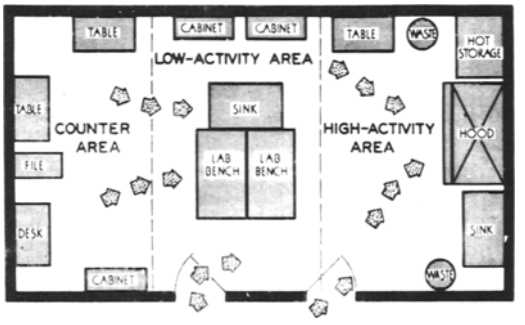


FIG. 1. Example of laboratory arrangement for tracer-level radiochemical operations (from Reference 7).

A smear technique may be used to distinguish between fixed and removable contamination. Some of the removable contamination is transferred to a smear, usually a piece of filter paper, as it is wiped across the surface. The filter paper may be used either dry or moistened to enhance the transfer of the contamination to the paper. The paper is then counted with a low energy beta detector. If a moistened smear is to be counted using an internal proportional counter, it must first be dried. A liquid scintillation counter may be used by placing the filter paper into a vial and covering it with a few milliliters of scintillation solution. Although smear results are not reproducible because transfer and counting efficiencies may vary by several orders of magnitude, the results do suggest whether or not contamination exists. The presence of contamination indicates poor work procedures and inadequate controls, and immediate steps are needed to identify and curtail the source of the contamination. Storage areas, sinks, hoods, laboratory door and refrigerator handles, and telephones should be routinely smeared, since these locations have a high potential for becoming contaminated. To simplify floor surveys, we have constructed a floor monitor which uses end-window GM tubes (1.4-1.8 $mg/cm^2$) connected to a portable survey meter equipped with a speaker (Fig. 3). Although this instrument cannot detect tritium, its efficiency for $^{14}C$ is nearly 10% under ideal conditions.

## AIR SAMPLING

Occasional monitoring of laboratory air and stack discharges may be required to control exposure to airborne $^{3}H$ and $^{14}C$ and to establish conformance with radiation protection standards. Flow-through ionization-chamber instruments (Fig. 4) are widely used

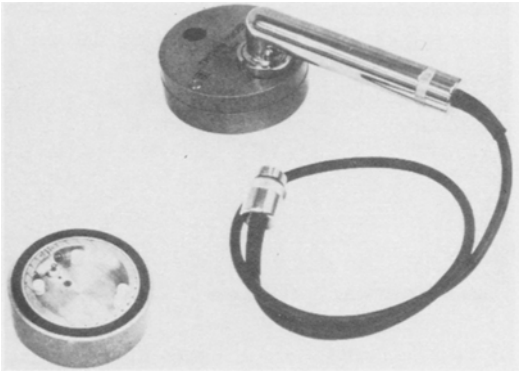

FIG. 2. Gas-flow open window probe for tritium surface-monitoring (Courtesy of Eberline Instrument Corp.).

FIG. 3. Floor monitor for $^{14}C$.

for this purpose. Since these instruments respond to charged particles of ions entering the chamber, they are usually equipped with filters and electrostatic precipitators to remove these interferences from the air stream. The flow-through monitor is therefore limited to measuring tritium and $^{14}C$ in the form of gases or vapors. Another limitation of these instruments is their relatively poor sensitivity which may make them impractical for monitoring at permissible levels for nonoccupational exposure (Table II).

Air monitoring can also be performed by collecting a sample of the contaminant from the air and then measuring the activity by liquid scintillation or other counting techniques. Proper sample collection requires prior knowledge of the physical and chemical form of the radionuclide to be measured. One simple but effective method for sampling $^{3}H$ as tritiated water or $^{14}C$ as carbon dioxide is by cold-trapping. Monitoring refrigerated storage areas for leaking tritium samples by counting a sample of freezer frost is a modification of this technique. Other collecting techniques include filtering, chemical scrubbing, absorption and adsorption.

## PERSONNEL MONITORING

Personnel exposures in a laboratory using $\mu$Ci to mCi amounts of $^{3}H$ or $^{14}C$ are usually determined by means of a bioassay program. Tritium in the body will usually be oxidized to water and excreted in saliva, sweat, exhaled moisture or urine. An estimate of the amount of tritium in the body can be made by counting a known volume of the excreted water (usually urine) in a liquid scintillation solution. Although color and chemical quenching occur, quite small amounts of tritium can be detected by this technique. A person containing a maximum permissible body-burden of 1000 $\mu$Ci of $^{3}H$ in the form of body water will excrete tritium at the rate of approximately 23 $\mu$Ci/liter (12). This value is based on a $^{3}H$ retention half-time of 12 days in standard man and will be influenced by the person's fluid intake. Although most of the tritium will be excreted with a relatively short biological half-time, a small fraction will be retained in the body much longer (13,14).

Most $^{14}C$-labeled organic compounds taken into the body are oxidized and the $^{14}C$ exhaled as $^{14}CO_2$. The amount of radionuclide contained in the body can be estimated by collecting a known volume of exhaled air, trapping the $CO_2$ in hyamine or phenethylamine (15) which are soluble in liquid scintillation solutions, and determining the $^{14}C$ activity. If the person contains a maximum permissible body-burden, (300 $\mu$Ci) he will exhale $^{14}C$ at a rate of 2 x $10^{-4}$ $\mu$Ci per liter of air (16).

Personnel monitoring of external radiation levels is not required in a laboratory handling up to several curies of $^{3}H$ or $^{14}C$, since the external radiation levels are so low. When very large quantities of activity are used, bremsstrahlung may result in significant external radiation levels and film badges or thermoluminescent dosimeters should be worn. These dosimeters may also be used for measuring the dose to the hands.

## DECONTAMINATION

Tritium or $^{14}C$ contamination on a smooth, nonporous and nonreactive surface can usually be removed with warm water, mild detergent and "elbow grease." Many organic solvents are also effective cleaning agents. Cleaning procedures sometimes remove only the upper layer of the contaminant or force the contaminant deeper into the material. A decontaminated

area should therefore be resurveyed for several days to determine if $^{3}H$ or $^{14}C$ are still present and diffusing back to the surface.

Surfaces that cannot be decontaminated should either be replaced or covered with a sealer. A sealer prevents spreading by fixing the contaminant to the surface; however it is only a temporary solution and should be used only if decontamination fails. The use of different colored paints as sealers may help identify the contaminated area.

Skin contamination can usually be removed with tepid water and mild detergents and should be flushed off the skin as soon as possible, because many $^{3}H$ and $^{14}C$ compounds readily cross the skin barrier. Vigorous scrubbing, solvents or harsh cleaning agents should not be used, because they may accelerate the penetration of the radionuclide into the body.

## WASTE DISPOSAL

The application for license asks the user to describe the disposal procedures he will follow. The permissible concentrations of $^{3}H$ and $^{14}C$ in effluents are quite high, and there should be little difficulty in complying with federal and state regulations. These concentrations, however, are maximum limits, and efforts should be made to maintain the levels as low as practicable (2).

### Liquids

The permissible concentrations for release of radioactive liquids to sanitary drains are $2 \times 10^{-2}$ $\mu$Ci/cc for $^{14}C$ and $1 \times 10^{-1}$ $\mu$Ci/cc for $^{3}H$. The maximum daily release must not exceed 10 mCi of $^{3}H$ and 1 mCi of $^{14}C$, and the total annual release must not exceed 1 curie. Actual concentration measurements are not necessary, if the licensee can show that the total activity released will be diluted by the water used in the laboratory to concentrations below the permissible levels. To verify conformance with regulations, calculations or records of concentration measurements must be maintained. High activity liquids should not be released indiscriminately to the sanitary sewer system but should be collected, mixed with an absorbent and disposed of with solid wastes.

The disposal of large numbers of liquid scintillation samples is a problem for $^{3}H$ and $^{14}C$ users. Many laboratories simply empty the vials into the sanitary drain. This procedure, however, increases the potential for contaminating the laboratory and a recent time-cost study (Martin, University of Colorado, unpublished) has shown that this method may be almost three times as expensive as burying the vials with the solid waste.


FIG. 4. Flow-through ionization chamber for monitoring concentration of airborne $^{3}H$ and $^{14}C$ (Courtesy of Johnson Laboratories, Inc.).

### Solids

Solid waste such as samples and contaminated items are usually buried at a commercial radioactive-waste burial site or on the licensee's property. Radioactive-waste-disposal firms, licensed by the AEC, are available to furnish a complete disposal service. Atomic Energy Commission regulations (2) authorize 12 burials per year of limited quantities (1 Ci of $^{3}H$ and 100 mCi of $^{14}C$ per burial) on the licensee's property. Burial must be at a minimum depth of 4 ft in a controlled area that is properly identified. Records of all burials must be maintained.

When specifically authorized in the user's license, incineration can be used to dispose of slightly contaminated combustibles and laboratory animals. Since most $^{3}H$ and $^{14}C$ compounds are converted to gases during burning, there is negligible radioactive ash. Some laboratories incinerate liquid scintillation samples, vials and all. One novel method of incineration (Johnson, Northwestern University, unpublished) uses the scintillation solvent as the fuel to fire the incinerator burner.

### Gases

Gaseous wastes generated during tracer operations will usually contain very small amounts of activity and may be released to the environment, provided the average annual concentration of $^{3}H$ does not exceed $2 \times 10^{-7}$ $\mu$Ci/cc and $1 \times 10^{-7}$ $\mu$Ci/cc for $^{14}C$. An average chemical fume hood exhausts ca. 1000 $ft^3$/min; therefore ca. 8 mCi of $^{3}H$ and 4 mCi of $^{14}C$ may be

discharged daily.

To prevent the release of radioactivity to the environment, air cleaning devices should be provided for procedures that may generate large amounts of airborne activity. Passing the waste gases through molecular sieve, silica gel or a chemical scrubber is usually adequate.

## SUMMARY

To summarize, elaborate control measures are not warranted for tracer operations involving relatively small amounts of $^{3}H$ and $^{14}C$. Adequate control will usually be accomplished by good personal hygiene habits, use of protective clothing and equipment and frequent monitoring of laboratory areas and personnel. The potential chemical, electrical and mechanical hazards present in tracer laboratories will often outweigh the radiological hazards; however the radiological hazards must not be taken lightly, because much is still unknown about biological pathways of certain organic compounds and the genetic and somatic effects of chronic low level exposure from internal sources.

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# Synthesis of Phosphoglycerides[1,2]

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## ABSTRACT

The synthesis of phosphoglycerides is reviewed. Emphasis is placed upon precursor and intermediate compounds, phosphatidic acid, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, monoacylphosphoglycerides, purification and proof of structure. Suggestions are made for large scale synthesis of phosphoglycerides based upon our experience.

## INTRODUCTION

The synthesis of phosphoglycerides can be achieved by the following methods: (a) deacylation and reacylation of an isolated native phosphoglyceride, (b) phosphorylation of diacylglycerols with the desired moiety, (c) acylation of phosphatidic acid with the desired group, (d) reaction of suitable phosphoryl moiety with 1,2 diacyl *sn* glycerol iodo (or bromo) hydrin, and (e) acylation of either 1 or 2-monoacylphosphoglyceride with the desired acid. In many of these methods protective groups for the phosphoryl and nitrogenous moieties must be introduced and removed. Finally, purification is always required.

Since Slotboom and Bonsen (1) have recently published a comprehensive review on the chemistry of phospholipids, this paper will be limited to discussions of: synthesis of intermediate compounds, phosphatidic acid, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, monoacylphosphoglycerides, purification and determinaton of purity. Synthesis of phosphoglycerides has also been reviewed by Van Deenen and De Haas (2) and by Baer (3).

The standard stereospecific numbering (*sn*) nomenclature has been used throughout.

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[2]Scientific Contribution No. 502, Agricultural Experimental Station, University of Connecticut, Storrs, Conn. 06268.

## SYNTHESIS OF INTERMEDIATES

### *sn*-Glycerol-3-Phosphorylcholine (GPC) and Ethanolamine (GPE)

These compounds can be synthesized (4-6)

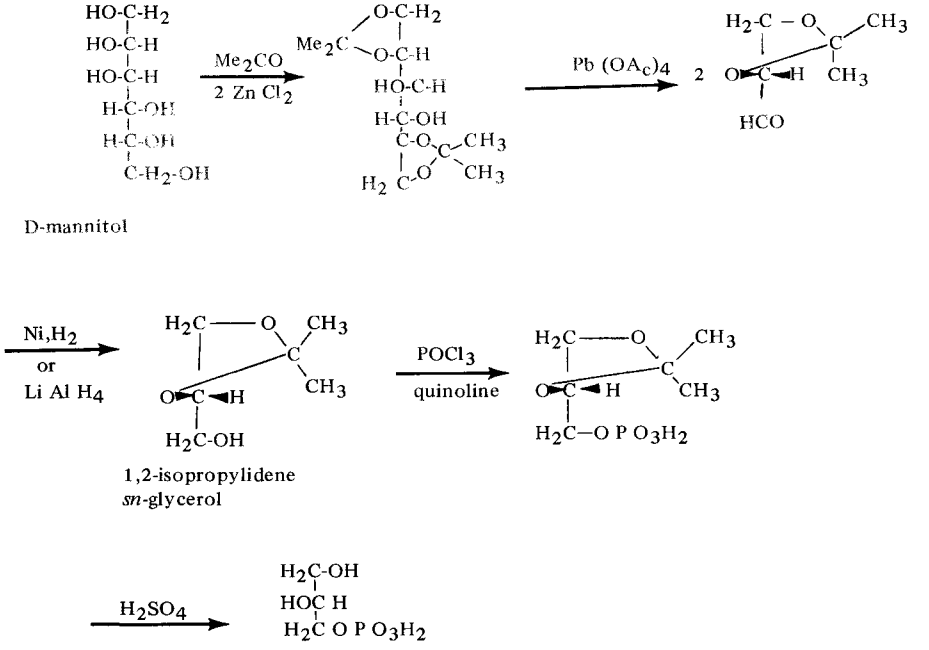


FIG. 1. Synthesis of 1,2-isopropylidene-*sn*-glycerol and *sn*-glycerol-3-phosphate (13).

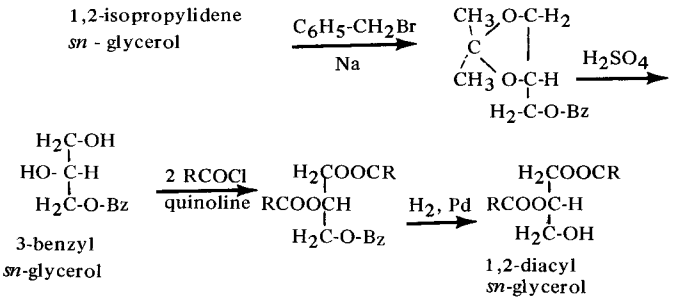


FIG. 2. Synthesis of saturated 1,2-diacyl-*sn*glycerol (17). Bz = benzyl.

but are more readily prepared from natural products (7-9). The deacylation reagent of choice is tetrabutylammonium hydroxide (10) as used by Chadha (8). In this procedure, egg yolk lecithins obtained from dried egg yolk solids are deacylated, and the resulting GPC recovered as the $CdCl_2$ adduct. The $CdCl_2$ can be removed when desired with mixed bed ion exchange resins. Although in the references above the egg yolk lecithin was purified on various types of columns, we have found that several precipitations from acetone will produce material of sufficient purity for deacylation (11). Deacylation as carried out by Chadha (8) does not require highly purified material as the impurities either remain behind during the methanolysis or during crystallization of the adduct. GPE has been similarly prepared from purified soybean phosphatidyl ethanolamine (12). Both GPC and GPE can be made simultaneously by deacylation of egg yolk powder and purified by column chromatography (9).

### *sn*-Glycerol-3-Phosphate (GP)

GP is synthesized from 1,2-isopropylidene *sn*-glycerol as described by Baer (13) and shown in Figure 1. Since 1,2-isopropylidene *sn*-glycerol is an important intermediate in the preparation of phosphoglycerides, the synthesis of this material from D-mannitol is also shown in Figure 1. By starting with D-mannitol, a readily available compound of known configuration, Baer was able to prepare phosphoglycerides of unequivocal structure. As these were then used to establish the structure of natural phosphoglycerides, and 1,2-isopropylidene *sn*-glycerol has been the basis for practically all syntheses of phsophoglycerides, the magnitude of the achievements by Baer is readily apparent.

L-mannitol, the precursor for 2,3-isopropylidene-*sn*-glycerol, is not commercially available, but was made by Fischer and Baer (14) and by Sowden (15) from L-arabinose which can be purchased.

### Enantiomeric Diacylglycerols

Syntheses of these compounds are described in a forthcoming review on synthetic glycerides (16) and hence will be discussed only briefly here. The first preparation of 1,2-diacyl-*sn*-glycerol was apparently that described by Sowden and Fischer (17) using 1,2-isopropylidene-*sn*-glycerol as the starting compound (see Fig. 2). The free hydroxyl group was benzylated, the ketal cleaved with $H_2SO_4$ yielding 3-benzyl-*sn*-glycerol which was acylated, and the protective benzyl group removed by hydrogenation. This latter step precludes the formation of unsaturated diacylglycerols.

Baer and Buchnea (18) prepared unsaturated diacylglycerols in much the same manner, starting with the benzyl ether. The double bonds in the oleic acid were protected by conversion to dibromostearic acid prior to acylation. The bromines were then removed by shaking with zinc dust in ether subsequent to hydrogenolysis. Diacid unsaturated diacylglycerols were obtained similarly with several additional steps (19). The 3-benzyl *sn*-glycerol was tritylated at the *sn*-1-hydroxyl, and the *sn*-2-hydroxyl acylated with either stearoyl or oleoyl chloride. The trityl group was removed with HCl, and the *sn*-2-acyl migrated to the *sn*-1-position. Again the *sn*-2-position was acylated, this time with a different acid. In either case the double bonds in oleic acid were brominated. Then the benzyl group was removed by hydrogenolysis, and the bromines with zinc dust. This reaction sequence is shown in Figure 3. The *sn*-1-benzyl ether can be obtained from 2,3-isopropylidene-*sn*-glycerol or from other sources, and then used for 2,3-diacyl-*sn*-glycerols.

Buchnea (20) has recently made a series of enantiomeric diacylglycerols in similar fashion. However a column of silicic acid-boric acid was employed for detritylation. Buchnea synthesized the 2,3-enantioners by reversing the sequence of events. The 1,2-isopropylidene *sn*-glycerol was acylated instead of benzylated. The ketal was removed with concentrated HCl at -15 C, and the *sn*-1-OH tritylated. The *sn*-2-OH was acylated, and the trityl group

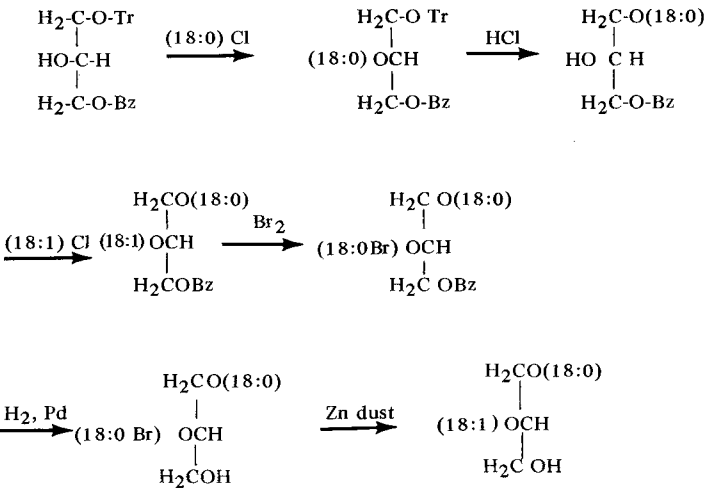


FIG. 3. Synthesis of a diacid 1,2-diacyl-*sn*-glycerol (19). Tr = trityl; Bz = benzyl; 18:0 = stearoyl; 18:1 = oleoyl; and 18:0Br = dibromostearoyl.

removed as before. With these methods Buchnea produced, for the first time, such compounds as 1-stearoyl-2-linoleoyl-*sn*-glycerol. This pairing of acyl groups is often observed in natural phosphoglycerides.

Lands and Zschocke (21) have synthesized 1-benzyl *sn*-glycerol by inversion of the *sn*-3-isomer. Gigg and Giff (22) described procedures for making 1-benzyl-*sn*-glycerol and *sn*-glycerol-2,3-carbonate from D-manitol.

Others have investigated the syntheses of enantiomeric diacylglycerols. Gigg and Gigg (23) obtained *sn*-glycerol-1,2-carbonate from 3-benzyl *sn*-glycerol by treatment with phosgene and hydrogenolysis (Fig. 4). Diacylglycerols are available via the tetrahydropyranyl ether route also shown in Figure 4. The carbonate is useful as a blocking group because it is removed by base, while most of the protective groups employed in glyceride synthesis are labile to acid. The specificity of pancreatic lipase for primary esters allows the synthesis of the diacid diacylglycerol as illustrated. The *sn*-2,3-enantiomers will result if the *sn*-1-benzyl ether is the starting compound.

Pfeiffer et al. (24), starting with 1,2-isopropylidene-*sn*-glycerol, blocked the free hydroxyl with trichloroethoxy carbonyl chloride (TCC) and, through a series of reactions utilizing both trityl and carbonate blocking groups, eventually obtained both diacylglycerol enantiomers. The TCC was removed with Zinc dust in acetic acid. Some 1,3-diacylglycerol was formed, and the possibility of both racemization and elaidinization must be considered. TCC was also utilized by Rakhit et al. (25) to make a series of 1,2-diacyl-*sn*-glycerols.

#### Glycerol Iodohydrins

These compounds are employed as acceptors of silver phosphoryl salts in one synthetic route for phosphoglycerides (2). They are achieved by the tosylation of either enantiomer of isopropylidene glycerol followed by replacement of the tosyl group with either iodine (26) or bromine (27). Bird and Chadha (27) found the bromohydrin to be less photosensitive than the iodohydrin and hence more convenient to use, although the rate of reaction was slower.

#### Fatty Acid Chlorides and Anhydrides

Oxalyl chloride is the preferred halogenating agent for fatty acids, because formation of color and polymerization do not occur (28). Fatty acid anhydrides are conveniently synthesized with the coupling agent, dicyclodihexylcarbodiimide (29). Caution must be exercised with this procedure, as the latter compound is a skin irritant.

### PHOSPHATIDIC ACID

This is an ephemeral compound in tissue, but is involved in the biosynthesis of both phospho- and triacylglycerols and hence should be more readily available.

Baer (30) utilized 1,2-diacyl-*sn*-glycerols as intermediates for saturated phosphatidic acids. The diacylglycerols were phosphorylated with diphenylphosphorylchloride, and the phenyl groups removed by catalytic hydrogenation. Later Baer and Buchnea (31) made dioleoyl phosphatidic acid by phosphorylation of the diacylglycerol with phosphorous oxychloride. Rakhit et al. (25) made the diacylglycerols via

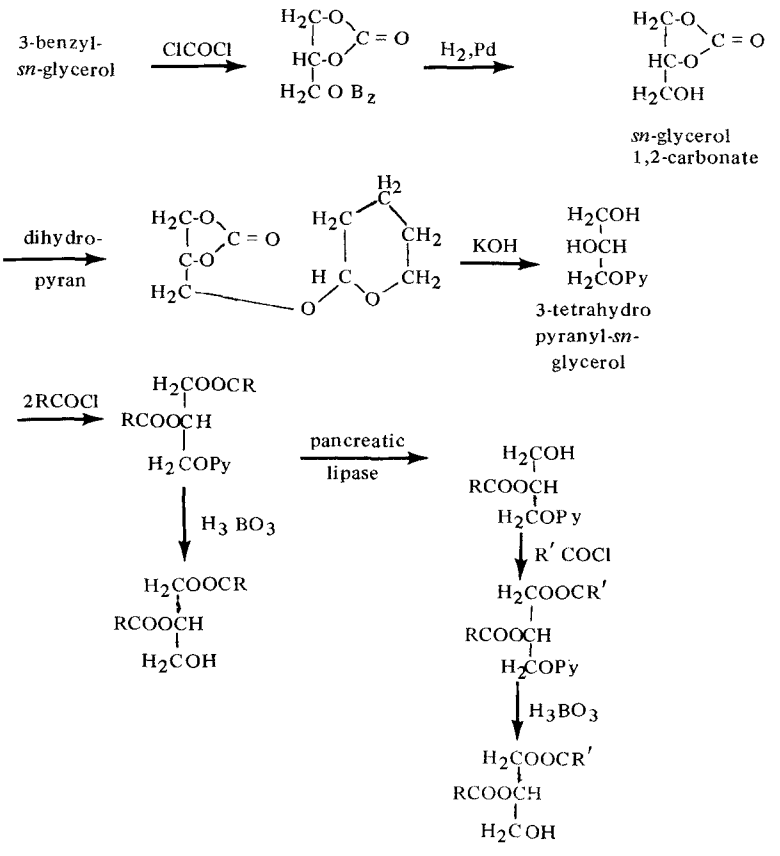


FIG. 4. Synthesis of mono- and diacid diacylglycerols (23), Bz = benzyl, and Py = tetrahydropyranyl.

the trichloroethoxy carbonyl chloride reaction with 1,2-isopropylidene-*sn*-glycerol, then similarly phosphorylated with phosphorous oxychloride.

Bonsen and de Haas (32), utilizing a protective group new to phosphoglyceride synthesis, silver-di-*t*-butylphosphate in the exchange reaction with 1-oleoyl-2-myristoyl-*sn*-glycerol iodohydrin, obtained the mixed acyl phosphatidic acid. The protective groups were removed with dry HCl. A minor disadvantage of this method is that several steps are required for the synthesis of the silver di-*t*-butylphosphate. The stereochemical integrity of the phosphatidic acid was established by reaction with phospholipase A.

Bird and Chadha (27) prepared phosphatidic acids from 1,2-diacyl-*sn*-glycerolbromohydrins and silver dibenzylphosphate. They describe a procedure for further purification of the latter compound.

Lapidot et al. (33) directly acylated *sn*-glycerol-3-phosphate with the appropriate acid anhydride in the presence of the tetraethylammonium salt of the acid. This was accomplished without the formation of large amounts of what were thought to be cyclic phosphates, as had been noted when acid anhydrides alone were the acylation agents.

## PHOSPHATIDYL CHOLINES (PC)

Baer and Kates (34) were the first to synthesize a genuine phosphatidyl choline identical to the *sn*-3 dipalmitoyl compounds isolated from various natural sources. This was done as shown in Figure 5. The 1,2-diacyl-*sn*-glycerol was phosphorylated, and the resulting reaction mixture treated with choline chloride. Phosphatidyl choline was obtained by purification through the reineckate, and the protective phenyl group removed by hydrogenolysis. In a later paper (35) an improved procedure for purification involving washing with various solvents was presented, but hydrogenolysis was still required. Continuing their syntheses of phosphatidyl cholines, Baer et al. (36) prepared the dioleoyl compound. In this method (Fig. 6) 1,2 isopropylidene-*sn*-glycerol was phosphorylated, then treated with ethylene chlorohydrin. The resulting compound was hydrogenated to

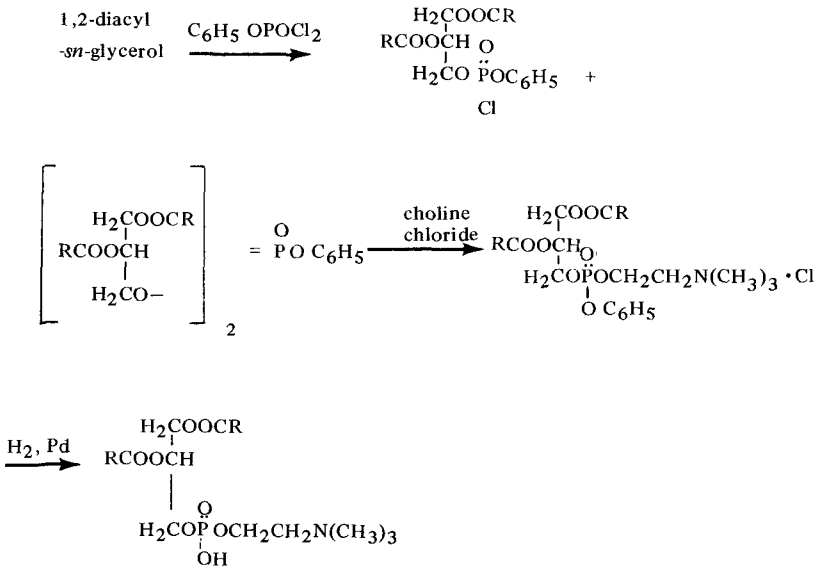


FIG. 5. Synthesis of saturated phosphatidyl choline (34,35).

remove the phenyl group, exposed to acid to cleave the ketal, and finally isolated as the barium salt. Acylation with oleoyl chloride was the next step followed by heating with trimethylamine. The desired compound was separated by silicic acid column chromatography. Shortly thereafter, still another improvement was made. Using the $CdCl_2$ complex of synthetic GPC, Baer and Buchnea (37) obtained improved yields of several diacyl PCs by direct acylation with the acyl chlorides. Synthetic GPC was employed, because the GPC obtained by the deacylation procedures in use at the time apparently was not pure. Baer and Kindler (38) developed yet another method for synthetic dioleoyl PC, which resembled the earlier procedure for synthesis from 1,2-diacyl-*sn*-glycerol except that phorphorous oxychloride replaced phenyl dichlorophosphate, thus avoiding the necessity for hydrogenolysis.

Cubero Robles and coworkers (9,39) avoided total synthesis by acylation of GPC obtained by the deacylation of egg yolk lecithin. Acylation was accomplished with the acid anhydride and an easily dissociable salt of the acid. Excellent yields, 70.5% for dioleoyl PC, were obtained. They also prepared 1-oleoyl-2-stearoyl-*sn*-PC by removing the *sn*-2-acid from the dioleoyl PC with snake venom phospholipase A, recovering the lysolecithin and reacylating with stearic anhydride. An additional hydrolysis indicated that the PC was essentially as named above.

We have employed the acylation technique above to produce large quantities of essentially pure dipalmitoyl and dilinoleoyl PCs (11). In both cases the yields were relatively good–75.8% and 66.7%. This is obviously the method of choice for preparing large quantities of monoacid PCs.

The deacylation of a monoacid PC by phospholipase $A_2$ and reacylation with another acid could be used to prepare mixed acid compounds. The availability of a polymer bound phospholipase which could be used repeatedly would reduce the expense. The idea of deacylation-reacylation is not original with Cubero Robles and van den Berg (39), having been tried by De Haas and van Deenen (40) and Hanahan and Brockerhoff (41). However these investigators started with synthetic PCs.

The reacylation of GPC as the $CdCl_2$ adduct with an acid chloride gave yields of 30-50% (37). These relatively poor to fair yields were found by Aneja and Chadha (42) to be caused by the presence of acyl-chloro-deoxyglycerophosphorylcholines. These impurities, difficult to remove, were formerly considered to be cyclic lysolecithins.

The preparation of mixed acid PCs might also be accomplished by hydrolysis of the monoacid compounds with purified pancreatic lipase. This enzyme hydrolyzes the *sn*-1-acid from all common types of phosphoglycerides, regardless of the nature of the fatty acid (43). Crude preparations cannot be employed, as they contain phospholipase A.

The very difficult problem of synthesizing

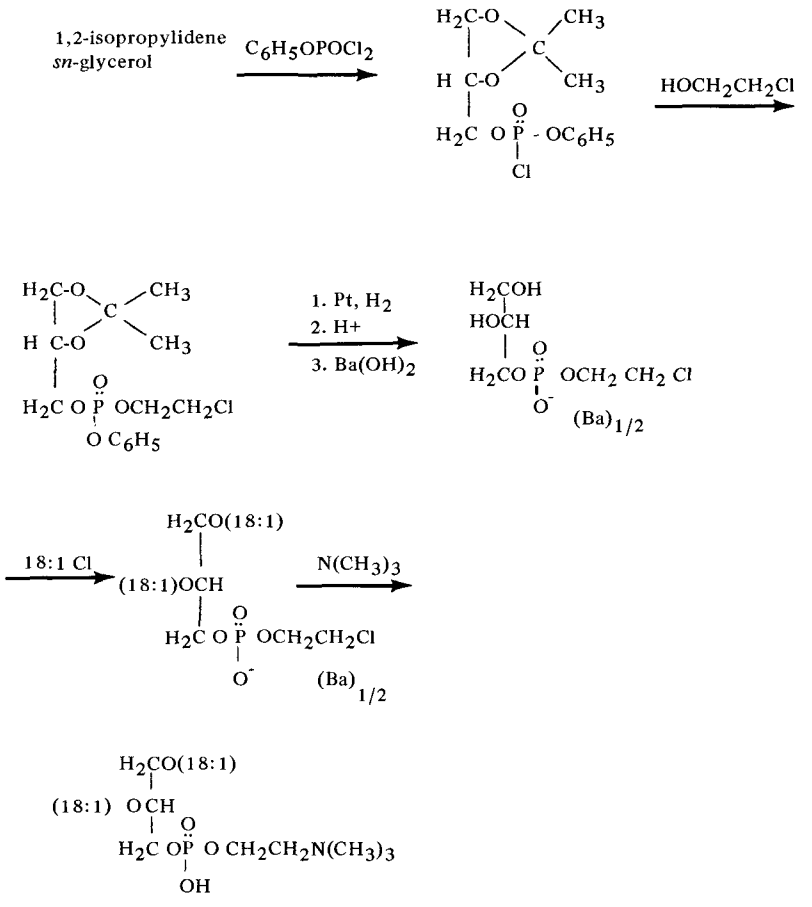


FIG. 6. Synthesis of unsaturated phosphatidyl choline (36).

mixed acid PC was approached quite differently by De Haas and van Deenen as shown in Figure 7. (44). 1-Stearoyl-2-oleoyl-*sn*-glycerol iodohydrin was phosphorylated with silver dibenzylphosphate, and the resulting compound converted to the silver salt. The choline moiety was introduced as the 2-bromoethyltrimethylammoniumpicrate. Several were prepared, and the sterochemical purity established by hydrolysis with phospholipase A. This group of investigators have developed many synthetic procedures based in general on the silver salt halogen exchange reaction and produced a large number of phosphoglycerides for further biochemical research. Their work is partially reviewed in References 1 and 2.

## PHOSPHATIDYL ETHANOLAMINES (PE)

Baer et al. (45), drawing upon their experience with PCs, prepared PE in much the same fashion. The amino function in ethanolamine was protected by a carbobenzoxy group, and both this and the phenyl group were removed by hydrogenolysis (Fig. 8). Again one of the compounds, distearoyl PE, was found to be identical with a PE isolated from hydrogenated egg yolk PEs, thereby establishing the *sn*-3-structure of the native phosphoglyceride. Then Baer and Buchnea (46), utilizing 1,2-dioleoyl *sn*-glycerol, produced the unsaturated PE. In this method the protective phthaloyl group was removed by hydrazinolysis. Wishing to avoid the synthesis of 1,2-diacyl-*sn*-glycerols, Baer and coworkers (5,6) developed an ingenious improvement. Direct phosphorylation of isopropylidene glycerol (*sn*-1,2; 2,3 or *rac*) was followed by addition of the phthaloyl protected ethanolamine. The ketal was removed, acid chlorides used to acylate the free hydroxyls, and the phthaloyl group removed by hydrazinolysis.

Daemen et al. (47) started with a mixed acid-1,2-diacyl-*sn*-glycerol iodohydrin to obtain

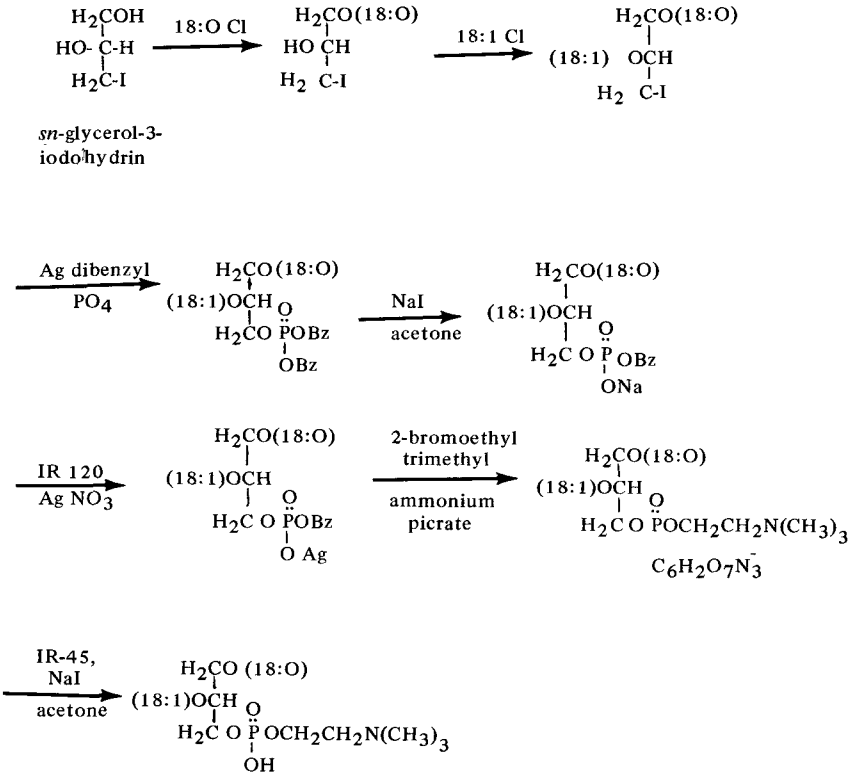


FIG. 7. Synthesis of diacyl phosphatidyl choline (44).

the mixed acid PE (Fig. 9). The silver salt-iodine exchange reaction was used to attach the protected ethanolamine. Later Chadha (48) employed the less photosensitive bromohydrin. The protective groups, e.g., benzyl and phthaloyl were removed by treatment with BaI and hydrazine. In an improved synthesis, silver benzyl-2-phthalimidoethyl phosphate was replaced by the *t*-butyloxycarbonyl derivative (49). Several steps were required for synthesis of both intermediates. Still another improvement employed *t*-butyl to protect the hydroxyl and *t*-butyloxycarbonyl for the amine (50). Both groups were removed with dry HCl. The HCl adduct of PE was converted to PE by treatment with Amberlite IR-120, and further purification achieved by crystallization.

Billimoria and Lewis (51) protected the amine function of ethanolamine with the trityl group which was removed with acid. The diacyl iodohydrin was converted to the silver benzyl phosphate which was exposed to tritylaminoethyl iodide.

In more recent years several newer protective groups and methods have been applied to the synthesis of PEs. Aneja et al. (52) attached the ethanolamine moiety to phosphatidic acid via trityl aziridine. Aneja et al. (12) also isolated PE from soybean phospholipids, phthaloylated the amine, deacylated the protected PE, and then reacylated with the acid anhydride. In much the same way Aneja et al. (53) prepared distearoyl PE from bacterial PE. The trityl group was used to protect the amine function. Aneja et al. (54) condensed phosphatidic acid with tritylaminoethanol using a new coupling agent, triisopropylbenzenesulfonyl chloride. The trityl group was removed by hydrogenolysis. Barzilay and Lapidot (55) employed the older coupling agent dicyclohexylcarbodiimide, to synthesize PE from phosphatidic acid and N-*t*-butoxycarbonyl ethanolamine. Dry HCl removed the protective group. More recently Barzilay and Lapidot (56) made use of another amino blocking group borrowed from the peptide chemists, to prepare PE. Ethanolamine was protected with *o*-nitrophenylsulfenyl chloride. The advantages of this compound are: a simple one-step condensation, stability during condensation and ease of removal with HCl or lithium thiosulfate.

Pfeiffer et al. (57) employed another new group, trichloroethoxy carbonyl chloride (TCC), to protect ethanolamine. The ethanol-

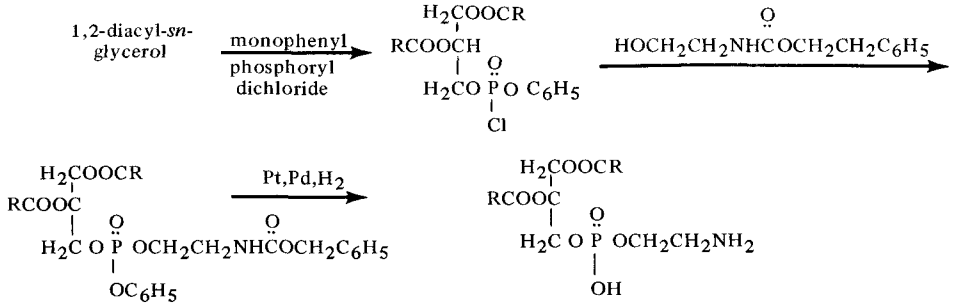


FIG. 8. Synthesis of saturated phosphatidyl ethanolamine (45).

amine was then converted to the phosphoryl chloride, and this was in turn reacted with 1,2-diacyl-*sn*-glycerol. The protective group was removed by shaking with zinc dust in acetic acid. Both Pfeiffer et al. (24) and Rakhit et al. (25) had utilized TCC in the synthesis of diacylglycerols. Rakhit et al. then prepared several PEs using *t*-butoxycarbonyl to protect the ethanolamine.

It appears that the synthesis of mono- and diacid PEs would be facilitated by the procedures described in the section on PCs: isolation of PE from natural sources (9,12), deacylation and reacylation (9,12), followed by hydrolysis with either purified pancreatic or phospholipase A and reacylation for the mixed acid PEs.

## PHOSPHATIDYL SERINES (PS)

Baer and Maurukas (58) prepared distearoyl PS as Baer et al. (45) had done earlier with PC (Fig. 10). The diacylglycerol was phosphorylated, and the phenylester of the phosphatidyl chloride was esterified with the benzyl ester of carbobenzoxyserine. All protective groups were removed by hydrogenolysis. De Haas et al. (59) accomplished the synthesis of a mixed acid PS by carrying out the silver salt-iodine exchange with a protected DL-serine and the appropriate iodohydrin. Dry HCl was employed to remove the protective groups. Turner et al. (60) blocked the amino of L-serine with anixyloxycarbonyl and the carboxyl with phthalimidomethyl, and then proceeded as had Baer and Maurukas (58). Turner et al. found that these groups were most desirable for the synthesis of unsaturated PEs than some of those previously mentioned. Finally, the procedure employed by Aneja et al. (54) for the synthesis of PE, which utilized triisopropylbenzenesulphonyl chloride as a coupling agent between phosphatidic acid and the appropriate nitrogenous moiety, was used to make PS.

## MONOACYLPHOSPHOGLYCERIDES

These compounds can be synthesized, but are best prepared by the action of purified pancreatic lipase (43) or phospholipase A (9) upon synthetic phosphoglycerides. The former enzyme produces the *sn*-2-monoacyl compound and the latter, the *sn*-1. Chemical synthesis of monoacylphosphoglycerides is discussed in the review by Slotboom and Bonsen (1).

## PURIFICATION AND DETERMINATION OF PURITY

Purification is accomplished by crystallization, which is usually limited to saturated phosphoglycerides or column chromatography. Conditions for crystallization are somewhat empirical depending upon the phosphoglyceride. For some examples see References 48, 51 and 52.

Many different methods of column chromatography have been utilized. A number of these

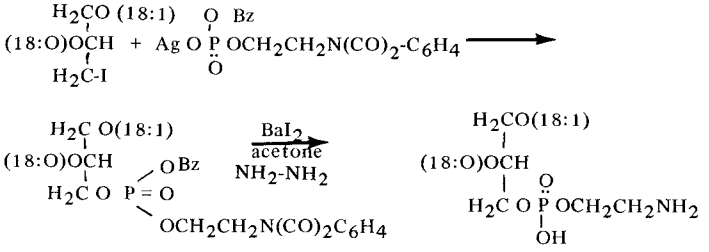


FIG. 9. Synthesis of diacid phosphatidyl ethanolamine (49).

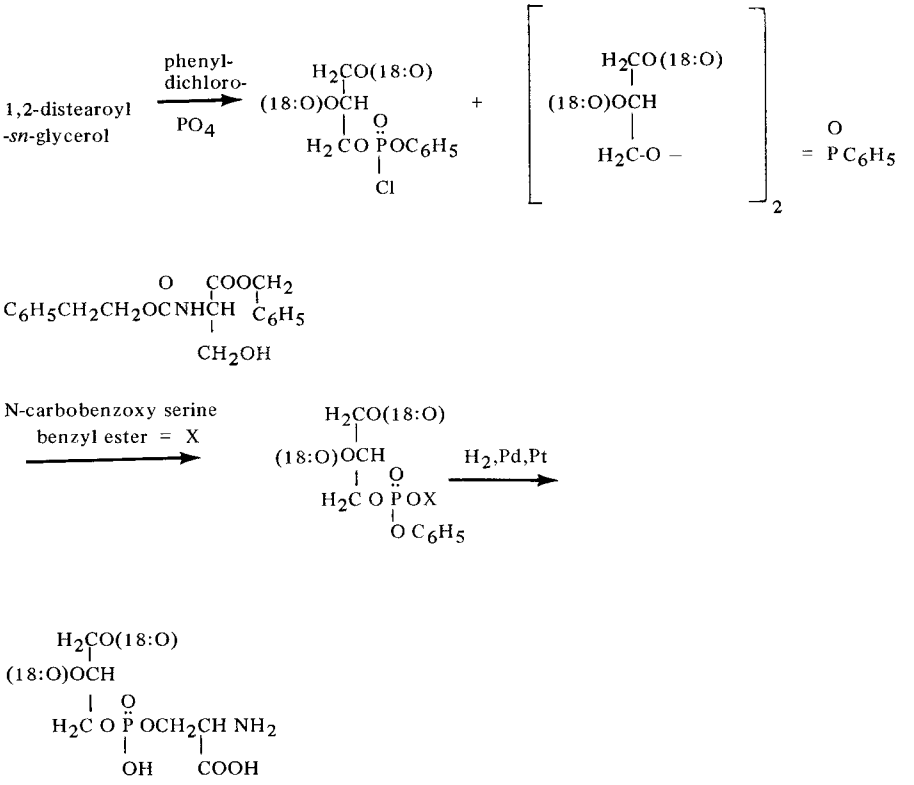


FIG. 10. Synthesis of phosphatidyl serine (58).

techniques are described by Rouser et al. (61) and in the appropriate papers listed in References.

Purity should be evaluated by the following methods: (a) single spot by two dimensional thin layer chromatography (62); (b) hydrolysis by phospholipase A followed by thin layer chromatographic examination of the products (63). If the phosphoglyceride is of the proper configuration, hydrolysis should be essentially complete after 18 hr, producing a 1-monoacyl-*sn*-glycerol and a free fatty acid formerly esterified to the *sn*-2-position (2,63). This method is not completely unequivocal, however, because snake venom phospholipase A will also hydrolyze a fatty acid esterified at the *sn*-1-position of an *sn*-2 ($\beta$) phosphoglyceride (64). Isomeric monoacylphosphoglycerides can be identified by various enzymatic manipulations. Thus the *sn*-1 monoacyl isomer is not attacked by phospholipase A and is by phospholipase C, whereas the *sn*-2-isomer is hydrolyzed by both enzymes. De Haas and van Deenen (65) were able to detect ca. 10% of either isomer by this method. A variation of this approach would involve reacylation of the monoacylphosphoglyceride with a marker acid followed by hydrolysis with purifed pancreatic lipase. If the new acid is digested by the enzyme, then it is located in the *sn*-1-position, and the structure is not that desired; (c) "correct" specific rotation. Traditionally, specific rotation has been utilized to ascertain the stereochemical integrity of synthetic phospholipids. Rotations of the synthetic product are compared to those reported in the literature or to that of a purified natural phosphoglyceride. Since the specific rotations reported have been relatively small, e.g., + 6.0 for PE (55), the significance of small differences remains open to question. A useful investigation would compare the purity, as determined by the enzymatic and optical rotation procedures. There is no doubt, however, that determinations of specific rotation are of value in ascertaining the purity of some of the initial and intermediate compounds involved in the synthesis of phosphoglycerides; (d) 1:1 molar ratio of phosphorous to nitrogen. While reassuring, these analyses are probably unnecessary if the synthetic compound is "pure" by criteria (a) and (b) above; and (e) proper fatty acids in the right positions as determined by gas liquid chromatography. The identity of the acids should be

checked before use in synthesis.

## DISCUSSION

Methods are available for the synthesis of many phosphoglycerides. However, except for monoacid PCs and PEs, techniques for producing large quantities of other and of mixed acid phosphoglycerides have not yet been developed. The use of pancreatic lipase and phospholipase A followed by reacylation is probably the most promising route to accomplish synthesis of large quantities of mixed acid phosphoglycerides. We hope that this review will encourage others to investigate the synthesis of these compounds.

## ACKNOWLEDGMENTS

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# SHORT COMMUNICATIONS

## Analysis of Prostaglandins in Rat Vesicular Glands


### ABSTRACT

Techniques are described whereby problems in the analysis of endogenous prostaglandins in rat vesicular glands are obviated by the use of powdered glands prepared by grinding the tissues in the frozen state. Freshly excised glands were cut into small pieces, frozen on dry ice and then pulverized to a fine powder. These powders were used as such or extracted with acetone. In the latter technique, the endogenous prostaglandins were quantitatively extracted into acetone, and most of the biological activity for prostaglandin synthesis was retained in the residual cake. Application of the techniques showed that the $E_2$ form was the major prostaglandin of the E series in rat vesicular glands.


Quantification of prostaglandins in animal tissues and body fluids is a difficult analysis, because these compounds are trace constituents occurring in concentrations of the order of only $10^{-9}$ to $10^{-7}$ moles per gram (1,2). Detection of biosynthetic acitivity of many tissues for the synthesis of prostaglandins is also a problem for the same reason. Procedures for the analysis of prostaglandins have been reviewed by Ramwell et al. (3), Shaw and Ramwell (4) and Anggard (1). In general, analytical measurements are applied to concentrates of prostaglandins obtained from homogenates of several grams or more of tissue. With small laboratory animals such as the rat, difficulties in the analysis of prostaglandins are compounded by the limited amount of tissue available, unless pooled samples from several animals are used.

In the course of studies on the vesicular glands of rats, we found that it was virtually impossible to obtain quantitative data on the concentration of prostaglandins with homogenates. Not only was it difficult to avoid mechanical loss of this tissue, but most of the activity of the gland was lost when the homogenization technique was used. These difficulties were largely obviated by grinding the tissues to a fine powder in the frozen state. By means of this technique, it was possible to measure endogenous prostaglandin concentration and detect biosynthetic activity for the synthesis of these compounds with the glands of single animals.

### EXPERIMENTAL PROCEDURES

Rat vesicular glands were obtained from male Sprague-Dawley rats weighing 200-250 g over a period of ca. 2 months. The animals were sacrificed under ether by withdrawal of blood from the aorta. The glands were excised, frozen on dry ice and stored at -20 C when not used immediately.

Prostaglandin reference standards and arachidonic acid were obtained from the Lipids Preparation Lab. of The Hormel Institute, and 8,11,14-eicosatrienoic-1-$^{14}$C acid (homo-γ-linolenic acid) from H.W. Sprecher, Department of Physiological Chemistry, Ohio State University. The chemical and radiochemical purity of the acid was greater than 96% as determined by gas

TABLE I

Analysis of Endogenous Prostaglandin E in Rat Vesicular Glands

| Ground powder technique (buffer-incubation procedure) | | | | Acetone-extraction technique (analysis of acetone extract) | | | |
|---|---|---|---|---|---|---|---|
| | | PGE | | | | PGE | |
| Animal | Weight of gland | Tissue, μg/g | μg/Gland | Animal | Weight of gland | Tissue, μg/g | μg/Gland |
| 1 | 0.384 | 672 | 258 | 5 | 0.499 | 524 | 261 |
| 2 | 0.652 | 206 | 134 | 6 | 0.780 | 450 | 351 |
| 3 | 0.844 | 843 | 711 | 7 | 1.167 | 482 | 562 |
| 4 | 0.878 | 265 | 233 | 8 | 1.810 | 169 | 306 |

TABLE II

Conversion of Arachidonic Acid to Prostaglandin $E_2$ by Rat Vesicular Glands

| Ground powder | | | | Acetone-extracted powder | | | |
|---|---|---|---|---|---|---|---|
| Animal | Arachidonic acid substrate, mg | $\mu$g $PGE_2$ per g tissue | Yield,% | Animal | Arachidonic acid substrate,mg | $\mu$g $PGE_2$ per g tissue | Yield,% |
| 1 | 2 | 166 | 8.3 | 4 | 2 | 93 | 4.6 |
| 2 | 1 | 74 | 7.4 | 5 | 1 | 21 | 2.1 |
| 3 | 1 | 23 | 2.3 | 6 | 1 | 12 | 1.2 |

liquid chromatography (5). Prostaglandin assay was carried out by UV absorption at 278 nm based on the method described by Nugteren et al. (6) and Yoshimoto et al. (7). An aliquot of the prostaglandin solution was diluted to 2 ml with ethanol. To this was added 2 ml 1 N NaOH with rapid mixing. The increase in absorption of 278 nm was continuously recorded until the plateau was reached, after 15 min at room temperature. The absorption was plotted as a function of time, and from the initial slope optical density at zero time was extrapolated. The total increase of absorption at 278 nm after alkali addition was calculated on the basis of the known molecular extinction coefficient of PGE compounds ($\Delta$ Absorbance = 1.00 corresponds to 39.4 $\mu$g prostaglandin per 3 ml reaction mixture) (6,7).

The thin layer chromatography (TLC) techniques for the identification and isolation of the prostaglandins of the E series and the subsequent resolution of these compounds into $E_1$, $E_2$, and $E_3$ by argentation-TLC were carried out as described by Green and Samuelsson (8) and Ramwell and Daniels (9). Radioactivity was measured with a Packard Tri-Carb scintillation counter.

#### Ground Powder Technique

Freshly excised or partially thawed glands are cut into small pieces, frozen on dry ice and pulverized in a procelain mortar by means of a pestle. The mortar is embedded in an acetone dry ice bath during this operation in order to insure that all fragments of the glands are maintained in the frozen state. The glands are reduced to a fine frozen powder which can be used directly for enzymatic synthesis of prostaglandins with the appropriate substrates or for the determination of endogenous prostaglandin concentration via the general buffer incubation procedure described in the text.

#### Acetone-Extracted Powder Technique

In this method the finely powdered glands obtained as described above are extracted with 30-50 volumes cold acetone (0 C). Acetone is removed by filtration through a Whatman No. 1 filter paper using a suction funnel under negative pressure. The filter cake is washed with another 30 volumes cold (0 C) acetone to removed occluded mother liquor, and dried under reduced pressure at room temperature. The prostaglandins of the E series are extracted essentially quantitatively into the acetone by this procedure, and virtually all of the enzyme activity for the synthesis of these compounds is retained in the cake.

#### Detection of Biosynthetic Activity of Rat Vesicular Glands for Synthesis of Prostaglandins of the E Series

Ground powder or acetone-extracted powder from 1 g vesicular gland (rat glands are ca. 400 mg to 1.8 g, depending on age of the animal) is added to 10 ml 0.1 N $NH_4Cl$ buffer adjusted to pH 8.5 with $NH_4OH$ after the addition of glutathione to give a concentration of 2 mg/ml buffer. For conversion of arachidonic acid to $PGE_2$, 1 mg substrate is used for powder from 1 g original gland. The substrate is emulsified with the complete buffer in a 25 ml beaker. Finally either the ground or acetone-extracted powder is added to the emulsion. As a control and to take into account the presence of residual or endogenous prostaglandins, one beaker is prepared without added substrate. Incubation is carried out in a water bath at 37 C with shaking under an atmosphere of air for 1 hr. The reaction is stopped by lowering the pH of the incubation mixture to 3 with 1 M citric acid. This mixture is extracted three times with 30 ml ethyl acetate. The combined ethyl acetate extracts are evaporated under a negative pressure, and the residue is dissolved in 5 ml 95% ethanol. The ethanol solution is stored at -20 C, and aliquots of this solution are used for identification and quantitative determinations of the prostaglandins.

## RESULTS AND DISCUSSION

Determination of the endogenous prostaglandin E concentration in the ground powders and in the acetone extract of ground powders

of the vesicular glands of several rats is summarized in Table I. The animals employed in the study were not litter mates, but merely selected from stock available at the time of the analysis which was carried out over a 2 month period. Hence it appears that the fairly large variation in the results does not reflect the error in the method so much as individual variation between animals. Likewise the difference in size of the vesicular glands indicates such a variation. For this reason the total amount of prostaglandin E per gland was also calculated. There appears to be less variation in these values than in the concentration per gram of tissue. Whether this observation is significant is not known. Regardless, it is evident that valid results can be obtained for the concentration of endogenous prostaglandins by the grinding techniques described, whereas homogenization as normally applied failed. The recovery of endogenous prostaglandins by the acetone extraction technique appears to be slightly lower than when the general $NH_4CL$ buffer-ethyl acetate proceure is used (Table I). However all of the prostaglandins can be recovered by exhaustive extraction with acetone. Hence either some synthesis occurs during incubation in the buffer, in the general procedure, or some loss of prostaglandins occurs in the acetone extraction technique. Regardless, the loss in the latter procedure is minimal, and that procedure is by far a simpler method. Analysis of the endogenous prostaglandin E fraction via argentation-TLC showed that the major component was the $E_2$ constituent.

The detection of biosynthetic activity of the glands for the synthesis of prostaglandins is illustrated in Table II. Although the yield of prostaglandin $E_2$ from arachidonic acid was low, it was nevertheless highly significamt; Because increasing substrate concentration increased yield, it is not unlikely that conversion may be increased further by adjustment of other conditions. There appears to be less synthetic acitivity in the powders extracted with acetone. A portion of the enzyme may have been inactiviated or extracted into the acetone. Nevertheless separation of endogenous prostaglandins from the enzyme systems was affected by acetone extraction.

Studies on the use of 8,11,14-eicosatrienoic-1-$^{14}$C acid (homo-$\gamma$-linolenic acid) as a substrate showed that the amount of it converted to $PGE_1$ was neglegible.

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# Presence of *trans*-6-Hexadecenoic Acid in the White Jellyfish *Aurelia aurita* Lamarck and in a Caribbean Gorgonian

**ABSTRACT**

An unusual fatty acid, *trans*-6-hexadecenoic, previously found in the lipids of marine turtles, the ocean sunfish and a sea anemone, is also present in the lipids of the white jellyfish and in a common Caribbean sublittoral gorgonian.

We have previously isolated *trans*-6-hexadecenoic acid from the depot fat of the (leatherback) marine turtle *Dermochelys coriacea coriacea* L. (1). Subsequently this acid has been found in two other marine turtle species (*Caretta caretta caretta* and *Lepidochelys olivacea* Kempi (2), in the ocean sunfish *Mola mola*

of the vesicular glands of several rats is summarized in Table I. The animals employed in the study were not litter mates, but merely selected from stock available at the time of the analysis which was carried out over a 2 month period. Hence it appears that the fairly large variation in the results does not reflect the error in the method so much as individual variation between animals. Likewise the difference in size of the vesicular glands indicates such a variation. For this reason the total amount of prostaglandin E per gland was also calculated. There appears to be less variation in these values than in the concentration per gram of tissue. Whether this observation is significant is not known. Regardless, it is evident that valid results can be obtained for the concentration of endogenous prostaglandins by the grinding techniques described, whereas homogenization as normally applied failed. The recovery of endogenous prostaglandins by the acetone extraction technique appears to be slightly lower than when the general $NH_4CL$ buffer-ethyl acetate proceure is used (Table I). However all of the prostaglandins can be recovered by exhaustive extraction with acetone. Hence either some synthesis occurs during incubation in the buffer, in the general procedure, or some loss of prostaglandins occurs in the acetone extraction technique. Regardless, the loss in the latter procedure is minimal, and that procedure is by far a simpler method. Analysis of the endogenous prostaglandin E fraction via argentation-TLC showed that the major component was the $E_2$ constituent.

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Studies on the use of 8,11,14-eicosatrienoic-1-$^{14}$C acid (homo-$\gamma$-linolenic acid) as a substrate showed that the amount of it converted to $PGE_1$ was neglegible.

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[Received July 20, 1972]

# Presence of *trans*-6-Hexadecenoic Acid in the White Jellyfish *Aurelia aurita* Lamarck and in a Caribbean Gorgonian

**ABSTRACT**

An unusual fatty acid, *trans*-6-hexadecenoic, previously found in the lipids of marine turtles, the ocean sunfish and a sea anemone, is also present in the lipids of the white jellyfish and in a common Caribbean sublittoral gorgonian.

We have previously isolated *trans*-6-hexadecenoic acid from the depot fat of the (leatherback) marine turtle *Dermochelys coriacea coriacea* L. (1). Subsequently this acid has been found in two other marine turtle species (*Caretta caretta caretta* and *Lepidochelys olivacea* Kempi (2), in the ocean sunfish *Mola mola*

(3) and in the sea anemone *Metridium dianthus* (4), where the corresponding alcohol was also found. Of these animals it has been reported that both the leatherback (5) and the sunfish (6) include jellyfish in their diets. Since the sea anemone belongs to the same phylum as jellyfish, we have therefore extended our investigations of the fatty acids of these animals to two other species of different classes.

*Aurelia aurita* Lamarck, or white jellyfish, caught off Eastern Passage, Nova Scotia, in late July 1970, were kept forzen *en masse* in flat trays until Septemper 1971. They were then crushed into walnut-sized lumps and freeze-dried, 3.3 kg yielding 35 g of dry material. An extraction by the method of Bligh and Dyer, with the addition of 28 g water (7), recovered 0.15 g of lipid (0.43% of dry weight). Thin layer chromatography (TLC) of the recovered lipid indicated the presence of obvious spots for phospholipids and triglycerides, with indications of small amounts of free fatty acids, sterol and sterol esters. The entire sample was saponified by AOCS Method Ca-6b-53, and unsaponifiable materials were removed. Esterification of the recovered fatty acids was carried out by refluxing with 6% $BF_3$-methanol for 10 min. TLC of the nonsaponifiables was carried out on silica gel Prekotes (Applied Science Laboratories) in an attempt to isolate the fatty alcohols, but if any were present the amounts were too small for successful isolation. Further TLC and gas liquid chromatography (GLC) steps and analyses were carried out as described earlier (1) with a view to estimating *trans*-6-hexadecenoic acid.

The gorgonian was collected from shallow water on December 8, 1971, at St. Thomas, Virgin Islands, but was not extracted in Halifax until December 13, 1971. During this time it was exposed to air in a plastic bag. The exterior layer had become dry and could easily be removed from the branched proteinaceous support matrix. A sample total of 88 g yielded 78 g of this outer layer which was extracted by the method of Bligh and Dyer. Dried 0.84 g of dark, shiny material (resembling a varnish) was recovered. TLC on a Kodak Chromagram silica gel sheet (hexane-diethyl ether-acetic acid 85:15:1) showed a high proportion of free fatty acids, small amounts of mono-, di- and triglycerides, and sterol, with sterol or wax esters. The entire sample was saponified, unsaponifiables were removed, and esters prepared by refluxing the recovered fatty acids with $BF_3$-methanol.

The nonsaponifiable materials from the saponification were subjected to preparative TLC carried out on Prekotes to obtain the free alcohols. These were acetylated by refluxing with acetic anhydride.

TABLE I

Principal or Unusual[a] Fatty Acids of the White Jellyfish *Aurelia aurita* and of a Caribbean Gorgonian as Weight Per Cent of Total Fatty Acids Identified[b]

| Fatty acid | Jellyfish | Gorgonian |
|---|---|---|
| 12:0 | NSA | 2.5 |
| 14:0 | 3.3 | 7.1 |
| 16:0 | 16.0 | 29.0 |
| *trans* 16:1ω10 | 2.0 | 0.9 |
| 16:1ω9 | 0.1 | NSA |
| 16:1ω7 | 4.6 | 4.0 |
| 7-Methyl-7-hexadecenoic ? | 0.3 | 1.3 |
| 18:0 | 6.4 | 11.4 |
| 18:1ω9 | 8.9 | 15.6 |
| 18:1ω7 | 2.6 | 0.8 |
| 18:1ω5 | 0.3 | NSA |
| 18:4ω3 | 0.1 | 4.4 |
| 20:0 | 1.2 | 0.3 |
| 20:1ω11 | 0.7 | Trace |
| 20:1ω9 | 4.8 | Trace |
| 20:1ω7 | 7.1 | 0.2 |
| 20:1ω5 | 1.1 | 0.1 |
| 20:1ω6 | 6.7 | 4.1 |
| 20:5ω3 | 8.5 | 0.5 |
| 22:0 | 0.1 | NSA |
| 22:1ω13 + 11 | 1.2 | NSA |
| 22:1ω9 | 3.2 | NSA |
| 22:1ω7 | 0.1 | NSA |
| 22:4ω6 | 0.6 | NSA |
| 22:5ω6 | 0.3 | NSA |
| 22:5ω3 | 1.6 | 0.5 |
| 22:6ω3 | 7.0 | 2.8 |
| Total | 88.9 | 85.6 |

[a]Respective weight percentages of less interesting fatty acids and tentatively identified minor fatty acids were: 13:0, NSA, 1.2; iso 14:0, 0.1, 0.6; 4,8,12-trimethyltridecanoic, 0.1, 0.3; 14:1ω9, 0.6, trace; 14:1ω7, 0.3, 0.4; iso 15:0, 0.6, 0.3; anteiso 15:0, 0.1, 0.1; 15:0, 1.0, 0.8; 15:1ω6, 0.1, 0.5; iso 16:0, 0.5, 0.1; 2,6,10,14-tetramethylpentadecanoic, trace, 0.3; iso 17:0, 0.7, 0.1; anteiso 17:0, 0.4, trace; 17:0, 0.7, 0.6; 3,7,11,15-tetramethylhexadecanoic, 0.1, 0.1; 17:1ω8, 0.1, 2.5; iso 18:0, 0.2, trace; 18:2ω6, 0.8, 0.8; 18:3ω6, 0.5, 0.1; 18:3ω3, 0.4, 0.2; 19:0, 0.7, trace; 19:1ω10, 0.2, 0.2; 20:2ω6, 1.0, 0.8; 20:2(5,13), 1.3, NSA; 20:4ω3, 0.4, NSA.

[b]NSA = No significant amount.

Most features of a summary fatty acid analysis for the white jellyfish total (Table I) lipid do not differ significantly from those reported for the pink jellyfish *Cyanea capillata* (8). The iso, anteiso and odd chain saturated acids and minor fatty acids such as 14:1, 18:2ω6, 18:3ω6, 20:3ω3, etc., were similar in types and proportion in the two species of jellyfish and accordingly have been presented as a footnote to Table I. An unusual nonmethylene-interruped eicosadienoic acid (9) amounted to 1.3%. The low proportion of polyunsaturated fatty acids is thought to be due to the effects of freeze-drying. In this form of sample

lipid extraction is facilitated, whereas in the alternative of proper $H_2O/MeOH/CHCl_3$ ratios for raw jellyfish (99% water) the volume to be manipulated is very large and filtration difficult even with filter aid (8).

Two per cent of the total fatty acids in the analysis of the total fatty acids from the white jellyfish was found to be *trans*-6-hexadecenoic acid. In the earlier study of the pink jellyfish *Cyanea capillata* this was probably included in the unknown designated as *c* in the unhydrogenated sample (8). By chance the hydrogenated methyl esters would have contained what is now suspected to have been a methyl 7-methylhexadecanoate peak in this position (see below) in similar proportion.

The *trans*6-hexadecenoic acid was present in the gorgonian in a measured proportion of 0.9% but may have been less in the living animal. Because of the elapsed time between the collection of the gorgonian specimen and the extraction of the lipids, some polyunsaturated fatty acids may have been lost to autoxidation. If this were the case, the low level of these fatty acids found in this sample would tend to increase the percentages of shorter chain acids or saturated acids, or both. Alternatively, the higher environmental temperature or the presence of wax esters could reduce the polyunsaturated fatty acid content. The lower proportions of 20:0 and 20:1 acids tend to support the two latter possibilities. In the analysis of the gorgonian alcohol acetates by GLC 14:0 (11%), 16:0 (32%), 18:0 (45%) and 18:1 (13%) were identified, but the sample was insufficient to identify a peak for the alcohol corresponding to the *trans*-6-hexadecenoic acid.

A study of the distribution of the *trans*-6-hexadecenoic acid in the tissues and organs of a leatherback turtle does not indicate any specific metabolic or functional value of this acid in higher animals (10). Recent observations on the facile accumulation of free fatty acids from seawater by marine invertebrates (11) suggest that the high proportions of long chain saturated acids (18:0, 20:0) in the jellyfish might reflect the high proportion of fatty acids "dissolved" in seawater (12), and it is not impossible that *trans*-6-hexadecenoic acid could also have an orgin exogenous to the coelenterates.

The tentatively identified 7-methyl-7-hexadecenoic acid listed in Table I has recently been found to amount to 2-3% of sunfish liver oil (3), but most marine lipids probably contain some of this acid as evidenced by the almost universal occurrence of minor (<1%) amounts of the methyl ester of 7-methylhexadecanoic acid in hydrogenated ester samples. The search for the former acid in the two coelenterate GLC runs was retrospective, but the leatherback turtle and ocean sunfish association with coelenterates seems to be confrimed by both unusual unsaturated fatty acids. Further studies are planned to follow these novel biological associations in greater detail.

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[Received June 6, 1971]

# Acyl Migration Solid Phase Isomerization of 1,2-Diglycerides to 1,3-Isomers

**ABSTRACT**

In mixtures of 1,2- and 1,3-diglycerides stored some degrees below their melting points, a rather rapid isomerization of almost all the 1,2- diglycerides to the 1,3-isomers occurred. This solid-phase

lipid extraction is facilitated, whereas in the alternative of proper $H_2O/MeOH/CHCl_3$ ratios for raw jellyfish (99% water) the volume to be manipulated is very large and filtration difficult even with filter aid (8).

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In mixtures of 1,2- and 1,3-diglycerides stored some degrees below their melting points, a rather rapid isomerization of almost all the 1,2- diglycerides to the 1,3-isomers occurred. This solid-phase

TABLE I

Solid-Phase Isomerization of 1,2-Diglycerides

| Starting mixture | Temperature C | Time, days | Ratio 1,3-/1,2 diglycerides | |
|---|---|---|---|---|
| | | | Before | After |
| Distearoyl | 65 | 2 | 75:25 | 98:2 |
| Stearoyl, palmitoyl | 60 | 2 | 75:25 | 97:3 |
| Stearoyl, palmitoyl | 20 | 210 | 75:25 | 95:5 |
| Stearoyl, palmitoyl[a] | 49 | 3 | 47:53 | 94:6 |
| Dipalmitoyl | 55 | 4 | 71:29 | 95:5 |
| Stearoyl, oleoyl | 35 | 5 | 75:25 | 94:6 |
| Palmitoyl, oleoyl | 29 | 10 | 71:29 | 90:10 |
| Dicapryl | 33 | 15 | 75:25 | 90:10 |
| Caproyl, palmitoyl | 26 | 7 | 71:29 | 90:10 |

[a]Crystallization residue.

isomerization leads to a substantial increase in the yield of saturated and unsaturated mono- and mixed-acid 1,3-diglycerides.

A recently published study by Lutton (1), in which the solid-phase isomerization of 1,2-diglycerides to 1,3-diglycerides from mixtures of both isomers was observed, has prompted us to report our findings more extensively.

Some years ago the need for larger quantities of pure 1,3-diglycerides in our laboratory was the reason for studying the addition of fatty acids to glycidyl esters (2). Although this addition reaction seemed to be 100% selective in the formation of the 1,3-isomers, the high temperature and long reaction period required to reach an acceptable degree of conversion led, in all cases, to equilibrium mixtures of 1,2- and 1,3-diglycerides (3). Addition of a quarternary "onium" salt such as tetraethylammonium bromide (4) decreased the reaction temperature and reaction time, however being still insufficient to avoid isomerizations.

It was during these studies that we discovered the isomerization phenomena of solid 1,2-diglycerides (5). When mixtures of 1,2- and 1,3-diglycerides—obtained, for instance, from the above mentioned reaction—were stored preferably some three degrees below their melting points, a rather rapid isomerization of almost all 1,2-diglycerides to 1,3-diglycerides occurred, the rate of which depended on temperature. After this isomerization, one rapid single crystallization from hexane or acetone gave the desired 1,3-diglycerides in high yields and purity.

Equilibrium mixtures of S(OH)S (1,3-distearoyl glyceride) and SS(OH) (1,2-distearoyl glyceride) could be isomerized at 65 C within 3 days to a mixture containing less than 2 or 3% 1,2-isomer. Compositions were determined by gas liquid chromatography analysis.

More time (3 or 4 weeks) was needed to decrease the 1,2-diglycerides content in S(OH)O (1-stearoyl-3-oleoyl-glyceride) and P(OH)O (1-palmitoyl-3-oleoyl-glyceride), because these diglycerides have lower melting points than their saturated analogs, and one is constrained to work at lower isomerization temperatures. During these isomerization reactions, the melting points will increase by some four degrees, so that the storage temperature can be adjusted correspondingly. A few examples are given in Table I.

This solid-phase isomerization can be extended to saturated and unsaturated mixed-acid diglycerides of the aliphatic series, including oxo acids, and furthermore to substituted and unsubstituted aromatic acyl groups such as *p*-nitrobenzoyl. In tritylated or benzylated monoglycerides (1,2- and 1,3-isomers), isomerization in the solid phase also occurs to give practically pure 1,3-isomers by acyl migration. The next example may be an illustration of this extension.

Glyceryl-1,3-di-*p*-nitrobenzoate was prepared by partial acylation of the α-mono glyceride of *p*-nitrobenzoic acid. The product was purified by crystallization until it contained only 1.1% 1,2-isomer; mp 136-137 C. Isomerization at 160 C gave the equilibrium mixture; mp 125 C; 1,2-/1,3-ratio 27.6:72.4. Solid-phase isomerization at 120 C for 2 days gave the initial pure product containing 1.5% 1,2-isomer (mp 135 C).

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[Received July 18, 1972]

# Effect of Neonatal Food Restriction and Hyperphenylalaninemia on Desmosterol to Cholesterol (D/C) Ratio in Developing Rat Brain

## ABSTRACT

The sterol content and the ratio of desmosterol to cholesterol (D/C ratio) in brains of undernourished and hyperphenylalaninemic rats were measured. Neonatal food restriction resulted in a high D/C ratio, but the total sterol content did not change significantly. Phenylalanine treatment had no appreciable effect on the D/C ratio, but the total sterol content was reduced. The results suggest that neonatal food restriction leads to a block at the desmosterol reductase step, while treatment with phenylalanine inhibits sterol synthesis at a step prior to desmosterol.

## INTRODUCTION

In rat brain desmosterol accumulates prior to the time when the rate of cholesterol deposition is most rapid (1-3). A subsequent decrease in brain desmosterol is accompanied by a rapid increase in desmosterol reductase activity (4). These findings indicate that the reduction of desmosterol to cholesterol may be the rate-limiting step for cholesterol biosynthesis in the brain. Since the deposition of cholesterol in brains of undernourished rats (5-7) and hyperphenylalaninemic rats (8) is slow, we considered it of interest to investigate the effect of neonatal food restriction and hyperphenylalaninemia on the ratio of desmosterol to cholesterol (D/C ratio). The results indicate that neonatal food restriction causes a high D/C ratio in the brain, but that hyperphenylalaninemia has no effect on the D/C ratio.

## MATERIALS AND METHODS

Litters of Sprague-Dawley rats were divided at 2 or 4 days of postnatal age into two groups of five pups each. Five pups of a litter were rendered either hyperphenylalaninemic or undernourished, and five pups served as controls. Hyperphenylalaninemia was induced from 5th day of age as described earlier (8). Pups were undernourished from 3rd day of age by restricting the time for nursing. They were separated from the mother from 8 A.M.-4P.M., a period of 8 hr. The pups were maintained at 30 C in light when away from the mother. At appropriate ages the animals were decapitated, and their brains (without cerebellum) were removed and weighed. Individual brains were frozen and lyophillized. Weighed portions of lyophillized tissue were saponified with 3 ml of 15% KOH w/v in 50% ethanol v/v at 80 C for 1 hr. The nonsaponifiable fraction containing sterols was extracted with two 10 ml portions of light petroleum ether (bp 30-60 C). The petroleum ether extract was washed twice with water and evaporated to dryness. The residue was dissolved in chloroform and the total sterol content was estimated by the method of Zlatkis, Zak and Boyle (9). This method has the advantages over the acetic anhydride sulphuric acid reagent, in that it measures both free and esterified sterols and is more sensitive. An aliquot of the petroleum ether extract was evaporated to dryness. Sterols in the residue were acetylated by adding acetic anhydride-pyridine reagent 1:2 v/v and allowing the mixture to stand overnight. The acetate derivatives of the sterols were separated by thin layer chromatography as described earlier (4). Zones corresponding to desmosterol acetate and cholesterol acetate were located by staining with iodine and eluted with two 5 ml portions of chloroform. The chloroform extracts were washed twice with water to remove silver nitrate from the chloroform layer and then evaporated to dryness. The sterol content of

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TABLE I

Effect of Neonatal Food Restriction and Hyperphenylalaninemia on Total Sterols and Ratio of Desmosterol to Cholesterol (D/C) in Brain

| Age of rats, days | Treatment[a] | Treatment period, days | Brain wt, g | Total sterols, mg/brain | D/C Ratio |
|---|---|---|---|---|---|
| 8 | Control (5) | 5 | 0.754 ± 0.021 | 3.39 ± 0.11 | 0.130 ± 0.007 |
| | Undernourished (5) | 5 | 0.698 ± 0.018[b] | 3.17 ± 0.24 | 0.253 ± 0.017[b] |
| 9 | Control (5) | 6 | 0.856 ± 0.031 | 3.74 ± 0.22 | 0.124 ± 0.021 |
| | Undernourished (5) | 6 | 0.828 ± 0.025 | 3.50 ± 0.11 | 0.221 ± 0.222[c] |
| 14 | Control (5) | 12 | 1.173 ± 0.036 | 9.28 ± 0.68 | 0.078 ± 0.010 |
| | Undernourished (5) | 12 | 1.100 ± 0.022[b] | 8.82 ± 0.25 | 0.107 ± 0.011[d] |
| 9 | Saline (5) | 5 | 0.747 ± 0.039 | 3.61 ± 0.28 | 0.195 ± 0.098 |
| | Phenylalanine (5) | 5 | 0.621 ± 0.051[b] | 3.28 ± 0.30[d] | 0.199 ± 0.099 |
| 10 | Saline (5) | 6 | 0.969 ± 0.036 | 4.78 ± 0.29 | 0.165 ± 0.012 |
| | Phenylalanine (5) | 6 | 0.849 ± 0.032[b] | 4.16 ± 0.23[c] | 0.153 ± 0.011 |
| 12 | Saline (5) | 8 | 1.118 ± 0.040 | 7.17 ± 0.41 | 0.141 ± 0.022 |
| | Phenylalanine (5) | 8 | 0.929 ± 0.072[b] | 6.20 ± 0.29[c] | 0.125 ± 0.005 |

[a]Figures in parentheses represent the number of rats in each group.
[b]$P<0.005$.
[c]$P<0.01$.
[d]$P<0.05$.

each zone was estimated as described above, using cholesterol acetate and desmosterol acetate as standard.

## RESULTS

Body weights of undernourished rats were significantly lower than weights of control animals (76%, 77% and 72% of control for rats undernourished 6, 7 and 12 days, respectively). As reported earlier (8) treatment with phenylalanine did not affect the body weight. There was a significant difference in brain weight between control and undernourished animals in all but one experiment. However undernourishment did not significantly change the total sterol content per brain (Table I). Both the brain weight and total sterol content per brain was significantly lower in phenylalanine treated than in control animals (Table I). Although the brains of undernourished animals did not have an abnormal total sterol content, the ratio of desmosterol to cholesterol was significantly higher than in the controls. Phenylalanine treatment did not influence the D/C ratio (Table I).

## DISCUSSION

Since the cholesterol in brain is primarily of endogenous origin, the reduced cholesterol content should be the result of a reduced synthesis of this sterol in brain. Cerebral cholesterol synthesis might be altered either by metabolic inhibition of any one step in the intermediary pathway of cholesterol synthesis, by reduction in the synthesis of enzymes involved in the rate-limiting steps for cholesterol biosynthesis, or by limitation in the availability of cofactors required for the reduction of desmosterol to cholesterol.

One might expect that the increased D/C ratio observed in undernourished rats reflects a block at the desmosterol reductase step. We have shown earlier that the desmosterol reductase activity of rat brain during development reached a maximum at 8-11 days of postnatal age, and that the enzyme required NADPH (4). It was suggested that the enzyme may be substrate-induced. Restriction of food during the period when the activity of desmosterol reductase is increasing rapidly might slow the induction of the enzyme by reducing the rate of formation of new protein. However cerebral NADPH levels which have been shown to increase during this period (10) may also be the limiting factor. Winick (11) has reported that both absolute and relative amounts of glucose metabolized via the HMP shunt pathway are reduced in rats born to and nursed by mothers malnourished by restricting protein. Agrawal, Fishman and Prenskey (12) have also recently suggested that there may be a block in the intermediary metabolism of glucose in the brains of undernourished rats. It is thus likely that neonatal food restriction could result in reduced cerebral NADPH levels.

The effect of phenylalanine treatment on cerebral sterols was different from that of neonatal food restriction. Although the cerebral sterol content is low in hyperphenylalaninemic rats there is no difference between the D/C

ratio of brains from control and hyperphenylalaninemic animals. This would suggest that in the hyperphenylalaninemic animals the block in cerebral sterol synthesis must occur prior to the formation of desmosterol. In this respect it is of interest to note the similarity between the rats treated with phenylalanine and the three myelin deficient genetic mutants of mice—the jimpy mouse, the quaking mouse and the myelin synthesis-deficiency mouse. Cell-free preparations from brains of all of these mutants (13) like those from hyperphenylalaninemic animals (14) show reduced incorporation of mevalonic acid to cholesterol, but there is no change in the cerebral D/C ratio.

As a final comment it may be noted that the difference in the effects of phenylalanine treatment and undernourishment on the D/C ratio make it unlikely that the condition of experimental hyperphenylalaninemia is associated with a nutritional deficiency.

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# Effect of Dietary Cholesterol and Fasting on Hepatic 3-Hydroxy-3-Methyl Glutaryl Coenzyme A Hydrolase

**ABSTRACT**

Hepatic HMG-CoA hydrolase activity has been shown to increase in rats fasted for 72 hr or fed for 14 days on 5% cholesterol supplemented diet. Cycloheximide treatment resulted in further activation of this enzyme in animals fed cholesterol. Significant activation of HMG-CoA hydrolase was also observed in rats fed normally and treated with cycloheximide and actinomycin D either individually or simultaneously.

It is well established that fasting, as well as cholesterol feeding, virtually abolishes hepatic cholesterologenesis (1,2). While indirect methods localized the control at mevalonate-NADP oxidoreductase (acylating CoA) (hydroxy methyl glutaryl CoA reductase, E.C. 1.1.1.34) (3,4), direct evidence for decrease in HMG-CoA reductase activity following cholesterol feeding or fasting was first provided by Linn (5). Although bile salts are inhibitors of HMG-CoA reductase (6), the possibility of their acting as regulators of cholesterol synthesis has been ruled out (7,8). Furthermore in vitro addition of cholesterol in solubilized form had no inhibitory effect on either crude or purified preparation of rat liver HMG-CoA reductase (5,9). This also suggested that cholesterol may not be the true regulator of cholesterol synthesis. Recently 3-hydroxy-3-methylglutaric acid (HMG) has been shown to inhibit cholesterol synthesis from acetate when administered to rats or added to liver slices or homogenates (10). HMG is also known to inhibit competitively bacterial HMG-CoA reductase (11) and exhibit hypocholesterolemic and hypolipedemic properties in rats (12,13). Since in vivo HMG-CoA hydrolase (E.C. 3.1.2.5) catalyzes the irreversible formation of HMG, it was the purpose of this investigation to determine whether cholesterol feeding and fasting has an activating effect on this enzyme.

Male albino rats weighing 80-100 g were maintained on Hind Lever basal diet (Hindustan

ratio of brains from control and hyperphenylalaninemic animals. This would suggest that in the hyperphenylalaninemic animals the block in cerebral sterol synthesis must occur prior to the formation of desmosterol. In this respect it is of interest to note the similarity between the rats treated with phenylalanine and the three myelin deficient genetic mutants of mice—the jimpy mouse, the quaking mouse and the myelin synthesis-deficiency mouse. Cell-free preparations from brains of all of these mutants (13) like those from hyperphenylalaninemic animals (14) show reduced incorporation of mevalonic acid to cholesterol, but there is no change in the cerebral D/C ratio.

As a final comment it may be noted that the difference in the effects of phenylalanine treatment and undernourishment on the D/C ratio make it unlikely that the condition of experimental hyperphenylalaninemia is associated with a nutritional deficiency.

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Male albino rats weighing 80-100 g were maintained on Hind Lever basal diet (Hindustan

TABLE I

Effect of Fasting, Dietary Cholesterol and Cycloheximide on Hepatic HMG-CoA Hydrolase[a]

| Source of homogenate | Specific activity, nmole SH/min/mg | Fraction of control,% fed normally |
|---|---|---|
| Fed normal | 2.67 ± 0.37 | 100 |
| Fasted | 5.70 ± 0.35[b] | 213 |
| Cholesterol-fed | 4.92 ± 0.40[c] | 184 |
| Cholesterol-fed and cycloheximide-treated | 6.75 ± 0.75[c] | 253 |
| One-half fed normally + one-half fasted | 4.00 ± 0.10 | 150 |
| One-half fed normally + one-half fed cholesterol | 4.02 ± 0.10 | 151 |
| One-half fed normally + one-half fed cholesterol and cycloheximide-treated | 5.71 ± 0.20 | 214 |

[a]Each value represents the mean ± SEM of six rats.
[b]$p < 0.02$.
[c]$p < 0.01$.

Lever Co., India) and water ad libitum. The group fed cholesterol received 5% cholesterol-supplemented diet for a period of 14 days. Animals in the fasted group were deprived of food but not water for 72 hr before sacrifice. To avoid differences due to diurnal variations, all the animals were killed between 10:30 and 11:00 AM. Actinomycin D and cycloheximide were administered intraperitoneally in saline at doses of 70 μg/100 g and 0.5 mg/100 g body wt., respectively, 4 hr pior to sacrifice. The animals were killed by a blow on head, excised quickly, and their livers removed, chilled, homogenized and centrifuged at 10,000 x g for 15 min to remove mitochondria. The supernatant was assayed for HMG-CoA hydrolase as described by Dekker et al. (14). Protein was estimated by the method of Lowry et al. (15).

