Composition of Uropygial Gland Secretions of Birds of Prey

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ABSTRACT

The chemical composition of the uropygial gland secretion of five species of birds of prey was investigated by gas liquid chromatography-mass spectroscopy technique, and the results are discussed from the chemotaxonomical point of view. The secretion is a complex mixture of monoester waxes, the fatty acids of which are mainly dimethyl-branched, with the first substituent in 2 position and the other near the methyl end of the molecule. Mono-, trimethyl-, and unbranched fatty acids also are observed. The wax alcohols are mainly mono- and dimethyl-substituted. Unbranched alcohols and traces of trimethyl-substituted alcohols also were detected. Chemotaxonomically, the birds of prey differ from all orders hitherto investigated. The degree of substitution increases from the *Falconidae* to the *Accipitridae*.

INTRODUCTION

During the last years, several investigations have shown that the chemical compositions of the uropygial gland secretion can be correlated to the bird's position in the natural system. This means that closely related birds possess qualitatively similar compositions in contrast to birds of different orders or even families. Thus, a chemotaxonomy of birds has begun to develop (1-3).

To determine whether there are relationships between the order *Strigiformes* (owls), which we have just investigated (4), and the *Falconiformes* (birds of prey), as discussed by various authors (5,6), the uropygial gland secretion of the latter was examined, and the results are presented in this paper. This attempt was started with five members of two families of birds of prey: kestrel (*Falco tinnunculus*), merlin (*Falco columbarius*), sparrow hawk (*Accipiter nisus*), goshawk (*Accipiter gentilis*), and buzzard (*Buteo buteo*).

PROCEDURES

The freshly extirpated uropygial glands from adult animals were preserved with acetone and frozen until the material was investigated. After evaporation of the solvent, the greasy material was distributed between chloroform/methanol/water (2:1:1, v/v/v, 40ml). The lower layer contained the crude lipids which were fractionated by column chromatography (SiO_2 Woelm, 9.1% water content), as described previously (7), and this procedure yielded wax esters, triglycerides, and lesser amounts of more polar compounds which were disregarded. Methanolysis of the waxes by 5% methanolic HCl yielded fatty acid methyl esters and free alcohols which were separated by column chromatography. The alcohols were oxidized by CrO_3 in cyclohexane/tertiary-butanol/acetic acid (8), and the resulting fatty acids were esterified. The structures of both fractions of methyl esters were elucidated by gas liquid chromatography-mass spectroscopy (GLC-MS) combination technique with a GNOM-MAT 111 mass spectrometer using a 9 m glass column with 3% OV 101 impregnation on Gas Chrom Q, as reported previously (9).

RESULTS

Although the triglycerides show the typical fatty acid pattern which occurs in most depot fats (mainly 16:0, 16:1, 18:0, 18:1, 18:2, and small amounts of 18:3), the composition of the wax fatty acids and alcohols is very complex, as can be seen from Tables I and II.

The mass spectroscopical identification of mono-, di-, tri-, and tetramethyl-substituted fatty acid methyl esters of the type occurring in the preen secretion of the birds investigated already is discussed elsewhere (10-13) or is analogous to those spectra. In Table III, the main characteristic fragments of the mass spectra which are useful for the identification of the compounds of Tables I and II are summarized.

These secretions are characterized by the occurrence of fatty acids with one methyl-branch in 2 position and a second or third branch near the other end of the molecule. Though 2-methyl- or 2,x-dimethyl- and 2,x,y-trimethyl-branched fatty acids are common as constituents of uropygial gland secretions, the relatively high content of 2,ω-1- or 2, (ω-n)-dimethyl-substituted compounds (with n = 2-4) is remarkable and may be taken as a chemotaxonomic parameter. The chain length of the fatty acids is unusually high and may be as large as C_{27}. We found a stepwise increase of the degree of substitution and an elongation of the carbon

TABLE I

Fatty Acid Composition of Uropygial Gland Waxes from Five Species of Birds of Prey[a]

Fatty acid	*Falco tinnunculus*	*Falco columbarius*	*Accipiter nisus*	*Accipiter gentilis*	*Buteo buteo*
Unbranched (total)	(0.9)	(5.3)	(---)	(1.6)	(0.5)
n-C_{13}	---	0.1	---	---	---
n-C_{14}	---	0.2	---	Trace	---
n-C_{15}	---	0.1	---	---	---
n-C_{16}	0.2	2.1	---	0.4	0.2
n-C_{18}	0.7	2.8	---	0.9	0.3
n-C_{20}	---	---	---	Trace	---
n-C_{22}	---	---	---	0.3	---
Monomethyl-substituted (total)	(37.1)	(30.8)	(20.7)	(14.3)	(0.9)
2-Methyl-substituted (total)	(37.1)	(29.1)	(20.7)	(12.7)	(0.8)
2-C_{12}	0.1	0.1	---	---	---
2-C_{13}	---	Trace	---	---	---
2-C_{14}	0.2	0.1	---	---	---
2-C_{15}	---	---	---	Trace	---
2-C_{16}	1.6	0.3	---	---	Trace
2-C_{17}	---	---	---	Trace	0.2
2-C_{18}	1.4	0.4	---	---	Trace
2-C_{19}	0.4	---	---	---	---
2-C_{20}	18.6	14.4	3.0	0.4	0.1
2-C_{21}	7.9	7.9	1.1	---	---
2-C_{22}	6.9	5.9	11.1	0.9	0.4
2-C_{23}	---	---	1.1	---	---
2-C_{24}	---	---	3.7	2.9	0.1
2-C_{25}	---	---	0.7	8.1	---
2-C_{26}	---	---	---	0.1	---
2-C_{27}	---	---	---	0.3	---
4-Methyl-substituted (total)	(---)	(1.2)	(---)	(0.7)	(---)
4-C_{12}	---	---	---	Trace	---
4-C_{14}	---	0.2	---	0.2	---
4-C_{15}	---	0.2	---	0.1	---
4-C_{16}	---	0.6	---	---	---
4-C_{17}	---	---	---	0.2	---
4-C_{18}	---	0.2	---	0.1	---
4-C_{20}	---	---	---	0.1	---
Other monomethyl-substituted (total)	(---)	(0.5)	(---)	(0.9)	(0.1)
6-C_{12}	---	---	---	Trace	---
6-C_{16}	---	---	---	0.7	---
12-C_{16}	---	0.2	---	---	0.1
6-C_{17}	---	---	---	Trace	Trace
6-C_{18}	---	---	---	Trace	---
12-C_{18}	---	0.1	---	---	---
16-C_{18}	---	0.2	---	---	---
6-C_{19}	---	---	---	0.2	---
Dimethyl-substituted (total)	(45.0)	(47.7)	(70.7)	(56.7)	(40.7)
2,x-Dimethyl-substituted (total)	(45.0)	(47.4)	(70.7)	(55.6)	(40.7)
2,6-/2,8-C_{12}	0.1	---	---	---	---

2,10-C_{12}	0.2	—	—	—	—
2,10-C_{14}	0.4	—	—	—	—
2,12-C_{14}	0.1	—	—	—	—
2,6-/2,8-/2,10-C_{15}	0.4	0.2	—	—	—
2,12-C_{15}	0.3	—	—	—	—
2,6-/2,8-/2,10-C_{16}	2.1	0.6	—	Trace	Trace
2,12-C_{16}	2.3	0.8	—	—	Trace
2,14-C_{16}	2.0	0.8	—	0.2	0.3
2,6-/2,8-/2,10-C_{17}	1.3	2.3	—	0.4	0.2
2,6-/2,8-/2,10-C_{18}	0.9	0.6	—	0.4	0.2
2,14-C_{18}	1.6	0.5	—	—	0.1
2,16-C_{18}	1.0	0.5	—	—	0.3
2,6-/2,8-/2,10-C_{19}	0.5	—	—	0.6	—
2,12-C_{19}	0.4	—	—	—	—
2,14-C_{19}	0.5	1.4	—	—	—
2,16-C_{19}	0.8	1.0	—	—	—
2,6-/2,8-/2,10-C_{20}	5.4	5.3	—	—	—
2,14-C_{20}	4.0	5.0	3.1	1.3	0.5
2,16-C_{20}	4.7	5.7	4.0	1.0	0.9
2,18-C_{20}	5.6	10.3	11.1	2.3	1.7
2,6-/2,8-/2,10-C_{21}	8.2	9.9	—	—	—
2,14-C_{21}	1.2	0.6	—	0.2	—
2,16-C_{21}	0.1	0.5	1.4	1.1	0.7
2,18-C_{21}	0.9	1.4	1.7	0.6	—
2,10-/2. 12-C_{22}	—	—	—	1.0	0.5
2,14-C_{22}	—	—	5.4	1.0	0.5
2,16-C_{22}	—	—	—	1.0	1.3
2,18-C_{22}	—	—	13.8	3.3	1.4
2,20-C_{22}	—	—	20.3	6.5	4.1
2,14-C_{23}	—	—	—	0.7	—
2,16-C_{23}	—	—	—	1.4	—
2,18-C_{23}	—	—	0.7	1.0	1.3
2,20-C_{23}	—	—	0.8	0.2	0.1
2,10-C_{24}	—	—	—	3.9	—
2,14-C_{24}	—	—	—	0.3	2.0
2,16-C_{24}	—	—	1.5	0.4	—
2,18-C_{24}	—	—	—	Trace	—
2,20-C_{24}	—	—	2.1	4.7	4.0
2,22-C_{24}	—	—	4.8	9.8	5.7
2,10-/2,14-C_{25}	—	—	—	1.2	—
2,16-C_{25}	—	—	—	1.4	—
2,18-C_{25}	—	—	—	0.2	—
2,20-C_{25}	—	—	—	1.1	1.0
2,22-C_{25}	—	—	—	0.4	1.7
2,24-C_{25}	—	—	—	0.7	—
2,14-/2,16-C_{26}	—	—	—	0.5	2.6
2,18-C_{26}	—	—	—	0.3	—
2,20-C_{26}	—	—	—	2.7	—
2,22-C_{26}	—	—	—	1.5	2.7
2,24-C_{26}	—	—	—	2.3	3.8
2,22-C_{27}	—	—	—	—	2.0
2,24-C_{27}	—	—	—	—	1.1
Other dimethyl-substituted (total)	(—)	(0.3)	(—)	(1.1)	(—)
4,8-C_{12}	—	—	—	Trace	—
4,10-C_{12}	—	—	—	0.1	—

4,8-C_{13}	—	—	—	Trace	—
4,6-C_{14}	—	—	—	Trace	—
4,10-C_{14}	—	0.1	—	0.5	—
4,8-C_{15}	—	—	—	Trace	—
4,6-C_{16}	—	0.2	—	0.3	—
10,14-C_{16}	—	—	—	0.1	—
6,14-C_{18}	—	—	—	0.1	—
10,16-C_{18}	—	—	—	Trace	—
Trimethyl-substituted (total)	(15.7)	(16.2)	(8.6)	(23.8)	(49.9)
2,6,10-C_{12}	0.1	—	—	—	—
2,6,8-/2,6,10-C_{14}	0.4	0.4	—	—	—
2,6,12-C_{14}	0.7	—	—	—	—
2,6,10-C_{15}	Trace	—	—	—	—
2,6,10-C_{16}	0.3	—	—	—	—
2,6,12-C_{16}	1.8	0.7	—	—	—
2,6,14-C_{16}	2.2	1.1	—	—	—
2,6,10-C_{17}	0.3	—	—	—	—
2,6,12-C_{18}	0.2	0.5	—	—	—
2,6,14-C_{18}	1.8	0.6	—	—	—
2,6,16-C_{18}	2.0	1.0	—	—	—
2,10,14-C_{18}	—	0.6	—	—	0.2
2,14,16-C_{18}	—	—	—	—	0.6
2,10,16-C_{18}	—	0.9	—	—	—
2,6,14-C_{19}	0.3	1.3	—	—	—
2,6,16-C_{19}	5.6	—	—	0.1	—
2,10,14-C_{19}	—	—	—	0.1	—
2,14,16-C_{19}	—	—	—	—	0.5
2,6,16-/2,8,16-C_{20}	—	6.7	—	1.0	—
2,14,18-C_{20}	—	—	3.7	3.5	3.0
2,10,14-/2,10,16-C_{20}	—	—	—	—	1.1
2,14,18-C_{21}	—	—	—	—	1.4
2,6,12-C_{21}	—	2.4	—	—	—
2,6,16-C_{21}	—	—	—	0.1	—
2,14,18-C_{21}	—	—	—	0.7	—
2,16,18-C_{21}	—	—	—	0.2	—
2,10,18-/2,14,18-C_{22}	—	—	—	2.8	4.0
2,10,20-/2,14,16-/2,14,20-/2,16,20-C_{22}	—	—	4.9	7.7	6.7
2,14,20-/2,16,20-C_{23}	—	—	—	1.1	2.6
2,14,20-/2,16,20-/2,18,20-C_{24}	—	—	—	3.2	4.2
2,18,22-C_{24}	—	—	—	—	7.8
2,14,18-C_{25}	—	—	—	—	3.0
2,14,22-C_{25}	—	—	—	3.1	—
2,16,22-C_{25}	—	—	—	0.2	—
2,18,22-C_{25}	—	—	—	—	0.8
2,14,18-C_{26}	—	—	—	—	3.5
2,14,20-/2.20,24-C_{26}	—	—	—	—	7.6
2,14,22-C_{26}	—	—	—	—	2.9
Tetramethyl-substituted (total)	(—)	(—)	(—)	(—)	(3.9)
2,10,12,14-C_{20}	—	—	—	—	1.1
2,10,14,16-C_{22}	—	—	—	—	1.7
2,14,18,22-C_{24}	—	—	—	—	1.1
Unidentified	(1.3)	(—)	(—)	(3.6)	(4.1)

[a]Determined as methyl esters in area percent of the gas liquid chromatographic peak. The number before the C indicates the branch position, the indices the chain length, e.g. 2,18-C_{20} means 2,18-dimethyl-eicosanoic acid.

TABLE II

Alcohol Composition of Uropygial Gland Wax from Five Species of Birds of Prey[a]

Alcohol	*Falco tinnunculus*	*Falco columbarius*	*Accipiter nisus*	*Accipiter gentilis*	*Buteo buteo*
Unbranched (total)	(32.1)	(10.3)	(13.8)	(3.4)	(1.0)
n-C_{12}	---	0.1	---	0.1	---
n-C_{14}	---	0.2	0.2	0.3	0.1
n-C_{15}	---	---	0.1	0.1	---
n-C_{16}	0.6	0.6	1.1	1.6	0.3
n-C_{17}	1.6	0.2	---	Trace	---
n-C_{18}	21.1	3.0	1.3	1.3	0.3
n-C_{19}	4.1	1.1	0.1	---	---
n-C_{20}	4.7	5.1	2.6	---	0.1
n-C_{21}	---	---	0.8	---	---
n-C_{22}	---	---	6.2	---	0.2
n-C_{24}	---	---	1.4	---	---
Monomethyl-substituted (total) (and ethyl-substituted)	(56.6)	(68.5)	(65.1)	(41.3)	(41.6)
2-ethyl-C_{12}	---	---	---	0.2	0.1
12-C_{14}	---	---	Trace	---	---
2-C_{16}	---	---	---	0.6	---
12-C_{16}	1.0	0.6	0.1	0.3	---
14-C_{16}	0.2	---	---	0.9	---
10-/12-C_{17}	2.8	3.1	---	---	---
14-C_{17}	0.7	---	---	---	---
15-C_{17}	1.2	0.8	---	---	---
10-/12-C_{18}	6.0	5.8	---	---	---
14-C_{18}	7.9	3.8	0.3	0.7	0.2
16-C_{18}	28.7	23.8	0.3	0.6	0.3
2-/6-/10-C_{19}	---	0.8	---	---	---
12-C_{19}	0.6	1.6	---	---	---
14-C_{19}	---	0.4	---	---	0.1
16-C_{19}	5.4	11.7	0.4	---	Trace
18-C_{19}	---	---	0.4	---	---
2-C_{20}	---	0.5	---	---	---
8-/10-/12-C_{20}	1.5	---	3.3	---	---
14-/16-C_{20}	0.6	7.0	10.2	0.7	0.7
18-C_{20}	---	8.6	15.9	0.3	---
14-/16-C_{21}	---	---	1.8	0.8	0.6
18-C_{21}	---	---	1.3	0.1	0.3
20-C_{21}	---	---	0.6	---	---

8-/10-/12-/14-C_{22}	---	---	7.0	3.6	0.9
16-/18-C_{22}	---	---	6.1	4.1	2.3
20-C_{22}	---	---	11.6	8.3	2.6
10-/12-C_{23}	---	---	---	1.6	1.1
14-/16-C_{23}	---	---	0.8	2.1	2.1
18-C_{23}	---	---	0.3	---	1.1
10-/12-/14-C_{24}	---	---	1.8	7.0	2.8
16-/20-C_{24}	---	---	0.6	2.5	4.3
22-C_{24}	---	---	1.9	5.3	9.6
14-/16-C_{25}	---	---	0.2	0.8	1.2
22-C_{25}	---	---	---	---	1.5
14-/16-C_{26}	---	---	0.2	0.8	2.2
22-C_{26}	---	---	---	---	1.9
24-C_{26}	---	---	---	---	5.7
Dimethyl-substituted (total)	(10.3)	(21.2)	(19.1)	(52.0)	(48.0)
2,12-/6,14-/10,14-C_{16}	0.2	---	---	0.7	---
12,16-/10,16-C_{18}	7.8	12.6	0.1	0.5	0.3
4,16-C_{18}	2.3	2.3	---	---	---
10,16-/12,16-C_{19}	---	2.8	---	---	---
2,16-C_{20}	---	1.7	---	---	---
2,18-C_{20}	---	1.8	---	---	---
12,16-C_{20}	---	---	---	0.6	0.8
14,16-C_{20}	---	---	---	---	0.7
14,18-C_{20}	---	---	5.0	---	---
10,16-/12,16-C_{21}	---	---	---	0.4	---
12,18-/14,18-C_{22}	---	---	4.1	5.5	3.7
12,20-/14,20-/16,20-C_{22}	---	---	6.7	13.0	5.4
10,14-/10,16-C_{23}	---	---	---	1.5	---
14,20-/16,20-C_{23}	---	---	---	0.8	2.9
16,22-C_{23}	---	---	---	3.8	0.7
10,14/10,16-C_{24}	---	---	---	5.0	---
10,20-C_{24}	---	---	---	1.8	---
14,20-/16,20-C_{24}	---	---	0.5	4.0	6.3
14,22-/16,22-/18,22-C_{24}	---	---	2.7	13.0	14.2
10,14-/10,16-/10,22-/12,22-/14,22-/18,22-C_{25}	---	---	---	0.7	3.0
14,22-/16,22-C_{26}	---	---	---	0.7	2.6
14,24-/16,24-C_{26}	---	---	---	---	7.4
Trimethyl-substituted (total)	(---)	(---)	(---)	(---)	(4.0)
2,18,22-C_{24}	---	---	---	---	4.0
Unidentified	1.0	---	2.0	3.3	5.4

[a]Determined as methyl esters after oxidation of the alcohols by CrO_3/AcOH and subsequent esterification with CH_3OH/HCl (further explanations in Table I).

TABLE III

Typical Fragments of Mass Spectra of Fatty Acid Methyl Esters

Position and kind of substitution	Mass numbers
2-Methyl	88; M−59
4-Methyl	87≫74; M−73; M−49
6-Methyl	M−76; small 129; M−50<M−76; 143→111→(93)
10-Methyl	129≈130; M−132; 199→167→149
12-Methyl	227→195→177
14-Methyl	255→223→205
15-Methyl	269→237→219
16-Methyl	283→251→233
18-Methyl	311→279→261
20-Methyl	339→307→289
22-Methyl	367→335→317
24-Methyl	395→363→345
2-Ethyl	102; M−59; M−28
2,6-Dimethyl	88; M−90; M−49; 157→125→(107)
2,8-Dimethyl	88; 185→153→135; 125
2,10-Dimethyl	88; 213→181→163; 153; 144; M−146
2,12-Dimethyl	88; 241→209→191; 181
2,14-Dimethyl	88; 269→237→219; 209
2,16-Dimethyl	88; 297→265→247; 237
2,18-Dimethyl	88; 325→293→275; 265
2,20-Dimethyl	88; 353→321→303
2,22-Dimethyl	88; 381→349→331
2,24-Dimethyl	88; 409→377→359
4,6-Dimethyl	87≫74; M−73; M−76; M−49; 157→125→(107)
4,8-Dimethyl	87≫74; M−73; 185→153→135
4,10-Dimethyl	87≫74; M−73; 213→181→163
6,14-Dimethyl	M−76; 143→111→(93); 269→237→219
10,14-Dimethyl	129≈130; M−132; 199→167→149; 269→237→219
10,16-Dimethyl	129≈130; M−132; 199→167→149; 297→265→247
10,20-Dimethyl	129≈130; M−132; 199→167→149; 353→321→303
10,22-Dimethyl	129≈130; M−132; 199→167→149; 381→349→331
12,16-Dimethyl	227→195→177; 297→265→247
12,18-Dimethyl	227→195→177; 325→293→275
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14,18-Dimethyl	255→223→205; 325→293→275
14,20-Dimethyl	255→223→205; 353→321→303
14,22-Dimethyl	255→223→205; 381→349→331
14,24-Dimethyl	255→223→205; 409→377→359
16,20-Dimethyl	283→251→233; 353→321→303
16,22-Dimethyl	283→251→233; 381→349→331
16,24-Dimethyl	283→251→233; 409→377→359
18,22-Dimethyl	311→279→261; 381→349→331
2,6,8-Trimethyl	88; M−90; 199→167→149
2,6,10-Trimethyl	88; M−90; 227→195→177; 137
2,6,12-Trimethyl	88; M−90; 255→223→205; 165
2,6,14-Trimethyl	88; M−90; 283→251→233; 193
2,6,16-Trimethyl	88; M−90; 311→279→261; 221
2,8,16-Trimethyl	88; 125; 185→153→135; 311→279→261
2,10,14-Trimethyl	88; 153; 213→181→163; 283→251→233
2,10,16-Trimethyl	88; 153; 213→181→163; 311→279→261
2,10,18-Trimethyl	88; 153; 213→181→163; 339→307→289
2,10,20-Trimethyl	88; 153; 213→181→163; 367→335→317
2,14,16-Trimethyl	88; 209; 269→237→219; 311→279→261
2,14,18-Trimethyl	88; 209; 269→237→219; 339→307→289
2,14,20-Trimethyl	88; 209; 269→237→219; 367→335→317
2,14,22-Trimethyl	88; 209; 269→237→219; 395→363→345
2,16,18-Trimethyl	88; 237; 297→265→247; 339→307→289
2,16,20-Trimethyl	88; 237; 297→265→247; 367→335→317
2,16,22-Trimethyl	88; 237; 297→265→247; 395→363→345
2,18,20-Trimethyl	88; 265; 325→293→275; 367→335→317
2,18,22-Trimethyl	88; 265; 325→293→275; 395→363→345
2,10,12,14-Tetramethyl-	88; 213→181→163; 255→223→205; 297→265→247
2,10,14,16-Tetramethyl-	88; 213→181→163; 283→251→233; 325→293→275
2,14,18,22-Tetramethyl-	88; 269→237→219; 339→307→289; 409→377→359

chain from the genus *Falco* via *Accipiter* to *Buteo*. In this investigation, only adult animals were used to avoid possible quantitative differences between animals of various ages (14).

The relatively simple fatty acid composition of the *Falconidae* differs significantly from the *Accipitridae*. No relationship to the *Strigiformes* (owls) is apparent. However, traces of 2-ethyl-dodecanol are detectable in the preen gland secretion of the goshawk and the buzzard, and related fatty acids have been shown to occur in the secretions of owls and tits (4,13).

For the alcohol components, there is also a parallel increase in the degree of substitution and in chain length from *Falco* via *Accipiter* to *Buteo*. Though small amounts of 2-methyl- and middle chain branched alcohols were detected, it is obvious that the methyl-branches are located mainly near the methyl end of the molecules. The alcohols correspond roughly to the fatty acids, containing an additional methyl group in 2 position. This structural similarity perhaps indicates a biosynthetic relationship between the alcohol and acid moieties of the uropygial waxes.

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Occurrence and Chemical Structure of Nonmethylene-Interrupted Dienoic Fatty Acids in American Oyster *Crassostrea virginica*

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ABSTRACT

The American oyster, *Crassostrea virginica*, was found to contain structurally homologous nonmethylene-interrupted dienoic (NMID) fatty acids. The major C_{20} and C_{22} nonmethylene-interrupted dienoic fatty acid isomers were shown to occur as two pairs of homologues 5,13-20:2 with 7,15-22:2 and 5,11-20:2 with 7,13-22:2. A combination of analytical procedures was required for conclusive structure determination.

INTRODUCTION

Interpretation of open tubular gas chromatograms of the methyl esters of fatty acids from marine molluscs in the region of 20:1 and 22:1 has been complicated by the presence of unidentified C_{20} and C_{22} fatty acids (1-5). These details are not apparent in conventional gas liquid chromatography (GLC) with packed columns (1,6-8). These components now have been identified in oysters as basically two homologous series of C_{20} and C_{22} nonmethylene-interrupted (NMID) fatty acids. They have been purified partially by a combination of silver nitrate-thin layer chromatography ($AgNO_3$-TLC) and preparative GLC. Structures were determined by oxidative and reductive ozonolyses combined with GLC and confirmed spectroscopically. The major isomers were found to be 5,13-20:2 and 7,15-22:2 along with lesser amounts of 5,11-20:2 and 7,13-22:2. There were many other minor isomers.

EXPERIMENTAL PROCEDURES

Oysters, *Crassostrea virginica*, were collected on several occasions between 1966 and 1970 near Ellerslie, Prince Edward Island, and transported to holding tanks at the Halifax Laboratory. Extraction of animals was carried out on total wet organic tissue with chloroform-methanol (9). Lipids were saponified by AOCS Method Ca-6b-53 (10). The fatty acids were recovered and converted to methyl esters by refluxing with 7% BF_3-MeOH.

Initial enrichment of NMID fatty acids (Scheme 1) was achieved by $AgNO_3$-TLC on Prekotes (Adsorbosil 5, from Applied Sciences Laboratories, State College, Pa.) previously immersed horizontally in a 10% solution of $AgNO_3$ in acetonitrile and dried. Development was with chloroform. Purification of chain lengths was achieved by preparative GLC using a packed stainless steel column (5% SE-30, 6 ft x 1/4 in. inside diameter, carrier gas He, column temperature 190 C), a heated collection unit (250 C), and glass collection tubes with local reheating to reduce losses from "fogging." Concentrates of fatty acid methyl esters then were identified structurally by GLC analysis of both oxidative (11) and reductive (12,13) ozonolysis degradation products. GLC of oxidative ozonolysis products was carried out on packed (10% EGSS-X on Gas Chrom P, Applied Science Laboratories) columns (6 ft x 1/8 in. inside diameter) at either 130 C for short chain methyl esters or at 180 C for dimethyl esters. Methyl ester preparations and reductive ozonolysis products were examined on stainless steel open tubular capillary columns (150 ft x 0.01 in. inside diameter) coated with butanediolsuccinate polyester (BDS) in Perkin-Elmer model 226 or 900 GLC apparatus. Column temperatures used were: for aldehydes, 60 C, for aldehyde-esters, 150 C; for long chain methyl esters, 170 C. Other operating conditions are described elsewhere (1,14). Acidic and aldehyde products were converted from wt percent to mole percent to facilitate comparison with the open tubular GLC peak proportions.

Mass spectroscopy was carried out on a DuPont CEC 21-110B double focusing mass spectrometer at 70 eV. The NMR spectra of NMID fatty acid methyl esters were obtained using a Varian A-60 spectrometer. Samples were dissolved in deuterated chloroform. IR spectra were obtained using a Perkin-Elmer model 237 spectrometer. Samples were run as a film on a salt block.

RESULTS AND DISCUSSION

The combination of $AgNO_3$-TLC and preparative GLC gave very effective separation of 20:2 and 22:2 NMID fatty acid methyl esters

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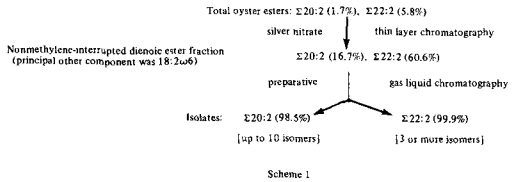
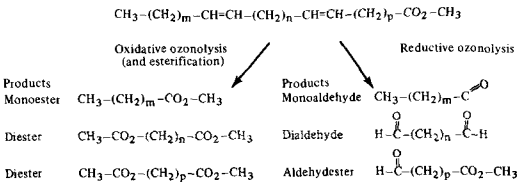


Scheme 1

Purification of Nonmethylene-Interrupted Dienoic Fatty Acid Methyl Esters of *Crassostrea virginica*



Scheme 2

Respective Products from Oxidative and Reductive Ozonolyses of Typical Nonmethylene-Interrupted Dienoic Fatty Acids

(Scheme 1). However, separation of individual isomeric species within these fractions could not be achieved. Attempts at partial purification of the isomers by low temperature $AgNO_3$-TLC (15) met with limited success. Fractions obtained from this procedure still contained a multiplicity of isomers but in proportions which differed greatly from the original "purified" sample composition. Analyses of these fractions were useful in confirming proposed structures (Table 1). Conclusions were drawn on the basis of quantitative data from degradative studies rather than from spectroscopic analyses of pure, single components or their derivatives. IR spectroscopy gave essentially identical spectra for both the C_{20} and C_{22} fractions purified by GLC. Both spectra were in all ways characteristic of fatty acid methyl esters (16) and had absorption maxima (~1700 cm^{-1}, ~2990 cm^{-1}) indicating the presence of *cis* unconjugated double bonds (17). NMR spectra confirmed IR findings. No CH_2 protons subject to shifts by more than one functional center were observed, suggesting the presence of two double bonds separated from each other and from the carboxyl function by several methylene groups.

Mass spectroscopy was used only to obtain a molecular ion for each chain length preparation (Scheme 1). These were respectively m/e 322 and m/e 350, corresponding to methyl eicosadienoate and methyl docosadienoate, respectively. An attempt was made to prepare trimethyl-silyloxy and methoxy derivatives of the

TABLE I

Mole Percentages of Oyster 20:2 and 22:2 Major Nonmethylene-Interrupted Dienoic (NMID) Isomer Component Peaks[a] as Recovered by Scheme 1 and after Additional Purification by Low Temperature Silver-Nitrate-Thin Layer Chromatography Compared to Products from Reductive Ozonolysis

Sample origin		Ozonolysis products			
A 20:2 NMID esters isolated from oyster		Aldehydes	Percent	Aldehyde esters	Percent
Isomer	Percent				
1	22.8	C_5	3.0	C_5	88.6
2	61.2	C_6	3.0	C_7	11.4
3	13.7	C_7	61.6		
4	2.3	C_8	1.6		
		C_9	24.5		
		C_{10}	6.6		
B 20:2 NMID repurified esters					
Isomer	Percent	Aldehydes	Percent	Aldehyde esters	Percent
1	53.6	C_7	49.3	C_5	86.9
2	29.7	C_9	39.5	C_7	13.1
3	16.6	C_{10}	11.3		
C 22:2 NMID esters isolated from oyster					
Isomer	Percent	Aldehydes	Percent	Aldehyde esters	Percent
1	22.6	C_7	78.5	C_7	100.0
2	75.7	C_9	19.7		
3	1.7	C_{10}	1.8		
D 22:2 NMID repurified esters					
Isomer	Percent	Aldehydes	Percent	Aldehyde esters	Percent
1	55.5	C_7	43.6	C_7	100.0
2	44.5	C_9	56.4		

[a]From open tubular gas liquid chromatographic analysis.

TABLE II

Comparison of Oxidative Fission Acidic (Methyl Ester) Fragments (Mole %) and Proportions of Theoretical Products from Two Major 20:2 and 22:2 Nonmethylene-Interrupted Dienoic (NMID) Acids Isolated from *Crassostrea virginica*

A	20:2 NMID methyl esters			Dimethyl esters		+	Methyl esters	
	Isomers	Percent		Theoretical (min)	Measured		Theoretical (min)	Measured
	5,11	22.8	C_5	42.0	28.1			
	5,13	61.2	C_6	11.4	7.1	C_6	---	14.1
	3	13.7	C_7	---	12.1	C_7	61.2	45.0
	4	2.3	C_8	30.6	40.5	C_8	---	5.9
			C_9	---	7.1	C_9	22.8	21.0
			C_{10}	---	3.0	C_{10}	---	13.3
			C_{11}	---	2.1			
B	22:2 NMID methyl esters			Dimethyl esters		+	Methyl esters	
	Isomers	Percent		Theoretical (min)	Measured		Theoretical (min)	Measured
	7,13	22.6	C_5	---	6.1			
	7,15	75.7	C_6	11.3	7.2	C_6	---	12.5
	3	1.7	C_7	49.2	45.2	C_7	75.7	46.7
			C_8	37.9	37.1	C_8	---	6.6
			C_9	---	---	C_9	22.6	25.0
			C_{10}	---	1.9	C_{10}	---	9.4
			C_{11}	---	1.2			

esters for mass spectroscopy, but the derivatives obtained were of questionable purity, gave complex mass spectra, and yielded no useful information beyond the inconclusive suggestion of a fragment of eight carbons from between ethylenic bonds.

Oxidative degradation of ozonides was somewhat more useful in defining the precise structure of the components. As Scheme 2 indicates, however, the products obtained from oxidative fission only suggest two alternative structures for each isomer, since the diesters may originate from either of two locations in the molecule.

Reductive degradation of ozonides, also outlined in Scheme 2, yields three different classes of components. These need only be matched up according to their relative abundance and the total chain length of the original esters to give all the analytical information required for complete identification. However problems can be encountered here also. The central dialdehyde fragments are subject to polymerization (18), before or after GLC, and may, for this and other reasons, give reduced yields. The aldehyde-ester fragment is more stable but potentially difficult to identify conclusively, since not all the necessary standards are readily available. Quantitation problems also arise with this method due to the multiplicity of isomers and innate differences in volatility, column loss, and differential detector sensitivity among breakdown products (13,19). For these reasons and because our starting material was a mixture, we have proposed conclusive structures for the major isomers and for the present prefer to only record the occurrence of the minor isomers and note possible degradation products.

Table I shows results from reductive ozonolysis. In the 20:2 NMID, representing four distinct isomeric esters, C_7 and C_9 dominate the product aldehydes, and the major aldehyde ester was C_5. There is insufficient C_7 aldehyde ester to combine with more than a fraction of the two major aldehydes. The total of the C_7 and C_9 aldehydes is very similar to the total amount of C_5 aldehyde ester. The mole percentage of C_7 aldehyde is the same as that of the second 20:2 NMID isomer, while the percentage of C_9 aldehyde is very similar to that of the first NMID isomer. The major 20:2 NMID isomer must, therefore, be 5,13-20:2, while the next most common isomer must be 5,11-20:2. These correspond to two (respectively b and a) of the three NIMD peaks (a,b, and c) observed primarily in gastropods, such as the moon snail *Lunata triseriata* or periwinkle *Littorina littorea* (1). The third unusual dienoic peak (c) observed in the gastropods probably corresponds to isomer 3, Table I. It may consist basically of the normal methylene interrupted 7,10-20:2 structure proposed previously on the basis of calculation of GLC retention time (1), as this accounts for the C_7 aldehyde ester and the unexpected C_{10} aldehyde, with perhaps minor amounts of other isomers. Isomer 4 perhaps could have an $\omega 5$ (for the C_5 aldehyde) and an $\omega 7$ (or $\omega 9$) combination, these or similar positional details accounting for the later elution relative to isomer 3 for reasons elaborated elsewhere (20,21). The ozonolysis products from the repurified low temperature $AgNO_3$-TLC fraction (Table I) provides further

quantitative support for the three major isomers.

Analogous reasoning with the 22:2 NMID isomers indicates that 7,15-22:2 is the most common and 7,13-22:2 the next most common isomer in the oyster lipids, although this may not be the case in other molluscs (1) or oyster samples (3). The fewer isomers permit a better fit for the fragments, and the homologues are in keeping with an origin in the two 20:2 NMID and simple chain extension by two carbon atoms.

The oxidative ozonolysis process applied to the same two mixtures of oyster isolates used for the reductive studies yielded dicarboxylic acid products (Table II), such as C_6 and C_8, from between the ethylenic unsaturations which could be combined with the C_5 diacid from the 20:2 NMID and C_7 from the 22:2 NMID (both identifiable as having original carboxylic acid functions from the C_5 and C_7 aldehyde ester fragments, Table I) in ca. the original isomer proportions discussed above for the C_{20} and C_{22} chain lengths. Losses of shorter chain products seem to have occurred, but it is not known if this occurred in recovery or is a GLC problem (19). The presence of ca. equal molar proportions of pimelic (di-C_7) and capric (mono-C_{10}) acids in the acidic 20:2 NMID products supported the normal methylene-interruped 7,10 structure for the third 20:2 isomer.

Open tubular GLC also reveals that there are relatively minor proportions of one or more dienoic components superimposed on the 18:1 complex (ω9,ω7, and ω5) from mollusc lipids (2). These have not been characterized as NMID acids, and a normal methylene-interrupted 5,8-18:1 homologue of the proposed 7,10-20:2 appears to be one possibility. The 20:2 and 22:2 NMID occur in both polar and nonpolar lipids of another oyster sample in varying proportions of 0.5-6.5% of total fatty acids in lipid fractions but ca. 2% for NMID 20:2 and 2-4% for 22:2 (3) or ca. the same proportions as in the sample examined in this study (Scheme 1).

Nonmethylene-interrupted unsaturation rarely occurs in fatty acids obtained directly from natural sources and is more often encountered as an intermediate stage in the catalytic hydrogenation of oils (22). Relatively large amounts of NMID fatty acids previously have been observed in a few plant species (23-26). In terrestrial animals, NMID fatty acids generally occur as trace components (27,28) often as the result of dietary factors (29,30). Novel 26:2 and 26:3 NMID recently have been found in marine sponges (31). The occurrence of somewhat similar components in shellfish need not indicate a close trophic relationship to the marine algae in their diet, even though some species of algae contain NMID fatty acids (23). An animal desaturase mechanism may be responsible for the production of these unusual compounds. Potential metabolic precursors for such compounds are likely to be the corresponding shorter chain monoethylenic ω9 (9-18:1, 11-20:1) and ω7 (11-18:1, 13-20:1) acids which are present in large amounts in the oyster and other molluscs (1-3,5). A discussion of the metabolic and taxonomic significance of these unusual components has been given previously by Ackman and Hooper (1).

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Presence of Phytosphingosine Combined with 2-Hydroxy Fatty Acids in Sphingomyelins of Bovine Kidney and Intestinal Mucosa

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ABSTRACT

A minor part of sphingomyelins of bovine kidney and small intestine has been shown by mass spectrometry to contain phytosphingosine in the earlier unknown combination with 2-hydroxy fatty acids.

INTRODUCTION

The existence in sphingomyelins from mammalian tissue of dihydroxy long chain bases (sphingosine and related bases) in combination with normal fatty acids has been known for a long time (1). A few years ago, it was shown that this phospholipid also may contain dihydroxy long chain bases in combination with 2-hydroxy fatty acids (2) or trihydroxy long chain bases (phytosphingosine and related bases) combined with normal fatty acids (3). This communication describes a molecular species of sphingomyelins with phytosphingosine and 2-hydroxy fatty acids, present in small amounts in bovine kidney and jejunum-ileum mucosa layer.

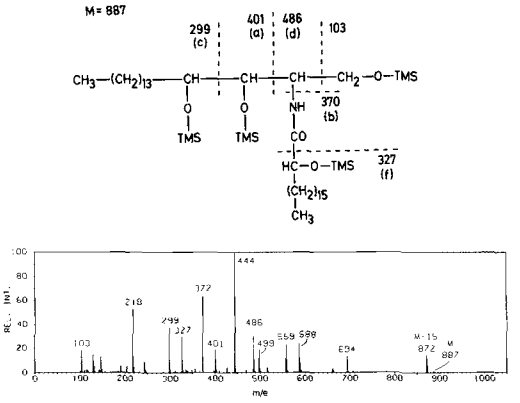

FIG. 1. Thin layer chromatograms of ceramides prepared from bovine kidney (B) and jejunum-ileum mucosa layer (C). The shorthand designation of ceramides refers to the reference fraction of synthetic ceramides (A) and d means dihydroxy, and t, trihydroxy covering the long chain base; h means 2-hydroxy concerning the fatty acid. Normal silica gel and arsenite impregnated gel were used as adsorbents and chloroform-methanol 95:5 (v/v) as developing solvent. Spots were made visible with the copper acetate reagent (9).

EXPERIMENTAL PROCEDURES

The preparation of sphingomyelins from bovine kidney (medulla zone) (4) and jejunum-ileum mucosa (2,5) has been described before. The pure phospholipid was degraded by phospholipase C treatment (6) into ceramide and phosphorylcholine. The different ceramide species were obtained pure by combined use of column chromatography (2,7) and preparative thin layer chromatography (TLC) (6,8). The most polar ceramide fraction migrated on normal and arsenite impregnated silica gel (8) as a synthetic ceramide sample with phytosphingosine and D2-hydroxyoctadecanoic acid (8) (Fig. 1). This ceramide also was analyzed as a trimethylsilyl derivative (10) by mass spectrometry using the direct inlet system of an MS 902 mass spectrometer (10). Mass spectra of the synthetic ceramide and of the natural samples are reproduced in Figure 2 and in Figures 3 and 4, respectively. The interpretation was based upon earlier published data (11-14), although the present molecular species has not been analyzed before. The ions at m/e 218 (fragment M-b-c), 299 (c), and 401 (a) are from phytosphingosine (Fig. 2), and 2-hydroxy fatty acid fragments are seen at m/e 327 (f), 372 (b+2), 444 (b+1+73), 486 (d), 499 (M-c-98), 599 (M-a+73), and 588 (M-c). The mol wt ions at m/e 872 (M-15) and 887 (M) also are present. This shows that mass spectrometry

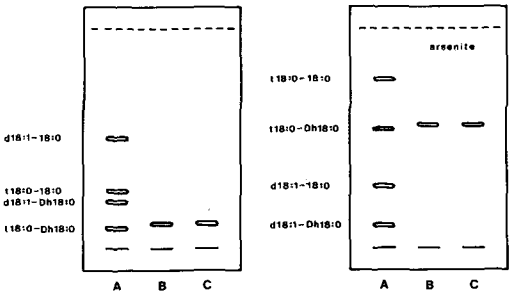

FIG. 2. Mass spectrum and structural formula of the trimethylsilyl derivative of a synthetic ceramide containing phytosphingosine and D2-hydroxyoctadecanoic acid. Letters in the formula are the same as used by Samuelsson, et al. (11-14). The sample evaporated at 165 C. Ion source temperate, 310 C; electron energy, 35 eV; filament current, 500 μA; and acceleration voltage, 6.5 kV. Peaks below m/e 100 were not reproduced. TMS = trimethylsilyl. Rel. Int. = relative intensity.

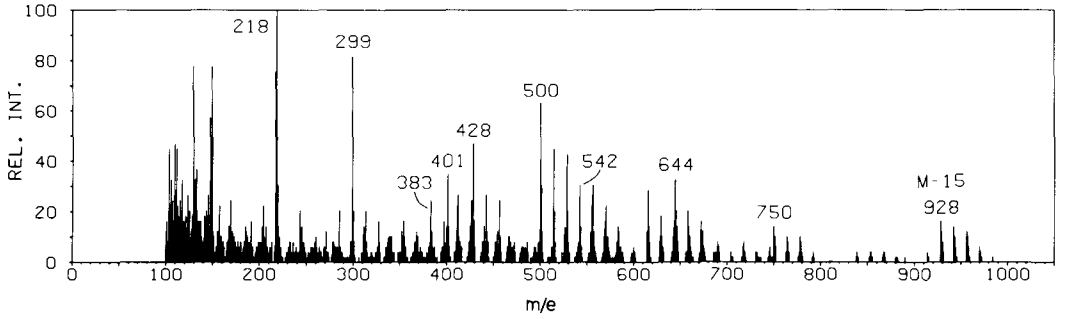

FIG. 3. Mass spectrum of the trimethylsilyl derivatives of polar ceramides from bovine kidney sphingomyelins. The sample evaporated at 150 C. Ion source temperature, 270 C; electron energy, 35 eV, filament current, 100 μA; and acceleration voltage, 8 kV. Peaks below m/e 100 were not reproduced. Rel. Int. = relative intensity.

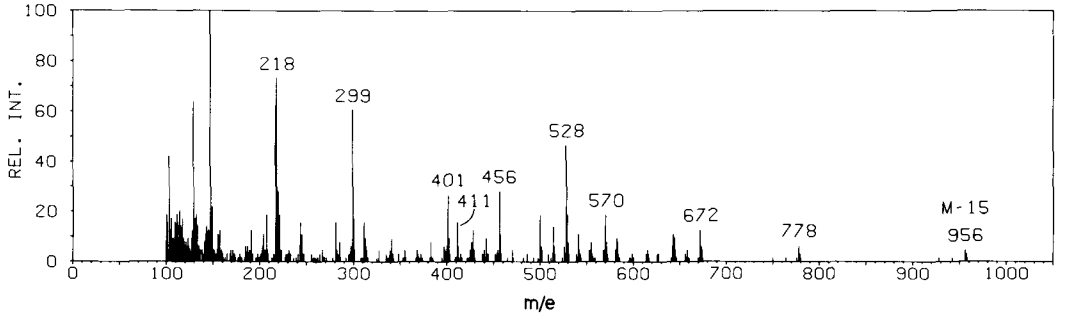

FIG. 4. Mass spectrum of the trimethylsilyl derivatives of polar ceramides from bovine jejunum-ileum mucosa layer. The sample evaporated at 165 C. Ion source temperature, 300 C; electron energy, 35 eV; filament current, 500 μA; and acceleration voltage, 6.5 kV. Peaks below m/e 100 were not reproduced. Rel. Int. = relative intensity.

gives specific information on ceramides with trihydroxy long chain bases and 2-hydroxy fatty acids, as shown before for other ceramide species (11-14).

RESULTS

The mass spectra of the ceramide fractions from bovine kidney and jejunum-ileum are very similar (Figs. 3 and 4). The dominating long chain base was phytosphingosine, seen at m/e 299 (c). In the kidney (Fig. 3), small amounts of the C_{17} (m/e 285) and C_{19} (m/e 313) homologues also were present. The fatty acids found were saturated 2-hydroxy fatty acids with 22, 23, and 24 carbon atoms. This clearly is shown by the groups of triplet peaks separated by 14 mass units (CH_2) at m/e 383, 397, and 411 (f); 428, 442, and 456 (b+2); 500, 514, and 528 (b+1+73); 542, 556, and 570 (M-a); 644, 658, and 672 (M-c); and 750, 764, and 778 (M-103-90). The peaks at 542, 556, and 570 (M-a) also could be b + 1 + 73 for C_{25}, C_{26}, and C_{27} fatty acids, respectively, but this is ruled out by the other fragment series, although small amounts of C_{25} hydroxy fatty acids are seen at m/e 584, 792, and 970 in the spectra of kidney (Fig. 3). Although conclusions concerning relative quantities of different species should be carefully drawn due to possible distillation effects in the ion source, the two series of peaks at m/e 750-792 (M-103-90) and m/e 928-970 (M-15) are the most suitable for quantitation in the present case. No fragments corresponding to short chain (C_{16}-C_{18}) fatty acids were found in the reproduced spectra or in spectra recorded at lower or higher probe temperatures. This is supported by TLC (Fig. 1), with only one spot seen just in front of the reference. Ceramides earlier have been shown to separate into two bands according to chain length (2,6,8). Finally, the existence of this novel ceramide species from mammalian sphingomyelins is confirmed by mol wt ions (M-15) at m/e 928, 942, and 956, respectively, for the three dominating fatty acids.

The small amounts of substance obtained (ca. 1% of total sphingomyelins) did not permit any further studies on the configuration or the possible existence of branched paraffin chains

(10). Synthetic ceramides having the D or L forms of the 2-hydroxy fatty acid are known to separate well on thin layer plates coated with either normal or arsenite impregnated silica gel (8). The ceramide fractions in this study had the same mobility as synthetic ceramides having the D configuration. This, together with the knowledge that 2-hydroxy fatty acids from bovine rennet stomach probably are of D configuration (2), suggests the same configuration of the 2-hydroxy fatty acids in sphingomyelins from bovine kidney and jejunum-ileum.

The difference between the two ceramide fractions is a small amount of long chain base homologues (C_{17} and C_{19}) in the kidney which is lacking in the jejunum-ileum. The kidney fraction had relatively more 22:0 and 23:0 than 24:0 fatty acids, while 24:0 dominated in the intestine.

The possibility that this ceramide species may be derived from contaminating glycolipid, which is known to contain these ceramides (15), is very unlikely. The sphingomyelin fractions are well separated from contaminating material, as seen by TLC, and are eluted later from silicic acid columns than less polar glycolipids or free ceramides. Furthermore, the enzyme preparation is not able to hydrolyze glycosphingolipid into ceramides.

DISCUSSION

The biological meaning of a variation in the number of hydroxy groups of ceramides, free or bound in glycolipids or sphingomyelins, is not known at the present time. However, the differences found between animal species and organs (16) may be shown to be linked to important variations in membrane associated phenomena, like permeability and translocation. It is noteworthy in this context that trihydroxy bases (phytosphingosine and related bases) seem to be preferentially combined with longer chain fatty acids (C_{22}-C_{24}), while dihydroxy bases (sphingosine and related bases) are linked to shorter chain (C_{16}-C_{18}), as well as longer chain acids (M.E. Breimer, K.-A. Karlsson, B.E. Samuelsson data to be published).

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Synthesis and Properties of Phosphatidyl Carnitine and Phosphatidyl β-Methylcholine

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ABSTRACT

rac-Phosphatidyl carnitine and rac-phosphatidyl β-methylcholine were synthesized by direct condensation of phosphatidic acid and the appropriate alcohols in the presence of 2,4,6-triisopropylbenzenesulphonylchloride and pyridine. Tetraphenylborates of the quarternary ammonium compounds β-methylcholine and carnitine benzyl ester were shown to be particularly convenient for synthesis in homogeneous phase. Physical and chemical properties of the two phosphoglycerolipids and some intermediates were described. Phosphatidyl carnitine and phosphatidyl β-methylcholine were hydrolyzed by phospholipase A_2 (phosphatide acyl-hydrolase, EC 3.1.1.4), pancreatic lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3), and phospholipase C from *Bacillus cereus* (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3). Neither hydrolysis nor transphosphatidylation of phosphatidyl carnitine and phosphatidyl β-methylcholine was achieved by phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4). The occurrence of phosphatidyl carnitine in embryonic chicken tissue was suggested by comparison with the synthesized compound. Phosphatidyl carnitine could not be detected in the tissue of rat embryos.

INTRODUCTION

In addition to soluble carnitine so called "lipid-bound carnitine" has been detected in animal tissues. In 1963, Mehlman and Wolf (1,2) demonstrated the existence of phosphatidyl carnitine (PCar) and phosphatidyl β-methylcholine (PMC) in chicken and rat embryos. Two years later, Khairalla and Mehlman (3), as well as Fritz (4), doubted the occurrence of PCar in these tissues while PMC was not mentioned. "Lipid-bound carnitine" was said to exist exclusively as long chain acyl carnitine. In the course of their larval state, different fly species incorporated carnitine or β-methylcholine into their phospholipids, if these compounds replaced the dietary choline (Bieber, et al., 5,6). When carboxy-^{14}C-carnitine or methyl-^{14}C-carnitine was added to the diet of *Phormia regina*, in addition to long chain acyl carnitine and PMC, a phospholipid was found containing labeled carnitine in both cases (7). Formation of PCar in *P. regina* larvae was shown to occur exclusively by Mehendale, et al., (8) when carnitine and N-trimethylaminopropanol were fed simultaneously. The occurrence of PMC together with PCar raised the question as to whether the PMC is formed by decarboxylation of PCar or whether free carnitine is decarboxylated and β-methylcholine incorporated via the Kennedy pathway. Carnitine decarboxylase was found in *P. regina* (9) and in other animal tissues. Despite the overall structural relationship between choline and its β-substituted derivatives, carnitine and β-methylcholine, there are remarkable differences corresponding to the chemical and enzymatic reactivity with respect to the secondary alcohol group.

In this paper, the chemical synthesis (Fig. 1), some physical and chemical properties, and the enzymatic hydrolysis of PCar and PMC are described. This synthesis opens up the possibility of clarifying unresolved problems of the biosynthesis, significance, and occurrence of these phosphoglycerolipids.

EXPERIMENTAL PROCEDURES

Materials

Carnitine chloride, sodium tetraphenylborate, and Silica Gel HR were purchased from Merck, Darmstadt, G.F.R. 2,4,6-triisopropylbenzenesulphonylchloride (TPS) was obtained from Riedel de Haen, Hannover, G.F.R. Phospholipase A_2 and D were purchased from Boehringer Mannheim GmbH, Mannheim, G.F.R., and phospholipase C of *Clostridium welchii* from Sigma Chemical Co., St. Louis, Mo. Phospholipase C was isolated from *Bacillus cereus*. Gas chromatographic analysis was carried out on a Hewlett-Packard 7620 A fitted with an integrator Hewlett-Packard 3370 B. Stationary phase was EGSSX 10% on Gas Chrom Q. ^{13}C-NMR spectra were recorded with a 60 MHz-instrument (Bruker HFX 60, Karlruhe, G.F.R.; frequency 15.086 MHz) equipped with a Siemens computer model 301. Hexafluorobenzene was used as reference substance for the phospholipids phosphatidyl choline (PC) and PCar dissolved in chloroform.

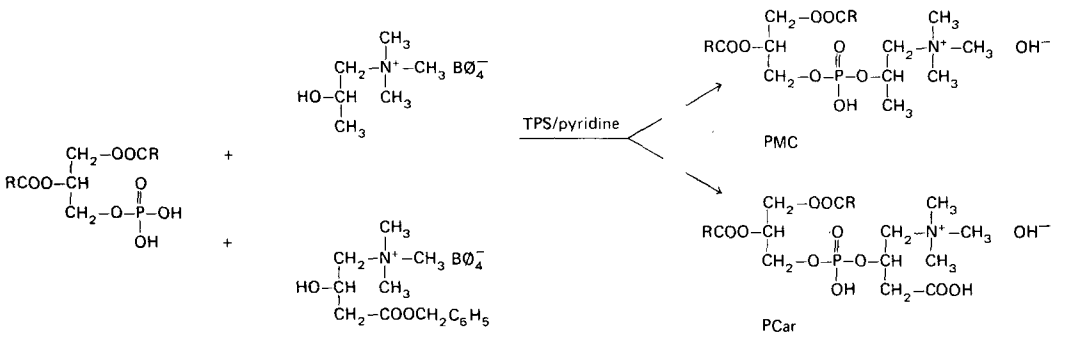

FIG. 1. Synthesis of phosphatidyl β-methylcholine (PMC) and phosphatidyl carnitine (PCar). (R = palmitoyl and $B\emptyset_4^-$ = tetraphenylborate.) TPS = 2,4,6-triisopropylbenzenesulphonylchloride.

PMC was measured in deuterochloroform.

DL-Carnitine Benzyl Ester Tetraphenylborate

Synthesis of DL-carnitine benzyl ester chloride: Finely powdered carnitine chloride (9.9 g; 50 mmole) were suspended in 150 ml benzyl alcohol. A dried HCl gas stream was passed through the heated suspension (70 C). After 4 hr, the clear solution was evaporated to dryness, the residue ground with ethyl ether, and filtered. The white crystals obtained were dissolved in 100 ml water, neutralized with Amberlite IR-45 ion exchanger, filtered, and the solvent removed under reduced pressure. The residue, recrystallized from acetonitrile, yielded 11.7 g (81%), mp 161 C.

Alternative synthesis of DL-carnitine benzyl ester chloride: Freshly distilled phenyldiazomethane (10) was added to an ice-cooled solution of carnitine chloride in methanol/water 4:1 (v/v) until the red color persisted. The compound was isolated as described above. Yield: 70%.

Synthesis of DL-carnitine benzyl ester tetraphenylborate: A solution of 6.84 g (20 mmole) sodium tetraphenylborate in 300 ml water was added to a solution of 5.75 g (20 mmole) carnitine benzyl ester chloride in 300 ml water with vigorous stirring. Stirring was continued for 1 hr, and the white solid was filtered off, washed several times with water, and recrystallized from methanol/water. Carnitine benzyl ester tetraphenylborate was obtained in a yield of 86%, mp 159 C. Analysis calculated for $C_{38}H_{42}BNO_3$ (572): C, 79.85; H, 7.41; N, 2.45. Found: C, 80.13; H, 7.61; N, 2.48.

β-Methylcholine Tetraphenylborate

Synthesis of β-methylcholine chloride: N-trimethylaminoacetone chloride was synthesized according to Major and Cline (11). Varying the method of the authors, the reduction of this ketone was achieved by sodium boronhydride in aqueous methanol.

Synthesis of β-methylcholine tetraphenylborate: Under vigorous stirring, a solution of 13.7 g (40 mmole) sodium tetraphenylborate in 200 ml water was added dropwise to a solution of 6.15 g (40 mmole) β-methylcholine chloride in 200 ml water. After the addition, stirring was continued for 1 hr. The white precipitate was filtered off and thoroughly washed with water. The dried β-methylcholine tetraphenylborate was recrystallized from methanol. Yield: 12.6 g (72%), mp 214-216 C. Analysis calculated for $C_{30}H_{36}BNO$ (437): C, 82.35; H, 8.23; N, 3.20. Found: C, 82.42; H, 8.40; N, 3.26.

rac-Dipalmitoylphosphatidyl Carnitine

Synthesis of rac-dipalmitoylphosphatidyl carnitine benzyl ester: rac-Dipalmitoylphosphatidic acid (650 mg; 1 mmole) and 910 mg (3 mmole) TPC were dried overnight in a desiccator over P_4O_{10} under reduced pressure (0.1 mm Hg). The dried compounds were mixed, and 25 ml absolute pyridine were added. Under rigorous exclusion of moisture, the reaction mixture was warmed cautiously until all phosphatidic acid was dissolved and then allowed to stand for 5 hr at room temperature. Water (5 ml) was added, and stirring was continued for 1 hr. The mixture was evaporated to dryness in vacuo; the residue was extracted twice with each 30 ml portions of ethyl ether. The combined extracts were cooled to 0 C, filtered, and brought to dryness. The white solid was recrystallizable from acetone. The pure phospholipid was isolated by preparative thin layer chromatography (TLC) or by column chromatography on silica gel (Mallinckrodt, St. Louis, Mo.) as stationary phase, with chloroform/methanol 10:1 (v/v) followed by 4:1 (v/v) as the eluants. Because of the lability of the phosphate carnitine ester bond, the column chromatography had to be carried out at 4 C. After column chromatography, a yield of

565 mg (64%) was obtained, mp 110 C (capillary). Analysis calculated for $C_{49}H_{89}NO_{10}P \cdot H_2O$ (901): C, 65.23; H, 10.28; N, 1.55; P, 3.44. Found: C, 64.83; H, 10.24; N, 1.50; P, 4.10.

Synthesis of rac-dipalmitoylphosphatidyl carnitine: Phosphatidyl carnitine benzyl ester (500 mg) was dissolved in 50 ml glacial acetic acid and hydrogenated under atmospheric pressure in the presence of 300 mg palladium/charcoal (10%). After 2 hr, the benzyl group was removed quantitatively. The catalyst was filtered off through Celite and washed with chloroform. The combined filtrates were evaporated to dryness under reduced pressure. The residue was recrystallized from chloroform/acetone. Analysis calculated for $C_{42}H_{83}NO_{10}P \cdot H_2O$ (811): C, 61.69; H, 10.65; N, 1.72; P, 3.81. Found: C, 61.90; H, 10.79; N, 1.74; P, 4.20.

Properties of phosphatidyl carnitine: PCar is a white crystalline substance. When melting, it shows several transition states, having a final mp at 212 C. the R_f values on Silica Gel HR were: chloroform:methanol:water, 65:25:4 (v/v/v), R_f = 0.12; chloroform:methanol:ammonia (25%), 60:30:10 (v/v/v), R_f = 0.30; and chloroform:methanol:glacial acetic acid:water, 62:25:8:4 (v/v/v/v), R_f = 0.36. IR and Laser-Raman spectra were similar to those of PC. The ^{13}C-NMR spectrum of dipalmitoylphosphatidyl choline published by Metcalfe, et al., (12) resembled that of dipalmitoylphosphatidyl carnitine due to the poor resolution of the methylene C-atom signals of the base (Fig. 2). The appearance of an additional signal shifted 8 ppm to lower field than the ester carbonyl C-atoms in the ^{13}C-NMR spectrum of PCar, however, proved the evidence of a free carboxylic function. According to Stothers and Lauterbur (13), the signal of the carboxylic C-atom of acetic acid is shifted 7 ppm to lower field than the ^{13}C-NMR signal of the carboxylic C-atom of acetic acid ethyl ester.

rac-Dipalmitoylphosphatidyl β-Methylcholine

Synthesis of rac-dipalmitoylphosphatidyl β-methylcholine: rac-Dipalmitoylphosphatidic acid (1.75 g; 2.7 mmole), 2.46 g (5.6 mmole) β-methylcholinetetraphenylborate and 2.39 g (7.7 mmole) TPS were dried, as described above. To the mixture of the dried compounds, 50 ml absolute pyridine was added, and the reaction mixture was warmed cautiously until a clear solution was obtained. The solution was allowed to stand 4 hr at room temperature; 5 ml water was added, and the mixture was stirred for 1 hr. After evaporation to dryness, the residue was extracted several times with ether, the combined filtrates were brought to dryness, and the residue was recrystallized from dioxane, yield: 1.58 g (75.6%). The product was purified by preparative TLC. Analysis calulated for $C_{41}H_{83}NO_8P \cdot H_2O$ (767): C, 64.30; H, 11.05; N, 1.83; P, 4.04. Found: C, 63.69; H, 10.97; N, 1.79; P, 4.50.

Properties of rac-phosphatidyl β-methylcholine: Like PCar, PMC is a white crystalline solid with a capillary mp of 218 C, showing several thermotropic transition states. TLC on Silica Gel HR gave the following R_f values: chloroform:methanol:water, 65:25:4 (v/v/v), R_f = 0.39; chloroform:methanol:ammonia (25%), 60:30:10, R_f = 0.70; and chloroform:methanol:glacial acetic acid:water, 65:25:8:4 (v/v/v/v), R_f = 0.65. The IR and ^{13}C-NMR spectra showed no remarkable differences with respect to PC. Figure 2 shows the ^{13}C-NMR spectra.

Enzymatic Hydrolysis

Hydrolysis by phospholipase A_2 from Crotalus terrificus terrificus (phosphatide acylhydrolase, EC 3.1.1.4): Following van Deenen and de Haas (14), 20 mg of either rac-PCar or rac-PMC in 1 ml 0.1 M borate buffer (pH = 7.0) was emulsified by ultrasonic treatment in the presence of $2.5 \cdot 10^{-3}$ M $CaCl_2$ and 8.2 mg sodium deoxycholate. After addition of the enzyme, the incubation mixture was allowed to stand for 2 hr at 37 C. Under these conditions, both substrates were cleaved ca. 50%.

Hydrolysis by pancreatic lipase from porcine pancreas (triacylglycerol acyl-hydrolase, EC 3.1.1.3): PCar or PMC (10 mg), 3 mg sodium deoxycholate, and 4.5 mg bovine serum albumine were suspended by ultrasonic treatment in a 0.1 M borate buffer (pH = 8.0) containing $5 \cdot 10^{-3}$ M $CaCl_2$. According to Slotboom, et al., (15) the incubation mixture was kept 5 hr at 30 C. Both phospholipids almost were hydrolyzed quantitatively.

Hydrolysis by phospholipase C from B. cereus or C. welchii (PC cholinephosphohydrolase, EC 3.1.4.3): As described by Graf and Stein (16), the incubation mixture consisted of 5 mg PCar or PMC, 0.98 ml ethyl ether, 0.02 ml ethanol and 0.02 ml 40 mM $CaCl_2$-solution. After addition of the enzyme, the reaction mixture was kept for 5 hr at 37 C and then 15 hr at room temperature. Neither PCar nor PMC was attacked by the enzyme of *C. welchii*. The enzyme from *B. cereus* hydrolyzed the two substrates. According to Slein and Logan (17), *B. cereus* contains three different phospholipase C-like activities, one cleaving sphingomyelin, a second splitting phosphatidyl inositol, and a third hydrolyzing PC and phosphatidyl ethanolamine. Because of

the structural similarity, it can be assumed that the third enzyme was also specific for PCar and PMC.

Hydrolysis by phospholipase D from cabbage leaves (PC phosphatidohydrolase, EC 3.1.4.4): PCar and PMC are two suitable substrates to investigate the question of whether phospholipids containing secondary alcohol components can be hydrolyzed by phospholipase D. Because both substrates were not attacked by phospholipase D it can be assumed that phospholipids with secondary alcohol moieties generally are not hydrolyzed. These results support the postulate of Menzei and Newburgh (7), according to whom a carnitine containing phospholipid isolated from *P. regina* larvae was assumed to be PCar, as it was not converted by phospholipase D. Enzymatic transphosphatidylation with PCar and PMC as phosphatidic acid donators to appropriate primary alcohols failed, because no intermediate phosphatidyl-S-enzyme-complex was formed in accordance to the hydrolysis experiments of Yang and Benson (18). As no enzymatic transfer of phosphatidic acid residue from PC to the secondary alcohols β-methylcholine and carnitine was found, the results of Dawson (19) obtained with secondary acceptor alcohols were confirmed.

Natural Occurrence of Phosphatidyl Carnitine

The question of natural occurrence of PCar was studied with 16 day old chicken embryos and rat embryos of 2 cm length. The tissues were frozen in liquid air, minced, and extracted according to Folch, et al. (20). The Folch extracts were separated by TLC on Silica Gel HR developed with chloroform:methanol:water 65:25:4 (v/v/v). The spot probably containing carnitine was scraped from the plate and extracted with chloroform/methanol 1:1 (v/v). The eluted lipids were rechromatographed using chloroform:methanol:ammonia (25%) 60:30:10 (v/v/v). From the extracts of chicken tissue, a weak spot which showed the same R_f value as synthetic PCar was isolated and extracted. Solvents were removed, and the residue was hydrolyzed with 6 N HCl for 18 hr at 105 C. In the hydrolysate, carnitine could be detected by paper chromatography. A chromatographically purified sample of PCar from chicken tissue was transesterified by 5% methanolic sulphuric acid. By gas chromatographic separation of the fatty acid methyl esters, the following fatty acid composition was found (peak area %): 16:0 = 43.2, 16:1n-7 = 4.8, 18:0 = 14.6, 18:1n-9 = 28.6, 18:2n-6 = 7.2, and 20:0 = 1.5.

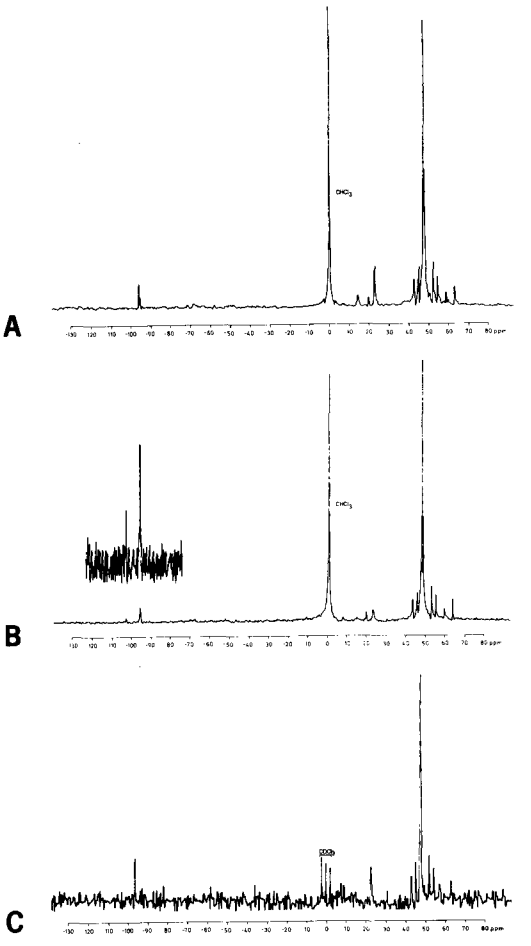

FIG. 2. ^{13}C-NMR spectra of phosphatidyl choline (PC) (A), phosphatidyl carnitine (PCar) (B), and phosphatidyl β-methylcholine (PMC) (C).

DISCUSSION

The phosphorylation of carnitine was found to be difficult because of the high polarity of the substance and the sterically unfavorable secondary hydroxy group which easily is subject to a β-elimation. Neither the reaction of phosphatidic acid monobenzyl ester silver salt with bromocarnitine picrate nor attempts to phosphorylate carnitine benzyl ester directly led to the expected products. Only the application of the direct condensation of phosphatidic acid with the protected alcohol component in the presence of pyridine/TPS, according to Aneja, et al., (21) was successful. Dicyclohexylcarbodiimide was also suitable as a condensing agent, giving, moreover, lower yields than TPS. By conversion of β-methylcholine chloride and carnitine benzyl ester chloride to the nonhydroscopic tetraphenylborates, the quarternary

ammonium salts became soluble in pyridine, allowing the reaction to be carried out in homogeneous phase. The condensation also was accomplished in heterogeneous phase at 60 C giving, however, a lower yield. Due to the close structural relationship between the phosphoglycerolipids PC, PCar, and PMC, the physical and chemical properties, and the IR, ^{13}C-NMR and Laser-Raman spectra were very similar.

According to the substrate requirements for phospholipase A_2 postulated by van Deenen and de Haas (14) and for pancreatic lipase by Slotboom, et al. (15), a hydrolysis of PCar and PMC had to be expected by these enzymes. Due to the high specificity of phospholipase C from *C. welchii* for the choline containing phospholipids PC and SP, there was no enzymatic breakdown of PCar and PMC. It was shown that PCar and PMC were not suitable substrates for phospholipase D. Furthermore, no transphosphatidylase activity was detected.

PCar isolated from embryonic chicken tissue showed a fatty acid composition which is characteristic of phosphoglycerolipids. The biosynthesis of PCar and PMC and the significance of carnitine and β-methylcholine incorporation are unresolved. It has not been proven conclusively that the detected carnitine containing phospholipid is the supposed 1,2-diacylphosphatidyl carnitine. The convenient synthesis of these natural products will contribute to the solution of these unresolved problems.

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Evaluation of Chromic Oxide, Glycerol Triether, and β-Sitosterol as Fecal Flow Markers in Two Species of Nonhuman Primates

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ABSTRACT

Recovery of β-sitosterol, glycerol triether (1-hexadecyl-2,3,-didodecyl glycerol triether), and chromic oxide was studied in African green monkeys and stumptail macaques consuming diets containing 0.75 mg/Cal cholesterol and 38% of calories as safflower oil or butter. Following oral administration of these compounds, feces were collected daily for 9 days. For all animals, excretion of β-sitosterol and glycerol triether paralleled one another almost exactly. Except for two animals, this was also true for chromic acid. Essentially 100% of the administered β-sitosterol and 90-95% of the glycerol triether were recovered; excretion of these markers virtually was complete by day 3. Ninety-two percent of the β-sitosterol was isolated in the nonsaponifiable lipid extract of the feces with less than 6% in the remaining aqueous phase. A maximum of 3.1% of the β-sitosterol and 1.8% of the glycerol triether were found in the blood. For stumptail macaques, the major excretory form of β-sitosterol was the 5β-derivative. African green monkeys were more variable; one animal excreted the bulk of the β-sitosterol unchanged while others excreted greater than 80% as the ring-saturated 5β-derivative. Animals consuming the safflower oil containing diet consistently excreted a greater percentage of the β-sitosterol unchanged, compared with those animals eating the butter containing diet. There was no evidence for steroid ring degradation in any of the animals used in this study.

INTRODUCTION

The study of whole body cholesterol metabolism by sterol balance techniques requires the use of either isotopic or chromatographic procedures or a combination of the two (1). In each of these procedures, all of the excreted cholesterol must be recovered in the feces as neutral or acidic steroids. Several investigators now have reported, however, that in certain human beings there may be considerable loss of cholesterol, presumably by breakdown of the steroid nucleus by intestinal bacteria (2-4). Similar losses of bile acids do not occur (2). Since β-sitosterol is degraded to the same extent as cholesterol (2,5), β-sitosterol can be used as an internal standard for correction of steroid ring breakdown in sterol balance studies (2).

Studies in man have been unsuccessful in identifying the degradation products of steroid ring breakdown or, for that matter, the process by which it occurs. Further studies have been hampered by the lack of an animal model in which to study this phenomenon. For this reason, we attempted to identify a species of nonhuman primate in which neutral steroid losses occur.

We knew from previous experiments that squirrel monkeys (*Saimiri sciureus*) do not degrade the steroid ring, as essentially 100% of dietary β-sitosterol is recovered in the feces (6). Squirrel monkeys, however, are small animals with a rapid intestinal transit time (less than 24 hr). Since degradation of the steroid ring hasbeen reported to be related to prolonged retention of fecal material in the colon (7), we wanted to know if larger nonhuman primates, with longer intestinal transit times, would degrade the steroid ring and, thus, serve as a useful animal model for this phenomenon. For these studies, we selected the African green monkey (*Cercopithecus aethiops*) and the stumptail macaque (*Macaca arctoides*).

TABLE I

Composition of Diets

Ingredients	Safflower oil (g)	Butter (g)
Non-fat dry milk solids	3360	3360
Complete vitamin mixture	240	240
Salt mixture	240	240
Sucrose	2400	2400
Gelatin	160	160
Butter	---	1976
Safflower oil	1600	---
β-sitosterol	---	4.8
Cholesterol[a]	27.8	23
Vitamin D_3	4.96	4.96

[a]0.75 mg cholesterol/Cal.

[1]This work was done during R.W. St. Clair's tenure as an Established Investigator of the American Heart Association.

TABLE II

Data on Nonhuman Primates Used in These Studies

Animal number	Serum cholesterol[a] (mg/dl)	Body wt (kg)	Diet[b]
Stumptail macaques			
S–1	401	12.5	Safflower oil
S–2	733	14.1	Butter
S–3	378	10.5	Safflower oil
S–4	399	11.4	Butter
African green monkeys			
1352	208	3.9	Safflower oil
1353	215	4.1	Safflower oil
1354	449	4.1	Butter
1355	260	4.5	Butter
1356	179	3.4	Butter
1357	194	4.3	Safflower oil

[a]Serum cholesterol concentration immediately prior to administration of the β-sitosterol, glycerol triether, and chromic oxide markers.

[b]See Table 1 for composition of the diets.

To facilitate this study, we compared the recovery of three markers: β-sitosterol (a sterol marker), glycerol triether (an oil-phase marker), an chromic oxide (a particulate-phase marker). Such a comparison also provides data as to the intestinal transit time for lipid in these species and data on the usefulness of these markers in sterol balance studies.

MATERIALS AND METHODS

Four adult male stumptail macaques and six adult male African green monkeys were purchased from Primate Imports, Port Washington, N.Y. The animals were maintained in our primate facility in individual stainless steel cages and were fed diets containing 0.75 mg/Cal cholesterol and ca. 38% of calories as either butter or safflower oil. The butter diet was supplemented with β-sitosterol so that the β-sitosterol contents of both diets were equal. The composition of these diets is shown in Table I. Information as to the diet consumed by each animal and the body wt and plasma cholesterol concentration at the time of this study are shown in Table II.

After 5-7 months on diet, the animals were sedated with Ketamine hydrochloride (Vetelar, Parke-Davis, Detroit, Mich.) (10 mg/kg intramuscularly) and were administered orally chromic oxide, β-sitosterol-4-^{14}C, and glycerol triether-^{3}H to study the transit time of these compounds through the intestinal tract and their recovery in the feces. β-Sitosterol-4-^{14}C was obtained from Amersham/Searle Co., Des Plaines, Ill, and glycerol triether-^{3}H, from New England Nuclear Corp., Boston, Mass. (Glycerol triether = 1-hexadecyl-2, 3-didodecyl glycerol triether [trivial name] or 1-hexadecoxy-2,3-didodecoxypropane [systematic name]). The ^{3}H-label of the glycerol triether was in the 9 and 10 positions of the hexadecyl moiety and was prepared in a manner identical with that used by Morgan and Hofmann (8). Both the β-sitosterol-4-^{14}C and the glycerol triether-^{3}H had a radiochemical purity of >99% after chromatography on Silica Gel G using Skellysolve B (Skelly Oil Co., Tulsa, Okla.), ethyl ether, acetic acid (146:50:4 v/v/v) as the developing solvent.

An emulsion containing 50 μCi (339 μg) of β-sitosterol-4-^{14}C and 71 μCi (353 μg) of glycerol triether-^{3}H was made by dissolving these compounds in a mixture of 0.5 ml acetone, 0.5 ml safflower oil, and 1 drop Tween 20 (Nutritional Biochemicals Corp., Cleveland, Ohio). Fifty-nine ml of a 6.8% aqueous solution of skim milk powder were added and the mixture sonicated for 10 min with a Polytron (Brinkmann Instruments, Westbury, N.Y.). The emulsion was administered to the monkeys within 2 hr of its preparation.

Five ml of this emulsion, containing 13.75 x 10^{6} dpm glycerol triether-^{3}H and 8.68 x 10^{6} dpm β-sitosterol-^{14}C, were administered to each animal by gastric tube. The gastric tube then was rinsed with 10 ml emulsion mixture without the radioactive markers. Immediately thereafter, each animal was given by mouth a no. 2 gelatin capsule containing a known amount (184-290 mg) of chromic oxide. Animals then were returned to their cages and fed their normal diet (Table II).

Feces were collected from each animal daily for 9 days and stored frozen until analyzed. The feces were mixed with several volumes of

water, weighed, and mixed in a blender until uniform. While still blending, to ensure that the mixture did not settle, aliquots were removed in duplicate and placed in previously weighed bottles. The wt of the homogenate to be analyzed was determined by reweighing the bottle. Thirty ml of redistilled ethanol and 3 ml of 10N NaOH were added and the mixture heated to boiling in a reflux apparatus for 1 hr. After addition of 10 ml water, the mixture was extracted 4 times with 50 ml each of redistilled Skellysolve B. This extract was taken to dryness at 45 C in a flash evaporator, dissolved in a known amount of chloroform and methanol (2:1 v/v), and an aliquot taken for determination of radioactivity.

We further analyzed the feces from each animal, collected on the day of maximum β-sitosterol excretion (Figs. 1 and 2), to determine whether all of the ^{14}C radioactivity in the feces from the ingested β-sitosterol-4-^{14}C was recovered as neutral steroid. For this, an aliquot of the Skellysolve B extract was taken to dryness at 45 C under a stream of nitrogen and separated by thin layer chromatography (TLC) on Silica Gel G, using ethyl ether and Skellysolve B (55:45 v/v) as the developing solvent. The radioactivity in the three neutral steroid fractions, as described by Miettinen and coworkers (9), then was compared with the total radioactivity applied to the thin layer plate.

One ml of the aqueous phase, after extraction of the feces with Skellysolve B, was dried under nitrogen, neutralized with hydrochloric acid, dissolved in 10 ml Aquasol (New England Nuclear Corp.), and counted for radioactivity.

With the exception of those samples counted in Aquasol, all other radioactivity determinations were done by dissolving the sample in 10 ml toluene containing 6 g diphenyloxazole/liter. All samples were counted in a Beckman LS-230 liquid scintillation counter to a 2-σ error of less than 2%, and quenching was corrected for by an external standard channels ratio technique.

Chromic oxide in feces was determined in duplicate by the method of Bolin, et al. (10).

A day and 3 days after administration of β-sitosterol-4-^{14}C, glycerol triether-^{3}H, and chromic oxide, blood samples were drawn and serum separated by centrifugation. Lipids were extracted in 20 volumes of isopropanol and one aliquot taken for determination of radioactivity and another for cholesterol determination (11).

RESULTS

Table III shows data on the recovery of β-sitosterol and glycerol triether in serum and

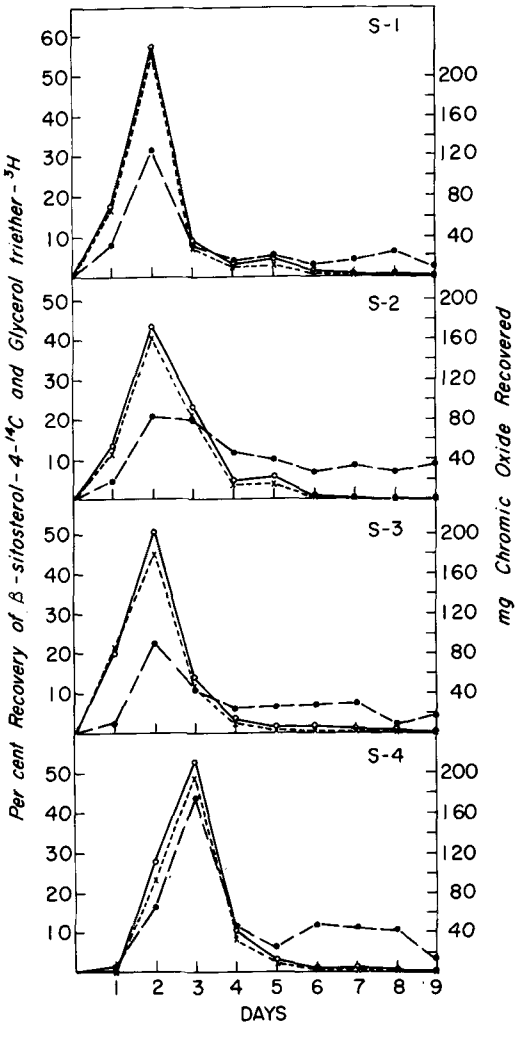


FIG. 1. Fecal recovery of β-sitosterol-4-^{14}C, glycerol triether-^{3}H, and chromic oxide in stumptail macaques. β-Sitosterol and glycerol triether values represent recovery in the nonsaponifiable lipid extract of the feces. o—o = β-Sitosterol-4-^{14}C, X---X = glycerol triether-^{3}H, and •– –• = chromic oxide.

feces collected for a total of 9 days after oral administration of these compounds. Virtually all of the administered β-sitosterol was recovered in both species of monkeys. Of the recovered β-sitosterol, greater than 92% was isolated in the nonsaponifiable lipid extract of the feces. Ca. 5% was in the remaining aqueous phase.

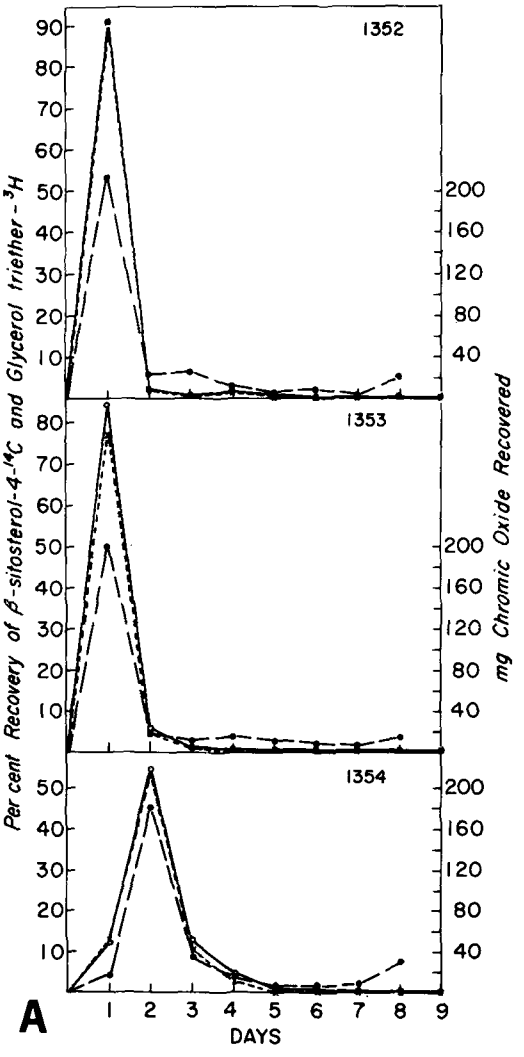
The total recovery of glycerol triether-^{3}H was significantly less than for total recovery of β-sitosterol in stumptail macaques but was not significantly different from the total recovery of β-sitosterol in African green monkeys. For both animal species, however, recovery of

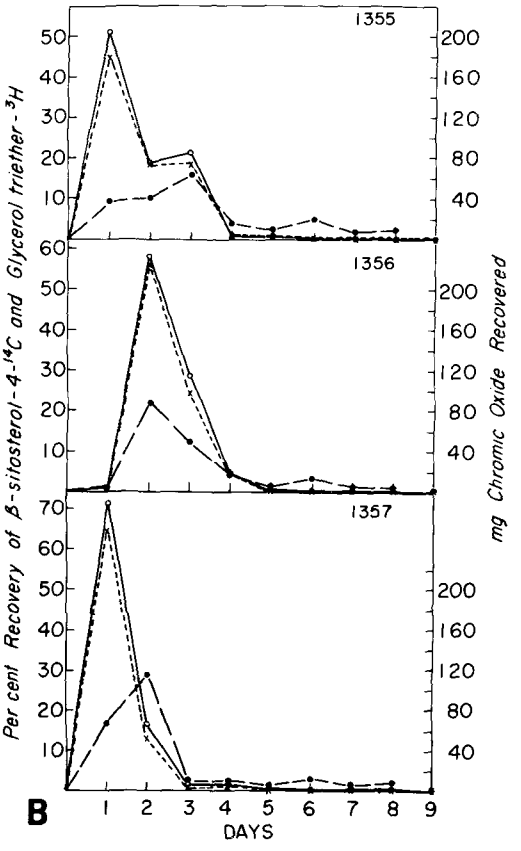
glycerol triether in the nonsaponifiable lipid extract was significantly less than for β-sitosterol. Part of this difference could be made up by the observation of slightly greater glycerol triether radioactivity in the aqueous fraction of the feces following saponification and extraction with Skellysolve B. This did not, however, account for the entire difference, as there were small amounts of glycerol triether (<10%) that were not recovered in any of the fractions analyzed.

Between 85-95% of the glycerol triether recovered in the lipid extract of the feces had chromatographic properties identical with authentic glycerol triether after separation on Silica Gel G, using ethyl ether and Skellysolve B (55:45 v/v) as the developing solvent.

The absorption of β-sitosterol and glycerol triether-^{3}H and their appearance in serum was measured at a day and 3 days following their oral administration. With the exception of two animals, the maximum radioactivity for both isotopes was found in the serum at day 1. Using the figure for maximum serum radioactivity (usually day 1), we calculated the amount of radioactivity in the serum by estimating blood serum volume as 4.5% of body wt (12). By this method, as shown in Table III, a maximum of 3.1% of the administered dose of β-sitosterol and glycerol triether could be accounted for in the serum. There was slightly less glycerol triether in the serum than there was β-sitosterol, but these differences were not statistically significant.

For each animal, the percentage of the administered β-sitosterol and glycerol triether recovered in feces daily for 9 days is shown in Figures 1, 2A, and 2B. Also shown in these





FIGS. 2A and 2B. Fecal recovery of β-sitosterol-4-^{14}C, glycerol triether-^{3}H, and chromic oxide in African green monkeys. β-Sitosterol and glycerol triether values represent recovery in the nonsaponifiable lipid extract of the feces. ○—○ = β-Sitosterol-4-^{14}C, X—X = glycerol triether-^{3}H, and ●– –● = chromic oxide.

TABLE III

Percentage Recovery of Administered β-Sitosterol-4-^{14}C and Glycerol Triether-^{3}H

	β-Sitosterol-4-^{14}C				Glycerol triether-^{3}H			
	Feces[a]				Feces			
Animal number	Nonsaponifiable lipid	Aqueous	Serum[b]	Total	Nonsaponifiable lipid	Aqueous	Serum	Total
Stumptail macaques								
S–1	94.5	8.0	1.6	104	87.0	6.5	0.42	93.9
S–2	93.0	6.1	3.1	102	81.8	5.8	---	87.6
S–3	91.2	7.4	2.9	101	82.3	4.0	1.5	87.8
S–4	97.3	4.9	0.9	103	83.8	3.1	1.8	88.7
Mean ±	94.0[c]	6.6	2.1	102[c]	83.7	4.8	1.2	89.5
SEM[d]	±1.3	±0.7	±0.5	±0.6	±1.2	±0.8	±0.4	±1.5
African green monkeys								
1352	96.9	4.4	0.01	101	96.3	6.5	0.09	103.0
1353	91.5	7.0	0.20	98.7	83.4	17.5	0.44	101.0
1354	86.0	5.7	1.3	98.0	80.1	9.6	1.2	90.9
1355	93.3	5.0	2.8	101	83.9	7.8	0.51	92.2
1356	92.3	4.9	0.41	97.6	84.9	7.5	0.39	92.7
1357	92.0	7.6	0.45	100	80.4	10.1	0.48	91.0
Mean ±	92.0[e]	5.8	0.9	98.6	84.8	9.8	0.52	95.1
SEM[d]	±1.4	±0.5	±0.43	±1.2	±2.4	±1.6	±0.15	±2.2

[a]Recovery of radioactivity in feces represented the total radioactivity recovered in the Skellysolve B fraction (nonsaponifiable lipid) and that remaining in the aqueous fraction after extraction with Skellysolve B.

[b]Radioactivity in the serum represents an estimate of total serum radioactivity based upon an estimate of the serum volume of 4.5% of body wt (12). The radioactivity values are based upon the maximum radioactivity of the serum, which usually occurred on day 1 after the administration of the isotopes.

[c]Significantly greater than for glycerol triether-^{3}H recovery in the same animals. $P < 0.001$.

[d]SEM = standard error of the mean.

[e]Significantly greater than for glycerol triether-^{3}H recovery in the same animals. $P < 0.05$.

TABLE IV

Distribution of Administered β-Sitosterol-4-^{14}C Radioactivity in Fecal Neutral Steroids[a]

Animal number	Day excreted[b]	Dietary fat	β-Sitosterol[c] (% of total neutral steroid radioactivity)	β-Sitostanol	β-Sitostanone
Stumptail macaques					
S–1	2	Safflower oil	30	63	6.8
S–2	2	Butter	11	84	1.4
S–3	2	Safflower oil	46	47	1.9
S–4	3	Butter	15	82	2.4
African green monkeys					
1352	1	Safflower oil	97	1.7	0.47
1353	1	Safflower oil	42	48	6.9
1354	2	Butter	17	80	2.2
1355	1	Butter	36	61	2.7
1356	2	Butter	10	88	1.4
1357	1	Safflower oil	58	34	7.7

[a]Recovery represents the percent radioactivity in each neutral steroid fraction after thin layer chromatography compared with the total radioactivity isolated from the entire thin layer plate.

[b]Represents the feces collected on the day of maximum β-sitosterol excretion.

[c]The β-sitosterol fraction includes cholesterol plus the other plant sterols; the β-sitostanol fraction includes the ring-saturated 5β derivatives of the other plant sterols and cholesterol. The β-sitostanone fraction includes the 3-keto derivatives of the other plant sterols and cholesterol.

figures is the recovery of chromic oxide. The chromic oxide data are presented as actual mg recovered/day, rather than as a percentage of the administered dose. This was necessary, since feces, when digested with strong oxidizing agents used for the chromic oxide procedure, often contained small amounts of color that exceeded that of the blanks. This results in little percentage error during the peak of chromic oxide excretion but does result in significant accumulative error during periods when presumably little, if any, of the administered chromic oxide is being excreted, such as days 4-9. For most sterol balance studies, the chromic oxide is administered daily, and, under these conditions, the error in the determination of chromic oxide is minimal.

Although we did not measure the total percentage of chromic oxide recovered, we do know that the peak of excretion of the chromic oxide, with the exception of animals 1355 and 1357, corresponded well with the excretion of β-sitosterol and glycerol triether.

For all animals, regardless of diet or species, the excretion of β-sitosterol and glycerol triether paralleled one another almost exactly. In stumptail macaques, the bulk of both isotopes was excreted on the second or third day following administration of the markers, while in African green monkeys, the bulk of excretion occurred on day 1 or 2.

To be assured that all of the radioactivity from the administered β-sitosterol that was measured in the fecal nonsaponifiable lipid extract was, indeed, in the form of neutral steroids, we separated an aliquot of the fecal nonsaponifiable lipid extract by TLC into the three neutral steroid classes (9). Results are shown in Table IV. For both species of monkeys, consuming either the safflower oil or butter containing diets, greater than 95% of the ^{14}C radioactivity of the fecal nonsaponifiable lipid extract was found as fecal neutral steroids. For the stumptail macaques, the major excretory form of β-sitosterol was as the ring-saturated 5β-derivative (β-sitostanol), although there were also substantial quantities of unaltered β-sitosterol and small amounts of the 3-keto derivative (β-sitostanone),

The African green monkeys were somewhat more variable, ranging from one animal that excreted the bulk of the β-sitosterol unchanged to others with greater than 80% excreted as the ring-saturated 5β-derivative.

For those animals consuming the safflower oil containing diet, we consistently recovered more of the β-sitosterol unchanged in the feces (an average of 66% for African green monkeys and 38% for stumptail macaques) than for those animals consuming the butter containing diet (an average of 21% for African green monkeys and 13% for stumptail macaques).

DISCUSSION

In this study, neither stumptail macaques nor African green monkeys degraded significant amounts of the steroid ring of β-sitosterol. This is similar to previous results obtained in squirrel monkeys (6).

To our knowledge, only the dog (13) and pig (14), in addition to some human beings, have

been reported to have losses of neutral steroids during intestinal transit. A more recent report, however, failed to confirm the losses of neutral steroids in the dog (15). This is not to say that other members of these species or other species, particularly larger nonhuman primates with longer intestinal transit times, might not share this phenomenon. In man, at least, losses of neutral steroid during intestinal transit appears to be highly individualistic, occurring in some individuals but not in others (2,3). Thus, it may not be possible to say with absolute certainty that *all* individuals of a certain animal species do not degrade the steroid ring simply because the individuals studied did not.

Davignon, et al., (7) showed that the longer the colonic contents remain in the colon, the greater are the chances of degradative losses. Since the intestinal transit time in both species of monkeys studied here, as well as for squirrel monkeys, is 3 days or less, there might not be adequate time for bacteria to act on the sterols of the intestinal contents. This may explain the lack of degradation in these nonhuman primates.

It is also possible that the composition of the diet may influence the extent of steroid ring degradation. Denbesten, et al., (16) showed, for example, that, in man, addition of lactose and bulk (as cellulose) to the diet reduced the loss of neutral steroids. The diets used in our studies, however, were semisynthetic diets low in bulk and, thus, might be expected to favor conditions for degradation.

Although differences in steroid ring degradation were not seen between animal species or diets consumed, there were distinct differences between dietary groups in the excretory form of β-sitosterol in the neutral steroid fraction of the feces. There was substantially more conversion of β-sitosterol to β-sitostanol in animals consuming the butter containing diet, compared with those eating the safflower oil containing diet. Whether this is due to dietary induced differences in intestinal microflora, subtle differences in intestinal transit time, or some other factors, is unknown.

Marsh and coworkers (14) have reported that, like man, the pig apparently can degrade the steroid ring. Unlike man, however, the products of degradation in the pig still remain in the feces and are associated with both the neutral and acidic fractions. After separation of the fecal neutral steroids by chromatography, some of these products of degradation stay at the origin of the thin layer plate while others migrate with the solvent front. This was not the case for either the stumptail macaque or the African green monkey, since greater than 95% of the radioactivity of the lipid extract of the feces was found in the neutral steroids after TLC (Table IV). Whether these observations suggest the operation of different mechanisms for steroid ring breakdown in different animal species is not known.

In this study, we employed three types of markers: β-sitosterol, glycerol triether, and chromic oxide. In all cases, the transit time of β-sitosterol and glycerol triether through the intestine was the same. In all but two cases, the passage of the chromic oxide corresponded exactly with that of the other two markers. In the two animals that were not the same, chromic oxide excretion reached a peak on the day after that of the other two markers. This might be explained by a difference in the passage through the gut of the particulate components of the feces or by a retention of the very dense chromic oxide in the intestine (7).

Although both compounds appeared to be >99% radiochemically pure at the time they were given to the monkeys, the recovery of the glycerol triether was consistently less than for β-sitosterol. One possible explanation for this observation is that small quantities of contaminants with the same chromatographic mobility as the triether could have been present in the sample. If these were lost during intestinal transit and did not appear in the nonsaponifiable lipid or aqueous phases of the feces or in the blood, then they would have escaped detection. It is also possible that some tritium was lost by exchange with hydrogen in vivo or in vitro during analysis. We studied the possibility of exchange in vitro by checking the stability of the glycerol triether-^{3}H by adding it to nonradioactive feces and carrying it through the extraction procedure. When this was done, greater than 95% of the triether was recovered following saponification and extraction with Skellysolve B. Consequently, the losses of glycerol triether-^{3}H must be occurring primarily in vivo.

For accurate sterol balance studies in man, it is necessary to utilize a fecal flow marker, such as chromic oxide, to correct for bile acid recovery, while β-sitosterol is used as an internal standard for correction of steroid ring breakdown. In nonhuman primates, particularly those consuming formula diets, it is difficult to administer chromic oxide precisely on a daily basis, since it separates readily from the other components of the formula diet.

Consequently, it would be an advantage to have a species of nonhuman primate that was known not to degrade the steroid ring. The African green monkeys and stumptail macaques

described in this report, as well as squirrel monkeys studied previously (6), appear to be such animals. Thus, dietary β-sitosterol, by virtue of its not being absorbed or degraded, would be the only internal standard needed for sterol balance studies and could be used to correct for both bile acid and neutral steroid recovery. This greatly simplifies the analytical, as well as the animal care, problems associated with sterol balance studies in these primates. It should be cautioned, however, that we have not shown that all individuals of these species fail to degrade the steroid ring and that this information about individual animals should be known before using β-sitosterol as the only internal standard.

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Triglyceride Composition of *Sapindus mukorossi* Seed Oil

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ABSTRACT

The fatty acid composition of *Sapindus mukorossi* seed oil was determined by spectrophotometry, urea complexation, and gas liquid chromatography (GLC). The percentages of individual acids were found to be: palmitic, 4.0; stearic, 0.2; arachidic, 4.4; oleic 62.8; linoleic, 4.6; linolenic, 1.6; and eicosenoic, 22.4. Triglyceride composition was calculated from the fatty acid compositions of the native oil and of the monoglycerides produced from it by pancreatic lipase hydrolysis. The oil is composed of 0.1, 2.1, 22.0, and 75.8% trisaturated, monounsaturated disaturated, diunsaturated monosaturated, and triunsaturated glycerides, respectively. The special characteristic of the *Sapindus mukorossi* seed oil is its content of 26.3 and 26.7% triolein and eicoseno-di-oleins, respectively.

INTRODUCTION

Sapindaceae, or the soapberry family, is a large one comprised of 12 genera. The seed kernels of the plants in this family are rich in fat which in some cases amounts to as high as 72% by wt of the kernels, and some of these species, such as *Schleichera trijuga*, are utilized commercially in the countries of origin as a source of triglycerides. *Sapindus mukorossi*, a handsome tree, cultivated throughout northwest India, produces hard, glossy, black seeds which contain 30% of kernel and yield a light yellow oil (1,2).

Seed fats of various species of this family have been examined earlier by different groups of workers (3-10). The unsaturated fatty acids in all cases have been reported to consist principally of oleic acid or a mixture of oleic and linoleic acids (60-80%). Lately Hopkins and Swingle (10) have indicated that eicosenoic acid occurs in most of the seed fats of the Sapindaceae family, and, in some cases, it amounts to 42% of the total acids. Three species of the *Sapindus* genus were found to contain 13, 15, and 17% of eicosenoic acid as a constituent of their seed fat (10). Another remarkable feature of these seed fats of the Sapindaceae family is the presence of a large proportion of arachidic acid as a constituent which in some species goes as high as 35%.

Mattson and Volpenhein (11,12) and Gunstone (13) had earlier shown that C_{20}-monoethenoid acid, like saturated acids, belongs to category I and is preferentially esterified at 1 and 3 positions of the glycerine molecule. Eicosenoic acid, thus, differs basically from C_{18} unsaturated acids. This observation adds special interest to the investigation of the glyceride compositions of the seed fats of the sapindaceae family because of the reported presence of arachidic and eicosenoic acids as major constituents in those seed fats. But the glyceride composition of the seed fats of this family had not yet been investigated extensively by the more recently developed techniques.

Two seed fats of Malayan species of the genus *Nephelium* and that of *Schleichera trijuga* were studied by Hilditch and Stainsby (14) and Weerakoon (15) utilizing the intensive crystallization technique. The inadequacy of the crystallization technique in the study of triglyceride composition, especially of those fats containing high percentages of unsaturated acids has been pointed out by Hilditch (16-18). Studies based upon more recently developed techniques like selective enzyme hydrolysis and thin layer chromatography (TLC) give more information about the triglyceride composition. In the present work, the seed oil of *S. mukorossi* of Indian origin has been studied for its fatty acid and triglyceride composition. The percentage fatty acid composition has been determined by gas liquid chromatography (GLC) of the methyl esters. GLC analysis was, however, preceded by segregation of the mixed fatty acids by urea complexation technique so that the minor acids, if there be any, would be promi-

TABLE I

Characteristics of Seed Oil and Mixed Fatty Acids of *Sapindus mukorossi*

Characteristics	Oil	Mixed fatty acids
Specific gravity, 25 C	0.9040	--
Refractive index, n_D, 28 C	1.4680	--
Percent free fatty acids (as oleic)	1.0	--
Saponification equivalent	279.9	287.6
Iodine value (Wij's 1/2 hr)	78.3	84.8
Acetyl value	21.1	--
Reichert value	1.4	--
Polenske value	0.3	--
Unsaponifiable (percent by wt)	1.1	--

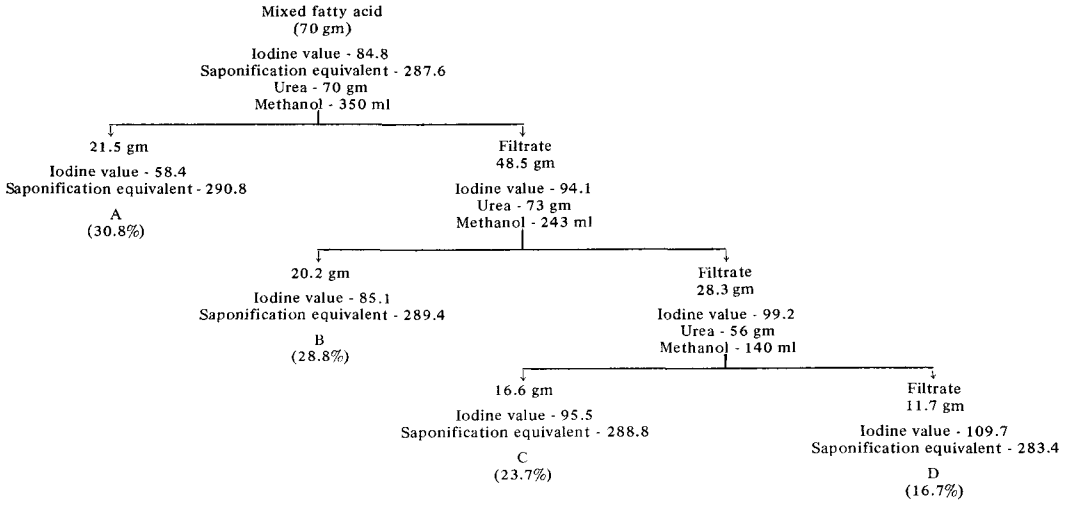

FIG. 1. Fractionation of mixed fatty acids by urea complexation. Computed values: saponification equivalent - 288.7, iodine value - 83.5.

nent enough in either of these fractions analyzed by GLC. The glyceride composition of the seed fat was determined by combination of the techniques of selective enzymatic hydrolysis, TLC and GLC.

Vanderwal (19-21), as well as Coleman and Fulton (22), suggested that the fatty acids in natural fats were distributed according to a 1,3 random, 2 random distribution pattern. Subbaram and Young (23) believed that the assumption of 1,3 random, 2 random distribution held true for a number of fats but could not be applied as a general rule to all fats. In the present investigations, studies were extended to determine the glyceride composition of the fully randomized product of the seed oil of *S. mukorossi* and to find out the changes in composition as effected by the interesterification process. This extension of study was carried out with the idea that a comparison of the triglyceride compositions of the native seed oil and of its randomized product might indicate whether, in the triglyceride molecules of the native seed oil, fatty acids were distributed randomly or not.

EXPERIMENTAL PROCEDURES

S. mukorossi seeds were obtained from established commercial suppliers in Calcutta, and identification of the species was confirmed in consultation with the Botanical Survey of India. The seeds were hulled, the shells were discarded, and the finely ground kernels were extracted with petroleum ether (bp 40-60 C).