There was a two-fold increase in hepatic HMG-CoA hydrolase activity in fasted and cholesterol-fed rats, and cycloheximide treatment resulted in further activation of this enzyme in cholesterol-fed animals (Table I). The activation in response to cycloheximide and cholesterol feeding could be due to different mechanisms, as prolonged cholesterol feeding produced no further activation (M. Saleemuddin, unpublished observations). Cycloheximide and actinomycin D treatment also resulted in activation of HMG-CoA hydrolase in

TABLE II

Effect of Actinomycin D and Cycloheximide on HMG-CoA Hydrolase[a]

| Source of homogenate | Specific activity, nmole SH/min/mg | Fraction of control,% fed normally |
|---|---|---|
| Fed normally | 2.34 ± 0.18 | 100 |
| Cycloheximide-treated | 3.90 ± 0.50[b] | 166 |
| Actinomycin D-treated | 3.32 ± 0.26[c] | 141 |
| Actinomycin D- and cycloheximide-treated | 4.96 ± 0.32[c] | 212 |
| One-half fed normally + one-half cycloheximide-treated | 3.35 ± 0.30 | 143 |
| One-half fed normally + one-half actinomycin D- treated | 3.01 ± 0.30 | 133 |
| One-half fed normally + one-half cycloheximide- and actinomycin D- treated | 4.50 ± 0.41 | 192 |

[a]Each value represents the mean ± SEM of six rats.
[b]$p < 0.05$.
[c]$p < 0.001$.

rats fed normally (Table II). Several examples, of such activation in response to these inhibitors, are available (16,17).

Based on the preceding evidence, very little can be said regarding the mechanism of activation of HMG-CoA hydrolase. Mixing experiments, however, tend to eliminate the possible existence of excess activator of this enzyme in livers of fasted, cholesterol-fed, actinomycin D-treated and cycloheximide-treated rats, as evidenced by the observed additive reaction rates (Tables I,II). Protein synthesis inhibition per se rather than specific induction, appears to be responsible for activation in response to actinomycin D and cycloheximide, because simultaneous treatment with these structurally different inhibitors did not prevent the activation. Furthermore our unpublished experiments have shown that actinomycin D and cycloheximide do not activate this enzyme in vitro over a wide concentration range. The works of Kenny (18) and Shambaugh et al. (19) have shown that actinomycin D and cycloheximide prevent the inactivation of rapidly turning enzymes. Recent studies indicate that, besides usual modes of regulations, enzyme degradation may be an important mechanism in controlling the activity of mammalian enzymes (20,21). The increase in specific activity of HMG-CoA hydrolase, in response to cycloheximide and actinomycin D, might be due to its decreased degradation together with a continued conversion of inactive precursor to active enzyme, since one would expect de novo synthesis to also be inhibited. The existence of such activation has been suggested for carbamyl phosphate synthetase (19) and glutamate dehydrogenase (22) of frog tadpole liver and for arylsulfatase of *Aspergillus nidulans* (23). The additive activation effect of actinomycin D and cycloheximide is difficult to explain and requires further study. The possible diurnal rhythm of the hydrolase is under investigation, because strong rhythmic change in activity could also be responsible for the observed activation, provided the activity was caught during a steep fall.

These observations suggest a possible role of HMG-CoA hydrolase in regulation of cholesterol biosynthesis. Increased acitivity of HMG-CoA hydrolase in cholesterol-fed or fasted animals would be expected to not only reduce the available pool of HMG-CoA for reduction to mevalonate, but HMG released would also competitively inhibit HMG-CoA reductase (10,11) and thus decrease cholesterol synthesis. Although the factor(s) directly responsible for observed change in HMG-CoA reductase in cholesterol-fed and fasted animals is yet to be found, competitive inhibition by HMG alone could not possibly be responsible for the observed decrease in activity, because reduced activity was also observed in washed microsomes at large dilutions and saturating substrate concentrations (5). Furthermore we could show significant activation of HMG-CoA hydrolase after only 2 weeks of cholesterol feeding and 72 hr fasting, while a marked decrease in HMG-CoA reductase has been reported after very short treatments (5). These findings suggest that, if competitive inhibition is responsible for in vivo inhibition of cholesterol biosynthesis, it could be a secondary control such as the post mevalonate inhibition of cholesterol biosynthesis which becomes effective after a prolonged cholesterol feeding (24).

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# Inhibition of Sterol Biosynthesis in Chlorella Ellipsoidea by AY-9944[1]

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## ABSTRACT

When *Chlorella ellipsoidea* was grown in the presence of 4 ppm AY-9944, complete inhibition of $\Delta^5$-sterol biosynthesis was achieved. However total sterol production remained unaltered. As a result a number of sterols accumulated that appear to be intermediates in sterol biosynthesis. These sterols were described and identified as (24S)-5α-ergost-8(9)-en-3β-ol, (24S)-5α-stigmast-8(9)-en-3β-ol, 4α-methyl-(24S)-5α-ergosta-8,14-dien-3β-ol, 4α-methyl-(24S)-5α-stigmasta-8,14-dien-3β-ol, 4α-methyl-(24S)-5α-ergost-8(9)-en-3β-ol and (24S)-4α-methyl-5α-stigmast-8(9)-en-3β-ol. The occurrence of these sterols in *Chlorella ellipsoidea* is the first time they have been noted in biological material. The accumulation of these sterols in treated cultures indicates that AY-9944 is an extremely effective inhibitor of the $\Delta^8 \rightarrow \Delta^7$ isomerase and the $\Delta^{14}$ reductase of these plants. The occurrence of small amounts of other sterols in treated cultures has led to a proposed pathway for the biosynthesis of sterols in *Chlorella ellipsoidea*.


## INTRODUCTION

It has been pointed out that the hypocholesterolemic drug, AY-9944 (*trans*-1,4-bis-[2-chlorobenzylaminomethyl] cyclohexane dihydrochloride), inhibits the biosynthesis of cholesterol in animals by preventing the reduction of 7-dehydrocholesterol to cholesterol (1-5). Since AY-9944 has been regarded as a quite specific $\Delta^7$-reductase inhibitor in animals and the effect

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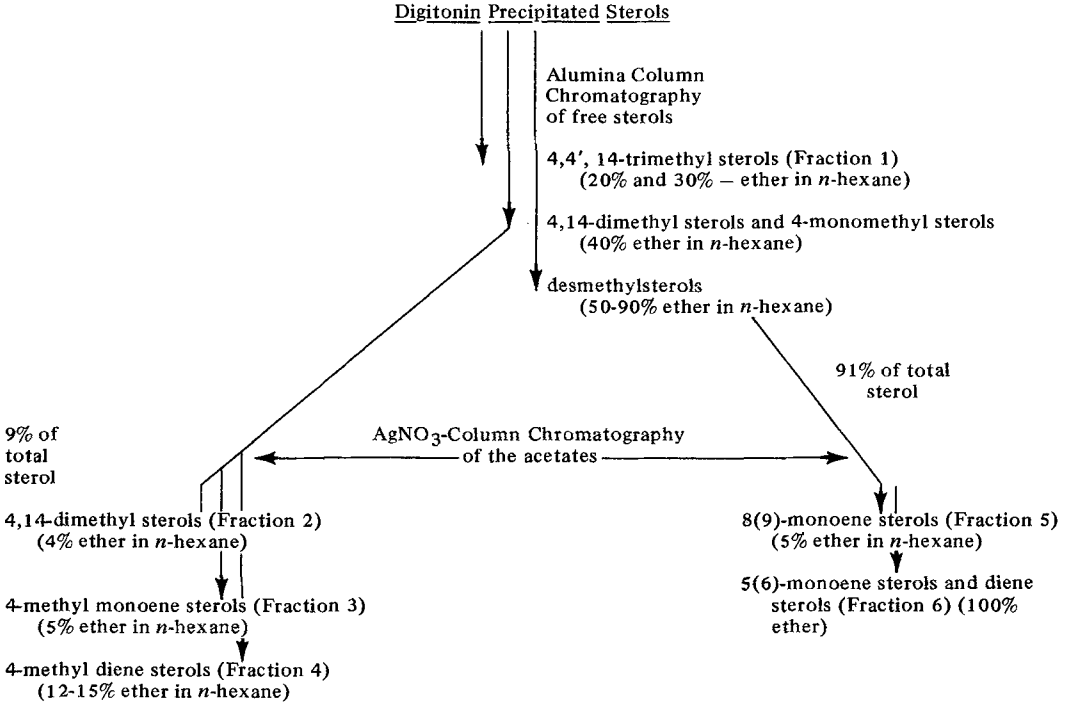


FIG. 1. Separation of *Chlorella ellipsoidea* sterols using alumina and $AgNO_3$-silica gel chromatography.

TABLE I

Relative Retention Times of Sterols Isolated from Control or AY-9944-Inhibited Cultures of *Chlorella ellipsoidea*

| Sterol acetates | Relative retention times on 4 gas chromatographic systems[a] | | | | |
|---|---|---|---|---|---|
| | SE-30[b] | SE-30[b,f] | QF-1[c] | Hi-Eff-8BP[d] | PMPE[e] |
| Brassicasterol | 1.12[h] | 1.12 | 1.09 | —[g] | — |
| $\Delta^5$-Ergostenol | 1.29 | 1.29 | 1.29 | 1.32 | 1.29 |
| Poriferasterol | 1.42 | 1.42 | 1.32 | 1.34 | 1.31 |
| Clionasterol | 1.63 | 1.63 | 1.56 | — | — |
| Cholesterol | 1.00 | 1.00 | 1.00 | — | — |
| $\Delta^{8,14}$-Ergostadienol | 1.32 | 1.32 | 1.24 | 1.44 | 1.38 |
| 24S-$\Delta^{8,14}$-Stigmastadienol | 1.66 | 1.66 | 1.48 | 1.74 | 1.65 |
| $\Delta^{8(9)}$-Ergostenol | 1.35 | 1.35 | 1.29 | 1.33 | 1.38 |
| 24S-$\Delta^{8(9)}$-Stigmastenol | 1.69 | 1.69 | 1.56 | 1.59 | 1.65 |
| 4$\alpha$-Methyl $\Delta^{8,14}$-ergostadienol | 1.50 | 1.54 | 1.38 | 1.54 | 1.42 |
| 4$\alpha$-Methyl 24S- $\Delta^{8,14}$-stigmastadienol | 1.87 | 1.93 | 1.67 | 1.87 | 1.69 |
| 4$\alpha$-Methyl $\Delta^{8(9)}$-ergostenol | 1.55 | 1.59 | 1.46 | 1.44 | 1.40 |
| 4$\alpha$-Methyl $\Delta^{8(9)}$-stigmastenol | 1.92 | 1.97 | 1.74 | 1.69 | 1.66 |
| 24-Dihydroobtusifoliol | 1.51 | 1.54 | 1.55 | 1.28 | 1.21 |
| 4$\alpha$, 14$\alpha$-Dimethyl 24S-$\Delta^8$-stigmastenol | 1.87 | 1.93 | 1.85 | 1.53 | 1.44 |
| 24-Methylene cycloartanol | 2.02 | 2.12 | 2.22 | — | — |

[a]Relative to cholesterol acetate.

[b]Column 1.8 m x 3.4 mm ID, 3% SE-30 on 100-120 mesh Gas Chrom Q, 20 psi, 244C.

[c]Column 2.8 m x 3.4 mm ID, 1% QF-1 on 100-120 mesh Gas Chrom Q, 25 psi, 231 C.

[d]Column 1.8 m x 3.4 mm ID, 3% Hi-Eff-8BP on 100-120 mesh Gas Chrom Q, 25 psi, 238 C.

[e]Column 1.8 m x 3.4 mm ID, 2% PMPE on 100-120 mesh Gas Chrom Q, 20 psi, 250 C.

[f]RRT is expressed as free sterol relative to free cholesterol.

[g]Data not obtained due to unresolved mixture on these columns.

[h]In all cases actual relative retention times, as given, are essentially identical to calculated relative retention times.

of AY-9944 on plant sterol biosynthesis was unknown, it was of interest to determine the effect of AY-9944 on the biosynthesis of $\Delta^5$-sterols in a unicellular green alga, *Chlorella ellipsoidea*. Assuming that AY-9944 acts similarly in algae and animals and assuming algal $\Delta^5$-sterols to be synthesized via the pathway expected for higher plants, we expected that AY-9944 treatment would alter sterol composition in favor of the $\Delta^{5,7}$- and $\Delta^7$-sterol intermediates. However, from our earlier data (6-8) and from the work described here, it seems certain that, rather than being a $\Delta^7$-reductase inhibitor, AY-9944 inhibits the reduction of the $\Delta^{14}$-bond of $\Delta^{8,14}$ sterol intermediates in the biosynthesis of sterols in *C. ellipsoidea*. In earlier reports (6,7) we identified two novel sterols, (24S)-5$\alpha$-ergosta-8,14-dien-3$\beta$-ol and (24S)-5$\alpha$-stigmasta-8,14-dien-3$\beta$-ol from AY-9944-treated cells of *Chlorella ellipsoidea*. In this paper we identify a total of 16 sterols from AY-9944-treated cultures—several of which have not been previously found in nature. A quantitative analysis of all sterols found in control and treated cells of *C. ellipsoidea* is reported, and a proposed scheme of sterol biosynthesis in this organism is presented.

## EXPERIMENTAL PROCEDURES

*Chlorella ellipsoidea* Gerneck, Indiana Culture Collection No. 247, was grown heterotrophically in 15-l carboys on basal inorganic medium plus 0.5% glucose. A constant air flow from an oil-free compressor provided oxygen and kept the cells in suspension. Upon inoculation 4 ppm (8.6 $\mu$M) AY-9944 was added to treated cultures. Sterols were extracted from freeze-dried cells with chloroform-methanol 2:1 and partially purified by digitonin precipitation as described by Doyle et al. (9). Further purification and separation was accomplished using alumina, $AgNO_3$-impregnated Silica Gel G and Anasil B column chromatography (7). This routine resulted in separation of the sterols as seen in Figure 1. Qualitative and quantitative gas liquid chromatographic (GLC) analyses, GC-mass spectrometric (MS) analyses, and physical data were also obtained as previously described (7).

## RESULTS AND DISCUSSION

Sterols were extracted from *C. ellipsoidea* control cultures and from those grown in the presence of 4 ppm AY-9944. GLC of the

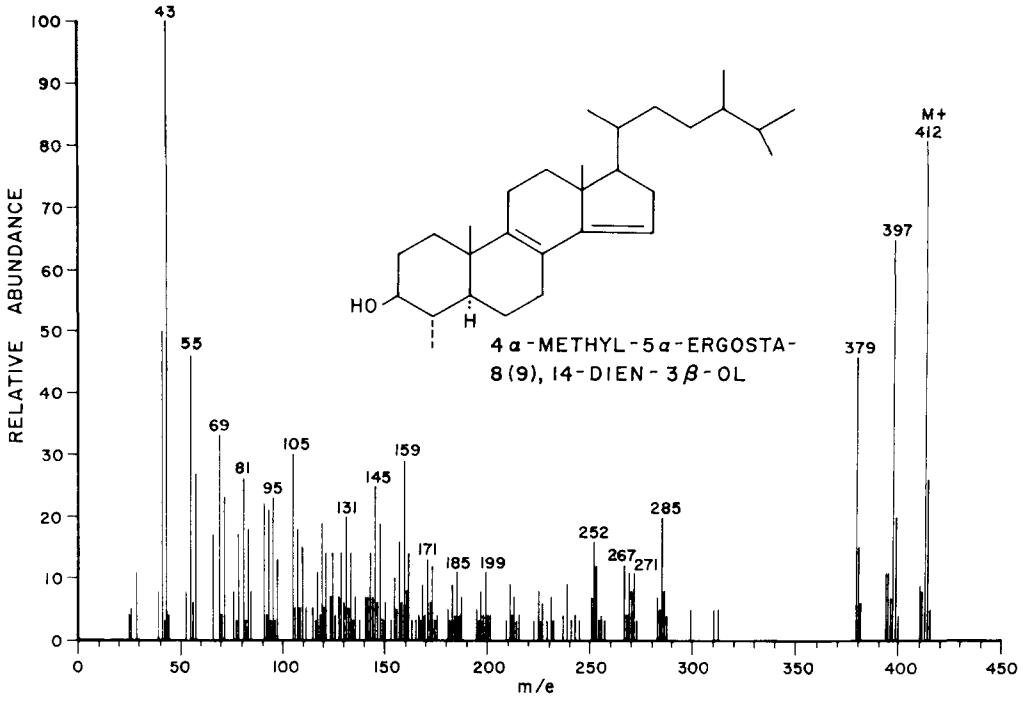


FIG. 2. Mass spectrograph of 4α-methyl-5α ergosta-8(9),14-dien-3β-ol.

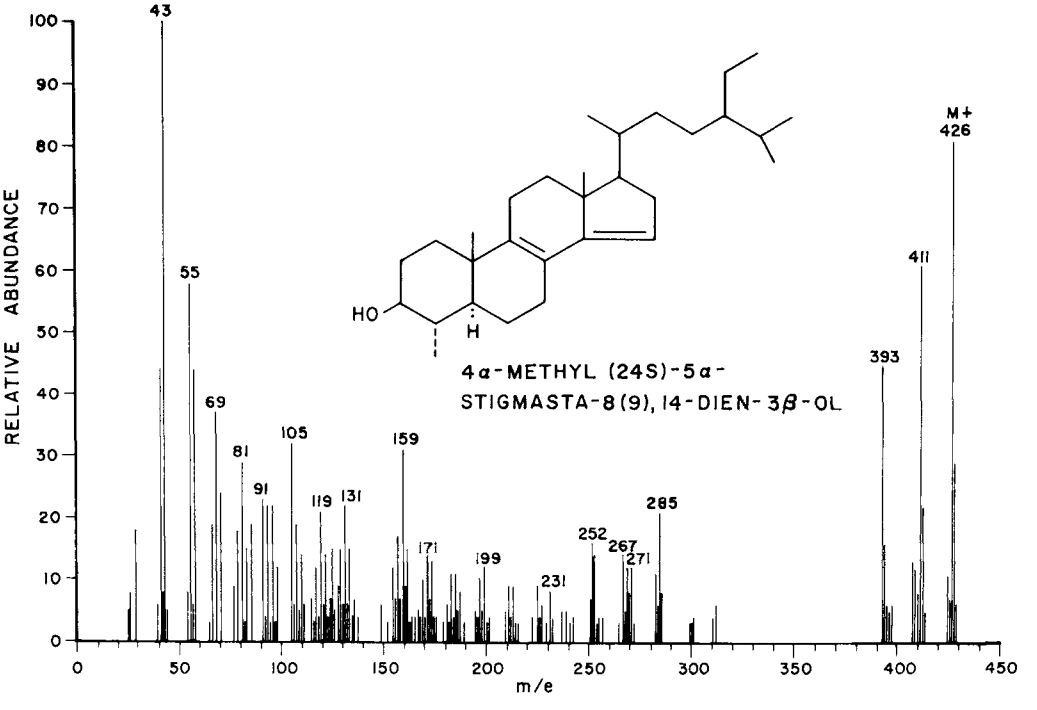


FIG. 3. Mass spectrograph of 4α-methyl (24S)-5α-stigmasta-8(9),14-dien-3β-ol.

TABLE II

A Quantitative Comparison of Sterols from Control and AY-9944-Treated Cultures of *Chlorella ellipsoidea*

| Sterols | Control | | AY-9944-treated | |
|---|---|---|---|---|
| | Per cent of sample | Dry wt, μg/g | Per cent of sample | Dry wt, μg/g |
| Brassicasterol | 5.7 | 181 | 0.0 | 0 |
| $\Delta^5$-Ergostenol | 21.9 | 696 | 0.4 | 13 |
| Poriferasterol | 65.6 | 2065 | 1.0 | 30 |
| Clionasterol | 6.8 | 216 | 0.1 | 3 |
| Cholesterol | 0.0 | 0 | 0.1 | 2 |
| $\Delta^{8,14}$-Ergostadienol | 0.0 | 0 | 26.4 | 831 |
| 24S-$\Delta^{8,14}$-Stigmastadienol | 0.0 | 0 | 43.2 | 1371 |
| $\Delta^{8(9)}$-Ergostenol | 0.0 | 0 | 6.1 | 193 |
| 24S-$\Delta^{8(9)}$-Stigmastenol | 0.0 | 0 | 13.2 | 415 |
| 4α-Methyl $\Delta^{8,14}$-ergostadienol | 0.0 | 0 | 4.0 | 126 |
| 4α-Methyl 24S $\Delta^{8,14}$-stigmastadienol | 0.0 | 0 | 3.1 | 97 |
| 4α-Methyl $\Delta^{8(9)}$-ergostenol | 0.0 | 0 | 0.4 | 13 |
| 4α-Methyl 24S- $\Delta^{8(9)}$-stigmastenol | 0.0 | 0 | 0.3 | 10 |
| 24-Dihydroobtusifoliol | 0.0 | 0 | 0.5 | 17 |
| 4α,14α-Dimethyl 24S-$\Delta^{8(9)}$-stigmastenol | 0.0 | 0 | 1.0 | 30 |
| 24-Methylene cycloartanol | tr[a] | tr | 0.1 | 2 |
| Total | 100.0 | 3157 | 99.9 | 3153 |

[a]tr=Trace; indicates less than 1 μg/g DW.

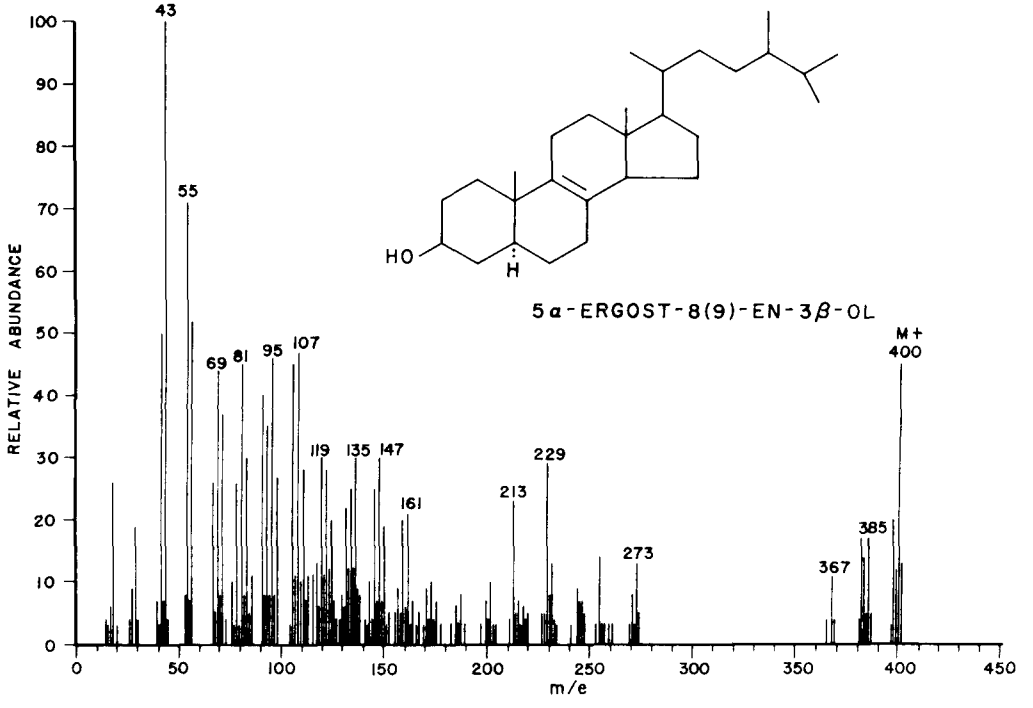


FIG. 4. Mass spectrograph of 5α-ergost-8(9)-en-3β-ol.

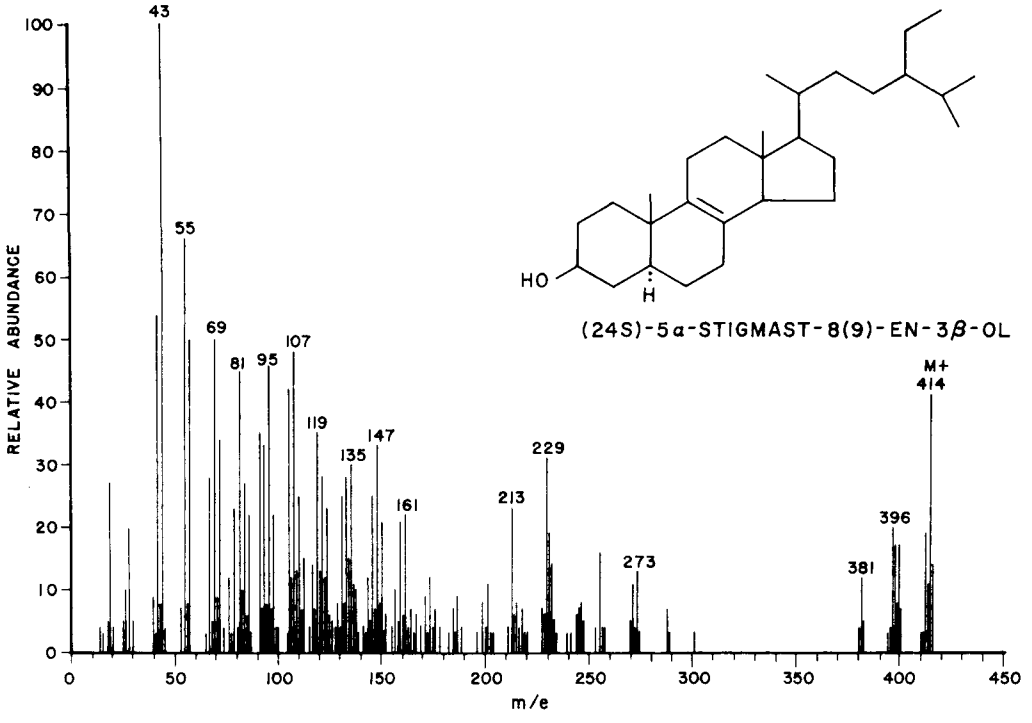


FIG. 5. Mass spectrograph of (24S)-5α-stigmast-8(9)-en-3β-ol.

digitonin-precipitated sterols from the inhibited cultures revealed two major peaks and two minor peaks with relative retention times (RRT) on four GC systems completely unlike those of controls and unlike any available authentic standards. Alumina column chromatography, followed by $AgNO_3$-silica gel chromatography of the sterol acetates resulted in a separation of the sterols into six fractions (Fig. 1). In several cases pairs of sterols were found that were C-28 and C-29 homologs. Although the sterols of each pair could not be further separated by thin layer or column chromatography, the compounds were well resolved by GLC and thus amenable to identification by GLC (four systems) and GC-MS.

#### Identification of 4α,14α-Dimethyl Sterols and 24-Methylene Cycloartanol

Fraction 1 from alumina column chromatography contained very small amounts of 24-methylenecycloartanol, although no cycloartenol was detected. Fraction 2 contained the two sterols, 4α,14α-dimethyl-(24S)-5α-ergost-8(9)-en-3β-ol (dihydroobtusifoliol) and 4α,14α-dimethyl-(24S)-5α-stigmast-8(9)-en-3β-ol. Identification of these sterols was achieved principally on the basis of relative retention data from four GC systems, which showed observed values to be indistinguishable from those reported by Doyle et al. (10). Evidence for a C-4 methyl substituent is provided by the behavior of the sterols on alumina chromatogrpahy and differences in behavior of the free sterols and sterol acetates on SE-30 (11). GC-MS analyses of these sterols also confirmed the above identifications.

#### Identification of 4α-Methyl $\Delta^{8(9)}$-Sterols

Identification of the sterols in fraction 3 as 4α-methyl-(24S)-5α-ergost-8(9)-en-3β-ol and 4α-methyl-(24S)-5α-stigmast-8(9)-en-3β-ol is based on their movement on the alumina and $AgNO_3$-silica gel columns, as well as the very close correlation of the observed and calculated RRTs in the four GC systems (Table I). The presence of a methyl at C-4 is demonstrated by the free sterol having a higher RRT on SE-30 than the corresponding sterol acetate (11).

To our knowledge this is the first reported occurrence of the identification of these two sterols from natural sources, although Anding et al. (12) have recently isolated an "undefined methyl sterol" which they feel may have the structure, 4α-methyl-ergost-8(9)-en-3β-ol.

#### Identification of 4α-Methyl Dienols

The GC-MS of the two sterols of fraction 4

provided evidence for a methyl at C-4. The spectra are identical below m/e 285, and differ only by 14 mass units above m/e 285 indicating the presence of homologs containing a methyl and an ethyl, respectively, at C-24 (Figs. 2,3). The parent ion peaks were at m/e 412 and 426; both have strong fragmentations at m/e 285, indicating loss of a saturated side chain from a diunsaturated nucleus containing an additional methyl. A strong peak is seen at m/e 252, which apparently corresponds to loss of the side chain and part of ring D, since this fragmentation is also seen at m/e 238 in $\Delta^{8,14}$-cholestadienol, $\Delta^{8,14}$-ergostadienol and (24S)$\Delta^{8,14}$-stigmastadienol, and appears to be a characteristic of sterols with this double bond system. The saturated nature of the sterol side chain, the methyl at C-4, and the placement of the double bonds at the 8 and 14 positions were verified by GLC (Table I). A decrease in RRT of the acetate compared to the free sterol is evidence of a methyl group at C-4. Actual RRT values were identical to those calculated by the method of Clayton (14), thus confirming the MS analyses and identifying the sterols of fraction 4 as 4α-methyl-(24S)-5α-ergosta-8,14-dien-3β-ol and 4α-methyl-(24S)-5α-stigmasta-8,14-dien-3β-ol. An UV absorption spectrum of a mixture of these sterols gave the expected absorption maximum at 251 nm.

#### Identification of Desmethyl Sterols

The two sterols of fraction 5, comprising 19% of the total sterols (Table II), are identified as (24S-5α-ergost-8(9)-en-3β-ol and (24S)-5α-stigmast-8(9)-en-3β-ol. The mass spectra show molecular weights of 400 ($C_{28}H_{48}O$) and 414 ($C_{29}H_{50}O$), respectively. As seen in Figure 4, the C-28 homolog has a fragmentation peak at m/e 273, suggesting a saturated side chain. The placement of the double bond at the $\Delta^{8(9)}$ position is done on the basis of the IR spectrum, mass spectrum and GLC retention data obtained from a mixture of these two sterols. The IR spectrum lacked bands characteristic of disubstituted and trisubstituted double bonds. The only points on a sterol structure where a tetrasubstituted double bond could occur are at the 8(9), 8(14) and 24(25) positions (providing an alkyl substituent is present at C-24). These are easily distinguishable on the four GLC columns used in this research. The optical rotation (free sterol $[\alpha]_D^{23}$ + 39; acetate, $[\alpha]_D^{23}$ + 28) data of this mixture are also within the expected range for $\Delta^{8(9)}$-sterols (13). Except for an increase in 14 mass units, the spectrum (Fig. 5) of the stigmastenol compound of fraction 5 is identical to that of its C-28 homolog. In addition to MS, direct evidence for the proposed structures comes from a comparison of calculated to actual RRT values on the four GC systems. On all systems the calculated values are within 1% of the actual values. These data allow identification of the sterols of fraction 5 as $\Delta^{8(9)}$-sterols and collectively exclude all other possibilities.

Two of the sterols of fraction 6 have been previously identified as (24S)-5α-ergosta-8,14-dien-3β-ol and (24S)-5α-stigmasta-8,14-dien-3β-ol (7). These two compounds comprised 69% of the total sterols isolated from the treated cultures (Table II).

Extremely small amounts (Table II) of $\Delta^5$-ergostenol, poriferasterol and clionasterol—the predominant sterols of control cultures of *C. ellipsoidea* (15)—were detected in AY-9944-grown cultures. All had relative retention times on GC that matched authentic standards. GC-MS analysis of poriferasterol and $\Delta^5$-ergostenol confirmed the identification of these sterols. All sterols identified in this work are assumed to have one 24S configuration, since the "normal" sterols of these organisms have been established to have the 24S configuration (15,16).

Mass spectrographic analysis and GC retention data confirmed the presence of small amounts of cholesterol and brassicasterol in the AY-9944-inhibited cultures. This is the first reported isolation of cholesterol from *Chlorella,* although its occurrence in *Oocystis* (17), *Ulva* and *Chaetomorpha* (18) has been reported.

As seen in the quantitative comparison of sterols from control and AY-9944-treated cultures (Table I), AY-9944 has a drastic effect on sterol composition which apparently has no influence on sterol production (3.2 mg/g dry wt in control and inhibited cultures). AY-9944 is virtually a 100% effective inhibitor of $\Delta^5$-sterol biosynthesis in *C. ellipsoidea.* The total of 5 mg of $\Delta^5$-sterols in treated cultures is calculated to approximate the amount of these sterols which was present in the untreated inoculum used to start the treated cultures.

Instead of the predicted accumulation of the immediate precursors to $\Delta^5$ sterols, i.e., $\Delta^{5,7}$ and $\Delta^7$ sterols, AY-9944 brought about an almost exclusive accumulation of $\Delta^{8(9)}$ and $\Delta^{8,14}$ sterols. An inhibition of the reduction of the $\Delta^{14}$ double bond and an inhibition in the $\Delta^8 \rightarrow \Delta^7$ isomerase are clearly suggested. It is certainly possible to state with confidence that AY-9944 must be more than a $\Delta^7$-reductase inhibitor in plants. It is also clear that any speculation concerning sites of AY-9944 inhibition must be accompanied by a postulated pathway involving the sterols which have ac-

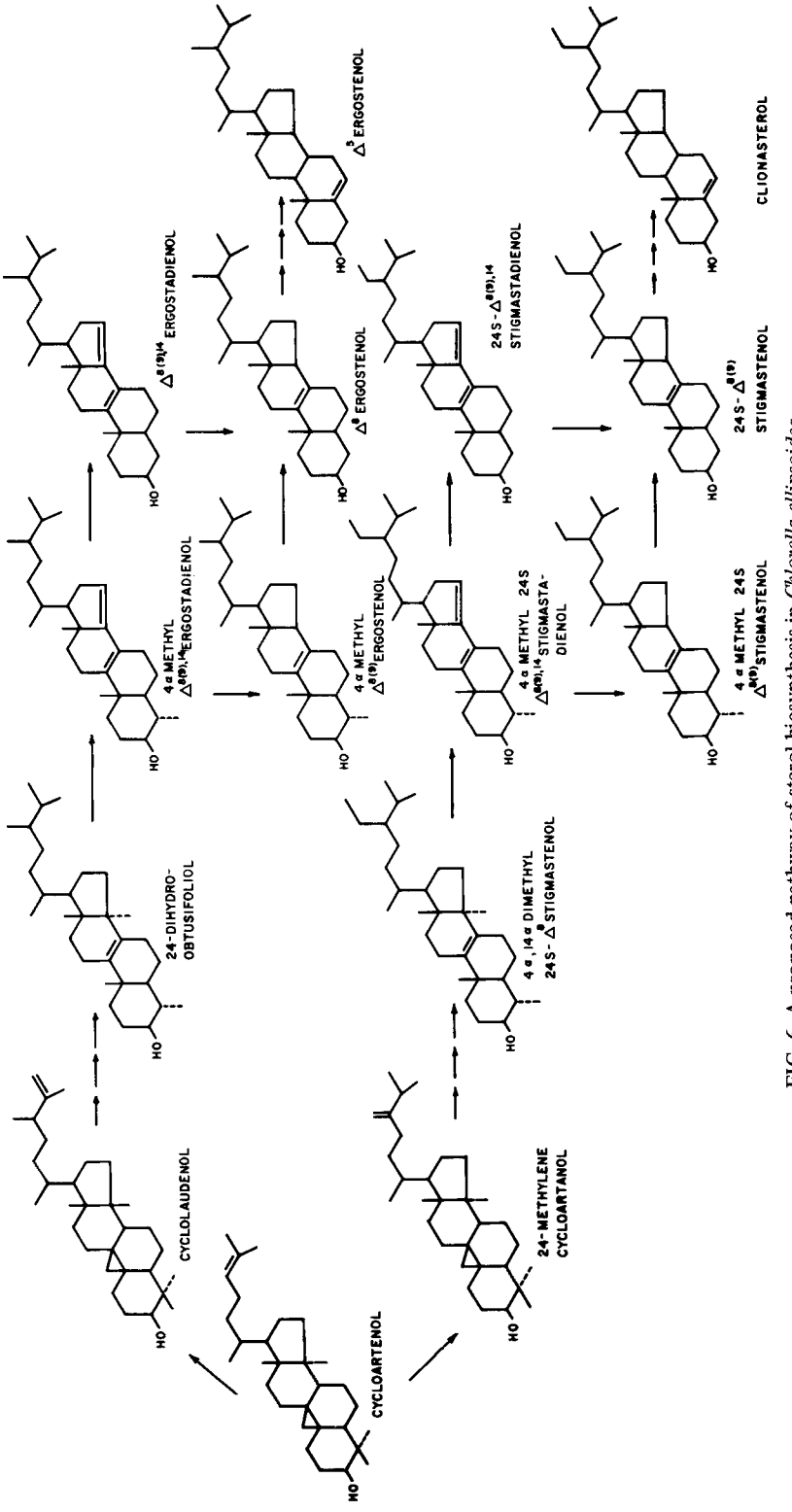


FIG. 6. A proposed pathway of sterol biosynthesis in *Chlorella ellipsoidea*.

cumulated. Accordingly the sterols described above are included in a proposed scheme for $\Delta^5$-ergostenol and clionasterol biosynthesis, although other reactions are possible (Fig. 6). Although no cycloartenol and cyclolaudenol were detected in *C. ellipsoidea,* inclusion of these sterols in the scheme reflects the current thought on sterol biosynthesis in plants. All other sterols in this scheme were found in *C. ellipsoidea.*

Strong support for such a sequence is taken from the substantial recent literature concerning the occurrence of $\Delta^{8,14}$-sterol dienes in biological material, and their suspected roles as intermediates in sterol biosynthesis. Frost and Ward (19) isolated 5α-stigmasta-8,14, 24(28)-trien-3β-ol from *Vernonia* seed oil. Canonica et al. (20), Akhtar et al. (21) and Watkinson et al. (22) have shown conclusively that cholesta-8,14-dien-3-ol is converted to cholesterol in rat liver homogenate enzyme system. Akhtar et al. (23) and Canonica et al. (24) have shown that the loss of a methyl at C-14 is accompanied by a simultaneous oxidation at C-15.

Assuming such a pathway exists in plants (*Chlorella* in particular), then the two suggested sites of inhibition just mentioned, the inhibition of the $\Delta^{14}$-reductase step and interruption of the $\Delta^8 \rightarrow \Delta^7$-isomerase step, should again be considered. A choice of the former as the primary site of inhibition is preferable when one considers that 77% of the accumulated sterols are $\Delta^{8,14}$-sterols and that a reduction of the 14(15) double bond is likely to require NADPH and be similar in other respects to the $\Delta^7$-reductase system reported in animals. An accumulation of a $\Delta^{8,14}$-sterol has also recently been observed as a result of AY-9944 treatment in an animal system (25).

However the accumulation of the $\Delta^{8(9)}$-sterol monoenes (21% of accumulated sterols), which appear after the sterol dienes in the postulated sequence, indicates an additional inhibition at the $\Delta^8 \rightarrow \Delta^7$-isomerase step by AY-9944. Alternatively, if AY-9944 inhibits only the isomerase reaction, then the equilibrium of the previous reaction (reduction of the C-14 double bond) must be strongly in favor of the sterol diene.

From Table II it is observed that the inhibited cultures produced practically no $\Delta^{22}$-sterols, while 77% of the total sterol of control cultures had a 22(23) double bond. Either AY-9944 has a secondary effect in preventing insertion of this double bond, or the introduction of the 22,23 double bond may not normally occur until later in the pathway.

Dealkylation of the methyl groups on the sterol nucleus in *C. ellipsoidea* appears to proceed as in higher plants, i.e., the 4α-methyl is the last group to be removed. However, in *C. emersonii* (sterols are $\Delta^7$), Doyle et al. (9) found that the 14α-methyl was removed last. Thus sterol composition and the pathway of biosynthesis of sterols in *Chlorella ellipsoidea* are much more similar to those of higher plants than is the case with *Chlorella emersonii.*

## ACKNOWLEDGMENTS

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# Clofibrate and Nafenopin (SU-13437): Effects on Plasma Clearance and Tissue Distribution of Chylomicron Triglyceride in the Dog

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## ABSTRACT

Adult male dogs, which received orally for 21 days either no drug, 75 mg/kg/day of clofibrate or 25 mg/kg/day of nafenopin, were injected iv with $^{14}$C-labeled chylomicron-lipid, essentially chylomicron-triglyceride fatty acid, at 0.2 g of fat per kilogram body weight. At times from 2-160 min after injection, samples of blood, liver, skeletal muscle and adipose tissue were removed, weighed and assayed for $^{14}$C-lipid content. Also, in other dogs treated for 14 days, samples of skeletal muscle and adipose tissue were removed before and after treatment by biopsy for measurement of lipoprotein lipase activity. Chylomicron-$^{14}$C was cleared more rapidly from the plasma of nafenopin-treated and to a lesser extent of clofibrate-treated dogs than controls. The $t_{0.5}$ for clearance was ca. 31 min for control, 12 min for nafenopin-treated and 20 min for clofibrate-treated dogs. The total skeletal muscle mass of nafenopin-treated animals took up 40-50% of the injected chylomicron-triglyceride during the first 20 min after injection, as compared with ca. 25-30% for clofibrate-treated and 20-25% for control dogs. Also in treated dogs lesser percentages of the cleared chylomicron-$^{14}$C appeared in the liver soon after injection and more in adipose tissue at later times. These observations could be related partially to a measured ability of clofibrate to increase adipose tissue lipoprotein lipase activity (ca. 50%) and of nafenopin to increase a lipase activity of skeletal muscle (ca. 20%). These data suggest that clofibrate and particularly nafenopin lower plasma triglycerides by increasing their clearance by the peripheral tissues.

## INTRODUCTION

Results of recent studies suggest that the hypolipidemic drugs clofibrate (ethyl-*p*-chlorophenoxyisobutyrate) and nafenopin (2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy] propionate) can lower plasma triglyceride levels, in part, by increasing their clearance from plasma by peripheral tissues (1-3). Clofibrate was seen to increase both the uptake of chylomicron-triglyceride fatty acid by rat adipose tissue in vitro (1) and the activity of lipoprotein lipase in this tissue (2), while nafenopin has been shown to enhance the clearance from plasma of an artificial fat emulsion in human patients (3). An ability of clofibrate and nafenopin to lower plasma triglyceride levels, through increasing triglyceride clearance by the peripheral tissues, could be important, since certain types of human hypertriglyceridemias could involve defects in the clearance of very low density lipoproteins from plasma (4-6). With the intention of further investigating this proposed mechanism for the hypotriglyceridemic action of clofibrate and nafenopin, the present study examines the influence of treatment with clofibrate and nafenopin upon the plasma clearance and tissue distribution of chylomicron-triglyceride in the intact dog and upon adipose tissue and skeletal muscle lipoprotein lipase activity.

## MATERIALS AND METHODS

### Preparation of Labeled Chylomicrons

Chylomicrons containing triglycerides labeled with $^{14}$C-fatty acids were prepared in 18 hr fasted dogs (male and female) fed ca. 100 $\mu$Ci of 1-$^{14}$C palmitate (specific activity 11 mCi/mM) dissolved in 25 ml of a 2:3 corn oil-milk emulsion. About 1 hr after feeding this mixture the dogs were anesthetized with sodium pentobarbital (30 mg/kg, iv); the thoracic duct was cannulated with a polyethylene tube, and chyle was collected dropwise for 6-8 hr in 40 ml centrifuge tubes immersed in an ice bath. The dogs were sacrificed at the end of the collection period. The chyle was allowed to clot and the chylomicrons were isolated by ultracentrifugation (70,000 x g for 40 min) and concentrated to 50 ml in saline as described by Havel and Fredrickson (7). Total lipid was extracted from aliquots of these emulsions, essentially as described by Ways and Hanahan (8). Twenty volumes of 2:1, rather than 10 volumes of 1:1 chloroform-methanol, were

used per volume of sample extracted. The chylomicron emulsions (prepared eight times) contained on the average 8.35 ± 1.51 (SE) g of fat and 66.7 ± 11.0 (SE) μCi of $^{14}C$ per 100 ml. The distribution of $^{14}C$ among the chylomicron lipids was determined in each preparation after separation of the lipids into major classes by a thin layer chromatography (9). An average of 94.1 ± 1.2 (SE) % of the chylomicron-$^{14}C$ was in triglycerides. The remainder of the $^{14}C$ was distributed as follows: 1.9% phospholipids, 1.8% cholesterol, 0.9% mono and diglycerides, 1.0% free fatty acids and 0.3% cholesterol esters. There was negligible nonlipid $^{14}C$ present with the chylomicrons. The chylomicrons, stored at 4 C, were used within 8 days of preparation. This storage procedure is similar to that employed by Nestel et al. (10).

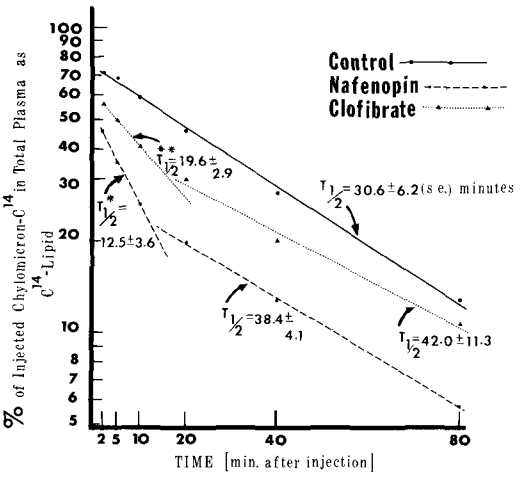



FIG. 1. Plasma disappearance of chylomicron-triglyceride. Dogs were injected iv with $^{14}C$-labeled chylomicron-triglyceride at 0.2 g of fat per kilogram body weight (ca. 1.6 μCi/kg body wt). Plasma lipid-$^{14}C$ content was determined at 2, 5, 10, 20, 40, 80 and 160 min after injection. Measurements are average of four untreated (control), four clofibrate (75 mg/kg/day, 21 days)-, and four nafenopin (25 mg/kg/day, 21 days)-treated dogs. *P($t$) of difference from control <0.05. **P($t$) of difference from control <0.17.

**Measurements of Plasma Clearance and Tissue Distribution of Chylomicron Triglyceride**

Adult male mongrel dogs (8-13 kg) which were separately caged and maintained on a Purina dog chow diet received either no drug (controls) or one oral dose by gelatin capsule of clofibrate (75 mg/kg/day) or nafenopin (25 mg/kg/day) for 21 consecutive days. These doses of clofibrate and nafenopin are each about three times the respective therapeutic dosage levels in man. Both clofibrate- and nafenopin-treated dogs lost ca. 10% of their body weight over the 3 week treatment period but otherwise appeared to tolerate either drug well. Food consumption, followed with several dogs, before and during treatment was unchanged. There was no evidence for a relationship between drug effects and loss of body weight. Blood samples collected before, during and at the conclusion of the treatment period were assayed for plasma cholesterol and triglyceride levels as described earlier (11). On day 22, food was removed from the dogs at 7:30 A.M. (to minimize absorption of dietary fat), and the dogs were anesthetized with sodium pentobarbital (30 mg/kg, iv) at about 11:30 A.M. A midline laparotomy was performed, and the trachea was cannulated. In order to collect respiratory carbon dioxide, the endotracheal tube was attached by means of an inhalation-exhalation valve system to a breathing bag and this to four glass columns (3 x 100 cm) in series; each was filled with glass beads and contained 90 ml of 15% NaOH. Air was pulled through the valve system, over the endotracheal tube, and through the breathing bag and columns.

The superficial muscles of both pelvic limbs, medial aspects, were exposed as were the femoral veins. $^{14}C$-Labeled chylomicrons, at 0.2 g of fat per kilogram body weight (ca. 1.6 μCi/kg body wt), were injected into the cannulated cephalic vein of a front leg and ca. 0.5 g samples of liver, mesenteric adipose tissue and skeletal muscle (either sartorius, cranial or caudal, or the gracilis) along with ca. 6 ml samples of venous blood were removed at 2,5,10,20,40,80 and 160 min postinjection. Tissues were taken in such a way as to avoid interference with circulation of regions sampled later. The solid tissue samples were immediately frozen at ca. -70 C. Microhematocrits were done on the blood samples, and the plasma was then separated by centrifugation and recovered. At the end of the experiment the dogs were sacrificed and the liver removed and weighed. The dose of chylomicron lipid chosen for this study is similar to the lower doses of chylomicron triglyceride (0.33 g/kg body wt) given to anesthetized dogs by Havel and Fredrickson (7). They observed that the removal of chylomicrons from plasma appeared to follow first order kinetics.

Within 24 hr after collection, the tissue samples were thawed, washed in saline, blotted dry, weighed, cut into small pieces and homogenized in 4 ml of ice cold saline with the Polytron homogenizer. Adhering adipose and connective tissue was carefully dissected away from the muscle samples prior to weighing and

TABLE I

*Effects of Treatment with Clofibrate or Nafenopin upon Plasma Triglyceride Levels*[a]

| Dog | Treatment | Plasma triglyceride levels, mg/100 ml | | | | | Dog | Treatment | Plasma triglyceride levels, mg/100 ml | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Day of measurement | | | | Per cent change | | | Day of measurement | | | | Per cent change |
| | | -2 | 10-12 | 15 | 22 | | | | -2 | 10-12 | 15 | 22 | |
| A | Clofibrate | 32.7 | 29.6 | — | lost | - 9 | C | Nafenopin | 59.2 | 19.6 | — | 18.3 | -69 |
| B | Clofibrate | 37.1 | 22.8 | — | lost | -39 | D | Nafenopin | 30.9 | 36.9 | — | 21.4 | -31 |
| G | Clofibrate | 33.9 | 23.6 | — | 19.4 | -43 | E | Nafenopin | 16.1 | 18.0 | — | 12.9 | -20 |
| H | Clofibrate | 20.0 | 13.6 | — | 13.5 | -33 | F | Nafenopin | 17.7 | 15.4 | — | 17.8 | + 1 |
| 6 | Clofibrate | 21.2 | — | 10.9 | — | -49 | 1 | Nafenopin | 22.2 | — | 11.8 | — | -47 |
| 7 | Clofibrate | 23.5 | — | 35.5 | — | +55 | 2 | Nafenopin | 18.5 | — | 9.8 | — | -47 |
| 8 | Clofibrate | 20.4 | — | 12.9 | — | -37 | 3 | Nafenopin | 34.2 | — | 12.1 | — | -65 |
| 9 | Clofibrate | 31.2 | — | 13.9 | — | -55 | 4 | Nafenopin | 27.5 | — | 9.0 | — | -67 |
| | | | | | | | 5 | Nafenopin | 21.6 | — | 9.1 | — | -58 |
| | Averages | 27.5 | 22.4 | 18.3 | 16.5 | -27 | | Averages | 27.5 | 22.4 | 10.4 | 17.6 | -45 |

[a]Dogs designated by letters were those used for the chylomicron-triglyceride uptake experiments and received orally for 21 days clofibrate (75 mg/kg/day) or nafenopin (25 mg/kg/day). Those designated by numbers were used for studies of lipoprotein lipase activity. These dogs received clofibrate or nafenopin for 14 days also at 75 or 25 mg/kg/day, respectively.

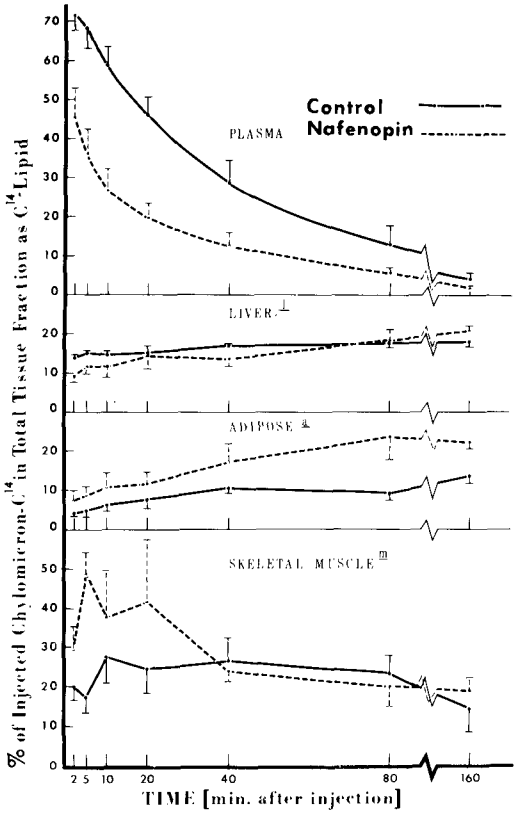
homogenization. The lipids of the total homogenates or plasma samples were extracted into 20 volumes of chloroform-methanol 2:1 as described for extraction of chylomicron lipid. The isolated total lipids were transferred with chloroform to scintillation counting vials, the chloroform evaporated, and the lipids assayed for $^{14}$C-radioactivity. All samples were counted for 10 or more minutes. Generally the lowest activities measured were ca. 1000 cpm. Efficiency was determined in all samples by addition of internal standard, $^{14}$C-toluene. The $^{14}$C radioactivity content of the tissue samples was expressed initially as dpm of lipid-$^{14}$C per gram or milliliter of tissue. Based upon the total liver weight and estimated 5% body wt as adipose tissue, 40% as skeletal muscle, and 8% as blood, values were calculated for the total lipid-$^{14}$C content of the entire plasma, liver, adipose tissue and skeletal muscle at each of the times studied. The estimates for adipose tissue and skeletal muscle weight as per cent body weight are identical to those made by Havel and Goldfien (12). The radioactivity content of the various tissue fractions was then expressed as the per cent of the injected chylomicron-$^{14}$C, essentially $^{14}$C-labeled chylomicron-triglyceride fatty acids, present in the tissue fraction as $^{14}$C-labeled lipid at each of the different times.

At the end of the experiment the sodium hydroxide solution in each of the four glass columns was transferred quantitatively to separate 500 ml volumetric flasks with water and diluted to volume. Aliquots (0.2 ml) of these solutions were transferred to scintillation counting vials containing 0.3 ml of water. The samples were dissolved in 15 ml of scintillation counting solution (5 g PPO and 0.3 g POPOP per liter of toluene) containing 8% v/v of "Bio-solv" (Beckman Co.). Quenching was determined by the addition of internal $^{14}$C standard. The $^{14}$C content of the respiratory $CO_2$ was measured and expressed as the per cent of the injected chylomicron-$^{14}$C oxidized to respiratory $CO_2$.

Chylomicron-triglyceride was used for these experiments rather than $^{14}$C-labeled very low density lipoprotein (VLDL) triglyceride, mainly because of the much greater practicality of preparing the rather large quantities of labeled lipoprotein triglyceride required. Further, the mechanisms and sites for clearance of both chylomicron and VLDL triglyceride from plasma are assumed to be similar (13).

### Assay for Lipoprotein Lipase Activity in Adipose Tissue and Skeletal Muscle before and after Treatment with Clofibrate or Nafenopin

Adult male dogs (10-19 kg), different from



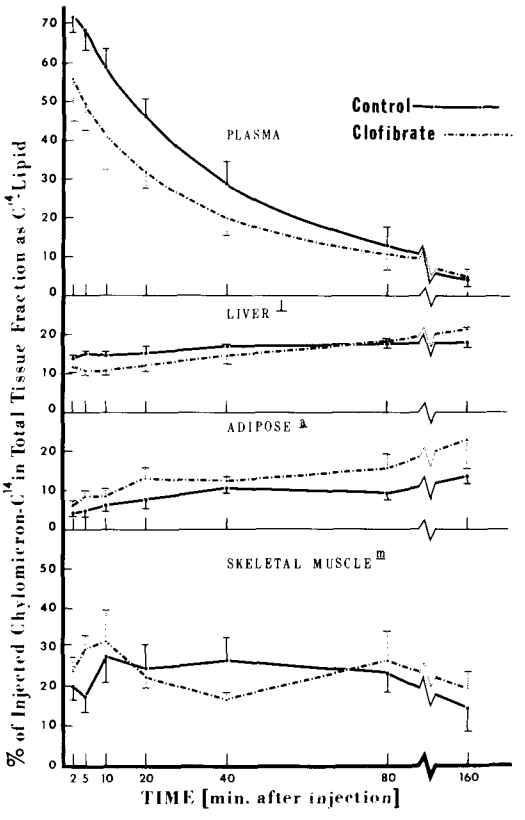




FIG. 2. (above, left) Plasma loss and tissue distribution of chylomicron-triglyceride in control and nafenopin-treated dogs. Dogs were injected iv with $^{14}C$-labeled chylomicron-triglycerides at 0.2 g of fat per kilogram body weight (ca. 1.6 $\mu$Ci/kg body wt). The lipid-$^{14}C$ content of the tissues was determined at 2, 5, 10, 20, 40, 80 and 160 min after injection. Each set of data is the average of four control or four nafenopin-treated dogs (25 mg/kg/day, 21 days). Bars are one SE. The data were analyzed by two-way analysis of variance.

| | Net treatment effect P(F) | Diff. at specific times— LSD test, P(t) $<$ 0.05 | Time effect (net ↑ or ↓ in % with time) P(F) |
|---|---|---|---|
| *l* | Not significant | 2,5,10($<$0.06),40,160($<$0.06) | $<$0.01 |
| *a* | $>$0.05 & $<$0.10 | 40, 80, 160 | $<$0.01 |
| *m* | $>$0.10 & $<$0.25 | 5, 20 | $<$0.05 |

FIG. 3. (above, right) Plasma loss and tissue distribution of chylomicron-triglyceride in control and clofibrate-treated dogs. Dogs were injected iv with $^{14}C$-labeled chylomicron-triglyceride at 0.2 g of fat per kilogram body weight (ca. 1.6 $\mu$Ci/kg body wt). The lipid-$^{14}C$ content of the tissues was determined at 2, 5, 10, 20, 40, 80 and 160 min after injection. Each set of data is the average of four control or four clofibrate-treated dogs (75 mg/kg/day, 21 days). Bars are one SE. The data were analyzed by two-way analysis of variance.

| | Net treatment effect P(F) | Diff. at specific times— LSD test, P(t) $<$ 0.05 | Time effect (net ↑ or ↓ in % with time) P(F) |
|---|---|---|---|
| *l* | $>$0.10 & $<$0.25 | 5, 10, 20, 160 | $<$0.01 |
| *a* | $>$0.05 & $<$0.10 | 160 | $<$0.05 |
| *m* | Not significant | 5, 40 | $<$0.05 |

those used in the chylomicron-triglyceride uptake experiments but similarly maintained, were anesthetized with sodium pentobarbital (30 mg/kg, iv), and a ca. 5 g cross section sample (ca. 3 cm wide) of the gracilis muscle (right hind leg) and ca. 1 g of subcutaneous adipose tissue from the area of the femoral vein were removed using sterile techniques, transferred to separate beakers of saline in an ice bath, and assayed for lipoprotein lipase (LPL)

TABLE II

Recovery of Injected Chylomicron-$^{14}$C as $^{14}$C-Labeled Lipid at Various Times after Injection

| | Total % of the injected $^{14}$C accounted for at various times after injection as $^{14}$C-labeled lipid[a,b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Minutes postinjection | | | | | | |
| Treatment[c] | 2 | 5 | 10 | 20 | 40 | 80 | 160 |
| Control | 108 ± 1 | 105 ± 5 | 107 ± 5 | 93 ± 3 | 84 ± 5 | 67 ± 4 | 62 ± 9 |
| Clofibrate | 101 ± 14 | 98 ± 9 | 92 ± 11 | 79 ± 6 | 64 ± 4 | 73 ± 7 | 76 ± 5 |
| Nafenopin | 90 ± 3 | 103 ± 7 | 83 ± 8 | 86 ± 12 | 65 ± 8 | 66 ± 5 | 68 ± 6 |
| Overall averages | 100 | 102 | 94 | 86 | 71 | 69 | 69 |

[a]Values are averages ± one SE.

[b]The totals were based upon the $^{14}$C-lipid content of the total plasma, liver, skeletal muscle, adipose tissue, and of $^{14}$C in the exhaled respiratory $CO_2$.

[c]Four dogs per group were treated orally with no drug (controls), 75 mg/kg/day of clofibrate or 25 mg/kg/day of nafenopin for 21 days.

activity as described below. The incision was sutured and 300,000 units of Benzathine Penicillin G were given intramuscularly. Beginning 2 days after removal of the tissue samples, the dogs orally received clofibrate or nafenopin in a gelatin capsule at 75 or 25 mg/kg, respectively, for 14 consecutive days. Fourteen days of treatment were used rather than 21, since 14 days administration of either drug was sufficient to produce a clear hypotriglyceridemic response. On day 15, samples of the same tissues but from the left leg were taken. Blood samples were obtained before and at the conclusion of drug treatment for measurement of plasma cholesterol and triglyceride levels.

Samples of adipose tissue (400-500 mg) were washed in ice cold saline, blotted dry, weighed, and homogenized for 30 sec via the Polytron homogenizer (at speed setting of 1.5) in 15 ml of the coconut oil-emulsion medium (buffered to pH 8.5) containing whole dog serum as described by Tolman et al. (2). After cutting away adhering connective tissue and adipose tissue, the muscle samples (800-1000 mg) were cut into small pieces, washed in saline, and homogenized in 15 ml of the medium for 30 sec with the Polytron homogenizer (at a speed setting of 2.5). The homogenates were transferred to 25 ml Erlenmeyer flasks and incubated via a Dubnoff metabolic shaker for 60 min at 37 C in air. Duplicate but usually triplicate 1 ml samples of the homogenates were taken before and after incubation and added to 10 ml of Dole's solution (14) containing 1 ml of water in a glass-stoppered test tube. The free fatty acids were extracted as described by Trout et al. (15) and measured via the automated colorimetric method of Lorch and Gey (16). Tissue and medium blanks were incubated simulataneously to determine inherent lipolytic activity. Lipase activity is expressed as μeq of free fatty acid (FFA) produced per gram tissue (wet weight) per hour.

### Calculations and Statistics

The half-life $t_{0.5}$ for clearance from plasma of $^{14}$C-labeled chylomicron-lipid was calculated for each dog by plotting the concentration of chylomicron-$^{14}$C present in plasma (expressed as the per cent of the injected chylomicron-$^{14}$C in the total plasma as $^{14}$C-lipid) on a log scale vs. time on an arithmetic scale (Fig. 1). Straight lines describing the disappearances were fitted by the method of least squares. The mean half-life ($t_{0.5}$) and standard error values for chylomicron-triglyceride disappearance from plasma of control, clofibrate- or nafenopin-treated dogs were calculated by averaging the individual $t_{0.5}$ values for the dogs in each group. With each dog, the disappearance of chylomicron-$^{14}$C from plasma was described by exponential processes which presumably indicate first order kinetics.

The uptake of chylomicron-triglyceride fatty acid by the various tissue fractions examined in control and drug-treated dogs was expressed graphically in terms of the per cent of the injected chylomicron-$^{14}$C present in the entire tissue fraction as $^{14}$C-lipid vs. time after injection. A two-way analysis of variance was used to statistically evaluate drug treatment and time effects. When a significant drug x time interaction was found, the differences between the measured uptake values for the control and drug-treated dogs at each time were statistically evaluated by the least significance difference test (LSD test) (17).

## RESULTS

### Effect of Drug Treatmen upon Plasma Lipid Levels

With the exception of one dog in each

group, treatment with clofibrate or nafenopin at 75 and 25 mg/kg/day, respectively, decreased the plasma levels of triglycerides in all dogs (Table I). On the average, nafenopin produced greater reductions of plasma triglycerides than clofibrate. Neither treatment with clofibrate nor nafenopin produced significant changes in the plasma levels of total cholesterol.

#### Clearance of $^{14}$C-Labeled Chylomicron-Triglyceride from Plasma

The disappearance of chylomicron-lipid, essentially chylomicron-triglyceride, from the plasma of control and drug-treated dogs is expressed graphically in Figure 1. When $^{14}$C-labeled chylomicron-triglycerides were injected iv into untreated dogs (controls), the disappearance of lipid-$^{14}$C from plasma between 2 and 80 min postinjection was described by a single exponential process with a half-life of ca. 31 min. In comparison, the clearance of chylomicron-triglyceride from plasma of both clofibrate- and nafenopin-treated animals appears to involve biexponential processes; a rapid, early disappearance seen from 2 to ca. 20 min postinjection, followed by another with a half-life similar to that measured for controls. The rapid phase of the plasma clearance in nafenopin-treated dogs ($t_{0.5}$ = 12.5 min) was significantly different from that measured for controls. The comparable difference between clofibrate-treated ($t_{0.5}$ = 19.6 min) and control dogs was significant at the 0.17 level. Another related difference between control and treated dogs was observed; in the 2 min immediately following injection of the labeled lipid ca. 50% of the injected radioactivity has already been removed from the plasma of the treated dogs as compared with ca. 30% removed from the plasma of control animals.

#### Distribution of Chylomicron-Triglyceride (Chylomicron-$^{14}$C) among Body Major Tissue Fractions

The distribution of the $^{14}$C-labeled chylomicron-triglyceride cleared from plasma of control and drug-treated dogs is described in Figures 2 and 3. The per cent of the injected chylomicron-$^{14}$C present in the tissue fractions as $^{14}$C-labeled lipid is plotted against time after injection. With both control and drug treated animals, skeletal muscle incorporated more of the injected chylomicron-$^{14}$C as $^{14}$C-labeled lipid than the other tissue fractions examined (Figs. 2 and 3). Skeletal muscle seems to be of primary importance for clearing chylomicron-triglyceride soon after injection, with adipose tissue and liver assuming greater roles with increasing time. For example, in the control dogs the skeletal muscle mass incorporated ca. 25% of the injected chylomicron-$^{14}$C between 10 and 80 min postinjection. This decreased to ca. 15% by 160 min. In contrast, the adipose tissue of control dogs contained less than 5% of the injected activity soon after injection but gradually incorporated increasing amounts, such that at 160 min it contained ca. 15% of the injected chylomicron-$^{14}$C. Unlike either skeletal muscle or adipose tissue, the total liver of control animals contained ca. 14% of the injected activity almost immediately after injection. This increased to ca. 18% by the end of the experimental period. It is important to note that at the first four times examined after injection—that is, at 2,5,10 and 20 min postinjection—one can account for essentially all of the injected chylomicron-$^{14}$C at each of these times by adding the per cent contribution of each tissue fraction studied (Table II). This would indicate that the estimates made for the contribution of the various tissue fractions to the total body weight and the calculations are reasonable. The inability to account for more than ca. 70% of the injected activity at the later times could relate to entry of chylomicron-triglyceride-$^{14}$C into tissues other than those examined and also to oxidation of $^{14}$C-labeled tissue lipids to water soluble metabolites.

Treatment of dogs with either clofibrate or nafenopin resulted in significant changes in the uptake of chylomicron-$^{14}$C by the tissue fractions examined. Nafenopin produced greater changes than clofibrate. During the first 20 min after injection of the labeled chylomicrons, there was a tendency toward greater incorporation of the injected chylomicron-$^{14}$C into the skeletal muscle of the nafenopin-treated dogs than controls (Fig. 2). Also, soon after injection significantly lower percentages of the chylomicron-$^{14}$C were taken up by the liver of nafenopin-treated animals. Slightly more was present in the livers of treated dogs by the end of the experimental period. The $^{14}$C-lipid content of the skeletal muscle in the treated dogs decreased sharply at ca. 20 min postinjection. This decrease coincided with an increase in corporation into the adipose tissue mass, such that between 40 and 160 min after injection significantly greater percentages of the injected radioactivity were found in the adipose tissue of the nafenopin-treated dogs. Thus, overall, nafenopin seemed to enhance the uptake of chylomicron-triglyceride by the skeletal muscle soon after injection and at later times stimulated uptake into adipose tissue. The effects of clofibrate upon the tissue uptake of labeled chylomicron-triglyceride are generally in the same direction as observed with nafenopin but less pronounced (Fig. 3).

TABLE III

Effects of Treatment with Clofibrate or Nafenopin upon Adipose Tissue and Skeletal Muscle Lipoprotein Lipase Activity

| | | Lipoprotein lipase activity (μeq FFA/g tissue, wet wt/hr)[a,b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Skeletal muscle[c] | | | Adipose tissue | | |
| Treatment | Dogs, *n* | Pretreatment | Posttreatment | Per cent Δ | Pretreatment | Posttreatment | Per cent Δ |
| Clofibrate | 4 | 8.93 ± 1.18 | 8.79 ± 0.71 | -2 | 28.9 ± 3.2 | 43.0 ± 3.5 | 49[d] |
| Nafenopin | 5 | 8.25 ± 0.43 | 9.93 ± 0.32 | 20[e] | 38.8 ± 9.8 | 37.0 ± 3.3 | -5 |

[a]Values are averages ± one SE. (FFA = free fatty acids.)

[b]Adipose tissue and skeletal muscle lipase activity was measured in tissue samples taken from the same dogs before and after 14 days oral treatment with clofibrate (75 mg/kg/day) or nafenopin (25 mg/kg/day).

[c]This lipase activity is probably not identical with lipoprotein lipase as defined by Korn (18).

[d]$P(t) < 0.02$ (paired $t$ test).

[e]$P(t) < 0.05$ (paired $t$ test).

Clofibrate treated dogs, in comparison to controls, appeared to incorporate relatively more of the injected chylomicron-$^{14}C$ into the skeletal muscle mass soon after injection (Fig. 3). Also, like nafenopin, less of the injected radioactivity appeared in the liver during the first 20 min and more at 160 min postinjection. Greater percentages of the injected chylomicron-$^{14}C$ were measured in the adipose tissue of clofibrate-treated dogs throughout the experimental period. The biggest difference was seen at 160 min.

Finally, treatment with clofibrate or nafenopin had no significant effects upon the oxidation in vivo of the chylomicron-triglyceride fatty acid to respiratory $CO_2$. Control dogs oxidized on the average 12.7 ± 5.5 (SE) %, clofibrate 7.6 ± 2.1%, and nafenopin treated dogs 9.8 ± 3.3% of the chylomicron-$^{14}C$ during the 160 min postinjection period.

The experiments to this point indicated that treatment with nafenopin and to a lesser extent clofibrate accelerated the removal of chylomicron-triglyceride from plasma and stimulated its uptake, initially by skeletal muscle and secondly by adipose tissue. Because tissue lipoprotein lipase activity is undoubtedly a major determinant of the rate at which chylomicron-triglyceride is cleared from the circulation, experiments were conducted to measure the influence of treatment with clofibrate and nafenopin upon the lipoprotein lipase activity of adipose tissue and skeletal muscle.

### Drug Effects upon Lipoprotein Lipase (LPL) Activity

As described in Materials and Methods, skeletal muscle and subcutaneous adipose tissue removed from the same dogs before and after treatment with clofibrate or nafenopin were assayed for lipoprotein lipase activity. The paired $t$ test was used to analyze for significant drug effects; that is, each dog served as his own control. On the basis of activity per gram of tissue (wet weight), adipose tissue possessed about four times as much lipase activity as skeletal muscle (Table III). However, as indicated below, the lipase activities measured for adipose tissue and skeletal muscle probably represent different enzymes. As reported in the literature (18), adipose tissue LPL was markedly inhibited by the addition of 1.0 M NaCl to the tissue homogenates (Table IV). The LPL activity of adipose tissue measured both before and after treatment with clofibrate or nafenopin was equally inhibited by NaCl. Furthermore, the adipose tissue lipase activity was greatly reduced when assayed in the absence of dog blood serum (Kreb's phosphate buffer substituted for the serum). Serum cofactor is another requirement for LPL activity. When examined, a dog's adipose tissue lipase activity was 50.8 μeq of FFA produced per gram tissue per hour in the presence of dog serum but only 10.0 in the absence of added serum. By contrast, the lipase activity of skeletal muscle was inhibited to a much lesser extent by NaCl than that of adipose tissue and was independent of a serum cofactor. The lipase activity measured with skeletal muscle, therefore, does not appear to be due to lipoprotein lipase as defined by Korn (18).

Clofibrate treatment had no apparent effects upon the skeletal muscle lipase activity, but it did result in a ca. 50% increase in the LPL activity of adipose tissue. Treatment with nafenopin led to a significant 20% increase in the lipase activity of skeletal muscle; however it had no effect upon LPL activity of adipose tissue (Table III).

## DISCUSSION

In comparison to control dogs, chylomicron-

TABLE IV

Inhibition of Skeletal Muscle and Adipose Tissue Lipase Activities by 1.0 Molar NaCl

| Treatment-state of dogs | No. of dogs | Per cent inhibition[a] | |
|---|---|---|---|
| | | Skeletal muscle | Adipose tissue |
| Pretreatment | 2 | 28 (24 and 32) | 82 (79 and 84) |
| Posttreatment-clofibrate[b] | 4 | 4 (-5 to 18) | 82 (79 to 84) |
| Posttreatment-nafenopin[c] | 1 | 22 | 86 |

[a]The values are average per cent inhibition of the lipase activities measured in the absence of the 1.0 molar NaCl. Values in brackets give actual or range of values.

[b]Clofibrate given orally for 14 days at 75 mg/kg/day.

[c]Nafenopin given orally for 14 days at 25 mg/kg/day.

triglyceride was removed more rapidly from the plasma of dogs treated with nafenopin or clofibrate. The observed ability of nafenopin to accelerate the clearance of chylomicron-triglyceride from plasma agrees closely with the recent findings reported by Boberg et al. (3). They observed that treatment of human patients with up to 600 mg of nafenopin per day for 1-3 months increased by almost 100% the rate of clearance of an artificial fat emulsion from plasma. They suggested that nafenopin lowers plasma triglyceride levels by increasing its clearance from blood. In the present study, chylomicron-triglyceride was cleared almost two and one-half times more rapidly from the plasma of nafenopin treated as compared with control dogs. Like nafenopin, studies with human patients given clofibrate suggest that clofibrate could lower triglycerides by influencing peripheral clearance mechanisms. Ryan and Schwartz (19) reported that clofibrate significantly depressed plasma triglyceride levels in humans without changing FFA turnover or triglyceride production. Westerfield and coworkers (20) recently observed that clofibrate greatly improved the handling of an exogenous fat load in rats. On the average, labeled chylomicron-triglyceride was removed ca. 50-60% more rapidly from the plasma of clofibrate-treated dogs than controls; however this difference was only of borderline statistical significance.

In further contrast with controls, the disappearance of chylomicron-$^{14}$C from the plasma of treated dogs (both nafenopin and clofibrate) seemed to be biexponential. This biexponential disappearance might reflect an accelerated catabolism of chylomicrons to lipoproteins of higher density or an initial rapid clearance of the larger chylomicron particles from plasma. Clearance of lipoproteins from plasma appears to involve a step-wise conversion of larger to smaller triglyceride-rich lipoproteins (21) with the larger lipoprotein particles being cleared more rapidly than the smaller ones (22,23). Thus, in treated vs. control dogs, at later times after injection greater fractions of the circulating labeled lipoproteins could be of higher density with the net disappearance of label from plasma at these times reflecting the slower turnover of these smaller particles. It is interesting to note that Fredrickson and coworkers (24) also observed that removal of $^{14}$C-triglyceride labeled chylomicrons from plasma of dogs could involve two phases.

The explanation for the disappearance of ca. 50% of the injected labeled lipid from the plasma of treated dogs and 30% from control dogs during the 2 min immediately following injection is unclear. However this observation may involve trapping of intact chylomicrons in the liver and skeletal muscle mass. As discussed below, essentially all of the chylomicron-$^{14}$C removed from plasma at 2 min postinjection was found in the skeletal muscle mass and in the liver. Furthermore, tissue uptake of intact chylomicrons has been demonstrated by others (25,26). Regardless of the clearance mechanism involved, drug treatment appeared to also accelerate this very rapid, very early removal of chylomicron-triglyceride from plasma.

In all groups of dogs studied, skeletal muscle and liver together accounted for virtually all of the chylomicron-$^{14}$C removed from plasma during the first 10-20 min after injection of this labeled lipoprotein. With more time, adipose tissue gradually took up increasing amounts of the chylomicron-$^{14}$C. Bragdon and Gordon (25) also observed that liver and skeletal muscle are of great importance in the early, rapid clearance from plasma of chylomicron-triglyceride fatty acids injected into rats. Uptake of chylomicron-triglyceride by muscle presumably involves trapping of intact chylomicrons followed by hydrolysis of triglyceride. The mechanism responsible for uptake by the liver is less clear. The rapid incorporation of chylomicron-$^{14}$C into liver may solely involve trapping of

chylomicrons in the space of Disse, since Felts and Berry (27) demonstrated that liver parenchymal cells do not directly take up and metabolize any significant amounts of circulating triglycerides.

The observed effects of drug treatment upon the uptake of chylomicron-triglyceride by the various tissue fractions examined, i.e., early stimulation into skeletal muscle and later into adipose tissue, could partially involve effects upon tissue lipase activities. As was reported by Tolman et al. (2) for the rat, treatment of the dog with clofibrate led to a significant increase in adipose tissue lipoprotein lipase activity. On the other hand, nafenopin stimulated a skeletal muscle lipase activity but did not noticeably influence the activity of adipose tissue LPL. Yet nafenopin did significantly increase the uptake of chylomicron-$^{14}$C by adipose tissue. This apparent paradox has not been explained; however one can speculate that the uptake by adipose tissue might be secondary to an accelerated catabolism of $^{14}$C-labeled chylomicrons at other tissue sites (notably skeletal muscle) with subsequent uptake of labeled metabolites by adipose tissue. Although the measured skeletal muscle lipase is different from adipose tissue LPL, the muscle enzyme did hydrolyze exogenous triglyceride, and, considering the entire dog, it would appear to be more abundant than the LPL of adipose tissue.

In conclusion, treatment of the dog with nafenopin and to a lesser extent clofibrate accelerated the clearance of chylomicron-triglyceride from plasma and appeared to stimulate its uptake initially by skeletal muscle and later by adipose tissue. These effects may be related in part to a clofibrate stimulation of adipose tissue LPL activity and a nafenopin-induced increase of a skeletal muscle lipase activity. The findings of this study may strengthen the rationale for using clofibrate and nafenopin in the treatment of hypertriglyceridemic patients, since Quartfordt and coworkers (4), Boberg et al. (3), Havel et al. (5) and Eaton (6) have independently suggested that a decreased triglyceride clearance by peripheral tissues could be the primary metabolic abnormality in certain types of human hypertriglyceridemias.

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# The Synthesis of Ceramide Galacturonate

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## ABSTRACT

Ceramide galacturonate was synthesized from ceramide galactoside by the sequential treatment with galactose oxidase and hypoiodite oxidation. The sole water soluble hydrolysis product was galacturonic acid.

The glycosphingolipids represent a family of substances which contain one or more carbohydrate units glycosidically linked to the primary hydroxyl group of ceramide. The number of sugars in the glycoside residue varies from one to at least seven and may contain from one to four different sugars (1). Uronic acid-containing sphingolipids have been reported to occur in plant tissues (2). The purpose of this report is to describe the synthesis of ceramide galacturonate by the stepwise oxidation of ceramide galactoside according to the following sequence:

ceramide galactoside
↓ galactose oxidase
ceramide 6-aldehydogalactoside [1]

ceramide 6-aldehydogalactoside
↓ 10-
ceramide galacturonate [2].

## MATERIALS AND METHODS

Ceramide galactoside was purified from bovine brain tissue as described previously (3). The oxidation of ceramide galactoside by galactose oxidase (Kabi, Sweden) and the isolation of the reaction product were carried out according to a published procedure (4). The further oxidation of the ceramide 6-aldehydogalactoside was accomplished by the hypoiodite oxidation method of Moore and Link (5) which is carried out in methanol. The dried sample was dissolved in methanol (2.4 ml) and five drops of chloroform in an Erlenmeyer flask, and placed on a magnetic stirrer in a 37 C room. Warmed (37 C) methanol (4 ml) containing 280 mg iodine was added while stirring. Then 4% methanolic KOH (3.5 ml) was added dropwise over a 10 min period. An additional aliquot of 4% methanolic KOH (5 ml) was then added. Sufficient hydrochloric acid was added to this oxidation mixture to adjust the pH to 3.0. Two volumes of chloroform were added, and the solution was extracted according to the procedure of Folch et al. (6). The final lower chloroform phase was evaporated to dryness and applied to a DEAE cellulose column (1.8 x 8 cm) in chloroform-methanol 2:1. The unreacted ceramide 6-aldehydogalactose was recovered in 10 bed volumes of chloroform-methanol 2:1, and the ceramide galacturonate was eluted in 10 bed volumes of 1 M $LiCl_2$ in chloroform-methanol 1:1. This eluate was dialyzed against 100 volumes of distilled water in the cold for 48 hr. A clear upper phase in the retentate was separated from the underlying emulsion by centrifugation. An aliquot of both phases was assayed for uronic acid. Acid hydrolysis of the final product with Dowex-50-$H^+$-0.1N HCl was accomplished according to the method of Davidson (7).

The uronic acid content was quantitated by the carbazole procedure of Dische (8) as modified by Gregory (9) with galacturonic acid as the colorimetric standard. Thin layer chromatography was on Analtech Silica Gel G plates with chloroform-methanol-water 24:7:1 as solvent. Descending paper chromatography of the uronic acids was on Whatman #1 paper with acetone-ethanol-isopropanol-0.05N borate pH 10.0 3:1:1:2 as solvent (10). An alkaline silver nitrate reagent was employed for the detection of reducing sugars on the chromatograms.

## RESULTS

The conversion of ceramide galactoside to

TABLE I

Identification of Galacturonic Acid in a Hydrolysis Product

| Compound | Rgal |
|---|---|
| Galactose | 1.0 |
| Glucuronic acid | 0.82 |
| Galacturonic acid | 0.74 |
| Hydrolysis product | 0.73 |

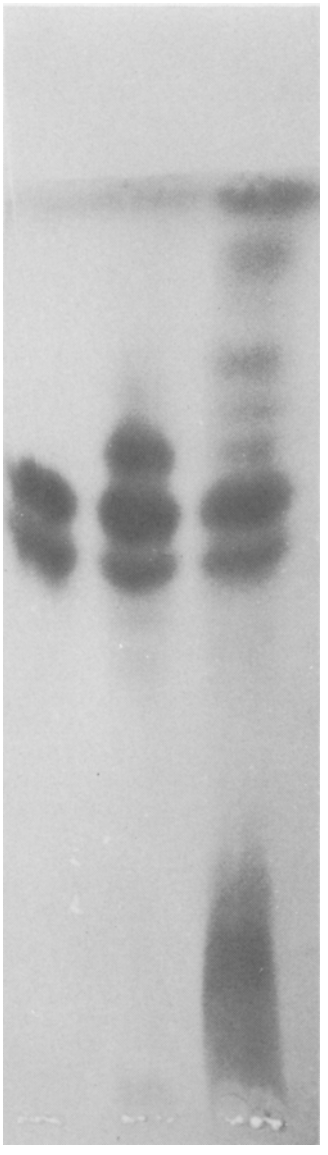

FIG. 1. Thin layer chromatogram of the two oxidation products: left lane starting ceramide galactoside; center lane galactose oxidase product; right lane hypoiodite oxidation products.

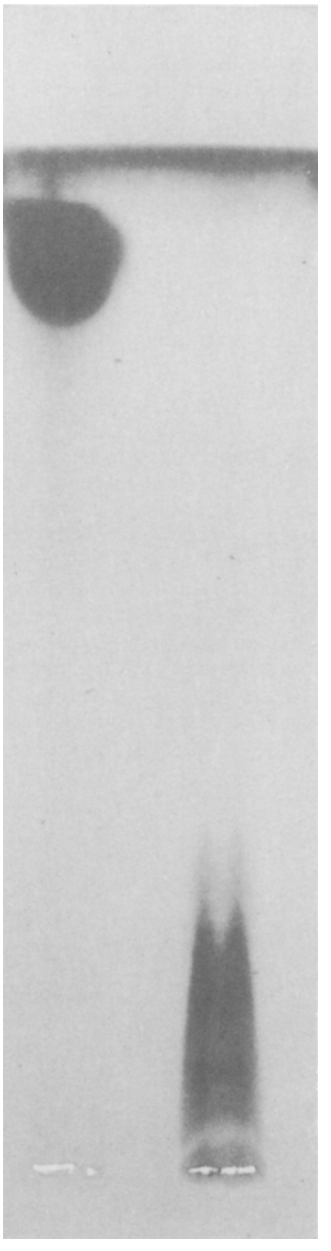

FIG. 2. Thin layer chromatogram of purified final product: left lane starting ceramide galactoside; right lane purified ceramide galacturonate.

the 6-aldehydo derivative was revealed by $AgNO_3$ spray reagent. The starting cerebroside reacted only faintly to this reagent due to the absence of a reducing group. When this material was subjected to further chemical oxidation by hypoiodate, a carbazole positive reacting compound was obtained. Thin layer chromatographic examination of the reaction mixture revealed the appearance of a polar product near the origin (Fig. 1). Colorimetric analysis indicated the formation of ca. 50 $\mu$moles of uronic acid starting with 110 $\mu$moles of ceramide galactoside. The final compound recovered in the $LiCl_2$ eluate of the DEAE cellulose column indicated the presence of a nonpolar material (Fig. 2). Examination of the phases in the retentate obtained after dialysis indicated that the uronic acid-containing product was present

solely in the underlying emulsion. IR spectra of the starting ceramide galactoside and the final product indicated the major difference was the appearance of a free carboxyl group at 1715-1730 $cm^{-1}$.

Paper chromatographic separation of the water soluble hydrolysis products revealed a single silver nitrate positive spot which cochromatogramed with authentic galacturonic acid as indicated in Table I. No other reducing compound was recovered from the hydrolysis mixture.

The presence of a simple uronic acid derivative of ceramide has not been reported to occur in biological material. Such materials or suitable derivatives may prove useful as specific inhibitors of individual acid hydrolases. Theoretically, similar uronic acid-containing modifications could be prepared for cer-glu-gal; cer-glu-gal-gal; cer-glu-gal (NANA)- galNAc-gal; and cer-glu-gal-galNAc-gal, where there is a terminal galactose which is susceptible to oxidation by galactose oxidase.

The evidence supporting the structure of the product formed as ceramide galacturonate is based upon the following: (a) a relatively polar product formed after hypoiodite oxidation of the ceramide 6-aldehydogalactose; (b) the established capacity for hypoiodite to oxidize an aldehyde group to the corresponding carboxyl group; (c) the appearance of a positive carbazole reaction, not given by either the parent ceramide galactoside or the immediate precursor; (d) the presence of galacturonic acid as the water soluble hydrolysis product as demonstrated by paper chromatography; and (e) IR spectroscopy.

ACKNOWLEDGMENTS

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# Biosynthesis of Brain Sphingolipids and Myelin Accumulation in the Mouse

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## ABSTRACT

Microsomal fractions were prepared from the brains of mice sacrificed at 13 age points between 5 and 48 days of age. Several enzymatic activities implicated in sphingolipid biosynthesis were assayed. The developmental pattern for the terminal step in galactosylceramide synthesis, transfer of galactose from UDP-galactose to ceramide, peaked sharply at 17-19 days of age. Thus maximal activity for biosynthesis of this relatively myelin specific lipid appears slightly before the time period of maximal rate of accumulation of myelin (21-23 days). These latter data were obtained by isolating myelin from mice at 10 age points from 9 to 45 days of age. A control enzymatic activity, transfer of glucose from UDP-glucose to ceramide to form glucosylceramide, was high at all age points with a broad peak between 20 and 35 days of age. Condensation of palmitoyl-CoA and serine to form ketodihydrosphingosine, a common precursor to both glucosyl- and galactosylceramide also followed a developmental pattern of high activity at all age points, but peaked slightly at ca. 10-12 days of age.

The quantitative importance of sphingoglycolipids in the central nervous system of higher organisms is well known and has generated considerable interest in the metabolism of these compounds. Of relevance to the present study are the observations that galactosylceramide (cerebroside) is localized primarily in myelin, where it may account for 19% of its dry weight (1). In contrast, gangliosides, which account for ca. 1.7% of the dry weight of grey matter (2), are localized largely in neuronal elements. A developmental study of several enzymatic activities involved in sphingolipid biosynthesis was carried out using a mouse brain preparation. This was correlated with data on the accumulation of myelin. Since the bulk of the brain galactosylceramide has a relatively slow turnover rate (3,4) we expected that enzymatic activities involved in its synthesis would correlate primarily with the rate of myelin accumulation. The developmental pattern expected for an enzyme responsible for an early step in ganglioside biosynthesis is more difficult to predict, since this class of compounds is relatively heterogeneous both with respect to its subcellular localization and with respect to the turnover rates of individual ganglioside species (5).

The following reactions were studied:

palmitoyl-CoA + serine→ketodihydrosphingosine [1]
ceramide + UDP-galactose→galactosylceramide [2]
LCB + UDP-galactose→psychosine [3]
ceramide + UDP-glucose→glucosylceramide [4]

The ketodihydrosphingosine resulting from the condensation of palmitoyl-CoA and serine [1] (6-8) is later reduced to dihydrosphingosine, a long chain base (LCB) found in all classes of sphingolipids. Following acylation by an acyl-CoA (9,10) the resulting ceramide can be galactosylated to form galactosylceramide. Galactosylceramides containing hydroxy fatty acids (HFA) and nonhydroxy fatty acids (NFA) are found in brain (11), and so experiments relating to [2] were carried out separately with HFA- and NFA-ceramide as precursor (12-14). The galactosylation of LCB [3] was also studied, since this has been proposed as a step in an alternate route for galactosylceramide biosynthesis (15,16). Another possible fate for ceramide is glucosylation to form glucosylceramide (17) a precursor of ganglioside.

## MATERIALS AND METHODS

Swiss-Webster mice were bred from stock obtained from Carworth, New City, N.Y. Litters were pruned to 7-8 animals at birth, sacrificed at the desired age, and microsomes were prepared (13) and stored at -50 C until used. In the case of younger age points, microsomes from several litters were pooled.

The enzyme assays were carried out as reported previously (18); for each assay a radioactive substrate and nonradioactive acceptor were incubated with microsomal fractions and the appropriate cofactors. After incubation the lipids were extracted and separated by thin layer chromatography using the appropriate solvent system. The lipid product being studied

was scraped from the plate and was quantitated by scintillation counting.

Myelin was isolated from pooled, freshly excised brains by the CsCl gradient procedure (19). The details of this isolation procedure, and the protein composition of the isolated myelin samples have been presented (20).

## RESULTS

### Assay Procedure

The variability for any of the biosynthetic assays was 10-20% for triplicate determinations of the same sample carried out at the same time. However, in comparing the many separately collected samples required for a developmental study, several possible complications had to be controlled. A decline of ca. 20% in enzymatic activity for synthesis of galactosylceramide or ketodihydrosphingosine was noted after 1 month of storage at -60 C. The rate of decay was increased by storage at -20 C, and loss of activity was also observed after several cycles of freezing and thawing. We also noted that enzymatic activity for galactosylceramide biosynthesis was significantly decreased when microsomes were obtained from mice that had been born and suckled as part of a large (more than 12 pups) litter, as compared to preparations from a smaller (6-8 pups) litter. Variability in batches of ceramide used as substrate was also a factor; differences of up to 20% in activity were observed for various samples of NFA-ceramide used as substrate for galactosylceramide synthesis. These included samples prepared by ourselves (12) and the product of Applied Science Labs., State College, Pa. The reason for this variation is not known, since all the ceramide samples were pure by thin layer chromatography (10).

To minimize variation, all results shown in the figures were obtained with the same group of microsomal fractions, collected over a period of 3 weeks and stored together at -60 C. All the assays for a particular enzymatic activity were carried out at the same time, and the handling of each microsomal preparation was identical. Several preliminary experiments were carried out with a lesser number of age points, and the shape of the curves in each case was identical to those shown. However the absolute level of enzymatic activity varied by as much as 40%, presumably due to the variables discussed above.

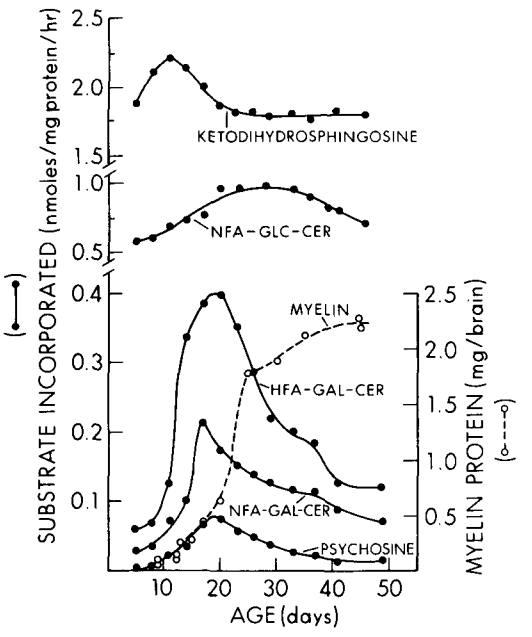


FIG. 1. Activity for biosynthesis of sphingolipids and accumulation of myelin during development. Incubation conditions for specific assays were: (a) Ketodihydrosphingosine. Incubation mixtures (6,18) of 0.13 ml containing 1 mg microsomal protein, 7.5 μmoles potassium phosphate buffer at pH 7.5, 25 μmoles L-serine and 0.1 μmole $^{14}$C-palmitoyl-CoA at 5 μC/μmole were agitated at 37 C for 15 min. (b) HFA-galactosylceramide. The lipid substrate, 0.24 mg HFA-ceramide was coated onto 25 mg Celite by evaporation from chloroform-methanol 2:1 (12). The incubation mixture (8,12) of 0.13 ml contained also 75 μmoles Tris-HCl at pH 7.4, 0.3 μmoles neutralized ATP, 0.15 μmoles EDTA, 0.15 μmoles dithiothreitol, 0.3 μmoles $MgCl_2$ and 12.5 nmoles $^{14}$C-UDP-galactose at a specific activity of 10 μC/μmole. Following addition of 0.4 mg microsomal protein the tubes were agitated for 120 min at 35 C. (c) NFA-galactosylceramide. As in (b), except that the lipid substrate NFA-ceramide was coevaporated onto the Celite with 0.25 mg crude lecithin, this additional polar lipid being necessary to optimize formation of NFA-galactosylceramide (13). (d) Psychosine. As in (b), except that dihydrosphingosine was substituted for ceramide. (e) Glucosylceramide. Incubation conditions were as in (b), except that $^{14}$C-UDP-glucose was substituted for the $^{14}$C-UDP-galactose.

Following incubation, lipids were extracted and separated by thin layer chromatography (18). Radioactivity in specific lipids was quantitated by scintillation counting. Myelin was isolated from mice of various ages and the amount of protein assayed as previously described (19,20).

Abbreviations: HFA-Gal-Cer, α-hydroxy fatty acid galactosylceramide; NFA-Gal-Cer, nonhydroxy fatty acid galactosylceramide; NFA-Glc-Cer, nonhydroxy fatty acid glucosylceramide.

### Galactosylceramides and Psychosine

Development of biosynthetic activity for formation of HFA- and NFA-galactosylceramide and for psychosine is shown in Figure 1. It is clear that the developmental pattern of these activities is the same—very low at 5 days of age, increasing at least 6-fold to a peak at 17

to 20 days of age and then declining slowly. In a series of such experiments the activities for galactosylation of HFA-ceramide and of dihydrosphingosine always coincided, although there is a significant quantitative difference in the rate of incorporation of galactose for the two substrates. Addition of dihydrosphingosine did not stimulate the synthesis of galactosylceramide. The shape of the curves for NFA-galactosylceramide synthesis was always similar to that of the other two curves, but some minor variations were noted. In four separate studies, the ratio of $^{14}$C-galactose incorporated at 17-20 days into HFA-galactosylceramide, NFA-galactosylceramide and psychosine was 7:3:1.

It has been reported that cerebrosides containing long chain fatty acids in the N-acyl position are localized preferentially in white matter (21). We therefore thought it possible that activity for formation of long chain and medium chain length galactosylceramides might vary independently. In one experiment (not shown) NFA-ceramide was incubated with UDP-galactose, and cerebrosides containing long chain (primarily $C_{24}$) and medium chain (primarily $C_{18}$) fatty acids in the N-acyl position were collected and quantitated separately (13). A similar experiment was carried out with HFA-ceramide. In each case the shape of the curves for galactosylation of HFA-long chain and HFA-medium chain length ceramides was identical.

#### Glucosylceramide

The development pattern of enzymatic activity for glucosylceramide biosynthesis (Fig. 1)—high activity at all age points with a broad peak from 20-35 days of age—is clearly different from that of galactosylceramide synthesis. In some experiments HFA-ceramide was used as an acceptor and proved to be as effective as NFA-ceramide at every age point. The product was assumed to be HFA-glucosylceramide, because its $R_f$ was slightly less than that of authentic NFA-glucosylceramide upon thin layer chromatography on both borate-impregnated (22) and normal (12) silica gel plates.

#### Ketodihydrosphingosine

The developmental pattern for synthesis of ketodihydrosphingosine (Fig. 1) is also markedly different from that for galactosylceramide synthesis—high for all age points but with a slight peak at 11 days.

#### Myelin Accumulation

Myelin accumulates rapidly between 10 and 30 days of age. The time of most rapid accumulation (ca. 21-23 days) lags by several days the peak of activity for galactosylceramide biosynthesis (Fig. 1).

## DISCUSSION

Interpretation of the results of the developmental study is straightforward. The time of peak enzymatic activity for galactosylceramide biosynthesis in the mouse (17-19 days) is close to that reported for maximal rate of accumulation of galactosylceramides and proteolipid (23) and for the time of most efficient uptake of injected glucose into galactosylceramide in vivo (24). Before the onset of rapid myelinogenesis (ca. 10 days of age) enzymatic activity is very low. These results for HFA-galactosylceramide biosynthesis are very similar to those obtained by Brenkert and Radin (25) using lyophilized whole homogenates of rat brain as an enzyme source. Our studies utilized microsomal fractions because of the difficulty of measuring ketodihydrosphingosine synthesis in the presence of the acyl hydrolases in whole brain homogenates. Both our results and those of Brenkert and Radin (25) differ somewhat from those of Shah (26), in that Shah reported almost zero enzymatic activity below 10 days of age and a peak of activity at 30 days of age in rats.

The marked preference for HFA-ceramide relative to NFA-ceramide, as substrate for galactosylceramide formation, previously noted by us (12), has been observed in other laboratories (14,27). However, by optimizing conditions for NFA-ceramide utilization, significant amounts of NFA-galactosylceramide can be formed (13,28). The developmental pattern for this enzymatic activity is very similar to that for HFA-galactosylceramide biosynthesis (Fig. 1), although in some experiments minor differences were noted. Such slight variations could quite possibly be accounted for by differing availability to a single enzyme system of the two substrates NFA- and HFA-ceramide, under the experimental conditions utilized. However, lacking other data, it is also possible that several enzymes are involved.

Enzymatic activity for galactosylation of LCB to form psychosine has been reported (15). Our data (Fig. 1), as well as that from other laboratories (29,30), indicate that activity of this enzyme in vitro peaks at about the time of maximal myelination. Although it had previously been thought that this compound was precursor to galactosylceramide (16), evidence has accumulated that the acylation of psychosine reported under the specified incubation conditions was not enzymatic (13,31). Our

data, showing that HFA-ceramide is a much better substrate for galactosylation than LCB at all age points, supports the hypothesis that the latter reaction is the result of poor substrate specificity under in vitro conditions. Indeed such a result might be expected, since even in vivo it appears that the same enzyme is required to handle galactosylation of a large number of ceramides differing in chain length, in the presence or absence of an α-hydroxy group of the fatty acid, in degree of fatty acid and LCB unsaturation etc.

Enzymatic activity for formation of galactosylceramide peaks ca. 3 days earlier than the peak of myelin accumulation. This is as expected; enzymatic activity must rise before accumulation of a product much further down the biosynthetic pathway.

The biosynthesis of glucosylceramide was initially studied by Basu et al. (17), and two developmental studies have been published recently (25,26). Shah (26), utilizing conditions similar to our, reported a peak of activity at 10-20 days of age in rat and a decline thereafter, although activity at all age points was considerable. Brenkert and Radin (25), working with a preparation derived from whole rat brain homogenate, reported an initial high level of activity which gradually declined by ca. 50% between 20 and 60 days of age. Our data (Fig. 1) indicate elevated activity between 20 and 30 days of age, although the activity is quite high at all age points. Possibly the difference in results relates to our use of microsomes instead of whole brain homogenate. There might be a considerable enzymatic activity for glucosylceramide biosynthesis in synaptosomal fractions, that would not be included in our microsomal fraction.

It is interesting to note that the enzyme system synthesizing glycosylceramide shows a lack of substrate specificity; both HFA- and NFA-ceramide can be utilized. A similar observation has been made by Brenkert and Radin (25). This lack of specificity is also found in vivo, where ca. 20% of the small pool of free glucosylceramide contains HFA (32), although this HFA-glucosylceramide is not incorporated into gangliosides.

The results for ketodihydrosphingosine biosynthesis have not been reported by others, but they are consistent with what we know about brain sphingolipid metabolism in general. Long chain bases are involved in biosynthesis of the rapidly turning over sphingomyelin and gangliosides of grey matter, as well as the more slowly metabolized sphingolipid of white matter. This enzymatic activity is high at every age point studied and is elevated even more in the range from 10 to 25 days—the time period of maximal brain development.

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# Tetra-Acid Triglycerides Containing a New Hydroxy Eicosadienoyl Moiety in *Lesquerella auriculata* Seed Oil[1]

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## ABSTRACT

The seed oil of *Lesquerella auriculata* contains 32% of a previously unknown fatty acid, 14-hydroxy-*cis*-11,*cis*-17-eicosadienoic acid; we propose for it the trivial name "auricolic acid." In addition, the oil contains 2% densipolic (12-hydroxy-*cis*-9,*cis*-15-octadecadienoic) acid, 10% lesquerolic (14-hydroxy-*cis*-11-eicosenoic) acid and 5% ricinoleic acid. The oil of *L. auriculata* is also unusual, because a large part of the total oil consists of triglycerides containing more than three acyl groups. These components were characterized by various chromatographic, spectroscopic and lipolytic techniques.

## INTRODUCTION

All species of *Lesquerella* analyzed so far produce seed oils containing hydroxy fatty acids (1,2). Those reported fall in two groups on the basis of oil composition, and these groups coincide reasonably well with geographical distribution. In general, species from western regions of the U.S. produce oils containing large amounts of lesquerolic (14-hydroxy-*cis*-11-eicosenoic) acid, while those from the Southeast produce oils in which densipolic (12-hydroxy-*cis*-9,*cis*-15-octadecadienoic) acid is the major component. Small amounts of ricinoleic acid occur in oils from both groups.

All species of *Lesquerella* known to produce densipolic acid as a major component fall in the auriculate-leafed group of the genus and have a chromosome number N = 8 (3). Two members of the group produce lesquerolic acid, but one has N = 9 and the other N = 7. The one member of the group not included in earlier publications (1,2) is *Lesquerella auriculata*, which has N = 8. This species is native to Texas and Oklahoma, and its western origin suggests that its oil should contain lesquerolic acid. On the basis of the chromosome number and morphology, however, Barclay et al. (3) speculated that the oil should contain densipolic acid. Analysis of the oil now reveals an unusual triglyceride structure and a previously unknown fatty acid that combines structural properties from both lesquerolic and densipolic acids.

## METHODS

Oil (3.7g) was extracted from the ground seed and analyzed as previously described (2,4). IR absorption was measured for films of oils on sodium chloride disks or for 1% solutions in either carbon disulfide or carbon tetrachloride. Ethanolic solutions were examined in 1 cm cells in a Beckman DK-2A for absorption in the UV region from 205-360 nm. NMR spectra were obtained from deuteriochloroform solutions with a Varian HA-100 spectrometer. Methyl esters from free acids and monoglycerides were prepared by a $BF_3$-methanol procedure (5). In samples containing estolide linkages, the normal 5 min saponification step in this procedure was extended to 3-4 hr. A Packard Model 7401 gas chromatograph used for analyses of methyl esters was equipped with both a 4 ft x 1/4 in. glass column packed with 5% Apiezon L on Chromosorb W-DMCS and with a 12 ft x 1/4 in. glass column packed with 5% LAC-2-R 446 on the same support. Analyses on the two columns were run simultaneously at an oven temperature of 200 C. In quantitating gas liquid chromatography (GLC) results for hydroxy esters, a correction factor was used that was determined by comparing detector responses for methyl ricinoleate and methyl oleate.

Lipolytic products were analyzed by GLC in either a Hewlett-Packard 5750 or an F&M 810 instrument equipped with a 2 ft x 1/8 in. stainless steel column packed with 3% OV-1 on Gas-Chrom Q (Applied Science Lab., Inc., State College, Pa.) and temperature-programed from 150 to 400 C at 4 C/min.

Methyl esters were ozonized in dichloromethane solutions, and the resulting products were reduced with triphenylphosphine (6). GLC of the fragments formed was similar to that of methyl esters, except that oven temperatures were linearly programed from 70 to 200 C at 6 C/min.

Monohydroxy esters were isolated by preparative thin layer chromatography (TLC) on 1

[1]Presented in part at the AOCS Meeting, New Orleans, April 1970.

[2]ARS, USDA.

mm Silica Gel G layers with hexane-ether 70:30 as the developing solvent. The isolated hydroxy esters were further fractionated on TLC plates coated with a layer of Silica Gel G containing 20% silver nitrate. The developing solvent was benzene-chloroform-ether 50:50:20. The hydroxy esters were hydrogenated in ethanol with palladium on charcoal as the catalyst.

*L. auriculata* oil was fractionated on a chromatographic column consisting of 100 g of Adsorbosil CAB 14 (Applied Science Lab., Inc.) in a 2 cm diameter column with hexane-benzene and benzene-ether as the gradient elution solvents. For smaller samples the same separations were achieved by preparative TLC on Silica Gel G with hexane-ether 70:30 as the developing solvent.

Lipolysis of glycerides was accomplished with either pancreatic lipase (EC 3.1.1.3) or castor bean lipase (EC 3.1.1.3). With pancreatic lipase, the method of Luddy and coworkers (7) was followed, except that a Sonifier Cell Disruptor (Heat Systems Co., Melville, N.Y.) equipped with a microtip on a 0.5 in. diameter step horn was used at an output of 50 w for 3 min for the emulsification. The hydrolysis with castor bean lipase was done at room temperature at pH 4.3 for 4 hr (8).

Mixtures from pancreatic lipolysis were fractionated by chromatography on an 85 cm bed of Sephadex LH-20 in a Sephadex SR 25/100 column. Tetrahydrofuran was the eluent at a flow rate of 0.5 ml/min. The effluent was monitored by a Waters differential refractometer.

Mass spectra were obtained with a Nuclide 12-90 spectrometer having an inlet temperature of 225 C and an ionization potential of 70 v.

Molecular weights in $CHCl_3$ were determined by vapor pressure osmometry with a Mechrolab Model 301A osmometer. Trierucin and trivernolin, greater than 95% purity, served as standards.

## RESULTS AND DISCUSSION

### Structure of New Hydroxy Acid

Initial examination indicated that the oil (32.6%) of *L. auriculata* seed was grossly similar to other *Lesquerella* oils (1,2). The predominant features were the strong hydroxyl absorption (2.85 $\mu$) in the IR and the polar components shown by TLC analyses. The oil had no significant reaction with HBr and no maximum in the UV.

GLC of methyl esters derived from the oil (Table I) revealed an unknown component, which exhibited equivalent chain lengths (ECL) (9) of 21.2 from the Apiezon L column and

TABLE I

Composition (%) of Chromatographic Fractions and Lipolysates from *Lesquerella auriculata* Oil

| Methyl ester[a] | Total oil | Fraction I (12.0%) | | | | Fraction II (60.6%) | | | | Fraction III (27.4%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Monoglycerides | Free acids | Estolides | Total | Monoglycerides | Free acids | Estolides | Total | Monoglycerides | Free acids | Estolides |
| 14:0 | Trace | 0.7 | 0.7 | — | 0.8 | 0.1 | 0.1 | Trace | 0.2 | 0.3 | Trace | 0.4 | 0.7 |
| 16:0 | 3.8 | 10 | 3.5 | 11 | 11 | 3.3 | 3.4 | 14 | 2.1 | 0.9 | 5.9 | 6.8 | 2.3 |
| 16:1 | 1.4 | 4.6 | 9.6 | 1.8 | 5.6 | 0.9 | 4.2 | 0.6 | 1.7 | 0.3 | 3.5 | 0.2 | 1.5 |
| 18:0 | 5.4 | 8.6 | 1.6 | 12 | 3.1 | 4.5 | 1.5 | 20 | 0.6 | 1.0 | 3.5 | 10 | 0.8 |
| 18:1 | 27 | 37 | 52 | 44 | 22 | 27 | 58 | 47 | 5.8 | 19 | 60 | 10 | 1.9 |
| 18:2 | 3.0 | 5.1 | 12 | 4.2 | 1.2 | 2.3 | 7.6 | 2.4 | 0.7 | 1.7 | 5.6 | 0.5 | 0.2 |
| 18:3 | 6.9 | 9.2 | 17 | 7.8 | 4.9 | 7.4 | 19 | 6.4 | 1.3 | 5.0 | 18 | 1.5 | 0.8 |
| 20:1 | 2.8 | 2.7 | 0.2 | 7.8 | 0.6 | 2.1 | 0.3 | 5.0 | 0.2 | 0.7 | 0.2 | 0.9 | 0.8 |
| 22:1 | 0.7 | 3.2 | — | 10 | 0.1 | 0.6 | — | — | 1.4 | Trace | — | — | 0.9 |
| 18:1-OH | 5.3 | 6.5 | — | — | 20 | 5.6 | 1.4 | — | 12 | 4.6 | 3.3 | 2.0 | 5.2 |
| 18:2-OH | 2.1 | Trace | — | — | 0.2 | 1.7 | 0.3 | — | 3.0 | 4.2 | — | 3.8 | 3.8 |
| 20:1-OH | 9.8 | 2.2 | — | — | 6.6 | 9.9 | 0.8 | 0.9 | 13 | 14 | — | 12 | 19 |
| 20:2-OH | 32 | 7.1 | — | — | 21 | 33 | 3.7 | 3.5 | 58 | 49 | — | 48 | 62 |

[a]18:1-OH = ricinoleic, 18:2-OH = densipolic, 20:1-OH = lesquerolic and 20:2-OH = auricolic acids. Traces of a number of other fatty acids are present.

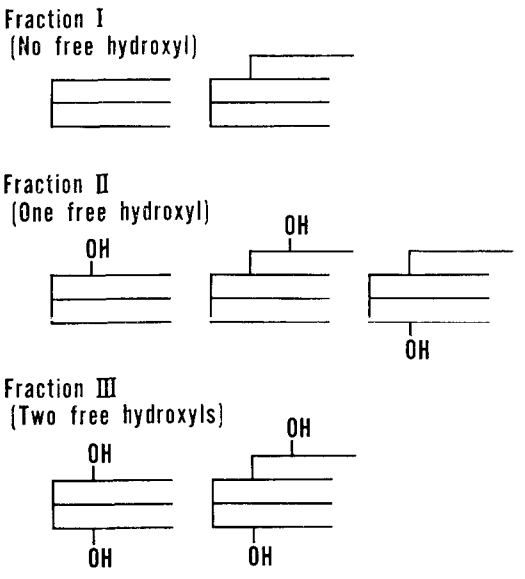


FIG. 1. Glyceride structures in *Lesquerella auriculata* oil.

27.2 from the LAC-2-R 446 column. Comparison of ECL's suggested that this component was a $C_{20}$ homolog of densipolic acid (12-hydroxy-*cis*-9,*cis*-15-octadecadienoic acid) (Apiezon L, ECL = 19.2; LAC-2-R 446, ECL = 25.2 [9,10]).

Preparative $AgNO_3$/TLC of the hydroxy ester fraction gave one band that included a mixture of the unknown component and methyl densipolate (95% and 5% by GLC). Failure to separate the unknown component from methyl densipolate strengthened the suggestion of structural similarity.

NMR analysis of the mixture gave results expected for a 20-carbon hydroxy ester with two double bonds. In addition, the NMR spectrum showed a well defined triplet at 9.05$\tau$, resulting from the effect of an $\omega$-3 double bond on the protons of the terminal methyl group (10).

Ozonolysis-GLC (6) showed only two fragments: $C_{11}$ aldehyde-ester and $C_9$ aldehyde-ester in a molar ratio of 20:1, which is in close agreement with the molar ratio of the unknown ester to methyl densipolate in the original mixture (19:1). These ozonolysis products and the NMR results show the double bonds in the 11,12 and 17,18 positions.

Mass spectroscopy of the hydrogenated esters defined the location of the hydroxyl group (11). Prominent peaks representing ions with m/e of 257, 228 and 225 showed the hydroxyl to be on the 14th carbon atom of the major component. The corresponding ions, 28 mass units less, were found for the small amount of hydrogenated methyl densipolate present.

The absence of IR absorption in the 10-11 $\mu$m (*trans* double bond) region for the unsaturated unknown ester and the results described above define its structure as the methyl ester of 14-hydroxy-*cis*-11,*cis*-17-eicosadienoic acid. We call this compound auricolic acid.

#### Glycerides of *Lesquerella auriculata*

Several seed oils containing hydroxy acids are known to have more than three fatty acids per glycerol molecule (12). That is, a hydroxy acid esterified to glycerol has its hydroxyl group acylated in turn. An unknown component observed in the TLC analysis of methyl esters prepared by the fast $BF_3$/methanol procedure (5) encouraged us to look for the estolide structure in *L. auriculata* oil. The unknown component migrated just above the normal hydroxy esters. When the esters were saponified for 3 hr and reesterified with $BF_3$/methanol, this component was no longer present. The unusual component, when isolated by preparative TLC, gave peaks with retention characteristics similar to those of diglycerides when subjected to GLC analysis on an OV-1 column.

The total oil was separated chromatographically into three major fractions: fraction I (12.0%), glycerides with the mobility of normal triglycerides; fraction II (60.6%), glycerides

TABLE II

Tetraglycerides in *Lesquerella auriculata* Oil Fractions[a]

| Fraction | Proportion of oil,% | Tetraglycerides in fraction, % | | | | |
|---|---|---|---|---|---|---|
| | | Pancreatic lipolysis | Castor lipolysis | Vapor phase osmometry | GLC of esters | NMR |
| I | 12.0 | 79 | 70 | 63 | 64 | 50 |
| II | 60.6 | 94 | 86 | 72 | 100 | 100 |
| III | 27.4 | 65 | 66 | 59 | 54 | 59 |
| Whole oil (calculated) | | 84 | 79 | 67 | 83 | 83 |

[a]Assuming tetraglyceride structures shown in Figure 1 and no penta- or larger glycerides; GLC = gas liquid chromatography.

with one free hydroxyl group; and fraction III (27.4%), glycerides with two free hydroxyl groups. Our experimental evidence supports the structures given in Figure 1 for the glycerides in these fractions.

Fraction I had no hydroxyl absorption in the IR, but upon analysis of the esters from this fraction by GLC, we found 16% hydroxy esters. Fractions II and III showed 50 and 71% hydroxy esters, respectively, both greater than would be expected for monohydroxy and dihydroxy triglycerides. These results suggested that *L. auriculata* seed oil contained triglycerides with at least four acids (tetraglycerides). Phillips and Smith (12) showed that triglycerides and tetraglycerides of *Monnina emarginata* oil could be separated by analytical TLC when benzene was the developing solvent. Using this procedure with *M. emarginata* seed oil as a standard, we found that fraction I had only two components, one migrating as triglycerides and the other as estolide glycerides. In a separate comparison with ergot oil, demonstrated by Morris and Hall (13) to contain polyestolide glycerides, the two components of fraction I migrated (TLC) like the two least polar ones from ergot, presumably the tri- and tetraglycerides.

NMR of the fractions of *L. auriculata* oil also indicated that tetraglycerides were present. When we compared the area of the α-glycerol protons (5.80τ) to the area of terminal methyl protons (9.05τ), we found ca. 3.5 acyl groups per molecule (50% tetraglycerides) in fraction I, 4.0 acyl groups per molecule (100% tetraglycerides) in fraction II and 3.6 acyl groups per molecule (59% tetraglycerides) in fraction III (Table II). Calculating the percentage of tetraglycerides from the amount of hydroxy acids in each fraction (neglecting molecular weight differences and assuming the tetraglyceride structures in Fig. 1) gave the following estimations: fraction I should contain ca. 64%; fraction II, ca. 100%; and fraction III, ca. 54%. Mean molecular weights of fractions I-III, as determined by vapor-phase osmometry (trierucin [mol wt 1054] and trivernolin [mol wt 927] as standards), were 1077, 1160 and 1149, respectively. On the basis of mean molecular weights of the acids present, molecular weights for possible tri- and tetraglycerides were calculated. Comparison of the calculated and determined results indicates that fraction I contains 63% tetraglyceride; fraction II, 72%; and fraction III, 59%.

Pancreatic lipase was used to locate the position on the glycerol molecule of the estolide structures. The hydrolysis produced estolides along with other fatty acids from the 1 and 3 positions. Since procedures generally used to fractionate lipolysates were inadequate, e.g., to separate methyl auricolylauricolate from diglycerides, we turned to chromatography on Sephadex LH-20. The lipolytic products from *L. auriculata* oil after reaction with diazomethane were separated as shown in Figure 2. Peaks were identified by either TLC, GLC or both. All the estolide methyl esters, with or without a free hydroxyl group, were eluted together well before the single-acid methyl esters, which also migrated as a unit. The monoglycerides were eluted after the single-acid methyl esters, polarity apparently overriding molecular size in affecting the speed with which the former traverse the column. The component following the unreacted glycerides consisted of diglycerides, and small amounts of diglycerides also accompanied the estolide methyl esters. Presumably, the faster moving component was the estolide diglycerides, and those diglycerides that migrated with the estolide esters were normal diglycerides. Most of the normal diglycerides were removed from the estolide esters by preparative TLC.

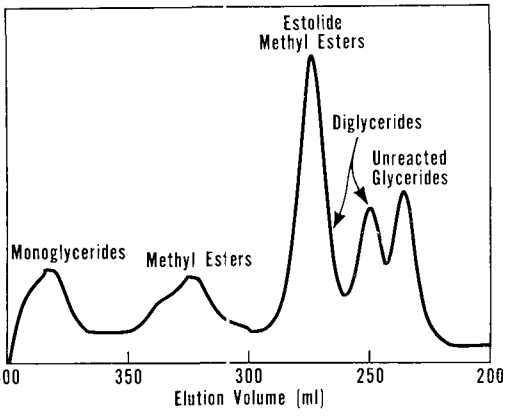


FIG. 2. Chromatography of the lipolysate of *L. auriculata* seed oil on Sephadex LH-20 with tetrahydrofuran as the eluting solvent.

Analysis of the methyl esters from the free acids, monoglycerides and saponified estolides from fractions I-III gave a general picture of the acyl distribution in the total oil (Table I). Since fraction I had no free hydroxyl groups, there should be no free hydroxyl groups in the lipolytic products of this fraction, and none were found. Neither were hydroxy methyl esters obtained from the monoglycerides. Therefore all the estolides were assumed to be on the outer glycerol positions. Fifty per cent (found 48%) of the methyl esters from cleavage of the estolides should be hydroxy esters. If we consider only the nonhydroxy acids, the lipo-

TABLE III

Estolides from Fraction I by Total Chain Length

| Chain length | Amount, wt% | |
|---|---|---|
| | Calculated[a] | Found (GLC)[b] |
| $C_{32}$ | 1.0 | 1.8 |
| $C_{34}$ | 17.2 | 26.2 |
| $C_{36}$ | 46.6 | 23.4 |
| $C_{38}$ | 34.7 | 47.6 |
| $C_{40}$ | 0.6 | 1.0 |
| Average chain length | 36.4 | 36.4 |

[a]For random combination of acids in the fraction.
[b]GLC = gas liquid chromatography.

lytic data appear to be typical for cruciferous oils (14,15). That is, the $C_{16}$ and $C_{18}$ unsaturated acids are found predominantly in the β-position of glycerol, and the saturated and long chain acyl groups in the outer positions.

The free acids were isolated from a lipolysate of fraction I by preparative TLC. They were methylated with diazomethane and analyzed by GLC. In addition to normal methyl esters, several components had chain lengths that corresponded to estolides containing from 32 to 42 carbon atoms. Since lipid species up to $C_{66}$ without free hydroxyl groups would have been detected by our GLC procedures, fraction I evidently contains no estolides with three fatty acids. In the absence of these, presumably larger estolides are also absent. This result supports the TLC evidence (comparison with *Monnina* and ergot oils) that fraction I contains no molecules larger than tetraglycerides.

When the estolide distribution (Table III) was compared with that calculated on the basis of a random distribution of the normal and hydroxy acids (Table I), a definite preference for combination of the longer normal acids with the longer hydroxy acids became apparent. The percentage of estolides in the free acids was 53 by GLC. If fraction I were all tetraglycerides, 67% would be present. Therefore this analysis indicates that 79% of fraction I was composed of tetraglycerides.

The above TLC and GLC methods are not directly applicable to fractions II and III because of the free hydroxyl groups. While polyestolides may possibly occur, the calculations for these fractions given in Table II are, for simplicity, based on the assumption that only tri- and tetraglycerides are present.

Assumption that relative refractometer responses for tri- and tetraglycerides were the same for all fractions enabled calculation of the percentages of the latter component in fractions II and III. These percentages (Table II) were calculated from the 79% found in fraction I by GLC and by comparison of the Sephadex column elution curves for the lipolysates of the three fractions.

Fractions I-III were also hydrolyzed by castor bean lipase so that another measure of the amount of tetraglyceride present could be made. This enzyme, like pancreatic lipase, hydrolyzes glycerol esters while leaving other esters generally intact. However unlike pancreatic lipase, castor lipase also attacks the β-carbon esters under the conditions employed. GLC of the lipolysate, after conversion of the free acids to methyl esters with diazomethane, revealed the relative amounts of estolide ester and single-acid esters. A theoretical tetraglyceride should result in 50% single-acid esters and 50% estolide esters by weight. Fraction I showed 35% estolide esters or 70% tetraglycerides; fraction II, 43% estolides or 86% tetraglycerides; and fraction III, 33% estolides or 66% tetraglycerides. These results are compared in Table II with those from pancreatic lipase hydrolysis and other methods.

ACKNOWLEDGMENTS

L.W. Tjarks performed NMR analyses; W.K. Rohwedder, mass spectroscopy; and C.W. McGrew, vapor-phase osmometry. C.F. Krewson (deceased), Eastern Regional Research Laboratory, Philadelphia, Pa., provided trivernolin, and H. Grynberg, Institute of General Chemistry, Warsaw, Poland, contributed trierucin.

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# Saturated and Monounsaturated Long Chain Hydrocarbon Profiles of Lipids from Orange, Grapefruit, Mandarin and Lemon Juice Sacs[1]

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## ABSTRACT

Saturated and monounsaturated long chain hydrocarbons were determined in four citrus fruit species—orange, grapefruit, mandarin and lemon. All species manifested their own intrinsic profiles. In the saturated fraction, iso and anteiso hydrocarbons comprised between 45 and 58% of the total hydrocarbons, while in the monounsaturated fraction the range was 2 to 22%. As a general rule, odd-numbered, iso hydrocarbons were always found at percentages higher than their anteiso homologs.

## INTRODUCTION

There is a paucity of information on the lipid composition of citrus fruits. To attest to this statement is the fact that two comprehensive reviews (1,2) on the chemical constituents of citrus fruits have devoted limited space to the subject of lipids. Studies which have been conducted have dealt mainly with general lipid compositional changes of commercially processed citrus juice during storage (3-5).

Paraffinic long chain hydrocarbons have been analyzed qualitatively in cold-pressed orange oil (6), but no information exists on the quantitative composition of these hydrocarbons within the juice sacs. The purpose of this study was to enhance our knowledge of citrus lipids through a comparative examination of the long chain hydrocarbons of orange, grapefruit, mandarin and lemon juice sacs. In this study hydrocarbon profiles were determined for the sweet orange (*C. sinensis* var. Lue Gim Gong), grapefruit (*C. paradisi* var. Thompson), mandarin (*C. reticulata* var. Dancy) and lemon (*C. limon* var. Eureka).

## MATERIALS AND METHODS

### Fruit Collection

The Lue Gim Gong orange and Dancy mandarin were obtained from Whitmore Experimental Farm (Plant Science Res. Div., USDA, Orlando, Fla.). Thompson grapefruit was obtained from Adams Citrus Nursery, Haines City, Fla., and Eureka lemon was obtained from a local market. All citrus fruits were at a legal stage of maturity according to regulations of the Departments of Agriculture of Florida and the U.S. (7). Fruit were harvested from trees according to the procedures of Sites and Reitz (8-10).

### Isolation of Juice Sac Lipids

Lipids were extracted from juice sacs and purified according to methods described previously (11-13). Quadruplicate extractions were run on a single batch of fruit from each species.

### Column and Thin Layer Chromatography

The total purified lipid (ca. 150-200 mg) was dissolved in $CHCl_3$ and percolated onto an 0.9 x 30 cm column containing 10 g Merck, 70-325 mesh silica gel (Brinkmann Instruments, Westbury, N.Y.). The neutral lipids that contained the long chain hydrocarbons were eluted with 200 ml $CHCl_3$.

The neutral lipid fraction was concentrated to a small volume and streaked on precoated Silica Gel G plates (20 x 20 cm, 500$\mu$, Analtech, Inc., Wilmington, Del.). These prewashed, nonactiviated plates were developed at room temperature in hexane-ethyl ether 90:10. The band corresponding to the hydrocarbon fraction was scraped from the plate and eluted with ethyl ether. This fraction was, in turn, restreaked on a silver nitrate-impregnated Silica Gel G plate (12) and developed in 2% ethyl ether in petroleum ether (30-60 C boiling range). This solvent system separated the saturated and monounsaturated hydrocarbons. After elution of these two fractions with ethyl ether, the monounsaturated fraction was dissolved in 1 ml hexane and hydrogenated under 60 lb/in.$^2$ at room temperature for 1 hr with 10 mg 10% Pd-C catalyst in a Parr apparatus.

### Gas Liquid Chromatography and Quantitation

Gas chromatographic analysis of the long chain hydrocarbons was determined with an F&M Model 5750 gas chromatograph equipped with a flame ionization detector. Hydrocarbons

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

[2]One of the laboratories of the S.E. Market. Nutr. Res. Div., ARS, USDA.

TABLE I

Saturated Long Chain Hydrocarbon Profiles of Citrus Juice Sacs (wt%)

| Carbon no. | Orange | | | Grapefruit | | | Mandarin | | | Lemon | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N[a] | I[b] | AI[c] | N | I | AI | N | I | AI | N | I | AI |
| 20 | 0.3[d] | T[e] | T | 0.2 | T | T | 0.6 | T | T | 0.1 | T | T |
| 21 | 0.9 | 0.1 | T | 0.4 | T | T | 0.5 | T | T | 0.5 | 0.2 | T |
| 22 | 2.8 | 0.4 | 0.1 | 1.5 | 0.2 | 0.1 | 1.7 | T | T | 1.3 | 0.6 | 0.4 |
| 23 | 17.4 | 11.1 | T | 10.6 | 12.7 | 0.1 | 12.4 | 3.5 | T | 6.9 | 6.6 | 0.4 |
| 24 | 6.0 | 2.3 | 7.4 | 4.9 | 3.4 | 7.4 | 6.6 | 1.7 | 3.0 | 4.7 | 1.7 | 4.2 |
| 25 | 14.8 | 11.3 | 1.9 | 12.5 | 14.6 | 2.4 | 19.4 | 9.7 | 1.4 | 14.5 | 10.0 | 1.2 |
| 26 | 1.9 | 0.9 | 6.2 | 2.3 | 1.2 | 6.3 | 4.7 | 1.0 | 5.4 | 3.7 | 1.9 | 5.8 |
| 27 | 2.5 | 3.5 | 0.6 | 5.7 | 4.2 | 0.5 | 10.0 | 2.9 | 0.7 | 10.1 | 4.3 | 1.1 |
| 28 | 0.4 | 0.3 | 1.8 | 1.0 | 0.5 | 1.6 | 2.6 | 0.5 | 1.8 | 2.7 | 0.7 | 2.4 |
| 29 | 0.9 | 0.9 | 0.2 | 1.7 | 1.2 | 0.1 | 4.0 | 1.0 | 0.3 | 6.3 | 2.7 | 0.4 |
| 30 | 0.3 | 0.1 | 0.6 | 0.4 | 0.2 | 0.5 | 0.9 | 0.3 | 0.6 | 0.9 | 0.3 | 0.9 |
| 31 | 0.8 | 0.3 | 0.1 | 0.5 | 0.3 | 0.1 | 1.1 | 0.2 | 0.1 | 1.4 | 0.2 | 0.1 |
| 32 | 0.2 | T | 0.2 | 0.1 | T | 0.1 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 | 0.1 |
| 33 | 0.2 | 0.1 | T | 0.2 | 0.1 | T | 0.4 | T | T | 0.2 | T | T |
| 34 | 0.1 | T | T | 0.1 | T | T | 0.1 | T | T | 0.1 | T | T |
| 35 | 0.1 | T | — | 0.1 | T | — | 0.2 | T | T | 0.1 | T | T |
| 36 | T | — | — | T | — | — | T | — | — | T | — | — |
| 37 | T | — | — | T | — | — | 0.1 | — | — | T | — | — |
| 38 | T | — | — | T | — | — | T | — | — | T | — | — |

[a]Normal chain hydrocarbon.
[b]Iso, general structure: $CH_3$-CH($CH_3$)-($CH_2$)$_x$-$CH_3$.
[c]Anteiso, general structure: $CH_3$-$CH_2$-CH($CH_3$)-($CH_2$)$_x$-$CH_3$.
[d]Mean of four to six determinations.
[e]Trace, less than0 0.1%.

were determined on a glass column (3.05 m long and 4 mm ID) coated with 3% SP-1000 (Supelco, Inc., Bellefonte, Pa.) on 100-120 mesh, Gas Chrom Q (Applied Science, State College, Pa.). The injection port and detector were at 275 C and the helium flow rate was 80 ml/min. The sample was injected on column at 165 C and programed for 5 min at 4 C/min, then 2 C/min for 24 min, then 3 C/min, up to 270 C, and finally held isothermally at this upper limit until the $C_{38}$ hydrocarbon eluted. A normal, iso and anteiso hydrocarbon standard from $C_{16}$ to $C_{36}$ was prepared as described previously (14). Long chain hydrocarbons were determined by comparative gas liquid chromatographic retention times against the above standard and by way of plots of retention times vs. equivalent carbon numbers. Mass spectra of citrus-branched hydrocarbons were determined previously by this laboratory (6). Quantitative results were obtained by triangulation measurement techniques and also by measurement of peak areas with the aid of a disc integrator.

## RESULTS AND DISCUSSION

The total hydrocarbon fraction represents 1-3% of the total extractable lipid from citrus juice sacs. Of the total lipid, saturated long chain hydrocarbons comprise: orange, 1.6%; grapefruit, 1.8%; mandarin, 1.2%; and lemon, 1.4%. Monounsaturated hydrocarbons represent: orange, 0.1%; grapefruit, 0.3%; mandarin 0.4%; and lemon, 0.1%. The remaining hydrocarbon fraction, generally comprising less than 1%, is a complex mixture of polyunsaturated hydrocarbons and other undefined components.

Mass spectral studies (6) have shown that citrus synthesizes linear, iso and anteiso hydrocarbons. To date, no definite evidence has been presented for the presence of multibranched or biterminally branched hydrocarbons, or both, in citrus. With sensitive gas chromatographic techniques, long chain hydrocarbons were detected in juice sacs up to the region $C_{45}$ to $C_{50}$. In this study quantitative measurements were conducted on the dominant hydrocarbon region, viz., between $C_{20}$ and $C_{38}$. Hydrocarbons above $C_{38}$ were never found higher than trace percentages and therefore were not tabulated in this study.

In Table I are shown the saturated hydrocarbon profiles of the four citrus species. For normal chain hydrocarbons (N column), the orange, grapefruit and mandarin show that the two most prominent members of this group are $C_{23}$ and $C_{25}$. For the Lue Gim Gong orange, $C_{23}$ is found at the highest percentage, while $C_{25}$ is most prominent in grapefruit, mandarin and lemon. This higher percentage of $C_{23}$ over

TABLE II

Monounsaturated Long Chain Hydrocarbon Profiles of Citrus Juice Sacs (wt%)

| Carbon no. | Orange | | | Grapefruit | | | Mandarin | | | Lemon | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N[a] | I[b] | AI[c] | N | I | AI | N | I | AI | N | I | AI |
| 20 | 0.8[d] | T[e] | 0.2 | 1.1 | T | 0.3 | 0.5 | T | 0.1 | 3.6 | T | 0.7 |
| 21 | 0.3 | T | 0.2 | 0.7 | T | 0.1 | 0.2 | T | 0.1 | 0.6 | T | 0.5 |
| 22 | 1.2 | 0.1 | 0.4 | 0.9 | T | 0.1 | 0.6 | T | 0.1 | 2.7 | 0.1 | 0.6 |
| 23 | 14.6 | 1.9 | 0.5 | 1.5 | 0.2 | 0.1 | 0.6 | T | T | 2.3 | 0.5 | 0.3 |
| 24 | 4.5 | 0.7 | 1.7 | 0.9 | 0.1 | 0.7 | 0.6 | T | 0.1 | 2.3 | 0.2 | 1.8 |
| 25 | 28.1 | 4.5 | 0.9 | 7.5 | 0.8 | 0.2 | 4.1 | 0.3 | 0.1 | 6.8 | 1.2 | 0.5 |
| 26 | 3.5 | 0.3 | 2.5 | 0.9 | T | 1.1 | 0.8 | T | 0.3 | 1.4 | 0.2 | 3.5 |
| 27 | 16.4 | 2.2 | 0.5 | 16.2 | 0.3 | 0.2 | 6.3 | 0.2 | 0.1 | 10.6 | 2.4 | 0.6 |
| 28 | 1.3 | 0.2 | 1.2 | 2.4 | T | 0.4 | 1.3 | T | 0.1 | 1.6 | 0.1 | 2.8 |
| 29 | 3.7 | 0.8 | 0.2 | 51.7 | 0.1 | 0.1 | 35.9 | 0.2 | 0.1 | 32.2 | 1.4 | 0.3 |
| 30 | 0.6 | 0.1 | 0.4 | 1.5 | T | 0.1 | 3.1 | T | T | 1.9 | 0.2 | 1.6 |
| 31 | 3.2 | 0.5 | 0.2 | 9.5 | 0.1 | T | 41.0 | 0.1 | T | 10.7 | 1.6 | 0.2 |
| 32 | 0.3 | 0.1 | 0.4 | T | — | — | 0.8 | — | T | 0.3 | 0.2 | 0.8 |
| 33 | 0.3 | 0.1 | 0.1 | 0.2 | — | T | 2.3 | T | T | 0.5 | 0.1 | 0.1 |
| 34 | 0.1 | T | 0.2 | T | — | — | T | — | — | T | — | — |
| 35 | T | T | T | T | — | — | T | — | — | T | — | — |

[a]Normal chain monoene.

[b]Iso, $CH_3$-CH($CH_3$)-$(CH_2)_x$-CH=CH-$(CH_2)_x$-$CH_3$, double bond location unknown.

[c]Anteiso, $CH_3$-$CH_2$-CH($CH_3$)-$(CH_2)_x$-CH=CH-$(CH_2)_x$-$CH_3$, double bond location unknown.

[d]Mean of four to six determinations.

[e]Trace, less than 0.1%.

$C_{25}$ has been shown previously for six other varieties of the sweet orange (14). The percentage difference of $C_{23}$ over $C_{25}$ generally ranges between 1 and 4%. The important point is that oranges, regardless of the variety and maturing season, consistently show a higher percentage of $C_{23}$ than of $C_{25}$. On the other hand, the higher percentage of $C_{25}$ over $C_{23}$ observed for Thompson grapefruit also appears characteristic to the grapefruit group as Foster, Redblush and Marsh Seedless (15); and Duncan and Burgundy grapefruit (unpublished data) show a similarly higher $C_{25}$ content over $C_{23}$. Whether the higher $C_{25}$ to $C_{23}$ content shown for the mandarin is intrinsic to this species is not known at present, as an insufficient number of mandarin varieties have been investigated. The lemon appears distinguishable from the other three citrus by the relatively low percentage of $C_{23}$ and by the noticeably higher percentage of $C_{29}$. Examination of three other lemon varieties, viz., Lisbon, Malta and Kusner (unpublished data) shows profiles similar to the Eureka lemon profile, and all lemons consistently show a higher $C_{27}$ percentage than $C_{23}$. Because of this latter characteristic, lemons are readily distinguished from the orange, grapefruit and mandarin species.

Iso hydrocarbons (column I) show profiles relatively similar to the normal group. The two major hydrocarbons are $C_{23}$ and $C_{25}$, with $C_{25}$ generally the most prominent in this branched group. For both the normal and iso groups, odd-numbered hydrocarbons predominate. Anteiso hydrocarbons (column AI) show that $C_{24}$ and $C_{26}$ predominate in this group. In the anteiso group even-numbered carbon chains predominate.

Scrutiny of Table I reveals some general patterns for branched hydrocarbons. For even-numbered hydrocarbons the percentage of anteiso structures is generally greater than their iso homologs. Conversely, odd-numbered hydrocarbons show an opposite relationship, i.e., iso structures are present at a larger percentage than their anteiso counterparts.

In Table II the percentage composition of the monounsaturated hydrocarbon fraction is shown. For the linear monounsaturated group, all profiles of the four species appear to differ vastly from one another. For the orange, $C_{23}$, $C_{25}$ and $C_{27}$ are most prominent, with $C_{25}$ predominating. The prominence of $C_{25}$ as the major monounsaturated hydrocarbon has also been shown for three other sweet oranges varieties (16) and thus, a posteriori, appears characteristic of the orange family. For Thompson grapefruit, $C_{25}$, $C_{27}$, $C_{29}$ and $C_{31}$ are most prominent, with $C_{29}$ comprising 50% of the total monounsaturated group. The prominence of $C_{29}$ has been shown for several varieties of grapefruit (15) and appears intrinsic to this citrus species. The Dancy mandarin shows $C_{29}$ and $C_{31}$ as dominant; however insufficient data is available to speculate as to whether this profile is typical of mandarins.

TABLE III

Isomeric Percentage Composition of Saturated and Monounsaturated Hydrocarbons

| | Saturated | | | | | | Monounsaturated | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Odd | | | Even | | | Odd | | | Even | | |
| Species | N | I | AI | N | I | AI | N | I | AI | N | I | AI |
| Orange | 37.6 | 27.3 | 2.8 | 12.0 | 4.0 | 16.3 | 66.6 | 10.0 | 2.6 | 12.3 | 1.5 | 7.0 |
| Grapefruit | 31.7 | 33.1 | 3.2 | 10.5 | 5.5 | 16.0 | 87.3 | 1.5 | 0.7 | 7.7 | 0.1 | 2.7 |
| Mandarin | 48.1 | 17.3 | 2.5 | 17.5 | 3.6 | 11.0 | 90.4 | 0.8 | 0.4 | 7.7 | 0.0 | 0.7 |
| Lemon | 40.0 | 24.0 | 3.2 | 13.7 | 5.3 | 13.8 | 63.7 | 7.2 | 2.5 | 13.8 | 1.0 | 11.8 |

The Eureka lemon shows $C_{29}$ as the most dominant alkene.

The monounsaturated branched hydrocarbons (iso and anteiso) reveal patterns similar to the saturated branched group. Information gleaned from this table reveals that even-numbered, anteiso monoenes are always found at percentages greater than their iso homologs. With few exceptions, normal monoenes are found at higher percentages than either of their iso or anteiso counterparts. In composite form the total percentages for the three isomeric structures for both the saturated and monounsaturated hydrocarbons are shown in Table III. In all citrus the isomer accumulated to the least extent in the saturated group is the odd-numbered, anteiso alkane, while even-numbered, iso alkenes accumulate least in the alkene fraction.

The hydrocarbon profiles reported in this paper for orange, grapefruit and lemon are each representative of their species. Examination of the saturated long chain hydrocarbon composition of 24 citrus varieties show that the property most useful in differentiating citrus is the ratio between $C_{23}$ and $C_{25}$. Table IV shows the $C_{23}/C_{25}$ ratio range for 14 varieties of orange, six varieties of grapefruit, four varieties of lemon and two varieties of mandarin. For normal hydrocarbons, all oranges show a higher percentage of 23 than 25 and therefore manifest ratio values greater than one. Grapefruit, lemons and mandarins show more 25 than 23, with the concomitant result that all values are below one. Each species shows a characteristic range with apparently no overlap with other species. The iso 23:25 ratios for the four species also show characteristic ranges. However there appears to be some overlap of the lemon values with grapefruit and mandarins. There are other hydrocarbon relationships among the various citrus species, but they are not as noticeable as the 23:25 ratio.

The biosynthesis of long chain hydrocarbons in higher plants appears to proceed by way of an elongation-decarboxylation pathway employing fatty acids as priming units (17,18). Branched acids are formed by employing isobutyrate, isovalerate and 2-methylbutyrate as initial primers. These priming units are derived from the amino acids valine, leucine and isoleucine (19). Isoleucine functions as the priming unit for anteiso, odd-numbered fatty acids, while leucine and valine function as units for iso, even-numbered and odd-numbered fatty acids. For anteiso, even-numbered fatty acids to occur, 3-methylvalerate would have to be present as a priming unit or the plant would have to possess enzymes capable of α-oxidation of anteiso, odd-numbered fatty acids, or both.

The enzymic synthesis of long chain hydrocarbons in citrus has not been investigated adequately. Investigations have shown that citrus synthesizes a multitude of saturated and unsaturated, branched chain fatty acids (11,12). Iso and anteiso, odd-numbered and iso, even-numbered fatty acids are formed in citrus; however no anteiso, even-numbered fatty acid has ever been detected (12). According to the elongation-decarboxylation mechanism, iso. even-numbered fatty acids would produce iso, odd-numbered hydrocarbons, and anteiso, odd-numbered hydro-

TABLE IV

The Normal and Iso 23:25 Ratio of the Saturated Hydrocarbon Fraction in Four Citrus Species

| | 23:25 Ratio range | | | |
|---|---|---|---|---|
| Hydrocarbon | Oranges | Grapefruit | Mandarins | Lemons |
| Linear | 1.05-1.34 | 0.85-0.94 | 0.62-0.69 | 0.30-0.47 |
| Iso | 0.96-1.34 | 0.52-0.86 | 0.37-0.54 | 0.43-0.67 |

carbons. If the elongation-decarboxylation mechanism is solely operative in citrus, no anteiso, odd-numbered hydrocarbon would be formed, because no anteiso, even-numbered fatty acid is detected in citrus. Perusal of Table I and II reveals the presence of anteiso, odd-numbered hydrocarbons although at low percentages relative to the other branched hydrocarbons. Because of the inexplicable occurrence of this type paraffin, the authors believe, a posteriori, that an additional hydrocarbon synthesizing mechanism may also be occurring in citrus fruit, and this may be the head-to-head condensation mechanism postulated by Kaneda (20).

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# Possible Mechanisms of Autoxidative Rancidity[1]

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## ABSTRACT

Rancidity appears as a very complex phenomenon if we consider the numerous reaction products that have been identified. Based on gas liquid chromatographic analysis of the aldehydes, alcohols, alkyl formates and hydrocarbons produced by the autoxidation of oleic acid and nonanal, an explanation of some possible reaction pathways is presented. Rancidity is described as a result of a succession of reactions, some of which are free radical in form, initiated by oleic monohydroperoxides. These yield aldehydes that are autoxidizable and also yield, by specific recurrent reactions, the wide variety of products encountered that are offensive in taste and odor.


[1]Presented at the AOCS-ISF World Congress, Chicago, September 1970.

## INTRODUCTION

The work of many scientists has led to the well accepted conclusion that autoxidation is mainly a chain reaction, and that during the transfer of energy from one excited molecule to another, oxygen is tied up as peroxidic free radicals (1,2).

Farmer and coworkers (3,4) have defined more precisely the sites of oxygen fixation in the fatty acid molecules, and many others have studied the processes of subsequent evolutions of the peroxides (5,6).

Surprisingly, very few attempts have been made to present an overall view of the entire phenomenon that would explain the wide variety of compounds encountered in rancid fats, or that arise during the autoxidation of simpler molecules (7).

At the French Institute for Research on Fats and Oils (ITERG) we tried to obtain new information about the mechanisms of fat

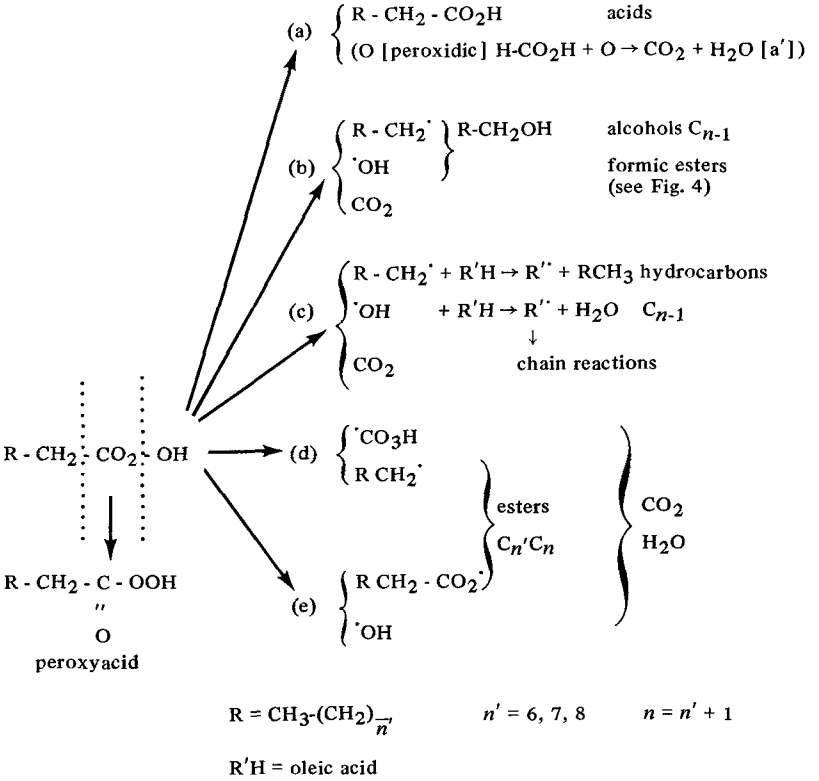


FIG. 1. Possible decomposition of the first hydroperoxidic products resulting from autoxidation of the aldehydic breakdown products of oleic hydroperoxides.

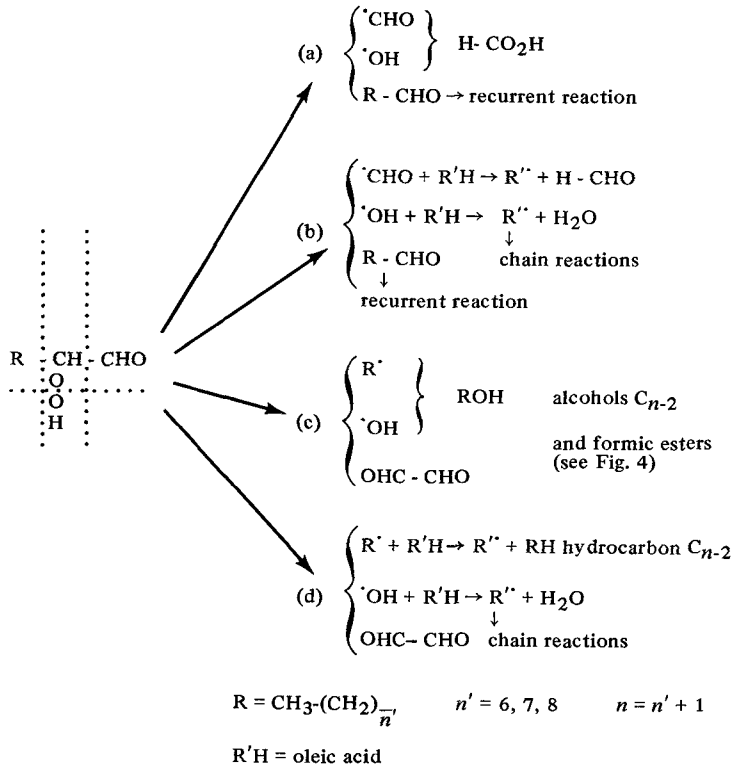


FIG. 2. Possible decomposition of the second hydroperoxide resulting from autoxidation of the aldehydic breakdown products of oleic hydroperoxides.

autoxidation (8). In order to obtain more reliable results we studied nearly pure fatty acids. Because saturated aldehydes with 8-11 carbon atoms can be expected to result from scission or further changes of the primary oleic hydroperoxides, the autoxidation of nonanal has been studied also.

## EXPERIMENTAL PROCEDURES

### Autoxidation

Autoxidation was performed at temperatures varying between ambient and 40 C. To accelerate the reaction, the autoxidizable materials were dispersed as thin film on glass wool fibers by soaking wads of glass wool in oleic acid (>98% pure) or nonanal (99% pure) and removing the excess material by centrifugation. The impregnated glass wool was placed into U-tubes, and purified air at constant temperature was blown through the porous mass. The air was passed through a dry ice trap at -60 C to condense the volatile autoxidation products, and then bubbled through a solution of aque-

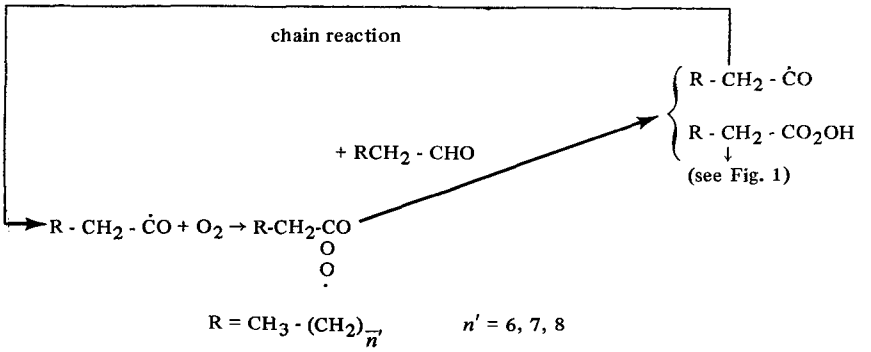


FIG. 3. Autoxidation of the aldehyde function.

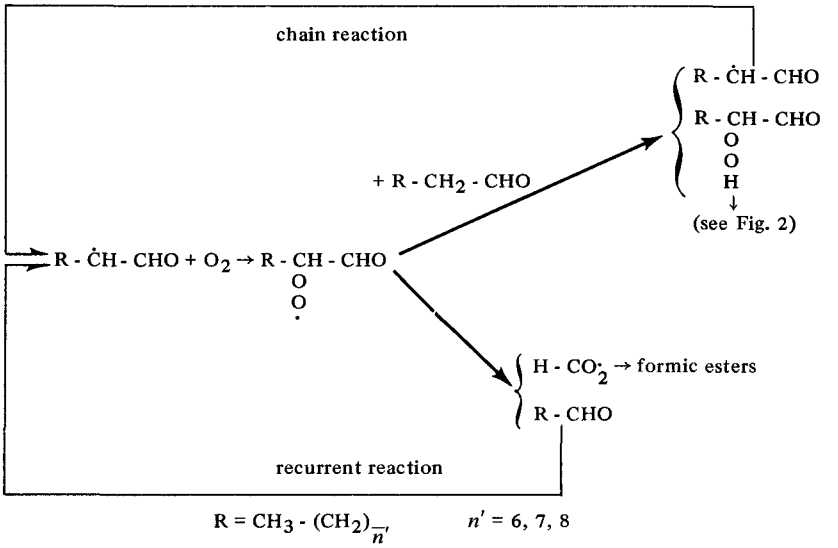


FIG. 4. Possible autoxidation of aldehyde on the α-carbon with breakdown leading to a recurrent reaction.

ous barium hydroxide to detect and measure carbon dioxide. The procedures and apparatus have been described in detail by Loury and Lechartier (9).

### Analytical Methods

The volatile compounds resulting from the autoxidation were fractionated and identified by various chromatographic techniques that have been described in detail earlier (10-12).

## RESULTS

The presence of carbon dioxide in the gas stream coming out of the dry ice trap was evident from the fact that the barium hydroxide solution became cloudy very quickly and that a precipate identified as barium carbonate soon formed in the solution.

Two distinct phases were present in the dry ice trap: an upper organic layer with a strong odor and a lower aqueous layer which exhibited an acidic reaction.

Besides water, which was an important product of the autoxidation, the aqueous layer contained formic, acetic and propionic acids. The organic layer contained series of saturated aliphatic aldehydes, alcohols, formate esters and hydrocarbons listed in Table I.

## DISCUSSION

Starting with autoxidized oleic acid, it can be anticipated that the 8-, 9-, 10- and 11-hydroperoxides predicted by Farmer could undergo scission of the C-C bonds adjacent to the hydroperoxide group to yield saturated aldehydes with 8-10 carbon atoms. Prior to our studies, the origin of shorter chain aldehydes and other aliphatic compounds could not be readily explained.

A comparison of the products obtained from autoxidized oleic acid and nonanal, in Table I, shows that the parent substances are the medium chain length aldehydes resulting from the scission of the hydroperoxides, and that the numerous reaction products offensive in taste and odor are derived from their degradation.

It is well known that aldehydes autoxidize in the usual way to yield peroxides and finally the corresponding acids (reaction a, Fig. 1). The peroxidic oxygen released may react with formic acid and decompose it into carbon dioxide and water (reaction a', Fig. 1).

If we consider the free radicals resulting from the breakdown of peracids, alcohols with one less carbon atom and carbon dioxide may be formed (reaction b, Fig. 1) and also formic esters. The same free radicals may initiate chain reactions to yield hydrocarbons with one less carbon atom and water (reaction c, Fig. 1). A wide variety of esters may be formed, along with water and carbon dioxide, by recombination of free radicals (reactions d and e, Fig. 1).

The occurrence of formic acid and the relatively high quantities of formic esters always identified in the volatile substances may be explained, when we postulate an α-peroxidation of the aldehydes. Although we were unable to ascertain this reaction experimentally we may suppose a resonance equilibrium between two limiting forms of the aldehyde

molecule (15):

$$R - CH_2 - \overset{+}{C}H - \overset{-}{O} \leftrightarrow R - CH_2 - CHO \leftrightarrow R - \overset{-}{C}H - \overset{+}{C}HOH$$

or more exactly between two limiting forms of the carbonyl free radical initiating the autoxidation chain:

$$R - CH_2 - \dot{C}O \leftrightarrow R - \dot{C}H - CHO$$

The first hybrid yields a peracid free radical by autoxidation, and chain transfer gives the peracid (Fig. 3):

$$R - CH_2 - \dot{C}O + O_2 \rightarrow R - CH_2 - \underset{\underset{\dot{O}}{O}}{CO}$$

The second hybrid is able to tie up oxygen on the α-carbon to yield the α-hydroperoxy aldehyde by a similar mechanism (Fig. 4):

$$R - \dot{C}H - CHO + O_2 \rightarrow R - \underset{\underset{\dot{O}}{O}}{CH} - CHO$$

The latter shows clearly the easy formation of formic acid and a new aldehyde by breakdown of the O-O and C-C bonds:

$$R - \underset{\underset{\dot{O}}{O}}{CH} - CHO \rightarrow H - CO_2H + R - CHO$$

(H: recurrent reaction)

This hypothetical hydroperoxide might then follow different ways of decomposition (16) as represented in Figure 2. By combination of free radicals (reaction a) formic acid occurs as well as an aldehyde with one less carbon atom leading to a recurrent reaction. These free radicals may initiate chain reactions, and formaldehyde, sometimes identified (17), and water may be formed (reaction b).

This hydroperoxide may decompose in other ways leading to alcohols with two carbon atoms less (reaction c), and combination with formic radicals (Fig. 4) may yield formic esters. These same free radicals may also initiate chain reactions and yield hydrocarbons with two less carbon atoms (reaction d) and water. In the last two reactions glyoxal, already identified, is formed.

The above considerations must be in accordance with the rule of autoxidation; that is to say, with the principle of chain reaction. Figures 3 and 4 show the two possible paths of the autoxidative reaction.

The recurrent reactions of autoxidation that give rise to the volatile products may be represented as follows (18):

$$P \rightarrow \underset{\downarrow}{\overset{H\text{-}CO_2H}{\uparrow}}\, a_n \rightarrow \underset{\downarrow}{\overset{H\text{-}CO_2H}{\uparrow}}\, a_{n-1} \rightarrow \underset{\downarrow}{\overset{H\text{-}CO_2H}{\uparrow}}\, a_{n-2} \rightarrow (\text{etc.})$$

$$A_n \qquad A_{n-1} \qquad A_{n-2}$$

P = Hydroperoxide of oleic acid.

$a_n$ = Aldehyde of scission, e.g., octanal, nonanal or decanal.

$a_n, a_{n-1}, a_{n-2}$ = Aldehydes, lower homologs of $a_n$.

$A_n, A_{n-1}, A_{n-2}$ = Acids.

$n$ = Number of C atoms.

This equation shows the formation of strongly offensive substances, e.g., propanal, butanal, starting from oleic acid, as well as their occurrence at ordinary temperatures and always in small quantities (degressive geometric progression).

We were unable to ascertain the presence of unsaturated aldehydes. This may be due either to the severe conditions of autoxidation we used, or to the fact that these aldehydes are engaged in other combinations during the primary stages of autoxidation, e.g., a dialkylperoxide resulting from the dimerization of the alkoxy-radical, the latter resulting from the decomposition of the hydroperoxide. This peroxidimer would break down into saturated aldehydes only:

$$R - \underset{\underset{\dot{O}}{O}}{CH}(R') \rightarrow R - CHO + R'' - CH(OR') - R'''$$

$$R'' - CH - R'''$$

$R = CH_3 - (CH_2)_6-$ or $CH_3 - (CH_2)_7-$

R′, R″, R‴ = other fragments of the oleic acid molecule.

It must be added that saturated aldehydes may combine to give cyclic substances, e.g., 2,4,6-trialkyl-trioxanes (13).

We came to the conclusion that autoxidative rancidity is a succession of reactions between free radicals having varied life spans, and produces volatile products which are offensive in taste and odor. The starting point is a peroxidation of the aldehydes resulting from the scission of the peroxides.

Admittedly, some of these proposals are speculative and may also be incomplete. Nevertheless they offer opportunities for new research on antioxidants, aimed at finding substances that could inhibit the peroxidation of $n$-aldehydes and prevent their degradation.

TABLE I

Products Identified by Gas Liquid Chromatography from Autoxidized Oleic Acid and Nonanal

| Class of products | Oleic acid[a] | Nonanal[b] |
|---|---|---|
| *n*-Aldehydes | $C_2$, $C_3$, $C_4$, $C_5$, $C_6$, $C_7$, $C_8$, $C_9$, $C_{10}$ | $C_6$, $C_7$, $C_8$, $C_9$ |
| *n*-Alcohols | $C_5$, $C_6$, $C_7$, $C_8$ | $C_6$, $C_7$, $C_8$ |
| *n*-Alkylformates | $C_2$, $C_3$, $C_4$, $C_5$, $C_6$, $C_8$ | $C_6$, $C_7$, $C_8$ |
| *n*-Hydrocarbons | $C_6$, $C_7$, $C_8$ | $C_6$, $C_7$, $C_8$ |

[a]Methyl oleate and glyceridic oils yield the same products (13).

[b]Further research is being conducted (14).

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# The Incorporation of 2-Aminoethylphosphonic Acid into Rat Liver Diacylglyceroaminoethylphosphonate

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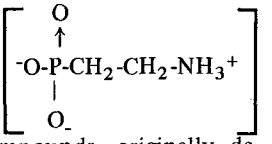
## ABSTRACT

Rats intravenously administered ($^{14}C$) 2-aminoethylphosphonic acid (AEP) incorporated ca. 16% of the total injected compound into liver lipids. Thin layer chromatography and selective chemical and enzymatic hydrolysis of the labeled lipids demonstrated that essentially all of the radioactivity was in one compound, diacylglycerol-AEP, the phosphonate analog of phosphatidylethanolamine. Lipids from kidneys, heart, skeletal muscle, adipose, pancreas and brain were examined and found to contain less than 2% collectively of the total injected radioactivity. The residues from the tissues contained ca. 3.2% of the total injected ($^{14}C$) AEP.

## INTRODUCTION

The alkylphosphonic acid derivative 2-aminoethylphosphonic acid  (AEP) and related compounds, originally detected in ciliated rumen protozoa (1), have been found in a variety of biotypes including a number of protozoa and marine invertebrates such as clams, oysters and sea anemones (for recent reviews see References 2 and 3). AEP has also been found in small amounts in a number of mammalian tissues, including goat liver (4), bovine brain (5) and human brain (6). In addition, the cholinephosphonate derivative N-trimethylaminoethylphosphonic acid was isolated from human aortic plaque lipids (7).

Kandatsu and coworkers (4,8) carried out studies on the incorporation of ($^{32}P$)AEP into rat tissues and goat liver. In the rat studies these workers found that ($^{32}P$)AEP administered

TABLE I

Extent of ($^{14}C$)AEP Incorporation into Rat Tissues

| Organ[a] | Amount of ($^{14}C$)AEP incorporated | |
|---|---|---|
| | dpm | %[b] |
| Liver (3) | | |
| Lipid | 239,500 (±67,100) | 16.3 (±3.6) |
| Residue | 44,130 (±33,180) | 2.90 (±2.04) |
| Skeletal muscle (3)[c] | | |
| Lipid | 81,130 (±70,240) | 5.33 (±4.32) |
| Residue | 4,060 (±590) | 0.27 (±.05) |
| Brain (2) | | |
| Lipid | 945 (±65) | 0.06 (±.004) |
| Residue | 49 (±9) | 0.003 |
| Adipose (1) | | |
| Lipid | 1,900 | 0.12 |
| Residue | 28 | 0.002 |
| Heart (1) | | |
| Lipid | 9,920 | 0.63 |
| Residue | 240 | 0.015 |
| Kidney (1) | | |
| Lipid | 9,940 | 0.63 |
| Residue | 860 | 0.055 |
| Pancreas (1) | | |
| Lipid | 1,620 | 0.10 |
| Residue | 52 | 0.003 |
| Whole blood (3) | 6,400 (±2,530) | 0.41 (±0.15) |
| Feces (1) | 0 | 0 |
| Urine (1) | 145,950 | 9.3 |

[a]The number in parentheses indicates the number of animals examined.

[b]% Refers to the percentage of total administered ($^{14}C$)AEP recovered in a given fraction.

[c]The weight of skeletal muscle was taken as 22% of the body weight.

intraperitoneally was incorporated into rat liver lipids and insoluble residues to the extent of 3.3 and 9.6%, respectively. Thin layer chromatography of the lipids revealed the presence of at least two labeled lipids, neither of which was identified (8). In the goat liver studies, intravenously administered ($^{32}$P)AEP was bound in both lipid and nonlipid fractions; again the AEP lipids were apparently not identified (4).

In this report we present evidence that the rat incorporates intravenously administered ($^{14}$C)AEP into liver phospholipids, specifically diacylglycerol-AEP (phosphonoethanolamine) (PNE). Very little, if any, radioactivity was found in any other liver lipids, and very little ($^{14}$C)AEP was detected in heart, kidney or brain lipids.

## MATERIALS AND METHODS

### Biochemicals

Nonradioactive AEP was purchase; from Calbiochem. Ethanolamine, O-phosphorylethanolamine, phospholipase D and phospholipase A (*Ancistrodon piscivorus* venom) were purchased from Sigma Chemical Co. ($^{14}C_{1,2}$)AEP, synthesized by the method of Kosolapoff (9) as described by Smith and Law (10), was a gift of J.H. Law, University of Chicago. The ($^{14}$C)AEP was radiochemically pure as determined by thin layer chromatography with authentic standards on Silica Gel G in solvent IV (see below) and scanning the developed plate for radioactivity on a Packard Model 7201 Radiochromatogram Scanner.

### Treatment of Animals and Preparation of Lipid Extracts

Young adult male albino rats, Holtzman strain, weighing 200-220 g were used. The animals were fed an ordinary laboratory diet ad libitum until 24 hr prior to injection of ($^{14}$C)AEP, during which time they fasted. After the 24 hr fast, each of three animals was intravenously injected via a dorsal tail vein with 1.55 (±0.14) x $10^6$ dpm ($^{14}$C)AEP (specific activity 10 $\mu$Ci/$\mu$mole) in 0.5 ml sterile 0.9% NaCl solution. The animals were allowed to eat ad libitum beginning 2 hr after the injection. The animals were decapitated 72 hr after injection; various tissues were removed and rinsed twice with cold 0.9% NaCl solution, then individually homogenized for 1 min in a Waring Blendor in 300 ml $CHCl_3$/MeOH 2:1. The homogenates were stirred for 16 hr at room temperature to extract the lipids. The insoluble residue from this extraction was re-extracted with 300 ml $CHCl_3$/MeOH 1:1 for 24 hr at room temperature. The combined 2:1 and 1:1 $CHCl_3$/MeOH extracts were evaporated under reduced pressure, redissolved in $CHCl_3$/MeOH 2:1 and washed with 0.1 M KCl as described by Folch et al. (11). The dried lipid was dissolved in 10 ml benzene and assayed for radioactivity

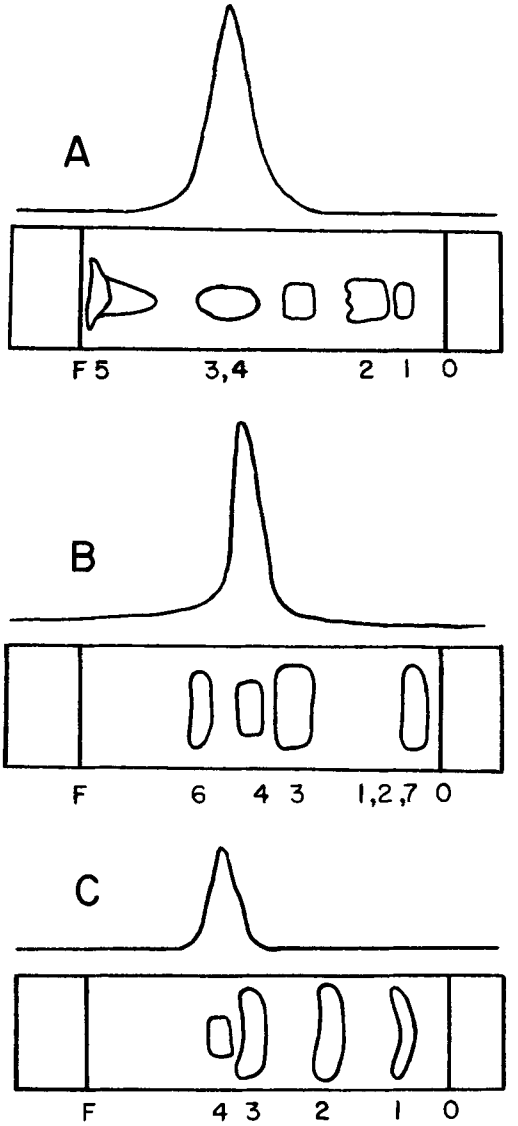



FIG. 1. Radiochromatograms of ($^{14}$C)AEP-labeled rat liver lipids: (A) chromatographic system of Wagner et al. (13); (B) chromatographic system of Thompson (14); (C) chromatographic system of Hori et al. (15). O and F indicate the origin and solvent front of the chromatograms, respectively; (1) lysolecithin; (2) lecithin; (3) phosphatidylethanolamine; (4) PNE; (5) neutral lipids; (6) cardiolipin; (7) lyso-PNE. Authentic nonradioactive *T. pyriformis* PNE was spotted at the origin in order to aid in localization of rat liver PNE. Without this carrier PNE, there was insufficient rat liver PNE present to visualize with the usual reagents.

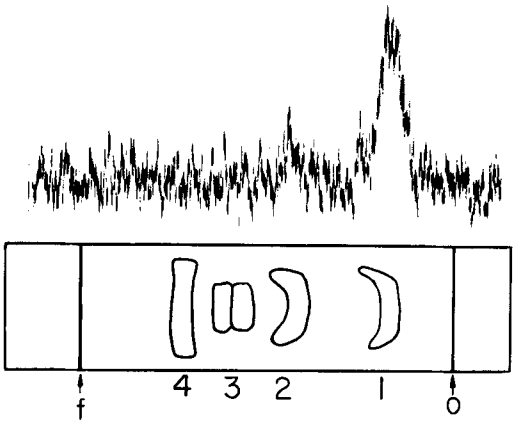



FIG. 2. Radiochromatogram of the water soluble products of the acid hydrolysis of ($^{14}$C)AEP labeled rat liver lipids (carrier *T. pyriformis* PNE added prior to hydrolysis); (1) AEP; (2) ethanolamine and deacylated glycerol-AEP; (3) and (4) unidentified compounds. The chromatographic system was that of Snyder and Law (16).

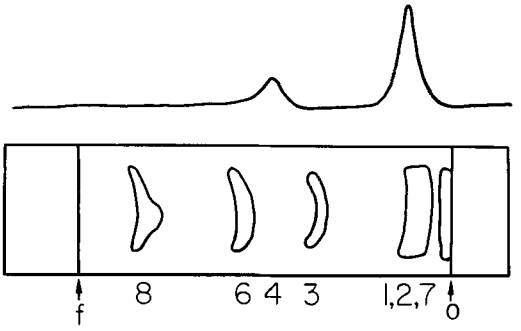



FIG. 3. Radiochromatogram of the $CHCl_3$ soluble material after phospholipase A treatment of ($^{14}$C)AEP-labeled rat liver polar lipids; (1), (2), (3), (4), (6) and (7) are as designated in Figure 1; (8) free fatty acids. The chromatographic system was that of Thompson (14). No carrier PNE was added; therefore there was no discernible spot for PNE on the chromatogram. The radioactivity detected at $R_f = 0.5$ cochromatographs with authentic PNE (compound 4, Fig. 1) and probably represents unhydrolyzed PNE.

as described previously (12).

Weighed samples of dried lipid-free tissue residues were solubilized with Protosol tissue solubilizer (New England Nuclear Corp.). Toluene scintillator was added, and the samples counted as described elsewhere (12). The samples were corrected for quenching by the addition of known amounts of ($^{14}$C) toluene and recounting.

#### Chromatography and Hydrolysis of Lipids

Thin layer chromatography was carried out on commercially prepared Silica Gel G analytical plates (E. Merck). The solvent systems used were: solvent I, $CHCl_3$/MeOH/$H_2O$ 65:25:4 (13); solvent II, $CHCl_3$/HOAc/MeOH/$H_2O$ 75:25:5:1.8 (14); solvent III, $CHCl_3$/MeOH/HOAc/$H_2O$ 100:20:12:5 (15); and solvent IV, isopropanol-acetone-58% $NH_4OH$-$H_2O$ 5:2:4:3 (16). Solvent I separates lipids into classes but does not separate PNE from phosphatidylethanolamine (PE), whereas solvents II and III both clearly separate PNE from PE. Solvent IV separates AEP from ethanolamine but not from O-phosphorylethanolamine. Radioactive lipids were detected on developed plates as described above. Phospholipids were detected with Dittmer and Lester's spray reagent (17); ninhydrin was used to visualize compounds containing free amino groups, and $I_2$ vapors were used to visualize all lipids.

Liver lipids were fractionated into polar and nonpolar fractions as described by White and Cox (18). Phospholipase D hydrolysis of the polar lipids was carried out as described by Yang et al. (19), and phospholipase A hydrolysis was done as described by Hildebrand and Law (20).

Acid hydrolysis of polar lipids was carried out in 6 N HCl for a total of 14.5 hr at 121 C in sealed tubes. Aliquots of the enzymatic and acid hydrolyzates were chromatographed on Silica Gel G plates with appropriate solvent systems. Carrier nonradioactive PNE (from *Tetrahymena pyriformis*) was added to the polar lipids prior to enzymatic and acid hydrolysis to aid in visualization of reaction products.

## RESULTS AND DISCUSSION

The total uptake of ($^{14}$C)AEP into rat liver lipids ranged from 11.3 to 21.6% of the injected radioactivity, with the average for the three animals being 16.3% (Table I). Incorporation of ($^{14}$C)AEP into skeletal muscle lipids was ca. 5.3%, while the incorporation into other tissue lipids was much lower, e.g., heart, 0.63%; kidney, 0.63%; brain, .06%. As was the case with the lipids, the liver residue was the most highly labeled residue fraction with an average incorporation of 2.9%. The other tissues ranged downward from 0.27%. Approximately 9% of the injected AEP was excreted via the urine.