The techniques used in the study of fatty acid composition consisted of urea complexation segregation, alkali-isomerization, and spectrophotometric determination of polyethenoid acids and GLC. For segregation of the mixed fatty acids by urea complexation, the method as advocated by Schlenk (24) was adopted. Determination of polyethenoid acids by alkali-isomerization, and spectrophotometry was carried out by the method suggested by Hilditch, et al. (25,26).

The triglyceride composition was based upon selective enzymatic hydrolysis. Lipolysis was carried out as suggested by Coleman (27) at pH 8.5 and 37.5 C using a purified pancreatic lipase preparation with the addition of Ca^{++} ions and bile salts. The partial glycerides were separated on a thin layer (0.3-0.4 mm) of silica by developing with a solvent system of n-hexane, diethyl ether, and acetic acid (80:20:0.25). The monoglyceride fraction detected with 2',7'-dichlorofluorescein was extracted with hot alcohol. The monoglyceride and the original triglyceride samples then were saponified, the free acids liberated, extracted, and converted to methyl esters by an acid catalyzed esterification process.

GLC was carried out with the Hewlett-Packard analytical gas chromatograph (model 700-R12) equipped with flame ionization detector. The column (6 ft x 1/4 in.), packed with 10% polyester diethylene glycol succinate on 60-80 mesh gas chrom Z, was operated at 160 C with a carrier gas flow of 40 ml/min. Peak areas were determined as the product of peak ht and the width at half ht. The wt percentages obtained were converted to mole percentages.

Eicosenate and linolenate formed a combined peak from the diethyleneglycol succinate column (10). The percentage content of eicosenate was calculated by subtracting the spectrophotometrically determined percentage of linolenic acid from the total percentage of eicosenoate and linolenate, as determined by GLC.

S. mukorossi seed oil was randomized at 30 C by the process suggested by Chakrabarty and Bhattacharya (28). A solution of 30 g of refined and bleached oil in 45 ml n-hexane was taken in a 3-necked flask, and to it was added, drop by drop, the catalyst sodium methoxide (methanolic solution, 0.4% w/w of the oil solution). Rearrangement was continued to the equilibrium state. After 60 min, the randomized sample was isolated by the usual procedure after destroying the catalyst with dilute hydrochloric acid.

RESULTS

On extraction with petroleum ether (bp 40-60 C) the seed kernels of *S. mukorossi*, yielded 32.6% of a light yellow oil with a characteristic smell. On analysis by standard procedures, the oil and the mixed fatty acids derived therefrom showed the characteristics given in Table I.

The mixed fatty acids (70.0 g), free from unsaponifiable portion, were next fractionated by urea complexation technique into 4 fractions (A-D), with increasing degree of unsaturation. The scheme of fractionation is shown in Figure 1.

Mixed fatty acids of *S. mukorossi* seed oil and also of the fractions (A-D) were next examined spectrophotometrically for the content of polyethenoid acids. Methyl esters of these mixed fatty acids then were analyzed by GLC. All the results are shown in Table II.

The fatty acid compositions of the 2-monoglycerides obtained from lipolysis of *S. mukorossi* seed oil and its interesterified product next were determined by GLC. The percentage content of linolenic acid in the monoglyceride fatty acids was not determined by spectrophotometric method and the fatty acid composition was based only upon GLC analysis. The content of linolenic acid in the native *S. mukorossi* seed oil was found to be low (1.6%), and all of the linolenic acid was assumed to be linked to the 2 position of the triglycerides. The percentage content of eicosenoic acid was obtained by subtracting 4.8%, i.e. 3 x 1.6, from the total percentage of linolenic and eicosenoic acids, as indicated by the combined peak. The results, along with the fatty acid composition (mole %) of the triglycerides, are given in

TABLE II

Fatty Acid Composition (wt %) of *Sapindus mukorossi* Mixed Fatty Acids and of Fractions A, B, C, and D Obtained by Urea Complexation

Sample	Percent yield	Saponification equivalent	Iodine value	Spectrophotometric analysis: Saturated (by difference)	Spectrophotometric analysis: Monoethenoid (as oleic)	Spectrophotometric analysis: Linoleic	Spectrophotometric analysis: Linolenic	Gas liquid chromatographic analysis: $C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$	$C_{16:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{22:1}$
Mixed fatty acid		287.6	84.8	14.7	78.1	5.6	1.6	4.0	0.2	4.4	-	-	62.8	4.6	1.6	22.4	-
A	30.8	290.8	58.4	38.7	58.3	2.5	0.5	9.9	3.4	18.3	2.5	-	40.6	2.9	0.5	21.6	0.3
B	28.8	289.4	85.1	10.0	86.4	2.8	0.8	4.1	0.1	0.2	-	-	66.6	2.1	0.8	26.1	-
C	23.7	288.8	95.5	4.4	87.2	6.3	2.1	0.9	-	-	-	0.4	68.7	7.1	2.1	20.8	-
D	16.7	283.4	109.7	2.8	78.6	12.5	6.1	0.3	-	-	-	0.6	70.4	12.7	6.1	9.9	-
Computed values (A-D)		288.7	83.5	16.3	76.6	5.2	1.9	4.5	1.1	5.7	0.8	0.2	59.7	5.3	1.9	20.8	0.1

TABLE III

Fatty Acid Composition (Mole %)

Sample	Fatty acids						
	$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$
Sapindus mukorossi triglyceride	4.5	0.2	4.1	64.1	4.7	1.6	20.8
2-Monoglycerides from							
Native seed oil	1.9	-	3.2	73.0	10.5	4.8	6.6
Randomized seed oil	6.0	0.4	7.8	62.7	7.7	4.8	10.6

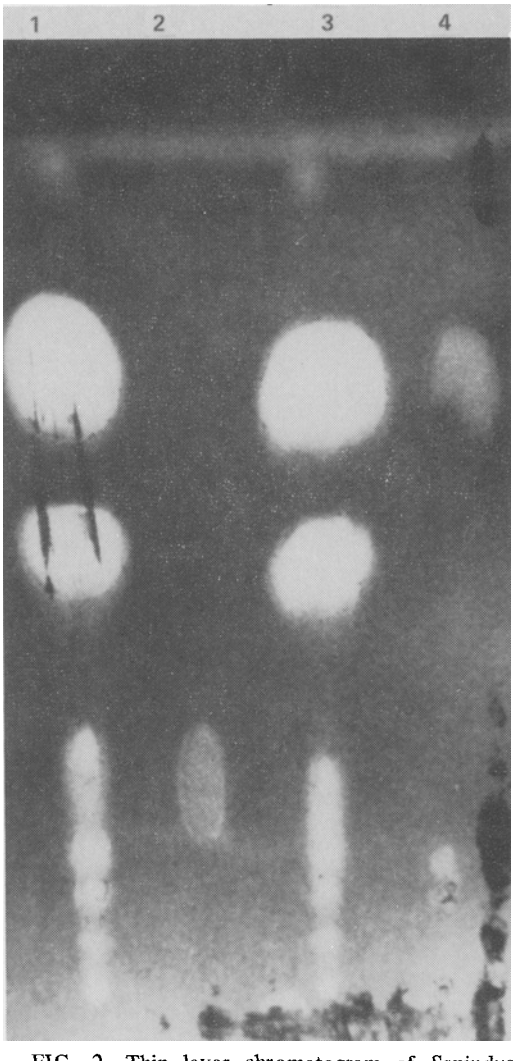

FIG. 2. Thin layer chromatogram of *Sapindus mukorossi* seed oil. 1,3 = seed oil, 2 = stearic acid, and 4 = tripalmitin. Solvent: n-hexane-diethyl ether-acetic acid (80 + 20 + 0.25). Detection by 2′,7′-dichlorofluorescein.

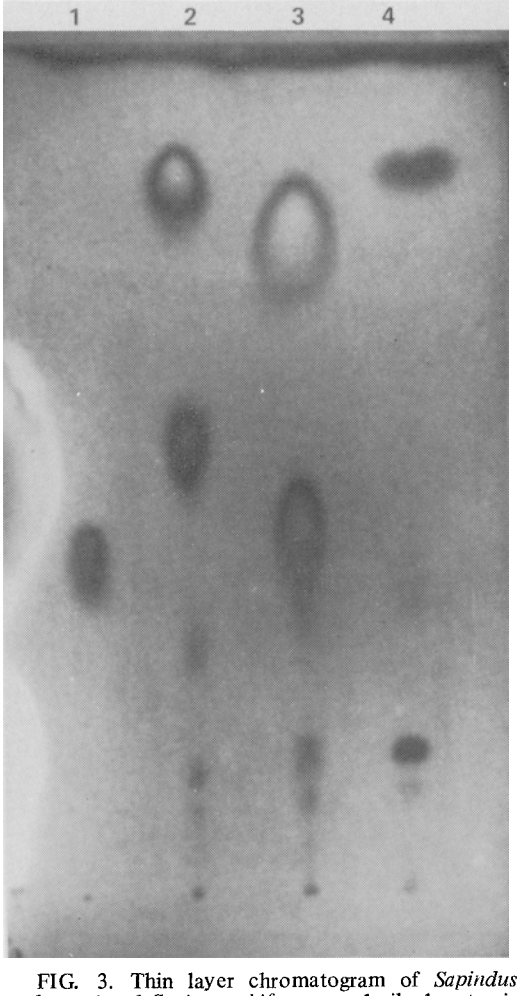

FIG. 3. Thin layer chromatogram of *Sapindus mukorossi* and *Sapium sebiferum* seed oils. 1 = stearic acid, 2 = *S. mukorossi* oil, 3 = *S. sebiferum* oil, 4 = tripalmitin. Solvent: n-hexane-diethyl ether-acetic acid (80 + 20 + 0.25. Detection by 2′,7′-dichlorofluorescein.

Table III.

The triglyceride compositions of *S. mukorossi* seed oil and its randomized product were next calculated from the fatty acid composition of the original triglyceride and the 2-monoglycerides formed using the assumptions of Vanderwal (29) and Coleman (27). While calculating, the fatty acids have been grouped as S-$C_{16:0}$,

TABLE IV

Triglyceride Composition[a] (mole %) of *Sapindus mukorossi* Seed Oil and Its Interesterified Product

Glycerides	Seed oil	Randomized product
Trisaturated (SSS)	0.1	0.1
1,2-disaturated 3-unsaturated (SSU)		
1,2-disaturated 3-olein (SSO)	0.7	1.1
1,2-disaturated 3 unsaturated (SSU)	-	0.1
1,2-disaturated 3-eicosenoin (SSE)	0.3	0.5
Total 1,2-disaturated 3-unsaturated (SSU)	1.0	1.7
1,3-disaturated 2-unsaturated (SUS)		
1,3-disaturated 2-olein (SOS)	0.8	0.2
1,3-disaturated 2-unsaturated (SUS)	0.2	0.1
1,3-disaturated 2-eicosenoin (SES)	0.1	-
Total 1,3-disaturated 2-unsaturated (SUS)	1.1	0.3
1,3-diunsaturated 2-saturated (USU)		
1,3-dioleo 2-saturated (OSO)	1.8	5.9
1-oleo 2-saturated 3-unsaturated (OSU)	0.1	0.6
1-oleo 2-saturated 3-eicosenoin (OSE)	1.7	4.9
1,3-diunsaturated 2-saturated (USU)	-	-
1-unsaturated 2-saturated 3-eicosenoin (USE)	0.1	0.2
1,3-dieicoseno 2-saturated (ESE)	0.4	1.0
Total 1,3-diunsaturated 2-saturated (USU)	4.1	12.6
1-saturated 2,3-diunsaturated (SUU)		
1-saturated 2,3-diolein (SOO)	9.2	4.9
1-saturated 2-oleo 3-unsaturated (SOU)	0.3	0.2
1-saturated 2-oleo 3-eicosenoin (SOE)	4.4	2.0
1-saturated 2-unsaturated 3-olein (SUO)	1.9	1.0
1-saturated 2,3-diunsaturated (SUU)	0.1	-
1-saturated 2-unsaturated 3-eicosenoin (SUE)	0.9	0.4
1-saturated 2-eicoseno 3-olein (SEO)	0.7	0.8
1-saturated 2-eicoseno 3-unsaturated (SEU)	-	-
1-saturated 2,3-dieicosenoin (SEE)	0.4	0.3
Total 1-saturated 2,3-diunsaturated (SUU)	17.9	9.6
Triunsaturated (UUU)		
Triolein (OOO)	26.0	26.3
1,2-dioleo 3-unsaturated (OOU)	1.6	2.6
1,2-dioleo 3-eicosenoin (OOE)	24.6	21.5
1,3-diunsaturated 2-olein (UOU)	-	0.1
1-unsaturated 2-oleo 3-eicosenoin (UOE)	0.7	1.1
1,3-dieicoseno 2-olein (EOE)	5.8	4.4
1,3-dioleo 2-unsaturated (OUO)	5.4	5.2
1-oleo 2,3-diunsaturated (OUU)	0.4	0.5
1-oleo 2-unsaturated 3-eicosenoin (OUE)	5.1	4.3
Triunsaturated (UUU)	-	-
1,2-diunsaturated 2-eiconsenoin (UUE)	0.2	0.2
1,3-dieicoseno 2-unsaturated (EUE)	1.2	0.9
1,3-dioleo 3-eicosenoin (OEO)	2.1	4.0
1-oleo 2-eiconseno 3-unsaturated (OEU)	0.1	0.4
1-oleo 2,3-dieicosenoin (OEE)	2.0	3.3
1,3-diunsaturated 2-eiconsenoin (UEU)	-	-
1-unsaturated 2,3-dieiconsenoin (UEE)	0.1	0.2
Trieicosenoin (EEE)	0.5	0.7
Total triunsaturated (UUU)	75.8	75.7

[a]The fatty acids have been grouped as, S-$C_{16:0}$, $C_{18:0}$, and $C_{20:0}$; O-$C_{18:1}$; U-$C_{18:2}$ and $C_{18:3}$; and E-$C_{20:1}$.

$C_{18:0}$ and $C_{20:0}$, O-$C_{18:1}$, U-$C_{18:2}$ and $C_{18:3}$, and E-$C_{20:1}$. The results are given in Table IV.

DISCUSSION

The results of the present work, along with the previous findings on the fatty acid composition of the seed fats of the *Sapindus* genus are shown in Table V.

The fatty acid composition of *S. mukorossi*, as determined in the present investigation, shows a general agreement with the previous findings on the seed oil of this species, especially in the content of saturated acids. *S. mukorossi* seed oil of Indian origin also has been

TABLE V

Fatty Acid Composition of Seed Fats of *Sapindus* Genus

Species	Habitat	Percent wt									
		Saturated acids					Unsaturated acids				
		$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$	$C_{24:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	C_{18}-epoxy acid
Sapindus drummondii (5)	U.S.	23.0	23.0	23.0	23.0	23.0	57.0	20.0	-	-	-
Sapindus drummondii (10)	U.S.	5.0	trace	3.0	-	-	55.0	16.0	4.0	17.0	-
Sapindus trifoliatus (3)	India	5.6	8.5	21.9	-	2.5	61.5	-	-	-	-
Sapindus trifoliatus (7)	India	5.4	8.5	20.7	2.1	-	55.1	8.2	-	-	-
Sapindus saponaria (10)	U.S.	5.0	4.0	7.0	-	-	65.0	6.0	-	13.0	-
Sapindus mukorossi (35)	U.S.	14.0	14.0	14.0	14.0	14.0	69.0	11.0	3.0	-	0.3
Sapindus mukorossi (10)	U.S.	5.0	1.0	5.0	-	-	54.0	14.0	6.0	15.0	-
Sapindus mukorossi (present work)	India	4.0	0.2	4.0	-	-	62.8	4.6	1.6	22.4	-

found to be rich in the content of eicosenoic acid. The present investigation, however, indicated that a sample of Indian origin contains less of linoleic and linolenic acids and more of oleic acid and eicosenoic acid than the seed oil from Western countries. These variations to the extent of 7.0% in the content of monoethenoid acids in the seed oils from two sources may be attributed to the environmental and ecological factors, rather than to the experimental techniques. Of course in the present case, the contents of polyethenoid acids were determined by both spectrophotometric and GLC analyses. None of the seed fats of the *Sapindus* genus, except that of *Sapindus trifoliatus* was, however, found to contain more than 10.0% of arachidic acid, which is a deviation from the general characteristics of the seed fats of the Sapindaceae family.

Not much work has, however, been carried out on the triglyceride composition of the seed fats from the Sapindaceae family. Only two seed fats from Malayan species of the genus *Nephelium* were studied by Hilditch and Stainsby (14) utilizing the crystallization technique. The glyceride composition of *S. trijuga* seed oil also was examined earlier by Weerakoon and Hilditch and Williams (15-18) by the intensive crystallization technique. Lately, the same seed oil was analyzed utilizing the technique of pancreatic lipase hydrolysis (30). The results of the earlier findings on the triglyceride composition of the seed fats of various species of this family, along with the present findings, are given in Table VI.

A special characteristic of the *S. mukorossi* seed oil is found to be its high content of triunsaturated glycerides (75.8%), of which 26.3% and 26.7% are contributed by triolein and eicoseno-di-olein, respectively. These percentages, however, agree closely with the data calculated for randomization from the fatty acid composition. But the effect of the process of randomization upon the native seed oil which actually accomplished a random distribution becomes evident only when the percentage contents of the positional isomers of different triglycerides, before and after randomization, are taken into account. Thus, on full randomization of the native seed oil, the percentage contents of 1,3-dioleo 2-saturated; 1-saturated 2,3-diolein; 1-oleo 2-saturated 3-eicosenoin; 1-saturated 2-oleo 3-eicosenoin; 1,2-dioleo 3-eicosenoin; 1,3-dioleo 2-eicosenoin; 1,3-dieicoseno 2-olein; 1-oleo and 2,3-dieicosenoin changed from 1.8, 9.2, 1.7, 4.4, 24.6, 2.1, 5.8, and 2.0 in the native seed oil to 5.9, 4.9, 4.9, 2.0, 21.5, 4.0, 4.4, and 3.3, respectively, in the randomized product.

TABLE VI

Glyceride Composition (mole %) of Seed Fats of Sapindaceae Family[a]

Species (reference)	Saturated acid content (mole %)	S_3	S_2U	SU_2	U_3
Nephelium mutabile (14)	55.4	1.5	63.0	35.0	trace
Nephelium lapaceum (14)	49.0	1.4	43.0	55.0	trace
Schleichera trijuga (15)	41.3	1.0	37.0	47.0	15.0
Schleichera trijuga (30)	27.4	0.5	16.7	47.3	35.5
Sapindus mukorossi (present work)	8.8	0.1	2.1	22.0	75.8

[a]S_3 = trisaturated, S_2U = monounsaturated disaturated, SU_2 = diunsaturated monosaturated, and U_3 = triunsaturated.

According to the theory of Gunstone (13), the 2 position of the triglyceride is esterified preferentially with C_{18} unsaturated acids. Present results agree fairly closely with that hypothesis but not completely, since the fatty acids acylating the 2 position were found to be composed of 88.3% of C_{18} unsaturated, 5.1% saturated, and 6.6% of $C_{20:1}$ acids. This analysis further confirms that $C_{20:1}$ acid is esterified preferentially at the 1 and 3 positions of the triglyceride, as suggested by Gunstone (13) and Mattson and Volpenhein (12), and its enrichment factor in the 2-monoglyceride has been found to be 0.3 (31,32).

The sponification equivalent of the seed oil of *S. mukorossi* was found to be less than the saponification equivalent of the mixed fatty acids obtained therefrom. Normally, this difference (7.7) is indicative of the presence of some esters of low mol wt acids. But Miesel and Polensky values (1.4 and 0.3, respectively) of the oil were not indicative of the presence of such lower fatty acids in the glycerides. On the other hand, chromatography of the *S. mukorossi* oil on the thin layer of silica gel, developed with the solvent system: n-hexane-diethyl ether-acetic acid (80:20:0.25), indicated the presence of two types of triglycerides of different polarity, as will be evident from the photorecords of the chromatogram of the oil (Fig. 2). The possibility of the presence of triglyceride containing epoxy acids is excluded by the GLC and TLC study of the methyl esters of mixed fatty acids (33,34). It also may be pointed out that Earle, et al., (35) on the basis of HBr uptake could detect only 0.3% content of C_{18} epoxy acid in the seed oil of *S. mukorossi*.

Sprecher, et al., (36) and Maier and Holman (37) showed that the seed oil of *Sapium sebiferum* (Miesel value, below 2; Polensky value, below 1) contained ca. 25% of a fraction which was little more polar than normal triglycerides. This fraction was characterized by the same authors as monoestolide triglycerides, i.e. (mono) hydroxy triglyceride esterified with ordinary fatty acids. Stillingia oil (seed oil of *S. sebiferum*) earlier had been reported (38) to have lower saponification equivalent than was necessary to conform with its acid composition. It subsequently was established (39,40) that ca. 5% wt of the component acids of stillingia oil consisted of deca-2,4, dienoic acid which recently has been shown (31) to be involved in the estolide linkage. The mono, di-, and triestolide triglycerides also have been detected along side with normal triglycerides in ergot oil (41). The seed oil of *S. mukorossi* gave a chromatogram similar to that of the oil of *S. sebiferum*, when both were chromatographed together on a thin layer of silica gel (Fig. 3). This observation indicates that the more polar component of the seed oil of *S. mukorossi* may be an estolide in character, and some lower hydroxy acid involved in the estolide linkage may be the factor behind the observed discrepancies between the sponification number of the seed oil of *S. mukorossi* and its mixed fatty acids. Further studies on the constitution of the more polar fraction of the seed oil of *S. mukorossi* are being carried out.

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Development of Hyperbetalipoproteinemia in Pigs Fed Atherogenic Diet

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ABSTRACT

Hormel miniature pigs were studied over a period of 24 weeks to observe the changes in serum lipoprotein pattern, cholesteryl ester, free cholesterol, and triglyceride in the atherogenic-fed pigs. These pigs were compared to age-related control animals in our breeding herd. Pigs fed the atherogenic diet (20% tallow, 3% cholesterol, and a bile supplement) exhibited a heterogeneous response but showed mean increases in cholesteryl ester (571 mg/dl) and free cholesterol (226 mg/dl), a slight increase in triglyceride (58 mg/dl), and a severe hyperbetalipoproteinemia. Three animals with the highest cholesteryl ester (all above 600 mg/dl) had resolvable β components in their 1.006 g/ml very low density lipoprotein fraction (type III), as well as huge increases in the S_f 12-20 low density lipoprotein fraction. The other four animals had substantial increases in S_f 0-20, and the three highest had much of their low density lipoprotein in the S_f 12-20, or "remnant" fraction. The test pigs all showed gross lesions in the aorta with an increase in cholesteryl ester and free cholesterol in the tissue as compared with control animals.

INTRODUCTION

The use of the pig as an experimental model

TABLE I

Serum Cholesteryl Ester, Free Cholesterol, and Triglyceride Content of Pigs Fed Control and Atherogenic Diets

Groups	Weeks	Cholesteryl ester (mg/dl)	Free cholesterol (mg/dl)	Triglycerides (mg/dl)
Control	12	82 ± 17[a]	5 ± 5	96 ± 24
	20	82 ± 13	15 ± 18	58 ± 14
	24	72 ± 12	20 ± 24	81 ± 24
Experimental	0	87 ± 5	7 ± 4	47 ± 6
	12	239 ± 22	44 ± 15	65 ± 13
	16	343 ± 25	117 ± 58	50 ± 12
	20	608 ± 117	157 ± 80	62 ± 7
	24	658 ± 152	233 ± 133	139 ± 17

[a]± Standard error of the mean.

TABLE II

Developing Lipoprotein Fractions of Pig Serum

Groups		Relative % of total lipoprotein		
		High density lipoprotein	Very low density lipoprotein	Low density lipoprotein
Control	12 weeks-UC[a]	53.97 ± 1.43[b]	10.39 ± 5.49	35.64 ± 5.62
	20 weeks-UC	52.53 ± 4.90	12.43 ± 5.97	35.05 ± 1.26
	24 weeks-AGE[c]	41.63 ± 1.01	18.14 ± 7.55	40.23 ± 5.19
Experimental	0 weeks-UC	61.50 ± 3.17	8.38 ± 3.03	30.12 ± 1.03
	12 weeks-UC	57.69 ± 4.14	0.98 ± 0.47	41.34 ± 3.93
	16 weeks-AGE	33.09 ± 3.26	2.70 ± 0.17	64.22 ± 3.30
	20 weeks-UC	30.61 ± 5.42	15.39 ± 6.28	54.00 ± 3.04
	24 weeks-AGE[d]	26.29 ± 4.56	2.98 ± 0.47	70.73 ± 12.49

[a]UC = Ultracentrifuge data.

[b]± Standard error of the mean.

[c]AGE = Electrophoresis data.

[d]In at least three cases, there is additional very low density lipoprotein (and reduced low density lipoprotein) because of the presence of "flating β" in the 1.006 g/ml fraction.

for the study of atherosclerosis has become increasingly prevalent due to the close similarity of the pig to the human in many respects, as well as the increasing scarcity of primates for experimental purposes. The realization that changes in the lipoprotein (LP) profile, especially in association with increased serum cholesterol and triglycerides (TG) values, are of fundamental importance in this disease has prompted studies in this direction.

Studies by Janado, et al., (1) have shown swine serum to contain all three classes of LP: high density lipoprotein (HDL), very low density lipoprotein (VLDL), and two classes of low density lipoproteins (LDL_1 and LDL_2). This has been confirmed (R.L. Jackson, private communication). Martin and coworkers (2,3) detected four major LPs by electrophoresis and all the major LP classes by ultracentrifugation. The HDL apoprotein of pig serum was found by Jackson, et al., (4) to have an amino acid content similar to that of the human. These workers, using the Hormel PIGmeePIG miniature pig, also showed that pig HDL had a decrease in the relative content of the apo LP-Gln-II as compared with human HDL, although LP-Gln-I, the major apo LP from human and pig HDL, was stated to have similar chemcial, immunological, physical, and physiological properties.

Calvert and Scott (5) found in pigs fed high cholesterol and high TG diets a rise in serum LDL and a minor rise in HDL. The pattern described differed from the typical type II in man in that the HDL was increased somewhat.

The present study was conducted in the interest of obtaining quantitative and descriptive data on the developing LP profile in the atherogenic-fed pig.

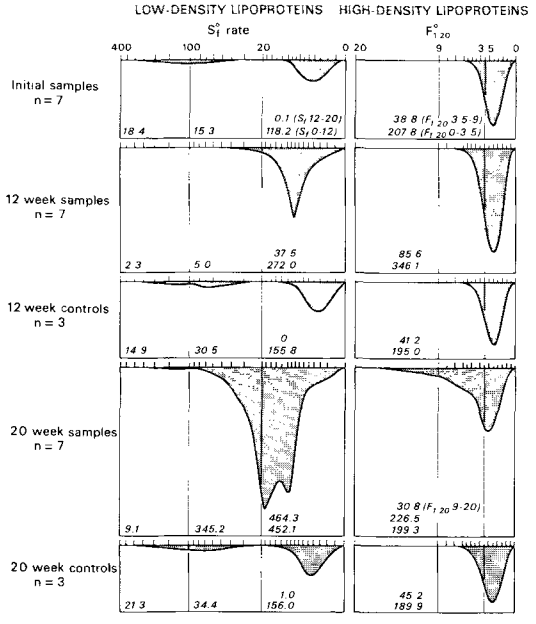

FIG. 1. Total low and high density serum lipoprotein spectra obtained at the three specified time intervals. Concentrations for the indicated lipoprotein classes are given in mg/dl serum.

MATERIALS AND METHODS

Seven weanling pigs, 10 weeks of age, were obtained from The Hormel Institute herd of PIGmeePIG miniature swine and fed an atherogenic diet for 24 weeks. Two females and five castrated males from three litters near the same age were used. Three female control pigs fed a nonatherogenic diet also were sampled. The atherogenic diet (6) contained corn, oats, and soybean oil meal fortified with vitamins and minerals to which was added 20% beef tallow, 3% cholesterol, and 1% hog gall extract (75% solids). The nonatherogenic diet was similar to the above diet without the cholesterol, bile, and tallow. The animals were bled at repeated intervals and total serum cholesterol (7), cholesteryl ester (CE), free cholesterol (FC) (8) and TG (9) were determined. The serum was analyzed at three samplings (at the Donner Laboratory) (0, 12, and 20 weeks) by ultracentrifugation methods (10) to determine the LDL and HDL spectra and by agarose-gel electrophoresis

TABLE III

Cholesteryl Ester and Free Cholesterol in Aortas of Pigs Fed Control and Atherogenic Diets

Groups	Cholesteryl ester (mg/100 g wet tissue)	Free cholesterol (mg/100 g wet tissue)
Control pigs, nonlesion[a]	26 ± 1.8[b]	84 ± 1.0
Atherogenic-fed pigs, nonlesion area	51 ± 19.6	131 ± 24.1
Atherogenic-fed pigs, lesion area	312 ± 192.5	211 ± 46.1

[a]Data from 22 age-related control pigs

[b]Standard error of mean.

of plasma and 1.006 g/ml VLDL fractions (11). Electrophoresis of serum lipoproteins was conducted at 16 and 24 weeks (at The Hormel Institute) using agarose gel slides stained with fat red 7B and quantified (11) by microdensitometry at 530 nm (model RFT, Transidyne Corp., Ann Arbor, Mich.). The aortic tissues of the atherogenic-fed pigs were divided into lesion areas and nonlesion areas and analyzed (8) for CE and FC.

RESULTS AND DISCUSSION

The serum CE, FC, and TG values are shown in Table I. The serum CE and FC of the pigs fed the nonatherogenic diet remained fairly constant at low levels, with the total serum cholesterol less than 100 mg/dl, which is ca. the normal value for this breed. The serum CE and FC values of the pigs fed the atherogenic diet rose markedly and drastically as experienced previously (6) with a major share of the serum cholesterol in the form of CE. The serum TG values of the control pigs remained in the normal range (50-100 mg/dl), while, in pigs fed the atherogenic diet, the values were elevated significantly only at the last sampling period of 24 weeks. This value (139 ± 17), although significantly higher than the normals (81 ± 24), is still not a severely elevated value. Table II shows the relative percentages of the LP fractions of the total LP in serum of the pigs during the experimental period. The experimental pigs acted as their own controls at the start of the experimental period (0 weeks). The values show a consistent pattern for the control pigs throughout the experiment with roughly an equivalent amount of HDL and LDL present and a much lower amount of VLDL. In the atherogenic-fed pigs, the LP pattern changed quite drastically with very large increases in LDL and a moderate and significant increase in HDL. Figure 1 shows the ultracentrifugal values in mg/dl of serum for the total LDL and HDL spectra. LP concentrations in the control pigs remained relatively constant in all fractions. However, the atherogenic-fed pigs showed a huge increase in LDL, particularly of the S_f 12-20, or "remnant" LP class. The VLDL also increased, especially in the S_f 20-50 range, but not as drastically as the LDL fraction. This total LDP spectrum suggests a similarity to type III hyperbetalipoproteinemia—one that should be correctable and reversible. A particular feature was the huge increase in the S_f 12-50 LDL and VLDL which were at extremely low levels in all control animals. Those atherogenic-fed pigs with substantial amounts of $S_f > 20$ exhibited a "floating β" component by agarose-gel electrophoresis in their isolated total VLDL fractions ($\sigma < 1.006$ g/ml). However, unlike the human type III LP profile, total HDL in the atherogenic-fed pigs significantly increased (almost two-fold) and exhibited a broader LP spectrum.

The CE and FC contents of the aortic tissue of the pigs are shown in Table III. The atherogenic-fed pigs all showed lesion areas by gross observation and, although not severe, were graded as grade i (6 pigs) and grade iii (1 pig) (12). The lesion area values for CE and FC, although quite variable (see large standard error) and not statistically different due, in part, to the relatively small number of animals available, did show a large increase in both fractions compared to the control pigs.

This experiment demonstrates the development of a severe hyperbetalipoproteinemia in the pig. Further studies should be extremely valuable, especially in the realm of intervention and potential regression of the atherosclerotic disease process.

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Lipids of Freshwater Dolphin *Sotalia fluviatilis:* Comparison of Odontocete Bioacoustic Lipids and Habitat

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ABSTRACT

The melon and jaw lipids of the freshwater dolphin *Sotalia fluviatilis* are composed mainly of isovaleroyl wax esters and diisovaleroyl triglycerides. The blubber fat contains only a trace of wax ester and is mostly tri- (long chain) and monoisovaleroyl triglycerides. Detailed gas liquid chromatographic analyses of the intact wax esters and triglycerides and of the derived fatty acids and fatty alcohols indicate common compositional patterns in the wax esters and triglycerides of the respective head lipids. Both odd and even long chain (C_{12}-C_{16}) iso-structures are prominent in the melon and jaw lipids, but only higher odd chain length iso-acids are major components in the blubber. *Sotalia fluviatilis* (family Delphinidae) and *Inia geoffrensis* (family Platanistidae) share the same freshwater habitat in the upper Amazon River, and both utilize echolocation to navigate and to find food. Comparison of their respective bioacoustical lipid compositions shows distinctive types of head fats, *Sotalia* being rich in iso-5:0 and *Inia* lacking iso-5:0. This indicates that isovaleric acid per se has no obligatory role in dolphin echolocation.

TABLE I

Lipid Class Composition (wt percent) of *Sotalia* Melon, Jaw, and Blubber Fats

Class	Subclass[a]	Melon	Jaw	Blubber
Wax esters	XV	21.6	47.8	ND[b]
	XX	0.3	1.0	Trace
Diacyl glyceryl ethers	XXX	ND	ND	Trace
Triglycerides	VXV	75.9	48.3	6.7
	XXV	2.0	2.8	40.5
	XXX	0.2	0.1	52.7

[a]X = long chain ($\geq C_8$) fatty alcohol or fatty acid. V = principally isovaleric acid.

[b]ND = not detected under the conditions employed.

INTRODUCTION

Members of the cetacean suborder Odontoceti (dolphins, porpoises, and toothed whales) utilize ultrasonic echolocation to navigate and to find food (1-3). Varanasi and Malins (4,5) and Blomberg (6) recently have postulated that the occurrence of large amounts of isovaleric acid in various odontocete head fats may facilitate echolocation by providing essential bioacoustic properties for sound transmission and refraction. Our recent finding (7) that the jaw fat of the Amazon River dolphin *Inia geoffrensis* (family Platanistidae) contains no isovaleric acid pointed out an inconsistency in this isovalerate/echolocation correlation among dolphins. However, we were uncertain whether this unexpected lipid composition represented the different familial evolutionary relationship of *Inia* or the adaptation of the animal to a freshwater environment. To help answer this

TABLE II

Carbon Number Distribution (mole percent) of Wax Esters in *Sotalia* Melon and Jaw Fats

Alcohol moiety if isovaleroyl wax ester	Carbon number	Melon fat	Jaw fat
Wax esters of short chain acids			
iso-14:0	19	0.2	0.3
n-14:0	19	0.7	0.8
iso-15:0	20	16.6	19.2
n-15:0	20	2.0	2.6
iso-16:0	21	47.4	38.3
n-16:0	21	22.2	28.2
iso-17:0	22	5.4	5.1
n-17:0	22	0.3	0.4
iso-18:0	23	0.2	0.1
n-18:0	23	3.6	2.9
n-20:0	25	Trace	Trace
	Total XV	98.4	97.9
Wax esters of long chain acids			
	24	Trace	Trace
	25	Trace	Trace
	26	Trace	Trace
	27	0.1	0.1
	28	Trace	0.1
	29	Trace	Trace
	30	0.1	0.1
	31	0.4	0.6
	32	0.4	0.5
	33	0.3	0.5
	34	Trace	Trace
	36	Trace	Trace
	Total XX	1.6	2.1

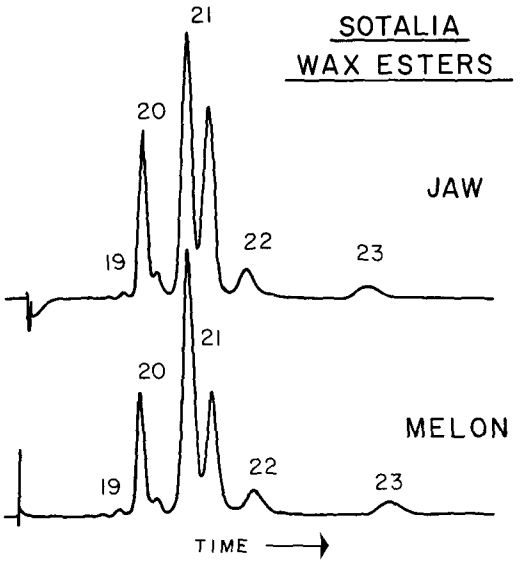

FIG. 1. Gas chromatograms of hydrogenated isovaleroyl wax esters (XV) from *Sotalia fluviatilis* jaw and melon fats. Peaks labeled according to carbon number, i.e. C_{21} = isovalerate (principally) esters of iso-16:0 and n-16:0 alcohols, etc. Operating conditions: stainless steel column 1.80 m x 2.5 mm inside diameter packed with 20% diethyleneglycol succinate polyester on 60-80 mesh Chromosorb W, 190 C, 25 ml/min. N carrier gas.

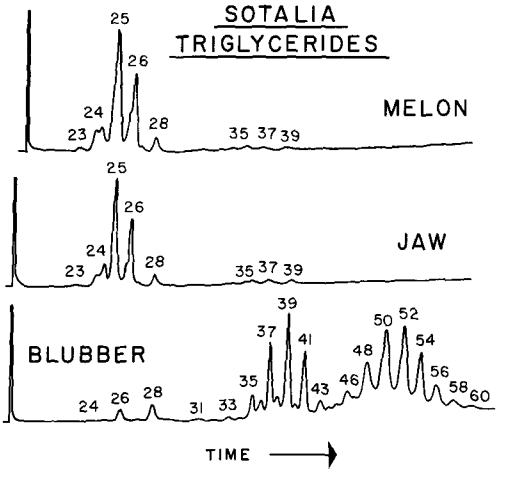

FIG. 2. Gas chromatograms of hydrogenated triglycerides from *Sotalia fluviatilis* melon, jaw, and blubber fats. Peaks labeled according to carbon number; C_{22}-C_{30} = VXV molecules, C_{31}-C_{47} = XXV; and C_{44}-C_{60} = XXX. Operating conditions: stainless steel column 0.60 m x 2.5 mm inside diameter packed with 3% JXR on 100-120 mesh Gas-Chrom Q, column temperature programed 160 → 360 C at 4.6 C/min., 100 ml/min. He carrier gas.

question, we now have examined the lipids of *Sotalia fluviatilis*, a dolphin that shares the common freshwater habitat of the upper Amazon River with *Inia* but is a member of the family Delphinidae in which many isovalerate-rich genera are known (4-6, 8-12). No previous studies on *Sotalia* lipids have been reported in the literature.

EXPERIMENTAL PROCEDURES

Materials

An adult 1.40 m female *S. fluviatilis* [MLF-255] from Marineland of Florida was sampled. The animal was caught in the Napo River near Iquitos, Peru, on November 4, 1968, and transported by air to Marineland, where it was maintained until its death on December 19, 1968. A transverse center section was cut from the melon and divided into three wedge-shaped pieces of equal size. The center piece was subdivided into top, middle, and bottom, and the middle portion was extracted for analysis. The fatty tissue within one of the mandibles was removed and extracted in its entirety. A portion of the blubber layer (8 mm thick from skin to muscle) was removed near the dorsal fin and sliced into inner and outer layers of equal thickness; the outer layer was extracted for analysis. Each fatty tissue was extracted by grinding in a Waring blender with distilled chloroform, slurrying with anhydrous Na_2SO_4, filtering, and stripping off the solvent on a rotary evaporator.

Triglyceride and wax ester standards for chromatography were prepared as previously described (13,14).

Methods

Samples were resolved into lipid classes by preparative thin layer chromatography (TLC) on 200 x 200 x 1.0 mm plates of Adsorbosil-1 (Applied Science Laboratories, State College, Pa.) developed in 87/12/1//petroleum ether/diethyl ether/acetic acid (11,13). Both hydrogenated and unhydrogenated forms of each sample were separated in this manner.

The intact wax ester and triglyceride fractions from the hydrogenated samples were analyzed for carbon number distribution by gas liquid chromatography (GLC) on short, packed JXR-silicone columns, as already described (7,13; Fig. 2). In addition, the hydrogenated short chain wax esters (XV) were analyzed on a packed, 1.80 m, diethyleneglycol succinate polyester (DEGS) column under conditions appropriate for fatty acid methyl esters (Fig. 1). Identification and quantitation procedures for such analyses have been described previously (13,15). The hydrogenated XV wax esters also were examined for short chain esters other

than isovaleroyl by open tubular gas chromatography (9).

Quantitation of lipid classes in the melon and jaw fat samples was accomplished by GLC of the total hydrogenated lipids (wax esters + triglycerides) on the JXR-silicone column and by observing the area ratio of the C_{21} wax ester and the C_{25} triglyceride peaks. Calibration factors (15) based upon GLC runs of known composition wax ester/triglyceride mixtures then were used to calculate the wt ratio of C_{21} wax ester to C_{25} triglyceride in the total sample. Since the carbon number distributions (wt percent) of the wax esters and triglycerides had been determined already, the lipid class composition (wt percent) could be calculated directly.

The wax ester and triglyceride fractions from the unhydrogenated samples were converted to alcohol acetates and fatty acid methyl esters (7) for detailed GLC study on 46 m x 0.25 mm inside diameter open tubular columns coated with butanediol succinate polyester or Apiezon L (7,13). Isovaleric acid was characterized and quantitated by GLC of the derived butyl esters on a 2.00 m x 3.0 mm inside diameter column packed with 5% SE-30 silicone (13). Presentation of fatty acid and fatty alcohol compositional data to two decimal places is solely for comparative purposes and does not imply this order of accuracy.

TABLE III

Carbon Number Distribution (mole percent) of Triglycerides in *Sotalia* Melon, Jaw, and Blubber Fats

Carbon number	Melon fat	Jaw fat	Blubber fat
Diisovaleroyl triglycerides			
22	ND[a]	0.2	ND
23	1.3	0.9	ND
24	14.3	15.4	0.1
25	53.2	43.8	0.5
26	25.4	29.1	2.0
27	0.3	0.3	0.1
28	2.7	4.6	4.0
30	Trace	0.1	ND
Total VXV	97.2	94.4	6.7
Monoisovaleroyl triglycerides			
31-34			1.3
35	1.8	2.8	2.6
36			1.7
37	0.4	1.4	9.1
38	0.1	0.1	1.2
39	0.3	1.1	13.1
40	ND	ND	0.6
41	Trace	0.1	7.3
42	ND	ND	0.5
43	Trace	Trace	1.4
45	ND	ND	1.0
47	ND	ND	0.6
Total XXV	2.6	5.5	40.4
Triglycerides without isovaleric acid			
44	Trace	Trace	0.5
46	Trace	Trace	2.6
48	0.1	Trace	8.0
50	Trace	Trace	14.1
52	Trace	Trace	14.2
54	Trace	Trace	8.3
56	ND	ND	3.8
58	ND	ND	1.3
60	ND	ND	0.1
Total XXX	0.2	0.1	52.9

[a]ND = not detected under the conditions employed.

RESULTS AND DISCUSSION

Lipid Composition

Lipid class compositions (Table I) show that *Sotalia* melon and jaw fats are mixtures of wax esters and triglycerides, while the blubber fat is almost entirely triglycerides. The level of wax esters in the jaw fat is more than twice that found in the melon. Diacyl glyceryl ethers are present in the blubber lipids but at a level of <1% as checked by TLC against authentic mixtures of known proportions. Isovaleroyl wax esters (XV) and diisovaleroyl triglycerides (VXV) are the predominent subclasses in the melon and jaw, but long chain (XXX) and monoisovaleroyl (XXV) triglycerides predominate in the blubber. The possible presence of triisovalerin was checked by both TLC and GLC, but none was found in any of the samples. The short chain wax esters, although designated XV for convenience, were found to contain small amounts of 2-methylbutyrate and isobutyrate esters when examined by open tubular GLC. Actual mole percentages were iso-5:0/anteiso-5:0/iso-4:0//96/2.9/1.1.

The high level of isovalerate wax esters and triglycerides in *Sotalia* head fats is typical for the Delphinidae family and clearly distinguishes the head fats from the blubber. Similar findings have been reported for eight other genera in this family by Litchfield, et al. (11,12). Although XV wax esters are prominent in all Delphinidae head fats examined to date, the levels of such wax esters and the relative amounts in the melon and jaw fats from the same animal seem to vary widely and follow no discernable pattern. However, the XV wax ester content of *Tursiops truncatus* (16) and *Globicephala melaena* (8) melon fats has been shown to vary widely with the exact location sampled within the melon, so perhaps the direct comparison of wax ester levels in Delphinidae head fat samples collected by different investigators is not justified.

The chain length and carbon number distri-

butions for the wax esters (Table II, Fig. 1) demonstrate the similarity of molecular species in both bioacoustic lipids. The carbon number distributions for triglycerides (Table III, Fig. 2) indicate that the VXV triglycerides are quite closely related in both melon and jaw lipids but are quite different from the VXV of the blubber. The XXV triglycerides have lower carbon numbers in the melon and jaw fats than in the blubber. Both trends point toward longer X fatty acid chains in the blubber triglycerides. Most other components are too minor to

TABLE IV

Fatty Acid Composition (mole percent) of *Sotalia* Melon, Jaw, and Blubber Fats

	Melon		Jaw		Blubber
Fatty acid	Wax esters	Triglycerides	Wax esters	Triglycerides	Triglycerides
iso-5:0	98.82	68.15	97.87	61.89	18.94
n-10:0	ND[a]	ND	ND	0.14	0.05
iso-11:0	ND	0.15	ND	0.24	0.17
anteiso-11:0	ND	ND	ND	0.21	0.10
n-11:0	ND	0.05	ND	Trace	0.05
iso-12:0	ND	0.09	ND	0.15	0.44
n-12:0	ND	0.12	ND	0.31	0.74
iso-13:0	ND	0.66	ND	0.54	0.47
anteiso-13:0	ND	0.02	ND	0.05	0.22
n-13:0	ND	0.08	ND	0.17	0.54
iso-14:0	0.03	2.35	0.04	2.04	0.91
n-14:0	0.26	2.71	0.28	4.07	4.81
iso-15:0	0.11	13.87	0.52	11.71	1.14
anteiso-15:0	0.02	0.74	0.02	0.55	0.39
n-15:0	0.05	0.40	0.05	0.63	1.43
iso-16:0	0.05	2.79	0.28	1.97	0.47
n-16:0	0.33	3.55	0.40	6.28	8.39
iso-17:0	0.02	0.27	0.08	0.60	0.25
anteiso-17:0	Trace	0.08	0.02	0.14	0.15
n-17:0	0.05	0.29	0.01	0.15	0.22
iso-18:0	0.01	Trace	Trace	Trace	0.05
n-18:0	0.03	0.53	0.03	0.53	1.36
Total saturated	99.8	96.9	99.6	92.4	41.3[b]
14:1ω9	ND	ND	Trace	0.09	0.30
14:1ω7	0.01	0.02	ND	0.10	0.67
14:1ω5	Trace	0.02	0.01	0.04	1.26
15:1ω?	ND	Trace	Trace	0.02	0.12
15:1ω?	0.01	0.03	0.01	0.03	0.30
16:1ω9	0.05	0.03	0.09	0.33	1.93
16:1ω7	0.07	1.77	0.14	3.33	19.25
16:1ω5	Trace	0.01	Trace	0.09	0.30
7-Methyl-hexadecenoic	ND	0.02	ND	0.10	0.20
17:1ω8	Trace	0.03	Trace	0.10	0.81
18:1ω11+9	0.07	0.74	0.12	2.03	19.58
18:1ω7	Trace	0.12	0.02	0.43	3.43
18:1ω5	ND	0.02	Trace	0.05	0.10
20:1ω11	ND	ND	ND	0.05	0.05
20:1ω9	ND	ND	ND	0.17	0.57
20:1ω7	ND	ND	ND	0.02	0.05
Total monoene	0.2	2.8	0.4	7.0	49.5[c]
18:2ω6	0.01	0.22	0.01	0.46	5.84
18:3ω3	Trace	0.07	Trace	0.19	1.38
Total polyene	<0.1	<0.3	<0.1	0.7	9.2[d]

[a]ND = not detected under conditions employed.

[b]Includes: n-19:0, 0.7%; and n-20:0, 0.15%.

[c]Includes: 12:1, 0.15%; 19:1, 0.12%; 22:1ω13+11, 0.20%; 22:1ω9, 0.07%; and 22:1ω7, trace.

[d]Includes: C_{16} polyene acids totalling 0.37%; 18:3ω6, 0.05%; 18:4ω3, 0.07%; 20:2ω6, 0.12%; 20:3ω6, 0.39%; 20:3ω6, 0.17%; 20:4ω6, 0.54%; 20:4ω3, 0.05%; and 20:5ω3, 0.25%. C_{22} polyene acids could not be detected.

permit confident comparisons.

A detailed study of the fatty acids in the wax esters and triglycerides (Table IV) also exposes the fundamental differences between lipid classes and concurrently the similarities within the two sets of lipids from the bioacoustic tissues. The extremely high isovaleric acid content of the wax esters made the study of the other fatty acids difficult; but, by isolating the XX band for study separately from the XV band (14), the GLC background noise was diminished. Isobutyric acid was a relatively minor component of wax ester acids compared to the 26.8 mole percent found in a jaw fat from *T. truncatus* (9). The various long chain saturated acids (normal and iso) are present in ca. parallel proportions in the respective jaw and melon wax esters and triglycerides, implying a common biosynthetic origin in the two tissues. This idea further is supported by the similar iso-5:0 contents of the wax esters (98.8% vs 97.9%) and triglycerides (68.2% vs 61.9%) in the fatty head tissues.

The greater importance of iso-15:0 and iso-13:0 relative to n-15:0 and n-13:0 acids, respectively, in *Sotalia* melon and jaw fats (Table IV) has been reported previously in bioacoustic lipids of the Delphinidae (*G. melaena* [8], *Tursiops gilli* [4], *T. truncatus* [9], *Stenella attenuata* [10]), the Monodontidae (*Delphinapterus leucas* [13]), and the Phocoenidae (*Phocoena phocoena* [17], *Neomeris* [*Neophocoena*] *phocoenoides* [18]) families. These fats all have major concentrations of isovaleric acid. On the other hand, the even numbered iso-12:0, iso-14:0, and iso-16:0 showed a different trend in genera where isovaleric acid is prominent, generally comprising less than the n-12:0, n-14:0, and n-16:0 levels, respectively. In the head lipids of *I. geoffrensis* (7,19) and *Physeter catodon* (20) where there is little or no isovaleric acid, there is quite a different relationship among these acids. Iso-13:0 and iso-15:0 are ca. equal to or less than the n-13:0 and n-15:0, while iso-12:0, iso-14:0, and iso-16:0 are ca. one-fifth to one-twentieth the n-12:0, n-14:0, and n-16:0 levels.

It is known that odd numbered iso-acids originate by chain elongation of isovaleroyl-CoA and even numbered iso-acids similarly from isobutyroyl-CoA in mammals (21). Hence, the higher levels of odd numbered iso-acids in the Delphinidae, Phocoenidae, and Monodontidae families are probably a result of their accelerated isovaleric acid metabolism which generates iso-5:0 from leucine (22). It should be noted, however, that the fraction of long chain saturated acids having the iso-structure (even + odd) is much lower in the nonisovalerate families (*Inia* jaw triglycerides = 0.13 [7], and *Physeter* melon triglycerides = 0.06 [20]) than in the isovalerate-rich families (*Sotalia* jaw triglycerides = 0.57; *Delphinapterus* melon triglycerides = 0.60 [13]; *Globicephala* melon wax esters = 0.69 [8]; and *Phocoena* jaw triglycerides = 0.39 [17]). This difference indicates a much lower level of chain elongation activity for branched chain C_4 and C_5 acids in the Platanistidae and Physeteridae than in the Delphinidae, Phocoenidae, and Monodontidae.

TABLE V

Fatty Alcohol Composition (mole percent) of *Sotalia* Melon and Jaw Wax Esters

Fatty alcohol[a]	Melon	Jaw
iso-14:0	0.28	0.25
n-14:0	1.16	1.27
iso-15:0	16.99	23.24
anteiso-15:0	0.74	Trace
n-15:0	2.29	2.79
iso-16:0	46.31	38.41
n-16:0	20.94	25.91
iso-17:0	4.58	3.54
anteiso-17:0	1.33	0.59
n-17:0	0.42	0.51
iso-18:0	0.23	0.17
n-18:0	0.57	0.73
Total saturated	95.8	97.4
16:1ω7	1.92	0.79
18:1ω11+9	2.23	1.80
Total monoene	4.2	2.6

[a]The shorthand notation is the same as is commonly used for fatty acids.

The fatty alcohols from the two bioacoustic fats of *Sotalia* are also very similar (Table V) and show good agreement with the carbon number distribution found by GLC of the intact XV wax esters (Table II). Iso-15:0, iso-16:0, and n-16:0 comprise over 84% of the alcohol chains in both the melon and jaw. The distribution of alcohol moieties does not resemble either the wax ester or the triglyceride fatty acids in their respective tissues, indicating considerable chain selectivity in the reduction of acids to alcohols.

Comparison of Bioacoustic Lipids and Habitat

The two dolphins *S. fluviatilis* (family Delphinidae) and *I. geoffrensis* (family Platanistidae) share a common freshwater habitat in the upper Amazon River, and both apparently utilize echolocation to navigate and to capture food in the muddy waters in which they live. Laboratory experiments have positively demonstrated echolocation by *Inia* (23); and there is good behavioral evidence (24,25) that *Sotalia*

also echolocates, based upon its emission of typical echolocation-type click trains when approaching a fish or other object underwater. Comparison of the bioacoustical lipid compositions reported here for *Sotalia* with those reported earlier for *Inia* (7,19) clearly indicates two distinctive types of head fats.

In *Inia* (7,19), the wax esters and triglycerides are dominated by the C_{12}, C_{14}, and C_{16} chain length fatty acids, while the fatty alcohols are mostly the straight C_{16} chains found in many marine animals, including the sperm whale *P. catodon* (20). Iso-acids of C_{10}-C_{18} chain lengths are present in small amounts, and isovaleric acid is essentially absent. Similar fatty acid compositions have been reported for *Platanista gangetica* melon (26), the only other member of the Platanistidae for which lipid data are available.

In *Sotalia*, however, an altenative type of bioacoustic lipid has evolved based essentially, but not exclusively, upon the C_5 isovaleric acid. Chain lengthening of the highly available iso-5:0 precursor in *Sotalia* alters the composition of the $\geq C_{10}$ fatty acids and fatty alcohols by incorporating much higher levels of the iso-structures. Thus, the freshwater *Sotalia* clearly has retained the typical isovalerate-rich bioacoustic lipids found in all the saltwater Delphinidae (11,12).

It is evident, therefore, that the familial evolutionary relationship, rather than the common freshwater environment, is the determining factor for bioacoustical lipid compositions in *S. fluviatilis* and *I. geoffrensis*. Since both animals apparently echolocate effectively, we conclude that isovaleric acid per se has no obligatory role in dolphin echolocation.

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Peanut Alkaline Lipase[1]

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ABSTRACT

Peanut alkaline lipase, (glycerol ester hydrolase EC 3.1.1.3), pH optimum 8.5, was isolated from acetone powders prepared from developing and germinated peanut seed (*Arachis hypogaea* L. var. NC-2). Enzyme activity/seed increased in successive developmental stages. The course of the hydrolytic reaction was linear with regard to enzyme concentration and all times tested up to periods exceeding 60 min. K_m for the reaction was determined to be 2.6 x 10^{-4}M. Molecular weight of peanut lipase, as estimated by Sephadex gel filtration and sodium dodecyl sulfate gel electrophoresis, was ca. 55,000.

INTRODUCTION

In the literature, studies on lipase are entangled confusingly with studies on esterases (1). Sarda and Desnuelle (2) indicate that lipases act only on substrates in heterogeneous solutions and do not act, or act very slowly, on water-soluble substrates. Using this criterion, enzymes that catalyze the hydrolysis of all but the most soluble substrates (Tweens, triacetin, and p-nitrophenylacetate) may be classified as true lipases.

In plants, lipase investigations have been most extensive on oleaginous seed where lipase activity generally is manifest upon germination. Peanut lipase investigations (3-7) have been few and almost exclusively concerned with lipase from germinated seed. Data from some of the studies indicate the presence of lipase in ungerminated peanuts, e.g. activity at 0 days germination (3,6,7). Also, Pickett and Holley (8) reported lipase activity in mature and immature cured peanuts.

This is a report of some characteristics of a partially purified lipase from maturing and germinated peanut seed, although data from developing seed only are presented, since the various characteristics examined were the same from the two sources.

MATERIALS AND METHODS

Materials

Peanut seed (*Arachis hypogaea* L. var. NC-2) were acquired from the North Carolina Peanut Belt Research Station, Lewiston, N.C. Ammonium sulfate (special enzyme grade) and tributyrin were obtained from Mann Research Laboratories, Orangeburg, N.Y., and corn oil was purchased commercially as Mazola corn oil. Sephadex G-150 and DEAE-Sephadex A-50 were from Pharmacia Fine Chemicals, Piscataway, N.J.; acrylamide (electrophoresis grade), N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylmethanediamine were from Eastman Organic Chemicals, Rochester, N.Y.; sodium dodecyl sulfate (SDS) was purchased from Matheson, Coleman, and Bell, Norwood, Ohio.

Enzyme Source

Immature peanut seed were hand-picked and shelled, and stage of development was estimated by the methods of Pattee, et al. (9). Seed of the same approximate age were grouped and stage of development verified by comparison of percent moisture of each sample with percent moisture of the various stages determined in preliminary studies.

Lipase from germinated peanuts was obtained from mature peanuts dusted with a fungicide and germinated in moist vermiculite at 25 C in the dark.

Purification

Acetone powders were prepared from all seed samples according to the procedure of Pattee and Swaisgood (10). Lipase was extracted from acetone powder by stirring for 1 hr at room temperature in 20 volumes of 0.9% NaCl. The slurry obtained was centrifuged at 11,700 x g for 15 min (this force and time were used through the 60% $(NH_4)_2SO_4$ precipitation) and filtered through Whatman no. 4 filter paper. This crude extract was used as an enzyme source in selected studies. The crude extract was taken to 40% saturation with solid $(NH_4)_2SO_4$, and, after standing for 1 hr at 6 C, the suspension was centrifuged. The precipitate obtained was assayed and discarded, and the supernatant was taken to 60% $(NH_4)_2SO_4$ saturation. The precipitate obtained after 1 hr at 60% $(NH_4)_2SO_4$ was pelleted by centrifugation, the supernatant decanted, and the precipi-

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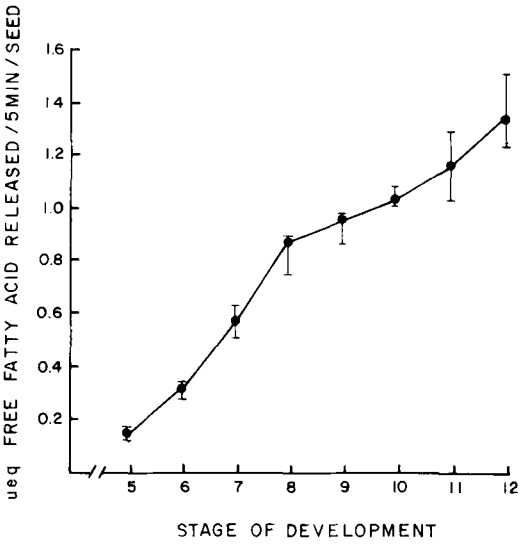

FIG. 1. Changes in lipase activity during development of peanut seed (tributyrin substrate).

tate redissolved in a small volume of 0.9% NaCl. This solution was used in selected studies after overnight dialysis against 0.9% NaCl. For further purfication, enough cold 95% ethanol (ETOH) was added to the dissolved 60% $(NH_4)_2SO_4$ precipitate to obtain a 20% ETOH solution which was placed in a cold room at 6 C for 1 hr and then centrifuged at 25,300 x g for 10 min (this force and time were used for the remainder of the purification). The resulting pellet was discarded. The supernatant was dialyzed against 0.9% NaCl for 3 hr, centrifuged, and lipase precipitated by 70% $(NH_4)_2SO_4$ saturation. The precipitate was pelleted by centrifugation, the supernatant decanted, and the pellet redissolved in a small volume of 0.1 M phosphate buffer, pH 7.0. This enzyme solution was dialyzed overnight against 0.1 M phosphate buffer, pH 7.0.

Gel Chromatography

The dialyzed 70% $(NH_4)_2SO_4$ precipitate was placed on a Sephadex G-150 column (2.5 cm x 45 cm) which had been equilibrated with 0.1 M phosphate buffer, pH 7.0. Reverse-flow elution was performed with the same buffer using a flow rate of 16 ml/hr. Two ml aliquots were collected automatically and protein elution was monitored continuously by absorbance at 280 nm. Active fractions were pooled and used for characterization studies.

Assay Procedure

The lipase assay was a modification of the titratable acidity procedure of San Clemente and Vadehra (11) in which the reaction mixture pH is held constant against acid production by the addition of suitable base. The reaction mixture normally consisted of 5 ml 0.01 M tributyrin-0.1% Tween 20 mixture, emulsified by high speed blending for 1 min; 2 ml enzyme solution; and 3 ml water. The constituents were mixed in a small reaction vessel and allowed to equilibrate, in regard to temperature and pH, for 5 min before beginning the assay. Standard assay conditions were 25 C and pH 8.5; activity was reported as μeq of free fatty acid released/5 min assay. Titration with 0.01 N NaOH was done automatically using a Radiometer pH meter, PHM 26, in connection with a Radiometer titrator, TTT11, and Radiometer autoburette, ABU12.

Blank assays were conducted to determine acid production by reactions other than enzymatic hydrolysis of the lipid substrate. Except for slight activity with Tween 20 and alkaline hydrolysis, which was noted and considered during determination of the higher ranges of the pH curve, no acid production was detected. Enzymatic hydrolysis was verified by thin layer chromatography of blank and standard tributyrin assays. Diglycerides present in the standard assay, but absent in the blank, indicated that substrate hydrolysis at pH 8.5 was a result of enzyme activity.

TABLE I

Purification of Lipase from Developing Peanut Seed

Fraction	Specific activity	Percent recovery	Purification
Crude extract	1.92		
40-60% $(NH_4)_2SO_4$	6.33	58.4	3.3
20% Ethanol	12.64	53.01	6.58
70% $(NH_4)_2SO_4$	17.99	46.72	9.37
Sephadex G-150	31.86	23.36	16.59

Protein Determination

Protein was estimated routinely by measuring absorbance at 280 nm and quantitated using the spectrophotometric method described by Layne (12).

Sodium Dodecyl Sulfate Gel Electrophoresis

Before gel electrophoresis, the pooled Sephadex G-150 fractions were concentrated using an Amicon pressure cell, and placed on a DEAE-Sephadex A-50 column (1.5 cm x 95 cm) equilibrated with 0.1 M phosphate buffer, pH 7.0. The column was developed with a linear gradient of 0-0.4 M NaCl in 0.1 M phosphate buffer, pH 7.0.

SDS gel electrophoresis of proteins in the active peak from DEAE-Sephadex A-50 was

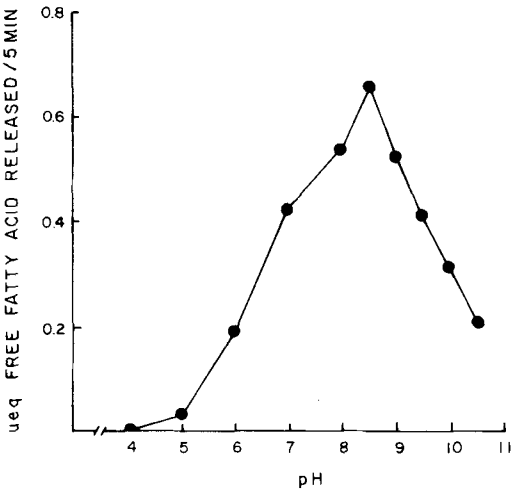

FIG. 2. Effect of pH upon peanut lipase activity (5.8 mg protein/assay, .01 M tributyrin substrate).

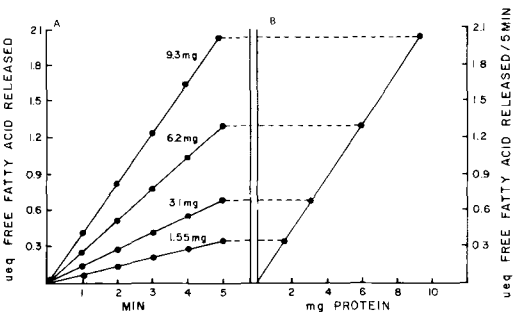

FIG. 3A. Effect of time upon rate of tributyrin hydrolysis by various peanut lipase concentrations. 3B Effect of enzyme concentration upon peanut lipase activity.

carried out according to the methods of Weber and Osborn (13), except that the procedure was conducted at room temperature using an apparatus that did not have a thermostatted jacket. Under these conditions, the marker dye moved three-fourths through the gel in 6-7 hr. Gels were stained with Coomassie brilliant blue and destained electrophoretically.

RESULTS AND DISCUSSION

Lipase Activity in Developing Seed

Lipase activity increased steadily as development proceeded from stage 5-stage 12, with stage 12 being an almost mature seed (Fig. 1). Data, which are presented as activity units/seed, indicate that activity was highest in the mature seed. However, conversion of the data to activity/g acetone powder would indicate that activity was highest in the immature seed. This comes about, because, at stage 6, many more seeds were needed to produce 1 g acetone powder than at stage 12 (64 and 2.85 seeds, respectively). Pickett and Holley (8) reported that cured immature seed appeared to contain more lipase than cured mature seed when activity was reported/g defatted seed material.

Kartha (14) found that lipase activity in seeds of *Azadiachta indica* was maximum, on a per seed basis, by the time oil development reached 45% of maximum and overall seed development reached 50%. With peanuts, however, activity increased past stage 9-10 when oil content approaches maximum value (15) and overall seed development is well beyond 50%.

Purification

The lipase obtained after extraction, precipitation, and chromatographic procedures represented a 14-17-fold purification with tributyrin substrate (Table I). Attempts to increase purification by stabilizing the enzyme with β-mercaptoethanol and 0.9% NaCl throughout the procedure were of little consequence.

pH Optimum

Optimum pH of lipase from peanut seed was 8.5 (Fig. 2). However, optimum pH reported for lipase from germinated peanut seed varied from acid to alkaline. Using different assay methods, St. Angelo and Altschul (5) reported an optimum pH of 4.6, Ramakrishnan (3) reported an optimum of 5.2 and Urs, et al., (4) reported maximum activity at ca. pH 8.

Rate of Hydrolysis

The rate of hydrolysis of a typical peanut lipase reaction using various enzyme concentrations and 0.01 M tributyrin as substrate is presented in Figure 3A. The reaction was linear with time for each enzyme concentration. A linear rate of hydrolysis was exhibited for over 60 min by some dialyzed 60% $(NH_4)_2SO_4$ precipitates (data not presented). The strict linearity with time denotes a zero order reaction indicating that substrate was not limiting and no inhibitory action was exhibited by the products. As with castor bean acid lipase (16), peanut lipase demonstrated a slower rate of hydrolysis with long chain triglycerides (corn oil) than with short chain triglycerides (tributyrin).

Enzyme Concentration

The results of varying enzyme concentration with a constant substrate concentration, when plotted, were linear and passed through the origin (Fig. 3B). The conditions for measuring lipase activity in other experiments generally fell within the values presented here.

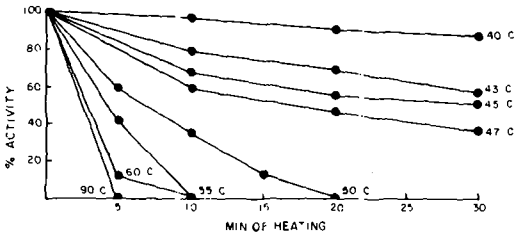

FIG. 4. Time course of heat inactivation of peanut lipase.

Substrate Concentration

The lipase activity curve produced by varying substrate concentration was a typical rectangular hyperbola. The Michaelis-Menten constant, K_m, as calculated using a Lineweaver and Burk double reciprocal plot, was 2.6 x 10^{-4}M. The value was the same with different concentrations of enzyme and with peanut lipase from developing and germinated seed. This value lies between the K_m of rapeseed lipase, 4.06 x 10^{-4}M (17), and the K_m of wheat germ lipase, 3.1 x 10^{-5}M (18).

Stability

Stability of the enzyme in acetone powder, held at -20 C, was examined over a 6 month period, and no apparent loss in activity was detected. Loss of activity in crude extracts was noticeable in 6-8 hr, but stability was extended to several days when the enzyme was purified partially by passage through Sephadex G-150.

To determine heat stability, 60% $(NH_4)_2SO_4$ precipitate dissolved in 0.9% NaCl was dialyzed overnight against 0.9% NaCl and subjected to preselected temperatures for various times (Fig. 4). Temperatures approaching 50 C drastically altered activity, but 30 min at 40 C had little effect. Peanut lipase appears to be more heat labile than castor bean lipase which lost only ca. 20% of its activity during 30 min at 60 C (19).

Mercuric Chloride Inhibition

The activity of peanut alkaline lipase was inhibited by $HgCl_2$, thus indicating the presence of sulfhydryl groups in the enzyme molecule. Activity was inhibited completely by 1.5 x 10^{-4}M $HgCl_2$ and over 60% by 1.6 x 10^{-5}M. Attempts were made to reverse the inhibition with a reaction mixture containing 0.01 M ethylenediaminetetraacetic acid and 0.05 M cysteine; however, acid production at alkaline pH by one or both precluded assay of the enzyme after treatment. Ory, et al., (16) found that castor bean lipase was inhibited completely by 5 x 10^{-4}M $HgCl_2$, and Singer (20) found that wheat germ lipase was inhibited by 1 x 10^{-3}M p-chloromercuribenzoate, as well as by other sulfhydryl reagents.

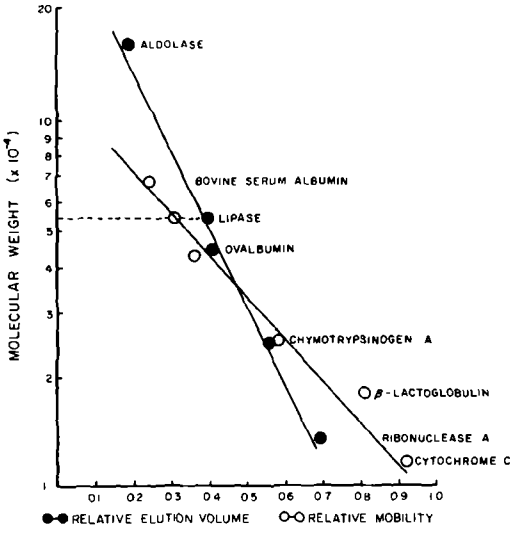

FIG. 5. Peanut lipase mol wt determination by Sephadex gel filtration (●–●) and sodium dodecyl sulfate gel electrophoresis (○–○).

Mol Wt

Mol wt of the enzyme was determined by SDS gel electrophoresis and Sephadex gel filtration. After elution from DEAE Sephadex A-50, lipase-active fractions yielded two bands on SDS gels. After standard proteins were run, and relative mobilities calculated and plotted vs mol wt, the two bands were determined to have mol wt of ca. 55,000 and 82,000. To determine which of the bands was peanut lipase, Sephadex gel filtration was performed. Elution volumes of standard proteins were determined by absorbance at 280 nm, and elution volume of lipase was determined by enzyme activity from a calibrated 2.5 cm x 45 cm reverse flow Sephadex G-150 column. A plot of relative elution volume vs mol wt indicated that lipase had a mol wt of ca. 55,000 (Fig. 5).

The mol wt of 55,000 reported here is, with one exception, in relative agreement with mol wts estimated for lipase from other sources. Downey and Andrews (21), employing gel filtration techniques, determined the mol wt of a triacetin-hydrolyzing wheat germ enzyme to be ca. 51,000 and found a mol wt of 42,000 for pig pancreatic lipase. Though not reporting a mol wt per se, Conners and Diffendall (22) suggested a mol wt of less than 50,000 for lipase from tobacco leaves, while Olney, et al., (23) associated lipase activity in *Vernonia anthelmintica* seed with a molecule of over

200,000 mol wt.

This study neither proved nor disproved the in vivo activity of peanut lipase. Any statement as to the physiological importance of lipase in developing seed would be purely speculative. However, the implications as to physiological preparation for germination are interesting, since several reports (3,6,7) indicate measurable lipase activity in dormant seed. In these reports (3,6,7), lipase activity increased upon germination but whether the increase was activation of already formed enzyme or de novo synthesis remains to be determined.

ACKNOWLEDGMENT

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SHORT COMMUNICATIONS

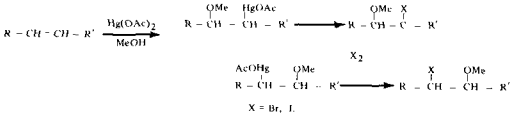
Location of Double Bonds in Long Chain Esters by Mass Spectroscopy of Methoxyhalogeno Derivatives Prepared from Methoxymercuriacetate Adducts

ABSTRACT

Methyl esters of mono-, di-, and triunsaturated long chain fatty acids react quantitatively with mercuric acetate in methanol to produce methoxyacetoxymercuri derivatives. Demercuration of these products with solutions of bromine or iodine in methanol yields methoxybromo or methoxyiodo derivatives readily isolable by thin layer chromatography. Mass spectra of the methoxyhalogeno derivatives of monounsaturated esters are characterized by intense peaks due to loss of halogen and cleavage adjacent to methoxy functions; the latter fragmentation allows the position of the original double bond to be established. The mass spectra of the methoxyhalogeno derivatives of polyunsaturated esters are more complex.

INTRODUCTION

Demercuration of methoxymercuriacetate derivatives of unsaturated long chain esters by reaction with sodium borohydride (1,2) leads to methoxylated derivatives whose mass spectra contain intense peaks due to cleavage adjacent to the methoxy functions (3,4). As a method for the location of the position of double bonds in long chain esters, this procedure is convenient (2,4), but, since each double bond leads to two isomeric derivatives, the mass spectra contain an excess of diagnostic peaks. An alternative demercuration procedure is the replacement of the mercuriacetate group by halogen (5) according to the following scheme:



It was thought that such methoxyhalogeno derivatives of long chain esters might give mass spectra showing intense peaks of the type $R(R').CH = \overset{+}{O} - Me$, but those due to structures of the type $R(R').CHX.CH = \overset{+}{O} - Me$ would be of much lower intensity.

RESULTS AND DISCUSSION

Methoxyiodo derivatives were prepared from methyl oleate, elaidate, linoleate, and linolenate; methoxybromo derivatives of the monounsaturated esters also were studied. Methoxymercuriacetate adducts were prepared by heating the reactants under reflux for 1 hr, as described previously (4,6). To the cold crude reaction mixture, 5% solutions of bromine or iodine in methanol were added until the color persisted, and the mixture then was kept at room temperature for 3 hr. The final mixture was partitioned between equal volumes of dichloromethane and 5% aqueous sodium sulphite, the organic layer washed with water and dried over anhydrous sodium sulphate. The crude reaction products were investigated by thin layer chromatography (TLC) on 0.5 mm layers of silica gel (Merk $PF_{254+366}$) using a solvent system of hexane-diethyl ether (90:10). In all cases, no unchanged unsaturated esters (R_f 0.60) were detected. The methoxybromo derivatives from methyl oleate and elaidate had identical $R_{methyl\ oleate}$ values of 0.77; the value for the corresponding iodo ester was 0.80. The iodo derivatives from methyl linoleate and linolenate had $R_{methyl\ oleate}$ values of 0.64 and 0.42, respectively, in this chromatographic system. Pure samples of the methoxyhalogeno esters were obtained by preparative TLC.

Mass spectra (70 eV) were determined on an AEI MS9 instrument. The mass spectrum of the methoxyiodo derivative from methyl oleate is shown in Figure 1; the spectrum of the corresponding derivative from methyl elaidate gave a practically identical spectrum. Loss of an iodine atom gives rise to an intense fragment having m/e 327, which successively loses the elements

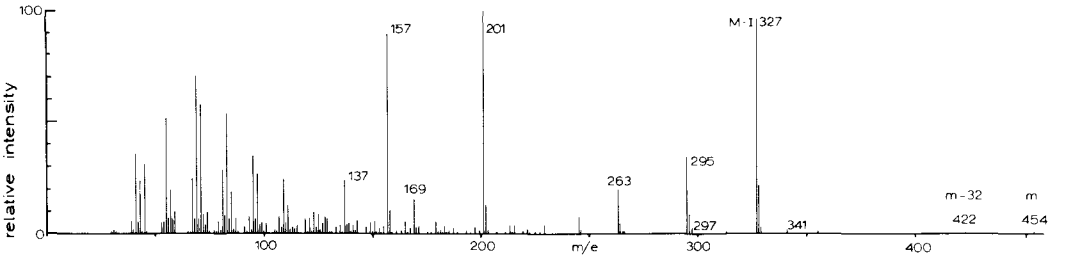


FIG. 1. Mass spectrum of the methoxyiodo esters derived from methyl oleate by methoxymercuration followed by demercuration with iodine.

of methanol to produce the fragments at m/e 295 and 263. The position of the original double bond is shown clearly by the intense peaks at m/e 157 [$CH_3 \cdot (CH_2)_7 \cdot CH=\overset{+}{O}-CH_3$] and 201 [$CH_3-\overset{+}{O}=CH \cdot (CH_2)_7 \cdot COOCH_3$]; the latter fragment successively loses the elements of methanol to produce the peaks at m/e 169 and 137. Peaks at m/e 297 and 341 attritibutable, respectively, to the ions $CH_3 \cdot (CH_2)_7 \cdot CHI \cdot CH=\overset{+}{O}-CH_3$ and $CH_3-\overset{+}{O}=CH \cdot CHI \cdot (CH_2)_7 \cdot COOCH_3$ are of very low intensity. The spectra of the methoxybromo derivatives from methyl oleate and elaidate contained the same major peaks as shown in Figure 1, the fragment having m/e 327 being formed by loss of a bromine atom from the molecular ion (m/e 407). The mass spectra of the methoxyiodo derivatives of methyl linoleate and linolenate had prominent peaks (m/e 483 and 639, respectively) due to loss of iodine atoms from the molecular ions and other peaks which indicated the position of the original double bonds. The overall spectra of the derivatives from these polyunsaturated esters were, however, much more complicated and less reproducible, changing considerably in pattern with the mass spectrometer temperature and other parameters.

Demercuration of methoxymercuriacetate derivatives of monounsaturated long chain esters by treatment with bromine or iodine, therefore, produces methoxybromo or methoxyiodo derivatives whose mass spectra contain peaks which enable the position of the original double bond to be established easily. The mass spectra are simpler than those of the methoxylated esters prepared by demercuration of long chain methoxymercuriacetates (4) and, indeed, contain the same diagnostic peaks as those found in spectra of dimethoxy esters produced by methylation of glycols prepared by hydroxylation of unsaturated esters (7). The mass spectra of the present derivatives (Fig. 1) have the advantage that the mol wt is given by the intense peak due to loss of the halogen atom. This present technique and the procedure involving demercuration with borohydride (4) both have the advantage that the chemistry is performed in a single flask. Indeed, these two procedures are quite complementary; the same crude methoxymercuriacetate can be divided and used for both demercuration methods. For routine experiments, demercuration with iodine is preferred from the point of view of safety and convenience. Methoxyiodo derivatives of polyunsaturated esters, such as methyl linoleate and linolenate, are formed readily by using the present procedure but their mass spectra do not allow the location of the original double bonds to be established so clearly.

Alkoxybromination of long chain monounsaturated esters also has been carried out by reaction with t-butyl hypobromite in the presence of various alcohols (8,9). It was noted that methyl hendec-10-enoate gave a mixture of isomeric alkoxybromo derivatives (9); since demercuration of methoxymercuriacetate adducts of this ester with borohydride leads only to the 10-methoxy esters (1,3), the procedure described in this paper should allow the facile synthesis of 11-halogeno-10-methoxyhendecanoates.

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Synthesis of Prostaglandins in Platelets of Hypercholesterolemic Rabbits

ABSTRACT

As a continuation of previous studies on the synthesis of prostaglandins in platelets of human venous blood, the present communication describes the synthesis of prostaglandins in experimental hypercholesterolemia induced in rabbits by dietary means. In such a condition, high serum level of cholesterol did not change the rate of prostaglandin synthesis.

INTRODUCTION

Recent studies have shown that human platelets synthesize prostaglandins (PGs) (1-3). Among the PGs synthesized are prostaglandins E_1 (PGE_1), E_2 (PGE_2), F (PGFs), and also probably PGAs and PGBs (2,4). The inhibiting effect of PGE_1 upon platelet aggregation induced by adenosine diphosphate (ADP) or other naturally occurring agents is 10 times the potentiating effect of PGE_2 upon this phenomenon (5-10). As both PGE_1 and PGE_2 are formed in almost equal amounts in the platelets, the balance in normal prostaglandin synthesis may be distorted in pathological conditions, such as hyperlipemia, affecting platelet function.

In previous studies (1,2), the biosynthesis of PGs in normal platelets was elucidated. This communication presents some data concerning the biosynthesis of PGs in hypercholesterolemia, mimicking cardiovascular diseases (11) and in which an abnormal platelet aggregation is thought to be a part of the pathogenic mechanism.

MATERIALS AND METHODS

Five albino white female rabbits weighing 3-3.5 kg fed a pellet diet (Kanino, optimal in every respect and obtained from Aktieselskabet Korn- og Foderstof Kompagniet, Viby J., Denmark) were studied for PG synthesis in their platelets during a period of normal feeding. The animals then were fed a diet containing cholesterol (dietary intake: 400 mg cholesterol/day for 40 days) and then evaluated again for their capability to synthesize PGs in the platelets in hypercholesterolemic state.

The cholesterol enriched diet was prepared by dissolving an appropriate amount of cholesterol in ether which then was poured over an appropriate amount of chicken pellets. By evaporation of ether at 45 C, cholesterol was adsorbed on the pellets. Cholesterol content was regulated so that in the week prior to

TABLE I

Serum-Cholesterol Level[a] in Rabbits Prior to and after 40 Days' Feeding on Pellet Diet with 400 mg Cholesterol/Day

Animal no.	290	359	454	614	733
Serum-cholesterol prior to diet	18.0	77.2	14.6	20.3	30.2[b]
Serum-cholesterol after 40 days on cholesterol diet.	[c]	985	386	506	765

[a]mg/100 ml.

[b]Slight hemolysis of serum.

[c]Not studied since the animal did not eat the cholesterol diet.

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[b]Slight hemolysis of serum.

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TABLE II

In vitro Biosynthesis of Prostaglandins in Platelets from Normal and Hypercholesterolemic Rabbits[a]

	No. of animal	Prostaglandins[b]			Platelet count/ml PRP
		PGE_1	PGE_2	PGF	
Prior to cholesterol diet	290	0.040	0.045	0.217	3.94×10^8
	359	0.064	0.023	0.258	5.48×10^8
	454	0.078	0.038	0.096	6.52×10^8
	614	0.028	0.015	0.102	4.50×10^8
	733	0.012	0.049	0.094	5.17×10^8
		0.044 ± 0.026	0.034 ± 0.013	0.153 ± 0.073	
After 40 days on cholesterol diet	359	0.128	0.047	0.128	4.56×10^8
	454	0.028	0.040	0.096	5.44×10^8
	614	0.059	0.024	0.135	4.31×10^8
	733	0.037	0.037	0.149	4.36×10^8
		0.063 ± 0.039	0.035 ± 0.008	0.127 ± 0.019	
		t = 0.728	t = 0.144	t = 0.721	
		NS	NS	NS	

[a]Biosynthesis is expressed as nmole 1-^{14}C-acetate incorporated/500 μliter platelet-rich plasma (PRP)/hr.
[b]PGE_1 = prostaglandin E_1, PGE_2 = prostaglandin E_2, and PGF = prostaglandin F_α. t = Statistical value and NS = not significant.

dietary experiment, the mean daily intake of pellets/rabbit could be estimated. From this figure, the amount of cholesterol in the pellets was regulated to give a daily intake of 400 mg cholesterol/rabbit/day. Control of the intake of cholesterol enriched food confirmed that all rabbits but no. 290 took the estimated amount of pellets.

Radioactive acetate, various PGs used as carriers in chromatographic processes, and necessary chemicals were obtained and used as described in our previous communications (1,2,12,13). Venous blood-anticoagulant mixture from rabbits, both on normal pellet diet and on cholesterol enriched diet, obtained by puncturing a marginal ear vein, was centrifuged at 270 g for 20 min, the supernatant transferred to another tube, and recentrifuged at 270 g for 10 min. This gave a platelet-rich plasma (PRP), free from other elements of blood. Platelet-free plasma (PFP) was prepared as described previously (2, note all figures concerning platelet count appearing as "x 10^9" should read "x 10^8"). The reaction mixture during incubation consisted of (A) PRP, 500 μliter; (B) PFP, 420 μliter; (C) glutathione, 55 μliter (55 μg); and (D) radioactive acetate, 2.5 μmoles in a total incubation volume of 1 ml. The air from the incubation tube was replaced by oxygen. The incubation temperature was 37 C, and incubation time was 1 hr. After the incubation was complete, to each incubation mixture were added 5 μg of each PG followed by an immediate reaction-termination and extraction, as described elsewhere (14). Thin layer chromatographic (TLC) separation of the material obtained from the incubation mixture was performed as described elsewhere (14). Thin layer chromatographic (TLC) separation of the material obtained from the incubation mixture was performed as described earlier (1,2). The zone due to the PGF compounds was extracted repeatedly with methanol, the extract centrifuged, and the supernatant transferred directly to a counting vial from which the solvent was evaporated completely to give a residue which was dissolved in 200 μliter methanol and counted for its radioactivity (1,2). The material present in the PGE zone was similarly extracted and then resolved further into PGE_1, PGE_2, and PGE_3 by argentation-TLC. The material present in each individual PGE zone was extracted as described earlier (12). The organic extract was placed directly into a counting vial and evaporated to a residue followed by assay of its radioactivity, as described above.

RESULTS AND DISCUSSION

Table I shows the serum cholesterol level in the 5 experimental animals prior to and after 40 days of cholesterol feeding. Apart from animal no. 290 which did not eat the cholesterol enriched diet, the remaining 4 animals increased their serum cholesterol level from 12-27-fold. Table II shows the biosynthesis of prostaglandins (PGE_1, PGE_2, and PGF) in platelets of animals prior to and after 40 days' cholesterol feeding. Great individual variations in the synthetic capability were found. Thus, the rate of formation of PGE_1 was within 0.012-0.078 nmole acetate incorporated by the platelets present in 500 μliter PRP/hr in rabbits on normal pellet diet, while the same for rabbits in hypercholesterolemic state was in the

range of 0.028-0.128 units (a unit represents 1 nmole acetate incorporated by 500 μliter PRP in 1 hr incubation). In case of PGE_2, these values were 0.015-0.049 and 0.024-0.047 units, respectively; and, for PGF, the values were within 0.094-0.258 units in normal rabbits as against 0.096-0.149 units in the hypercholesterolemic state. Statistical evaluation reveals no significant differences in normal and hypercholesterolemic rabbits. Concerning the proportion between PGE_1 and PGE_2, this ratio was 1.3 in the normal group and 1.8 in the hypercholesterolemic group. This difference in ratio was, however, not significant.

The present communication indicates that, in rabbits as in humans, both PGE_1 and PGE_2 are formed. Since, on a molecular basis, PGE_1 inhibits platelet aggregation ca. 30 times more effectively than PGE_2 promotes it (15), the relative rates of biosynthesis of the two prostaglandins effectively should inhibit aggregation. From experimentally induced hypercholesterolemia in animals and observation of hypercholesterolemic cases in human clincs, it has been suggested that the pathological elevation of blood cholesterol may lead to coronary thrombosis. Hypothetically, the coronary thrombosis could be due to an altered rate of PG synthesis in platelets, such that more PGE_2 is synthesized in hypercholesterolemic than in normal subjects and, therefore, platelet aggregation promoted. We have found no confirmation for this idea, since our data indicate that at least diet induced hypercholesterolemia in rabbits does not significantly alter PG synthesis in platelets. Our continuing studies with hypercholesterolemic humans should reveal whether the same observation holds true for humans.

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Effect of Dietary Linolenic and Linoleic Acids upon Growth and Lipid Metabolism of Rainbow Trout[1] (*Salmo gairdneri*)

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ABSTRACT

Nine diets, each containing different levels of linoleic acid (18:2ω6) and linolenic (18:3ω3) were fed to duplicate groups of rainbow trout for 14 weeks. The growth rate, feed efficiency, accumulated mortality, and fatty acid composition of neutral fat and phospholipids of these groups of fish were determined. The growth was slow in the groups of fish receiving diets containing (A) low concentration of 18:3ω3 and (B) high concentration (5%) of 18:2ω6. The accumulated mortality was high in these groups of fish. The diet containing 1% 18:3ω3 alone supported rapid fish growth with low mortality. The feed efficiency of this diet was also high. The metabolism of 18:2ω6 and 18:3ω3 in fish and their conversion to more unsaturated fatty acids typical of fish lipids was investigated.

INTRODUCTION

Recent investigations have shown that the essential fatty acid (FA) requirement of rainbow trout differed from that of the land animals. It was reported that the FAs of the ω3 family were required by trout for normal growth (1,2), while linoleic acid (18:2ω6) of the ω6 family accentuated the deficiency symptoms, such as abnormal heart conditions and shock syndrome (3). However, the growth response of the fish to diets containing both ω3 and ω6 FAs has not been investigated thoroughly.

Analysis of the lipids extracted from a number of commercial trout rations showed that most of the rations contained a high percentage of ω6 FAs and a low percentage of ω3 FAs. The ω3/ω6 ratio of these rations varied 0.14-0.86 (4). It appears that these rations may not be providing adequate amounts of required FAs to support optimum growth.

The purpose of this experiment is to determine the effect of various levels of dietary ω3 and ω6 FAs upon growth, mortality, and FA composition of rainbow trout.

[1]Technical Paper 3854 Oregon Agricultural Experiment Station.

MATERIALS AND METHODS

Rainbow trout, immediately after hatching, were fed a fat-free diet for 1 month. Fifty randomly selected fish then were stocked in each of 18 fiberglass tanks, 75 liter capacity. Water temperature was 11.5 C and the flow rate ca. 8 liters/min.

Diet ingredients, such as casein, gelatin, dextrin, and α-cellulose, were extracted with warm isopropanol to remove trace lipids. Ethyl laurate, ethyl linoleate, and ethyl linolenate were obtained from commercial sources. Gas liquid chromatographic (GLC) analysis showed them to be at least 99% pure. Nine diets were prepared. The composition and calorie content of these diets were identical except for the lipid component. The diet composition is shown in Table I, and the lipids in each diet are listed in Table II. Each diet was fed to duplicate lots of fish three times daily. Food was offered as long as the fish continued to feed. The fish were weighed every 2 weeks. Feed consumption and mortality were recorded. The experiment was terminated at the end of 14 weeks. Five fish were taken from each lot, and the FA composition of the lipid extracted from the whole fish

TABLE I

Composition of Trout Diets

Component	Wt (%)
Casein	51.0
Gelatin	9.0
Dextrin	16.8
Lipid[a]	6.0
Mineral mix[b]	4.0
Carboxymethylcellulose	1.4
α-Cellulose (Alphacel-NBC)	8.6
Vitamin mix[c]	2.0
Choline chloride	1.0
Vitamin E concentrate (330 international units/g as dl- -tocopheryl acetate)	0.2

[a]Lipid composition listed in Table II.

[b]Bernhart-Tomerelli Salt Mix, modified by adding NaF and $CoCl_2$ at 0.0002 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 25,000 and 4000 international units/kg, respectively.

TABLE II

Effect of Dietary Lipids upon Growth, Feed Efficiency, and Mortality of Rainbow Trout

Diet no.	Lipid composition (%) 18:3ω3	18:2ω6	12:0	Average final wt (g)[a]	Feed efficiency[b] (gain/feed)	Accumulated mortality (%)
1	0.1	0	5.9	1.75*	0.60	26.0
2	0.5	0	5.5	3.26**	0.94	14.0
3	1.0	0	5.0	4.84***	1.14	4.0
4	0.1	1.5	4.4	3.45**	0.89	26.0
5	0.5	1.5	4.0	4.00****	0.98	6.0
6	1.0	1.5	3.5	4.03****	0.99	2.0
7	0.1	5.0	0.9	1.98*	0.65	44.0
8	0.5	5.0	0.5	1.75*	0.58	34.0
9	1.0	5.0	0	1.75*	0.61	39.0

[a]Average fish wt at the end of 14 weeks. Values with the same symbol are not significantly different ($P<0.01$).

[b]Feed efficiency is defined as units of wt gained/unit of dry feed consumed.

TABLE III

Effect of Dietary Lipids upon Growth Rate of Rainbow Trout

Diet	Average fish wt (g) in weeks[a] 0	2	4	6	8	10	12	14
1	0.43	0.48	0.61	0.75	0.98	1.10	1.39	1.75
2	0.44	0.49	0.75	1.07	1.49	1.90	2.46	3.26
3	0.44	0.57	0.90	1.35	1.94	2.54	3.62	4.84
4	0.44	0.58	0.87	1.21	1.71	2.14	2.72	3.45
5	0.44	0.59	0.89	1.30	1.81	2.36	3.19	4.00
6	0.44	0.57	0.88	1.26	1.79	2.34	3.15	4.03
7	0.44	0.57	0.80	1.05	1.29	1.58	1.63	1.99
8	0.44	0.57	0.78	1.03	1.25	1.32	1.44	1.75
9	0.43	0.53	0.70	0.93	1.24	1.23	1.42	1.80

[a]Average of two lots.

was determined. The lipid extraction and GLC methods used in this study were described previously (1).

RESULTS AND DISCUSSION

The growth rate of fish receiving diets 1, 2, and 3, in which no ω6 was added, was dependent upon the respective level of 18:3ω3 in the diet. Diet 1 contained only 0.1% 18:3ω3, and the growth rate of the fish was extremely slow. In diet 2, the 18:3ω3 FA was increased to 0.5%. The growth rate of the fish was significantly ($P<0.01$) higher than the diet 1 fish. The highest growth rate was achieved by the group of fish receiving diet 3, in which 1% 18:3ω3 was added. These results are shown in Tables II and III.

Diets 4, 5, and 6 contained the same percentages of 18:3ω3 as diets 1, 2, and 3, respectively; but each diet was supplemented with 1.5% of 18:2ω6 FA. The average final wt (Table II) of diets 4 and 5 fish was significantly ($P<.01$) higher than that of diets 1 and 2 fish, respectively. These results indicated that fish growth was improved by supplementing 1.5%ω6 FA to diets insufficient in ω3. However, no additional growth was observed with the same level of 18:2ω6 supplementation to diet 3 which was sufficient in ω3 FA.

Diets 7, 8, and 9 again contained 0.1, 0.5, and 1.0% 18:3ω3, respectively. Each of these diets were supplemented with 5% of 18:2ω6. Fish receiving these diets all grew poorly with accompanying high mortality rates. It appeared that trout could not tolerate diets containing high percentages of ω6 FA. These data verified the finding of Lee, et al. (1) that an experimental diet with 10% corn oil as the only lipid source supported poor trout growth.

Table II also shows that the accumulated mortality was high in the groups of fish receiving diets 1 and 4. These diets contained only 0.1% ω3 FA. As the ω3 FA level increased to 0.5% in diets 2 and 5, the fish mortality was reduced greatly. The mortality was reduced fur-

TABLE IV

Influence of Dietary Lipids upon Percentages of Certain Phospholipid Fatty Acids in Trout[a]

Fatty acids	Diets								
	1	2	3	4	5	6	7	8	9
18:0	4.6	5.1	5.9	6.6	6.9	7.2	8.1	9.3	8.9
18:1ω9	34.9	30.0	26.9	19.3	20.5	20.0	9.9	10.8	11.5
18:2ω6[b]	3.5	2.8	2.1	10.5	9.4	8.0	16.9	17.7	17.6
18:3ω6	-	-	-	0.5	0.6	0.5	2.3	2.1	2.1
18:3ω3	-	0.8	1.8	0.2	0.8	1.2	-	0.6	1.3
20:1ω9	2.6	1.8	1.3	1.4	1.1	1.0	0.2	0.2	0.3
18:4ω3	-	0.6	1.0	-	0.3	0.4	-	0.3	0.6
20:2ω9	2.2	2.3	1.3	0.5	0.6	0.4	-	-	-
20:2ω6	-	-	-	1.8	1.4	1.1	2.4	2.7	2.0
20:3ω9	6.0	5.3	2.2	1.2	0.8	0.4	-	-	-
20:3ω6	-	-	-	2.8	2.7	2.0	3.6	3.5	3.1
20:4ω6	0.9	0.5	0.3	6.1	5.0	4.4	9.8	9.2	8.0
20:4ω3	-	-	0.3	-	-	0.4	-	-	-
20:5ω3	0.4	1.0	1.3	0.3	0.4	0.8	0.5	0.9	1.2
22:4ω6	-	-	-	0.5	-	-	0.9	1.0	0.5
22:5ω6	-	-	-	14.5	7.5	4.5	18.1	10.8	6.7
22:5ω3	-	-	1.2	-	-	1.0	-	-	0.2
22:6ω3	6.4	14.0	19.8	6.1	13.7	18.3	6.3	11.0	15.3
Total ω3	6.8	16.4	25.4	6.6	15.2	22.1	6.8	12.8	18.6
Total ω6	4.4	3.3	2.4	36.7	26.6	20.5	54.0	47.0	40.0

[a]Average of analysis of duplicate lots.

[b]Other isomers may be present.

TABLE V

Influence of Dietary Lipids upon Percentages of Certain Neutral Lipid Fatty Acids in Trout[a]

Fatty acids	Diets								
	1	2	3	4	5	6	7	8	9
18:0	2.7	3.1	3.0	3.7	4.3	4.1	4.6	4.2	4.7
18:1ω9	35.0	29.6	26.7	21.9	22.5	23.7	8.6	6.8	10.5
18:2ω6[b]	1.4	1.4	1.2	14.3	14.9	13.8	43.9	49.8	43.0
18:3ω6	-	-	-	0.6	0.8	0.5	6.5	6.6	5.5
18:3ω3	-	1.5	4.3	0.4	2.2	4.1	0.3	1.8	3.6
20:1ω9	2.3	1.6	1.2	1.3	1.1	0.6	0.7	-	-
18:4ω3	-	1.0	1.4	-	1.0	0.8	-	1.2	2.5
20:2ω9	1.0	1.6	1.0	0.4	0.5	0.4	0.2	-	-
20:2ω6	-	-	-	0.8	1.0	0.5	2.0	1.7	1.6
20:3ω9	0.5	0.8	0.4	-	-	-	-	-	-
20:3ω6	-	-	-	0.6	0.6	0.6	3.1	2.9	2.2
20:4ω6	-	-	-	1.2	1.1	1.0	6.4	6.1	4.3
20:4ω3	-	-	-	-	0.2	-	-	-	-
20:5ω3	-	-	-	-	0.3	0.3	0.3	0.4	0.8
20:4ω6	-	-	-	-	-	-	0.5	0.2	0.4
22:5ω6	-	-	-	1.3	0.8	0.2	5.1	2.7	2.0
22:5ω3	-	-	-	-	-	0.1	-	-	-
22:6ω3	-	1.1	2.2	0.8	1.9	2.7	1.3	2.8	4.3
Total ω3	0	3.6	7.9	1.2	5.6	8.0	1.9	6.2	11.2
Total ω6	1.4	1.4	1.2	18.8	19.2	16.6	67.5	70.0	59.0

[a]Average of analysis of duplicate lots.

[b]Other isomers may be present.

ther in the groups of fish fed diets 3 and 6 to which 1% ω3 FA was added. The accumulated mortality was highest in the groups of fish fed diets containing 5% 18:2ω6 (diets 7, 8, and 9).

A possible explanation for the excessive mortality may be the high dietary percentage of lipids of ω6 family (1). The added factor of ethanol derived from the hydrolysis of FA

ethyl esters in the diet also may make a contributions (5). Assuming an intake of 3.2 g dry diet for 100 g body wt of fish (diet 9), the consumption of ethanol would amount of 28.6 mg or 286 mg/kg body wt. However, no information was found on the effect of ethanol upon FA metabolism in fish.

The FA composition of the phospholipids and neutral lipids extracted from the whole fish is shown in Tables IV and V. A high concentration of 20:3ω9 FA was present in the phospholipids of diets 1 and 2 fish, which is an indication of essential FA deficiency (6,7). The growth rate of these groups of fish was low. The 20:3ω9 FA was less in the groups of fish fed diets 4 and 5. These two diets contained the same levels of 18:3ω3 as diets 1 and 2 but were supplemented with 1.5% 18:2ω6. The addition of 18:2ω6 at this level appeared to satisfy partially the metabolic needs of the fish, and the growth rate of diets 4 and 5 fish was higher than the groups of fish fed diets 1 and 2, respectively.

No 20:3ω9 was detected in the tissues of fish fed diets 7, 8, and 9, in which 5% of 18:2ω6 was added. The poor growth exhibited by these groups seemed to be the result of a dietary imbalance of 18:3ω3 and 18:2ω6 FAs.

By examination of the body FA composition, the metabolism of dietary 18:2ω6 in trout can be seen in fish fed diets 4, 5, 6, as well as diets 7, 8, 9. Through carbon chain elongation and desaturation, the 18:2 was converted into 18:3, 20:2, 20:3, 20:4, and 22:5 FAs of the ω6 family. The dietary 18:3ω3, on the other hand, was converted into 18:4, 20:4, 20:5, 22:5, and 22:6 FAs of the ω3 family. The conversion of 18:2ω6 into 22:5ω6 was inhibited to a certain extent by dietary 18:3ω3. Diets 4, 5, and 6 contained the same level (1.5%) of 18:2ω6, but the level of 18:3ω3 in these diets varied. In diet 4, in which only 0.1% 18:3ω3 was added, the concentration of 22:5ω6 and total ω6 fatty acids in phospholipids was 14.5% and 36.7%, respectively. As the level of 18:3ω3 increased in diets 5 and 6; the concentration of 22:5ω6, was well as total ω6 FAs, decreased accordingly.

The metabolism of 18:3ω3 was inhibited by dietary 18:2ω6 but to a lesser degree. This can be seen in diet series 2, 5, 8 or 3, 6, 9, in which the level of 18:3ω3 was constant, while the level of 18:2ω6 varied. The concentration of 22:6ω3 and total ω3 FAs in phospholipids decreased as the 18:2ω6 levels in the diets increased. These results were in accord with the rat experiments conducted by Holman and coworkers (6,8), wherein the competitive inhibition between linoleate and linolenate was demonstrated.

The FA composition of body lipids reflects the dietary lipids (9). Hligh body ω6 FAs were found in the fish on diets 7, 8, 9. The level found was abnormally high when compared to fish in the wild state. These diets supported poor fish growth. Presumably, increasing the level of 18:3ω3 in these diets would have reduced the incorporation of ω6 FAs into the phospholipids of the fish and better fish growth would have occurred.

The results support the conclusion that rainbow trout grow best on diets high in lipids of the ω3 family and low in lipids of the ω6 family. Diets 3, 5, and 6 support this contention. These diets, in addition to producing rapid growing fish with low mortality, exhibited high feed efficiency.

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Nature of Alkanes in Beef Heart Lipids

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ABSTRACT

n-Alkanes have been found to be the major saturated hydrocarbon components in the fatty tissues on beef heart. These alkanes consist of a homologous series C_{14}-C_{35}, with the C_{29} and C_{31} n-alkanes being most abundant. C_{16}, C_{19}, and C_{20} isoprenoid alkanes also were identified. A C_{17} isoprenoid alkane tentatively was identified. The fatty tissues on beef heart contained 32 μg/g saturated hydrocarbons. The distribution pattern of these saturated hydrocarbons is in marked contrast to the alkane distribution in beef liver where branched and cyclic alkanes are predominant. The enrichment and the similarity of the n-alkane distribution in the fatty tissues on heart and in pasture plants may have implications for the physiological aspects of hydrocarbons in the diet.

INTRODUCTION

Trace amounts of saturated hydrocarbons generally are regarded to be ubiquitous in nature. The occurrence of alkanes in several animal and plant species and tissues has been summarized (1), and it became apparent that relatively few studies have been made concerning the occurrence and, particularly, the physiological role of saturated hydrocarbons in mammalian tissues (2-7). Studies of beef liver (1) and beef brains (8) showed distributions of alkanes (chiefly branched and cyclic) which are different from the distribution in pasture plants. In addition, these results suggest preferences in the accumulation of certain classes of hydrocarbons in different anatomical sites of the same species. In an attempt to better understand the occurrence and role of alkanes in mammals, part of another organ, i.e. the fatty tissues on the beef heart muscle, was analyzed.