Thin layer chromatography of the liver lipids in solvents I, II and III and scanning of the developed plates for radioactivity revealed the presence of only one radioactive lipid (Fig. 1). In solvent I radioactivity was found in a fraction that cochromatographed with PE and PNE (Fig. 1A), whereas in solvents II and III, both of which separate PNE from PE, radio-

activity was found only in a lipid that cochromatographed with authentic *T. pyriformis* PNE (Figs. 1B and 1C). A striking feature of these observations is the absence of detectable radioactivity in choline-containing lipids, specifically diacylglycerolcholine phosphonate, the phosphonic acid analog of lecithin. This observation is remarkably similar to those of Smith and Law (10,21), who found that *T. pyriformis* incorporated ca. 10% of the exogenously supplied ($^{14}$C)AEP into PNE (or its alkyl glyceryl ether derivative) but none into choline lipids.

Treatment of the rat liver polar lipids with phospholipase D did not release any $^{14}$C from the labeled lipids into the $H_2O$ soluble fraction, although the experimental conditions were such that almost all of the PE present was converted to phosphatidic acid. This is consistent with the presence of a direct P-C bond in PNE. On hydrolysis of the liver polar lipids with 6 N HCl for 14.5 hr at 121 C, the water soluble radioactivity cochromatographed with authentic AEP (Fig. 2). After treatment of the polar lipid with phospholipase A, all of the radioactivity chromatographed with authentic lyso-PNE (Fig. 3). These results demonstrate conclusively that the ($^{14}$C)AEP is present in the form of PNE. Furthermore the results indicate that the rat probably does not have the ability to cleave the P-C bond in AEP; otherwise one would expect some redistribution of the $^{14}$C into other compounds such as fatty acids. That this was not the case is indicated by the absence of $^{14}$C in the neutral lipid fraction in Figure 1 and the free fatty acid fraction (compound 8) in Figure 3.

These observations indicate that the rat can incorporate AEP into liver PNE with little, if any, conversion of PNE to the phosphonate analog of lecithin. These studies are of importance, in that AEP is found in milligram amounts in common human foodstuffs such as edible shellfish, e.g., oysters and clams, and in smaller amounts in tissues of ruminants such as cattle and goats. Further studies on the in vivo and in vitro metabolism of this and related compounds are underway in our laboratory.

ACKNOWLEDGMENTS

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# Anticoagulant Activity of Glycol Analogs of Phosphatidylserine

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## ABSTRACT

Three different analogs of phosphatidylserine were made by the combination of protected serine with phosphorus oxychloride and various diolmonooleates. These analogs of phosphatidylserine were 2-oleoylethyleneglycol-1-phosphoryl-L-serine, 1-oleoyl-2-desoxylysoglycero-3-phosphoryl-L-serine and 1-oleoyl-hexane-1, 6-diol-6-phosphoryl-L-serine. They were solubilized with sodium desoxycholate and tested in the Hicks-Pitney and antithromboplastin tests. Like the phosphatidylserines, they were found to have anticoagulant acitivity.

## INTRODUCTION

The anticoagulant activity of phosphatidylserine both natural (1,2) and synthetic (3,4) prompted an investigation of the activity of phosphatides having various diols in place of the glycerol of phosphatidylserine. All of the compounds reported here were unsaturated in the fatty acid portion. None of these have been made before, although a lauroylglycolphosphoryl-L-serine has been made (5). Pfeiffer and coworkers (6) have made various glycol analogs of phosphatidylethanolamine containing unsaturated acids. The importance of unsaturation in the fatty acids of phosphatidylserine and phosphatidylethanolamine in relation to their activity in tests of blood coagulation has been discussed before (3,4).

The preparation of glycol monooleates is easy by the method of Hartman and coworker (7,8). Using this method we have made the monooleates of ethylene glycol, 1,3-propanediol and 1,6-hexanediol. The three monooleates were combined with phosphorus oxychloride and the phthalimidomethyl ester of *t*-butyloxycarbonyl-L-serine or of anisyloxycarbonyl-L-serine to give three protected phospholipids. From these the protecting groups were removed to give oleoylglycolphosphoryl-L-serine and the corresponding derivatives of the other glycols.

## EXPERIMENTAL PROCEDURES AND RESULTS

### Materials and Methods

These were the same as in earlier papers from this laboratory (4,9). Oleic acid of better than 99% purity was purchased from the Hormel Institute, Austin, Minn.

### Monooleate of Glycols

The product of the Hartman reaction was chromatographed on washed Silicar CC-7 (Mallinckrodt Chemical Works) using solutions of ether in hexane to elute the column. The pure material was eluted from the column with 20% ether in hexane after the removal of a mixture

TABLE I

Analyses of Products

| Product | Carbon | | Hydrogen | | Nitrogen | | Phosphorus | | Formula |
|---|---|---|---|---|---|---|---|---|---|
| | Theory | Found | Theory | Found | Theory | Found | Theory | Found | |
| Monooleate of ethylene glycol | 73.57 | 73.71 | 11.73 | 11.95 | --- | --- | --- | --- | $C_{20}H_{38}O_3$ |
| Monooleate of 1,3-propanediol | 74.07 | 73.98 | 11.84 | 11.92 | --- | --- | --- | --- | $C_{21}H_{40}O_3$ |
| Monooleate of 1,6-hexanediol | 75.33 | 75.53 | 12.12 | 12.15 | --- | --- | --- | --- | $C_{24}H_{46}O_3$ |
| Ethylene glycol PS[a] | 54.96 | 54.60 | 9.03 | 8.65 | 2.79 | 2.65 | 6.16 | 5.89 | $C_{23}H_{44}O_8NP$[d] |
| Propanediol PS[b] | 56.78 | 56.69 | 9.14 | 9.03 | 2.76 | 2.68 | 6.10 | 5.94 | $C_{24}H_{46}O_8NP$ |
| Hexanediol PS[c] | 58.05 | 57.88 | 9.56 | 9.34 | 2.51 | 2.49 | 5.55 | 5.36 | $C_{27}H_{52}O_8NP$[d] |

[a] 2-Oleoylethyleneglycol-1-phosphoryl-L-serine.
[b] 1-Oleoyl-propane-1,3-diol-3-phosphoryl-L-serine.
[c] 1-Oleoyl-hexane-1,6-diol-6-phosphoryl-L-serine.
[d] Hemihydrate.

TABLE II

Anticoagulant Activities of Diol Analogs of Phosphatidylserine in the Modified Hicks-Pitney Test (1) vs. Coagulant Phosphatides

| Substance tested | Micrograms in incubation mixture | Incubation time, min: 2 | 4 | 6 | 8 |
|---|---|---|---|---|---|
| | | Substrate clotting time, sec | | | |
| Propane glycol phosphorylserine | 50 | >90 | 101 | 90 | 58 |
| | 25 | >90 | 94 | 33 | 10 |
| | 10 | >90 | 47 | 8 | 8 |
| Ethylene glycol phosphorylserine | 50 | 108 | 99 | 91 | 73 |
| | 38 | 82 | 50 | 36 | 35 |
| | 25 | 45 | 20 | 11 | 9 |
| | 10 | 46 | 11 | 8 | 8 |
| Hexane diol phosphorylserine | 50 | 93 | 78 | 77 | 66 |
| | 25 | 86 | 74 | 64 | 22 |
| | 10 | 90 | 66 | 10 | 9 |
| Controls | | | | | |
| Crude phosphatides (10) | 6 | 56 | 8 | 8 | 8 |
| Crude phosphatides + deoxycholate | 6 + 50 | >90 | 30 | 8 | 8 |
| Deoxycholate | 50 | >90 | 75 | 60 | 42 |
| Buffered saline | — | >90 | 80 | 59 | 46 |

with 10% ether in hexane. The product showed only one spot when examined by thin layer chromatography (TLC) on Silica Gel H using chloroform-acetone 88:12. The analyses of the products are shown in Table I.

### Glycol Phosphorylserines

The monooleate of 1,3-propanediol was combined with phosphorus oxychloride and the phthalimidomethyl ester of *t*-butyloxycarbonyl-L-serine exactly as described in our previous publications (3,4,9). The protecting groups were removed as described and the 1-oleoyl-propane-1,3-diol-3-phosphoryl-L-serine was purified by column chromatography on two silicic acid columns and one DEAE cellulose (acetate) column until it was pure by TLC and paper chromatography (3,4,9). The glycolphos-

TABLE III

Anticoagulant Activity of Diol Analogs of Phosphatidylserine in the Antithromboplastic and Recalcification Tests (1)

| Substance tested | Microgram in clotting test | Clotting time, sec: Antithromboplastin test | Recalcification test |
|---|---|---|---|
| Propane glycol phosphorylserine | 200 | 140 | >50,000 |
| | 100 | 39 | >50,000 |
| | 50 | 20 | 1730 |
| | 25 | 16 | 720 |
| Ethylene glycol phosphorylserine | 200 | 75 | > 5000 |
| | 100 | 20 | 2190 |
| | 50 | 16 | 780 |
| | 25 | 15 | 530 |
| Hexane diol phosphorylserine | 200 | 190 | > 7200 |
| | 100 | 37 | > 7200 |
| | 50 | 21 | 1200 |
| | 25 | 17 | 765 |
| Controls | | | |
| Buffered saline | — | 16 | 480 |
| Sodium desoxycholate solutions | 25-200 | 14-18 | 480-585 |

phorylserines from the monooleate of ethylene glycol and 1,6-hexanediol were made in the same manner except that sometimes the phthalimidomethyl ester of anisyloxy-carbonyl-L-serine was employed (4). For analysis the compounds were dried only at room temperature in vacuo. This did not suffice for all the compounds and the analytical results include water. A similar result has been reported by Pfeiffer et al. (6). Analytical data are shown in Table I.

**Anticoagulant Acitivity of the Phosphatidylserine Analogs**

All of the diol phospholipids were solubilized in sodium desoxycholate solution (1). They were tested for anticoagulant activity in three tests of blood coagulation previously employed by us (1): (a) the modified Hicks-Pitney test which evaluates the ability of the substance being tested to overcome the strong coagulant activity of a crude brain phosphatide fraction (10); (b) the antithromboplastin test which indicates the ability of the substance tested to prolong the short coagulation times obtained when plasma is clotted by the addition of brain thromboplastin and $Ca^{++}$; and (c) the recalcification test in which plasma is clotted by $Ca^{++}$ without the addition of lipid coagulants. The latter test is very sensitive and gives extremely prolonged clotting times or complete inhibition of clotting in response to anticoagulant substances.

In the Hicks-Pitney test (Table II) two of the diol phospholipids showed detectable anticoagulant activity at a concentration of 10 $\mu$g/ml in the test system. Higher concentrations produced complete inhibition. The diol analogs inhibited the action of brain thromboplastin in the antithromboplastin test and showed anticoagulant activity in the recalcification test (Table III). In all tests the propyleneglycol and hexanediol phospholipids had greater anticoagulant activity than the ethylene glycol analog.

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# Some Monomethyl-Branched Fatty Acids from Ruminant Fats: Open-Tubular GLC Separations and Indications of Substitution on Even-Numbered Carbon

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## ABSTRACT

Isomeric methyl esters of fatty acids in three groups ($C_{15}$, $C_{17}$, $C_{19}$) have been isolated from ruminant fats. Basic structural analysis by physiochemical techniques indicated that these odd-numbered fatty acids were even chain with a single methyl branch on the chain. High resolution open-tubular gas liquid chromatographic studies indicate that, with the exception of iso acid impurities in these fractions, only even-numbered carbons of the fatty acid chains bear the methyl branch.

## INTRODUCTION

Ruminant fats are notorious for the complexity of the mixture of fatty acids from which they are constituted (1-5). In recent years the three important isoprenoid acids 4,8,12-TMTD (=4,8,12-trimethyltridecanoic), 2,6,10,14-TMPD (=2,6,10,14-tetramethylpentadecanoic or pristanic) and 3,7,11,15-TMHD (=3,7,11,15-tetramethylhexadecanoic or phytanic) have been recognized as important among the unusual fatty acid components and thoroughly documented (6-10). The iso and anteiso acids have been proposed as a means of identifying and classifying such fats (11).

Application of coupled gas chromatography-mass spectrometry to butter fat identified many fatty acids present in trace amounts, including a number of even chain acids with single methyl branches in positions other than the iso and anteiso positions (5). A further study of this type recently carried out on human milk produced similar results (12), and indicated that the single methyl branches not of the iso and anteiso series would be found on both odd and even carbon atoms of many chain lengths.

As a result of collaborative studies based on high resolution open-tubular gas liquid chromatography (GLC) of fractions of odd-numbered (total) even chain length fatty acids isolated from ruminant fats, we have formulated alternative views on the location of single methyl branches on the basic even chain length fatty acids in these fats. Although the structural evidence is indirect we feel that, in view of the publication of the findings of Ryhage and colleagues (5,12), our data suggesting specific methyl substitution only on even-numbered carbons in the chains should be presented at this time.

## MATERIALS AND METHODS

The procedures followed for the isolation of the fractions studied were various combinations of conventional lipid separations with preparative GLC. Attention was concentrated on certain well defined fractions, for which one preparation exemplifies the technology. Details on other fractions may be obtained from the authors.

### B 104 ($C_{15}$) and Y 103 ($C_{17}$)

Fatty acid methyl esters prepared from butterfat (sample B/36,37), 20 kg, were distilled and a nominal "$C_{14}$" cut of 2.2 kg was collected. After partial removal of 4,8,12-TMTD (8) the bulk of the "$C_{14}$" sample was crystallized from acetone at low temperatures. The noncrystallizable residue, 194.6 g,

TABLE I

Optical Rotations for Representative Samples from Each Chain Length in $CHCl_3$ at 21 C

| Fraction | Chain length | Concentration, g/ml | Observed rotation | $[\alpha]_D^{21}$ |
|---|---|---|---|---|
| B104 | $C_{15}$ | 0.010 | -0.008 | -4.00 |
| H115 | $C_{17}$ | 0.007 | -0.006 | -4.28 |
| H183 | $C_{19}$ | 0.00675 | -0.002 | -1.48 |

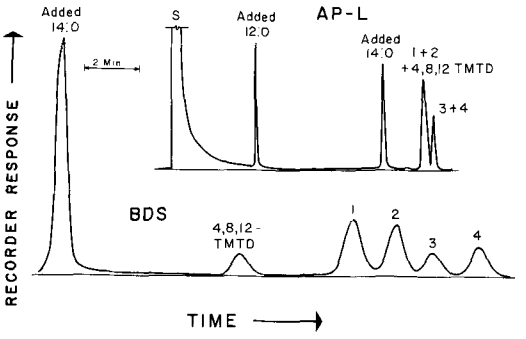


FIG. 1. Gas liquid chromatography of methyl esters of isomeric monomethyl-branched fatty acids (4,8,12-TMTD) isolated from butterfat (sample B-104). Butanediol succinate polyester (BDS) analysis on two 150 ft x 0.01 in. columns coupled in series. Temperature 150 C, helium 50 psig. AP-L analysis on one column of the same dimensions operated at 180 C and 40 psig.

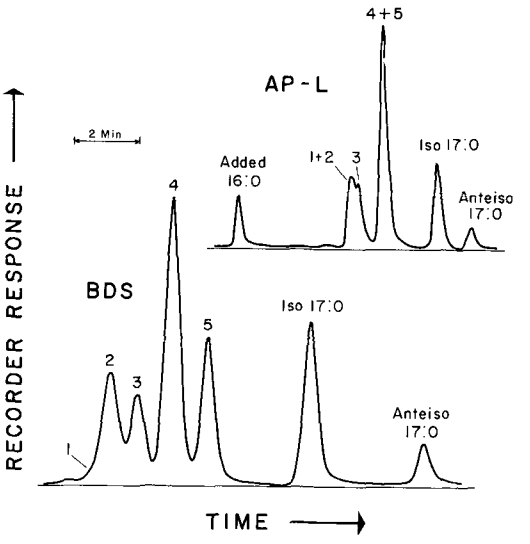


FIG. 2. Gas liquid chromatography of methyl esters of isomeric monomethyl-branched fatty acids isolated from sheep perinephric fat (sample Y-103). BDS analysis on one 300 ft x 0.01 in. column. Temperature 130 C, helium 80 psig. AP-L analysis on one column 150 ft x 0.01 in. operated at 180 C and 40 psig.

was hydrogenated, and the crystallization repeated to remove all solids. After removal of nonsaponifiables and reesterification of acids, 5 g of methyl esters were recovered. The esters were subjected to preparative GLC (20% Apiezon-L), and the fraction nominally containing 4,8,12-TMTD was collected and treated with urea. An adduct fraction (B 103) subjected to preparative GLC (20% EGA = ethylene glycol adipate polyester) gave a fraction, designated B104, of 0.105 g.

Fraction Y 103 (0.013 g) was also isolated from butterfat by the same techniques employed for the other samples.

#### H 115 ($C_{17}$), H 183 ($C_{19}$) and H 162 ($C_{17}$)

The initial processing steps of sheep perinephric fat (sample N/558) have been described elsewhere (13). Distillation of hydrogenated "liquid" methyl ester concentrate (H100) gave 20 fractions and a residue. From the 15th fraction an isolate of 0.064 g, designated H 115, was eventually obtained.

The 19th and 20th fractions and a distillation residue were combined. Methyl esters (4.8 g) prepared from purified fatty acids were subjected to fractionations of various types, with a final recovery of "component" H 183 of 0.020 g.

An additional fraction, H 162, was collected from preparative GLC on AP-L as an earlier and less pure fraction from the preparation of H 115. It included a number of components in the $C_{15}$ to $C_{17}$ range.

#### 11-Cyclohexylundecanoic Acid

This material was isolated from sheep perinephric fat (14). Retention data in equivalent chain lengths (ECL) (15) were: $H_3PO_4$ modified EGA in packed column, 19.12 at 207 C, 18.84 at 178 C; AP-L in packed column, 18.00 at 207 C, 17.82 at 180 C; BDS in open-tubular column, 18.40 at 150 C, AP-L in open-tubular column, 17.92 at 190 C.

#### Open-Tubular Gas Liquid Chromatography

All columns were of stainless steel and were purchased ready-coated with butanediol succinate polyester (BDS) or AP-L from the Perkin-Elmer Corp., Norwalk, Conn., and were used in either Model 226 or Model 900 GLC units of Perkin-Elmer manufacture. BDS-coated columns employed were 150 ft long (ca. 50 m) or 300 ft long (alternatively two columns 150 ft long were coupled with a Swagelok [Crawford Fitting Co.] zero volume connector) and 0.01 in. (0.25 mm) ID. AP-L-coated columns were all 150 ft long and 0.01 in. ID. The injection ports were operated at 250 C, and the manifold (Model 900) at 270 C. Other details are given in figure captions. Because of the scarcity of sample and the need for ultimate resolution, fine splits were employed and attenuations were normally 1, 2 and 4 (or 5) X. Injections were in neohexane, effected by Hamilton 701-N syringe with a back-up of 1 $\mu$l of pure solvent.

#### Physicochemical Studies

Optical rotations were measured in chloro-

form, 20 mm tube, in a Bendix-NPL Automatic Polarimeter Type 143, Model D. IR spectroscopy was carried out with a Perkin-Elmer 137 E spectrophotometer. Nuclear magnetic resonance analyses were made with a Varian T-60 Analytical NMR spectrometer.

## RESULTS

A variety of physicochemical studies were carried out on the purified fractions. Mass spectroscopy indicated that the structures could be saturated methyl esters, respectively, of $C_{15}$ (B104) $C_{17}$ (H115, Y103) or $C_{19}$ (H183) acids. Fraction H162 was shown by open-tubular GLC to contain $C_{15}$ and $C_{17}$ isomeric constituents corresponding to those in B104, H115 and Y103. IR spectroscopy confirmed all of the fractions as structurally methyl esters of fatty acids and eliminated other potential structures. Nuclear magnetic resonance showed that, based on one methoxy group ($-O-CH_3$), two methyl groups ($-CH_3$) were present, and confirmed the absence of other unusual structures. Optical rotations for H 115, H 183 and B 104 are given in Table I. There was insufficient sample to study Y 103.

The results of the examination of fractions B104, Y103 and H183 on open-tubular GLC on both polar (BDS) and nonpolar (AP-L) liquid phases can be compared in Figures 1-3. A surprising feature of these analyses was the collapse of the four main unknown peaks visible on BDS into two peaks on AP-L. The solution to this development depended on the collection of a sufficient number of samples to provide a reasonably complete spectrum of methyl branch positions on even-numbered straight chain fatty acids (15). This information is summarized in Figure 4 and discussed elsewhere (15,16).

The crucial point is the shift in retention time for a 4-methyl substituent from that corresponding to an $\omega 5$ methyl substituent on BDS to that corresponding to an $\omega 4$ on AP-L (Fig. 4). The significance of the "$\omega$" notation vis à vis the terminal methyl group is developed in detail elsewhere (15,16).

The relevance of this is most easily visualized from a discussion of B104 (Fig. 1). Initial considerations, based on the literature (5), required that each of the peaks 1-4 in the BDS analysis represents one of a series of fatty acids. Since it was known that the iso ($\omega 1$) and anteiso ($\omega 2$) acids had been eliminated, it was apparent that the next possible isomer ($\omega 3$) would also be eliminated by preparative GLC (ECL on BDS of 14.55 for $\omega 3$ vs. 14.58 for $\omega 1$; see Fig. 4). Accordingly, peak 4 (Fig. 1)

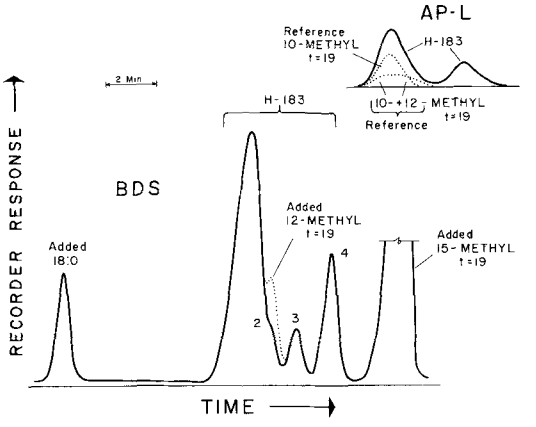


FIG. 3. Gas liquid chromatography of methyl esters of isomeric monomethyl-branched fatty acids isolated from sheep perinephric fat (sample H-183). BDS analysis on one 150 ft x 0.01 in. column. Temperature 150 C, helium 40 psig. AP-L analysis on one column of the same dimensions operated at 190 C and 60 psig. Dotted lines are traced from analyses carried out under identical conditions.

could be the next possibility and thus be $\omega 4$; ECL 14.45 on BDS. A standard became available in the form of a bacterial lipid cyclopropanoid fatty acid (17,18), which after hydrogenolysis (18,19) yielded a known pair of methyl-branched fatty acids, respectively, 9-methyl- and 10-methylhexadecanoate in structure, and a minor pair characterized by inference as 9-methyl- and 10-methyltetradecanoates (15).

With the use of this secondary standard, it could be confirmed that peak 4 of B104 coincided with 10-methyltetradecanoate ($\omega 4$) on both BDS (Fig. 5) and on AP-L (Fig. 6). Very careful consideration of the envelopes of mixed GLC runs, and of superimposed GLC runs of both substances on a number of columns, strongly suggested that methyl 9-methyltetradecanoate ($\omega 5$), if not completely absent, was at least a very minor proportion of all components, or had been completely masked in highly efficient GLC analyses on BDS (Fig. 1) or AP-L (Fig. 6; insert) by other components such as peak 4 on BDS or the tail of peak 2 on AP-L. Subsequently, a plant cyclopropanoid acid (20) yielded presumed methyl 5-methyl- and 6-methyltetradecanoates ($\omega 9$ and $\omega 8$). Although these did not separate from each other they coincided with peak 1 of B104 (Fig. 1). The remaining peak of B104, no. 2, since it is not the 9-methyl ($\omega 5$) acid yet is well separated from peak 1, is plausibly methyl 8-*methyltetra*decanoate ($\omega 6$ in structure). The spacing (compare Fig. 1) is such as to indicate that in B104 *only* the even-numbered carbons

TABLE II

Isomeric Monomethyl-Branched Fatty Acids Reported for Samples of Butterfat and Human Milk and Identified Essentially by Mass Spectroscopy and Those Identified in Ruminant Fats (Excluding Iso and Anteiso) from Purely GLC Considerations (NR = No Record)

| Methyl branch position | Butterfat peak no. for | | | Human milk peak no. for | | | Ruminant fats[a] peak no. for | | |
|---|---|---|---|---|---|---|---|---|---|
| | t=15 | t=17 | t=19 | t=15 | t=17 | t=19 | t=15 | t=17 | t=19 |
| | | | | | | | (B104) | (Y103) | (H183) |
| 2 | NR | NR | NR | 31-34[b] | NR | NR | NR | NR | NR |
| 3 | NR | NR | NR | NR | NR | NR | NR | NR | NR |
| 4 | NR | NR | NR | 31-34[b] | 52 | NR | 3 | 4 | 3 |
| 5 | NR | NR | NR | 31-34[b] | NR | NR | NR | NR | NR |
| 6 | NR | NR | NR | 31-34[b] | NR | NR | 1 | 1 | 1 (?) |
| 7 | 25-26 | NR | NR | 29-30 | 50-51[c] | NR | Absent | NR | NR |
| 8 | 25-26 | 39-40 | NR | 29-30 | 50-51[c] | NR | 2 | 2 | 1 (?) |
| 9 | 25-26 | 39-40 | NR | NR | NR | NR | Absent | Absent | NR |
| 10 | 25-26 | 39-40 | NR | NR | NR | 69 | 4 | 3 | 1 |
| 11 | 30 | 39-40 | NR | NR | NR | 69 | Absent | Absent | NR |
| 12 | 25-26 | 39-40 | NR | 36 | 53 | NR | — | 5 | 2 |
| 13 | 25-26 | NR | NR | 35 | NR | NR | — | Absent | Absent |
| 14 | — | 39-40 | NR | — | 55 | NR | — | — | 4 |
| 15 | — | 39-40 | NR | — | 54 | NR | — | — | NR |
| 16 | — | — | 55 | — | — | NR | — | — | — |
| 17 | — | — | — | — | — | NR | — | — | — |

[a]This paper, see Figures 1-3.
[b]Includes 4,8,12-TMTD.
[c]Includes 2,6,10,14-TMPD.

in the tetradecanoate fatty acid chain are methyl groups, substituted, viz., at the 4, 6, 8 and 10 carbons, with these eluting in the sequence 6-methyl, 8-methyl, 4-methyl and 10-methyl isomers.

Most of the work on H115 (and H162) was preliminary in nature and is represented by the superior GLC work on Y103 illustrated in Figures 2, 7 and 8. The H series examined had been freed of the iso and anteiso 17:0 components. Figure 2 illustrates the collapse on AP-L of BDS peak 1 (a small shoulder on the leading edge of peak 2) with peaks 2 and 3 into one peak, and BDS peaks 4 and 5 into another peak, exactly analogous to the behavior of B104. In addition to the methyl 9-methyl- and 10-methylhexadecanoates, pure methyl 10-methylhexadecanoate (21) was available, and a presumptive methyl 7-methyl- and 8-methylhexadecanoate pair which are not resolvable on even very efficient BDS open-tubular columns (20).

A further microbial methyl 10-methyloctadecanoate was accompanied by methyl 8-methylhexadecanoate (22,23). This $C_{17}$ ester partially separated on very efficient BDS columns (ca. 50,000 theoretical plates) from the methyl 9-methylhexadecanoate of the pair (methyl 9-methyl- and 10-methylhexadecanoates) (Fig. 9). Peak 2 of Y103 coincided with methyl 8-methylhexadecanoate. As shown in Figure 7A, peak 3 of Y103 on BDS coincides with methyl 10-methylhexadecanoate. This is confirmed in Figure 7B, but careful comparison of the valley between peaks 2 and 3, and that between the methyl 9-methyl- and 10-methylhexadecanoates, strongly suggests that methyl 9-methylhexadecanoate is essentially absent from Y103. Since H115 showed the same four components (or five if shoulder 1 is included) this lack of the 9-methyl isomer probably applies to H115 as well. The ECL values indicate that peak 4 is methyl 4- methylhexadecanoate ($\omega$12) and that peak 5 is methyl 12-methylhexadecanoate ($\omega$4). The behavior of this class of sample on AP-L is illustrated further in Figure 8 where the behavior of H115, Y103 and methyl (9-methyl- and 10-methylhexadecanoates) is compared. The origin of the small peak ahead of the first large peak of Y103 is not clear, but it is much diminished in the purer H115. The removal of the iso and anteiso components by preparative GLC also reduced the later-eluting components in H115 compared to Y103. Preparative GLC of H162 showed that peak 5 (of Y103; compare Fig. 2) was virtually eliminated along with the iso and anteiso $C_{17}$ acids and peak 4 greatly reduced. It was also noteworthy that the peaks numbered 1-4 in the BDS analyses of B104 (from butterfat) were clearly represented in this crude sheep fat sample in the same proportions to

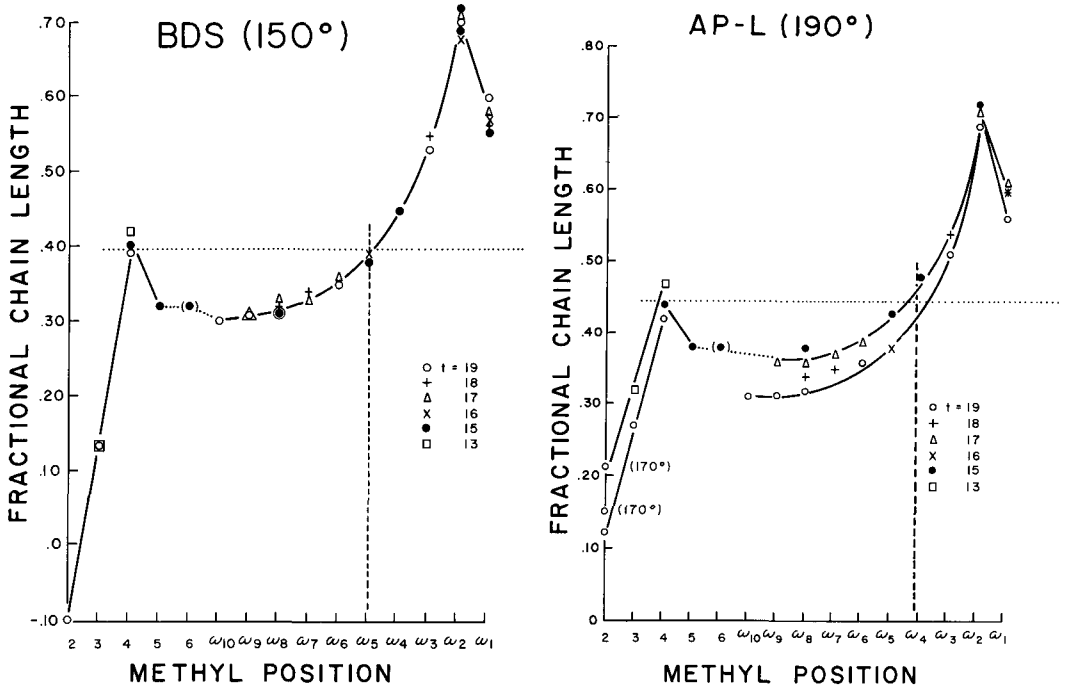


FIG. 4. Experimental fractional chain length (FCL) values for open-tubular gas liquid chromatography columns coated with BDS, operated at 150 C, and AP-L, operated at 170-190 C, for analyses of various monomethyl-branched fatty acids with substituents at diverse points in the chains. Dotted sections indicate an area of certain isomers where the C $n$ substituents ($n > 4$) overlap into the $\omega n$ substituents ($n \geq 8$). Note straight lines suggested for $C_2$, $C_3$ and $C_4$. Two sets of lines or curves are implied by the AP-L results, the upper based essentially on $t$ = 15 and $t$ = 13 data, the lower essentially on $t$ = 19 data. Dotted and dashed lines show how $C_4$ fractional chain length values relate to $\omega 5$ fractional chain length value.

each other immediately after the 4,8,12-TMTD peak.

The peaks of Y103 appear to be, in order: 1, methyl 6-methyl-; 2, methyl 8-methyl-; 3, methyl 10-methyl-; 4, methyl 4-methyl-; and 5, methyl 12-methylhexadecanoates (respectively, $\omega 10$, $\omega 8$, $\omega 6$, $\omega 12$ and $\omega 4$). Not only was there no indication of a methyl 9-methylhexadecanoate ($\omega 7$) isomer, for which a standard was available, but the peak shapes of Y103 strongly suggest the absence of 11-methylhexadecanoate ($\omega 5$) as well as of 13-methylhexadecanoate ($\omega 3$).

Extensive GLC studies of sample H183 are summarized in Figure 3. The sample as received had no methyl 15-methyloctadecanoate. An authentic material was added in the BDS analysis to illustrate the isomer spacing. The shoulder in the BDS analysis labeled 2 was enlarged by addition of authentic 12-methyloctadecanoate and reference methyl 9-methyl- and 10-methyloctadecanoates, and methyl 10-methyloctadecanoate could not be differentiated from the edge of peak 1. Authentic methyl 4-methyloctadecanoate coincided with peak 3. On AP-L the elution order for methyl 9-methyl-, 10-methyl- and 12-methyloctadecanoates was correct and fitted within the envelope of the first peak of the AP-L pair for H183. The methyl 4-methyloctadecanoate fell into the second AP-L peak for H183. This behavior is entirely compatible with peak 1 containing methyl 6-methyl-, 8-methyl- and 10-methyloctadecanoates (respectively, $\omega 12$, $\omega 10$, $\omega 8$). As shown in Figure 4, these would show little or no separation from each other (or from isomers with neighboring odd-numbered carbon substituents). As the curve for fractional chain length (FCL) (15,16) values swings upward, some separation is indicated; hence the partial resolution as peak 2 of the methyl 12-methyloctadecanoates ($\omega 6$). The spacing for this material (as added) for peak 4, if methyl 4-methyloctadecanoate ($\omega 4$), and for the added 15-methyloctadecanoate ($\omega 3$) is obviously correct for a smooth curve relationship. The FCL values on BDS agree (for example, the ECL of 14.45 for methyl 10-methyltetradecanoate vs. 18.44 for peak 4 strongly supports the view that the latter is methyl 14-methyloctadecanoate, both being $\omega 4$). Because of the longer chain length there is less

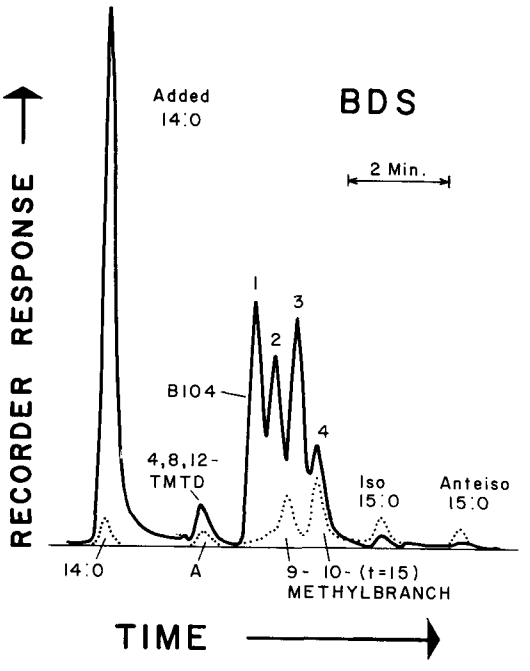


FIG. 5. Gas liquid chromatography on BDS of B104 methyl esters (see Fig. 1), and bacterial reference material (15) under the same conditions. Two 150 ft x 0.01 in. columns in series operated at 150 C and 50 psig.

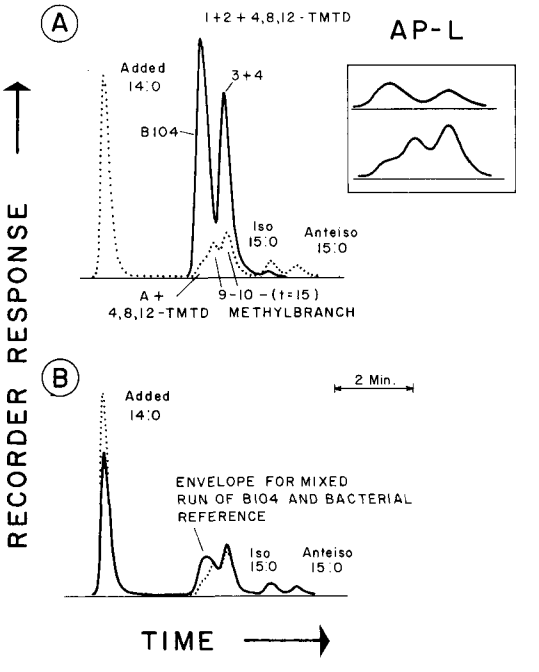


FIG. 6. Gas liquid chromatography on AP-L of B104 methyl esters (see Fig. 1) and bacterial reference material (15) under the same conditions separately or mixed. One 150 ft x 0.01 in. column at 170 C and 40 psig helium. Insert shows the parts of the two samples of most interest at 150 C and 40 psig helium.

assurance of the absence of methyl branches on odd-numbered carbons in the chain, but the peak width of peaks 3 and 4 and other factors suggest that the presence of methyl 13-methyloctadecanoate ($\omega$5) is at least unlikely.

## DISCUSSION

The butterfat analysis published in 1967 (5) did not differentiate the esters of methyl-branched acids by peaks except in one instance. As shown in Table II, the methyl 11-methyltetradecanoate ($\omega$3) reportedly elutes as peak 30 after the majority of the $t$ = 15 isomers in peak 30, and also apparently after the methyl 12-methyltetradecanoate ($\omega$2) in peak 25/26. In the methylhexadecanoates ($t$ = 17) no peak discrimination is reported, although the components listed span a fractional chain length (FCL) range of ca. 0.3 to 0.7. No methyl esters of 4-methyl-branched acids were reported, but fatty acids with methyl branches in the 9 and 11 positions are listed for both the $t$ = 15 and $t$ = 17 series, and in the 7 position only for the $t$ = 15 series.

The more recent human milk analysis (12) lists peaks in order by number from a GLC separation on diethylene glycol adipate polyester (DEGA) in the support-coated open-tubular (SCOT) column. In actual polarity DEGA normally corresponds approximately to that of BDS. The isomer elution order of methyl 4-methylhexadecanoates ($\omega$12) in peak 52 after the 8-methyl ($\omega$8) isomer in peaks 50-51, and before the 12-methylhexadecanoate ($\omega$4) in peak 53, is in agreement with our proposal. The elution of the methyl 4-methyltetradecanoate in a comparable position is also possible, but no doubt the interpretation of the mass spectroscopy results was hindered by an excessive proportion of 4,8,12-TMTD. As shown in Figure 4, a 2-methyl substituted acid could not fall in this position (peaks 31-34), although it is so listed in this report. The elution order for the methyl 5-methyl-, 6-methyl (peaks 31-34) and 7-methyl- and 8-methyl- (peaks 29-30) tetradecanoates is also the reverse of that which might have been expected, since the methyl 7-methyl- and 8-methyltetradecanoates are $\omega$7 and $\omega$6, respectively, which in longer chain lengths would give them a reasonably longer retention time than the other pair of isomers ($\omega$9 and $\omega$8). However, as the latter are in this instance proximal to the methyl 4-methyltetradecanoate, this may have an overriding influence and therefore the elution order may be

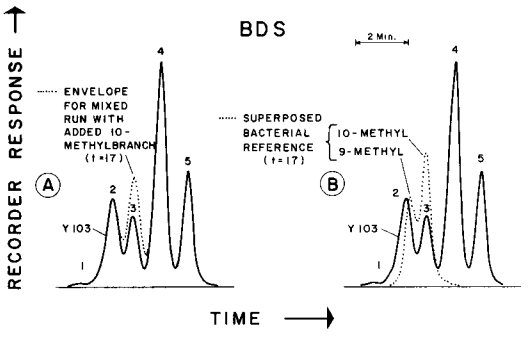


FIG. 7. Gas liquid chromatography on BDS of Y103 (except for iso and anteiso 17:0, see Fig. 2) and methyl 9-methyl- and 10-methylhexadecanoate standards. Conditions were identical to those for Figure 7.

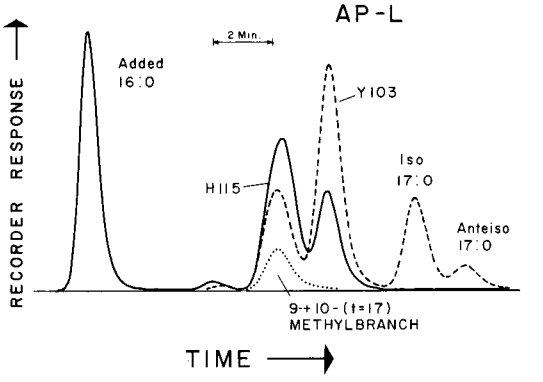


FIG. 8. Gas liquid chromatography on AP-L of Y103 (see Fig. 2), H115 and methyl 9-methyl- and 10-methylhexadecanoates. One 150 ft x 0.01 in. column operated at 180 C and 80 psig helium.

that described. Two $t$ = 15 acids with methyl branches on odd carbons of the chain (5- and 7-methyl, $\omega$9 and $\omega$7) and one $t$ = 17 (7-methyl, $\omega$9) are reported for the human milk.

The elution order for several series of isomeric methyl-branched alkanes on a Polysev (phenoxy aromatic) liquid phase has been observed to be (where complete) $\omega$6, $\omega$5, $\omega$4, $\omega$2, $\omega$3 (24). This is in accord with the basic structural elution schemes summarized in Figure 4.

Our results indicate pointedly the absence from ruminant fats of 7-methyl-, 9-methyl- and 11-methyltetradecanoic acids, of 9-methyl-, 11-methyl- and 13-methylhexadecanoic acids, of 13-methyl- and probably of 15-methyloctadecenoic acid. This high degree of specificity for the occurrence of methyl substitution on even-numbered carbons in the fatty acid chains is indicated only by GLC data. We suggest that the difficulty of quantitative interpretation of the mass spectra from multicomponent mixtures of this type may have led to undue emphasis on the presumed probable occurrence of fatty acids with the methyl branch on odd-numbered carbons in ruminant (butter) fat (5) and perhaps also in human milk except for 7-methylhexadecanoic acid and its metabolic homologues. These may have an exogenous origin in human milk, as 7-methylhexadecanoic acid is universally found in fish fats prominent in the North Europen diet (R.G. Ackman, S.N. Hooper, L. Safe and S. Safe, unpublished results).

The proposal that these methyl-branched fatty acids arise by specific methyl group insertion at the even-numbered carbons of pre-existing ethylenic double bonds is in accord with accepted theories (12). It is, however, difficult to reconcile the presence of 4-methyloctadecanoate with this hypothesis. We therefore propose that in ruminant biochemistry the random inclusion in normal fatty acid biosynthesis of a propionate group (condensing at the 2-carbon [25]), in lieu of an acetate group, would be equally, if not more, positionally specific and would result in the same overall effect. This especially would permit the presence of our 4-methyloctadecanoic acid as well as other unusual acids, e.g., 2-methyltetradecanoic and 2-methylpentadecanoic, reported from human milk (12).

Preparative GLC would remove the 2-methyl substituted acids from the more highly purified samples investigated in this study because of their positions remote from the peak (on packed column) of interest (see Fig. 4). Coincident cyclohexyl acids would be removed for

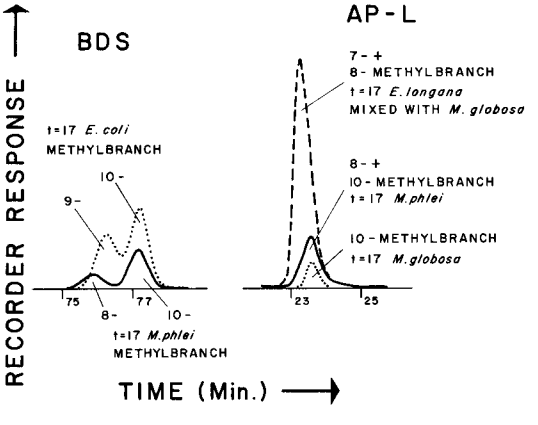


FIG. 9. Gas liquid chromatography (GLC) studies to demonstrate the extent of resolution of methyl 8-, 9- and 10-methylhexadecanoates on a 300 ft BDS-coated open-tubular GLC column (compare Fig. 9), and the inferior extent of resolution of methyl 7-, 8- and 10-methylhexadecanoates on an efficient 150 ft AP-L coated open-tubular GLC column.

similar reasons. Propionic acid is well known as a component of rumen liquor. The proportion of propionic acid to acetic acid in ruminant plasma is lower than in the rumen (26), but apparently higher than is found in the serum of humans (27). The amounts and relative proportions of volatile fatty acids in rumen are controlled by diet (28,29). This in turn should affect the level in ruminant plasma (30), but propionic acid appears to be approximately as important as butyric acid in both cow and sheep plasma (30). There is a tentative identification of propionic acid in human milk (4). We therefore feel that propionic acid incorporation into fatty acids provides an adequate basis for our hypothesis on the origin of these monomethyl-branched acids in fats of ruminants, and that the concept could be extended to humans as a source supplementing intake of preformed monomethyl-branched fatty acids from dietary sources.

Other instances of methyl branch substitutions, specifically on even-numbered carbons of alkane chains, are found in waxes of vernix caseosa (31) and in the preen gland waxes (including the alcohol moiety) of the tufted duck (32) and other aquatic birds. The biochemistry of the condensation to form these chains is discussed in some detail in these and related papers.

The possibility cannot be disregarded that a part of these monomethyl acids is associated with the biosynthesis or catabolism of cyclopropane fatty acids of (total) odd numbers of carbons. Microorganisms with suitable fatty acids exist in rumen, and the appropriate enzymatic specificity to produce a methyl substituent on an even-numbered carbon in an even-numbered chain as a byproduct or alternate to completion of a cyclopropane ring has been considered (33). It has also been suggested that methyl-branched fatty acids could supplement *cis*-monoethylenic acids in microbial membranes (34). Hydrogenolysis of cyclopropane rings in the rumen could accompany other biochemical activities of a reducing nature, but to account for our findings would have to be also based on a highly stereospecific process such as is known for biohydrogenation of ethylenic unsaturation (35,36).

## ACKNOWLEDGMENTS

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SHORT COMMUNICATION

# Brain Cholesterol: XV. Incorporation of Specifically Labeled Glucose


## ABSTRACT

Glucose-1 and glucose-6 labeled with tritium or carbon-14 were incorporated in vivo into brain and liver sterol. Differential incorporations of glucose labeled in both positions were compared. A higher than expected incorporation of C-1 from glucose was noted.


## INTRODUCTION

There is a considerable amount of evidence indicating that glucose is practically the sole carbon substrate supporting normal brain metabolism and function in vivo (1,2). In our preliminary data with specifically labeled glucose, a high incorporation of glucose-1-carbon into tissue cholesterol was measured (3,4). The following is a report of experiments giving additional data for the differential incorporation of specifically labeled glucose.

## MATERIALS AND METHODS

Twenty Swiss Webster male mice (20-25 g) were divided into four groups and injected intraperitoneally with mixtures of glucose-$H^3$ and glucose-$C^{14}$. Tritated compounds were injected at 100 $\mu$C level, while carbon-14 compounds were given at levels of 10 $\mu$C. Purity of the injected precursors was established by radioactive scan of a developed paper chromatogram.

In the first series of experiments the incorporations of glucose-1-$C^{14}$:glucose-6-$H^3$ and glucose-6-$C^{14}$:glucose-6-$H^3$ into two groups of mice at five time periods were compared. In another series the same carbon-14 labeled sugars were compared to glucose-1-$H^3$ incorporation instead of glucose-6-$H^3$. Animals were anesthetized with ether and cardiac punctures made at 30, 60, 120, 240 and 480 min before killing the animals. Liver and brain were surgically removed, washed with isotonic saline, blotted dry and immediately frozen. Prior to analysis the samples were thawed and homogenized in 10-15 volumes of chloroform-methanol 2:1. The sterol fraction was isolated by column chromatography (5) and the double-labeled sterol assayed for radioactivity in a liquid scintillation counter as described previously (6).

## RESULTS

The fractionated sterol was not contaminated by other neutral lipids as determined by thin layer chromatography. However the purified sterol fraction was not measured quantitatively, since molar ratios and not specific activities (SA) were compared. Due to the differences in specific activity of precursor used, radioactivity (DPM) was corrected to molar incorporation of glucose to nullify this difference in precursor SA. Tables I and II contain molar ratio values obtained from brain tissue; for simplicity of presentation, liver tissue

TABLE I

Mole Ratio of Precursor Incorporated into Brain Cholesterol

| Time, min | Glc-1-$H^3$[a] | Glc-1-$C^{14}$[a] | Glc-1-$C^{14}$ / Glc-1-$H^3$ | Glc-1-$H^3$[a] | Glc-6-$C^{14}$[a] | Glc-6-$C^{14}$ / Glc-1-$H^3$ |
|---|---|---|---|---|---|---|
| 30 | 81 | 34,020 | 420 | 189 | 93,217 | 493 |
| 60 | 105 | 50,329 | 479 | 242 | 93,444 | 386 |
| 120 | 213 | 158,329 | 743 | 92 | 25,263 | 275 |
| 240 | 211 | 135,064 | 640 | 179 | 78,798 | 440 |
| 480 | 12 | 4,395 | 366 | 227 | 89,948 | 396 |

[a] $\mu$Mole x $10^{-10}$.

values are given in Figure 1. Data from plasma and red blood cells are not presented, since their values represent complex interactions of sterol transport into plasma from various tissue pools. These latter values were not amenable to simple interpretation.

In Table I the data for glucose incorporation into brain cholesterol are given. Two comparisons were made. The incorporation of glucose-1-$C^{14}$ and glucose-6-$C^{14}$ was compared to that of glucose-1-$H^3$ in two separate groups. Using this comparative method, the molar ratio for glucose-1-$C^{14}$:glucose-1-$H^3$ incorporation is equal (30 and 480 min values) or greater than molar ratio of glucose-6-$C^{14}$:glucose-1-$H^3$, the greatest difference being apparent at the 120 min interval.

In a second experiment another comparison of glucose-1-$C^{14}$ to glucose-6-$C^{14}$ incorporation was made. However in this instance glucose-6-$H^3$ instead of glucose-1-$H^3$ was made the common denominator for carbon-14 incorporation molar ratio comparison (Table II). While the contrast was not as high as in the first series of experiments, the ratio of C-1 carbon from glucose entering the sterol brain pool is equal to or greater than C-6.

Similar comparisons were made for sterol biosynthesis in liver tissue (Fig. 1). The results for this tissue are similar, but are even more marked than brain values. As with brain data, values at 120 and 240 min indicate marked incorporation of C-1 (glucose) as compared to C-6 (glucose) when both are equated to glucose-1-$H^3$ incorporation. Also, as in the brain, the incorporation of C-1 into liver sterol was slightly more, as compared to C-6, when adjusted to glucose-6-$H^3$ incorporation.

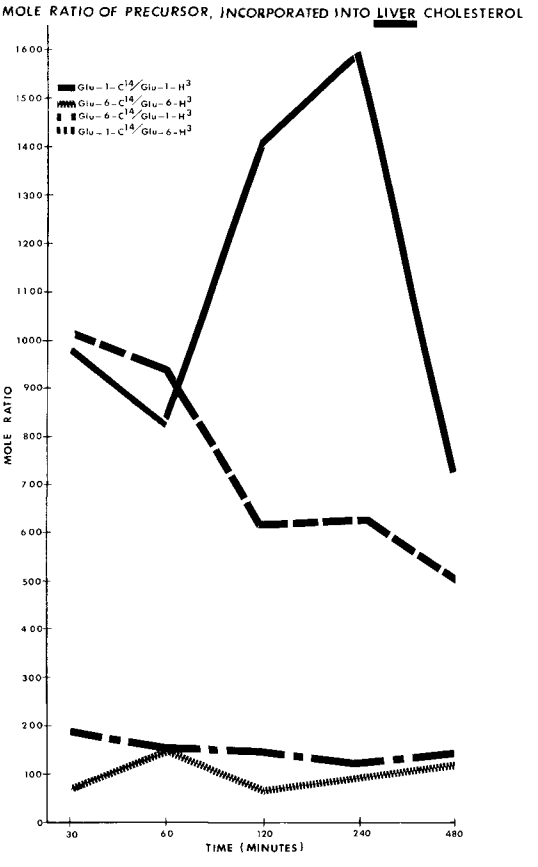


FIG. 1. Specifically labeled glucose $C^{14}/H^3$ was incorporated into liver cholesterol. Molar ratios were compared at various time intervals.

## DISCUSSION

The interpretation of data obtained from glucose-1 or glucose-6 labeled precursors is not without anomalies or exceptions (7-9). The relative contribution of C-1 and C-6 to incorporation experiments was discussed by Felts et al. (10). A further complication to data interpretation is the question of isotope discrimination. Enrichment due to such effects of tritium relative to $C^{14}$ in a product during metabolism of formate $H^3$-$C^{14}$ (11) and glucose $H^3$-$C^{14}$ have been described (12). However in our experimental protocol such enrichment would be the same in both $H^3$ groups and therefore should not be a factor.

With the above limitations in mind, our derived data indicate a different incorporation of C-1 from glucose into tissue sterol as compared to C-6. Unless a large isotope discrimination factor is present, the results in this report suggest, but do not prove, that fixation of $CO_2$ into the pathway for sterol (cholesterol) synthesis may occur.

Precedents for such a pathway are supported by early workers where isovaleric acid was shown to be a precursor to tissue cholesterol (13). The incorporation of labeled carbon dioxide during isovalerate metabolism in liver slices and homogenates is known to be dependent upon the availability of biotin to the animal (14,15). Also, Jacobsohn and Corley (16) reported that biotin is required for the incorporation of $\beta$-methylcrotonate into cholesterol, but not acetate. Other authors have noted the differential incorporation of C-1 and C-6 into lipids (17 and references therein). No explanation of their results was offered.

A review of past literature as well as our present experimental results suggests that alternate pathways other than acetate for cholesterol biosynthesis probably exist. A role for

TABLE II

Mole Ratio of Precursor Incorporated into Brain Cholesterol

| Time, min | Glc-6-$H^3$[a] | Glc-1-$C^{14}$[a] | Glc-1-$C^{14}$ / Glc-6-$H^3$ | Glc-6-$H^3$[a] | Glc-6-$C^{14}$[a] | Glc-6-$C^{14}$ / Glc-1-$H^3$ |
|---|---|---|---|---|---|---|
| 30 | 390 | 61,440 | 158 | 230 | 20,599 | 90 |
| 60 | 535 | 79,075 | 148 | 245 | 36,830 | 150 |
| 120 | 617 | 82,808 | 134 | 648 | 60,908 | 93 |
| 240 | 321 | 47,555 | 145 | --- | --- | --- |
| 480 | 439 | 66,000 | 150 | 666 | 96,849 | 145 |

[a]$\mu$Mole x $10^{-10}$.

$CO_2$ in the incorporation of leucine or $\beta$-methyl-crotonyl into sterol has been indicated (18,19). A similar suggestion as an explanation of our results with specifically labeled sugars is not inappropriate. However more definite experiments need to be performed, since the use of glucose-$H^3$ for quantitative tracer work is always open to question (20).

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# Thermophilic Fungi: IV. The Lipid Composition of Six Species

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## ABSTRACT

The lipid composition of six thermophilic fungi *(Myriococcum albomyces, Mucor miehei, Papulaspora thermophila, Rhizopus* sp., *Thielavia thermophila* (+), *Thielavia thermophila* (-), and *Torula thermophila)* was examined. The relative per cent total lipids (4.9-26.3%), neutral lipids (55.5-88.3%), polar lipids (11.7-44.6%) and the fatty acid profile of each lipid fraction was determined. The predominant fatty acids were 16:0, 18:0 and 18:2, and lesser amounts of 12:0, 14:0, 15:0, 16:1, 16:2, 17:0 and 18:3 were present. The total lipids contained an average of 0.96 double bonds per mole fatty acid (unsaturation index [USI]) the neutral lipids 0.86 USI and the polar lipids 0.84 USI, excluding the values for *Torula thermophila.* These data show a high degree of saturation and are consistent with data reported for other fungal thermophiles. *Torula thermophila* possessed abnormally high USI values (1.15-1.50) and was cultured at three different temperatures (25, 45 and 51 C). As the culture temperature of *Torula thermophila* increased, the USI decreased. The USI of the polar lipids of *Torula thermophila* at 25, 45 and 51 C were 1.50, 1.28 and 1.11, respectively. Thus the membrane lipids of this fungus appear unusual for a thermophile.

## INTRODUCTION

Two hypotheses have been proposed that attempt to explain the ability of life to exist at relatively high temperatures. The first is termed the dynamic hypothesis and the second the stable component hypothesis (1,2). Considerable experimental evidence has been accumulated in support of the latter, which states that the prime factor that imparts temperature tolerance is the presence of protective agents or an intrinsic stability of cellular constituents.

These hypotheses have been derived primarily from data collected from experiments conducted with thermophilic bacteria. Recently the lipid composition of nine thermophilic fungi has been compared to that of morphologically similar mesophilic fungi of the same genera with the hope that insight into the unique character of thermophily might be gained (3,4). It was found that the fatty acids of the thermophiles as a group were generally more saturated than the mesophiles. A comparison made within genera showed that both the neutral and polar lipid fractions of the thermophiles were more highly saturated than those of their mesophilic counterparts. This article presents the lipid composition of six additional species of thermophilic fungi and compares these data with the lipid composition of other thermophilic fungi.

TABLE I

Total Dry Weight, Lipid Weight, Per Cent Lipid and Per Cent Neutral and Polar Lipids of Thermophiles

| Organism | Total dry wt, mg | Lipid wt, mg | Per cent lipid | Per cent neutral lipid | Per cent polar lipid |
|---|---|---|---|---|---|
| *Myriococcum albomyces* | 416.6 | 68.6 | 16.5 | 88.3 | 11.7 |
| *Mucor miehei* | 1840.8 | 89.7 | 4.9 | 55.4 | 44.6 |
| *Papulaspora thermophila* | 1504.8 | 146.0 | 9.7 | 67.4 | 32.6 |
| *Rhizopus* sp. | 702.9 | 115.8 | 16.4 | 69.9 | 30.1 |
| *Thielavia thermophila* (+) | 397.4 | 104.4 | 26.3 | 66.8 | 33.2 |
| *Thielavia thermophila* (-) | 988.5 | 195.5 | 19.8 | 74.9 | 25.1 |
| *Torula thermophila* (25 C)[a] | 1178.3 | 86.5 | 5.8 | 67.3 | 32.7 |
| *Torula thermophila* (45 C) | 690.7 | 103.3 | 15.0 | 66.4 | 33.6 |
| *Torula thermophila* (51 C)[b] | 995.8 | 105.2 | 7.8 | 70.6 | 29.4 |

[a]Grown for 17 days.

[b]Grown for 9 days. The maximum temperature for growth is 51 C for this isolate.

TABLE II

Fatty Acid Profile of Total, Neutral and Polar Lipids of Thermophilic Fungi

| Organism | Lipid[a] fraction | 12:0 | 14:0 | 15:0 | 16:0 | 16:1 | 17:0 | 16:2 | 18:0 | 18:1 | 18:2 | 18:3 | Per cent ECL >20.6 and no. of components |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Myriococcum* | T | tr[b] | 0.5 | 0.1 | 17.8 | 1.2 | 0.1 | 0.4 | 11.1 | 40.8 | 26.6 | 0.4 | 1.0 (6) |
| *albomyces* | N | tr | 0.5 | tr | 17.6 | 1.1 | 0.4 | 0.1 | 11.3 | 42.7 | 25.0 | 0.3 | 0.8 (6) |
| | P | tr | 0.3 | tr | 31.7 | 0.7 | tr | tr | 5.7 | 45.6 | 16.0 | --- | <0.1 (1) |
| *Mucor* | T | tr | 0.6 | 0.5 | 18.2 | 1.3 | 0.2 | tr | 4.3 | 59.9 | 10.5 | 1.0[c] | 3.7 (5) |
| *miehei* | N | tr | 0.8 | 0.3 | 18.7 | 1.1 | tr | tr | 7.3 | 60.7 | 8.7 | 0.5[c] | 1.8 (4) |
| | P | tr | 0.5 | 0.6 | 21.2 | 1.2 | tr | tr | 0.9 | 62.5 | 12.0 | 0.7[c] | 0.5 (1) |
| *Papulaspora* | T | tr | 0.3 | 0.1 | 23.9 | 2.4 | 0.1 | tr | 6.7 | 36.8 | 25.7 | 0.4 | 4.1 (7) |
| *thermophila* | N | tr | 0.5 | tr | 24.9 | 2.5 | tr | tr | 8.8 | 42.1 | 20.8 | 0.3 | <0.1 (1) |
| | P | tr | 0.2 | 0.1 | 27.3 | 3.9 | tr | tr | 2.7 | 36.8 | 27.4 | tr | 1.6 (4) |
| *Rhizopus* | T | 0.4 | 0.5 | 0.4 | 17.9 | 2.2 | tr | tr | 12.0 | 39.4 | 17.3 | 4.7[c] | 5.3 (8) |
| sp. | N | 0.8 | 0.6 | 0.3 | 21.0 | 1.6 | 0.1 | tr | 18.5 | 35.4 | 11.6 | 2.8[c] | 7.2 (8) |
| | P | 2.5 | 2.0 | 3.9 | 25.8 | 3.5 | 2.7 | 1.7 | 1.5 | 48.5 | 6.0 | 0.5[c] | 1.3 (6) |
| *Thielavia* | T | tr | 0.2 | 0.1 | 28.7 | 1.0 | 0.2 | 0.1 | 6.7 | 10.0 | 49.7 | 1.8 | 1.5 (5) |
| *thermophila* (+) | N | tr | 0.2 | 0.2 | 32.8 | 0.7 | tr | tr | 7.4 | 10.5 | 47.1 | 1.2 | <0.1 (4) |
| | P | tr | 0.4 | 0.2 | 43.3 | 0.6 | tr | tr | 7.6 | 9.0 | 28.2 | 0.2 | 10.5 (2) |
| *Thielavia* | T[d] | tr | 2.9 | 1.3 | 31.1 | tr | tr | tr | 5.1 | 16.2 | 41.4 | tr | 2.0 (3) |
| *thermophila* (-) | N[d] | tr | 5.2 | 4.8 | 36.0 | tr | tr | --- | 6.8 | 15.7 | 31.3 | tr | 0.2 (2) |
| | P | tr | 1.8 | 1.0 | 31.2 | 1.6 | tr | tr | 1.9 | 13.0 | 43.9 | tr | 5.6 (1) |
| *Torula* | T | tr | 0.2 | tr | 15.9 | 0.7 | tr | tr | 3.7 | 42.0 | 22.1 | 13.6 | 1.8 (4) |
| *thermophila* (25 C) | N | tr | 0.8 | tr | 17.3 | 1.3 | tr | tr | 4.5 | 46.3 | 17.5 | 10.8 | 1.4 (3) |
| | P | tr | 0.1 | tr | 16.0 | 0.7 | tr | tr | 1.5 | 29.3 | 31.9 | 18.8 | 1.5 (4) |
| *Torula* | T | tr | 0.2 | 0.1 | 26.6 | 0.8 | tr | tr | 5.0 | 17.1 | 44.8 | 4.9 | 0.4 (5) |
| *thermophila* (45 C) | N | tr | 0.6 | 0.3 | 27.4 | 0.8 | 0.2 | tr | 5.8 | 17.8 | 42.5 | 4.7 | <0.1 (2) |
| | P | tr | 0.1 | 0.2 | 26.2 | 0.9 | 0.2 | 0.1 | 2.8 | 14.8 | 48.3 | 5.3 | 1.2 (4) |
| *Torula* | T | 0.4 | 1.0 | 0.3 | 24.6 | 0.5 | 0.4 | 0.2 | 2.1 | 24.2 | 44.5 | 0.7 | 0.9 (4) |
| *thermophila* (51 C) | N | 0.6 | 1.4 | 0.3 | 23.7 | 0.6 | 0.4 | 0.2 | 2.4 | 24.6 | 43.7 | 0.3 | 1.2 (4) |
| | P | tr | 0.1 | 0.3 | 31.3 | 0.3 | 0.3 | 0.2 | 1.4 | 22.6 | 41.2 | 1.8 | 0.5 (2) |

[a]T = total, N = neutral, P = polar.

[b]tr = trace.

[c]*Gamma* linolenic acid.

[d]Lipid fraction contains unknown nonfatty acid components: $X_1$ having ECL values of 16.15 and 15.01 on 15% DEGS and 2% OV-1 and $X_2$ having ECL values of 22.95 and 17.05 on 15% DEGS and 2% OV-1, respectively.

TABLE III

Unsaturation Indices of Total Lipids and Neutral and Polar Lipids of Thermophiles

| Organism | USI Total | USI Neutral | USI Polar | USI P / USI N |
|---|---|---|---|---|
| *Myriococcum albomyces* | 0.97 | 0.95 | 0.78 | 0.82 |
| *Mucor miehei* | 0.85 | 0.81 | 0.90 | 1.11 |
| *Papulaspora thermophila* | 0.92 | 0.87 | 0.96 | 1.10 |
| *Rhizopus* sp. | 0.90 | 0.69 | 0.69 | 1.00 |
| *Thielavia thermophila* (+) | 1.16 | 1.09 | 0.66 | 0.60 |
| *Thielavia thermophila* (-) | 0.99 | 0.78 | 1.02 | 1.31 |
| Average | 0.96 | 0.86 | 0.84 | 0.99 |
| *Torula thermophila* (25 C) | 1.28 | 1.15 | 1.50 | 1.30 |
| *Torula thermophila* (45 C) | 1.22 | 1.18 | 1.28 | 1.08 |
| *Torula thermophila* (51 C) | 1.16 | 1.14 | 1.11 | 0.97 |

## EXPERIMENTAL PROCEDURES

The organisms studied were from the culture collection of the Department of Biology and were: *Myricoccum albomyces* (No. E5), *Mucor miehei* (No. E21), *Papulaspora thermophila* (No. Ph II-1-1), *Rhizopus* sp. (No. F II-51), *Thielavia thermophila* (+) (No. R85W), *Thielavia thermophila* (-) (No. R46W2) and *Torula thermophila* (No. F15).

Culture flasks containing 50 ml of medium were prepared as previously described (3). Flasks were incoluated with mycelium, spores or mycelium spore mixtures and were incubated in stationary culture at 45 C for 4 days. In a separate experiment one of the species, *Torula thermophila,* was grown at three temperatures–25, 45 and 51 C for 4-17 days.

The contents of six culture flasks of each species, selected for apparent uniformity in development, were filtered into a tared fiberglass mat and washed with 150 ml of distilled water. The wet mycelium plus fiberglass mat was placed in a Virtis flask and homogenized in 20 volumes v/w chloroform-methanol 2:1 v/v for 3 min. The homogenate was allowed to stand for 30 min and filtered through another tared fiberglass mat. The mycelial mat was washed with an additional 50 ml chloroform-methanol 2:1 v/v and the filtrates combined. The filtrates were evaporated to dryness under a vacuum; the lipid residues were dissolved in 20 ml chloroform-methanol 2:1 v/v and washed according to Folch et al. (5). The purified lipid extract was evaporated to dryness under $N_2$ and weighed. The mycelium, including the fiberglass mat, was dried at 105 C for 24 hr and then weighed.

A weighed portion of the fungal lipids was fractionated into neutral and polar lipids on a silicic acid column (ATF silicic acid, 100-200 mesh). Neutral lipids were eluted with 40 ml ethyl ether and the polar lipids with 40 ml methanol followed by 30 ml chloroform-methanol-water 65:25:4 v/v/v. The latter two fractions were combined. Recovery of applied lipids to the silicic acid column averaged 92.5%.

Methyl esters of the fatty acids were prepared by transesterification with 12.5% boron-trifluoride in methanol (6). The methyl esters were analyzed by gas liquid chromatography as described previously (7). Identifications were made by comparison of retention times of unknowns with methyl ester standards (Supelco, Inc., Bellefonte, Pa.).

## RESULTS AND DISCUSSION

The weight of the dried extracted mycelia, the weight of the lipids, the per cent lipids based upon the weight of the dried extracted mycelia plus the weight of the lipids, and the per cent neutral and polar lipids are presented in Table I. The per cent lipids ranged from 4.9% in *Mucor miehei* to 26.3% in *Thielavia thermophila* (+). The per cent neutral lipids ranged from 55.4% to 88.3%. The polar lipids varied from 11.7% to 44.6%. These values fall within the range found for other thermophiles studied (4).

The relative percentage of fatty acids found in the total, neutral and polar lipids of the six thermophiles studied is shown in Table II. The most prevalent fatty acids were 16:0, 18:0, 18:1 and 18:2, and lesser amounts of 12:0, 14:0, 15:0, 16:1, 16:2, 17:0 and 18:3 were present. These thermophiles contained varying amounts of longer chain fatty acids (ECL >20.6), and as yet these acids have not been identified. Generally the polar lipid fractions were richer in 16:0 and 18:1 and lower in 18:0 than the neutral lipid fractions.

The unsaturation indices (USI) (number of double bonds per mole of fatty acid) of the

total lipids and the neutral and polar lipid fractions are presented in Table III. These values do not include the contribution of the methyl esters with a retention time of greater than 20.6 where information was available. For example the total lipids of *Mucor miehei* and *Rhizopus* sp. possessed 2.0% and 1.0% 20:4 (arachidonic), respectively, which would contribute to the USI values. Excluding *Torula thermophila,* the USI of the total lipids varied from 0.85 to 1.16 (average 0.96), the neutral lipid from 0.69 to 1.09 (average 0.86) and the polar fractions from 0.69 to 1.02 (average 0.84). These data showing a high degree of saturation are consistent with previously reported USI values for thermophilic fungi which possessed an average USI of 0.95 neutral lipids and 0.89 polar lipids (4). The ratio USI polar lipid-USI neutral lipids is presented in Table III and shows no tendency toward preferential incorporation of unsaturated fatty acids into either fraction. The USI of the neutral and polar lipids of *Rhizopus* sp. are low when compared to the USI of the total lipids, and may reflect loss of material on the silicic acid column or partial degradation of the unsaturated fatty acids of the neutral or polar lipid fractions on storage.

*Torula thermophila* has a relatively wide temperature growth range and was cultured at three different temperatures (25, 45 and 51 C) to observe what changes in the lipid composition occur, and these data are presented in Tables I-III. It is interesting to note that at 25 C the major fatty acid present in *Torula thermophila* is 18:1, while at 45 and 51 C the major fatty acid present is 18:2, in contrast to most other thermophiles examined. Perhaps in this organism the mixed-function oxygenase enzyme system converting 18:1 to 18:2 is not as operable at 25 C as it is at 45 and 51 C.

The USI of the lipids of *Torula thermophila* decrease as culture temperature increases from 25 to 51 C (Table III). This phenomenon is particularly evident in the polar lipid fraction. This inverse relationship has been found with many other microorganisms (1,9-11); however exceptions do exist (12,13). These data lend further strength to the hypothesis that the more saturated nature of the polar lipids of thermophiles provide the organism with the greater thermostability needed to grow and survive at elevated temperatures (4,8).

The USI of the lipids of *Torula thermophila* were unusually high in all fractions at all culture temperatures. For example the USI of the polar lipids were 1.50, 1.28 and 1.11 at 25, 45 and 51 C, respectively. Thus the lipids of *Torula thermophila* were significantly more unsaturated than the 14 other thermophiles examined. Other isolates of *Torula thermophila* should be examined to determine if they also possess this high degree of unsaturation. One investigator has reported (14) that no observable taxonomic difference exists between *Humicola grisea* var. *thermoidea* and *Torula thermophila;* however, in the isolates examined in this study, there was a significant difference in the fatty acid composition. The thermostability of *Torula thermophila* does not seem to be explainable solely by its fatty acid composition, and therefore additional studies are needed to determine if the lipid composition of the membranes is unusual.

ACKNOWLEDGMENT

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# Effects of Surface Concentration, Metals and Acid Synergists on Autoxidation of Linoleic Acid Monolayers on Silica

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## ABSTRACT

To approximate in a model system the autoxidation of monomolecular layers of lipids on the cell surfaces of freeze-dried foods, the autoxidation of presumed monolayers of linoleic acid adsorbed from solution onto silica gel has been studied as a function of time and α-tocopherol and acid synergist content. The method of Honn, Bezman and Daubert was used, modified by the substitution of linoleic acid for soybean oil and the use of gas chromatography to follow oxygen disappearance at 80 C. It was found that adsorption of linoleic acid onto silica gel from petroleum ether solution conforms to a Langmuir isotherm, consistent with the formation of a monolayer. Confirming the finding of Honn et al. with soybean oil, it was found that the most rapid uptake of oxygen occurred at a linoleic acid-silica ratio close to that for the monolayer. Without included antioxidant, oxidation commences at a nearly linear rate without observable induction period. Time for consumption of one-half mole of oxygen per mole of linoleic acid is ca. 60 min on acid-washed silica. If very small amounts of α-tocopherol are included in the layer, virtually no oxygen uptake measurable in this system occurs during an induction period, the length of which is approximately proportional to tocopherol content. The inflection point at the commencement of rapid oxidation is very sharp; the ensuing oxidation rate approximates that of the unprotected acid. The induction period of linoleic acid with the same tocopherol content is as much as 100% longer when exposed in monolayer than in a bulk form. However the rate after commencement of rapid oxidation is 8-10 times greater in the monolayer. Acid washing of the silica reduced its iron content by 75%. Acid washing also reduced by 60% the rate of autoxidation without α-tocopherol and increased the length of the induction period four-fold when α-tocopherol was present. The effect of pretreatment of the silica by adsorption of the acid synergists, ascorbic, phosphoric, citric and ethylenediamine tetraacetic acid was qualitatively similar to the effect of acid washing. The synergists extended the induction period in increasing order as listed, EDTA producing a 100-fold extension. For ascorbic acid the rate reduction and increase of induction period were not found on unwashed silica and were dependent on the extent of washing. These findings are consistent with synergist sequestration of metals in a complex that is ineffective in new chain generation by peroxide decomposition.

TABLE I

Comparison of Linoleic Acid Adsorption as Determined by Gravimetry or Extraction and Titration

| Initial solution concentration, mg/2 ml aliquot | | | Final solution concentration, mg/2 ml aliquot | | | Gravimetric assay of solutions, mg | Extraction and titration of absorbed acid, mg | | |
|---|---|---|---|---|---|---|---|---|---|
| Mean | N | S.D.[a] | Mean | N | S.D.[a] | Mean[b] | Mean[b] | N | M.D.[c] |
| 237 | 3 | ±0.5 | 209 | 4 | ±1.2 | 279 | 370 | 2 | ±4.0 |
| 118 | 3 | 0.6 | 95 | 4 | 1.4 | 232 | 300 | 2 | 0.5 |
| 58 | 3 | 0.8 | 35 | 4 | 0.06 | 229 | 254 | 2 | 1.5 |
| 23 | 3 | 0.7 | 3.8 | 4 | 0.17 | 197 | 199 | 2 | 1.0 |
| 12 | 3 | 0.3 | 0.33 | 4 | 0.06 | 115 | 114 | 2 | 2.5 |

[a]Standard deviation.

[b]Mean weight of linoleic acid per gram uncoated, acid-washed silica. Calculated as 10 (initial-final concentration).

[c]Mean deviation.

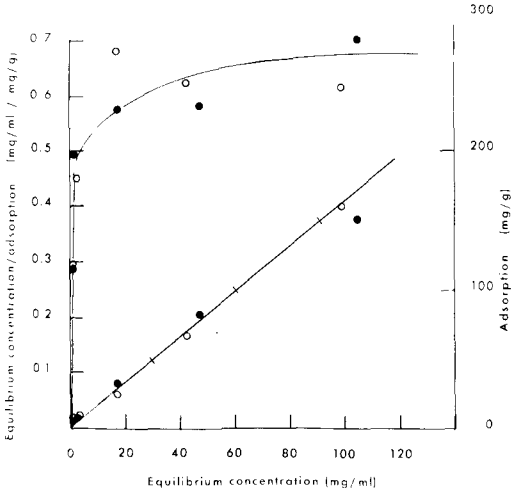


FIG. 1. Conventional (———) and Langmuir (—//—) plots of adsorption of linoleic acid on Silica Gel G at 22 C as function of equilibrium concentration of petroleum ether solution.

## INTRODUCTION

Investigators of autoxidation of lipids, whether concerned with antioxidant prevention of rancidity in foods or the metal facilitation of drying in oils, have in general exposed the lipid in a form with a relatively low surface to volume ratio. For example paint films, although thin, are thousands of molecular diameters in thickness. In order to study each stage and product of autoxidation in as homogeneous a form as possible, it is important to increase the ratio of surface to volume. Diffusion restrictions do not then produce so many different stages of autoxidation at one time.

Autoxidation of hydrocarbon films of greatly reduced thickness on surfaces of high specific area had been studied by a few workers prior to 1950 (1-4). Lea (5) and Dubouloz and Laurent (6) used films of lipid deposited on filter paper to study rancidity in fats. Taufel (7), in 1952, studied lipid autoxidation on chromatography paper, while Spruyt (8), in 1955, employed glass beads as a means of surface expansion. Togashi et al. (9), in 1961, studied the autoxidation of thin films of lipid deposited on glass and dried gelatin surfaces. It was estimated that the films were still several hundred molecules in thickness, although they contained only 0.1 mg/cm$^2$.

Honn et al. (10), in 1951, made the first precise study of the relation of depth of lipid layers to rate of autoxidation on silica. They found that there was an optimum oil-solid ratio for maximum autoxidation rate that was a function of specific surface area of the adsorbent. Surface area measurements suggested that this oil-solid ratio was approximately that for a monolayer of the lipid.

Leermakers and coworkers (11,12) have recently made extensive study of the photochemical reactions of compounds bound to a silica gel surface.

Because very pure and well characterized silica gels of high specific area are now available, we used the techniques of Honn et al. and Leermakers and coworkers to study autoxidation in monomolecular lipid films spread on silica. In theory each molecule is nearly equally exposed to oxygen, the pores of the silica offering only a small diffusion barrier. We wished to determine the effects on autoxidation of the presumed limited migrational mobility of lipids, antioxidants, metals and synergists. We report here the effects of metal ions and the so-called acid synergists (ascorbic, citric, phosphoric and polyamino acids) on autoxidation of linoleic acid in this system.

## EXPERIMENTAL PROCEDURES

### Materials

A detailed description of the procurement and purification of Silica Gel G, synthetic and natural tocopherol, linoleic acid, petroleum ether and dry nitrogen have been described (13). The physical characteristics of the adsorbent were also outlined.

In studies of fatty acid and synergist adsorption, the 95% ethanol used in fatty acid extraction was U.S.I./U.S.P. brand; the absolute ethanol was U.S.I. brand (U.S. Industrial Chemicals Co., N.Y.).

For autoxidation experiments, absolute ethanol was used for synergist adsorption and was glass-distilled before use. In some experiments with citric acid and disodium ethylenediaminetetraacetate ($Na_2EDTA$), distilled, deionized water was used (ILLCO-WAY De-ionizer, Illinois Water Treatment Co., Rockford, Ill.; conductivity 1.2 $\mu$mho/cm at 25 C).

Acid washing of silica and glassware was done with nitric and hydrochloric acids which were ACS Reagent Grade (Fisher Scientific Co.; iron, 0.00002%; and Allied Chemical; iron, 0.00001%, respectively).

For analysis of metal content of silica, we used hydrofluoric acid (49%) which was ACS Reagent Grade (suitable for electronic use, Fisher Scientific Co.; iron, 0.00005%). Sulfuric acid was ACS Reagent Grade (Allied Chemical; iron, 0.00002%). Acid-washed platinum crucibles were used for solution of silica.

All the synergist acid compounds were used as received from the supplier. l-Ascorbic acid

TABLE II

Reduction of Linoleic Acid Adsorption by Acid Synergist Adsorption

| Adsorbates other than linoleic acid | Linoleic acid adsorbed,[a] mg | | | Synergist adsorbed, mg | Mole per cent synergist | Reduction in adsorption, mg | Percentage reduction in adsorption |
|---|---|---|---|---|---|---|---|
| | Mean | N | M.D.[b] | | | | |
| None | 196.5 | 2 | ±0.5 | 0.0 | 0.0 | 0 | 0 |
| 95% ETOH | 188.5 | 2 | ±0.5 | 0.0 | 0.0 | 8.0 | 4.1 |
| Ascorbic acid and ETOH | 184.5 | 2 | ±3.5 | 2.93 | 2.5 | 12.0 | 6.1 |
| Phosphoric acid and ETOH | 174.5 | 2 | ±1.5 | 2.66 | 3.7 | 22.0 | 11.2 |
| Citric acid and ETOH | 177.5 | 2 | ±1.5 | 3.14 | 2.4 | 19.0 | 9.7 |

[a]Linoleic acid was adsorbed from a solution containing 250 mg in 22 ml petroleum ether onto 1 g acid-washed silica.

[b]Mean deviation.

was from Nutritional Biochemicals Corp., Cleveland (analysis at these laboratories showed iron, less than 0.00018%); citric acid monohydrate was Certified ACS Grade (Fisher Scientific Co.; supplier's analysis, iron, 0.00002%); phosphoric acid was Certified ACS Grade (Fisher Scientific Co.; supplier's analysis, iron, 0.003%); and $Na_2EDTA$ was Baker Analyzed Reagent (J.T. Baker Chemical Co., Phillipsburg, N.J.; supplier's analysis, iron, 0.01%). Iron added by the synergist was never more than 0.3 ppm on a silica basis.

### Methods

Acid washing of silica gel, adsorption of lipids onto activated silica gel from redistilled petroleum ether, solvent removal, air oxidation at 80 C, and monitoring of oxygen uptake by headspace gas analysis have been described (13).

For determination of adsorption isotherms, linoleic acid at various concentrations in 20 ml redistilled, deoxygenated petroleum ether was adsorbed at 22 C onto 1 g acid-washed silica in a 50 ml glass-stoppered, round bottom flask, the supernatant liquid decanted, and the equilibrium concentration and amount adsorbed determined by gravimetric and extraction-titration methods described below.

For determination of autoxidation rate as a function of surface concentration, two methods of adsorption were used: (a) Various concentrations of linoleic acid in 20 ml petroleum ether were shaken with a uniform charge of 1 g acid-washed silica, in a 50 ml round bottom flask, and the supernatant liquid was decanted before solvent removal. The adsorbed fatty acid was estimated by extraction and titration. Observed oxygen uptake rates (made on duplicate samples) were normalized on the basis of 244 mg/g silica, the linoleic acid content per flask most like that of method b; (b) At a constant concentration of 11.5 mg/ml in 21 ml petroleum ether, linoleic acid was adsorbed onto amounts of acid-washed silica varying from 0.25 to 5.0 g. None of the solution was decanted, and all of the solvent was removed, so that each flask finally contained 241 mg linoleic acid. Thus no normalization of rates was necessary, and rates are comparable to those of method a.

Oxygen uptake rate was expressed as its reciprocal—time required for uptake of a specified amount of headspace oxygen. Unless specified otherwise, the surface concentration of linoleic acid used in the remaining experiments (acid wash, synergists) was ca. 185 mg/g or 80% of theoretical monolayer coverage (see Results). This was one molecular equivalent of the oxygen content of the flasks used.

Solution loss from adsorption flasks was reduced to less than 1% by a Teflon spray and a tight wire binding of the ground glass stoppers to the flasks.

If the flasks were to be held for a period after solvent removal and prior to oxidation, they were stoppered and wrapped in Parafilm and stored at -10 C to prevent premature displacement of nitrogen by air.

In long experiments, where more than ca. 25 headspace gas samples were withdrawn through the rubber serum-bottle stopper, the syringe needle must be kept very sharp, its orifice clean, and its plunger tight-fitting. The needle insertion point must be varied. If these precautions are taken, headspace gas readings are highly reproducible and leakage is undetectable.

The Bishov-Henick method of headspace gas analysis has the advantage that oxygen is measured directly, rather than total or compensated headspace gas volume change, as in Warburg manometry. Also, we required a non-

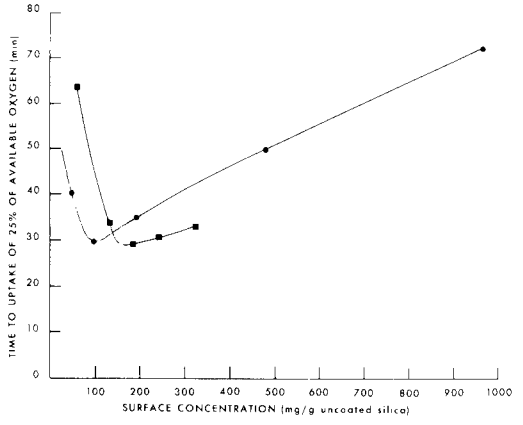


FIG. 2. Relation of autoxidation rate to weight ratio of linoleic acid to silica. ▪ Acid concentration varied. 62 ± 0.5 C. • Silica charge varied. 59 ± 0.5 C.

liquid measuring system which cannot be obtained easily in Warburg manometry. Determinations by gas analysis are rapid and do not mix and deplete the headspace gas by rather large amounts as in the method of Honn et al. (10).

We have reported results as per cent oxygen in headspace air. Using this convention, a linear absolute rate of oxygen uptake will appear slightly nonlinear. The headspace oxygen content at an actual uptake of half the available oxygen (20.9%) will not be the expected 10.45% but 11.7% of headspace air $\left(\frac{10.45}{10.45 + 79.10} \times 100\right)$. If desired, absolute uptake of oxygen can be obtained from the initial flask volume either by expressing oxygen as per cent of initial nitrogen or by a simple correction applied to per cent headspace air readings.

Synergist compounds (ascorbic acid, citric acid and phosphoric acid) were adsorbed from solution in redistilled absolute ethanol (ca. 2.5 mg acid per 5 ml alcohol on 1 g acid-washed, heat-activated silica). Methods of adsorption by shaking and solvent removal were similar to those for lipid adsorption. The flasks with adsorbed synergists were then reactivated at 110 C for 1 hr before proceeding with the normal lipid adsorption and oxidation procedures. Because of the very small solubility of $Na_2EDTA$ in ethanol, in one experiment citric acid monohydrate (2.8 mg/5 ml/g silica) and $Na_2EDTA$ (5.2 mg/5 ml/g silica) were adsorbed from distilled, deionized water onto acid-washed silica, and the water removed on a rotary evaporator before activation and lipid adsorption.

We used gravimetric determination of the amount of adsorbed linoleic acid by the method of Brooks (14). We removed solvent from measured volumes of equilibrated adsorbing solution in a vacuum desiccator at room temperature.

As an additional check, adsorbed linoleic acid was eluted from the silica and estimated by titration. The lipid-coated silica was stirred 5 min with 20 ml 95% ethanol, centrifuged, and the clear supernatant decanted. This procedure was repeated twice more with 10 ml 95% ethanol. The combined extracts were titrated with standardized 0.102 N alcoholic KOH and phenolphthalein indicator (15), using as a blank a similarly prepared and titrated ethanol extract of uncoated silica.

The amounts of synergist acids adsorbed were estimated by ethanol extraction and titra-

TABLE III

Extractable and Total Iron in Silica Used for Monolayer Studies of Fatty Acid Autoxidation

| Iron | Unwashed Silica G | | | Silica G washed by one repetition of acid-wash procedure (4 washes) | | | Silica G washed by two repetitions of acid-wash procedure (8 washes) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean, μg/g silica | N | Average error | Mean, μg/g silica | N | Average error | Mean, μg/g silica | N | Average error |
| Extractable iron[a] | 324 | 1 | | 32 | 1 | | 2 | 1 | |
| Extractable iron[b] | 301 | 1 | | 28 | 1 | | 4 | 1 | |
| Total iron[c] | 407 | 2 | ±10 | 124 | 2 | ±2 | 111 | 2 | ±2 |

[a]Extractable iron determined by collecting acid washings from 1st, 4th and 8th wash repetitions, respectively, using normal acid-wash procedure (13) and reading each on atomic absorption spectrophotometer.

[b]Extractable iron determined by adding 32 ml $HNO_3/HCl/H_2O$ 1:1:2 to 8 g silica, stirring the slurry over a boiling hot water bath, settling 30 min, centrifuging 15 min, decanting supernatant and reading on atomic absorption spectrophotometer.

[c]Total iron determined by total solution of silica in $HF/H_2SO_4$ in platinum crucible, total evaporation of $SiF_4$ and acids, solution of residue in 10 ml 10% HCl and reading on atomic absorption spectrophotometer.

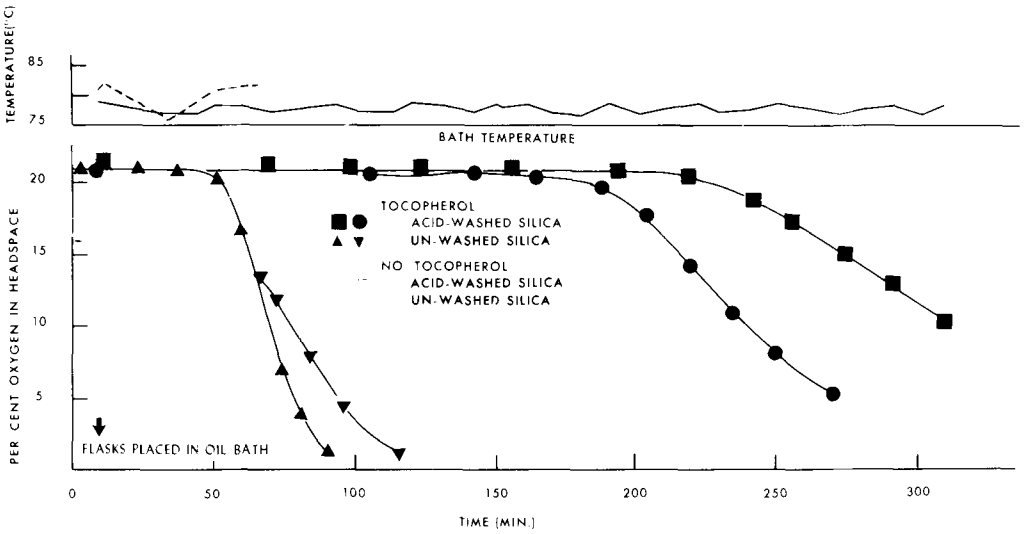


FIG. 3. Effect of acid wash of silica on autoxidation of linoleic acid with and without 0.05 mole per cent tocopherol and deposited as monolayer on silica. Ratio of tocopherol to linoleic acid 0.049 mole per cent.

tion as above. When amounts of combined adsorbed fatty acid and synergists were being measured, the extracted synergist acid was determined by identical titration methods on a sample not coated with lipid. The number of equivalents of synergist were subtracted from total equivalents (both normalized to 1 g uncoated silica) to obtain fatty acid equivalents.

Measurement of extractable and total metal content of silica was by flame atomic absorption spectrophotometry after extraction in $HCl/HNO_3/H_2O$ 1:1:2 v/v or by silica solution in $HF/H_2SO_4$ by the method of Trent and Slavin (16). A Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer was used.

## RESULTS AND DISCUSSION

### Adsorption of Linoleic Acid on Silica

In general, adsorption from solution onto silica is not found to lead to layers more than one molecule thick, although there are exceptions, as noted by Brooks (14). In all the exceptions, multilayer films were formed only on activated carbons, and typically from binary solutions. O'Connor (17) found evidence from the wetting of flamed silica plates that such a surface was covered with a double layer of cetyl trimethyl ammonium bromide after dipping into solutions at concentrations above the critical micelle concentration.

The theoretical treatment of adsorption from solutions at equilibrium is still only partly satisfactory, but Brooks (14) has found that the Langmuir isotherm is well obeyed by long chain fatty acid adsorption, giving a value of the weight ratio for the theoretical saturated monolayer and for the equilibrium concentration at half-saturation.

We found that the gravimetric method of assessing fatty acid concentration in the equilibrium solution gave results in good agreement with the method of extraction and titration at the two lower equilibrium solution concentrations (Table I). A plot of linoleic acid adsorption in two of several reproducible experiments is shown in Figure 1. The same figure also shows the data plotted in the Langmuir isotherm convention (14, p. 532). The slope and intercept of the best fitting line for the lowest four points of experiment A gave a value of 230 mg/g as the monolayer weight ratio and $8 \times 10^{-4}$ M as the half-saturation equilibrium solution concentration. The data of experiment B gave a value of 250 mg/g and $2 \times 10^{-3}$ M.

At equilibrium concentrations above the lowest two, Table I shows that the coated silica after solvent removal holds substantially more linoleic acid than while immersed in the solvent. About 1-2 ml of solution is entrapped after decantation of the supernatant solution. This contributes significant amounts of non-equilibrium adsorption of any lipid at higher concentrations. For this reason we have used in most studies an initial concentration of 10 mg/ml, similar to the second to the lowest of Table I. Although only ca. 75% of a saturated monolayer is adsorbed, there is little risk of nonhomogeneous adsorption and multilayer formation.

TABLE IV

Decrease in Rate of Autoxidation of Linoleic Acid Monolayer on Silica Due to Acid Synergist with and without Antioxidant[a]

| Autoxidation rate and induction period | I Ascorbic acid[b] on unwashed silica | | | II Ascorbic acid[b] on silica washed by one repetition of acid-wash procedure | | | III Ascorbic acid[b] on silica washed by two repetitions of acid-wash procedure | | | IV Citric acid[c] on silica washed by two repetitions of acid-wash procedure | | | V Phosphoric acid[d] on silica washed by two repetitions of acid-wash procedure | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean, min | N[e] | Mean deviation | Mean, min | N[e] | Mean deviation | Mean, min | N[e] | Mean deviation | Mean, min | N[e] | Average error | Mean, min | N[e] | Mean deviation |
| Control: time to consumption of 56% available oxygen without synergist or antioxidant | 35 | 2 | ±16 | 42.6 | 5 | ±11.6 | 55 | 2 | ±6 | 55 | 1 | | 28 | 1 | |
| Per cent decrease in rate with synergist added | 4.5 | 2 | ±0.5 | 45.5 | 5 | ±8.2 | 63 | 2 | ±2 | 45 | 1 | | 47 | 1 | |
| Control: induction period (time to reach headspace oxygen reading of 20.0% with antioxidant[a] but without synergist) | | | | 174 | 1 | | 130 | 2[f] | ±54 | 114 | 2[f] | ±14 | 75 | 2[f] | ±12 |
| Per cent increase in induction period with synergist added | | | | 12 | 1 | | 93 | 2[f] | ±23 | 429 | 2[f] | ±105 | 197 | 2[f] | ±76 |

[a]Tocopherol concentration: 0.05-0.09 mole per cent of linoleic acid concentration in initial solution.

[b]Ascorbic acid concentration: 1.6-1.8 mole per cent of linoleic acid concentration in initial solution.

[c]Citric acid concentration: 1.4 mole per cent of initial linoleic acid concentration.

[d]Phosphoric acid concentration: 2.7 mole per cent of initial linoleic acid concentration.

[e]Number of experiments with two replicate samples per experiment where replicates deviate $<10\%$.

[f]The two experiments, results of which are combined in this figure, were done 3 years apart on different batches of silica, both washed by two repetitions of the standard acid-wash procedure.

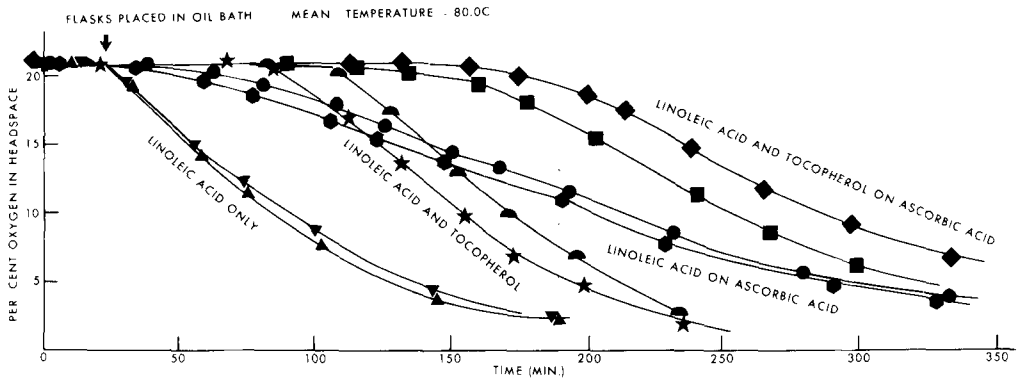


FIG. 4. Synergism of ascorbic acid in the autoxidation of linoleic acid containing 0.05 mole per cent tocopherol deposited as monolayer on silica. Molar ratio of tocopherol to linoleic acid 5.15 x $10^{-4}$. Ascorbic acid to linoleic acid 1.67 x $10^{-2}$.

#### Effect of Acid Synergists and Solvent on Adsorption of Linoleic Acid

At the surface concentrations of the acid synergists used in this study (2-4 mole per cent of the linoleic acid), Table II shows that they had a measurable but small effect on adsorption of the linoleic acid. Ethanol alone also has some effect. Since ethanol was adsorbed onto silica in the control samples for experiments where synergists were adsorbed from ethanol, the decrease in oxidation rate due to reduced linoleic acid adsorption could be no more than 2-7% (the difference between adsorption reduction with and without synergist). The oxidation rate data have not been corrected for these small amounts, since rate changes observed were proportionately much greater. The effect of $Na_2EDTA$ on fatty acid adsorption was not studied, but oxidation studies on silica coated with either $Na_2EDTA$ or citric acid showed equal maximum oxygen uptake.

#### Determination of Lipid-Solid Ratio for Maximum Autoxidation Rate

In order to confirm in our system the finding of Honn et al. that there exists a surface concentration which gives maximum autoxidation rate on silica, silica was coated by two different methods to produce the weight ratios of silica and linoleic acid shown in Figure 2. Oxygen uptake was studied at 62 ± 0.5 C for method a and 59 ± 0.5 C for method b.

In the silica monolayer system, linoleic acid without antioxidant begins an immediate, nearly linear uptake of oxygen at 80 C (Figs. 3,4,7). This rate gradually diminishes in a quasi-first order manner. It is designated below as the rapid rate and is reported in most of these studies as *tau*, time to consumption of 56% of available headspace oxygen, or a headspace oxygen reading of 10.4%.

However for the maximum rate studies we plotted time elapsed to 25% uptake of available oxygen, the linear portion of uptake. For method a (variable linoleic acid charge) maximum rate is at a surface concentration of 175 mg/g. Maximum rate for method b (variable silica load) is at 100 mg/g. This difference is not surprising, because the second method involves much nonequilibrium adsorption. The value from the first method is similar to that obtained in several previous experiments and is preferable.

The finding of a maximum rate at a concentration close to that found for the predicted monolayer for linoleic acid supports the finding of Honn et al. (10) that soybean oil has a very low oxidation rate at very low ratios of lipid to silica. Honn et al. make the hypothesis that these low rates at low loadings are caused by interruptions in the monolayer, with resultant chain termination. This hypothesis suggests that isolated soybean or linoleic acid molecules are relatively anchored, since migrational mobility would permit chain propagation.

If chain propagation is inhibited or prevented at very low loadings of linoleic acid on silica, the system would offer a unique opportunity to study the initiation reaction.

#### Effect of Metal Ion Concentration on Autoxidation Rate

Since new chain generation by peroxide decomposition is known to be partially a function of metal catalysis, we analyzed the silica for both extractable and total iron content. Total copper content of unwashed silica was found to be below 1 ppm, and no further analysis was made for it. (Copper has been found to be at the most 10 times as active as iron in peroxide decomposition [20]).

Table III shows the results. About 100 ppm

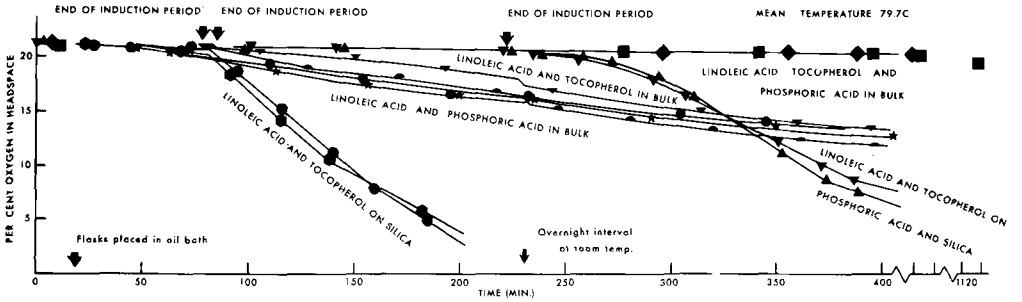


FIG. 5. Synergism of phosphoric acid in the autoxidation of linoleic acid containing 0.05 mole per cent tocopherol deposited as monolayer on silica. Molar ratio of tocopherol to linoleic acid 5.14 x $10^{-4}$. Phosphoric acid to linoleic acid 2.53 x $10^{-2}$.

of iron are not extractable out of a total of 400 ppm in unwashed silica. From 2-4 ppm iron remain extractable from the silica after exhaustive washing.

Autoxidation rate of linoleic acid alone on acid washed silica (Fig. 3) is 40% of that on unwashed silica. With tocopherol present in the linoleic acid monolayer, a very low uptake of oxygen occurs during a tocopherol-dependent induction period. The end of this period is taken in these studies as a headspace reading of 20.0% or an uptake of ca. 5 mole per cent of available oxygen. Figure 3 also shows with data from another experiment that this induction period is lengthened more than four-fold by acid washing. These effects have been repeated in several experiments, although the extent varies between different batches of silica.

In a gravimetric study on four samples, acid washing caused a mean increase of 6% in the amount of linoleic acid adsorbed on silica from similar concentrations in solution, for which Figure 2 has not been corrected. The effect of correction would be to increase the difference, since corrected rates would be lower and induction periods longer.

As will be discussed below, acid washing also has a large effect on the extent of rate reduction and induction period increase caused by ascorbic acid.

We assume that the effect of acid-wash is largely upon iron since there is at least 400 times as much iron as copper and most organic pro-oxidants would not be much affected. If valid, the assumption implies that metal ion promotion of peroxide decomposition with resultant new chain generation is effective in a dry medium where both metal and lipid are presumably anchored and can migrate little.

### Effect of Acid Synergists on Autoxidation Rates

When used without antioxidant, all the acid synergists tested in the silica system reduced rapid autoxidation rate without producing an induction period. They all increased induction period when antioxidant was present, if the silica was sufficiently acid-washed (Table IV).

Ascorbic acid was the more intensively studied (Fig. 4). It greatly reduced rapid rate, but only if the silica was acid-washed as the first two columns of Table IV show. The effect on induction period is even more sensitive to acid washing (columns II and III, Table IV). Virtually no induction period effect is pro-

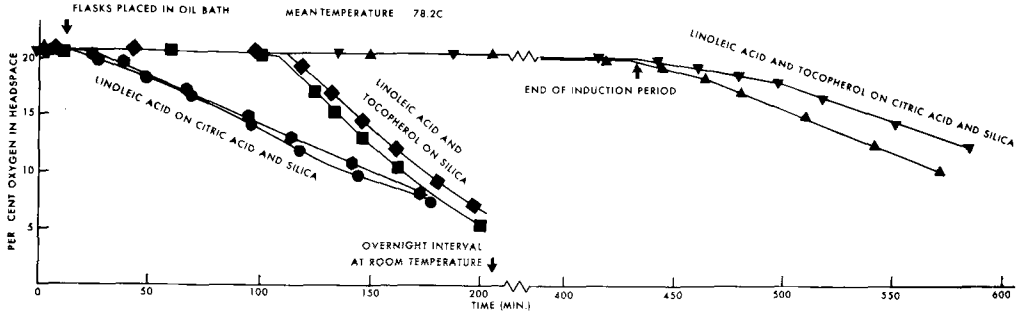


FIG. 6. Synergism of citric acid in the autoxidation of linoleic acid containing 0.05 mole per cent tocopherol deposited as monolayer on silica. Molar ratio of tocopherol to linoleic acid 5.10 x $10^{-4}$. Citric acid to linoleic acid 1.43 x $10^{-2}$.

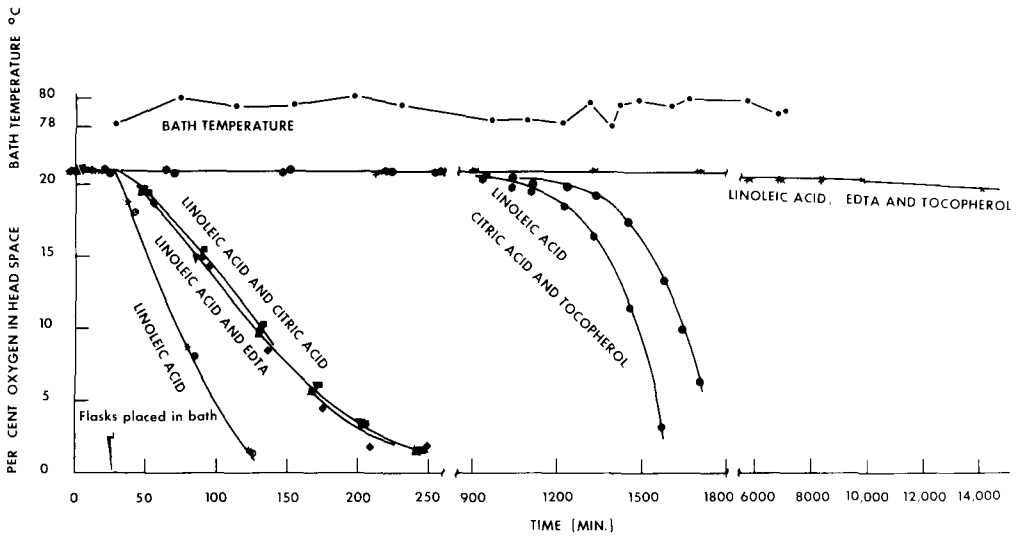


FIG. 7. Comparative synergism of citric acid and $Na_2EDTA$ in the autoxidation of linoleic acid monolayer containing 0.06 mole per cent tocopherol. Molar ratio of tocopherol to linoleic acid 5.6 x $10^{-4}$. Citric acid to linoleic acid 1.45 x $10^{-2}$. $Na_2EDTA$ to linoleic acid 1.50 x $10^{-2}$.

duced on unwashed or partially washed silica.

Phosphoric and citric acids (Figs. 5 and 6 and Table IV) produce less effect on rapid rate than ascorbic acid. They are much more effective in increasing induction period, doubling and quadrupling it, respectively. The smaller effect of synergists and antioxidants on oxidation of the bulk oil (not deposited on silica) is also shown in Figure 5.

When the effects of citric acid and $Na_2EDTA$ were compared in an experiment using doubly acid-washed silica without antioxidant (Fig. 7 and Table V), both were found to decrease rapid rate by ca. 50%. However, when antioxidant was added, induction period with citric acid was 17.3 hr at 80 C, whereas with $Na_2EDTA$ it was 170 hr, a 10-fold increase and an extraordinarily long induction period at this temperature. Thin layer chromatography of the ethanol extract of the lipid-coated silica at 140 hr showed virtually no degradation of the linoleic acid. No discoloration or rancid odor whatever could be detected, in great contrast to the contents of the other experimental flasks, which had browning and very rancid odors.

Tables IV and V show that all the synergist acids studied reduce rapid rate by 50-60%. For ascorbic acid, the effect is very much greater after purification of the silica by metal extraction procedures. Since the most highly washed silica still has an appreciable iron content (Table III), the rate reducing effect seems largest at a high ratio of synergist acid to metal.

Thus, in this system the synergist acids by themselves have a pronounced effect on rate. Except for ascorbic acid, this is contrary to their usual lack of pronounced effect alone in other systems (18, but note 19 and 20), which is the reason for their designation as synergists with the chain terminating antioxidants. Since with ascorbic acid, acid washing of silica is necessary before this effect is seen, it may be that the lack of effect in the usual system is due to the relatively high concentration of metal prevailing in most refined oils (21). In effect this may nearly totally complex the synergist, in the presence of a residual, uncomplexed metal ion concentration sufficient to maintain a maximum new chain generation rate, so that a step not affected by metal ions becomes rate-limiting. A similar ineffectiveness of ascorbic acid and a maximum autoxidation rate, at concentrations of added iron above 2 ppm, was found by Morris et al. (20).

All of the synergist acids studied also increase antioxidant-dependent induction period. In the order of effectiveness they rank: ascorbic < phosphoric < citric <<< $Na_2EDTA$. This order is somewhat similar to that of their stability constants or solubility products (phosphoric acid) with ferric or cupric ion in buffered aqueous solution (22).

Thus both acid washing and the synergist acids reduce rapid autoxidation rate and increase induction period. These effects are consistent with a hypothesis that in the dry silica system, where the lipid is presumably anchored, sequestration of metals in the higher oxidation

TABLE V

Comparative Effect of Citric Acid and Disodium Ethylenediaminetetraacetic Acid on Linoleic Acid Monolayer Autoxidation

| Time | Control | | | Citric acid[a] | | | $Na_2EDTA$[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean, min | N | M.D.[c] | Mean, min | N | M.D.[c] | Mean, min | N | M.D.[c] |
| Time of consumption of 56% available oxygen without antioxidant | 45 | 2 | ±1 | 100 | 2 | ±4 | 96 | 2 | ±2 |
| Induction period[d] (time to reach headspace oxygen of 20.0% with antioxidant) | | | | 1,033 | 2 | ±90 | 10,170 | 2 | ±1,800 |

[a]1.45 mole per cent of linoleic acid in adsorbing solution. Doubly acid-washed silica.

[b]1.50 mole per cent. Doubly acid-washed silica.

[c]Mean deviation.

[d]Tocopherol present at 0.06 mole per cent of linoleic acid in adsorbing solution.

state (23) occurs during the period of synergist adsorption from alcohol or water solution.

However, in aqueous solution, stability constants and solubility products often involve several equilibria. To extrapolate their relative magnitudes and effects on redox potentials to another solvent, alcohol or lipid, is dubious. It is probable that in any system, ascorbic acid has much less affinity for metal ions than EDTA. It is also much less effective as a synergist. On the other hand, from solubility and stability constant data, one would expect phosphoric acid to reduce effective iron concentration more than citric acid. Citric acid, however, is the more potent synergist. In addition, the rapid rate reduction is quite similar for all the synergists studied, whereas the induction period increase differs greatly. If synergists act by metal sequestration, it follows that metal-induced new chain generation must affect autoxidation rate much more at low peroxide values (induction period) than at high (during rapid rate). Nonetheless the dry, presumably anchored surface phase of a monolayer on silica is unattractive for other proposed mechanisms of synergist activity. For example, evidence has been presented (24,25) for the view that synergists directly inhibit the "pro-oxidant" activity observed during the induction period with high concentrations of primary antioxidants (26). We have also observed this activity at 4 mole per cent tocopherol concentration (13), but we feel that the postulated antioxidant catalysis of peroxide decomposition (25) may be more simply explained by "reduction activation" of hydroperoxide (27) by trace metals themselves previously reduced by the antioxidant during lipid adsorption. The reduction of ferric iron in organic solvents by tocopherol, for example, is well known.

Ascorbic acid is a special case, since in aqueous solution it is known to be a reducing agent as well as an acid and a metal sequestrant. Depending on the conditions, it also can act either as a pro- or an antioxidant (28,29). In general, the pro-oxidant effect occurs in aqueous solution and is considered to depend on its reduction of iron or copper ions, which in the reduced form accomplish peroxide "activation" (27,30). The active form of the molecule is the anion or dianion, the neutral acid being inactive (31). Since in the present system water as a phase is precluded, it is suggested that here the sequestering action during adsorption is the basis for rate reduction and synergism.

We found no evidence for an induction period and hence for a primary antioxidant, reduction or chain-terminating function for ascorbic acid in our system, nor for a pro-oxidant action. The effect on both rapid rate and induction period was similar to that for the other acid synergists, differing only in extent. It appears likely that the reason that ascorbic acid does not function as a chain-terminating hydrogen donor in lipid oxidation is that its oxidation requires the anion that is stabilized in aqueous solution and not in the nonpolar lipid phase. The unusual characteristics of ascorbic acid emphasize the fact that all four of the synergists studied are qualitatively alike in activity yet differ greatly in chemical nature. They have in common only the fact that their solutions are acid and that they are metal sequestrants. Since in our system water activity is very low and since even in water solution $Na_2EDTA$ gives a pH between 4.3 and 5.3 (32), only very weakly acidic, any inhibitor action based on acidity alone seems unlikely. Also,

Dutton et al. (19) have shown that nonacidic metal sequestrants like sorbitol have an effect similar and equal to citric acid.

We therefore feel that our data, and in particular the dependence of both the rapid rate reduction and the induction period synergism of ascorbic acid on acid washing of the silica, are consistent with synergist sequestration of metal ions during both rapid phase and induction period. In totally different systems, both Morris et al. using liquid lard (20) and Dutton et al. using soybean oil (19) found similar effects of the acid synergists on rapid rate as well as on induction period. The conclusions of both are summed up by the latter's statement, "These data and other data presented here pose the question of whether many of the so-called synergists may not display synergistic effects in part because of their ability to complex pro-oxidant catalyst [metals] as well as because of their 'acid activation' of 'inhibitols' [primary antioxidants]."

## ACKNOWLEDGMENTS

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# Arachidonic and Eicosapentaenoic Acids in Developing Gametophores and Sporophytes of the Moss, *Mnium cuspidatum*

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**ABSTRACT**

The fatty acid composition of different parts of the moss, *Mnium cuspidatum*, which contains up to 35% arachidonic acid in its lipids, was studied through the annual cycle and especially during the period of rapid development of the reproductive parts. The content of 20:4ω6 was highest in summer and lowest in winter; but for 20:5ω3, the reverse was found. Levels of the acids, 20:5ω3, 18:3ω3 and 16:3ω3 showed parallel fluctuations through the seasons of the year, and functionally they may substitute for each other. In contrast, 20:4ω6 is at a high level when 18:2ω6 is low. The latter acid accumulates in storage or dormant tissue and may be a reserve to form arachidonic acid for specific requirements in cell membranes when rapid growth resumes.

Plants are generally limited in their synthesis of highly unsaturated ω6 and ω3 acids to the $C_{18}$ chain length, but lipids of mosses, liverworts and ferns contain arachidonic and eicosapentaenoic acids at levels up to 40% of total fatty acids. Extensive investigations have indicated that linolenic acid has structural functions in chloroplasts of higher plants (1,2), while in animals the highly unsaturated acids are important constituents of other cell membranes (3). Little effort has been made to explore the function of 20:4 and 20:5 acids in spore-producing plants, although such studies would be likely to contribute to the understanding of lipid functions in both seed plants and vertebrate animals.

Work in this direction has been initiated in this laboratory. Preliminary data indicated that *Mnium cuspidatum* has a particularly high content of arachidonic acid. The fatty acid composition of different tissues of this and other lower plants has been reported recently (4). We are describing here the seasonal changes of fatty acid composition in *M. cuspidatum*. From the data of one annual cycle, it became apparent that changes in fatty acids are most drastic from April to June at the time when the reproductive parts of the moss are developing rapidly. Analyses of fatty acids in gametophores and sporophytes were then made at close time intervals. Lipid classes and their fatty acid composition will be the topic of a subsequent publication.

## EXPERIMENTAL PROCEDURES

Mats of *M. cuspidatum* covering ca. 2 $ft^2$ were cut from one location at the Jay C. Hormel Nature Center, Austin, Minn. Good replication of values from samples of the same date in different years and from duplicate harvesting on the same day showed that the sampling error does not affect the overall results.

The state of development of the plant tissues was determined by examination with a dissecting microscope. Gametophores and sporophytes

TABLE I

Fatty Acids in Gametophore Lipids of *Mnium cuspidatum*[a]

| Fatty acid | Calendar month, 1970 | | | | | | | | 1971 |
|---|---|---|---|---|---|---|---|---|---|
| | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 3 |
| 16:0 | 18.5 | 18.3 | 14.2 | 20.0 | 19.7 | 16.5 | 16.5 | 14.3 | 16.1 |
| 16:1 | 1.1 | 0.9 | 1.5 | 0.7 | 1.3 | 1.1 | 1.4 | 1.9 | 1.9 |
| 16:3ω3 | + | + | + | + | 2.0 | 2.3 | 5.0 | 6.1 | 5.2 |
| 18:0 | 3.6 | 2.9 | 1.6 | 1.0 | 0.8 | 0.7 | 0.8 | 1.1 | 0.9 |
| 18:1 | 7.4 | 7.8 | 9.6 | 5.7 | 4.2 | 6.0 | 4.9 | 5.1 | 2.3 |
| 18:2ω6 | 7.4 | 8.2 | 9.5 | 9.7 | 9.8 | 11.9 | 13.6 | 14.3 | 8.0 |
| 18:3ω3 | 14.0 | 14.3 | 12.3 | 12.9 | 16.4 | 19.8 | 20.6 | 21.7 | 22.6 |
| 20:4ω6 | 28.2 | 35.3 | 33.6 | 36.2 | 32.7 | 28.2 | 24.1 | 19.7 | 24.8 |
| 20:5ω3 | 10.4 | 6.0 | 5.3 | 7.9 | 7.8 | 9.9 | 9.8 | 11.2 | 13.2 |

[a]Per cent of total fatty acids.

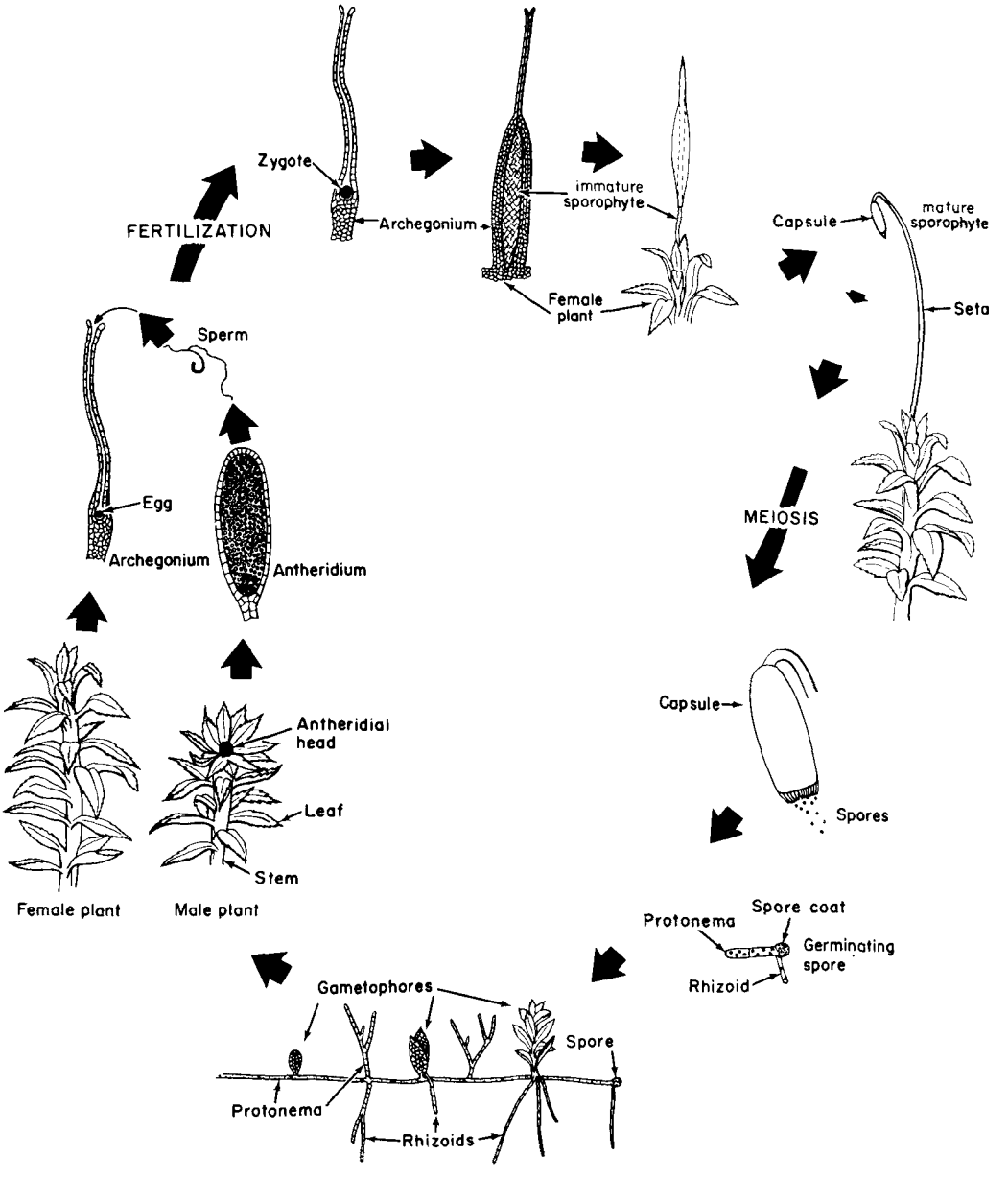


FIG. 1. The life cycle of *Mnium cuspidatum*. (Reproduced from "Methods in Developmental Biology," 1967, courtesy W.T. Doyle and publisher, T.Y. Crowell Co., N.Y.)

were separated from the rest of the material and weighed immediately in screw-cap vials. Chloroform-methanol 2:1 v/v was then added. Tissue could be stored in this solvent at -17 C for several months without marked change in lipid or fatty acid composition. For extraction, the tissue was homogenized with this solvent in a Sorvall Omni-mixer. An aliquot of the slurry was taken to dryness to determine moisture content of the tissue by difference from its fresh weight. The main portion of the slurry was filtered and the fiber re-extracted with new solvent. Filtrates were shaken with 0.2 volumes of aqueous 0.9% NaCl, and the lipids recovered from the chloroform phase by evaporating the solvent in a Rotovac at less than 40 C. Traces of water were removed by high vacuum or by adding methanol for repeated evaporation. Lipids were weighed in teflon-lined screw-cap tubes and stored in chloroform or chloroform-

TABLE II

Fatty Acids and Other Components in Developing Gametophores

| Month/day | 4/5[a] | 4/16[b] | 4/23 | 4/30 | 5/7 | 5/14 | 5/21[c] | 5/28[c] | 6/4[d] | 6/11[d] | 6/18[e] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Water, % of fresh wt | 32 | 71 | 76 | 74 | 71 | 71 | 75 | 74 | 78 | 83 | 81 |
| Lipid, % of dry wt | 4.6 | 5.3 | 6.6 | 5.2 | 5.4 | 5.7 | 5.4 | 5.5 | 5.7 | 5.5 | 5.3 |
| Chlorophyll, % of lipid | 8.6 | 5.2 | 6.1 | 6.1 | 5.6 | 5.2 | 8.3 | 8.2 | 9.7 | 9.1 | 10.8 |
| Fatty acid, % | | | | | | | | | | | |
| 16:0 | 17.7 | 20.2 | 19.9 | 20.5 | 21.4 | 19.9 | 18.1 | 18.5 | 15.6 | 15.5 | 17.2 |
| 16:1 | 1.6 | 1.4 | 1.1 | 1.2 | 1.1 | 0.9 | 1.2 | 1.3 | 1.3 | 1.1 | 1.2 |
| 18:0 | 0.8 | 3.5 | 3.8 | 3.7 | 3.9 | 3.4 | 2.9 | 3.5 | 3.0 | 3.3 | 2.6 |
| 18:1 | 9.4 | 8.9 | 7.6 | 7.6 | 6.6 | 6.2 | 6.0 | 6.4 | 5.4 | 5.9 | 5.8 |
| 18:2ω6 | 10.2 | 6.9 | 9.6 | 6.6 | 8.8 | 8.3 | 7.9 | 7.1 | 9.4 | 9.0 | 10.0 |
| 18:3ω3 | 22.8 | 15.1 | 15.6 | 14.8 | 13.9 | 13.9 | 16.0 | 14.7 | 15.7 | 13.0 | 12.8 |
| 20:4ω6 | 21.5 | 26.7 | 27.0 | 26.7 | 27.0 | 30.2 | 31.8 | 29.0 | 30.7 | 30.6 | 30.3 |
| 20:5ω3 | 9.6 | 5.2 | 7.0 | 5.7 | 5.4 | 4.5 | 5.4 | 5.1 | 4.7 | 4.1 | 4.3 |

[a]Gametophores from 1970.
[b]Developing gametophores, 1971.
[c]Sperm and egg developed.
[d]Zygote formed.
[e]Stoloniform shoots appearing.

methanol 2:1 under $N_2$ at -17 C. Chlorophyll was determined spectrophotometrically at 652 mμ (5) in a Bausch and Lomb Spectronic 20 and was calculated as weight per cent of lipid.

Fatty acid compositions were determined from samples of at least 10 mg lipids. After saponification and extraction of unsaponifiables with petroleum ether (bp 30-60 C)-diethyl ether 1:1, the acids were recovered and esterified with diazomethane (6). The methyl esters were purified from pigments and other contaminants by thin layer chromatography on Silica Gel H, 0.5 mm thick, with petroleum ether (bp 60-70 C)-diethyl ether-acetic acid 80:20:1. Gas liquid chromatography (GLC) of the esters was carried out on ethylene glycol succinate (Applied Science Laboratories, Inc., State College, Pa.) and on cycloheptaamylose propionate (7). The former phase was superior for most separations, but superpositions occurred with 16:3 and 18:1, as well as with 20:5 and 24:0. The latter phase resolved these pairs. Cross reference of equivalent chain lengths and peak areas afforded identifications and quantifications. The results were checked by GLC of hydrogenated aliquots.

## RESULTS

Arachidonic acid in gametophores is at the lowest level in midwinter, increases during early spring and nearly doubles its level by midsummer (Table I). Linoleic acid, a potential precursor of arachidonic, decreases from December to March, while arachidonic acid increases. Linolenic acid is lowest in summer, increases in autumn and stays at high values during the winter months. Eicosapentaenoic acid essentially follows the pattern of linolenic acid but at a lower level.

Fatty acids, water and chlorophyll were determined at weekly intervals from April to June (Table II). New gametophores appeared in mid-April, antheridium and archegonium developed in May, and fertilization of the egg took place in early June. Stoloniform shoots appeared later in June. Figure 1 explains these details in the life cycle of the moss.

Water content of growth from the preceding year (sampled April 5) is less than half of that in the new growth (April 16), and in the latter it increases gradually over the whole period into June. A high level of chlorophyll was retained through the winter (April 5). Chlorophyll in the new growing gametophore is relatively low, but reaches the higher level by the end of May. The amount of lipid remains rather constant between 5 and 6% dry wt.

New gametophores contain more arachidonic but less linolenic and eicosapentaenoic acids than gametophores that had developed before the winter. The level of arachidonic acid rises to 30% of total fatty acids before sperm and egg cells are detectable (May 21). This level of arachidonic acid remains constant from mid-May to mid-June, during which period chlorophyll doubles. Linoleic, linolenic and eicosapentaenoic acids are, in contrast to arach-

TABLE III

Fatty Acids and Other Components in Developing Sporophytes

| Month/day | 11/16[a] | 4/16[b] | 4/23 | 4/30 | 5/7 | 5/14[c] | 5/21[d] | 5/28[d] |
|---|---|---|---|---|---|---|---|---|
| Water, % of fresh wt | | 72 | 79 | 81 | 75 | 85 | 84 | 81 |
| Lipid, % of dry wt | 5.1 | 4.8 | 4.4 | 5.5 | 4.2 | 9.6 | 3.9 | 3.7 |
| Chlorophyll, % of lipid | 1.7 | 1.3 | 1.9 | 1.5 | 1.6 | 3.4 | 0.6 | 0.8 |
| Fatty acid, % | | | | | | | | |
| 14:0 | 1.8 | 3.6 | 2.9 | 2.7 | 3.3 | 1.5 | 5.5 | 5.4 |
| 16:0 | 16.9 | 27.6 | 28.0 | 32.1 | 35.5 | 20.6 | 41.1 | 42.1 |
| 16:1 | 1.0 | + | + | + | + | 0.5 | + | + |
| 16:3 | + | 3.5 | 3.4 | 3.8 | 5.5 | 3.6 | 3.3 | 4.1 |
| 18:0 | 3.0 | 2.0 | 1.8 | 1.4 | 0.6 | 0.5 | 1.9 | 2.3 |
| 18:1 | 14.7 | 6.0 | 4.3 | 2.8 | 1.2 | 6.0 | 5.5 | 2.0 |
| 18:2ω6 | 11.9 | 9.5 | 10.2 | 12.6 | 13.8 | 29.6 | 13.5 | 11.4 |
| 18:3ω3 | 12.4 | 12.2 | 11.4 | 14.2 | 13.4 | 16.4 | 8.8 | 8.7 |
| 20:4ω6 | 23.0 | 23.5 | 24.3 | 20.6 | 15.4 | 11.8 | 11.8 | 13.2 |
| 20:5ω3 | 4.3 | 1.8 | 1.3 | 1.2 | 0.5 | 1.4 | -- | 0.7 |
| 24:0 | 4.3 | 6.0 | 8.5 | 4.2 | 3.7 | 1.5 | 4.1 | 4.8 |

[a]Immature sporophyte developed before winter season.

[b]Maturing sporophytes.

[c]Spores have matured and are ready for dispersal.

[d]Seta and capsules with some residual spores.

idonic acid, at a higher level in old growth than in new.

Sporophytes of *M. cuspidatum* (Fig. 1) in this area begin development after fertilization and are visible on the female plant by September. Without apparent change during the winter, they begin growth again in April. Both the lipid and chlorophyll levels rise sharply with maturation of the spores (Table III). After dispersal of the mature spores, analysis of the capsules showed that they contain much less lipid and chlorophyll than the spores.

The fatty acid composition of sporophytes changes greatly during their development (Table III). During April, before separation of spores from capsules, arachidonic acid receded from 24% in the immature sporophyte to ca. 15%. Spores contain much more lipid than capsules, but arachidonic acid is at equal percentage in both. In contrast to 20:4, the acids 18:2 and 18:3 increase slightly during development of the sporophytes, but during the last week before dispersal the level of linoleic acid is doubled. Eicosapentaenoic acid is relatively high before winter, but decreases during the development in spring.

## DISCUSSION

Arachidonic acid is present in all species so far analyzed of the classes *Hepaticae* (liverworts), *Musci* (mosses), *Lycopsida* (club mosses) and *Filicineae* (ferns) (4,8-15), mostly at a level of 5-10% of total fatty acids. However up to 35% arachidonic acid has been found in *Mnium cuspidatum*. Because of this and its local availability, *M. cuspidatum* had been selected for more intensive study. Arachidonic acid was found in all tissues of this moss, including rhizoids, but was highest in gametophores and stoloniform shoots(4).

The chloroplasts of these lower plants are known to be different from those of higher plants in their mode of reproduction (16) and in their resistance to extreme conditions that would disrupt chloroplasts of higher plants (17). Photosynthetic acitivity in *M. cuspidatum* is mainly in gametophores and stoloniform shoots. Arachidonic acid is at a particularly high level in these parts of the plant. Arachidonic acid has also been identified in lipids of isolated chloroplasts from several mosses and ferns (12) and may be essential for the properties mentioned above. The samples of *M. cuspidatum* analyzed here show a relatively high content of chlorophyll during winter when the level of arachidonic is at 20% of total acids. The acid is present also in mature spores where the reproduction of chloroplasts is particularly important. However, in immature gametophores, arachidonic acid increases from ca. 20% to 30% before chlorophyll rises from 5% to 10%. The data are in agreement with the concept that arachidonic acid is associated with the photo-

synthetic apparatus, but they indicate also that this is not the only function arachidonic acid may have.

Other functions are likely in view of the occurrence of 10% arachidonic acid in rhizoids of *Mnium* (4) which do not contain chlorophyll. In addition, arachidonic acid is at equal level in lipids of spores and capsules. The former have a high amount of chlorophyll, in reference to lipids, whereas the latter have a much lower amount. Stoloniform shoots do not have any role in sexual reproduction, but contain arachidonic acid at the same level as the gametophores (4). Therefore arachidonic acid may be correlated, not only with photosynthesis and reproduction, but also with other properties characteristic for these plants, such as resistance to extreme environmental conditions.

Linoleic acid is accumulated in the gametophore in later summer and fall. The high level is maintained over winter, and, similarly, the acid serves in the spring to produce 20:4ω6 during the time of rapid cell division. The level of 20:4ω6 is 30% or more throughout the summer, while 18:2ω6 is low. During this period, gametophores are developing rapidly, antheridium and archegonium are maturing, fertilization is occurring, and the new sporophytes are beginning to develop. Then, in October when very little new growth is occurring, 20:4ω6 declines as 18:2ω6 increases. Similarily, the sporophytes mature in early spring, and as the spores are formed, 20:4ω6 declines while 18:2ω6 accumulates. The highest level of 18:2ω6 is reached when the spores are mature. As in gametophores, the supply of 18:2ω6 may serve for the synthesis of 20:4ω6 when the latter acid is required for rapid cell division after germination of the spores.

Similar speculations about function may apply to 20:5ω3 acid which occurs at levels between 4% and 13% in *M. cuspidatum*. However a characteristic difference between the ω3 and ω6 acids seems to be that, through the seasons, the amount of 20:5ω3, 18:3ω3 and 16:3ω3 follow a rather similar pattern, and they may substitute for each other. The fluctuations of 20:4ω6 and 18:2ω6 are opposite to each other, and 20:4ω6 may be distinct in its function from 18:2ω6.

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# Toxicity of Fatty Ozonides and Peroxides

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## ABSTRACT

Studies on the acute toxicity of the ozonides and hydroperoxides of methyl linoleate are reported. High purity preparations of these compounds were injected intravenously or administered orally to adult male rats. The lethal dose by iv injection of these compounds was virtually the same—0.07 mmol/100 g body wt. No deaths were caused in a 24 hr period by single oral dosages of these compounds of ca. 10-fold that causing death by the iv route. The major effect of these compounds was on the lungs. The lungs became enlarged from edema and accumulation of fluid, and the animals died of lung congestion and injury similar to the effects of ozone toxicity. There was no destruction of vitamin E in the tissues of animals given lethal dosages of ozonides or peroxides intravenously, but significant changes occurred in fatty acid composition of the lipids of the serum and lung. Arachidonic acid increased at the expense of linoleic and oleic acids in these tissues. Only small amounts of peroxidic and TBA positive substances were detected in lung and serum, indicating that the injected ozonides and hydroperoxides were destroyed in the tissues.

## INTRODUCTION

The toxicity of ozone has been studied extensively because of its importance as an air pollutant (1,2). Long term exposure to low concentrations of ozone (0.1-1 ppm) increases neonatal mortality and causes chronic lung injury in laboratory animals, while exposure to high concentrations (greater than 1 ppm) causes severe lung injury resulting in death from pulmonary edema (3-11). The mechanism of the action of ozone in animal tissues has not been elucidated. It has been suggested (5,12-16) that cell damage occurs via the reaction of free radicals mainly because of similarities to the effects of high energy radiation on animal tissues. Stokinger (5) and Mountain (16) have suggested that free radicals may be generated by the reaction of ozone with sulfhydryl groups. Another hypothesis is that free radicals are produced via lipid oxidation which is catalyzed by the action of ozone (12,17). The latter concept is based mainly on the relationship of a vitamin E deficiency to an increased susceptibility of animals to acute ozone toxicity (18-21).

Ozone reacts instantaneously with double bonds to form ozonides (22). Therefore, because polyunsaturated fatty acids are primary components of phospholipids of lipoproteins, which are important in lipid transport and membrane structure, fatty ozonides may be a primary product of the attack of ozone in animal tissues. The present study was undertaken to assess the toxicity of methyl linoleate ozonide and determine its effect on animal tissues. For comparison, and because lipid oxidation has been implicated in ozone toxicity, studies were also carried out on the

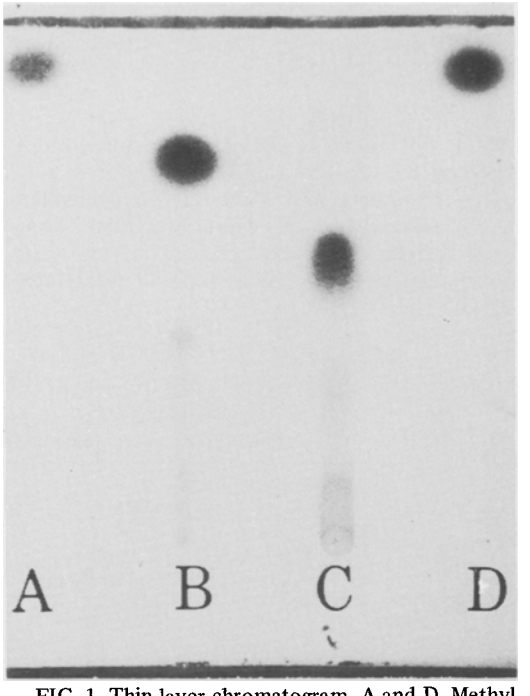


FIG. 1. Thin layer chromatogram. A and D, Methyl linoleate; B, methyl linoleate ozonide preparation; C, methyl linoleate hydroperoxide preparation; adsorbent, Silica Gel G (A.G. Merck, Darmstadt, Germany); solvent system, petroleum ether-ethyl ether-acetic acid 80:20:1.

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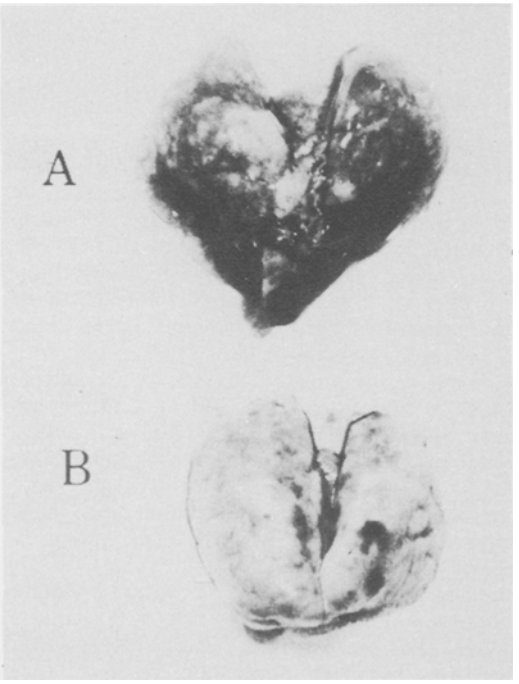


FIG. 2. Lungs. A, Lung from an adult male rat 24 hr after iv injection (tail vein) of 20 mg, per 100 g body wt, of methyl linoleate hydroperoxide emulsified in 0.2 ml of rat serum. B, Lung of an adult male rat (fed a diet containing 10% fresh safflower oil) of the control group.

toxicity of methyl linoleate hydroperoxides. Hydroperoxides are the primary product of lipid autoxidation and, although the mechanism of the action of oxidized fats in animal tissues has not been elucidated, the toxicity of fatty hydroperoxides has been well demonstrated (23-26).

## EXPERIMENTAL PROCEDURES

### Materials and Methods

Methyl linoleate hydroperoxide was prepared from pure methyl linoleate purchased from the Lipids Preparation Laboratory of The Hormel Institute. It was allowed to autoxidize in the dark at 0 C to a peroxide value of ca. 600 me/kg under conditions that give predominately hydroperoxides as described previously (27). Isolation and purification of the hydroperoxides were also carried out as described previously (27). The final preparation had a peroxide value of 6085 me/kg, determined by KI reduction (27), vs. the theoretical value for pure linoleate hydroperoxide of 6135 me/kg; TBA value (28) of the preparation was zero. Thin layer chromatography (TLC) (Fig. 1) also showed that the preparation was pure except for a small amount of material at the origin.

Methyl linoleate ozonide was prepared in batches of 100 mg by reacting pure methyl linoleate with an excess of a pentane solution of 0.3 M ozone at -65 C (29). After a reaction period of 1 min, the excess ozone was removed by bubbling nitrogen through the solution. The purity of this preparation was estimated at greater than 90% as determined by KI reduction (27) and as illustrated by TLC in Figure 1.

Mature male rats, 185-250 g, were purchased from Dan Rolfsmeyer Co., Madison, Wis., and fed ad libitum a sucrose-casein diet containing all of the required minerals and vitamins, and 10% safflower oil (30).

The toxicity of the linoleate ozonide and hydroperoxide preparations was determined by iv injection of these preparations emulsified with rat serum. The volume of each injection was 0.2 ml/100 g body wt. Pure methyl linoleate, injected also in the form of an emulsion

TABLE I

Toxicity of High Purity Methyl Linoleate Hydroperoxide and Ozonide Preparations Injected Intravenously[a] into Adult Male Rats

| Methyl linoleate hydroperoxide preparation | | | Methyl linoleate ozonide preparation | | |
|---|---|---|---|---|---|
| Dose,[b] mg/100 g body wt | Number of animals | Survivors after 24 hr | Dose,[c] mg/100 g body wt | Number of animals | Survivors after 24 hr |
| 22-40 | 8 | 0 | 26-40 | 8 | 0 |
| 20 | 8 | 4 | 25 | 6 | 2 |
| 12-19 | 12 | 12 | 24 | 6 | 3 |
| | | | 18-23 | 9 | 9 |

[a]Injected into the tail vein in 0.2 ml rat serum per 100 g body wt of animals weighing 185-240 g.

[b]1 mmol = 326 mg.

[c]1 mmol = 342 mg.

TABLE II

Analyses of Lung Tissues of Animals Receiving Intravenous Injections[a] of Hydroperoxides or Ozonides of Methyl Linoleate

| Dose | Number of animals | Triglyceride level, mg/g | Weight of lung, g | TBA, OD/g tissue |
|---|---|---|---|---|
| None | 6 | 11.0[b] ±1.7 | 1.58 ±0.21 | 0 |
| Hydroperoxide, iv 18 mg/100 g body wt | 6 | 4.3 ±2.4 | 2.40 ±0.5 | .275 |
| Ozonide, iv 23 mg/100 g body wt | 4 | 5.9 ±1.3 | 2.60 ±1.0 | 0.250 |

[a]Injected into the tail vein in 0.2 ml rat serum per 100 g body wt of animals weighing 185-200 g.

[b]M ± SD.

with rat serum, was used as the control and was innocuous.

Tissue and serum lipid analyses were carried out on animals sacrificed by withdrawal of blood from the aorta 24 hr after receiving sublethal doses of ozonides or hydroperoxides. The lipid was extracted with chloroform-methanol 2:1 and recovered after extraction of nonlipid substances (31). Fatty acid composition was determined on methyl esters prepared by interesterification of the lipid with methanol, using HCl as a catalyst (32). These analyses were carried out by gas liquid chromatograph, equipped with a flame ionization detector, and a 6 ft x 1/4 in. column packed with Gas Chrom P, containing 8% EGSS-X (Applied Science Laboratories, Inc.).

Vitamin E was determined by a spectrophometric method (33) using the Emmerie-Engel color reaction on the nonsaponifiable fraction obtained by careful saponification and extraction under an atmosphere of nitrogen. Triglycerides (TG) were determined by the method of Van Handel and Zilversmit (34) which employs the chromotopic acid color reaction. TBA values of serum and hemolyzed red cells were determined as described by Bunyan et al. (35). The method of Donnan (28) was used for the determination of tissue TBA values. This method differs from that employed by Bunyan et al. (35) by using a lower pH.

## RESULTS

The toxicity of the linoleate ozonide and hydroperoxide preparations were similar as illustrated in Table I. The amounts of these preparations, which were acutely toxic when injected intravenously, appeared to be critical inasmuch as even 1 mg more or less appeared to be highly significant. The results in Table I were obtained on the basis of a 24 hr survival time, but the hydroperoxide preparation reacted much faster than the ozonide preparation. Death, when it occurred, was generally within 6 hr after injection of the hydroperoxide preparation. Another difference between the reaction of the peroxide and the ozonide preparations was in the color of the blood. The blood became very dark after injection of lethal doses of the ozonide preparation, indicating a deficiency of oxygen. There was no change in the color of the blood on injection of hydroperoxide preparations, although the animals also suffered the same visible symptoms of respiratory distress as animals injected with the ozonide preparations. Respiratory distress was evidenced by rapid heart beat, morbidity and coughing or gasping. The similarity of the toxic effects of the two preparations suggested a high degree of similarity between the action of the hydroperoxides and the ozonides. Furthermore, the lungs were the only organs visibly affected by the injection of either preparation. A typical lung of animals, injected with the hydroperoxide preparation, is compared with the lung of a normal animal in Figure 2. Although the defective lung shown in Figure 2 was taken from a hydroperoxide injected animal, outwardly it appeared to be the same as those produced by injections of the ozonide preparation. Injection of either the ozonide or hydroperoxide preparation caused development of edema and severe hemorrhages, as shown in Figure 2. The size of the lung was also greatly increased by the toxic actions of the hydroperoxide and ozonide preparations, as shown in Table II. Presumably the increase in size of the lung was caused by edema and accumulation of fluid because the level of TG was decreased (Table II). TBA reacting substances were detected in the lungs of animals injected with either the hydroperoxide or ozonide preparations (Table II). Material giving a positive

TABLE III

Vitamin E Analyses of Tissues of Animals Receiving Intravenous Injections[a] of Methyl Linoleate Ozonides or Hydroperoxides

| Dosage | Lung, μg/g | Liver, μg/g | Heart, μg/g | Serum, μg/ml |
|---|---|---|---|---|
| None | 25[b] ±10.70 | 34 ±5.40 | 50 ±11.80 | 18 ±2.86 |
| Hydroperoxide, iv 18 mg/100 g body wt | 24 ±10.90 | 43 ±8.40 | 50 ± 6.86 | 24 ±4.20 |
| Ozonide, iv 23 mg/100 g body wt | 23 ± 4.10 | 32 ±5.24 | 46 ± 1.69 | 15 ±1.50 |

[a]Injected into the tail vein in 0.2 ml rat serum per 100 g body wt of animals weighing 185-240 g.

[b]M ± SD, six animals.

peroxide value was also detected in the serum of the animals injected with these preparations and in the lungs of animals injected with the ozonide preparation. However it appeared that most, if not all, of the original ozonides and hydroperoxides were destroyed in the tissues.

No deaths were recorded in the 24 hr period subsequent to giving oral dosages by intubation into the stomach of the ozonide or hydroperoxide preparations of ca. 10-fold—those which gave an acute toxicity by the iv route. Gross examination of the organs of these animals revealed no visible effect on the tissues at the end of 24 hr except for the intestines. The intestines were enlarged and appeared irritated. These animals also suffered a noticeable loss of appetite and had diarrhea. However no TBA or peroxide substances were detected in the serum, lungs, hearts or livers of these animals. There also was no destruction of vitamin E in these tissues, and the lungs were normal in size and appearance.

Intravenous injections of the ozonide or hydroperoxide preparations also did not cause any destruction of vitamin E in the tissues (Table III). Accordingly there was no effect of injections of the ozonides or hydroperoxide preparations on the susceptibility of the erythrocytes of these animals to dialuric acid-induced hemolysis carried out as described by Bunyan et al. (35). TBA values of the hemolyzed erythrocytes in these experiments were zero in spite of the fact that the serum contained TBA-positive substances. Likewise there was no effect on the swelling properties of liver mitochondria of these animals, as might have been expected had the stores of vitamin E in the tissues been depleted (36,37).

Changes in fatty acid composition of the lipid of the lung occurred in both the hydroperoxide and ozonide injected animals as shown in Table IV. These results showed that the percentage of arachidonic acid (20:4) in the lung was about doubled by the toxic effects of both the ozonide and hydroperoxide preparations. The increase in 20:4 appeared to occur mainly at the expense of oleic (18:1) and linoleic (18:2) acids. Similar changes of even greater magnitude occurred in the serum of animals injected with the ozonide preparation (Table IV). However the hydroperoxide preparation had little effect on the fatty acid composition of the serum lipids. Oral dosages of the ozonide preparation also caused similar changes in the fatty acid of both the serum and lung lipids.

## DISCUSSION

The present study indicates that the toxicity of fatty ozonides and hydroperoxides injected intravenously is similar in many respects to that produced by ozone. The primary attack occurs on the lung, and death occurs from lung injury as a result of the same symptoms—congestion and hemorrhages. Other symptoms common to ozone toxicity observed in these animals were those of respiratory distress. Although the toxicity of hydroperoxides and ozonides injected intravenously appeared to be the same as that of ozone, in vivo oxidation did not appear to be involved, inasmuch as there was no destruction of vitamin E, and there was no effect on the susceptibility of red blood cells to dialuric acid-induced hemolysis.

These observations are in accord with the studies by Olcott and Dolev (23), which showed that tocopherol or ethoxyquin had no effect on the acute toxicity of hydroperoxides injected intraperitoneally.

Failure to detect appreciable amounts of KI-reducing substances (peroxide values) in the tissues indicated that both hydroperoxides and ozonides reacted with tissue constituents; that no destruction of vitamin E occurred appeared to rule out in vivo oxidation in the tissues of

TABLE IV

Fatty Acid Composition of Lung and Serum Lipids of Animals Receiving Intravenous Injections[a] of Methyl Linoleate, Hydroperoxide or Ozonide Preparations

| Fatty acid composition, wt % | Serum | | | Lung | | |
|---|---|---|---|---|---|---|
| | Control, none | Hydroperoxide, iv 18 mg/100 g body wt | Ozonide, iv 23 mg/100 g body wt | Control, none | Hydroperoxide, iv 19 mg/100 g body wt | Ozonide, iv 23 mg/100 g body wt |
| 14:0 | | | | 2.6 ±0.55 | 3.1 ±0.47 | 2.8 ±0.79 |
| 16:0 | 17.6[b] ±1.13 | 18.7 ±0.98 | 20.3 ±0.37 | 29.2 ±2.00 | 30.0 ±1.89 | 26.6 ±2.13 |
| 16:1 | 3.0 ±0.28 | 2.6 ±0.44 | 1.5 ±1.00 | 6.4 ±0.47 | 6.4 ±0.91 | 11.1 ±3.44 |
| 18:0 | 9.0 ±1.09 | 9.0 ±0.98 | 10.8 ±1.52 | 6.6 ±0.95 | 7.4 ±1.11 | 6.8 ±1.6 |
| 18:1 | 13.8 ±3.70 | 16.1 ±2.17 | 9.8 ±1.82 | 21.6 ±1.70 | 15.3 ±2.83 | 15.6 ±1.44 |
| 18:2 | 38.8 ±3.09 | 34.8 ±3.11 | 26.6 ±4.83 | 25.0 ±2.45 | 21.5 ±1.95 | 20.1 ±3.67 |
| 20:4 | 17.6 ±4.80 | 18.6 ±4.20 | 30.8 ±3.59 | 6.5 ±1.00 | 14.1 ±0.57 | 10.8 ±1.00 |

[a]Injected into the tail vein in 0.2 ml rat serum per 100 g body wt of animals weighing 185-240 g.
[b]M ± SD.

these animals. Likewise in vivo oxidation was not initiated or catalyzed by the injection of ozonides or hydroperoxides. It has been suggested (14,19,20) that in vivo oxidation is initiated by radicals formed by reactions of ozone in the tissues. However the present study shows that the absence of ozonides or hydroperoxides in the tissues does not necessarily mean that they are not formed or that their formation is prevented by biological antioxidants, because obviously they would be destroyed as rapidly as they are produced. Thus, considering that the toxic effects of ozonides are very similar to those of ozone and the rapidity of the reaction of ozone with the double bonds of unsaturated fatty acids, ozonides may be produced in the lung by the action of ozone in the atmosphere. Likewise the formation of hydroperoxides in vivo oxidation cannot be precluded in spite of the fact that vitamin E may function as a biological antioxidant in this reaction. It is significant, however, that the acute toxicity of ozonides or hydroperoxides did not appear to involve the erythrocytes or the liver mitochondria. That these tissues may be targets of the toxic reaction of ozonides and hydroperoxides in vitamin E-depleted animals as in ozone toxicity is under investigation and may provide more definitive information on the role of vitamin E in the protection of these tissues against oxidation.

The toxicity of fatty peroxides has been demonstrated in feeding tests (38-40), as well as by injection or oral administration (24-26) and on ip injection (23). The method of administration might be expected to have a profound influence on the type of toxicity that is manifested. The emphasis in the present study was on iv injection because of the interest in possible relationships with ozone toxicity. Although the toxic effects of oral administration of hydroperoxides, as well as ozonides, were less severe in our studies than in those obtained by Olcott and Dolev (23), the effects appeared to be the same.

No evidence was obtained in the present study on the mechanism of the reaction of ozonides or hydroperoxides in the tissues. However the marked effects on fatty acid composition indicated that they reacted with tissue constituents involved in lipid metabolism. It seems paradoxical that the percentage of arachidonic acid, which is highly susceptible to oxidation, should be increased by the toxic action of ozonides and peroxides, which are strong oxidizing agents. Similar effects on fatty acid composition have been observed in animals depleted of their stores of vitamin E (41-44) and in the toxic action of ozone (21). It appears that, whereas the phenomenon is related to in vivo oxidation under these conditions, in ozonide and hydroperoxide toxicity it is caused by the direct reaction of these compounds inasmuch as there was no destruction of vitamin E in the tissues. Several explanations (21,41,43) have been advanced for this phenomenon. Because the lower unsaturated fatty acids are decreased in reference to arachidonic acid, it would appear that the effect is on the enzyme systems involved in the interconversions of these acids, as opposed to the relative susceptibility of unsaturated fatty acids to chemical oxidation.

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# Long Chain Hydrocarbon Profiles of Duncan Grapefruit, Dancy Mandarin and Their Hybrids

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## ABSTRACT

Saturated and monounsaturated long chain hydrocarbons were determined in Duncan grapefruit, Dancy mandarin and their three hybrids: Seminole, Orlando and Minneola. The four most prominent hydrocarbons in the saturated group were *n*-$C_{23}$ and $C_{25}$, and iso $C_{23}$ and $C_{25}$. In the monounsaturated group Duncan, Dancy and Seminole showed $C_{29}$ as dominant, while $C_{25}$ predominated in Orlando and Minneola. Seminole, Orlando and Minneola accumulated a noticeably higher percentage of branched chain monoenes than either of their parents. The saturated and monounsaturated hydrocarbon profiles of Orlando and Minneola were completely different from their parents.

## INTRODUCTION

This laboratory has recently undertaken an extensive study of the lipid composition of citrus fruits (1-6). During investigations of the long chain hydrocarbon profiles of oranges and tangors (6), grapefruit (7) and lemons (unpublished data), specific patterns emerged that were intrinsic for each citrus species. Because of the specific nature of these profiles, the possibility that these long chain hydrocarbon patterns could be utilized in differentiating citrus hybrids appeared promising.

In 1905 Webber and Swingle (8) designated hybrids of the mandarin and grapefruit as tangelos. Tangelos are a highly diverse group of citrus manifesting characteristics that are both typical of their parents and intermediate between them. The three tangelos used in this study, viz., Seminole, Orlando and Minneola, resulted from a cross of Duncan grapefruit (*C. paradisi*) with Dancy mandarin (*C. reticulata*). Duncan is the oldest grapefruit variety in Florida, having been brought to Florida from the West Indies around 1830 (9). Dancy was introduced in Florida from Tangiers around 1867 and is currently the most important mandarin variety in the U.S.

In the present study, long chain hydrocarbon profiles were determined for the two parents, viz., Duncan and Dancy, and their three tangelo offspring. From these observed profiles, a chemotaxonomic pattern relating offspring to parents was sought.

## MATERIALS AND METHODS

### Isolation and Purification of Juice Sac Lipids

Duncan grapefruit, Dancy mandarin and the three tangelos, viz. Orlando, Minneola and Seminole, were obtained from Whitmore Experimental Farm (Plant Science Research Division, USDA, Orlanda, Fla.). The five citrus were cut in half and the intact juice sacs carefully separated from core, peel, seeds and carpellary membrane with the aid of a citrus spoon. The juice sacs were freeze-dried to a powder and stored at -18 C. Lipids were extracted and purified from 20 g juice sac powder by methods described previously (1,2,6). Quadruplicate extractions were run on a single batch of fruit from each cultivar.

### Column and Thin layer Chromatography (TLC)

The total purified lipid (ca. 200 mg) was

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TABLE I

Total Lipid and Hydrocarbon Concentrations of Duncan Grapefruit, Dancy Mandarin, Seminole, Orlando and Minneola Tangelos (mg/20 g dry wt)

| Variety | Total lipid | Hydrocarbon fraction | | |
|---|---|---|---|---|
| | | Saturated | Monoene | Complex |
| Duncan | 193.3±8.4 | 3.8±0.3 | 0.4±0.1 | 0.2±0.0 |
| Dancy | 177.2±2.5 | 2.2±0.2 | 0.7±0.2 | 0.7±0.1 |
| Seminole | 198.5±2.4 | 2.5±0.1 | 0.6±0.2 | 0.4±0.2 |
| Orlando | 169.1±7.3 | 2.6±0.2 | 0.2±0.1 | 0.4±0.0 |
| Minneola | 194.9±8.5 | 3.2±0.4 | 0.5±0.2 | 0.3±0.2 |

dissolved in $CHCl_3$ and percolated onto a 0.9 x 30 cm column containing 9 g Baker, 60-200 mesh silica gel (J.T. Baker Chemical Co., Phillipsburg, N.J.) which had previously been washed with 100 ml $CHCl_3$. The neutral lipids, which contained the long chain hydrocarbons, were eluted with 200 ml $CHCl_3$. The neutral lipid fraction was concentrated to a small volume and streaked on precoated Silica Gel G plates (20 x 20 cm, 500$\mu$, Analtech, Inc., Wilmington, Del.). These plates were washed previously with $CHCl_3$/MeOH/acetic acid 65:30:5 prior to development of the neutral lipid fraction. The neutral lipid fraction was developed on these prewashed plates at room temperature in chambers lined with filter paper in hexane-ethyl ether 92:8. The band corresponding to the long chain hydrocarbon fraction was scraped from the plate and eluted with ethyl ether. The hydrocarbon fraction was, in turn, streaked on a silver nitrate-impregnated Silica Gel G plate (3), developed with 2% ethyl ether in petroleum ether and visualized under UV light after spraying with Rhodamine 6G. This system separated the hydrocarbon fraction into three fractions, viz., saturated, monounsaturated and a complex fraction containing all other components. This latter fraction was obtained by eluting all components found between the monounsaturated fraction and the origin on the TLC plate and contained pigments, polyunsaturated hydrocarbons and undefined hydrocarbons. The bands corresponding to the saturated and monounsaturated hydrocarbons were scraped from the plate and eluted with ethyl ether. The eluates were evaporated to dryness and the residues weighed. Procedural blank runs were made on blank TLC plates as a check against artifact inclusion. The monounsaturated fraction was dissolved in 1 ml hexane and hydrogenated under 60 lb/in.$^2$ at room temperature for 1 hr with 10 mg 10% Pd-C catalyst in a Parr apparatus.

### Gas-Liquid Chromatography (GLC) and Quantitation

Gas chromatographic analysis of the long chain hydrocarbons was performed with a Hewlett Packard Model 7610A high efficiency gas chromatography equipped with flame ionization detectors. Hydrocarbons were determined on a glass column (3.05 m long and 4 mm id) packed with 3% SP-1000 (Supelco, Inc., Bellefonte, Pa.) on 100/120 mesh, Gas Chrom Q. The injection port and detector were at 190 C and 275 C, respectively, and the helium flow rate was 80 ml/min. The sample was injected on column at 165 C and programed at 4 C/min for 5 min, then 2 C/min for 20 min, then 3 C/min up to 270 C and finally held isothermally at this upper limit until the $C_{35}$ hydrocarbon eluted. Mass spectra of citrus-branched hydrocarbons were previously determined by this laboratory for Valencia orange oil (10). Although mass spectra were not determined for the hydrocarbons of the citrus reported in this paper, the authors have no reason to believe that the mass spectra of these hydrocarbons would be any different than those previously reported for citrus. In addition, a normal, iso and anteiso hydrocarbon standard from $C_{16}$ to $C_{36}$ was prepared from normal and branched fatty acids of Temple oranges as described previously (6). Hydrocarbons were determined by comparative GLC retention times against the above standard and by way of plots of retention times vs. equivalent carbon numbers. Quantitative results were obtained by triangulation measurement techniques and also by measurement of peak areas with the aid of a disc integrator.

## RESULTS AND DISCUSSION

The lipid content and hydrocarbon concentrations of the five citrus are shown in Table I. Total lipids extracted from 20 g juice sac powder for the two parents, Duncan grapefruit and Dancy mandarin, show the percentage of lipid as 0.97% and 0.89%, respectively. The percentage lipid for the tangelos are: Orlando (0.85%), Minneola (0.97%) and Seminole (0.99%). Saturated hydrocarbons represent 1.2-2.0% of the total lipid, while monounsaturated hydrocarbons comprise 0.1-0.4%.

Saturated long chain hydrocarbon profiles are shown in Table II. Only hydrocarbons between $C_{20}$ and $C_{35}$ are quantified in this group. Hydrocarbons greater than $C_{35}$ are detected in these five citrus cultivars but at trace percentages (below 0.01%). Values in Table II and III are reported to one-hundredth of a per cent. The purpose of reporting at this low percentage is to show the relative abundance of minor components and their importance in better understanding the chemotaxonomic hydrocarbon patterns of citrus (6,7). For the normal chain hydrocarbons (N column), Duncan show $C_{23}$ and $C_{25}$ to be dominant with $C_{25}$ predominating. All grapefruit examined to date show this pattern, and thus it appears intrinsic to this citrus species (7). In citrus, hydrocarbons may differ by only a percentage or two as in the case of grapefruit. The important point, however, is the fact that they differ consistently. Regardless of the grapefruit's maturing season, i.e., fall, winter or early spring, all grapefruit consistently show more $C_{25}$ than $C_{23}$. The other parent, Dancy,

TABLE II

Saturated Long Chain Hydrocarbon Profiles of Citrus Juice Sacs (wt %)

| Carbon no. | Duncan | | | Seminole | | | Orlando | | | Minneola | | | Dancy | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N[a] | I[b] | AI[c] | N | I | AI | N | I | AI | N | I | AI | N | I | AI |
| 20 | 0.23[d] | tr[e] | tr | 0.48 | tr | tr | 0.38 | tr | tr | 0.15 | tr | tr | 0.23 | tr | tr |
| 21 | 0.36 | 0.01 | tr | 0.91 | tr | tr | 0.90 | tr | tr | 0.77 | tr | tr | 0.50 | 0.03 | tr |
| 22 | 1.73 | 0.09 | 0.04 | 2.60 | 0.22 | 0.15 | 2.51 | 0.21 | 0.10 | 2.74 | 0.17 | 0.08 | 1.75 | 0.04 | 0.04 |
| 23 | 12.53 | 10.10 | tr | 14.15 | 13.08 | tr | 14.10 | 17.93 | tr | 16.37 | 17.06 | tr | 12.44 | 3.55 | tr |
| 24 | 6.29 | 2.81 | 6.79 | 5.93 | 2.98 | 7.21 | 4.96 | 4.34 | 7.81 | 5.66 | 3.83 | 7.56 | 6.62 | 1.70 | 3.03 |
| 25 | 13.87 | 14.58 | 1.99 | 14.04 | 12.71 | 2.73 | 9.73 | 15.97 | 3.42 | 10.42 | 14.76 | 3.20 | 19.51 | 9.75 | 1.38 |
| 26 | 2.66 | 1.58 | 6.22 | 2.22 | 0.96 | 5.00 | 1.22 | 0.99 | 4.86 | 1.22 | 1.05 | 4.90 | 4.70 | 0.99 | 5.41 |
| 27 | 4.32 | 4.25 | 0.65 | 2.72 | 2.87 | 0.53 | 1.49 | 2.76 | 1.10 | 1.31 | 3.11 | 0.84 | 10.12 | 2.94 | 0.70 |
| 28 | 0.92 | 0.39 | 1.81 | 0.83 | 0.22 | 0.97 | 0.48 | 0.14 | 0.85 | 0.42 | 0.17 | 1.03 | 2.64 | 0.50 | 1.78 |
| 29 | 1.37 | 1.13 | 0.19 | 1.72 | 0.60 | 0.17 | 0.79 | 0.46 | 0.13 | 0.50 | 0.53 | 0.14 | 4.02 | 0.98 | 0.31 |
| 30 | 0.41 | 0.19 | 0.49 | 0.73 | 0.37 | 0.37 | 0.43 | 0.17 | 0.23 | 0.40 | 0.21 | 0.26 | 0.92 | 0.32 | 0.60 |
| 31 | 0.67 | 0.31 | 0.07 | 1.58 | 0.24 | 0.15 | 0.65 | 0.18 | 0.04 | 0.46 | 0.14 | 0.11 | 1.11 | 0.23 | 0.14 |
| 32 | 0.24 | 0.06 | 0.15 | 0.05 | tr | 0.04 | 0.14 | tr | 0.11 | 0.14 | 0.05 | 0.14 | 0.26 | 0.09 | 0.13 |
| 33 | 0.21 | 0.11 | tr | 0.29 | tr | tr | 0.28 | tr | tr | 0.10 | tr | tr | 0.35 | 0.04 | 0.01 |
| 34 | 0.11 | tr | 0.02 | 0.03 | --- | --- | 0.11 | --- | --- | tr | --- | --- | 0.05 | tr | tr |
| 35 | 0.05 | tr | --- | 0.15 | --- | --- | 0.03 | --- | --- | tr | --- | --- | 0.09 | tr | tr |
| Odd | 33.38 | 30.49 | 2.90 | 35.56 | 29.50 | 3.58 | 27.97 | 37.30 | 4.69 | 29.93 | 35.60 | 4.29 | 48.14 | 17.52 | 2.54 |
| Even | 12.59 | 5.12 | 15.52 | 12.87 | 4.75 | 13.74 | 10.23 | 5.85 | 13.96 | 10.73 | 5.48 | 13.97 | 17.17 | 3.64 | 10.99 |
| Total | 45.97 | 35.61 | 18.42 | 48.43 | 34.25 | 17.32 | 38.20 | 43.15 | 18.65 | 40.66 | 41.08 | 18.26 | 65.31 | 21.16 | 13.53 |

[a]Normal chain hydrocarbon.

[b]Iso, general structure $CH_3$-CH($CH_3$)-($CH_2$)$_x$$CH_3$.

[c]Anteiso, general structure $CH_3$-$CH_2$-CH($CH_3$)-($CH_2$)$_x$$CH_3$.

[d]Values represent the mean of five to seven determinations; coefficient of variation (CV) determined for several mean ranges (MR) showed the following: MR 0.01-0.10; CV 10-35%; MR 0.1-1.0; CV 5-10%; MR 1.0-5.0; CV 3-5%; and MR Above 5.0; CV less than 2%.

[e]Trace, less than 0.01%.

TABLE III

Monounsaturated Long Chain Hydrocarbon Profiles of Citrus Juice Sacs (wt %)[a]

| Carbon no. | Duncan | | | Seminole | | | Orlando | | | Minneola | | | Dancy | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | I | AI | N | I | AI | N | I | AI | N | I | AI | N | I | AI |
| 20 | 0.75 | tr | 0.14 | 1.06 | tr | 0.45 | 3.04 | tr | 0.49 | 1.14 | tr | 0.49 | 0.54 | tr | 0.10 |
| 21 | 0.83 | tr | 0.17 | 0.77 | 0.05 | 0.34 | 0.66 | 0.52 | 0.94 | 1.17 | 0.11 | 0.34 | 0.21 | tr | 0.08 |
| 22 | 1.97 | tr | 0.21 | 1.63 | 0.07 | 0.27 | 1.53 | tr | 0.78 | 1.83 | 0.15 | 0.49 | 0.60 | 0.01 | 0.12 |
| 23 | 4.05 | 0.44 | 0.22 | 5.39 | 2.97 | 0.45 | 8.87 | 4.09 | 1.38 | 9.38 | 3.19 | 1.07 | 0.56 | 0.03 | 0.04 |
| 24 | 3.38 | 0.37 | 0.90 | 3.09 | 0.88 | 2.51 | 6.14 | 0.62 | 2.08 | 5.31 | 0.85 | 2.86 | 0.62 | 0.03 | 0.14 |
| 25 | 10.85 | 1.49 | 0.76 | 13.25 | 3.68 | 0.76 | 28.52 | 7.92 | 1.52 | 29.59 | 6.31 | 2.00 | 4.07 | 0.34 | 0.11 |
| 26 | 1.54 | 0.13 | 1.60 | 2.73 | 0.18 | 1.96 | 2.16 | 0.92 | 3.48 | 2.76 | 0.69 | 1.90 | 0.75 | 0.02 | 0.31 |
| 27 | 15.68 | 0.94 | 0.57 | 8.99 | 1.34 | 0.60 | 11.26 | 2.58 | 0.64 | 12.36 | 2.79 | 0.70 | 6.34 | 0.16 | 0.08 |
| 28 | 2.10 | tr | 0.52 | 1.47 | 0.11 | 0.86 | 1.00 | 0.11 | 0.83 | 1.11 | 0.20 | 1.44 | 1.31 | 0.02 | 0.13 |
| 29 | 41.15 | 0.10 | 0.10 | 22.07 | 0.18 | 0.17 | 5.26 | 0.21 | 0.17 | 4.02 | 0.34 | 0.16 | 35.88 | 0.12 | 0.06 |
| 30 | 1.23 | 0.57 | 0.67 | 1.67 | 0.51 | tr | 0.27 | 0.30 | 0.09 | 1.95 | 0.07 | 0.21 | 3.05 | tr | 0.02 |
| 31 | 6.46 | tr | tr | 18.66 | tr | tr | 1.35 | 0.07 | 0.08 | 2.44 | 0.33 | 0.14 | 40.99 | 0.06 | tr |
| 32 | tr | --- | --- | 0.30 | 0.05 | tr | tr | --- | --- | tr | --- | --- | 0.81 | --- | 0.02 |
| 33 | 0.11 | --- | --- | 0.53 | --- | --- | 0.12 | --- | --- | 0.11 | --- | --- | 2.27 | tr | tr |
| 34 | tr | --- | --- | tr | --- | --- | tr | --- | --- | tr | --- | --- | t | --- | --- |
| 35 | tr | --- | --- | tr | --- | --- | tr | --- | --- | tr | --- | --- | t | --- | --- |
| Odd | 79.13 | 2.97 | 1.82 | 69.66 | 8.22 | 2.32 | 56.04 | 15.39 | 4.73 | 59.07 | 13.07 | 4.41 | 90.12 | 0.71 | 0.37 |
| Even | 10.97 | 1.07 | 4.04 | 11.95 | 1.80 | 6.05 | 14.14 | 1.95 | 7.75 | 14.10 | 1.96 | 7.39 | 7.88 | 0.08 | 0.84 |
| Total | 90.10 | 4.04 | 5.86 | 81.61 | 10.02 | 8.37 | 70.18 | 17.34 | 12.48 | 73.17 | 15.03 | 11.80 | 98.00 | 0.79 | 1.21 |

[a]For abbreviations see Table II.

show $C_{23}$, $C_{25}$ and $C_{27}$ to be the dominant normal hydrocarbons with $C_{25}$ predominating. Insufficient data have been collected on mandarins to determine whether this pattern is specific for all mandarins. The Orlando and Minneola tangelos appear to have profiles quite different from either of their parents with respect to the normal chain group. Both Orlando and Minneola show $C_{23}$ and $C_{25}$ dominating, but $C_{23}$ is more prominent. Seminole differs from its two sister hybrids by showing relatively higher percentages of longer chain hydrocarbons, i.e., in the region $C_{26}$-$C_{31}$. The normal chain profile of Seminole more closely resembles Duncan grapefruit than Dancy mandarin.

Examination of the iso group (column I) reveals that $C_{25}$ manifests the highest relative percentage for the parents, Duncan and Dancy, while $C_{23}$ predominates in all three tangelos. While the tangelos show moderately high percentages for iso $C_{23}$ and $C_{25}$, Dancy shows a markedly lower relative per cent for these two branched hydrocarbons. For the anteiso group (column AI), $C_{24}$ and $C_{26}$ predominate in all five citrus; however, while $C_{24}$ is dominant in Duncan and the three tangelos, $C_{26}$ dominates in Dancy.