METHODS AND RESULTS

Extraction and Isolation of Saturated Hydrocarbons

All solvents used in the analyses were redistilled from spectral-grade or pesticide-grade reagents using 12 cm columns packed with Raschig rings. All glassware and Teflon fittings were cleaned in a mixture of hot concentrated H_2SO_4/HNO_3(85:15, v/v). As a further precaution against contamination, a complete procedure blank was made under conditions identical to those used for the beef heart fatty tissue analysis. The blank run revealed no hydrocarbons or other compounds, confirming that no contaminations occurred in the laboratory.

The beef heart was obtained from a freshly butchered animal. The organ was handled with and wrapped in solvent-cleaned aluminum foil, packed in dry ice during transport, and then immediately dissected. As an added precaution against contamination the outer ½ cm fatty tissue was discarded. The remaining fatty and connective tissues on the heart muscle (246 g) were used for the analysis. The fatty tissue was cut into ~1 cm sized pieces and macerated in portions in a modified Waring blender with ca. 450 ml chloroform/methanol (2:1, v/v). (All lubricants previously had been removed from the blender and acid-cleaned Teflon gaskets installed.) Aliquots of this mixture were next passed through a medium-porosity Büchner funnel.

After filtration, the solution was flash evapo-

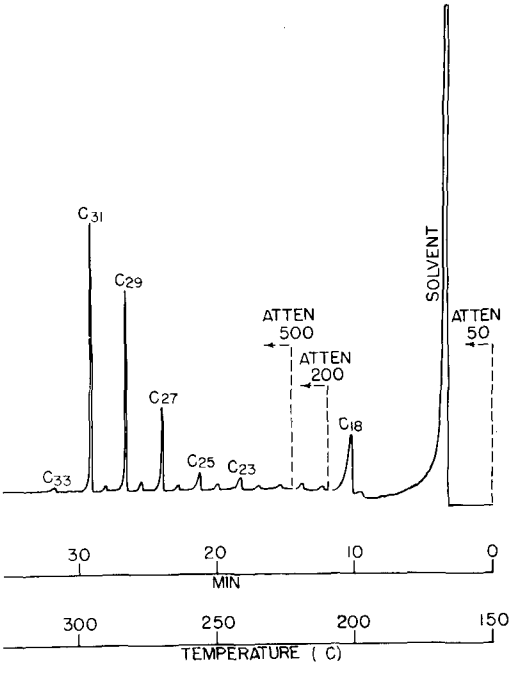

FIG. 1. Gas chromatogram of unsieved hydrocarbons–Dexsil-300 column.

rated at 55 C in 100 ml portions. The final residue consisted of 200 g yellow liquid. This residue then was saponified in 70 g portions by refluxing each of the aliquots for 1 hr in a solution of 600 ml benzene/methanol (2:3, v/v), 60 ml H_2O, and 33 g KOH. The KOH solution was prewashed with hexane to remove any contaminating hydrocarbons. After saponification, the benzene and methanol solutions were resolved in a separatory funnel, and the methanol fraction then was washed twice with benzene. The combined benzene fractions, which contained the hydrocarbons, were flash evaporated to give a residue of 10 ml light yellow liquid. This liquid was next extracted with 200 ml hexane and washed again with 100 ml 5% aqueous KOH to remove remaining traces of soap. The hexane was evaporated under electronic grade N_2 (passed through molecular sieves) to yield 2.05 g light yellow oil.

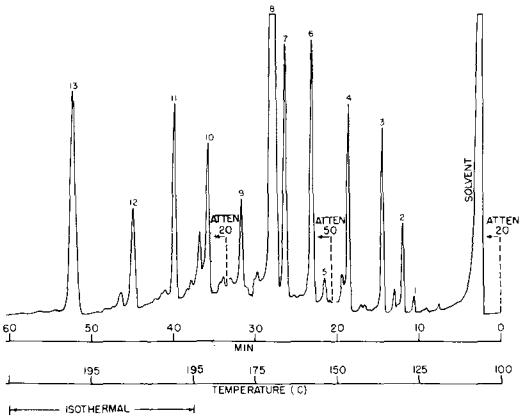

FIG. 2. Gas chromatogram of unsieved hydrocarbons (low mol wt portion)—OS-138 column. Hydrocarbons: (1) n-C_{14}, (2) C_{16} isoprenoid, (3) n-C_{15}, (4) n-C_{16}, (5) pristane, (6) n-C_{17}, (7) phytane, (8) n-C_{18}, (9) n-C_{19}, (10) n-C_{20}, (11) n-C_{21}, (12) n-C_{22}, and (13) n-C_{23}.

The oil was poured onto a prewashed alumina column (120 g Woelm neutral, Waters Associates, Framingham, Mass.) and eluted with n-hexane. At room temperature the eluate yielded an 8.9 mg mixture of a colorless, waxy solid and viscous liquid after evaporation under N_2. Further chromatography on prewashed silica gel (100 g of Adsorbosil-CAB 140-200 mesh, Applied Science Laboratories, State College, Pa.) by elution with n-hexane yielded at room temperature a mixture of 7.9 mg white crystals and a colorless oil after evaporation under N_2.

Before analysis, two-thirds of the hexane eluate from the silica gel column was separated into branched/cyclic and normal fractions by the use of Linde 5Å molecular sieve pellets, preactivated at 250 C for 2 weeks. The solu-

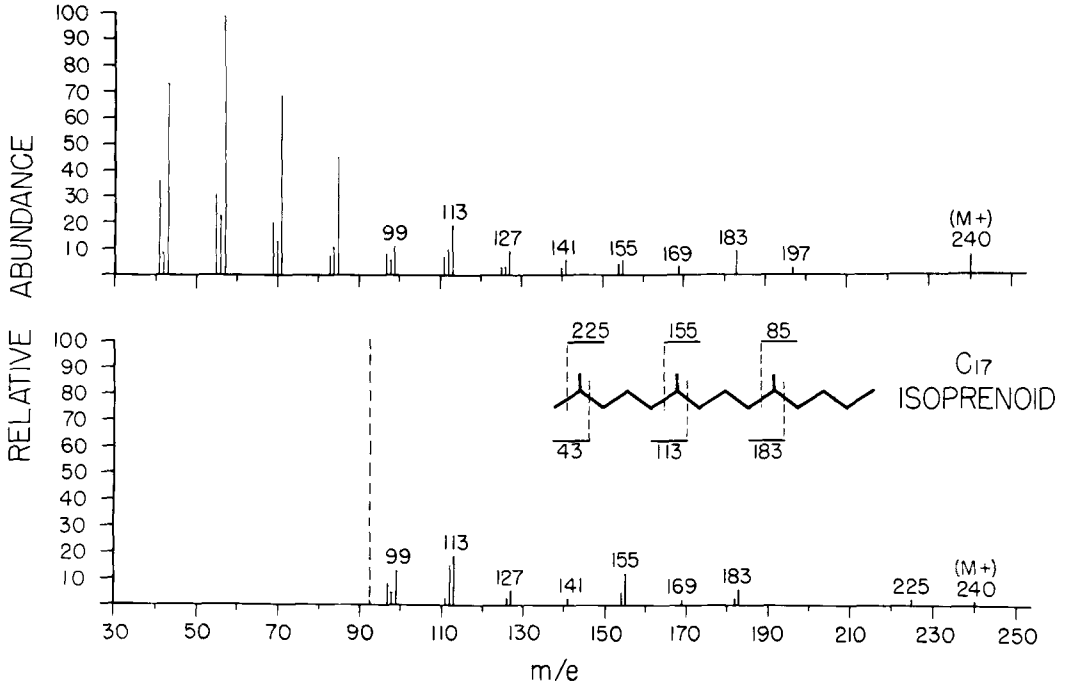

FIG. 3. Mass spectrum of C_{17} branched alkane. Lower spectrum is the standard (9).

tion, together with the molecular sieve pellets, was refluxed in benzene for 1 week. The benzene, which contained the branched alkanes, then was decanted. n-Alkanes were removed from the molecular sieve pellets by refluxing with n-hexane for 1 week after the benzene solution was removed.

Identification of Saturated Hydrocarbons by Combined Capillary Gas Chromatography-Mass Spectrometry

Identifications of the hydrocarbons were made by injecting the sieved and unsieved fractions into a Perkin-Elmer model 226 gas chromatograph directly connected to a Hitachi RMU-6E mass spectrometer through a molecular separator. The gas chromatograph was equipped with a 50 ft, 0.02 in. inside diameter, Dexsil-300 support coated open tubular (SCOT) column and programed at 150-300 C at 5 C/min (Fig. 1). For better separation, the unsieved material was programed on a 100 ft, 0.02 in. inside diameter, OS-138 (polyphenylether) SCOT capillary column at 100-195 C (Fig. 2). Rapid mass spectral scans were recorded of each peak as it emerged from the gas chromatograph.

Interpretation of Mass Spectra

Mass spectra of the unsieved fraction revealed a complete homologous series of saturated hydrocarbons C_{14}-C_{35} showing fragmentation patterns typical of n-alkanes. Three isoprenoids also were identified: C_{16} (2,6,10-trimethyltridecane), C_{19} (2,6,10,14-tetramethylpentadecane), and C_{20} (2,6,10,14-tetramethylhexadecane). The branched/cyclic fraction revealed an additional gas chromatographic peak whose mass spectrum indicated a branched C_{17} alkane. This branched alkane had fragment ions at m/e = 99, 113, and 183 which suggest an isoprenoid structure. Although this mass spectrum showed some variation from a published spectrum (9) of C_{17} isoprenoid (Fig. 3), such differences could be caused by imperfect gas chromatographic separation and by instrumental variations between mass spectrometers. Consequently, this compound tentatively was identified as a C_{17} isoprenoid alkane. No branched or cyclic compounds having mol wts higher than C_{20} were indicated by the mass spectra. The C_{19} isoprenoid (pristane) was identified by comparison with a standard; the C_{20} (phytane) and C_{16} isoprenoids were identified by comparison with published spectra (10,11).

DISCUSSION

The hydrocarbons in fatty tissues on beef heart muscle consisted mainly of n-alkanes (n-C_{14}-n-C_{35}). A strong odd carbon preference in the region n-C_{21}-n-C_{33} was observed. n-C_{31} was the most abundant alkane, followed by n-C_{29} and n-C_{27} (Fig. 1). Among the branched chain compounds, phytane predominated, followed by the branched C_{17} hydrocarbon.

Total yield of saturated hydrocarbons from the bovine heart fatty tissue was 32 μg/g. This yield and the distribution pattern differed from those of beef liver (1) which contained 4 μg saturated hydrocarbon/g liver, consisting of predominantly branched/cyclic alkanes (C_{19} and C_{20}) and only trace quantities of normal alkanes. In contrast to the heart alkanes, C_{18}-C_{22} were the most abundant n-alkanes in liver. The analysis of a third tissue, beef brains (8), yielded dominantly branched alkanes C_{10}-C_{30} (identifications were based upon gas chromatographic retention times and on IR spectroscopy).

n-Alkanes in beef heart fatty tissues appear to have been partially absorbed from ingested plant material; such plants have similar n-alkane distributions (5).

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Studies of Lipid Class and Fatty Acid Profiles of Rat Mammary Tumors Induced by 7,12-Dimethylbenz(a)anthracene

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ABSTRACT

The lipid class and fatty acid composition profiles of mammary glands of female rats fed a nutritionally adequate diet are compared to those of tumors induced in the mammary glands by intravenous injection of dimethylbenz(a)anthracene of animals fed the same diet. Ca. 95% of the lipids of the mammary glands of the control group of animals consisted of triglycerides; glycolipids and phospholipids were present in only minor amounts. In contrast, the lipids of the mammary tumors contained much lower amounts of neutral lipids and higher concentrations of phospholipids. The glycolipid fraction was a minor component of both tissues but differed greatly in composition. The composition of the phospholipid and neutral lipid fractions, particularly the latter, of the mammary tumors also differed from that of the mammary glands of the control animals. The neutral lipids of the tumor tissues contained elevated levels of free fatty acids and cholesterol and much lower concentrations of triglyceride compared to the mammary gland lipids. Differences also were observed in the fatty acid composition of tumor and mammary gland lipid. The greatest differences occurred in the concentrations of polyunsaturated fatty acids which were generally much higher in the tumor lipids.

INTRODUCTION

The invasion of malignant tumor growth to adjacent normal tissue is believed to involve the action of cell surface membranes and intercellular contact (1-4). Phospholipids are major constituents of cell membranes and may be important in tumor growth (5-7); glycolipids appear to be involved in tumor growth by virtue of their antigenic properties (8-10). Since the lipids of various tissues differ in fatty acid composition (11), the properties of phospholipids in the matrix of surface structures can vary by virtue of differences in their constituent fatty acids, as evidenced by physical studies (12-14).

Endocrine effects upon the lipid and fatty acid composition of mammary gland and tumor

TABLE I

Analysis of Rat Mammary Gland and Tumor Tissue Lipid Fractions[a]

Fatty acids	Neutral lipid		Glycolipid[b]		Phospholipid	
	Control	Tumor	Control	Tumor	Control	Tumor
Percent wt of total lipid	98.6 ± 1.0[c]	64.1 ± 15.7	0.4 ± 0.2	2.5 ± 1.5	1.1 ± 0.8	33.5 ± 15.1
14:0	1.1 ± 0.3	0.8 ± 0.1	2.2	1.3	0.4 ± 0.1	0.4 ± 0.3
16:0	18.5 ± 1.5	15.8 ± 1.3	16.4	12.6	21.3 ± 2.4	21.5 ± 2.5
16:1	4.4 ± 0.3	3.2 ± 0.7	4.6	3.0	1.2 ± 0.2	1.1 ± 0.7
18:0	4.7 ± 0.2	6.2 ± 2.0	9.4	10.0	18.3 ± 0.6	16.8 ± 1.8
18:1	37.9 ± 2.5	32.9 ± 4.3	27.5	14.1	14.5 ± 1.5	14.5 ± 5.4
18:2	26.3 ± 3.0	22.9 ± 7.0	14.7	6.9	13.8 ± 2.1	8.0 ± 2.9
18:3	2.5 ± 0.4	2.0 ± 0.3	2.3	1.0	0.5 ± 0.1	0.4 ± 0.1
20:3	0.1 ± 0.1	0.6 ± 0.4	1.1	2.4	1.4 ± 0.2	2.1 ± 0.3
20:4	0.9 ± 0.1	7.3 ± 5.5	5.1	9.1	13.8 ± 3.5	20.0 ± 5.6
20:5	0.3 ± 0.1	0.7 ± 0.4	2.7	7.2	0.4 ± 0.2	0.8 ± 0.6
22:3	0.3 ± 0.1	0.5 ± 0.4	2.7	12.3	0.7 ± 0.3	1.5 ± 0.8
22:4	0.2 ± 0.1	1.6 ± 1.2	1.5	3.7	3.3 ± 3.1	3.5 ± 0.6
22:5	0.6 ± 0.3	1.5 ± 0.6	1.4	1.9	1.4 ± 0.3	1.8 ± 0.9
22:6	1.0 ± 0.4	2.7 ± 1.4	1.5	4.1	4.9 ± 3.3	3.2 ± 1.0
Others (<1%)	1.3 ± 0.5	1.4 ± 0.8	6.9	10.4	3.8 ± 1.1	4.8 ± 3.4

[a]Lipid content of tumor tissue = 30.7 ± 13.2 mg/g wet tissue. Lipid content of mammary glands of control animals 601 ± 120 mg/g wet tissue.

[b]Fatty acid analysis carried out on pooled fractions from tumors of six animals and glands of four healthy animals.

[c]Mean ± standard deviation.

tissue in rats have been observed by Rees, et al. (15). In these studies, major differences were observed in the amount and fatty acid composition of the triglycerides. Reported here is a comparison of the lipid class and fatty acid profiles of mammary glands of female rats of the Sprague-Dawley strain fed a nutritionally adequate diet and tumors induced in this organ by intravenous administration of 7,13-dimethylbenz(a) anthracene (DMBA) of animals fed the same diet.

MATERIALS AND METHODS

Induction of rat mammary tumors: Weanling female rats of the Sprague-Dawley strain, obtained from ARS/Sprague-Dawley, Madison, Wis., were housed in individual cages and fed ad libitum a basic casein-sucrose diet containing vitamins and minerals in the required amounts and 10% by wt of corn oil (16).

Tumors were induced in the mammary glands of the animals by a modification of the procedure described by Huggins, et al. (17). A lipid emulsion (0.2 ml) of 1% (w/v) DMBA was injected into the caudal vein of animals at ages of 50, 53, and 56 days. The DMBA emulsion was prepared by dissolving 50 mg in 1.5 ml corn oil by warming and stirring the mixture until a clear solution was obtained. The final emulsion was obtained by mixing 3.5 ml fresh rat serum vigorously with the solution. The animals were sacrificed ca. 3 months after the last injections of DMBA when they showed external symptoms of the presence of mammary tumors.

Mammary tumors measuring 1-4 cm in diameter were dissected, cleaned of adhering tissue, and quick-frozen on dry ice immediately after the animals were sacrificed. The mammary glands were removed from 6 animals that had been maintained as a control group at ca. the same time, frozen on dry ice, and stored at -20 C until analyzed. The lipids were extracted from the tissues under an atmosphere of nitrogen by homogenization in chloroform-methanol, 2:1, and the nonlipid impurities were removed by aqueous extraction of a solution of the lipid in chloroform and petroleum ether, as previously described (14). The amount of lipid in each tissue was determined from an aliquot of this solution by gravimetric analyses.

Fractionation of the lipid into neutral lipids, glycolipids, and phospholipids was carried out using columns of silicic acid treated with ammonium hydroxide, as previously described (18). The neutral lipids were eluted with chloroform, the glycolipids with acetone, and the phospholipids with methanol after flushing acetone out of the column with chloroform. The

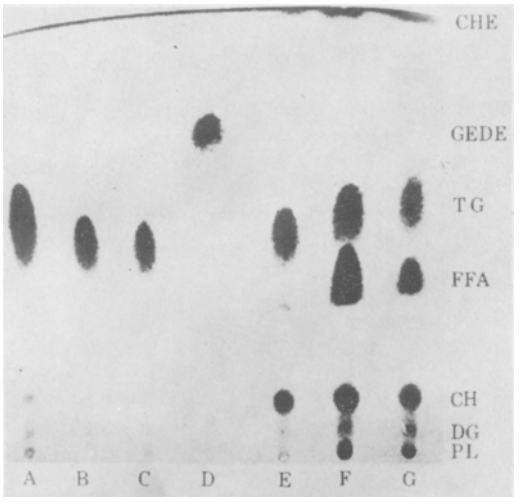

FIG. 1. Thin layer chromatogram of neutral lipid fractions. A, B, and C, rat mammary gland; D, glyceryl ether diester; E, F, and G, rat mammary tumor. Silica Gel H, 0.3 mm thick, solvent system, Skelly F:diethyl ester:acetic acid, 90:10:1. CHE = cholesteryl esters, GEDE = glyceryl ether diester, TG = triglyceride, FFA = free fatty acid, CH = cholesterol, DG = diglyceride, and PL = polar lipid.

fractionation was carried out on a column 39 x 1 cm containing 17.5 g adsorbent with samples of the order of 65 mg. The relative amount of each fraction was determined by gravimetric analysis.

Fatty acid composition was determined by gas liquid chromatography (GLC) of methyl esters prepared by interesterification with methanol by heating a small sample of 1-10 mg at 95 C for 4 hr in sealed tubes containing nitrogen using HCl as a catalyst. The GLC analyses were carried out with an Aerograph gas chromatograph equipped with a 6 ft x 1/8 in. column packed with 10% EGSS-X on Gas Chrom P at 195 C at a flow rate of carrier gas N_2 of 40 ml/min using a flame ionization detector. Fatty acid composition was determined by comparison of retention volumes with authentic methyl ester standards. Quantitative analysis was made on the basis of the proportionalities of the peak areas. Precision of the method was ca. ± 5% for the major components.

Thin layer chromatography (TLC) of the lipid classes was carried out using Silica Gel G or H (Merck, A.G., Darmstadt, Germany) coated plates. The specific conditions used for these analyses and the components that were identified are described in the figures.

RESULTS

The lipids of the mammary glands of the

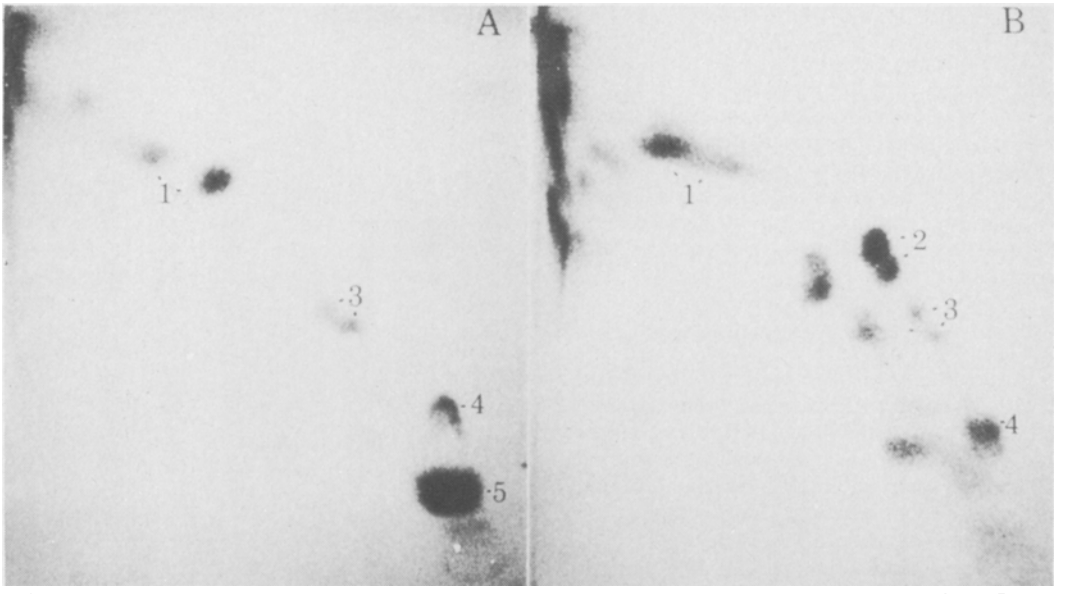

FIG. 2. Two dimensional thin layer chromatogram of glycolipid fractions. A. Rat mammary gland. B. Rat mammary tumor. 1. Ceramide monohexoside. 2. Ceramide dihexoside. 3. Ceramide trihexoside. 4. Ceramide polyhexoside. 5. Ganglioside. Silica Gel H, 0.3 mm thick, solvent system, first direction (y-direction), chloroform:methanol:water, 65:25:4, second direction (x-direction), chloroform:acetone:methanol:acetic acid:water, 5:2:1:1:0.5.

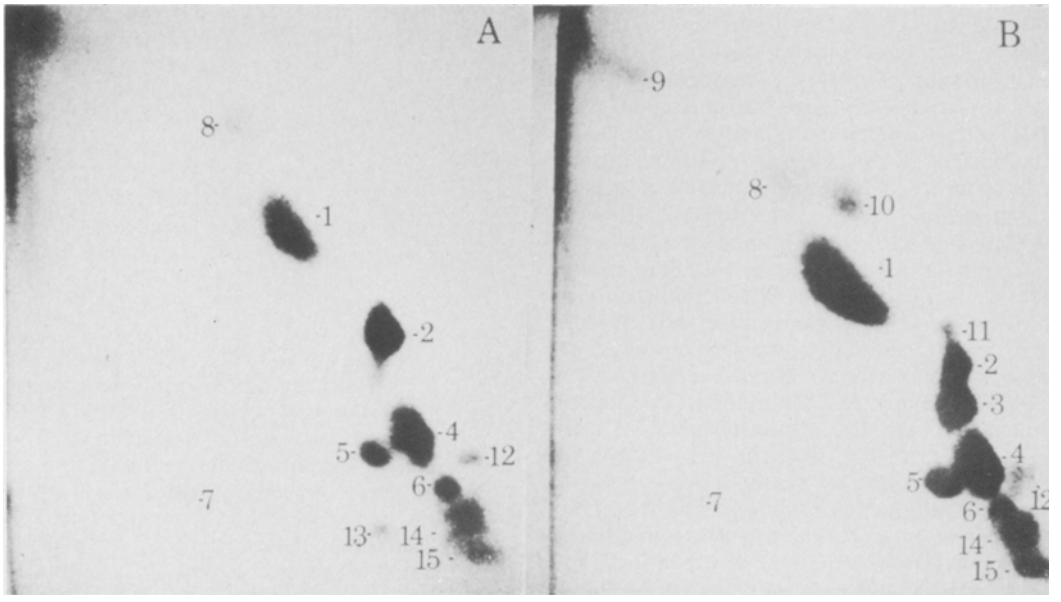

FIG. 3. Two dimensional thin layer chromatogram of the phospholipid fractions. A. Rat mammary gland. B. Rat mammary tumor. 1. Phosphatidyl ethanolamine. 2. Phosphatidylcholine. 3. Lysophosphatidylethanolamine. 4. Sphingomyelin. 5. Phosphatidyl inositol. 6. Lysophosphatidyl choline. 7. Phosphatidic acid. 8-15. Unidentified. Silica Gel H, 0.3 mm thick, solvent system, (y-direction), chloroform:methanol:ammonium hydroxide, 65:35:5; (x-direction), chloroform:acetone:methanol:acetic acid:water, 5:2:1:1:0.5.

control animals and the tumor tissue differed greatly in composition, as shown in Table I. The amount of lipid in the tumor tissues was only ca. one-twentieth of those of the mammary glands. This difference was largely a reflection of the relative amounts of the neutral and phospholipid fractions. The phospholipid fraction was only a minor component of the

mammary gland lipid, but a major component of the tumor lipid. The glycolipid fraction was greater in the lipid of the tumors than the mammary glands but was a minor component in both lipids. The composition of the tumor lipids was more variable than the lipids of the mammary glands of the control animals, as evidenced by the higher standard deviation undoubtedly because of differences in the individual tumors as indicated, at least in part, by the differences in their sizes.

Large differences existed in the fatty acid composition of the lipids of the tissues as shown in Table I. The greatest differences appeared to be in the relative amounts of long chain unsaturated fatty acids. There were fairly large variations in the fatty acid composition of the tumor lipids, as indicated by the standard deviations, but the same general pattern was evident in all of the fractions (Table I).

TLC showed that the composition of the lipid fractions of the tumor tissue differed greatly from their counterparts isolated from the lipids of the mammary glands of the control group. The differences were greater in some tumors than others; however, the pattern of the differences was similar. Typical differences in the neutral lipids are shown in Figure 1.

The neutral lipids of the tumor tissues contained a much higher percentage of cholesterol and free fatty acids. No glyceryl ether diesters were detected in the lipids of either the tumors or the mammary glands of the control animals. Quantitative analyses of the composition of the neutral lipid fraction by TLC-charring-densitometry technique (18) showed that the mammary gland lipid of the control animals consisted of ca. 90% triglyceride, 6% free fatty acid, and less than 1% cholesterol. Tumor lipid varied considerably in composition; the average of the two samples F and G, Figure 1, was 20.5% for triglyceride, 57.2% free fatty acid, and 15.2% cholesterol. Small amounts of cholesterol esters also were detected in the tumor lipid, as shown in Figure 1, but were not measured.

The composition of the glycolipid fractions of the two tissues differed both quantitatively and qualitatively, as illustrated in Figure 2. The tumor tissue contained less ganglioside and appeared to be devoid of some of the components of this fraction observed in the lipid of the mammary glands of the control animals. Differences also were observed in the cerebroside and polyhexoside fractions. In addition, the glycolipid fraction of the tumor lipid appeared to contain a number of unknown constituents. Comparison of the two dimensional chromatograms of the TLC of the phospholipids also showed some differences in the composition of this fraction, but the differences appeared to be mostly quantitative (Figure 3).

DISCUSSION

The present study, like that of Rees, et al., (15) showed that large differences existed in the relative amounts of triglycerides and phospholipids of the mammary glands of rats and the tumors induced in this gland by DMBA. The neutral lipids of the tumors contained much higher concentration of free fatty acids and cholesterol than that of the mammary glands of the control group of animals. Some differences also were present in the composition of the phospholipids, but the differences in the composition of the glycolipid fractions were the most interesting and significant. The concentration of the glycolipid fraction was elevated in the tumor lipids, but it was a minor component of the lipid of both tissues. The striking feature of the differences between the composition of this fraction of the mammary gland tissue of the control animals and mammary tumor tissue was its similarity to the differences observed in this fraction between human liver and hepatoma tissue previously reported (19). The similarity of the differences in these fractions was even more remarkable considering the large general differences between the lipids of these organs and their neoplasms from two widely different mammalian species, human and the rat. The significance of the glycolipid fraction in tumor growth is not known. However, glycolipids have been reported in the reactions involving cell surface antigens and are important in contact inhibition phenomenon in the regulation of cell proliferation (3,8). The difference in fatty acid composition between the mammary gland lipid and mammary tumors also may be important in tumor growth, inasmuch as the properties of the host molecules are influenced by the constituent fatty acids (5,12-14,20).

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Influence of Dietary Fatty Acids on Membrane Properties and Enzyme Activities of Liver Mitochondria of Normal and Hypophysectomized Rats

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ABSTRACT

Studies are reported on the effects of diets containing fatty supplements with (A) a high concentration of arachidonate (46% concentrate of ethyl arachidonate), (B) a high concentration of linoleate (corn oil), and (C) an essential fatty acid deficient, fully saturated fat (hydrogenated coconut oil) upon lipid composition, membrane permeability, and enzyme activities of liver mitochondria of normal and hypophysectomized rats. The fatty supplements produced differences in the fatty acid composition of the liver mitochondria; hypophysectomy, in addition, influenced the neutral and phospholipid composition. Permeability, indicated by swelling properties, correlated generally with the degree of unsaturation and essential fatty acid content of the lipid of the mitochondria of the normal animals. The fatty supplements also influenced the enzyme acitivites of the mitochondria of the normal animals. The mitochondria of the hypophysectomized animals were less responsive to the differences in the dietary fat in both their swelling properties and enzyme activities. Although the relationship was complex, it appeared that the hypophysis was involved in the functions of essential fatty acids in liver mitochondria.

INTRODUCTION

The effects of dietary fat upon the lipid composition of mitochondria have received considerable attention in relation to the role of polyunsaturated fatty acids, particularly essential fatty acids (EFA), in the structure and function of membranes (1-8). The importance of phospholipids as structural components of membranes has long been recognized. It is now apparent that these compounds also are required for maximum activity of a number of membrane-bound enzymes (9-19). In this study, a comparison is made of the effects of diets containing fatty supplements differing widely in their fatty acid composition, particularly in EFA, upon lipid composition, membrane permeability, and enzyme activities of liver mitochondria of normal and hypophysectomized rats.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were housed in individual cages and fed ad libitum a basic fat-free diet (20) supplemented with 10% corn oil (CO group), 10% hydrogenated coconut oil (HCO group), or 10% of a concentrate of ethyl arachidonate, 46% arachidonic acid (AR group). The ethyl arachidonate concentrate was prepared from the fatty acids of fresh pig liver by low temperature fractional crystallization after removal of the nonsaponifiable material. The same diets were fed to corresponding groups of rats hypophysectomized 2-4 days after weaning (Hormone Assay Labs, Chicago, Ill.). Each group contained 10 animals. The animals were sacrificed starting ca. 10 weeks after weaning. The livers were excised and homogenized in 10 volumes of 0.24 M sucrose containing 20 mM Tris-HCl, pH 7.4. Mitochondria were isolated by established procedures (21) with only a few modifications. To reduce the microsomal contamination, the mitochondria were spun down at 4500 rpm for 8 min and subsequently at 7500 rpm for 2 min on a Sorvall centrifuge with an SS 34 rotor and washed twice at the same centrifugal force. This procedure reduced microsomal contamination to less than 10%, as measured by the specific activity of glucose-6-phosphatase. Contamination of the mitochondria with the lysosomal fraction was also very minor as judged from the acid phosphatase activity. The preparation of outer and inner mitochondrial membrane was performed by the swelling and shrinking procedure (21) followed by 3 hr sucrose-density centrifugation in an SW 39 rotor at 29000 rpm using a Beckman model L-2 ultracentrifuge. The mitochondria suspension (1.5 ml, 15-19 mg/ml) was treated with potassium phosphate for 30 min and with adenosine 5'-triphosphate (ATP) for 5 min at 0 C and layered on top of 4 ml 1.18 M sucrose.

All fractions were monitored for purity by

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TABLE I

Major Fatty Acids of Dietary Fat and Mitochondria of Normal and Hypophysectomized Rats (ca. 10 Weeks after Weaning) Fed a Basic Fat-Free Diet Supplemented with Corn Oil (CO), Hydrogenated Coconut Oil (HCO), or Ethyl Arachidonate Concentrate (AR)

	(Percent wt)								
	CO group			HCO group			AR group		
	Mitochondria			Mitochondria			Mitochondria		
Fatty acid	Normal	Hypox	Dietary fat	Normal	Hypox	Dietary[a] fat	Normal	Hypox	Dietary[b] fat
16:0	20.0 ± 0.9[c]	15.3 ± 0.4	12.5	17.2 ± 3.1	16.7 ± 0.3	8.5	21.8 ± 1.6	17.8 ± 0.4	
16:1	3.0 ± 1.5	2.2 ± 0.4		10.2 ± 1.9	10.9 ± 1.0		3.1	1.2 ± 0.2	0.2
18:0	18.8 ± 0.9	19.0 ± 0.4	2.5	18.7 ± 1.3	18.1 ± 0.4	9.5	15.3 ± 2.2	21.0 ± 0.3	
18:1	9.2 ± 0.3	8.5 ± 1.4	30.0	20.8 ± 0.9	25.3 ± 2.1		8.1 ± 0.7	10.3 ± 0.6	0.8
18:2	21.6 ± 0.8	25.4 ± 1.3	54.0	5.4 ± 1.2	4.5 ± 1.3		12.0 ± 1.6	10.0 ± 3.4	6.5
20:3		1.7 ± 0.3		13.1 ± 4.8	12.6 ± 1.0			1.8 ± 0.1	7.4
20:4	20.5 ± 0.5	23.0 ± 4.9		9.1 ± 0.1	7.3 ± 3.1		33.4 ± 4.4	30.1 ± 2.2	46.2
22:5	1.4 ± 0.6	0.3		1.0 ± 0.5	0.9		1.6 ± 0.1	1.4 ± 0.5	4.4
22:6	3.7 ± 0.3	3.1 ± 0.4		0.8 ± 0.2	2.0 ± 1.2		6.1 ± 0.7	9.2 ± 2.5	10.3

[a]Other fatty acids were 8:0 = 6.2; 10:0 = 6.3; 12:0 = 49.0; and 14:0 = 20.0.

[b]Other fatty acids were 18:3, 9.4%; 20:5, 3.4%; and unidentified minor components.

[c]Mean ± standard deviation.

TABLE II

Lipid Class Analysis of Mitochondria of Normal and Hypophysectomized (2-5 Days after Weaning) 10 Week Old Rats Fed a Basic Fat-Free Diet Supplemented with 10% Corn Oil (CO), Hydrogenated Coconut Oil (HCO), or an Ethyl Arachidonate Concentrate (AR)

	Percent wt of total lipid					
	CO group		HCO group		AR group	
Lipid classes	Normal	Hypox	Normal	Hypox	Normal	Hypox
Unidentified[a]	2.3	2.4	6.4	0.9	2.5	---
Cholesterol esters	1.3 ± 0.4[b]	1.1 ± 0.3	2.8 ± 1.8	3.6 ± 1.4	0.2 ± 0.1	2.1 ± 0.9
Triglycerides	2.0 ± 1.7	1.9 ± 1.1	5.1 ± 4.4	3.1 ± 2.1	0.5 ± 0.1	6.6 ± 1.3
Fatty acids	2.2 ± 0.9	3.3 ± 2.2	4.1 ± 1.6	2.2 ± 0.3	1.3 ± 0.4	1.5 ± 1.0
Cholesterol	3.7 ± 2.7	5.9 ± 2.7	8.9 ± 7.8	6.9 ± 1.0	0.3 ± 0.1	4.2 ± 1.1
Neutral lipids	11.5 ± 1.7	14.6 ± 1.6	27.3 ± 3.9	16.7 ± 1.5	4.8 ± 0.5	14.4 ± 1.0
Polar lipids[c]	88.5 ± 7.0	85.4 ± 6.0	72.7 ± 7.6	83.3 ± 5.8	95.2 ± 2.1	85.6 ± 7.1

[a]Mixture of minor components.

[b]Mean ± standard deviation.

[c]Determined by the difference in the amount of total lipid and the sum of the neutral lipids.

electron microscopy.

For fatty acid and lipid analysis, the mitochondria were extracted by the Folch procedure (22). The fatty acids were analyzed as their methyl esters by gas liquid chromatography (GLC) using an Aerograph instrument equipped with a flame ionization detector and an 8 ft x 1/8 in. column packed with 10% EGSS-X on Chromosorb W at 200 C. Nitrogen was used as the carrier gas and the percent distribution of the fatty acids was obtained by triangulation of the peak areas. The methyl esters were prepared by interesterification by heating 1-10 mg lipid with 3 ml dry 6% HCl (by wt) in methanol under a nitrogen atmosphere in a sealed glass tube in a boiling water bath for 2 hr. The lipid classes were analyzed by a combination of column and thin layer chromatography (TLC), as previously described (23), in which the charring densitometry technique was used for quantification of individual components using the appropriate reference standards. The unknown components among the neutral lipids were not identified and were grouped together in the analysis. The polar lipid fraction was determined by the difference between the total sample and the total amount of the neutral lipid fraction.

Fresh mitochondria, less than 5 hr old, were suspended in 0.25 M sucrose containing 20 mM Tris-HCl buffer, pH 7.4, a system in which no swelling occurred at 25 C. The extent of swelling was followed by the decrease in optical density at 520 nm using a Beckman DU model spectrophotometer connected to a Haake thermostat to maintain constant temperature of 25 ± 1 C. To minimize variations, the protein concentration was controlled, and the equilibration time at 25 C was standardized at 3 min prior to the addition of the swelling agents. The concentration of the swelling agents was chosen so that maximum swelling was reached in less than 20 min. The swelling properties of the mitochondria of the hypophysectomized and

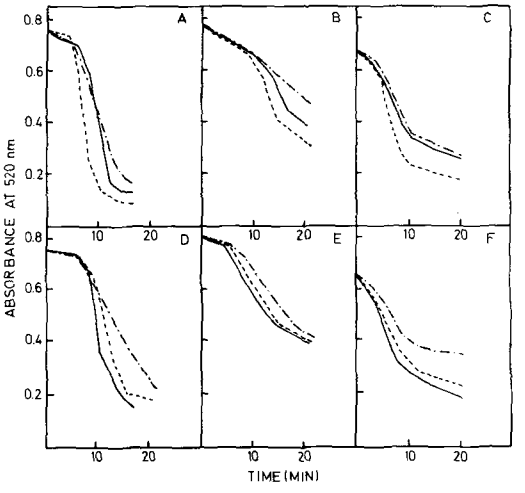

FIG. 1. Swelling of liver mitochondria of normal (A,B,C) and hypophysectomized (D,E,F) rats fed the three different diets: (——) corn oil (CO), (–.–.–) hydrogenated coconut oil (HCO), and (—) arachidonate concentrate (AR). Mitochondrial protein in mg: A, 0.46; B, 0.40; C, 0.46; D, 0.59; E, 0.68; and F, 0.60. The mitochondria were suspended in 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.4. The swelling was measured as decrease in optical density at 520 nm at temperature 25 C after the addition of: (A,D) 10 mM glutathione (A, 0.02-0.10 and D, 0.05-0.18 A_{520} units); (B,E) 2 mM K_2HPO_4 (B, 0.01-0.10 and E, 0.02-0.16 A_{520} units); (C,F) mitochondria suspended in 75 mM NH_4Cl, 20 mM Tris-HCl, pH 7.4, 2 μM Rotenone, 0.5 μM valinomycin (C, 0.01-0.05 and F, 0.02-0.10 A_{520} units). Probability differences between the HCO and the AR groups for both the normal and hypophysectomized animals are: (A) $P < 0.025$, (B) $P < 0.01$, (C) $P < 0.10$, (D) $P > 0.50$, (E) $P > 0.5$, and (F) $P < 0.10$.

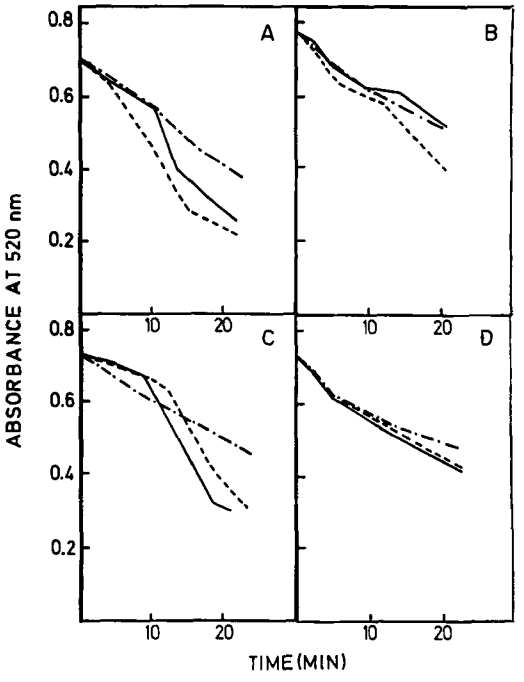

FIG. 2. Swelling of the liver mitochondria of normal (A,B) and hypophysectomized (C,D) rats fed three different diets: (——) corn oil (CO), (–.–.–) hydrogenated coconut oil (HCO), and (– –) arachidonate concentrate (AR). Mitochondrial protein in mg: A, 0.40; B, 0.46; C, 0.68; and D, 0.60. The mitochondria were suspended in 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.4. The swelling was measured as decrease in optical density at 520 nm at temperature 25 C after the addition of: (A,C) mitochondria suspended in 0.1 M NH_4Cl and 2 mM Tris-HCl, pH 8.3 (A, 0.01-0.04 and C, 0.01-0.13 A_{520} units); (B,D) mitochondria suspended in 65 mM NH_4Cl, 20 mM Tris-HCl, pH 7.4, 1.5 μM ethylenediamine tetraacetic acid and 80 mM NaN_3 (B, 0.01-0.09 and D, 0.02-0.14 A_{520} units). Probability differences between the HCO and the AR groups for normal and hypophysectomized animals are: (A) $P < 0.005$ and (C) $P < 0.10$.

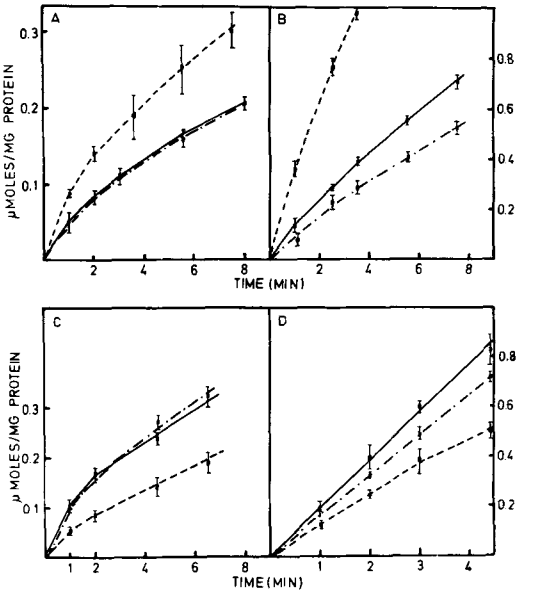

FIG. 3. L-Glutamate dehydrogenase of liver mitochondria matrix of normal (A) and hypophysectomized (C) rats kept on either a corn oil (CO) (——), a hydrogenated coconut oil (HCO) (–.–.–), or an arachidonate concentrate (AR) containing diet (– –). The enzyme activity was measured as increase in optical density at 340 nm at 37 C. The cuvettes contained in 2.0 ml: 0.5 mg (1.0 mg) mitochondrial matrix protein; 50 mM phosphate buffer, pH 7.4; 30 mM nicotinamide; 0.4 mM potassium cyanide (KCN) 13 mM L-glutamate; and the reaction was started with 0.7 mM diphosphopyridine nucleotide, oxidized form (DPN). β-Hydroxybutyrate dehydrogenase of liver mitochondria inner membrane of normal (B) and hypophysectomized (D) rats. The enzyme activity was measured as increase in optical density at 340 nm at 37 C. The cuvettes contained in 2.0 ml: 0.5 mg (0.25 mg) mitochondrial inner membrane protein; 20 mM Tris-HCl buffer, pH 8.5; 30 mM nicotinamide; 1.0 mM ethylenediamine tetraacetic acid; 0.4 mM KCN; 50 mM sodium (DL) β-hydroxybutyrate; and the reaction was started with 0.7 mM DPN.

normal animals were determined using glutathione, phosphate, sodium azide, NH_4Cl plus valinomycin, and NH_4Cl under alkali conditions as swelling agents. The conditions used with each agent are described in the legends of the figures.

ATP, diphosphopyridine nucleotide, oxidized form (DPN), diphosphopyridine nucleotide, reduced form (DPNH), triphosphopyridine nucleotide, oxidized form (TPN), triphosphopyridine nucleotide, reduced form (TPNH), glucose-6-phosphate, β-glycerophosphate, β-hydroxybutyrate acid, L-glutamic acid, nicotinamide, and cytochrome C were purchased from Sigma Chemical Co., St. Louis, Mo.

Enzyme measurements: glucose-6-phosphatase, acid phosphatase, cytochrome C oxidase, TPNH cytochrome C reductase, succinate cytochrome C reductase, 6-phosphogluconate dehydrogenase, β-hydroxybutyrate, and L-glutamate dehydrogenase were measured as described by Beaufay, et al. (24). Mitochondria-located ATPase was assayed according to Sandoval, et al., (25) except for arsenate which was omitted. The enzyme was stimulated by 2,4-dinitrophenol and inhibited by oligomycin. Lipid peroxidase was analyzed according to the procedure of Placer, et al. (26). Protein was determined by the Lowry method (27) and phosphorus by the Bartlett procedure (28).

RESULTS

The fatty acid composition of the dietary fat

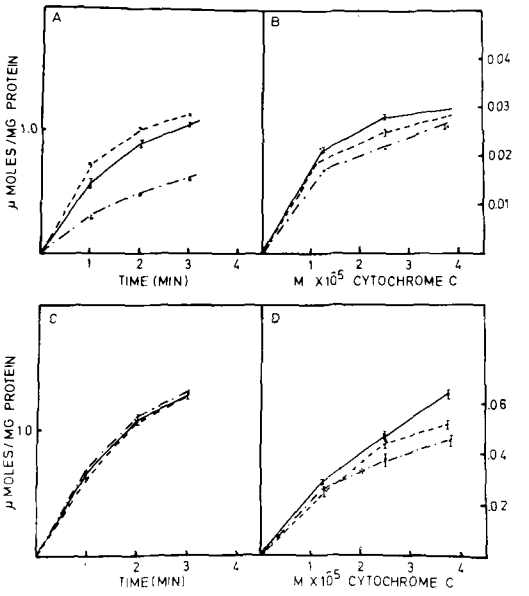

FIG. 4. Cytochrome C oxidase of liver mitochondria of normal (A) and hypophysectomized (C) rats kept on either a corn oil (CO) (———), a hydrogenated coconut oil (HCO) (–.–.–), or an arachidoncate concentrate (AR) (—) containing diet. The enzyme activity was followed as decrease in optical density at 550 nm at 37 C. The cuvettes contained in 2.0 ml: 0.05 mg (A) and 0.06-0.064 mg (C) mitochondrial protein; 30 mM phosphate buffer, pH 7.4; and 50 μM reduced cytochrome C. Succinate cytochrome C reductase of mitochondria of normal (B) and hypophysectomized (D) rats. The enzyme activity was followed as increase in optical density at 550 nm at 37 C. The cuvettes contained in 2.0 ml: 0.1 mg (B) and 0.2 mg (D) mitochondrial protein; 50 mM phosphate buffer, pH 7.4; 30 mM nicotinamide; 0.2 mM potassium cyanide (KCN); cytochrome C (oxidized) at the indicated amounts; and 5.5 mM succinate.

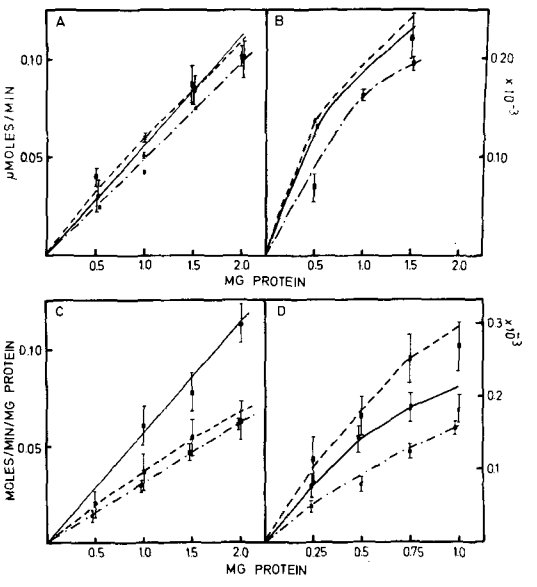

FIG. 5. Adenosine triphosphatase (ATPase) of mitochondria of normal (A) and hypophysectomized (C) rats kept on either a corn oil (CO) (———), a hydrogenated coconut oil (HCO) (–.–.–), or an arachidonate concentrate (AR) (—) containing diet. The enzyme activity was measured by determining the liberated inorganic phosphate from ATP after incubating the mixture for 15 min at 37 C. The incubation mixture contained in 1.0 ml: 40 mM KCl, 0.1 mM ethylenediamine tetraacetic acid, 5 mM ATP, and between 0.5 and 2.0 mg mitochondrial protein. The reaction was stopped by adding 2 ml 10% trichloroacetic acid and centrifuged, and then an aliquot was taken for phosphate analysis. Lipid peroxidase of liver mitochondria of normal (B) and hypophysectomized (D) rats. The photometric measurement was carried out at 548 nm using the method of Placer, et al., (26) with a slight modification. The incubation mixture contained in 0.5 ml: 0.25 M Tris-maleate buffer, pH 7.2; 2 mM arachidonic acid as sodium salt; and mitochondrial protein in the indicated amounts. The incubation was carried out at 37 C for 30 min.

produced well defined differences in the fatty acid composition of the mitochondrial lipids as shown in Table I. The HCO supplement produced a fatty acid composition typical of an EFA deficiency, elevation of 20:3, 18:1, and 16:1 and a corresponding decrease in 18:2 and 20:4 compared to the CO supplement. However, the mitochondria of the HCO group were not depleted entirely of their stores of essential fatty acids. The fatty acid composition of the mitochondria of the AR group differed mainly from that of the CO group by the higher level of 20:4 and lower level of 18:2. There was little effect of hypophysectomy upon the fatty acid composition produced by the fatty supplements, except for a small amount of 20:3 that was detected in the AR and CO groups.

The fatty supplements also produced differences in the relative amounts of neutral and phospholipids in the mitochondrial lipid; the neutral lipid was highest in the animals receiving the HCO supplement, which was the most saturated, and the lowest in the groups receiving the AR supplement, which was the most unsaturated. These differences essentially were erased by hypophysectomy by an increase in the relative amount of neutral lipids in the animals receiving the CO and AR supplements (Table II).

The swelling properties of the mitochondria of the normal animals were influenced by the fatty supplements with all of the swelling agents, except sodium azide (Figs. 1 A,B,C and 2 A,B). In general, the effect was greatest with the mitochondria of the AR group and least with those of HCO group, and the differences were significant between these groups with most of these agents. The swelling properties of the mitochondria of the hypophysectomized

animals also were influenced by the dietary supplements but to a lesser degree than those of the normal animals, and the particular stimulatory effect of the AR supplemented diet was absent, inasmuch as it was generally ca. the same or less than the CO supplement (Figs. 1 D,E,F and 2 C). The differences in the swelling properties of the mitochondria of the three groups of either the normal or hypophysectomized animals when sodium azide was used as the swelling agent were not significant (Fig. 2 B,D). While the AR supplement appeared to have a slight stimulatory effect with this agent, the responses to the HCO and CO supplement were essentially the same.

Studies of mitochondrial enzymes showed that, in general, the activities of the enzymes increased with increased unsaturation of the fatty supplements. The activities of L-glutamate dehydrogenase, a matrix located enzyme, and β-hydroxybutyrate dehydrogenase, located in the inner membrane, were increased greatly by the AR supplement compared to the CO and HCO supplements (Fig. 3 A,B). The effect of the dietary fatty supplements upon these enzymes was much different in hypophysectomized animals. In fact, the activity of these enzymes appeared to be suppressed by the AR supplement.

The activity of cytochrome C oxidase was greater in the mitochondria of the normal animals receiving the AR and CO supplement than in those of the HCO group (Fig. 4 A). This effect also was erased by hypophysectomy as shown in Figure 4 C. The activity of succinate cytochrome C reductase of the mitochondria of the HCO group was lower than that of the other groups, and the differences were similar in the mitochondria of the hypophysectomized animals; but the activity of this enzyme did not appear to be related to EFA content, inasmuch as the CO and AR group were essentially the same (Fig. 4 B and 4 D, respectively).

The activity of ATPase of the mitochondria was influenced by fatty supplements in the normal animals relative to their degree of unsaturation and EFA content. It was also lowest in the mitochondria of the hypophysectomized animals that received the HCO supplement, but the relationship between the CO and AR supplements was reversed by hypophysectomy (Fig. 5 A and C). With lipid peroxidase, the activity varied relative to the EFA content of the fatty supplement in both the normal and hypophysectomized animals, as shown in Figure 5 B and D, respectively.

DISCUSSION

Effects of dietary fat upon membrane structure-function have been related mainly to studies on the effect of EFA through the use of diets devoid of these acids (1-3) or by refeeding starved animals diets high in carbohydrates devoid of EFA (29-33). The fatty supplements used in the present study varied widely in their composition and produced wide differences in the fatty acid and lipid class composition of mitochondria. However, the mitochondria of the HCO group of animals were not depleted entirely of their stores of linoleic and arachidonic acids. Hence, the mitochondria of the three groups varied in their EFA content and also in lipid unsaturation. Hypophysectomy did not alter greatly the fatty acid composition of the mitochondria of the three groups but did affect the distribution of the fatty acids among the lipid classes, inasmuch as it influenced the relative amounts of neutral and polar lipids. In general, the rate of translocation of anions and cations, as measured by swelling, was slower in the mitochondria of the normal animals with the lowest EFA content. The swelling was also generally the lowest in the mitochondria of these animals after hypophysectomy but was inconsistent in the mitochondria of the hypophysectomized animals receiving the AR and CO supplements. Hence, the swelling properties appeared to be related to the EFA content, either directly or relative to the fatty acid distribution among the lipid classes through a function of the hypophysis. Comparison of the mitochondria swelling properties between the HCO- and CO-fed animals did not exhibit the wide differences as in some reports, but the swelling amplitude under the conditions employed in this study seem more likely to correlate with physiological phenomenon than long term (5 hr) swelling experiments. Sodium azide is known to uncouple oxidative phosphorylation (34). Hence, the absence of an effect of this agent relative to the different dietary supplements may be explained by the general impairment of mitochondrial function.

The enzyme activities of the mitochondria appeared to follow the same pattern as the swelling properties. With the normal animals, the enzyme activities of the mitochondria appeared to be related to their EFA content as reflected by the difference in the fatty acid composition of the supplements. There did not appear to be any well defined relationship between the enzyme activities and the EFA content of the mitochondria of the hypophysectomized animals, except for lipid peroxidase. However, the enzyme activity was generally lower in the mitochondria of the hypophysectomized animals that received the HCO supplement. Hence, as with the swelling properties,

the hypophysis appeared to be involved in the role of EFA in the enzyme activity of the liver mitochondria, inasmuch as the fatty acid composition was not particularly influenced by hypophysectomy.

ACKNOWLEDGMENTS

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On Methylating Activity of L-(Methyl-^{14}C)-Methionine in Metabolism of Phospholipids by Insect *Ceratitis capitata*

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ABSTRACT

The methylating activity of L-(methyl-^{14}C)-methionine in different stages of development of the insect *Ceratitis capitata* was studied in a series of in vitro and in vivo experiments. Larval and pharate adult homogenates of the insect were used in the in vitro conditions, and the utilization of the methyl group of methionine for the synthesis of different classes of phospholipids was evaluated. Incorporation of radioactivity in lipids by pharate adult homogenates was significantly higher than that by larval homogenates. In both cases, phosphatidyl ethanolamine showed the highest levels of radioactivity incorporation. Free bases from total lipid hydrolysates were resolved and identified by paper chromatography, and the labeling was investigated by radioactivity scanning of paper chromatograms. Significant differences were observed in the activity of both stages of development of the insect. Larval and pharate adult homogenates incorporated mainly the labeled methyl groups into ethanolamine. Monomethyl ethanolamine was the only methyl derivative that appeared in the hydrolysates of lipids synthesized by larval homogenates, whereas mono-, di- and trimethyl ethanolamine clearly were detected in those synthesized by pharate adult homogenates. Administration of L-(methyl-^{14}C)-methionine to larvae confirmed the existence of methylation reactions in the metabolic activity of the insect.

INTRODUCTION

It generally is accepted that transmethylation reactions have no signification in the phospholipid metabolism among insects (1). Thus, no evidence for transmethylation products was obtained by the labeling found in fractionated tissue extracts of *Tribolium confusum, Phormia regina*, and other insects reared on diets containing labeled methyl donors (2).

Nevertheless, larvae of *Phormia regina* and *Musca domestica* can synthesize large amounts of phosphatidyl-N,N'-dimethyl ethanolamine when dimethyl aminoethanol is added to the diet (3,4).

On the other hand, by the injection of L-(methyl-^{14}C)-methionine to larvae of *Vitula edmandsae serratilineela* the presence of a transmethylation system capable of methylating phosphatidyl ethanolamine (PE) to form phosphatidyl-N-methyl ethanolamine, phosphatidyl-N,N'-dimethyl ethanolamine, and phosphatidyl choline (PC) was indicated (5).

In a series of previous experiments, the levels and specific radioactivities of the main phospholipid classes from *Ceratitis capitata* at different stages of development were determined when 5 day old larvae were fed on diets containing either ^{3}H-glycerol, ^{32}P-orthophosphate, or ^{14}C-acetate (6). The clear tendency to equalize the specific activities of PC, PE, and phosphatidyl serine suggested the possibility of participation of base conversion mechanisms during the metamorphosis of the insect.

The purpose of this contribution is to investigate further the methylating activity of L-(methyl-^{14}C)-methionine either when it was fed to larvae of *C. capitata* or when it was incubated in the presence of larval or pharate adult homogenates of the insect.

MATERIALS AND METHODS

Rearing of Insects

Larval and pharate adult *C. capitata* (Wiedemann) were used. Diet, temperature, and humidity conditions during culturing were controlled carefully. Culturing of the insect was carried out under the conditions previously described (7).

Preparation of Homogenates

Larvae were reared until 2-3 days before the larval-pupal apolysis and were starved 3-4 hr before used. Pharate adults were collected 5 days beyond the larval-pupal apolysis. Both larvae and pharate adult were washed carefully with distilled water before use in the experiments. Larvae and pharate adults were homogenized directly with 3 vol cold homogenizing buffer (0.35 M sucrose-0.05 M tris, pH 7.4) in a Potter-Elvehjem glass homogenizer with a Teflon pestle. Homogenates were handled according to the method described (8). Floating lipids were removed from larval and pharate adult

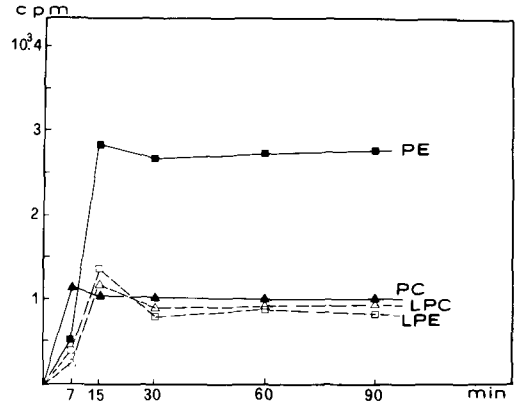

FIG. 1. Radioactivity incorporated into different classes of phospholipids by larval homogenates of *Ceratitis capitata* from L-(methyl-^{14}C)-methionine. PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, LPC = lysophosphatidyl choline, and LPE = lysophosphatidyl ethanolamine.

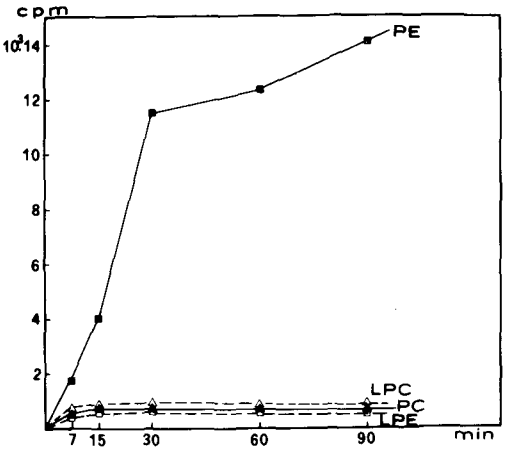

FIG. 2. Radioactivity incorporated into different classes of phospholipids by pharate adult homogenates of *Ceratitis capitata* from L-(methyl-^{14}C)-methionine. PE = phosphatidyl ethanolamine, LPC = lysophosphatidyl choline, PC = phosphatidyl choline, PC = phosphatidyl choline, and LPE = lysophosphatidyl ethanolamine.

homogenates used as enzyme preparations in the in vitro experiments.

In vitro Assay Mixtures

The assay mixture for incorporating L-(methyl-^{14}C)-methionine contained/1 ml: adenosine 5′-triphosphate, 2.4 mg; α-glycerophosphate, 2.6 mg; $MgCl_2$, 2.0 mg; $NaHCO_3$, 1.6 mg; NaCl, 7.2 mg; KH_2PO_4, 0.70 mg; tris-base, 0.8 mg; sodium penicillin, 0.4 mg; streptomycin sulphate, 0.4 mg; phosphatidyl-N,N′-dimethyl ethanolamine, 4 mg; and sodium desoxycholate, 8 mg. The mixture was sonicated for 15 sec. To 0.5 ml mixture was added 0.5 ml respective homogenate containing 10-12 mg proteins and 0.1 ml (12.5 μCi) of labeled methionine (specific activity, 56 mCi/mmol; The Radiochemical Centre, Amersham, England). Incubations were carried out in a shaker at 37 C for different times.

In vivo Assays

Five day old larvae (5 g) were starved 5 hrs and fed afterwards for 3 hr on 0.5 g diet containing 250 μCi L-(methyl-^{14}C)-methionine. After this time, 1 g pool of larvae were washed carefully and submitted to the extraction of lipids. The rest of the pool of larvae were left to pupate under the ordinary conditions of diet and collected as 5 day old pharate adults. Insects at this stage of development also were submitted to the general procedure of extraction of lipids.

Extraction of Lipids

At the end of the incubation, the reaction was stopped by the addition of chloroform, and total lipids were obtained according to the method of Bligh and Dyer (9). Larvae and pharate adults from the in vivo experiments also were extracted according to the procedure of Bligh and Dyer (9). Phosphorous was estimated according to the method previously described (10) based upon the procedure of Bartlett (11).

Fractionation of Classes of Lipids

The total lipids (3 g) were separated on thin layer plates prepared by coating a slurry of 65 g Silica Gel G (E. Merck, Darmstadt, Germany) in 140 ml distilled water to a thickness of 0.3 mm. The solvents used were chloroform-methanol-water (65:25:4, v/v/v) as the first solvent and butanol-acetic acid-water (60:20:20, v/v/v) in the second direction. Lipids were visualized by placing the plates in iodine vapor, and, following evaporation of the iodine, the spots were removed carefully and used for either P determination or radioactivity estimation.

Hydrolysis of Lipids

Total lipids were hydrolyzed with N HCl (0.1 ml/mg lipids) at 100 C for 16 hr. After hydrolysis, the mixture was extracted with 3 vol heptane (3 times), and the aqueous solution was analyzed by paper chromatography. Whatman no. 1 paper, impregnated in N KCl solution and dried, was used for paper chromatography. The upper phase of the mixture phenol-n-butanol-80% formic acid-KCl saturated water (50:50:3:10, w/v/v/v) was used as developing system. Location of radioactive

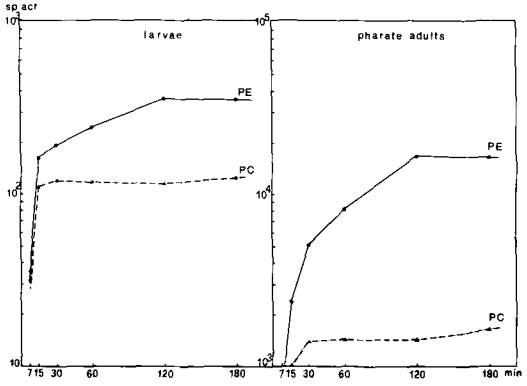

FIG. 3. Specific activities (sp act) (logarithmic scale) of phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) vs time of incubation with larval and pharate adult homogenates of *Ceratitis capitata*.

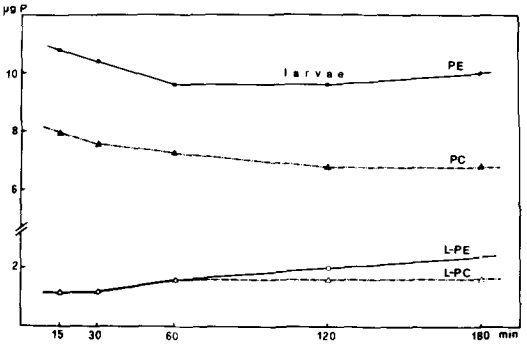

FIG. 4. Changes of the quantitative composition (µg P/10 mg proteins) of different phospholipid classes during incubation of L-(methyl-^{14}C)-methionine with larval homogenates of *Ceratitis capitata*. PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, LPE = lysophosphatidyl ethanolamine, and LPC = lysophosphatidyl choline.

spots on papers was carried out by autoradiography using Valca, H-27, films.

Measurements of Radioactivity

For the radioassay of individual classes of lipids, the spots from the thin layer plates were transferred to scintillation vials containing 10 ml scintillation solution (4 g diphenyloxazole [PPO], 0.1 g diphenyloxazole-benzene [POPOP], toluene to 1 liter).

Paper chromatograms were cut into 3 mm strips, and the radioactivity present in each one was determined by liquid scintillation counting. Data given in the figures represent the mean values of three individual determinations ($p<0.01$). Radioactivity was measured in a Nuclear Chicago model 6766 liquid-scintillation spectrometer. Identification was carried out from R_f data of the literature and using pure standard samples.

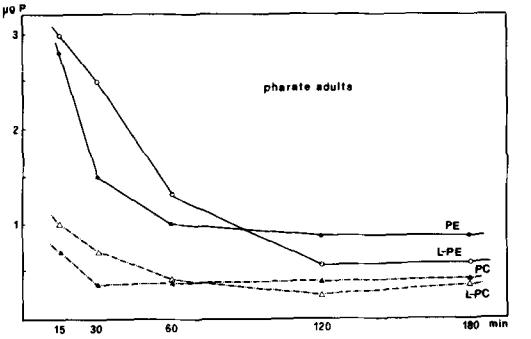

FIG. 5. Changes of the quantitative composition (µg P/10 mg proteins) of different phospholipid classes during incubation of L-(methyl-^{14}C)-methionine with pharate adult homgenates of *Ceratitis capitata*. PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, LPE = lysophosphatidyl ethanolamine, and LPC = lysophosphatidyl choline.

RESULTS AND DISCUSSION

In vitro and in vivo series of experiments were carried out to test the behavior of L-(methyl-^{14}C)-methionine as methyl donor during different phases of development of the insect *C. capitata*.

The in vitro experiments consist in a time study of incorporation of radioactivity into the main phospholipid classes, PE, PC, and their lysoderivatives (lysophosphatidyl ethanolamine [LPE] and lysophosphatiyl choline [LPC]) by either larval or pharate adult homogenates of the insect.

Figures 1 and 2 show the distribution of the incorporated radioactivity by larval and pharate adult homogenates, respectively. Incorporation took place rapidly, and PE accounted in both cases for the highest levels; however, incorporation was noticeably higher in the pharate adult homogenates than in those of the larval stage. PC exhibited low levels of radioactivity, and the PE/PC content ratio was higher in the pharate adult than in the larval homogenates. The incorporation of radioactivity in the lysoderivatives was the same as that of PC in both homogenates.

These findings clearly show that larval and pharate adult homogenates of the insect exhibit a different incorporation capacity of the radioactivity from L-(methyl-^{14}C)-methionine into the different phospholipid classes. This fact stresses again the previous findings on the different metabolic activity of the two stages of development of the insect (8,12,13).

The labeled carbon atom of the methyl group of the methionine was incorporated into PE, mainly by pharate adult homogenates, in agreement with results of Moulton, et al., (14)

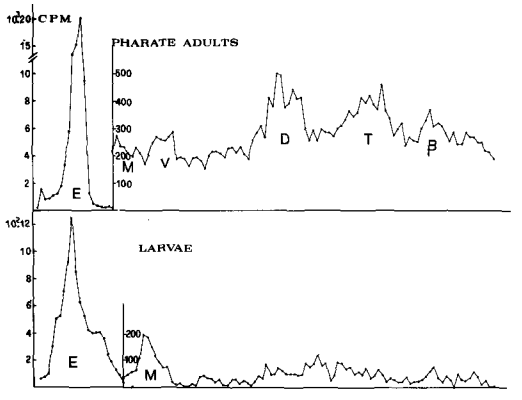

FIG. 6. Scanning of radioactivity on paper chromatograms after separating the bases from lipid hydrolysates. (E = ethanolamine, M = monomethyl ethanolamine, D = dimethyl ethanolamine, T = choline, V = valine, and B = β-methyl choline). In vitro incorporation of radioactivity from L-(methyl-^{14}C)-methionine by larval and pharate adult homogenates of *Ceratitis capitata*.

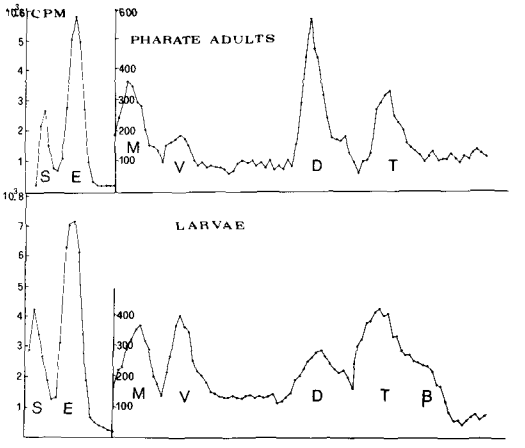

FIG. 7. Scanning of radioactivity on paper chromatograms after separating the bases from lipid hydrolysates. (S = serine. Others as in Figure 6). In vivo incorporation of radioactivity from dietary L-(methyl-^{14}C)-methionine in larvae and pharate adults of *Ceratitis capitata*.

using *M. domestica* larvae. Thus, the results obtained with *C. capitata* are consistent with the idea that one carbon reactions that occur in vertebrate metabolism also are performed in insects. On the other hand, the low levels of radioactivity which appeared in PC allow one to conclude that the in vitro methylation pathway for PC synthesis, although clear, was not of much quantatative significance.

In Figure 3, the specific activities of PE and PC vs time are given.

These results are consistent with the higher incorporation of the methionine methyl group into PE than into PC and confirm both the incorporation of the methyl group into the (1 C) pool and the ability of homogenates to perform transmethylation reactions from methionine using ethanolamine or PE as acceptors.

Figures 4 and 5 show the results of the quantitation of the phospholipids during the time course experiments using larval and pharate adult homogenates, respectively. Since the contents of both PE and PC undergo only a slight decrease during the experiments with larval homogenates, levels of labeled PC (Fig. 3) could be a measure of the synthesis of the phospholipid through the methylation pathway. Patterns of variation of the contents of PE and LPE during the time course experiments using pharate adult homogenates (Fig. 5) showed a sharp decrease during the first 60 min of incubation. PC and LPC showed a low variation during the experiment. The concentrations of phospholipids in the enzyme preparations used in these in vitro experiments differ from the composition of the insect (10) because of the elimination of a layer of the less polar floating lipids during the preparation of the homogenates. From Figures 3 and 5, it is clear that, in spite of the net diminution of the contents of PE (Fig. 5) during the incubation with pharate adult homogenates, the specific activity of the phospholipid was higher than that exhibited in the experiments using larval homogenates (Fig. 3). Thus, it can be concluded that the synthesis of PE from methionine methyl groups can not counterbalance the net degradation of the phospholipid content and that transmethylation reactions occur more efficiently in the pharate adult than in the larval stage of development of the insect under the in vitro conditions. These findings could explain, at least partially, the tendency to equalize the specific activities of PE and PC during development of the insect when larvae were fed on several labeled precursors (6).

To learn more about the methylation reactions in the in vitro experiments, aliquots of both total lipid extracts were hydrolyzed, and the bases were separated and identified by paper chromatography. Figure 6 shows the radioactivity patterns of labeled bases from the phospholipids synthesized in the in vitro experiments using either larval or pharate adult homogenates of the insect. In agreement with the findings on the distribution of radioactivity in phospholipids, ethanolamine exhibited the highest levels of incorporation in both stages of development of the insect; however, the levels achieved in the presence of pharate adult homogenates were much higher than those

using larval homogenates. Labeling of methyl ethanolamines was very scarce with larval homogenates, monomethyl derivative being the only one that exhibited a significant incorporation. Using labeled methionine, it is clear from Figure 6 that an increased proportion of labeled monomethyl and dimethyl ethanolamine, choline and β-methyl-choline was detected following the incubation with pharate adult homogenates. Evidence of labeled β-methyl-choline was gained by means of in vivo experiments (15) in which larvae were fed on (methyl-^{14}C)-choline and L-methionine simultaneously; paper chromatography of total lipid hydrolysates led to a radioactive spot coincidental with the position of a pure standard.

These results support further the more active methylation capacity of the pharate adult homogenates.

Figure 7 shows the patterns of labeling of bases from lipids synthesized in vivo when larvae were fed on L-(methyl-^{14}C)-methionine; bases also were analyzed from lipids of pharate adults coming from further development of an aliquote of the pool of labeled larvae. Both patterns are very similar from a qualitative point of view, ethanolamine and serine being the most labeled bases. Larvae exhibited slightly higher levels of radioactivity in choline than those present in the pharate adult stage.

These results prove the presence of the three methylated ethanolamines in both stages of development of the insect, larvae and pharate adults. These experiments indicated the existence in *C. capitata* of a transfer of methyl groups from methionine to PE in a similar way to the described process in the vertebrate tissues (16). Evidence also is given of the presence of methylated intermediates to the synthesis of PC. Nevertheless, in which proportion the operating mechanism for the synthesis of the labeled PC is either the direct methylation of PE or the incorporation of methylated ethanolamines by the cytidine 5′-triphosphate pathway remains an open question.

Paper chromatography of the phospholipid hydrolysates shows clearly the presence of a radioactive ninhydrine-positive spot that was identified as valine. This fact agrees again with the participation of the methionine methyl groups in the (1 C) metabolic patterns of the insect. Since free amino acids are not carried over the lipid extracts, the presence of valine in the aqueous solution after the hydrolysis of phospholipids indicates the previous existence of phosphatidyl valine.

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Decomposition of Unsaturated Fatty Acid Hydroperoxides by Hemoglobin: Structures of Major Products of 13L-Hydroperoxy-9,11-octadecadienoic Acid

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ABSTRACT

13L-Hydroperoxy-9,11-octadecadienoic acid was decomposed rapidly in the presence of hemoglobin. The product consisted of five major compounds, i.e. 13-keto-9,11-octadecadienoic acid, 13L-hydroxy-9,11-octadecadienoic acid, *erythro*-11-hydroxy-12,13-epoxy-9-octadecenoic acid, *threo*-11-hydroxy-12,13-epoxy-9-octadecenoic acid, and 9DL-hydroxy-12,13-epoxy-10-octadecenoic acid.

INTRODUCTION

Heme compounds catalyze decomposition of fatty acid hydroperoxides, and free radical intermediates generated during this process initiate oxygenation of unsaturated fatty acids (1). Since heme compounds frequently occur in proximity to oxygen and unsaturated lipids in biological systems, it is probable that heme catalysis is of wide importance. Some aspects of this process, e.g. kinetics, relative effectiveness of different heme compounds, and the effect of inhibitors have been studied in detail. On the other hand, little is known about the structures of the oxygenated fatty acid derivatives that are formed during heme catalysis. In the present work, the product formed on decomposition of 13L-hydroperoxy-9,11-octadecadienoic acid by hemoglobin was studied. The structures of five compounds have been determined. After submission of this paper for publication there appeared a paper by Gardner, et al. (2) in which nine oxygenated fatty acids were identified following treatment of an isomeric mixture of linoleic acid hydroperoxides with an Fe(III)-cysteine couple.

MATERIALS AND METHODS

[1-^{14}C]13L-Hydroperoxy-9,11-octadecadienoic acid (70 μCi/mmole) was prepared as previously described (3). The preparation contained 4-5% of the isomeric 9-hydroperoxide.

erythro- And *threo*-11,12-dihydroxy-1-octadecanols were obtained by *trans*-hydroxylation of *trans*- and *cis*-vaccenic acids, respectively, with performic acid followed by reduction with $LiAlH_4$ (3). Hemoglobin (bovine, mixture of methemoglobin, and oxyhemoglobin) was purchased from Sigma Chemical Co., St. Louis, Mo.

Oxidative ozonolysis of (-)-menthoxycarbonyl derivatives and analysis of the esterified product by gas liquid chromatography (GLC) was performed as previously described (4).

Thin layer chromatography (TLC) of fatty acid hydroperoxides and their reaction products was carried out with plates coated with Silica Gel G. The organic layer of an equilibrated mixture of ethyl acetate-2,2,4-trimethylpentane-water (50:100:100, v/v/v) was used as solvent. Separation of *erythro*- and *threo*-11,12-dihydroxy-1-octadecanols was achieved by TLC on sodium arsenite-impregnated plates and methanol-chloroform (3:97, v/v) as solvent (3). Spots and bands were located by spraying with 2',7'-dichlorofluorescein and viewing by UV. Radioactivity on TLC plates was determined with a Berthold Dünnschichtscanner II.

GLC was carried out with an F&M biomedical gas chromatograph model 402 and columns of 1% SE 30 on Gas Chrom Q (long chain compounds) and 5% QF-1 on Gas Chrom Q (ozonolysis products). Mass spectra were recorded with an LKB 9000 instrument equipped with a column of 1% OV-1 on Supelcoport (Supelco, Bellefonte, Pa.).

RESULTS

[1-^{14}C]13L-Hydroperoxy-9,11-octadecadienoic acid (0.5 mg) in 6 μliter ethanol was added to 1 ml solutions of hemoglobin (0.15%, 0.5%, 1.5%, and 5%, w/v) in 0.1 M potassium phosphate buffer at 37 C. The mixtures were incubated at 37 C for 5 min and subsequently diluted with water, acidified, and extracted twice with diethyl ether. The ether phase was washed until neutral and dried over $MgSO_4$. Evaporation of the ether gave a residue (ca. 60% of the added radioactivity) that was treated with diazomethane and subjected to TLC. Radioactivity assay showed that the fatty acid hydroperoxide had disappeared in all incubations and that five major compounds were present and together constituted ca. 90% of the recovered radioactivity (Fig. 1). Use of high concentrations of hemoglobin (1.5 and 5%)

appeared to favor formation of compound V. To obtain sufficient amounts of compounds I-V for structural work, batches of 8 mg [1-^{14}C]13L-hydroperoxy-9,11-octadecadienoic acid were incubated with 500 mg hemoglobin in 10 ml buffer and the esterified product subjected to preparative TLC (Fig. 1). The product contained the following percentages of the 5 major compounds: compound I, 11% (R_f = 0.75); compound II, 25% (R_f = 0.60); compound III, 10% (R_f = 0.48); compound IV, 14% (R_f = 0.43); compound V, 31% (R_f = 0.38).

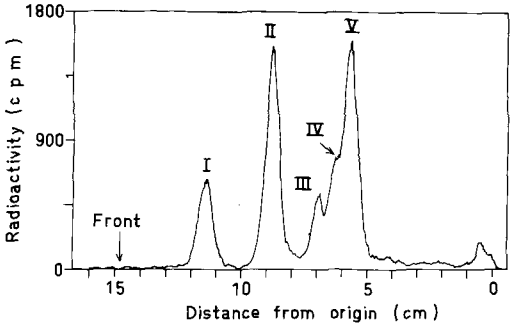


FIG. 1. Thin layer radiochromatogram of esterified material isolated after incubation of 8 mg [1-^{14}C]13L-hydroperoxy-9,11-octadecadienoic acid with hemoglobin. Solvent system: organic layer of ethyl acetate-2,2,4-trimethylpentane-water (50:100:100, v/v/v). I, II, III, IV, and V = major compounds.

STRUCTURE OF COMPOUND I

The UV spectrum of compound I showed an absorption band with λ_{max}^{EtOH} = 278 nm suggesting a conjugated dienone chromophore. GLC analysis showed a single peak with equivalent chain length corresponding to C-20.1 (column, 1% OV-1, column temperature, 190 C). The mass spectrum recorded on this peak (Fig. 2) showed ions of high intensity at m/e 308 (M), 277 (M-31; loss of $\cdot OCH_3$), 252 (M-56; β-cleavage with loss of CH_2=CH-CH_2-CH_3), 237 (M-71; α-cleavage with loss of $\cdot[CH_2]_4CH_3$), 209 (M-99; α-cleavage with loss of $\cdot\overset{\overset{O}{\|}}{C}$-$[CH_2]_4CH_3$), 177 (209-32), and 151 ($[(CH{=}CH)_2$-$\overset{\overset{O}{\|}}{C}$-$(CH_2)_4CH_3]^+$).

Catalytic hydrogenation of compound I (0.2 mg; 5% palladium-on-carbon) afforded a tetrahydro derivative, the mass spectrum of which was identical with that of authentic methyl 13-ketooctadecanoate (5,6). Finally, oxidative ozonolysis performed on compound I (0.2 mg) followed by esterification afforded a major compound identified by GLC as dimethyl azelate. Compound I, thus, possessed a keto group at C-13 and a double bond at Δ^9. The second double bond was placed at Δ^{11} by the UV data which had shown the presence of a conjugated dienone structure.

The structure of compound I was, therefore, methyl 13-keto-9,11-octadecadienoate.

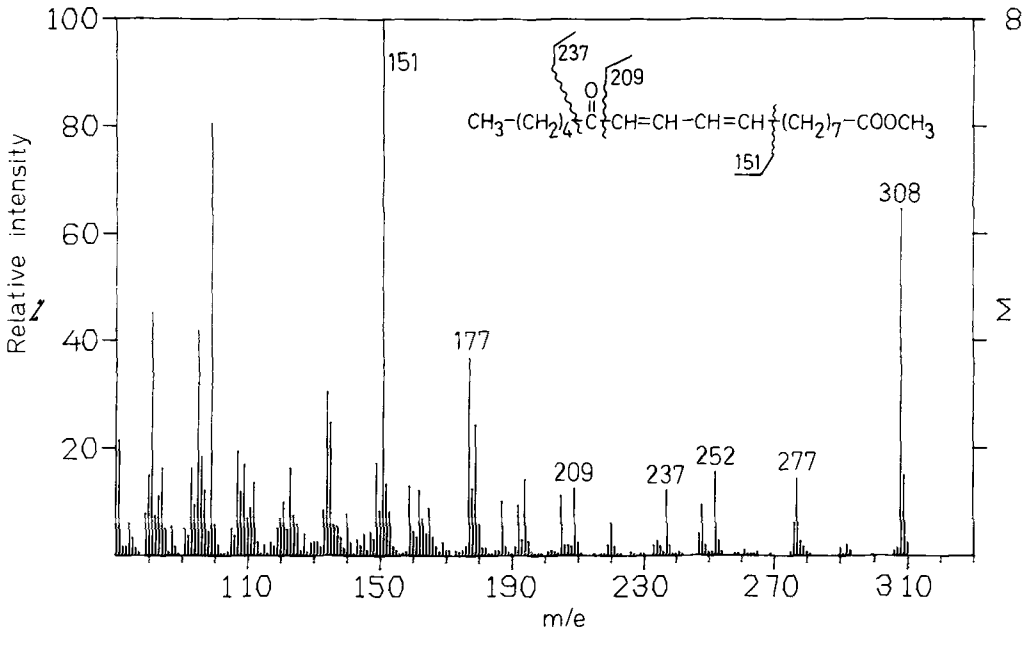


FIG. 2. Mass spectrum of compound I.

TABLE I

Thin Layer Chromatography of Dihydroxyoctadecanols Formed on Reduction of Hydrogenated Derivatives of Compounds III and IV

Compound	R_f	Position of hydroxyls[a]
threo-11,12-Dihydroxy-1-octadecanol	0.59	1,11,12
erythro-11,12-Dihydroxy-1-octadecanol	0.47	1,11,12
Compound III hydrogenated and reduced	0.47	1,11,12
	0.29	1,11,13
Compound IV hydrogenated and reduced	0.59	1,11,12
	0.47	1,11,13 (Major isomer)
		1,11,12 (Minor isomer)

[a]Determined by mass spectrometric analysis of the trimethylsilyl derivatives.

STRUCTURE OF COMPOUND II

UV spectrometry showed an absorption band with λ_{max}^{EtOH} = 234 nm indicating the presence of one pair of conjugated double bonds. The mass spectrum of the trimethylsilyl (TMSi) derivative of compound II (C-19.9) showed ions of high intensity at m/e 382 (M), 311 (M-71; α-cleavage with loss of $\cdot[CH_2]_4CH_3$), 225 ($[(CH{=}CH)_2\text{-}CH(OTMSi)\text{-}(CH_2)_4CH_3]^+$), 186, 143, and 130, indicating a C_{18} ester carrying a TMSiO group at C-13. Catalytic hydrogenation afforded methyl 13-hydroxyoctadecanoate as shown by GLC-mass spectrometry and comparison with authentic material (5,6). Oxidative ozonolysis performed on the menthoxycarbonyl derivative of compound II yielded dimethyl azelate and the menthoxycarbonyl derivative of methyl 2L-hydroxyheptanoate.

The structure of compound II was, thus, methyl 13L-hydroxy-9,11-octadecadienoate.

STRUCTURE OF COMPOUND III

No absorption band in the range 220-320 nm was present in the UV sprectrum of compound III, showing that the conjugated double bonds of the starting hydroperoxide were not retained. The mass spectrum of the TMSi derivative (C-20.7) showed ions at 398 (M), 327 (M-71; loss of $\cdot[CH_2]_4CH_3$) and 285 (M-113; loss of $\cdot CH\text{-}CH\text{-}[CH_2]_4CH_3$ with O) (3). Catalytic hydrogenation shifted the molecular ion to m/e 400, showing the presence of one double bond. The dihydro derivative was not affected by treatment with sodium borohydride. This was in agreement with the presence of an epoxy group at C-12,13 but not with a keto group at any of these positions. The double bond was placed at Δ^9 by oxidative ozonolysis which afforded *inter alia* dimethyl azelate. Finally, the dihydro derivative of compound III (ca. 0.5 mg) was refluxed with 50 mg of $LiAlH_4$ in 5 ml tetrahydrofurane for 18 hr. The product contained comparable amounts of 11,12-dihydroxy-1-octadecanol and 11,13-dihydroxy-1-octadecanol as shown by GLC-mass spectrometry (3), demonstrating conclusively the presence of a hydroxyl group at C-11 and an epoxy group at C-12,13 in compound III.

To obtain information about the configuration at carbons 11 and 12, the above mixture of dihydroxyoctadecanols was subjected to TLC with sodium arsenite-impregnated plates. Two peaks of labeled material appeared (R_f = 0.47 and 0.29; references, *threo*-11,12-dihydroxy-1-octadecanol, R_f = 0.59, and *erythro*-11,12-dihydroxy-1-octadecanol, R_f = 0.47). The more polar material (R_f = 0.29) was due to 11,13-dihydroxy-1-octadecanol as shown by mass spectrometric analysis of the TMSi derivative (ions of high intensity were present at m/e 503 [M-15; loss of $\cdot CH_3$]), 428 (M-90; loss of TMSiOH), 357 (M-[90+71]; loss of TMSiOH plus $\cdot[CH_2]_4CH_3$), 331 (M-187; loss of $\cdot CH_2\text{-}CH[OTMSi]\text{-}[CH_2]_4CH_3$), and 173 ($TMSiO^+{=}CH\text{-}[CH_2]_4CH_3$) (3). The less polar material (R_f = 0.47) similarly was analyzed and found to be 11,12-dihydroxy-1-octadecanol (the mass spectrum showed ions of high intensity at m/e 503 (M-15), 433 (M-85; loss of $\cdot[CH_2]_5CH_3$), 331 (M-187; loss of $\cdot CH[OTMSi]\text{-}[CH_2]_5CH_3$), and 187 ($TMSiO^+{=}CH\text{-}[CH_2]_5CH_3$) (3). This must be the *erythro*- isomer according to the TLC data. No radioactivity was detected at the position of the *threo*- isomer of 11,12-dihydroxy-1-octadecanol (Table I).

On the basis of these experiments, Compound III was assigned the structure methyl *erythro*-11-hydroxy-12,13-epoxy-9-octadecenoate.

STRUCTURE OF COMPOUND IV

The UV spectrum of compound IV did not

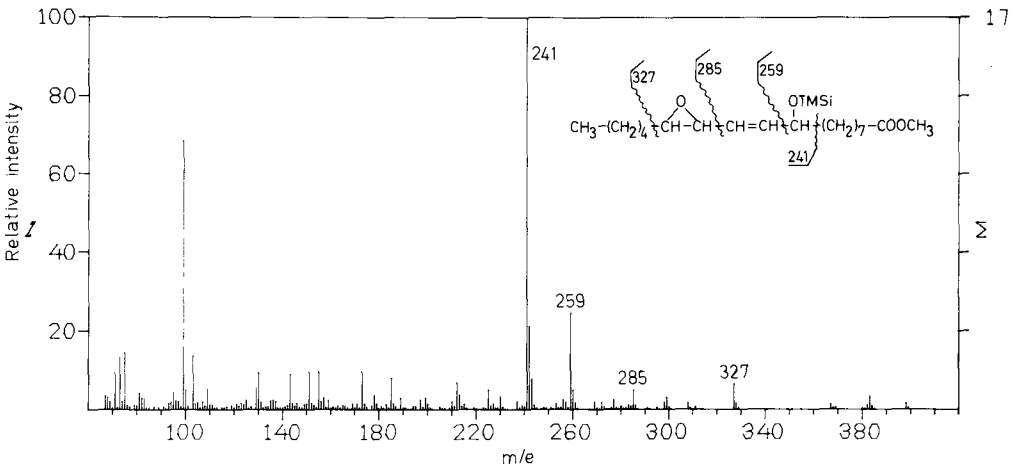


FIG. 3. Mass spectrum of the trimethylsilyl derivative of compound V.

show any absorption band in the range 220-320 nm demonstrating that the conjugated double bonds of the precursor were not retained. The mass spectrum of the TMSi derivative (C-20.7) was almost identical with that of the corresponding derivative of compound III and of methyl 11-hydroxy-12,13-epoxy-9-octadecenoate (3). One double bond was present in compound IV, since catalytic hydrogenation shifted the molecular ion from m/e 398 to m/e 400. Oxidative ozonolysis afforded *inter alia* dimethyl azelate, demonstrating that the double bond was present at Δ^9. These experiments suggested that compound IV was a stereoisomer of compound III. The relative configuration at carbons 11 and 12 was determined by TLC analysis of the derived dihydroxyoctadecanols as described above. Two peaks of radioactivity appeared (R_f = 0.59 and 0.47; coinciding with the references, *threo*- and *erythro*-11,12-dihydroxy-1-octadecanols, respectively [Table I]). The less polar material derived from compound IV was due to 11,12-dihydroxy-1-octadecanol as shown by GLC-mass spectrometry. This must be the *threo*-isomer according to the TLC data. The more polar material (R_f = 0.47) consisted mainly of 11,13-dihydroxy-1-octadecanol but also 11,12-dihydroxy-1-octadecanol. From the relative intensities of the ions at m/e 173 and m/e 187 in several mass spectra recorded on the common peak of the TMSi derivatives and from the relative amounts of the two reduction products isolated by sodium arsenite TLC (Table I), it was calculated that compound IV was ca. 80% *threo*- and 20% *erythro*-isomers of methyl 11-hydroxy-12,13-epoxy-9-octadecenoate.

STRUCTURE OF COMPOUND V

The conjugated double bonds of 13L-hydroperoxy-9,11-octadecadienoic acid were not retained in compound V, since the UV spectrum showed no absorption band in the range 220-320 nm. The mass spectrum of the TMSi derivative (C-21.5) (Fig. 3) showed ions of high intensity at m/e 327 (M-71; loss of $\cdot[CH_2]_4CH_3$), 285 (M-113; loss of $\cdot CH\text{-}CH\text{-}[CH_2]_4CH_3$), 259 (M-139; loss of $\cdot CH{=}CH\text{-}CH\text{-}CH\text{-}[CH_2]_4CH_3$), and 241 ($TMSiO^+{=}CH\text{-}CH{=}CH\text{-}CH\text{-}CH\text{-}[CH_2]_4CH_3$).

Treatment of compound V (0.5 mg) with glacial acetic acid at 70 C for 2 hr yielded a more polar compound (R_f = 0.10-0.15) that was converted into the TMSi derivative and analyzed by mass spectrometry. The mass spectrum showed an ion at m/e 470 (M-60; elimination of CH_3COOH) as well as intense ions at m/e 387 (M-143; loss of $\cdot CH[OCOCH_3]\text{-}[CH_2]_4CH_3$), 259 ($TMSiO^+{=}CH\text{-}[CH_2]_7\text{-}COOCH_3$), and 173 ($TMSiO^+{=}CH\text{-}[CH_2]_4CH_3$), indicating a mixture of monounsaturated C_{18} esters carrying one acetoxy group and two TMSiO groups. Although the positions of the oxygen functions were not analyzed further, it was clear that compound V readily incorporated one molecule of acetic acid in agreement with the presence of an epoxy group.

Oxidative ozonolysis performed on the menthoxycarbonyl derivative of Compound V afforded *inter alia* the menthoxycarbonyl derivatives of dimethyl 2L-hydroxy-(52%) and 2D-hydroxy-(48%) sebacates. This demonstrated the presence of double bond at Δ^{10}, as well as the presence of a hydroxyl group at C-9 (52% 9D, 48% 9L).

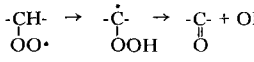
The structure of compound V was, therefore, methyl 9DL-hydroxy-12,13-epoxy-10-octadecenoate.

DISCUSSION

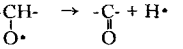
The mechanism proposed (1) for heme catalysis consists of initial formation of an activated coordination compound from the fatty acid hydroperoxide and the heme compound. Subsequent homolytic scission of the O-O bond affords oxy-fatty acid and oxy-heme radicals that may initiate autoxidation of unsaturated fatty acids. The fact that phenolic antioxidants inhibit heme catalyzed autoxidation supports the view that free radicals are involved.

The product formed on decomposition of linoleic acid hydroperoxides by heme compounds has earlier been characterized by UV spectrometry (7). This showed a decreased absorption at 233 nm and increased absorption at 285 nm, indicating partial disappearance of the conjugated diene structure and formation of a conjugated dienone. TLC revealed a "complex range of products" (7). Notably, the same complex mixture apparently was formed when linoleic acid hydroperoxides were treated with transition metals salts or exposed to heat.

The present work describes isolation and structure determination of five major compounds present in the product formed on incubation of 13L-hydroperoxy-9,11-octadecadienoic acid with hemoglobin (Fig. 4). The least polar compound (compound I) was shown to be 13-keto-9,11-octadecadienoic acid (methyl ester). Its strong absorption at 278 nm explained the increased absorption around this wavelength earlier observed in products of heme catalysis. Formation of 13-keto-9,11-octadecadienoic acid from the starting hydroperoxide probably occurs by expulsion of OH· from the 13-peroxy radical:



The same mechanism was postulated for formation of 13-keto-9,11-octadecadienoic acid from 13-hydroperoxy-9,11-octadecadienoic acid incubated anaerobically with linoleic acid and soybean lipoxygenase (8). An alternate mechanism consists of elimination of H· from the 13-oxy radical:



For the formation of 13L-hydroxy-9,11-octadecadienoic (parent acid of compound II), two mechanisms seem possible, i.e. addition of a hydrogen atom to the 13-oxy radical (9):

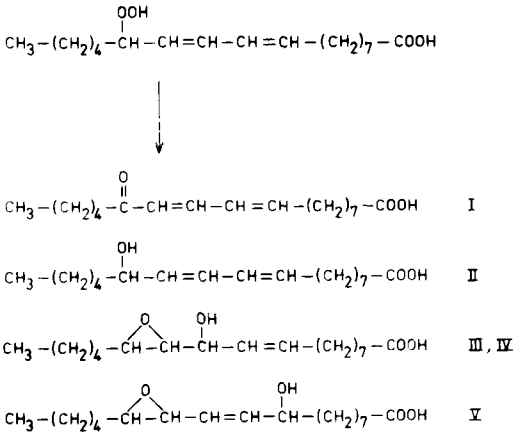
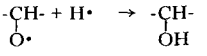
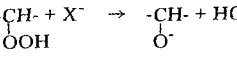


FIG. 4. Structures of the five major oxygenated fatty acids formed from 13L-hydroperoxy-9,11-octadecadienoic acid on incubation with hemoglobin.



and reduction of the hydroperoxide group as a result of attack by nucleophilic groups present in the hemoglobin molecule:

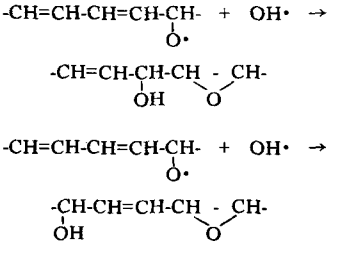


Compounds III and IV were shown to be stereoisomers of 11-hydroxy-12,13-epoxy-9-octadecenoic acid (methyl esters). Compound III was found to be the pure *erythro*-isomer, whereas compound IV was mainly (ca. 80%) the *threo*-isomer. The reason for the presence of *erythro*-isomer (ca. 20%) in compound IV is not known. Possibly, this isomer differed from compound III with respect to the configurations at the epoxy group and the Δ^9 double bond. The 11,12-dihydroxy-1-octadecanols derived from compounds III and IV were accompanied by comparable amounts of two isomeric 11,13-dihydroxy-1-octadecanols of unknown configurations (Table I). It is planned to correlate the latter compounds with the diastereoisomeric pair of 11,13-dihydroxy-1-octadecanol (11D,13D+11L,13L and 11D,13L+11L,13D). Knowledge of the configuration at C-11,13 of compounds III and IV coupled with the configuration at C-11,12 (Table I) will allow assignment of the configuration of the epoxy group (*cis* or *trans*) of compounds III and IV.

threo-11-Hydroxy-12,13-epoxy-9-octadecenoic acid recently was isolated from the product formed on heat treatment of 13L-hydroperoxy-9,11-octadecadienoic acid (3). 11-Hydroxy-12,13-epoxy-9-octadecenoic acid and its isomer, 11-hydroxy-9,10-epoxy-12-octa-

decenoic acid, also were formed during autoxidation of linoleic acid (3).

Compound V was identified as 9DL-hydroxy-12,13-epoxy-10-octadecenoic acid (methyl ester). This acid has been isolated previously after incubation of linoleic acid with flour doughs (10). The mechanism in the formation of compounds III-V probably consists of addition of OH• to the oxy-fatty acid radical without (compounds III and IV) or with (compound V) shift of the Δ^9 double bond:



In the case of *threo*-11-hydroxy-12,13-epoxy-9-octadecenoic acid formed by heat treatment of 13L-hydroperoxy-9,11-octadecadienoic acid (3), isotopic studies showed that the major part of the epoxide was formed by a reaction involving elimination of the distal oxygen of the hydroperoxide group and incorporation of OH (ion or radical) from the aqueous solvent. Similar studies on the formation of the compounds described in the present paper are in progress.

ACKNOWLEDGMENT

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Effects of Administration of Hypolipidemic Agent, 2,2‴-[(1-Methyl-4, 4-Diphenylbutylidene)bis(p-Phenyleneoxy)] Bistriethylamine Oxalate (SQ 10,591) upon Cholesterol Esterification by Aorta, Adrenal, and Testes of Cholesterol-Fed Rabbits

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ABSTRACT

Administration of 2,2‴-([1-methyl-4, 4-diphenylbutylidene] bis(p-phenyleneoxy]) bistriethylamine oxalate (SQ 10,591) at 20 mg/Kg daily for 5 days to cholesterol-fed rabbits resulted in no change in aortic microsomal cholesterol esterification with a palmitoyl coenzyme A substrate or of aortic mitochondrial cholesterol esterification with a palmitate substrate. Esterification by both reactions in the adrenal was much higher than in either aorta or testes. Adrenal and testicular mitochondrial esterification and testicular microsomal esterification were inhibited significantly after SQ 10,591 administration. In vitro addition of 0.0001 M SQ 10,591 significantly inhibited both microsomal and mitochondrial cholesterol esterification in aorta, adrenal, and testes.

INTRODUCTION

Of the arterial accumulation of cholesteryl esters occurring during atherogenesis, up to half may be attributable to local esterification (1). Increases in cholesterol esterifying activity have been observed in atherosclerotic arteries in several species (2-4); in pigeons fed an atherogenic diet, the increase in cholesterol esterification seems to precede development of demonstrable lesions (5). In both rabbit and swine arteries, there appear to be two distinct enzymatic esterifying systems—an acyltransferase mechanism, requiring adenosine 5'-triphosphate (ATP) and coenzyme A (CoA) and localized primarily in the microsomal compartment, and an enzyme not requiring cofactors, with optimal activity at low pH (6,7). Previous studies utilizing swine arterial preparations (7) have demonstrated that the acyltransferase esterification in the microsomal fraction, and the low pH direct reaction in the mitochondrial fraction both could be inhibited by in vitro addition of 2,2‴-([1-methyl-4,4-diphenylbutylidene] bis [p-phenyleneoxy]) bistriethylamine oxalate (SQ 10,591). In the present study, the latter compound was administered to cholesterol-fed rabbits to determine if any in vivo inhibitory effects upon esterification could be observed in the early atherosclerotic aorta. Since steroidogenic tissues also have a significant rate of cholesterol esterification, activity was assayed in adrenal and testes to determine any effects of the administered SQ 10,591.

MATERIALS AND METHODS

Two groups of 5 male New Zealand white rabbits each, 4 months of age, were maintained for 2 months on a stock commercial diet (Purina) to which was added 1% cholesterol + 3% hydrogenated coconut oil. One group of these rabbits then was injected subcutaneously daily for 5 days with 20 mg/Kg SQ 10,591 (oxalate salt of 2,2‴-([1-methyl-4, 4-diphenylbutylidene-bis (p-phenyleneoxy)] bistriethylamine) in sterile sesame oil. The other group of rabbits served as controls and were injected with sesame oil on the same schedule as the SQ 10,591 treated group. All rabbits were killed by intravenous administration of sodium pentobarbital 18 hr after their last treatment. Aortas, adrenals, and testes were removed and dissected free of surrounding connective tissue. The advential layer of the aortas was discarded. The tissue then was minced with an Arbor tissue press (Harvard Apparatus Co., Millis, Mass.) suspended in 0.25 M sucrose + 0.001 ethylenediaminetetraacetic acid (EDTA) (100 mg tissue/ml solution) and further homogenized at 2000 rpm. The homogenates were centrifuged for 10 min at 500 x g and the supernatant fluid centrifuged at 12,000 x g for 15 min and then recentrifuged at 18,000 x g for 15 min (the latter pellet was discarded). The 12,000 x g mitochondrial pellet was resuspended in an equal volume of 0.25 M sucrose and recentrifuged (this pellet was used for all mitochondrial assays). The 18,000 x g supernatant fluid was centrifuged at 104,000 x g for 60 min. This microsomal pellet was resuspended in 0.15 M potassium chloride (KCl) and recentri-

TABLE I

Synthesis of Cholesteryl Esters by Aortic, Adrenal, and Testicular Microsomes from Control and SQ 10,591 Treated Cholesterol-Fed Rabbits[a]

Microsomes from:	Control	SQ 10,591	
Aorta	616 ± 80	715 ± 83	NS
Adrenal	20,190 ± 2,200	22,350 ± 1,795	NS
Testes	719 ± 63	454 ± 43	P<0.01

[a]Incubations were done for 1 hr in 0.2 ml phosphate buffer, pH 7.4, with 0.5 μc, 1.0 nmole of a palmitoyl-1-^{14}C coenzyme A substrate, and 0.5 mg microsomal protein. Figures given are mean pmoles substrate esterified/mg protein/hr ± standard deviations of incubations from five separate preparations; pmoles esterified are calculated assuming no equilibrium with endogenous substrate. NS = nonsignificant difference.