Table II reveals some general patterns for citrus hydrocarbons. For odd-numbered hydrocarbons, the percentage of iso structures is always greater than their anteiso homologs. Conversely, even-numbered hydrocarbons show an opposite relationship, i.e., anteiso structures are generally greater than their iso homologs. In collective form, the total percentages for the three isomeric structures in this saturated group are shown at the bottom of Table II. Scrutiny of these collective data reveals some interesting patterns. Dancy is the only cultivar to accumulate more linear saturated hydrocarbons than branched (iso and anteiso combined). While Duncan, Seminole and Dancy show the normal group to comprise the highest percentage, Orlando and Minneola show the iso group as most prominent. The percentage ratios for the three isomeric structures for Seminole tangelo are very similar to Duncan grapefruit. The hydrocarbon profile of Seminole also reflects this close similarity with Duncan. The relationship normal > iso > anteiso is observed for Duncan, Seminole and Dancy, while Orlando and Minneola manifest iso > normal > anteiso. Because of the accumulation of large percentages of branched structures by all three tangelos, it appears that the greater extent of branched chain hydrocarbon fromation in tangelos is more typical of Duncan grapefruit than Dancy mandarin. In all five cultivars, odd-numbered anteiso paraffins accumulate to the least extent.

The percentage composition of the monounsaturated fraction is shown in Table III. This table above all shows the great divergence of the three tangelos from their parents. In the normal group of Duncan, $C_{29}$ predominates overwhelmingly, while for Dancy $C_{29}$ and $C_{31}$ share equal prominence. Orlando and Minneola show $C_{25}$ as dominant in the normal group, while Seminole shows $C_{29}$ as most prominent. Orlando and Minneola possess similar profiles, while Seminole shows marked differences from its sister hybrids. The difference lies in Seminole's accumulation of larger percentages of linear $C_{29}$ and $C_{31}$. All five cultivars show $C_{25}$ as the most prominent iso paraffin. The percentages for the branched paraffins in the three tangelos are all noticeably higher than their parents, Duncan and Dancy. For the anteiso group, $C_{24}$ and $C_{26}$ predominate in all five citrus.

A breakdown of the total percentages for the three isomeric structures in this monounsaturated fraction is shown at the bottom of Table III. One of the most noticeable features of these collective data are the very low percentages of branched hydrocarbons (iso and anteiso) accumulated by Dancy. Normal odd-numbered hydrocarbons comprise the largest segment of the monounsaturated group, while even-numbered, iso structures comprise the least in all five cultivars. The tangelos are differentiated readily from the parents by the presence of moderately high percentages of branched structures. Seminole differs from its sister hybrids, Orlando and Minneola, by showing lower relative percentages for all branched structures.

In three previous papers (6,7,11), the authors have speculated on possible mechanisms for hydrocarbon synthesis in citrus, and from all available evidence generally favor the elongation-decarboxylation pathway postulated by Kolattukudy (12-14). Citrus fruits synthesize a multitude of saturated and unsaturated branched chain fatty acids (1,3). In Duncan grapefruit (1) saturated iso fatty acids comprise 0.68% and anteiso acids 0.10% of the total fatty acid fraction. The saturated branched hydrocarbons reported in this paper for Duncan show percentages of 35.61 and 18.42 for iso and anteiso hydrocarbons, respectively. The vast difference in percentage of branched chain fatty acids and hydrocarbons is also shown for Dancy mandarin. Dancy synthesizes 0.34% and 0.05% of saturated iso and anteiso branched fatty acids, respectively (1). However the percentages of saturated iso and anteiso hydrocar-

bons formed by Dancy are 21.16 and 13.53, respectively. The reason for this large difference between the percentages of branched fatty acids and branched hydrocarbons is not understood. Since hydrocarbons are an end product of fatty acid metabolism, there is no reason to expect some quantitative relationship between a precursor (branched fatty acid) and its end product (branched hydrocarbon).

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# Lipids of Human Myocardium

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## ABSTRACT

The major lipid classes, including some phospholipids, and their fatty acid profiles have been measured in portions of left ventricular muscle and psoas muscle obtained at autopsy. Atrial appendages and ventricular muscle removed during cardiac surgery were examined also. The proportions of the individual phospholipids were the same in all the muscles, having an excess of phosphatidyl ethanolamine and phosphatidyl serine compared with the serum. Their fatty acid profiles resembled those obtained from other locations. The triglyceride content of the myocardium was relatively constant (except in the atrial appendage) but did rise slightly with increasing obesity. The free fatty acid concentration in the myocardium was relatively high and had a variable fatty acid profile.

## INTRODUCTION

Lipids are important for the myocardium, because more than half the energy requirements of human myocardium are met by the catabolism of lipids (1) and lipid deposition occurs in the myocardium in pathological conditions. In the present study the lipid compositions of various parts of the myocardium, in autopsy and operative specimens, have been compared. The results have been related to the lipids in a skeletal muscle and to age, sex and cause of death.

## MATERIALS AND METHODS

### Myocardium

*Autopsy*: Myocardium was obtained at 60 routine autopsies, most of which were carried out within 24 hr of death, on subjects who had died from a variety of causes including road traffic accidents, poisoning and heart failure. No muscle which appeared to be infarcted was selected. The material was kept at -20 C until processing. After thawing, pieces of myocardium weighing 5-15 g were cut out from selected sites. Large arteries and obvious fat deposits were not included except for some auricular specimens in which fat was intimately mixed with muscle. In some instances psoas muscle was also obtained. Not all the specimens were submitted to complete analysis; the numbers involved are indicated where appropriate.

*Operative*: Five atrial appendages and five pieces of papillary muscle or ventricular wall, or both, weighing between 1 and 2 g were obtained at the time of cardiac surgery on eight patients. The specimens were placed at -20 C within 5 min of excision. Only tissue removed obligatorily in the course of the operations was used.

### Lipid Extraction

The pieces of myocardium and psoas muscle were either taken directly from the refrigerator or re-frozen on solid carbon dioxide. They were sliced with a scalpel into fragments less than 1 mm thick and weighed in a round-bottomed flask before being freeze-dried. When drying

TABLE I

Comparison of Various Lipids in Interventricular Septum and Psoas Muscle

A. Milligrams per Gram Wet Weight, Mean ± SEM

| Muscle | TL[a] | PL | TG | FFA | CE | C |
|---|---|---|---|---|---|---|
| Septum | 26.5±1.0 | 13.2±0.7 | 3.5±0.66 | 2.7±0.32 | 0.4±0.04 | 0.9±0.06 |
| Psoas | 31.0±2.9 | 7.2±0.5 | 14.4±2.1 | 1.9±0.16 | 0.4±0.06 | 0.6±0.03 |

B. Per Cent Total Phospholipid, Mean ± SEM

| Muscle | PE | PS | PC | Sp | LPC |
|---|---|---|---|---|---|
| Septum | 26.5±1.5 | 8.5±0.5 | 49.2±0.9 | 6.4±0.5 | 8.6±0.8 |
| Psoas | 25.2±0.8 | 11.4±0.5 | 48.7±1.2 | 6.8±0.6 | 7.9±0.8 |

[a]TL = Total lipid, PL = phospholipid, TG = triglyceride, FFA = free fatty acids, CE = cholesterol esters, C = free cholesterol, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, PC = phosphatidyl serine, PC = phosphatidyl choline, Sp = sphingomyelin, LPC = lysophosphatidyl choline.

TABLE II

Percentage of Individual Fatty Acids in the Lipid Classes of the Interventricular Septum (Mean ± SEM)

| Lipid class | | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 20:4 |
|---|---|---|---|---|---|---|---|---|
| TG | (27)[a] | 4.1±0.3 | 29.9±0.9 | 5.7±0.5 | 8.4±0.6 | 45.5±1.1 | 5.3±0.6 | 0 |
| FFA | (27) | 1.5±1.2 | 25.9±1.2 | 3.0±0.4 | 11.4±0.7 | 28.9±1.7 | 11.7±1.0 | 16.5±1.7 |
| PS | (22) | 0.6±0.2 | 10.5±1.9 | 2.2±0.3 | 47.3±3.0 | 17.3±1.8 | 5.3±0.5 | 13.5±1.6 |
| PE | (23) | 0.6±0.2 | 7.4±1.2 | 1.8±0.4 | 21.5±1.8 | 12.8±2.1 | 9.5±1.6 | 26.1±2.7 |
| PC | (26) | 1.1±0.4 | 30.8±1.3 | 1.9±0.3 | 9.4±0.7 | 18.3±0.8 | 14.3±1.7 | 11.0±1.3 |
| Sp | (20) | 1.6±0.3 | 35.9±2.3 | 4.1±0.6 | 24.0±2.2 | 16.0±1.3 | 8.1±1.1 | 2.7±0.7 |
| LPC | (23) | 2.3±0.5 | 36.5±3.1 | 4.8±1.0 | 14.9±1.1 | 14.2±1.3 | 7.1±0.8 | —[b] |
| CE | (13) | 3.2±1.0 | 28.7±2.1 | 9.1±1.7 | 8.4±2.0 | 35.0±5.1 | 12.2±2.2 | 0 |

[a]For abbreviations see Table I. Number in parentheses is number of specimens.

[b]Results too variable for analysis. Usually absent.

was complete, 100 ml chloroform-methanol 2:1 was added to each flask and extraction carried out for 30 min at room temperature with a magnetic stirrer. The flask was kept overnight at 4 C, the residue filtered off and extraction completed with a further 100 ml chloroform-methanol mixture at room temperature for 30 min. The combined extract was washed with 0.2 volumes 0.5 $NaH_2PO_4$ in a separating funnel overnight and the chloroform phase removed.

### Lipid Analysis

Duplicate aliquots of the extract were taken for estimating total lipids gravimetrically and total phospholipids from the phosphorus content. Neutral lipids and individual phospholipids were quantitated by thin layer chromatography (TLC) on silicic acid (2). The published method was modified in several ways. A Quickfit plate spreader was employed set at 0.5 mm. From the thin layer chromatograms of neutral lipids, the zone of free fatty acids was eluted with two 5 ml volumes of diethyl ether. The extract was dried, methylated with diazomethane and then estimated with the hydroxylamine reaction as used for triglyceride. For the separation of phospholipids, the plates were prepared using Silica Gel H in a 0.001 M $Na_2CO_3$ solution, and the solvent mixture was chloroform-methanol-water-acetic acid 50:30:8:4. A band running before phosphatidyl ethanolamine was considered to be largely phosphatidyl serine by comparison with standards. Recoveries from these systems exceed 95% (2).

*Gas chromatography*: Lipid classes for fatty acid analysis were obtained by TLC as described above, except that only the edges of the plates were stained, to locate the lipids, and the unstained zones between scraped off. Except for the free fatty acids described above, the lipids were eluted from the silicic acid by shaking with 2 x 5 ml freshly prepared methanolic HCl. The decanted solution was kept at 85 C under an air-cooled reflux condenser for 4 hr (neutral lipids) or 6 hr (phospholipids). After cooling, the methyl esters were extracted by adding 5 ml water and shaking with 2 x 5 ml petroleum ether (bp 40-60 C). The combined extract was washed with water and left to stand over a small quantity of $Na_2SO_4/NaHCO_3$ 2:1 for a few hours before storage at -20 C.

The methyl esters were separated by gas liquid chromatography. Earlier specimens were examined with a Pye argon chromatograph using 10% diethylene glycol adipate as stationary phase. Later a Pye 104 chromatograph with hydrogen flame detectors was employed. Glass columns packed with acid washed 100-120 mesh Celite, treated with hexamethyldisilazane and coated with 10% diethylene glycol succinate were used. With few exceptions the operating conditions were 150 C, nitrogen flow 50 ml/min, hydrogen flow 50 ml/min and air flow 700 ml/min.

Peaks were identified by comparison with National Heart Institute fatty acid standard mixtures and occasionally by chromatography on a nonpolar column. Quantitation was by triangulation. When the standard mixtures, which included arachidonic acid, were analyzed the results agreed with the stated composition with a relative error of less than 12% on almost all occasions. This analytical system gives a retention time of many hours for docosahexaenoic acid, and the resultant peak was too indistinct for definite detection or quantitation. Other analytical techniques were as described previously (3,4).

### Recoveries

For the autopsy specimens the mean overall recovery, i.e., the sum of the individual lipids compared with the total measured gravimetrically, was 78% for myocardium and 79% for

TABLE III

Lipid Classes in Operative Specimens of Atrial Appendage and Ventricle (Means with Ranges in Brackets)[a]

| Specimen | mg/g Wet wt | | | | Per cent | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | TL | PL | TG | FFA | PE | PS | PC | Sp | LPC |
| Atrial appendage ($n$=5) | 45.1 (15.5-97.0) | 19.0 (5.3-32.0) | 21.1 (3.2-66.7) | 7.3 (1.6-13.0) | 21.6 (16-25) | 10.8 (0-21) | 40.0 (25-51) | 8.6 (0-13) | 19.0 (10-44) |
| Ventricle ($n$=5) | 27.6 (7.9-60.6) | 10.6 (3.1-15.5) | 7.9 (1.0-17.8) | 3.9 (0.4-10.7) | 27.8 (25-35) | 8.0 (0-15) | 42.4 (31-54) | 9.2 (0-19) | 12.6 (4-21) |

[a]For abbreviations see Table I.

psoas. This low recovery may be partly accounted for by the protein component of the protein-lipid complexes extracted into chloroform-methanol (5). The mean overall recovery of phospholipids compared with the phosphorus content of the extracts was 84.6% for myocardium and 83.8% for psoas, but this is to be expected because not all the phospholipids of the myocardium are identified by the system used here. For example cardiolipin, which constitutes 10% of the phospholipids in bovine myocardium (5,6) was not included. Plasmalogens were not separated by this system, but considerable amounts are present in myocardium (7). Evidence of this was found in the gas chromatographic analyses, in that only 80% of the peaks in phosphatidyl ethanolamine and 87% in phosphatidyl choline could be identified as methyl esters. Most of the other peaks were consistent with dimethyl acetals derived, presumably, from plasmogens.

For the operative specimens the phospholipid recoveries were similar to those for the autopsy material, being 83% for ventricular tissue and 79% for the atrial appendage. However, although the overall recovery for the operative ventricular specimens was again low at 81%, that for the atrial appendages was 104%. This is unexplained, but the variation between the specimens was very large and the total numbers small (Table III).

## RESULTS

### Autopsy Specimens

*Comparison of ventricular sites*: In the first 16 hearts the amounts of total lipid, total phospholipid, neutral lipid classes, individual phospholipids and fatty acid profiles in ventricular myocardium taken from the septum, the left ventricular tip and the lateral left ventricular wall were compared. The mean values were almost identical with considerable individual variation, and therefore subsequent examinations were confined to the septum, as this site can be sampled easily without risk of including pericardial lipid deposits. Also, for fatty acid analyses (below), the results from the three sites were pooled.

*Comparison of interventricular septum and psoas muscle*: The results from 29 subjects aged 17-66 years and including six women were examined. No relationships were observed between age, sex or cause of death. The results are shown in Table I. Because of missing values the total number of observations is reduced to between 22 and 27 under the various headings.

With the exception of phosphatidyl serine, which was slightly more abundant in the psoas, the phospholipids were virtually identical in the two muscles. As the values are percentages the $t$ test would not be valid and was therefore not applied.

TABLE IV

Fatty Acids in the Operative Specimens (Means with Ranges in Brackets)

| Specimen | Per cent | | | | | |
|---|---|---|---|---|---|---|
| | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 |
| TG ($n$=7) | 6.0 (4.8-7.7) | 34.7 (25.8-42.3) | 8.3 (3.3-17.0) | 8.1 (2.9-18.1) | 36.7 (26.1-48.7) | 5.4 (2.7-13.6) |
| FFA ($n$=5) | 8.6 (0-33.3) | 36.9 (29.4-43.3) | 1.8 (0-3.3) | 20.9 (16.4-27.0) | 21.7 (26.1-40.5) | 4.4 (0-12.3) |

[a]No 20:4 was found in TG. Two specimens of FFA contained 20:4 in the proportions of 5.0% and 7.3%. For abbreviations see Table I.

The concentrations of the lipid classes, with the exception of the cholesterol esters, were all different in the septum and the psoas ($P < 0.05$). The most striking difference was the relatively increased total phospholipid and free fatty acid with low triglyceride in the septum compared with the psoas.

When the triglyceride content of the psoas and septum was compared (Fig. 1) there was a slight but significant tendency for the septum triglyceride to rise with increase in psoas triglyceride ($n = 22$, $r = 0.60$, $P < 0.01$). Substantial amounts of triglyceride were found in the septum of only one subject. If he is omitted the relationship is still significant ($n = 21$, $r = 0.48$, $P < 0.05$).

*Fatty acid profiles of the lipid classes*: These were determined on a total of 27 specimens from 15 hearts. The ages of the subjects ranged from 17 to 77 years, and three were women. No correlation was observed between the results and age, sex or cause of death and the values were therefore pooled. Missing values reduced the total number of observations as indicated (Table II).

#### Operative Specimens

The results from the papillary muscles and ventricular wall were pooled. The total amount of lipid present was similar to that in the autopsy specimens, but the triglyceride was higher (Table III). The amounts of cholesterol present were too small to measure accurately. In the atrial appendages there was much more lipid of all classes. The phospholipids from ventricle and atrium were similar to each other and to the autopsy specimens, with one exception—the lysophosphatidyl choline was higher in the operative specimens and particularly in the atrial appendages.

Some of the specimens yielded little lipid material, and it was not possible to obtain satisfactory fatty acid analyses of the separated phospholipids. There were seven satisfactory fatty acid analyses of triglyceride and five of free fatty acid. As the values varied widely, the results for material from both sites were pooled (Table IV). The composition of the triglyceride was similar to that in the autopsy specimens, but the free fatty acid differed in that there was more stearic acid. Arachidonic acid was absent from three specimens and only present in small amounts in the other two.

## DISCUSSION

The lipids of the myocardium, particularly the phospholipids, are known to be complex, but most of the published analyses have been of animal tissues (5,6,8). Studies on human myocardium are scant (9,10); a recent comprehensive report (7) was concerned with separated microsomes and mitochondria, but these contain only ca. 5% of the total lipid present (6).

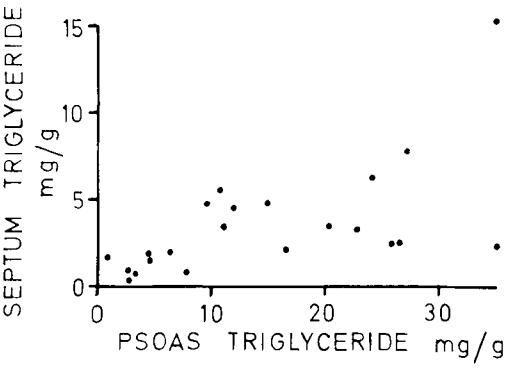


FIG. 1. The relationship between the concentration of triglyceride in the interventricular septum and psoas muscle in 22 subjects.

It is impossible to free muscle tissue from interspersed fat cells, and presumably these cells are responsible for the high triglyceride content of psoas and also for its previously reported (11) correlation with the degree of obesity of the subject. The data in Figure 1 show a similar relationship for the triglyceride in the septum but to a less marked degree. However, to judge from the triglyceride content of the psoas, the present series does not seem to have contained any grossly obese individuals because the highest level observed was ca. 35 mg/g; whereas in the previous study (11), admittedly using a different method, levels as high as 115 mg/g were found.

The concentration of phospholipid in the septum was about twice that in the psoas, but the proportions of the individual phospholipids were the same in the two sites. They differed from serum phospholipids in having a much higher content of phosphatidyl ethanolamine and phosphatidyl serine (2). In the operative specimens there was a high proportion of lysophosphatidyl choline, particularly in the atrial appendages. Although the TLC method used does not include a correction for "tailing," which would tend to increase the apparent proportion of this phospholipid, the same technique was applied also to the autopsy material, and the high levels in the operative specimens remain unexplained. The fatty acid profiles of the triglyceride and the phospholipids had a similar appearance to those of the same lipids from other locations, e.g., serum (4).

The free fatty acid concentration in the septum was high but similar to that reported in rat (12) and ox (13) heart. The most marked feature of its fatty acid composition (Table II) was the high arachidonic acid content. On general grounds the free fatty acid might be expected to be derived from triglyceride, but as the latter was virtually free from arachidonic acid this could only be partially true in the present instance. However in the operative specimens the free fatty acid, although present in even higher concentration than in the autopsy specimens, was usually free from arachidonic acid. This suggests that some of the free fatty acid present in the autopsy material was derived from hydrolysis of phospholipids during autolysis.

The results in the operative specimens were more variable than in the autopsy ones. The operative samples were found to be just large enough for chemical analysis, so that pathological examinations could not be carried out. Although obvious macroscopic defects were avoided, it must be assumed that the tissues were abnormal as they were removed from hearts requiring surgery, but the numbers were too small for the variations to be analyzed further.

There was a particularly high level of triglyceride in some of the atrial appendages. The stearic acid content of the free fatty acid was approximately double that in the autopsy material; such a high level is not found in depot or serum triglyceride and free fatty acid; its significance is not clear.

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# Cholesterol-Cerebroside Interaction: the Role of α-Hydroxy Fatty Acids

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## ABSTRACT

The esterification of cholesterol by the plasma phosphatidyl choline-cholesterol acyltransferase reaction was studied by two methods, radioisotopic and colorimetric, in the presence of cerebroside, ceramide, or methyl esters of lignoceric or α-hydroxy lignoceric acid. The radioisotopic method measures esterification of exogenous labeled cholesterol which must be taken up into the lipoprotein-bound pool prior to its utilization as a substrate. The colorimetric method measures esterification of endogenous lipoprotein-bound free cholesterol since the exogenous labeled cholesterol is negligible in concentration. Cerebroside and ceramide containing α-hydroxy fatty acids reduced the utilization of exogenous labeled cholesterol as substrate, but had no effect on lipoprotein-bound exogenous cholesterol esterification. Cerebroside and ceramide containing no α-hydroxy fatty acid had no effect on exogenous labeled cholesterol esterification. The methyl esters of lignoceric acid and α-hydroxy lignoceric acid had no effect on the esterification of exogenous cholesterol in plasma. There is a decrease in esterification of exogenous labeled cholesterol with increasing concentration of α-hydroxy fatty acid ceramide. Increasing the concentration of exogenous cholesterol tends to counteract the effect of the ceramide on cholesterol esterification. There was little effect on exogenous cholesterol esterification when the α-hydroxy fatty acid ceramide was exposed to plasma before adding the labeled cholesterol. The findings demonstrate an interaction between free cholesterol and cerebroside or ceramide containing α-hydroxy fatty acids, but the nature of the interaction is not elucidated.


## INTRODUCTION

Cholesterol has been shown to be esterified in plasma by a reaction described by Glomset (1), in which a fatty acid is transferred from the β-position of phosphatidyl choline to cholesterol to form cholesteryl esters. The enzyme activity is associated primarily with the α-lipoproteins (1-3).

In this study we have measured the extent of esterification of cholesterol by the plasma phosphatidyl choline-cholesterol acyltransferase reaction by two methods. The radioisotopic method utilizes exogenous 4-$^{14}$C-cholesterol esterification as a measure of extent of reaction. The value by this method is less than that determined by the net increase in cholesterol esters after incubation measured by a colorimetric method. Exogenous labeled cholesterol must be taken up by lipoprotein sites prior to its utilization as substrate (4). The time required for the entry of exogenous cholesterol into the endogenous lipoprotein-bound pool results in a relative reduction in the extent of its esterification.

An interaction between cholesterol and cerebroside and ceramide containing α-hydroxy

TABLE I

Interaction of Cerebrosides with Free Cholesterol[a]

| Sample | mg% Cholesterol esterified, colorimetric method | P Value | mg% Cholesterol esterified, radioisotopic method | P Value |
|---|---|---|---|---|
| Control (6) | 36.08 ± 0.41 | — | 12.65 ± 0.65 | — |
| Cerebroside (8), 100 mg% | 36.09 ± 0.55 | N.S. | 4.12 ± 0.25 | <0.01 |
| α-OH FA Cerebroside (4), prepared from 100 mg% | — | — | 7.12 ± 0.09 | <0.01 |
| Non-α-OH FA cerebroside (4), prepared from 100 mg% | — | — | 12.65 ± 0.65 | N.S. |

[a]The numbers in parentheses after each sample represents the number of separate assays performed. The values are expressed as the standard error of the mean. P values were determined by the student's *t* test.

TABLE II

Interaction of Ceramides with Free Cholesterol[a]

| Sample | mg% Cholesterol esterified, colorimetric method | P Value | mg% Cholesterol esterified, radioisotopic method | P Value |
|---|---|---|---|---|
| Control (5) | 32.51 ± 7.85 | — | 18.39 ± 2.80 | — |
| Ceramide (4), 50 mg% | 32.84 ± 3.99 | N.S. | 13.88 ± 1.96 | <0.05 |
| α-OH FA Ceramide (5), 50 mg% | 33.54 ± 1.91 | N.S. | 2.85 ± 0.96 | <0.001 |
| Non-α-OH FA Ceramide (4), 50 mg% | 33.45 ± 4.17 | N.S. | 17.29 ± 0.42 | N.S. |

[a]See Table I.

TABLE III

Effect of Fatty Acid Methyl Esters on Serum Cholesterol Esterification[a]

| Sample | mg% Cholesterol esterified, radioisotopic method | P Value |
|---|---|---|
| Control (5) | 26.93 ± 1.99 | — |
| Methyl cerebronate (5), 100 mg% | 28.86 ± 1.59 | N.S. |
| Methyl lignocerate (5), 100 mg% | 30.01 ± 1.41 | N.S. |

[a]See Table I.

fatty acids is demonstrated by a reduction in the utilization of exogenous 4-$^{14}$C-cholesterol as substrate in the presence of these compounds. No reduction in esterification of lipoprotein-bound cholesterol occurred as determined by the colorimetric method.

## MATERIALS AND METHODS

Chemicals were obtained from the following sources: (a) cerebroside, Supelco, Inc., Bellefonte, Pa., no. 6008; (b) ceramide, Pierce Chemical Co., Rockford, Ill., no. 319401; (c) α-hydroxy fatty acid ceramide, Supelco, Inc., Bellefonte, Pa., no. 6022; (d) non-α-hydroxy fatty acid ceramide, Supelco, Inc., Bellefonte, Pa., no. 6021; (e) methyl lignocerate, Applied Science Laboratories, Inc., State College, Pa., no. 20402; (f) methyl cerebronate, Applied Science Laboratories, Inc., State College, Pa., no. 21699; (g) 4-$^{14}$C-cholesterol, specific activity 30.6 mc/mM, purity greater than 97%, New England Nuclear Corp., Boston, Mass., NEC-018; (h) cholesterol, grade A, Calbiochem, San Diego, Calif., no. 2281.

TABLE IV

Interaction of α-Hydroxy Fatty Acid Ceramide with Serum[a]

| Sample | mM Cholesterol esterified | P Value |
|---|---|---|
| Control (3) | 0.47 ± 0.02 | — |
| Ceramide, 1 mM (3) (added first to serum) | 0.37 ± 0.01 | <0.10 |
| Ceramide, 1 mM (3) (added to cholesterol) | 0.04 ± 0.00 | <0.001 |

[a]Each sample was performed in triplicate, as indicated by the number in parentheses after each sample. The values expressed are the standard error of the mean. Exogenous cholesterol concentration was 0.005 mM in each assay. P values were determined by the student's *t* test.

Cerebroside was separated into α-hydroxy fatty acid and non-α-hydroxy fatty acid cerebrosides by silica gel thin layer chromatography (TLC), using chloroform-methanol-water 65:25:4 v/v/v. Lipids were outlined with iodine vapor in triplicate control TLC determinations. Discarding an inter-area between them, the corresponding areas for the two bands of cerebroside were removed from the TLC plates used to prepare the purified cerebrosides. Subsequent TLC determinations showed only one band each for the α-hydroxy fatty acid cerebroside and the non-α-hydroxy fatty acid cerebroside. The amount of prepared cerebroside incubated with each assay was that recovered from 2 mg of the original cerebroside mixture.

Two mg cerebroside mixture, or the purified cerebroside recovered from 2 mg cerebroside mixture, was coated to the side of an incubation vial with 0.1 μc 4-$^{14}$C-cholesterol ($3.27 \times 10^{-3}$ μM or 1.26 μg), by evaporating the organic solvents under a stream of nitrogen. Two milliliters pooled human serum was added to each assay.

The experiment was also performed with ceramide mixture, α-hydroxy fatty acid cer-

amide, or non-α-hydroxy fatty acid ceramide, except that the final concentration of each assay was 50 mg%.

A similar experiment was performed using methyl esters of lignoceric acid and α-hydroxy lignoceric acid (cerebronic acid). The final concentration of each methyl ester was 100 mg%.

Pooled serum was exposed to 1 mM α-hydroxy fatty acid ceramide prior to transfer of the serum to vials containing 0.1 μc labeled cholesterol coated to the wall. Esterification was compared both to controls and to samples where the α-hydroxy fatty acid ceramide and labeled cholesterol were allowed to interact prior to incubation with serum.

The effect of increasing amounts of ceramide on exogenous labeled cholesterol was studied at concentrations of exogenous cholesterol of 0.005 mM and 0.05 mM. Additional unlabeled cholesterol was added to the radioisotopic cholesterol so that the amount of radioactivity was the same at both concentrations.

The effect of increasing amounts of exogenous cholesterol was studied with concentrations of α-hydroxy fatty acid ceramide of 0.1 mM and 1 mM.

In each experiment the pooled human serum was incubated at 37 C for 24 hr. A 1 ml aliquot of serum was extracted with 24 volumes of chloroform-methanol 2:1 v/v, and fractionated by the Hirsch and Ahrens method (5) or the Leeder and Clark adaptation (6) of the Hirsch and Ahrens method. Radioactivity was determined in a scintillation system using 5 g/liter PPO (2,5-diphenyloxazole, Packard Instrument Co.) and 0.1 g/liter dimethyl POPOP (1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, Packard Instrument Co.), in toluene.

Free and ester cholesterol concentrations were determined by the colorimetric method of Rosenthal et al. (7) before and after incubation. The value obtained by the colorimetric method is the net increase in cholesteryl ester concentration after incubation. Using the radioisotopic method, the extent of esterification of cholesterol is obtained by multiplying the per cent of total radioactivity recovered in the cholesteryl ester fraction times the original free cholesterol concentration.

## RESULTS

Esterification of exogenous 4-$^{14}$C-cholesterol by the plasma phosphatidyl choline-cholesterol acyltransferase reaction was reduced significantly when incubated in the presence of cerebroside or ceramide containing α-hydroxy fatty acids (Tables I and II). No decrease was observed with cerebroside or ceramide containing no α-hydroxy fatty acids. Similarly, no reduction in esterification of exogenous 4-$^{14}$C-cholesterol was observed when incubated in the presence of the methyl esters of lignoceric acid or α-hydroxy lignoceric acid (Table III).

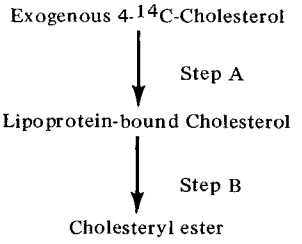


FIG. 1. Esterification of exogenous cholesterol by plasma. Both ceramide and cerebroside, having an α-hydroxy fatty acid, produce their effect on the esterification of labeled cholesterol at Step A, by preventing the uptake of labeled cholesterol by plasma lipoproteins. The radioisotopic method measures the sum of Steps A and B, i.e., uptake and esterification. The colorimetric method measures only esterification of lipoprotein-bound cholesterol (Step B), since the concentration of exogenous labeled cholesterol is negligible. There is no decrease in esterification of cholesterol in the presence of cerebrosides or ceramides measured by the colorimetric method. The decrease in esterification by the radioisotopic method in the presence of ceramide or cerebroside containing α-hydroxy fatty acids is due to a decrease in uptake of exogenous cholesterol by lipoprotein sites (Step A).

Much less reduction in esterification was observed when pooled serum was exposed first to 1 mM α-hydroxy fatty acid ceramide prior to the addition of labeled cholesterol, compared with the simultaneous addition of these compounds. There was a slight but marginally significant decrease in exogenous labeled cholesterol esterification, compared to controls, when the ceramide was added first (Table IV).

Increasing amounts of α-hydroxy fatty acid ceramide resulted in decreasing exogenous cholesterol esterification with concentrations of exogenous cholesterol both at 0.005 mM and at 0.05 mM (Fig. 2A).

Increasing amounts of exogenous cholesterol tended to reverse the decrease in exogenous cholesterol esterification resulting from α-hydroxy fatty acid ceramide at concentrations of 0.1 mM and 1 mM (Fig. 2B).

There was no effect on esterification of endogenous lipoprotein-bound free cholesterol, as measured by the colorimetric method, when incubated with cerebroside, ceramide or fatty acid methyl esters (Tables I-III).

The interaction of cerebroside and ceramide containing α-hydroxy fatty acids with labeled

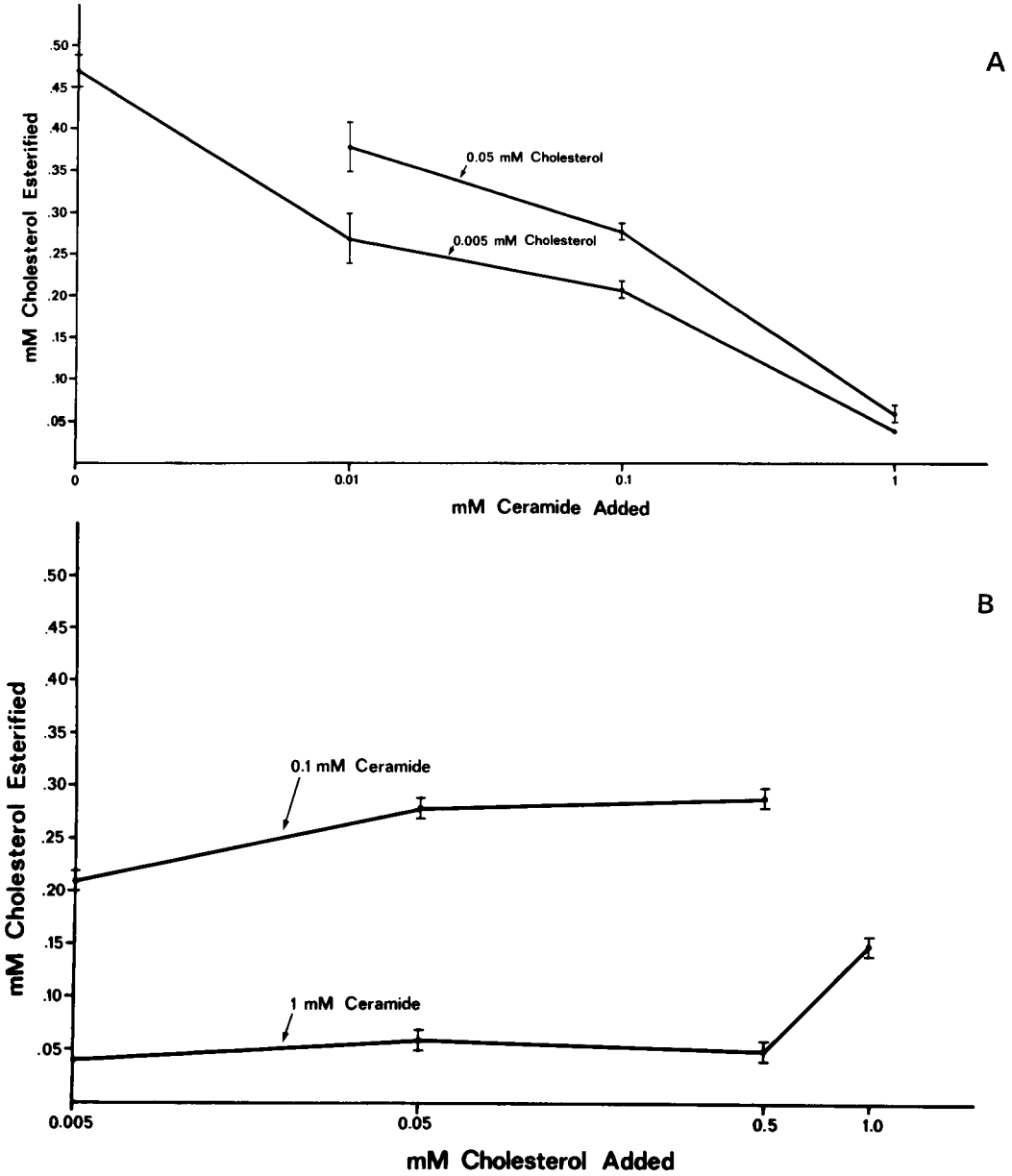


FIG. 2. Plasma cholesterol esterification is measured by the radioisotopic assay for exogenous cholesterol, in the presence of α-hydroxy fatty acid ceramide. The results are expressed as the standard error of the mean of three samples.

free cholesterol prevents its uptake by the plasma lipoprotein sites necessary for esterification (Fig. 1). Cerebroside and ceramide do not act as enzyme inhibitors, since there is no effect on cholesterol esterification measured colorimetrically. The reduction in esterification as measured by the radioisotopic method is the result of binding the labeled free cholesterol by cerebroside and ceramide containing α-hydroxy fatty acids, and preventing them from entering the lipoprotein-bound cholesterol pool.

## DISCUSSION

An interaction between free cholesterol and cerebroside or ceramide containing α-hydroxy fatty acids is demonstrated. No interaction is observed with cerebroside or ceramide containing no α-hydroxy fatty acids. It is reasonable to propose that the interaction between

cholesterol and cerebroside or ceramide containing α-hydroxy fatty acids is polar. One explanation is that the α-hydroxy fatty acid in amide bond to sphingosine provides an environment favorable for hydrogen bonding with the hydroxyl group of cholesterol.

The observed decrease in esterification of exogenous cholesterol by α-hydroxy fatty acid ceramide is not the result of binding of lipoprotein sites by the ceramide, since prior exposure of serum to the ceramide does not decrease markedly the esterification of exogenous labeled cholesterol.

Similarly, the decrease in exogenous labeled cholesterol esterification can be decreased further by increasing the concentration of α-hydroxy fatty acid ceramide and can be partially reversed by increasing the concentration of exogenous cholesterol. These observations indicate that there is an interaction between free cholesterol and α-hydroxy fatty acid ceramide.

An interaction between cholesterol and cerebroside has been suggested by other observations, although the role of α-hydroxy fatty acids was not elucidated. Jones and coworkers have shown that feeding cerebroside to hypercholesterolemic patients resulted in a reduction in their plasma cholesterol concentration (8,9). Rosenheim and Webster have shown that feeding cerebroside to animals results in increased coprostanol excretion (10). Coprostanol is derived from cholesterol in the intestine (11), and cerebroside has been shown to be unnecessary for the enzymatic conversion (12). Furthermore total sterol excretion is increased when cerebroside is ingested. Kerasin, the non-α-hydroxy fatty acid cerebroside, has no effect; but in one subject, the ingestion of phrenosin (α-hydroxy fatty acid cerebroside) resulted in increased coprostanol and other sterol excretion (10). On the basis of our findings, the binding of cholesterol by cerebroside containing α-hydroxy fatty acids may result in decreased absorption of cholesterol from the intestine, resulting in the observed reduction in plasma cholesterol concentration and the corresponding increase in fecal excretion of sterols.

Cholesterol and cerebroside are both components of the myelin sheath. The interaction between cholesterol and α-hydroxy fatty acid cerebroside may result in a greater cohesiveness of the myelin sheath. During development, when no stainable cerebroside is present, esterified cholesterol is demonstrable. When cerebroside becomes detectable by histochemical techniques, virtually all the cholesterol is in the free form (13). The cholesterol-cerebroside interaction, in which α-hydroxy fatty acids are required, may then occur, and contribute to the integrity of the myelin sheath.

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# Specificity of *Geotrichum candidum* Lipase with Respect to Double Bond Position in Triglycerides Containing *cis*-Octadecenoic Acids

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## ABSTRACT

Fifteen approximately random, mixed, triacylglycerols, each of which contained 12:0, 14:0, 9,12-18:2, 16:0 and only one positional isomer of *cis* 18:1 (Δ2 through Δ16), were synthesized. These mixtures were used as substrates for the lipase from the microorganism *Geotrichum candidum* to define the specificity of the enzyme for unsaturated fatty acids. Comparatively small quantities of the 18:1 isomers, other than 9-18:1, were hydrolyzed. Relatively large amounts of 18:2 were released from all substrates. There was no preference between 9-18:1 and 18:2. The positional isomers other than 9-18:1 accumulated in the di- and monoacylglycerols.

## INTRODUCTION

A lipase elaborated by the microorganism *Geotrichum candidum* has been found to attack triglycerides containing common fatty acids, specifically hydrolyzing fatty acids having *cis*-9- and *cis,cis*-9,12-unsaturation (1,2). The enzyme was found to hydrolyze oleate, linoleate and palmitoleate from synthetic triacylglycerols, but not petroselinate (*cis*-6-), vaccenate (*cis*-11-) or *trans* isomers (3). Other positional isomers of 18:1 were unavailable at the time of that study, and the full scope of the enzymes specificity for unsaturation has not been tested. Nearly random triacylglycerols containing all of the positional isomers of 18:1 have been synthesized, each containing 12:0, 14:0, 16:0, 18:2 and only one positional isomer of 18:1 (Δ-2 through Δ-16). These triglycerides were chosen because each would contain three fatty acids that were ostensibly not hydrolyzed by the lipase, one acid that is known to be hydrolyzed, and one isomer of 18:1 to be tested. All acids would be present in nearly equal proportions, and gas liquid chromatography (GLC) could be used for quantitative analysis of the discrimination for or against a given isomer. Each triacylglycerol was hydrolyzed by *G. candidum* lipase, the products of hydrolysis were recovered and the fatty acids were identified and measured by GLC. The fatty acid contents of each fraction were normalized to an added internal standard of 17:0. The hydrolysis of each isomer of 18:1 was related to the hydrolysis of the internal standard, 18:2, which is known to be easily hydrolyzed by the *Geotrichum* lipase.

## EXPERIMENTAL PROCEDURES

### Synthesis of Mixed Triacylglycerols

A series of nearly random, mixed triacylglycerols was synthesized, each to contain approximately equal amounts on a weight basis of 12:0, 14:0, 16:0, 9,12-18:2 and one isomer of 18:1. The synthesis was carried out with each of the *cis* isomers of 18:1 from Δ2 through Δ16, keeping the other constituents the same. The 9,12-18:2 was included in the mixture as an internal standard that would be hydrolyzed from the triacylglycerol by *Geotrichum* lipase, and any activity upon the isomeric 18:1 acids could be related to the activity upon 18:2. All methyl octadecenoate isomers, except 9-18:1 and 6-18:1, were obtained from Gunstone and Ismail (4). The 12:0, 14:0, 16:0 and 9,12-18:2 acids obtained from the Lipids Preparation Laboratory of The Hormel Institute, University of Minnesota, were checked and found to be better than 99% pure.

Saponification of each isomeric methyl ester was carried out on 293 μl with 0.3 g potassium hydroxide, 12 ml methanol and 2 ml water. The mixture was refluxed until monitoring of the mixture by thin layer chromatography (TLC) indicated no methyl ester remaining. The mixture was then cooled and diluted with 20 volumes water, acidified to pH 2 with concentrated hydrochloric acid and extracted three times with chloroform. The combined extracts containing theoretically 250 mg acid, were dried over anhydrous sodium sulfate.

The following synthesis of triacylglycerol is

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essentially that of Mattson and Volpenhein (5). Mixed fatty acid chlorides were prepared by refluxing a mixture containing 250 mg each of 12:0, 14:0, 16:0, 9,12-18:2 and one isomer of 18:1 in dry benzene with 0.64 ml oxalyl chloride. Periodically an aliquot of the reaction mixture was reacted with 5 $\mu$l methanol (distilled over magnesium) plus 5 $\mu$l dry pyridine to form methyl esters. The progress of the reaction was assayed by the relative sizes of the spots for free acid and methyl esters separated by TLC using petroleum ether (bp 30-60 C)-ether-acetic acid 70:30:1. After the free fatty acids were all converted to acyl chlorides, the benzene and excess oxalyl chloride were removed under vacuum for 4 hr at 70-75 C. Then 15 ml chloroform, which had been washed with water and dried over anhydrous sodium sulfate, was added to the fatty acid chlorides with 0.18 ml anhydrous pyridine and 0.091 ml anhydrous glycerol. Alternatively, the glycerol was added step-wise to avoid an excess which might lead to the formation of mono- and diacylglycerols. The mixture was stirred and refluxed until monitoring by TLC indicated no fatty acid chlorides. Twenty volumes of petroleum ether-ethyl ether 1:1 were then added to the reaction mixture. The ether solution was washed three times with water, twice with 1% hydrochloric acid and twice again with water before being dried over anhydrous sodium sulfate.

Pure triacylglycerols were obtained by preparative TLC of the ether extract on 3 mm cakes of Silica Gel H, developed twice in petroleum ether-ether 90:10. The isolated bands were collected and extracted with chloroform to obtain the triacylglycerols. An aliquot of each preparation was transesterified to ascertain the fatty acid composition. The remainder was sealed under vacuum in ampules for storage prior to use.

During storage a yellow hue developed, possibly due to oxidation. Lipolysis was inhibited until the substance was removed by elution through a column of alumina (6). A similar inhibition of pancreatic lipase when linoleate acylglycerols were the substrates has been noted.

#### Digestions and Analyses

Digestions of triacylglycerol substrates by *G. candidum* lipase were conducted in duplicate with a control minus the enzyme. The enzyme was obtained from *G. candidum* (7) and stored at -20 C. Each digestion was carried out on 20-25 mg substrate, weighed into a 25 ml Erlenmeyer flask. After addition of 8 ml *tris* buffer, (0.25 M, pH 8.2) containing 1% gum arabic and 0.5 ml of 0.1 M $CaCl_2$, the aqueous

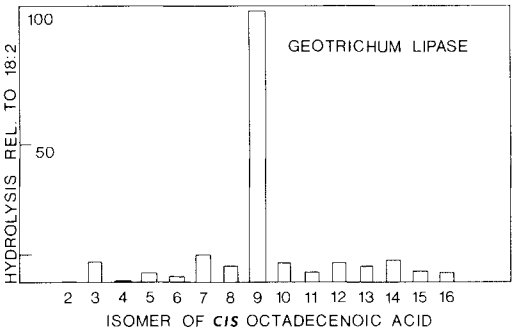


FIG. 1. Yields of isomeric 18:1 acids hydrolyzed from triglycerides by *Geotrichum* lipase, expressed as per cent of the yield of linoleic acid from the same triglyceride preparations.

mixture was warmed until the substrate melted and then was emulsified with a Branson Sonifier. The tip of the sonifier was rinsed with ethyl ether, and the emulsified substrate was held at 37 C for 30 min to remove the ethyl ether.

To each of two flasks was added 5 ml *tris* buffer containing 10 mg *G. candidum* lipase, and the digestion was allowed to proceed for 30 min at 37 C. The digestions were terminated by the addition of 1 ml 20% $H_2SO_4$ and were extracted with redistilled $CHCl_3$ (3). After removal of the solvent, the digestion products were separated by preparative TLC on 20 x 20 cm glass plates coated with 0.5 mm Silica Gel G. The plates were developed in petroleum ether (30-60 C)-ethyl ether-acetic acid 90:30:2 to separate triacyl-, diacyl- and monoacylglycerols. After brief exposure to $I_2$ vapors, the visible bands were scraped and extracted with $CHCl_3$/MeOH 9:1. An internal standard, methyl heptadecanoate, was added to each sample, and the solvent was removed. The contents of each were transferred to a Babcock milk test bottle with ca. 0.7 ml heptane. To each sample was added 5 ml of 2.5 N Na methoxide, and the bottle was shaken vigorously and allowed to stand for 15 min. The free fatty acid fraction was esterified by 3 hr of exposure at room temperature to 5 ml methanol containing 20% dry HCl. The bottles were then filled with distilled $H_2O$ and centrifuged at 6000 rpm for 10 min, bringing the heptane layer to the top of the narrow neck of the bottle. A portion of the layer was then injected directly into a gas liquid chromatograph.

Two GLC instruments were used: a Barber Colman, Selecta-System 5000 with a stainless steel column (10 ft x 1/4 in.) packed with 18% DEGS on Chromosorb W and F & M Model 810 with a stainless steel column (10 ft x 1/8 in.)

TABLE I

Per Cent Composition of the Fatty Acids Released by Hydrolysis with *Geotrichum* Lipase from Triglycerides Containing Isomeric *cis*-18:1 Acids[a]

| 18:1 Isomer | 12:0 | 14:0 | 16:0 | 18:1 | 18:2 |
|---|---|---|---|---|---|
| Δ2 | 16.5 | 10.3 | 14.6 | 0 | 58.6 |
| Δ3 | 9.0 | 8.6 | 13.9 | 3.1 | 64.8 |
| Δ4 | 7.8 | 5.4 | 7.3 | 0 | 79.5 |
| Δ5 | 8.3 | 5.0 | 8.2 | 2.7 | 75.8 |
| Δ6 | 9.4 | 6.8 | 14.4 | 2.0 | 67.4 |
| Δ7 | 10.2 | 5.5 | 6.8 | 6.6 | 70.9 |
| Δ8 | 8.0 | 3.7 | 9.2 | 4.3 | 74.8 |
| Δ9 | 3.5 | 9.1 | 17.5 | 50.6 | 15.3 |
| Δ10 | 7.9 | 4.6 | 6.6 | 6.9 | 74.0 |
| Δ11 | 7.7 | 6.7 | 7.8 | 4.3 | 73.5 |
| Δ12 | 22.8 | 11.3 | 15.4 | 6.7 | 43.8 |
| Δ13 | 11.7 | 8.2 | 17.6 | 6.1 | 56.4 |
| Δ14 | 17.2 | 8.6 | 12.4 | 5.1 | 56.7 |
| Δ15 | 6.5 | 4.2 | 10.3 | 3.9 | 75.1 |
| Δ16 | 10.1 | 8.3 | 12.0 | 1.9 | 67.5 |

[a]Minor components deleted.

containing 15% DEGS on Chromosorb W. The instruments were operated at 185 C and 175 C, respectively.

We found that 16-18:1 could not be separated from 18:2 on either a DEGS column or on an EGS column. Each fraction from the 16-18:1 digestion was therefore separated after conversion to methyl esters, by TLC on a 20 x 20 cm plate coated with 0.4 mm Silica Gel G containing $AgNO_3$; 55 g/15 g w/w. After development with petroleum ether (30-60 C)-ethyl ether 9:1 and exposure to $I_2$ vapors, the saturated, monounsaturated and diunsaturated esters were recovered and analyzed by GLC. An internal standard of methyl heptadecanoate was added to the mono- and diunsaturated ester samples for quantification. By this means, 16-18:1 is separable from 18:2. The procedure was tested on the digestion products from the Δ-6-triacylglycerol, yielding quantitative results almost identical to those obtained by GLC alone.

The areas under each peak in the GLC chart were converted to mole units in relation to the internal standard 17:0 taken as unity, and then calculated as mole per cents.

## RESULTS

The fatty acid compositions (mol %) of the original triacylglycerols indicated that relatively small quantities of 2-18:1 and 3-18:1 were incorporated into the triacylglycerols, possibly due to the proximity of the double bond to the carboxyl group or to the *cis* configuration or to both. The incorporation of 18:2 was likewise nonuniform. The lowest proportions occurred when 18:2 was "competing" with 9-18:1 and 12-18:1. It is clear that acylation of 18:2 was not always equimolar, and there was also considerable variation in the contents of saturated acids, indicating nonequimolar acylation. A subsequent study of the hydrolysis of these preparations by pancreatic lipase revealed that the distribution of 18:1 isomers and 18:2 was nonrandom. However these deviations from randomness and equimolarity do not negate the phenomenon of specificity studied here, because the measure of specificity is based upon the proportions of a given acid in the fatty acids hydrolyzed.

If the specificity of *G. candidum* lipase is as our earlier results indicated (3), we should find large proportions of 9-18:1 and 18:2 in the free fatty acids, and relatively small proportions of these acids in the mono-, di- and residual triacylglycerols. The compositions of the residual triacylglycerols, not reported here, reflected such specificity of the lipase. With the exception of 9-18:1, which decreased as expected in the residual triacylglycerols, the rest of the 18:1 isomers either increased or remained much the same when compared to the original triacylglycerols. The 18:2 content was lower in every case, and in general the amounts of saturated acids increased, indicating that those molecules containing 9-18:1 and 18:2 were preferentially hydrolyzed. The ratios of 9-18:1 to 18:2 were almost the same in original and residual triacylglycerols, suggesting that these two acids were hydrolyzed at the same rates.

The analyses of the free fatty acids (Table I) indicate that 9-18:1 was released in much larger quantities than any of its isomers. Neighboring isomers were hydrolyzed to a low degree (Fig. 1). With the exception of the substrate con-

taining 9-18:1, much more 18:2 was hydrolyzed than any of the 18:1 isomers. In the original triacylglycerol and in the free fatty acids from the 9-18:1 triglyceride, the per cent of 18:2 of total unsaturated acids is about the same, suggesting that there was no preference between 9-18:1 and 18:2. The total saturated acids hydrolyzed ranged from 30 to 50%, demonstrating that the enzyme(s) does not discriminate completely against saturated acids (2). The degree of total hydrolysis ranged from 12 to 15%, or ca. 30 to 40% of the unsaturated acids.

The diacyl glycerols contain minimal amounts of 9-18:1 and 18:2, and conversely larger proportions of the other isomers of 18:1 and of the saturated acids. The general trend noted in the diacylglycerols was also seen in the monoacylglycerols, except for some low concentrations of 12:0 which may have been hydrolyzed from diacylglycerols to form monoacylglycerols.

## DISCUSSION

The remarkable specificity of *G. candidum* lipase for *cis*-9-fatty acid unsaturation has been confirmed by study involving all positional isomers of *cis* 18:1. The enzyme hydrolyzed very little of the other 14 positional isomers of 18:1, when *cis*-9,*cis*-12-18:2 was present in the triacylglycerol substrate and was hydrolyzed extensively. There was no preference between 9-18:1 and 18:2. The hydrolysis of the isomeric 18:1 acids was generally less than the hydrolysis of the saturated acids in the triglycerides. The diacylglycerols and the monoacylglycerols were enriched in the 18:1 positional isomers other than 9-18:1. This study illustrates an extreme case of enzyme specificity related to double bond position. The lipolytic enzyme, *Geotrichum* lipase has a high degree of discrimination in favor of acids having a *cis* double bond in the $\Delta 9$ position.

## ACKNOWLEDGMENTS

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# The Effect of Dietary Supplementation of Cholesterol and Its Subsequent Withdrawal on the Liver Lipids and Serum Lipoproteins of Chickens[1]

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## ABSTRACT

Two groups of male chickens were fed either a control diet (group N) containing a standard poultry ration admixed with 10% corn oil or a cholesterol diet (group C) in which the control diet was supplemented with 1% cholesterol. After 6 weeks on the diets, a negligible amount of very low density lipoprotein (VLDL) was found in the serum from control animals. On the other hand, the serum VLDL from the cholesterol-fed birds was the predominant lipoprotein and carried 72% of the total serum lipids. Surprisingly this lipoprotein from cholesterol-fed animals was very low in triglycerides (6%) and high in total cholesterol (77%). While the level of serum low density lipoprotein was unaffected by the ingestion of cholesterol, the concentration of total lipids and phospholipids in the high density lipoprotein decreased in cholesterol-fed animals. The greatest change in liver lipids from animals fed cholesterol was found in the cholesterol esters, whereas the unesterified cholesterol, triglyceride and phospholipid varied slightly or remained constant. In normal animals the distribution of cholesterol between the liver and the serum was about equal, whereas in the cholesterol-fed birds the liver accounted for 80% of the cholesterol found in the liver-serum pool. In order to determine how the hypercholesterolemic bird responds to the withdrawal of cholesterol from the ration, a diet-exchange experiment was conducted. In this study the birds that were originally fed the cholesterol diets (group C) for 6 weeks were placed on the control diet (group CN) and the birds fed the control diet (group N) for 6 weeks were given the cholesterol diet (group NC). At periodic intervals, 1, 3, 7 and 14 days following the change of diets, 3-5 animals from each group were sacrificed, and analyses performed on their serum lipoproteins and liver lipids. Within one day after the diet substitution, there was a 31-fold increase and a 46% decrease, respectively, in the serum VLDL concentration in groups NC and CN as compared with their corresponding steady state values (groups N and group C, respectively). The liver cholesterol increased 4-fold and decreased 40%, respectively, in the two groups NC and CN as compared with the values obtained before the diet substitution. It is suggested that the concentration of cholesterol in the liver is the principal factor controlling cholesterol metabolism in chickens fed a hypercholesterolemic diet.


## INTRODUCTION

The serum cholesterol levels of many species differ in their response to dietary cholesterol. For example, the rat (1) and guinea pig (2) exhibit only a nominal increase in their serum cholesterol concentration upon ingestion of a cholesterol-containing diet as compared with the enormous elevation in serum cholesterol observed with the rabbit (3) and the chicken (4-7).

Although the effect of dietary cholesterol on serum cholesterol levels has been investigated extensively in several species, the serum lipoproteins of most species, other than those of humans, have not been characterized sufficiently. Very little is known concerning the nature and magnitude of the compositional changes in the liver and serum lipoproteins of the hypercholesterolemic chicken when placed on a normal poultry ration. In the present study, three classes of lipoproteins were isolated by preparative ultracentrifugation from normal and cholesterol-fed chickens and characterized on the basis of their lipid composition, protein content and by disc electrophoresis. The alteration in liver lipids and in serum lipoproteins in normal and hypercholesterolemic chickens was investigated at periodic

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TABLE I

Composition of Serum Lipoproteins and Liver of Chickens Fed Control and Cholesterol Diets[a,b]

| Description | Diet | mg Lipoprotein per 100 ml serum or mg/ gm wet wt liver | | % Protein in lipoprotein | % Lipid Class in Total Lipid of each lipoprotein class or liver | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Lipoprotein | Total lipids | | PL | FC | | CE | TG |
| VLDL | Control | 6.6±3.3 | 4.4±2.7 | 10.9 | 21.5 | | (46.4)[c] | | 32.1 |
| | 1% Cholesterol | 1085.5±104.5 | 967.1±103.0 | | 17.5 | 19.3 | | 57.2 | 6.0 |
| LDL | Control | 80.6± 10.3 | 57.0± 11.9 | 29.3 | 32.6 | | (34.4) | | 33.0 |
| | 1% Cholesterol | 76.7± 16.2 | 58.8± 14.6 | 23.3 | 21.3 | | (59.1) | | 19.6 |
| HDL | Control | 353.5± 37.3 | 155.1± 19.1 | 56.1 | 54.6 | 6.6 | | 34.2 | 4.6 |
| | 1% Cholesterol | 320.0± 36.4 | 128.5± 19.7 | 59.8 | 28.7 | 9.5 | | 53.2 | 8.6 |
| Liver | Control | | 37.7± 1.3 | | 58.5 | 7.4 | | 0.8 | 33.3 |
| | 1% Cholesterol | | 118.4± 13.8 | | 18.7 | 6.7 | | 54.3 | 20.3 |

[a]PL = phospholipid; FC = unesterified cholesterol; CE = cholesterol esters; TG = triglyceride; VLDL = very low density lipoprotein; LDL = low density lipoprotein; and HDL = high density lipoprotein.

[b]Values are the means of six chickens (five chickens for liver determinations) fed for 6 weeks on the diet ± SD. All animals were fed until sacrifice.

[c]The numbers in parenthesis are the total cholesterol value. FC and CE values were not determined in these cases.

intervals after substitution of their diets, control for cholesterol and vice versa.

## MATERIALS AND METHODS

Male chickens, New Hampshire-Columbian cross, 1 day old, were divided into two groups and were given a control (group N) and cholesterol diet (group C), respectively, for periods varying from 6 to 8 weeks before sacrifice. The control diet contained 90% of a complete poultry ration and 10% of corn oil. The cholesterol diet contained the same constituents as the control diet plus 1% of cholesterol (Nutritional Biochemicals Corp.). The animals had free access to food and water at all times. In the diet exchange experiments, chickens previously fed the control diet for 6 weeks were switched to the cholesterol diet (group NC), while birds fed the cholesterol diet for 6 weeks were transferred to the control diet (group CN). The animals were fed ad libitum until sacrifice 1, 3, 7 and 14 days after the change of diets.

The chickens were anesthetized using either diethyl ether or sodium pentobarbital (Diamond Laboratories) and the blood was obtained by cardiac puncture. The tissues used in this study were quickly excised and rapidly processed for lipid and protein analyses. Blood was allowed to clot at ambient temperatures and the serum was obtained by centrifugation. A Beckman Model L2-65 Preparative Ultracentrifuge was used to separate the different lipoproteins from each other according to the method of Havel et al. (8). The very low density lipoproteins ([VLDL] $d < 1.019$ g/ml) and low density lipoproteins ([LDL] 1.019-1.063 g/ml) were isolated by centrifuging for 1.5 x $10^8$ g-min while the high density lipoproteins ([HDL] 1.063-1.21 g/ml) were separated by centrifuging for 1.7 x $10^8$ g-min. The temperature of isolation was 11 C, and a 40.3 rotor was used in all cases. Separation of the VLDL from the LDL in hypercholesterolemic animals was difficult because of both the bulk and the tendency of this VLDL to stick to the walls of the centrifuge tube. The soluble portion was removed with an antigen pipet and the insoluble part with the aid of a narrow spatula. Although the VLDL was removed as carefully as possible, this handling problem still caused some contamination in the next step, i.e., in the LDL fraction.

The lipid extraction was accomplished according to the method of Folch et al. (9). Protein determinations were made according to the method of Lowry et al. (10) employing bovine serum albumin (Sigma Chemical Co.) as the standard. Occasionally the VLDL and LDL fractions from the cholesterol-fed birds were quite turbid, and the protein determinations would therefore be unreliable. To remove the

TABLE II

Protein and Lipid Composition of VLDL During Diet Change[a]

| Diet change | Days after change | mg/VLDL Fraction/100 ml serum[b] Lipoprotein | Protein | PL | TC | TG | Weight ratio, CE/FC |
|---|---|---|---|---|---|---|---|
| 1% Cholesterol to control | 0 | 1085.5±104.5 | 109.4±16.4 | 175.7±27.8 | 766.6±179.0 | 59.9±10.0 | 2.97 |
| | 1 | 587.0±116.6 | 73.2±13.2 | 118.5±23.7 | 420.1± 96.4 | 22.8±14.5 | 2.93 |
| | 3 | 581.5± 85.6 | 75.5±11.5 | 102.8±10.0 | 347.4± 42.6 | 55.8±38.2 | 2.70 |
| | 7 | 83.8± 21.6 | 28.0± 3.2 | 13.6± 3.9 | 38.0± 15.7 | 4.1± 1.3 | — |
| | 14 | 9.0± 4.0 | 1.0± 0.2 | 1.5± 1.1 | 3.4± 1.6 | 3.1± 1.1 | — |
| Control to 1% cholesterol | 0 | 6.6± 3.3 | 1.0± 0.2 | 1.2± 0.5 | 2.6± 0.8 | 1.8± 1.8 | — |
| | 1 | 203.7± 16.3 | 46.9±14.0 | 40.0± 3.0 | 110.3± 18.5 | 11.8± 6.6 | 2.77 |
| | 3 | 557.5± 62.3 | 78.4±13.7 | 94.5± 7.6 | 337.8± 36.5 | 46.8±14.7 | — |
| | 7 | 449.2± 61.5 | 72.0± 2.4 | 80.3± 2.8 | 292.0± 10.3 | 28.7±16.9 | 3.18 |
| | 14 | 774.7±183.8 | 80.3±17.6 | 114.6±43.1 | 495.5±174.2 | 84.4±61.3 | 3.25 |

[a]For abbreviations see Table I.

[b]Values are the means ± SD. Five chickens were used for each determination for the 0 and 1st day after diet change, while three chickens were used for each determination for the 3rd, 7th and 14th day after diet change.

turbidity, a modification (11) of the method of Lowry et al. (10) was employed. In brief, the method consisted of spotting the sample on filter paper (1.6 cm diameter), drying, elution of some of the lipid with petroleum ether (bp 30-60 C), elution of the protein on the filter paper with 1N sodium hydroxide followed by the standard procedure of Lowry et al. (10). Phospholipids (PL) were estimated by the method of Bartlett (12) using dipalmitoyl lecithin (Sigma Chemical Co.) as the standard. For the separation of phospholipids, the thin layer chromatographic (TLC) technique described by Skipski et al. (13) was employed. Total cholesterol (TC) was determined according to a modification of the method of Zuckermann et al. (14). Unesterified cholesterol (FC) and cholesterol ester (CE), as well as the CE/FC ratios, were determined according to a modification of the procedure of Beukers et al. (15) using TLC to separate the two cholesterol forms. Triglycerides were separated by TLC, scraped off the plate, eluted with chloroform and then determined by the method of Amenta (16) using tripalmitin as the standard.

The lipoprotein concentration was obtained by summing the concentrations of the protein and lipid constituents (PL, TG, FC, CE) found in the respective fraction. Likewise, the total lipid concentration of either the liver or lipoprotein fraction was obtained by adding the concentrations of the separate lipid classes. The TC concentrations of the liver-serum pools were calculated to give the concentrations that would be present in a 1.0 kg chicken. This was determined by assuming that the serum was 7% (17) and the liver was 2.5% (18) of the body weight of the chicken.

The amount of ingested cholesterol that was not absorbed in the cholesterol-fed chickens was determined by subtracting the amount of cholesterol per unit body weight in the feces of control birds from that found in the feces of the cholesterol-fed birds. The sodium salts as well as the acids of cholic, desoxycholic, taurocholic, choleic, glycocholic and glycotauric acids were found not to interfere with the fecal cholesterol determinations. From the quantity of unabsorbed cholesterol in the feces and the amount of feed consumed per day by the chickens on the cholesterol diet, it was estimated that the chicken absorbed ca. 83% of the ingested cholesterol.

The disc electrophoretic procedure was the same as method C of Narayan et al. (19). The acrylamide concentration of the main gel was 3.75% and its length was 1.25 in. On top of the main gel was a spacer gel of 0.5 in. length. Electrophoresis was terminated when the tracking dye penetrated 0.75 in. into the main gel.

## RESULTS

### Composition of Serum Lipoproteins and Liver

The composition of the serum lipoproteins and the liver of the chickens fed the control and cholesterol diets is presented in Table I. A striking difference was seen in the VLDL fractions of animals on the two diets. In the normal animals the VLDL fraction comprised ca. 1.5% of the total serum lipoproteins, while in the cholesterol-fed animals the VLDL fraction constituted 73% of the total serum lipoproteins. The low percentage of triglycerides

TABLE III

Protein and Lipid Composition of LDL During Diet Changes[a]

| Diet change | Days after change | Lipoprotein | mg in LDL/ 100 ml Serum[b] Protein | PL | TC | TG |
|---|---|---|---|---|---|---|
| 1% Cholesterol to control | 0 | 76.7±16.2 | 17.1±4.3 | 13.3±4.3 | 36.8±9.7 | 12.2±3.4 |
| | 1 | | 24.9±4.2 | 23.2± 8.3 | 42.8± 9.9 | |
| | 3 | | 20.5±1.2 | 19.2± 1.4 | 34.6± 3.3 | |
| | 7 | | 32.4±2.8 | 38.9±11.2 | 57.4±14.0 | |
| | 14 | | 25.3±1.1 | 24.2± 3.4 | 28.8± 4.1 | |
| Control to 1% cholesterol | 0 | 80.6±10.3 | 21.5±2.9 | 17.9± 4.6 | 18.9± 2.9 | 18.1±5.3 |
| | 1 | | 20.6±4.6 | 13.6± 4.1 | 25.3± 6.6 | |
| | 3 | | 15.9±4.8 | 12.3± 4.0 | 23.8±10.0 | |
| | 7 | | 16.2±1.1 | 11.2± 0.8 | 21.7± 2.8 | |
| | 14 | | 16.3±0.8 | 11.7± 0.5 | 23.7± 1.6 | |

[a]For abbreviations see Table I.

[b]See footnote to Table II.

(6%) and the very large amount of total cholesterol (77%) found in the VLDL fraction of the cholesterol-fed chickens makes this fraction unique among VLDL fractions from other species, which usually have the percentages of these two lipid classes reversed. The percentage of phospholipid found in the HDL fraction decreased ca. 50% in the cholesterol-fed animals as compared to the normal birds. In normal chickens, 84% and 77% of the serum PL and TC, respectively, were present in the HDL fraction, whereas 78% and 87% of the PL and TC, respectively, were found in the VLDL fraction of the cholesterol-fed birds. The total lipid concentration of the liver from the cholesterol-fed chickens was three times greater than that found in the livers from normal birds. Most of this increase was due to the increase in the liver cholesterol ester content, which comprised only 0.8% of the lipids in normal birds, while it increased dramatically to 54% of the lipids in the livers of cholesterol-fed chickens.

#### Diet Interchange and VLDL Composition

Dramatic changes were observed in the VLDL composition when normal and cholesterol-fed animals had their respective diets switched (Table II). There was a 31-fold increase and a 46% decrease in the serum VLDL from groups NC and CN, respectively, after one day of diet substitution. The TC concentration in VLDL increased 43-fold and decreased 45% after the first 24 hr with the groups NC and CN, respectively. There was ca. 3.0 times as much CE as FC by weight in all the VLDL fractions. The determinations of PL composition indicated that phosphatidyl choline was present to the extent of 70% at all stages in the diet interchange studies. The plateau reached in the lipoprotein concentration between 3 to 7 and 1 to 3 days in the group NC and CN animals, respectively, is difficult to interpret. It is possible that at these periods in the diet substitution, lipid is being taken up or released depending on the diet, from extra-hepatic sources (viscera, muscle, etc.). Within 14 days, the chickens on the CN diet had VLDL concentrations that were almost the same as those found in normal birds. The chickens on the NC diet had VLDL levels that were ca. 71% of that found in animals fed the cholesterol diet for 6 weeks, indicating that after the initial rapid increase in the VLDL concentration there is a more gradual increase when normal birds are placed on the cholesterol diet.

#### Diet Interchange and LDL Composition

The changes observed in the LDL concentrations due to diet substitutions were far less striking than those seen in the VLDL fraction (Table III). No component, protein, TC, PL or TG changed by more than 2 to 3-fold. These changes were probably of minor significance to the animal, since the LDL fraction contains only from 4 to at most 20% of any one of these components, except TG, in the serum. Some of the observed changes may also have been due to the difficulty in separating the very turbid VLDL from the LDL in the hypercholesterolemic birds during centrifugation.

#### Diet Interchange and HDL Composition

It appeared that only the protein and PL moieties were very sensitive to the dietary

TABLE IV

Protein and Lipid Composition of HDL During Diet Changes[a]

| Diet change | Days after change | mg HDL in 100 ml Serum[b] | | | | | Weight ratio, CE/FC |
|---|---|---|---|---|---|---|---|
| | | Lipoprotein | Protein | PL | TC | TG | |
| 1% Cholesterol to control | 0 | 320.0±36.4 | 180.9±18.6 | 35.7± 7.7 | 77.9±13.5 | 10.7±2.0 | 5.63 |
| | 1 | 308.9±19.6 | 192.7±16.8 | 53.6±11.4 | 77.1± 9.3 | 12.0±1.0 | --- |
| | 3 | 347.0±19.6 | 200.7± 9.5 | 53.2± 5.7 | 84.8± 5.1 | 8.4±2.0 | 5.90 |
| | 7 | 383.1±29.4 | 213.1±17.6 | 88.0± 6.0 | 72.6± 5.2 | 9.4±2.0 | 8.19 |
| | 14 | 391.7± 3.0 | 197.9± 5.0 | 111.3± 2.4 | 73.1± 7.9 | 9.3±2.1 | 7.26 |
| Control to 1% cholesterol | 0 | 353.5±37.3 | 215.3±30.7 | 97.9±35.2 | 73.0±11.9 | 88.3±2.4 | 5.18 |
| | 1 | 331.1±17.0 | 180.4±13.4 | 71.8± 3.0 | 61.2± 5.1 | 6.5±1.3 | 5.72 |
| | 3 | 249.1±37.8 | 150.3±24.2 | 34.8± 8.1 | 49.7± 8.5 | 7.0±2.2 | 6.72 |
| | 7 | 249.0±13.2 | 149.4± 4.4 | 39.9± 1.4 | 48.8± 7.8 | 8.8±1.5 | 7.20 |
| | 14 | 239.1±17.7 | 138.4±10.7 | 40.8± 4.3 | 49.5± 4.5 | 10.4±2.2 | --- |

[a]For abbreviations see Table I.

[b]See footnote to Table II.

changes (Table IV). Within 3 days of the dietary substitutions, the change in the HDL phospholipid levels became significant ($P < 0.05$). In animals from group NC, the HDL phospholipid levels decreased to approximately a third of their normal value. In chickens from group CN, the phospholipid content increased; until after 2 weeks on the new diet, the level was three times greater than that of the initial level. The change in HDL phospholipid was found to be nonselective among the different phospholipid classes. About 80% of the HDL phospholipids were comprised of phosphatidyl choline. The protein content of HDL decreased by 35% after 2 weeks for the chickens of group NC, while it increased only slightly in the same time period for the animals in group CN. After 2 weeks on the diets, the total cholesterol content of HDL decreased 32% in birds in group NC, whereas it remained essentially the same in chickens in group CN. The weight ratio of CE/FC in the HDL fraction (5.2-8.2, mole ratio 3.0-4.7) was about twice that found in the VLDL fraction (2.7-3.3, mole ratio 1.6-1.9) regardless of the diet that was fed to the birds.

#### Polyacrylamide Electrophoresis

Disc electrophoresis demonstrated that a large portion of VLDL from cholesterol-fed birds did not penetrate the spacer gel. Whether this lack of penetration into the spacer gel was due to large VLDL particles or due to aggregration of VLDL during the concentrating step in disc electrophoresis cannot be answered with certainty at this time. With VLDL samples from control birds, the gels were clear and indicated the absence of both spacer and main gel components with the quantity of sample that was used. The serum LDL from both groups penetrated the spacer gel and resolved into two bands. With HDL, three components were observed in the serum from both groups. Both Sudan Black B stain, as well as Amido Black 10B stain were employed; however, except for the spacer band from serum VLDL in group C, no new components were observed in the gel patterns with all lipoprotein samples from group C as compared with those from group N.

#### Diet Interchange and Liver Composition

Almost all the changes in the liver lipid composition that occurred in chickens as a result of diet substitutions were due to variations in the cholesterol ester levels in the liver (Table V). The phospholipid levels remained constant at ca. 21 mg/g wet liver weight, ireespective of the type or duration of the diet, while the triglyceride and free cholesterol concentrations increased from one and one-half to three times their original value. The liver cholesterol ester level, on the other hand, increased 184-fold during the 2 week period for chickens in groups NC and decreased by 95% during the same time period for animals in group CN. The rapid changes in liver total cholesterol concentrations are exemplified by the 4-fold increase and 40% decrease seen in animals from groups NC and CN, respectively, after only one day on the new diets.

#### Serum-Liver Cholesterol Pool

When the distribution of total cholesterol between the serum and liver was determined, the liver was found to contain 74% and 54% of the TC in this pool in the cholesterol-fed and normal chickens, respectively (Table VI). Al-

TABLE V

Alterations in Liver Lipid Composition After a Dietary Substitution[a]

| Diet change | Days after diet change | Number of animals used | mg in g Liver[b] | | | | Weight ratio, CE/FC |
|---|---|---|---|---|---|---|---|
| | | | PL | TC | TG | FC | |
| Control to 1% cholesterol | 0 | 5 | 22.1±1.0 | 3.1±0.2 | 12.6±1.0 | 2.8 | 0.10 |
| | 1 | 5 | 20.3±1.5 | 12.5±1.5 | | 4.9 | 1.54 |
| | 3 | 3 | 19.3±0.5 | 26.4±3.2 | | 6.7 | 2.96 |
| | 7 | 3 | 22.8±1.3 | 32.7±2.3 | | 4.7 | 5.90 |
| | 14 | 3 | 21.9±1.0 | 61.2±4.6 | | 6.0 | 9.22 |
| 1% Cholesterol to control | 0 | 5 | 21.9±1.3 | 71.6±9.0 | 19.9±7.3 | 7.9 | 8.11 |
| | 1 | 5 | 21.3±0.6 | 43.2±10.2 | | 5.0 | 7.59 |
| | 3 | 3 | 22.2±0.1 | 51.1±12.6 | | 5.6 | 8.08 |
| | 7 | 3 | 20.3±1.0 | 13.2± 0.5 | | 5.1 | 1.59 |
| | 14 | 3 | 21.3±1.7 | 5.7± 0.1 | | 2.7 | 1.12 |

[a]For abbreviations see Table I.

[b]Values are the mean ± SD.

though both serum and liver TC concentrations increased when normal animals were placed on the cholesterol diet (group NC), the proportion of the TC found in the liver as a function of the liver-serum TC pool increased from 54% to 69% within 24 hr on the new diet. The amount of TC present in the liver increased to 79% of the liver-serum TC pool after 2 weeks on the cholesterol diet. On the other hand, in animals in group CN the proportion of the TC present in the liver to that in the liver-serum TC pool varied slowly during the first 7 days, followed by a precipitous drop in the value on the 14th day.

## DISCUSSION

### Serum VLDL and HDL

Chickens fed a cholesterol diet for 6 weeks had almost 10 times the serum cholesterol levels of animals fed a control diet for the same length of time (Table VI). Almost all of this increase was accounted for by the increase observed in the VLDL fraction of the cholesterol-fed birds (Table I) and is consistent with previous literature (7). This lipoprotein fraction is present at very low levels in normal birds (7,20,21). Much smaller changes were observed in the LDL fraction of the cholesterol-fed animals. The HDL concentration was lower in the cholesterol-fed than in the control diet-fed birds, mainly due to a large decrease in phospholipid concentration (Table IV). Decreases in the HDL concentration were observed when rats were fed cholesterol diets (22,23). Leveille and Sauberlich (6) observed a 45% decrease in the PL concentrations of serum HDL from chickens fed a cholesterol-corn oil diet for 4 weeks. In order to explain the decrease in HDL in hypercholesterolemic birds, it may be postulated that there is either a transfer of the protein-PL complex of HDL or of the individual components, protein and phospholipid separately. It may be possible that some apoproteins may be shifted into the VLDL in the hypercholesterolemic state, although no evidence for a common polypeptide was found in normal birds (20). In both human and the rat, VLDL and HDL are known to contain some common polypeptides (24-26). Decreased HDL synthesis could also account for the lower HDL concentration found in cholesterol-fed birds, although no evidence for this was found (27).

French et al. (28) described a cholesterol-induced, cholesterol-rich VLDL fraction in the rabbit. It was suggested that the accumulation of VLDL was caused by the inability of lipoprotein lipase to hydrolyze the cholesterol esters present in rabbit serum chylomicrons. Considering the high percentage of cholesterol (77%) in the VLDL fraction, this may have been what occurred in the chickens on the cholesterol diet.

Chylomicrons have not been detected in normal chickens (20,21). Several authors (29,30) have obtained data that indicated all nutrients, including fatty acids, were absorbed by the portal vein of the chicken and not by the poorly developed lymphatic system. Nonetheless, whatever the source of the VLDL fraction found in the cholesterol-fed chicken, cholesterol ester and not triglycerides, as found in most species, was the predominant hydrophobic lipid (Table II).

TABLE VI

Distribution of Total Cholesterol (TC) between Serum and Liver with Diet Change

| Diet change | Number of days on diet change | Number of animals used | mg TC per 1000 g Chicken ± SD | | Liver TC x 100 |
|---|---|---|---|---|---|
| | | | Serum | Liver | (Liver + serum) TC |
| 1% Cholesterol to control | 0 | 5 | 637±134[a] | 1790±225 | 73.8 |
| | 1 | 5 | 355± 61 | 1080±255 | 75.3 |
| | 3 | 3 | 327± 27 | 1278±315 | 79.6 |
| | 7 | 3 | 118± 19 | 330± 13 | 73.7 |
| | 14 | 3 | 95± 14 | 143± 3 | 60.9 |
| Control to 1% cholesterol | 0 | 5 | 65± 8 | 78± 5 | 54.5 |
| | 1 | 5 | 138± 15 | 313± 38 | 69.4 |
| | 3 | 3 | 288± 16 | 660± 80 | 69.6 |
| | 7 | 3 | 255± 5 | 818± 58 | 76.2 |
| | 14 | 3 | 398±121 | 1530±115 | 79.4 |

[a]Values are the mean ± SD.

#### Liver Lipid Composition

The liver PL concentration was unaffected by dietary changes, indicating that probably most, if not all, was necessary to maintain structural integrity. When the diet was changed from a control to the cholesterol diet, the liver TC concentration increased twice as fast as that of the serum after 1 day on the cholesterol diet, after which the increase was about the same as for serum (Table VI). Considering the liver-serum TC concentrations as one pool, it can be seen that the liver contains 75-80% of TC when the animals are on the cholesterol diet and 54% when the animals are on the control diet. This central role of the liver is probably due to its ability to eliminate body cholesterol as bile salts and neutral sterols via the feces.

#### Cholesterol Metabolism

When considering cholesterol metabolism, several factors should be taken into account: absorption, transport, synthesis, deposition and excretion. Absorption of cholesterol was estimated at 83% of the ingested amount when the chickens were on the cholesterol diet and is close to the value of 77% absorption reported for the cholesterol-fed rabbit (3). This percentage of absorption would mean that the chickens absorbed daily ca. 800 mg cholesterol. Assuming that the chicken synthesized cholesterol at the same rate as did the rat (31), a value of about 13-15 mg can be calculated to be synthesized daily by a 600 g chicken. Thus synthesis contributes very little to the total cholesterol present in the cholesterol-fed chickens. Further, cholesterol synthesis probably decreased substantially in response to increased cholesterol absorption (32,33).

The liver-serum pool is known to be the most miscible pool with exogenous cholesterol (34). This is shown by the 13-fold increase in the TC concentration of the liver-serum TC pool in the cholesterol-fed compared to control chickens (Table VI). The liver, which exhibited the greatest response to the cholesterol diet among the tissues investigated (intestine, adipose tissue and erythrocytes exhibited only a slight increase (18) in total cholesterol on the cholesterol diet), is probably the main deposition site of the exogenous cholesterol.

After 1 day on the CN diet, almost 1 gm TC is removed from the liver-serum TC pool, presumably as excretion products (neutral sterols and bile salts, Table VI). As the percentage of liver TC gradually decreased from 75-80% to 65% of the liver-serum TC pool during the 2 weeks of the dietary exchange experiments, much less TC was removed from the liver-serum pool. It therefore appeared that the liver TC (mainly CE) concentration controlled the rate of TC removal from this pool. Nishida et al. (35) observed that an average of 9.4% and 9.2% of the intraperitoneally injected cholesterol-4-$^{14}$C was excreted from chickens fed a high protein and high protein plus cholesterol diet, respectively, and concluded that the excretion of cholesterol and its metabolites is unaffected by the level of cholesterol in the diet. If the liver-serum TC pool in their experiments were the same as those found in the present work, it would be ca. 13 times as large in cholesterol-fed birds as the same pool in normal birds (Table VI). This would then mean that the labeled cholesterol was diluted by a factor of 13 in the cholesterol-fed birds. In order to account for the almost identical per cent excretion of

labeled cholesterol from chickens on both diets, a 13-fold increase in cholesterol excretion (as bile salts and neutral sterols) is necessary. This interpretation is consistent with the data obtained by Wilson who observed an enormous increase of 14-fold in neutral sterol and 3-fold in bile acid excretion in rats fed a cholesterol diet (36). It is therefore suggested that in the hypercholesterolemic chicken, cholesterol metabolism is controlled mainly by the liver TC concentration.

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# SHORT COMMUNICATIONS

## Sulfated and Nonsulfated Bile Acid in Human Serum


### ABSTRACT

The presence of sulfated bile acid in serum of healthy persons and patients with hepatobiliary diseases was recognized by using an Amberlite XAD-2 column for extraction of bile acid and utilizing a Sephadex LH-20 column for separation of sulfated bile acid (sulfate of either taurine or glycine conjugate). and nonsulfated bile acid (taurine and glycine conjugate. The values of nonsulfate determined in this study were almost similar to the level of total serum bile acid reported by previous workers. A small amount of sulfated bile acid was found in normal serum, and the percentage of sulfated to total bile acid was ca. 10%. In patients with hepatobiliary diseases, both levels of sulfated and nonsulfated bile acid in serum rose, but sulfate did not increase in parallel to the level of nonsulfate. A remarkable increase of serum-sulfated bile acid could be observed in patients with cholestasis, while a slight elevation was noted in patients with chronic hepatocellular insufficiency. The percentage of sulfated to total bile acid was from 1.8 to 21.2% in patients with hepatobiliary diseases. Sulfated bile acid in serum consisted of only dihydroxycholanoic acid, deoxycholic and chenodeoxycholic acid. As no solvolysis was not carried out in previous works, bile acid sulfate in serum as described in this study was not determined at all.


It is well known that serum bile acid increases and large amounts of bile acid, consisting of glycine conjugated, taurine-conjugated and free bile acid (1-6), are excreted into the urine of patients with hepatobiliary diseases. A recent gas chromatographic method is very useful for analysis of serum bile acid. However it is not yet possible to make direct determination of conjugated bile acid by gas chromatography, so that no study has been made about other forms of bile acid conjugates in human serum.

Recently we found the presence of a new form of bile acid conjugates, sulfate of either glycine- or taurine-conjugated bile acid, in urine of patients with hepatobiliary diseases by using an Amberlite XAD-2 column for extraction of bile acid and a Sephadex LH-20 column for separation of sulfated and nonsulfated bile acid (unpublished).

In this paper the presence of sulfated bile acid in serum of healthy persons and patients with hepatobiliary diseases is described.

Six patients with hepatobiliary diseases and five healthy persons were studied. The age, sex, diagnosis and results of liver function tests are summarized in Table I. Two milliliters of pathological serum or 7 milliliters of normal serum were diluted with 9 volumes of 0.1N NaOH in 0.9% NaCl solution. The sample of serum was applied on a small Amberlite XAD-2 column, and after washing with water bile acids were eluted with ethanol (7). The ethanol eluate was evaporated to dryness, and the residue was applied on Sephadex LH-20 column. The glycine and taurine conjugate was eluted with 70 ml of chloroform-methanol 1:1 v/v containing 0.01 mole/liter of sodium chloride (nonsulfate fraction), and sulfate of either glycine or taurine conjugate with 50 ml of methanol (sulfate fraction) (8-10). The sulfate fraction was subjected to solvolysis, saponification, extraction with ether after acidification, and methylation. The resulting materials were purified on an aluminum oxide column and analyzed by gas chromatography using QF-1 column after conversion into trifluoroacetate of bile acid methyl ester (3). The nonsulfate fraction was analyzed without solvolysis.