TABLE II

Synthesis of Cholesteryl Esters by Aortic, Adrenal, and Testicular Mitochondria from Control and SQ 10,591 Treated Cholesterol-Fed Rabbits[a]

Mitochondria from:	Control	SQ 10,591	
Aorta	41 ± 3.7	36 ± 2.4	NS
Adrenal	772 ± 60	506 ± 55	P<0.01
Testes	58 ± 9.0	31 ± 3.2	P<0.01

[a]Incubations were done in 0.2 ml citrate-phosphate buffer, pH 5.0, with 0.25 μc, 5.0 nmoles of a palmitic-1-^{14}C substrate, and 0.5 mg mitochondrial protein. Figures given are pmoles substrate esterified/mg protein/hr ± standard deviations of five separate determinations; pmoles esterified are calculated assuming no equilibrium with endogenous substrate. NS = nonsignificant difference.

fuged. The washing (supernatant fluid) from this centrifugation was discarded.

For assay of cholesterol esterifying activity, 0.2 ml aliquots of each microsomal fraction were mixed with 0.2 ml aliquots of 0.2 M phosphate buffer, pH 7.4, plus a palmitoyl-1-^{14}C CoA substrate (New England Nuclear, Boston, Mass.; 0.05 μc, 1.0 nmoles), and incubated for 1 hr at 37 C. Control aliquots were heated at 90 C for 10 min and other unheated controls stopped at zero time or incubated for 1 hr with buffer plus substrate but no tissue. In some experiments, $^{14}CO_2$ was collected in a center well containing 20% NaOH. The incubations were stopped by addition of chloroform/methanol, 2:1, and the lipids extracted by the procedure of Folch, et al. (8). Samples were evaporated to dryness under an N_2 stream, dissolved in chloroform, and the lipid fractions separated by thin layer chromatography on Silica Gel H, 0.5 mm plates using an ascending solvent mixture of hexane-ethyl ether-acetic acid, 120:20:1. The free and esterified cholesterol bands were scraped and eluted with 2 successive portions of chloroform/hexane, 4:1 by mixing at 40 C for 1 hr. Radioactivities in these fractions were determined by liquid scintillation counting using a diphenyloxazole-diphenyloxazole-benzene (PPO-POPOP) in toluene scintillation solution and a Packard model 3314 automatic refrigerated liquid scintillation counter. Quenching was monitored by the channels ratio technique.

TABLE III

Effect of In Vitro Addition of SQ 10,591 upon Synthesis of Cholesteryl Esters by Aortic, Adrenal, and Testicular Microsomal and Mitochondrial Preparations from Cholesterol-Fed Rabbits[a]

Tissue fraction	Control	SQ 10,591 0.0001 M
Microsomes		
Aorta	613 ± 71	180 ± 22
Adrenal	20,500 ± 1,830	6,190 ± 780
Testes	794 ± 110	214 ± 56
Mitochondria		
Aorta	43 ± 6.7	10 ± 4.3
Adrenal	888 ± 98	272 ± 36
Testes	63 ± 4.1	12 ± 2.7

[a]Microsomal incubations and calculations were done as described in Table I. Mitochondrial incubations and calculations were done as described in Table II. SQ 10,591 was dissolved in the buffers. Tissues for this group of experiments were all obtained from the sesame oil-injected control group. All differences between control and SQ 10,591 groups were significant at P<0.001.

An additional esterification reaction was assayed by incubating 0.2 ml aliquots of each mitochondrial fraction with 0.2 ml 0.2 M citrate-phosphate buffer pH 5.0, containing 0.25 μc, 5.0 nmoles of a palmitate-1-^{14}C substrate (New England Nuclear) added in 10 μliter acetone. Because of the slower rate of this reaction, these mixtures were incubated for 18 hr at 37 C, and samples then were processed as in the assay described above. Control aliquots were heated at 90 C for 10 min, and other unheated controls stopped at zero time or incubated for 18 hr with buffer plus substrate but no tissue.

Protein content of each fraction was analyzed by the method of Lowry, et al., (9) adapted for the autoanalyzer.

The probabilities that apparent differences in the data were due to chance was calculated by the t-test.

RESULTS AND DISCUSSION

In Table I are shown the rates of microsomal

incorporation of the palmitoyl CoA substrate into aortic, adrenal, and testicular cholesteryl esters. Esterification was markedly higher by adrenal microsomes than either aortic or testicular microsomes. SQ 10,591 administration had no significant effect upon esterification by aortic or adrenal microsomes but did decrease testicular microsomal esterification.

Mitochondrial synthesis of cholesteryl esters from the palmitate substrate at pH 5.0 without added cofactors is shown in Table II. As in the microsomes, esterification by the adrenal mitochondria occurred more rapidly than in either aortic or testicular mitochondria. Significant inhibition of adrenal and testicular mitochondrial cholesterol esterification was induced by SQ 10,591 administration.

Effects of in vitro addition of SQ 10,591 in 10^{-4}M concentration to microsomal and mitochondrial preparations are shown in Table III. SQ 10,591 markedly inhibited both microsomal esterification with the palmitoyl CoA substrate and mitochondrial esterification with the palmitate substrate in aorta, adrenal, and testes.

No significant alterations in oxidation of either the palmitoyl CoA or palmitate substrate to CO_2 were induced by either in vivo administration or in vitro addition of SQ 10,591, indicating that observed effects of SQ 10,591 upon rates of esterification were not due to diminished substrate concentrations.

Administration of SQ 10,591 to rats previously has been shown to inhibit the conversion of polyisoprenol pyrophosphates to cholesterol (10). Formation of liver and serum cholesterol esters is inhibited to a greater extent than free cholesterol synthesis, suggesting possible inhibitory effects of SQ 10,591 upon esterification (10). Addition of SQ 10,591 to incubated preparations of swine aortic microsomes and mitochondria has been found to inhibit esterifying activity in both of these fractions (7). Similar in vitro inhibition of esterification was observed in the aortic microsomal and mitochondrial fractions from the cholesterol-fed rabbits of the present experiments, but no effect could be demonstrated after in vivo administration of 20 mg/Kg SQ 10,591, a dosage level which did inhibit cholesterol ester biosynthesis from acetate in rats (10).

Decreased incorporation of palmitate into adrenal mitochondrial cholesteryl esters was observed in the present experiments both after administration of SQ 10,591 and after in vitro addition to incubated preparations. In a previous study, SQ 10,591 administration produced no change in incorporation of acetate into rat adrenal cholesteryl esters (10). At 10^{-5}M concentrations, SQ 10,591 has been noted to inhibit both adrenocorticotropin activated and cyclic adenosine 5′-monophosphate activated rat adrenal steroidogenesis (11). If rates of adrenal cholesterol esterifying activity are regulated by mechanisms similar to those in the ovary, a reduced local steroid concentration would have been expected to activate, rather than inhibit, cholesterol esterification (12). A more probable mechanism of inhibition by SQ 10,591 may be related to its cationic amphipathic, or surface-active properties, which, like a structurally similar compound SKF 525-A (diethylamino diphenylvalerate HCl), may inhibit enzyme activity directly or may bind to subcellular membranes and limit diffusion of substrates to the sites of esterification (13,14).

In the testes, incorporation of palmitoyl CoA into microsomal cholesterol esters and palmitate into mitochondrial cholesterol esters both were inhibited after administration of SQ 10,591. In vitro addition of SQ 10,591 also inhibited both types of esterification, similar to the effects previously observed in rabbit ovarian preparations (15). The mechanisms of inhibition may be similar to those discussed above for the adrenal. Testicular microsomal esterification appears to be more sensitive to SQ 10,591 administration than is adrenal microsomal esterification; this possibly may be related to the diminished pituitary gonadotropin stimulation of the testes observed by Lerner, et al., (10) after SQ 10,591 administration.

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Lipid and Fatty Acid Composition of Testes of Quaking Mice

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ABSTRACT

Testes of quaking mice (sterile mutants) and of controls were analyzed for major lipid classes and fatty acid composition. Of the main lipid classes, only cholesterol esters differed significantly in concentration between the two groups (1.01 for quakers vs 0.69 mg/g wet wt of tissue for controls). The concentration of triglycerides was 4.5-5.0, that of total phosphatides 18-19, and that of free cholesterol 1.9-2.0 mg/g for mutants and controls. The concentrations of phosphatidyl ethanolamine and of sphingomyelin were both lower in quaking than in normal mice, but only the change in the former was statistically significant. Phosphatidyl choline was the major phosphatide (43-45% of total phosphatides) followed by phosphatidyl ethanolamine (24-26%) and sphingomyelin, phosphatidyl serine, and phosphatidyl inositol (all ca. 7% of total phosphatides). Minor differences between the mutants and controls were observed in concentrations of fatty acids of major lipid classes. The mutants, sterile because of faulty spermatid differentiation, had normal quantities of 22:6 w3 and 22:5 w6. These data are consistent with the hypothesis that the 22-carbon polyenes are associated with the formation of spermatids, rather than with their final differentiation into spermatozoa.

INTRODUCTION

The male quaking mouse (qk/qk) has been reported to be sterile because of faulty spermatid differentiation (1). Lipid abnormalities have been found in the central nervous system (2,3) and in organs, such as thymus, spleen, and kidney (4) of this animal. However, lipids or the testis of the quaking mouse have not been studied. Our interest in the role of lipids and fatty acids in male reproductive tissue led us to consider the possibility of a defect in lipid composition and metabolism in the testis of this mutant. Accordingly, we have determined the major lipid classes and fatty acid composition of testes of the quaking mouse and compared the values with those of controls.

METHODS

The mice were C 57BL/6J males obtained from the Jackson Laboratory, Bar Harbor, Maine. Controls were furnished by the same supplier (qk/+ or +/+, indistinguishable). The animals were maintained on Purina chow until their death by decapitation. Testes were removed quickly and chilled in ice, after which they carefully were cleaned of all adhering tissue and weighed. A small amount of tissue was used from each of several testes from the various groups of quaking and control mice for histological examination. These were fixed in Bouin's media and stained with periodic Schiff stain (PAS) and counter-stained with Wright's iron hematoxylin. The balance of the tissues was either hydrolyzed with alcoholic potassium hydroxide for total fatty acid separation by methods reported previously (5) or homogenized in Folch mixture (chloroform:methanol 2/1) for extraction of total lipid. Aliquots of the extracts were used for determinations of triglyceride (6), phospholipid phosphorus (7), and of free and esterified cholesterol (8). Classes of lipids wre separated by thin layer chromatography (TLC) using the solvent system: petroleum ether-ethyl ether-glacial acetic acid, 80:20:1 (v/v/v). Individual phosphatides were separated using Supelcosil 42A (Supelco, Bellefonte, Pa.) for two dimensional TLC using the solvent systems: (A) chloroform-methanol-28% ammonia, 65:25:5 (v/v/v) and (B) chloro-

TABLE I

Fatty Acid Composition of Testicular Total Lipids of Quaking Mice

Fatty acid	Percent of total fatty acids
16:0[a]	29.9 ± 0.78[b]
16:1	1.48 ± 0.09
18:0	6.95 ± 0.22
18:1	15.8 ± 0.82
18:2	8.11 ± 0.89
20:3	1.17 ± 0.04
20:4	11.9 ± 0.37
22:4	1.67 ± 0.21
22:5	11.0 ± 0.43
22:6	7.01 ± 0.26
24:4 } 24:5 }	2.79 ± 0.25

[a]Number of carbons in chain: number of double bonds.

[b]Mean of 6 samples ± standard error of the mean; testes of 2-5 mice/sample.

form-acetone-methanol- acetic acid-water, 6:8:2:2:1 (all on a volume basis). After developing and charring lightly with sulfuric acid, the identified spots were scraped quantitatively into test tubes and phosphorus quantitated (7). All analyses of lipid fractions were done at least in duplicate. Fatty acids from total lipids and from separated lipid classes were methylated with boron trifluoride-methanol (9) for analysis by gas liquid chromatography (GLC). GLC analyses were done as reported previously (5). Standard methyl esters obtained from Applied Science Laboratories, State College, Pa., were used to establish retention times and for calibration of the flame ionization detectors. Hydrogenation of methyl esters was done (10), and the hydrogenated derivatives were reanalyzed by GLC to establish the total amount of fatty acids of a particular chain length. Further identifications were made by reference to fatty acid methyl esters obtained from rat and human testes and previously characterized by chemical means.

Testes from 2-5 mice were pooled for each sample analyzed to obtain sufficient lipid. Four different sets of mice were obtained for these studies, but, in most cases, the results from more than one set have been grouped in a table for calculation of means and for statistical evaluation. The ages of the animals are given with each set of data.

RESULTS

The ages of the mice in the first set ranged from 41-124 days. During this time, the body wt of controls increased from 16-27 g, while that of the quaking mice increased from 13-21 g. Wt of the testes of control mice increased from 0.07 at 41 days to 0.11 g/testis but that of testes of quakers showed little or no increase from the 41 day wt of 0.06 g/testis. Total fatty acid concentration was constant throughout this period for both groups (14.7 ± 1.26 mg/g for controls vs. 13.7 ± 0.57 mg/g for quaking mice). The fatty acid composition of total lipids did not differ significantly between the two groups. In Table I is given the composition for testes of quaking mice. The means of pools of all ages are given, since there was no consistent change in the concentration of any fatty acid during this entire age period. Only those fatty acids present in a concentration of 1% or greater are listed. Among those fatty acids present in concentrations less than 1% were 14:0, 18:3, 20:2, 20:5, 22:3, and 22:5 w3 (the isomer reported in the table is 22:5 w6). Ca. twice as much 24:5 as 24:4 was present. Several of the samples were hydrogenated and reanalyzed by GLC. The completely hydrogenated samples had amounts of saturated fatty acids consistent with the amounts of individual components of the same carbon number in the nonhydrogenated samples.

Lipid composition was determined in 2 sets of mice of ages 83-111 days. The results are given in Table II. Most of the lipid was phosphatide, and free fatty acids were present only in trace quantities. There were no significant differences between controls and quakers in the concentrations of phosphatide, triglycerides, or free cholesterol. However, there was a statistically significant greater amount of esterified cholesterol in testes of the quaking mice.

Ca. 90% of the total phosphatide phosphorus was accounted for in 5 identified phosphatides given in Table II. Most of the phosphatides in testes was phosphatidyl choline and phosphatidyl ethanolamine, but considerable concentrations of phosphatidyl serine, phosphatidyl inositol, and sphingomyelin also were present. No significant differences between controls and mutants were found for any of these, except phosphatidyl ethanolamine. Although the difference in concentration of sphingomyelin was not statistically significant, it is important to indicate that in every case except one the concentration was greater in the testes of the controls than in the quakers. In addition to these major phosphatides, small amounts of cardiolipin and lysophosphatides were present.

The fatty acids obtained from each of the main classes of testicular lipids of mice aged 83-111 days were analyzed by GLC. The results are given in Table III. Very few differences were found between controls and quakers which proved statistically significant. These included 18:1 of phosphatides; 20:4 and 22:4 of cholesterol esters; and 14:0, 18:0, 18:2, and 22:5 w3 of triglycerides. In addition to the fatty acids reported in Table III, there were small amounts of numerous components among which were tentatively identified 15:0, 20- and 22-carbon mono-, di-, and trienes; and 18:3. There were also small but significant amounts of components with retention times beyond 24:5, but these were not identified or studied further.

Histological examination of the sections revealed that very few spermatids with tails were present in the testes of the quaking mice, although these were abundant in those of controls.

DISCUSSION

The role of lipids, and particularly polyenoic

TABLE II

Lipid and Phosphatide Composition of Testes of Quaking and Control Mice

Lipid class	mg/g Wet wt testis[a]	
	Controls	Quaking
Triglycerides	4.80 ± 0.52	4.59 ± 0.30
Phosphatides	19.0 ± 0.54	18.2 ± 0.60
Free cholesterol	1.98 ± 0.03	1.90 ± 0.21
Esterified cholesterol	0.69 ± 0.07	1.01 ± 0.09 (P=0.17)
Ratio: esterified cholesterol / free cholesterol	0.35 ± 0.04	0.53 ± 0.07 (P=0.061)
	Percent of total phosphatides[a]	
	Controls	Quaking
Phosphatidyl choline[b]	42.7 ± 1.3	45.7 ± 1.2
Phosphatidyl ethanolamine[b]	26.4 ± 0.52	24.3 ± 0.89 (P=.05)
Sphingomyelin	7.24 ± 0.43	6.41 ± 0.30
Phosphatidyl serine	7.69 ± 0.33	7.63 ± 0.19
Phosphatidyl inositol	6.88 ± 0.35	6.80 ± 0.59

[a]Mean ± standard error of the mean of 8 samples; testes from 2-5 mice were pooled for each sample.

[b]Includes plasmalogens, if present.

TABLE III

Fatty Acids of Phospholipids, Triglycerides, and Cholesterol Esters from Testes of Control and Quaking Mice

Fatty acid	Percent of total fatty acids					
	Phosphatides[a]		Triglycerides[a]		Cholesterol esters[a]	
	Controls	Quaking	Controls	Quaking	Controls	Quaking
12:0	tr	tr	1.1 ± 0.28	0.89 ± 0.20	2.3 ± 0.38	3.0 ± 0.59
			(P = .001)			
14:0	0.81 ± 0.13	0.80 ± 0.07	1.3 ± 0.09	2.1 ± 0.14	4.1 ± 0.31	4.1 ± 0.79
16:0	29.2 ± 0.94	30.4 ± 1.3	16.7 ± 0.93	18.2 ± 0.35	15.3 ± 0.81	14.8 ± 1.6
16:1	1.3 ± 0.14	1.5 ± 0.11	5.1 ± 0.30	4.8 ± 0.17	6.8 ± 0.35	6.4 ± 0.32
			(P = .001)			
18:0	8.0 ± 0.47	8.9 ± 0.44	2.7 ± 0.14	4.2 ± 0.17	7.2 ± 0.35	6.3 ± 0.36
	(P = .008)					
18:1	12.2 ± 0.40	14.3 ± 0.52	25.0 ± 1.4	22.7 ± 1.0	18.4 ± 1.1	17.8 ± 1.3
			(P = .03)			
18:2	2.7 ± 0.11	3.0 ± 0.26	22.3 ± 1.8	16.2 ± 2.2	3.5 ± 0.28	3.1 ± 0.3
20:3 w6	1.4 ± 0.11	1.3 ± 0.06	0.73 ± 0.08	0.86 ± 0.11	0.67 ± 0.09	0.44 ± 0.08
					(P = 0.008)	
20:4	14.2 ± 0.52	13.0 ± 0.79	1.7 ± 0.18	1.7 ± 0.21	1.7 ± 0.12	1.2 ± 0.13
					(P = .01)	
22:4	1.4 ± 0.07	1.4 ± 0.10	1.6 ± 0.13	2.1 ± 0.92	2.5 ± 0.18	1.6 ± 0.24
22:5 w6	12.3 ± 0.96	10.8 ± 0.75	5.9 ± 0.92	6.7 ± 0.84	3.9 ± 0.48	3.2 ± 0.53
			(P = .05)			
22:5 w3	0.51 ± 0.06	0.55 ± 0.09	1.1 ± 0.14	1.7 ± 0.28	2.7 ± 0.36	1.88 ± 0.31
22:6	8.50 ± 0.80	7.0 ± 0.62	2.9 ± 0.44	3.7 ± 0.41	6.7 ± 0.90	5.1 ± 0.91
24:4	1.1 ± 0.11	1.1 ± 0.13	0.8 ± 0.17	1.1 ± 0.22	2.7 ± 0.40	2.7 ± 0.72
24:5	1.5 ± 0.16	1.5 ± 0.13	1.3 ± 0.11	1.7 ± 0.22	3.9 ± 0.49	3.0 ± 0.31

[a]Mean ± standard error of the mean of 8 samples for phosphatides and triglycerides and of 10 samples for cholesterol esters; testes from 2-5 mice were pooled for each sample.

fatty acids, in the male reproductive system has attracted increasing attention. In the rat, it was shown that the testicular concentration of 22:5 w6 increased dramatically with sexual maturation of the animal (11). Furthermore, in conditions in which the testes were caused to degenerate by means of surgical cryptorchidism or by use of cadmium chloride, the concentration of this polyene decreased to low values (12). Because the quaking mouse has a defect in spermiogenesis, it might be a useful animal in the study of the role of lipids in spermatogenesis. Our studies show only minor differences in lipid and fatty acid composition of testes of quaking compared to control mice. In the mouse, 22:6 w3 is a prominent component of testicular fatty acids in addition to 22:5 w6 (which is the chief 22-carbon polyene in rat testes). Both of these polyenes were present in testicular lipid fractions of quaking mice in concentrations not significantly different from controls. Bennett, et al., (1) has proposed that the defect in testis of the quaking mouse is one of spermatid differentiation, and our limited histological examinations confirm this morphological observation. Our results are consistent with the hypothesis that testicular 22:5 w6 and 22:6 w3 are related to the production of spermatids, rather than to the final differentiation into spermatozoa.

Some differences between testicular lipids of quaking and control mice were noted in these studies. Minor differences were found in fatty acid concentrations in particular lipid classes (for example, 14:0, 18:0, and 18:2 in triglycerides), but these cannot be related to any morphological changes. Moreover, these differences were small and may not represent important alterations. A meaningful change was an increase in the amount of esterified cholesterol (but not free cholesterol) in testes of quaking mice. Generally, esterified cholesterol increases during impairment of the spermatogenic process (13). Sphingolipids in the central nervous system were reported reduced compared to other structural components (2,3). In our studies, phosphatidyl ethanolamine was the only phosphatide which was decreased significantly in testes of quaking mice, and this decrease was only borderline ($P = 0.05$). The concentration of sphingomyelin was lower in the mutants than in controls in all cases but one, but the difference in the means was not statistically significant ($P = 0.1$). A greater number of analyses eventually might show this difference to be significant. Analysis of the fatty acid pattern of individual phosphatides and particularly of sphingomyelin of testes of quaking and control mice may show alterations not seen in the fatty acid pattern of total phosphatides, but these studies have not yet been done. A reduction in the C_{20}-CoA elongation system has been shown in microsomes from brain of quaking mice (14).

A glycerol-base (rather than a sphingosine base) glycolipid present in testes of various animals was reported to be reduced greatly in amount in testes of the w/w^v mouse, a sterile mutant deficient in germinal cells (15). The concentration of this glycolipid in testes of the quaking mouse has not yet been determined.

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R.F. Sellers, Department of Anatomy, prepared the histological sections, and M.-C. Orgebin-Crist, Department of Obstetrics-Gynecology, interpreted the histological studies. This work was supported by Research Grant 1 RO1-HD 07694 from the U.S. Public Health Service.

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SHORT COMMUNICATIONS

Separation of Polyunsaturated Fatty Acids by Argentation Thin Layer Chromatography

ABSTRACT

A single step, silver nitrate thin layer chromatographic procedure which separates a methyl ester mixture containing 0-6 double bonds is described. Purity of each ester recovered from the silver nitrate plate was 98-99%. Recovery of the esters ranged from 100% for saturates to 77% for pentaenes.

INTRODUCTION

Various procedures are available for separation of fatty acid methyl esters. The most common is gas liquid chromatography which gives excellent quantitative data but does not lend itself readily to the recovery of appreciable amounts of the esters. Large scale separation of fatty acid methyl esters has been accomplished by column chromatography, but this method is time consuming and does not separate fatty acids with more than two double bonds. Silver nitrate thin layer chromatography (TLC) has been used extensively to fractionate methyl esters according to their degree of unsaturation. However, there is presently no simple method available that allows the separation on the same plate of samples containing 0-6 double bonds. We have developed a simple TLC procedure that allows this separation.

EXPERIMENTAL PROCEDURES

Purified fatty acid methyl esters (Nu-Chek Prep) were used to prepare a near equimolar mixture of the following fatty acids: 18:0, 18:1, 18:2, 18:3, 20:4ω6, 20:5ω3, and 22:6ω3. Thin layer plates (5 x 20 cm) were coated with a 0.25 mm thick slurry of silver nitrate impregnated silica gel (35 ml 4% [w/v] $AgNO_3$ to 15 g Silica Gel G [E. Merck, Darmstadt, Germany]). The plates were allowed to air dry for ca. 45 min, activated for 30 min at 110 C, and stored in a light-tight, dessicated container. Methyl esters (950 μg) were applied to each plate as a narrow band and the plates developed twice in a solvent system containing hexane-diethyl ether (anhydrous)-acetic acid (94:4:2). After the first development, the plate was dried under a stream of nitrogen. Following the second development, the plate was dried again under nitrogen, sprayed lightly with a 0.1% solution of 4',5'-dibromofluorescein (Eastman Kodak Co., Rochester, N.Y.) in isopropanol, dried under nitrogen, and placed in a tank saturated with NH_4OH vapors. After ca. 5 min, pink spots on a yellow background appeared which were very pronounced when viewed under UV light. Individual bands were scraped into a 7 ml tube, and a known amount of methyl arachidate (20:0) was added to each tube to determine the recovery of each methyl ester by the internal standardization procedure of Blank, et al. Methanol (2 ml) containing 1% acetic acid was added to each tube which was sealed tightly and placed in a boiling water bath for 5 min. After cooling and centrifugation, the methanol layer was removed and the silica gel reextracted with the acetic acid-methanol solu-

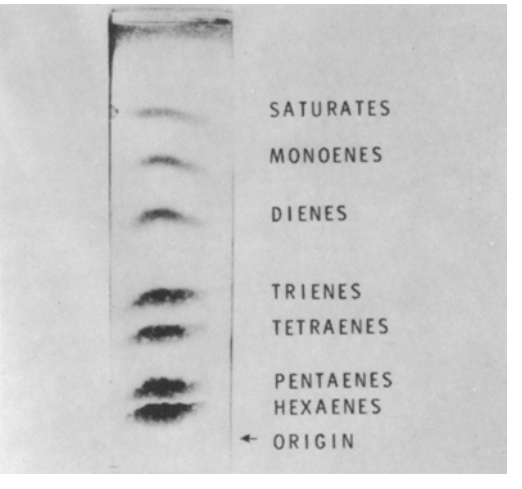

FIG. 1. Thin layer chromatogram of methyl ester mixture described in text. The individual bands were visualized by spraying with 55% H_2SO_4 containing 0.6% $Na_2Cr_2O_7$ and heating at 200 F for 20 min.

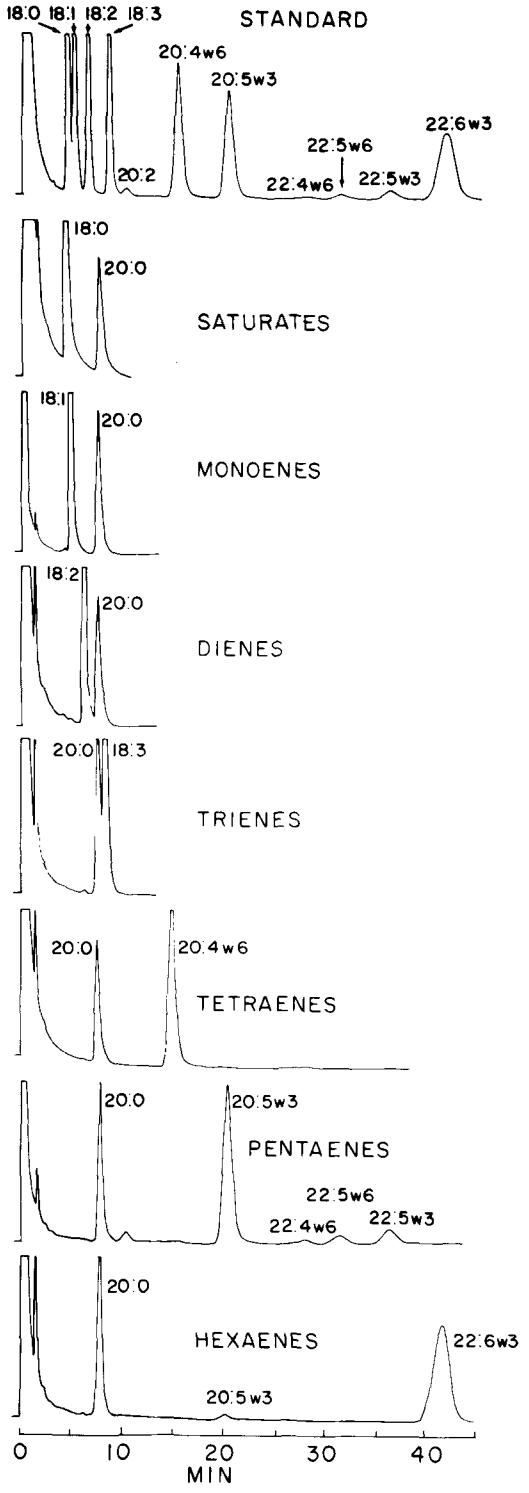

FIG. 2. Gas liquid chromatographic elution pattern of individual bands from a 4% silver nitrate thin layer plate. Conditions are described in the text.

tion. The extracts were combined and taken to dryness under a stream of nitrogen and a small amount of CS_2 added as the solvent for gas chromatography.

Gas chromatography was carried out on a Varian model 2100 gas chromatograph using a 6 ft glass column packed with 15% EGSS-X on 100-120 mesh Gas Chrom P (Applied Science Laboratories, State College, Pa.) and equipped with a hydrogen flame ionization detector. A model CRS-100 digital integrator (Infotronics, Houston, Tex.) was used to quantitate peak areas. Usually ca. 10 μg methyl ester was injected.

Figure 1 shows a representative thin layer plate that was developed in the solvent system. There is a clear separation of all bands. We have observed that when the relative humidity increases to above 50%, the bands do not migrate as far as they usually do, and cross contamination of pentaenes with hexaenes becomes a problem. We get the best separation when the room humidity is ca. 42-44%.

A composite of a representative gas chromatographic analysis of each band is shown in Figure 2. Clear separation of the bands was obtained as evidenced by the low amount of cross contamination. Saturates were uncontaminated. There was less than 1% cross contamination in monoenes, dienes, trienes, and tetraenes and only 2% contamination in pentaenes and hexaenes. The percent recovery of saturates was 100%. The recovery of the other esters ranged from 77% (pentaenes) to 86% (trienes). The relatively high percent (84%) recovery of 22:6ω3 indicates that little autooxidation of the methyl esters occurred during the time the plate was exposed to air.

We have applied this system to the separation of methyl esters of retinal fatty acids and have observed the same percent recovery and purity as obtained for the standard mixture.

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1. Blank, M.L., B. Verdino, and O.S. Privett, JAOCS 42:87 (1965).

[Received October 4, 1974]

Simple, Rapid Method for Detecting Phase Transitions of Lipids

ABSTRACT

A simple rapid method was worked out for studying the physical and structural state of lipids by measuring the refractive indices of a layered lipid film. Melting processes of n-paraffins and phase transitions of phospholipids and the lecithin-cholesterol system also can be followed as a function of temperature. In accordance with data from the relevant literature, the measured refractive indices show that cholesterol lowers the phase transition temperature of lecithin. By isorefraction curves, the most ordered structure of the layers is indicated to occur at the equimolar ratio of cholesterol and lecithin. The method may be applied to study the effect of lipid-soluble agents upon lipid structure and is applicable for routine investigations and industrial purposes as well.

INTRODUCTION

The study of phase transitions of artificial lipid bilayers and biomembranes requires relatively difficult physical methods, such as differential scanning calorimetry, X-ray diffraction, NMR, electron spin resonance, microscopy, and electron microscopy (1-5). Structural birefringence also indicates the physical state of lipid layers (3). According to condensor theory (6), the sign of birefringence depends upon the spatial arrangement of the elements in a composite body. The birefringence is positive if the composite body is built up from thin rods parallel with the long axis and negative when the elements are discs and are situated perpendicularly to the long axis. Indeed, usually fibrous structures, such as cellulose, etc., ex-

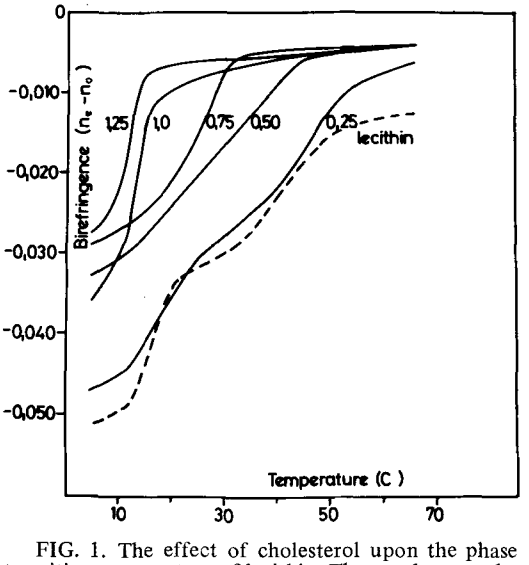
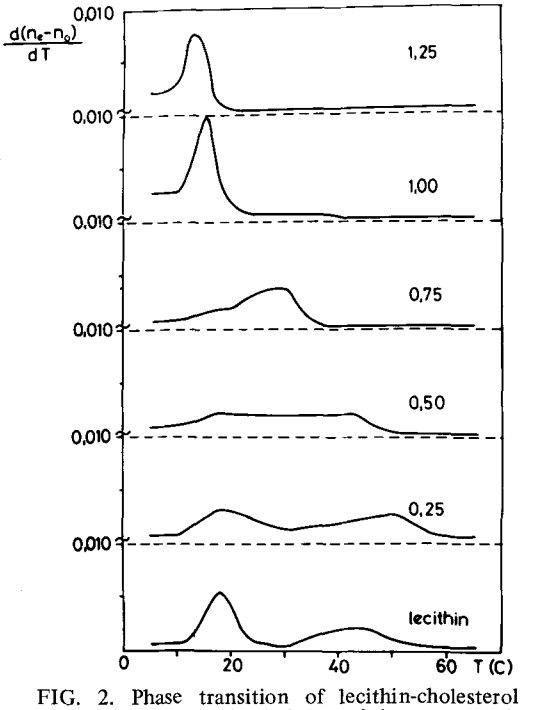


FIG. 1. The effect of cholesterol upon the phase transition temperature of lecithin. The numbers on the curves mean the molar ratio of cholesterol.



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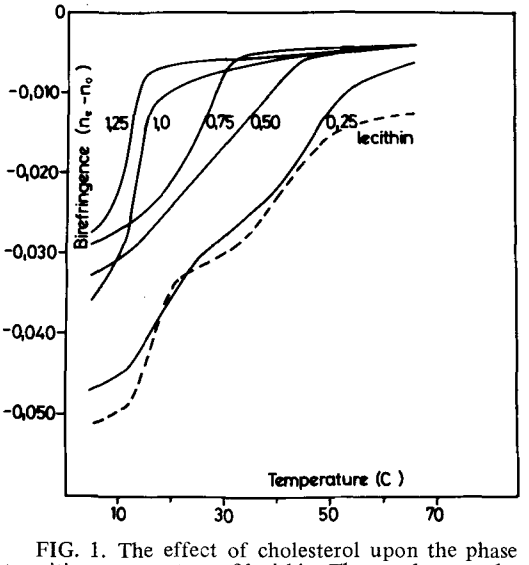

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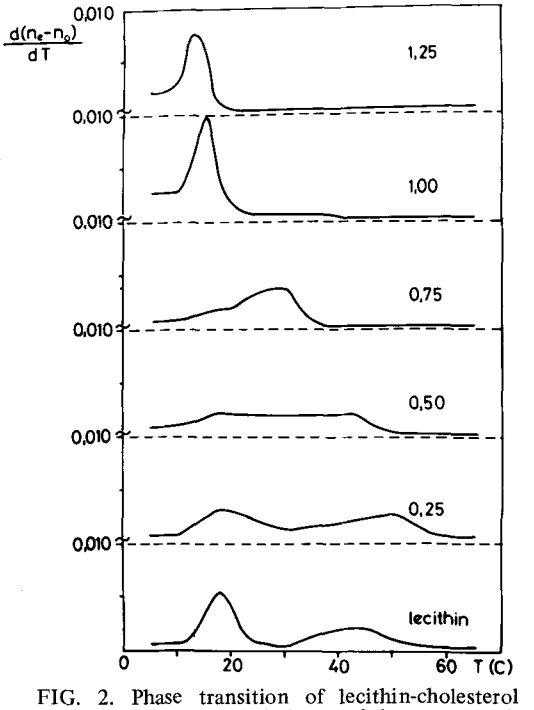

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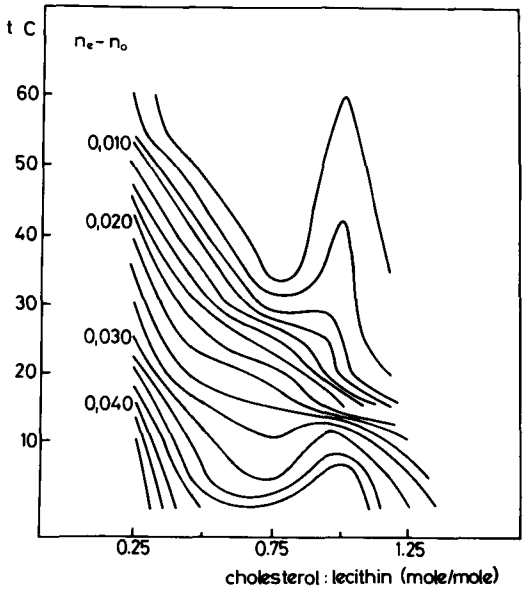

FIG. 3. Isorefraction curves.

hibit a positive birefringence, while the lamellar structure of the chloroplast, for example, gives a negative one.

If the biorefringence is obtained refractometrically as the difference of the extraordinary and ordinary refractive indices ($n_e - n_o$), the measurement is independent of the thickness and parallelism of the layer. In this paper, data proving the effectiveness of refractometry in studying phase transitions of lipids are presented.

EXPERIMENTAL PROCEDURES

Method

100 μliter 2% (w/v) Chloroform solution of lipids was dropped onto the upper prism of an Abbe refractometer (Zeiss GmbH, type G). On evaporating the solvent, a homogeneous lipid sheet appeared on the surface of the prism, and it was swollen by a wet N_2 stream for 10 min. Then, the prisms were closed and sealed with silicone-grease. The temperature was raised slowly and checked at the site of the lipid layer by a thermopile. The values of the refractive indices were read off at every degree throughout the temperature range between 5-65 C.

Materials

The n-paraffins (hexadecane, mp 18.5 C; octadecane, mp 28 C) were purchased from Fluka, Ltd., Buchs, Switzerland. The lecithin was purified from egg yolk by the method of Singleton (7), and its purity was checked by thin layer chromatography in the solvent system chloroform:methanol:water, 65:25:5, and according to Turner (8). The preparation gave only one spot by both methods used. The cholesterol was the product of Reanal (Hungary), and it was recrystallized three times from ethanol.

RESULTS AND DISCUSSION

It is known that n-paraffins exhibit two refractive indices in their crystallized state (below their mp), while only the ordinary index is observable in the melted state (9). The transition at the mp is very sharp and can be detected by our method with an accuracy of ±0.5 C.

Layered lecithin, immediately after evaporation of the solvent, did not show two refractive indices, hence no birefringence. However, after 10 min swelling in a wet N_2 atmosphere, a structural reorganization took place and both indices became observable. The change in birefringence of these layers was considerable if the temperature was raised, particularly in the temperature ranges of 15-20 C and ca. 45 C. It is important to note that differential thermal analysis of egg yolk lecithin gave a transition ca. 20 and 30 C (10,11) and a smaller endothermic change at ca. 50 C (11). In the control experiments with 50% (w/v) chloroform solution of lecithin, only the ordinary index appeared, and it was decreasing in a monotonic fashion with increasing temperature.

The effect of cholesterol upon the phase transition of lecithin was pronounced, as indicated by a shift of change in birefringence along the temperature axis (Fig. 1). As is known, cholesterol condenses the phospholipids in monolayers (12,13) and lowers the phase transition temperature between the crystalline and liquid crystalline phases (1).

The latter phenomenon is seen in Figure 1: the greater the molar ratio of cholesterol in lecithin, the lower the temperature of phase transition, as indicated by the sudden change in birefringence.

Considering the known mechanism of phase transition and the effect of cholesterol, as well as the nature of textural birefringence, the curves of Figure 1 can be understood if we assume that their initial values at low temperatures are characteristic of the crystalline phase. On the other hand, small absolute values in the higher temperature range can be attributed to the liquid crystalling phase. Sharp rises in the curves represent phase transitions in the lipid structure.

The first derivatives of the curves in Figure 1 were obtained by graphical differentiation and

are presented in Figure 2. It is evident that, in the presence of small quantities of cholesterol in lecithin (0.25 and 0.50 molar ratios), the 20 and 45 C transitions of lecithin are eliminated and the phase transition occurs over a wider range of temperature than at higher molar ratios ("intermediate fluid" state of phospholipids).

Some new aspects of the phase transition phenomena may be revealed if the temperatures corresponding to a fixed value of birefringence are plotted vs the molar ratio of cholesterol and lecithin. Thus a set of isorefraction curves are obtained (Fig. 3). At the equimolar ratio of cholesterol and lecithin, a higher temperature is necessary to reach the same value of birefringence as at other ratios. This may reflect a strictly ordered layer of lipid molecules at the equimolar ratio and may be indicative of complex formation between lecithin and cholesterol.

On the basis of these investigations, this simple and rapid method of refractometry seems to be suitable for studying the effect of lipid-soluble and other membrane-active agents upon the physical state and structure of lipid layers.

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Hydrocarbons from Males, Females, and Larvae of Pecan Weevil: *Curculio caryae* (Horn)

ABSTRACT

As part of a program to identify as many as possible of the components of the pecan weevil, *Curculio caryae* (Horn), the hydrocarbons from males, females, and larvae were isolated by solvent extraction and column chromatography and subjected to gas liquid chromatography-mass spectrometry analysis. n-Alkanes from C_{14}-C_{32} in the larvae and unsaturated and branched chain hydrocarbons from C_{20}-C_{32} in males and females were found. There are no significant differences between the hydrocarbons of the male and female pecan weevils.

INTRODUCTION

The adult pecan weevil, *Curculio caryae* (Horn), attacks green maturing nuts in late summer and damages them by making feeding and oviposition punctures. The insect then undergoes an extended life cycle which takes 2-3 years to complete (1). The extended life cycle of the pecan weevil indicates that the role of lipids in this insect is a central one. The importance of lipids in the biochemistry of

are presented in Figure 2. It is evident that, in the presence of small quantities of cholesterol in lecithin (0.25 and 0.50 molar ratios), the 20 and 45 C transitions of lecithin are eliminated and the phase transition occurs over a wider range of temperature than at higher molar ratios ("intermediate fluid" state of phospholipids).

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INTRODUCTION

The adult pecan weevil, *Curculio caryae* (Horn), attacks green maturing nuts in late summer and damages them by making feeding and oviposition punctures. The insect then undergoes an extended life cycle which takes 2-3 years to complete (1). The extended life cycle of the pecan weevil indicates that the role of lipids in this insect is a central one. The importance of lipids in the biochemistry of

insects has been stressed in recent reviews (2,3). Also, extensive studies in the order Coleoptera have been conducted (4-7). Our investigation was designed to add to the body of general knowledge of insect hydrocarbons and specifically to further the understanding of the hydrocarbons of Coleoptera.

TABLE I
Hydrocarbon Composition of Pecan Weevil Larvae

Hydrocarbon[a]	Mol wt	Percent[b]
n-Tetradecane	198	T[c]
n-Pentadecane	212	T[c]
n-Hexadecane	226	0.2
n-Heptadecane	240	0.3
n-Octadecane	254	0.5
n-Nonadecane	268	0.5
n-Eicosane	282	0.7
n-Heneicosane	296	0.8
n-Docosane	310	1.3
n-Tricosane	324	2.4
n-Tetracosane	338	1.9
n-Pentacosane	352	2.9
n-Hexacosane	366	2.1
n-Heptacosane	380	1.5
n-Octacosane	394	10.3
n-Nonacosane	408	2.1
n-Triacontane	422	24.9
Branched $C_{30}H_{62}$[d]	422	30.8
$C_{31}H_{64}$	436	2.1
$C_{32}H_{66}$	450	3.8

[a]Mass fragmentation patterns of these compounds corresponded with those published (10).

[b]Percent ot total hydrocarbons.

[c]Trace, present in concentrations of less than 0.1%.

[d]Five most abundant fragment ion values plus parent peak observed or inferred. (57, 43, 71, 85, 41, and 422).

The adult pecan weevils were newly emerged and unfed. The larvae were last instar and about to enter diapause. The experimental procedures were the same as those employed previously (8,9) with these few exceptions. The mass spectra were obtained from a Hewlett-Packard 5930 quadrupole mass spectrometer interfaced with a 5700 A gas chromatograph from a 20 x 1/8 in. 1.5% SE-30 gas chromatographic column at ionizing voltage 70 eV. The gas chromatograph unit was programed from 120-260 C at 4 C/min rate. The final temperature was maintained for 20 min.

RESULTS AND DISCUSSION

The lipid content of the larvae is among the highest ever reported for any stage of any insects (7). This high amount of lipid can be attributed to the larvae feeding on the lipid-rich meats of the pecan, which has been its habitat and sole source of food. The buildup of lipids permits the larvae to survive the long diapause period underground. The hydrocarbon content of the larvae is in the normal range.

The percent total lipids and hydrocarbons of the adult male and female is within the normal range for most other in-season insects. Also, the

TABLE II
Hydrocarbon Composition of Male and Female Adult Pecan Weevils

Hydrocarbon	Mol wt	Male (%)[a]	Female (%)[a]
Eicosene	280	0.2	---
1,3-Henicosadiene[b]	292	0.6	---
3-Heneicosene[b]	294	1.7	1.6
2-Heneicosene[b]	294	---	0.5
1,3-Docosadiene[b]	306	1.8	1.8
3-Docosene[b]	308	---	0.6
Tricosene	322	2.5	2.6
Tetracosene	336	1.7	1.7
Pentacosene	350	0.3	0.3
Hexacosene	364	0.5	0.4
Heptacosene	378	6.8	6.8
4-Methyl hexacosane	380	5.7	5.6
Octacosene	392	1.1	2.5
5-Methyl heptacosane	394	4.6	4.6
4-Methyl octacosane	408	11.5	11.5
5-Methyl nonacosane	422	c	c
11-Methyl nonacosane	422	c	c
13-Methyl nonacosane	422	c	c
15-Methyl nonacosane	422	c	c
$C_{31}H_{62}$	434	1.7	1.7
$C_{32}H_{64}$	448	2.2	2.2

[a]Percentage of total hydrocarbons.

[b]The geometric isomers of the unsaturated hydrocarbons could not be assigned from the mass spectral data (*cis-trans*).

[c]One gas liquid chromatograph peak contained these four hydrocarbons (36.8%) whose structures were assigned from the mass spectra by the method of Nelson (11).

insects would have just recently emerged from an extended period underground during which they undoubtedly had depleted their lipid reserves for survival. Females contain a slightly larger amount of lipid than males, which is a common occurrence in Coleoptera and in other insects. It is presumed that this higher quantity of lipid is used for reproductive purposes. The investigation of the pecan weevil lipid constituents was done in this laboratory (7).

Table I presents the hydrocarbons of the pecan weevil larvae for which structures were determined. They accounted for 89.1% of the gas liquid chromatographic (GLC) peaks which were presumed to be hydrocarbons. All of the hydrocarbons in the series from C-14-C-32 were present and each, except one, were n-alkyl. In this present work, GLC conditions were adequate to elute hydrocarbons up to C-36, but none above C-32 were observed. The presence of normal chain saturated hydrocarbons probably can be attributed to the larvae feeding on the lipid-rich meats of the pecan. n-Octacosane, n-triacontane, and a branched chain triacontane are the major hydrocarbons of the larvae; they account for 66% of the total hydrocarbons.

Table II lists the total hydrocarbon composition of male and female adult pecan weevils and their percentage distribution. These accounted for 79.7% and 81.2% of the total hydrocarbons amenable to GLC-mass spectrometry in the male and female pecan weevil, respectively. All of the hydrocarbons in series C_{20}-C_{32} were present in both males and females; all were unsaturated and branched chain hydrocarbons. Five other aliphatic hydrocarbons (C_{13}-C_{19}) also were found in a previous study on the pecan weevil volatiles (12). The mixture of C_{30} branched chain hydrocarbons in both the male and female which eluted as one GLC peak was resolved by examination of the mass spectral data according to the method of Nelson (11). The C_{30} hydrocarbons in this mixture included 5-, 11-, 13-, and 15-methyl nonacosane.

In summary, both the larvae and adults contain hydrocarbons through C-32. In contrast, larvae contain only n-alkyl saturated hydrocarbons, except for one branched C-30 hydrocarbon, while adults of both sexes contain only n-alkyl unsaturated and branched chain saturated hydrocarbons. No significant differences between males and females were observed.

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Enzymic Synthesis of 1-Alkyl-2-Acyl-*sn*-Glycero-3-Phosphorylethanolamine through Ethanolaminephosphotransferase Activity in the Neuronal and Glial Cells of Rabbit in Vitro

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ABSTRACT

The transfer of radioactivity from cytidine-5'-diphosphate ethanolamine into 1-alkyl-2-acyl-*sn*-glycerophosphorylethanolamine of neuronal and glial cells from adult rabbit brain cortex has been investigated in vitro. The synthesis of 1-alkyl-2-acyl-*sn*-glycerophosphorylethanolamine in both cell populations was stimulated 23-25-fold by the addition of 6 mM alkylacylglycerol. The neuronal cell-enriched fraction was found to possess/unit protein a 1.7-1.8-fold ethanolaminephosphotransferase activity (EC 2.7.8.1), as compared to the glial fraction, when saturating concentrations (6 mM) of alkylacylglycerols were added in the incubation system. The neuronal/glial ratio was 2.6-2.8 in the absence of lipid acceptor or with low concentrations of alkylacylglycerol. Under most favorable conditions, 6.4 and 3.3 nmoles 1-alkyl-2-acyl-*sn*-glycerophosphorylethanolamine/mg protein/30 min was obtained for neurons and glia, respectively. Various kinetic properties of the 1-alkyl-2-acyl-*sn*-glycerophosphorylethanolamine synthesizing phosphotransferase activity were found to be similar both in neurons and glia.

INTRODUCTION

1-Alkyl-2-acyl-*sn*-glycero-3-phosphorylethanolamine (alkylacyl-GPE) has been shown to be synthesized from cytidine-5'-diphosphate ethanolamine (CDPE) and 1-alkyl-2-acyl-*sn*-glycerols (alkylacylglycerols) (1). Brain microsomes also possess this capacity (2). This ethanolamine lipid accounts for ca. 3% of the total ethanolamine phospholipids (EPG) from brains (3-6); its synthesis, in addition, is thought to be important, since alkylacyl-GPE represents in animal tissues the natural precursor of 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphorylethanolamine (alkenylacyl-GPE), the ethanolamine plasmalogen. It has been shown, in fact, that the biosynthesis of plasmalogens in mammalian systems involves the introduction of long chain alcohols into 1-*O*-alkyl glycerolipids and the oxidation of alkylacyl-GPE to the corresponding alkenylacyl derivative (7,8). It also has been demonstrated, in addition, that the *O*-alkyl synthesizing enzymes in brain are most active during the early stages of growth and development (9), and this finding supports the concept of *O*-alkyl glycerolipids being the precursors of plasmalogens in nervous tissue, since the time of active myelination during development coincides with the maximum plasmalogen accumulation.

Neuronal and glial cells recently have been studied in vitro in a variety of biochemical reactions (10), including phospholipid synthesis (10-14). No reports have appeared as yet, however, on the ability of these cells to perform alkylacyl-GPE synthesis. A study of the enzymic properties of the de novo mechanism for alkylacyl-GPE synthesis both in neuronal and glial cell fractions seems, therefore, of value to understand its physiological function during development and its relationship to plasmalogen formation. An experimental study concerning this problem is reported in the present work.

EXPERIMENTAL PROCEDURES

Preparation of Neuronal and Glial Cell Fractions

The neuronal and glial fractions were prepared from white rabbits (1.5-1.8 kg body wt), as reported elsewhere (13). They were analyzed for purity by light microscopy, electron microscopy (15-18), and marker enzyme tests (11,13), and treated before incubation exactly as reported previously (14).

Chemicals and Labeled Substrates

Materials: Materials were obtained as described previously (10). Pancreatic lipase (steapsin) was from Nutritional Biochemical Corp., Cleveland, Ohio, bile salts from Difco Laboratories, Detroit, Mich.; and Silica Gel HR from E. Merck A.G., Darmstadt, W. Germany. The labeled substrate, more precisely CMP-[1,2-^{14}C] ethanolamine phosphate (labeled CDP-ethanolamine), was synthesized and purified, as reported elsewhere (10). Before

using, the product was checked for its purity, as described previously (10,13).

Diacylglycerols: The 1,2-diacyl-*sn*-glycerols (diacylglycerols) from soybean lecithin, brain choline phosphoglycerides, and heart phospholipids were prepared, purified, and analyzed as reported by Binaglia, et al. (14). They were stored, if necessary, for a few days at -20 C in diethyl ether. The sonicated dispersions were prepared just before use, as reported elsewhere (10).

Alkylacylglycerol: This lipid acceptor was prepared from ratfish (*Chimera monstrosa*) liver oil. The method of preparation, purification, and elution was described elsewhere (2). Only trace amounts of the 1,3 isomer of alkylacylglycerol were observed on Silica Gel HR by thin layer chromatography (TLC) carried out in n-hexane-diethyl ether (2:1, by volume), which resolves the 1,2 and 1,3 products. The percent composition of acyl groups in the 2 position was the following (triplicate determinations):14:0 (traces), 16:0 (17.81%), 16:1 (2.76%), 18:0 (2.92%), 18:1 (75.25%), 18:2 (0.12%), and 20:1 (1.63%). These data agree well with previous findings (2). The eluate containing the pure alkylacylglycerol was emulsified in 50 mM Tris-HCl buffer (pH 7.60) containing 0.1% Tween-20 and 0.05% purified bovine serum albumin (5-fold crystallyzed, Serva, Heidelberg, W. Germany). Sonication was carried out as reported elsewhere (2) just before use. Stable emulsions up to 45 mM were obtained.

Analytical Methods

Gas-liquid chromatography (GLC): Methyl esters of the acyl groups of the alkylacylglycerols were prepared by acid methanolysis (15). GLC was carried out at 210 C with a Carlo Erba (Milan, Italy) Fractovap GV gas chromatographer, equipped with 150 x 0.3 cm steel columns. The stationary phase was 20% ethylene glycol adipate (EGA) on sylanized Chromosorb P, and the carrier gas was nitrogen. The responses from the flame ionization detector were quantitated by the triangulation method. The peak areas of the methyl esters were divided by their mol wt and the fatty acid composition calculated as moles percent.

Diradylglycerol concentration: The diacylglycerol concentration was estimated as described elsewhere (10). The alkylacylglycerol concentration was determined by GLC of the fatty acid released from position 2 of the isolated glycerol. Methyl ester derivatives of the acyl groups were prepared by acid methanolysis with 2% H_2SO_4 in methanol-benzene (1:1, by volume) for 4 hr at 65 C. After extraction with n-hexane, the fatty acid and 1-alkyl glycerol mixture was concentrated under vacuum and subjected to TLC on Silica Gel G with n-hexane-ethyl ether (9:1, by volume) as the solvent to separate the methyl esters, which were successively extracted from the silica gel with chloroform:methanol (2:1 by volume) and analyzed by GLC, as reported above. The peak areas were quantitated by adopting myristic acid (14:0) as an internal standard. This fatty acid molecule was practically absent in the alkylacylglycerol preparation. Final calculation was made as explained above.

Protein was determined according to Lowry, et al., (16) with crystalline bovine serum albumin as a standard (10). Phospholipid P was determined in the lipid extract according to Ernster, et al. (17).

Incubation

Incubation for 30 min at 40 C normally was carried out in the following incubation mixture (0.15 ml final volume):labeled CDPE (specific activity of 0.89-1.7 nCi/nmole) at the concentration values indicated in each table or figure (normally 0.70-1.1 mM); Tris-HCl buffer, pH 8.0 (50 mM); alkylacylglycerol (6 mM), dissolved in Tween-20 (0.024% final concentration) and albumin (0.012%); $MnCl_2$ (10 mM); and neuronal protein (150-200 μg) or glial protein (300-400 μg). Components were added at +2 C in the indicated order. The final pH value (measured at the end of the incubation period) was 8.1. When indicated, other diradylglycerols were added in place of the alkylacyl derivative. Each incubation was carried out as described previously (14) and the incubation mixture treated as follows.

Isolation and Assay

Isolation of phospholipid: Total lipid was extracted, isolated, and determined, as reported previously (14). 2-Monoacyl-*sn*-GPE (derived from ethanolamine plasmalogen) and diacyl-*sn*-GPE were separated, analyzed, and counted as reported by Binaglia, et al. (14). Identification of lipid was confirmed by using reference phospholipid standards which always were included in each experiment on the same plate. The diacyl-*sn*-GPE spot was eluted with three portions of chloroform-methanol-acetic acid-H_2O (50:39:1:10, by volume), by using 3 ml mixture each time. The solution was dried under a stream of nitrogen at 30 C and the residue taken up with small amounts of chloroform. The mixture of diacyl-GPE then was resolved by solvent partition after saponification, as reported elsewhere (2). Iodine vapor, ninhydrin spray, phosphomolibdate reagent,

TABLE I

Incorporation of CMP-[1,2-^{14}C]-ethanolamine phosphate (CDPE) into Diacyl-Glycerophosphorylethanolamine (-GPE), Alkenylacyl-GPE and Alkylacyl-GPE of Neuronal and Glial Cell Dispersions from Rabbit Brain in Absence and Presence of Added Alkylacylglycerol Preparations[a]

Synthesized lipid	Fraction	Addition	Activity[b]	A/B[c]
Diacyl-GPE	Neurons	---	1.74	2.1
	Glia	---	0.84	
Alkenylacyl-GPE	Neurons	---	1.18	2.1
	Glia	---	0.57	
Alkylacyl-GPE	Neurons	---	0.22	2.7
	Glia	---	0.08	
Diacyl-GPE	Neurons	Alkylacylglycerol	5.57	2.6
	Glia	Alkylacylglycerol	2.13	
Alkenylacyl-GPE	Neurons	Alkylacylglycerol	3.38	2.5
	Glia	Alkylacylglycerol	1.34	
Alkylacyl-GPE	Neurons	Alkylacylglycerol	5.14	2.6
	Glia	Alkylacylglycerol	2.01	

[a]Neuronal or glial homogenates were incubated under standard conditions for 30 min. at 40 C with 3.2 mM-alkylacylglycerol and 0.7 mM-CDPE (specific activity of 1.4 nCi/nMol). Incorporation was examined as described in the text.

[b]nMoles/mg protein/30 min.

[c]A = neurons and B = glia.

and radiochromatography with a radiochromatoscanner were used for detection of spots.

Determination of phospholipids: The identified labeled lipid spots, scraped off from the TLC plates, or the lipid containing solutions were counted as reported elsewhere (10). Recovery of labeled lipid samples was ca. 90-95%. The amount of synthesized phospholipid was calculated by dividing the estimated nCi by the specific activity of the incubated precursor. The results, expressed as nmol of synthesized lipid, then were converted into nmol x mg protein^{-1} x 30 min^{-1}.

RESULTS

Incorporation of CDPE into Ethanolamine Lipids of Neuronal and Glial Cells

Experiments were performed to determine the capacity of neuronal and glial cell preparations to synthesize EPG from CDPE in the absence of added lipid acceptors. Table I shows that the neuronal and glial cell fractions in absence of added acceptors incorporated a substantial amount of CDPE into diacyl-GPE and alkenylacyl-GPE but small radioactivity into alkylacyl-GPE. In terms of specific activity, the neurons displayed a two-three-fold increase of activity for all the three EPG subclasses. Moreover, both neurons and glia displayed a higher rate of synthesis of diacyl-GPE as compared to that of the other EPG.

In the presence of 3.2 mM-alkylacylglycerol, a 23-25-fold stimulation was found either in glia or neurons for the synthesis of its specific product, the alkylacyl-GPE. The neuronal fraction again displayed a higher activity as compared to glia with a 2.6-fold increase, which was of the same order of that shown in the absence of added alkylacylglycerol (Table I). This finding indicates presumably that the addition of alkylacylglycerol stimulates equally well in both fractions the synthesis of alkylacyl-GPE.

The increased uptake of labeled CDPE into alkylacyl-GPE following alkylacylglycerol addition was not specific, since a consistent stimulation (two-three-fold increase of labeling) of diacyl- and alkenylacyl-GPE synthesis was obtained on adding the alkylacylglycerol (Table I). This finding was substantiated also by the procedure adopted by us to prepare the alkylacylglycerol used in the present work. It was checked to ensure that no traces of diacylglycerol or alkenylacylglycerol were present in the alkylacylglycerol preparation and, as reported under "Methods," that only trace amounts of isomers were present in the product. This result, which was comparable to that observed on adding diacyl or alkenylacylglycerol (14), also was obtained on examing alkenylacyl-GPE synthesis in rat brain microsomes (2), although this did not apply to diacyl-GPE. On increasing the Tween-20 final concentration up to 0.04%, definite inhibition for all CDPE incorporations was observed.

Incubations have been carried out also with isolated rabbit brain microsomes to compare the results with those of Table I and with

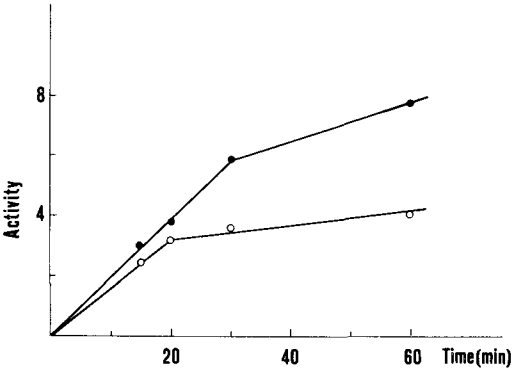

FIG. 1. The effect of the time of incubation on the synthesis of alkylacyl-glycero-3-phosphorylethanolamine by neuronal and glial cell homogenates of rabbit brain in vitro. The incubation system contained (in a final volume of 0.15 ml) 50 mM Tris-HCl buffer (pH 8.0), labeled 0.81 mM CMP-[1,2-^{14}C]ethanolamine phosphate (CDPE) of 1.7 nCi/nmol specific activity, 6 mM alkylacylglycerol, 0.025% Tween-20, 0.012% bovine serum albumin, 12.3 mM cysteine, 10 mM $MnCl_2$ and 1.07 mg/ml or 1.03 mg/ml glial (o—o—o) or neuronal (•—•—•) protein, respectively. Temperature 40 C. The time of incubation was varied as shown. Activity reported as nmoles/mg protein.

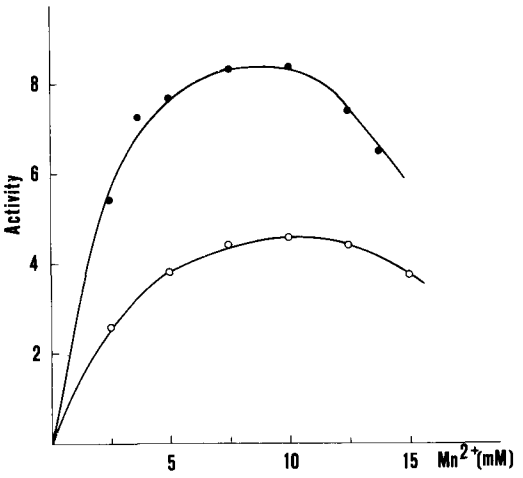

FIG. 2. The effect of Mn^{2+} ions on the incorporation of labeled cytidine-5'-diphosphate ethanolamine into alkylacyl-glycero-3-phosphorylethanolamine of neuronal (•—•—•) and glial (o—o—o) fractions of rabbit brain in the presence of 6 mM alkylacylglycerol. Incubation for 30 min was carried out as reported in Figure 1. CDPE concentration was 0.82 mM (specific activity of 0.888 nCi/nmole). Activity reported as nmoles/mg protein/30 min.

published findings (2). Under the experimental conditions reported in Table I, a 23-fold, 3-fold, and 2.9-fold stimulation of the endogenous syntheses of alkylacyl-, alkenylacyl- and diacyl-GPE, respectively, was found on adding 3.2 mM- alkylacylglycerol, and this result compares well with the data reported in Table I. The stimulation of alkylacyl-GPE synthesis was lower than that reported previously (2), but, in our experiments, nonsaturating alkylacylglycerol concentrations were used. Conversely, a stimulation of diacyl-GPE synthesis by added alkylacylglycerol was observed with the present study in whole brain microsomes, and this was not noticed in the previous report (2).

Characteristics of Lipid Labeling

Incubation of CDPE with neurons and glia produced only labeled EPG. The radioactivity of EPG was found entirely in the base moiety of the phospholipid at any time of incubation, as observed by previously described hydrolytic methods (18). In all the experiments reported in the forthcoming sections, the radioactivity of isolated lipids will be referred only to that present in alkylacyl-GPE.

Properties of Ethanolaminephosphotransferase Activity (EC 2.7.8.1) of Glia and Neurons Synthesizing Alkylacyl-GPE

pH: Under standard incubation conditions and 6 mM-alkylacylglycerol, the optimum of pH of the CDPE-alkylacyl ethanolaminephosphotransferase activity (EC 2.7.8.1) was found to be around 8.0 in both cell fractions, with results similar to those observed when syntheses of diacyl-GPE and alkenylacyl-GPE were examined (10,14). Higher phosphotransferase activity was found above pH 8.4-8.5 but with more scattering of data. Owing to these data, the pH value of 8.0 has been used throughout the successive experiments. At this pH value and 6 mM-alkylacylglycerol, a neuronal/glial activity ratio of 1.64 (6.20 and 3.78 nmoles/mg protein/30 min in neurons and glia, respectively) has been found on using the above mentioned experimental conditions.

Effect of enzyme concentration and time of incubation: During the incubation period of 30 min, the increase in incorporation of the labeled CDPE into the alkylacyl-GPE was proportional to the increase in glial protein added over the range of 0.3-2.0 mg/ml and in neuronal protein 0.25-1.3 mg/ml. At any value of protein concentration, neuronal/glial ratio of phosphotransferase activity was in the range of 1.6-1.7. The incorporation of radioactivity into alkylacyl-GPE was somewhat reduced on using higher protein concentration, as observed in parallel experiments. In all the forthcoming sections, the glial and neuronal protein concentration will not exceed values higher than 2.0 and 1.0 mg/ml, respectively.

The rate of synthesis of alkylacyl-GPE in neurons was proportional in time up to 30-40

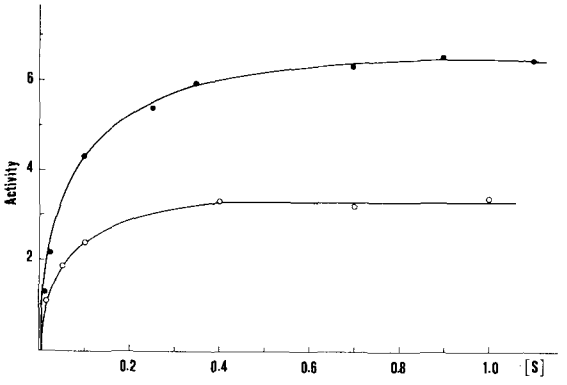

FIG. 3. The effect of cytidine-5'-diphosphate ethanolamine (CDPE) concentration upon the synthesis of alkylacyl-glycero-3-phosphorylethanolamine by neurons (•—•—•) and glia (○—○—○) from rabbit brain. Each tube contained the equivalent of 1.03 mg/ml and 1.07 mg/ml neuronal and glial protein, respectively. Incubation conditions were similar to those reported in Figure 1. The concentration of CDPE (1.71 nCi/nmole) was varied, as indicated. Incubation was carried out at 40 C for 20 min. The activity is expressed as nmoles/mg protein/30 min. [S] = CDPE, mM.

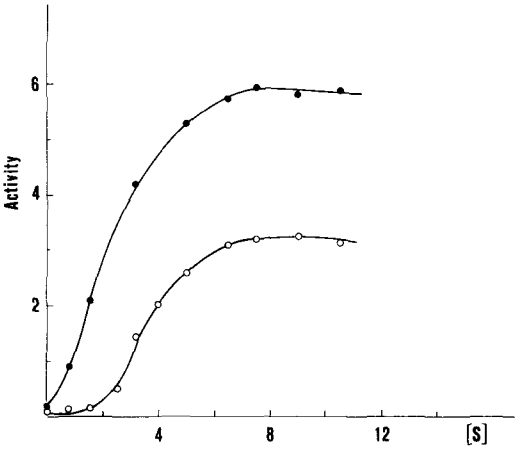

FIG. 4. The effect of the concentration of alkylacylglycerol upon the velocity of the ethanolamine-phosphotransferase reaction in the presence of excess cytidine-5'-diphosphate ethanolamine (CDPE). Neurons (•—•—•) and glia (○—○—○) were incubated in amounts corresponding to 0.53 mg protein/ml and 1.23 mg/ml, respectively, in the incubation medium reported in Figure 1. CDPE concentration was 0.81 mM (specific activity of 0.88 nCi/nmol) and time of incubation 20 min. Alkylacylglycerol was added, as indicated. The amounts of Tween-20 and serum albumin always were kept constant to obtain final concentration values of 0.025% and 0.012%, respectively. The activity is expressed as nmoles/mg protein/30 min. [S] = alkylacylglycerol, mM.

min of incubation (Fig. 1), whereas the activity in glia retained linearity only during the first 20 min of incubation. Protein concentration in both fractions was ca. 1 mg/ml. Concomitantly, the neuronal/glial ratio of CDPE incorporating activity increased from 1.2 at 15 min of incubation to 1.6 and 1.9 at 30 and 60 min, respectively. These last results are somewhat different from those observed for diacyl-GPE (10) and alkenylacyl-GPE (14) syntheses. The incubation time was limited to 30 min for all the successive experiments.

Divalent cations: No incorporation of radioactivity into alkylacyl-GPE was observed in the absence of either Mn^{2+}- or Mg^{2+}-ions. This result confirms previous work obtained with neurons and glia concerning the synthesis of diacyl- and alkenylacyl-GPE (10,14). Earlier studies carried out with nonnervous and nervous tissues already had shown the requirement of these cations in the reaction (1,2,18-21). Synthesis of alkylacyl-GPE was equally absent in whole brain microsomes when these cations were omitted (2). Both Mg^{2+}- and Mn^{2+}-ions stimulated CDPE incorporation into neuronal and glial alkylacyl-GPE. Mn^{2+}-ions were, however, much more active at low concentrations than Mg^{2+}-ions, thus confirming previous results (10,14). No activity was detectable up to 7-8 mM-Mg^{2+}-ions, this finding being rather typical for the neuronal activity. The optimal Mn^{2+}-ion concentration was found to be around 10 mM (Fig. 2), and this concentration was used throughout our experiments. Neuronal/glial ratio at 10 mM Mn^{2+}-ions was around 1.7-1.8.

K_m for CDPE: Figure 3 shows that, in both neurons and glia, the enzyme activity:CDPE concentration relationship follows an almost typical Michaelis-Menten equation in the presence of saturating alkylacylglycerol. The system apparently was saturated by ca. 0.7-0.8 mM substrate concentration in neurons and 0.4 mM in glia. A Lineweaver-Burk double reciprocal plot indicated a K_m value for CDPE of 3.9 x 10^{-5}M and 3.1 x 10^{-5}M in neurons and glia, respectively. These values are not different from those obtained in the same cell fractions with diacylglycerols, as lipid acceptors (10,14). V_{max} values for neuronal and glial activities have been calculated to be ca. 6.4 and 3.3 nmol/mg protein/30 min, respectively, with a neuronal/glial ratio of ca. 1.9. Saturating CDPE concentrations always have been used throughout this work.

K_m for CDPE has been measured also in whole rabbit brain microsomes incubated with alkylacylglycerol in the conditions reported in Figure 3. A K_m value of 2 x 10^{-5}M was observed, which is rather similar to that noticed for the neuronal and glial enzyme, although it is noticeably lower than that reported previously

for the rat brain microsomes incubated with similar lipid acceptor (2).

K_m for alkylacylglycerol: Figure 4 shows that, on increasing the alkylacylglycerol concentration in the incubation system, an increase of the alkylacyl-GPE formation occurred after an initial inert phase which was much more evident in glia, as compared to neurons. Maximum reaction rates apparently were detected at ca. 6-8 mM concentration in both cell populations. At this value of saturating alkylacylglycerol concentration, a neuronal/glial ratio of ca. 1.8-2.0 was obtained, which was very similar to that observed in other experimental conditions (Figs. 2 and 3), but considerably lower than that noticed when nonsaturating alkylacylglycerol amounts were used (Table I), as visible also from Figure 4. Saturating concentrations of alkylacylglycerol always have been used during our work, except in the experiments depicted in Table I.

It has been impossible to examine whether higher concentrations of alkylacylglycerols would have inhibited the incorporation of CDPE into the correspondent phospholipid, as examined for diacyl and alkenylacylglycerols (10,14), since no stable emulsions of this lipid acceptor, in the final incubation system, were obtained at concentrations higher than 8-10 mM.

On inspecting Figure 4, it is clear that, in the reaction rates for the synthesis of alkylacyl-GPE in neurons, half of the V_{max} values were obtained at 2.2 mM alkylacylglycerol concentration, with corresponding V_{max} values of ca. 6 nmoles/mg protein/30 min. Corresponding values for glia were ca. 4 mM and 3 nmoles/mg protein/30 min. The apparent values of K_m found for alkylacylglycerol in neurons and glia in the present work are not different from those reported in both cell populations for alkenylacylglycerol (14). In addition, the value found in neurons is rather similar to those reported in the same cells for diacylglycerol (10) and in whole brain microsomes for diacyl and alkenylacylglycerols (18,19).

DISCUSSION

The presence of an enzyme which catalyzes the reaction between labeled CDPE and added alkylacylglycerol to produce labeled alkylacyl-GPE has been demonstrated clearly with the present work in separated neuronal and glial cells. A noticeable stimulation of the ethanolaminephosphotransferase activity (23-25-fold stimulation), of the same magnitude of that produced for plasmalogen synthesis by added alkenylacylglycerol (14), was, in fact, observed on adding either to glia or to neurons excess alkylacylglycerol. The physiological significance of this reaction (2) also is related to the fact that endogenous levels of alkylacylglycerol have been detected in nervous tissue (22). Moreover, the enzymic synthesis of ethanolamine plasmalogens through the desaturation of synthesized alkylacyl-GPE has been reported to occur in brain tissue by Blank, et al., (23) and Horrocks and Radomińska-Pyrek (24), and, owing to this result, the finding reported in the present work of the occurrence in neurons and glia of an alkylacyl-GPE synthesizing reaction acquires even more importance for the significance of the metabolic reactions in these cell populations.

Glial cells, therefore, seem less efficient than neurons on a protein basis in carrying out in vitro the de novo synthesis of alkylacyl-GPE either in the absence or in the presence of the proper lipid acceptor. This finding is not due, in our opinion, to different rates of penetration in vitro of precursors to the sites of synthesis, as explained with previously reported results (10). On the other hand, if the alkylacyl-GPE synthesizing activity is referred to deoxyribonucleic acid (DNA) content (and presumably to number of cells) rather than to protein, then a higher activity in glia is obtainable as compared to neurons, since the protein/DNA ratio in glial cells is ca. three times that in neurons (25). This consideration might indicate that glial cells are endowed/unit cell of a higher potentiality for alkylacyl-GPE synthesis than neurons, and, presumably, this could be reflected in a higher rate of synthesis of ethanolamine plasmalogens in glia as compared to neuronal cells through the successive step catalyzed by the desaturase activity, a problem which is currently under investigation in our laboratory. This assumption does not seem to be unwarranted, since, at 20 min interval from incubation with CDPE and alkylacylglycerol, a certain decrease in alkylacyl-GPE synthesis takes place in glia as compared to neurons (Fig. 1), and this finding could imply that an active subsequent desaturation of alkylacyl-GPE into alkenylacyl-GPE is taking place in the glial homogenates after that time.

Saturating concentrations of any 1-radyl-2-acyl-*sn*-glycerols are necessary for assessing neuronal/glial ratios of transferase activity (10,14, and present work). This consideration is verified by the findings reported in the present work that a different neuronal/glial ratio, higher than that reported in the present work under saturating concentrations of alkylacylglycerol, is obtained when low amounts of lipid acceptor are incubated (Table I and Fig. 4).

The characteristics of the system for alkylacyl-GPE synthesis in neurons do not appear to differ significantly from those of glia, apart from the described quantitative differences. Moreover, CDPE in neuronal and glial cells has a similar K_m value in the presence of saturating alkylacylglycerol concentrations, which is rather similar to those found in both cell populations for diacyl- and alkenylacyl-GPE synthesis on using saturating amounts of the proper lipid acceptors. This indicates, presumably, as observed also for whole brain microsomes (2,18), that the transfer of CDPE to different diradylglycerols is catalyzed by the same enzyme protein, either in glia or in neurons. It must be added also, in connection with these considerations, that the increased uptake of labeled CDPE into diacyl-, alkenylacyl-, and alkylacyl-GPE following the addition of the proper diradylglycerol was not completely specific (14 and present work), since stimulation was observed also for other EPG subclasses. Probably these unspecific stimulations may be due either to formation of complex micelles which activate the endogenous synthesizing system or to a detergent-like activity of the diradylglycerols which stimulate the membrane-bound enzymic system. The described finding currently is being investigated in this laboratory.

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Relationship between Lipids in Plasma and Skin Secretions of Neonatal Calf with Particular Reference to Linoleic Acid

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ABSTRACT

A study has been made on the lipid composition of the skin secretions and plasma of the neonatal calf. A significant proportion of the skin surface lipids was comprised of triglycerides. Saturated fatty acids comprised the major proportion of the fatty acids of the skin surface triglycerides at birth. Immediately after birth, the proportion of the saturated fatty acids decreased, and there was a concomittant increase in the proportion of 18:1. Some 3-4 weeks after birth, the proportion of 18:2 Δ *cis*-9, *cis*-12 in the skin surface triglycerides increased to ca. 14%, and there was a decrease in the proportion of 18:1. The 18:2 was shown to be confined almost entirely to the 2 position of the triglycerides. During the first 5 weeks after birth, the concentrations of the cholesteryl esters and phospholipids in the plasma increased markedly and were accompanied by a rapid increase in the proportion of 18:2 within these 2 fractions. The results are discussed in relation to the known role of 18:2 in the metabolism of biological systems.

INTRODUCTION

Large differences exist between the lipid compositions of the various major tissues in the adult and new-born ruminant (1). By far the most important of these differences involves the levels of essential fatty acids in the tissues (2,3). Very low levels of C18 polyunsaturated fatty acids are found in the tissues and plasma of the ruminant at birth, in spite of the considerable concentrations of these acids that circulate in the maternal plasma; relatively high tissue concentrations of the C20 trienoic acid normally associated with essential fatty acid deficiency also are observed. However, in spite of the low concentrations of linoleic acid in ruminant milk (ca. 1% or less of the total calories) (4), the deficiencies of linoleic acid in the tissues of the new-born ruminant are soon rectified when the animal begins to suckle.

Recent investigations into the fatty acid compositions of the lipids found on the skin surface of the ox have shown the presence of ca. 10% of linoleic acid (5). This acid was, furthermore, found to be associated predominantly with the triglyceride fraction which comprised a significant proportion of the total skin surface lipids. In view of the various metabolic roles known to be played by linoleic acid in biological systems (6), the presence of high concentrations of linoleic acid (ca. 20% of the total fatty acids) in the triglyceride fraction isolated from the skin surface lipids of cattle seemed to be of significance in relation to the part thought to be played by the skin surface secretions in affording some protection against bacterial or other exogenous agents (7). As it already has been shown that considerable differences exist in the general level of linoleic acid present in the body of the young and adult ruminant (2,3), the presence, or otherwise, of polyunsaturated fatty acids in the skin surface lipids of the new-born ruminant during the critical period immediately after birth seemed to be of importance and worthy of investigation.

EXPERIMENTAL PROCEDURE

The experimental animals were new-born Ayrshire calves weighing between 30-40 kg at birth. The handling and dietary treatment of each calf was similar to that normally carried out commercially. Thus, each calf was allowed to remain with and suckle its mother for 24 hr after birth, after which time it was removed and housed in an individual stall at an environmental temperature of 22 C. For the first week after birth, the diet consisted solely of the dam's milk fed to appetite. After this time, each calf was offered an increasing amount of a proprietary concentrate mixture; by the end of the fifth week, the amount of the concentrate mixture being consumed by each calf was ca. 1 kg.

As soon after birth as possible, hair was clipped from large areas along the dorsal surface of both sides of each animal and these areas were thoroughly washed with soap and water, followed by thorough washing with water to remove all traces of the soap solution. The skin surface lipids from these areas of each animal then were collected at 0, 3, 7, 14, 21, 28, 35, and 42 days after birth using the method described previously (5). After filtration of the skin washings and the removal of the solvent on a rotary film evaporator, the lipid residues from

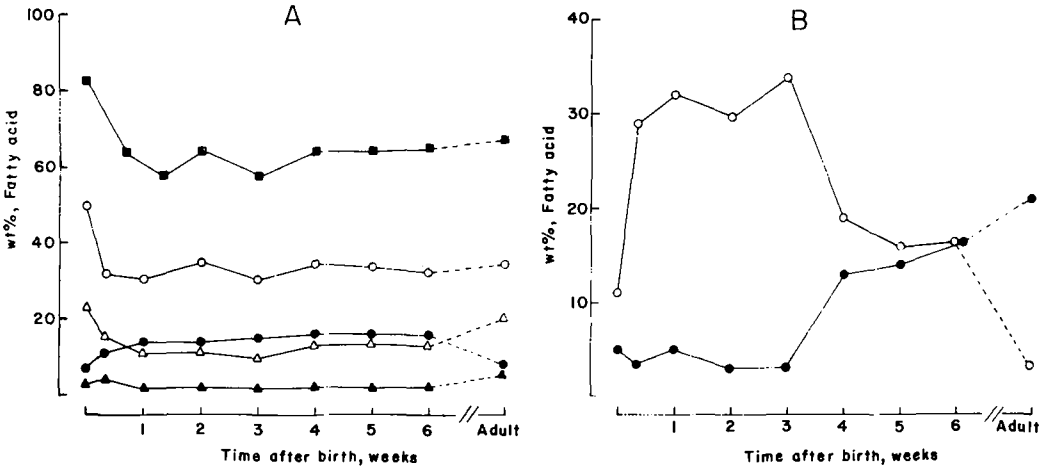

FIG. 1. The fatty acid concentrations (wt %) of the skin surface triglycerides of the calf after birth. A. Saturated fatty acid: □—□ =total saturated fatty acids, ○—○ = 16:0, △—△ =14:0, ●—● = 18:0, and ▲—▲ =12:0. B. Unsaturated fatty acids: ○—○ = 18:1 and ●—● = 18:2.

TABLE I

Wt Percentages of Major Fatty Acids in Plasma of Calf after Birth

Fatty acid	Days after birth								
	0	1	2	3	5	7	14	24	35
16:0	31.0	26.0	24.8	26.1	20.3	24.2	21.7	15.5	17.3
16:1	9.5	6.0	5.3	4.5	3.7	5.2	4.1	3.6	4.8
18:0	12.2	12.9	14.6	13.8	16.7	14.0	14.6	15.8	15.6
18:1	41.6	43.6	37.6	34.6	30.8	32.0	30.0	25.9	25.8
18:2	2.7	6.9	10.3	12.6	18.7	17.1	25.6	32.6	32.0
18:3	0.1	2.0	3.6	4.7	7.2	5.0	2.8	3.9	2.6
20:3 (n-9)	1.0	1.0	2.8	0.9	0.6	0.3	>0.1	>0.1	>0.1
20:4 (n-6)	1.8	2.4	1.0	2.8	2.0	1.9	1.3	2.4	2.2
mg fatty acid/100 ml plasma	66	80	116	93	124	137	76	92	130

four animals were pooled to obtain larger samples. Blood samples were obtained by means of heparinised "Vacutainer" tubes (Becton-Dickinson, Ltd., Rutherford, N.J.) from the jugular vein of each calf at 0, 1, 2, 3, 5, 7, 14, 24, and 35 days after birth. The lipids were extracted from the plasma by the method of Nelson and Freeman (8) and pooled as above.

The lipid classes of both the skin washings and the plasma then were separated on thin layer chromatoplates of Kieselgel G (E. Merck, Darmstadt, Germany). The major lipid classes of the skin washings were separated on the thin layer chromatoplates using the three stage development system of hexane:ether:acetic acid (80:20:1 v/v/v) to half plate, then full development with hexane:ether (95:5 v/v) followed by full development with pure hexane, as outlined by Nicolaides, et al. (9). The major lipid classes of the plasma were separated using a solvent system of hexane:ether:formic acid (80:20:1 v/v/v). The lipid classes were visualized by spraying with a 0.1% (w/v) solution of 2,4-dichlorofluorescein in methanol followed by exposure under UV light. After identification of the lipid classes, the bands of silica gel were placed in scintered glass funnels. The cholesteryl ester, unesterified fatty acid and triglyceride fractions were eluted with 20 ml diethyl ether and the phospholipids with 20 ml methanol.

The positional distribution of the fatty acids between the 1,3 and 2 positions of the skin surface triglycerides was investigated by hydrolysis with pancreatic lipase according to the method of Luddy, et al. (10) followed by separation of the products on thin layer chromatoplates of Silica Gel G using hexane:ether:formic acid (80:20:1 v/v/v) as a solvent system and elution of the bands with chloroform:methanol (95:5 v/v).

Methyl esters of the fatty acids were pre-

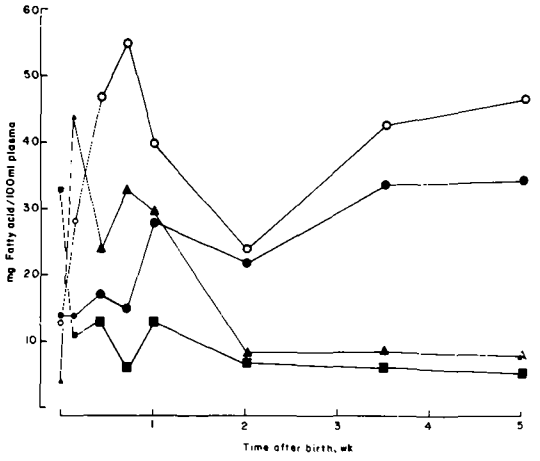

FIG. 2. The concentrations of the major lipid fractions (total fatty acids) in the plasma of the calf after birth. o—o = Phospholipids, •—• = cholesteryl esters, ▲—▲ = triglycerides, and ■—■ = unesterified fatty acids.

pared by transmethylation using 5% hydrochloric acid in dry methanol (11). Gas liquid chromatographic analysis of the fatty acid methyl esters was carried out on a 2 m column of 15% EGSS-X on 100-120 mesh Gas Chrom P fitted to a Perkin Elmer F11 gas chromatograph with dual flame ionization detectors. Appropriate standards were used for identification in all thin layer and gas liquid analyses. Wherever possible, reactions were carried out under nitrogen, and 2,6-di-t-butyl-p-cresol was added to solvents to minimize autoxidation. Solvents were distilled before use.

RESULTS

Fatty Acid Composition of Skin Surface Triglycerides

The concentrations of the major fatty acids in the triglyceride fraction isolated from the skin surface of the calf from the day of birth up to 6 weeks after birth are shown in Figures 1A and B. Results for the fatty acid composition of the triglyceride isolated from the skin surface of the adult animal also are included. It can be seen that, by far, the major proportion of the fatty acids contained in the triglycerides of the skin surface is composed of the saturated fatty acids 14:0, 16:0, and 18:0. At birth, saturated fatty acids constituted over 80% of the total fatty acids present in the triglycerides of the skin surface lipids and were composed of 12:0 (3%), 14:0 (23%), 16:0 (50%), and 18:0 (7%). Within 3 days after birth, the concentration of saturated fatty acids had decreased to ca. 60% of the total fatty acids present and remained at this level for the remaining period of the trial. The decrease in the proportion of the saturated fatty acids between birth and the third day after birth was entirely due to a decrease in the proportion of 14:0 and 16:0. The concentration of 18:0 over the period almost doubled. Up to and including the second week after birth the decrease in the proportion of total saturated fatty acids present in the skin surface triglycerides exhibited immediately after birth was accompanied by a compensatory increase in the proportion of 18:1, the level of which increased from ca. 10% up to ca. 30% of the total fatty acids present. The concentration of 18:2 during this period did not exceed 4%. However, samples of skin surface lipids obtained at the fourth week after birth showed a very marked increase in the proportion of 18:2 Δ *cis*-9. *cis*-12 present which was maintained for the remainder of the experiment; this increase in the proportion of 18:2 was accompanied by a concomittant decrease in the proportion of 18:1. Structural identification of the 18:2 as the Δ *cis*-9, *cis*-12 acid was obtained by a combination of techniques, including partial hydrogenation with hydrazine hydrate and oxidation with potassium permanganate/periodate; this has been fully discussed elsewhere (5). Quantification of the fatty acids contained in the triglyceride fraction by means of an internal standard (12) showed that, at birth, the triglycerides accounted for ca. 15% of the total fatty acids contained in the skin surface extract and that, by 6 weeks after birth, they accounted for ca. 35% of the total fatty acids present.

Composition of Plasma Lipids

The concentrations of the total plasma fatty acids and the concentrations of the fatty acids associated with each of the major lipid fractions of the plasma of the calf during the first five weeks after birth are shown in Table I and Figure 2, respectively. The increase in the concentration of the total plasma fatty acids observed over the complete five week period could be accounted for solely by increases in the concentrations of the cholesteryl ester and phospholipid fractions. By the fifth week after birth, the fatty acids of these 2 fractions comprised some 86% of the total plasma fatty acid concentration. An initial rapid rise in the concentration of the triglyceride fraction was confined to ca. the first week after birth, after which time its concentration returned to a level similar to that observed at birth. The high initial concentration of the unesterified fatty acid fraction decreased rapidly after birth.

The changes in the plasma fatty acid composition of the calf over the first 5 weeks after

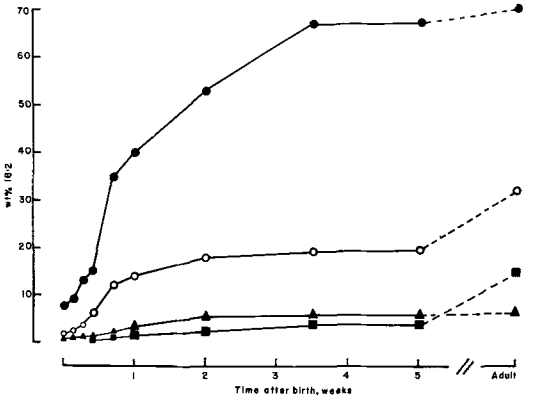

FIG. 3. The concentrations of 18:2 in the major plasma lipids of the calf after birth. ●—● = Cholesteryl esters, ○—○ = phospholipids, ▲—▲ = triglycerides, and ■—■ = unesterified fatty acids.

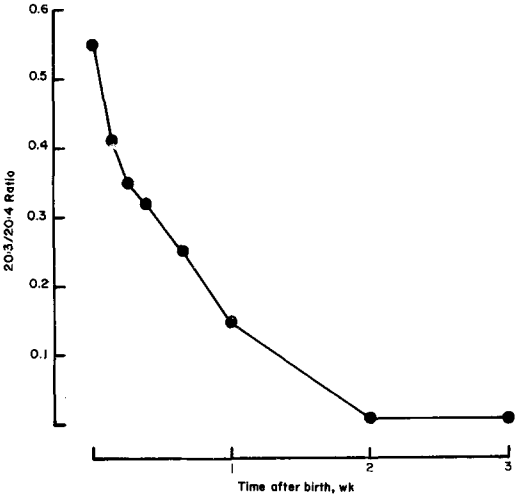

FIG. 4. The ratios of 20:3 (n-9)/20:4 (n-6) in the total plasma lipids of the calf after birth.

birth are given in Table I. During this period, it can be seen that there is a significant increase in the concentration of 18:2 *cis*-9, *cis*-12. Figure 3 shows the distribution of the changes in the concentration of 18:2 between the major plasma lipids obtained from the calves during the 5 weeks after birth. The immediate and marked rise in the concentration of 18:2 circulating in the plasma of the calves was accounted for almost entirely by increases in the 18:2 concentration of the cholesteryl ester and phospholipid fractions. The concentrations of 18:2 in the triglyceride and unesterified fatty acid fractions of the plasma only underwent minimal increases above the concentrations found at birth. Even by the fifth week after birth, the concentration of 18:2 in the triglyceride and unesterified fatty acid fractions was only ca. 5% compared to the levels of 20 and 60%, respectively, for the phospholipid and cholesteryl ester fractions. This distribution of 18:2 between the major plasma lipids is similar to that found in the adult animal (Fig. 3). The changes in the concentration of 18:2 in the plasma immediately after birth were accompanied by concomittant changes in the 20:3/20:4 ratios (Fig. 4).

DISCUSSION

The possible physiological significance of the high concentrations of 18:2 Δ *cis*-9, *cis*-12 in triglycerides present on the skin surface of the ox already has been suggested (5). Although significant concentrations of triglycerides are present in the skin surface lipids of the newborn ox, the present investigations clearly show that it is some 3 weeks after birth before these skin surface triglycerides attain a fatty acid composition similar to that found for the skin surface triglycerides of the adult. The fatty acid composition of the skin surface triglycerides of the ox undergoes two major changes during the period between birth and maturity. Thus, immediately after birth, there is a distinct decrease in the proprotions of the saturated fatty acids with a concomittant increase in the proportion of 18:1. The composition then remains relatively constant for at least 3 weeks after birth during which time the proportion of 18:2 is relatively small. At ca. 3-4 weeks after birth the proportion of the 18:2 then increases to a level similar to that found in the adult (5). This increase in the concentration of 18:2 takes place at the expense of 18:1, the proportion of the other acids remaining relatively constant.

Although the time sequence may be different, these changes in the 18:2 content of the skin surface triglycerides may be related to the changes in the concentration of 18:2 that occur throughout the tissues of the ruminant immediately after birth (2,3). Thus, although very low concentrations of 18:2 were found in the plasma lipids of the calves at birth, there were pronounced increases in the 18:2 contents of the plasma lipids immediately after birth. This conforms with the observations on the changes in the 18:2 metabolism by the neonatal ruminant originally made with lambs (2,3,13) and subsequently confirmed with calves (14).

However, unlike the triglycerides of the skin surface, the triglycerides of the plasma showed only a minimal increase in the concentration of 18:2 above that found at birth; the large increase in the concentration of 18:2 which occurred in the plasma lipids was confined entire-

TABLE II

Composition of Fatty Acids in 1,3 and in 2-Position of Triglycerides from Skin Surface Lipids

Fatty acid	Wt %		% Distribution	
	1,3	2	1,3	2
12:0	2.3	0.6	78.5	21.5
14:0	24.5	12.3	66.6	33.4
16:0	44.5	19.1	69.9	30.1
18:0	18.9	5.5	77.3	22.7
Total saturated	90.2	37.5		
16:1	1.3	2.5	34.7	65.3
18:1	4.5	3.1	59.2	40.8
18:2	4.1	55.7	6.87	93.2
18:3	-	1.2		100
Total unsaturated	9.9	62.5		

ly to the cholesteryl ester and phospholipid fractions.

The low concentrations of 18:2 observed in the plasma lipids of the calves at birth were accompanied by small concentrations of 20:3, 20:3/20:4 ratios of ca. 0.5 were found for the total plasma lipids (Table I and Fig. 4). Although the presence of 20:3 together with 20:3/20:4 ratios greater than 0.4 have been observed in the plasma lipids of the new-born lamb (2), previous observations with calves (13,14) have failed to detect the presence of 20:3 in the total plasma lipids. In the present work, the increase in the concentration of 18:2 that occurred in the plasma lipids of the calves immediately after birth was accompanied by a rapid decrease in the ratio of 20:3/20:4. The significance of these changes in the metabolism of the polyunsaturated fatty acids by the neonatal ruminant has been fully discussed elsewhere (1).

In view of these findings, it is unlikely that the fatty acids that occur in the triglyceride fraction of the skin surface lipids originate directly from those of the plasma triglycerides; similarly, as the unesterified fatty acid fraction of the plasma also contains only small concentrations of 18:2, its participation in the formation of the skin surface triglyceride also may be considered as unlikely. An important role of the cholesteryl esters and phospholipids in the provision of the necessary fatty acids for the formation of the skin surface triglycerides must, therefore, be implicated. Indirect evidence for the possible involvement of the phospholipid fraction may be obtained from investigations into the distribution of the fatty acids in the skin surface triglycerides of the adult ox; hydrolysis by pancreatic lipase has shown that the 18:2 is almost wholly confined to the 2 position (Table II). Although the occurrence of 18:2 in the triglycerides of various ruminant tissues has been shown to be confined mainly to the 2 position (15), its specificity for this position in these tissues would appear to be less than the almost total specificity for position 2 displayed by the skin surface triglycerides. The specificity of 18:2 for the 2 position in ruminant milk fat triglycerides is higher than that for the tissue triglycerides (16); however, its value is still lower than that found in the triglycerides of the skin surface. Although cholesterol esterase activity has been shown to be present in the skin, evidence for cholesteryl ester hydrolase activity in the skin has yet to be demonstrated (17).

Although the precise function of the skin surface lipids in unknown, it is now generally accepted that they afford some protection against the environment, in particular against invasion of the skin surface by bacterial or other exogenous agents; furthermore, the importance of the lipids of the skin surface under certain environmental stresses has been indicated (7). It also has been suggested that the protection afforded by the skin surface lipids depends, not only upon the quantity of material present, but also upon the chemical composition (7). The presence on the skin surface of 18:2 Δ *cis*-9, *cis*-12 with its various metabolic properties, in particular its antibacterial activity (18) and its role in the regulation of moisture loss through the skin (6), is, therefore, of particular importance. Recently, the superior tolerance to heat exposure and disease by Zebu cattle when compared to cattle of European breeds has been correlated with the increased quantities of lipid found on the skin surface of Zebu cattle, and, in this connection, it is interesting to note that this increased tolerance to heat and infection has been correlated with increased bodily levels of 18:2 (19).

The present observations show that it is some 4 weeks after birth before the 18:2 con-

tent of the skin surface lipids reaches the level found in the adult. As the 18:2 in the skin surface lipids may have considerable influence on the ability of the new-born calf to regulate both microbiological infestations of the skin and moisture loss through the skin, it could be that the skin surface lipids have a considerable significance in the ability of the new-born calf to survive under adverse circumstances.

ACKNOWLEDGMENTS

M. Smith supplied the skin surface washings, and F. Campbell provided technical assitance.

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Some Unusual Fatty Acids of *Rhizobium*

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ABSTRACT

A number of unusual fatty acids were identified after isolation from *Rhizobium*. They include 11-methyl-octadec-11-enoic, 12-methoxy-11-methyl- and 11-methoxy-12-methyloctadecanoic, and 11-methoxy- and 13-methoxynonadecanoic acids.

INTRODUCTION

Bacteria are known to contain major proportions of branched chain and other substituted fatty acids not found normally, except in very small amounts, in more highly specialized organisms. Thus, Agre and Cason (1) identified tuberculostearic (10-methyloctadecanoic) acid in the tubercle bacillus. Iso- and anteiso acids were identified in *Bacillus subtilis* by Kaneda (2).

The identity of many more complex branched chain acids of bacterial origin has been described in detail by Asselineau (3). Other frequently found substituted fatty acids are cyclopropane fatty acids, e.g. lactobacillic (4), as well as hydroxy and methyl branched hydroxy acids (3).

While analyzing the fatty acids of a variety of strains of *Rhizobium*, we found a number of gas liquid chromatographic (GLC) peaks, corresponding to 20-30% of the total fatty acids, which defied identification by R_f values. Some of these components did not brominate or hydrogenate and were thought to be branched chain fatty acids. The presence of cyclopropane substituted acids also was suspected. In addition, we found a considerable amount of octadecenoic acid. This often has been described as "oleic," but the existence of oleic acid in bacteria rarely has been confirmed. On the other hand, *cis*-vaccenic acid (*cis*-octadec-11-enoic) has been identified in bacteria by a number of authors (3). We, therefore, also proved the identity of the octadecenoic acid found in *Rhizobium*.

MATERIALS AND METHODS

Bacteria: A culture of *Rhizobium NZP2037* was grown in 100 liters yeast-mannitol-broth (5) containing 0.5% mannitol in a stainless steel fermenter under sterile conditions at 28 C for 40 hr. The culture was stirred and sterile air circulated during incubation. At the end of this period, the cell density was 11.3×10^8/ml. The cells were separated by continuous centrifugation with a Sharples centrifuge (virus rotor type B24) at speeds of $3.0\text{-}3.8 \times 10^4$ revs/min. Ca. 90% of the cells were, thus, recovered. When freeze-dried, the yield was 28.1 g.

Extraction of lipids: The dry cells were resuspended in 100 ml water and the lipids extracted from 5 equal aliquots with 10 volumes 2:1 chloroform-methanol after sonic disintegration (3 times for 2 min) with a 100 watt measuring and scientific equipment sonicator. The total lipid yield was 3.27 g (11.6%). Poly-β-hydroxybutyrate was removed from the

TABLE I

Gas chromatographic Data of Unknown Fatty Acids and Their Composition (% wt) in Urea Nonadduct Obtained after Hydrogenation

	Equivalent chain length of methyl esters[a]			
Unknown	EGSS-X (185 C)	EGA (200 C)	Percent	Subsequently identified as:
1	14.56	14.66	28.7	12-Methyltetradecanoic acid
2	18.22[b]	18.20[b]	21.4	11-Methyloctadec-11-enoic acid
3	19.65	19.47	21.0	*cis*-11, 12-Methyleneoctadecanoic acid
4	21.00	20.69	12.7	12-Methoxy-11-methyl- and 11-methoxy-12-methyloctadecanoic acid
5	21.90	21.57	4.3	11-Methoxynonadecanoic and 13-methoxynonadecanoic acid
			88.1	

[a]EGSS-X = ethylene glycol succinate methyl silicone polymer and EGA = ethylene glycol adipate.
[b]Equivalent chain length of unhydrogenated fatty acid.

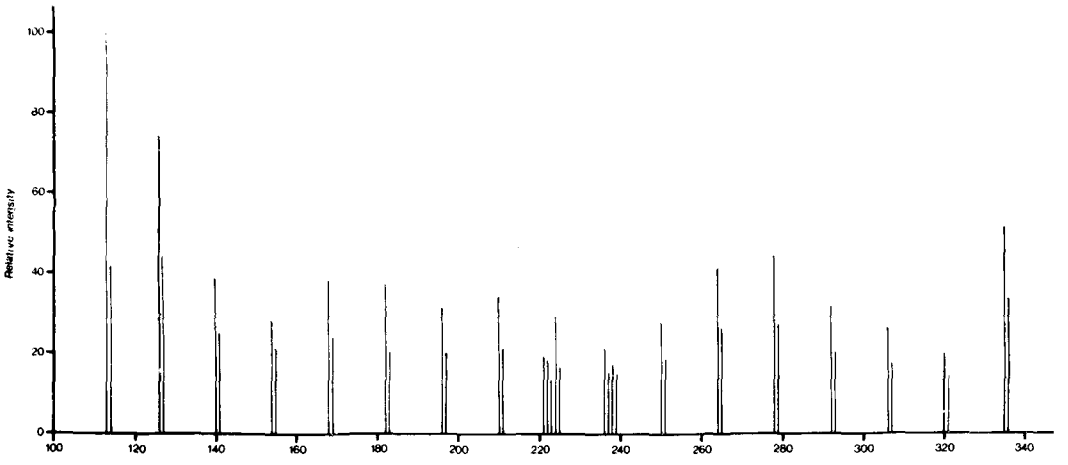


FIG. 1. Mass spectrum of the pyrrolidide of vaccenic acid (n-octadec-11-enoylpyrrolidine).

lipid by precipitation with diethyl ether from chloroform solution, using 10 vol ether. This purification was repeated twice leaving an ether-soluble residue of 833 mg (25.5%). This residue was saponified with N/2 NaOH in ethanol, and the nonsaponifiables were removed with ether. The aqueous solution then was acidified, and the fatty acids recovered and converted to methyl esters with BCl_3-methanol complex (Brian, et al., [6]). The yield of methyl esters was 388 mg.

Isolation of unknown acids: It had been found previously that most of the unknown components did not change their retention times on GLC after hydrogenation by the micromethod of Appelqvist (7). Half the available methyl esters were, therefore, hydrogenated in a Parr bomb in methanol at 20 C under 3 atmospheres using PtO_2 as catalyst. Completion of the reaction was confirmed by GLC.

The hydrogenated methyl esters were fractionated into straight and branched chain fractions by urea adduct formation using 6 ml saturated urea in methanol at 20 C, as described by Ackman and Hooper (8). The nonadduct was collected and the adduct treated twice more with 2 ml urea solution. All nonadducts were combined. Most of the methyl stearate still present was removed by a final urea adduct precipitation from 1 ml solution. The final yield of nonadduct methyl esters was 21%.

GLC of methyl esters: The mixture of unknown fatty acids was analyzed with an F&M (model 5750) gas chromatograph fitted with flame ionization detectors. The column used was 2 m x 2.3 mm inside diameter stainless steel packed with 5% ethylene glycol succinate methyl silicone polymer (EGSS-X)

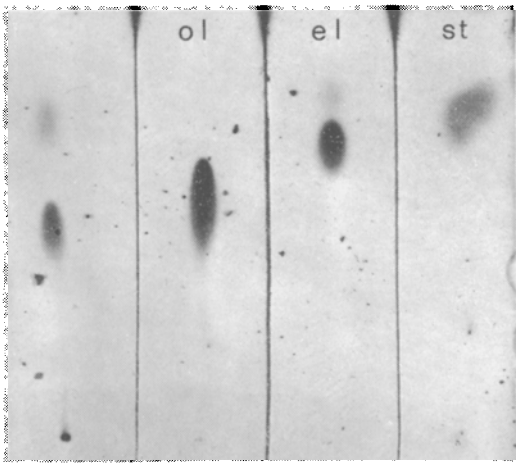


FIG. 2. Argentation thin layer chromatogram of recovered methyl vaccenate with authentic methyl oleate (*cis*), methyl elaidate (*trans*), and methyl stearate. ol = Oleate, el = elaidate, and st = stearate.

coated on 100-120 mesh Chromosorb W (acid washed, silanized). N_2 was used as carrier gas at 12 ml/min, and the temperature was 185 C.

Preparative GLC was carried out with the same instrument fitted with a stream splitter and a 2 m x 4 mm inside diameter aluminium column packed with 100-120 mesh Chromosorb W coated with 40% (w/w) ethylene glycol adipate (EGA). N_2 (25 ml/min) was the carrier gas, and the separation was carried out at 200 C. The fractions were collected as described by Schlenk and Sand (9) using repeated injections.

Pyrrolidides and methoxy derivatives: Pyrrolidides and methoxy derivatives of monoenoic acids were prepared by the methods of Andersson and Holman (10) and Minnikin, et al.,

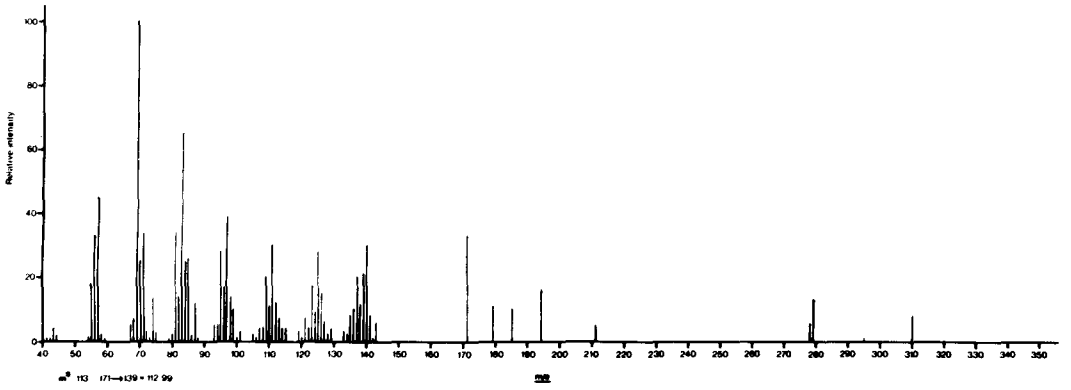


FIG. 3. Mass spectrum of *unknown 2* identified as methyl 11-methylvaccenate (methyl 11-methyloctadec-11-enoate).

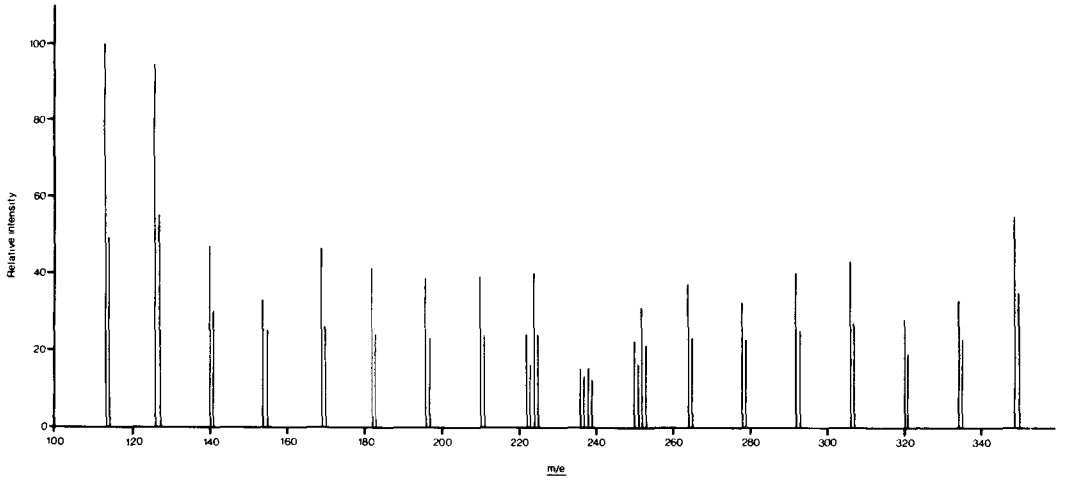


FIG. 4. Mass spectrum of the pyrrolidide of *unknown 2* (11-methyloctadec-11-enoylpyrrolidine).

(11) respectively.

Mass spectrometry: The mass spectrometer used was an AEI MS 30 double beam instrument coupled via a membrane separator to a PYE 104 gas chromatograph. The latter was provided with the analytical column described above and He was used as carrier gas. The spectra were run at 70 EV.

Proton magnetic resonsance (PMR) spectra: Spectra were obtained with a Varian T-60 spectrometer. They were determined in CCl_4 using trimethylsilyl (TMS) as reference compound.

IR spectra: Spectra were determined in a Perkin Elmer (model 137) spectrometer with CCl_4 as solvent.

RESULTS AND DISCUSSION

The urea nonadduct was analyzed by GLC, and the composition and GLC data are summarized in Table I. Octadecenoic and the unknown fatty acids were identified as described below.

Octadecenoic acid (18:1): The compound, identified by its gas chromatographic behavior as a methyl octadecenoate, gave a mass spectrum identical to that of an authentic sample of methyl oleate. The position of the double bond could not, however, be inferred from this, as positional isomers of unsaturated esters may give similar mass spectra (12). It recently was shown (10) that the position of the double bond can be deduced by examination of the mass spectra of pyrrolidides of unsaturated fatty acids. A sample of 18:1 was, therefore, converted to its pyrrolidide and its mass spectrum determined (Fig. 1). Application of the empirical rule formulated by Andersson and Holman (10) establishes the position of the double bond as between carbons 11-12.

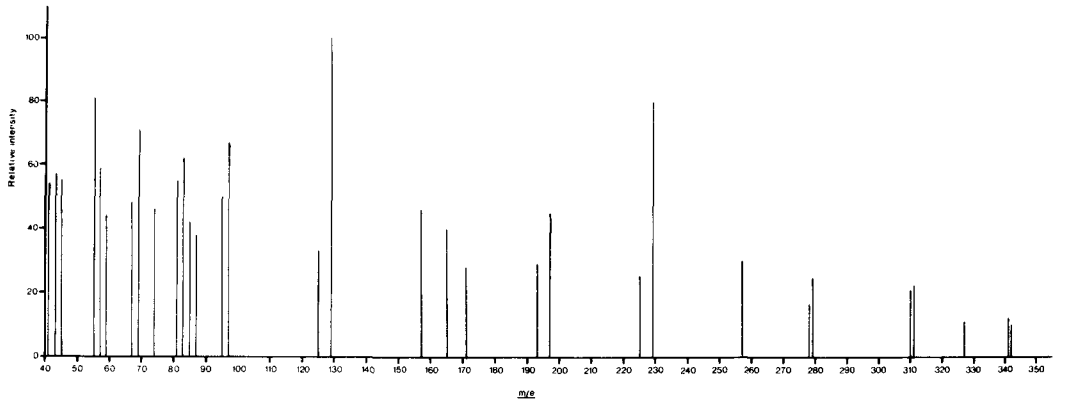


FIG. 5. Mass spectrum of *unknown 4*, identified as mixed methyl 12-methoxy-11-methyl- and 11-methoxy-12-methylstearates.

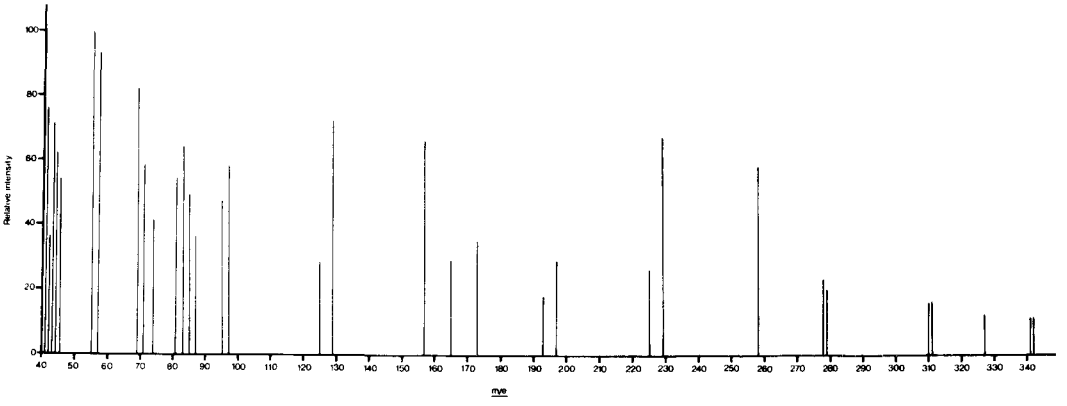


FIG. 6. Mass spectrum of *unknown 5*, identified as mixed methyl 11- and 13-methoxy-nonadecanoates.

Argentation chromatography (13) established the configuration of the double bond as *cis* (Fig. 2). The compound is, therefore, methyl *cis*-vaccenate.

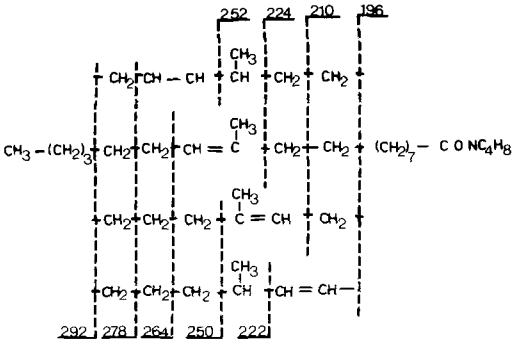
Unknown 1: This was identified as 12-methyltetradecanoate by comparing the mass spectrum of its ester with that of an authentic sample.

Unknown 2: This material was resistant to hydrogenation under conditions used (7) but was reduced with PtO_2 under 3 atmospheres H_2. Furthermore, this compound could not be brominated under the conditions which caused bromination of the cyclopropane ring of lactobacillic acid. Its mass spectrum (Fig. 3) showed a molecular ion at m/e 310, together with peaks at m/e 279, 278 (loss of OCH_3, CH_3OH), and 194 (loss of 116 atomic mass units), suggesting a nonadecenoate. The mass spectrum of the reduced material showed a molecular ion at m/e 312 with ions at m/e 297 (M^+-CH_3), 283 ($M^+-C_2H_5$), 281 (M^+-OCH_3), and 269 ($M^+-C_3H_7$) establishing the product to be a methyl nonadecanoate. In the lower mass region, this spectrum was identical to that of methyl 11-methyloctadecanoate (14) with enhanced peaks at m/e 213 ($^+CH(CH_3)-(CH_2)_9-CO_2CH_3$), 181 ($213-CH_3OH$), 163 ($213-CH_3OH-H_2O$), 185, 186, and 187 ($^+(CH_2)_9CO_2CH_3$ + 0, 1, 2, H), thus showing the existence of a methyl branch at carbon 11.

To locate the double bond, the unsaturated methyl ester (unknown 2) was converted to its pyrrolidide and its mass spectrum recorded (Fig. 4). It is interesting to note the ease with which, in contrast to the case of the original methyl ester, the existence of an 11-methyl branch also can be deduced from this spectrum from the enhanced C_{10} and C_{12} fragments and diminished C_{11} fragment (analogously to the well established case for branched saturated methyl esters).

The position of the double bond can be

established as being between carbons 11-12 as follows:

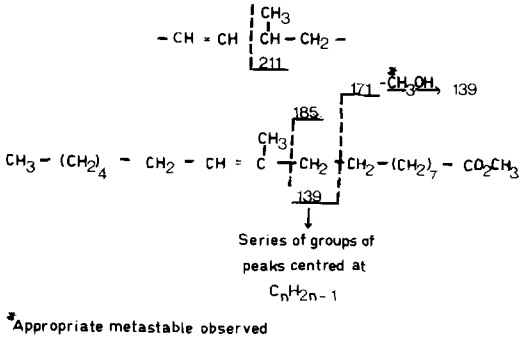


Scheme 1

Fragmentation of the pyrrolidide of *unknown 2* (11-methyloctadec-11-enoylpyrrolidine).

Direct fragmentation of the unrearranged pyrrolidide leads to the formation of intense ions at m/e 224, 210, 196... and 264, 278, 292.... Fragmentation, subsequent to double bond migration toward the nonpolar end of the molecule, leads to the fragment of m/e 252. Fragmentation subsequent to double bond migration toward the polar end of the molecule leads to the formation of ions of m/e 250 and 222. (Note that ions of m/e 224, 252, 250, and 222 are formed by cleavage of bonds to tertiary carbon atoms and so would be expected to have enhanced intensities.)

This formulation is consistent with the greater difficulty of hydrogenation observed (trisubstituted double bond) and allows rationalization of the observed mass spectrum of the original compound (Fig. 3), assuming in this instance that fragmentation, being facilitated by the methyl branch, occurs with little double bond migration, as shown below:



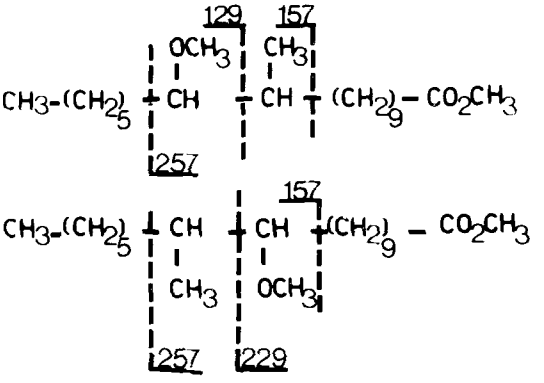
*Appropriate metastable observed

Scheme 2

Fragmentation of methyl 11-methylvaccenate (methyl 11-methyloctadec-11-enoate).

Unknown 3: The identity of lactobacillic acid was established by comparing the equivalent chain length on EGSS-X and the mass spectrum with those of an authentic sample. Hydrogenation by the method of Appelqvist (7) did not affect the acid, but bromination caused its disappearance from the gas chromatogram. The configuration about the cyclopropane ring was established as *cis* by the PMR spectrum (15) which showed a peak at 0.31 ppm upfield of TMS and one at 0.64 ppm downfield of TMS.

Unknowns 4 and 5: The mass spectra of unknowns 4 and 5 were very similar (Figs. 5 and 6). Both showed molecular ions at m/e 342 with prominent ions at 341 (M^+-1), 327 (M^+-CH_3), 311, 310 (M^+-OCH_3, CH_3OH), 279, and 278 ($M^+-CH_3OH-OCH_3$, CH_3OH) identifying them as methoxy nonadecanoates (16). This was confirmed by the PMR spectra which showed the presence of a second OCH_3 resonance (at 3.26, 3.23δ in unknowns 4 and 5, respectively) upfield of the carbomethoxy OCH_3 (at 3.62, 3.60δ) and by a carbon-oxygen stretching band in the IR spectrum (1095 cm^{-1}). The lower mass portions of the mass spectra indicate that both unknowns 4 and 5 are, in fact, mixtures.

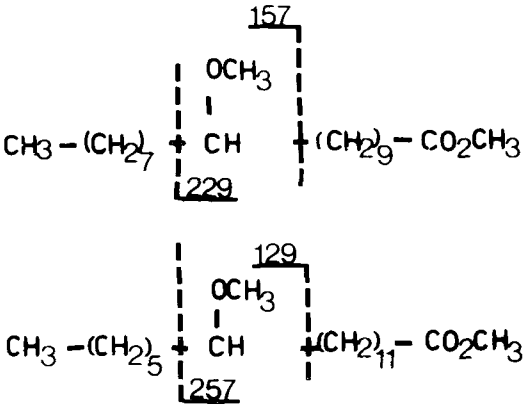


Scheme 3

Fragmentation of methyl 12-methoxy-11-methyl- and 11-methoxy-12-methylstearates.

Unknown 4: The peaks at m/e 257, 229, and 157 are consistent with the structure methyl 11-methoxy-12-methylstearate. The peaks at 257, 129, and 157 are consistent with the structure methyl 12-methoxy-11-methylstearate. These compounds would not be separable under the GLC condition employed.

Unknown 5 (Scheme 4): Strong peaks at m/e 229, 157 are consistent with the structure of methyl 11-methoxynonadecanoate. The peaks at 129, 257 indicate the structure methyl 13-methoxynonadecanoate. In addition, the equivalent chain length with EGSS-X and EGA as liquid



Scheme 4

Fragmentation of methyl 11- and 13-methoxy-nonadecanoates.

phases differed by close to one carbon unit from those of synthetic methoxyoctadecanoate prepared from methyl vaccenate. The equivalent chain length of the synthetic acid was 21.00 on EGSS-X and 20.70 on EGA. Unknown acids 2, 4, and 5 appear to be related closely to lactobacillic. To ensure that they were not artifacts, the conclusion of Brian, et al., (6) that lactobacillic acid was converted quantitatively to its methyl ester with BCl_3-methanol complex was checked with an authentic sample. In addition 12-hydroxystearic acid was reacted with BCl_3-methanol reagent, and no artifacts were detected by GLC. Finally, it should be pointed out that we also have found these acids in the free fatty acids which constitute a major proportion of the lipids of *Rhizobium NZP 2037* and which previously had been isolated and converted directly to methyl esters with diazomethane and in naturally occurring methyl esters from the same organism.

We consider that 11-methyl-octadecenoate, 12-methoxy-11-methyl- and 11-methoxy-12-methylstearate, 11-methoxy nonadecanoate, and 13-methoxy nonadecanoate are the expected products of ring opening of lactobacillic acid with OH^- (or a derivative thereof) as the e^- donor and subsequent methylation. 11-Methyloctadecenoate could be formed by elimination from the intermediate which led to the formation of 12-methoxy-11-methylstearate.

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Dealkylation of 24-Ethylsterols by *Tetrahymena pyriformis*

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ABSTRACT

When *Tetrahymena pyriformis* was incubated with sitosterol ([24R]-24-ethylcholest-5-en-3 β-ol]) or its *trans*-Δ^{22}-derivative (stigmasterol), the C-24-dealkylated product, cholesta-5,7,*trans*-22-trien-3 β-ol, was obtained in both cases. 24(S)-24-Ethylcholesta-5,7,*trans*-22-trien-3 β-ol also was found to be a metabolite. When sitosterol was the substrate, 24(R)-24-ethylcholesta-5,7-dien-3 β-ol was obtained as a third product. Identifications were made by mass spectroscopy, quantitative chromatography, labeling with ^{14}C, and by other means. The dealkylated product (cholestratrienol) represented 30% of the sterols isolable after incubation. The administration of sterols to this organism did not induce sterol biosynthesis, since 2-^{14}C-mevalonate failed to yield radioactive sterol in the presence of added stigmasterol.

INTRODUCTION

Organisms with a clearly defined nervous system either directly biosynthesize sterols lacking a substituent at C-24, or they dealkylate ingested 24-alkylsterols to give, for instance, cholesterol from sitosterol. Carnivorous animals may lack the dealkylation mechanism, even if the capacity for sterol biosynthesis is absent. A more thorough discussion both of this subject and of the biological role of sterols is provided by Nes (1). The most thoroughly examined cases of dealkylation are with arthropods (2-5) in which metabolism appears to proceed by reversal (6-12) of the alkylation process found in plants (13-25). In its simplest form, the plant route begins with a Δ^{24}-sterol which yields a $\Delta^{24(28)}$-24-substituted sterol which is then reduced at the $\Delta^{24(28)}$-bond to the 24-alkylsterol, e.g. desmosterol to 24-methylenecholesterol to 24-methylcholesterol, if we neglect the problems of sequencing with other events. The introduction of the 24-methylene group actually occurs in the examined cases immediately after cyclization of squalene oxide to give 24-methylenecycloartenol or 24-methylene-24,25-dihydrolanosterol. The exact place in the sequence at which reduction or a second C_1-transfer occurs is not entirely certain. However, the sterols given (desmosterol, etc.) will proceed experimentally through the sequence (15,16). A second alkylation at the $\Delta^{24(28)}$-stage yields a 24-ethylidenesterol, e.g. isofucosterol, which, on reduction, gives the common 24-ethylsterols. In insects, both 24-methylenecholesterol and desmosterol have been identified as intermediates along with 24-ethylidinecholesterol in the conversion of sitosterol to cholesterol (6-12). In the protozoan, *Tetrahymena pyriformis*, however, which, like insects, lacks the capacity to biosynthesize sterols de novo (26,27), neither 24-methylene nor 24-ethylidene derivatives of cholesterol were dealkylated (28). This was surprising in view of preliminary evidence that sitosterol is converted by the organism to cholestatrienol (29). Since the fact and mechanism of dealkylation bear strongly both upon the role of sterols in living systems and upon their phylogeny (1), absolute proof for dealkylation in *T. pyriformis* was undertaken. Proof was obtained both with labeled and unlabeled substrates indicating that *T. pyriformis* accomplishes the dealkylation in a different manner than do insects. This may mean that the ciliated protozoal group represented by *T. pyriformis* is phylogenetically divergent from the line leading to arthropods.

Nomenclature used in this paper is: cholesterol, cholest-5-en-3 β-ol; sitosterol, (24R)-24-ethylcholest-5-en-3 β-ol; campesterol, (24R)-24-methylcholest-5-en-3 β-ol; stigmasterol, (24S)-24-ethylcholesta-5, *trans*-22-dien-3 β-ol; stigmastatrienol, (24S)-24-ethylcholesta-5,7, *trans*-22-trien-3 β-ol; cholestatrienol, cholesta-5,7, *trans*-22-trien-3 β-ol; 24-methylenecholesterol, 24-methylcholesta-5,24(28)-dien-3 β-ol; fucosterol, 24-ethylcholesta-5, *cis*-24(28)-dien-3 β-ol; and isofucosterol, 24-ethylcholesta-5, *trans*-24(28)-dien-3 β-ol; desmosterol, cholesta-5,24-dien-3 β-ol. It is worth noting that, while, as a result of the Sequence Rule, the designation for the same absolute configuration at C-24 is inverted in sterols saturated at C-22,23 compared to their corresponding Δ^{22}-derivatives, all of the 24-alkylsterols used in the investigation possessed the α-configuration at C-24. By the Sequence Rule, this is designated R in the saturated cases and S in the Δ^{22}-derivatives.

EXPERIMENTAL PROCEDURE

Materials and Methods

Commercial stigmasterol from soybeans was purified by crystallization and adsorption chromatography. Examination by gas liquid chromatography (GLC) and by mass spectroscopy showed the sample used for incubation had the assigned structure and consisted of a single component. [4-^{14}C]-Sitosterol was purchased from Amersham-Searle Corp., (Des Plaines, Ill.) and had a specific activity of 1.1 x 10^8 dpm/mg. It was diluted with pure sitosterol. The sample (5.62 x 10^5 dpm/mg) used for incubations showed a single peak of radioactivity when submitted to GLC. When it was submitted to thin layer chromatography (TLC) in benezne-ether (8:2, v/v) and the plate was scanned for radioactivity, a single peak was obtained. [2-^{14}C]-Mevalonate (as the dibenzylethylene-diamine salt) was purchased from New England Nuclear Corp., (Boston, Mass.). Incubations were performed as previously described (28,30) with *T. pyriformis*, Type W, at 28 C for 40 hr in several 500 ml flasks each containing culture medium and ca. 3 mg sterol. The culture medium was composed of distilled water containing 2% (w/v) of yeast extract (Difco), and it was 9 x 10^{-5}M in iron-ethylenediaminetetraacetic complex. The substrates were added to the culture medium in a solution of ethanol. Cell counts showed that, after incubation, there were ca. 2 x 10^8 cells/flask. The cells were separated from the medium by centrifugation, and, unless otherwise noted, data are derived from the cells which were lyophilized prior to continuous extraction with acetone in a Soxhlet apparatus. The acetone extract contained ca. 110 mg/incubation flask. Phospholipids were precipitated from a chloroform solution (1 ml) of the latter material at -20 C by the addition of 20 ml acetone. The remaining lipid (70 mg) was saponified under nitrogen in 25 ml of 10% KOH in ethanol at room temperature for 16 hr. This yielded 20 mg neutral lipid containing tetrahymanol, sterol, and other substances. The sterol was isolated by chromatography on a thin layer of silica gel developed with benzene-ether (8:2, v/v). After acetylation at room temperature with acetic anhydride in pyridine, the sterol was submitted to chromatography on a thin layer of silica gel impregnated with 10% (w/w) of silver nitrate. A single development with chloroform-ligroin-acetate (100:40:0.75, v/v) separated Δ^5-, $\Delta^{5,22}$-, $\Delta^{5,7}$-, and $\Delta^{5,7,22}$-sterols from each other with increasing rates of movement being in inverse order to that given for the double bond structure. In addition, Δ^{22}-sterols lacking a substituent at C-24 moved slower than those with a substituent. The result of these correlations is that cholestatrienol had the slowest rate of movement of any of the compounds and monoenic Δ^5-sterols the fastest. GLC was performed at 235 C with 0.94% of silanized XE-60 (Analabs, North Haven, Conn.) on Chromosorb W with helium as the carrier gas. The instrument used was equipped with a stream splitter allowing 90% of the effluent to pass through a proportional counter for radioactivity and 10% through a flame ionization detector for mass. Mass and radioactivity were recorded on separate strip charts, and retention times for the mass and radioactive peaks consequently were obtained simultaneously but individually. When free sterols were used, labeled cholesteryl acetate served as the standard. Mass spectroscopy was performed by Morgan-Schaffer Corp., Montreal, Canada. For further information on instrumentation, as well as on standard sterols and thier physical constants, our earlier publications (e.g. 16, 17, 28, 30, 31, and references cited therein) should be consulted.

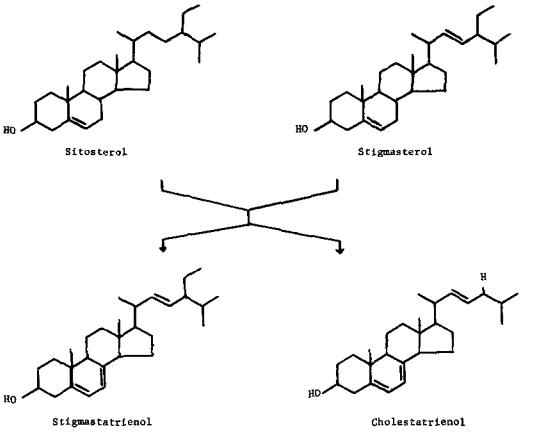
RESULTS

After incubation with stigmasterol, cellular neutral lipids were separated by TLC on silica gel into fractions representing pentacyclic triterpenoids, sterols, and other lipids. The sterol fraction was acetylated and further resolved by argentation TLC. Three bands were observed with R_f values of 0.42 (band 1), 0.16 (band 2), and 0.08 (band 3). Band 1 corresponded to the position for 24-ethyl-$\Delta^{5,22}$-steryl acetates, and GLC showed the eluted material contained a substance with a relative retention time of 1.39. Authentic stigmasteryl acetate had a relative retention time of 1.38 and band 1 must have represented a small amount of unchanged substrate.

Bands 2 and 3, which represented most of the material, had R_f values expected of $\Delta^{5,7,22}$-trienic steryl acetates with and without a 24-ethyl group, respectively. GLC of the eluted materials showed each to be composed of essentially single components with relative retention times of 1.61 and 1.07, respectively. The latter is the value of authentic cholestatrienyl acetate. Authentic stigmastatrienyl acetate was not available, but a calculated relative retention time can be obtained by multiplying the value for cholestatrienyl acetate by the increment (sitosterol/cholesterol) for the 24-ethyl group (1.56). The calculated value is 1.67 which is within experimental error of the observed value. UV spectroscopy showed that both sterols had the 3 characteristic absorption

peaks for $\Delta^{5,7}$-sterols at 272, 283, and 294 nm with a shoulder at 263 nm. These structural assignments were confirmed by mass spectroscopy. Stigmastatrienyl acetate (MW 452) from band 2 showed a molecular ion at m/e 452 of very low intensity with a strong peak for M^+ less acetic acid at m/e 392. A peak of moderate intensity at m/e 253 corresponded to M^+ less acetic acid less the 139 mass units for the $C_{10}H_{19}$-side chain, as would be expected for allylic cleavage at the C-17,20-bond brought about by the Δ^{22}-bond. Moderate peaks at m/e 143 and 157 were consistent with fragments consisting of rings A and B lost due to cleavages through ring C induced by the $\Delta^{5,7}$-system (32). The spectrum, in fact, was substantially the same as that of ergosteryl acetate, except for peaks of ions which retain the side chain, e.g. M^+ less acetic acid and M^+ less acetic acid less methyl. The spectrum of the sterol from band 3 confirmed that it was the corresponding 24-dealkylated compound (cholestatrienyl acetate, MW 424). A peak of low intensity appeared at m/e 424 for M^+ and a strong one at m/e 364 for M^+ less acetic acid. Allylic loss of the side chain and fragmentation through ring C due to the $\Delta^{5,7}$-system were evident by moderate peaks at m/e 253, 157, and 143.

Since sterol biosynthesis conceivably could have been induced by incubation with stigmasterol, the cholestatrienol could have arisen de novo. This was contraindicated by an incubation with [2-^{14}C]-mevalonate in the presence of stigmasterol. The neutral lipids were chromatographed on a thin layer of silica gel as previously, and the plate was scanned for radioactivity. A strong peak appeared in the triterpenoid region for tetrahymanol but none in the sterol region. Direct evidence for dealkylation then was obtained with a labeled 24-ethylsterol. Radioactive stigmasterol was not available, but [4-^{14}C]-sitosterol was. In neither the labeled sitosterol nor the unlabeled stigmasterol was it possible to detect cholesterol. Incubation of the [4-^{14}C]-sitosterol (8.4 x 10^6 dpm) led to the isolation of 5.2 x 10^6 dpm in the total lipid extract of the cells. The remaining label appeared in the medium. After separation into phospholipids and other lipids, the former contained 2 x 10^4 dpm (probably as contamination) and the latter 5.1 x 10^6 dpm. The other lipids were saponified leading to 4.8 x 10^6 dpm in the neutral fraction. After acetylation, an aliquot was chromatographed on a thin layer of silica gel impregnated with silver nitrate as previously. The regions corresponding to Δ^5-steryl and $\Delta^{5,22}$-24-ethylsteryl acetates (band 1), the region in between the latter and that corresponding to triene (band 2), the region corresponding to stigmastatrienyl acetate (band 3), the region (band 4) in between the latter and the one next, and the region corresponding to cholestatrienyl acetate (band 5) were eluted and submitted to scintillation counting. The respective results were 15,900; 36,335; 506,000; 70,000; and 381,000 dpm. In another experiment, an aliquot (1.4 x 10^6 dpm) of the neutral lipids after argentation chromatography led to 40,000; 43,000; 590,000; not measured; and 418,000 dpm, respectively, in the five bands. The substance in band 1 was submitted to GLC with continuous counting of the effluent. A single peak with the retention time of sitosterol was observed on the radioactive recorder. Similar examination of band 4 showed two radioactive products. One had a retention time (1.61) the same as stigmastatrienyl acetate. The other, with a somewhat longer retention time (1.84), probably was the 22,23-dihydro derivative derived from 7,8-dehydrogenation as the only metabolic step. The calculated value for the retention time is 1.89 (1.61 divided by 0.85 which is the contribution for introduction of the Δ^{22}-bond). The material from band 5 showed single peaks of mass and radioactivity which were coincident and possessed the retention time of cholestatrienyl acetate (1.08 realtive to cholesteryl acetate) proving that the dealkylated product was derived from the alkylated substrate.



Scheme 1

Metabolism in *T. pyriformis*

DISCUSSION

The combination of the mass spectral, chromatographic, and labeling data can leave little doubt that a sterol lacking the alkyl group at C-24 was formed after incubation of a 24-ethylsterol with *T. pyriformis*. In addition, as ex-

pected (28-30), dehydrogenation occurred at C-7,8 and C-22,23. The sequence of events is uncertain. Among the alternatives which are consistent with the available information are (A) sitosterol to 7-dehydrositosterol to 7,22-bisdehydrositosterol (stigmastatrienol) to 7,22-bisdehydrocholesterol (cholestatrienol) corresponding to sequential introduction of the Δ^7-bond, the Δ^{22}-bond, and reductive dealkylation at C-24, and (B) sitosterol to 22-dehydrositosterol (stigmasterol) to 7,22-bisdehydrositosterol and than as in alternative (A) corresponding to introduction of the Δ^7-bond after introduction of the Δ^{22}-bond. It remains for further work to elucidate how the reductive dealkylation occurs and what, if any, influence the Δ^{22}-bond has upon it. It is improbable that $\Delta^{24(28)}$- or $\Delta^{22,24(28)}$-intermediates are involved, since 24-methylenecholesterol and isofucosterol both lead to their $\Delta^{5,7,22,24(28)}$-tetraenic derivatives without formation of cholestatrienol and since fucosterol leads to its $\Delta^{5,7,24(28)}$-trienic derivative without formation of cholestatrienol (28). In the fucosterol case, steric hindrance inhibits introduction of the Δ^{22}-bond (28).

The failure of the $\Delta^{24(28)}$-substrates to undergo dealkylation differentiates dealkylation in *T. pyriformis* from dealkylation in insects (6-12) and presumably from arthropods in general. This, together with the presence in *T. pyriformis* of the 22,23-dehydrogenase which also has not been identified in animals, may place this organism outside of a direct phylogenetic line to the animal kingdom.

It is interesting to note that the only organisms, other than some species of *Tetrahymena* which do not biosynthesize sterols, are nonphotosynthetic prokaryotes (bacteria [33,34] and mycoplasmas [35]) which are presumably insufficiently evolved and two groups of eukaryotes (arthropods and two fungal genera [36-38]). Animals phylogenetically lower than arthropods, e.g. marine annelids (39), do biosynthesize sterols, and, in the fungal case, all other examined genera of fungi do so. This information tentatively is interpreted to mean that sterol biosynthesis was lost rather than never possessed by the divergent eukaryotes, but, both in the animal (40) and in the plant (38,41,42) examples, sterol must be provided from an exogenous source for completion of the life cycle. *T. pyriformis* and some of its close relatives (43) are, thus, the only eukaryotic forms of life known which fail to biosynthesize sterols and still require none (tetrahymanol acting in the sterol's role) which places this organismic type vertically in the hierarchy somewhere in between the bacterial and mycoplasmic prokaroytes and the lower eukaryotes which possess the sterol pathway. In a horizontal direction, *T. pyriformis* stands also in a median position between plants and animals (44). Its preference for dealkylated sterols, evidenced by the conversion of sitosterol and stigmasterol to cholestatrienol, is an animal-like quality, but 22,23-dehydrogenation is a plant-like quality. The failure to reduce a $\Delta^{5,7}$-diene to a Δ^5-monoene also is found among nonphotosynthetic plants, notably fungi, rather than among animals.

ACKNOWLEDGMENTS

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Positional Analysis of Isovaleroyl Triglycerides Using Proton Magnetic Resonance with $Eu(fod)_3$ and $Pr(fod)_3$ Shift Reagents: I. Model Compounds

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ABSTRACT

The proton magnetic resonance spectra of isomeric triglycerides of isovaleric and palmitic acids in the presence of the downfield and upfield chemical shift reagents $Eu(fod)_3$ and $Pr(fod)_3$ were studied. The resonance profiles of the γ-protons on the isovaleroyl chains are distinctive for each of the four possible triglyceride isomers at low shift reagent/triglyceride ratios. With either reagent, the well defined γ-methyl isovalerate doublets can be used to identify 2-isovaleroyl and 1,3-isovaleroyl structures and to analyze isomeric mixtures. This technique will be useful for positional analysis of natural cetacean triglycerides containing isovaleric acid.

INTRODUCTION

Early applications of proton magnetic resonance (PMR) to the study of lipid structure were fairly limited in scope, since the signals resolved represented only a small fraction of the total proton population (1). However, the recent advent of lanthanide chemical shift reagents now has provided NMR chemists with greatly improved resolution of such signals, so that considerable additional structural information now can be obtained from the PMR spectra of fatty acid methyl esters (2,3) and triglycerides (4-6).

Characterization of the unusual isovaleroyl lipids found in dolphin, porpoise, and toothed whale fats has interested this group (7) and other laboratories (8,9) in recent years. The discovery by Pfeffer and Rothbart (4) that PMR with chemical shift reagents can differentiate 2-oleo-1,3-dipalmitin from *rac*-1-oleo-2,3-dipalmitin raises the possibility that positional analysis of mixed-acid, isovaleroyl, cetacean triglycerides also might be possible using a similar approach. Therefore, we have carried out detailed PMR structural studies on various synthetic isovaleroyl triglycerides and their appropriate mixtures. Our results with both the downfield shift reagent $Eu(fod)_3$ and the upfield shift reagent $Pr(fod)_3$ demonstrate that the positional isomers of mixed-acid isovaleroyl triglycerides can be identified positively by the characteristic resonance signals of their γ-isovalerate protons and that analysis of isomeric mixtures by PMR is quite feasible.

EXPERIMENTAL PROCEDURES

Synthesis of Isovaleroyl Triglycerides

The anhydrides of isovaleric acid (J.T. Baker Chemical Co., Phillipsburg, N.J.) and palmitic acid (Lachat Chemicals, Chicago, Ill.) were pre-

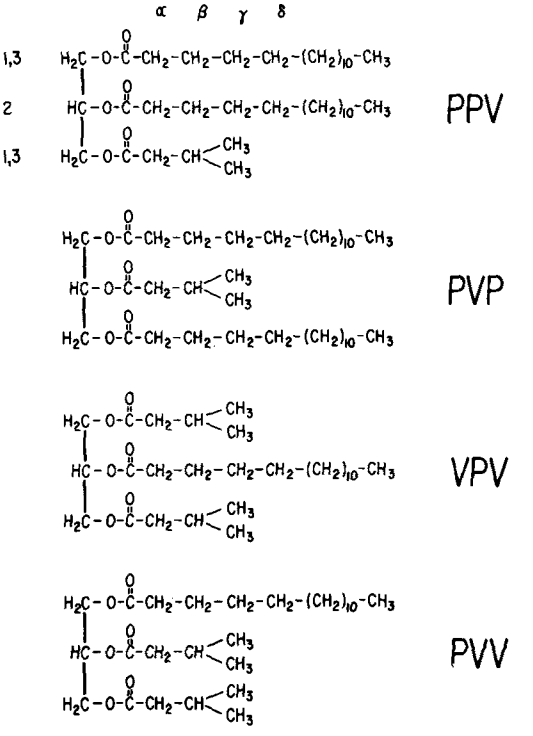

FIG. 1. Structural formulas and nomenclature for the synthetic monoisovaleroyl (PVP and PPV) and diisovaleroyl (VPV and PVV) triglyceride positional isomers used in this study. The usual numerical nomenclature is employed for positions on the glycerol moiety, while the Greek letters α, β, γ, and δ designate the carbon positions on the fatty acid chains. The PPV and PVV preparations studied were racemic mixtures, but only one enantiomer has been drawn here.

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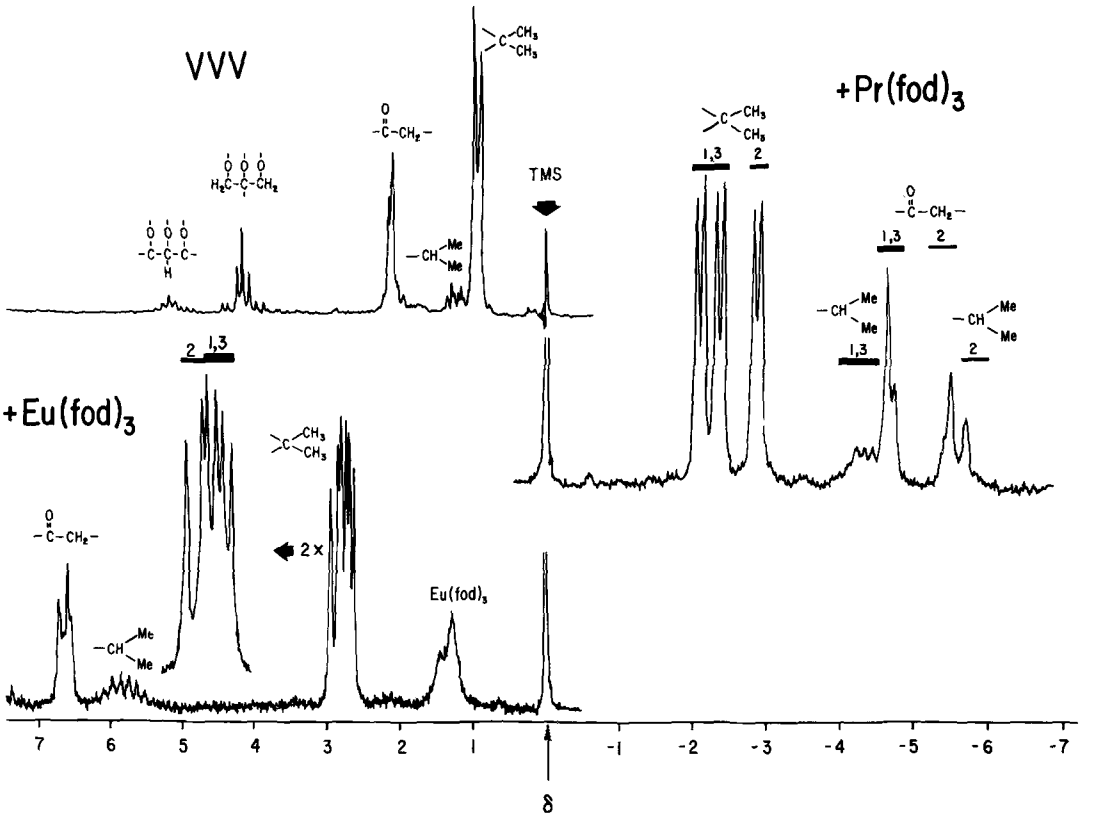

FIG. 2. (*Top*) 60 MHz Proton magnetic resonance spectrum of triisovalerin (VVV) in CCl_4. (*Middle*) Proton spectrum of VVV with upfield chemical shift reagent $Pr(fod)_3d_{27}$; mole ratio Pr/VVV ≅ 1.5. (*Bottom*) Spectrum of VVV with downfield shift reagent $Eu(fod)_3d_{30}$; mole ratio Eu/VVV ≅ 1.5. Sample size = 114 μM VVV.

pared by the procedure of Selinger and Lapidot (10). Dry potassium isovalerate and potassium palmitate were synthesized from the respective acids as described by Kester, et al. (11). Triisovalerin (VVV) was prepared by acylation of glycerol with isovaleroyl anhydride as outlined by Privett and Nutter (12). Trilaurin (Nu-Chek-Prep, Elysian, Minn.) and tributyrin (Fisher Scientific Co., Fairlawn, N.J.) were purchased and used as received.

Synthesis of the four isomeric diacid triglycerides shown in Figure 1 were carried out using the method of Mitchell (13) with certain modifications. This procedure utilizes chloropropanols to form the glycerol moiety and does not involve acylated intermediates with free hydroxyl groups; hence, acyl migration during synthesis and consequent isomeric impurities are avoided.

The symmetrical diisovaleroyl triglyceride (VPV) was synthesized by dissolving 1,3-dichloropropane-2-ol (Eastman Organic Chemicals, Kingsport, Tenn.) and 20% excess palmitoyl anhydride in benzene containing ~1% $HClO_4$ and shaking 15 min at 25 C (14). The resulting dichloroester was purified by thin layer chromatography (TLC) and then reacted with 100% excess dry potassium isovalerate in hexamethylphosphoric triamide (HMPA) (previously dried over type 4A molecular sieves) at 60 C for ~72 hr under nitrogen with occasional shaking.

The symmetrical monoisovaleroyl triglyceride (PVP) was obtained by acylating 1,3-dichloropropane-2-ol with 100% excess isovaleroyl anhydride in 1/1 benzene/pyridine for 3 hr at 100 C in a sealed ampule (12). The monoester product was isolated by TLC and then reacted with 100% excess dry potassium palmitate for 4 hr at 150 C in HMPA solution in a sealed ampule.

The nonsymmetrical, racemic triglycerides palmito-diisovalerin (PVV) and dipalmitoisovalerin (PPV) similarly were synthesized starting with 3-chloropropane-1,2-diol (Chemical Samples Co., Columbus, Ohio). Initial attempts to use 2,3-dichloropropane-1-ol proved unsatisfactory due to difficulty in reacting the potassium salt with the second chlorine sub-

stituent. Diol acylations with the respective acid anhydrides were carried out as described above; and then the monochlorodiester was reacted with 140% excess potassium soap in HMPA solution at 150 C for 3 hr in a sealed ampule.

Each synthetic triglyceride was purified by preparative TLC. The PVP and PPV were each recrystallized from methanol containing the minimal amount of diethyl ether necessary for solution. All triglyceride products analyzed 98+% pure by TLC and by gas liquid chromatography of the intact molecule.

PMR Procedures

The lanthanide chemical shift reagents employed were the deuterated "fod" derivatives (*tris*-[1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dione]) of europium III and praseodymium III. These $Eu(fod)_3d_{30}$ (Norell Chemical Co., Landisville, N.J.) and $Pr(fod)_3d_{27}$ (Merck, Sharp and Dohme, Montreal, Canada) reagents were stored under reduced pressure over P_2O_5 and used as received after a preliminary spectral shift analysis on triisovalerin proved satisfactory.

All PMR spectra were obtained using a 60 MHz Varian T-60 PMR spectrometer operated at room temperature in the standard manner. Analyses were run on 68-169 μM triglyceride dissolved in 0.4 ml distilled CCl_4 containing 0.5-1.0% tetramethylsilane (TMS) as an internal standard. After recording the PMR spectrum of the pure triglyceride, the appropriate shift reagent was added in 15-35 mg increments; the spectrum was recorded again after each addition until the molar ratio of shift reagent to triglyceride reached ~3.0. Identification of individual PMR signals involved comparison of integrations and multiplicities observed to the expected values, examination of results published elsewhere (4-6), and intercomparison of all triglyceride standards tested (VVV, VPV, PVV, PPV, PVP, trilaurin, and tributyrin).

Since the mole ratio of shift reagent to triglyceride cannot be duplicated exactly from run to run, resonance frequencies cited in this paper should be taken as relative and not absolute values.

RESULTS AND DISCUSSION

Triisovalerin

The PMR signals of isovalerate protons first were studied using VVV where no long chain acyl groups are present. Spectra of pure triisovalerin, VVV plus $Pr(fod)_3$, and VVV plus $Eu(fod)_3$ were examined (Fig. 2).

In the absence of any chemical shift reagent,

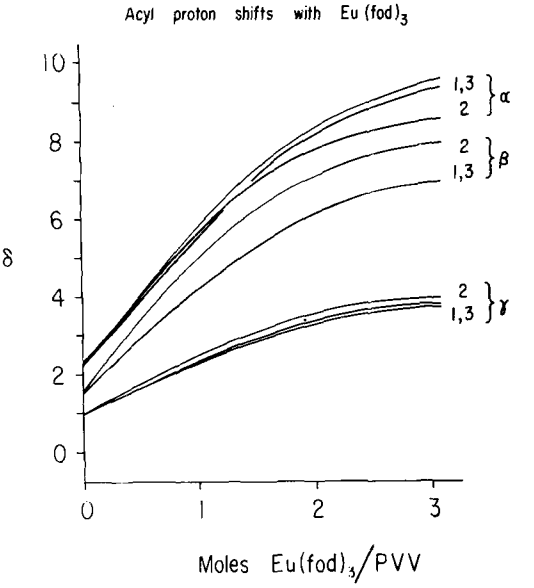

FIG. 3. Downfield chemical shifts induced in palmito-diisovalerin (PVV) proton resonance signals with increasing amounts of $Eu(fod)_3d_{30}$. Sample size = 95 μM PVV in CCl_4.

the three types of acyl protons in VVV each yield independent signals; but the resonances of the three isovalerate moieties are coincident: γ (1.0 δ, 18 H), β (1.3 δ, 3 H), and α (2.1 δ, 6 H).

With added $Pr(fod)_3$, the VVV proton spectrum is shifted upfield beyond the TMS signal, and resolution of the 1,3 and 2 position acyl proton signals is obtained. The most prominent feature is the three well resolved doublets of the terminal γ-methyl groups. The 2-γ-methyl doublet (-2.9 δ, 6 H) is located further upfield than the two 1,3-γ-methyl doublets (-2.4 δ, -2.1 δ, 6 H each). The appearance of two 1,3-γ-methyl doublets, instead of the expected one doublet, with VVV probably indicates unequal magnetic environments for the two $-CH_3$ groups of each isovalerate chain. Evidence for this is the PMR spectrum of tributyrin with $Pr(fod)_3$ which shows only one 1,3-γ-methyl signal, indicating no preferential coordination at the *sn*-1 or *sn*-3 end of the molecule. Apparently, the coordination of shift reagent at the 1,3 ester linkages restricts normal rotation of these external isovalerate chains. Moreover, the absence of such a phenomenon with the 2-γ-protons suggests that the coordination effects differ at the 2 and 1,3 chains. All α- and β-proton resonance signals are shifted even further upfield as a result of their greater proximity to the shift reagent coordination site. The protons on the chain esterified at the 2 position

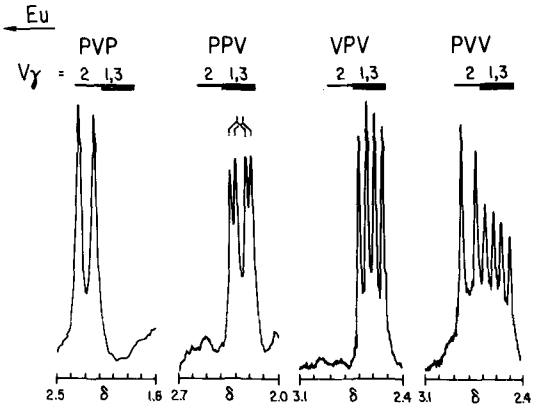

FIG. 4. Resonance signals for the isovaleroyl γ-protons of monoiisovaleroyl (PVP and PPV) and diisovaleroyl (VPV and PVV) triglycerides in the presence of $Eu(fod)_3d_{30}$. Sample size = 68-95 μM triglyceride in CCl_4; mole ratio Eu/triglyceride ≅ 1.0-1.2.

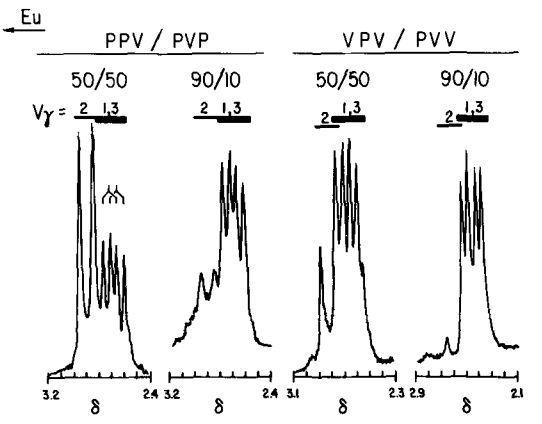

FIG. 5. Isovaleroyl γ-proton resonances of specific mixtures of isomeric triglycerides in the presence of $Eu(fod)_3d_{30}$. Ten percent of the isomer having isovaleric acid at the 2 position can be detected easily by this method. Sample size = 104-169 μM triglyceride in CCl_4; mole ratio Eu/triglyceride ≅ 1.0-1.3. PVP and PPV = monoisovaleroyl and VPV and PVV = diisovaleroyl triglyceride isomers.

again show a greater net shift than those on the external chains.

The PMR spectrum of VVV with $Eu(fod)_3$ shows a similar pattern of resonance signals, but the shift is now in the downfield direction and of less amplitude than with $Pr(fod)_3$. The three doublets of the γ-methyl groups are observed again, but signal separation is less complete. The doublet shifted furthest (2.9 δ, 6 H) represents the 2-γ-methyl protons, while the two overlapping doublets at 2.8 δ and 2.7 δ represent the 1,3-γ-methyl protons similar to the VVV + $Pr(fod)_3$ spectrum. The α- and β-proton resonances are shifted even further downfield, but resolution of the 1,3 and 2 position signals is not observed here.

Since the PMR spectra of VVV with both $Eu(fod)_3$ and $Pr(fod)_3$ could differentiate between isovalerate esterified at the 1,3 and 2 positions, we proceeded to the synthesis and NMR study of isomeric diacid triglycerides containing palmitic and isovaleric acids.

Isomeric Triglycerides with $Eu(fod)_3$

The downfield chemical shifts induced in the acyl protons by adding varying amounts of $Eu(fod)_3$ to PVV triglyceride are summarized in Figure 3. The shifts observed approximate a linear function of the $Eu(fod)_3$/PVV mole ratio over the 0.0-1.8 range, but the slopes decrease at higher $Eu(fod)_3$ levels, indicating a saturation effect.

The α-protons are shifted the greatest amount and eventually are resolved into three signals representing 1,3-palmitate, 1,3-isovalerate, and 2-isovalerate. The 2-α- and 1,3-α-isovalerate plots cross in the same manner as Almqvist, et al., (5) reported for long chain acids, but the crossover point in Figure 3 is not at as high a $Eu(fod)_3$/triglyceride ratio as they observed. This same α-signal crossover phenomenon also was found with VPV, PPV, and PVP in the 1.4-1.6 ratio range.

The β- and γ-proton signals show lesser shifts in accordance with their greater distances from the $Eu(fod)_3$/PVV coordination site. The 2 and 1,3 signals, however, still can be differentiated in both cases.

Comparison of the spectra used to construct Figure 3 with similar spectra and proton shift graphs for VPV, PPV, and PVP clearly indicated that the γ-doublet signals would be the most useful for distinguishing positional isomers of isovaleroyl triglycerides. The isovalerate γ-signals show good resolution, high intensities, and simple multiplicities at low $Eu(fod)_3$/triglyceride ratios. Although the isovalerate γ-methyl proton signals partially overlap the palmitate γ-methylene proton resonances, the latter signals are of considerably lower intensity and greater multiplicity and, thus, do not interfere with isomer identification. The β-methine proton signals of the isovalerate chain are of little analytical use, because they are resolved poorly, have low signal intensities, and possess complex multiplicities. The use of α-methylene proton signals to distinguish triglyceride positional isomers has been described by Pfeffer and Rothbart (4), but the crossover phenomenon described above can only be avoided at high $Eu(fod)_3$/triglyceride ratios where decreased resolution due to signal broadening be-

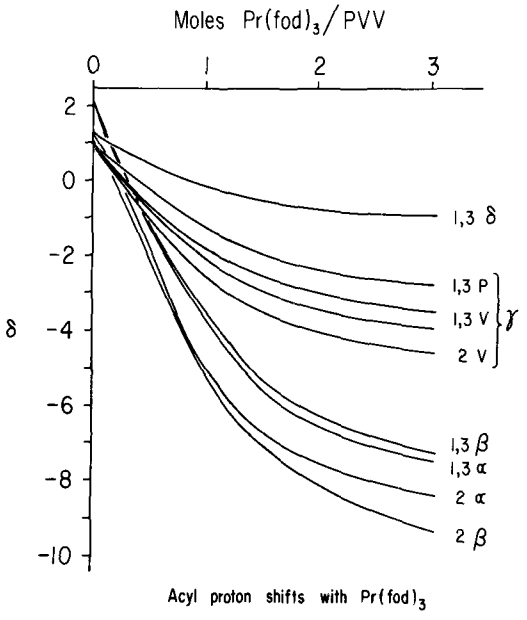

FIG. 6. Upfield chemical shifts induced in palmitodiisovalerin (PVV) proton resonance signals with increasing amounts of $Pr(fod)_3d_{27}$. Sample size = 94 μM PVV in CCl_4.

comes a problem. In addition, α-proton resonances of isomeric mixtures of isovaleroyl triglycerides are more complex and difficult to interpret than γ-proton signals.

The γ-methyl proton signals obtained with each of the four isovaleroyl triglyceride isomers at ca. 1/1 $Eu(fod)_3$/triglyceride ratios are illustrated in Figure 4. The 2-γ-methyl isovalerate protons in PVP show a doublet (2.30 δ, 6 H). The 1,3-γ-methyl isovalerate protons in PPV are shifted a lesser amount and produce two overlapping doublets (2.28 δ, 2.24 δ, 6 H total) each of one-half the expected signal intensity. Analogous patterns are found with the diisovaleroyl molecules. VPV exhibits the same two overlapping 1,3-γ-methyl doublets (2.64 δ, 2.58 δ, 6 H each) found with PPV but with twice the intensity, reflecting the doubling of the 1,3-isovalerate population in this triglyceride. The isovaleroyl γ-methyl proton signals of PVV resemble the sum of the PVP and PPV spectra. The 2-γ-methyl doublet (2.80 δ, 6 H) is shifted the greatest amount followed by the two overlapping 1,3-γ-methyl doublets (2.61 δ, 2.55 δ, 6 H total) of lower intensity. All four spectra in Figure 4 corroborate our initial assignment of separate 2- and 1,3-γ-methyl proton signals with VVV (Fig. 2).

Since the γ-proton signals of the isovalerate moieties differ according to their position in the triglyceride molecule, these signals may be

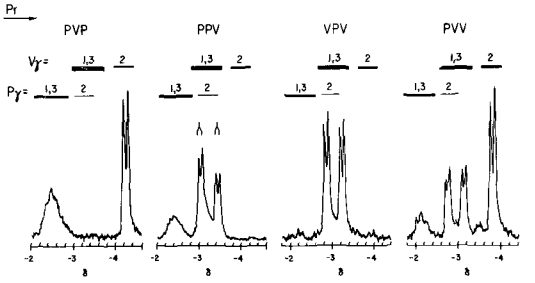

FIG. 7. Resonance signals for the isovaleroyl and palmitoyl γ-protons of monoisovaleroyl (PVP and PPV) and diisovaleroyl (VPV and PVV) triglycerides in the presence of $Pr(fod)_3d_{27}$. Sample size = 94-111 μM triglyceride in CCl_4; mole ratio Pr/triglyceride ≅ 1.6-2.2.

employed to determine the presence or absence of isovaleric acid at the 2 or 1,3 positions in triglyceride mixtures. Figure 5 (left) presents the γ-proton spectra for 50/50 and 90/10 mixtures of PPV and PVP; as expected, three doublets are observed (similar to the PVV spectra). The 2-γ-methyl doublet for isovalerate (2.90 δ) is clearly evident in both cases, even though it coincides with the palmitate γ-methylene signals. Hence 2-isovalerate esters can be detected below the 10% level in PPV/PVP mixtures. The spectra of 50/50 and 90/10 mixtures of VPV and PVV also are given in Figure 5 (right). Here, the 2-γ-methyl isovalerate doublet partially overlaps one of the 1,3-γ-methyl isovalerate doublets. Apparently, the 1,3-γ-doublets of VPV shift slightly further downfield than those of PVV, probably due to a slight difference in coordination geometry between the symmetrical and nonsymmetrical diisovalerate structures. Nevertheless, the remaining peak of the 2-γ-methyl doublet clearly is resolved and can be used to detect as little as 10% of the 2-isovalerate structure in VPV/PVV mixtures.

Isomeric Triglycerides with $Pr(fod)_3$

The acyl proton shifts observed for PVV with the upfield shift reagent $Pr(fod)_3$ are presented in Figure 6. As expected, the least shielded protons show the greatest upfield shift; but this creates an area of multiple resonance crossover in the 0.0-0.5 mole ratio range. Hence, informative $Pr(fod)_3$/PVV spectra only are obtained at higher ratios. The 2 acyl proton signals are shifted further than the corresponding 1,3 signals. The eventual overlap of the α- and β-signals can be attributed to their relative positions in the spectrum of pure PVV. Similar studies with VPV, PPV, and PVP gave acyl proton shift graphs very similar to Figure 6.

Once again the γ-methyl signals prove to be the most useful for structural analysis of iso-

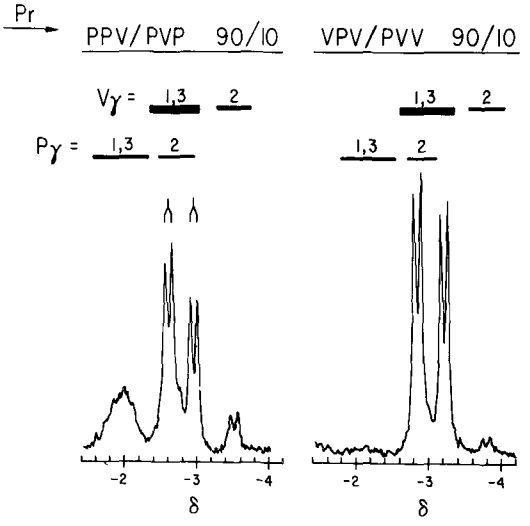

FIG. 8. Isovaleroyl and palmitoyl γ-proton resonances of specific mixtures of isomeric triglycerides in the presence of $Pr(fod)_3d_{27}$. Ten percent of the isomer having isovaleric acid at the 2 position can be detected easily by this method. Sample size = 112-140 μM triglyceride in CCl_4; mole ratio Pr/triglyceride ≅ 1.5-1.6. PVP and PPV = monoisovaleroyl and VPV and PVV = diisovaleroyl triglyceride isomers.

valeroyl triglycerides. The nonequivalence of resonance frequencies for the 2- and the 1,3-γ-protons of isovalerate are quite apparent in Figure 6; and the difference here with $Pr(fod)_3$ is greater than was observed with $Eu(fod)_3$ (Fig. 3).

Detailed study of the γ-proton signals of the four isovaleroyl triglyceride isomers at 1.6-2.2 $Pr(fod)_3$/triglyceride ratios (Fig. 7) confirmed the additional resolution with $Pr(fod)_3$. Three distinct sets of γ-signals are observed in the four isomers studied: the multiplet of the palmitate 1,3-γ-methylene protons, the nearly coincident palmitate 2-γ-methylene and isovalerate 1,3-γ-methyl signals, and the isovalerate 2-γ-methyl resonances. PVP exhibits a doublet at -4.25 δ characteristic of its 2-γ-methyl protons; and PPV shows two resolved doublets at -3.05 δ and -3.47 δ representing the isovalerate 1,3-γ-protons. VPV produces the same two isovalerate doublets as PPV but with double the signal intensity, while PVV shows both the two 1,3-doublets and the single 2-doublet of the isovalerate γ-protons. Fortunately, the coincident palmitate 2-γ-methylene proton signal observed in the PPV and VPV spectra is of sufficiently lower intensity and greater multiplicity that it does not interfere with identification of the isovalerate doublets. All four spectra in Figure 7 corroborate our initial assignments for γ-methyl protons in VVV (Fig. 2).

Mixed isomers of isovaleroyl triglycerides also can be analyzed clearly by PMR with $Pr(fod)_3$. Figure 8 shows our results with 90/10 PPV/PVP and 90/10 VPV/PVV mixtures. The upfield doublet at -3.52 δ in the PPV/PVP spectrum illustrates the ease with which 10% 2-isovalerate structure can be identified. With the VPV/PVV mixture, the same doublet also can detect as little as 10% 2-isovalerate present. The sensitivity of this doublet to 2-isovalerate is identical in both mixtures, but the greater strength of the 1,3-isovalerate signal in the VPV/PVV mixture makes the 2-γ-methyl doublet appear relatively smaller in the second spectrum.

It is apparent, therefore, that the γ-proton signal patterns achieved with $Pr(fod)_3$ provide a useful means to identify positional isomers in mixed-acid, isovaleroyl triglycerides. The greater signal separation with $Pr(fod)_3$ compared with $Eu(fod)_3$ makes $Pr(fod)_3$ the reagent of choice for this purpose. However, since one reagent produces an upfield and the other a downfield shift, the two techniques complement each other; and analyses with both $Pr(fod)_3$ and $Eu(fod)_3$ can provide a useful verification of any structural assignment.

These experiments on model mixtures indicate that the positional location of isovaleric acid in the natural fats of various dolphins and propoises could be determined easily by PMR spectroscopy with shift reagents. Such experiments are now underway and will be reported in the near future.

ACKNOWLEDGMENTS

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New Application of High Pressure Reversed-Phase Liquid Chromatography In Lipids

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ABSTRACT

High pressure reverse-phase liquid chromatography has been used to separate saturated fatty acids, their methyl esters, polyunsaturated fatty acids, and triglycerides. Rapid separation of fatty acids differing in chain length and number of double bonds has been accomplished. Analysis time was less than 10 min in most cases. The high pressure reverse chromatography resulted in better separations of polyenoic acids than can be accomplished by conventional argentation silicic acid column chromatography. The analyses were carried out on a chemically bonded reverse phase packing, VYDAC reverse phase.

INTRODUCTION

In 1950, Howard and Martin (1) reported

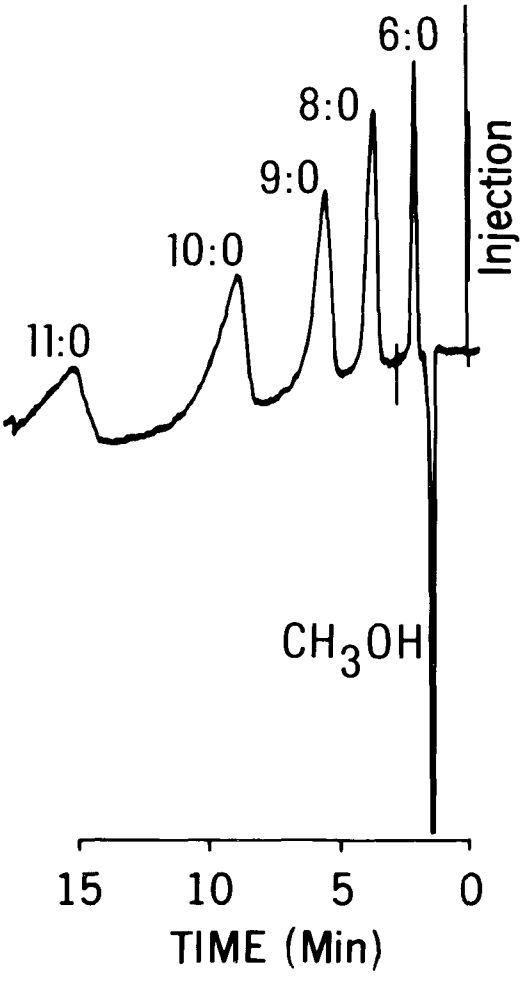

FIG. 1. High pressure liquid chromatography of methyl esters of fatty acids. Eluting solvent, MeOH/water (60:40). Pressure, 1000 psig. Flow rate, 1.0 ml/min. Temperature, 50 C. Sample size, ca. 300 μg.

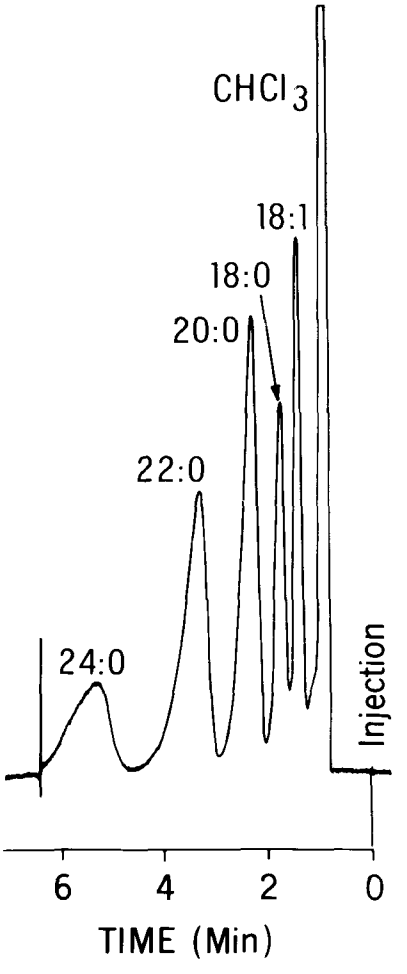

FIG. 2. High pressure liquid chromatography of methyl esters of fatty acids. Eluting solvent, MeOH/water (90:10). Pressure, 1500 psig. Flow rate, 1.7 ml/min. Temperature, 60 C. Sample size, ca. 200 μg each component.

the column chromatographic separation of fatty acids on dimethyldichlorosilane (DMCS) treated Celite 545 coated with the stationary phase heptane. The eluting system was a mixture of acetonitrile and water. In their separation, they were able to resolve saturated fatty acids differing by two carbon numbers. At that time, such a separation was unique and revolutionary. In 1963, Privett, et al., (2) used liquid-liquid partition chromatography for the preparation of pure methyl linolenate from linseed oil methyl esters. In his work, the stationary phase was heptane, and the mobile phase was a mixture of acetonitrile-methanol (85:15 v/v).

In this study, VYDAC reverse phase support (3) is used as the column packing. VYDAC reverse phase is a controlled surface porosity chromatography packing containing octadecyl silane groups chemically bonded to the support. The bonded octadecyl hydrocarbon groups act as a permanent stationary phase. Presaturation of the mobile phase is unnecessary with this packing. The eluting systems used here are mixtures of methanol and water in various proportions.

EXPERIMENTAL PROCEDURES

Apparatus: A du Pont 820 liquid chromatograph (Instruments Products Division, E.I. du Pont de Nemours & Co., Wilmington, Del.) was used throughout this study. The model 820 is equipped with both UV and refractive index detectors for monitoring the column eluant. In

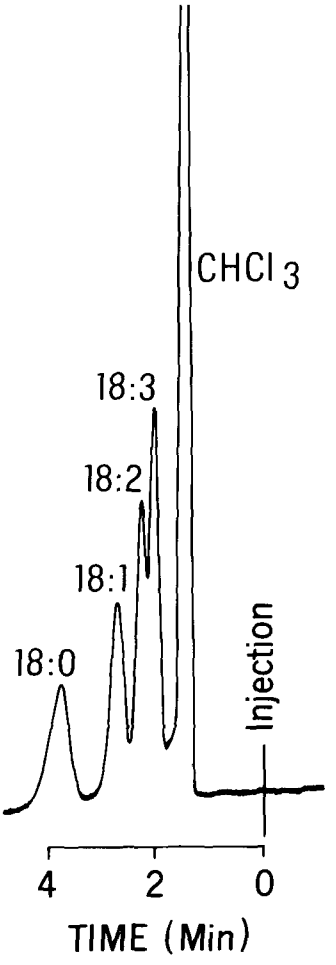

FIG. 3. High pressure liquid chromatography of methyl esters of polyunsaturated fatty acids. Eluting solvent, MeOH/water (90:10). Pressure, 800 psig. Flow rate, 1.0 ml/min. Temperature, 35 C. Sample size, ca. 300 μg each component.

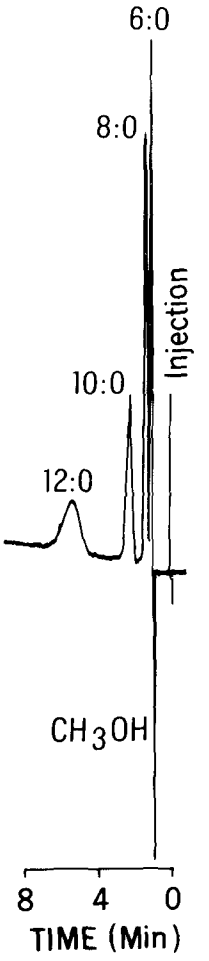

FIG. 4. High pressure liquid chromatography of fatty acids. Eluting solvent, MeOH/water (60:40). Pressure, 1500 psig. Flow rate, 1.5 ml/min. Temperature, 50 C. Sample size, ca. 300 μg each component.

this study, the refractive index detector was used, as most of the lipids used in this study are not sensitive to 254 nm UV wavelength.

A stainless steel column 1 meter long with internal diameter 2 mm, packed with VYDAC reverse phase support (35-44 μ) (Applied Science Laboratories, State College, Pa.) was used. Specific operating conditions for high pressure liquid chromatography (HPLC) are described in the text.

Eluants: Reagent grade methanol and freshly distilled water were used in making up the necessary ratios for eluant solvent systems.

Chemicals: All the fatty acids, polyunsaturated fatty acids, their methyl esters, and triglycerides used in this study were 99% pure (Applied Science Laboratories). They all were dissolved in reagent grade chloroform or methanol.

RESULTS AND DISCUSSION

Figure 1 illustrates a separation of short chain methyl esters of fatty acids ranging from methyl caproate (6:0) to methyl undecanoate (11:0). (The operating conditions of the chromatograph are listed in each figure.) A mixture of methanol and water in 60:40 (v/v) ratio was used as an eluant. By increasing the amount of methanol in the eluting system to a ratio of 80:20 (v/v), the longer chain methyl esters of fatty acids ranging from 11-15 can be eluted. A separation of methyl esters ranging from hexanoic to pentadecanoic acids (6:0-15:0) has been achieved in an isocratic system of methanol and water (80:20 v/v). On VYDAC

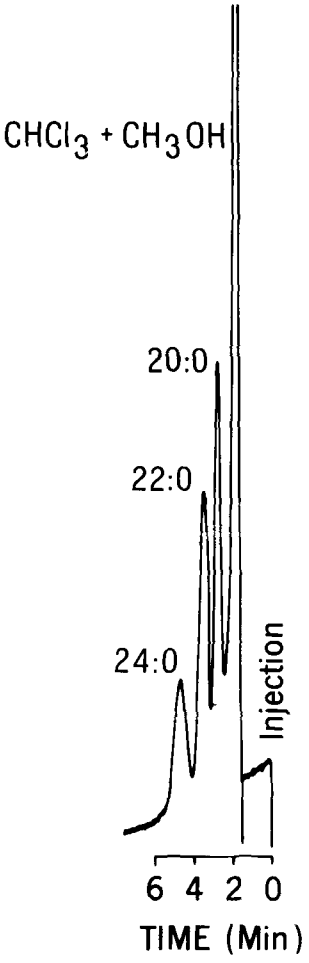

FIG. 5. High pressure liquid chromatography of fatty acids. Eluting solvent, MeOH/water (90:10). Pressure, 500 psig. Flow rate, 0.75 ml/min. Temperature, 50 C. Sample size, ca. 500 μg each component.

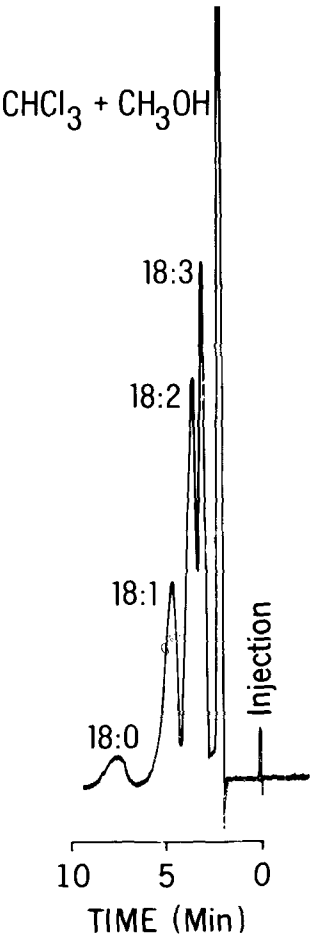

FIG. 6. High pressure liquid chromatography of polyunsaturated fatty acids. Eluting solvent, MeOH/water (80:20). Pressure, 500 psig. Flow rate, 0.7 ml/min. Temperature, 60 C. Sample size, ca. 100 μg each component.

reverse phase support, the separation of methyl esters of fatty acids differing by one carbon number has been achieved.

On further increasing the proportion of the methanol in the eluting system methanol and water to 90:10 (v/v), the chain length of the esters eluted can be extended up to methyl lignocerate (24:0), as illustrated in Figure 2. In Figure 2, methyl oleate (18:1) with one double bond eluted off the column before methyl stearate (18:0) with no double bond. It is evident from this separation (Fig. 2) that high pressure reverse phase liquid chromatography can achieve separations of methyl esters of unsaturated fatty acids.

In Figure 3, the methyl esters of 18 carbon fatty acids were separated according to the number of double bonds. This is a good illustration of reverse phase liquid chromatography, as the most polar compound, methyl linolenate with three double bonds, eluted first and the last compound to appear, methyl stearate, is the least polar compound of the series, because it has no double bonds. This is the reverse concept of conventional argentation chromatography where the least polar compound, methyl stearate, will elute first.

Figures 4 and 5 show separations of free fatty acids ranging from hexanoic to dodecanoic acids (6:0-12:0) and from arachidic to lignoceric acids (20:0-24:0), respectively. The solvent system for elution of the short-chain fatty acids is the same as that for their corresponding methyl esters. Even though free acids can be

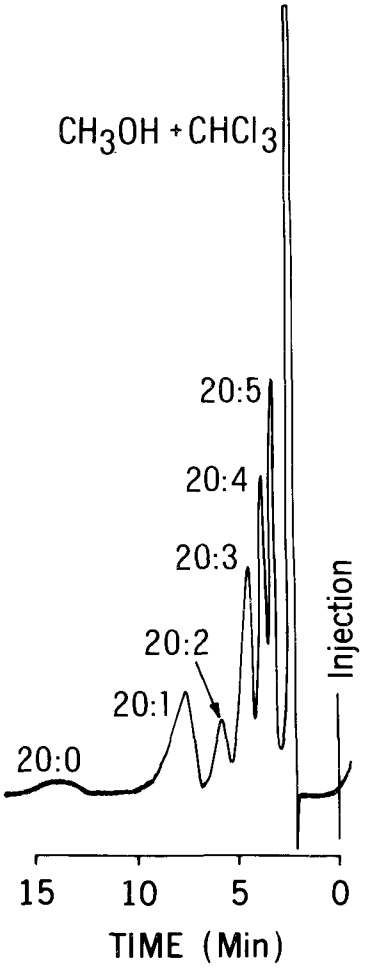

FIG. 7. High pressure liquid chromatography of polyunsaturated fatty acids. Eluting solvent, MeOH/water (80:20). Pressure, 1000 psig. Flow rate, 0.7 ml/min. Temperature, 60 C. Sample size, ca. 100 μg each component.

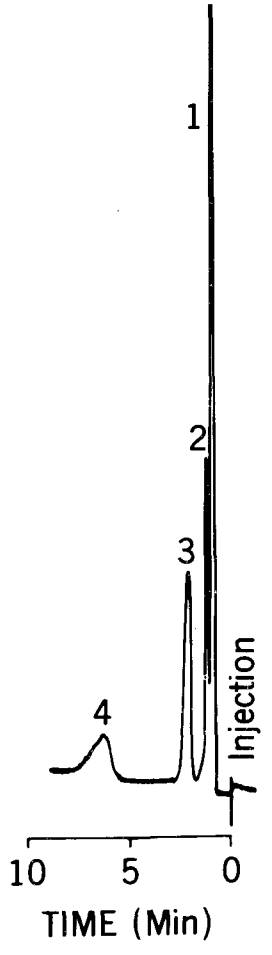

FIG. 8. High pressure liquid chromatograph of triglycerides. Eluting solvent, MeOH/water (90:10). Pressure, 1000 psig. Flow rate, 1.2 ml/min. Temperature, 60 C. Sample size, ca. 200 μg each component. Peak identification: 1. solvent peak, $CHCl_3$; 2. tricaprylin; 3. tricaprin; and 4. trilaurin.

eluted as free fatty acids in gas liquid chromatography (GLC), the system is good only up to 20 carbon acids. Free Fatty Acid Analyzing Phase (FFAP) stationary phase for GLC of free fatty acids can be used but is only good up to arachidic acid (20:0). However, in Figure 5, fatty acids ranging from arachidic acid (20:0) to lignoceric acid (24:0) are separated. This is a case where HPLC is truly a complementary tool to GLC.

The success in separating methyl esters of polyunsaturated acids (Fig. 3) warranted an investigation of the free acids. Separations of the 18 and 20 carbon polyunsaturated fatty acids were obtained and are illustrated in Figures 6 and 7. Figure 6 shows the separation of the 18 carbon fatty acids ranging from linolenic acid to stearic acid. The order of elution is the same as their methyl esters, but the eluting solvent system is 20% water in methanol instead of 10% water in methanol. This is due to the fact that the acids are more polar than their methyl esters. In reverse phase chromatography, the more hydrocarbon-like the compound is, the more it will be retained, and the more methanol will be required to elute methyl esters of fatty acids from the column. Therefore, in the case of free fatty acids, less methanol is needed to elute them from the column.

Figure 7 illustrates the separation of biologically important 20 carbon fatty acids, such as 5,8,11,14,17-eicosapentaenoic acid (20:5); 5,8,11,14-eicosatetraenoic acid (20:4); and 11,14,17-eicosatrienoic acid (20:3) which are the precursors of prostaglandins E_1, E_2, and E_3. This separation suggests that HPLC possibly can be utilized to monitor the synthesis of prostaglandins. A preliminary study of the separation of triglycerides also was done. The initial results were promising, and a separation of tricaprylin to trilaurin is demonstrated in Figure 8. The eluting solvent system is a mixture of methanol and water in the ratio of 90:10. For higher homologues of triglycerides, more methanol may be need for the separation.

The main objective of our work is to extend the use of HPLC in lipids. Since most of the lipids do not have chromophores, a refractive index detector has been used for detection. Most of the separations were done by an isocratic solvent system. If one chooses carefully the proper solvent system, the above separations can be improved further on a reverse phase column.

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Effect of Low Methionine, Choline Deficient Diets upon Major Unsaturated Phosphatidyl Choline Fractions of Rat Liver and Plasma[1]

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ABSTRACT

To see how the metabolism of specific phosphatidyl choline fractions might be affected when only a limited source of methyl groups was available, rats were fed for 7 days a low methionine, choline-deficient diet or one supplemented with either choline or methionine. Prior to killing, they were injected with ^{14}C-methyl methionine and liver and plasma phosphatidyl choline isolated and separated by argentation chromatography into 3 major unsaturated fractions. Fatty acid composition and radioactivity of the fractions were determined. Deficient rats had reduced total liver phosphatidyl choline when compared with the supplemented groups, but the proportions of 20:4 and 22:6 fatty acids in the total phosphatidyl choline were unchanged. Plasma phosphatidyl choline also was reduced sharply by the deficiency, as was its proportion of 20:4 fatty acid. Specific activities of the liver 22:6, 20:4, and 18:2 phosphatidyl choline fractions showed that deficient rats had less radioactivity in their 20:4 and 18:2 phosphatidyl choline than did the supplemented animals. Plasma phosphatidyl choline fractions presented a similar pattern. Feeding methionine or choline nearly doubled radioactive methyl group incorporation into the 20:4 phosphatidyl choline fraction of liver and plasma, while incorporation into the 22:6 phosphatidyl choline was reduced or unchanged. The results suggested that, in the rat, limited availability of methyl groups altered the metabolism of liver and plasma phosphatidyl choline fractions. Methionine, as a source of labile methyl groups, appears necessary for the normal synthesis of certain unsaturated phosphatidyl choline fractions (particularly 20:4 phosphatidyl choline). Transmethylation of phosphatidyl ethanolamine molecular species to the corresponding phosphatidyl choline species may be an important reaction in normal lipid metabolism and transport. Relative affinities for incorporation of the labeled methyl groups into the phosphatidyl choline fractions of either deficient or supplemented rats were: 22:6>20:4>18:2.

INTRODUCTION

It has been proposed that an important factor in the liver triglyceride accumulation that accompanies a choline deficiency in the rat results from impairment in either the availability or metabolism of the choline containing phosphatides for lipoprotein formation (1,2).

It now is recognized that phosphatidyl choline (PC) is not a homogeneous substance but consists of molecular species differing from each other, principally by the unsaturated fatty acid esterified at the 2 position (3,4).

The evidence suggests that those species containing oleic and linoleic acids are derived mainly by a direct reaction between cytidine diphosphate (CDP)-choline and a diglyceride, whereas those containing arachidonic acid and the more highly unsaturated fatty acid (docosahexaenoic) may be synthesized by the sequential methylation of phosphatidyl ethanolamine (PE) (5-7). In addition, enzymatic acylation of lysophosphatidyl choline with the long chain, polyunsaturated fatty acids (8) could be another source of these phosphatide species. The relative importances of each of the pathways have not been established.

Knowledge about the metabolism of the PC species has prompted a number of investigations which indicate that choline deficiency may alter the normal pathways of formation of one or more of the hepatic PC species (9-11). Lombardi, et al., (12) and Chen, et al., (13) have reported that young rats made choline deficient for only 24 hr exhibit liver PC with more arachidonic acid than do controls. They suggested that, during the early stages of choline deficiency, a specific PC subfraction may not be available for lipoprotein synthesis. In later studies with more severely choline-deficient rats, Beare-Rogers (14,15) showed that, while liver PC fatty acid composition remained relatively unaffected, the proportion of

[1]A preliminary report of this study was presented at the Federation of American Societies for Experimental Biology, Atlantic City, April, 1974.

TABLE I

Composition of Diets

Ingredients	Diet (%)			
	-Choline	-Choline +Methionine	+Choline	+Choline +Methionine
Soya protein[a]	20.0	20.0	20.0	20.0
Cerelose	65.2	65.5	64.9	65.2
Fortified oil[b]	1.0	1.0	1.0	1.0
Cottonseed oil	9.0	9.0	9.0	9.0
Salt mix[c]	3.5	3.5	3.5	3.5
Vitamin mix[d]	1.0	1.0	1.0	1.0
Choline choride	–	–	0.33	0.33
Methionine	–	0.28	–	0.28

[a]Soya assay protein (General Biochemicals, Chagrin Falls, Ohio), vacuum dried, contains 93% protein and 14.72 mg methionine/g protein (2.74 g methionine/kg diet).

[b]Fortified oil, 100 g of premix contains in mg: vitamin A acetate, 340; vitamin D, 10; and α-tocopherol acetate, 66.6.

[c]Salt mix (19).

[d]Vitamin mix contains in mg/100 g diet: thiamine•HCl, 0.5; riboflavin, 0.5; pyridoxine•HCl, 0.25; calcium pantothenate, 2.0; meso-inositol, 10.0; biotin, 0.01; vitamin K, 0.05; folic acid, 0.02; nicotinamide, 2.5; and vitamin B_{12} (0.1%), 0.002.

arachidonic acid in the PE decreased, while that of the docohexaenoic acid increased. This observation has been confirmed (16), and more recent studies by Lyman, et al., (17) have shown that the proportion of arachidonic acid, but not its absolute amount, was decreased in liver PE of choline-deficient rats because of a large absolute increase in PE docosahexaenoic acid. Increased biological half-lives of the PE subfractions from choline deficient rats suggested that the conversion of PE to PC was impaired possibly because of limited availability of methionine methyl groups for methylating the PE. The possibility that methionine might play an active role in determining the liver PC composition of choline-deficient rats was indicated by Beare-Rogers (18) who recently reported that supplementary methionine was more effective than choline for increasing the liver PC arachidonic acid in rats fed a choline-deficient diet.

In view of the fact that the polyunsaturated subfractions of hepatic PC seem most affected by choline deficiency and that a source of methyl groups may be necessary to maintain their normal distribution and proper concentration, we initiated an investigation of the effect of methionine and choline upon the synthesis by the methylation of PE of the major liver and plasma PC fractions in rats fed a low methionine choline-deficient diet.

EXPERIMENTAL PROCEDURES

Male, Long-Evans rats (Horton's Laboratories, Oakland, Calif.) weighing ca. 150 g each were separated into 4 dietary groups and allowed free access to water and their semipurified diets, which differed only in their choline and methionine content (Table I).

The animals were fed the semipurified control diet for 4 days prior to an experiment. They then were fed their respective experimental diets for 7 days, at which time the animals were injected intraperitoneally with 8 μC $^{14}CH_3$-methionine (in 0.9% saline)/100 g body wt (Amersham/Searle, Arlington Heights, Ill.). The rats were anesthetized 2 and 5 hr after the injections with Diabutal (sodium pentobarbital) and blood withdrawn from the heart. Equal amounts of blood from 3 rats were pooled according to the time period and the diet. Plasma was obtained after centrifuging in chilled, heparinized centrifuge tubes. Livers were removed, weighed, rapidly frozen on solid carbon dioxide, and lyophilized prior to lipid extraction.

Lipid Extraction

Plasma volume was measured and the plasma extracted by adding it dropwise to 100 ml chloroform-methanol (2:1, v/v) which contained 0.1 mg hydroquinone in 95% ethanol. The plasma samples were extracted further by gentle shaking for 1 hr, filtered, and reextracted with 100 ml chloroform-methanol (2:1). The extract volume was reduced to ca. 75 ml in a rotary vacuum evaporator (Calif. Laboratory Equipment Co., Emeryville, Calif.) and washed with an equal volume of distilled water. The mixture was centrifuged and the aqueous phase removed by aspiration. The lipid phase was taken to dryness and extracted into redistilled petroleum ether (boiling range 30-55 C) for

storage at -20 C until analysis. All solvents and chemicals were reagent grade.

Lyophilized livers were pulverized and extracted twice with the chloroform-methanol mixture described above. Solvents were removed under vacuum, and the lipids were extracted into redistilled petroleum ether and stored at -20 C until analyzed.

Phospholipid Separation and Analyses

Phospholipids were separated by thin layer chromatography (TLC) on washed Silica Gel H (20) using chloroform-methanol-acetic acid-water (25:15:4:2, v/v/v/v) (21). Phospholipid bands were identified under UV light after spraying with 0.4% 2',7'-dichlorofluorescein in absolute methanol. Phosphorus and radioactivity were determined on each band. Phosphorus in plasma extracts was determined by the method of Bartlett (22) while that of liver was determined by that of Sumner (23). Radioactivity of the silica gel bands scraped from the TLC plates was counted in a scintillator-dioxane mixture. Unfractionated lipid extracts and extracts of the PC subfractions obtained by argentation chromatography were counted in toluene with added scintillator.

Fractionation of Liver and Plasma PC

PC was isolated by TLC with the solvent system of Skipski, et al. (21) in sufficient amount for separation into subfractions by argentation TLC. The PC fractions were eluted according to the procedure described by Arvidson (24) and were stored in methanol at 0 C until rechromatography. Silver-impregnated plates were spread to a thickness of 0.5 mm with a slurry containing 6.25 g $AgNO_3$ and 50 g Silica Gel H and were activated immediately prior to use in a 175 C oven for 18 hr. Ca. 9 mg of PC was applied to a 20 x 20 cm TLC plate as a narrow band with a Radin-Pelick streaker (Applied Science Laboratories, State College, Pa.). The plates were developed in chloroform-methanol-water, (65:25:4, v/v/v). Bands were located by spraying with 0.25% Rhodamine 6G in redistilled 95% ethanol and viewing under UV light. Areas of fluorescence, as well as a plate blank, were scraped into centrifuge tubes and eluted 3 times with methanol, then decanted into 250 ml round bottom flasks. The eluates were evaporated under vacuum and transferred to graduated tubes with a small amount of methanol (less than 2 ml) and finally rinsed with petroleum ether. Solid choline chloride was added to each tube to make sure the lower phase was saturated and the methanol layer extracted three more times with petroleum ether. Aliquots of the extract were taken for counting and for fatty acid analysis. Recovery of the fractions relative to the amount of original PC averaged 94% for liver and 95% for plasma. Resolution of the PC fractions was similar to what we have reported previously (25). The fractions most completely resolved were the 18:2 and 20:4 PC.

Fatty Acid Analyses

Methyl esters of the fatty acids were prepared by refluxing the PC in methanol, acidified with H_2SO_4, for at least 30 min. One volume of water was added, and the methyl esters then were extracted into petroleum ether. Gas chromatography of the methyl esters was performed on a dual column Varian/Aerograph model 1700 chromatograph, equipped with hydrogen flame detectors and silanized diethyleneglycol succinate (DEGS) columns. Calbration of the instrument has been described earlier (20). Characterization of the fatty acids was made by gas chromatography of the methyl esters on a polar (20% DEGS) and a nonpolar column (3% SE-30). Hydrocarbon chain length and unsaturation were confirmed by rechromatographing hydrogenated samples and by comparing log plots of the relative retention times with those of known standards. The n3 and n6 families of fatty acids were differentiated using log plots (26). An internal standard of heptadecanoic acid was used in each sample, so that a quantitative evaluation of the fatty acids present could be made.

Radioactivity Determinations

Radioactivities of the PC and its subfractions were determined in a Beckman model LS-100 liquid scintillation counter. Bands of phospholipid, separated by TLC, were scraped into counting vials and counted in a solution of 0.4% 2,5-diphenyloxazole in dioxane containing 10% naphthalene and diluted with 0.2 volume of water. Efficiency of counting ^{14}C in the dioxane mixture was 75%.

Where applicable, statistical comparisons were made using the *t* test as described by Snedecor and Cochran (27). P values less than 0.05 were considered to be the lowest level of significance.

RESULTS

Distribution of liver and plasma PC is shown in Table II. Since the rats were young adults at the start of the experiment, there were no important differences among the groups in regard to food intake, growth, or liver size. Although its concentration was reduced, total liver phospholipid was not affected appreciably by

TABLE II

Liver and Plasma Phosphatidyl Choline Distribution and Concentration from Rats Fed Their Respective Diets 7 Days

Diet	Liver[a]					Plasma		
	Wet wt (g)	mg PL/g liver	Percent PE	Percent PC	mg PC/g liver	mg PL/100 ml	Percent PC	mg PC/100 ml
-Choline	8.6 ± 0.2[b]	28.0 ± 0.8[c]	31 ± 1	39 ± 1	11.0 ± 0.2[d]	58.3 ± 5.2[e]	45 ± 4	26.7 ± 2.8[f]
-Choline +methionine	8.0 ± 0.2	32.8 ± 0.6	26 ± 1	47 ± 2	15.4 ± 0.2	83.1 ± 8.4	54 ± 7	45.6 ± 6.5
+Choline	7.2 ± 0.2	35.0 ± 1.1	23 ± 1	49 ± 1	17.0 ± 0.2	98.1 ± 5.6	53 ± 1	52.8 ± 4.3
+Choline +methionine	8.6 ± 0.3	31.2 ± 0.6	21 ± 1	51 ± 1	15.9 ± 0.1	110.0 ± 18	57 ± 8	64.1 ± 14.7

[a]PL = phospholipid; PE = phosphatidyl ethanolamine; and PC = phosphatidyl choline.

[b]Mean ± standard error of the mean of eight rats/group.

[c]Significantly lower than supplemented groups, $p<0.05$.

[d]Significantly lower than supplemented groups, $p<0.001$.

[e]Mean ± standard error of the mean of two or more separate pools of blood plasma from three rats/pool.

[f]Significantly lower than supplemented groups, $p<0.01$.

choline deficiency, but, relative to the groups being fed choline or methionine, PC concentration in deficient rats was reduced significantly, while hepatic PE was increased. This effect of choline deficiency has been reported previously (15,17). Concentrations of total plasma phospholipids, as well as those of PC were lower in the choline-deficient rats than in those animals supplemented with either methionine or choline. The differences were most pronounced for the PC.

Fatty acid composition of the liver and plasma PC from the different groups is shown in Table III. Choline deficiency had no significant effect upon the proportion of either arachidonic or docosahexaenoic acids in the liver PC. However, as previously noted by us (17) and by others (28), proportions of stearic acid were higher and those of linoleic acid lower when deficient rats were compared with the groups getting the supplements. The significance of these fatty acid changes are not known.

The fatty acids of the plasma PC in all the groups differed from those of the liver, principally, by having a higher proportion of linoleic acid and a lower proportion of arachidonic acid. These differences were intensified by choline deficiency, which increased the proportion of linoleic acid and reduced that of arachidonic acid even further when compared with the supplemented groups.

Differences in the proportions of linoleic and arachidonic acids of the PC reflect changes in the molecular species of the PC. To evaluate quantitative aspects of the changes observed, three major fractions, each characterized by its content of either docosahexaenoic (22:6), arachidonic (20:4), and linoleic (18:2) acids, were isolated from the liver and plasma PC from each group of animals. Table IV shows the percent and concentration of each of the liver and plasma PC fractions. The lower concentration of hepatic PC in the deficient animals occurred mostly from a reduction in the 20:4 and 18:2 fractions. The concentration of 22:6 PC fraction also was depleted by the deficiency, but its reduction exerted only a negligible quantitative effect upon the PC. The apparent higher proportion of the liver 22:6 PC in deficient animals resulted from the decline in other fractions. These kinds of changes resembled those reported by Miller and Cornatzer (29) for liver microsomes and mitochondria from rats severely depleted of choline and methyl groups.

All the PC fractions in the plasma of deficient rats, except the 22:6 PC, were low compared to supplemented groups. Proportionately, however, the concentration of the 20:4 PC fraction appeared affected more than was the 18:2,

TABLE III

Fatty Acid Composition of Liver and Plasma Phosphatidyl Choline from Rats Fed Their Respective Diets 7 Days

Diet	Fatty acid (wt %)					
	16:0	18:0	18:1	18:2	20:4	22:6
			Liver			
-Choline	21.0 ± 0.3[a,b]	26.6 ± 0.4[c]	2.8 ± 0.2[b]	12.8 ± 0.3[c]	28.4 ± 0.6[b]	3.1 ±0.2[b]
-Choline +methionine	21.1 ± 0.6	22.8 ± 0.8	3.7 ± 0.1	17.2 ± 0.5	27.3 ± 0.9	2.9 ± 0.2
+Choline	22.0 ± 0.4	22.2 ± 0.4	4.3 ± 0.1	16.6 ± 0.7	26.8 ± 0.7	3.2 ± 0.2
+Choline +methionine	20.8 ± 1.3	23.8 ± 0.7	3.0 ± 0.8	15.0 ± 0.7	28.6 ± 1.4	3.0 ± 0.3
			Plasma			
-Choline	22.0 ± 0.4[d,b]	20.3 ± 0.3[b]	4.3 ± 0.1[b]	33.6 ± 1.7[e]	14.9 ± 1.0[f]	2.1 ± 0.3[b]
-Choline +methionine	21.4 ± 0.7	20.5 ± 0.9	4.0 ± 0.6	28.5 ± 0.3	20.2 ± 1.0	2.6 ± 0.2
+Choline	23.6 ± 0.4	21.3 ± 1.3	3.6 ± 0.2	30.4 ± 1.6	17.4 ± 1.1	2.0 ± 0.3
+Choline +methionine	20.8 ± 0.3	22.4 ± 0.8	3.8 ± 0	24.2 ± 1.5	22.3 ± 1.5	2.7 ± 0.3

[a]Mean ± standard error of the mean of eight rats/group.

[b]Not significantly different from supplemented groups.

[c]Significantly different from supplemented groups, $p<0.01$.

[d]Mean ± standard error of the mean of two or more separate pools of blood plasma from three rats/pool.

[e]Significantly higher than methionine supplemented groups, $p<0.01$.

[f]Significantly lower than methionine supplemented groups, $p<.001$.

although, quantitatively, the latter was the major plasma PC fraction. Upon correction of the deficiency by either methionine or choline, the 20:4 fraction was increased more than was the 18:2, although concentrations of both plasma fractions were increased. This was particularly apparent when both choline and methionine were fed.

The percent of radioactivity administered as $^{14}CH_3$-methionine and recovered in the liver and plasma PC is shown in Table V. The average uptake of radioactivity by the liver PC of the choline-deficient animals was ca. 75% of that of the supplemented groups but was not consistently lower than the supplemented rats. Methyl group utilization in the synthesis of plasma PC, however, was only ca. 32% of the supplemented groups.

Specific activities of the major liver and plasma PC fractions were determined in all groups at 2 and 5 hr intervals after injecting the rats with $^{14}CH_3$-methionine (Table V). These time intervals were chosen as the result of a preliminary experiment which showed that peak incorporation of the label into liver and plasma PC occurred around 2 hr for the 22:6 PC fraction, then declined rapidly thereafter. Maximum incorporation into the other liver PC fractions also occurred by 2 hr, but, for plasma, it was later or around 5 hr. Individual specific activities for the hepatic 20:4 and 18:2 PC fractions were maintained relatively constant during these periods, indicating that exchange of the nitrogen base (30) or transacylation reactions (31) were probably of minor importance.

Table V shows that, in all groups, the 22:6 fraction of both liver and plasma PC had the highest specific activity of all the fractions at both time periods. The high specific activity of this hepatic PC fraction has been noted previously (16).

Although choline deficiency increased the specific activity of this liver PC fraction compared with other groups, it significantly depressed the uptake of radioactivity by the 20:4 and 18:2 PC fractions. Supplementation with either choline or choline and methionine prevented the high initial incorporation of methyl groups by the liver 22:6 fraction, and the incorporation of the label by the 20:4 and 18:2 PC fractions was increased significantly.

The 22:6 PC fraction from plasma of choline-deficient rats did not exhibit the early, unusually high specific activity, seen in the liver, even though specific activities of this fraction were higher than the other plasma PC fractions. Methionine or the combination of methionine and choline increased the specific activities of the 20:4 and 18:2 PC fractions considerably above those in the deficient groups. This was particularly true at the 5 hr period. Choline supplementation seemed to

TABLE IV

Major Unsaturated Fractions of Rat Liver and Plasma Phosphatidyl Cholines from Animals Fed Their Respective Diets 7 Days

Diet	Phosphatidyl choline fractions					
	22:6 PC[a]		20:4 PC		18:2 PC	
	Liver					
	Percent PC	mg Fraction/g liver	Percent PC	mg Fraction/g liver	Percent PC	mg Fraction/g liver
-Choline	13.5 ± 1.5[b]	1.48 ± 0.03[c]	56.9 ± 1.7	6.26 ± 0.13[d]	24.2 ± 0.5	2.66 ± 0.06[d]
-Choline +methionine	9.8 ± 0.8	1.51 ± 0.02	50.0 ± 2.1	7.71 ± 0.09	34.0 ± 1.5	5.24 ± 0.06
+Choline	11.4 ± 0.4	1.94 ± 0.02	52.0 ± 1.8	8.85 ± 0.09	29.2 ± 1.5	4.97 ± 0.05
+Choline +methionine	9.8 ± 0.7	1.55 ± 0.02	55.6 ± 2.4	8.82 ± 0.11	29.9 ± 1.2	4.74 ± 0.06
	Plasma					
	Percent	mg Fraction/100 ml	Percent	mg Fraction/100 ml	Percent	mg Fraction/100 ml
-Choline	7.1 ± 2.2	1.75 ± 0.84[e]	24.6 ± 2.1	6.70 ± 1.4[d]	62.2 ± 3.4	16.3 ± 0.7[f]
-Choline +methionine	8.2 ± 0.6	3.78 ± 0.66	35.0 ± 0.5	16.0 ± 1.9	52.5 ± 2.0	23.9 ± 2.1
+Choline	8.8 ± 1.1	4.65 ± 0.85	31.6 ± 4.8	16.7 ± 1.6	53.9 ± 3.2	28.7 ± 4.7
+Choline +methionine	6.0 ± 0.5	3.65 ± 0.44	45.8 ± 2.7	29.5 ± 6.5	43.6 ± 1.2	27.7 ± 5.3

[a]Fractions designated according to predominate unsaturated fatty acid. The 22:6 fraction had ca. 15% 20:4 and 22:5 fatty acids. The 18:1 phosphatidyl choline (PC) fraction was only 4-6% of the total PC, and its incorporation of radioactivity was less than half that of the 18:2 PC, so data for it were not presented.

[b]Mean ± standard error of the mean of eight rats/group.

[c]Significantly lower than group supplemented only with choline, $p<0.001$.

[d]Significantly lower than supplemented groups, $p<0.001$.

[e]Not significantly different from supplemented groups.

[f]Significantly lower than supplemented groups, $p<0.005$.