The results of the quantitative estimation of bile acid sulfate in serum of patients with various hepatobiliary diseases and that of healthy persons are given in Table II. The values of nonsulfate determined in this study were almost similar to the level of total serum bile acid reported by previous workers (1-6). A small amount of sulfated bile acid was found in normal serum, which consisted of only dihydroxycholanoic acid, deoxycholic and chenodeoxycholic acid. The percentage of sulfated to total bile acid was 5.5 to 13.8% in five normal subjects.

In patients with hepatobiliary diseases, both levels of sulfated and nonsulfated bile acid in serum rose, but sulfate did not increase in parallel to the level of nonsulfate. A remarkable

TABLE I

Clincial Findings of Patients and Healthy Persons

| Case | Age | Sex | Diagnosis | Liver function test[a] Kunkel, units | T. Bilir., mg/dl | A l-Ph, units | SGOT, units | SGPT units |
|---|---|---|---|---|---|---|---|---|
| S.S. | 27 | F | Acute hepatitis | 6.1 | 14.5 | 9.4 | 348 | 243 |
| Y.F. | 51 | M | Chronic hepatitis | 12.3 | 2.7 | 18.0 | 150 | 145 |
| J.S. | 50 | M | Cirrhosis | 18.8 | 1.1 | 53.4 | 45 | 45 |
| Y.I. | 53 | M | Papilla Vater's carcinoma | 3.5 | 27.0 | 76.9 | 147 | 88 |
| H.W. | 66 | F | Primary biliary cirrhosis | 34.0 | 12.4 | 30.6 | 84 | 19 |
| M.G. | 31 | M | Metastatic carcinoma of liver | 10.5 | 3.0 | 45.4 | 200 | 91 |
| I.M. | 34 | M | Normal | 3-10 | 0.3-1.0 | 2.7-10.0 | 5-40 | 3-35 |
| H.Y. | 30 | M | Normal | | | | | |
| K.K. | 19 | M | Normal | | | | | |
| M.S. | 24 | F | Normal | | | | | |
| M.Y. | 24 | F | Normal | | | | | |

[a]T. Bilir., total serum bilirubin concentration; A1-Ph, serum alkaline phosphatase (in King-Armstrong units); SGOT, serum glutamic oxalacetic transaminase; and SGPT, serum glutamic pyruvic transaminase.

increase of serum-sulfated bile acid could observed in the patient with papilla Vater's carcinoma, biliary cirrhosis and metastatic carcinoma of the liver, while a slight elevation was noted in the patient with chronic hepatitis and liver cirrhosis. The increased sulfate consisted of only dihydroxycholanoic acid, deoxycholic and chenodeoxycholic acid. The percentage of sulfated to total bile acid varied from 1.8 to 21.2%, and that of sulfated to total dihydroxycholanoic acid was 32.6% in metastatic carcinoma of the liver, 29.7% in biliary cirrhosis, 18.8% in papilla Vater's carcinoma, 6.0% in liver cirrhosis and 3.1% in chronic hepatitis. These values in patients with cholestasis seem significantly higher than those in patients with

TABLE II

The Levels of Nonsulfated and Sulfated Bile Acid in Serum of Healthy Persons and Patients with Hepatobiliary Diseases

| Case | S.S. | Y.F. | J.S. | Y.I. | H.W. | M.G. | I.M. | H.Y. | K.K. | M.S. | M.Y. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nonsulfated bile acid, μg/ml[a] | | | | | | | | | | | |
| DC § | Trace | 1.37 | (-) | (-) | (-) | 0.46 | 0.18 | Trace | 0.35 | 0.41 | 0.17 |
| CDC § | 28.03 | 14.30 | 17.98 | 14.00 | 28.20 | 4.81 | 0.07 | 0.92 | 0.15 | 0.33 | 0.08 |
| C § | 11.98 | 3.47 | 9.95 | 55.85 | 34.61 | 4.24 | Trace | 0.14 | 0.18 | 0.18 | (-) |
| Total | 39.91 | 19.14 | 27.93 | 69.85 | 62.81 | 9.51 | 0.25 | 1.06 | 0.68 | 0.92 | 0.25 |
| Sulfated bile acid, μg/ml | | | | | | | | | | | |
| DC. | (-) | 0.06 | (-) | (-) | 0.94 | 0.30 | 0.03 | (-) | 0.04 | 0.09 | 0.04 |
| CDC. | 1.50 | 0.44 | 0.51 | 3.24 | 11.00 | 2.26 | (-) | 0.16 | (-) | Trace | Trace |
| C. | (-) | (-) | Trace | (-) | (-) | (-) | (-) | (-) | (-) | (-) | (-) |
| Total | 1.50 | 0.50 | 0.51 | 3.24 | 11.94 | 2.56 | 0.03 | 0.16 | 0.04 | 0.09 | 0.04 |
| Total bile acid, μg/ml | | | | | | | | | | | |
| | 41.42 | 19.64 | 28.44 | 73.09 | 74.75 | 12.07 | 0.28 | 1.22 | 0.72 | 1.01 | 0.29 |
| Per cent sulfated to total bile acid | | | | | | | | | | | |
| | 3.6 | 2.5 | 1.8 | 4.6 | 16.0 | 21.2 | 10.7 | 13.1 | 5.5 | 9.0 | 13.8 |
| Per cent sulfated to total dihydroxycholanoic acid | | | | | | | | | | | |
| | 5.1 | 3.1 | 6.0 | 18.8 | 29.7 | 32.6 | 10.7 | 14.8 | 7.4 | 10.8 | 13.8 |

[a]§DC, deoxycholic acid; §CDC, chenodeoxycholic acid; and §C, cholic acid.

chronic hepatocellar insufficiency.

The sulfated bile acid fraction obtained after Sephadex LH-20 column (the patient Y.I.) was subjected to alkaline hydrolysis without solvolysis, and analyzed by gas chromatography. No peak of bile acid was detected except trace amount of chenodeoxycholic acid. As no solvolysis was carried out in previous works, bile acid sulfate in serum as described in this study was not determined at all.

According to our previous report, large amounts of bile acid sulfate were found in urine of patients with hepatobiliary diseases (unpublished). Daily excretion of total bile acid in urine was 13.21 mg per day for the patient Y.I. and 61.10 mg/day for the patient S.S. The sulfated bile acid in their urine occupied 57.1% of total bile acid in the former case and 93.3% in the latter case, respectively. The sulfate in urine consisted of both di- and trihydroxycholanoic acid. The percentage of sulfated to total bile acid in serum of these patients was 4.6% for the case Y.I. and 3.6% for the case S.S. Only dihydroxycholanoic acid was identified in serum-sulfated bile acid fraction. Therefore there was large discrepancy between sulfated bile acid concentration in urine and that in serum, and bile acid composition of serum sulfate was different from that of urinary sulfate. These findings suggest that sulfated bile acid might be more easily excreted into urine than the nonsulfated form.

Recently, $^{14}$C-labeled glycolithocholic acid sulfate and $^{14}$C-labeled taurolithocholic acid sulfate were recovered from bile following the oral administration of $^{14}$C-labeled lithocholic acid to human (11,12), and sulfated bile acids in bile of rat after intraperitoneal injection of $^{14}$C-labeled lithocholic acid were identified (13). These findings indicated that the sulfation of bile acid probably occurs in the liver.

Further investigation of the clinical significance of sulfated bile acid in serum should be conducted.

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# Preparation and Characterization of Prostanoyl Carnitine

**ABSTRACT**

A method is described for the preparation of prostanoyl carnitine from prostanoic acid and L-carnitine. The crystalline compound exhibits an IR spectrum and chemical reactivity characteristic of long chain acyl carnitine esters and undergoes $\beta$-oxidation in rat liver mitochondria in the absence of exogenous carnitine and ATP. This suggests that it may be an intermediate in the transport of prostanoic acid across the mitochondrial membrane. The data supply additional evidence that prostaglandin-like substances are converted to the carnitine ester prior to transport in the mitochondrion.

chronic hepatocellar insufficiency.

The sulfated bile acid fraction obtained after Sephadex LH-20 column (the patient Y.I.) was subjected to alkaline hydrolysis without solvolysis, and analyzed by gas chromatography. No peak of bile acid was detected except trace amount of chenodeoxycholic acid. As no solvolysis was carried out in previous works, bile acid sulfate in serum as described in this study was not determined at all.

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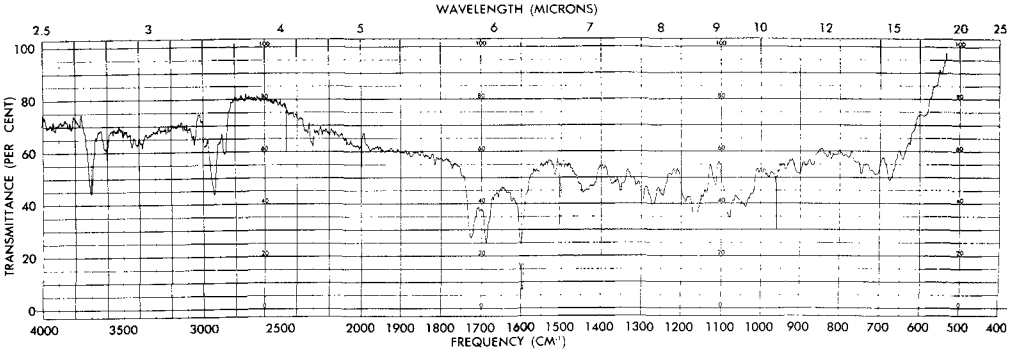


FIG. 1. IR spectrum of prostanoyl carnitine in methylene chloride was recorded on a Perkin Elmer 421 IR spectrophotometer with methylene chloride in the reference beam. A polystyrene marker for 1601 $cm^{-1}$ is indicated.

Prostaglandins, $C_{20}$ unsaturated fatty acids (1), undergo $\beta$-oxidation in rat liver mitochondria (2) and in other tissues (3,4). It has recently been suggested that prostaglandins may require exogenous carnitine and ATP for oxidation (5). Additional evidence for this hypothesis would be provided by investigation of the oxidation of the carnitine derivative of a prostaglandin.

Carnitine esters of long chain fatty acids are best prepared from the corresponding acid chloride (6). The number of reactive groups and degree of unsaturation of the prostaglandins, however, render specific chlorination to the acid chloride difficult. In the present paper we report the synthesis of prostanoyl carnitine from prostanoic acid, a saturated unsubstituted prostaglandin analogue, and its oxidation by isolated mitochondria.

Cyclopentane heptanoic acid, 2-octyl (prostanoic acid), 5 mg (1 mg/ml in absolute ethanol) was bubbled with chlorine gas for 60 min at 0 C in the presence of 5 mg L-carnitine hydrochloride (Sigma Chemical Co.). Moisture was excluded and the mixture continuously stirred. A further 10 mg of L-carnitine was added and stirring continued for 60 hr at room temperature. The reaction mixture was then added to 15 ml dry acetone and cooled to 0 C for 2-3 hr to removed unreacted carnitine. The supernatant was decanted and 20 ml anhydrous ether (0 C) added. After 3 hr a further 20 ml ether was added to incipient turbidity. The mixture was dessicated overnight at -20 C, at which point a clear oil had formed. The supernatant was discarded and 10 ml ether (-20 C) added to effect crystallization. After complete crystallization at -20 C the product was collected and recrystallized from acetone by addition of ether. The product was washed with ether and dried over phosphorous pentoxide under vacuum. The synthesis gave ca. 15% yield based on the amount of carnitine added. The presence of ester bonds in the crystalline product was verified by the ferric hydroxamate method (7).

The compound was further characterized as a carnitine ester by its IR spectrum, in methylene chloride (Fig. 1). L-Carnitine shows an OH stretching band at 3260 $cm^{-1}$, an absorption band of C=O, characteristic of a secondary OH function at 1100 $cm^{-1}$ and a C=OOH function at 1725 $cm^{-1}$. In the case of acyl carnitine esters the bands at 3260 and 1100 disappear, and C=O stretching bands of the acyl groups are found at ca. 1200 $cm^{-1}$. The C=O stretching band of the C(=O)OH function found at 1725 $cm^{-1}$ in L-carnitine is displaced to 1710-1720 $cm^{-1}$, and an equivalent absorption band due to the C(=O)OR function appears at ca. 1740 $cm^{-1}$ (6,8). As can be seen in Figure 1, the

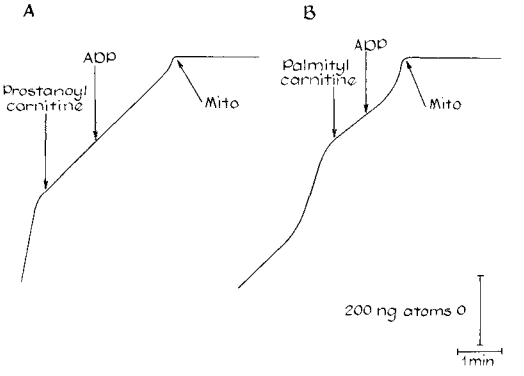


FIG. 2. The oxidation of prostanoyl carnitine by isolated rat liver mitochondria was followed as described in Reference 3. At the points indicated, mitochondrial protein (~5 mg), ADP (1 $\mu$mole), A. prostanoyl carnitine (300 nmoles) and B. palmityl carnitine (500 nmoles) were added. The electrode calibration (10) in nanogram atoms of oxygen is indicated.

compound exhibits all the characteristics of a carnitine ester.

Microelemental analysis indicated the presence of C,H,0,N in the ratios 14:4:5:1 by weight, which approximates theoretical values. In addition it did not discolor $KMnO_4$ solutions, indicating the absence of crotonbetaine, commonly formed in the synthesis of carnitine esters.

Following accepted nomenclature, the compound is described as (2[1]-*n*-octyl)-cyclopentaneheptanoyl carnitine and in this paper is assigned the trivial name of prostanoyl carnitine. Prostanoyl carnitine forms white rod-shaped crystals that are insoluble in water, soluble in absolute ethanol and sparingly soluble in methylene chloride. The melting point is 29 C, and the compound is hydroscopic.

Fatty acyl carnitine derivatives are believed to be intermediates in the transport of acyl groups across the mitochondrial membrane prior to oxidation (9); addition of the carnitine derivative of a long chain fatty acid to intact mitochondria eliminates the requirement for exogenous carnitine and ATP.

The oxidation of prostanoyl carnitine by isolated rat liver mitochondria was followed using a membrane-covered Clark-type oxygen electrode. Addition of 300 nmoles to ADP-stimulated mitochondria, with an oxygen consumption of 118.1 ng atoms of oxygen per minute per milligram protein, resulted in an immediate increase in oxidation (245.2 ng atoms of oxygen per minute per milligram protein). This oxidation could not be further increased by addition of carnitine (0.5 $\mu$moles) and ATP (0.1 $\mu$moles). This is in contrast to the oxidation of prostanoic acid and prostaglandins, which exhibits an absolute requirement for exogenous carnitine and ATP. Figure 2 shows the similarity between the oxidation of palmityl carnitine (Sigma Chemical Co.) and prostanoyl carnitine. In this system, therefore, prostanoyl carnitine behaves as a typical acyl carnitine ester.

$\beta$-Oxidation is a major pathway in the metabolism of prostaglandins (2), and the evidence reported indicates that this oxidation is characteristic of long chain fatty acids. The oxidation of prostanoyl carnitine may provide additional evidence for the involvement of carnitine. However there are considerable differences in structure between prostanoic acid and the prostaglandins, and further investigation is necessary using the carnitine derivatives of native prostaglandins. The oxidations of prostanoic acid and prostaglandins are carnitine-dependent and similar, suggesting that extrapolation of this data to the prostaglandins may indeed be possible.

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# Autoxidation of Fatty Acid Esters in the Presence of a Heavy Metal Catalyst "Salcomine" (Co[II]-bissalicylalethylenedi-imine): I. Effect of Catalyst upon Rate of Consumption of Oxygen and Decomposition of Hydroperoxide

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## ABSTRACT

The autoxidation of fatty acid esters in the presence of a heavy metal chelate, "Salcomine" (Co[II]-bissalicylalethylenedi-imine), has been studied. Both antioxidative and prooxidative effects have been observed. When the concentration of the catalyst is decreased or the temperature is increased, the induction period becomes shorter and under some conditions disappears. It was shown that the decomposition of hydroperoxides is affected by salcomine; a first order reaction is involved with Arrhenius parameters E = 21,000 cal, and A = $8.99 \times 10^{10}$ $sec^{-1}$. The solubility of oxygen in fatty acid esters above 30 C, with and without salcomine, is the same. Theoretical aspects of the reaction mechanism, that are consistent with the experimental results, are proposed.


## INTRODUCTION

The well known free radical chain mechanism for the autoxidation of unsaturated fatty acid esters was proposed 25 years ago (1,2). The subject has been studied extensively, and the reactions involved have been reviewed and discussed many times (3,4).

The initiation reaction, which needs to occur only to a negligible extent to trigger the chain mechanism, is believed to require direct addition of oxygen to the substrate (5-7) and may involve a singlet oxygen (8). During propagation an alkyl free radical (R·) reacts with oxygen to give a hydroperoxide free radical (ROO·), which can abstract a hydrogen from a substrate molecule (RH) to give a hydroperoxide (ROOH) and a new R· free radical, to continue the chain. Termination is by interaction of two free radicals.

The acceleration of the rate of autoxidation of fatty acid esters by metal catalysts has been the subject of an increasing number of studies during the last decade. However the mechanisms of this phenomenon have not been fully clarified or explained in terms of modern physical-chemical concepts. Also, the decomposition of hydroperoxides has not been studied directly, and the fate of the catalyst itself during autoxidation often remains unsolved. The main difficulties are the complex nature of

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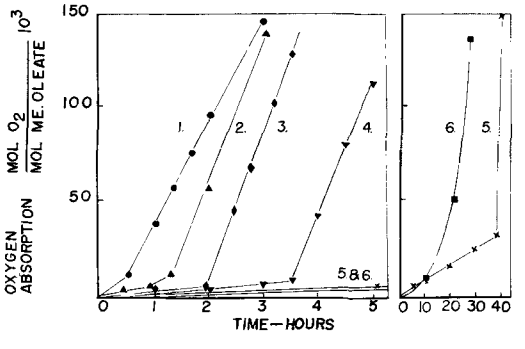



FIG. 1. Autoxidation of methyl oleate. 3.5 g of ester in each case with salcomine as follows: 1, 0.5 mg; 2, 1.0 mg; 3, 2.0 mg; 4, 4.9 mg; 5, 19.7 mg; 6, none. Temperature 80 C.

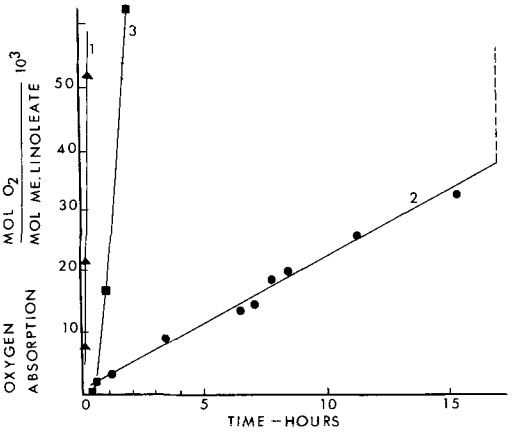



FIG. 2. Autoxidation of methyl linoleate. 1, 1.9 g of ester, 1.1 mg of salcomine; 2, 9.2 g of ester, 151.2 mg of salcomine; 3, 9.2 g of ester, no salcomine. Temperature 64 C.

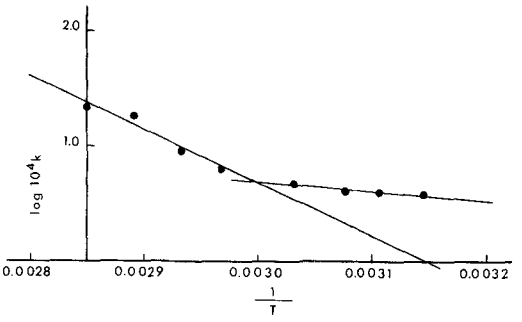



FIG. 3. A plot of $\frac{1}{T}$ vs. log $10^4k$ for the decomposition of fatty acid ester hydroperoxide catalyzed by salcomine.

the autoxidation reaction itself, and the complicated chelation and other reactions of the heavy metal catalysts in the autoxidation environment.

Under certain conditions, the effects of heavy metal catalysts on autoxidation have been reported to be either rpooxidant, as has been shown for ions of Co, Mn, Fe, Cu (9-12), and for heme compounds (13,14), or antioxidant shown for Cobalt heptoate (15), salts of Cu(III) (16), Co(II)-bissalicylalethylenedi-imine (17-19), Cobalt stearate (20), Cu ions (21), Fe(III) (22) and Cytocrome C (23). Inversion from pro- to antioxidant effects has been demonstrated with heme compounds (24,25). Also, it has been suggested that the heavy metal catalysts may activate the oxygen atoms in autoxidation (20,26,27).

In this study an attempt has been made to increase our understanding of the situation by following the autoxidation of fatty acid esters in the presence of Co(II)-bissalicylalethylenediimine ("Salcomine"), a Co(II)-compound already in chelate form and of known configuration. It was hoped that in this manner the kinetically paradoxical presence and absence of prooxidative phenomena in the same system containing a heavy metal catalyst could be demonstrated more clearly.

## MATERIALS AND METHODS

Salcomine was prepared by the method of Diehl and Hach (28). It absorbed 4.47 wt % oxygen (0.47 mol/atom of Co) at room temperature, and was similar to the most active form studied by Calvin et al. (29).

The methyl esters of fatty acids were obtained from The Hormel Institute, Austin, Minn., or prepared from technical fats or fatty acids by interesterification, esterification and distillation. If not otherwise indicated in the figures, they were more than 99% pure. Neither

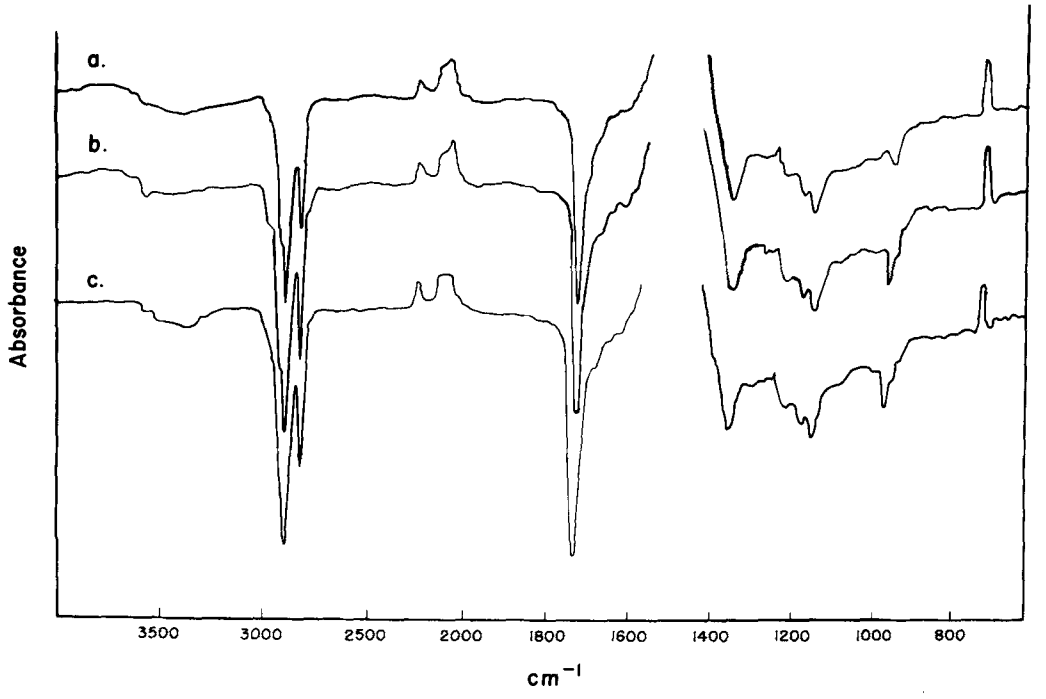



FIG. 4. IR spectra of components separated by thin layer chromatography from the methyl linoleate hydroperoxide concentrate (PV 3841). Curve a: closest to the start; curve c: closest to the front.

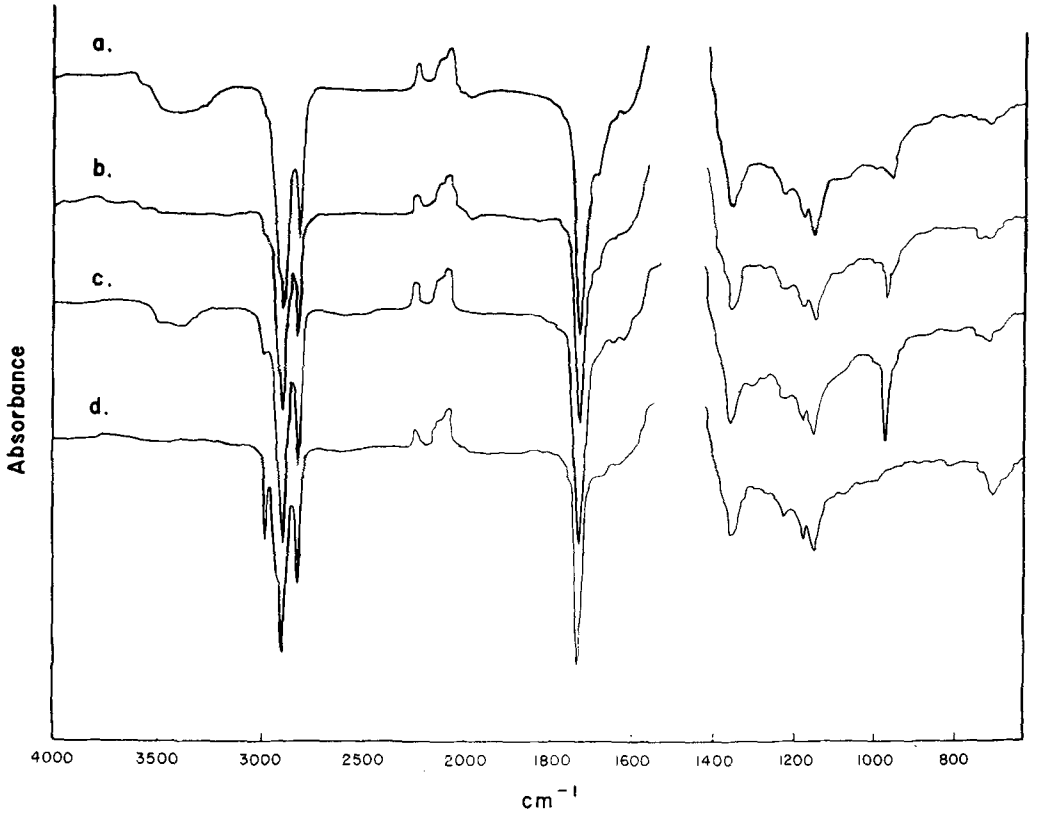



FIG. 5. IR spectra of components separated by thin layer chromatography from the uncatalyzed autoxidation of methyl linoleate. Curve a: closest to the start; curve c: closest to the front; curve d: unoxidized methyl linoleate.

free fatty acids nor peroxides could be detected in the preparations by conventional methods.

Gas liquid chromatography determinations were made with a Beckman GC-2 instrument on a 360 x 0.600 cm column of 10% Resoflex R-446, at 208 C, or with a F&M Scientific Corp. chromatograph, model 609 (hydrogen flame ionization), column 244 x 0.635 cm, 8% ethylene glycol succinate (Applied Science Laboratories, State College, Pa.), column temperature 175 C.

IR spectra were obtained with Perkin-Elmer model 237 B.

Peroxide values were determined by the procedure of Wheeler (30), under nitrogen.

The solubilities of salcomine at various temperatures in methyl linoleate were determined by stirring an excess of salcomine in the ester under vacuum. The absorbance of filtered samples of the clear linoleate solutions, dissolved in tetrahydrofuran, was measured at 386 nm, and the concentration of salcomine was read from a standard curve.

The kinetic experiments were made using a modified form of the apparatus of Kern and Willersin (31). The reading accuracy of the gas burette was ± 0.05 $cm^3$. The reaction vessel had a magnetic stirrer, and could be equipped with a rubber septum through which samples could be withdrawn for measuring the rate of decomposition of hydroperoxides. The tests were made in diffuse daylight at constant pressure (1 atm).

The solubilities of oxygen in oxygen-free fatty acid esters (obtained by heating under vacuum) were determined at selected temperatures with salcomine present (138.7 mg oxygen free salcomine in 25 ml fatty acid esters) and with salcomine absent. The solubilities were measured immediately after stirring began and before autoxidation started.

For the autoxidation studies, salcomine was weighed into the flask and held at 1 mm Hg or less for 1 hr at 80 C (or 15 min if it had been preheated and evacuated). After addition of the methyl ester, traces of peroxides that it might contain were destroyed by stirring the contents of the reaction vessel for 5 min under vacuum

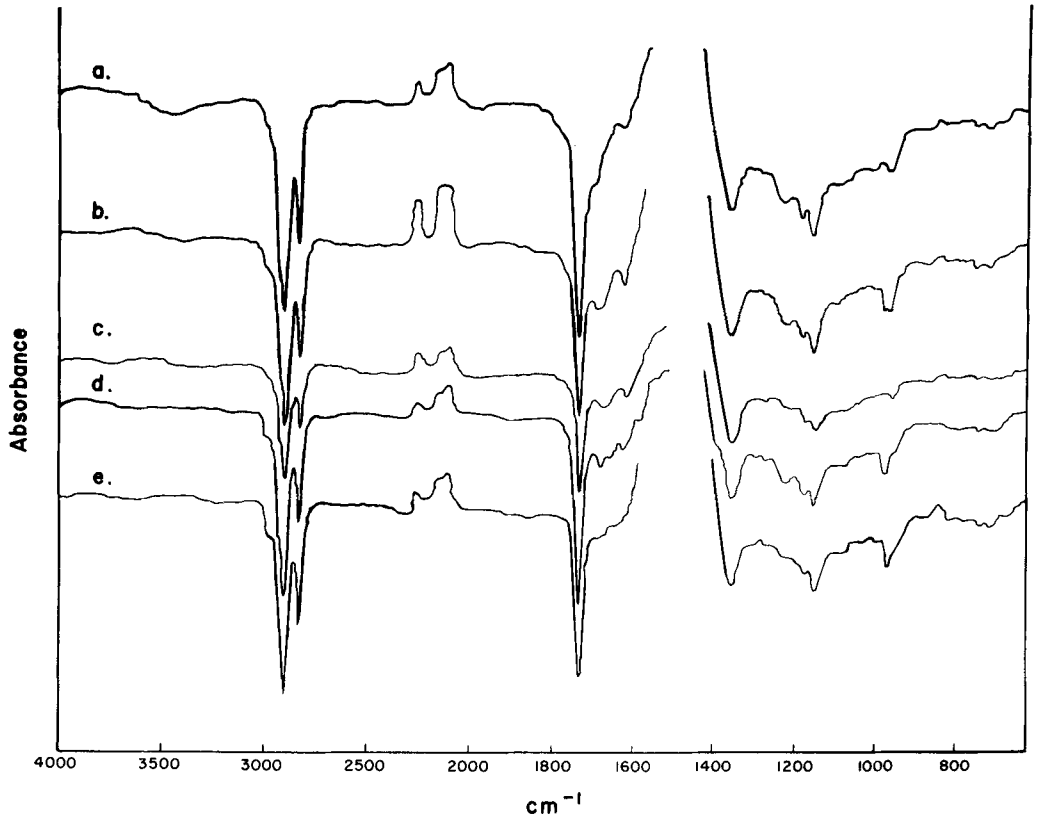



FIG. 6. IR spectra of components separated by thin layer chromatography from the methyl linoleate autoxidized with salcomine. Curve a: closest to the start; curve e: closest to the front.

at the appropriate experimental temperature before oxygen was admitted. This precaution was taken with all polyunsaturated methyl esters but not with methyl oleate.

The decomposition of hydroperoxides in the presence of salcomine was studied with a mixture containing 91.5% methyl oleate and 8.5% methyl linoleate that had been preoxidized at 80 C to a peroxide value of 120 me/kg. Oxygen free salcomine was added to preoxidized methyl esters (2.5 g salcomine/1000 g methyl esters), and aliquots were stirred at temperatures ranging from 45 to 78 C. Helium gas was constantly bubbled through the reaction mixture by means of a long injection needle. Samples were removed through another permanently positioned injection needle, and peroxides were determined immediately.

## RESULTS AND DISCUSSION

### Solubility Measurements

The solubility of oxygen free salcomine in methyl linoleate increased gradually from 760 mg/liter at 60 C to 880 mg/liter at 90 C, and similar values were obtained with mixtures of fatty acid esters. In experiments corresponding to curves 4 and 5 in Figure 1 and Curve 2 in Figure 2, and also in the tests to determine the rate of decomposition of hydroperoxides, the amount of salcomine used exceeded its solubility.

The solubility of oxygen in fatty acid esters varied only slightly between 50 and 90 C, and remained unchanged when salcomine was present. The values of 4.0-4.2 $cm^3$/25 ml agree well with those obtained by Bateman et al. (32). Not until the temperature was lowered to 30 C did the effect of salcomine become evident when the chelate absorbed 48.5% of its capacity for oxygen. Since all the autoxidations were performed above 50 C, it is obvious that salcomine cannot be a carrier of oxygen in these reactions. This conclusion is supported further in that other compounds, of similar configuration but unreactive towards oxygen, have a similar effect upon autoxidation (33).

### Autoxidation Measurements

The effect of salcomine upon the total rate

TABLE I

Decomposition of Fatty Acid Methyl Ester Hydroperoxides[a] at Different Temperatures, Catalyzed by Co(II)-bissalicylalethylenedi-imine[b]

| Temperature | $t$ sec | $p$ | $10^4k$[c] | log $10^4k$ | Temperature | $t$ sec | $p$ | $10^4k$ | log $10^4k$ |
|---|---|---|---|---|---|---|---|---|---|
| 78 C | 420 | 38.9 | 22.48 | 1.352 | 57 C | 360 | 87.5 | 3.706 | 0.568 |
| | 900 | 16.5 | 20.01 | 1.301 | | 900 | 63.1 | 5.116 | 0.708 |
| | 1440 | 4.6 | 21.37 | 1.330 | | 1440 | 42.2 | 5.980 | 0.776 |
| | | | Mean | 1.327 | | | | Mean | 0.684 |
| 73 C | 480 | 39.5 | 19.33 | 1.286 | 52 C | 900 | 71.5 | 3.709 | 0.569 |
| | 960 | 16.8 | 18.56 | 1.268 | | 1800 | 47.0 | 4.182 | 0.621 |
| | 1680 | 5.5 | 17.27 | 1.237 | | 2820 | 27.7 | 4.548 | 0.657 |
| | | | Mean | 1.263 | | | | Mean | 0.617 |
| 68 C | 540 | 59.4 | 9.636 | 0.984 | 49 C | 540 | 80.5 | 4.014 | 0.604 |
| | 1440 | 33.9 | 7.515 | 0.876 | | 1680 | 52.4 | 3.849 | 0.585 |
| | 2050 | 13.4 | 9.847 | 0.994 | | 2580 | 35.1 | 4.052 | 0.607 |
| | | | Mean | 0.951 | | | | Mean | 0.598 |
| 64 C | 540 | 75.6 | 5.202 | 0.716 | 45 C | 840 | 73.7 | 3.618 | 0.556 |
| | 1260 | 42.9 | 6.723 | 0.828 | | 1920 | 51.0 | 3.501 | 0.544 |
| | 2040 | 22.3 | 7.354 | 0.868 | | 3240 | 28.8 | 4.428 | 0.646 |
| | | | Mean | 0.803 | | | | Mean | 0.582 |

[a]Oleic acid methyl ester 91.5%, linoleic acid methyl ester 8.5%; peroxide value 120 meq/kg.

[b]2.5 g Salcomine per 1000 g substrate.

[c] $k = \frac{2.303}{t} \times \log\frac{100}{p}$; where $p$ = % unreacted, $t$ = time elapsed.

of autoxidation may be seen in Figures 1 and 2. If the amount of salcomine is small, little or no induction period can be detected; however the antioxidative effect becomes more and more pronounded with increasing amounts of salcomine. As expected, the rates of oxidation in the presence of salcomine increased with temperature during the induction period (curves not shown), and the latter became shorter. The rates after the end of the induction period were not affected. The autoxidation of methyl oleate and linoleate in the absence of salcomine is different from the catalyzed reactions, as may be seen also in Figures 1 and 2.

Since the initiation period is prolonged when the amount of salcomine is increased, the chelate has antioxidative properties. On the other hand, if comparison is made between normal autoxidation and autoxidation catalyzed with samll amounts of salcomine, its effect is also prooxidative. On this basis similarities exist between salcomine and heme compounds, which possess both pro- and antioxidative properties (24,25). Further reference to this subject may be found in a recent review (34).

### Hydroperoxide Decomposition Measurements

Under the same reaction conditions used to study the decomposition of hydroperoxides in the presence of salcomine, no decomposition could be detected when the catalyst was omitted. With salcomine, as can be seen from the constants given in Table I, the rates of decomposition correspond to a first order reaction. In Figure 3, log $10^4k$ and $\frac{1}{T}$ have been plotted.

Since both dissolved and undissolved salcomine (1:2.3) were present in the reaction mixture, the difference in the slope of the curve below and above 60 C points to a heterogeneous catalysis below 60 C. The computed Arrhenius parameters above 60 C are, E = 21,000 cal, and A = $8.99 \times 10^{10}$ $sec^{-1}$. As comparable values for fatty acid hydroperoxides were not found, the values given by Molyneux for homolytic uncatalyzed cleavage of organic peroxides (35) may be compared: E = 32,000-38,000 cal, and A = $10^{13}$ to $10^{16}$ $sec^{-1}$ – values that are considerably higher than those with salcomine as would be expected.

### Mechanism of Hydroperoxide Decomposition

The reaction products from the autoxidation of methyl linoleate, with and without salcomine, were separated from the unreacted ester by thin layer chromatography (under nitrogen blanket with Silica Gel H, E. Merck A.G., Darmstadt, Germany, petroleum ether-ethyl ether-acetic acid 75:25:1).

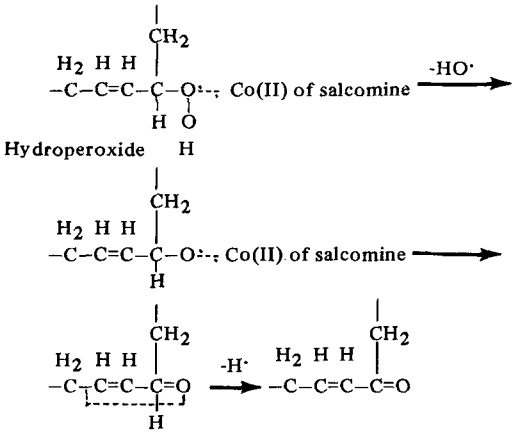
Figure 4 shows the IR spectra of the fractions (corresponding to spots of the thin layer chromatography, everything collected, curve a from the start) obtained from a hydroperoxide concentrate (PV 3841), which had been purified by counter-current extraction (from The Hormel Institute). The hydroperoxide group can be detected clearly between 3100 and 3600 $cm^{-1}$. The absorption is weak from 1600 to 1700 $cm^{-1}$, where the $\alpha$-$\beta$

unsaturated carbonyl group has its characteristic bands (36-38), which have been especially assigned to carbonyl compounds formed in the autoxidation of linoleic acid (36).

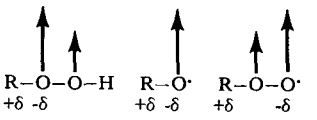
The spectra from uncatalyzed autoxidation of methyl linoleate in Figure 5 are mostly similar to those of the hydroperoxide concentrate. However evidence of carbonyl conjugation is apparent in curve c. Curve d represents pure methyl linoleate.

In Figure 6 are seen the spectra from the autoxidation catalyzed by salcomine. In curves b, c and d, the bands 1600-1700 $cm^{-1}$ are almost close in strength to the absorption of the ketodiene isolated by Vioque and Holman (36). On the other hand the hydroperoxide or hydroxyl absorption from 3100 to 3600 $cm^{-1}$ is practically absent except in curve a. Similar observations have been made about the autoxidation of cyclohexane (39), and ethylbenzene (40); with rhodium and iridium catalysts the reaction is led to the corresponding $\alpha$-$\beta$ unsaturated ketones. Undoubtedly the formation of unsaturated ketones is strongly favored if salcomine catalyzes the autoxidation of methyl linoleate.

To explain this kind of observation it must be assumed that, because of the inductive effect of the adjacent carbon chain, the electron density of the hydroperoxide group is highest on the oxygen atom closest to the carbon chain, thus forming the point in the molecule where the catalyst salcomine makes its contact. The electron density on RO· oxygen is obviously too high to allow a stable contact and decomposition of the hydroperoxide results. However the catalyst is temporarily linked to the radical and the reaction leads, via a resonance stabilized intermediate, to a carbonyl compound:




Qualitatively the charge distribution and electron density of the molecular species in question, compared to each other, can be presented as follows:




The height of the arrow is an indication of the realtive electron densities on each oxygen atom. The removal of the hydrogen atom moves the negative charge to the right in the peroxy radical.

The reaction is thus guided by salcomine, which "lends" its electron. In some other cases, depending upon the stability of the transition state complex (affected by the substrate, the catalyst and the enivronment), a ketone and alcohol may be formed simultaneously, as is known to happen in the catalytic autoxidation of $\beta$-pinene (41).

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# Synthesis of Prostaglandins (PGE, PGF, PGA and PGB) in Human Platelets

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## ABSTRACT

Human blood platelets, which previously have been shown to possess the necessary enzymic system for the synthesis of prostaglandins E, are also able to synthesize other prostaglandins, namely PGF, PGA and PGB. The synthesis and degradation rate of the prostaglandins were studied by chase experiments. Their synthesis rate was ca. 0.025 nmol acetate incorporated into $PGE_1$ (and $E_2$) per $10^9$ platelets, while their degradation rate per hour was ca. 13% (PGE) and 8% (PGF) of the initial amount.

## INTRODUCTION

Platelet aggregation seems controlled by prostaglandins (PGs), since $PGE_1$ hampers this process and $PGE_2$ enhances it (1,2). These effects of PGs may be mediated through the cyclic-AMP system (3,4). In a recent publication we demonstrated that human platelets possess the whole enzymic system necessary for PGE synthesis and that the synthetic capability was so high that pharmacologically effective concentrations of PGEs could be accumulated in platelets (5). The present communication demonstrates that platelets are capable of synthesizing prostaglandins other than PGEs. Furthermore, this paper contains a trial for an evaluation, separately, of the synthesis and degradation rate of the prostaglandins in human platelets.

## MATERIALS AND METHODS

### Preparation of Platelet-Rich Plasma (PRP)

Blood was collected from normal human donors who had not taken drugs such as aspirin during the previous week. Trisodium citrate (3.8% w/v adjusted to pH 7.4 with citric acid) was used as blood anticoagulant in the ratio of 1:9 v/v. All glassware was siliconized. The blood was centrifuged at 270 g for 15 min, and the supernatant transferred to another tube and recentrifuged at 270 g for 10 min. This gave a platelet-rich plasma (PRP) free from erythrocytes and leucocytes (5). About 15 ml platelet-free plasma was also prepared by centrifuging the blood with the anticoagulant at 2500 g for 20 min.

### Preparation of Acetate-1 $^{14}$C

Sodium acetate (The Radiochemical Centre, Amersham, Buckinghamshire, England, specific activity 62 mCi/mmol, total activity 250 $\mu$Ci) was adjusted to 102 $\mu$mol acetate by adding nonradioactive acetate (AR, BDH) and diluted to a volume of 1000 $\mu$l with distilled water.

### Glutathione (Reduced Form, GSH, Biochemical, BDH)

14 mg glutathione was dissolved in 2 ml redistilled water, stored in a refrigerator and used within a week.

### Prostaglandins (PGs)

Prostaglandins A and B, E and 19-OH-PGA and PGB, together with prostaglandins F, were obtained from human seminal plasma as described by Hamberg and Samuelsson (6). Since the prostaglandins were used as carriers in different chromatographic resolutions, they were not added as individual compounds to the materials that were to be resolved by chromatographic methods. They were used rather as a class of chromatographically identical compounds, viz., PGA and PGB as one class, $PGE_1$, $PGE_2$ and $PGE_3$ as another, and lastly, 19-OH-(PGA and PGB) together with prostaglandins F as the third class. Reference prostaglandin $E_1$ was obtained as a gift from the Unilever Research Laboratories, Vlaardingen, The Netherlands.

### Metabolic Studies

All incubations (type A and B) were done in siliconized pyrex glass tubes at 37 C with a continuous passage of water-saturated oxygen (11 ml/min).

*A) Incubation in plasma medium:* Platelet-free plasma was added to the PRP so as to adjust the final concentration of the platelets to 1.0 x $10^9$/ml. Besides the platelets, the incubation mixture consisted of 1.08 $\mu$mol glutathione and 15 $\mu$mol acetate in a total incubation volume of 6 ml. The time of incubation was 2 hr during which a continuous passage of oxygen was maintained.

*B) Incubation in plasma for chase experiments:* Five incubations in duplicate were done. In each case the incubation mixture consisted of 1.72 x $10^9$ platelets, 0.308 $\mu$mol glutathione and 4.3 $\mu$mol radioactive acetate in a total volume of 1.72 ml. After 2 hr incubation, a

TABLE I

The Specific Incorporation of $^{14}$C-Acetate into Various Prostaglandins and Lipid Fractions in Human Platelets

| | Crude column chromatographic fractions[a] | | Thin layer chromatographic fractions after separation of crude chromatographic fractions by thin layer chromatography |
|---|---|---|---|
| Lipid | Elution mixture | Total incorporation, nmol acetate/$10^9$/hr | Incorporation in prostaglandin, nmol acetate/$10^9$/hr |
| Neutral lipid | Benzene-ether 95:5 v/v | 0.0907 ± 0.0063 | |
| PGA + PGB | Ethylacetate-benzene 3:7 v/v | 0.1583 ± 0.0094 | 0.0262 ± 0.0012 |
| PGE | Ethylacetate-benzene 6:4 v/v | 0.0580 ± 0.0039 | 0.0251 ± 0.0030 ($E_1$); 0.0209 ± 0.0012 ($E_2$); 0.0018 ± 0.0005 ($E_3$) |
| PGF | Ethylacetate-benzene 8:2 v/v | 0.0356 ± 0.0042 | 0.0260 ± 0.0033 |
| Phospholipids | Methanol | 0.6616[b] ± 0.0460 | |
| Total | | 1.0051 ± 0.0698 | 0.1000 ± 0.0092 |

[a]Two experiments.

[b]Not exclusively due to the phospholipids (PL).

measured large excess of "cold" acetate (non-radioactive), in a concentration equal to 100 times the original acetate concentration, was added. In the first two tubes the incubation was stopped immediately by adding 1.9 ml saline and 3.8 ml absolute ethanol (zero time chase), whereas with other incubation tubes, incubation was continued for 30, 60, 90 and 120 min.

In both types of incubation (A and B), the reactions were initiated by adding radioactive acetate. They were terminated by adding 5 ml of a mixture of chloroform-methanol in the case of A, while saline and absolute ethanol were added in the case of B (see above).

#### Extraction of Incubation Mixture A

The mixture was extracted with additional 30 ml of the chloroform-methanol mixture mentioned above at 4 C overnight and filtered through a Whatman no. 40 (ashless) filter paper, after which the paper was washed twice with 5 ml chloroform. The filtrate was concentrated at 60 C under nitrogen to a residue which was extracted with chloroform and filtered in a desiccator (5). The filtrate was evaporated to a residue under nitrogen at 60 C.

#### Extraction of Incubation Mixture B

In the case of chase experiments, the incubation mixture was extracted by a slightly modified method of Unger et al. (7). This was essential because the high concentration of acetate either made the incubation medium alkaline enough to prevent the prostaglandins being extracted with the chloroform-methanol mixture or caused them to bind more firmly to the precipitated serum albumin (7). The steps of extraction were as follows: (a) To the incubation medium 1.9 ml saline and 3.8 ml absolute ethanol were added and the mixture mixed. (b) The pH of the medium was adjusted to 3.0 by adding 400 µl of formic acid. (c) The mixture was hereafter extracted twice with chloroform, each time the volume of chloroform being 8 ml. (d) To the combined extracts 2 ml water was added twice to remove the acetate and formic acid. (e) The organic phase was evaporated under oxygen-free nitrogen at 60 C. (f) The residue was dissolved in 10 ml chloroform, followed by evaporation as above to aid the removal of formic acid. (g) The residue thus obtained was subsequently used for the column chromatographic resolution.

#### Column Chromatography

This was performed after the method described by Hamberg and Samuelsson (6). Silicic acid (Mallinckrodt, 100 mesh powder AR grade) was activated at 115 C overnight, prior to its use in the column. 2.5 g of the adsorbent was used to prepare a column, 1 x 6 cm, in benzene-ether 95:5. About 50 ml of this solvent were allowed to drain from the column before applying the material. The extracts obtained from the incubations were mixed with the various prostaglandins and then applied to the top of the column, which was eluted successively with the following solvent systems: (i) benzene-ether 95:5, 150 ml; (ii) ethylace-

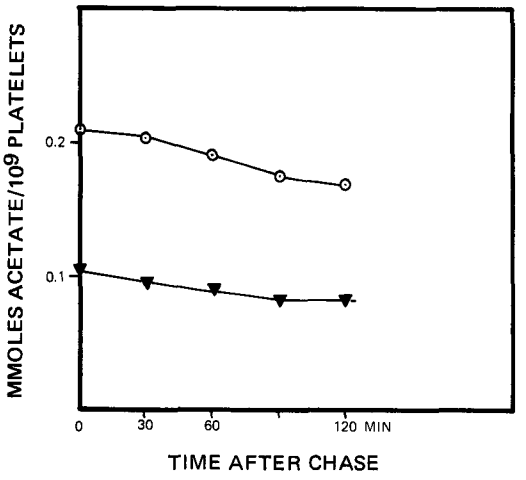


FIG. 1. The $^{14}C$-labeled acetate remaining for 120 min in prostaglandins formed in human platelets during a previous period of 2 hr incubation. Conditions of incubation: platelet-rich plasma (PRP), glutathione 55 μg/ml, platelets 1.0 x $10^9$/ml in a total volume of 1.72 ml and 2.5 μmol acetate/ml. Incubation time: 120 min. Ordinate: Acetate remaining in PGs (nmol acetate/$10^9$ platelets). The zero values on the ordinate indicate the amounts of acetate incorporated as nmol acetate/$10^9$ platelets/2 hr. Abscissa: Chase time (min). After 120 min of incubation, "cold" acetate (100 x 2.5 μmol/ml) was added, and incubations (in duplicate) were continued for additional times as described in the text (Chase experiment). The SD value of the individual incubations at 30, 60, 90 and 120 min were between 0.79 and 0.93% of the individual value. The combined SD value of the means at 30, 60, 90 and 120 min were 1.2, 1.2, 1.2, 1.3 and 1.3% of the mean value indicated in the figures, both for PGE and PGF. The upper curve represents the PGE and the lower curve the PGF component.

tate-benzene 3:7, 150 ml; (iii) ethylacetate-benzene 6:4, 150 ml; (iv) ethylacetate-benzene 8:2, 100 ml; and (v) methanol, 100 ml.

The reason for resorting to the intermediary step of column chromatography is that in incubations with plasma the quantity of triglycerides, cholesterol, other neutral lipids and polar lipids was too high to be resolved directly by thin layer chromatography (TLC). Thus, from the incubation extract, the neutral lipids were first eliminated, the elution of the prostaglandins followed in the next steps, and finally the prostaglandins were resolved by TLC. The polar lipids stayed on the column until elution with methanol.

The solvent from each eluate was distilled off under reduced pressure and the residual mass dissolved in ether. The solvent was evaporated under nitrogen, and the residue thus obtained was used in the TLC resolution. In the case of extracts from the chase experiment, the column (1 x 6 cm) was prepared in ethylacetate-benzene 3:7, and after mixing the material with the prostaglandins the elution was done as follows: (a) ethylacetate-benzene 3:7, 100 ml; (b) ethylacetate-benzene 6:4, 150 ml; (c) ethylacetate-benzene 8:2, 100 ml.

### Thin Layer Chromatography

TLC was performed on Silica Gel G (0.25 mm) (Merck, Darmstadt, W. Germany) using the equipment of Desega Werke (W. Germany). The plates were activated at 110 C for 30 min and stored in a desiccator until use. The materials from (ii) and (iv) were cochromatographed separately, together with reference prostaglandins A and B, and 19-OH (PGA and PGB) together with the PGF compounds respectively. The solvent system used for the development of the plates consisted of ethylacetate-iso-octane-acetic acid-water 110:20:10:100, equilibrated for 1 hr before using the organic phase (8). The prostaglandin zones were shown by exposing the plates to iodine. PGA and PGB have Rf values 0.78, whereas 19-OH-(PGA and PGB) and PGF have an Rf value 0.34 in this solvent system.

The material from (iii) was cochromatographed with reference PGE compounds on silver nitrate-treated Silica Gel G (0.25 mm, silver nitrate-Silica Gel G 1:25 w/w). The solvent system consisted of ethylacetate-acetic acid-methanol-iso-octane-water 110:10:15:10:100, equilibrated for 1 hr before using the organic phase (6). The spots due to the reference prostaglandins E and the zones due to them were shown by spraying the plate with water. The zones were marked. The materials from (b) and (c) were resolved by TLC, by cochromatographing with prostaglandins E and F, respectively.

### Assay of Radioactivity

In every case except the marked zones, the remaining portion of the plate was divided into zones 1.5 cm wide, which were also assayed for radioactivity together with the zones due to the prostaglandins. The material from (i) consisted chiefly of neutral lipids, and the material from (v) was due mainly to polar lipids. These were not resolved further by TLC and were used, as such, for counting their radioactivity.

The radioactivity was measured in a Beckman liquid scintillation counter (model 230) with 10 ml fluor prepared by mixing 8.0 g butyl-PBD(2-[4'-butylphenyl]-5-[4'-biphenyl]-1,3,4-oxdiazole) and 0.5 g of 2-(4'-biphenyl)-6-phenylbenzoxazole (Beckman Instruments Inc., Fullerton, Calif.) in a liter of toluene. The counting efficiency was 69.4%. Corrections for

quenching were performed with an external standard.

## RESULTS

Table I demonstrates that, besides PGE, human platelets are able to synthesize PGA, PGB and PGF. Of the label incorporated into PGs, the major part of the labeled material eluted in crude fraction from the silicic acid columns ends up in PGA and PGB (15.8%), followed by PGE (5.8%) and PGF (3.6%). As in our previous publication (5), nearly equal label is found in $PGE_1$, $PGE_2$, PGA, PGB and PGF. Thus 0.025 nmol acetate was incorporated into $PGE_1$, 0.021 nmol into $PGE_2$ and only 0.002 nmol into $PGE_3$/hr/$10^9$ platelets. Finally, both PGF, PGA and PGB contained 0.026 nmol/hr/$10^9$ platelets.

Figure 1 demonstrates the decline in radioactive label of PGEs after initiation of the chase experiment. The elimination appears to be nearly linear as a function of time. After the end of 2 hr of chase, nearly 25% of the PGEs was degraded ($p \leqslant 0.001$). In the case of PGF, the elimination followed a similar pattern, and at the end of 2 hr 15% of the PGF was eliminated ($p \leqslant 0.001$).

## DISCUSSION

The present communication extends our previous findings (5) by showing that human platelets form not only PGEs but also PGA, PGB and PGF. Smith and Willis (9) claimed that $PGF_{2\alpha}$ is formed in the platelets; this idea is supported by our present findings.

From a biological point of view, the presence in the thrombocytes of $PGE_1$ and $PGE_2$ in concentrations high enough to influence the aggregation (5) is of interest since Kloeze (2) recently showed $PGE_1$ to inhibit this process, contrary to $PGE_2$ which enhances it. However the influence of $PGE_2$ on an equimolecular basis, compared to the effect of $PGE_1$, is ca. 10 times lower where the efficiency of platelet aggregation is concerned. At the same time, one cannot ignore the endogenous formation of nonradioactive $PGE_2$ from arachidonate and nonradioactive $PGE_1$ from di-homo-γ-linolenic acid. However accepting an endogenous synthetic rate of these prostaglandins proportionally similar to the present finding using radioactive acetate, the effect of $PGE_1$ should be greater than that of $PGE_2$, thus normally hampering the aggregation phenomenon.

Since the proportion of the radioactivities of crude PGA and PGB to that of PGE was found to be 3:1, but 1:2 in purified fractions, the PGA and PGB in the crude fractions must contain significant amounts of impurities which, however, could not be identified.

From the chase experiments, it is obvious that both PGEs and PGF are degraded in plasma, and this may explain the findings of Nakano and Prancan (10) who have shown PGE to be enzymically degraded slowly into less polar metabolites in the rat plasma.

The rates of synthesis and degradation of PGs in thrombocytes may thus suggest a control function in thrombocyte aggregation. The protective function of dietary margarine and vegetable oils, enriched in essential fatty acids of the ω-6 series, on cardiovascular casualties in humans (11,12) may thus in part be explained by their transformation into PGEs in the thrombocytes.

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# Occurrence of Ethanolamine- and Choline-Containing Plasmalogens in Adipose Tissue

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## ABSTRACT

Analysis of phospholipids in porcine, bovine and rat adipose tissue revealed a relatively high level of plasmalogens (*O*-alk-1-enyl lipids). About 50% of the ethanolamine phospholipids in the pig and beef samples consisted of alk-1-enyl-acylglycerophosphorylethanolamine, and the corresponding value for the rat sample was near 35%. In the ethanolamine and choline phospholipid fractions, the *O*-alk-1-enyl moieties were almost exclusively 16:0, 18:0 and 18:1, whereas the acyl moieties had chain lengths ranging from 16 to 22 carbon atoms with a high degree of unsaturation.

## INTRODUCTION

It has been known for some time that adipose tissue contains small but significant quantities of alk-1-enyldiacylglycerols (1,2), which are often referred to as "neutral plasmalogens." A recent communication from this laboratory reported very high levels of ethanolamine plasmalogens (alk-1-enylacylglycerophosphorylethanolamine) in pig adipose tissue (3). In this report we describe the plasmalogen content of the phospholipids from porcine, bovine and rat adipose tissue, and we report also on the nature of the acyl- and alk-1-enyl moieties present in the ethanolamine and choline phospholipid fractions from these tissues.

## EXPERIMENTAL PROCEDURES

Porcine and bovine adipose samples were obtained from a local slaughter house, kept over ice and extracted within 1 hr of removal. Epididymal fat pads of rats were removed and the fat extracted immediately. For each 10 g tissue, 50 ml water was added, and the method of Bligh and Dyer (4) was then used for extraction. The lipid samples were stored in chloroform at -20 C.

Aliquots of the total lipids were oxidized and the total phosphorus determined (5). Total phospholipids were calculated by multiplying the phosphorus value (mg/g tissue) by 25. Aliquots of the total lipid extract were also chromatographed on 20 x 20 cm thin layers of Silica Gel HR (slurried with 5% sodium bicarbonate) in chloroform-methanol-acetic acid-water 50:25:8:3. The lane containing the sample was exposed to HCl fumes for 5 min to hydrolyze the alk-1-enyl ether moieties (6,7). The plates were then dried over KOH in a vacuum desiccator and developed in a second direction in chloroform-methanol-ammonia 60:35:5. The separated components were charred after exposure to $H_2SO_4$ and the charred spots removed for phosphorus assay.

[1]On leave from the New Zealand Medical Research Council.

TABLE I

Phospholipid Content and Composition of Adipose Tissue Samples[a]

| Component | Pig | Beef | Rat |
|---|---|---|---|
| Total phospholipids, μg/g wet wt | 0.10 | 0.08 | 0.14 |
| | | Per cent of total lipid P | |
| Origin | 2.1 | 0.5 | 0.7 |
| Lysophosphatidylcholine | --- | --- | 0.2 |
| Sphingomyelin | 7.5 | 9.8 | 8.2 |
| Phosphatidylcholine plasmalogen | 3.7 | 3.1 | 4.2 |
| Phosphatidylcholine | 42.4 | 42.1 | 46.5 |
| Phosphatidylinositol | 1.6 | 3.0 | 2.1 |
| Phosphatidylserine | 3.4 | | |
| Phosphatidylethanolamine plasmalogen | 18.0 | 20.0 | 11.4 |
| Phosphatidylethanolamine | 13.1 | 17.6 | 23.4 |
| Solvent front | 8.1 | 4.1 | 3.6 |
| Recovery | 95 | 92 | 84 |

[a]Each value represents a mean value of duplicate samples from a single lipid extract.

TABLE II

Composition of Acyl and Alk-1-enyl Moieties from Adipose Tissue Phospholipids[a]

| Component | | Mole % | | | | | |
|---|---|---|---|---|---|---|---|
| | | Pig | | Beef | | Rat | |
| | | EP[b] | CP[c] | EP | CP | EP | CP |
| Methyl esters | 16:0 | 3.0 | 14.1 | 3.0 | 17.4 | 6.9 | 21.6 |
| | 16:1 | 0.5 | 1.9 | 0.4 | 1.7 | 1.0 | 1.4 |
| | 17:0 | 0.6 | 0.9 | 0.3 | 1.2 | 0.7 | 0.5 |
| | 17:1 | 0.4 | 0.4 | | 0.5 | 0.5 | 0.3 |
| | 18:0 | 20.4 | 29.3 | 13.2 | 24.1 | 19.0 | 25.5 |
| | 18:1 | 13.9 | 22.7 | 7.3 | 17.4 | 16.6 | 18.5 |
| | 18:2 | 18.1 | 20.2 | 3.4 | 7.8 | 11.5 | 18.3 |
| | 18:3 | 0.2 | 0.2 | 0.5 | 1.3 | 0.5 | 0.1 |
| | 20:1 | --- | --- | 0.2 | --- | --- | --- |
| | 20:2 | --- | 0.2 | 0.7 | --- | --- | 0.2 |
| | 20:3 | 0.8 | 0.6 | 5.4 | 6.6 | --- | 4.0 |
| | 20:4 | 18.6 | 6.9 | 21.6 | 8.4 | 21.2 | 9.6 |
| | ? | 0.4 | --- | 2.1 | 2.6 | --- | --- |
| | 22:2 | 2.3 | 0.2 | 8.5 | 3.7 | 1.3 | 0.5 |
| | 22:3 | 0.2 | --- | 1.5 | 0.5 | 0.7 | 0.4 |
| | 24:1 | --- | 0.6 | 0.2 | 0.1 | 0.2 | --- |
| | 22:5 | 1.1 | 0.3 | 11.0 | 3.0 | 2.7 | 0.7 |
| | 22:6 | 0.4 | 0.3 | 1.9 | 0.6 | 5.5 | 1.4 |
| Dimethylacetals | 16:0 | 4.6 | 0.7 | 4.9 | 1.2 | 4.7 | 0.33 |
| | 16:1 | 0.6 | 0.1 | 0.9 | 0.4 | 0.4 | 0.15 |
| | 17:0 | 0.6 | --- | 0.6 | 0.2 | 0.4 | 0.05 |
| | 17:1 | --- | --- | 0.1 | --- | --- | --- |
| | 18:0 | 9.6 | 0.3 | 11.0 | 0.9 | 3.3 | 0.11 |
| | 18:1 | 3.6 | --- | 1.0 | --- | 2.4 | --- |
| | 18:2 | 0.3 | --- | 0.2 | --- | 0.4 | --- |
| Total dimethylacetals | | 18.7 | 1.4 | 18.7 | 2.7 | 11.6 | 0.7 |
| Total plasmalogens | | 37.4 | 2.8 | 37.4 | 5.4 | 23.2 | 1.4 |

[a]Each value represents a single determination.

[b]EP = Ethanolamine phospholipid fraction.

[c]CP = Choline phospholipid fraction.

Phospholipid-rich fractions were prepared from aliquots containing 1-2 g total lipid in chloroform by adsorbing the phospholipids onto 2 g silicic acid (Brosil HA) in a 50 ml screw-cap centrifuge tube. The tubes were capped, shaken for 1 min and then centrifuged at 1500 rpm for 2 min. The chloroform solution was decanted, and the silicic acid was washed three times with 20 ml volumes of chloroform using the same procedure. The phospholipids were released by washing the silicic acid with three 20 ml volumes of methanol. Although the phospholipids were quantitatively recovered in the methanol, these extracts were contaminated with an equal quantity of neutral lipids. The combined methanol extracts were taken to dryness, dissolved in chloroform and spotted on preparative thin layer plates that were developed with chloroform-methanol-acetic acid-water 50:25:8:4. The plates were sprayed with dichlorofluorescein, and the ethanolamine and choline phospholipid fractions were located under UV light and extracted from the silica gel with 2% acetic acid in methanol. These fractions were then heated in a sealed tube for 15 min at 100 C with 0.5 ml of 7% HCl in methanol (8). The tubes were cooled in ice and 2 ml hexane was added, followed by 1 ml of 2.5 N sodium hydroxide. The hexane layer was removed and the aqueous portion extracted three more times with hexane. The combined hexane extracts contained the methyl esters of the fatty acids and the dimethylacetals of the fatty aldehydes freed from the alk-1-enyl linkages. These derivatives were separated by thin layer chromatography using benzene as the solvent (9).

Gas liquid chromatographic analyses were carried out on samples of the mixed methyl esters and dimethylacetals, the purified methyl esters and the purified dimethylacetals. Appropriate correction factors for the chain length and detector response were used to calculate the total mole per cent of each component. The response for each methyl ester and dimethylacetal was taken as being proportional to the carbon number of the aliphatic chain (10,11). The conditions used for gas liquid chromatography have been described elsewhere (3). The plasmalogen content was obtained by

doubling the total mole per cent of the dimethylacetals.

## RESULTS AND DISCUSSION

The phospholipid content and the percentage of phosphorus in each phospholipid fraction are listed in Table I. The quantity of the total phospholipids was similar for each sample. The values are slightly lower than the 0.20 μg/g tissue observed for the white adipose tissue from the mouse (12), but they are several times less than those reported for the adipose tissue of fetal lambs (13). However the adipose tissue of the fetal lamb is predominantly brown adipose tissue (14). Brown adipose tissue differs from the white adipose tissue in containing less lipid and more abundant cytoplasm (15,16). Consequently the phospholipid content tends to be higher both as an amount per gram tissue and as a proportion of the total lipid (12,15,17). The brown adipose lipid of the mouse contains 1-2% phospholipids (12).

The data in Table I also show that the ethanolamine phospholipids consist of 54, 53 and 34% plasmalogen for the pig, beef and rat adipose samples, respectively. The value for the pig is similar to that reported earlier (3). The corresponding values for the choline phospholipids given in the same order are 8.0, 6.9 and 8.2.

Table II lists as moles per cent the acyl and alk-1-enyl compositions of the ethanolamine and choline phospholipids, and the total mole per cent of plasmalogen in these fractions. The values for the ethanolamine phospholipids are lower than those determined by the two dimensional thin layer chromatographic procedure. This appears to be a result of different animals used for the two analyses. When the same samples of bovine brain and bovine kidney ethanolamine phospholipids were assayed, no differences in the plasmalogen content were detected with the two methods.

The following trends were noted for all animals with respect to the acyl moieties. The ethanolamine phospholipids contained less palmitate relative to stearate, and a higher proportion of polyunsaturated fatty acids, than the choline phospholipid fractions. The ethanolamine phospholipids also tended to have less of the $C_{16}$ relative to the $C_{18}$ alk-1-enyl moieties than the choline phospholipids, which is consistent with previous observations (18) made for a number of other tissues.

Bovine adipose tissue appears to differ from bovine cardiac tissue, in that the former contains only low amounts of choline plasmalogens. In the bovine cardiac tissue, the content of choline plasmalogens is even greater than that of the ethanolamine plasmalogens (19).

Our results extend our previous observations (3) and suggest that the phospholipids from adipose tissue, like those from nervous tissues, cardiac and skeletal muscle, and kidney, are particularly rich in the ethanolamine plasmalogens (18).

## ACKNOWLEDGMENTS

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# Synthesis and Biological Characterization of 10,11-Methylene-9-Nonadecene; an Analog of Sterculic Acid[1]

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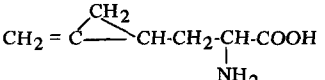
**ABSTRACT**

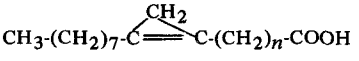
A method is presented for the preparation of 10,11-methylene-9-nonadecene, a structural analog of the biologically active 9,10-methylene-9-octadecene (sterculene). When tested for its ability to inhibit stearyl CoA desaturase in vivo, 10,11-methylene-9-nonadecene failed to alter the stearic-oleic acid ratio in the yolks of eggs from hens that had been fed 75 mg per day for 8 days. The compound did not give the Halphen test nor react rapidly with silver nitrate or methyl mercaptan in alcohol.

## INTRODUCTION

Hypoglycin [1] and sterculic acid [2] (n=7) are structurally similar in the part of each molecule that is concerned with their respective biological activities; yet these activities are diverse.



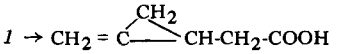
*1*



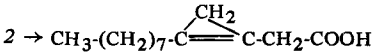
*2*

It has been postulated that sterculic acid and its analogs inhibit stearyl CoA desaturase by irreversibly adding to the sulfhydryl groups of the enzyme (1-3). The activity of hypoglycin has been attributed to its ability to tie up intramitochondrial CoA and consequently inhibit $\beta$-oxidation of fatty acids (4).

If the studies of the metabolism of hypoglycin (5) and cyclopropanoid compounds (6,7) are indicative of the metabolism of cyclopropenoid compounds, the end metabolic products of hypoglycin and sterculic acid would only differ in the length of the aliphatic side chain and the location of the double bond (*3* and *4*).



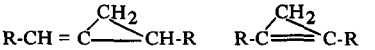
*3*



*4*

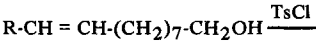
Fogerty et al. (8) have recently shown that when $n$ = 6, 7 or 8 in structure *2*, the compound inhibits the in vitro stearyl CoA → oleate desaturation; but when $n$ = 9, this inhibition was not observed.

The present work is an effort to determine the importance of a different structural alteration on the biological activity of cyclopropenes. We asked: if the double bond is moved from within the ring (as in *2*) to a position adjacent to it (as in *1*), will the compound still retain biological activity? To answer this question, 10,11-methylene-9-nonadecene *5*, an analog of the biologically active (9,10) sterculene *6* was synthesized and tested.

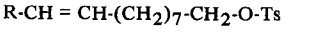


*5* *6*

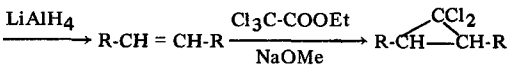
R is $CH_3$-$(CH_2)_7$- in all formulas. The synthesis was based on the following scheme:



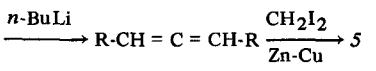
*7*



*8*



*9* *10*



*11*

[1]Contribution 1972 from the Arizona Agricultural Experiment Station.

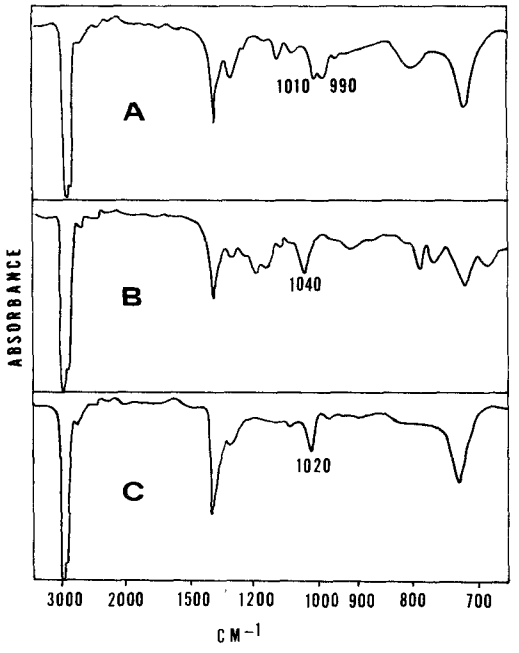



FIG. 1. IR spectra of 10,11-methylene-9-nonadecene (A) and the products of its reactions with $Br_2$ (B) and $H_2$ (C).

## MATERIALS AND METHODS

### Gas Liquid Chromatography (GLC)

Methyl esters: 8 ft, 1/4 in. column, 15% DEGS on Chromosorb W, 195 C, 80 ml He/min, quantitation by peak area measurement. All other compounds: 4 ft, 1/4 in. column, 20% DC 200 on Chromosorb P, 245 C, 80 ml He/min.

### Thin Layer Chromatography (TLC)

Olefins, dichlorocyclopropane: 10% $AgNO_3$ on silica gel, benzene-cyclohexane 1:4. Alcohols, tosylates: silica gel, Skelly Solve B-Ether-HAc 90:10:1. Compounds located with $I_2$, $KMnO_4$, $H_2SO_4$.

### Spectra

IR: Perkin-Elmer Infracord 137B, 0.5 mm cells, $CS_2$ soln. NMR: Varian A-100, $CCl_4$. Mass: Hitachi Perkin-Elmer RMV-Ge.

### Analysis

Schwartzkopf Analytical Laboratory.

### Oleyl Alcohol (*7*)

Commercial material was distilled through a spinning band column, bp 135-145 C/0.2 mm.

### 9-Octadecene (*9*)

Oleyl alcohol was tosylated in dry pyridine on an ice bath (11) and the crude product crystallized from 10 parts of methanol at -16 C. The purified tosylate was reduced with $LiAlH_4$ in boiling tetrahydrofuran (12), and 9-octadecene was purified on a 20% $AgNO_3$/silica gel column by elution with Skelly Solve F (low boiling petroleum ether: p.e.) and by distillation, bp 100 C/10.15 mm.

### 1,1-Dichloro-2,3-Dioctylcyclopropane (*10*)

9-Octadecene was treated with ethyl trichloroacetate and sodium methoxide in dry heptane as described by Kenney et al. (13). The reaction was explosive, however, when scaled up (0.2-0.4 mole) because of an induction period. A controllable reaction resulted when it was initiated slightly above room temperature instead of on an ice bath (13) and trichloroacetate addition carefully restricted until the mixture began to reflux. Slow additions of ester and vigorous stirring gave the best results. The crude product was diluted with p.e. and filtered through 10 parts of aluminum oxide to remove oxygenated compounds. Unreacted 9-octadecene was removed by urea clathration (ethanol-product 4:1 v/w); mole/mole urea/product 6:1). *10* was obtained in >95% purity (GLC); its IR spectrum was as described (13).

### 9,10-Nonadecadiene (*11*)

The allene was prepared by a described procedure (14) that was modified to minimize dimerization. *n*-Butyllithium (0.224 mole, 22% in hexane) was stirred mechanically under $N_2$ on an ice bath, while 0.075 mole *10* in 200 ml ether was added over 3 hr. After two more hours on the ice bath, water was cautiously added and the product extracted with p.e. Drying ($MgSO_4$), evaporation of solvent and vacuum distillation gave 9,10-nonadecadiene free of *10* but still containing traces of 9-nonadecene. The product was freed of the latter with a 20% $AgNO_3$/silica gel column and p.e. as eluant. IR: sharp peak at 1950 $cm^{-1}$ (allene). GLC retention times: $9 < 11 \ll 10$. TLC $R_f$: $10 > 11 > 9$.

### 10,11-Methylene-9-Nonadecene (*5*)

The procedure of Rawson and Harrison (15) was modified to prevent spiropentane formation. Zinc dust (0.3 mole) and $Cu_2Cl_2$ (0.3 mole) in ether (200 ml) were refluxed and stirred 30 min under $N_2$. Allene (*11*) (0.15 mole) in 50 ml ether was then added slowly, followed by addition of $CH_2I_2$ (0.12 mole) in 50 ml ether over a 1 hr period. Samples were then withdrawn every 0.5 hr, added to dilute HCl and immediately analyzed (GLC retention times: *11* < *5* < dioctylspiropentane). The

reactions were run less than 4 hr to avoid formation of appreciable spiropentane; they were usually stopped after ca. 25% conversion of allene to the methylenecyclopropane (*5*). Since the allene could be recovered and separation of *5* from spiropentane was difficult, this proved to be the most economical method.

Unreacted $CH_2I_2$ was distilled from the reaction mixture (32 C/0.4 mm) after removal of solvent. The residue was placed on a 20% $AgNO_3$/silica gel column (column dry wt/product wt 100:1) and rapidly eluted with 5% ether in p.e. to separate *5* from *11* and spiropentane. Traces of nitrated products were removed from *5* by distillation.

#### Analysis

Purified *5* rapidly absorbed $Br_2$ changing the methylenecyclopropane doublet at 990 and 1010 $cm^{-1}$ in the IR to a single peak at 1040 $cm^{-1}$ (cyclopropane) (Fig. 1). It also rapidly absorbed 1 mole $H_2$ (5% Pd/C, EtOAc) to yield a cyclopropane, the spectrum of which was superimposable on that of dihydrosterculene (Fig. 1). Permanganate-periodate oxidation (16) gave *n*-octanoic acid as the only product (IR, GLC Me ester). NMR spectrum: -C<u>H</u> = at $\tau$ 4.3-4.5, -C<u>H</u>$_2$-CH = at $\tau$ 7.8-8.0, ring methylene protons at $\tau$ 9.3-9.5. Mass spectrum: m/e at 278 (parent peak), 264 ($M^+ - CH_2$), 126 and 152 (rupture at double bond). Elementary analysis: Calculated for $C_{20}H_{38}$: 86.33% C, 13.67% H. Found: 86.14% C, 13.85% H.

#### Reactions

a) With methanethiol (1): Solutions of *5* and *6* (0.133 mM) in 0.33 ml ethanol containing 0.4 mM $CH_3SH$ were prepared and analyzed periodically by GLC. After 6 hr 70% of *6* had reacted; none of *5* had reacted.

b) Halphen test (17): *5* did not give the characteristic red color that develops when cyclopropenes are heated with $CS_2$ and S in pyridine.

c) With $AgNO_3$ (18): *5* did not give the immediate black precipitation of metallic silver that occurs when cyclopropenes are treated with alcoholic $AgNO_3$ at room temperature.

#### Biological Evaluation

Capsules containing 75 mg methyl sterculate, sterculene (*6*) or 10,11-methylene-9-octadecene (*5*) in 50 mg corn oil or 125 mg corn oil alone were each administered daily in duplicate to white Leghorn hens for 8 days. Eggs were collected from each pair of hens after 2, 5 and 8 days of capsule feeding. The yolk lipids were saponified, the fatty acids esterified (7% $BF_3$/MeOH) and the fatty acid compositions determined by GLC. Twelve eggs were collected just prior to the feeding regimen to establish a normal fatty acid composition.

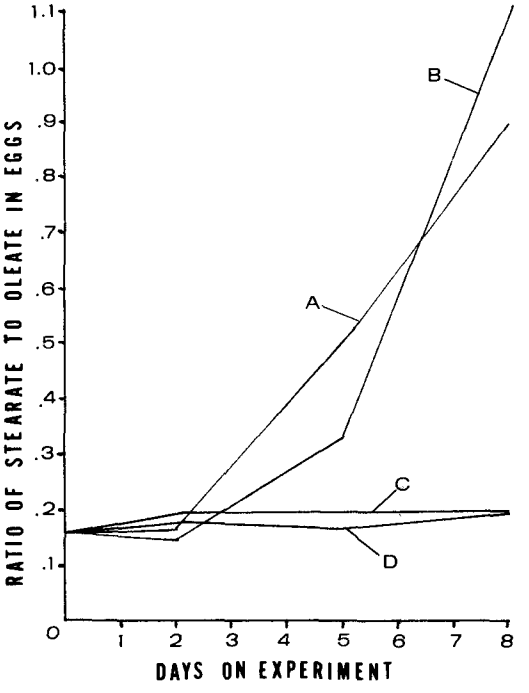



FIG. 2. Changes in the ratio of stearic to oleic acid in yolks of eggs from hens fed: (A) methyl sterculate, (B) sterculene, (C) 10,11-methylene-9-nonadecene and (D) corn oil only.

## RESULTS AND DISCUSSION

The synthesis of 10,11-methylene-9-nonadecene (*5*) from 9-octadecene (*9*) was chemically straightforward; the greatest difficulties were encountered during the separation of products from the various reactions. Urea clathration, column chromatography and vacuum distillation proved to be the best techniques. Although the final product (*5*) did not react with alcoholic $AgNO_3$, prolonged exposure to a 20% $AgNO_3$/silica gel column in p.e. (>24 hr) gave some decomposition with the formation of nitrated derivatives similar to those described by Johnson et al. (19). The lack of reactivity to alcoholic silver ion may be due to alcohol complexes of the ion. Babb and Gardner reported that the smaller molecule, 2,2-dimethylmethylenecyclopropane was also stable to alcoholic silver perchlorate (20).

The final step in the reaction sequence, addition of methylene to the allene *11*, required some exploration before the best condi-

tions were found. Too long an exposure of *11* to $CH_2I_2$ and Zn-Cu couple or too high a ratio of the reagents to the allene led to an appreciable production of dioctylspiropentane, a result also described for smaller allenes (21-23). When the reaction was arrested after 25% of *11* had been converted to *5*, spiropentane content of the mixture was still negligible and *5* could be separated from *11* by rapid elution through a silver nitrate column. The recovered allene was then distilled and used again.

The physical and chemical properties of *5* correspond to its assigned structure. Bromination and hydrogenation yield a cyclopropane and oxidation gives only octanoic acid. The IR (24), NMR (25) and mass spectra are all consistent with a structure possessing a double bond adjacent to a cyclopropyl ring.

A methylene cyclopropane is chemically and biologically distinct from a cyclopropene. Compound *5* failed to add methanethiol rapidly, give the Halpen test or precipitate silver from alcoholic silver nitrate. All of these reactions readily occur with sterculene (*6*).

The results of the feeding tests are shown in Figure 2. Methyl sterculate and sterculene altered the ratio of stearic to oleic acid as expected (10,26) from 0.1 to 1 in those eggs from hens fed the two compounds for 8 days. 10,11-Methylene-9-nonadecene was completely inert in its ability to cause this change. The chemical and biological data show what happens when a double bond is moved from within a three-membered ring to a position adjacent to it. The concomitant relief of strain makes the methylenecyclopropane much less reactive in vitro and in vivo than the corresponding cyclopropene.

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# Pyrolysis of Saturated Triglycerides

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## ABSTRACT

Saturated monoacid triglycerides were pyrolyzed in a flowing atmosphere of helium at 630-650 C, and the products were collected at low temperature. They were fractionated by thin layer chromatography, according to polarity, and were further resolved and analyzed by gas chromatography, mass spectrometry and IR spectrometry. Alkanes, alkenes, ketones, esters, free acids and cyclic products were identified. Among the latter are probably lactones and substituted dioxanes and dioxolanes, for which structures have been postulated. The parent acid of the triglyceride and the cyclic residual products, after loss of one acid group from the triglyceride, were prominent products. Many products of pyrolysis had structures similar to the major ions produced in the mass spectrum of the triglyceride.

## INTRODUCTION

The mass spectrum of a substance that has low vapor pressure and, consequently, must be heated to high temperature to be vaporized is the composite of the spectrum of the intact substance plus the spectra of fragments generated by pyrolysis in the instrument. Even with more volatile substances, this statement is true to some degree because of encounter of the sample with the glowing filament. Ideally, electron impact yields ionized molecules which are fragmented to give ions from which the original structure can be deduced. Similarly, thermal excitation yields neutral fragments from which the original structure can be deduced. For example, the pyrolysis pattern of branched hydrocarbons can be interpreted in a manner analogous to the interpretation of the mass spectrum of the hydrocarbons (1). In the case of substances with low volatility, such as a lecithin, the mass spectrum is often composed of the spectra of the pyrolysis products. In such a case, one might learn more by deliberate pyrolysis than by attempted mass spectrometry.

Thermal degradation of fats also has practical importance because it takes place in cooking and food processing. This practical importance has been the motivation for numerous studies of pyrolysis of fats (triglycerides) in the presence of and in the absence of air (2-5). The present study was addressed to two problems—the analysis of lipid structure and the identification of products that might influence the nutritional or biological value of fats. The study was conducted on four highly purified monoacid triglycerides, and the products were characterized by thin layer chromatography (TLC), gas chromatography (GLC), IR spectrometry and mass spectrometry (MS).

## EXPERIMENTAL PROCEDURES

Pyrolysis was performed in a stainless steel tube 60 cm x 1 mm at 630-650 C in a stream of helium at a flow rate of 65 ml/min, which allowed the sample to remain in the heated region for 0.2 sec. The neat triglyceride was injected in 10 $\mu$l portions through a gas chromatographic injection port maintained at 305 C. The products of pyrolysis were collected in a 2 liter vessel, the exit of which was plugged with glass wool, followed by a cold finger cooled in dry ice plus acetone. The products were dissolved in chloroform and stored under nitrogen at -20 C, as were the subsequently isolated fractions.