TABLE V

Specific Activities of Liver and Plasma Phosphatidyl Choline Fractions from Rats Fed Their Respective Diets 7 Days

Diet	Percent of ^{14}C methyl incorporated into total PC	Phosphatidyl choline fractions[a]					
		22:6 PC		20:4 PC		18:2 PC	
		2 hr	5 hr	2 hr	5 hr	2 hr	5 hr
				Liver			
-Choline	11.0 ± 1.3[b]	131 ± 15.6[c,d]	72.7 ± 7.5[e]	32.3 ± 2.0[f]	27.3 ± 0.6[g]	18.2 ± 1.2[d]	17.4 ± 0.7[f]
-Choline +methionine	14.8 ± 0.3	89.4 ± 9.8	67.0 ± 4.3	54.8 ± 6.2	40.2 ± 4.2	25.4 ± 2.8	23.9 ± 1.9
+Choline	14.7 ± 0.5	81.2 ± 10.2	56.6 ± 2.1	43.3 ± 3.1	35.6 ± 0.8	30.8 ± 4.4	22.0 ± 0.5
+Choline +methionine	16.5 ± 0.9	79.1 ± 7.7	60.2 ± 8.0	45.3 ± 5.4	43.1 ± 0.8	28.3 ± 1.8	25.5 ± 1.2
				Plasma			
-Choline	0.20	66.3[h]	72.1	16.8	27.2	4.6	13.0
-Choline +methionine	0.64	93.9	69.0	37.3	50.5	11.6	20.6
+Choline	0.63	55.3	48.3	24.4	28.4	6.2	12.6
+Choline +methionine	0.62	65.2	60.4	25.9	45.7	9.6	21.8

[a]Expressed as cpm/μg fatty acid in phosphatidyl choline (PC) fraction.

[b]Significantly lower only for methionine supplemented groups, $p<0.05$. Mean from eight rats.

[c]Mean ± standard error of the mean of four rats/time interval.

[d]Significantly different only for groups supplemented with choline or choline and methionine, $p<0.05$.

[e]Significantly higher only for group supplemented with choline, $p<0.05$.

[f]Significantly lower than supplemented groups, $p<0.05$.

[g]Significantly lower than supplemented groups, $p<0.01$.

[h]Mean of two separate pools of blood plasma from three rats/pool. Insufficient number of samples for statistical comparisons.

TABLE VI

Relative Specific Activities of Liver and Plasma Phosphatidyl Choline Fractions from Rats Fed Their Respective Diets 7 Days

	Phosphatidyl choline fractions					
	22:6		20:4		18:2	
Diet	2 hr	5 hr	2 hr	5 hr	2 hr	5 hr
			Liver			
-Choline	3.04 ± 0.20[a,b,c]	2.44 ± 0.28[c]	0.77 ± 0.03[d]	0.91 ± 0.02	0.43 ± 0.02	0.58 ± 0.01
-Choline +methionine	1.95 ± 0.16[c]	1.89 ± 0.08[c]	1.20 ± 0.13[d]	1.12 ± 0.04[d]	0.56 ± 0.06	0.66 ± 0.02
+Choline	2.00 ± 0.10[c]	1.69 ± 0.03[c]	1.08 ± 0.06[d]	1.06 ± 0.02[d]	0.76 ± 0.08	0.66 ± 0.02
+Choline +methionine	1.93 ± 0.27[c]	1.70 ± 0.17[c]	1.06 ± 0.05[d]	1.08 ± 0.02	0.68 ± 0.02	0.66 ± 0.03
			Plasma			
-Choline	5.14[e]	3.32	1.30	1.25	0.36	0.50
-Choline +methionine	3.79	2.41	1.50	1.77	0.47	0.72
+Choline	3.20	2.06	1.41	1.21	0.36	0.54
+Choline +methionine	3.10	1.63	1.35	1.24	0.46	0.59

[a]Relative specific activity = $\frac{\text{specific activity of fraction}}{\text{specific activity of total phosphatidyl choline}}$.

[b]Mean ± standard error from 4 rats/time interval.

[c]Significantly higher than 20:4 phosphatidyl choline, $p<0.02$.

[d]Significantly higher than 18:2 phosphatidyl choline, $p<0.02$.

[e]Average of two separate pools of blood plasma from three rats/pool at each time interval.

have less of an effect upon this parameter than did methionine, and, by the 5 hr period, specific activities of the plasma 20:4 and 18:2 fractions were no higher than those of the deficient group. By 5 hr after administering the radioactive methionine, specific activities of most of the plasma PC fractions had continued to rise, so that they were similar to their respective hepatic fractions.

To see whether certain PC fractions may have been formed preferentially when methionine or choline was made available, the relative specific activities of each fraction were calculated and are shown in Table VI. In all groups, the 22:6 PC of both liver and plasma had a disproportionately high relative activity. This was especially evident in the choline-deficient animals. Relative specific activities of the plasma 22:6 PC fraction consistently were higher than those of the liver, indicating, possibly, there may have been some selective formation and secretion of this PC fraction by the liver, even when availability of methyl groups was limited.

The relative specific activities of the liver 20:4 PC fraction from choline-deficient rats were appreciably lower than those of the supplemented groups, at least during the early period of time. Relative specific activities of this plasma fraction in the deficient group were slightly higher than those of the liver but were still less than the specific activities seen after supplementation. With supplementation, relative specific activities of the plasma 20:4 PC fraction were quite similar to those of the liver. The lowest relative specific activities of the PC fractions determined were in the 18:2 PC fraction of both the liver and plasma. These data indicate, therefore, that the highest proportions of the methyl lable, when methyl groups are made available to choline-deficient rats, are used in the synthesis of the 22:6 and 20:4 PC fractions with much less appearing in the 18:2 PC. The relative affinities of incorporation of the labile methyl groups into the PC fractions are 22:6>20:4>18:2. If rats are deficient in methyl groups, the proportion of methylation of the 22:6 fraction is increased, relative to the other fractions.

DISCUSSION

The conversion of PE to PC by sequential methylation is the only mechanism known by which choline-deficient rats can synthesize PC de novo. This reaction depends upon the availability of labile methyl groups and is thought to lead to the synthesis of PC species containing polyunsaturated fatty acids, particularly arachidonic and docosahexaenoic. Therefore, we might expect changes in the proportions of these PC fractions in the livers of rats fed the choline-deficient diet. Since no effect upon the polyunsaturated fatty acid pattern of the liver PC was evident (Table III), sufficient methionine must have been provided by the diet (0.27%) to maintain resonably normal PC fatty acid patterns but at the expense of a reduced liver PC concentration.

On the other hand, the concentration of plasma PC was not only reduced by the deficiency to nearly half that of the supplemented groups, but the proportion of arachidonic acid in this phospholipid was lowered significantly, relative to the supplemented rats. The lower proportion and concentration of this fatty acid indicates that all the PC fractions in the plasma were not depleted uniformly, and the deficiency affected the arachidonoyl fraction to a greater extent than it did the others (Table IV).

There still exists controversy regarding whether the conversion of PE to PC is impaired in choline-deficient rats (11,32). Evidence by Rytter and Cornatzer (9) suggested that the conversion of PE to PC may have been reduced in choline-deficient rats. More recently, Skurdal and Cornatzer (33) have shown that the activity of microsomal PE methyltransferase, the enzyme necessary for the methylation of PE, was decreased in choline-deficient rats. The data in Table V also are consistent with the view that, in choline deficiency, PC synthesis is impaired. In the absence of supplementary methionine or choline, methyl group incorporation into PC was reduced appreciably in the 20:4 and 18:2 PC fractions of both liver and plasma. When either methionine or choline was fed, the much higher specific activity of the predominant 20:4 PC fraction, when compared with the smaller 18:2 fraction, was indicative of a high rate of methyl group utilization for its synthesis.

These results suggest the rat synthesizes an appreciable amount of its liver 20:4 PC by methylation of PE. When choline is omitted from the diet and the supply of labile methyl is limited, overall synthesis of the liver PC fractions is reduced to a level just necessary to maintain the integrity of the membranous structures of the liver cell, a situation accomplished by decreased secretion of PC as a part of the lipoproteins, into the blood. The reduced proportion of the plasma 20:4 PC fraction may result from a selective retention of 20:4 PC by the liver, since most of the limited labile methyl would be used for synthesis of this large PC fraction.

Another liver PC fraction that might be ex-

pected to be affected by a choline deficiency is the 22:6 fraction, since its synthesis also has been associated with the methylation pathway (34). Previous studies have shown that a 22:6 PE fraction accumulated in rat liver PE under the conditions of choline deficiency (14,17). However, the results in Table III and IV show that the proportion of this fatty acid in liver and plasma PC was unchanged by the dietary treatments and the deficiency had only a small effect upon its concentration in the liver. Within 2 hr after administering the labeled methionine to the deficient rats, however, the 22:6 liver fraction had a specific activity higher than the choline supplemented groups. This occurred even though the size of the 22:6 PC pool into which it entered was similar to that of the control (Table V). Thus, while the incorporation of labeled methyl groups into the other PC fractions of deficient animals decreased, it increased in the 22:6 fraction. Methionine or choline supplements shifted the patterns of incorporation from the 22:6 PC toward the 20:4 PC fraction.

The rapid decline in radioactivity of this fraction suggests it is either quickly incorporated into the lipoproteins and secreted into the blood or else is converted into other PC fractions by transacylation reactions. Tinoco, et al., (34) proposed that the newly synthesized choline could be hydrolyzed and transferred to another PC fraction, while the diacylglyceride was reutilized for the synthesis of PE. Such a mechanism might explain the relatively high specific activity and the abnormal accumulation of the 22:6 PE fraction in the livers of choline-deficient rats when administered radiolabeled ethanolamine (17).

The initially high specific activity of the liver 22:6 PC fraction was not apparent in the respective plasma PC fraction from deficient rats (Table V). However, when results were expressed as relative specific activities (Table VI), deficient animals, at both time periods, had a higher proportion of radiolabel in both their liver and plasma 22:6 PC than did the other groups.

Relative specific activities of the 18:2 liver and plasma PC fractions in all groups were lower than the other two PC fractions. Formation of this PC fraction is thought to be primarily by condensation of CDP-choline with a diglyceride (31), so its uptake of radioactivity would result principally from acylation-deacylation reactions and exchange with some of the radiolabeled choline.

It was expected that feeding choline to the deficient animals would have diluted the choline labeled with ^{14}C-methyl and thereby reduce the incorporation into the liver and plasma PC fractions. However, specific activities of liver PC fractions were relatively unaffected, when choline was supplemented (Table V), compared with rats fed methionine or choline and methionine. Feeding choline increased the amount of liver PC, while maintaining its fatty acid composition (Table III) the same as the other supplemented groups. Choline must not have been used directly for synthesizing the specific PC fractions, nor must it have exchanged significantly with the other unsaturated fractions of PC, since the specific activities of the individual liver PC fractions remained quite stable. Possibly, choline spared the limited dietary methionine, which increased the conversion of PE to PC to that seen when methionine was fed. It is doubtful that choline served as a methyl donor, since there is evidence this reaction does not occur readily (35).

Plasma PC levels increased appreciably with choline supplementation (Table II). However, the specific activities of the plasma fractions were low compared with those supplemented with methionine (Table V). By 5 hr, specific activities of the plasma 20:4 and 18:2 fractions resembled those of the deficient groups. Only when methionine was added to the diet did the specific activities of the plasma 20:4 fraction and, to a lesser extent, those of the 18:2 increase until they were comparable to the specific activities of their respective liver fractions. It appeared that the supplemental choline provided a pool of PC for export into the plasma whose origin may not have been the same as when methionine methyl groups were provided.

These results have shown that a deficiency of labile methyl groups induced in rats by a diet deficient in choline and methionine interferes with the normal synthesis of the 22:6 and 20:4 subfractions of PC. A principal effect of the deficiency appears to be to reduce methylation of PE and synthesis of the liver 20:4 PC fraction. Presumably, the 20:4 PC synthesized is conserved and used principally for maintaining hepatic cell membranes with only limited amounts made available for lipoprotein synthesis. Under the same conditions, the liver 22:6 PC synthesis increases, apparently, because of a greater affinity of this fraction for the available labile methyl groups.

The experiments reported here were conducted in rats which had an established fatty liver, so did not show whether the availability of the liver 20:4 PC fraction was related to the fat infiltration normally associated with a choline deficiency in rats. Although there is evidence suggesting that a correlation may exist (14), a cause and effect relationship between

liver fat infiltration and the reduction in total hepatic 20:4 PC has not been demonstrated.

For the present, however, it can be said that methionine, as a source of labile methyl groups, is important in regulating and maintaining the proportions of specific unsaturated PC fractions and transmethylation of PE to PC may be essential for normal lipid metabolism and transport.

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Effects of Phenobarbital upon Bile Acid Synthesis in Two Strains of Rats

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ABSTRACT

Rats of the Wistar and Sprague-Dawley strains were injected with sodium phenobarbital (100 mg/kg body wt/day) for 8 days. Fecal bile acid excretion was measured on days 6 and 8 of the experiment, and biliary bile acid composition, hepatic microsomal cholesterol, 7α-hydroxylase, and 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase were determined at the end of the study. In the Wistar rat, injection of phenobarbital produced a doubling of fecal bile acid output (controls, 5.3 mg/rat/day; treated rats, 10.6 mg/rat/day) and a two-threefold increase in cholesterol 7α-hydroxylase. The fecal bile acid output of Sprague-Dawley rats increased 20% in response to phenobarbital (controls, 9.5 mg/rat/day; treated rats, 11.6 mg/rat/day). The activity of cholesterol 7α-hydroxylase remained unchanged. In both strains, phenobarbital treatment produced a decrease in the proportion of cholic acid in total biliary bile acids (controls, 85%; treated groups, 65%). This was associated with a decrease of 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase activity by ca. 50%. Biliary cholesterol concentrations were reduced in phenobarbital treated rats of both strains, but liver cholesterol concentrations remained unchanged. The drug produced a 25% increase in liver wt, on the average.

INTRODUCTION

The administration of pharmacological amounts of phenobarbital produces a proliferation of the hepatic endoplasmic reticulum (1,2). In addition, the barbiturate induces microsomal enzymes involved in the hydroxylation and demethylation of certain drugs (1). The enzyme cholesterol 7α-hydroxylase is reported to reside within the endoplasmic reticulum (3,4). This enzyme, which is thought to be rate-limiting for bile acid synthesis from cholesterol, is not induced by phenobarbital in rats of the Sprague-Dawley strain (5). Shefer, et al., (6) and Wada, et al., (7) confirmed these results in the Sprague-Dawley strain but found that phenobarbital enhanced cholesterol 7α-hydroxylase in male Wistar strain rats. In Sprague-Dawley rats, phenobarbital produces a decrease in the activity of the microsomal 12α-hydroxylase, an enzyme of possible importance in the regulation of the ratio of cholic acid to chenodeoxycholic acid in bile (5,8). The response of this enzyme to phenobarbital in the Wistar rat has not been studied.

The present study was designed to find out, for the first time, whether the observed differential effect of phenobarbital upon cholesterol 7α-hydroxylase in the two strains of rats also was associated with a similar differential effect upon bile acid production. For this purpose, the effect of phenobarbital upon the activity of cholesterol 7α-hydroxylase and bile acid output was measured in the same animals. In addition, for the first time, we have correlated the amount of biliary cholic acid with the microsomal 12α-hydroxylase activity in phenobarbital-treated rats.

EXPERIMENTAL PROCEDURES

Treatment of Animals

Male Wistar rats weighing 225-235 g were obtained from the Otisville Laboratory, New York City Health Department. Male Sprague-Dawley-derived rats of ca. the same wt were purchased from Charles River Breeding Laboratories, Wilmington, Mass. The animals were placed into metabolic cages which allow for quantitative feces collection and were fed a stock diet of Rockland rat chow supplemented with 5% corn oil. The rats had access to water ad lib.

At 9 a.m., the rats were injected intraperitoneally with sodium phenobarbital (Merck, Darmstadt, Germany) (25 mg/ml) dissolved in 0.9% sodium chloride. The dose was 100 mg/kg (25 mg/250 g) of body wt (1 ml sodium phenobarbital solution). Control animals were injected with 1 ml 0.9% sodium chloride solution. The animals were injected daily during the 8 day experimental period. Feces were collected on day 6 and day 8. On day 8, the rats were weighed and anesthetized with Diabutal (Diamond Laboratories, Des Moines,

Iowa), and cannulas were inserted into the common bile ducts. Bile was collected for 1 hr. The rats were killed by exsanguination, and their livers were excised and weighed. A suitable portion of liver was used for the preparation of the microsomal fraction.

Preparation of Liver Microsomes

The liver tissue was blotted dry on sterile gauze, weighed, and put into a 15 ml centrifuge tube in the amount of homogenizing medium necessary to obtain a solution of ca. 40% (w/v) concentration. Temperature was maintained between 0-5 C during preparation of the microsomes. The homogenizing medium contained 0.3 M sucrose, 75 mM nicotinamide, 2 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM mercaptoethanol. The tissue was homogenized with a loose fitting Teflon pestle (radial clearance, 0.5 mm). The whole homogenate was centrifuged at 11,000 x g for 10 min to sediment nuclei, cell debris, and the mitochondrial fraction. The supernatant solution was centrifuged at 100,000 x g. The supernatant solution was decanted, and the sedimented microsomal pellet was homogenized in the same tube with homogenizing medium to give a solution of 40% (w/v) concentration. The microsomal suspensions were used for the assay of cholesterol 7α-hydroxylase and 12α-hydroxylase. The microsomal protein concentration was determined by the method of Lowry, et al., (9). A typical preparation had a concentration of ca. 10 mg/ml.

Labeled Compounds

(4-^{14}C)-Cholesterol (New England Nuclear, Boston, Mass.) was purified by column chromatography on silicic acid (Bio-Rad Laboratories, Richmond, Calif.) in the presence of unlabeled 7α- and 7β-hydroxycholesterol as previously described (10).

^{3}(H)-7α-Hydroxy-4-cholesten-3-one was synthesized by the method of Björkhem from (^{3}H)-chenodeoxycholic acid (New England Nuclear), specific radioactivity 800 mCi/mmole, by electrolytic coupling with isovaleric acid, oxidation of the resulting 3α,7α-dihydroxy-5β-cholestane with aluminum tert-butoxide and dehydrogenation of the 7α-hydroxy-5β-cholestane-3-one with selenium dioxide (11).

Reference Compounds

5α-Cholestane (Applied Science Laboratories, State College, Pa.) was used as an internal standard for gas liquid chromatography (GLC).

3α,7α-Dihydroxy-12 keto-5β-cholanoic acid was used as internal standard to correct for analytical losses of fecal bile acids.

7α-Hydroxy-4-cholesten-3-one (unlabeled) was prepared in the same manner as the labeled compound (11).

7α,12α-Dihydroxy-4-cholesten-3-one was synthesized as described by Berséus, et al., (12) by electrolytic coupling of cholic acid with isovaleric acid.

For the preparation of 7α-hydroxycholesterol, a mixture of 7α- and 7β-hydroxycholesterol was obtained by the reduction of 7-ketocholesterol (Schwarz-Mann, Orangeburg, N.Y.) with sodium borohydride, followed by separation of the epimers by preparative thin layer chromatography (TLC) on silica gel (Analtech, Wilmington, Del.) (13). 7α-Hydroxycholesterol was purified further by column chromatography on deactivated silicic acid (13).

Assay of Cholesterol 7α-Hydroxylase Activity

Incubations of the microsomal enzyme were carried out using (4-^{14}C)-cholesterol as substrate. The substrate consisted of (4-^{14}C) cholesterol (5 x 10^{5} dpm, 5 x 10^{-6} M, 2 nmoles/tube) and unlabeled cholesterol (100 mmoles/tube) which was solubilized with cutscum (Fisher Scientific Co., Springfield, N.J.) as previously described (14). The assay system contained, in a total volume of 0.5 ml: 0.15 ml of an NADPH-generating system (70 mM potassium phosphate buffer, pH 7.4; 4.5 mM $MgCl_2$; 1.25 mM nicotinamide adenine dinucleotide phosphate, oxidized form [NADP]; 2.5 mM glucose-6-phosphate; 5 units glucose-6-phosphate dehydrogenase); 0.15 ml medium containing the solubilized cholesterol substrate; and 0.2 ml microsomal protein. Incubation was carried out in the dark at 37 C for 20 min. The reaction was stopped by the addition of 15 volumes of methylene chloride-ethanol (5:1; v/v). The reaction product, 7α-hydroxycholesterol, was extracted, separated by TLC, and the radioactivity determined. Enzyme activity was expressed as dpm incorporated into 7α-hydroxycholesterol/mg protein/min. The mass of 7α-hydroxycholesterol formed also was measured directly by an isotope derivative method (15).

Assay of Microsomal 12α-Hydroxylase Activity

The procedure used was that of Einarsson with modifications (16). The substrate, (^{3}H)-7α-hydroxy-4-cholesten-3-one (150 nmoles; specific activity, 550 dpm/nmole) was mixed with 0.3 ml bovine albumin solution (containing 0.6 mg albumin) and left at room temperature for 10 min before incubation. The standard assay system contained in a volume of

2.2 ml: 1 ml NADPH-generating system (70 mM potassium phosphate buffer, pH 7.4; 2.25 mM $MgCl_2$; 1.25 mM $NADP^+$; 2.5 mM glucose-6-phosphate, 5 units glucose-6-phosphate dehydrogenase); 150 nmoles (^{3}H)-7α-hydroxy-4-cholesten-3-one mixed with 0.6 mg albumin; and 0.5 ml microsomal suspension (ca. 5 mg protein).

Incubations were carried out in air at 37 C for 20 min with shaking and terminated by the addition of 2 ml methanol. Water (2 ml) was added, and the reaction products were extracted twice with 20 volumes of chloroform-methanol (3:1; v/v).

TLC

The chloroform extract was evaporated under nitrogen and applied to a Silica Gel G (Analtech) thin layer plate with unlabeled 7α-hydroxy-4-cholesten-3-one and 7α, 12α-dihydroxy-4-cholesten-3-one added as carriers. The plates were developed at room temperature using ethyl acetate-benzene (6:4; v/v). The compounds were made visible by exposing the plate to iodine vapors (7α,12α-dihydroxy-4-cholesten-3-one, R_f 0.36; 7α-hydroxy-4-cholesten-3-one R_f = 0.78) and counted for radioactivity. Enzyme activity was expressed as pmoles 7α, 12α-dihydroxy-4-cholesten-3-one formed/mg protein/min. A zero-time control was run for each experiment.

The TLC separations of the bile acids were carried out as previously described (17-19).

GLC

Separations of the trimethylsilyl ethers of the fecal bile acids and neutral sterols by GLC were carried out as previously described (17-19).

Biliary bile acids were analyzed as the trifluoroacetate derivatives prepared with trifluoroacetic anhydride (Pierce Chemical Co., Rockford, Ill.). Quantitation of the trifluoroacetates was carried out on a 3% QF-1 column on 100-120 mesh Supelcoport (Supelco, Bellefonte, Pa.) using external standards. Operating temperatures for the column, inlet, and detector were 230 C, 245 C, and 245 C, respectively.

Methods for Isolation and Quantitation of Acidic Steroids from Feces

The methods for the isolation of the fecal bile acids have been described in detail (17,18). Quantitation of the material in the acidic steroid fraction was carried out by GLC of the trimethylsilyl ethers. 5α-Cholestane was added as an internal standard. Correction for losses during the procedure was made using 3α,7α-dihydroxy-12-keto-5β-cholanoic acid as an internal standard. β-Sitosterol was used to correct for fecal flow (17).

Liver and Biliary Cholesterol Concentration

These methods have been described in detail (17). Quantitation was carried out by GLC using the trimethylsilyl ether derivatives.

Isolation and Quantitation of Biliary Bile Acids

A sample of bile (0.1 ml) was mixed with 2 ml 2N NaOH and heated in an autoclave for 3 hr at 16 psi. The solution was cooled in ice and acidified with HCl to pH 1-2. The bile acids were extracted with chloroform. Solvents were evaporated and the bile acid methyl esters prepared using methanol-5% HCl as previously described (17). Quantitation of the biliary bile acids was carried out by preparation of the trifluoroacetates.

Measurement of Radioactivity

For radioactivity determinations, samples were placed into scintillation vials containing 0.3 ml water, and 14 ml scintillation solution containing 5 g 2,5-diphenyloxazole and 100 g naphthalene/liter dioxane was added. The samples were counted in a Beckman LS 200B liquid scintillation system. Corrections for background, crossover, and quench were made where required.

RESULTS

Rats of both strains were injected with sodium phenobarbital daily during the 8 day experimental period. Control animals of both strains were studied concurrently. The animals in each group had similar initial wt (229-236 g). The Sprague-Dawley rats gained an average of 56 g during the experimental period, while the Wistar rats gained only 21 g on the average. The average daily food intake of the Sprague-Dawley rat was 27 g/day, while the Wistar rats on the same diet ingested only 20 g/day. The average daily fecal output of the Sprague-Dawley rats was 8.5 g/day and of the Wistar rats was 5.2 g/day.

The results in Table I compare the cholesterol concentrations in the liver and bile of the rats at the time of sacrifice. Liver cholesterol concentrations were similar in the phenobarbital and control groups of both strains of rat. Biliary cholesterol concentrations were significantly lower in the phenobarbital-treated animals (0.12 mg/ml in Wistar group and 0.14 mg/ml in the Sprague-Dawleys) as compared to the controls (0.21 mg/ml in the Wistars and 0.23 mg/ml in the Sprague-Dawley rats). Plasma

TABLE I

Effects of Phenobarbital upon Cholesterol Concentration in Liver, Plasma, and Bile

Number of animals and treatment[a]	Liver wt (g)	Liver cholesterol concentration[b] (mg/g)	Bile cholesterol concentration[b] (mg/ml)	Plasma cholesterol concentration[b] (mg/100 ml)
Wistar-phenobarbital treated				
5				
Average ± SEM[c]	13.5[d] ± 0.50	1.98 ± 0.04	0.12[d] ± 0.00	71 ± 2.8
Range	12.7 – 15.3	1.87 – 2.10	0.11 – 0.13	65 – 80
Wistar-control				
3				
Average ± SEM	9.9 ± 0.83	2.06 ± 0.33	0.21 ± 0.03	69 ± 1.3
Range	8.8 – 11.5	1.61 – 2.70	0.16 – 0.24	67 – 70
Sprague-Dawley-phenobarbital treated				
5				
Average ± SEM	17.5[e] ± 0.69	1.90 ± 0.32	0.14[e] ± 0.01	80 ± 2.7[f]
Range	17.0 – 18.6	1.57 – 2.20	0.12 – 0.16	74 – 88[f]
Sprague-Dawley-control				
3				
Average ± SEM	13.5 ± 0.50	2.10 ± 0.16	0.23 ± 0.04	86 ± 3.0[f]
Range	12.5 – 14.2	1.94 – 2.27	0.21 – 0.24	70 – 97[f]

[a]Wistar and Sprague-Dawley rats were fed a stock diet of rat chow supplemented with corn oil. The rats were injected with sodium phenobarbital (100 mg/kg body wt) each day during the 8 day experimental period.

[b]The cholesterol concentrations in liver, bile, and plasma were determined at the end of the experiment.

[c]SEM = standard error of the mean.

[d]Differs from Wistar control group ($p<0.01$).

[e]Differs from Sprague-Dawley control group ($p<0.01$).

[f]Values reported are for rats studied under similar conditions.

cholesterol of the Wistar rats was not changed significantly by phenobarbital administration.

The fecal acidic steroid output was determined on day 6 and day 8 of the experimental period. These results are summarized in Table II. A significant increase in daily fecal acidic steroids was observed in the Wistar rats treated with phenobarbital (5.3 mg/rat/day to 10.6 mg/rat/day, $p<0.01$). In the Sprague-Dawley strain, phenobarbital administration caused only a slight increase in the fecal acidic steroid output (9.5 mg/rat/day to 11.6 mg/rat/day, $p>0.01$).

The activity of the rate-limiting enzyme of bile acid synthesis (cholesterol 7α-hydroxylase) was measured in both strains of rats and is summarized in Table II. In the Wistar strain treated with phenobarbital, we found the cholesterol 7α-hydroxylase activity to be enhanced, averaging 2-3 times that of the controls (13.5 picomoles 7α-hydroxycholesterol formed/mg protein/min in treated rats and 5.8 in the controls) (6). Similar enhancement of enzyme activity (22 pmoles/mg protein/min for the treated rats as compared to 10.5 for controls) was obtained when using the isotope derivative method (15). The Sprague-Dawley rats showed no significant differences in enzyme activity between control and phenobarbital-treated animals. The slight decrease in enzyme activity in the treated group was within the error limits of the assay. The Sprague-Dawley strain does not show the increased enzyme activity for cholesterol 7α-hydroxylase observed in the Wistar strain.

The activity of the microsomal 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase was compared to the percent of cholic acid in the bile of the phenobarbital and control rats. The results are summarized in Table III. A 55% decrease in the 12α-hydroxylase activity was found in the phenobarbital-treated Wistar rats, and a slightly smaller decrease (40%) was found in the phenobarbital-treated Sprague-Dawley rats compared to the controls. The contribution of cholic acid to total biliary bile acid decreased from 85% in

TABLE II

Effects of Phenobarbital upon Fecal Acidic Steroid Output and Cholesterol 7α-Hydroxylase Activity

Animal and treatment	Number of animals	Number of fecal pools	Daily acidic steroid output[a] mg/rat/day	Cholesterol 7α-Hydroxylase activity[b] (day 8) pmoles/mg protein/min
Wistar-phenobarbital treated	5	10		
Average ± SEM			10.6[c] ± 0.87	13.5[c] ± 1.1
Range			8.0 – 13.2	11.6 – 17.1
Wistar-control	3	6		
Average ± SEM			5.3 ± 0.51	5.8 ± 0.4
Range			4.1 – 7.3	4.6 – 6.4
Sprague-Dawley-phenobarbital treated	5	10		
Average ± SEM			11.6 ± 0.52	10.4 ± 1.1
Range			8.55 – 13.05	8.7 – 14.1
Sprague-Dawley-control	3	6		
Average ± SEM			9.5 ± 1.25	12.1 ± 0.4
Range			5.25 – 13.23	10.6 – 13.5

[a]Rat feces were dried and weighed, and an aliquot was extracted with ethanol to remove the neutral and acidic steroids as described previously (17). The acidic steroids were isolated and purified by thin layer chromatography. Quantitation was accomplished by gas liquid chromatography of the trimethylsilyl ether derivatives. The values reported were corrected for losses using 3α, 7α-dihydroxy-12-keto-5β-cholanoic acid as internal standard as described.

[b]The activity of cholesterol 7α-hydroxylase ± standard error of the mean (SEM) of Wistar and Sprague-Dawley rats was determined at the end of the 8 day experimental period.

[c]Differs from Wistar control group ($p<0.01$).

both strains of control animals to ca. 65% in the phenobarbital-treated groups. The following bile acids predominated in the bile of both strains: cholic, chenodeoxycholic, deoxycholic, α-muricholic, and β-muricholic.

DISCUSSION

It has been suggested that phenobarbital is potentially useful in the treatment of gallstones, since it produces a significant lowering of the relative cholesterol concentration in the bile in treated animals (20-22). We have confirmed this finding: biliary cholesterol concentrations of treated rats of both strains were decreased significantly. This decreased biliary cholesterol has been explained on the basis of an increased bile-salt independent flow (22). It also has been suggested that phenobarbital increases bile acid pool size in the rat (23). The increase in pool size along with the decreased biliary cholesterol concentration seems to indicate that phenobarbital could increase cholesterol solubility in bile. Other studies have shown, however, that phenobarbital administration does not reduce the saturation of cholesterol in the bile of the hamster (21) or in man (20) and that its value as a therapeutic agent in gallstone disease should be reconsidered. In the rat, we also have found that the cholesterol concentrations in both liver and plasma remained near normal after phenobarbital, though the wt of the liver increased significantly (ca. 25%). The increase in liver wt has been attributed to a proliferation of the smooth endoplasmic reticulum (1-3).

Cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis, is associated with the endoplasmic reticulum (3). Its activity has been reported to reflect the rate of bile acid synthesis in vivo (17). The present study shows that phenobarbital administration increased the fecal bile acid output in the Wistar rat by 100% but only by 20% in the Sprague-Dawley strain. The activity of the rate-limiting enzyme of bile acid synthesis was measured in both strains of rat using the isotope incorporation method. Cholesterol 7α-hydroxylase activity/mg protein was found to be elevated in the Wistar strain (6) but remained near normal levels in the Sprague-Dawley strain. Thus, the activity of cholesterol 7α-hydroxylase seems to reflect the biosynthetic state in vivo.

The rats in this study also have been examined to determine whether a correlation

TABLE III

Activity of Microsomal 7α-Hydroxycholest-4-en-3-one 12α-Hydroxylase and Amount of Cholic Acid in Bile

Animal and treatment	12α-Hydroxylase activity[a] pm/mg protein/min	Amount of cholic acid in bile[b] percent of total
Wistar-phenobarbital treated		
Average ± SE[c]	51[d] ± 5.3	65[d] ± 2.3
Range	43-61	57-70
Wistar-control		
Average ± SE[c]	113 ± 3.5	85 ± 1.0
Range	109 - 116	83-87
Sprague-Dawley-phenobarbital treated		
Average ± SE[c]	33 ± 5.2	66[e] ± 5.6
Range	23-46	51-73
Sprague-Dawley-control		
Average ± SE[c]	47 ± 6.5	85 ± 1.0
Range	38-55	82-86

[a]The activity of the microsomal 12α-hydroxylase of both strains of rat was determined at the end of the experimental period (day 8).

[b]The total biliary bile acids were determined by gas liquid chromatography of the trifluoroacetate derivatives of the bile acid methyl esters. The numbers represent the average amount of cholic acid (and its bacterial metabolite deoxycholic) in the bile of the phenobarbital and control animals.

[c]SE = standard error.

[d]Differs from Wistar control group ($p<0.01$).

[e]Differs from Sprague-Dawley control group ($p<0.01$).

existed between the activity of the microsomal 12α-hydroxylase and the amount of cholic acid in the bile. It had been reported that the 12α-hydroxylase was considered an important factor which might regulate the amount of cholic acid in the bile. In our study, Wistar and Sprague-Dawley rats treated with phenobarbital showed a decrease in specific acitivity of 12α-hydroxylase of 55% and 40%, respectively, as compared to control animals in each strain. The phenobarbital also produced an acute change in the composition of the biliary bile acid pool. The amount of taurocholic acid in the bile decreased from an average of 85% of total bile acids in the control animals to ca. 65% in the phenobarbital-treated group. It, therefore, appears that the measurement of the in vitro activity of the microsomal 12α-hydroxylase may have value in estimating the relative proportion of taurocholic acid in the bile of the rat. Einarsson and Johansson (5) reported the importance of the 12α-hydroxylase in regulating cholic acid formation. Others also have reported that the activity of 12α-hydroxylase decreased with phenobarbital treatment, but the composition of the biliary bile acids was not measured (8,24). The activity of the microsomal 12α-hydroxylase is known to be affected by the thyroid state. In rats, treatment with thyroid hormone decreased the activity of the 12α-hydroxylase (25,26). The decrease in activity was not sufficient to account for the drastic reduction in the taurocholic acid level of the bile; the authors concluded that the ratio of 12α/26-hydroxylase

$$\frac{\text{7α-hydroxycholest-4-en-3-one 12α-hydroxylase}}{\text{3α, 7α-dihydroxy-5β-cholestane 26-hydroxylase}}$$

activity determined the amount of cholic acid in the bile (15). In contrast, the decrease of 12α-hydroxylase activity in the phenobarbital-treated rats alone seems to be sufficient to account for the moderate decrease in biliary cholic acid secretion (Table III).

The biliary bile acids were analyzed as the trifluoroacetate derivatives. The majority of the total biliary bile acids were trihydroxy bile acids (ca. 88% in the control groups and ca. 72% in the phenobarbital-treated rats). Using Wistar rats, Paumgartner, et al., (27) reported that the trihydroxy bile acid fraction of the bile comprised only 35% of the biliary bile acids in both the phenobarbital and control animals. In these experiments, no change in biliary bile acid composition was observed with phenobarbital treatment of 3 or 7 days. The reason for the

failure of phenobarbital to alter the biliary bile acid composition in these experiments might be attributed to the lower dose of phenobarbital used (4 mg/100 g body wt compared to 10 mg/100 g body wt in our experiments).

From these experiments in the Wistar and Sprague-Dawley rats, it seems likely that the in vitro assay of cholesterol 7α-hydroxylase activity reflects the state of bile acid synthesis in vivo and that the in vitro activity of the microsomal 12α-hydroxylase reflects the proportion of cholic acid present in the bile.

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Incorporation of Radioactive Polyunsaturated Fatty Acids into Liver and Brain of Developing Rat

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ABSTRACT

The incorporation of radioactivity from orally administered linoleic acid-1-^{14}C, linolenic acid-1-^{14}C, arachidonic acid-$^{3}H_8$, and docosahexaenoic acid-^{14}C into the liver and brain lipids of suckling rats was studied. In both tissues, 22 hr after dosing, 2 distinct levels of incorporation were observed: a low uptake (from 18:2-1-^{14}C and 18:3-1-^{14}C) and a high uptake (from 20:4-$^{3}H_8$ and 22:6-^{14}C). In adult rats, the incorporation of radioactivity into brain lipids from 18:2-1-^{14}C and 20:4-^{3}H was considerably lower than the incorporation into the brains of the young rats. In the livers of the suckling rats, the activity from the 18 carbon acids was associated mostly with the triglyceride fraction, whereas the activity from the 20:4-$^{3}H_8$ and 22:6-^{14}C was concentrated in the phospholipid fraction. In the brain lipids, the activity from the different fatty acids was associated predominantly with the phospholipids. In the liver and brain phospholipid fatty acids, some of the activity in the 18:2-1-^{14}C and 18:3-1-^{14}C experiments was associated with 20 and 22 carbon polyunsaturated fatty acids; however, radioactivity from orally administered 20:4-$^{3}H_8$ and 22:6-^{14}C was incorporated intact into the tissue phospholipid to a much greater extent compared with the incorporation of radioactivity into 20:4 and 22:6 in the experiments where 18:2-1-^{14}C and 18:3-1-^{14}C, respectively, were administered. Possible reasons for these differences are discussed. Rat milk contains a wide spectrum of polyunsaturated fatty acids, including linoleate, linolenate, arachidonate, and docosahexaenoate. During the suckling period in the rat, there is a rapid deposition of 20:4 and 22:6 in the brain. The results of the present experiments suggested that dietary 20:4 and 22:6 were important sources of brain 20:4 and 22:6 in the developing rat.

INTRODUCTION

Long chain polyenoic fatty acids are of particular interest in relation to the mammalian brain, since, in a wide variety of mammals, the brain grey matter phosphoglycerides are characterized by the presence of large amounts of 20:4ω6, 22:4ω6, and 22:6ω3 and by low levels of 18:2ω6 and 18:3ω3 (1). In the laboratory rat, a significant proportion of brain development occurs during the suckling period (2) and more than 70% of the long chain polyenoic fatty acids (20:4 and 22:6) in the rat brain are laid down by the end of the suckling period (3,4). Rat milk contains both linoleate and linolenate, as well as their longer chain metabolic products, such as 20:3ω6, 20:4ω6, 22:4ω6, 20:5ω3, 22:5ω3, and 22:6ω3 (4,5).

To investigate the possible role of dietary polyenoic fatty acids in contributing to brain lipids, we have examined the tissue uptake of a series of radioactive fatty acids which were administered orally to suckling rat pups. Some preliminary results of these experiments have been published elsewhere (5,6).

METHODS AND MATERIALS

Animals and Diets

Rat pups, 16-17 days old, were used in these experiments. They were bred from female rats of the Wistar strain which were maintained on a semisynthetic diet (7). The diet contained 14.4% of its calories as fat, which was a mixture of soybean oil and linseed oil (SBOL) (5:1, v/v) and the linoleic:linolenic ratio in the diet was 3.3:1. The animals were mated when ca. 4 months old. During the first 24 hr after birth, large litters were reduced to 9 pups, and, if the size of the litter fell below 6 during the suckling period, the litter was not used. Owing to the association between litter size, body growth, and the extent of brain development in rats (2), the litter size was controlled in the above manner so that results from different litters could be compared. Three adult female rats (300 g) also were used after they had been on the above diet for 12 months.

Radioactive Experiments

Linoleic acid-1-^{14}C (61 mCi/mmole), α-linolenic acid (60 mCi/mmole), and arachidonic

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TABLE I

Incorporation of Radioactivity from Labeled Fatty Acids into Liver and Brain Lipids of Suckling and Adult Rats

Time	Isotope	Liver lipids: Percent dose	Liver lipids: Specific[a] activity	Brain lipids: Percent dose	Brain lipids: Specific activity
Suckling rats[b]					
22 hr	18:2-1-^{14}C (6)[c]	2.69 ± 0.24	155 ± 14.5	0.44 ± 0.05	29.1 ± 3.17
	20:4-$^{3}H_8$ (9)	14.9 ± 1.58	1050 ± 120	2.06 ± 0.36	157 ± 28.0
	18:3-1-^{14}C (3)	3.29 ± 0.32	151 ± 8.89	0.29 ± 0.03	18.5 ± 2.74
	22:6-^{14}C (4)	19.8 ± 1.32	1360 ± 153	2.71 ± 0.79	197 ± 57.0
48 hr	18:2-1-^{14}C (4)	1.84 ± 0.08	81.9 ± 4.74	0.42 ± 0.02	23.7 ± 1.84
	20:4-$^{3}H_8$ (3)	7.84 ± 0.68	341 ± 37.7	1.66 ± 0.19	88.9 ± 5.33
	18:3-1-^{14}C (3)	0.85 ± 0.15	60.6 ± 10.9	0.36 ± 0.05	26.1 ± 4.71
Adult rats[d]					
22 hr	18:2-1-^{14}C (3)	4.17 ± 0.17	54 ± 2.6	0.039 ± 0.003	1.2 ± 0.3
	20:4-$^{3}H_8$ (3)	19.3 ± 0.10	222 ± 0.4	0.134 ± 0.004	3.4 ± 0.1

[a]Specific activity = dpm/mg lipid/μCi dose.

[b]18:2-1-^{14}C And 20:4-$^{3}H_8$ were injected simultaneously in 6 and 3 rats at 22 and 48 hr, respectively. The dose ratio ($^{3}H/^{14}C$) was 1.2:1 at 22 hr and 1.3:1 at 48 hr.

[c]The numbers in parenthesis represent the numbers of rats used. The results are shown as the mean ± standard error of mean.

[d]Isotopes injected simultaneously; the dose ratio ($^{3}H/^{14}C$) was 0.4:1.

acid-1-^{14}C (54 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, U.K. Arachidonic acid-$^{3}H_8$, methyl ester (32 mCi/mmole) was a gift from Unilever Research, Vlaardingen, The Netherlands, and docosahexaenoic acid-^{14}C, methyl ester (1.3 mCi/mmole) was prepared biosynthetically in this laboratory (8). Between 1-4 μCi each isotope was given to each rat, except in the 22:6-^{14}C experiments where 0.03 μCi was used. A known amount of a radioactive fatty acid (in solvent) was introduced into a vial containing 0.3 ml olive oil, and the solvent was evaporated with a stream of N_2. The oil and isotope mixture then was drawn into a glass syringe (with a blunt 18 gauge needle) and administered orally to unanesthetized animals. The isotope content of the residue in the syringe and vial was estimated, giving by difference the actual dose. This usually amounted to ca. 70% of the activity introduced into the vial (see above). In some experiments, linoleic-1-^{14}C and arachidonic-$^{3}H_8$ were administered simultaneously. The radiochemical purity of the isotopes, determined by gas liquid chromatography (GLC) of the methyl esters, was better than 98%, except for the 22:6-^{14}C where 7% of the activity was associated with 22:5ω3.

Lipid Extraction and Liquid Scintillation Counting

After dosing, the pups were returned to their mothers and sampled at 22 and 48 hr. Animals were killed by decapitation and the liver and brain quickly removed, washed in ice-cold saline, blotted dry, and weighed. These tissues then were extracted in chloroform-methanol (2:1, containing 10 mg/liter 2:6-di-*tert*-butyl-*p*-cresol as an antioxidant). The lipid extracts were washed according to the Folch procedure (9), and the total lipid wt was estimated by weighing dried aliquots of the lipid extract. Aliquots of the total lipids also were assayed for radioactivity by liquid scintillation counting using a Packard Tri-Carb model 3000 scintillation spectrometer. The scintillation solution consisted of 4 g diphenyloxazole (PPO) and 0.2 g diphenyloxozole-benzene (POPOP)/liter toluene. The counting efficiency for carbon 14 was normally ca. 75% and for double-label experiments (^{14}C and ^{3}H), ca. 23% for ^{3}H and ^{14}C in the mixed channel, and 25% for ^{14}C in the carbon only channel. The efficiency of counting was determined by use of internal standards of n-hexadecane-1-^{14}C and n-hexadecane-1,2-^{3}H (The Radiochemical Centre, Amersham, U.K.).

Aliquots of tissue lipids were separated by thin layer chromatography (TLC) using Silica Gel G as the adsorbent and light petroleum (bp 40-60)-diethyl ether-glacial acetic acid (85-15-5 or 90-10-1) as the solvents. The fractions were detected under UV light after spraying the TLC

TABLE II

Percentage Distribution[a] of Isotopes in Liver and Brain Lipid Fractions[b]

Isotope	18:2-1-^{14}C	20:4-$^{3}H_8$	18:3-1-^{14}C	22:6-^{14}C
Liver lipid fraction				
Triglyceride	69 ± 3	20 ± 2	82 ± 2	28 ± 3
Phospholipid	25 ± 3	78 ± 2	14 ± 2	70 ± 3
n[c]	6	6	4	4
Brain lipid fraction				
Cholesterol	14 ± 1	1 ± 0.3	26 ± 1	ND[d]
Phospholipid	84 ± 1	96 ± 1	72 ± 1	ND
n	6	4	4	

[a]The results are expressed as a percentage of the total isotope recovered from five different lipid fractions of the liver and brain (cholesteryl ester, triglyceride, free fatty acids, cholesterol + diglyceride, and phospholipid). The distribution was checked each time using two solvent systems (see "Methods and Materials"). The results are shown as mean ± standard error of mean.

[b]The pups were killed 22 hr after dosing with the different fatty acids.

[c]The number of separate experiments.

[d]ND = not determined.

plates with a methanolic solution (0.2%) of dichlorofluorescein. The radioactivity in the different fractions separated by TLC (phospholipids [PL], cholesterol, free fatty acids, triglycerides [TG], and cholesteryl esters) was measured by the elution of the samples from silica gel with 1 ml hyamine hydroxide (1 M in methanol) and 10 ml scintillation solution (5). This method proved unsatisfactory when estimating the distribution of ^{14}C and ^{3}H together in double-label experiments because of the quenching of ^{3}H by the hyamine. In these experiments, the TLC fractions were eluted from the silica gel with 40 ml solvent (chloroform-acetone-methanol-water, 10-1-5-0.1), and the samples were counted following evaporation of the solvents. Recoveries of 95.3 ± 0.2% for ^{14}C and 94.1 ± 0.2% for ^{3}H (mean ± standard error of mean for 12 determinations) were obtained.

Brain lipids also were treated with chloroform-0.2 N methanolic sodium hydroxide (2:1) (10) to convert ester-bound fatty acids to methyl esters. The lipid extract from this reaction was separated on TLC using light petroleum (bp 40-60)-diethyl ether-glacial acetic acid (90-10-1) as the solvent, and the fractions (fatty acid methyl esters, cholesterol, and alkali-stable lipids) were eluted from the silica gel and assayed for radioactivity.

The distribution of the radioactivity in the fatty acids of tissue TG and PL was determined by the separation and fraction collection of the methyl esters of these lipids using a preparative GLC (5). The methyl esters were prepared as described previously (4), and the preparative GLC was carried out using a glass column 2.1 m in length x 7 mm inside diameter packed with 10% ethylene glycol succinate methyl silicone polymer (EGSS-X) on Diatomite C-AW 60-70 mesh (W.G. Pye and Co., Cambridge, U.K.) at 190 C. The carrier gas flow rate was 170 ml/min.

Fatty acid fractions were decarboxylated by the Schmidt procedure as described by Goldfine and Bloch (11).

RESULTS

Incorporation of Activity from Radioactive Fatty Acids into Total Lipids of Liver and Brain

In the 16-17 day old rat pups (22 and 48 hr after dosing), there was a substantially greater incorporation of radioactivity from 20:4-$^{3}H_8$ and 22:6-^{14}C into the total lipids of liver and brain by comparison with the incorporation of activity from 18:2-1-^{14}C and 18:3-1-^{14}C (Table I). With all fatty acids, there was a greater recovery of activity in the liver lipids compared with brain lipids.

In adult rats, there was also a greater recovery of activity from 20:4-^{3}H in liver and brain lipids by comparison with the uptake of ^{14}C from linoleic acid-1-^{14}C (Table I).

The percentage of the dose of 18:2-1-^{14}C and 20:4-$^{3}H_8$ recovered in the brain lipids of the adult rats was less than 10% of the values obtained in the suckling rats, whereas the recovery of the dose in the liver lipids was of the same order of magnitude in adult and suckling rats. The values obtained for the adult rats are in close agreement with the results published in the literature for the recovery of activity from orally administered 18:2-1-^{14}C

TABLE III

Percentage Distribution[a] of Radioactivity in Fatty Acids of Liver Triglycerides

Fatty acid fraction[b]	Wt%[c]	18:2-1-^{14}C 22 hr	18:3-1-^{14}C 22 hr	20:4-$^{3}H_8$ 22 hr	22:6-^{14}C 22 hr
Solv.-14:0	5.7	---[d]	---	---	---
16:0-16:1	21.6	---	---	---	---
18:0-18:1	22.0	---	---	---	---
18:2	19.4	93 ± 1.2[e]	---	---	---
18:3-20:1	3.0	3.6 ± 0.4	80 ± 1.7	---	2
20:2-20:3	1.5	1.6 ± 0.3	---	1.4 ± 0.2	---
20:4	4.5	---	2.5 ± 0.4	79 ± 0.7	1
22:1-20:5	2.4	---	7.5 ± 0.8	3.1 ± 0.4	3
22:4-22:5	1.2	---	---	15 ± 0.6	1
22:5	5.6	---	5.6 ± 0.9	1.5 ± 0.5	8
22:6	8.0		1.8 ± 0.3		86
n		(3)	(3)	(3)	(1)[f]

[a]The results are expressed as the percentage of radioactivity in a fraction relative to the total radioactivity collected for all fractions.

[b]Under the gas liquid chromatographic conditions used the 18:3-20:1 fraction would include 18:3ω6, 18:3ω3, 20:0, and 20:1; the 20:2-20:3 fraction would include 20:2ω9, ω6, 18:4ω3, and 20:3ω6; fraction 20:4 includes 20:4ω6, 20:3ω3, and 22:0; fraction 22:1-20:5 includes 22:1, 20:4ω3, and 20:5ω3; and fraction 22:4-22:5 includes 22:4ω6, 22:5ω6, 24:0, and 24:1.

[c]Fatty acid composition (wt %) of liver triglycerides from 16-17 day old rats.

[d]Less than 1%.

[e]The number of separate analyses is shown in parenthesis and the mean value ± standard error of mean is shown. Fractions with greater than 10% of the total activity are underlined.

[f]In the 22:6-^{14}C experiment the liver TG from four animals were pooled prior to analysis.

and 20:4-1-^{14}C in liver and brain lipids of adult rats (12-14).

Recovery of Radioactivity in Whole-Body Lipids of 16-17 Day Old Suckling Rats

The recovery of radioactivity from the whole body lipids, 22 hr after dosing of the pups with 20:4-1-^{14}C, 22:6-^{14}C, 18:2-1-^{14}C, and 18:3-1-^{14}C, was (as percent of administered dose): 80.4 ± 2.1, 65.4 ± 3.2, 46.8 ± 2.0, and 51.1 ± 2.4, respectively (mean ± standard error of mean for 3 animals in each group, except the 22:6-^{14}C group where 4 animals were used). The arachidonic acid value was significantly greater ($p<0.05$) than the other three values.

Distribution of Radioactivity in Liver and Brain Lipid Fractions

In the liver lipids, radioactivity from the 4 different fatty acids was found in either the TG or PL with less than 5% of the activity being associated with the free fatty acids, cholesterol, or cholesteryl esters. Twenty-two hr after dosing, the activity from the 18 carbon acids (18:2 and 18:3) was concentrated in the TG, whereas, for the longer chain acids (20:4 and 22:6), the activity was associated predominantly with the PL (Table II).

In the brain lipids, the radioactivity from the different fatty acids mostly was found in the PL fraction, but, with the carboxyl-labeled fatty acids (18:2 and 18:3), some 14-26% of the activity also was found in the TLC-fraction corresponding to cholesterol (Table II). Diglycerides have similar R_f values to cholesterol; however, cleavage of the brain glyceride-ester lipids (see "Methods and Materials") showed that this activity was still associated with cholesterol. Although not reported in Table II, the distribution in the liver and brain lipids at 48 hr was very similar to the distribution shown for 22 hr.

Distribution of Radioactivity in Fatty Acids of TG and PL

There were differences in the distribution of the isotope in the individual fatty acids of the three fractions examined (liver TG, PL, and brain PL).

In the liver TG and PL, the majority of the radioactivity was associated with the fatty acid which had been administered (Tables III and IV). An exception to this occurred in the liver PL in the linolenic acid-1-^{14}C experiment where most of the activity was associated with the 22:6, 22:5, and 20:5 fractions. In the brain PL fatty acids in the linoleic acid-1-^{14}C experi-

TABLE IV

Percentage Distribution[a] of Radioactivity in Fatty Acids of Liver Phospholipids

Fatty acid fraction[b]	Wt %[c]	18:2-1-^{14}C		18:3-1-^{14}C		20:4-$^{3}H_8$		22:6-^{14}C
		22 hr	48 hr	22 hr	48 hr	22 hr	48 hr	22 hr
Solv.-14:0	0.6	---[d]	---	---	---	---	---	---
16:0-16:1	24.4	---	---	3.5 ± 0.6	4.4 ± 0.3	---	---	1
18:0-18:1	24.4	1.1 ± 0.3[e]	1.1 ± 0.3	3.7 ± 0.5	7.0 ± 0.3	---	---	1
18:2	9.3	83 ± 1.5	65 ± 3.7	---		---	---	---
18:3-20:1	0.1	2.3 ± 0.5	2.8 ± 0.8	8.7 ± 0.8	8.1 ± 0.8	---	---	1
20:2-20:3	0.9	2.1 ± 0.2	2.5 ± 0.6	1.0 ± 0.1	---	---	---	---
20:4	19.4	7.4 ± 1.0	23 ± 3.8	5.9 ± 0.3	5.4 ± 0.4	94 ± 1.1	93 ± 1.7	---
22:1-20:5	---	---	1.1 ± 0.3	11 ± 0.7	4.5 ± 0.4	4.4 ± 0.9	4.8 ± 1.4	1
22:4-22:5	0.5	---	---	2.9 ± 0.7	2.1 ± 0.2	1.3 ± 0.1	1.4 ± 0.3	2
22:5	3.0	1.5 ± 0.3	1.9 ± 0.4	26 ± 1.5	19 ± 2.4	---	---	3
22:6	14.7			36± 3.7	44 ± 3.1	---	---	90
n		(6)	(4)	(3)	(3)	(8)	(3)	(1)[f]

[a,b,d,e,f]See footnotes Table III.

[c]Fatty acid composition (wt %) of liver phospholipids from 16-17 day old rats.

ment, the radioactivity was associated with the 16:0 + 16:1, 18:0 + 18:1, 18:2, and 20:4 fractions (Table V), whereas, in the linolenic acid-1-^{14}C experiment, the activity mostly was found in the saturated and monounsaturated fatty acids and a little in the 22:6 fraction. In the arachidonic acid-$^{3}H_8$ and docosahexaenoic acid-^{14}C experiments, most of the activity was associated with the 20:4 and 22:6 fractions, respectively.

In the linolenic acid-1-^{14}C experiment, very little radioactivity was associated with the linolenate fraction of the brain PL (Table V). However, in the brain PL, 18:3ω3 amounts to only 0.1% of the total fatty acids. The calculated relative specific activity (RSA) of the brain linolenate was 0.09. This value was similar to the calculated RSA for brain 18:2, 20:4, and 22:6 in the 18:2-1-^{14}C, 20:4-$^{3}H_8$, and 22:6-^{14}C experiments. In these experiments, the RSA were found to be 0.10, 0.13, and 0.13, respectively. (The RSA was calculated as follows: for example, in the 18:2-1-^{14}C experiment, 22 hr after dosing, percent dose in brain lipids = 0.44% (Table I); activity in brain PL as percent of total brain lipids = 84% (Table II); activity in brain PL 18:2 as percent of total PL fatty acids = 31% (Table V); and the wt percent of 18:2 in brain PL fatty acids = 1.2%. Therefore, RSA = 0.44 x 0.84 x 0.31 ÷ 1.2 = 0.10).

Incorporation of Radioactivity from 18:2-1-^{14}C, 18:3-1-^{14}C, and 20:4-1-^{14}C into Brain Lipids

In view of the difference in the incorporation of radioactivity from 1-^{14}C fatty acids (18:2 and 18:3) and ^{3}H-labeled arachidonic acid into brain cholesterol (Table II) and saturated and monounsaturated fatty acids (Table V), it was decided to study the incorporation of radioactivity from 20:4-1-^{14}C into brain lipids. In this experiment, 3 animals were used, and it was found that 3% of the activity in the brain lipids was associated with cholesterol (1% in the 20:4-$^{3}H_8$ experiment, Table II), and ca. 6% of the ^{14}C in brain PL was found in saturated and monounsaturated fatty acids (0.2% in ^{3}H experiment). These differences may, in part, be accounted for by the use of the different isotopes (20:4-^{3}H vs 20:4-^{14}C) which could result in a selective loss of ^{3}H to body water relative to ^{14}C during the oxidation of arachidonic acid to acetyl CoA and CO_2 and during the recyclization of the isotope to other compounds, e.g. fatty acids and cholesterol.

When the results for three different carboxyl-carbon labeled fatty acids (18:2, 18:3, and 20:4) were calculated as a percentage of the dose appearing in different fractions of the brain lipids, the incorporation of ^{14}C into cholesterol and saturated plus monounsaturated fatty acids was the same for each fatty acid (Table VI). The differences in the incorporation of radioactivity from these three 1-^{14}C fatty acids into the total lipids of the brain could be accounted for entirely by the differences in the incorporation of the ^{14}C into the polyunsaturated acids of the brain.

Decarboxylation Studies

In both the linoleic acid-1-^{14}C and linolenic acid-1-^{14}C experiments, the low activity in the carboxyl carbon atoms of the 20:4ω6, 20:5ω3, and 22:5 + 22:6ω3 fractions (Table VII)

TABLE V

Percentage Distribution[a] of Radioactivity in Fatty Acids of Brain Phospholipids

Fatty acid fraction[b]	Wt %[c]	18:2-1-^{14}C		18:3-1-^{14}C		20:4-$^{3}H_8$		22:6-^{14}C
		22 hr	48 hr	22 hr	48 hr	22 hr	48 hr	22 hr
Solv.-14:0	1.5	---[d]	---	---	---	---	---	---
16:0-16:1	28.8	21 ± 1.0[e]	21 ± 1.8	39 ± 1.8	36 ± 0.6	---	---	---
18:0-18:1	32.4	14 ± 0.6	17 ± 0.8	26 ± 1.1	32 ± 2.6	---	---	7
18:2	1.2	31 ± 0.8	18 ± 1.4	1.8 ± 0.2	2.0 ± 0.2	---	---	
18:3-20:1	0.4	2.0 ± 0.4	2.1 ± 0.3	4.4 ± 0.5	2.5 ± 0.1	---	---	
20:2-20:3	0.5	4.7 ± 0.5	4.2 ± 0.4	---	---	---	---	3
20:4	13.1	24 ± 0.9	31 ± 2.8	3.0 ± 0.4	1.6 ± 0.6	85 ± 1.1	83 ± 3.1	
22:1-20:5	0.1	1.2 ± 0.2	1.2 ± 0.2	3.1 ± 0.2	1.3 ± 0.1	2.3 ± 0.4	3.3 ± 0.7	2
22:4-22:5	4.0	2.9 ± 0.2	4.3 ± 0.3	2.4 ± 0.3	2.7 ± 0.2	11 ± 1.1	12 ± 1.0	6
22:5	0.3	1.0 ± 0.2	1.0 ± 0.1	15 ± 1.9	2.3 ± 0.1	1.0 ± 0.2	1.1 ± 0.2	4
22:6	14.3				15 ± 1.1			78
n		(6)	(4)	(3)	(3)	(5)	(3)	(1)[f]

[a,b,d,e,f]See footnote Table III.

[c]Fatty acid composition (wt %) of brain phospholipids from 16-17 day old rats.

TABLE VI

Incorporation of Radioactivity from Carboxyl-Labeled Fatty Acids into Total Lipids, Cholesterol, and Fatty Acids of Brain

	Percentage of dose recovered in brain lipids			
			Phospholipid fatty acids[a]	
Isotope	Total lipid	Cholesterol[b]	16:0 to 18:1	18:2 to 22:6
18:2-1-^{14}C	0.47 ± 0.12[c]	0.07 ± 0.002	0.16 ± 0.001	0.25 ± 0.01
18:3-1-^{14}C	0.29 ± 0.03	0.07 ± 0.007	0.19 ± 0.03	0.03 ± .004
20:4-1-^{14}C	2.58 ± 0.14	0.07 ± 0.003	0.16 ± 0.02	2.35 ± 0.12

[a]The fatty acid methyl esters from brain phospholipids were fractionated using preparative gas liquid chromatography (see "Methods and Materials"), and two fractions were collected: from 16:0-18:1 and from 18:2-22:6.

[b]Separated from total lipids by TLC (see "Methods and Materials").

[c]The results are from 3 separate experiments with each isotope (t = 22 hr) and are presented as the mean ± standard error of mean.

suggested that the ingested fatty acids were incorporated intact into their respective longer chain metabolites.

In the brain, the carboxyl-carbon activity in the 16 carbon fatty acids was very close to the value predicted for de novo synthesis from an acetate molecule with only one of its two carbon atoms labeled. Such a molecule would be produced by β-oxidation of an even chain fatty acid labeled in the carboxyl position. In the 18 carbon saturated and monounsaturated acids of the brain, the result for the carboxyl-carbon activity suggested that a combination of de novo synthesis from acetate and chain elongation of 16 carbon acids was responsible for the labeling of these acids.

Incorporation of Radioactivity from Linoleic Acid-1-^{14}C and Arachidonic Acid-$^{3}H_8$ into Tissue 20:4

There was a greater incorporation of radioactivity from the exogenous arachidonate (-$^{3}H_8$) into tissue 20:4 compared with the endogenous formation of arachidonate from linoleic acid-1-^{14}C (Table VIII). Similarly, by calculation of the amount of activity in the 22:6 of liver and brain PL from 22:6-^{14}C compared with the incorporation of ^{14}C into 22:6 from linolenic acid-1-^{14}C, it was shown that the former compound yielded 95 times and 59 times as much activity in the 22:6 of liver and brain, respectively.

Linoleic acid is the chief precursor of arachidonate, and a possible reason for the difference

TABLE VII

Decarboxylation Studies on Fatty Acids Collected from Preparative Gas Liquid Chromatography

Fatty acid fraction	Relative carboxyl activity[a] 18:2-1-^{14}C (3)[b] Liver phospholipid 22 hr	Brain phospholipid 22 hr	18:3-1-^{14}C (3) Liver phospholipid 22 hr	Brain phospholipid 22 hr
16:0 + 16:1	---[c]	0.13	---	0.13
18:0 + 18:1	---	0.17	---	0.19
18:2	0.91	0.99	---	---
18:3	---	---	0.96	---
20:4	0.08	0.03	---	---
20:5	---	---	0.05	---
22:5+22:6	---	---	0.03	0.03

[a]Radioactivity in the -COOH group/activity in total fatty acid.

[b]The number of separate experiments is shown in parenthesis, and the results are presented as the mean. The relative carboxyl activity also was determined for the liver and brain fatty acids at 11 and 48 hr after dosing rats with linoleic acid-1-^{14}C, and the results were almost identical to those shown for 22 hr.

[c]Not determined.

in the $^{3}H/^{14}C$ ratio in the tissue arachidonate could be the dilution of the linoleic-1-^{14}C during dosing.

In the preceding double-label experiments, the 18:2-1-^{14}C and 20:4-$^{3}H_8$ were given to the pups in 0.3 ml olive oil. This oil contained 8% of its fatty acids as 18:2, and, therefore, 0.3 ml oil would provide ca. 23 mg linoleate. This would dilute the specific activity of the 18:2-1-^{14}C in the dose relative to the 20:4-$^{3}H_8$. To test the effect of the specific activity of the dose upon the uptake of ^{3}H-20:4 and ^{14}C-18:2 into tissue lipids, pups were dosed with the olive oil-isotope mixture to which was added 14, 43, or 86 mg methyl arachidonate (Table IX). The addition of the cold arachidonate was associated with a small decrease of the $^{3}H/^{14}C$ ratio in the total lipids of liver and brain; however, it had little effect upon the $^{3}H/^{14}C$ ratio in the arachidonate fraction of the liver and brain PL. Therefore, even when the specific activity of the 20:4-$^{3}H_8$ was lower than the specific activity of the 18:2-1-^{14}C (experiments 3 and 4, Table IX), the ^{3}H incorporation was still significantly greater than the ^{14}C incorporation into tissue 20:4.

In these experiments, the diet fed to the dams had a linoleic to linolenic ratio of 3.3 to 1. The incorporation of radioactivity from linoleic acid-1-^{14}C and arachidonic acid-$^{3}H_8$ into tissue lipids also was studied in pups whose mothers were fed a diet in which the linoleic to linolenic ratio was 48:1 (Table X). The feeding of this diet (which was almost completely devoid of linolenic acid) was associated with an increased conversion of linoleic to arachidonic acid in the liver PL (decreased $^{3}H/^{14}C$ in 20:4). However, there was no change in the $^{3}H/^{14}C$ ratio in the 20:4 of the brain PL.

TABLE VIII

Incorporation of Radioactivity from 20:4-$^{3}H_8$ and 18:2-1-^{14}C into Liver and Brain Arachidonate[a]

Time (hr)	n[c]	Ratio of $^{3}H/^{14}C$[b] Liver phospholipid 20:4	Brain phospholipid 20:4
22	6	140 ± 11[d]	22 ± 2.1
48	3	73 ± 10	14 ± 2.3

[a]Methyl arachidoante was isolated from the total phospholipid methyl esters by preparative gas liquid chromatography (see "Methods and Materials").

[b]This ratio = $^{3}H/^{14}C$ of collected 20:4 ÷ $^{3}H/^{14}C$ of dose. The dose ratio was 1.2:1 in the 22 hr experiment and 1.3:1 in the 48 hr experiment.

[c]Number of separate experiments.

[d]Results are presented as the mean ± standard error of mean.

DISCUSSION

In the developing rat, the incorporation of radioactivity from 20:4-$^{3}H_8$ and 22:6-^{14}C into liver and brain total lipids was very much greater than the incorporation of radioactivity from 18:2-1-^{14}C and 18:3-1-^{14}C. In the liver, this difference could be accounted for by the faster incorporation of radioactivity from the longer chain acids into PL and TG. In the brain, the differences could be explained entirely by a faster incorporation of radioactivity from the longer chain acids (20:4 and 22:6) into the PL fraction.

Previous workers have demonstrated that radioactivity from 18:2-1-^{14}C, 18:3-1-^{14}C, and 20:4-1-^{14}C is incorporated into the adult rat brain (12-15). This finding was confirmed in

TABLE IX

Incorporation of Radioactivity from 20:4-$^{3}H_8$ and 18:2-1-^{14}C into Tissue Lipids and Tissue Arachidonate: Effect of Dilution of Dose with Carrier Methyl Arachidonate

Experiment	Isotopes	Carrier oil: Olive oil	Carrier oil: Me 20:4	Ratio of $^{3}H/^{14}C$[a]: Liver total lipid	Liver phospholipid 20:4	Brain total lipid	Brain phospholipid 20:4
1[b]	18:2-1-^{14}C plus 20:4-$^{3}H_8$	0.3 ml[c]	0 mg	4.6	140	3.1	22
2[d]		0.3	14	4.3	78	2.9	18
3		0.3	43	4.6	140	2.4	19
4		0.3	86	4.0	150	1.9	20

[a]This ratio = $^{3}H/^{14}C$ in fraction ÷ $^{3}H/^{14}C$ in dose.

[b]Six animals in this experiment (dose ratio $^{3}H/^{14}C$ = 1.2:1). Data from Table VIII.

[c]Estimated 18:2 content of 0.3 ml oil = 23 mg.

[d]In experiments 2-4, there was one pup/experiment (dose ratio $^{3}H/^{14}C$ = 1.3:1). In these experiments, the olive oil-isotope mixture contained three different levels of methyl arachidonate (Me 20:4, >99% pure, NuChek Prep.).

the present experiments (Table I) where it also was demonstrated that there was a greater uptake of radioactivity from 20:4-$^{3}H_8$ by comparison with 18:2-1-^{14}C in the adult brain.

For all fatty acids, the uptake of radioactivity by the adult rat brain was markedly lower than the uptake in the developing rat brain (Table I). Although this difference might be accounted for by a difference in the permeability of brain membranes in rats of different ages, it also could be a reflection of the rapid rate of deposition of 20:4ω6, 22:4ω6, and 22:6ω3 in the developing rat brain (3,4).

Although radioactive 20:4 and 22:6 were isolated from the liver and brain lipids following the dosing of the pups with 18:2-1-^{14}C and 18:3-1-^{14}C, respectively, in the liver, at least, the bulk of the radioactivity was still present as the administered fatty acid. Thus, the comparison of the uptake of the radioactivity from orally administered 20:4-$^{3}H_8$ with the appearance of radioactivity in 20:4 from orally administered 18:2-1-^{14}C showed that, in both liver and brain, most of the radioactivity in the 20:4 fraction was derived from the orally administered 20:4 (Table VIII). This pattern also was observed when the 22:6 formation from 18:3-1-^{14}C was compared with the direct incorporation of 22:6-^{14}C.

Several factors may have influenced the different uptake of radioactivity from linoleic acid-1-^{14}C compared with arachidonic acid-$^{3}H_8$. First, in the initial experiments, the specific activity of the 18:2-1-^{14}C was lower than that of the 20:4-$^{3}H_8$ during dosing. When the arachidonic acid-$^{3}H_8$ specific activity was reduced below that of the linoleic acid-1-^{14}C, the radioactivity in the liver and brain 20:4 was still mostly derived from the arachidonic acid-$^{3}H_8$. Second, the SBOL diet ("Methods and Materials") contained both linoleic and linolenic acids, and it is known that linolenic acid can reduce the conversion of linoleic to arachidonic acid (16-18). In the absence of dietary linolenate (SSO diet, Table X), there was an increased formation of arachidonate (^{14}C) from linoleic acid-1-^{14}C in the liver PL; however, despite this, the majority of the radioactivity in the liver and brain PL arachidonate was derived from the orally administered 20:4-$^{3}H_8$.

Although linoleic acid is converted to arachidonic acid in the body, to explain the consistently greater uptake of dietary (exogenous) arachidonate by liver and brain lipids compared with the endogenous formation of 20:4 from dietary linoleate, the factors discussed below should be taken into account:

Linoleic acid is oxidized to CO_2 faster than arachidonic acid (19), and this may explain the

TABLE X

Effect of Dietary Fatty Acids upon Incorporation[a] of Radioactivity from 18:2-1-^{14}C and 20:4-$^{3}H_8$ into Liver and Brain Fatty Acids

Fatty acid fraction	Liver phospholipid		Brain phospholipid	
	Diet SBOL[b]	Diet SSO[c]	Diet SBOL	Diet SSO
18:2	83[d]	44	31	24
18:3-20:1	2.3	3.3	2.0	1.7
20:2-20:3	2.1	2.6	4.7	6.6
20:4	7.4	35	24	16
$^{3}H/^{14}C$ in 20:4	140 ± 11[e]	28 ± 3	22 ± 2	22 ± 1

[a]The pups were killed 22 hr after dosing with 18:2-1-^{14}C and 20:4-$^{3}H_8$. The dose ratio ($^{3}H/^{14}C$) in the soybean oil and linseed oil (SBOL) experiment was 1.2:1 and 0.7:1 in the safflower seed oil (SSO) experiment.

[b]SBOL diet (see "Methods and Mateials"), results from Tables IV, V, and VIII.

[c]SSO diet = same gross composition as SBOL diet, except that fat was supplied as safflower seed oil. Linoleic to linolenic ratio in this diet was 48:1 and in the SBOL diet, 3.3:1. Mean of results from three animals.

[d]The results are expressed as the percentage of radioactivity in a fraction relative to the total radioactivity collected for all fractions.

[e]This ratio = $^{3}H/^{14}C$ in collected 20:4 ÷ $^{3}H/^{14}C$ in dose.

retention of 80% of the ^{14}C in the whole body lipids following the dosing of pups with 20:4-1-^{14}C compared with a retention of only 47% of the ^{14}C when pups were dosed with 18:2-1-^{14}C.

The turnover of linoleic acid is faster than arachidonic acid in tissue lipids (20,21); this may mean that linoleic acid is more frequently exposed to the β-oxidation enzymes than arachidonic acid.

In the liver, linoleic acid-1-^{14}C was preferentially incorporated into TG, whereas arachidonic acid-$^{3}H_8$ was concentrated in the PL. If it is assumed that this distribution applied to all tissues and organs, then it would result in a dilution of the linoleic acid-1-^{14}C relative to arachidonic acid-$^{3}H_8$ owing to the different pool sizes of TG and PL in the whole animal.

The slow rate of conversion of linoleic acid to arachidonic acid may, in part, be accounted for by the substrate competition and end-product inhibition of the enzymes involved in this process (16,17). In the present experiments, substrates and end-products are found in the pups' diet (4,5).

The greater uptake of ^{14}C from 22:6-^{14}C compared with 18:3-1-^{14}C into the tissue lipids of developing rats may be accounted for by similar processes to those described above.

In view of these observations, it would be wrong to equate 1 molecule of dietary linoleic and linolenic acids with 1 molecule of dietary arachidonic and docosahexaenoic acids, respectively. Thus, although only relatively small amounts of these longer chain acids (20:4ω6 and 22:6ω3) are present in the pups' diet (milk) during this period of brain development, quantitatively they may be of considerable importance in supplying tissues with longer chain fatty acids.

A significant amount of the radioactivity in the brain lipids was associated with cholesterol and the saturated and monounsaturated fatty acids following the administration of carboxyl-labeled fatty acids (Tables II, V, and VI). It was calculated that, as a proportion of the administered dose of 18:2, 18:3, and 20:4 (all 1-^{14}C), the extent of labeling of cholesterol, saturated, and monounsaturated fatty acids in the brain was 8, 4, and 5 times, respectively, greater than the labeling of the same compounds in the liver. In these experiments, the radioactivity in the carboxyl-carbon of brain 16:0 and 16:1 suggested that these fatty acids were being synthesized de novo from acetate. This acetate was most probably derived by the β-oxidation of the fed carboxyl-labeled fatty acid. Acetate is an efficient precursor of cholesterol and saturated and monounsaturated fatty acids, and, in suckling rats, the brain has a marked preference over the liver for incorporating carboxyl-labeled acetate into lipids (22,23). This preference may be explained by the observations that, during the suckling period, the rate of synthesis and chain elongation of fatty acids in the brain is greater than that of the liver (24,25).

The high content of 20:4, 22:4, and 22:6 relative to 18:2 and 18:3 in rat brain is well recognized, and, indeed, this pattern has been observed in the brains of a number of different mammalian species (1). This pattern could originate because of a rapid desaturation and

chain elongation of 18:2 and 18:3 in the brain. Alternatively, it may be a result of a specific uptake of longer chain polyunsaturated fatty acids by the brain. The results of the present experiments demonstrate that the polyunsaturated fatty acids of the developing rat brain are derived from two sources, one endogenous and the other exogenous. The endogenous source is that formed in the liver from dietary precursors (18:2ω6 and 18:3ω3) and supplemented in part by that formed in the brain in situ. A larger source (exogenous) is the preformed dietary polyunsaturated acids (20:4ω6 and 22:6ω3).

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Polar Lipids of *Macrophomina phaseolina*[1]

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ABSTRACT

Macrophomina phaseolina was grown on a defined medium at three different carbon/nitrogen ratios. The lipids of the mycelia and the sclerotia were extracted; fractionated into polarity groups; and separated by thin layer, column, and gas liquid chromatographies. Sclerotia contained higher levels of neutral lipids and lower amounts of polar lipids than mycelia. The neutral lipid content of sclerotia increased, up to 77% of total lipids, and phospholipids decreased as carbon/nitrogen ratio increased from 10 to 320. The glycolipid content was not altered significantly by changes in carbon/nitrogen ratios. Although cardiolipin could not be detected in sclerotial polar lipids, both sclerotia and mycelia contained similar phospholipid profiles with major quantitative differences. Phosphatidic acid and phosphatidyl glycerol were major components of sclerotia, whereas phosphatidyl ethanolamine and phosphatidyl inositol were the major phosphatides of mycelia. Phosphatidyl choline was present in both mycelia and sclerotia. The fatty acid distribution did not show any particular pattern of saturation or unsaturation due to differences in carbon/nitrogen ratio. However, mycelial lipids tended to contain C24:1, C18:3, and C22:1 as major fatty acids, whereas the major fatty acids in sclerotial lipids were C18:2, C18:1, C22:1, C20:0, and C16:1. Saturated fatty acids were present in lesser concentrations.

INTRODUCTION

It has been established firmly that phospholipids in membranes are in a constant state of metabolic flux, with the rate of synthesis and degradation often varying with the physiological activity of the organism. Sclerotial development in some fungi may be regarded as a response to unfavorable conditions which keeps the organism alive until favorable conditions return. The sclerotia formed by *Macrophomina phaseolina* (Tassi) Goid. are multicellular propagules, with each cell containing the necessary organelles required for germination and growth (1). To date, the sclerotial lipids of relatively few fungi have been examined (2-7) and only with regard to their fatty acid composition. In the present investigation, we studied the polar lipids and the total fatty acid contents of *M. phaseolina* extracted from both the mycelial and sclerotial stages grown on three different carbon/nitrogen (C/N) ratios.

MATERIALS AND METHODS

M. phaseolina was grown on defined medium contained sucrose 11.6, 92.5, and 370 g (C/N ratios of 10, 80, and 320, respectively); sodium nitrate, 3 g; potassium dihydrogen phosphate, 1 g; magnesium sulfate, 0.5 g; potassium chloride, 0.5 g; ferrous sulfate, 0.01 g; and distilled water, 1000 ml. Portions of the culture medium (25 ml) were dispensed into 250 ml flasks, autoclaved for 15 min, seeded with agar plugs containing *M. phaseolina*, and incubated at 22 C for 5 days. Sclerotia of *M. phaseolina* were harvested from cultures grown on media previously described but solidified with agar (2%), dispensed in 9 cm Petri dishes covered with sterile cellophane, and incubated at 22 C. After 21 days, the sclerotia were scraped from the cellophane, passed through a 250μ mesh screen, and ground in a Bronwill homogenizer for 1 1/2 min in 20 ml isopropanol using 5 ml 0.45-0.50 mm glass beads.

Materials: Sephadex G-25, coarse, beaded, was purchased from Pharmacia Fine Chemicals, New Market, N.J. Silicic acid was the Unisil 100-200 mesh product of Clarkson Chemical Co., Williamsport, Pa. All chemicals used were AR grade. All solvents were redistilled, and butylated hydroxytoluene was added to prevent autoxidation. Chloroform was stabilized by distillation into enough methanol to give a final concentration of 0.25% (v/v). Concentrated ammonium hydroxide was prepared by bubbling gaseous ammonia into ice-cold distilled water in a plastic bottle until 28% by wt of ammonia was introduced. Solvents for extraction and column chromatography were degassed under reduced pressure and reequilibrated to atmospheric pressure with pure nitrogen.

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TABLE I

Separation of Lipids of *Macrophomina phaseolina* into Polarity Groups by Silicic Acid Column Chromatography

Silicic acid column fraction	Percent of total lipids[a]					
	Carbon/nitrogen 10		Carbon/nitrogen 80		Carbon/nitrogen 320	
	Mycelia	Sclerotia	Mycelia	Sclerotia	Mycelia	Sclerotia
Neutral lipids	13.1 (1.8)	18.7 (0.78)	5.9 (0.24)	54.3 (0.51)	10.1 (0.32)	75.2 (1.49)
Glycolipids	61.4 (8.2)	13.6 (0.57)	58.8 (2.37)	26.3 (0.25)	47.8 (1.51)	16.5 (0.35)
Phospholipids	23.1 (3.1)	65.3 (2.72)	33.3 (1.34)	19.4 (0.18)	36.9 (1.16)	5.1 (0.10)
Percent recovery	97.6	97.6	98.0	100.0	94.8	96.8

[a]Figures in parentheses represent percent lipid content on the basis of oven-dry wt of tissue.

Extraction and fractionation of lipids: Lipids of the mycelia were extracted by the hot isopropanol procedure (8) as modified by Wassef and Hendrix (9). Sclerotial lipids were extracted by grinding sclerotia in isopropanol in a Bronwill homogenizer under liquid CO_2 (Bronwill Scientific, Rochester, N.Y.). The sonicate then was treated for lipid extraction, as in the case of mycelia mentioned above. The extracts from 2-5 g (mycelia or sclerotia) were applied to a 2.5 x 15 cm Sephadex G-25 column to separate lipids from nonlipid contaminants (10). Lipids were separated into polarity groups by silicic acid column chromatography (10).

The method described by Rouser, et al., (10) was used for wt determination by the electrobalance procedure and for preventing autooxidation of lipids. Wts were determined on fraction 1, chloroform-methanol (19:1 v/v) saturated with water from the Sephadex columns, and all fractions separated by the silicic acid columns.

Analytical procedures: The total lipids (fraction 1 from Sephadex G-25 column) were hydrolyzed and the fatty acids esterified by heating in 4% methanolic/sulfuric acid in a sealed tube overnight at 75-80 C. The fatty acid methyl esters were extracted with n-hexane and then fractionated by gas liquid chromatography (GLC) using a Varian Aerograph 2100 gas chromatograph equipped with a flame ionization detector. The column was a 6 ft glass, U-shaped column with an inside diameter of 2 mm, packed with 10% diethyleneglycol succinate (DEGS) on Chromosorb G support (100-120 mesh). The column temperature was programed between 60-180 C at 8 C/min. The flame ionization detector was operated at 150 C with 35 ml/min hydrogen and 400 ml/min air. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min.

Qualitative and quantitative analyses of the total lipid extracts (Sephadex G-25 fraction 1) and silicic acid column fractions were made by the two dimensional thin layer chromatography (TLC) system of Rouser, et al., (10) and the silicic acid-impregnated paper chromatographic procedure of Marinetti (11,12). Specific sprays and dip reagents were used according to Kates (8,13). The identities of the phospho- and glycolipids were established by comparison of R_f values and cochromatography of appropriate standards (obtained from Supelco, Bellefonte, Pa.). The lipids were visualized on TLC by exposure to I_2-vapors, and the plates were photographed (by Brinkmann photocopier, Brinkmann Instruments, Westbury, N.J.). Individual lipid components were aspirated from the plates. Glycolipids were measured quantitatively by the procedures described by Dittmer and Wells (14) or by Kates (13). Total phospholipid-phosphorus was determined on TLC plates as described by Rouser, et al., (15) and on paper chromatography as described by Kates (8).

RESULTS

Total lipid yield: The amount of total lipids extracted from mycelia was markedly higher than those extracted from sclerotia. At a C/N ratio of 10, the total lipids accounted for ca. 13.1% of the mycelial dry wt, whereas they accounted for only 4.2% in sclerotia. At a C/N ratio of 80 and 320, the mycelial lipid content dropped to 4.0 and 4.8%, respectively. Sclerotial lipids also decreased to 0.9 and 1.9% at C/N ratios of 80 and 320, respectively.

Lipid polarity groups: Mycelial and sclerotial lipids were separated into polarity groups by

TABLE II

Fatty Acid Composition of Total Lipids of *Macrophomina phaseolina*

	Percent of total fatty acids					
	Carbon/nitrogen 10		Carbon/nitrogen 80		Carbon/nitrogen 320	
Fatty acid	Mycelia	Sclerotia	Mycelia	Sclerotia	Mycelia	Sclerotia
C8:0	trace	---	trace	---	1.7	---
C10:0	6.6	5.8	9.2	3.8	37.2	trace
C12:0	---	---	---	---	trace	---
C14:0	---	---	---	---	1.8	---
C16:0	2.9	13.1	2.7	trace	2.6	trace
C16:1	trace	17.2	trace	trace	trace	trace
C18:0	4.6	trace	4.6	6.4	trace	1.8
C18:1	trace	trace	trace	24.0	trace	16.2
C18:2	trace	49.0	1.3	3.5	trace	18.9
C18:3	30.8	12.9	15.9	1.3	25.0	23.4
C20:0	---	1.9	1.2	---	trace	38.4
C20:1	trace	trace	trace	trace	trace	trace
C22:1	14.3	---	10.8	52.4	13.5	trace
C24:1	39.0	trace	48.8	---	16.1	---

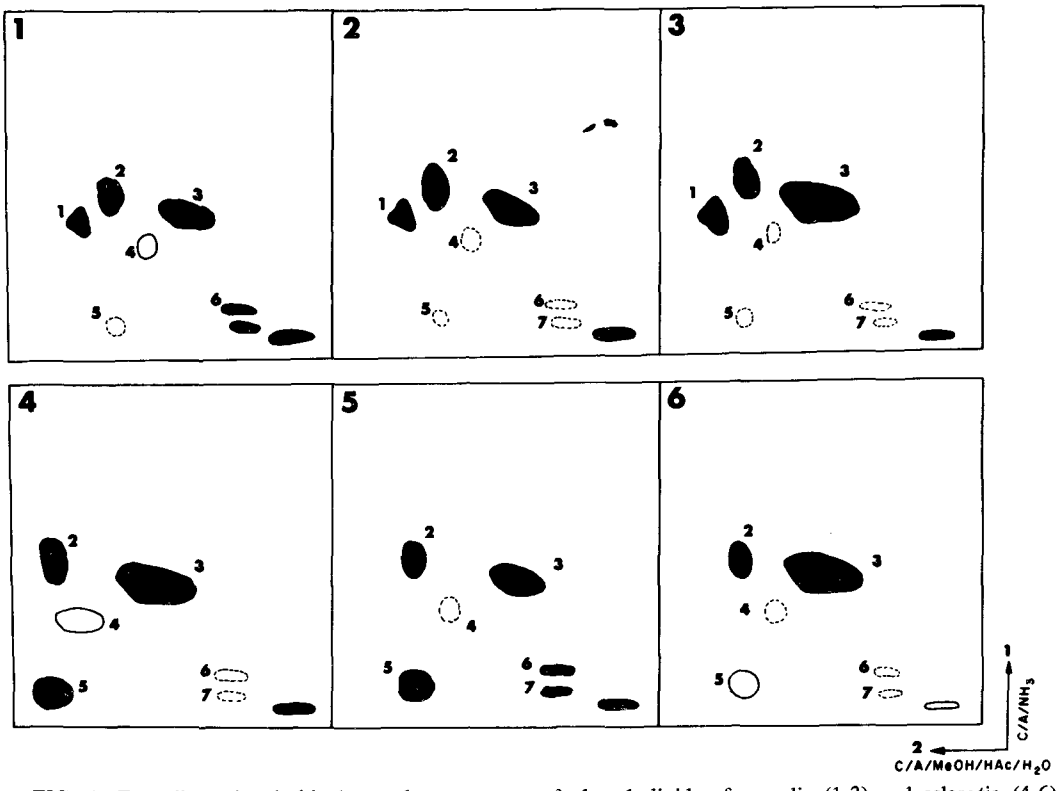

FIG. 1. Two dimensional thin layer chromatogram of phospholipids of mycelia (1-3) and sclerotia (4-6) grown on carbon/nitrogen ratio of 10, 80, and 320, respectively. 1. Diphosphatidyl glycerol (cardiolipin). 2. Phosphatidyl ethanolamine. 3. Phosphatidyl choline (lecithin). 4. Phosphatidyl glycerol. 5. Phosphatidic acid. 6. Phosphatidyl inositol. 7. Phosphatidyl serine and origin. Staining behavior and quantitative composition are given in Table III. Developing solvents are those described in ref. 10. Compounds present in higher, medium, and lower concentrations are represented by shaded, circled, and dotted lines, respectively.

silicic acid column chromatography. Mycelial neutral lipids constituted 13.1, 5.9, and 10.1% of the total lipid content (1.8, 0.2, and 0.3% on tissue dry wt basis) for organisms grown on media with C/N ratios of 10, 80, and 320, respectively (Table I). Except for C/N 10, these

TABLE III

Chromatographic Analysis of Phosphatides of *Macrophomina phaseolina*

Spot[a] no.	Ninhydrin	Periodate	Percent of total lipid-phosphorus						Tentative identity of compounds
			Carbon/nitrogen 10		Carbon/nitrogen 80		Carbon/nitrogen 320		
			P, Percent of total		P, Percent of total		P, Percent of total		
			Mycelia	Sclerotia	Mycelia	Sclerotia	Mycelia	Sclerotia	
1	−ve	−ve	7.2	0.0	9.1	0.0	12.9	0.0	Cardiolipin
2	+ve	−ve	22.9	13.6	32.6	18.3	20.5	15.6	Phosphatidyl ethanolamine
3	−ve	−ve	32.8	50.6	47.1	24.0	61.6	76.5	Phosphatidyl choline
4	−ve	+ve	4.1	11.5	1.7	3.4	<1.0	<1.0	Phosphatidyl glycerol
5	−ve	−ve	<1.0	9.6	<1.0	18.6	<1.0	3.9	Phosphatidic acid
6	−ve	−ve	11.7	1.0	<1.0	13.0	<1.0	<1.0	Phosphatidyl inositol
7	+ve	−ve	9.8	1.9	<1.0	15.2	<1.0	<1.0	Phosphatidyl serine
8	+ve	+ve	11.4	11.8	9.5	7.6	5.0	4.1	Origin

[a]See Figure 1.

values were seven-nine times higher for the sclerotial neutral lipids than for mycelial neutral lipids (Table I). The main neutral lipid constituents tentatively were identified as diglycerides and free fatty acids. Mycelia were considerably higher (two-five times) in glycolipids than were sclerotia (Table I). These differences were especially notable when lipid content was expressed on a tissue dry wt basis (Table I). Monogalactosyl and digalactosyl diglycerides tentatively were identified as the main constituents of the glycolipid fractions. The phospholipids comprised ca. one-third of the total lipids extracted from the mycelia of *M. phaseolina* grown at C/N ratios of 80 and 320. Ca. two-thirds of the total lipids extracted from sclerotia produced on media with a C/N ratio of 10 were phosphatides. This value decreased to ca. 20% at C/N 80 and 5% at C/N 320 (Table I).

Fatty acid composition: The fatty acid composition of the total lipids extracted from sclerotia and mycelia of *M. phaseolina* grown on C/N 10, 80, and 320 is presented in Table II. Qualitatively, mycelial lipids has similar fatty acid compositions at all C/N ratios. The major fatty acids were C18:3, C22:1, C24:1, and C10:0 with minor and trace amounts of C8:0, C16:0, C16:1, C18:0, C18:1, C18:2, and C20:0. The fatty acid composition of sclerotial lipids differed considerably from the mycelial lipids and varied with C/N ratio. While C16:0, C16:1, C18:2, and C18:3, were the major fatty acids extracted from sclerotia grown at C/N 10, the C18:1, C22:1 and C18:1, C18:2, C18:3, and C20:0 fatty acids were the major components isolated from sclerotia grown at C/N of 80 and 320, respectively (Table II). Trace amounts of other fatty acids were detected.

Phospholipid analysis: The complex mixture of phospholipids obtained in fraction 4 of the silicic acid column (S4) could not be resolved completely into individual components by one dimensional paper chromatography (11-13). A more effective procedure, however, was attained by chromatography on two dimensional TLC as shown in Figure 1; staining behavior and quantitative composition of mycelial and sclerotial phosphatides are given in Table III. Under all the growth conditions employed, mycelial phosphatides contained 7-13% diphosphatidyl glycerol (cardiolipin), but sclerotia lacked this compound (Fig. 1, Table III). On the other hand, sclerotial phospholipids contained 4-18-fold as much phosphatidic acid as mycelial phospholipids (Table III). Higher proportions of phosphatidyl ethanolamine also were present in the lipids of the mycelia as compared to the sclerotia in all C/N ratios

(Table III). Phosphatidyl choline was present at lower levels in mycelial phosphatides than in the phosphatides of sclerotia, except at a C/N of 80. Phosphatidyl inositol and phosphatidyl glycerol concentrations were 3 and 12 times higher in sclerotia grown at a C/N 10 than in mycelia. These concentrations decreased, however, as C/N ratio increased (Table III). Changes in phosphatidyl serine levels in mycelia and in sclerotia could not be connected to differences in the C/N ratio.

DISCUSSION

The results reported herein are the first to compare the lipids extracted from mycelia and sclerotia of a single fungal species grown on three different C/N ratios.

Mycelia and sclerotia of *M. phaseolina* differ considerably in lipid content. Neutral lipids were low in mycelia and high in sclerotia. The higher neutral lipid content in sclerotia was not unexpected in view of the fact that sclerotia are structures which exist in a dormant condition for long period of time. Since storage lipids are usually neutral, it is conceivable that the neutral lipids of sclerotia may serve as carbon and energy reserves.

The glycolipid containing fraction of the mycelia was exceptionally high in *M. phaseolina*, and study of the nature of the individual components is warranted.

The phospholipid content was expected to be higher in mycelia than in sclerotia due to the presence of active phospholipases accompanying fungal maturation (16,17). We cannot find a satisfactory explanation for the lower phosphatide content of mycelia grown at C/N 10 compared to the corresponding sclerotia. The seven phospholipids found in mycelia (Table III) have been shown previously to be normal phospholipid constituents in fungi present at varying concentrations, depending upon several physiological conditions (16,17). There are considerable differences, however, in the phospholipid levels of sclerotia and mycelia grown at the same C/N ratio (Table III). Cardiolipin could not be detected in sclerotial phosphatides; its importance in membrane structure and function remains to be seen. The concentrations of phosphatidic acid were higher, and phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl serine lower in sclerotia than in mycelia (Table III). These results suggest that phospholipases are present and active in mycelia and are able to hydrolyze phospholipids to phosphatidic acid as the mycelia produces sclerotia. There were no lysophosphatides detected in mycelia or sclerotia suggesting that phospholipase A_2 might be absent or inoperative.

The fatty acid composition of Eukaryotes has been a subject of interest for many investigators. In *Euglena gracilis* (18), ciliate protozoa (19), yeasts (20), basidiomycetes (21-23), and imperfect fungi (24), the fatty acids of the polar lipids have been shown to be more unsaturated than those of the neutral lipids. The situation in phycomycetous fungi (16) and flagellate protozoa (25) is different, since neutral and polar lipids show no differential fatty acid distribution. No conclusive pattern of distribution appears to exist in the mycelial and sclerotial lipids in *M. phaseolina*. Whether this observation is a feature of sclerotia forming fungi or whether it is an effect of the C/N ratio growing conditions remains to be determined. It would be of interest to compare fatty acid composition of neutral and polar lipids of the mycelial and sclerotial stages of *M. phaseolina*.

It is interesting to speculate on the implications of some of the differences in lipid composition of the mycelium and the sclerotia found in *M. phaseolina*. Distinct differences in the wt percent of the neutral and polar lipids between the sclerotia and the mycelia were shown. The complete characterization of all lipid components might provide a degree of insight into the survival mechanism of sclerotia. Furthermore, it might develop a better understanding of this resistant propagative unit in terms of how much catabolizable material is present which can be used as a source of energy for germination. In many respects, sclerotia may be compared with seeds regarding lipid composition. High concentrations of lipids in sclerotia probably function in a manner similar to those of seeds. Lipids not only provide the energy required for germination but also the energy required to maintain a low level of respiration for extended periods of time, a prerequisite for dormant, resistant propagative units, such as sclerotia.

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Distribution of Fatty Acids during Germination of Cottonseeds

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ABSTRACT

Gas chromatographic determination of the fatty acids in the seeds of cottonseed (*Gossypium sp.*) showed linoleic, palmitic, oleic, and stearic acids, with linoleic acid being the major component. Changes in the composition of fatty acids during various stages of germination were measured in the cotyledons and in the roots. A decrease in the content of all the fatty acids and an 8-fold increase in the moisture content of the cotyledons were observed during the 10 days of germination. There were no significant changes in the fatty acid contents of the roots with the exception of those in linoleic acid which increased by 50% during 4-10 days of germination. The possible significance of these changes is discussed.

INTRODUCTION

The U.S. is one of the biggest producers of soybean seeds and cottonseeds. Both of these are rich in protein and lipid, and these facts have created renewed interest in the studies regarding their chemical composition and genotypes. Hoffpauir, et al., (1) studied cottonseeds and reported that, among the different species, the percent of germination decreased with an increase in the content of free fatty acids in the seeds. The developing roots have an intricate system of membranes which are major sites of lipid accumulation. Also, studying fatty acid composition of the roots and cotyledons separately may give an insight into the metabolic interrelationship between the two organs.

Joshi, et al., (2) reported a decrease in the percent of palmitic and oleic acids in soybean cotyledons from 6-12 days of germination, while the percent of linoleic acid increased during the same period. A decrease in the percent of oleic acid during germination was interpreted to suggest that it was the immediate precursor for the synthesis of linoleic acid. Crombie, et al., (3) reported the composition of the fatty acid of *Citrullus vulgaris* seeds during germination and concluded that oleic was metabolizing rapidly. Grindley (4) reported the sequence of formation of fatty acids in the developing cottonseeds and observed that the percent of linoleic acid increased during maturation. Also, a gradual increase in the percent of total lipids occurred during the same period. The present report concerns the distribution of the fatty acids in cotyledons and roots of cottonseeds during the initial 10 days of germination.

MATERIALS AND METHODS

Seed

Cottonseeds were obtained from Wyart-Quarles Seed Co., Raleigh, N.C. All of the studies reported in this paper were conducted on three different batches of McNair 210.

Germination and Extraction of Lipid

Germination of the seeds was conducted in the dark at 37 C. At 4, 7, and 10 days following germination, the seedlings were separated into cotyledons and roots and were dried by the vacuum oven method prescribed by the Association of Official Analytical Chemists (5). The dried material was ground in a Waring blender and stored in a freezer.

Lipid material was extracted from an aliquot of the powder with petroleum ether using a Soxhlet apparatus. In the oilseeds, the triacylglycerols are not bound and comprise over 95% of the lipids which are extracted almost completely by the petroleum ether. The solvent was removed by evaporation at room temperature under a steady stream of nitrogen, and the extract was weighed and stored at refrigerator temperature.

Preparation of Derivatives

The fatty acids were obtained from the glycerides by alkaline hydrolysis (6), and the methyl esters were prepared for gas liquid chromatographic (GLC) analysis by the method of

TABLE I

Percentages of Lipid in Cottonseed Components during Germination

Seed component[a]	Days of germination		
	4	7	10
Cotyledons	22.5	17.0	9.8
	23.7	17.7	9.6
Roots	10.5	8.9	5.8
	11.1	9.8	5.6

[a]Seeds contained 24.4% lipid before germination. Glycerides comprised over 95% of this fraction. All determinations were carried out in duplicate.

TABLE II

Composition of Cotyledons (100 Seedlings)

Component[a]	Days of germination			
	0	4	7	10
Fresh wt (g)	9.4	10.7	19.0	26.3
	9.0	8.8	20.5	27.2
Dry wt (g)	8.4	4.8	4.5	4.5
	8.1	4.7	4.4	3.5
Moisture (%)	10.7	55.2	75.9	82.9
	10.0	48.0	78.5	87.2
Total fatty acids (mg)	2046	1065	812	440
	2501	1053	820	406
Palmitic (mg)	570	303	201	113
	697	299	208	110
Stearic (mg)	42	34	17	11
	52	33	18	11
Oleic (mg)	286	223	141	58
	352	222	146	57
Linoleic (mg)	1145	505	442	252
	1400	499	458	245

[a]The fatty acids were identified as methyl esters by comparing their retention time with that of authentic samples. The composition of the fatty acids was calculated on the basis of their gas chromatographic areas by the triangulation procedure. All the determinations were conducted on duplicate random samples of 100 seeds each.

TABLE III

Composition of Roots (100 Seedlings)

Component[a]	Days of germination		
	4	7	10
Fresh wt (g)	2.3	6.9	11.1
	2.4	6.3	11.0
Dry wt (g)	0.6	0.7	1.3
	0.6	0.6	1.0
Moisture (%)	73.9	89.9	88.3
	75.0	90.5	90.9
Total fatty acids (mg)	59	65	74
	61	61	72
Palmitic (mg)	21	22	22
	23	19	19
Stearic (mg)	1	1	1
	1	1	1
Oleic	9	7	9
	8	6	9
Linoleic (mg)	28	35	42
	29	35	43

[a]Procedure for the determination of fatty acids was the same as described in Table II.

Huston and Albro (7). Analysis of the lipid by the double development thin layer chromatographic (TLC) procedure of Skipski, et al., (8) showed that it contained less than 1% of the material as free fatty acids.

GLC

Methyl ester fractions obtained by the above procedure were diluted with n-heptane, and aliquots containing 0.05-0.1 mg mixture were injected into a Beckman GC 2A gas chromatograph. A 0.6 cm x 7.0 ft stainless steel column filled with 20% polydiethylene glycol succinate on acid-washed Chromosorb W was operated at a temperature of 220 C with helium as carrier gas and a current of 200 ma. Elution time was ca. 10 min. A standard solution containing a mixture of authentic samples of fatty methyl esters was run under identical experimental conditions prior to running samples. The retention times of the unknown samples of methyl esters were compared with the standards for identification purposes.

RESULTS

The results on the total lipid material at different stages of germination presented in Table I show that the seeds, prior to germination, contained 24.4% lipid which gradually changed to 9.6-9.8% in cotyledons and to 5.6-5.8% in roots after 10 days of germination.

In Table II are presented the results of the moisture contents and the levels of fatty methyl esters of cotyledons during various stages of germination. The cottonseeds, prior to germination, contained largely linoleic acid followed by palmitic, oleic, and stearic acids. During the 10 days of germination, the contents of all the 4 fatty acids decreased significantly in the cotyledons. At the end of this period, linoleic acid was still the major fatty acid, followed by palmitic, oleic, and stearic acids. The moisture content of the cotyledons increased throughout the germination, while the dry wt of the cotyledons decreased to one-half at 4 days of germination, mainly due to shedding of the seed

coat. The changes in fatty acid composition of the roots during 4-10 days of germination are presented in Table III. At 4 days of germination, the roots contained linoleic acid as the major component followed by palmitic, oleic, and stearic acids. At any given period of germination, the percentages of moisture in the roots were higher than in the cotyledons, while the dry wt and the fatty acid contents were lower in the roots than in the cotyledons. There were no significant changes in the fatty acid contents of the roots, with the exception of linoleic acid which increased by 50% during 4-10 days of germination.

DISCUSSION

The unsaturated fatty acids of cottonseeds were linoleic and oleic acids which together comprised over 75% of the total fatty acids. The remaining acids were palmitic and stearic acids. Cottonseed oil has been classified as "linoleic-rich" because linoleic acid alone constitutes over 50% of the total fatty acids.

Dutton and Mounts (9) studied photosynthesizing flax, soybean, and safflower plants exposed to $^{14}CO_2$ for a 1 hr period at seed-setting stage. Of the C_{18} unsaturated fatty acids, oleic acid was the first to acquire the radioactivity which subsequently and successively appeared in linoleic and linolenic acids. Gridley (4) used a combination of iodine value and the spectrophotometric method of Brown, et al., (10) and reported a decrease in the concentration of oleic and saturated acids and an increase in linoleic acid and total oil content of cottonseeds during maturation. The results of the present investigation show a gradual decrease in the percent of lipid material in the cotyledons and in the roots during the initial 10 day period of germination. Linoleic acid content of the cotyledons decreased by 80% during the 10 days of germination, while it increased by 50% in the roots during 4-10 days of germination. Whether the increase in linoleic acid in the roots was caused by a transfer from the cotyledons cannot be determined by the results of the present investigation. All other fatty acids decreased in the cotyledons, while they remained unchanged in the roots.

Soybean seedlings contained linolenic acid in the cotyledons and in the roots, but linolenic acid was absent from the cotton seedlings, while both types of seedlings contained linoleic acid as the major component (2). The differences in the pattern of fatty acids in cotyledons and roots during germination indicate the importance of analyzing individual components of the seedlings. A more detailed study of the enzyme kinetics of the desaturation and saturation reactions in the cell-free preparations using tracer techniques may explain the nature of the precursors of the linoleic acid in the roots of the cottonseed.

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Lipids of Cultured Hepatoma Cells: VI. Glycerolipid and Monoenoic Fatty Acid Biosynthesis in Minimal Deviation Hepatoma 7288C[1]

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ABSTRACT

1-^{14}C-Acetic, 1-^{14}C-palmitic, or 1-^{14}C-stearic acid was incubated with minimal deviation hepatoma 7288C cells grown in culture to assess: de novo fatty acid synthesis, oxidation, desaturation, and elongation of saturated fatty acids, as well as the ability of media fatty acids to serve as precursors of cellular glycerolipids. Distribution of radioactivity in the individual lipid classes and the various fatty acids of triglyceride, phosphatidyl choline, and phosphatidyl ethanolamine was determined. The radioactivity among the monoenoic acid isomers derived from triglyceride, phosphatidyl choline, and phosphatidyl ethanolamine was analyzed by reductive ozonolysis. Only small amounts of the labeled substrates were oxidized to carbon dioxide. Except for labeled stearic acid, which also was incorporated heavily into phosphatidyl inositol and phosphatidyl serine, most radioactivity was recovered in triglyceride, phosphatidyl choline, and phosphatidyl ethanolamine. Synthesis of cholesterol and long chain fatty acids from labeled acetic acid demonstrated that these cells can perform de novo synthesis of fatty acids and cholesterol. Both labeled palmitic and stearic acids were desaturated to the corresponding Δ9 monoenes, and palmitic and palmitoleic acids were elongated. The hexadecenoic acid fraction isolated from triglyceride, phosphatidyl choline, and phosphatidyl ethanolamine, when acetic or palmitic acid was the labeled substrate, showed that greater than 70% of the labeled acids were the Δ9 isomer. Radioactivity of the octadecenoic acid fraction derived from labeled acetic or palmitic acids was nearly equally divided between the Δ9 isomer, oleic acid, and the Δ11 isomer, vaccenic acid. Desaturation of labeled stearic acid produced only oleic acid. These data demonstrate that the biosynthesis of vaccenic acid in these cultured neoplastic cells proceeds via the elongation of palmitoleic acid. The relatively high level of vaccenic acid synthesis in these cells suggests that the reported elevation of "oleic acid" in many neoplasms may result from increased concentration of vaccenic acid.

INTRODUCTION

The significance of elucidating the role of lipid metabolism in the neoplastic cell, as well as the advantages offered by the cultured minimal deviation hepatoma cell (HTC) as a model system for studying neoplasia, has been discussed previously (1,2). Of the several studies wherein the HTC cell was utilized for study of lipid biosynthesis, no information has been obtained as to the relative contribution of the pathways for fatty acid and glycerolipid biosynthesis (3,4). Those studies reported incorporation of the fatty acid substrate into total cellular lipid and thus ignored the important information, such as interconversion of the fatty acid substrates into particular lipid classes. We have shown that HTC cells have a high capacity for the incorporation and interconversion of exogeneous palmitic acid into glycerolipid (2), and, in the present communication, we have utilized data from that previous communication for comparative purposes. The present results demonstrate that these HTC cells possess the capacity for the de novo fatty acid synthesis, as well as possess an appreciable stearic acid desaturase activity. Furthermore, isomeric analysis of the octadecenoic acid fraction synthesized from labeled acetic or palmitic acid revealed that these cells synthesize significant quantities of vaccenic acid.

EXPERIMENTAL PROCEDURES

7288C HTC were cultured on Swim's 77 medium (Grand Island Biological Co., Grand Island, N.Y., catalog no. H-20) supplemented with 20% bovine serum and 5% fetal calf serum. At confluency, the medium was decanted, and 20 ml Swim's 77 medium (without serum) and either 1-^{14}C-acetic (1.55 μCi), 1-^{14}C-palmitic

[1]Presented at the 65th Meeting of American Society of Biological Chemists, Minneapolis, June 1974.

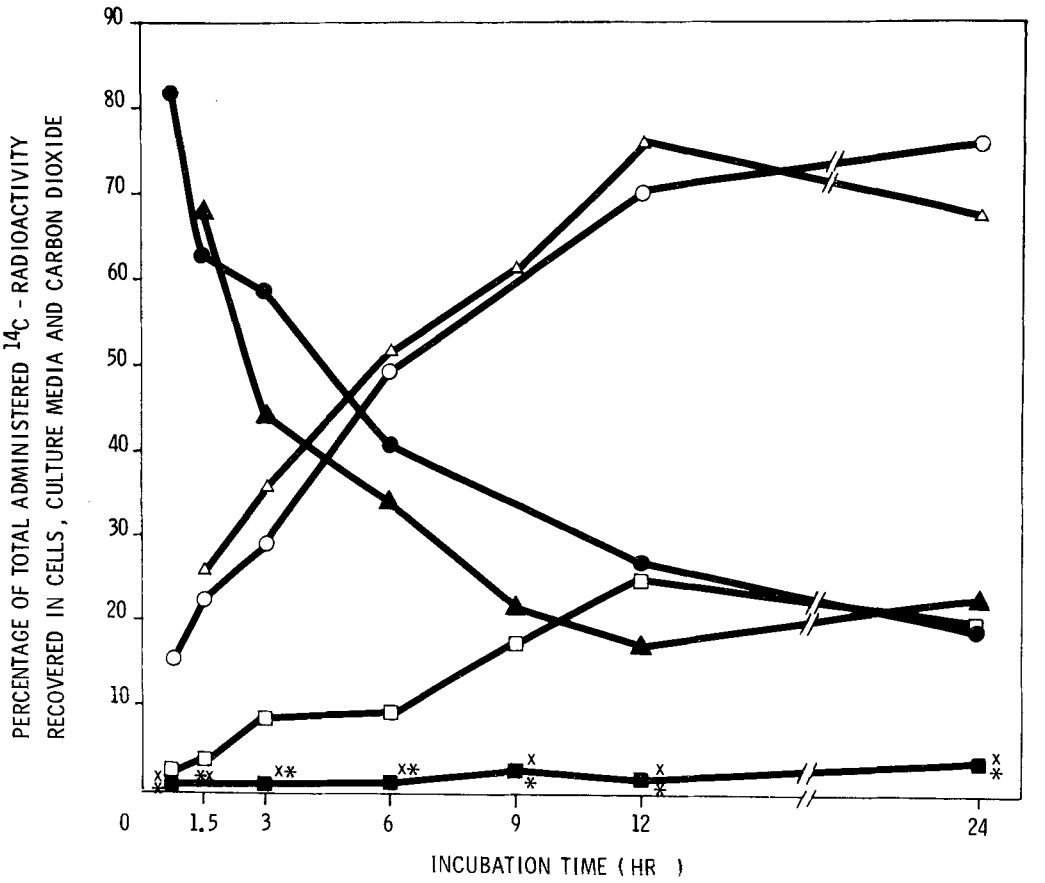

FIG. 1. Percentage of total administered radioactivity recovered in cellular lipid, lipids of culture media, and CO_2 at various incubation times. Culture conditions, isotope administration, extraction, and quantification of radioactivity are given in the text. Symbols represent: open = cells; closed = culture media; x and * = CO_2 from 1-^{14}C-acetic and 1-^{14}C-stearic acid, respectively. 1-^{14}C-acetic acid (□, ■, x); 1-^{14}C-palmitic acid (○, ●, ND = not determined); and 1-^{14}C-stearic acid (Δ, ▲, *).

(1.99 μCi), or 1-^{14}C-stearic (1.48 μCi) acid were added to the culture flasks and incubated for 0.75, 1.5, 3, 6, 9, 12, and 24 hr. Collection of radio-labeled CO_2 evolved by the cells when incubated with labeled acetic or stearic acid was accomplished by connecting a 1 x 3 in. length of silicone tubing from the mouth of the incubation flask to a wide mouth scintillation vial which contained 1 in. sq. piece of folded filter paper saturated with 0.25 ml ethanolamine. At termination of the incubation, the vial and contents were counted as described previously (2). Lyophilized cells and media were extracted twice by the Bligh and Dyer procedure (5) to obtain total lipids. Neutral lipids were isolated from polar lipids by silicic acid chromatography (6). Individual neutral lipid and phospholipid classes were resolved by thin layer chromatography (TLC) and the distribution of radioactivity determined. Triglyceride, phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) fractions were isolated by TLC and converted to methyl esters. The methyl esters were fractionated according to degree of unsaturation and chain length by preparative gas liquid chromatography (GLC) and the radioactivity in the individual esters for each lipid class determined. The details of these procedures have been described previously (2).

Monoene methyl esters resolved by preparative GLC were converted to ozonides by a modification of the Beroza and Bierl procedures (7). Ca. 2 ml CS_2 saturated with ozone at -70 C was added to the isolated radioactive monoene fraction which also contained 50 μg standard monoene carrier mixture comprised of equal amounts of the methyl esters of oleate, vaccenate, erucate, and nervonate. Excess ozone was driven off immediately by bubbling nitrogen through the solution. Dry nitrogen was

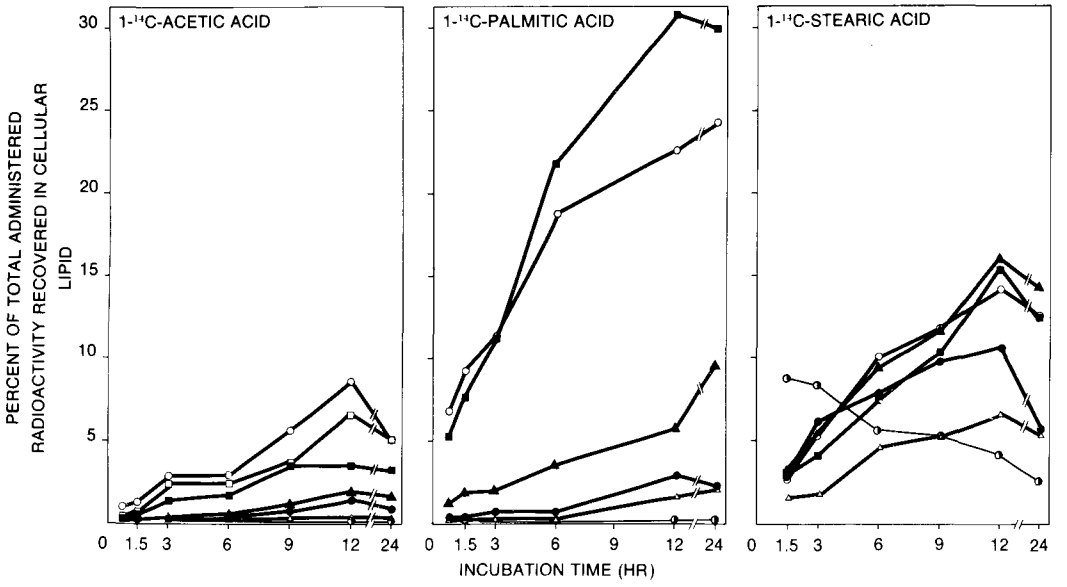

FIG. 2. Percentage of total administered radioactivity recovered in individual cellular lipid classes at various incubation times. Culture conditions, isotope administration, extraction, and quantification of the lipids are given in the text. Symbols represent: □—□ = cholesterol, ■—■ = triglycerides, ○—○ = choline phosphoglycerides, ●—● = inositol phosphoglycerides, Δ—Δ = serine phosphoglycerides, ▲—▲ = ethanolamine phosphoglycerides, and ◑—◑ = free fatty acid.

used to reduce the volume of solvent to 10 μliter or less. Triphenylphosphine (250 μg) was added to the ozonide mixture and injected into an Aerograph model A90-P chromatograph equipped with a thermal conductivity detector. Aldehydes and aldesters resulting from the cleavage of the ozonides were resolved on a 5 ft x 0.25 in. stainless steel column packed with 3% SE-30 coated on Varaport-30 (100-120 mesh) support. The column temperature was programed manually from 50-240 C. Injector, detector, and collector temperatures were maintained at ca. 265, 250, and 210 C, respectively. The flow rate of the helium carrier gas was maintained at 55 ml/min. Radioactive aldehydes and aldesters emerging from the gas chromatograph were trapped by means of a collecting device described by Wood and Reiser (8). Several small blocks of dry ice were placed on the trapping tube to ensure maximum recovery of the resolved radioactive aldehydes. Aldehyde and aldester fractions were rinsed from the collection tubes into scintillation vials with 15 ml scintillation solution (9) and the radioactivity counted in a Beckman LS-100C liquid scintillation spectrometer.

Tissue culture flasks, culture medium, fatty acid methyl esters, lipid standards, and solvents were purchased from those sources previously described (2). 1-^{14}C-Acetic acid (specific activity 54.7 mc/mM) and 1-^{14}C-palmitic acid (specific activity 55.2 mc/mM) were purchased from Nuclear Chicago, Des Plaines, Ill., and 1-^{14}C-stearic acid (specific activity 54.3 mc/mM) was purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Incorporation of Radioactivity into Total Lipids

The time-course incorporation of 1-^{14}C-acetic, 1-^{14}C-palmitic, and 1-^{14}C-stearic acids into cellular lipids, CO_2, and the amount of radioactive label remaining in the culture media are given in Figure 1. The two long chain fatty acids were incorporated to the same extent in an almost linear fashion for the first 12 hr. Decreases in media radioactivity with time were found to accompany the incorporation of the long chain fatty acid into cellular lipid. Uptake and conversion of labeled acetate into cellular lipid were reduced relative to the long chain acids, and peak incorporation occurred at the twelfth hr, the same as for labeled stearate. Radioactivity recovered as media lipid when acetic acid was the labeled substrate accounted for only 2-3% of the administered radioactivity. Radioactivity recovered as carbon dioxide was minimal throughout the 24 hr.

Incorporation of Radioactivity into Lipid Classes

The percentage of administered radioactivity

in individual cellular lipid classes is given in Figure 2. With few exceptions, the percentage of radioactivity continued to increase in all lipid classes through the twelfth hr. Generally, the ratio of radioactivity in a class to other classes remained somewhat constant during the first 12 hr. Acetate was the only substrate that was incorporated into cholesterol to any significant degree. Triglycerides, PC, and PE contained the highest percentages of radioactivity. When palmitic acid was the labeled substrate, slightly greater than 50% of the isotopic label was recovered in total phospholipid and, of that, 60-70% was isolated in PC. Between 80-90% of the isotopic label from palmitic acid recovered in the neutral lipid fraction was triglyceride. When stearic acid was the labeled substrate, radioactivity incorporated into glycerolipids during the first 9 hr was ca. equally distributed between the phosphatides of choline, ethanolamine, inositol, and triglyceride. Phosphatidylserine (PS) also contained a much higher level of radioactivity when stearate was the substrate than from acetate or palmitate.

Incorporation of Radioactivity into Fatty Acids

The specific radioactivities of hexadecanoic (16:0), hexdecenoic (16:1), octadecanoic (18:0), and octadecenoic (18:1) fatty acids from triglyceride, PC, and PE classes are given in Figure 3 for each labeled substrate. Mass data used in calculating the specific activities were taken from data obtained previously in this laboratory. Since the cells used in these studies were grown to confluency on Swim's 77 medium supplemented with 25% serum, mass data on the quantities of individual fatty acids in each class/given number of cells was calculated from cells grown on Swim's 77 medium supplemented with 25, 15, and 10% serum reported previously (1). The quantities and compositions of cellular lipids from these three media were very similar, and previous studies have shown that the class compositions and fatty acid compositions from HTC cells exhibit only marginal changes under the severest of growth conditions (10,11). Hexadecanoic and hexadecenoic acids of PC possessed the highest specific activity throughout the entire incubation period when acetate was the substrate. The specific activity of octadecanoic acid synthesized from labeled acetate in PC was ca. equal to that in triglyceride but was slightly greater than that in PE. Specific activities for octadecenoic acid in the three lipid classes were ca. equal. When palmitic acid was the labeled substrate, specific activities of fatty acids were the highest in triglyceride, intermediate in PC and lowest in PE. When stearic acid was the labeled substrate, the isotope was confined primarily to the octadecanoic and octadecenoic acid fraction. During the first 12 hr, the specific activities of each 18-carbon fatty acid in each lipid class were ca. the same.

Radioactivity in Monoenoic Fatty Acid Isomers

The distributions of radioactivity in the hexadecenoic and octadecenoic isomeric acids isolated from triglyceride, PC, and PE fractions at various time periods are given in Tables I, II, and III for acetate, palmitate, and stearate substrates, respectively. The percentages given are a comparison of radioactivity obtained from the aldester fragments. When acetate was the substrate, some 30-40% of the radioactivity was in the aldehyde fragments. Generally, the isomeric distributions did not show a change with time. Furthermore, triglycerides contained 90% of the $\Delta 9$ hexadecenoic isomer, palmitoleic acid, whereas PC and PE exhibited lower percentages (70-85%) of palmitoleic acid offset by higher percentages of the $\Delta 11$ isomer. Labeled palmitic acid gave rise to a higher percentage of palmitoleic acid in all three lipid classes, and, as expected, stearic acid radioactivity was not found in shorter chain fatty acids at significant levels. Ca. 70% of the radioactivity in the 18:1 fraction, when acetate was the substrate, was found in the $\Delta 11$ isomer, vaccenic acid (Table I). All lipid classes contained the same proportion of oleic and vaccenic acids. When palmitate was the substrate, oleic and vaccenic acids of each class contained ca. equal amounts of radioactivity. Essentially all of the labeled stearate converted to the corresponding monoene was oleic acid (Table III).

Data Evaluation

The experiments described were carried out with ca. 20×10^6 cells/flask for each time period. Because of the large number of hrs required to analyze each sample in the detailed manner described, only one flask/time period was used. Data from acetate experiments carried out with cells grown on Swim's 77 medium supplemented with 25% serum agreed well with data from corresponding time periods of this study. A further indication of the degree of reproducibility is demonstrated in the distribution of radioactivity of the isomeric hexadecenoic and octadecenoic acids. Since the differences between time periods were small, the time periods may be considered as multiple determinations. Only data that show a continuous and sustained change from one time period to another and are clearly outside marginal limits have been emphasized.

The geometrical configuration of monoene

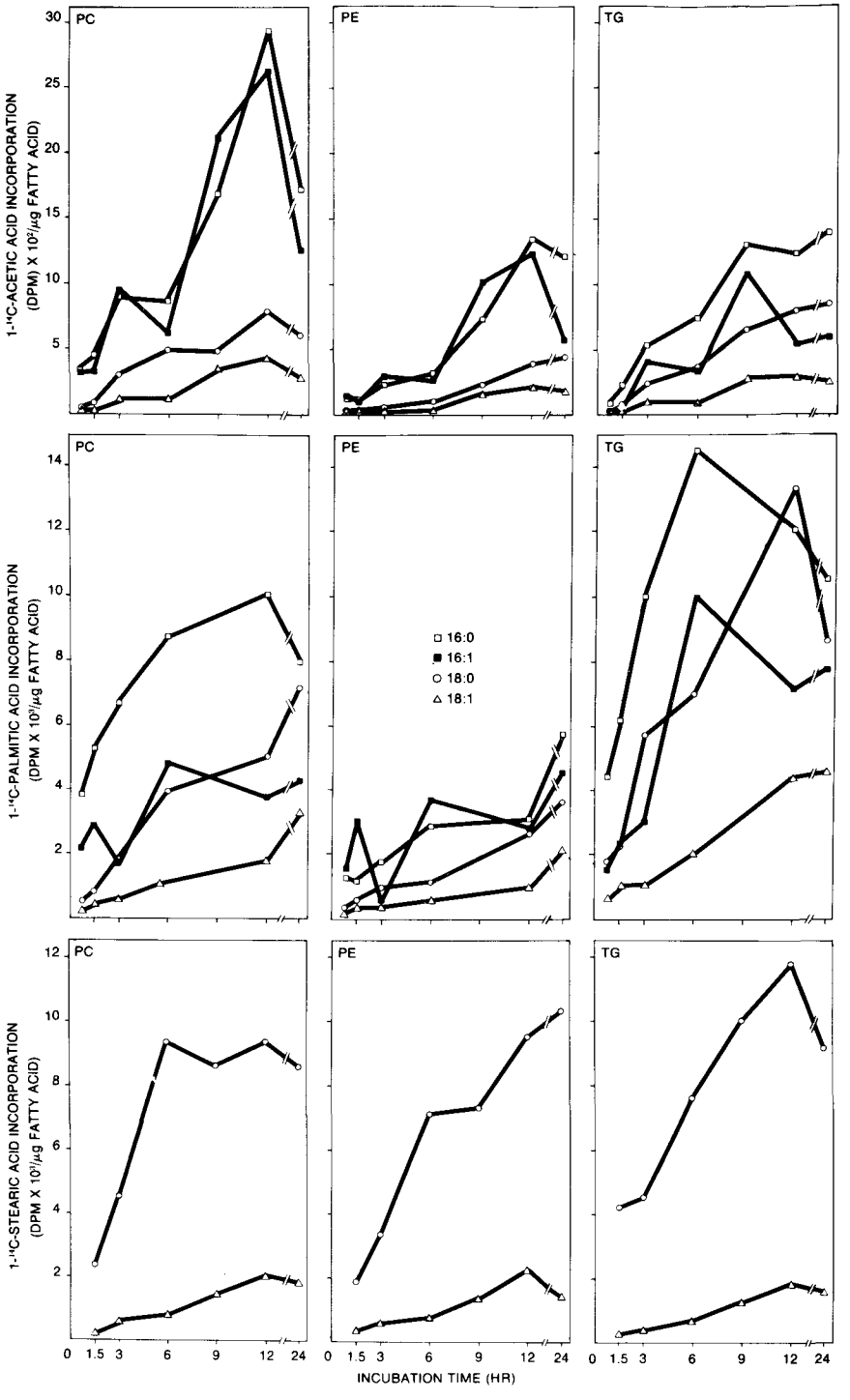

FIG. 3. Incorporation of radioactivity from tracer fatty acids into fatty acid esters of choline phosphoglycerides (PC), ethanolamine phosphoglycerides (PE), and triglycerides (TG). Values given are disintegration/min incorporated/μg glyceride fatty acid. Culture conditions, isotope administration, extraction, and quantification of the glycerolipid fatty acids are given in the text. Symbols represent: □——□ = hexadecanoic, ■——■ = hexadecenoic, ○——○ = octadecanoic, and Δ——Δ = octadecenoic.

TABLE I

Percentage Distribution of ^{14}C-Radioactivity Recovered in Isomeric Monoenoic Acids Drived from Lipid Class of Cultured Minimal Deviation Hepatoma Cells Incubated with 1-^{14}C-Acetic Acid[a]

Incubation time (hr)	Percentage of ^{14}C-radioactivity[b]					
	PC		PE		TG	
	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
	Hexadecenoic acid					
3	84	16	73	27	95	5
6	71	29	ND		ND	
9	87	13	79	21	94	6
12	72	28	88	11	93	7
24	67	33	70	30	92	8
	Octadecenoic acid					
3	23	76	29	71	31	69
6	29	71	43	57	38	62
9	22	78	28	72	27	73
12	26	74	36	64	29	71
24	34	66	40	60	43	57

[a]Culture conditions, isotopic administration, extraction, and quantification of the monoenes are given in the text.

[b]The percentages compare only the amounts of ^{14}C-radioactivity recovered in the aldester fragments. PC = choline phosphoglycerides, PE = ethanolamine phosphoglycerides, TG = triglycerides, and ND = not determined.

TABLE II

Percentage Distribution of ^{14}C-Radioactivity Recovered in Isomeric Monoenoic Acids Derived from Lipid Classes of Cultured Minimal Deviation Hepatoma Cells Incubated with 1-^{14}C-Palmitic Acid[a]

Incubation time (hr)	Percentage of ^{14}C-radioactivity[b]					
	PC		PE		TG	
	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
	Hexadecenoic acid					
0.75	ND		ND		81	19
1.5	97	3	ND		88	12
3.0	93	7	94	6	86	14
6.0	94	6	ND		97	3
12.0	95	5	87	13	98	2
	Octadecenoic acid					
0.75	42	58	53	47	52	48
1.5	35	65	49	51	ND	
3.0	37	63	54	46	45	55
6.0	49	51	67	32	61	39
12.0	51	49	56	44	57	43

[a]Culture conditions, isotopic administration, extraction, and quantification of the monoenes are given in the text.

[b]The percentages compare only the amounts of ^{14}C-radioactivity recovered in the aldester fragments. PC = choline phosphoglycerides, PE = ethanolamine phosphoglycerides, TG = triglycerides, and ND = not determined.

double bonds was not determined, but it is likely that they are of the *cis* configuration. Vaccenic acid from rat liver mitochondria (12) and rat adipose tissue (13) was of the *cis* configuration.

DISCUSSION

The incorporation of radioactively labeled acetate, palmitate, and stearate was followed into individual fatty acids of individual lipid classes of HTC cells as a function of time. Unlike studies that measure the radioactivity of a substrate incorporated into total lipids, the present study allows one to examine a number of metabolic questions: (A) the ability of the HTC cell to carry out de novo fatty acid biosynthesis; (B) the fate of exogenous long chain fatty acid vs endogenously synthesized fatty acids and class specificity of fatty acids; (C) the cells ability to oxidize, elongate, and desaturate fatty acids; and (D) the route of isomeric monoenoic acid biosynthesis.

Labeled acetate was incorporated into long chain fatty acids (Fig. 3) of several cellular lipid classes (Fig. 2). The lower percentage of acetate radioactivity incorporation into cellular lipids, than observed for palmitate or stearate, may reside in differences of acyl-CoA synthetase activities. Well to highly differentiated hepatoma microsomes have been shown to have a higher activity toward palmitate than butyrate (14). The data indicate that HTC cells have the ability to carry out de novo fatty acid biosynthesis. The possibility that acetate was incorporated only into fatty acids through elongation was discounted when the aldehydes from monoenoic acid ozonide cleavage contained 30-40% of the radioactivity. The high degree of ^{14}C-radioactivity in the free sterol fraction was shown by TLC analysis to correspond to an authentic cholesterol standard. Recent studies in this laboratory have confirmed that greater than 95% of the free cellular sterol in these HTC cells was cholesterol. There are numerous reports suggesting that Morris minimal deviation hepatoma grown in vivo has lost the capacity of "feedback control" for cholesterol biosynthesis (15). However, it has been reported that there is regulation of cholesterol biosynthesis in HTC cells (3,16).

Numerous investigators have reported that tumor cells cultured in animal serum incorporate free fatty acids from the medium and subsequently convert them into cellular lipid (17-22). The results presented in Figure 1 demonstrate that the percentage of the administered labeled palmitic and stearic acids incor-

TABLE III

Percentage Distribution of ^{14}C-Radioactivity Recovered in Octadecenoic Acids Derived from Lipid Classes of Cultured Minimal Deviation Hepatoma Cells Incubated with 1-^{14}C-Stearic Acid[a]

Incubation time (hr)	Percentage of ^{14}C-radioactivity[b]					
	PC		PE		TG	
	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
6.0	99	1	99	1	99	1
12.0	98	2	98	2	99	1
24.0	97	3	98	2	99	1

[a]Culture conditions, isotopic administration, extraction, and quantification of the monoenes are given in the text.

[b]The percentages compare only the amounts of ^{14}C-radioactivity recovered in the aldester fragments. PC = choline phosphoglycerides, PE = ethanolamine phosphoglycerides, and TG = triglycerides.

porated into cellular lipid were similar. Comparison of exogenous 1-^{14}C-palmitate and the desaturation produce (16:1) specific activities for all three lipid classes show triglycerides to be the highest and PE the lowest, whereas 16:0 and 16:1 specific activities were the highest in PC in the acetate experiments (Fig. 3). Similarly, the specific activity of 18:0 from the palmitate experiment was much higher in triglycerides than the other two classes. The data indicate preferential incorporation of exogenous palmitate into triglycerides and palmitate from de novo biosynthesis into PC. This interpretation is not complicated by degradation and resynthesis, because radioactivity was not found at significant levels in shorter chain acids when labeled palmitate and stearate were the substrates. In contrast to palmitate, labeled stearate and the desaturation product (18:1) was not incorporated preferentially into any of the three classes (Fig. 3). The high level of labeled stearate incorporated into phosphatidyl inositol (PI) and PS (Fig. 2), together with the data that indicated these two phospholipid classes in these cells contain a high level of 18:0 (10), suggests that labeled 18:0 and 18:1 was distributed among all classes on the basis of 18:0 and 18:1 mass in each class. The significance of these results cannot be evaluated fully until similar data are available from normal hepatocytes. However, it presently appears that some media fatty acids are handled differently from others and that some endogenous biosynthesized fatty acids exhibit class specificity different from exogenous acids. These studies also demonstrate the need to examine individual lipid classes as opposed to following incorporation into total lipids.

These hepatoma cells possess an active fatty acid elongation system. Labeled palmitate was elongated to stearate which showed preferential incorporation into triglyceride and PC (Fig. 3). The 18:1 fraction from the palmitate experiments also represented chain elongation, ca. 50% which occurred after desaturation of the 16:0 substrate (Table II). Significant amounts of radioactivity also were found in the triglyceride eicosenoic acid fraction at the longer time periods of the palmitate experiments. However, when stearate was the substrate, low radioactivities were observed in the eicosenoic acid fractions of all lipid classes at all time periods. The reason stearate and its desaturation product oleic acid were not incorporated into eicosenoic acids is not because this fraction does not contain the Δ11 isomer. The Δ11 isomer has been shown to account for 30-50% of the eicosenoic acid in triglyceride, PC, and PE of these cells (23).

Previous reports from this laboratory have shown that both neutral lipid and phospholipid classes of these HTC cells contained high levels of monoenoic fatty acids which increased as the level of serum and lipid in the growth media used to culture the cells was reduced (1,10,11). It also was established that the octadecenoic acid fraction derived from individual lipid classes contained high levels of vaccenic acid (23). These data are confirmed by the distribution of radioactivity in the isomeric octadecenoic acids of Tables I and II. The distribution of radioactivity from both the acetate and palmitate experiments did not show any octadecenoic isomer lipid class preference. Ca. equal amounts of radioactivity in oleic and vaccenic acids from the palmitate experiment agreed well with the mass data (23). Ca. twice as much radioactivity was found in the aldester fragment of vaccenic acid as oleic when acetate was the substrate (Table I). When the values are corrected for the additional acetate unit in Δ11 aldester, the values still are elevated. One explanation would be that palmitoleic acid synthesized de novo from acetate is used in preference to the palmitoleic acid, arising from the desaturation of exogenous labeled 16:0, for elongation to vaccenic acid.

The distribution of radioactivity in the isomeric hexadecenoic acids agreed well with previously reported mass data (23). As expected, the Δ11 hexadecenoic isomer contained little radioactivity when palmitate was the substrate (Table II). PC and PE contained a higher percentage of acetate radioactivity in the Δ11 hexadecenoic acid than triglycerides which is not understood at this time. The Δ11 hexadecenoic acid probably was derived from the elongation of Δ9 tetradecenoic acid. It is of

interest to note that despite a significant level of Δ11 hexadecenoic acid, the Δ13 octadecenoic isomer was not measurable, indicating some specificity of the elongation system. This is in agreement with mass data (23).

It is of interest that the distribution of radioactivity in the isomeric monoenes did not change significantly with respect to time (Tables I, II, and III). The data rule against one isomer reaching a certain level before the synthesis of the other is initiated but suggest that both isomers are synthesized at ca. the same time at a ratio that is dependent upon the substrate, i.e. 50% oleate–50% vaccenate from palmitate (Table II) and 33% oleate–66% vaccenate from de novo palmitate via acetate (Table I).

The predominance of palmitoleic, when palmitate was the substrate (Table II), and oleic acid, when stearate was the substrate (Table III), demonstrated that the desaturase system of hepatoma cells is exclusive for the Δ9 position. This is the same specificity exhibited by rat liver (13). Results of the palmitate and acetate experiments also show that vaccenic acid in the HTC cells arise from the elongation of palmitoleic acid. The route of vaccenic acid biosynthesis in these hepatoma cells appears to be the same as reported to occur in rat liver mitochondria by Holloway and Wakil (12). Several laboratories (24-26) in addition to this one (1,10,11) have reported high levels of "oleic acid" in the ester-linked lipids in neoplasms, but none of the reports established the isomeric composition of the 18:1 acid. The earlier report (23) plus the present communication appear to be the first to characterize the isomeric composition of the monoenes derived from individual lipid classes of a neoplastic tissue. The present data using labeled fatty acid precursors confirm the previous finding that vaccenic acid represents a significant quantity of the acyl moieties of the glycerolipids. It is possible that the reported increased levels of oleic acid observed in most tumor lipids are not all oleic acid and, conceivably, vaccenic acid contributes significantly to the increased level of octadecenoic acid fraction in neoplasms. At present, the function of vaccenic acid in mammalian cells is unknown. The full significance of these observations may become apparent when similar studies are performed on normal hepatocytes.

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Hepatoma, Host Liver, and Normal Rat Liver Neutral Lipids as Affected by Diet

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ABSTRACT

Groups of normal and hepatoma (7288CTC) bearing rats were maintained on normal chow and fat-free diets for 4 weeks. Normal liver, host liver, and hepatoma neutral lipids were examined in detail and compared. Water content, unaffected by diet was: hepatoma, 82%; host liver, 71%; and normal liver, 67%. The fat-free diet had no effect upon the hepatoma neutral lipids but elevated the triglyceride level in normal and host liver, shifted the triglyceride carbon number distribution to lower mol wt, and elevated the percentage of monoenoic acids in triglycerides and cholesteryl esters. Host triglyceride concentrations were ca. half, and cholesterol levels were reduced moderately relative to normal liver values. Hepatoma cholesterol levels were higher and triglyceride concentrations lower than normal and host liver values. Hepatoma triglycerides differed dramatically from liver and were characterized by increased concentrations of high mol wt species and a fivefold increase in the percentage of C-20 and C-22 fatty acids. The percentage of C-20 and C-22 fatty acids in hepatoma cholesteryl esters also increased ca. fivefold relative to liver. The data indicate that the systems that regulate triglyceride and cholesteryl ester fatty acid composition in liver do not control the compositions of these lipid classes in this hepatoma. The unchanged high level of essential fatty acids in the hepatoma lipids from the fat-free fed animals demonstrates the hepatoma's ability to absorb and conserve specific fatty acids.

INTRODUCTION

Hyperlipemia and the loss of lipid from the carcass of host animals bearing transplantable tumors are well documented observations. The literature up to 1956 regarding the mobilization and utilization of host lipids as a result of neoplasia has been reviewed by Haven and Bloor (1). At that time, it generally was believed that neutral lipid fatty acids, primarily saturated acids, were oxidized to meet energy requirements, while essential fatty acids were conserved, and the net result led to similarities in the concentrations of tumor and host lipids.

Since the mid 1950's, interest in the changes that occur in lipids of animals bearing tumors has decreased, while the intensity of research in the study of tumor lipids has increased. The few reports that have appeared on the lipid composition of host lipids do not lead to the same conclusions. Carruthers (2) found that the total neutral lipids of carcasses from mice bearing Krebs-2 carcinoma decreased slightly at the fifth week and total phospholipids increased but the fatty acid composition of individual neutral lipid classes did not change. Similarly, Carruthers (3) reported that three other transplantable mammary carcinomas (Walker carcinoma W 256, Walker carcinoma W256R, and methyl cholanthrene-induced mammary carcinoma TMC) did not cause a significant change in the fatty acid composition of host muscle or liver neutral lipids, and the marginal changes in the fatty acids of the total phospholipids were not consistent among all three tumor bearing hosts. Contrarily, Lankin (4) has reported that the lipid class composition of liver from mice bearing Ehrlich ascites carcinoma cells changed with tumor development: triglycerides and steryl esters reached maximum concentration at the fifth day of tumor growth. Neifakh and Lankin (5) have reported further that host livers from animals bearing Walker carcinoma and hepatoma 22 contain much higher levels of oleic, linoleic, and arachidonic acids than normal livers or the tumors (except linoleic acid in the Walker carcinoma).

Because of discrepancies in the literature regarding the effect of a tumor upon the quantitative and qualitative changes that occur in the host lipids and the lack of a detailed study comparing normal, host, and tumor lipids at the molecular species level, the present study was initiated. This investigation is part of a larger study to compare the lipid structure and metabolism of a minimal deviation hepatoma (7288C), commonly called HTC cells, grown in tissue culture; hepatoma 7288CTC, a solid hepatoma resulting from the reintroduction of HTC cells into a host; and normal liver (6). The data presented here compare quantitatively the

TABLE I

Comparison of Dry Matter, Neutral Lipid, and Phospholipid Levels of Normal Liver, Host Liver, and Hepatoma from Rats Maintained on Normal and Fat-Free Diets

Hepatic tissue[b]	Quantities (mg/g wet wt)[a]			
	Dry matter	Total lipid	Neutral lipid	Phospholipid
Rat liver, normal, chow diet (6)	325 ± 2	54.2 ± 4.2	17.6 ± 3.2	36.6 ± 2.2
Rat liver, normal, fat-free diet (5)	344 ± 2	77.6 ± 3.8	46.6 ± 3.4	31.4 ± 2.8
Rat liver, 7288CTC host, chow diet (3)	291 ± 4	46.1 ± 2.6	9.9 ± 1.0	36.2 ± 1.9
Rat liver, 7288CTC host, fat-free diet (3)	285	39.8	22.8	17.0
7288CTC Hepatoma, chow diet (4)	183 ± 5	25.8 ± 1.2	11.6 ± 0.5	14.2 ± 1.3
7288CTC Hepatoma, fat-free diet (3)	162	22.4	11.6	10.9

[a]Values without standard deviations represent the analysis of a single pooled sample from three animals, whereas value accompanied by standard deviations represent the mean of individual analysis of three-six animals/group.

[b]The number in parentheses indicates the number of animals in each group.

results obtained from detailed analyses of the neutral lipids derived from normal rat liver, host liver, and hepatoma 7288CTC as affected by normal and fat-free diets.

METHODS AND MATERIALS

Groups of three-six male Buffalo rats weighing 175-225 g were selected at random and placed on either Purina lab chow (a single lot) or a fat-free diet consisting of ca. 20% casein, 50% sucrose, 15% cellulose, 4% salts, and a vitamin supplement (Nutritional Biochemicals Corp., Cleveland, Ohio, catalog no. 450P). At the time the animals were placed on the diets, they were implanted bilaterally intramuscularally in the hind limbs with hepatoma 7288CTC. After 4-5 weeks, ca. 1 week before death of the host animals, the control and hepatoma bearing animals were sacrificed. Individual tumors and livers from the chow-fed hepatoma bearing rats and normal animals on each diet were handled separately up to the point where the neutral lipid composition was determined. Hepatomas and host livers from the fat-free fed rats were pooled, and each hepatic tissue analyzed as a single sample.

Tissues were weighed, lyophilized, and reweighed; total lipids were extracted twice by the Bligh and Dyer procedure (7), and the percentage of water, dry matter, and lipid was calculated. Neutral lipids were separated from the polar lipids by silicic acid chromatography (8) and the percentages of each fraction determined gravimetrically.

The percentage distribution of neutral lipid classes in the neutral lipid fraction was determined by high temperature gas liquid chromatography (GLC) of the intact lipids (9-11). Neutral lipid classes were resolved by thin layer chromatography (TLC) on adsorbent layers of Silica Gel G developed in a solvent system of hexane-diethyl ether-acetic acid (80:20:1, v/v/v). Chromatoplates used for qualitative and quantitative estimations were sprayed with sulfuric acid, charred, and documented by photography. Triglycerides and steryl ester bands were located by viewing chromatoplates sprayed with Rhodamine 6G under UV light. Adsorbent bands containing the triglycerides and steryl esters were scraped directly into Teflon-lined screw cap culture tubes (16 x 100 mm) and converted to methyl esters by acid catalyzed transesterification (11). Esters from the steryl ester fraction were cleaned up by TLC before analyzing by GLC. Analytical conditions were as described previously (11). Identities of fatty esters were based upon analyses before and after hydrogenation and cochromatography with fatty acid standards. The use of classical names for unsaturated esters does not imply that double bond position and configuration were examined.

The sources of standards, solvents, reagents, etc., were the same as given previously (6).

RESULTS

Quantity of Lipids

The composition of normal liver, host liver, and hepatoma from rats maintained on normal and fat-free diets is given in Table I. Diet had little effect upon dry matter but significantly affected the neutral lipid-phospholipid ratios of the livers from normal and tumor bearing animals. Normal liver contained the highest level of dry matter; hepatoma contained ca. one-half as much dry matter as normal liver, and host liver values were intermediate. Neutral lipid levels nearly tripled in the normal fat-free diet animals over chow-fed animals, but phospholipid levels were nearly equal. The high level of neutral lipid in the liver of rats maintained on the fat-free diet was reduced one-half when the

TABLE II

Comparison of Neutral Lipid Class Levels Found in Normal Liver, Host Liver, and Hepatoma 7288CTC as Affected by Diet

Hepatic tissue	Quantity (mg/g wet wt)[a]				
	DG	Sterol	FFA	TG	Steryl E.
Rat liver, normal, chow diet	0.4	3.4	0.9	11.0	1.1
Rat liver, normal, fat-free diet	1.4	2.9	1.9	38.8	1.8
Rat liver, host, chow diet	0.2	3.0	0.3	6.6	0.8
Rat liver, host, fat-free diet	0.1	1.4	-	20.6	1.9
Hepatoma 7288CTC, chow diet	0.4	4.0	1.4	4.0	1.6
Hepatoma 7288CTC, fat-free diet	0.2	3.9	0.2	5.1	1.9

[a]Quantities were determined by high temperature gas liquid chromatography. Values represent the mean of duplicate analysis of a single pooled sample of tissue from three-six animals in each group (see Table I for exact numbers). A dash (-) indicates the level was too low to measure quantitatively. Abbreviations: DG = diglycerides, sterol = primarily cholesterol, FFA = free fatty acids, TG = triglycerides, and steryl E. = primarily cholesteryl esters.

TABLE III

Determined Carbon Number Distributions of Triglycerides Derived from Normal Rat Liver, Host Liver, and Hepatoma 7288CTC of Animals Maintained on Normal and Fat-Free Diets

Tissue	Carbon number percentages[a,b]									
	46	48	50	52	54	56	58	60	62	64
Rat liver, normal, chow diet		2.2	14.3	52.0	19.5	10.7	1.4	T[c]		
Rat liver, normal, fat-free diet	1.2	15.5	42.6	37.8	3.0					
Rat liver, 7288CTC hepatoma host, chow diet		0.9	13.9	55.2	20.6	7.8	1.4			
Rat liver, 7288CTC hepatoma host, fat-free diet		5.8	33.8	49.1	10.3	0.9				
7288CTC Hepatoma, chow fed host		11.9	13.0	19.7	21.7	13.8	9.7	5.8	2.6	0.8
7288CTC Hepatoma, host fed fat-free diet		10.4	9.8	15.8	17.9	15.1	14.2	9.8	4.7	1.7

[a]Percentages determined by high temperature gas liquid chromatography.

[b]Percentages represent the analyses of a single pooled sample of tissue from three-six animals in each group.

[c]T denotes detectable quantities of less than 0.5%.

animals were bearing hepatomas. Hepatoma phospholipid concentrations were one-third to one-half normal liver phospholipid levels.

Quantity of Neutral Lipid Classes

Absolute quantities of neutral lipid classes for normal liver, host liver, and hepatoma from animals on normal and fat-free diets are given in Table II. Free sterols, primarily cholesterol as determined by GLC, triglycerides, and cholesteryl esters were the major classes. Lipid class concentrations given in Table II show the effect of the fat-free diet upon normal and host liver neutral lipids, which contrasts the lack of a significant effect upon the hepatoma. Both normal and host livers from animals fed the fat-free diet contained ca. four times more triglycerides than the corresponding chow-fed groups. Livers from host animals contained ca. half the concentration of triglycerides as livers from normal animals on the respective diets. The hepatoma contained higher concentrations of cholesterol and reduced levels of triglycerides relative to normal or host liver concentrations. Cholesteryl esters, which appeared to be elevated from composition data, were in line with values from both normal and host liver.

Triglycerides

Determined carbon number distributions of triglycerides derived from the various hepatic tissues are given in Table III. The fat-free diet caused a dramatic shift in the carbon number distribution to lower mol wt species in both normal and host livers but had little or no effect upon the hepatoma triglycerides. The determined carbon number percentages for both normal and host liver of chow-fed animals

TABLE IV

Quantitative Analysis of Triglyceride Fatty Acids Derived from Normal Rat Liver, Host Liver, and Hepatoma 7288CTC

	Fatty acid percentages[a,b]											
Hepatic tissue	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:2	20:4	24:0 + 22:4	22:5	22:6
Rat liver, normal, chow diet	0.8	27.0	5.0	2.2	33.2	23.7			1.2		1.1	2.6
Rat liver, normal, fat-free diet	1.3	37.8	14.2	1.9	43.8	0.8						
Rat liver, 7288CTC hepatoma host, chow diet	0.3	29.1	2.1	4.3	30.4	28.2			2.7		0.3	1.5
Rat liver, 7288CTC hepatoma host, fat-free diet	0.9	35.8	7.4	3.8	48.2	3.5			0.3			
7288CTC Hepatoma, chow fed host	0.4	12.4	1.3	12.1	29.1	18.4	3.6	1.7	7.8	4.8	4.4	2.8
7288CTC Hepatoma, host fed fat-free diet	0.5	14.1	2.3	12.1	28.8	15.0	3.6	2.1	9.1	4.4	3.6	3.5

[a]The difference between the sum of the percentages in any row and 100% represents the sum of percentages of other minor fatty acids not given in the table.

[b]Percentages represent the mean of duplicate analyses of a single pooled sample of tissue from three-six animals in each group.

were similar, but the values differed from random distribution calculated values in each case. Triglycerides of normal animals fed the fat-free diet exhibited a carbon number distribution slightly different from the triglycerides of host animals on the same diet. The carbon number distribution of hepatoma triglycerides differed dramatically from normal and host liver distributions irrespective of diet. Hepatoma triglycerides exhibited a wide carbon number range that extended into the very high mol wt species. Determined and random distribution calculated values for the hepatoma triglycerides covered the same range, but the lack of agreement of carbon numbers 48 and 54 indicated that not all of the fatty acids exhibit a random distribution.

The fatty acid composition of hepatoma, host liver, and normal liver triglycerides from animals maintained on normal and fat-free diets is given in Table IV. Diet affected the composition of normal and host animals' livers as expected but had no effect upon the hepatoma triglyceride composition. The compositions of normal and host liver triglycerides were similar for the respective diets. Triglycerides from the fat-free diet groups were practically devoid of polyunsaturated acids, which had been replaced with monoenoic acids. Hepatoma triglycerides differed dramatically from the compositions of normal or host animals irrespective of diet. The hepatoma triglycerides contained a large number of long chain acids that were absent or present in only trace amounts in normal or host liver.

Steryl Esters

Composition of the steryl esters, primarily cholesteryl esters, as determined by GLC, from hepatoma, normal liver, and host liver of animals maintained on normal and fat-free diets is given in Table V. The fatty acids found in normal and host liver steryl esters differed from the hepatoma, which was not affected by diet. Except for an increase in palmitic acid, the composition of the steryl ester fraction from normal and host animals was similar for the respective diets. Monoenoic acids replaced the polyunsaturated acids in the steryl ester fractions from normal and host animals on the fat-free diet. Hepatoma steryl esters, like the triglycerides from this neoplasm, contained a large number of fatty acids not present in normal or host livers. Cholesteryl esters from the hepatoma grown on rats maintained on a fat-free diet contained the same relative percentages of polyunsaturated acids as the steryl esters from chow-fed hosts.

Data Evaluation

Most of the data presented here were obtained from the analysis of a pooled sampled from each experimental group. However, because of only minor differences in the percentage class compositions and fatty acid compositions between control and host animals on the same diet, they may be considered as duplicate experiments for assessing reproducibility. Data on the neutral lipids of normal livers from older rats maintained on chow and fat-free diets were in close agreement with the data presented in these studies. The lack of a significant diet effect upon the hepatoma neutral lipids appears to be genuine. This is supported by the fact that 7288C hepatoma cells, the origin of the host grown hepatoma used in this study, cul-

TABLE V

Quantitative Analyses of Steryl Ester Fatty Acids Derived from Normal Rat Liver, Host Liver, and Hepatoma 7288CTC

	Fatty acid percentages[a,b]											
Hepatic tissue	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:2	20:4	22:4 + 24:0	22:5	22:6
Rat liver, normal, chow diet	T[c]	39.9	2.5	9.2	21.8	14.9			8.0			
Rat liver, normal, fat-free diet	T	37.5	11.6	7.9	40.3	T			2.7			
Rat liver, 7288CTC hepatoma host, chow diet	T	58.7	T	7.0	16.9	10.8			5.6			
Rat liver, 7288CTC hepatoma host, fat-free diet	0.6	44.1	8.8	5.6	33.1	2.9			4.6			
7288CTC Hepatoma, chow fed host	1.2	8.4	1.2	6.8	22.8	12.8	5.0	2.6	16.8	6.4	10.6	3.3
7288CTC Hepatoma, host fed fat-free diet	1.3	7.6	3.4	5.6	22.2	9.8	4.2	4.5	21.2	4.3	9.3	4.9

[a]The difference between the sum of the percentages in any row and 100% represents the sum of percentages of other minor fatty acids not given in the table.

[b]Percentages represent the mean of duplicate analyses of a single pooled sample of tissue from three-six animals in each group.

[c]T denotes detectable quantities of less than 0.5%.

tured on a variety of media showed only marginal changes in the neutral lipids relative to the standard stock culture medium (6).

DISCUSSION

Effect of Hepatoma upon Host

Generally, an observed difference between host liver and normal liver from animals on the same diet represents a direct or indirect effect of the hepatoma. The nutritional state of host and normal animals also must be taken into account. Therefore, the validity of an observation is considerably strengthened when results from two dietary regimens show the same trend.

The increased water content of the host liver is in agreement with previously reported data which indicate that hepatic and most other tissues of tumor bearing animals exhibit increased water content (12-15). Lankin (4) has shown that the triglycerides and cholesteryl esters of livers from mice bearing Ehrlich ascites cells change considerably with time after implantation. Host livers showed ca. 50% decrease in triglycerides, a moderate decrease in cholesterol, and no significant change in cholesteryl esters in this study (Table II). Total or fractionated neutral lipids from other tissues of tumor bearing animals show no recognizable pattern of change (2,3,12-15).

Ca. 50% loss of liver triglycerides in tumor bearing animals (Table II) resulted in little change in the carbon number distribution, relative to control animals (Table III). These data indicate that the various species of triglycerides were catabolized without apparent selectivity. This conclusion is supported by the similarity in the triglyceride fatty acid compositions between host and control animals on the same diet (Table IV). Except for higher levels of cholesteryl palmitate in tumor bearing animals, liver cholesteryl ester species were similar in host and control animals on the same diet (Table V). These data are in agreement with those reported by Carruthers (3) which indicated no change in liver total neutral lipid fatty acid composition between control animals and animals bearing transplantable mammary carcinomas. Neifakh and Lankin (5) have shown with two transplantable tumors, Walker carcinoma and hepatoma -22, that host liver total lipids contained more polyunsaturated acids than those from control animal livers. The present study indicated that, if indeed, there is a rise in concentration of polyunsaturated fatty acids of tumor bearing animals, it does not occur in the triglycerides and cholesteryl esters.

Effect of Diet upon Liver and Hepatoma Lipids

Livers of normal and tumor bearing rats maintained on the fat-free diet accumulated triglycerides (Table II). Both the triglycerides and cholesteryl esters contained elevated percentages of C-16 and C-18 monoenoic acids (Tables IV and V). The changes in liver lipids that occur in rats and mice maintained on fat-free or linoleic acid-deficient diets have been well documented (16,17). Triglycerides and cholesteryl esters did not accumulate eicosatrienoic acid which becomes elevated in total lipids of animals maintained on an essential fatty acid-deficient diet for a prolonged time (18).

The fat-free diet had little or no effect upon the quantity of individual hepatoma neutral lipid classes or upon the fatty acid composition of hepatoma triglycerides and cholesteryl esters. These results are in agreement with the data we obtained with hepatoma cells cultured on a medium supplemented with varying levels of serum and lipids (6,11). These mass data also agree with results obtained by several investigators (19-21) with labeled fatty acid precursors that indicated hepatoma fatty acid biosynthesis was not responsive to dietary regulation. The lack of dietary regulation of cholesterol biosynthesis in hepatomas has been well documented, and the subject recently has been reviewed by Sabine (22).

Liver vs Hepatoma

There is a temptation to attribute all differences between hepatomas and liver to neoplasia. The circulation, availability of nutrients, removal of metabolic products, etc., are different in the liver from a transplantable hepatoma growing in the hind limb of a rat and probably are contributing factors. Whether differences exist can depend upon what basis the data is calculated: wet wt, dry wt, relative percentages, etc. This becomes very important when the hepatoma contains only 55-65% of the dry matter as host or normal liver (Table I). Relative percentages suggested that steryl esters were increased in the hepatoma but, when calculated on a wet-wt basis, the concentration in liver and hepatoma was ca. equal (Table II).

Cholesterol concentrations were higher in the hepatoma, and triglyceride levels were lower than in liver. The hepatoma triglycerides were characterized by an increased percentage of the high mol wt species. The occurrence of high mol wt species of triglycerides in host grown tumors has been demonstrated in a wide variety of tumors (23). Hepatoma triglycerides were characterized further by the higher percentage of C-20 and C-22 unsaturated fatty acids: 25% in the hepatoma vs less than 5% in liver. High mol wt species containing C-20 and C-22 polyunsaturated acids, characteristic of phospholipid, could arise via the acylation of diglyceride species normally found in liver phospholipids (24,25). Diglycerides from the triglycerides and phosphatidyl choline were found to be identical in Ehrlich ascites cells (26), which led to the postulation that neoplasms may lack diglyceride selectivity for the biosynthesis of some glycerides. The present data supports this hypothesis.

One of the most intriguing aspects of this study was the demonstrated ability of the hepatoma to obtain the necessary essential fatty acids from the host animals on the fat-free diet to maintain the same approximate level of polyunsaturated acids in the triglycerides and cholesteryl esters as in these lipids from the chow-fed animals. What is more important is that this took place without the dramatic change in the fatty acid compositon of the hepatoma neutral lipids that was observed in the liver neutral lipids from animals on the fat-free diet. These data illustrate the ability of the hepatoma to absorb and conserve specific fatty acids.

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Carduus nigrescens Seed Oil—A Rich Source of Pentacyclic Triterpenoids[1]

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ABSTRACT

Pentacyclic triterpene alcohols (3%), their acetates (18%), and their long chain fatty acid esters (11%), together with triterpene acids (18%), represent ca. 50% of the oil from the seed and pericarp of the thistle *Carduus nigrescens* Vill. (Compositae). Along with the usual fatty acids, alkaline hydrolysis of this oil gave triterpene alcohols, some of which were identified by gas chromatography-mass spectrometry. Composition of the triterpenoid fraction, as indicated by gas chromatography of the corresponding acetates, was: α-amyrin (6%), β-amyrin (15%), lupeol plus ψ-taraxasterol (3%), erythrodiol (6%), and oleanolic acid (3%). Several components, representing 16% of the oil, were not identified. The content of pentacyclic triterpenoids is the largest found in plant seed oils.

INTRODUCTION

Carduus nigrescens Vill. is a member of the family Compositae, tribe Cynareae. Genera of this tribe are commonly known as thistles. The seed and pericarp of *C. nigrescens* contain 41% oil. Preliminary analysis of this oil in our laboratory indicated the presence of large amounts of nonglyceride components not usually associated with seed oils (unpublished results). In addition to maxima usually associated with triglycerides, the IR spectrum showed a strong absorbance at 8.1 μm (1230 cm^{-1}) which is characteristic of acetates. Gas liquid chromatography (GLC) revealed constituents which had retention characteristics resembling those of triterpene alcohols, triterpene acetates, and long chain fatty acid esters of triterpenes, as well as the common triglycerides.

Only a few references appear in the literature (1-3) that deal with the analysis of seed oils of plants of the genus *Carduus*, but none of these discuss the nonglyceride components. Pentacyclic triterpenoids have been reported in various plant parts of the Cynareae (4-6), but in only one publication have they been reported in the seed oils. In 1967, Mikolajczak and Smith (6) found 40% pentacyclic triterpene alcohols in the seed oils of *Jurinea anatolica* and *J. consanguinea.*

This paper describes the composition of *C. nigrescens* seed oil and identifies its major triterpenoid constituents.

EXPERIMENTAL METHODS

Reference Materials

The following authentic compounds were used as reference materials in thin layer chromatography (TLC), GLC, and mass spectrometry: acetates of α-amyrin, β-amyrin, ψ-taraxasterol, lupeol, and methyl oleanolate. α-Amyrin acetate was prepared (6) from the free alcohol purchased from K&K Laboratories, Plainview, N.Y. β-Amyrin acetate was donated by P. de Mayo, University of Western Ontario. Acetates of ψ-taraxasterol and lupeol were presented by E.R.H. Jones, Oxford University. Acetyl methyl oleanolate was prepared from oleanolic acid given by R.M. Parkhurst, Stanford Research Institute, Menlo Park, Calif.

Extraction and Analysis of Oil

Seed extraction: C. nigrescens seeds (6.17 g, including pericarp) were ground and extracted 6 hr in a Soxhlet extractor with petroleum ether (bp 35-60 C). Solvent was removed under vacuum at 25-30 C to give a bright yellow viscous oil (2.53 g).

Methylation of free acids: Free acids in the original oil were converted to methyl esters by treatment with an ethereal solution of diazomethane (7).

GLC analysis: GLC of the original oil and the methylated oil was carried out with a Hewlett-Packard model 5750 gas chromatograph equipped with a 3 ft x 1/8 in. stainless steel column packed with 3% OV-1 on 100-120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.). The temperature was programed at 4 C/min 100-400 C. The injection port was operated at 300 C and the flame ionization detector at 360 C. Retention data are reported as relative retention time (RRT) with β-amyrin acetate as the reference compound (Fig. 1). Methyl esters were identified by their equivalent chain length (8). Quantities of all components are expressed as GLC area percentages.

[1]Presented at the AOCS Fall Meeting, Philadelphia, September 1974.

[2]ARS, USDA.

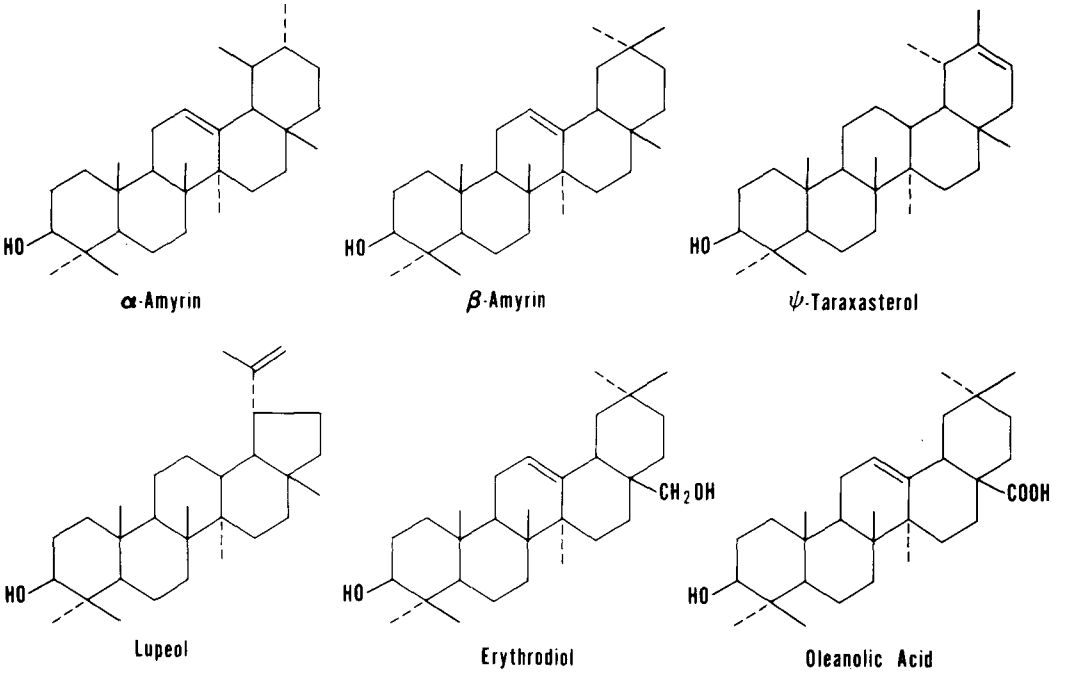

FIG. 1. Pentacyclic triterpenoids of *Carduus nigrescens* seed oil.

Preparation and Analysis of Mixed Methyl Esters

Preparation: Mixed methyl esters of the constituent acids in *C. nigrescens* seed oil were prepared by dissolving a 100 mg portion of the oil in 3 ml benzene and refluxing 4 hr with 5 ml 0.5N sodium hydroxide in methanol. Upon cooling, the alkaline solution was extracted with hexane to remove alcohols and other unsaponifiable material. Hexane extracts containing the alcohols were set aside for later examination. To the alkaline solution was added 5 ml boron trifluoride-methanol reagent of Metcalfe, et al. (9). After refluxing the mixture for 10 min, 50 ml aqueous saturated sodium chloride was added. The esters were extracted with hexane, and solvent was evaporated to give a sample of methyl esters.

GLC analysis: GLC of the mixed methyl esters was conducted on an F&M model 402 gas chromatograph as described by Kleiman, et al. (10).

Acetates of Triterpene Alcohols

Preparation: The hexane extracts set aside during the methyl ester preparation were evaporated to provide a mixture of alcohols, which was treated by the acetylation procedure described by Mikolajczak and Smith (6).

Preparative TLC: Preparative TLC of the mixed acetates was carried out on glass plates coated with 1 mm layers of Silica Gel G impregnated with 20% silver nitrate. The developing solvent was benzene-hexane (40:60). Sample bands were located by viewing the plates under long-wave UV light after they had been sprayed with a 0.2% solution of 2′,7′-dichlorofluorescein. Individual fractions were eluted with benzene-hexane (1:1). Fractions were numbered consecutively on the basis of increasing R_f.

Analytical TLC: Analytical TLC of the mixed acetates and of the fractions from preparative TLC was conducted with benzene-

TABLE I

Composition of Diazomethane-Treated *Carduus nigrescens* Seed Oil

Component	RRT[a]	GLC[b] area, %
Methyl esters of free fatty acids	0.10-0.67	19
Unknowns	0.70-0.80	1
Triterpene alcohols	0.90-0.95	3
Acetates of triterpene alcohols	1.00-1.02	18
Acetates of triterpenoid acid methyl esters	1.07-1.13	18
Diglycerides	1.18-1.27	4
Long chain fatty acid esters of triterpene alcohols	1.33-1.57	11
Triglycerides	1.62-1.86	26

[a]Relative retention time: The ratio of the retention time of an individual component to that of β-amyrin acetate.

[b]GLC = gas liquid chromatography.

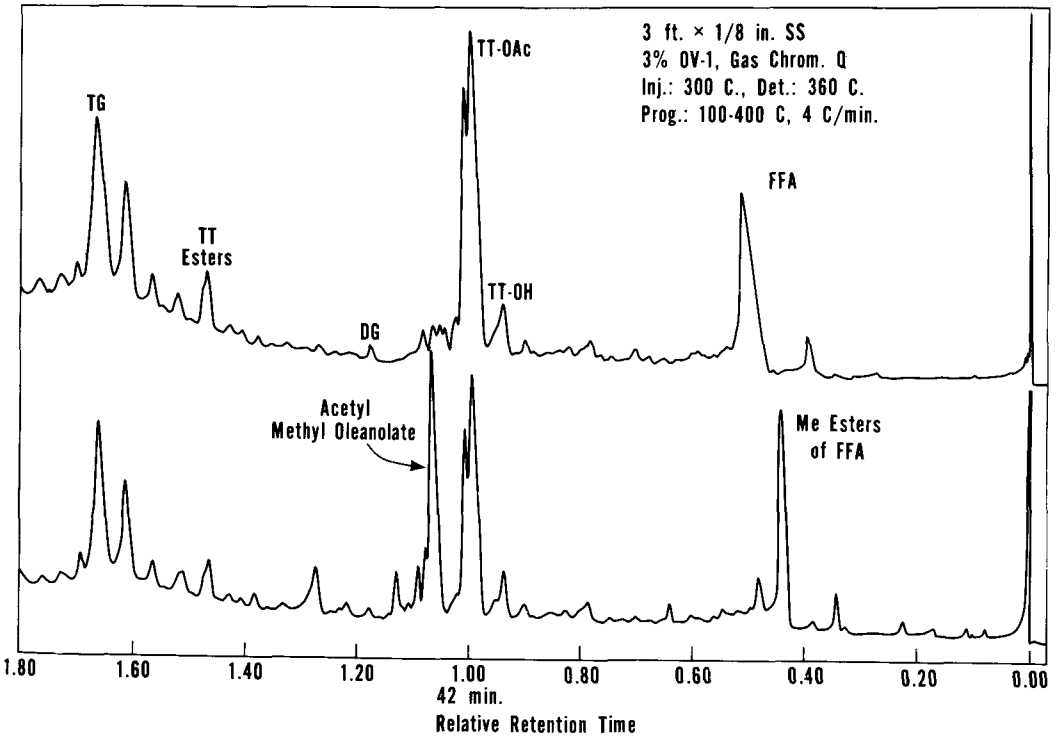

FIG. 2. Gas liquid chromatogram of *Carduus nigrescens* seed oil. Top: Original oil. Bottom: Oil after treatment with diazomethane. TG = triglycerides; TT esters = long chain fatty acid esters of triterpene alcohols; TT-OAc = triterpene acetates; FFA = free fatty acids; Me esters of FFA = methyl esters of free fatty acids.

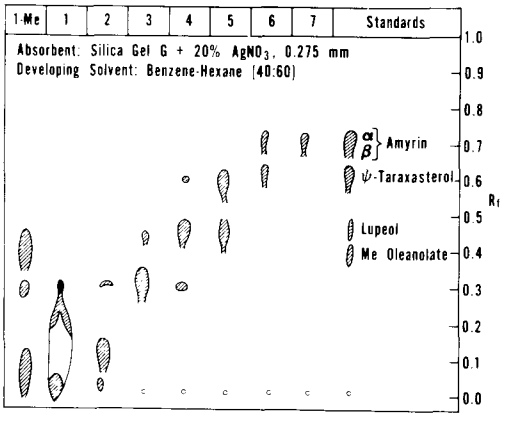

FIG. 3. Analytical thin layer chromatogram of triterpenoid acetate fractions (1 Me = fraction 1 after treatment with diazomethane).

hexane (40:60) on glass plates coated with 0.275 mm layers of Silica Gel G impregnated with 20% silver nitrate. The spots were visualized by charring with a sulfuric acid-dichromate solution. R_f values of individual components were compared with those of authentic reference compounds.

GLC analysis: The mixed acetates and preparative TLC fractions were examined by GLC with the Hewlett-Packard model 5750 instrument as described above. Fractions which appeared to contain free acids were treated with diazomethane and reexamined by GLC and TLC.

GLC-coupled mass spectrometry (GC–MS): Mass spectra of the acetylated fractions were obtained with a DuPont 21-492-1 mass spectrometer. Samples were introduced into the mass spectrometer through a Bendix 2600 gas chromatograph and a stainless steel jet separator. The gas chromatograph was equipped with a 3 ft x 1/8 in. stainless steel column packed with 3% Dexsil 300 on Gas Chrom Q. The temperature was programed at 4 C/min 230-350 C. The transfer line and jet separator were held at 280 C, and the mass spectrometer source was kept at 260 C. The filament current was 250 μA and the ionizing voltage, 70 eV.

Total Methyl Ester and Acetate Mixture

A 100 mg sample of the oil in 3 ml benzene was refluxed 3 hr with 10 ml 5% anhydrous HCl-methanol. The product, isolated by conventional methods, contained methyl esters and

TABLE II

Mass Spectra of Acetates of Triterpenoid Alcohols from *Carduus nigrescens* Seed Oil

Alcohol	Mass spectrum, *m/e* (relative intensity)
α-Amyrin	189(16), 203(11), 218(100), 249(1.4), 408(2.7), 453(1.2), 468(M^+)(4.4)
β-Amyrin	189(12), 203(28), 218(100), 249(1.3), 408(1.6), 459(0.59), 468(M^+)(2.4)
Lupeol	189(100), 203(28), 204(27), 218(21), 229(11), 249(10), 365(7.8), 393(6.3), 408(11), 453(3.2), 468(M^+)(20)
ψ-Taraxasterol	189(100), 203(12), 204(5.6), 218(5.0), 229(7.3), 249(10), 365(4.9), 393(4.6), 408(17), 453(1.9), 468(M^+)(13)
Erythrodiol	189(24), 203(100), 216(63), 276(9.3), 406(6.2), 466(11.9), 526(M^+)(0.8)
Unknown C	55(59), 69(87), 81(100), 93(60), 119(60), 135(55), 145(31), 161(24), 175(26), 189(83), 203(38), 215(15), 276(4.6), 406(4.6), 423(13), 466(49), 526(M^+)(2.3)
Methyl oleanolate	189(34), 203(100), 249(12), 262(71), 437(2.8), 452(8.5), 512(M^+)(2.8)

triterpene alcohols; it was treated by the acetylation procedure used previously (6) and was analyzed by GLC.

IR Analysis

IR spectra were determined with Perkin-Elmer Infracord model 137 and model 377 instruments. The oil was analyzed as a thin film on NaCl disks. All other samples were run as 1% CCl_4 solutions in 1 mm NaCl cells.

RESULTS

Composition of Oil After Methylation

GLC of the oil after methylation (Table I) shows, in addition to triglycerides, a variety of components ranging from long chain fatty acid esters of triterpene alcohols to methyl esters of fatty acids. The major peak among the long chain triterpene esters had the same RRT (1.50) as α- or β-amyrin palmitate. In addition, a peak was found whose retention time corresponded to that of an authentic sample of acetyl methyl oleanolate (Fig. 2).

Mixed Methyl Esters from Saponification

Saponification of the oil, followed by removal of unsaponifiables and subsequent treatment of the alkaline portion with boron trifluoride-methanol, gave methyl esters of common acids whose GLC analysis showed (amounts in parentheses are area percent): 12:0(1), 14:0(2), 16:0(12), 18:0(4), 18:1(26), 18:2(43), 18:3(5), 20:0(2), 20:1(2), 22:0(0.4), and 22:1(3).

Triterpene Alcohols

GLC analysis of the acetylated triterpene alcohol fraction indicated the presence of a complex mixture of triterpenoids. GC-MS of this mixture yielded spectra for the major components of the fraction; however, some minor components did not give sufficiently intense spectra for meaningful interpretation. Therefore, the acetate mixture was fractionated by preparative TLC. Seven fractions were separated with wt percentages as follows: 1(59), 2(5.4), 3(6.5), 4(7.6), 5(3.3), 6(4.2), and 7(13.6). There was considerable overlap in composition among these TLC bands (Fig. 3), but GC-MS of these partially purified materials provided usable spectra for minor triterpenoid components. Fractions 6 and 7 each were composed primarily of α- and β-amyrin acetates which were identified by TLC, GLC, and MS (Table II). In contrast, the acetates of ψ-taraxasterol in fractions 5 and 6 and lupeol in fractions 4 and 5 gave identical GLC retention times and similar mass spectra. However, they were readily distinguishable by analytical TLC. The similarities in the mass spectra and the GLC retention times were also observed with authentic samples of lupeol and Ψ-taraxasterol acetates. Their spectra resembled the mass spectrum obtained by Budzikiewicz, et al., (11) for ψ-taraxasterol acetate. Lupeol can be isomerized to ψ-taraxasterol, and certain transformation products can be derived from both of these compounds through acid-catalyzed solvolysis reactions (12). Possibly, related interconversions occur during the rigors of GLC and MS and are responsible for the observed similarities.

The major component of TLC fraction 3 had a mass spectrum (Fig. 4) closely resembling that published for erythrodiol diacetate (11). The major component of fraction 2 (unknown C, Table III) gave a mass spectrum (Fig. 4) in which the fragmentation pattern and molecular ion were the same as those observed for erythrodiol diacetate, but the relative intensities for the ions of *m/e* 189 and 203 were different. The peak at *m/e* 276 might be furnished by retro Diels-Alder fragmentation of ring C (11). These observations suggested that unknown C is an isomer of erythrodiol diacetate with a 12,13-double bond. To our knowledge, only one other mass spectrum has been published for

TABLE III

Composition of *Carduus nigrescens* Seed Oil after Methanolysis and Acetylation

Component	RRT[a]	R_f of acetate	GLC[b] area, %
Methyl esters of fatty acids	0.15-0.67		50
Unknowns	0.68-0.90		10
Unknown A	0.96		1
β-Amyrin acetate	1.00	0.70	15
α-Amyrin acetate	1.01	0.70	6
ψ-Taraxasterol acetate	1.04	0.60	3
Lupeol acetate	1.04	0.45	
Acetyl methyl oleanolate	1.06	0.40	3
Erythrodiol diacetate	1.10	0.30	6
Unknown B	1.12		2
Unknown C	1.14	0.10	1
Unknown D	1.17		2
Unknown E	1.18		1

[a]Relative retention time: The ratio of the retention time of an individual component to that of β-amyrin acetate.

[b]GLC = gas liquid chromatography.

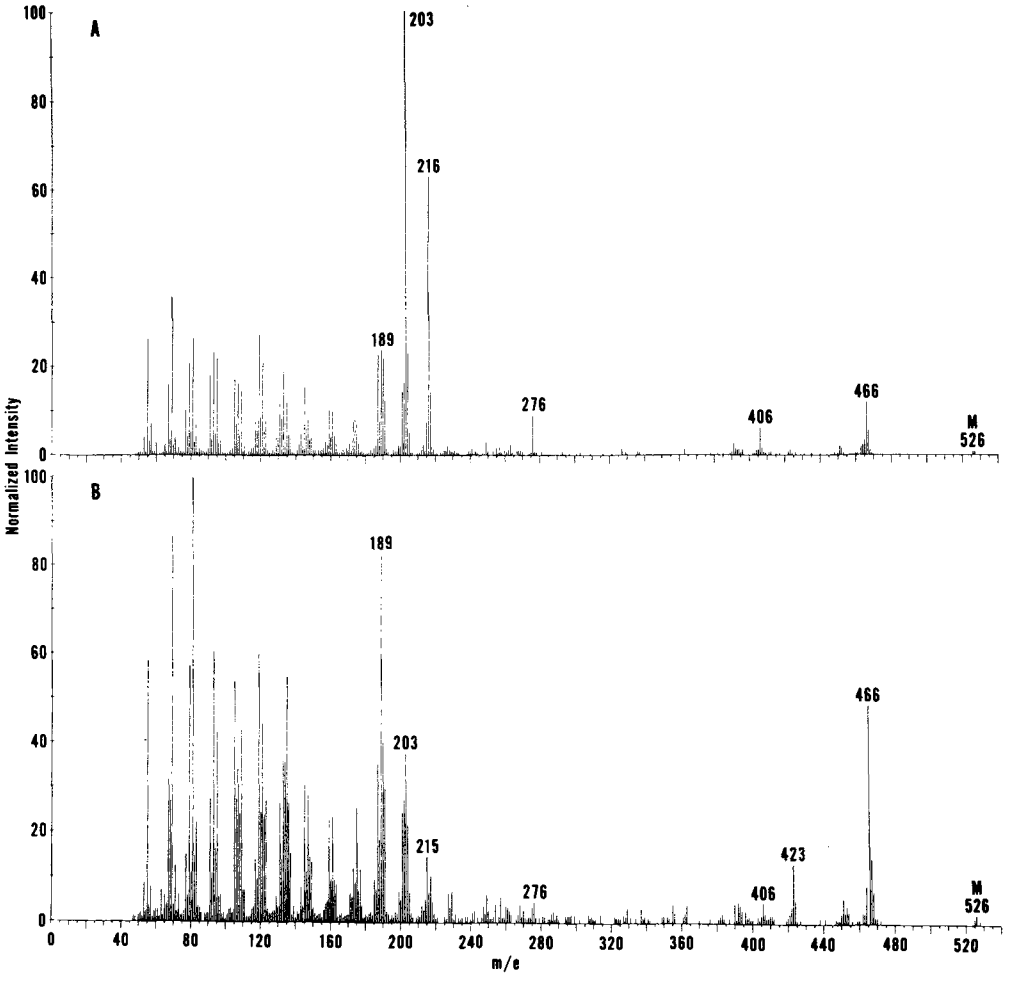

FIG. 4. Mass spectra of acetates of: (A) erythrodiol and (B) unknown C from *Carduus nigrescens* seed oil.

an isomeric Δ^{12}-ursene or oleanenediol diacetate—that of 30-hydroxy-β-amyrin diacetate (11). However, the relative intensities of its peaks do not match those in the spectrum of unknown C.

IR and GLC showed that free acids were present in the mixed acetates and were concentrated in TLC fraction 1; evidently, this acidic material was carried along during extraction of "unsaponifiables" with hexane. After methylation of the mixed acetates and fraction 1, analytical TLC revealed a spot at R_f 0.40 that was not originally observed. GLC showed a new component in the esterified mixed acetates, RRT 1.06, which constituted the major portion of the esterified fraction 1. The mass spectrum of this material closely resembled that of a known sample of acetyl methyl oleanolate. The mass spectrum of the corresponding acetyl derivative of methyl ursolate, an isomeric triterpene, did not match the spectrum of any component in this mixture.

Hydrocarbons and Sterols

Hydrocarbons may have been present in small amounts in the unsaponifiable fraction of the oil, but they were not investigated. Neither were sterols found in many plant seed oils (13-16) identified.

DISCUSSION

Pentacyclic triterpenoids have been found as minor components in many common plant seed oils (13-16) and often represent the greater part of the unsaponifiable portion of these oils. They were usually associated with hydrocarbons, sterols, and other terpene alcohols, especially with cycloartenol and 24-methylenecycloartanol.

Jacini, et al., (13) as well as Fedeli and Jacini (14) reported the composition of the nonglyceride components of 18 common vegetable oils and found 2 that contained α-amyrin and 10 with β-amyrin. Itoh, et al., in 1973 (15) studied the unsaponfiables of 19 common vegetable oils and reported α-amyrin in 6 and β-amyrin in 17 of these. In 1974, the same group (16) reported α-amyrin, β-amyrin, and lupeol in eight other seed oils. In all these cases, the pentacyclic triterpenes were minor constituents (less than 1% of the oil). In 1961, Vioque and Morris (17) reported that oleanolic acid occurs in olive oil. Our results show that this triterpene acid occurs in *C. nigrescens* seed oil as an acetate, but with the carboxyl groups free.

At the 50% level, *C. nigrenscens* seed oil has the highest concentration of pentacyclic triterpenoids of any known seed lipid. The highest reported previously was 40% in *J. anatolica* and *J. consanguinea* (6). *C. nigrescens* seed oil is also one of the rare examples of a seed oil in which nonglyceride components predominate.

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Mass Spectrometric Determination of Positions of Double Bonds in Polyunsaturated Fatty Acid Pyrrolidides

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ABSTRACT

Low resolution mass spectra of pyrrolidides of isomeric octadecadienoic acids and other polyunsaturated straight chain fatty acids are presented and discussed. The spectra of the pyrrolidides contain mainly ions from the polar part of the molecule and give spectra that are specific for each isomer. The interpretation follows, in most cases, the rule developed for monounsaturated fatty acid pyrrolidides.

INTRODUCTION

The location of double bonds in fatty acids by mass spectrometry has been approached in many ways which have been reviewed by Zeman, et al., (1,2). Pyrrolidides recently were suggested as suitable derivatives for mass spectrometry of unsaturated fatty acids (3), and the mass spectra of a series of pyrrolidides of monoenoic fatty acids recently was investigated (4). Later work showed that several tertiary amides (5,6) give easily interpretable mass spectra from which the double bond position could be deduced easily by the rule developed for pyrrolidides of monoenoic fatty acids (4). Of the amides studied, pyrrolidides were most suitable (6) for further investigation of fatty acids. This paper discusses the mass spectra of pyrrolidides of some polyenoic fatty acids with special reference to the isomeric series of methylene interrupted octadecadienoic fatty acids.

EXPERIMENTAL PROCEDURES

Methyl esters of methylene interrupted 18:2 fatty acids were obtained from the preparations of Christie and Holman (7); the *cis,cis*-9,15-18:2 and *trans,trans*-9,11-18:2 were submitted by H.J. Dutton; and the remaining polyenoic fatty acids were prepared by the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn. The pyrrolidides were prepared in quantitative yield on a microscale by a procedure described previously (4). Mass spectra were obtained on Hitachi Perkin-Elmer RMU-6D and LKB 9000 single focusing instruments operating at an ionization potential of 70 eV.

The samples were introduced through an all glass heated inlet system at 170-190 C (RMU-6D) or via a gas liquid chromatography-mass spectrometry (GC-MS) combination (LKB 9000) at 240 C. The GLC was performed on a F & M model 810 instrument furnished with an 8 ft x 1/8 in. aluminum column packed with 10% Silar 10C on 100/120 Gas Chrom Q (Applied Science Laboratories, State College, Pa.). Column temperatures were kept isothermal between 230 and 250 C, and the flow rate was 15 ml argon/min. The GLC conditions on the LKB 9000 instrument were: a 6 ft x 1/8 in. glass column filled with 3% OV-1 on Chromosorb W (HP) 80/100 (Applied Science Laboratories) at 230 C and helium as carrier gas.

RESULTS AND DISCUSSION

All mass spectra showed simple cleavage patterns, as was the case with monoenoic acids (3,4), with the base peak *m/e* 113 obtained through a McLafferty rearrangement (3). Each fragment in the high mass region was derived through a direct cleavage from the molecular ion which was indicated by mestastable ions (4). The molecular ions varied in intensity from 7% (5,8-14:2) to 47% (9,12-18:2) of the base peak. When the 18:1 series was compared with the methylene interrupted 18:2 series, it was obvious that the patterns of intensities of the molecular ions were similar and were governed by the positions of the double bonds. Thus, the lowest intensities for the molecular ions of the two series occurred for 5-18:1 (5.3%) and 5,8-18:2 (8.8%). In the 18:2 series, each isomer gave a fragmentation pattern which distinguished it from other isomers. Key fragments for all compounds investigated are given in Table I. In Figure 1, the spectra of *cis,cis*-6,9-18:2 and *cis,cis*-9,12-18:2 are shown. Here, the double bonds were indicated by the intervals of 12 atomic mass units (amu) within the regular series of 14 amu intervals according to the rule, which was formulated in a previous paper (4), for pyrrolidides of monoenoic fatty acids: *"If an interval of 12 atomic mass units, instead of*

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TABLE I

Key Fragments in Spectra of Pyrrolidides of Polyunsaturated Fatty Acids

Pyrrolidide	m/e	Rel. int.[a]	m/e	Rel. int.	m/e	Rel. int.	m/e	Rel. int.	m/e	Rel. int.	m/e	Rel. int.	m/e	Rel. int.	m/e	Rel. int.	m/e	Rel. int.	Molecular peak m/e	Molecular peak Rel. int.
cis,cis-4,7-18:2	124	1.3	126	3.3	138	1.3	139	2.3	152	11.0	153	7.8	166	4.7	178	2.4	192	3.5	333	16.0
cis,cis-5,8-18:2	140	1.1	152	.7	154	.6	166	2.9	168	1.1	178	.6	180	1.2	192	.5	206	.8	333	8.8
cis,cis-6,9-18:2	154	3.8	166	3.9	168	3.2	178	1.0	180	10.0	192	1.0	194	6.3	206	2.4	220	3.8	333	27.0
cis,cis-7,10-18:2	168	4.5	180	4.7	182	3.3	192	1.0	194	13.0	206	1.4	208	6.5	220	2.7	234	4.5	333	28.0
cis,cis-8,11-18:2	182	5.4	194	2.9	196	2.3	206	1.6	208	15.0	220	2.4	222	7.4	234	3.2	248	3.1	333	43.0
cis,cis-9,12-18:2	196	3.4	208	2.8	210	1.9	220	1.9	222	10.0	234	2.7	236	6.8	248	2.4	262	3.1	333	47.0
cis,cis-10,13-18:2	210	2.4	222	1.9	224	1.6	234	1.9	236	7.8	248	1.5	250	6.8	262	1.7	276	2.3	333	40.0
cis,cis-11,14-18:2	224	2.4	236	1.6	238	1.8	248	1.2	250	6.0	262	1.4	264	5.8	276	1.9	290	2.7	333	41.0
cis,cis-12,15-18:2	238	2.4	250	1.4	252	1.5	262	1.2	264	5.9	265	2.7	290	2.2	291	.7	304	3.0	333	45.0
cis,cis-13,16-18:2	238	5.2	250	1.0	252	1.9	262	.9	264	1.5	265	.6	290	3.5	291	1.0	304	1.8	333	43.0
cis,cis-14,17-18:2	252	5.4	260	.8	262	.8	264	.8	276	1.0	278	1.3	290	1.3	292	4.4	304	1.6	333	8.4
cis,cis-5,8-14:2	140	1.2	152	.9	154	.8	166	2.2	168	1.0	178	.5	180	1.1	192	.5	206	.8	277	7.1
cis,cis-7,10-16:2	168	4.9	180	4.0	182	2.7	192	.8	194	12.0	206	1.0	208	5.2	220	2.4	234	3.0	305	16.0
cis,cis-9,15-18:2	196	2.6	208	2.2	210	1.8	222	3.4	264	7.7	276	1.0	278	1.5	290	1.8	304	2.5	333	19.0
cis,cis,cis-6,9,12-18:3	154	3.9	166	3.4	180	6.5	194	8.7	206	2.5	220	8.8	234	3.1	246	3.8	260	3.4	331	23.0
cis,cis,cis-9,12,15-18:3	196	3.3	208	2.6	222	5.3	236	5.1	248	2.7	262	7.7	276	2.9	288	3.5	302	3.1	331	40.4
cis,cis,cis-8,11,14-20:3	182	4.5	194	3.2	208	9.2	222	6.3	234	3.2	248	8.6	262	3.5	274	4.1	288	3.2	359	38.0
trans,trans-8,10-18:2	180	1.9	182	2.1	192	.5	194	1.5	196	1.0	206	1.1	208	1.3	210	.7	220	2.1	333	23.0
trans,trans-9,11-18:2	194	.7	196	1.1	206	.6	208	1.0	210	.5	220	.8	222	.7	224	.6	234	1.4	333	10.0
trans,trans-10,12-18:2	208	2.0	210	3.1	220	1.8	222	2.1	224	2.2	234	2.4	236	2.1	238	1.6	248	2.1	333	23.0
cis,cis,cis,cis-5,8,11,14-20:4	140	1.7	152	.9	154	1.0	166	2.8	168	1.6	180	3.4	192	.6	194	.7	206	2.4		
	208	1.6	218	.8	220	1.5	232	2.1	246	2.4	258	.5	260	.7	272	1.0	286	.9	357	6.5

[a]Rel. int. = relative intensity.

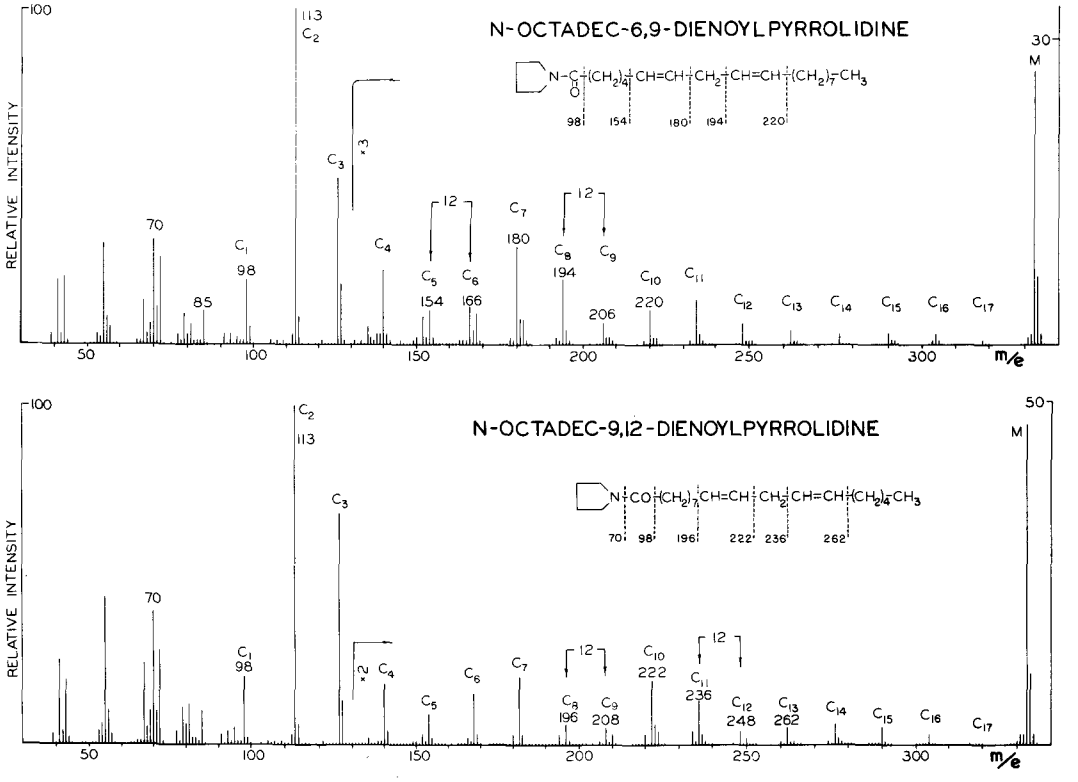

FIG. 1. Mass spectra of N-octadec-6,9-dienoylpyrrolidine and N-octadec-9,12-dienoylpyrrolidine.

the regular 14, is observed between the most intense peaks of clusters of fragments containing n and n-1 carbon atoms of the acid moiety, a double bond occurs between carbon n and n+1 in the molecule." This rule could be applied to most of the compounds listed in Table I, the exceptions being the dienoic pyrrolidides which have a double bond in positions 4,15,16 or 17 in a methylene interrupted system.

The spectrum of the 4,7-18:2 isomer had characteristic peaks at *m/e* 124 and *m/e* 126 together with *m/e* 139 and *m/e* 152 indicating the double bond in position 4. This was likewise true for the pyrrolidides of 4-18:1 and 4-10:1 (4). The double bond in position 7 was shown by the interval of 12 amu between carbons 6 and 7 (*m/e* 166 and *m/e* 178) according to the rule. The 12,15-18:2, 13,16-18:2 and 14,17-18:2 isomers had spectra which distinguished them from each other. The 12,15-18:2 isomer followed the rule, but the key fragments, representing 12 carbons of the chain, *m/e* 250 and *m/e* 252, were of almost the same intensity. Other details that distinguished the two isomers from each other were: the cluster of peaks that contained 13 carbons of the fatty acid chain of 12,15-18:2 was stronger than the corresponding peaks, e.g. *m/e* 265, for the 13,16-18:2 isomer. In the spectrum of the 13,16-18:2 isomer, the cluster of peaks that contained 15 carbons of the chain, e.g. *m/e* 291, was stronger than the corresponding cluster for the 12,15-18:2 isomer. Finally, for the spectrum of the 14,17-18:2 isomer, the interval of 12 amu, typical for the 12,15-18:2 and 13,16-18:2 isomers, was moved one carbon closer to the methyl end, e.g. *m/e* 278 and *m/e* 290 to *m/e* 292 and *m/e* 304.

The spectra of lower homologues of methylene interrupted dienoic acids represented by 5,8-14:2 and 7,10-16:2 followed the rule completely as can be seen in Table I. Also, a fatty acid with the two double bonds separated by four methylene groups, 9,15-18:2, gave a mass spectrum (Fig. 2) from which the double bonds could be located via the rule.

The mass spectra of pyrrolidides of three trienoic acids, 6,9,12-18:3, 9,12,15-18:3, and 8,11,14-20:3, were also interpretable according to the rule developed for monoenoic acids. This is shown for the 9,12,15-18:3 isomer in Figure 2. However, some of the key fragments, e.g. *m/e* 248 and *m/e* 250, in the spectrum of 6,9,12-18:3 were of intensities almost equal to those of the spectrum of the 12,15-18:2 isomer.

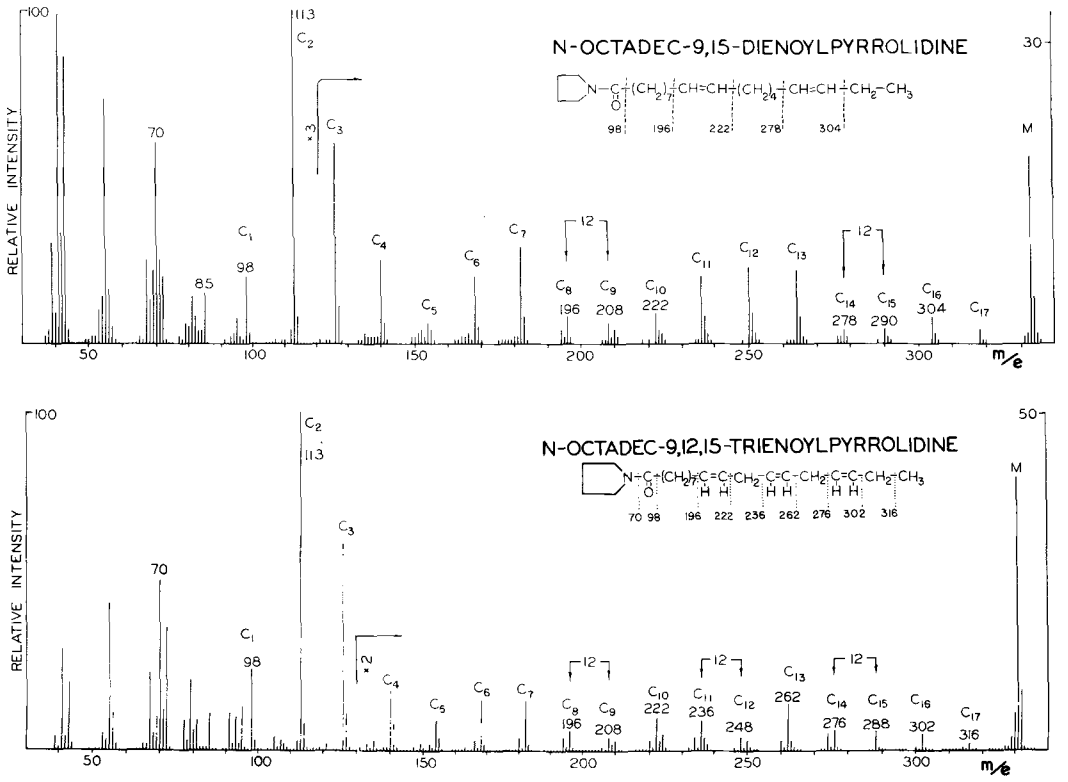

FIG. 2. Mass spectra of N-octadec-9,15-dienoylpyrrolidine and N-octadec-9,12,15-trienoylpyrrolidine.

A tetraenoic fatty acid, 5,8,11,14-20:4, also was investigated, and its pyrrolidide derivative gave a mass spectrum which could be interpreted partially according to the rule. In this case, key diagnostic fragments, e.g. *m/e* 152 vs *m/e* 154 and *m/e* 192 vs *m/e* 194, were of almost equal intensities.

In the spectra of pyrrolidides which have methylene interrupted double bonds in the middle of the chain, the peak cluster lying between the intervals of 12 amu was observed to be more intense than its surrounding peaks. For example, *m/e* 180 in the spectrum of the pyrrolidide of 6,9-18:2; *m/e* 222 in the spectrum of the pyrrolidide of 9,12-18:2; and *m/e* 222 and 262 in the spectrum of the pyrrolidide of 9,12,15-18:3 were high peaks in the general profile of the mass spectrum range involving the double bonds. This phenomenon facilitates the location of methylene interrupted polyunsaturation when it is difficult to distinguish whether intervals are 12 or 14 amu. In the mass spectrum of the pyrrolidides of a polyunsaturated fatty acid, *the presence of a peak relatively more intense than the peak clusters which flank it and which are involved in probable intervals of 12 amu indicates the presence of a methylene interrupted system. If the prominent peak contains m carbons of the fatty acid residue, the methylene carbon in the molecule was at position m + 1.*

Three fatty acids with conjugated unsaturation in the middle of the chain, i.e. 8,10-18:2, 9,11-18:2, and 10,12-18:2, also were investigated. In Table I, the key fragments in their spectra are listed. In each case, the double bond closer to the carbonyl was indicated by the interval of 12 amu according to the rule, but the remote double bond was, however, not in agreement with the rule. The conjugated system was indicated by another interval of 12 amu immediately following the first interval of 12 amu.

To test the use of pyrrolidides in the interpretation of unknown fatty acids with GLC and MS, corn oil triglycerides were investigated in a previous paper (6), and the double bonds were located without difficulties by using the rule developed for the monoenoic fatty acids. An 18 carbon fatty acid with one actylenic bond and three double bonds also was investigated (8), and, by applying our rule, the double bonds were found at positions 9,12 and 15, which later was confirmed by other analytical

methods. Interpretation of the mass spectrum of a triglyceride of this fatty acid revealed the same double bond positions when the same logic as used with pyrrolidides was applied. Recently, the mass spectrum of the pyrrolidide of an unknown polyunsaturated acid was studied by Joseph (9). Interpretation of the spectrum suggested the structure to be 3,6,9,12,15-18:5, and this structure was confirmed by other analytical methods.

This work shows that pyrrolidides offer advantages for the mass spectrometric analysis of polyenoic fatty acids. The derivatives are easy to prepare, and no chemical modification of the unsaturation in the fatty acid chain is necessary, as is the case with most of the other methods developed for this purpose (1,2). Although spectra of all the polyunsaturated fatty acid pyrrolidides are not interpretable according to the rule developed for the monoenoic acids, it is noteworthy that all spectra differ from each other. With enough reference compounds, there should be no problem in identifying each positional isomer by mass spectrometry.

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Effect of Inhibitors and Phenobarbital Pretreatment upon Hepatic Lipid Peroxidation during Protein and Riboflavin Dietary Stress in Male Rats

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ABSTRACT

Male young albino rats divided into three groups were maintained on the following diets. The normal group was maintained on 13% casein, 45% corn starch, 31% sucrose, 6% salt mixture, 4% peanut oil, and 1% vitamin mixture. The low protein group animals recieved only 5% casein, and the riboflavin-deficient group was fed normal diet, except that the riboflavin was absent from the vitamin mixture and ordinary casein was replaced by 13% vitamin-free casein. The effects of various inhibitors upon triphosphopyridine nucleotide, reduced form-linked lipid peroxide formation by the supernatant fraction of liver at 9000 x g from rats fed a normal diet, a low protein diet, or a riboflavin-deficient diet for 2,4, and 7 weeks were investigated. A significant decrease in triphosphoryridine nucleotide, reduced form-linked and ascorbate-induced lipid peroxidation was noticed in rats fed on low protein and riboflavin-deficient diet. Glutathione inhibited the triphosphopyridine nucleotide, reduced form-linked lipid peroxidation in rats from all three groups. However, the observed response was variable due to the nature of the diet. The magnitude of inhibition was greater in low protein-fed animals than in animals from the riboflavin-deficient and control groups. Cytochrome C inhibited peroxide formation, but the inhibition was greater in rats from the low protein and riboflavin-deficient groups than in animals from the normal group. Tocopherol exhibited the antioxidant property in all three groups of rats. Deoxycholate inhibited lipid peroxide formation in all the three groups.

INTRODUCTION

The microsomal membrane has been used in many laboratories as a model in the studies of lipid peroxidation, and the antioxidants have been used to protect the membrane system from peroxidative damage (1-3). The biological membrane system, especially subcellular organelles, are labile to lipid peroxidation (4). The process may be part of universal diseases, such as some phases of atherosclerosis, neural ceroid lipofuscinosis, or Batten's syndrome. The chemical deteriorative effect of any of these diseases might be slowed by the use of increased

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TABLE I

Composition of Diet

Ingredients	Normal (%)	Low protein (%)	Riboflavin deficient (%)
Casein	13	5	---
Vitamin free casein	---	---	13
Corn starch	45	53	45
Sucrose	31	31	31
Ground nut oil	6	6	6
Salt mixture[a]	4	4	4
Vitamin mixture[b]	1	1	1[c]

[a]Salt mixture of Hegsted, et al. (19), with the following modifications: $CuSO_4 \cdot 5H_2O$, 3.266 g, $CoCl_2 \cdot 6H_2O$, 2.16 g, and NaF, 0.432 g.

[b]Vitamin mixture of Schultze, (20). 100 International unit (IU) vitamin A, 20 IU vitamin D, and 0.5 mg vitamin E/day/rat also were added to the diet along with peanut oil. Vitamin-free casein was obtained from Calbiochem, Lucerne, Switzerland, containing 0.5 μg riboflavin g casein.

[c]Riboflavin vitamin-free mixture.

amounts of dietary antioxidants (5,6).

Lipid peroxidation also can be produced intracellularly under a variety of conditions (7,8). Accumulated lipid peroxides which can not be decomposed by catalysis lead to a number of harmful effects to the cells (9). In vitro peroxidation is markedly stimulated in the presence of Fe^{++}, Cu^{++}, or ascorbate (10-13) but inhibited in the presence of Zn^{++} (14). Antioxidants like α-tocopherol, vitamin k_1, k_3, and k_5 and metal chelators, such as α-dipyridyl and 8-hydroxy quinoline, abolish lipid peroxidation (15-17). Earlier, we reported (18) about lipid peroxidation and drug hydroxylation during riboflavin deficiency.

To our knowledge, there has been no report regarding the effect of various inhibitors upon lipid peroxidation during riboflavin and protein deficiency. Therefore, the present studies report the in vitro effect of glutathione (GSH), cytochrome C, α-tocopherol, and sodium deoxycholate upon the lipid peroxidation during various stages of protein and riboflavin deficiency in male rats. The effect of phenobarbital pretreatment upon lipid peroxidation also was studied.

MATERIALS AND METHODS

Haffkine strain young albino rats initially weighing 40-50 g were obtained from Haffkine Institute in Bombay, India. They were placed individually in wire bottom cages at room temperature and kept away from exposure to pharmacologically active compounds. The rats were fed a synthetic diet ad libitum for 1 week prior to the initiation of the experiments. After screening their growth response to the synthetic diet, they were divided into three groups: normal, low protein, and riboflavin-deficient and pair-fed for 7 weeks. The composition of the diet was as shown in Table I.

Phenobarbital pretreatment: At the end of 2,4, and 7 weeks of the feeding period, 5 rats from each group were injected intraperitonially with phenobarbital sodium (50 mg/kg body wt) for 5 successive days.

Tissue preparation: The animals were fasted overnight and sacrificed by decapitation 24 hr after the last injection. The livers immediately were excised, weighed, washed with 0.9% ice-cold saline, minced, and homogenized (1:10 w/v) in 0.25 M ice-cold sucrose in a Teflon pestle glass homogenizer. All further tissue preparations were performed at -2 C unless otherwise mentioned. The homogenates were centrifuged at 9000 x g for 20 min in a refrigerated centrifuge (Remi K-24) at -2 C. The 9000 x g supernatant fraction protein was used as enzyme protein in all assays and was measured according to the biuret method (21).

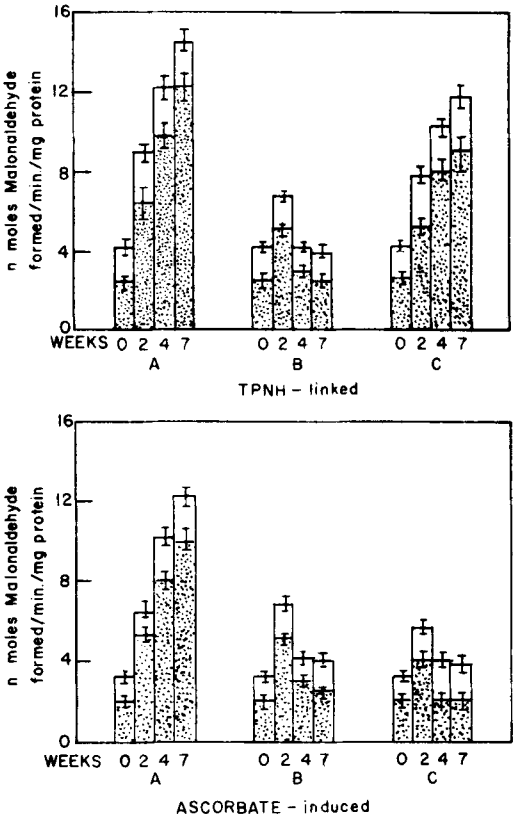

FIG. 1. Effect of diet and phenobarbital treatment upon triphosphopyridine nucleotide, reduced form (TPNH)-linked and ascorbate-induced lipid peroxidation in young male rats. A = normal, B = low protein, C = riboflavin deficient, ▭ = normal, and ▨ = phenobarbital treated.