The products of pyrolysis were separated into classes on the basis of polarity by preparative TLC using 2 mm thick cakes and 0.7 mm thick plates, using solvent systems ranging from 100% petroleum ether (30-60 C) to petroleum ether-diethyl ether-acetic acid 50:50:2. It was generally necessary to chromatograph each compound two or three times to obtain reasonable purity. The isolated classes of compounds were then analyzed by either mass spectrometry, IR spectrometry, comparative TLC, comparative GLC or a combination of these. The mass spectra were recorded using a Hitachi RMU-6D mass spectrometer operated at 70 eV. The samples were introduced via the direct inlet or via the gas chromatographic inlet. The IR spectra were recorded with a Perkin-Elmer Model 21 IR spectrophotometer, and samples were examined as neat liquid films when possible, or as 2% solutions in tetrachloroethylene or in carbon disulfide using a 0.1 mm cavity cell. The gas chromatographic columns were 10% SP1000 on Gas Chrom P 60-80 mesh, 10 ft x 1/4 in., 10% OV1 on Gas Chrom P 80-100 mesh, 6 ft x 1/4 in., 20% EGS + 2% phosphoric acid on Gas Chrom P 60-80 mesh, 6

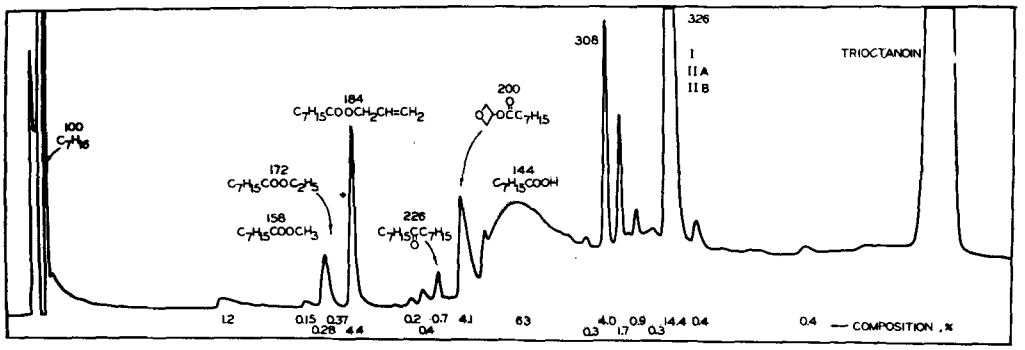



FIG. 1. Gas chromatogram of products of pyrolysis of trioctanoin on a column 6 ft x 1/4 in. packed with 10% OV1 on Gas Chrom P 60-80 mesh, programed from 40-265 C at 6 C/min. The sample was washed with sodium carbonate to remove most of the free acids. The percentage composition obtained from an analytical run on a sample from which acids were not removed is displayed along the baseline. Information obtained from gas liquid chromatography-mass spectrometry run with a larger sample is displayed above the tracing.

ft x 1/4 in., and 10% SP2250 on Gas Chrom P 60-80 mesh, 10 ft x 1/4 in. Helium was used as the carrier gas, and the detector was either a hydrogen flame or the total ion monitor of the mass spectrometer.

Trioctadecanoin, trihexadecanoin, tritetradecanoin and trioctanoin were purchased from The Hormel Institute Lipids Preparation Laboratory and were checked by TLC of the triglycerides and by GLC of the methyl esters of the acids to confirm that the purity was greater than 99%, with respect to lipid class and to fatty acid composition.

## RESULTS AND DISCUSSION

The pyrolysis of trioctadecanoin, trihexadecanoin, tritetradecanoin and trioctanoin yielded, in each case, mixtures of products, the patterns of which were similar on TLC for all the triglycerides. Mass spectra of corresponding compounds from the four triglycerides revealed them to be homologs. Structural analysis of the products from the three smaller triglycerides supported the conclusions which are given in detail only for trioctadecanoin.

Many of the pyrolysis products were similar in composition and structure to the structures postulated for the major ion fragments observed (6,7) in the mass spectra of the corresponding triglycerides. Some additional substances were formed, presumably because of the bimolecular reactions occurring at the relatively higher pressures present during pyrolysis.

Figure 1 shows the gas chromatogram of the pyrolysis products of trioctanoin, indicating the sequence in which the major products appear in GLC. Trioctanoin was chosen for this illustration because it and all of its pyrolysis products passed through the GLC-MS system at reasonable temperatures, whereas with the larger triglycerides, the products of higher molecular weight did not all emerge, and GLC-MS could not be practiced upon the full range of products. Therefore the products from pyrolyses of long chain triglycerides were separated by TLC into groups by polarity and were examined in groups by GLC-MS or by MS using the direct insertion probe. Typical TLC separations of the products are shown in Figure 2.

### Hydrocarbons

The hydrocarbons from trioctadecanoin, for example, were isolated from the pyrolysis mixture by preparative TLC using 2 mm plates developed in light petroleum ether (30-60 C)-diethyl ether 95:5. The isolated hydrocarbon material, which was ca. 17% of the pyrolysis products by weight, was chromatographed alongside authentic tetracontane and found to have the same $R_f$ value. The combined hydrocarbons were separated and identified by comparative GLC and by GLC-MS. The hydrocarbons consisted of two classes of compounds, the *n*-alkanes and the *n*-alkenes. A mass spectrum of one representative alkene had a molecular ion at m/e 224, a base peak at m/e 43, and a series of ions very similar to that found in the spectrum of authentic 1-hexadecene. Mass spectroscopy does not permit locating the double bond in olefins, but, in the pyrolysis of methyl esters of saturated fatty acids, most of the olefins produced are 1-alkenes (8). By analogy, 1-alkenes should also be formed from triglycerides. The last and most abundant member of the alkane series had a spectrum identical to the

spectrum of heptadecane, within experimental error.

Both even and odd chain lengths were present for both the homologous alkanes and alkenes, although the even chain members were slightly more abundant. The alkenes were much more abundant than the alkanes by a factor of 20 to 1; except heptadecane was considerably more abundant than heptadecene, and probably arises by decarboxylation. Each series formed a straight line when the log retention time in GLC was plotted against carbon number. GLC-MS confirmed the presence and identity of members from $C_{11}$ to $C_{17}$ in both series. The yield of hydrocarbons increased with increasing temperature of pyrolysis, and at 800 C hydrocarbons were the major product formed.

#### Ketones

The symmetrical ketone, $C_{17}H_{35}COC_{17}H_{35}$, represented 0.7% of the pyrolyzed material from trioctadecanoin. The GLC of this material indicated that small amounts of shorter ketones were also present, but these totaled less than 1% of the major member. The latter was isolated by TLC and identified by comparative TLC using a long chain symmetrical ketone standard and by MS. The spectrum was identical to an authentic spectrum of 18-pentatricosanone within experimental error. The formation of the symmetrical ketone is also known to occur when a fatty acid is pyrolyzed (9).

The methyl ketone, 2-nonadecanone, was a rather minor product which was identified by TLC and MS comparisons. The spectrum agreed well with the known spectrum of 2-nonadecanone. The proportion of 2-nonadecanone formed under our conditions was considerably less than reported by Crossley et al. (10).

#### Esters

Four simple esters were isolated from the pyrolysis mixture of trioctadecanoin and identified: methyl octadecanoate (0.4%), ethyl octadecanoate (0.3%), propyl octadecanoate (0.1%) and allyl octadecanoate (4.4%). The allyl esters were the fourth most abundant product of the pyrolysis of long chain triglycerides, and allyl butanoate was also identified as a major product in the pyrolysis of tributanoin. Each of these esters had the same $R_f$ value and mass spectrum as the corresponding authentic compound. In each case, the alcohol moiety of the ester must arise from the glycerol moiety of the triglyceride. Allyl stearate may arise by direct loss of two acyloxy groups from the triglyceride, or by secondary pyrolysis of structures such as Ia, Ib, Ic, Id, IIa, IIb or IIc.

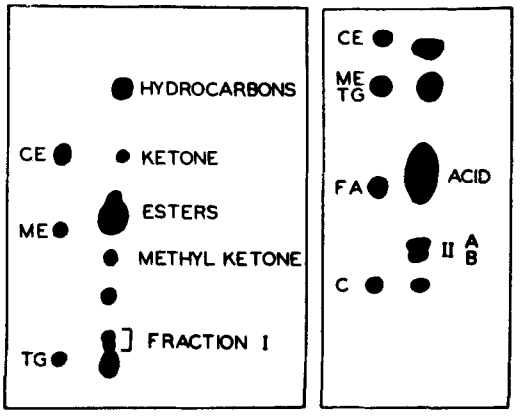
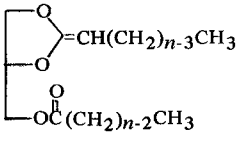


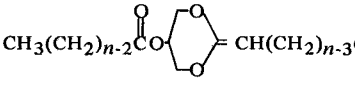

FIG. 2. Tracing of thin layer chromatographic separations (charred) typically used for isolation of the products of differing polarity. Left plate was developed in petroleum ether (30-60 C)-ether 95:5. Right plate was developed in petroleum ether (30-60 C)-ether-acetic acid 70:30:1. The standard contained cholesteryl ester, methyl ester, triglyceride, fatty acid and cholesterol.

#### Cyclic Compounds

The ion $[M\text{-}RCO_2]^+$ is the base peak of the spectrum of some triglycerides and is intense in the spectra of all others (6,7). In the spectrum of trioctadecanoin, this ion is m/e 607. In the pyrolysis of triglycerides, a major product is the free acid, which should leave a product equal to M-RCOOH. From trioctadecanoin this product would have MW 606. TLC of the pyrolysis products of trioctadecanoin separated two groups of isomers of different polarity, both of which had molecular weights of 606. The less polar fraction (fraction I), which preceded triglycerides in TLC had IR and mass spectra which suggest a structure closely related to the one proposed by Aasen et al. (6) for $[M\text{-}RCO_2]^+$. The possible structures may have a five-membered ring involving the 1 and 2 positions of glycerol (Ia), a six-membered ring involving the 1 and 3 positions of glycerol (Ib), or may be acyclic (Ic and Id).




Ia




Ib

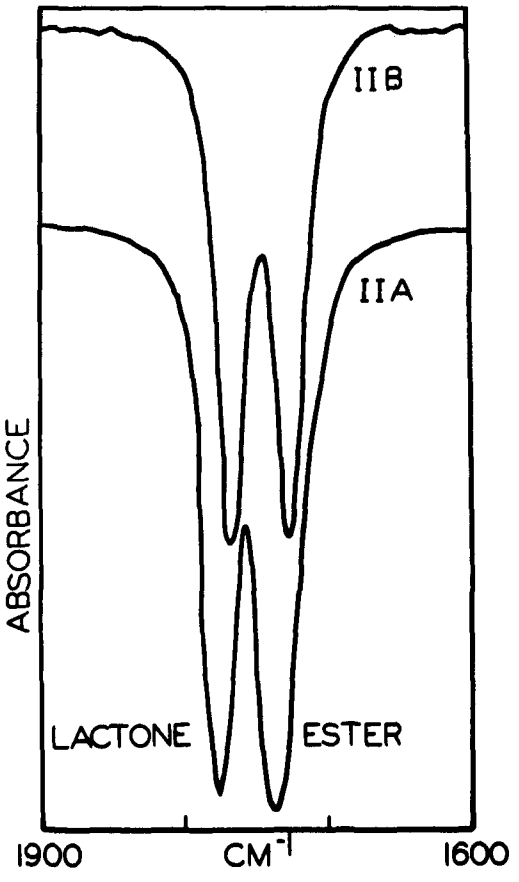
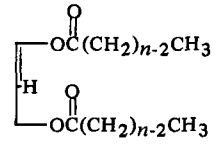
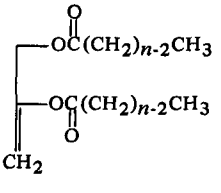



FIG. 3. Portions of the IR spectra of IIa and IIb which indicate the lactone structures.



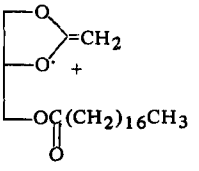

Ic




Id

The IR spectrum of fraction I eliminated structures Ic and Id, because the carbonyl stretching frequencies of compounds having double bonds adjacent to the ether oxygen have absorptions above 1770 $cm^{-1}$. The IR spectrum of the substance obtained experimentally had an absorption at 1730 $cm^{-1}$, which is normal for an ester carbonyl. Other diagnostic absorptions observed were 1665 $cm^{-1}$ for the carbon-carbon double bond stretching frequency, and a broad band at 1150 $cm^{-1}$ caused by the ether absorptions.

The mass spectrum of fraction I had a small proportion of molecular ion (0.1%) at m/e 606, and its base peak was m/e 267, $[RCO]^+$. Also present were peaks at m/e 533$[M-73]^+$, m/e 382$[RCO + 115]^+$, m/e 341$[RCO + 74]^+$, m/e 323$[M-RCO_2]^+$, m/e 284$[RCO_2H]^+$, m/e 309$[M-RCO_2CH_2]^+$ and the series $[98 + 14n]^+$ with $n = 1$ to 15, but with the first nine members predominant. In the mass spectrum of fraction I, the ion m/e 382, $[RCO + 115]^+$, corresponds to M minus the alkyl chain. Because $[RCO + 128]^+$ does not appear in the spectrum of I, the position of its double bond may be adjacent to the ring, favoring cleavage to form $[RCO + 115]^+$. The postulated structure for ion m/e 382 is shown as A.



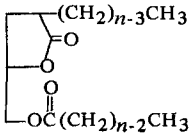

A $[RCO + 115]^+$ 382

A second parallel series, $[97 + 14n]^+$, is also present in the spectra. This series could be formed by the loss of a hydrogen atom from $[98 + 14n]$. The ion $[RCO + 74]^+$ at m/e 341 is probably the same as the one observed in the spectrum of triglycerides by Aasen et al. (6). The breaking of two bonds plus a proton rearrangement would be required in its formation.

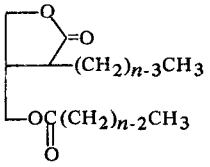
Two substances (fractions IIA and IIB), which follow triglycerides in TLC and which also have molecular weights of 606, were harvested separately by TLC. IR spectra of both compounds revealed two carbonyl absorptions, one at 1730 $cm^{-1}$ for normal ester carbonyl absorption and the other at 1770 $cm^{-1}$ consistent with the carbonyl absorption of a five-member lactone ring (Fig. 3). Neither spectrum revealed absorption for carbon-carbon double bond stretching, as was observed in the IR spectrum of I. Each spectrum had a broad band at 1150 $cm^{-1}$, indicating the presence of C-O-C stretching bonds. The only differences between the spectra of these two similar substances are the absorption at 960 $cm^{-1}$ in one

of them and absorptions at 830 $cm^{-1}$ and 1025 $cm^{-1}$ in the other, possibly indicating differences in ring structure.

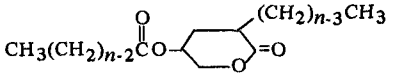
Loss of RCOOH from the triglyceride could lead to three lactone structures (IIa, IIb and IIc), plus their geometrical isomers. IIc should have a spectrum in which the lactone absorption is very close to the absorption due to a normal ester carbonyl 1730 $cm^{-1}$, whereas IIa and IIb should have spectra in which the lactone absorption is near 1770 $cm^{-1}$. Thus structures IIa and IIb are preferred for the two partially separable substances. Each of these structures, containing two asymmetric carbon atoms, may exist as four stereoisomers.



IIa



IIb



IIc

The mass spectra of the two compounds IIA and IIB (mol wt 606) have similar fragmentation patterns but show significant differences in intensities of prominent ions. The ion $[M\text{-}RCO_2CH_2]^+$ at m/e 309 was more intense in the spectrum of the less polar isomer (IIA) than in the spectrum of the more polar isomer (IIB). The cleavage of a substituent group on a carbon adjacent to the ring oxygen is favored (11), suggesting that the less polar substance may have structure IIa. The hydrogen atom on carbon 2 of glycerol is more acidic in structure IIa than in structure IIb, because of the electronegativity of the adjacent oxygen in IIa. This enhanced acidity should promote the loss of RCOOH, leaving $M\text{-}RCOOH^+$. The relative intensity of this ion, m/e 322, in the spectrum of the less polar substance was 99%, whereas in the spectrum of the more polar substance, it was 27%. Thus, the structure for the less polar substance (IIA) is postulated to be IIa and the more polar isomer (IIB) to be IIb, consistent with the assignment based on IR evidence.

TABLE I

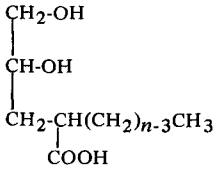
Fatty Acids Formed by Pyrolysis of Trioctadecanoin

| Equivalent chain length | Acids | Per cent |
|---|---|---|
| --- | <10 carbons | .05 or less |
| 11.00 | 11:0 | .09 |
| 11.42 | 11:1 | .29 |
| 12.00 | 12:0 | .19 |
| 12.41 | 12:1 | .78 |
| 13.00 | 13:0 | .30 |
| 13.42 | 13:1 | 1.15 |
| 14.00 | 14:0 | .28 |
| 14.42 | 14:1 | 1.35 |
| 14.64 | | .09 |
| 15.00 | 15:0 | .29 |
| 15.42 | 15:1 | 1.35 |
| 16.00 | 16:0 | .26 |
| 16.42 | 16:1 | 1.96 |
| 17.00 | 17:0 | .15 |
| 17.42 | 17:1 | .48 |
| 18.00 | 18:0 | 90.9 |

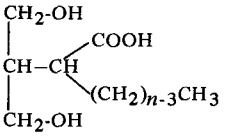
The isomeric substances, IIA and IIB, represent residues after one acid group is lost from the triglyceride, but these substances have mass spectra which differ considerably from those of diglycerides. In the spectrum of glycerol dioctadecanoate (m/e 606) M-18 is 0.6%, and the molecular ion (m/e 624) does not appear. The major ions at m/e 322 and 309 in spectra of IIA and IIB do not appear; nor do the minor ions in the spectrum of the diglyceride. The major ion $RCOOH^+$ at 284 in the spectrum of the diglyceride is minor in the spectra of IIA and IIB. Moreover IIA and IIB have a much larger $R_f$ value on TLC plates than does a diglyceride. Thus diglyceride is not a possible structure of IIA or IIB. Compounds of mol wt 606 (I, IIA and IIB) represent ca. 14% of the products of pyrolysis other than the hydrocarbons. The lactones IIA and IIB represent the major portion of the 14%.

Major lactone products have not been previously reported; nor have substituted dioxolane or dioxane structures, such as postulated here for fraction I, been observed. Structures such as Ia and Ib should be acid labile and should hydrolyze to yield glycerol and an acid. The lactone structures IIa and IIb should hydrolyze to branched dihydroxy acids in which the former glycerol residue is joined by a carbon-carbon bond to the fatty acid moiety at the $\alpha$

position of the latter (III and IV). The metabolic fates or biological activities of such structures are not known.

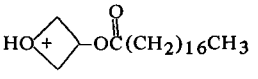


IV

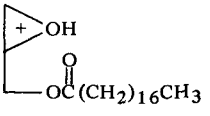


III

One substance isolated by TLC from the pyrolysis products had a mass spectrum that suggested it was similar to the structures B and C proposed for the ion $[RCO + 74]^+$ produced by electron impact upon triglycerides.



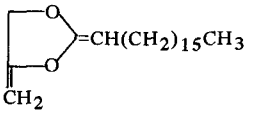
B



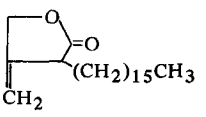
C

The molecular ion was at m/e 340, and the base peak was at m/e 267, corresponding to $RCO^+$; m/e 116 and the series m/e $[98 + 14n]^+$ were prominent ions. The ion m/e 116 is considered to be formed by a McLafferty type rearrangement to give the ion D which, in turn, could yield m/e 98 by loss of water. The highest member of the series $98 + 14n$ could originate by expulsion of water from the molecular ion, and lower homologs could be formed by further cleavage of the alkyl chain.

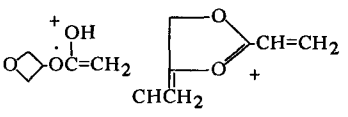
Another very minor component was characterized only by its mass spectrum. The spectrum had a strong molecular ion (22%) at m/e 322 and a series $[98 + 14n]^+$ descending from it and terminating at m/e 98. The base peak was at m/e 111. The spectrum had no other peaks except for the normal hydrocarbon fragments at low mass values. From this information, four structures are proposed—V and VI and their six-member ring isomers. These structures could arise by the loss of $RCO_2H$ from the compounds I, IIA and IIB. Because m/e 111 is its base peak, structure V is favored, for ion E would be a stable resonating structure. Structure VI would be expected to cleave next to the ring by the McLafferty mechanism, yielding primarily m/e 98, ion F.



V



VI



D 116 E 111 F 98

**Free Acids**

The most abundant product of the pyrolysis was fatty acid, most of which was the parent acid of the triglyceride. The acids amount to 63% of the pyrolyzed material (excluding hydrocarbons), and, of the acids, 91% was the parent acid. The other 9% was distributed among the saturated and monoenoic shorter chain acids.

All members of the series were present, the monoenoic acids being more abundant than the corresponding saturated acids. The position of the double bond was not established, but most of the homologous methyl esters had equivalent chain length (ECL) values 0.42 units greater than their saturated counterparts on a 10 ft 10% SP1000 column. This is a greater increment than the ECL increment of normal monoenes with the double bond near the center of the chain. These acids probably have double bonds near the point of scission, in analogy to the structures found in the pyrolysis of methyl esters (8).

The acids were identified by mass spectra in comparison with authentic spectra, and by TLC and comparative GLC of their methyl esters. The diacids reported by Crossley et al. (10) were not found in this study of pyrolysis.

ACKNOWLEDGMENTS

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# Observations on the Role of Vitamin E in the Toxicity of Oxidized Fats

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## ABSTRACT

Studies are reported on the relative effects of in vivo oxidation produced by diets devoid of vitamin E and the consumption of oxidized fat. Rats of the Sprague-Dawley strain were raised from weaning on a sucrose-casein diet containing minerals and vitamins in the required amounts, supplemented with 10% of safflower oil, menhaden oil, hydrogenated coconut oil or no fat. Animals of ca. 185 g of the group fed the 10% safflower oil were then switched for 4 weeks to safflower or menhaden oil-supplemented diets that were allowed to oxidize by exposing them to room temperature in the dark for 2-8 days. For comparison with effects of in vivo oxidation, animals were raised from weaning on similar fresh diets devoid of vitamin E. Consumption of oxidized fat was accompanied by loss of weight, effects on the size of the organs, changes in triglyceride levels and production of TBA-reacting substances in the tissues. There was no effect on the induced swelling of liver mitochondria or the susceptibility of erythrocytes to hemolysis in these animals. Growth was also suppressed in the animals fed the vitamin E-free diets, and in vivo oxidation in these animals produced marked effects on the membrane properties of erythrocytes and liver mitochondria.

## INTRODUCTION

The toxicity of oxidized fats has been investigated extensively (1-9), but because of many ramifications the primary mechanism of the action of these fats in animal tissues has not been delineated. There is a growing body of evidence, based on studies (10-14) of the toxicity of various products of autoxidized fats, that cellular damage occurs, indicating a breakdown of membrane structure. Because a primary function of vitamin E is believed to be its role as a biological antioxidant protecting cells against membrane damage (15-21), it was decided to investigate its role in the toxicity of oxidized fats.

## EXPERIMENTAL PROCEDURES

### Materials and Methods

Weanling male rats of the Sprague-Dawley strain were raised on a basic sucrose-casein diet containing minerals and vitamins in the required amounts (22) supplemented with 10% hydrogenated coconut oil, 10% safflower, 10% menhaden oil or no fat (fat-free diet). Corresponding diets devoid of vitamin E were prepared by leaving the tocopherol acetate out of the vitamin mixture and removing the tocopherols and related compounds from the fatty supplements by separating the nonsaponifiable fractions. The fats were fed as ethyl esters in these experiments.

Oxidized diets were prepared by allowing the safflower and menhaden oil-supplemented diets to oxidize at room temperature in the dark after they were mixed with all of the ingredients except the minerals and vitamins. At the desired stage of oxidation, as determined by peroxide and TBA values, the diets were placed in a nitrogen atmosphere at -20 C to prevent further oxidation. The vitamins and minerals were added daily to the diets which were fed ad libitum.

TBA values were determined on tissues by the method of Donnan (23) and on serum and hemolyzed red cells by the method of Bunyan

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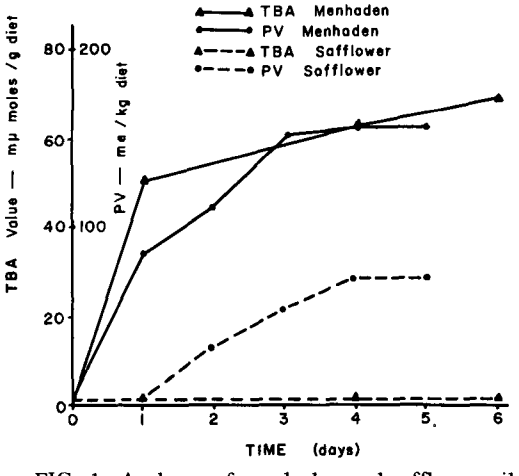


FIG. 1. Analyses of menhaden and safflower oil diets during storage at room temperature.

TABLE I

Analysis of Fat of Oxidized Diets[a]

| Major fatty acids[b] | Safflower oil | | Menhaden | |
|---|---|---|---|---|
| | Fresh | Oxidized[c] | Fresh | Oxidized[d] |
| 14:0 | | | 7.3 | 13.0 |
| 16:0 | 7.6 | 9.8 | 12.8 | 19.5 |
| 16:1 | | | 12.0 | 20.2 |
| 17:0 | | | 2.6 | 3.0 |
| 18:0 | 3.2 | 4.8 | 2.9 | 3.3 |
| 18:1 | 13.5 | 16.0 | 15.7 | 19.7 |
| 18:2 | 75.6 | 69.4 | 4.7 | 3.3 |
| 18:3 | | | 5.1 | 4.6 |
| 20:1 | | | 5.1 | 2.3 |
| 20:5 | | | 14.2 | 4.9 |
| 22:6 | | | 12.8 | 3.0 |
| TBA, O.D./g diet | 0 | .0 | 0 | 2.7 |
| PV, me/kg diet | 0 | 74 | 0 | 98 |

[a]See text for details.

[b]Shorthand designation: number before colon = chain length; number after colon = number of double bonds.

[c]Storage at room temperature for 8 days.

[d]Storage at room temperature for 2 days.

et al. (24). Peroxide values were determined by KI reduction (25). Triglyceride (TG) levels were determined by the method of Van Handel and Zilversmit (26). Vitamin E contents of tissues and serum were determined via a spectrophotometric method (27) using the Emmerie-Engel color reaction on nonsaponifiables obtained by saponification and extraction, in which all solutions were protected from atmospheric oxygen by an atmosphere of nitrogen.

For determination of fatty acid composition, the lipids were extracted with chloroform-methanol 2:1 and, after extraction of the nonlipid impurities, interesterified with methanol using HCl as a catalyst (28). The methyl esters were analyzed by gas liquid chromatography (GLC) using an F & M Model 1609 gas chromatograph equipped with a flame ionization detector and 6 ft x 1/4 in. column packed with EGSS-X (Applied Science). The column was operated at 185 C and a flow rate of carrier gas ($N_2$) of 75 ml/min. Quantitative analysis was determined directly from the proportionalities of peak areas.

Susceptibility of erythrocytes to dialuric acid-induced hemolysis was determined as described by Bunyan et al. (24). Swelling properties of liver mitochondria were measured as described by Stancliff et al. (29), except that an incubation time of 1 hr at 25 C in 10 $\mu$m/ml solutions of ferrous ammonium sulfate was used and measurements were made every 10 min.

## RESULTS

The course of oxidation of the diets is illustrated in Figure 1. The menhaden oil diet oxidized much faster than the safflower oil diet as evidenced by the large increase in both peroxide and TBA values. No TBA-reacting substances were produced in the oxidation of the safflower oil diet, and the peroxide value plateaued at a much lower level than that of the menhaden oil diet. In order to avoid excessive oxidation, the menhaden oil diet was allowed to oxidize for only 2 days. On the other hand, the safflower oil diet was allowed to oxidize for 8 days in order to achieve an appreciable degree of oxidation. Fatty acid composition, as well as TBA and PV values of the diets used in this study, are shown in Table I. Although the linoleic acid content of the safflower oil diet was reduced to some extent, the fatty acid composition was not greatly changed from that of the original oil. On the other hand, large changes occurred in the percentage of the polyunsaturated fatty acids of the menhaden oil during the oxidation of this diet.

The effect of the oxidized diets on growth and food consumption is shown in Figure 2. The depression in growth in the animals receiving the oxidized fat was due partly to a reduction of food intake. However there also was a deleterious effect of the diets as evidenced by changes in the percentages of the organs of the total body weight (Table II),

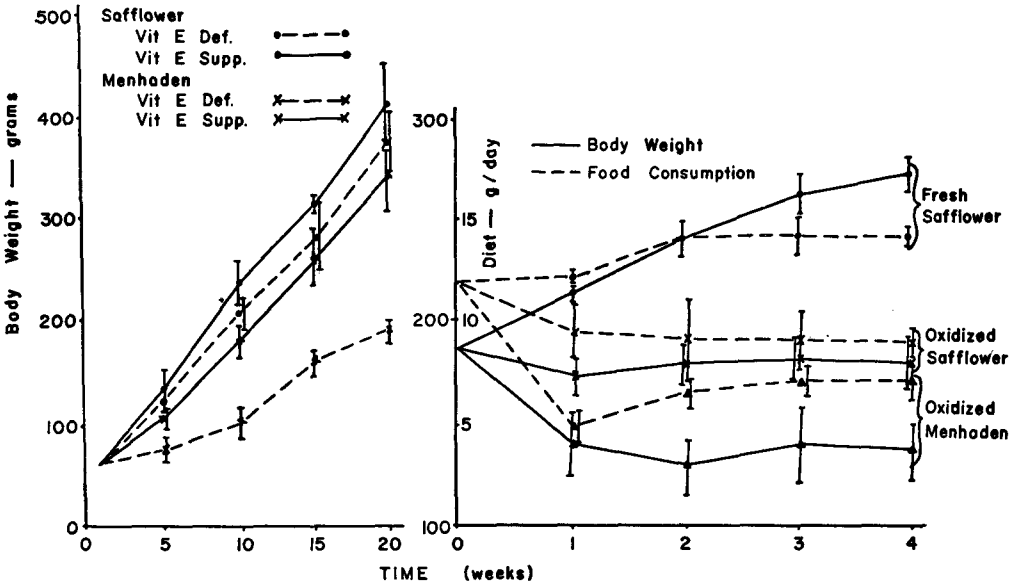


FIG. 2. Growth of animals fed fresh and oxidized diets and food consumption of animals switched to oxidized diets for 4 weeks. Vertical bars = standard deviation of 8-10 animals per group. $p < 0.001$ Vitamin E deficient menhaden to other groups.

detection of TBA-reacting substances and changes in TG levels of the tissues (Table III). Growth was also depressed in the animals fed the diets devoid of vitamin E (Fig. 2), especially the animals fed the more highly unsaturated menhaden oil ethyl esters. There was virtually no effect on the size of the organs in the animals fed the vitamin E deficient diets, but tissues of the hearts, fat pads and lungs contained products of lipid oxidation as evidenced by positive TBA values. Hence it appeared that the suppression of the growth of these animals was due to in vivo oxidation or, indirectly, to products of the reaction.

There was an appreciable decrease in weights of the organs of the animals receiving the oxidized diets over the 4 week feeding period (Table II). However a better indication of the effect of the oxidized diets was the percentages of the organs of the total body weight (Table II). The relative size of the livers were only slightly changed, but lung, heart, fat pads and reproductive organs were particularly effected by the oxidized diets. The percentage of the body weight of the lung and heart increased; the fat pads and the vesicular glands were greatly decreased. The vesicular glands of animals fed fresh and oxidized diets are shown in Figure 3 to illustrate the pronounced effect of the oxidized diets on these organs over the relatively short feeding period. The weight of the testes, likewise, decreased particularly in the animals fed the oxidized menhaden oil diet.

Examination of the triglyceride (TG) levels of the organs (Table III) showed large changes in the amount of fat in the tissues. The level of TG was much lower in the serum, lung and liver tissues and much higher in those of the heart and fat pads. Low TG levels in serum and liver are not unexpected because of the low food consumption. However the decrease in TG of the lung, at the same time that the percentage of this organ of the body weight decreased, was significant in view of other studies (30) which showed that in animals given intravenous injections of hydroperoxides, the lungs became enlarged from accumulation of fluid and edema. On the other hand, the increase in the level of TG in the heart and fat pads, together with a decrease in the percentage of these organs of the total body weight, indicated an abnormal accumulation of fat in these organs.

Although both the oxidized menhaden and safflower oil diets had relatively high peroxide values, no peroxides were detected in the organs or serum of the animals receiving these diets. TBA-reacting substances were detected in the tissues of animals fed both diets, but these substances did not appear to be those fed, because there was little of these substances produced in the oxidation of the safflower oil diet.

#### Effect of Vitamin E

There was no destruction of vitamin E in the diets because it was added in the form of the

TABLE II

Weight and Percentage of Body Weight of Organs of Adult Rats Fed Diets Containing Oxidized Fats for Four Weeks[a]

| | Dietary Fat | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh safflower | | Oxidized safflower | | | Oxidized menhaden | | |
| Organ | Wt, g | Body wt, % | Wt, g | $p$[b] | Body wt,%[a] | Wt, g | $p$[b] | Body wt, % |
| Liver | 11.19** ±99 | 4.2 | 5.92 ±58 | <0.005 | 3.4 | 5.32 ±.33 | <0.005 | 3.9 |
| Lung | 1.95 ±18 | 0.73 | 1.90 ±25 | <0.8 | 1.1 | 1.43 ±15 | <0.05 | 1.1 |
| Heart | 1.03 ±04 | 0.38 | 0.75 ±02 | <0.001 | 0.43 | 0.68 ±03 | <0.001 | 0.50 |
| Fat pad | 2.98 ±08 | 1.11 | 1.02 ±39 | <0.001 | 0.59 | 0.52 ±20 | <0.001 | 0.38 |
| Vesicular glands | 1.13 ±20 | 0.42 | 0.63 ±13 | <0.005 | 0.36 | 0.15 ±02 | <0.001 | 0.11 |
| Testes | 4.05 ±05 | 1.52 | 4.10 ±47 | <0.1 | 1.80 | 1.30 ±05 | <0.001 | 0.96 |
| Body/wt | 267 ±14 | | 172 ±22 | <0.005 | | 135 ±12 | <0.001 | |

[a]Eight to ten animals in each group.
[b]To animals fed the fresh safflower oil diet.
[c]M±SD.

acetate. Olcott and Mattill (31) showed that vitamin E acetate does not function as a chemical antioxidant, nor is it oxidized in animal diets. This observation was confirmed in accessory experiments in this study in which tocopherol acetate was added to the diets prior to storage at room temperature. The addition of the vitamin E acetate to the diets in these experiments had no effect on the formation of peroxides (Fig. 1), nor was it oxidized. Analyses of the tissues (Table III) showed that no destruction of vitamin E occurred in the ani-

TABLE III

Triglyceride (TG), TBA and Vitamin E Content of Tissues of Adult Rats Fed Diets Containing Oxidized Fat for Four Weeks[a]

| | Dietary fat | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Fresh safflower[b] | | Oxidized safflower | | | Oxidized menhaden | | | |
| Tissues | TG, mg/g | Vitamin E, μg/g | TG, mg/g | $p$, to fresh safflower | TBA, mμ mol/g | TG, mg/g | $p$, to fresh safflower | TBA, mμ mol/g | Vitamin E, μg/g |
| Lung | 13.0[c] ±0.2 | 25 ±10.7 | 11.1 ±1.6 | <0.2 | 3.6 | 7.5 ±1.0 | <0.005 | 4.1 | 25 ±6 |
| Heart | 3.2 ±1.3 | 50 ±11.8 | 3.7 ±0.65 | <0.02 | 3.9 | 7.3 ±2.3 | <0.005 | 6.2 | 52 ±4 |
| Liver | 7.9 ±1.1 | 34 ±5.4 | 5.7 ±0.5 | <0.05 | 0 | 3.6 ±0.18 | <0.005 | 0 | 24 ±2 |
| Fat pad | 130 ±10 | | 176 ±4 | <0.005 | 11.0 | 204 ±4 | <0.002 | 12.4 | |
| Serum | 570[d] ±40 | | 225[d] ±10 | <0.01 | 2.8 | 240[d] ±10 | <0.01 | 10.0[e] | |

[a]Eight to ten animals in each group.
[b]Not significantly different from that obtained with menhaden oil group (Fig. 2) (TBA values of tissues negative).
[c]M±SD.
[d]μg/ml.
[e]mμ moles/ml.

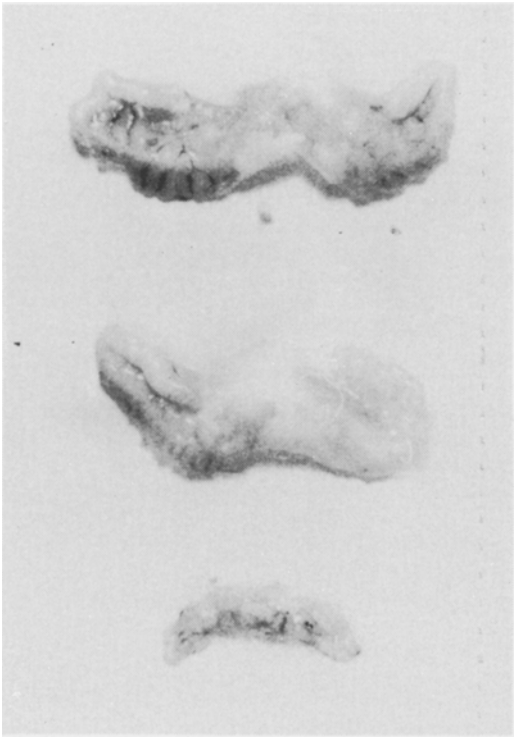

FIG. 3. Vesicular glands of A (upper), normal rats (fed fresh safflower oil); B (middle), animals from same group fed the oxidized safflower oil diet for 4 weeks; C (lower), animals from the same group fed oxidized menhaden oil diet for 4 weeks.

mals fed the oxidized diets. Further indication that vitamin E was not involved in the toxic effects of the oxidized fat was indicated from a study of the susceptibility of red blood cells to dialuric acid-induced hemolysis (Fig. 4).

These studies showed that increased susceptibility to hemolysis occured only in animals fed the vitamin E deficient diets. Moreover it was only with the animals fed these diets that the hemolyzed erythrocytes gave positive TBA values. There was no effect on the susceptibility to hemolysis of red blood cells of the animals fed the oxidized diets, in spite of the fact that the serum of these animals contained positive TBA-reacting substances (Table III). Hence it appeared that vitamin E protected the membrane lipids of the red blood cells of the animals fed the oxidized diets from in vivo oxidation.

The swelling properties of liver mitochondria were influenced by both fatty acid composition of the diet and vitamin E deficiency, as illustrated in Figure 5. Because the increase in unsaturation of the diet conferred increased swelling properties on the liver mitochondria in the presence of ferrous ammonium sulfate, it was evident that the swelling, under these conditions, correlated with the susceptibility of the membrane lipids to oxidation. That swelling was increased generally by a vitamin E deficiency indicated further that it protected the lipid in the mitochondria membrane from in vivo oxidation. This assumption was supported by determination of TBA values on samples of mitochondria taken at various intervals during the swelling measurements (Table IV).

No significant differences were detected between the swelling properties of the mitochondria of the animals fed the oxidized safflower or menhaden oil diets and the corresponding fresh diets (Fig. 5). Apparently the products of the oxidation of the dietary fat did not promote or catalyze in vivo oxidation of the lipids of the liver mitochondria membranes. Likewise, the composition of the dietary fat was not altered so as to influence its susceptibility to oxidation in the tissues. The unsaturation of the dietary fat of the menhaden oil-supplemented diet was decreased significantly. Hence conceivably there could be a lesser effect on swelling with this diet than the corresponding fresh diet. That no effect was observed indicated further that the products of oxidation of the diet had little or no influence on the in vivo oxidation or destruction of vitamin E in the tissues. It was not determined whether any of the products of the oxidation of the dietary fat were incorporated into liver mitochondria or erythrocytes, under the conditions employed.

## DISCUSSION

Although vitamin E did not appear to have any effect on the toxic reactions of oxidized fats, it protected the membrane of erythrocytes and liver mitochondria against in vivo oxidation in the animals fed the diets containing oxidized fats. Evidence to this effect was obtained in the studies on the susceptibility of erythrocytes to hemolysis and swelling properties of liver mitochondria. Because the products of fat oxidation of the ingested oxidized fat did not have any apparent effect on the susceptibility of erythrocytes to hemolysis, nor on the swelling properties of liver mitochondria, it will be interesting to determine if any oxidation products are incorporated into these tissues. The fact that no differences in the susceptibility of red cells to hemolysis and that no TBA values were produced in hemolyzed red cells of animals fed the fat-free diet lacking in vitamin E demonstrated that the effect on hemolysis was due to in vivo oxidation and not to a deficiency of the vitamin per se. Likewise, because swelling was related to the degree of

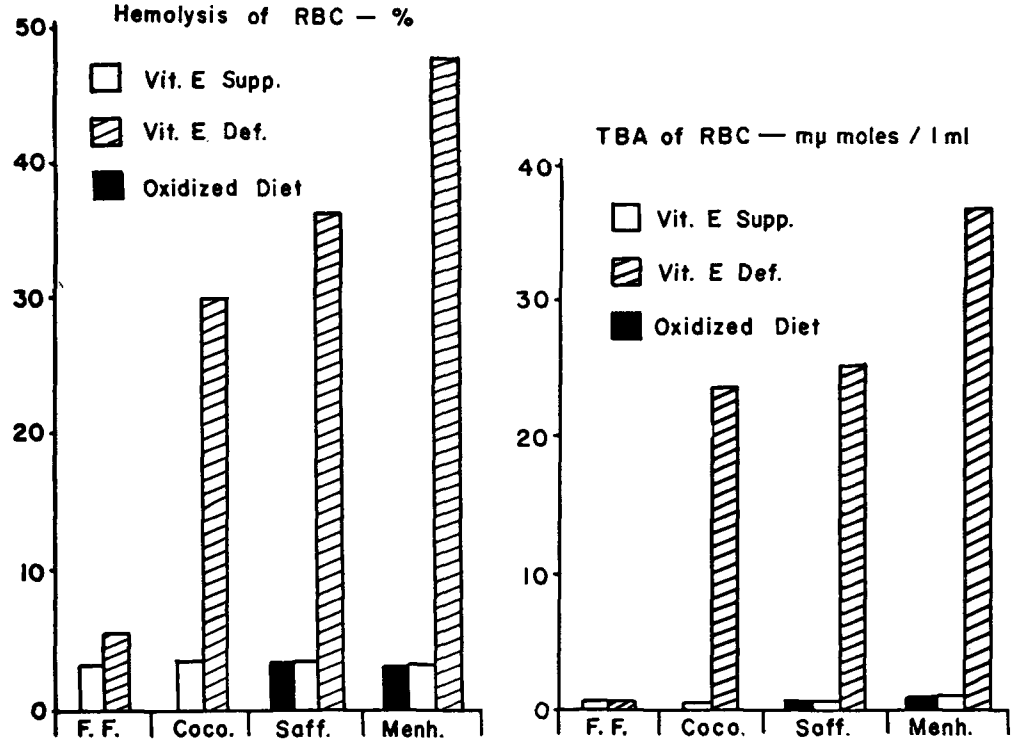


FIG. 4. Effect of oxidized and vitamin E deficient diets on the susceptibility of red blood cells of rats to dialuric acid-induced hemolysis. $p < 0.001$ Vitamin E deficient to vitamin E supplemented.

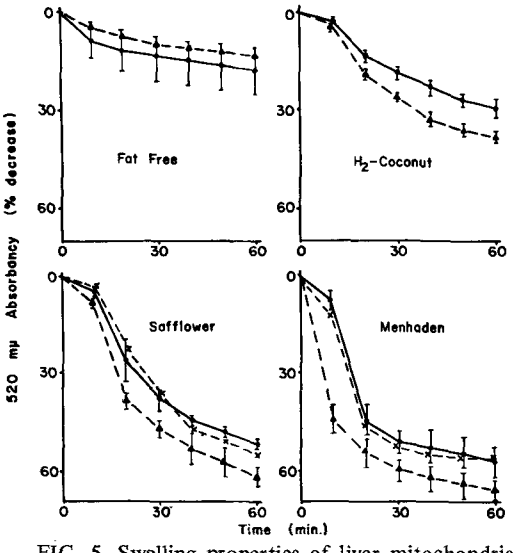


FIG. 5. Swelling properties of liver mitochondria of animals fed fresh (.——.), vitamin E deficient (△—△) and oxidized (x— x) diets. Swelling was determined in dilute solutions (10 μm/ml) of ferrous ammonium sulfate. Vertical bars = standard deviation of analysis on four to six animals. x—— ——x average of two animals. Swelling is significantly greater in the deficient groups except those fed the fat-free diet ($p < 0.002 - 0.001$).

unsaturation of the diet, it was apparent that increased swelling in these animals was due to in vivo oxidation.

Although the toxicity of oxidized fat apparently involves damage to cells, the reactions appeared to be different from in vivo oxidation that occurs in a vitamin E deficiency. The toxic reactions of oxidized fats appeared to be nonspecific insofar as their attack on the tissue is concerned. The mechanism of these reactions also appears to be different from those involved in in vivo oxidation. Ingestion of oxidized fat did not appear to promote in vivo oxidation because there was no apparent destruction of vitamin E in any of the tissues analyzed.

The accumulation of fat in hearts and fat pads may indicate that one of the effects of oxidized fats in the diet is the impairment of lipid transport or the mobilization of fat by an effect on lipoprotein synthesis. It has been demonstrated in in vitro studies (32) that lipoproteins are highly susceptible to alteration of both structure and function by reaction with oxidizing agents. Moreover α-tocopherol does not appear to inhibit the oxidation of sulfhydryl groups (33).

The absence of peroxides in the tissues of animals is in accord with reports (6,34-36) that

these compounds are decomposed during absorption from the gut. It also has been shown that fatty peroxides injected intravenously are readily catabolized (37) and that they serve as substrates for certain enzyme systems (33,38-40). Hence, if peroxides are formed in vivo from the ingestion of oxidized fat, they would be readily decomposed or detoxified (33). On the other hand, TBA-reacting substances including malonaldehyde have been detected in the tissues (16,29,41,42) and are generally taken as evidence of in vivo oxidation. The origin of these compounds in the animals fed oxidized fats is not known, but it is of interest that none are found in the liver. Thus these compounds are probably also metabolized. Hence, although some aspects of the fate of oxidized fat consumed in the diet are beginning to be unravelled, it is evident that much has yet to be learned of the reactions of these compounds in the tissues.

TABLE IV

TBA Values of Liver Mitochondria during Swelling Experiments in Ferrous Ammonium Sulfate Solution (mμmol/mg protein, average of four determinations)

| Time, min | Dietary fat | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Menhaden oil | | Safflower oil | | Hydrogenated coconut oil | | Fat-free | | | |
| | Vitamin E | No vitamin E | Vitamin E | No vitamin E | Vitamin E | No vitamin E | Vitamin E | No vitamin E | | |
| Zero | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| 30 | 6 | 16.4 | 4.6 | 6 | 0.08 | 0.21 | 0.15 | 0.07 | | |
| 60 | 8.3 | 23.4 | 10.3 | 15.5 | 0.15 | 0.31 | 0.19 | 0.12 | | |

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# Autoxidation of Fatty Acid Esters in the Presence of a Heavy Metal Catalyst "Salcomine" (Co[II]-bissalicylalethylenedi-imine): II. Formation of a Reaction Product from a Peroxy Radical and Salcomine

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## ABSTRACT

The formation of ionic Co(II)-bissalicylalaethylenedi-imine complexes of peroxy radicals of methyl esters of fatty acids was followed by means of UV spectra. Typical compounds were isolated and purified, and analyzed for Co content. IR spectra were determined. The mechanism of the autoxidation of fatty acid methyl esters in the presence of "Salcomine" (Co[II]-bissalicylalethylenedi-imine) has been outlined, and its effect is explained as an oxidative addition reaction, resulting from coordinative unsaturation.


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## INTRODUCTION

The kinetics of the autoxidation and decomposition of hydroperoxides of fatty acid methyl esters with salcomine were described earlier (1). Relative to the antioxidative effect of heavy metal catalysts under some conditions, it has been proposed by Heaton and Uri (2) that this phenomenon is due to a reaction in which the metal in its lower valence state reduces a free radical.

$$M^{n+} + RO_2\cdot \longrightarrow M^{n+1} + RO_2^-$$

In a nonpolar environment this reaction should, according to Ingold (3), be written,

$$MA_n + RO_2\cdot \longrightarrow ROOMA_n$$

where $MA_n$ is the reduced form of catalyst. It is usually difficult to isolate any reaction product of this kind because the amount is too small or the compound too unstable. The formation of a compound from salcomine and a free radical can be expected, and in this study its fate

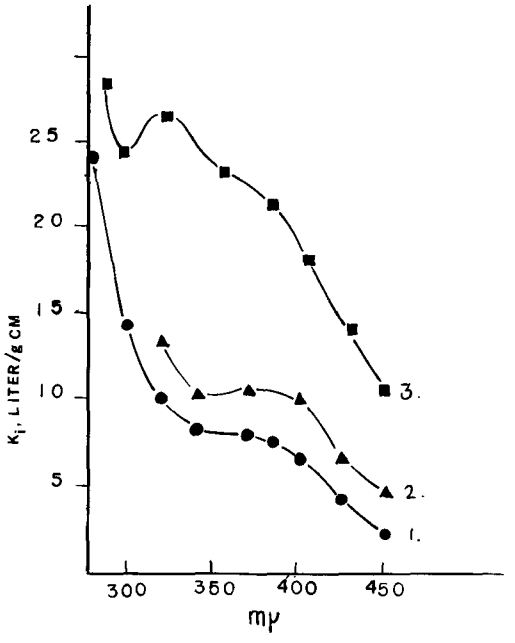



FIG. 1. UV absorption of purified salcomine-radical samples 1 (curve 1) and 2 (curve 2). Salcomine, curve 3. Solvent tetrahydrofurane.

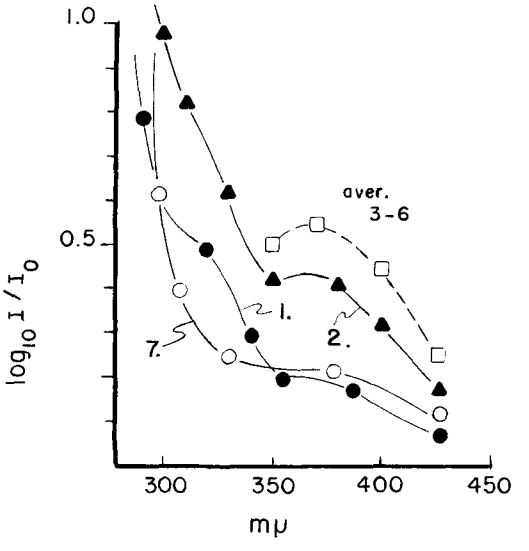



FIG. 2. UV absorption of the autoxidation mixture of methyl linoleate and salcomine. Solvent tetrahydrofurane. Dilution, 1-6 1:100; 7 1:1000.

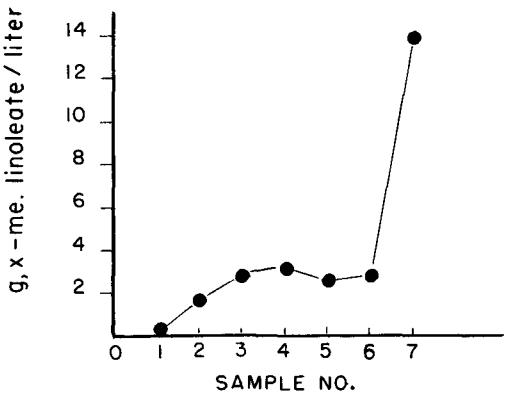



FIG. 3. The amount of the suggested salcomine-radical complex in the autoxidation samples 1-7.

during the autoxidation of fatty acid esters was followed.

## MATERIALS AND METHODS

The preparation of "Salcomine" (Co[II]-bis-salicylalethylenedi-imine), and fatty acid methyl esters has been described previously (1). Tetrahydrofurane, used as a solvent for UV measurements, was purified by distillation.

UV measurements were made with a recording Beckman instrument. The IR spectra were obtained with Perkin-Elmer model 237 B.

Isolation of the test material required special precautions. It was noticed, when fatty acid methyl esters were autoxidized in the presence of salcomine, that this compound underwent a series of color changes. The salcomine usually became darker, and simultaneously a greenish color was observed. If the quantity was small enough (ca. 10 mg/4 ml or less), it was observed that the reddish brown salcomine turned first into an almost clear solution, but after a moment a precipitate formed. If there was an excess of salcomine, it was difficult to observe this change because of unchanged salcomine in the reaction mixture.

Salcomine that had precipitated in autoxidation reactions, and that used in more detailed studies, originated in three different experiments. The samples were: (a) Transformed salcomine collected, after the end of the induction period had been reached, from a series of experiments with a mixture of fatty acid methyl esters (26.7% methyl linolenate, 11.7% methyl linoleate and 61.8% of methyl oleate, autoxidized at 55-90 C). (b) Transformed salcomine obtained from an autoxidation experiment with methyl linoleate (purity >99%) after the end of the induction period had been reached in a test conducted at 64 C. (c)

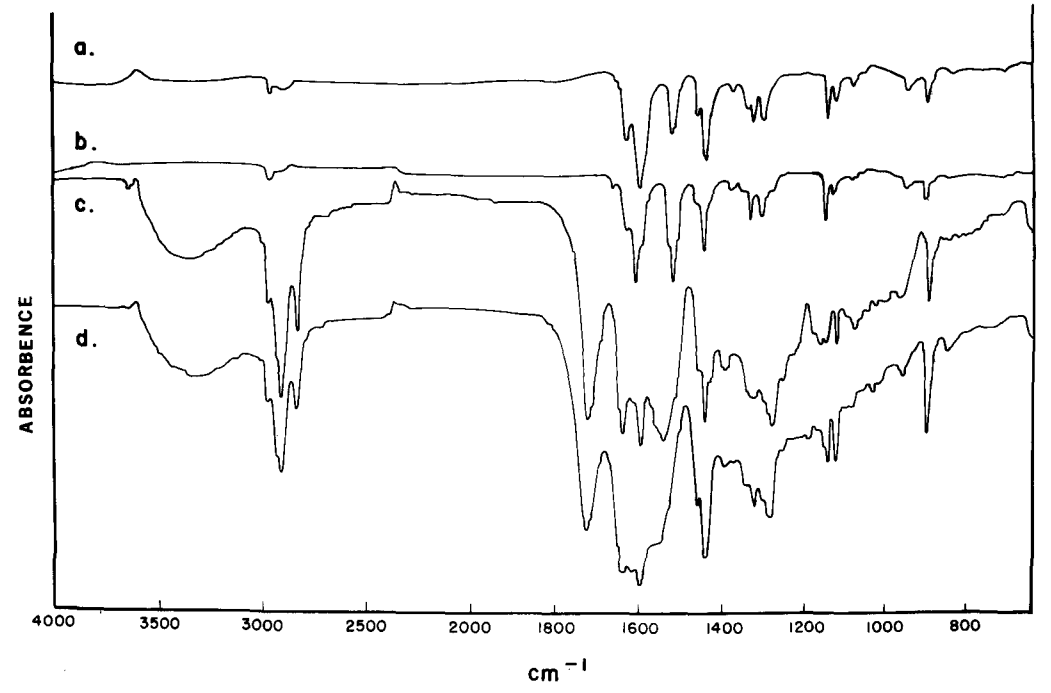



FIG. 4. IR spectra of: salcomine with $0_2$, a; salcomine without $0_2$, b; transformed salcomine, sample 1, c; transformed salcomine, sample 3, d. In $CS_2$.

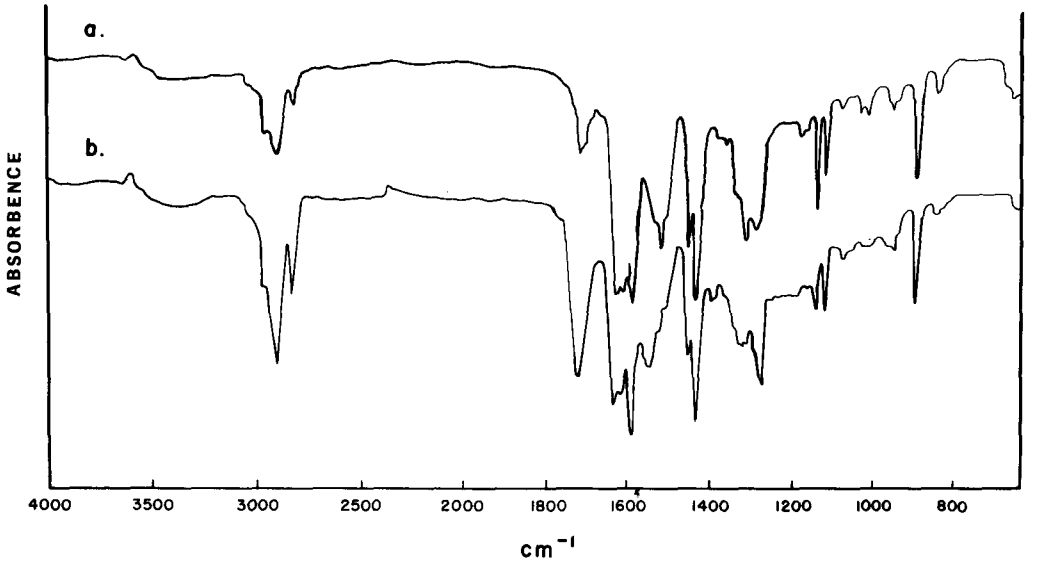



FIG. 5. IR spectra of transformed salcomine, sample 2. Partly reacted a, fully reacted b. In $CS_2$.

Transformed salcomine after the end of the induction period had been reached in a test with methyl oleate (purity >99%) at 80 C. To free the samples from accompanying methyl esters, benzene and Skelly F petroleum ether were employed as solvents.

Sample 1 (originally a few hundred milligrams) was washed four times with 30 ml benzene. After drying under vacuum it was a yellowish, finely grained material, and completely different from the original salcomine. When examined under a microscope, it was observed that the needle-like structure of salcomine had disappeared, and small yellowish flakes and lumps had formed. The compound had almost entirely lost its ability to bind oxygen (observed: 0.18 wt % in 24 hr, at room temperature, in air).

Sample 2 was washed first with petroleum ether. The recovered 62.3 mg was then treated with 3 ml benzene, the insoluble part was separated by centrifuging (22.7 mg), and from the benzene solution, 31.4 mg was precipitated by adding 20 ml petroleum ether. The substance was greenish in appearance. In solvent it was in the form of large flakes, but upon drying it agglomerated and lost its flake-like properties.

Sample 3 was also washed first with petroleum ether (20 ml). The precipitate, 18.2 mg, was then treated with 3 ml benzene, centrifuged, and again washed with 2.5 ml benzene and 10 ml petroleum ether. After this treatment 14.4 mg remained. The substance had a yellowish color and resembled the substance obtained from sample 1 in solubility and in general appearance.

Thus it was observed that the transformed sample 2 originating from autoxidized methyl linoleate was soluble in benzene (excluding a residue of 22.7 mg), but insoluble in petroleum ether. On the other hand transformed salcomine sample 3, recovered from the autoxidation of methyl oleate, was insoluble both in benzene and petroleum ether. Transformed salcomine a was obtained as benzene insoluble (also insoluble in petroleum ether) from a mixture of autoxidized methyl linolenate, methyl linoleate and methyl oleate.

Co analyses, made by incinerating the samples, gave values as follows: sample 1: Co 8.3%, N 3.18%; sample 2: Co 9.75%; sample 3: Co 9.8%.

## RESULTS AND DISCUSSION

The UV absorption spectrum of salcomine (free of oxygen) is indicated in Figure 1. Salcomine with oxygen had a similar spectrum. It resembles that of di-salicylaldehyde-propylenediamine-Ni(II)-chelate which has been described by Subbarao et al. (4). The spectra of samples 1 and 2 are also given in the Figure 1. These curves are similar, but different from that of salcomine.

The changes taking place in the region 280-440 mμ during the autoxidation of methyl linoleate catalyzed by salcomine at 64 C are seen in the Figure 2. Since methyl linoleate had no noticeable absorption in this region, the observations must be due to changes occurring

in the configuration of salcomine.

Assuming that the amount of dissolved salcomine remains constant (that is, the same as was in the solution in the beginning of the test when the reaction mixture [9.2 g me-linoleate and 151.2 mg salcomine] is stirred under vacuum for 1 hr at 64 C [Fig. 2, curve 1]), a curve for the amount of the suggested salcomine-free radical complex (which is formed in the reaction) in samples 1-7 is obtained (Fig. 3, computed at 386 m$\mu$). It is obvious that a new substance is being formed in increasing quantities. After the end of the induction period the amount produced is greatest (sample 7).

The IR spectra of purified samples of transformed salcomine a, b and c, described earlier, were also obtained. However salcomine has limited solubility in $CHCl_3$. The characteristic bands can be distinguished clearly (Fig. 4, a and b, salcomine). The presence of oxygen in salcomine is accompanied by the disappearance of the band at 1510 $cm^{-1}$.

A closer inspection of the spectra of transformed salcomine samples (Fig. 4, c and d; Fig. 5, a and b) reveals how the bands at 1590 $cm^{-1}$, 1460 $cm^{-1}$ and 880 $cm^{-1}$, which are typical for salcomine, are still present, giving reason to believe that the salcomine part of the molecule is unaltered. The peaks at 1725 $cm^{-1}$, and ca. 2900 $cm^{-1}$ belong to the ester carbonyl and the carbon chain, respectively. Besides the mentioned "old" characteristics in the spectra, new bands also become apparent at 1550 $cm^{-1}$ and 1275 $cm^{-1}$. Since metal chelates formed from acetyl acetone and $\beta$-diketones, which have at least partly ionic structure, absorb in these regions (5-7), it is possible that the observed bands of transformed salcomine result from a similar configuration.

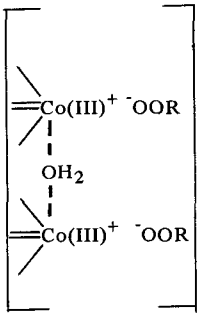
The aborption between 3100 and 3600 $cm^{-1}$, seen in the spectrum of the transformed salcomine, belongs to the peroxide group (8).

In the spectrum of the benzene insoluble residue, obtained in the purification of the sample 2, the bands at 1725 $cm^{-1}$ and 2900 $cm^{-1}$ are visible (Fig. 5, curve a) but weaker than in the spectra of other samples. Salcomine that has reacted only partially must be involved in this case.

In the light of their IR spectra all purified samples of salcomine transformed in autoxidation appear to be very similar. Also their Co contents are nearly the same. In its outward appearance and solubility, sample 1 is similar to sample 3, which was obtained from the autoxidation of methyl oleate.

Collectively, the data provide information about the configuration of the compounds formed in autoxidation from the fatty acid methyl esters and salcomine. In combination it has been shown that: (a) Cobalt content is 8.3-9.8% and nitrogen 3.18%. (b) The molecule has properties of salcomine. (c) The molecule has properties of the fatty acid ester. (d) The molecule does not absorb oxygen. (e) There is a peroxide group in the molecule. (f) There is an ionic type bond in the molecule.

The simplest compound that meets these requirements has the following configuration.




Salcomine-radical compound. (Bonding has been designated only for the Co part.)

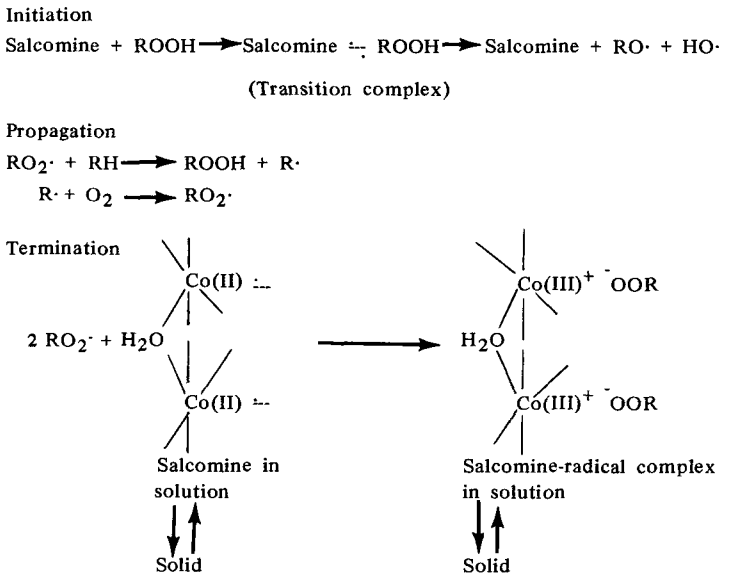
The calculated values for Co and N are: Co 8.91%, and N 4.23%. The observed values conform closely: Co 8.3-9.8%, and N 3.18%. The suggested configuration also meets other requirements quite closely, and it is evident that in the autoxidation of fatty acid esters in the presence of salcomine, this compound and peroxy-radicals form ionic complexes:

$ROO\cdot$ + Co(II) of salcomine $\rightarrow$

$ROO^-$ $^+$Co(III) of salcomine
(Stable complex)

The reactions of salcomine during autoxidation have counterparts in other chelate chemistry. Coordinative unsaturation leads to oxidative addition reactions as has been described by Collmann in a recent article (9). In the case of salcomine there is involved incomplete oxidative addition when the hydroperoxides decompose in its presence (1), and complete oxidative addition in the reaction salcomine + $ROO\cdot$. Because these reactions are associated with a half-filled coordination position, they belong to a rather special subclass of oxidative addition reactions.

The combined effects of salcomine upon the autoxidation of fatty acid methyl esters may be represented by the following scheme:




In addition, of course, there are the usual chain terminating reactions involving R· and $RO_2$·.

The aim of the studies performed with salcomine has been to correlate the theories of autoxidation, the chemical kinetics and the coordination chemistry involved in the autoxidation catalyzed by heavy metals (VIII group and Cu, Cr, Mn). It is important to take into consideration what transformations the catalyst may undergo during the reaction. Ions may become bound in forming coordination compounds (10-13), depending on many surrounding factors such as solvent, temperature and polarity. By using salcomine as a model compound, it was possible to study the reactions under more fixed conditions and elucidate some details.

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# SHORT COMMUNICATIONS

## A Simplified Gas Chromatographic Procedure for Analysis of Lipoxygenase Reaction Products

**ABSTRACT**

A simple and very sensitive technique was devised to analyze lipoxygenase reaction products by direct gas chromatography. Results showed that peanut lipoxygenase oxidizes linoleic acid at the C-13 position exclusively.

Lipoxygenase (E.C. 1.13.1.13) is the biological catalyst most often implicated as a major cause of deterioration in the quality of foods. The occurrence of lipoxygenase is widespread in many fruits and vegetables, including legumes and cereal grains (1-4). Comprehensive reviews on the chemistry and properties of the enzyme are available (3,4).

Lipoxygenase is highly specific for the peroxidation of unsaturated fatty acids containing the *cis*-1,*cis*-4-pentadiene configuration, such as linoleic, linolenic and arachidonic acids, their esters and triglycerides, but not the esters or triglycerides of oleic acid (5). The principal product is an optically active *cis-trans* conjugated hydroperoxide (6).

Several investigators have reported that lipoxygenase isolated from grains and oilseeds oxidizes linoleic acid, in varying degrees at either or both the C-9 and C-13 positions (7-12). The two isomeric hydroperoxides obtained were 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid and 13-hydroperoxyoctadecadienoic acid. The methods used in determining the site of attack by the enzyme previously involved several extensive procedures: extraction of the reaction products, separation by column or thin layer chromatography, analysis by gas liquid chromatography and sometimes mass spectroscopy.

Recently Dupuy et al. (13) developed a rapid, direct gas chromatographic (GC) technique for the analysis of volatile components in vegetable oils, which was subsequently modified for the analysis of volatiles from raw and roasted peanuts (14). Our report describes another application of this GC technique—the immediate analysis of enzyme reaction products. The aqueous reaction mixture is analyzed directly; no special sample preparation or derivatives are required. Also described is the ability to indicate with a high degree of probability the site of attack on linoleic acid by peanut lipoxygenase. This technique could be applicable to the study of other enzyme systems involving metabolism of fatty acids.

Peanut lipoxygenase was prepared as described previously (15). Linoleic acid and methyl linoleate were obtained from Applied Science Laboratories. The reaction medium consisted of 0.1 ml of substrate (linoleic acid or methyl linoleate), 0.04 ml of Tween-20 emulsifier, and 15 ml of 0.2 M phosphate buffer, pH 6.0. After sonicating for 10 sec, 5 ml of the enzyme preparation (ca. 18 mg protein/ml) was added, and the reaction was allowed to proceed at 25 C with stirring. After 1 hr a portion of the reaction medium was removed and analyzed by direct gas chromatography. Controls consisting of reaction media minus either substrate or enzyme were run under similar conditions. A MicroTek 2000 MF gas chromatography equipped with flame ionization detectors and a 1 ft x 1/4 in. OD stainless steel column packed with Porapak Q and a 2 ft x 1/4 in. OD stainless steel column packed with Porapak P were used in this work. The helium carrier gas flow rate was 60 ml/min. The electrometer attenuation was 10 x 1.

A 100 $\mu$l portion of the reaction mixture was added on top of a volatile-free glass wool plug in the liner of the injection port of the gas chromatograph as described previously (13). Additional glass wool was used to cover the sample. The glass liner with sample was inserted in the heated injection port. It was immediately secured in positon by tightening the retainer nut, and the injection port system was sealed with the septum and septum nut. The volatile components were rapidly eluted from the sample and swept onto the GC column as the carrier gas was forced to flow through the sample. The temperature of the column oven was programed from 60 to 200 C at 5 C/min after an initial hold period of 5 min. The initial temperature of the injection port was 100 C, but it gradually increased to ca. 110 C as the temperature of the column oven was increased

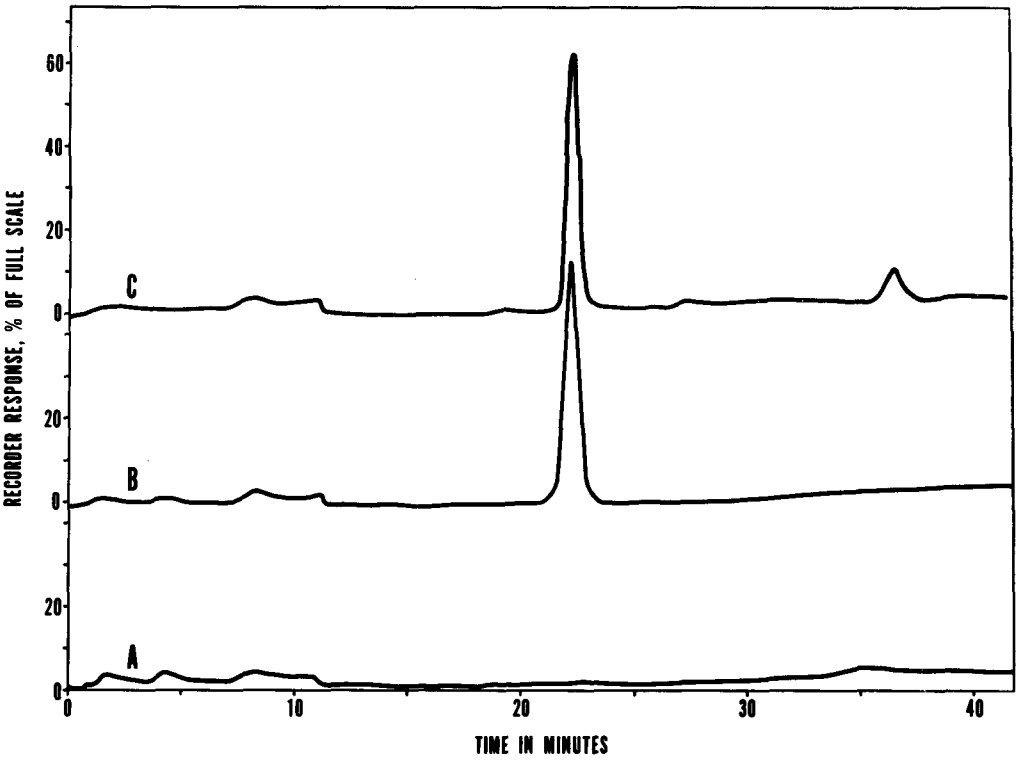


FIG. 1. Gas chromatograms of volatile reaction products produced from the oxidation of linoleic acid or its methyl ester by peanut lipoxygenase. Curve A is the profile obtained when the enzyme was omitted from the complete reaction mixture; curve B is the profile obtained with linoleic acid as substrate in the complete reaction mixture (enzyme, emulsified substrate, and buffer); curve C is the profile obtained when methyl linoleate was used as substrate in the complete reaction mixture.

to 200 C. After the chromatographic analysis was completed, the liner with the spent sample was removed from the injection port.

Representative chromatograms with linoleic acid and its methyl ester as substrates obtained on the Porapak P column are shown in Figure 1. When the enzyme was omitted from the complete reaction medium, no volatile components were eluted as shown in curve A. When linoleic acid was used as the substrate in the complete reaction medium, only one volatile component was eluted as shown in curve B. With methyl linoleate as substrate, two volatile components were eluted as shown in curve C.

The volatile component eluted from the reaction mixture containing linoleic acid as substrate had the same retention time as hexanal on the Porapak P and Porapak Q columns. The first volatile component eluted from the methyl linoleate reaction mixture had the same retention time as hexanal, and the second had the same retention time as methyl dodecanoate. No cleavage product was observed when oleic acid was used as substrate, confirming that these end products are the result of a lipoxygenase-catalyzed reaction (4).

Since only one component, hexanal, was obtained as the principal secondary reaction product formed by enzymatic oxidation of linoleic acid and only two components (hexanal and the C-12 methyl ester residue) from methyl linoleate oxidation, we conclude that peanut lipoxygenase attacks the C-13 position and not the C-9 position. This would be similar to the findings of Dolev et al. (7) who reported that soybean lipoxygenase attacks only the C-13 position of linoleic acid, and to Christopher and Axelrod (10), who isolated two isozymes from soybean, only one of which attacked the C-13 position. The other isozyme attacked both positions equally. Peanut lipoxygenase differs from that in flaxseed, which attacks both positions in ratios of 80% at the C-13 position and 20% at the C-9 positions (8), and from that in corn which attacks predominately (83%) the C-9 position and to a lesser degree (17%), the

C-13 (9).

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# Simultaneous Partial Purification of Sterol Ester Hydrolase and Lipase from Rabbit Pancreas

**ABSTRACT**

Sterol ester hydrolase and lipase from rabbit pancreatic aqueous extracts were partially purified simultaneously by a rapid method consisting of an initial 50% ammonium sulfate precipitation, followed by chromatography with sodium taurocholate on a 10% agarose gel column (0.5 million exclusion limit), followed by precipitation of both enzymes with heparin and $MnCl_2$. Sterol ester hydrolase was 39-fold purified and lipase 42-fold purified, as compared to the initial extract. The enzymes could be partially separated by a DEAE ion exchange procedure, and exhibited different quantitative responses to several activators and inhibitors.

In the course of purification of a sterol ester hydrolase (E.C.3.1.1.13) from rabbit pancreas, it was noted that lipase activity (glyceryl ester hydrolase, E.C.3.1.1.3) closely paralleled the activity of the former enzyme, particularly when nondelipidated preparations were used. Lipase alone has been used in the purification of several enzymes to free them from bound lipid material (1-3), and for this purpose it may be particularly useful to have available a combined preparation of lipase and sterol ester hydrolase. The procedure reported is a rapid method for the simultaneous partial purification of these enzymes.

Whole portions of pancreas obtained from New Zealand White rabbits were homogenized in a Sorvall Omnimixer with 10 times their volume of 0.05 M Tris buffer, pH 9.0 at 0 C.

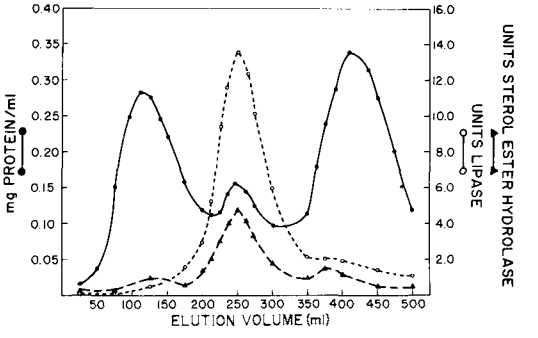


FIG. 1. Purification of sterol ester hydrolase and lipase by agarose gel column chromatography.

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# Simultaneous Partial Purification of Sterol Ester Hydrolase and Lipase from Rabbit Pancreas

**ABSTRACT**

Sterol ester hydrolase and lipase from rabbit pancreatic aqueous extracts were partially purified simultaneously by a rapid method consisting of an initial 50% ammonium sulfate precipitation, followed by chromatography with sodium taurocholate on a 10% agarose gel column (0.5 million exclusion limit), followed by precipitation of both enzymes with heparin and $MnCl_2$. Sterol ester hydrolase was 39-fold purified and lipase 42-fold purified, as compared to the initial extract. The enzymes could be partially separated by a DEAE ion exchange procedure, and exhibited different quantitative responses to several activators and inhibitors.

In the course of purification of a sterol ester hydrolase (E.C.3.1.1.13) from rabbit pancreas, it was noted that lipase activity (glyceryl ester hydrolase, E.C.3.1.1.3) closely paralleled the activity of the former enzyme, particularly when nondelipidated preparations were used. Lipase alone has been used in the purification of several enzymes to free them from bound lipid material (1-3), and for this purpose it may be particularly useful to have available a combined preparation of lipase and sterol ester hydrolase. The procedure reported is a rapid method for the simultaneous partial purification of these enzymes.

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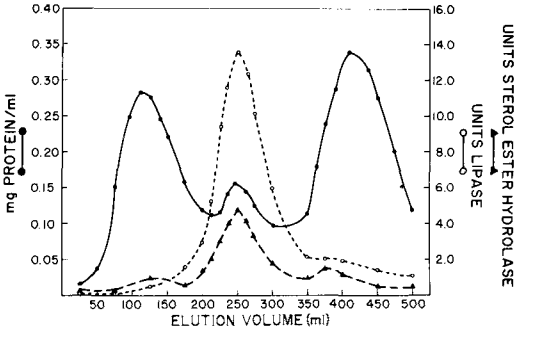


FIG. 1. Purification of sterol ester hydrolase and lipase by agarose gel column chromatography.

TABLE I

Purification of Rabbit Pancreatic Sterol Ester Hydrolase and Lipase

| Step | Protein, mg | Sterol ester hydrolase | | | | Lipase | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Total activity units[a] | Yield, % | Specific activity units/mg protein | Fold purification | Total activity units[b] | Yield, % | Specific activity units/mg protein | Fold purification |
| Initial extract | 139 | 31.0 | 100 | 0.22 | -- | 87.3 | 100 | 0.63 | -- |
| Ammonium sulfate, ppt | 74 | 28.3 | 91 | 0.38 | 1.7 | 76.6 | 88 | 1.04 | 1.7 |
| Agarose 0.5 m chromatography | 4.8 | 19.2 | 62 | 4.00 | 18.2 | 59.0 | 68 | 12.3 | 19.5 |
| Heparin-$MnCl_2$, ppt | 2.1 | 18.0 | 58 | 8.57 | 39.0 | 55.9 | 64 | 26.6 | 42.2 |

[a]Units of sterol ester hydrolase are expressed as the number of μmoles of free fatty acid released per hour from the cholesteryl-4-$^{14}$C oleate substrate under the assay conditions described.

[b]Units of lipase activity are the number of μmoles of free fatty acid released per hour from the glyceryl-tri-1-$^{14}$C-palmitate under the assay conditions described.

All subsequent procedures were done on ice or in a cold room at 0-4 C. The homogenate was centrifuged at 13,000 rpm (27,300 g) for 30 min and the fat layer removed with a Pasteur pipet. The precipitate was rehomogenized in 10 times its volume of the same Tris buffer and then recentrifuged. The combined supernatants were frozen for 18 hr or more, thawed and then recentrifuged as above to remove a small precipitate formed on freezing. This supernatant was then made to 50% saturation with solid ammonium sulfate, stirred with a magnetic bar for 15 min and centrifuged at 27,300 g for 20 min.

A glass column 2.5 x 100 cm was packed to 80 cm with Bio Gel A (10% Agarose Gel, 100-200 mesh, 0.5 million exclusion limit); Vo of this column as determined by Blue Dextran was 140 ml. The ammonium sulfate precipitate dissolved in the eluting mixture was added to the column, and the proteins eluted with 0.1 M Tris, pH 9.0 containing 0.02 M NaCl and 10 mg/ml sodium taurocholate. The enzyme-containing fractions were passed through an ultrafiltration membrane (Amincon, PM 30), and washed 2 times with equal volumes of 0.1 M NaCl to remove the taurocholate. The residue, concentrated to one-fifth its original volume, was made to 0.2% with heparin (Sigma Grade I) and to 0.03 M with $MnCl_2$. This was then mixed 15 min on ice and centrifuged at 27,300 x g for 20 min. The precipitate was suspended in 0.1 M NaCl, passed through the ultrafiltration membrane, washed 2 times with equal volumes of 0.1 M NaCl, and the residue suspended in 0.1 M NaCl containing 10 mg/ml sodium taurocholate (this preparation remained turbid).

Sterol ester hydrolytic activity was assayed by incubation of 1.0 ml aliquots of the fractions with 1.0 ml of 0.2 M Tris buffer, pH 6.8 containing 10 mg/ml sodium taurocholate and 0.1 ml cholesteryl-4-$^{14}$C oleate in acetone (Amersham-Searle, 0.05 μc, 1.0 μmoles/0.1 ml); duration of the incubations was 5-30 min at 37 C. Similar aliquots of the fractions preheated at 90 C for 15 min were incubated as controls. The reactions were stopped by addition of 10 ml ethanol-acetone 1:1. The mixtures were then shaken, centrifuged 10 min at 2500 rpm and 1.0 ml of 1% digitonin in 50% ethanol added to duplicate aliquots. After standing overnight the cholesteryl digitonides were separated by centrifugation, washed with ether, dissolved in 1.0 ml methanol and the radioactivity of the total and free cholesterol fractions determined by liquid scintillation counting in a 2,5-diphenyloxazole-1,4-bis-(5-phenyloxazolyl-2)benzene-toluene scintillation

solution. Quenching was monitored by subsequent addition of internal standards.

Lipase was assayed by the radioactivity method of Kaplan (4). Each aliquot contained 1.0 μmole, 0.03 μc of glyceryl-tri-$^{14}$C-palmitate (Amersham-Searle) and was incubated for 5-30 min at 37 C. Lipase was also assayed titrimetrically by the method of Marchis-Mouren et al. (5), after incubation of the enzyme preparations with 7.5 ml of a purified 5% olive oil emulsion substrate at 25 C for intervals of 5-30 min.

Protein was analyzed by the method of Lowry et al. (6) as adapted for the autoanalyzer.

Results at each step of the purification sequence are indicated in Table I, and a typical agarose gel chromatography result is shown in Figure 1. The sterol ester hydrolase and lipase activities both appeared immediately following the exclusion volume. Addition of the lipoprotein precipitants heparin and $MnCl_2$ resulted in precipitation of virtually all of both enzyme activities. The activities of sterol ester hydrolase and lipase paralleled each other at each step of the purification sequence. Purification at the end of the heparin-$MnCl_2$ stage was ca. 39-fold for sterol ester hydrolase and 42-fold for lipase.

The relative activities of pancreatic sterol ester hydrolase vs. lipase cannot be accurately determined in these experiments due to the different physical properties and degrees of dispersion of substrates used in the assays. The amount of triglyceride substrate used in the radioactive lipase assay was such as to approximate the amount of cholesteryl ester substrate used in the sterol ester hydrolase assay. Assay of the lipase preparations by a titrimetric method, using a much higher concentration of an emulsified triolein substrate than the triglyceride in the radioactive method, gave activities up to 300 times higher than the latter method; this was probably due to the greater interfacial area of substrate available for interaction with the enzyme. It was not possible to prepare a comparable concentration of emulsified cholesteryl ester substrate.

Rechromatography of the column fractions containing both enzymes on a Sephadex G-200 column and on another Bio Gel A 0.5 m column, using in both cases the same elution buffer as before but without added sodium taurocholate, resulted in the enzyme activities appearing predominantly in the exclusion volumes. The latter fractions appeared turbid, and it is possible that removal of the taurocholate resulted in the formation of insoluble aggregates of the lipoprotein enzymes.

Chromatography of the active fraction on a Sephadex G-200 column with taurocholate added to the elution buffer resulted in the appearance of both enzymes in the exclusion volume; the latter pattern together with their elution characteristics on the Bio-Gel A column described above suggests that the enzymes in the presence of taurocholate had molecular weights between 200,000-500,000.

Lipase could be partially separated from sterol ester hydrolase activity by a batch DEAE ion exchange procedure. The ammonium sulfate precipitate after dialysis was dissolved in 0.005 M Tris pH 8.0 containing 2.0 mg/ml sodium taurocholate. Diethylaminoethyl cellulose (Bio-Rad Cellex D, standard capacity) was added to the above preparation in a ratio of 0.1 gm/ml, mixed for 1 hr on ice and then centrifuged at 27,300 x g for 30 min. Mixing with 0.2 M NaCl in the Tris-taurocholate solution for 1 hr and recentrifuging resulted in the elution of 12% of the sterol ester hydrolase activity and 59% of the lipase. Subsequent mixing with 0.4 M NaCl in the same buffer resulted in elution of 76% of the sterol ester hydrolase and 15% of the lipase.

Activation and inhibition studies also revealed different properties of the two enzymes. Addition of 0.001 M $NH_4Cl$ resulted in stimulation of the sterol ester hydrolase activity to 160% and lipase to 108%. $ZnCl_2$ at a concentration of 0.001 M inhibited sterol ester hydrolase to 52% of the original activity and lipase to 80%; *p*-chloromercuribenzoate, 0.001 M, inhibited sterol ester hydrolase to 73% and lipase to 54%; 1.0% Lubrol PX (ethylene oxide condensate of fatty alcohols) inhibited sterol ester hydrolase to 96% and lipase to 5% of its original activity.

Rat pancreatic cholesterol esterase interacts directly with cholic acid to form a hexamer of 400,000 mol wt (7), which is in the same approximate range as rabbit sterol ester hydrolase in solution with sodium taurocholate in the present study. However the rat enzyme dissociated into units of 65-69,000 mol wt in the absence of cholic acid, whereas without taurocholate the rabbit enzyme of the present study appeared to aggregate into larger particles than with this bile salt. The rat cholesterol esterase preparation had been delipidated by acetone extraction early in the purification procedure, and the greater amount of lipid associated with the rabbit enzyme may be the reason for its different properties. Studies of pig pancreatic lipases and cholesterol esterases have shown that nondelipidated preparations of both types of enzymes show molecular weights >800,000; after delipidation these enzymes have molecular weights in the range of 15-40,000 (8-10).

Lipase and sterol ester hydrolase are similar in that both are able to act on emulsified or micellar substrates, both require bile salts as essential cofactors, and in the presence of lipids both apparently form high molecular weight lipoprotein complexes that enable their parallel purification by the sequence described above.

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