Lipid peroxidation: The triphosphopyridine nucleotide, reduced form (TPNH)-linked lipid peroxidation was assayed in a medium containing 50 mM Tris-HCl buffer, pH 7.4; 1 mM $MnCl_2$; 50 mM nicotinamide; 0.2 mM triphosphopyridine nucleotide, oxidized form (TPN^+); 0.5 mM sodium DL-isocitrate; 50 μliter isocitrate dehydrogenase; 0.2 mM adenosine 5′-phosphate (ADP); 30 μM Fe^{++}; and 2 mg/ml protein (9000 x g) in a final volume of 15 ml. Ascorbate-induced lipid peroxidation was carried out in a same medium, but nicotinamide was omitted, and the TPNH generating system was replaced by 1 mM ascorbate. The incubations were carried out in a Dubnoff metabolic shaker under air at 37 C for 15 min. Aliquots (2 ml) were removed at 0, 2, 5, 7, 10, 12, and 15 min. intervals, and the reaction was terminated by addition to tubes containing 2 ml 10% ice-cold trichloroacetic acid. The precipitated

TABLE II

Percent Inhibition in Enzymatic Lipid Peroxidation Due to Diet and Phenobarbital Pretreatment in Young Male Rats

Group	Feeding period (weeks)	TPNH[a]-linked lipid peroxidation		Ascorbate-induced lipid peroxidation	
		Percent difference for malonaldehyde formation due to diet	Percent difference due to phenobarbital treatment with individual control	Percent difference for malonaldehyde formation due to diet	Percent difference due to phenobarbital treatment with individual control
Normal	2	--	−29.0	--	−24.0
	4	--	−25.0	--	−20.0
	7	--	−17.0	--	−19.0
Low protein	2	−24.0[b]	−25.0	−12.0	−30.0
	4	−65.0[c]	−29.0	−60.0[c]	−50.0
	7	−72.0[c]	−38.0	−69.0[c]	−49.0[c]
Riboflavin-deficient	2	−13.0	−33.0	−11.0	−21.0
	4	−19.0	−21.0	−16.0	−25.0
	7	−19.0[b]	−24.0	−22.0	−15.0

[a]TPNH = triphosphopyridine nucleotide, reduced form.
[b]= $P<0.01$.
[c]= $P<0.001$.

protein was removed at 2000 x g for 10 min. The malonaldehyde formation was estimated from the protein-free supernatant by the thiobarbituric acid reaction (22). The TPNH-linked lipid peroxidation was estimated in the presence of various concentrations of GSH, cytochrome C, tocopherol, and deoxycholate during the various stages of deficiency.

Oxygen consumption: This was measured by Warburg's method (23) in a medium containing 50 mM Tris-HCl buffer, pH 7.4; 0.5 mM TPNH; and 5 mg protein in a final volume of 3.0 ml. The reaction was carried out at 37 C up to 30 min.

RESULTS

The liver wt of animals fed on low protein diet were decreased significantly, whereas, in riboflavin-deficient animals, a slight decrease in liver wts was noted at the end of 2 and 4 weeks feeding period and a significant decrease in liver wt was noted during the chronic deficiency of riboflavin.

Phenobarbital pretreatment stimulated the liver growth in all animals, irrespective of deficiency levels. The magnitude of stimulation was higher in both low protein and riboflavin-deficient animals, as compared to their control animals. The protein content of the supernatant fraction gradually was lowered due to the low protein diet, and the decrease was maximal at the end of 7 weeks. A slight increase in liver protein content was noted in the riboflavin-deficient animals. The percent increase in supernatant fraction protein was 7.0 at the end of 7 weeks of riboflavin deficiency.

The phenobarbital-induced effect upon supernatant fraction protein was noticed even in deficient animals.

TPNH-linked and ascorbate-induced lipid peroxidation: TPNH-linked and ascorbate-induced lipid peroxidations were markedly lowered in animals fed on low protein and riboflavin-deficient diets (Fig. 1). The percentage decreases in TPNH-linked and ascorbate-induced lipid peroxidations were 72.5, 18.7, 68.6, and 22.4, respectively, in low protein and riboflavin-deficient animals at the end of chronic deficiency (7 weeks) (Table II). The decrease in TPNH-linked and ascorbate-induced lipid peroxidation was more pronounded in low protein animals at the end of 2, 4, and 7 weeks feeding period than in riboflavin-deficient animals.

Effect of GSH upon TPNH-linked lipid peroxidation: Addition of GSH to the incubation medium inhibited lipid peroxidation in all animals (Fig. 2). A complete inhibition was

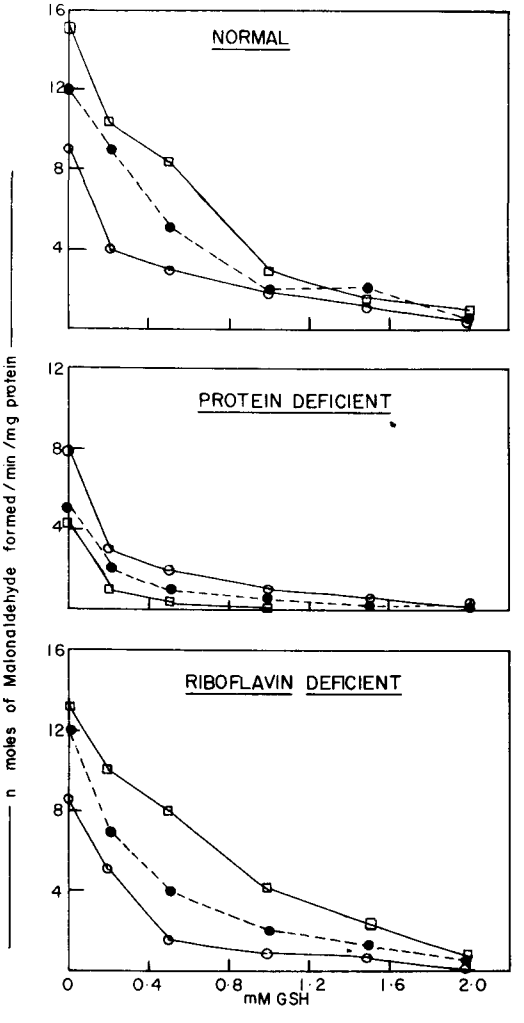

FIG. 2. Effect of glutathione (GSH) on triphosphopyridine nucleotide, reduced form-linked lipid peroxidation in normal, protein, and riboflavin-deficient young male rats. □—□ = 7 weeks feeding, ●—● = 4 weeks feeding, and ○—○ = 2 weeks feeding.

observed in the presence of 1 mM GSH in animals fed on low protein diet for 7 weeks. However, the percent inhibition was only 63.0 in riboflavin-deficient animals. In the presence of 2.0 mM GSH, the percentage inhibition was 85.0 and 94.0, respectively, in riboflavin-deficient animals and control animals at the end of 7 weeks feeding period.

Cytochrome C and TPNH-linked lipid peroxidation: The TPNH-linked lipid peroxidation was inhibited by cytochrome C in all animals irrespective of their dietary status (Fig. 3). A drastic inhibition was noted in the presence of 10 μM cytochrome C in animals from the low protein and riboflavin-deficient groups, whereas

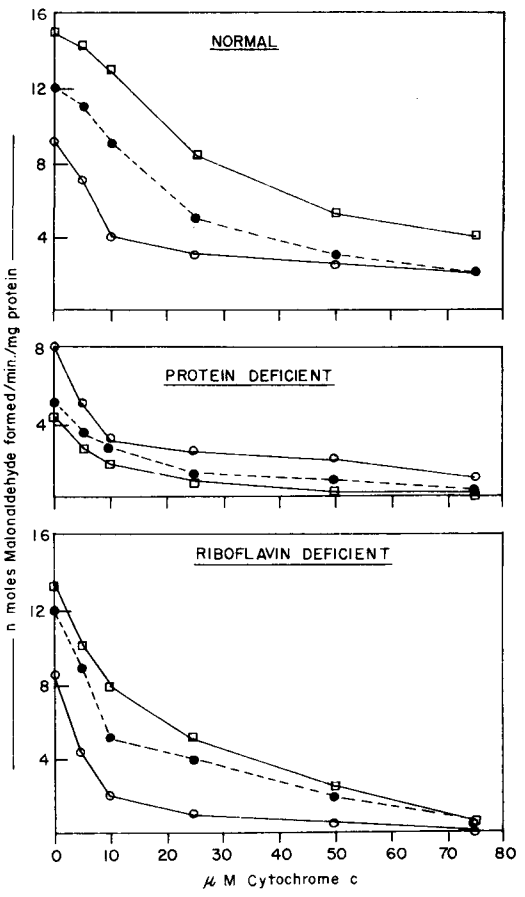

FIG. 3. Effect of cytochrome C upon triphosphoryridine nucleotide, reduced form-linked lipid peroxidation in normal, protein and riboflavin-deficient young male rats. □—□ = 7 weeks feeding, ●—● = 4 weeks feeding, and ○—○ = 2 weeks feeding.

25 μM cytochrome C was required to have similar inhibition in control animals. The percent inhibition due to 25 μM cytochrome C was 75.0, 54.0, and 40.0 in animals from low protein, riboflavin-deficient, and control groups, respectively. In the presence of 75 μM cytochrome C, an almost complete inhibition of TPNH-linked lipid peroxidation was noticed in the deficient group, whereas only 60.0% inhibition was noticed in control animals.

α-Tocopherol and TPNH-linked lipid peroxidation: TPNH-linked lipid peroxidation was blocked due to the presence of tocopherol in all animals (Fig. 4). Complete protection was noticed in the presence of 100 mM α-tocopherol in animals fed the low protein diet for 2 and 4 weeks. However, the same protection was obtained in the presence of 50 mM α-tocopherol at the end of the 7 weeks feeding period.

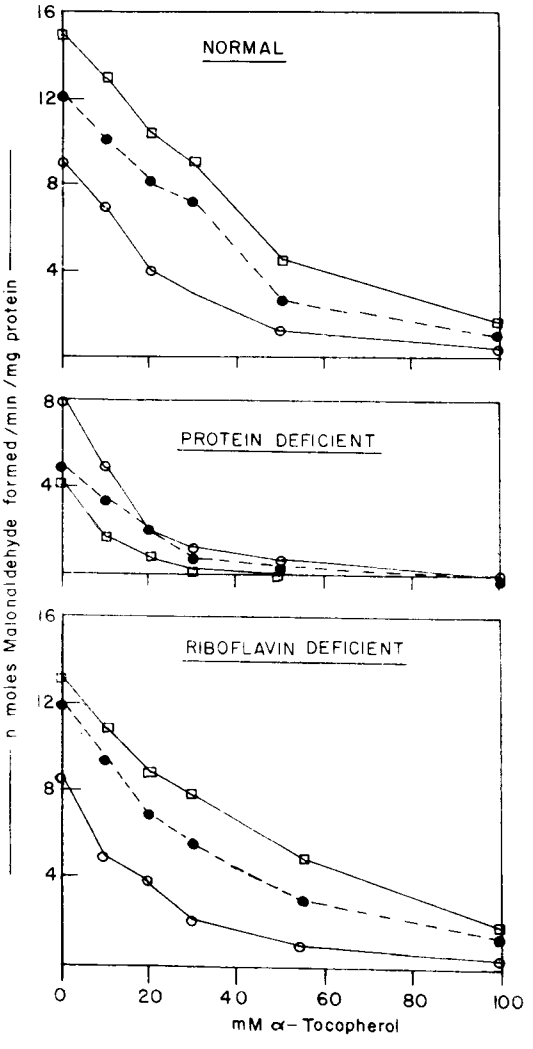

FIG. 4. Effect of tocopherol upon triphosphopyridine nucleotide, reduced form-linked lipid peroxidation in normal, protein, and riboflavin-deficient young male rats. □—□ = 7 weeks feeding, ●---● = 4 weeks feeding, and ○—○ = 2 weeks feeding.

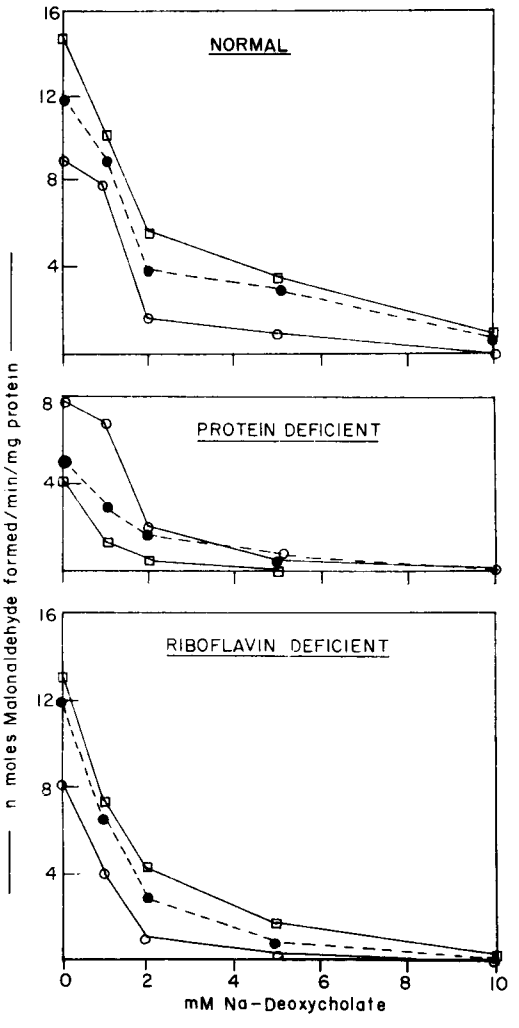

FIG. 5. Effect of sodium deoxycholate upon triphosphopyridine nucleotide, reduced form-linked lipid peroxidation in normal, protein, and riboflavin-deficient young male rats. □—□ = 7 weeks feeding, ●—● = 4 weeks feeding, and ○—○ = 2 weeks feeding.

Animals from riboflavin and control groups showed similar responses in the presence of α-tocopherol. The requirement of antioxidant for protection against lipid peroxidation was higher at the end of the 7 weeks feeding period than after 2 or 4 weeks. The inhibition in presence of 100 mM α-tocopherol was 70.0 and 74% in riboflavin-deficient and control animals, respectively, at the end of 7 weeks feeding period.

Deoxycholate and TPNH-linked lipid peroxidation: Deoxycholate inhibited the lipid peroxide formation in all the three groups of animals irrespective of the nature of the diet (Fig. 5). The inhibition due to deoxycholate increased as the protein deficiency increased, but the magnitude of inhibition in both riboflavin-deficient and control animals was lower than the low protein fed animals. The percentage inhibition due to 2 mM deoxycholate was 67, 75, and 66 in riboflavin-deficient animals during 2, 4 and 7 weeks deficiency levels, respectively.

In control animals, the decrease was less than in riboflavin-deficient animals at the end of 4 and 7 weeks feeding period. The percentage inhibition was 67 and 60 at the end of 4 and 7 weeks, respectively.

Phenobarbital pretreatment and oxygen consumption: Oxygen consumption was decreased significantly in animals fed on low protein diet

(Table III). In riboflavin-deficient animals, a slight increase in oxygen consumption was observed up to the end of 4 weeks, and a slight decline was observed at the end of 7 weeks of riboflavin deficiency. In all animals, a marked stimulation in oxygen uptake was observed due to the phenobarbital pretreatment. The magnitude of stimulation at the end of 7 weeks was more in animals from the protein-deficient group than in animals from the riboflavin-deficient and control groups.

DISCUSSION

Previous studies (18) from this laboratory have indicated that the peroxidation of subcellular lipids was influenced due to the dietary status of the animals. The present results indicate that TPNH-linked lipid peroxidation was inhibited by GSH, cytochrome C, vitamin E, and deoxycholate and that the magnitude of inhibition due to all compounds was affected due to the dietary status of the animals. Wills (24) indicated that the liver microsomal fraction clearly contains an iron compound essential for the formation of lipid peroxide in the presence of ascorbate or TPNH. The observed different pattern of inhibition due to cytochrome C may be due to the different levels of flavins caused by the deficient diet (25). The deoxycholate causes membrane disintegration and inhibits lipid peroxide formation. The observed inhibition pattern in different dietary groups could be due to different levels of polyunsaturated fatty acids and the levels of electron transport components. The lowering of polyunsaturated fatty acids during riboflavin deficiency in rats has been reported (26). The decrease in microsomal electron transport components during protein (27) and riboflavin (25) deficiencies also has been noticed. Vitamin E could protect against the lipid peroxide formation by acting as an antioxidant and also may act by preventing the initial loss of hydroperoxidation or peroxide bond or by scavenging the free radicals formed (28).

TABLE III

Effect of Low Protein, Riboflavin Deficiency, and Phenobarbital Pretreatment upon Oxygen Consumption in Young Male Rats

Group	Feeding period (weeks)	Oxygen consumption nmoles/min/mg protein		Percent difference due to diet	Percent of individual control due to phenobarbital pretreatment
		Control	Phenobarbital treated		
Normal	2	23.0 ± 2.0[a]	31.0 ± 4.0	---	133.0[b]
	4	25.0 ± 3.0	41.0 ± 3.0	--	160.0[b]
	7	32.0 ± 1.0	51.0 ± 2.0	--	156.0[b]
Low protein	2	16.0 ± 3.0	24.0 ± 2.0	−29.0[c]	115.0[c]
	4	30.0 ± 2.0	31.0 ± 1.0	−16.0[c]	103.0
	7	20.0 ± 3.0	37.0 ± 3.0	−38.0[c]	185.0[b]
Riboflavin-deficient	2	24.0 ± 2.0	32.0 ± 3.0	±4.0	132.0[c]
	4	30.0 ± 1.0	38.0 ± 2.0	±18.0	126.0[c]
	7	30.0 ± 4.0	41.0 ± 4.0	−6.0	134.0[c]

[a] Mean ± standard error of mean (5 rats in each group)
[b] P<0.01.
[c] P<0.001.

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Fatty Acid Synthetase of Bovine Mammary: Properties and Products

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ABSTRACT

Fatty acid synthetase purified (20 times) from lactating bovine mammary tissue had an approximate mol wt of 485,000. The enzyme had a high content of acidic and hydrophobic amino acid residues; 62 ± 4 sulhydryl groups and one 4'phosphopantetheine residue/mole of enzyme. The enzyme was relatively stable when stored (3 mg/ml) in potassium phosphate buffer (250 mM), containing dithiothreitol (5 mM) at -5 C or at -30 C or as a lyophilized powder at -30 C. Preincubation at 37 C in presence of dithioltreitol (5 mM) was necessary for obtaining maximum activity at the optimum pH of 6.8. Maximum specific activity of the isolated enzyme was 55 nmoles acetyl-coenzyme A $min^{-1}mg^{-1}$ incorporated into fatty acids. Butyryl-coenzyme A or acetyl-coenzyme A (30 μM), malonyl-coenzyme A (65 μM), and nicotinamide adenine dinucleotide phosphate, reduced form (300 μM) were required for optimum fatty acid synthesis. Malonyl-coenzyme A decarboxylase activity (5 nmoles $min^{-1}mg^{-1}$) associated with the enzyme permitted fatty acid synthesis in the presence of nicotinamide adenine dinucleotide phosphate, reduced form and malonyl-coenzyme A. The enzyme utilized acetyl-coenzyme A, butyryl-coenzyme A, and hexanoyl-coenzyme A as primers, with butyryl-coenzyme A giving the maximum rate of fatty acid synthesis. Apparent Km values of 22, 6.7, 3, 22, and 20 μM were obtained for malonyl-coenzyme A, acetyl-coenzyme A, butyryl-coenzyme A, hexanoyl-coenzyme A, and nicotinamide adenine dinucleotide phosphate, reduced form, The fatty acid synthetase was inhibited by N-ethylmaleimide, iodoacetamide, and progressively inhibited by increasing concentrations of long chain acyl-coenzyme A, i.e. palmityl-coenzyme A and myristyl-coenzyme A. This inhibition was relieved by bovine serum albumin or β-lactoglobulin (3 mg/ml). Palmitic acid was the major product of bovine mammary fatty acid synthetase. However, small amounts of fatty acids, 4:0-14:0 inclusive, also were synthesized. The pattern of fatty acids was altered by varying malonyl-coenzyme A to acetyl-coenzyme A ratios and by increasing the enzyme levels in the assays. At high concentrations of enzyme (0.5 mg/ml), greater amounts of short and medium chain fatty acids were generated.

INTRODUCTION

Ca. 40% of the fatty acids of cows' milk is synthesized de novo by mammary fatty acid synthetase (1,2). These consist of butyric 22, hexanoic 12, octanoic 6.5, decanoic 8, dodecanoic 10, tetradecanoic 21, and hexadecanoic 19, mole percent, respectively. Most of the 4:0, 6:0, and 8:0 acids are acylated in *sn*-3, and the remainder occur in position *sn*-2 and *sn*-3 of milk triglycerides (3-5).

The specific mechanisms controlling their orderly synthesis and the physiological-biochemical significance of these fatty acids in relation to the synthesis of triglycerides for secretion have not been elucidated. During lactogenesis, the fatty acid products of bovine mammary fatty acid synthetase (FAS) change from mostly long chain (16:0, 18:0) to short and medium chain lengths, typical of those found in milk (6,7). Thus, studies of the changes in properties of isolated FAS may reveal whether intrinsic properties of the FAS change during this period or if other factors are responsible for modulating product composition. Carey and Dils (8) have reported the presence of a specific protein in rabbit mammary which modified the chain length of the fatty acids synthesized by rabbit mammary FAS.

While establishing standardized procedures for monitoring changes in mammary FAS during lactogenesis, we studied the properties of FAS isolated from lactating cow mammary tissue. The results of these studies are reported herein and compared with data obtained for this enzyme from cow, rat, rabbit and goat mammary (9-12).

MATERIALS AND METHODS

Materials: 1-^{14}C-acetyl-coenzyme A (CoA),

TABLE I

Purification of Fatty Acid Synthetase from Bovine Mammary Gland

Fraction	Protein (mg)	Specific activity[a] (nmoles/mg)	Total enzyme activity	Recovery (%)	Purification factor
Particle-free supernatant	1,300	1.8	2,340	100	—
20-40% Ammonium sulfate fraction	520	3.0	1,560	66	1.7
Calcium phosphate gel fraction	360	4.0	1,440	60	2.2
Diethylaminoethyl cellulose peak	30	20.0	600	26	11.1
Ammonium sulfate fraction	10.5	25.0	260	11	14.0
Sephadex	6.0	35.0	210	9	19.5

[a]nmoles Acetyl-coenzyme A, incorporated into fatty acids/min/mg protein. The supernatant was obtained from mammary homogenate corresponding to ca. 35 g original mammary tissue which contained roughly 110-120 mg fatty acid synthetase, of which ca. 50% was lost during preparative centrifugation. Thus, mammary tissue, from a cow yielding 20 kg/g milk/day, contained ca. 7.3 μmoles fatty acid synthetase per kg.

2-^{14}C-malonyl-CoA, 1,3-^{14}C-malonyl-CoA, and 1-^{14}C-butyryl-CoA were purchased (New England Nuclear, Boston, Mass.). Acyl-CoA species of different chain lengths were obtained from P-L Biochemicals (Milwaukee, Wis.). Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), nicotinamide adenine dinucleotide, reduced form (NADH), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Calcium phosphate gel, prepared by the method of Keilin and Hartree (13), and ammonium sulfate were obtained from Nutritional Biochemicals (Cleveland, Ohio). Bis-acrylamide, acrylamide, and riboflavin were products of Eastman Kodak (Rochester, N.Y.). Sephadex and Sepharose were purchased from Pharmacia (Piscataway, N.J.), diethylaminoethyl (DEAE) cellulose from Schwarz/Mann Research Lab (Orangeburg, N.Y.); dithiothreitol (DTT) from Calbiochem (La Jolla, Calif.); 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), N-ethyl-maleimide (NEMI), and iodoacemaide were obtained from Aldrich Chemicals (Milwaukee, Wis.). An automated fraction collector (ISCO, Omaha, Neb.) was used to monitor column effluents for protein.

Purification of bovine mammary FAS: All potassium phosphate buffers (pH 6.8) contained ethylenediaminetetraacetic acid (EDTA) (3 mM) and dithiothreitol (5 mM). Preparative procedures were carried out at 5 C. DEAE, Sepharose, and Sephadex column chromatographies were done at 25 C.

Mammary tissue was obtained from lactating cows immediately after slaughter and chilled to 5 C. Connective and lymph tissues were removed, and the epithelial tissue was ground in a meat grinder, diluted twofold with potassium phosphate buffer (100 mM, pH 6.8), and homogenized in a Waring blender for 30 sec at 5 C. This preparation was rehomogenized in a Waring blender with a polytron assembly. This homogenate was centrifuged in a refrigerated Sorvall centrifuge using an GSA rotor (r = 5.75 in.) at 15,000 x g for 20 min at 4 C. The supernatant was decanted and strained through 2 layers of cheese cloth to remove the fat layer and centrifuged in a Beckman model L2-65 preparative ultracentrifuge using a type-21 fixed angle rotor at 44,000 x g for 75 min. The final supernatant was used as such, stored at -30 C, or quickly frozen in a round bottom flask by means of a dry ice-acetone bath and was lyophilized in a Virtis freeze-drier (at 0.03 mm Hg pressure at 24 C for 24 hr). The resulting powder was ground to a fine consistency and stored in sealed vials at -30 C.

The protocol of Hsu, et al., (14) was used in the purification of FAS from the high speed supernatant. Most of the FAS activity was obtained in a protein fraction precipitated from the 100,000 g supernatant using ammonium sulfate at 25-40% saturation. This protein was dissolved in 80 ml potassium phosphate buffer (5 mM, pH 6.8) containing DTT (5 mM) and EDTA (3 mM). This solution was purified further by adding calcium phosphate gel (0.5 mg/mg protein), gently stirring for 2 min, and then centrifuging (5000 g for 4 min) to remove the calcium phosphate gel and adsorbed protein. The resultant supernatant solution was applied to a column of DEAE cellulose (20 cm x 3.0 cm) prepared and washed as described (14). Following elution of inactive protein with 50 mM buffer, the FAS was eluted with concentrated buffer (250 mM). After pooling the eluate (60-90 ml fractions), the enzyme was precipitated with ammonium sulfate at 35% saturation and recovered by centrifugation

(18,000 g for 10 min), and the precipitate was dissolved in 1 ml potassium phosphate buffer (250 mM, pH 6.8). This solution was applied to a column (22 x 2 cm) of Sephadex G-200 previously equilibrated with 250 mM potassium phosphate buffer. The FAS activity was eluted with the same buffer in a volume of 2 ml at a relative retention volume of 1.3.

For estimation of mol wt, the FAS from the Sephadex column was passed through a column (60 x 1.5 cm) of Sepharose 6B that previously was calibrated with apoferritin, BSA, and α-lactalbumin.

The isolated enzyme was very unstable and denatured extremely easily precipitating as long strands of protein in dilute buffer following agitation or stirring. Because of its instability, the enzyme was assayed immediately; frozen to -30 C for short term storage, or freeze-dried and stored at -30 C.

Assay for FAS activity: Enzyme activity was measured using radiochemical assays. Assay tubes contained potassium phosphate buffer, 100 mM (pH 6.8); DTT, 5 mM; EDTA, 3 mM; 1-^{14}C-acetyl-CoA, 30 μM (15 x 10^4 cpm); malonyl-CoA, 65 μM; NADPH, 300 μM; and 50 μg enzyme in a total volume of 1 ml. Reaction mixtures, without malonyl-CoA, were preincubated at 35 C for 7 min to obtain maximum activity.

Reactions were started with malonyl-CoA and terminated, after 5 min, by adding 0.05 ml perchloric acid (60%) and 0.5 ml ethanol. We added 5 μmoles mixture of carrier fatty acids (4:0-16:0 inclusive) to the assay tubes and extracted products 3 times with 1.5 ml aliquots of hexane. Extracts were pooled, and hexane was evaporated at 4 C under nitrogen to a final volume of 2 ml to minimize loss of volatile short chain fatty acids. Radioactivity in duplicate aliquots (100 μliter each) was determined using a Packard TriCarb (model 3385) liquid scintillation spectrophotometer.

A unit of enzyme activity is the amount of enzyme required to incorporate 1 nmole of acetyl-CoA into fatty acids/min at 35 C.

Products of the enzyme were analyzed by thin layer chromatography (TLC) as described (15). Radioactivity in individual fatty acids was quantified by radio-gas chromatography using conditions previously established (3,16). The response of the radioactive monitor was calibrated using standard solutions of 1-^{14}C-butyric, 1-^{14}C-hexanoic, and 1-^{14}C-myristic acid.

Protein was determined by the method of Lowry, et al., (17) using the system outlined by Smith and Abraham (10) where necessary.

Sucrose density centrifugation: Sucrose density gradient centrifugation of the enzyme using

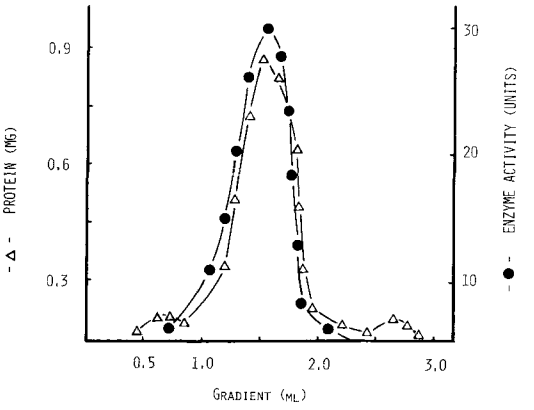

FIG. 1. Sucrose density gradient profile of bovine mammary fatty acid synthetase carried out as described in "Materials and Methods."

linear sucrose gradients of 5-20% sucrose in potassium phosphate buffer (250 mM, pH 6.8) was carried out in a Beckman SW39 swinging bucket rotor at 35,000 rpm for 16 hr at 5 C, as described by Smith and Abraham (10). After centrifugation, aliquots (0.2 ml) were collected from the bottom of the centrifuge tube with a 22 gauge needle. These were diluted to 0.8 ml and assayed for enzyme activity and protein content.

Amino acids: The amino acid composition of bovine mammary FAS was determined using a Beckman model 120 amino acid analyzer. Samples of the isolated enzyme were dialyzed against water, freeze-dried, hydrolyzed with 6N HCl for 22 hr, and analyzed by the conventional procedure as outlined in the Beckman manual (18). For quantification of cystine and estimation of 4'phosphopantetheine (from the taurine and β-alanine residues), the enzyme first was oxidized with performic acid, the hydrolyzed and analyzed as described (19). Tryptophan was determined by the procedure of Spies and Chambers (20).

The number of sulhydryl groups/mole purified enzyme was determined by the procedure of Ellman (21) using 5,5'dithiobis (2-nitrobenzoic acid) DTNB and conditions as described by Kumar, et al. (22).

Polyacrylamide disc gel electrophoresis was used to examine homogeneity of the enzyme. Discs, varying in gel concentration, were made by the method of Hedrick and Smith (23), and conditions used by Carey and Dils (11) were followed for electrophoresis of the enzyme. Fixing and staining of protein band(s) were achieved using Coomassie blue and diffusion destaining with trichloroacetic acid (12%).

Malonyl-CoA decarboxylase: Malonyl-CoA

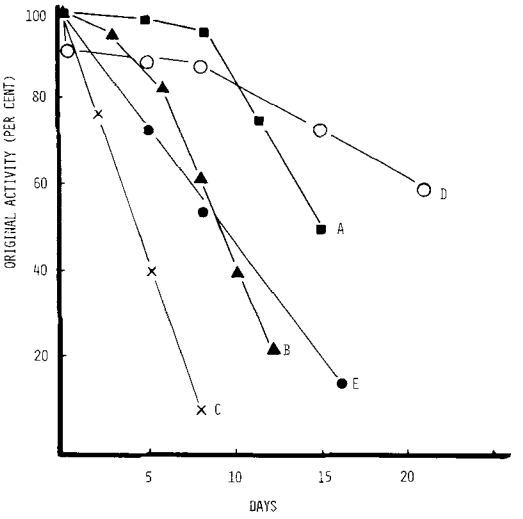

FIG. 2. Stability of bovine mammary fatty acid synthetase during storage. Purified fatty acid synthetase (3 g/ml) in 0.25 M potassium phosphate containing dithiothreitol (5 mM), ethylenediaminetetraacetic acid (3 mM) stored at -5 C (A), 21 C (B), and C same as B but protein at 0.5 mg/ml. Samples D and E represent freeze-dried enzyme (A) stored at -30 C and 21 C, respectively. Assays were as described in "Materials and Methods."

decarboxylase (EC 4.1.1.9) activity associated with the isolated FAS was measured by the release of $^{14}CO_2$ from 1,3-^{14}C-malonyl-CoA (60 μM, 5 x 10^5 cpm) using the standard FAS assay system minus NADPH. Incubations were carried out (10 min at 35 C) in small, sealed Warburg flasks containing 0.5 ml 1N ethanolic KOH in the center well. The reaction was started by the addition of enzyme and stopped by injecting 0.2 ml perchloric acid (60%). The contents of the center well containing evolved $^{14}CO_2$ were transferred quantitatively to a scintillation vial; 10 ml Aquasol (New England Nuclear) was added and the radioactivity determined in a Packard (model 3385) liquid scintillation spectrometer.

RESULTS

Purification: The FAS mostly was located in the 100,000 g supernatant, i.e. 1-3 units/mg protein, though activity was associated with the microsomal fraction (0.1-0.2 units/mg protein). A 20-fold purification and a 9% yield of FAS was obtained using mammary tissue from a cow producing 700 g milk fat/day (Table I). The activity in the supernatant fractions was quite variable, and supernatants from cows yielding 300, 700, and 900 g milk fat/day showed activities of 0.8, 1.8, and 2.3 nmoles acetate incorporated into fatty acids/mg protein/min. The specific activity of the isolated FAS also varied with stage of lactation, and an average value of 39 (range 25-55) was obtained for 5 animals yielding from 300-1200 g fat/day. This was within the range obtained by other workers for FAS from mammary tissue of rat (10), rabbit (11), cow (9,24,25) and guinea pig (26).

Purity: The isolated FAS was eluted as a single protein peak of constant specific activity from Sephadex G200 in 1.3 ml potassium phosphate buffer (250 mM) immediately after the void volume ($\frac{Ve}{Vo}$ 1.2). Both protein and FAS activity emerged from the calibrated Sepharose 6-B column as a single symmetrical peak. From plots of the logs of the mol wt of marker proteins (α-lactalbumin BSA and apoferritin) against their respective elution volumes from the Sepharose column, a mol wt of 485 x 10^3 daltons was estimated for FAS.

Enzyme activity and protein were coincident in the sucrose gradient following centrifugation (Fig. 1). The FAS was recovered in a volume of 1 ml with its peak at 1.6 ml, while the peak of the marker protein (pyruvate kinase) occurred at 2.3 ml gradient as it was tapped from the centrifuge tube. A sedimentation value of 13.3 S for the FAS corresponding to an approximate mol wt of 483 x 10^3 daltons was calculated. The isolated enzyme appeared as 2 protein bands with R_f values of 0.3 and 0.5 following gel electrophoresis using a gel concentration of 5.5%. The mobility of the major protein band was affected inversely by gel concentration.

Stability: Enzyme activity was stable for ca. 3 months when the supernatant solution was stored at -30 C. The purified enzyme was unstable, and its stability was affected by concentration of both protein and buffer, storage temperatures, and the presence or absence of thiol reducing agents (DTT). Storage of FAS (3 mg/ml) at -5 C in phosphate buffer (250 mM) containing EDTA (3 mM) and DTT (5 mM) ensured stability for 12 days (Fig. 2). Saturation of this solution with nitrogen prolonged stability slightly, i.e. up to 15 days, after which activity diminished but storage at -30 C markedly prolonged retention of activity, e.g. 80% after 2 months. The omission of DTT, low concentrations of enzyme (0.3–1 mg/ml), or use of dilute buffers (<100 mM) accentuated instability of FAS at all temperatures. Lyophilization resulted in an initial loss of 10% of activity, but, during subsequent storage at -30 C, the enzyme was more stable than frozen solutions of FAS stored at -30 C.

Amino acid composition: The enzyme had a high content of hydrophobic residues and acidic amino acids, especially glutamic acid (Table II). Glutamic acid and proline were noticeably

TABLE II

Amino Acid Composition of Mammary Fatty Acid Synthetases from Cow, Rat, and Rabbit

Amino acid	Moles/10^5 g enzyme		
	Bovine	Rat[a]	Rabbit[b]
Lysine	37.9	32.0	25.5
Histidine	19.8	23.0	14.0
Arginine	31.5	39.0	34.0
Aspartic acid	63.3	62.0	47.0
Threonine	37.3	41.0	26.5
Serine	51.7	59.6	35.5
Glutamic acid	104.3	84.7	71.0
Proline	55.0	47.5	42.6
Glycine	59.1	63.0	49.0
Alanine	57.1	68.0	62.0
Half-cystine	12.8	11.7	---
Valine	49.6	55.8	45.0
Methionine	13.0	14.4	---
Isoleucine	30.5	28.2	19.0
Leucine	90.7	95.6	70.4
Tyrosine	18.5	18.2	10.0
Phenylalanine	24.1	26.3	18.7
Tryptophan	16.9	16.3	---

[a]Data of Smith and Abraham (10).

[b]Data of Carey and Dils (11).

higher in the bovine compared to FAS from the rat and rabbit mammary gland (10,11), whereas the content of hydrophobic amino acids were quite similar in bovine and rat mammary FAS.

The number of cysteic acid residues obtained following performic acid oxidation was 64 ± 6 (n=4), whereas, titration, using DTNB (22), indicated 62 ± 4 (n=4) thiol groups/mole enzyme. From the amino acid composition, a minimum mol wt of 484 x 10^3 was calculated.

Enzymatic properties of FAS: The rate of incorporation of 1-^{14}C-acetyl-CoA into fatty acids was directly proportional to protein concentration up to 150 μg (Fig. 3). Using 50 μg FAS and standard assay concentrations (acetyl-CoA, 30 μM; NADPH, 300 μM; and malonyl-CoA, 65 μM), the rate of fatty acid synthesis was linear for 12 min. Malonyl-CoA, acetyl-CoA, and NADPH were required substrates for optimum synthesis of fatty acids. NADH was not a good replacement for NADPH. Dithiothreitol (DTT) markedly stimulated (2 times) the FAS activity. Significant quantities of fatty acids were synthesized in the absence of added acetyl-CoA, indicating the presence of malonyl-CoA decarboxylase activity associated with the FAS. This was confirmed by appropriate experiments (Table III). Thus, bovine mammary FAS, like the enzyme from rabbit mammary (27,28), pigeon liver (29), and yeast (30), possesses malonyl-CoA decarboxylase activity.

Bovine FAS had a pH optimum of 6.8, and its activity remained above 90% in the pH range

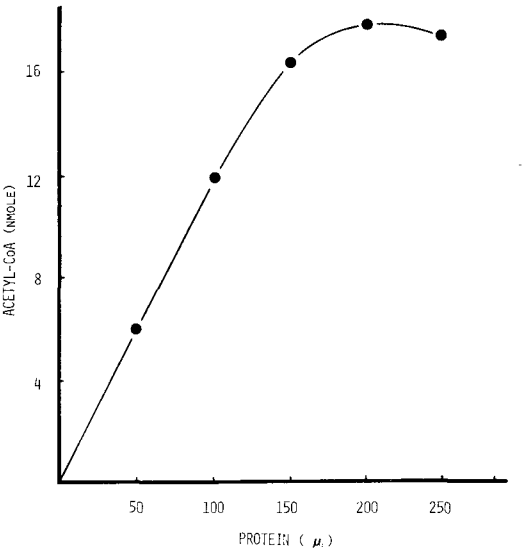

FIG. 3. Relationship between rate of incorporation of acetyl-coenzyme A into fatty acids and the concentration of fatty acid synthetase (FAS). Complete assay had malonyl-coenzyme A, 65 μM; acetyl-coenzyme A, 30 μM; nicotinamide adenine dinucleotide phosphate, reduced form, 0.3 mM; ethylenediaminetetraacetic acid, 3 mM; 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane, 5 mM; potassium phosphate buffer (0.1 M, pH 6.8) 100 mM, and varying levels of FAS protein in 1 ml. Each was incubated for 5 min at 35 C and analyzed as described in "Materials and Methods."

6.2-7.3. This is similar to the pH optima of FAS from mammary and liver tissues of other species studied (10-12, 29).

NADPH was the only effective hydrogen donor. NADH (300 μM) supported only 10% activity compared to NADPH. The apparent Km for NADPH was 20 μM which is comparable to the Km values of 14 and 34 μM obtained for other mammary FAS (10,24).

The effect of increasing malonyl-CoA concentration (Fig. 4) revealed a slight depression of activity above 80 μM. This was eliminated at higher concentrations of acetyl-CoA (30 μM). Maximum rates of incorporation were observed at malonyl-CoA concentrations of 65 μM. The absence of marked inhibition by malonyl-CoA is consistent with behavior of rat and rabbit mammary FAS but in contrast to the behavior of pigeon liver FAS which was somewhat inhibited by malonyl-CoA at 75 μM (29).

Because of the ability of lactating bovine mammary tissue to elongate several short chain fatty acids (31,32), we studied the ability of purified FAS to utilize several primers (Fig. 5). Butyryl-CoA was the preferred primer, being twice as effective as acetyl-CoA at equimolar concentrations. Hexanoyl- and octanoyl-CoA were effective primers, though some inhibition

TABLE III

Evidence of Non-NADPH[a] Dependent Malonyl Coenzyme A Decarboxylation by Bovine Mammary Fatty Acid Synthetase[b]

Treatment	$^{14}CO_2$ Released	Malonyl-CoA incorporated into fatty acids (nmoles/min/100 μg protein)
Flask A + malonyl coenzyme A −NADPH	8.4	1.0
Flask B + malonyl-coenzyme A + NADPH	13.1	5.8
Flask C + malonyl-coenzyme A + Acetyl-coenzyme A + NADPH	40.2	36.0

[a]NADPH = nicotinamide adenine dinucleotide phosphate, reduced form.

[b]All assay flasks contained 1,3-^{14}C-malonyl-coenzyme A (100 μM, 50 x 10^3 cpm) and fatty acid synthetase (100 μg). Flask B contained NADPH (300 μM), and Flask C contained NADPH (300 μM) and acetyl-coenzyme A (30 μM). Incubations were for 5 min at 35 C. The $^{14}CO_2$ was trapped in ethanolic KOH (1N). The radioactivity in the $^{14}CO_2$ and in the extracted fatty acids was determined as described in "Materials and Methods."

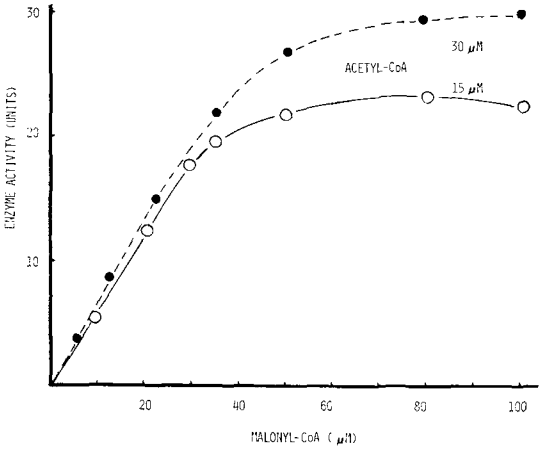

FIG. 4. The effect of varying malonyl-coenzyme A levels upon the rate of fatty acid synthesis by bovine mammary fatty acid synthetase at two concentrations of acetyl-coenzyme A. Assay as in "Materials and Methods."

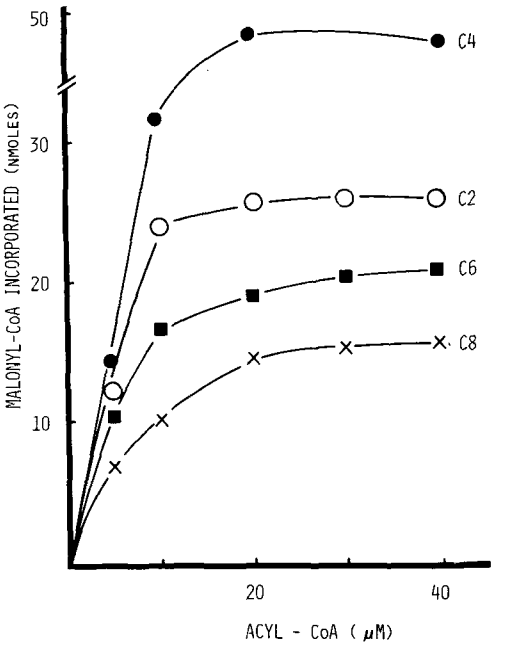

FIG. 5. The effect of different acyl-coenzyme A primer molecules upon the activity of bovine mammary fatty acid synthetase as measured by incorporation of 1-^{14}C-malonyl-coenzyme A into fatty acids. Assays as described in "Materials and Methods."

occurred above 40 μM. The FAS from rat and goat mammary also used these primers for fatty acid synthesis (10,12). Lin and Kumar (33) reported that a crude FAS from several tissues (rat, bovine, and rabbit mammary and liver) synthesized fatty acids two to three times as rapidly when butyryl-CoA was provided as primer.

Kinetic characteristics: Lineweaver-Burk plots yielded Km values of 22 μM, 6.7 μM, 3 μM, and 22 μM for malonyl-, acetyl-, butyryl-, and hexanoyl-CoA, respectively (Fig. 6A,B and C). The affinity of malonyl-CoA for FAS was intermediate between that reported for the mammary FAS from rat (13 μM), rabbit (29 μM), and guinea pig (27 μM) but quite similar to that from another cow (18.5 μM) (9-11, 26).

The Km of bovine FAS for acetyl-CoA was lower than those of rat, rabbit, guinea pig and goat mammary FAS which were 22, 9, 24 and 9 μM, respectively (10-12, 26).

Bovine mammary FAS showed a marked affinity for butyryl-CoA consistant with its

superiority as a primer, and the observed Km was comparable to that for rat (3 μM) and goat (5 μM) mammary FAS (10,12). The Km values obtained for acetyl-CoA and butyryl-CoA with our enzyme were ca. half those reported by Maitra and Kumar (25), but, in both studies, the Km for acetyl-CoA was twice that for butyryl-CoA.

The observed maximum velocities (Vmax) revealed that butyryl-CoA gave ca. twice the rates as acetyl-CoA and that malonyl-CoA was utilized at ca. seven times the rate of acetyl-CoA.

Inhibition: The involvement of sulfhydryl groups in the activity of bovine mammary FAS was indicated by the inhibitory effects of N-ethylmaleimide (1 mM) and iodoacetamide (1 mM) which reduced activity by 85 and 50%, respectively, and by the marked stimulation by DTT. This is consistent with the behavior of FAS from other sources (34).

Acyl-CoA species exerted progressively greater inhibitory effects with increasing wt and concentration. The 90% inhibition by 50 μM palmityl-CoA or myristyl-CoA was relieved by BSA or bovine β-lactoglobulin at 3 mg/ml. The latter protein occurs abundantly in milk but has no apparent biochemical function.

Products: The purified FAS from lactating bovine mammary tissue produced free fatty acids, and neither radioactive acyl thioesters (acyl-CoA) nor ester lipids were detected in the lipid extracts by paper or TLC. Palmitic acid was the principal product of bovine mammary FAS. The level of protein in the assays influenced the chain length of products (Table IV). At low enzyme (protein) levels, palmitic acid was the major product though significant quantities of myristic and butyric acid also were synthesized. When protein was increased, the production of palmitic acid decreased with a concomitant increase in levels of short and medium chain fatty acids. This observation is important, because, to date, isolated mammary FAS at low concentrations (10-20 μg/ml) have failed to synthesize products similar to those produced by the enzyme in vivo.

By using varying amounts of malonyl-CoA in the presence of fixed quantities of acetyl-CoA (30 μM), the chain length of fatty acid products was varied in a manner similar to that observed by other researchers (25,27,35-38). At low malonyl-CoA levels (10-20 μM), short chain (4:0,6:0, and 8:0) fatty acids were generated, and, as malonyl-CoA levels were increased up to 100 μM, the chain length of the products increased progressively. At no malonly-CoA to acetyl-CoA ratio was a typical pattern of milk fatty acids obtained. However, when larger

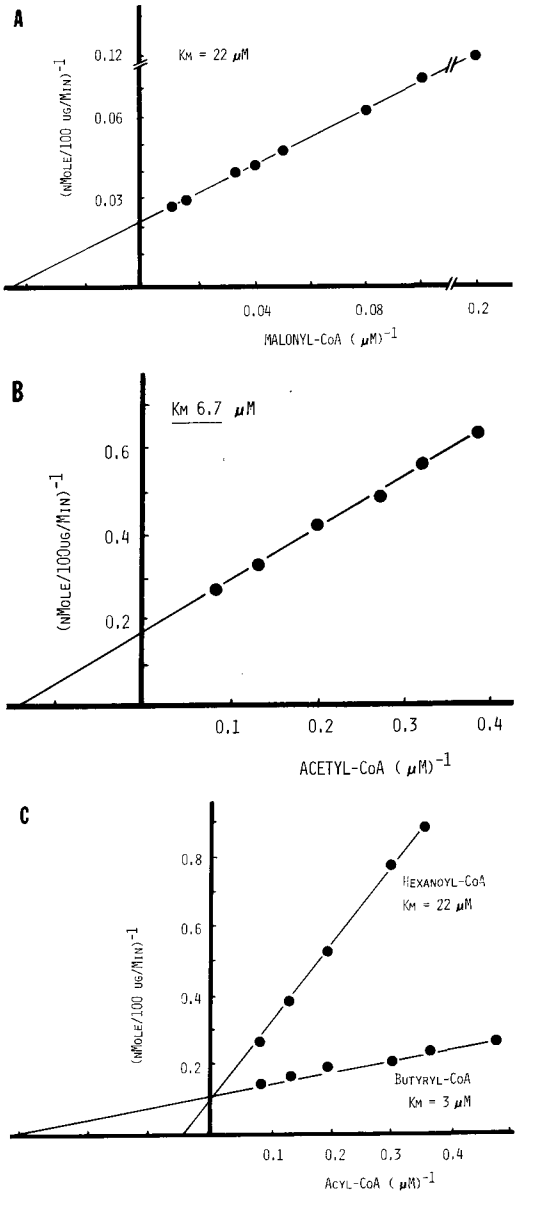

FIG. 6. Lineweaver-Burk plots for malonyl-coenzyme A (A), acetyl-coenzyme A (B), and butyryl-coenzyme A and hexanoyl-coenzyme A (C). Varying quantities of each substrate were incubated with isolated bovine mammary fatty acid synthetase using the standard assay as in "Materials and Methods."

quantities (>0.5 mg) of crude enzyme were used with acetyl-CoA (50 μM) and possibly limiting amounts of malonyl-CoA (50 μM), a series of short and medium chain fatty acids were produced which were more typical of those produced in vivo.

TABLE IV

Distribution of Radioactivity in Fatty Acids Synthesized by Different Concentrations of Isolated Mammary Fatty Acid Synthetase[a]

Fatty acid		Fatty acid synthetase level (μg) 50	100	250	500
Butyric	(C4)	5.1	7.5	8.0	11.2
Hexanoic	(C6)	2.0	5.7	4.7	5.8
Octanoic	(C8)	1.9	4.9	5.0	5.1
Decanoic	(C10)	1.8	4.2	4.8	5.2
Dodecanoic	(C12)	1.6	5.6	6.8	8.5
Tetradecanoic	(C14)	8.1	15.8	19.7	23.9
Hexadecanoic	(C16)	79.4	56.3	51.0	40.3
Octadecanoic	(C18)	T[b]	T	T	T

[a]Assays contained 1-^{14}C-acetyl-coenzyme A (30 μM); malonyl-coenzyme A (65 μM); nicotinamide adenine dinucleotide phosphate, reduced form (300 μM). dithiothreitol (3 mM); ethylenediaminetetraacetic acid (1 mM); and fatty acid synthetase in 1 ml potassium phosphate buffer (100 mM, pH 6.8) were incubated at 35 C for 5 min.

[b]T = trace.

DISCUSSION

The FAS from various mammary tissues are quite similar (Table V). All are apparently multienzyme complexes with comparable mol wt, except the FAS from rabbit mammary.

The purification achieved was higher, whereas the recovery was lower than that reported for other cow mammary preparations (9,24,25), but, in general, was of the same order as reported for other species (10-12,26). The maximum specific activity obtained (55 nmoles acetyl-CoA incorporated $mg^{-1}min^{-1}$) was comparable to that of FAS isolated from lactating mammary tissue of other species. Because of the activity of malonyl-CoA decarboxylase, the observed specific activity cited above may be low by 10-15%.

The estimated mol wt, 485 x 10^3, was lower than that (530 x 10^3) reported by Maitra and Kumar (9) and greater than that (450 x 10^3) found by Knudsen (24) for FAS from lactating bovine mammary gland. The corresponding values for mammary FAS from rat, rabbit, and guinea pig were 478, 900, and 400 x 10^3 daltons, respectively (10,11,26).

The stability of bovine FAS when stored at -30 C was consistent with the reports of Maitra and Kumar (9) and Knudsen (24). Knudsen (24) stated that purified cow mammary FAS in phosphate buffer (250 mM) containing DTT and EDTA was unstable at 4 C but stable for 2 months only when stored at -90 C. Our data indicated that cow FAS was quite stable for 12 days when stored at -5 C at 3 mg/ml, but storage at 5 C or 21 C resulted in gradual loss of activity. Like the FAS from rat (39,40) and rabbit (11), mammary cow FAS preparations were more unstable when stored at low protein concentrations in buffers of low ionic strength, and instability was more pronounced in the absence of DTT. In contrast to most findings, Maitra and Kumar (9) observed that loss of cow FAS activity was more rapid at room temperature than at 4 C and that stability was not affected by protein concentration nor by the inclusion of DTT above 1 mM.

Preincubation of the enzyme was necessary for maximum activity. Smith and Abraham (10,39) attributed this to reassociation of enzyme subunits during preincubation. However, Maitra and Kumar (9) found no evidence of

TABLE V

Comparison of Properties of Fatty Acid Synthetase from Mammary Tissue of Lactating Animals

Properties	Cow		Goat	Rat	Rabbit	Guinea pig
Molecular wt (x 10^3)	485	503	–	478	910	400
S_{20W}x10^{-13}s	13.3	13.5	12.4	13	16.5	12.3
Dissociation	+	–	+	+	+	
Sulfhydryl groups/mole	62	78	–	56	58	57
Phosphopantetheine/mole	+	–	–	1	–	–
pH optimum	6.8	6.8	6.8	6.7	6.6	6.0
Km (μM)						
Acetyl-coenzyme A	6.7	12.5	9.2	22	9.0	24
Butyryl-coenzyme A	3.0	6.6	5.2	3	–	25
Hexanoyl-coenzyme A	22.0	–	–	50	–	–
Malonyl-coenzyme A	22.0	18.5	–	13	29	27
NADPH[a]	20.0	6.8	–	34	–	–
Primer specificity	C4>C2>C6		–	C4>C2	C4>C2>C6	–
Product(s)	C4-C16		C16	C16	C16	C16
Reference	present study	(25)	(12)	(10)	(11)	(26)

[a]NADPH = nicotinamide adenine dinucleotide phosphate, reduced form.

dissociation of FAS from cow mammary. Strong and Dils (26) reported that loss in activity of FAS from guinea pig was not because of dissociation, and they reported that thiol groups (DTT) aided restoration of activity.

The stimulatory effect of DTT, the protective effect of nitrogen during storage, and inhibition by NEMI and DTNB all indicated that thiol groups are required for activity by bovine FAS. Presumably, the thiol moieties of phosphopantetheine and cysteine are involved according to the mechanisms proposed for the FAS from other tissues (34,41,42).

Bovine FAS had substrate requirements similar to FAS from other tissues. The specificity for NADPH is typical of FAS from animal tissues (34), and the apparent Km (22 μM) is much lower than the estimated intramammary concentration of NADPH (100 μM).

The FAS showed broad specificity with respect to "primers" for fatty acid synthesis, and its ability to use acetyl-, butyryl-, and hexanoyl-CoA is consistent with observations of its behavior in intact mammary tissue (31,32). The utilization of different acyl-CoA species as primers by bovine FAS indicates that the initial acyltransferase (loading enzyme) is not very specific. This observation is particularly interesting in the case of bovine mammary FAS, because these same acyl groups dissociate or are transferred from the enzyme during the normal biosynthetic sequence in vivo.

The preference for butyryl-CoA by bovine FAS is significant and consistent, because in vivo ruminant mammary tissue may absorb up to 3 moles of β-hydroxybutyric acid/day during lactation. This mostly is utilized for fatty acid synthesis predominantly as the initial (methyl end) four carbons of those fatty acids synthesized in the mammary gland (2,43,44). Lin and Kumar (33) described an active β-reductive enzyme system in mammary tissue supernatant which was capable of converting both acetyl-CoA and β-hydroxybutyryl-CoA to butyryl-CoA. In vivo, the supply of β-hydroxybutyrate to mammary tissue affects the rate of milk fat synthesis (2), and, conceivably, this may occur by directly governing the supply of primer (butyryl-CoA) to the FAS. Furthermore, though the concentration of butyryl-CoA is much lower than acetyl-CoA in lactating bovine mammary tissue (5 vs 15 μM), the apparent Kms for the FAS also are lower, i.e. 3 vs 6.7 μM for the butyryl- and acetyl-CoA, respectively, thereby ensuring efficient binding of the preferred primer.

Negligible inhibition of bovine FAS by malonyl-CoA occurred at concentrations up to 150 μM in contrast to pigeon liver FAS which was more sensitive to inhibition by malonyl-CoA (41). Conceivably, the malonyl-CoA decarboxylase associated with the mammary FAS of bovine and other species renders it less susceptible to inhibition by excess malonyl-CoA. The Km for malonyl-CoA was above the estimated malonyl-CoA level in mammary tissue, ie. 22 and 15-20 μM, respectively. Malonyl-CoA concentration may be limiting for mammary fatty acid synthesis in vivo, and significantly Mellenberger, et al., (7) showed a close correlation between the enzyme producing malonyl-CoA, i.e. acetyl-CoA:CO_2 ligase, and fatty acid synthesis in bovine mammary tissue. Furthermore, the activity of the acetyl-CoA ligase, whose Km is 50 μM for acetyl-CoA (45), may be limited by the intracellular concentration of acetyl-CoA which is 15-20 μM (46). These conditions, together with the malonyl-CoA decarboxylase, are significant, because they may not only limit the rate of fatty acid synthesis in mammary tissue but, importantly, they may maintain a low malonyl-CoA to acetyl-CoA ratio in the vicinity of the FAs and thereby influence chain lengths of the fatty acid products.

It has been well demonstrated in vitro that both the rate of fatty acid synthesis and the patterns of fatty acids made depend upon the concentration of malonyl-CoA and the malonyl-CoA to acetyl-CoA ratio (24,27,35-37). The malonyl-CoA concentration affects the rate of acyl chain elongation (condensation step) and, hence, overall rate of synthesis (34). In bovine mammary tissue, the concentration of primers (acetyl-CoA, butyryl-CoA, 20 and 10 μM, respectively) and the relatively low level of malonyl-CoA (15-20 μM) conceivably may retard the condensation step and allow the transfer of the growing acyl chains to the "leaving" site on the FAS, i.e. facilitate transacylase activity according to the mechanism proposed by Sumper, et al., (37), Plate, et al., (41) and Phillips, et al. (42). In studies with the isolated FAS, we, like others (8,10,11,25,27,36,38), found that the average chain length of the fatty acids synthesized was shortened by reducing the malonyl-CoA to primer ratio, whereas, at high malonyl-CoA levels, chain elongation occurred. However, we never simulated the pattern of products synthesized in intact tissue. In general, research has eliminated substrate ratios as the sole factors causing the production of the fatty acids peculiar to mammary tissue (8.27,35,36).

Several factors may be involved in determining the unique yet constant pattern of fatty acids synthesized by bovine mammary FAS.

The possibility of acyl thioesterase governing the specific chain lengths of mammary fatty acids was eliminated by the studies of Smith and Abraham (35). Transacylation of growing fatty acids conceivably may facilitate their continued production of FAS in vivo, and the concurrent acylation of decanoic and dodecanoic acids enhanced their biosynthesis by rabbit mammary supernatant (8). In bovine mammary tissue, the synthesis of short chain fatty acids occurs only in lactating tissue, and these acids mostly are acylated in position *sn*-3 of the secretory triglycerides (5,6,16), suggesting a relationship between the termination of acyl chain growth on the FAS of lactating tissue and the transacylases specific for position *sn*-3 of diacylglycerides.

Evidence for the existence of a discrete specifier protein, which modulated the synthesis of typical milk fatty acids by rabbit mammary FAS, has been reported (8). This effect was observed at high protein concentrations in vitro (20-30 mg/ml), which were ca. half the concentration found in vivo. This observation was significant and may help explain some of the discrepancies between products of FAS produced in vitro and in intact tissue or whole homogenates.

Under conditions contrived for classical kinetic behavior, i.e. low enzyme levels and saturating amounts of substrates, palmitic acid is the principal product of isolated mammary FAS. This contrasts with products obtained in vivo, where, in each species, a characteristic pattern of short and medium chain fatty acids is made (1, 10-12). One possible source of disparity between products synthesized in vitro and in vivo is the tremendous difference in enzyme to substrate ratios. In our assays, we used concentrations of 0.1, 30, and 65 μM enzyme, acetyl-CoA, and malonyl-CoA, respectively, whereas, in vivo, their estimated tissue concentrations are 10, 15, and 20 μM, respectively. While metabolic fluctuations and compartmentalization in tissue may alter these ratios between enzyme and substrates, the dissimilarity between the two situations is drastic. In vivo, the substrate to enzyme ratios are ca. one to two, whereas, in assays (in vitro), substrates, i.e. acetyl-CoA and malonyl-CoA, may be 300 and 600-fold greater than the enzyme concentration. This may be a significant factor influencing the nature of products obtained in vitro. Increasing the level of FAS in our assays markedly altered the products, i.e. increasing quantities of short and medium chain fatty acids were synthesized. These data indicated that FAS per se at high concentrations may influence chain length of products, though previous work with relatively low levels of FAS precluded an inherent mechanism in FAS for specifying chain termination. Carey and Dils (8) obtained evidence for a specific protein in rabbit mammary. Presumably, this factor is associated closely with mammary FAS, and, possibly, quantities of it remained associated with our FAS preparation and modified chain termination, particularly when we increased the enzyme concentration in our assays. If this specifier factor is a discrete protein, then it functions only in lactating mammary tissue, and its characteristics must differ among rat, rabbit, and ruminant, because it specifies termination at different chain lengths in each species and in ruminants, but the factor in the goat must be different from that of the bovine, because it specifies a higher concentration of decanoic acid in the goat.

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Metabolism of Linolenic Acid in Developing Brain: I. Incorporation of Radioactivity from 1-^{14}C Linolenic Acid into Brain Fatty Acids

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ABSTRACT

Twelve-thirteen day old rats were given 1-^{14}C linolenic acid by intraperitoneal injection. Fatty acids were isolated from the brains of animals sacrificed at the end of 8 and 48 hr and 15 and 45 days. Eight hr after the tracer, radioactivity was found neither in 18:3 nor its end-product, 22:6, and palmitate was the most highly radioactive component. At longer intervals, 22:6 seemed to retain much of the radioactivity, whereas palmitate showed a precipitous decline in radioactivity. Initial oxidation of linolenate and sparing of the linolenate complexed with polar lipids are discussed.

INTRODUCTION

Brain lipids contain a significant amount of polyunsaturated fatty acids (1-5), and, although both 18:2 ω6 and 18:3 ω3 fatty acids are found in negligibly small amounts, their metabolic products, 20:4 ω6 and 22:6 ω3, occur in rather large amounts. Working with chick embryo, Miyamoto, et al., (6) showed that radioactive 18:3 ω3 was taken up by the brain and readily converted to 22:6 ω3. Earlier work from this laboratory (7) showed that 18:3 ω3 was taken up directly into the brain even by adult rats and that there seemed to be no restriction to this uptake by the blood brain barrier system. However, data regarding metabolic turnover of linolenic acid (after initial uptake) covering an extended period that includes the critical period of growth and development is scanty. Therefore, questions, such as half-life, oxidation to acetate followed by synthesis, elongation, and desaturation to form other fatty acids, remain unanswered. The present study was undertaken to obtain this information, which may be useful ultimately to assess the importance of the ω3 fatty acids in the physiological function of the brain.

MATERIALS AND METHODS

Animals

Thirty-four albino Wistar rats, 12-13 days old, were used in this study. Twenty-one suckling rats were given 5 μc each of 1-^{14}C linolenic acid (all *cis* 9,12,15-octadecatrienoic acid, 52.5 mc/mM; DHOM Products, Hollywood, Calif.) as the albumin complex by intraperitoneal injection, and 11 of these rats were sacrificed 8 hr after the tracer; the remaining 10 were sacrificed 48 hr after the tracer. Seven other baby rats were given 10 μc and sacrificed 15 days later. Finally, 6 rats were given 25 μc each and allowed to survive for 45 days before sacrifice. All results were corrected for variation in dose.

Extraction, Isolation of Lipids, and Fatty Acid Fractionation

At the end of each period, the animals were sacrificed by decapitation; brain tissue was excised; and lipids were extracted by the methods of Folch, et al. (8). Cholesterol was separated from the pooled brain total lipids by use of SiO_2 (silica gel powder, 60-200 mesh, Baker, Phillipsburg, N.J.) column chromatography; 10% ether in pentane eluted the cholesteryl esters and triglycerides that occur in trace amounts in the brain and 40% ether in pentane eluted free cholesterol. The cholesterol was purified further by recrystallizing once and tested for purity by thin layer chromatography (TLC) using 16% ether in pentane with 1% acetic acid as solvent. The polar lipids were methylated to obtain total fatty acid methyl esters, which first were separated into classes, according to degree of unsaturation (9), and then into pure individual fatty acid methyl esters by preparative gas liquid chromatography (GLC) (4). The purified fatty acids were decarboxylated to determine the relative carboxyl activity (% RCA), as described previously (4).

RESULTS

The specific radioactivity (cpm/mg) of individual fatty acids over an extended period of 45 days after administration of 1-^{14}C linolenic acid is shown in Table I. Although the starting tracer material was radioactive linolenic acid, just 8 hr after the tracer, it was palmitate (not 22:6) that had the highest specific activity.

However, this activity decreased sharply over the extended period of 45 days. The maximum decrease was between 48 hr-15 days after injection. The metabolic end-product of 18:3 is 22:6, and Table I shows that 22:6 is the most highly active component throughout the experimental period, with the exception of the initial 8 hr period. In fact, at the end of 45 days, the specific activity of 22:6 is over 3 times that of most other fatty acids.

Specific activity of 18:0 does not rise as dramatically as does that of either 16:0 or 22:6, but, at the same time, radioactivity is retained over a longer period. It is surprising to find that the specific activity of oleic acid (18:1) shows a steady increase up to 48 hr after tracer administration with a very minor increase in the next 13 days in contrast to all other fatty acids, which show considerable decreases during this same period. In the later period, between 15-45 days, there is a decrease in radioactivity of 18:1 similar to that of other fatty acids. Arachidonic acid (20:4) has very low specific activity throughout the experimental period.

The values for % RCA, shown in Table I, reveal that the palmitate carboxyl carbon has ca. 13% of the total radioactivity, which is very close to the theoretical value (12.5%) for de novo synthesis of palmitate from acetate, which is a breakdown product of the injected radioactive linolenate (4,10). Stearic and oleic acids both show decreasing values of % RCA, as the period between injection and sacrifice increases. The reason for this fall in % RCA has been discussed in detail by Kishimoto and Radin (10). Arachidonate (20:4 ω6) has a very high % RCA, in distinction to the low % RCA of 22:6.

The percent composition of fatty acids by gas liquid chromatography (GLC) showed that there was almost equal percent of arachidonate and of docosahexaenoate in the suckling rat brain, but, gradually, the ratio reversed as the rats aged to 28 days, with very little change at the end of 58 days.

Figure 1 shows the relative specific radioactivity of fatty acids expressed as a product of the percent composition (GLC) and the specific activity (cpm/mg). This takes into consideration the changes in fatty acid composition during aging. The relative specific activity of palmitate is 3 times that of 22:6 or 18:0 and very much higher than that of 18:1, 8 hr after injection. However, there is a considerable drop in radioactivity of palmitate in the next 48 hr, in contrast to the increase in the activity of 22:6. At the end of 45 days, 22:6 has retained a considerable amount of radioactivity. The

TABLE I

Percent Composition, Specific Radioactivity, and Percent Relative Carboxyl Activity (RCA) of Brain Fatty Acids Following 1-^{14}C Linolenic Acid Injection

Brain fatty acid	13 Day old suckling rats 8 hr after tracer			15 Day old suckling rats 48 hr after tracer			28 Day old young rats 15 days after tracer			58 Day old adult rats 45 days after tracer		
	Percent composition GLC[a]	Specific activity cpm/mg	Percent RCA[b]	Percent composition GLC	Specific activity cpm/mg	Percent RCA	Percent composition GLC	Specific activity cpm/mg	Percent RCA	Percent composition GLC	Specific activity cpm/mg	Percent RCA
16:0	26.6	400	13.4	25.1	345	12.8	26.0	94	14.3	19.1	24	20.7
18:0	18.9	175	19.5	19.8	183	14.0	23.8	130	7.6	23.9	34	12.8
18:1	15.9	117	21.5	15.3	213	15.2	18.5	205	10.1	22.0	51	11.9
20:4	17.9	24	55.4	18.1	33	85.6	11.5	13	98.8	12.3	9	81.1
22:6	16.6	199	5.0	18:3	399	5.6	16.6	199	3.2	17.4	171	2.8

[a]Other fatty acids from the gas liquid chromatogram (GLC) run were included in the calculation but not shown in this table.

[b]$\%\ RCA = \frac{\text{Radioactivity of the carboxyl carbon}}{\text{Radioactivity of the total fatty acid}} \times 100$.

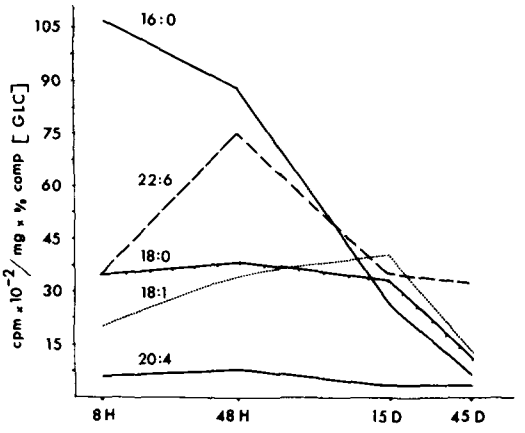

FIG. 1. Relative specific activity (cpm/mg x % composition by gas liquid chromatography) of brain fatty acids at different intervals following intraperitoneal injection of 1-^{14}C linolenic acid.

half-life of palmitate in terms of radioactivity (a result of the breakdown of linolenate to acetate followed by resynthesis) is ca. 5-6 days. In the case of 22:6, the maximum activity was observed at 48 hr after injection (but it could be anytime between 8-48 hr), and, in ca. 13 days, this activity was reduced by 50%. However, the decay curve flattens out between the fifteenth and forty-fifth days. The stearate and oleate curves are very close to each other throughout the period, except during the initial 8 hr. As expected, the radioactivity of 20:4 ω6 was low during the entire period of 45 days, since 18:3 is not a precursor of the ω6 family. The low activity comes from radioactive acetate units involved in chain elongation of endogenous nonradioactive 18:2.

DISCUSSION

The distribution of radioactivity (% RCA) in palmitate isolated from the brain is close to the theoretical value for de novo synthesis. Undoubtedly, the injected linolenic acid was degraded to provide radioactive acetate for this synthesis. This raises the question of the extent of β-oxidation in the brain. The in vitro work by Vignais, et al., (11) some time ago showed that β-oxidation of palmitate by brain mitochondria was slow and ca. one-sixth of that occurring in the liver. Later work by Beattie and Basford (12) showed that this low oxidation rate could be enhanced by higher concentrations of certain cofactors but still concluded that the brain was capable of only a limited amount of β-oxidation. Since this is not a source of energy for the brain, it appeared to be of little importance. Another cause of the low β-oxidation was attributed to a low concentration of carnitine, which acts as a carrier in the transport of long chain fatty acids into the mitochondria (13). Against this in vitro experimental background, the results of the in vivo experiments should have indicated very poor de novo synthesis of palmitate or cholesterol from an injected fatty acid, such as linolenic acid. However, the present results, as well as those observed when oleate (14) and linoleate (15) were the tracer fatty acids, show that palmitate always had considerable radioactivity. Hajra and Radin (16) also noticed some β-oxidation of long chain fatty acids following intracerebral injection. Palmitate administered to suckling rats results in considerable radioactivity in brain cholesterol (17). Thus, β-oxidation must occur in the brain itself, rather than reflect reactions occurring elsewhere in the body. However, it must be pointed out that β-oxidation occurring in the liver and elsewhere in the body could provide some acetate for synthetic reactions in the brain; also newly formed fatty acids could enter the brain via circulating blood, as shown earlier (7,14,15). High specific activities of palmitate and cholesterol then could result from a direct transport of preformed components via the circulating blood, but the relative proportions of these reactions occurring in vivo remain unknown. In general, Goransson (18) was unable to notice any differences in the oxidation of essential and nonessential fatty acids; Coots (19) found a sparing of 20:4, but Brown and Tappel (20) found that, for some unexplained reasons, linolenate was oxidized at a very much higher rate by carp liver mitochondria. In the present study, 8 hr after injection of linolenate, high radioactivities appeared both in palmitate and cholesterol, indicating considerable β-oxidation to acetate. The molecules that escape this rapid oxidation, probably those that are esterified to polar lipids, retain quite a sizeable amount of radioactivity. When 18:3 ω3 is converted to 22:6 ω3 (21), the C_1 carbon atom of 18:3 becomes C_5 of 22:6. Thus, in 22:6 ω3, most of the activity is not in the carboxyl carbon resulting in low % RCA. This low % RCA persists even up to 45 days, indicating that the original 18:3 has retained considerable radioactivity. This may be interpreted as sparing the supply of 18:3 (built up by preferential uptake and accumulation of 18:3 in the immediate postnatal period of the rat [22,23]) during the rapid growth associated with the critical period of brain development, lipid deposition, and myelination.

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Metabolism of 1-^{14}C Linolenic Acid in Developing Brain: II. Incorporation of Radioactivity from 1-^{14}C Linolenate into Brain Lipids

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ABSTRACT

Metabolism of 1-^{14}C linolenic acid was studied in growing animals by injecting the tracer intraperitoneally into 12-13 day old suckling rats and following up the results by sacrificing groups of animals at 8 hr, 48 hr, 15 day, and 45 day intervals. In the first 15 days, there was a greater decrease in radioactivity of brain total lipids compared to the later period, although the earlier age period is characterized by lipid deposition rather than breakdown. Since the 18:3 ω3 family of fatty acids occurs largely in the brain total phosphatidyl ethanolamine fraction, we expected that, in the initial period, total phosphatidyl ethanolamine would be the most highly radioactive component. However, results showed that 8 hr after the tracer phosphatidyl choline had the highest specific radioactivity. When the total phosphatidyl ethanolamine fraction was resolved into diacyl and alk-1-enyl species, it was found that radioactivity was not distributed evenly between the two species. There was a progressive increase in radioactivity of the alkenyl and a decrease in the diacyl species. Forty-eight hr after the tracer, however, the radioactivity of phosphatidyl ethanolamine increased and at 45 days remained slightly higher than phosphatidyl choline. Radioactivity of cholesterol, a result of synthesis from acetate undoubtedly derived from the breakdown of tracer linolenate, was also high 48 hr after tracer and remained high until 45 days.

INTRODUCTION

Both linoleic (18:2 ω6) and linolenic acids (18:3 ω3) have been considered as essential fatty acids, because most mammalian systems cannot synthesize these fatty acids from commonly available precursors. The effects arising from lack of linoleate in the diet have been documented extensively (1). However, this is not the case with linolenate. The absolute need of linolenic acid for normal physiological growth and development has been shown only in the case of insects (2) and trout (3,4). In the case of rats, Tinoco, et al., (5) found that linolenic acid was not essential for normal growth and whole tissue lipid analysis did not show any differences between controls and those fed an 18:3-deficient diet. However, Crawford and Sinclair (6) have criticized this conclusion and have questioned that analysis of total head fatty acids, for example, can be used as evidence of a deficiency of linolenate metabolites in brain ethanolamine phosphoglycerides. Certain organs, such as adrenals (7,8), testis (9,10), and brain (11), are rich in polyunsaturated fatty acids. Further, Bernsohn and Stephanides (12) have postulated a relationship between lack of ω3 polyunsaturated fatty acids and multiple sclerosis. Thus, essentiality of 18:3 may not be general but could be specific to certain aspects in some organs. That the fatty acids of the linolenate family have some as yet unclear role in brain development is inferred from the fact that animals that have low 22:6 ω3 in the brain at birth exhibit a marked tendency to accumulate this fatty acid during early development even when the level of 18:3 ω3 family fatty acids in the milk is quite low (13), suggesting a preferential accumulation of the 18:3 μ3 family fatty acids in the immediate postnatal period of the rat (14). Recent work from our laboratory showed this to be true even in older animals (15). Rapid conversion of 18:3 to 22:6 ω3 has been demonstrated in the chick embryo (16), as well as in adult rats (17). However, the metabolic fate of ω3 polyunsaturated fatty acids over an extended period, including the period of critical growth and development, has not been studied thoroughly. This work was undertaken to examine the fate of the injected linolenic acid and to follow the distribution of its radioactivity over an extended period from 12-60 days in rats.

MATERIALS AND METHODS

The number, dose administered, and the

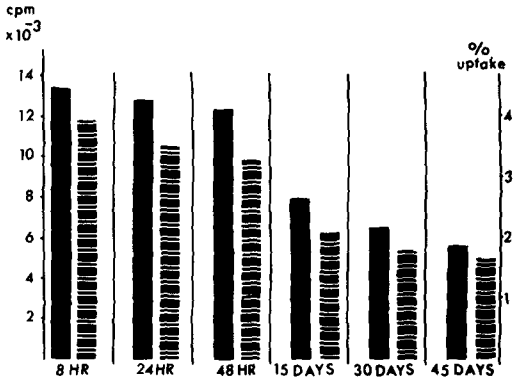

FIG. 1. Brain radioactivity:cpm/mg total lipid x total lipid in mg/g brain fresh wt.

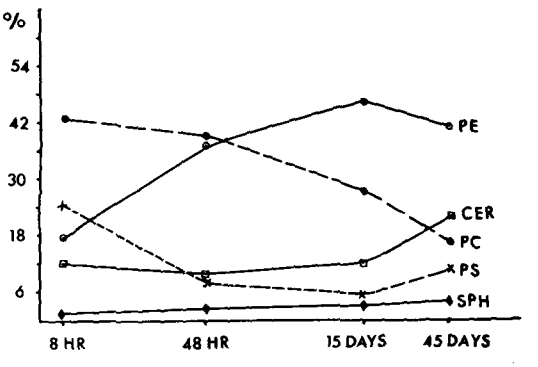

FIG. 2. Percent distribution of radioactivity in various polar lipids of the brain as determined by thin layer chromatography.

$$\% \text{ Uptake} = \frac{\text{cpm/g brain fresh wt}}{\text{dose administered } (\mu c/\text{g body wt})} \times 100.$$

experimental treatment of the animals was exactly the same as previously described (18).

Lipid Extraction and Fractionation

Blood was collected from decapitated animals; the brain and liver were excised, washed in distilled water, blotted to remove excess water, and weighed. Tissue from each group of animals was pooled and lipids extracted according to the method of Folch, et al., (19), as described previously (18,20). The total polar lipids were fractionated according to the methods described by Rouser, et al. (21). Thin layer chromatography (TLC) of polar lipids, for radioactivity distribution studies and for checking purity of samples, was carried out using chloroform:methanol:acetic acid:water (100:50:16:8 v/v) as developing solvent. Visualization was affected by brief exposure to iodine fumes. The areas were scraped directly into scintillation vials containing aquasol for radioactivity determination (22). The crude glycolipid fraction obtained by acetone and 10% methanol-in-chloroform elution from a silicic acid column was purified further by mild alkaline hydrolysis at 37 C for 30 min, essentially as described by Hoshi, et al. (23). The resulting cerebroside fraction was subjected to methanolysis by refluxing for 20 hr in 0.5 N H_2SO_4 in 90% methanol. The methyl esters first were extracted with ether:pentane (1:1 v/v) and the hydrolysate carefully neutralized to pH 8. The sphingosine then was extracted with ether. The fatty acid methyl esters further were fractionated on a SiO_2 column using 3-5% ether in pentane to elute unsubstituted fatty acid esters and 15-20% ether in pentane to obtain the hydroxy fatty acid methyl esters. The ethanolamine phosphoglyceride fraction was separated into the diacyl and 1-alk-enyl, 2-acyl component (plasmalogen) by taking advantage of the instability of the ether linkage to acid fumes (24,25). The ethanolamine phosphoglyceride fraction was spotted on a 5 x 20 cm TLC plate and the spotted area exposed to 12 N HCl fumes (from a small beaker) for 5 min. The plate was evacuated in a vacuum desiccator for ca. 4 hr to remove all acid fumes. The standards were spotted alongside the original spot and the plate developed in chloroform: methanol:acetic acid:water (100:50:16:8 v/v). This gave clear separation between unreacted diacyl phosphatidyl ethanolamine (PE) lyso PE, and aldehydes (split from plasmalogens). The lyso and aldehyde area counts together gave the radioactivity of the original plasmalogen component.

RESULTS

The radioactivity of brain total lipids is expressed as a product of specific activity (cpm/mg) and concentration (mg total lipid/g brain wet wt). The percent uptake was calculated as cpm/g brain x 100, divided by the cpm of injected dose/g body wt (Fig. 1). It was observed that, during the early period, between 8-48 hr, there was only ca. 8% decrease in brain total radioactivity. However, by 15 days, the radioactivity had decreased to 60% of the original. The decrease of the original radioactivity in the next 2 successive 15 day periods was much less, to 49 and 43%, respectively.

The polar lipid fraction was examined for radioactivity distribution by TLC (Fig. 2). There was an increase in the radioactivity of PE up to 15 days after administration of radioactive linolenic acid and only a slight decrease afterwards. In contrast, the radioactivity of phosphatidyl choline (PC), initially much

TABLE I

Specific Radioactivity (cpm/mg) of Brain Polar Lipids at Different Intervals Following Intraperitoneal Injection of 1-^{14}C Linolenic Acid to 13 Day Old Rats

Interval between dose and sacrifice	Brain polar lipids (specific activity, cpm/mg)					
	Cholesterol	Phosphatidyl choline	Phosphatidyl ethanolamine	Phosphatidyl serine	Cerebroside	Sphingomyelin
8 hr	290	226	160	147	161	59
48 hr	382	200	215	156	116	34
360 hr (15 days)	204	88	83	83	56	61
1080 hr (45 days)	113	33	59	60	32	28

TABLE II

Percent Distribution of Radioactivity in Ethanolamine Phosphoglyceride and Cerebroside Components at Different Intervals Following 1-^{14}C Linolenic Acid Injection

Interval between dose and sacrifice	Percent distribution of radioactivity				
	Ethanolamine phosphoglycerides		Cerebroside		
	Di acyl ethanolamine phosphoglyceride	1 Alk 1′ enyl, 2 acyl ethanolamine phosphoglyceride	Unsubstituted fatty acid methyl esters	Substituted (hydroxy) fatty acid methyl esters	Sphingosine
8 hr	62.2	37.8	---	---	---
48 hr	56.3	43.7	61.0[a]	17.0[a]	22.0[a]
15 days	50.4	49.6	28.9	42.7	28.4
45 days	42.4	57.6	35.1	31.5	33.4

[a]8 and 48 hr sample pooled.

higher than that of PE, decreased continuously over a period of 45 days. In the case of phosphatidyl serine (PS), there was an initial decrease in radioactivity followed by a slight increase after 15 days. Cerebrosides showed almost no change in radioactivity up to 15 days, but, at 45 days, there was a substantial increase in radioactivity. Sphingomyelin was associated with the lowest amount of radioactivity which seemed to increase marginally during the entire period. Table I gives the specific activities (cpm/mg) of various components isolated by column chromatography and of a purity determined by TLC. PC was the most highly radioactive component, but, surprisingly, PE and cerebrosides had almost equal specific activities 8 hr after the injection. Except for an increase in specific activity of PE and possibly PS, between 8-48 hr after the tracer injection, the other components show a gradual decrease in specific activities. The decrease is maximal for PC and minimal for sphingomyelin. The specific activity of PE is ca. equal to that of PS at the end of 15 and 45 days, and, at the end of 45 days, it is almost double that of PC or cerebrosides. The specific activity of cholesterol was higher than that of PC 8 hr after injection and remained considerably higher than other components even after 45 days.

The cerebrosides isolated from a SiO_2 column, although they gave a spot corresponding exactly to that of a known standard on TLC, lost some of the activity after mild alkaline hydrolysis. After complete degradation, it was found that the early samples (8 and 48 hr after hydrolysis from 12-13 day old rats) contained more radioactivity in the unsubstituted fatty acids than in hydroxy fatty acids, whereas, in later samples, the hydroxy fatty acids contained more radioactivity. At the end of 45 days, the unsubstituted and hydroxy fatty acids and sphingosine contained ca. equal amounts of radioactivity (Table II). The ethanolamine phosphoglyceride fraction is composed of both the diacyl form and the 1-alk-enyl 2 acyl (plasmalogen) form. After separation of these two components, it was observed that, initially, the diacyl form had almost two-thirds of the total radioactivity. However, as the interval between dose and sacrifice increased, the radioactivity of the plasmalogen component increased, and, at the end of 45 days, it had ca. 15% more activity than the diacyl form.

The relative specific activity of the various polar lipids can be expressed as a product of cpm/μM and the concentration in μM/g brain wet wt. This would take into account the fact that relative amounts of various polar lipids of

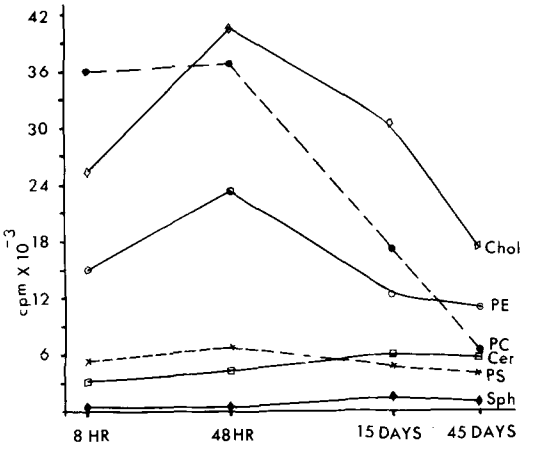

FIG. 3. Relative specific activity of brain lipids cpm/μM/g brain fresh wt.

the developing brain change considerably as shown by Wells and Dittmer (26). For calculations, the values reported by Wells and Dittmer were used (Fig. 3). The steep rise in relative radioactivity of PE and cholesterol is seen clearly from this curve. Cholesterol again has retained a considerable amount of radioactivity even after 45 days. There is a slow steady increase in the radioactivities of sphingolipids, sphingomyelin, and cerebroside, whereas PS shows only marginal differences. The half-life (in terms of ^{14}C activity) of cholesterol is estimated as 25.5 days, for PC, 11.3 days, whereas those of sphingomyelin and PS would be very much longer.

DISCUSSION

The retention of radioactivity in the brain long after its disappearance from other organs, such as the liver, has been observed by many, including ourselves (27,28). However, the amount of radioactivity retained in the brain seems to depend upon the fatty acid tracer. For example, in our earlier studies (28), when radioactive palmitate was used as the tracer, the brain total lipid radioactivity showed a marginal increase between 24 hr-1 month after dose, whereas the present study with radioactive linolenate shows a considerable drop between 48 hr-15 days. It is interesting to note that the drop in radioactivity during the first 15 days after 1-^{14}C linolenate is considerably greater than for the later periods and that this is despite the rapid build up of lipids in the brain known to occur in the early period of growth (26,29). This drop in activity agrees with similar findings by Kishimoto and Radin (29) after radioactive acetate administration. Thus,

a portion of brain lipids undergoes turnover even during the period of rapid deposition (29).

The percent distribution of radioactivity shown in Figure 2 indicates that, in the initial period, PC has a much larger proportion of the total radioactivity than any other polar lipid component. Since a polyunsaturated fatty acid (18:3) was used as a tracer, one normally would have expected PE to be the most active component, because it has been shown that brain PE (also the acyl component of plasmalogen) contains the highest polyunsaturated metabolic products (30,31). It was found that injected linolenic acid and its product 22:6 $\omega 3$ were oxidized rapidly to acetate, providing substrates for synthesis of highly active palmitate (18) in the brain, and, since brain PC has relatively larger amounts of palmitate (29), this might explain the initial higher radioactivity of PC.

The specific activities (cpm/mg) of various components (Table I) could be misleading in certain cases. For example, the specific activity of cerebroside is relatively high in the initial period, but it is known that 10-12 day old rat brain contains very small amounts of cerebroside (26,32) and so the total radioactivity contributed by cerebrosides in the whole brain is not significant. The high amount of radioactivity of cerebroside in the initial period may again be due to the fact that cerebrosides in the early period of development are richer in palmitate, rather than the characteristic longer chain (24:0) fatty acids of the mature brain cerebroside (23), and 8 and 48 hr after injection of palmitate was, indeed, highly labeled (18).

The distribution of radioactivity in the different components of the cerebrosides and ethanolamine phosphoglycerides (Table II) shows a gradual increase with age in radioactivity of the alkenyl acyl and a decrease in the diacyl form. This increase in radioactivity can be explained on the basis of earlier observations of Wells and Dittmer (26) that plasmalogen content of the brain increases with age and that their acyl compoents are rich in polyunsaturated fatty acids (29). It was observed that, as the interval between dose and sacrifice increased, 22:6 $\omega 3$ increased in radioactivity (18); this, in turn, increased radioactivity of the plasmalogen directly in the acyl and indirectly in the alkenyl side chains. The cerebroside hydroxy acids increase with age (23), as does their radioactivity (Table II), and, at the end of 45 days, the radioactivity seems to be ca. equally distributed in all three components.

A much better perspective to ^{14}C uptake and retention by various lipid components is gained from Figure 3 in which the radioactivity is expressed in terms of $\mu M/g$ brain wt. The rapid oxidative degradation of the radioactive linolenic acid and its product 22:6 $\omega 3$ to acetate would lead to synthesis of highly radioactive cholesterol, as well as palmitate. In turn, the palmitate would impart increased radioactivity to PC. However, after this initial metabolic degradation, the linolenic acid (now converted to 22:6 $\omega 3$) is incorporated into PE and retains appreciable activity even after 45 days. This suggests that 22:6, once incorporated into phospholipids, is less susceptible to catabolic changes at all times. One has to appreciate the complexity of the turnover rates from the heterogeneous nature of the brain tissue, multicomponent structure of the complex lipids, and profound changes that occur during a relatively short critical period of growth (33-35). Another peculiarity of the brain lies in the fact that there is a considerable amount of recycling of labeled precursors and products (36). For example, the observation that exogenous palmitic acid was handled differently from that synthesized de novo (28) can be explained as stemming from some, as yet unclear, compartmentalization.

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Isolation of a Pure Isomer of Linoleic Acid Hydroperoxide

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ABSTRACT

A mixture of positional isomers of linoleic acid hydroperoxide was produced from the oxidation of linoleic acid by lipoxygenase from corn or soybean. Chromatography on a column of silicic acid separated 13-hydroperoxy-11,9-octadecadienoic acid in 99+% purity from the mixture obtained by soybean lipoxygenase oxidation of linoleic acid. Attempts at isolation of pure 9-hydroperoxy-10,12-octadecadienoic acid from hydroperoxides obtained by corn lipoxygenase oxygenation of linoleic acid were partially successful with isolation of the 9-hydroperoxide in 97% purity.

INTRODUCTION

Pure positional isomers of linoleic acid hydroperoxides (LOOH) are difficult to obtain. Autoxidation of linoleic acid results in a 1:1 ratio of 9-hydroperoxy-10,12-octadecadienoic acid (9-LOOH) to 13-hydroperoxy-9,11-octadecadienoic acid (13-LOOH), which are stereochemically racemic as well (1). On the other hand, the enzyme, lipoxygenase, often selectively forms one specific positional and optical isomer of hydroperoxy-*cis,trans*-octadecadienoic acid. The specificity of most lipoxygenases usually does not totally select for production of one positional isomer, but one nearly always predominates. Although Dolev, et al., (2) reported production of 100% 13-LOOH by soybean lipoxygenase, most other investigators report an isomeric mixture. With soybean lipoxygenase, the ratio of the 13-LOOH to 9-LOOH usually varies from 92:8 (3) to 70:30 (4), and even pure isoenzymes do not yield pure isomeric hydroperoxides (5). Selective formation of the 9-LOOH over the 13-LOOH resulted from corn germ (6), potato (7), and a number of cereal (8,9) lipoxygenase studies. This author has never observed the exclusive formation of either 9-LOOH or 13-LOOH, except for the formation of 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid by *Dimorphotheca sinuata* lipoxygenase at pH 6.9 (10).

Because lipoxygenases from exotic sources, like *D. sinuata*, are not readily available, it would be more convenient to use lipoxygenases from either corn germ or soybeans to prepare hydroperoxides from g samples of linoleic acid and then to isolate the positional isomers of LOOH by a chromatographic method. Previously, a silicic acid column was used routinely to isolate half g quantities of mixed LOOH isomers from unreacted linoleic acid and other oxidation products (6,11). In the present study, it was found that, by fractionating the hydroperoxide peak, 13-LOOH and 9-LOOH are separated partially. A technique has been developed whereby one isomeric hydroperoxide is enriched by using lipoxygenases specific for either the 9- or 13-carbon and then by using the chromatographic method reported previously (6,11), except that elution is extended with a different solvent series, 13-LOOH and 9-LOOH are isolated in 99+% and 97% purity, respectively.

[1]ARS, USDA.

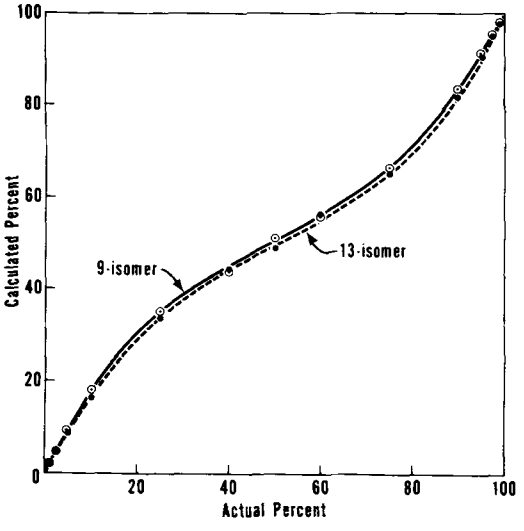

FIG. 1. Thin layer char densities from chromatography of standard mixtures of methyl 9-hydroxy- and methyl 13-hydroxystearates. "Calculated" values are taken from areas of the densitograms, and "actual" values are the standard mixtures prepared.

METHODS

Oxidation of Linoleic Acid

The oxidation of linoleic acid by soybean lipoxygenase (lipoxygenase type 1, Sigma Chemical Co., St. Louis, Mo., 134,000 units/mg) resulted in an isomeric ratio of 89%

TABLE I

Chromatographic Separation of Isomeric Linoleic Acid Hydroperoxides Derived from Oxidation of Linoleic Acid[a] by Soybean Lipoxygenase

Pooled fractions	Total wt, mg	Wt of 9-isomer,[b] mg	Percent 9-isomer	Wt of 13-isomer,[b] mg	Percent 13-isomer
55-65	171	Trace	Trace	171	99+
66-76	191	8	4	183	96
77-87	131	16	12	115	88
88-100	67	33	49	34	51
Total peak	560	62	11	498	89

[a]The total oxidation mixture derived from 720 mg linoleic acid was applied to the column.
[b]Calculated from percentage values.

13-LOOH and 11% 9-LOOH. The reaction mixture (480 ml total volume) was 5.4 mM linoleic acid (99+% pure from The Hormel Institute, Austin, Minn.), 0.09% Tween 20, 5.0 mM borate, and 0.04 mg lipoxygenase/ml. Before lipoxygenase was added, the reaction solution was adjusted to pH 10 with KOH. To minimize by-product formation, care was taken to ensure good oxygenation by using a vessel with a sintered glass bottom through which pure oxygen was bubbled. The oxidation proceeded at 21 C for 40 min at which time the reaction was adjusted to pH 4 with HCl and immediately extracted with $CHCl_3$-CH_3OH 2:1. The $CHCl_3$ layer was washed several times with water.

The oxidation of linoleic acid by corn germ lipoxygenase yielded an isomeric ratio of 91% 9-LOOH and 9% 13-LOOH. Lipoxygenase from corn germ was purified essentially as outlined by Kalbrener, et al., (12) with the exception that corn germ flour was extracted with water instead of phosphate buffer. The reaction mixture (total volume 200 ml) was 8.0 mM linoleic acid, 0.27% Tween 20, 0.05 M phosphate (pH 6.9), and a concentration of lipoxygenase equivalent to 60 mg corn germ flour (before purification)/ml. Other reaction conditions and the method of product extraction were the same as those for the soybean lipoxygenase reaction.

Column Chromatography

The column (inside diameter, 2.5 cm) was packed with a slurry of 50 g Mallinckrodt silicic acid (100 mesh, analytical reagent) in isooctane. The sample was slurried with 2 g silicic acid in hexane and layered on top of the column. The elution series were as follows: 70 ml 10% anhydrous ether, 200 ml 15% ether, 500 ml 20% ether, and 700 ml 25% ether in hexane. The column was pressurized with N_2 at 3 psi; 10 ml fractions were collected, and the fractions were monitored for LOOH by absorp-

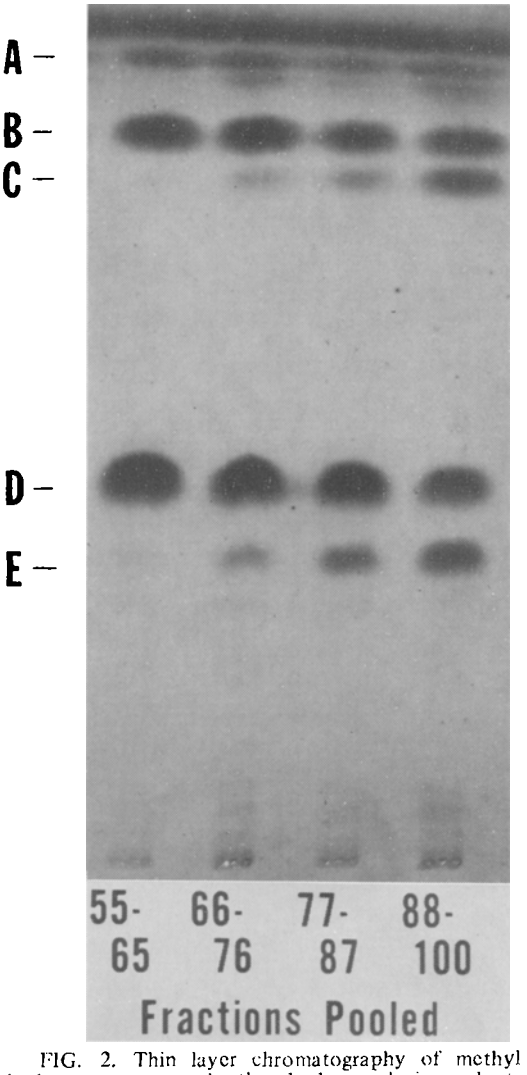

FIG. 2. Thin layer chromatography of methyl hydroxystearates and other hydrogenolysis products derived from linoleic acid hydroperoxides eluting from a column of silicic acid. Products are methyl esters of: stearate (A), 13-oxostearate (B), 9-oxostearate (C), 13-hydroxystearate (D), and 9-hydroxystearate (E).

TABLE II

Chromatographic Separation of Isomeric Linoleic Acid Hydroperoxides Composed of Equal Quantities of 9- and 13-Hydroperoxyoctadecadienoic Acids[a]

Pooled fractions	Total wt, mg	Wt of 9-isomer,[b] mg	Percent 9-isomer	Wt of 13-isomer,[b] mg	Percent 13-isomer
83-87	3.7	0.1	2	3.6	98
88-92	8.9	0.7	8	8.2	92
93-97	10.4	7.0	67	3.4	33
98-102	8.7	7.5	86	1.2	14
103-107	3.8	3.3	86	0.5	14
108-115	2.2	2.0	89	0.2	11
Total peak	38	19	49	19	51

[a]A portion of fractions 88-100 (Table I) was used for the separation.

[b]Calculated from percentage values.

tion of the diene at 232 nm.

Isomeric Composition

The isomeric ratios of 13-LOOH to 9-LOOH were determined by thin layer chromatographic (TLC) char-densitometry of their methyl hydroxystearate derivatives. Methyl hydroxystearates and other hydrogenolysis by-products, methyl oxostearates and methyl stearate, were synthesized from LOOH by reduction with H_2-Pd (10% Pd on charcoal) in methanol followed by esterification by diazomethane. This derivative was applied to a 250 μ Silica Gel G TLC plate, and the plate was developed twice with hexane-ether (70:30) (R_f 0.38, 13-isomer; R_f 0.30, 9-isomer). The isomeric identity of each methyl hydroxystearate was checked by mass spectrometry (13) after scraping from TLC plates. Char-densitometry was done by the procedure of Downing (14). Relative percentages of the isomers were calculated from the measured areas of the peaks taken from the densitograms, and, from this calculated percentage, the actual percentage was obtained from a standard curve (Fig. 1). The standard curve was constructed from densitograms of various mixtures of methyl 9- and methyl 13-hydroxystearates prepared from pure samples of the isomers. The calculated percentages of the isomeric methyl hydroxystearates from replicate densitograms had a standard deviation of 1.1.

RESULTS AND DISCUSSION

Isolation of 13-LOOH

To improve the separation of 9- and 13-LOOH, a different solvent series was used in a chromatographic method previously investigated for the isolation of mixed LOOH isomers (6,11). The probability of obtaining pure 13-LOOH was increased further by an LOOH mixture enriched in 13-LOOH. Any overlap in chromatography of 9- and 13-LOOH would tend to be decreased by prior enrichment. A 13-LOOH to 9-LOOH ratio of 89:11 was achieved with soybean lipoxygenase oxidation of linoleic acid, this mixture being the sample applied to the column. The LOOH peak was split into four parts and analyzed for its isomeric distribution (Table I). Essentially pure 13-LOOH was obtained in fractions 55-65. Although evidence for 9-LOOH in these fractions could not be detected in TLC densitograms or by mass spectroscopy, presence of traces was discerned visually on charred TLC plates (Fig. 2). Figure 2 shows the separation of the products after hydrogenation and methyl esterification of the LOOH samples. Hydroxystearic, oxostearic, and stearic acids commonly are observed as the hydrogenolysis products of LOOH.

To determine if the method would apply to isomeric mixtures less enriched with 13-LOOH, a 1:1 mixture of 9- to 13-LOOH (pooled fractions 88-100, Table I) was chromatographed. The LOOH peak from chromatography of this sample was fractionated into 6 parts, and analyses of these fractions showed a 98% pure 13-LOOH (Table II). However, in later fractions, 13-LOOH eluted appreciably with 9-LOOH. This behavior seemed anomalous, because the percentage of 13-LOOH stabilized and did not decrease any further. The anomalous behavior may be due to the sample used for separation, pooled fractions 88-100 (Table I) which were late eluting fractions from previous chromatography of LOOH. These pooled fractions may have contained an appreciable content of the *trans,trans* diene isomer; the presence of this isomer probably would account for the observed trailing of 13-LOOH. Morris, et al., (15) reported that *trans,trans* isomers of methyl hydroxyoctadecadienoates

TABLE III

Chromatographic Separation of Isomeric Linoleic Acid Hydroperoxides Derived from Oxidation of Linoleic Acid[a] by Corn Germ Lipoxygenase

Pooled fractions	Total wt, mg	Wt of 9-isomer,[b] mg	Percent 9-isomer	Wt of 13-isomer,[b] mg	Percent 13-isomer
69-75	24	12	50	12	50
76-82	106	95	90	11	10
83-89	94	89	95	5	5
90-96	41	40	97	1	3
97-103	16	16	97	0.5	3
104-113	4.4	4.2	95	0.2	5
Total peak	285	259	91	26	9

[a]The total oxidation mixture derived from 450 mg linoleic acid was applied to the column.

[b]Calculated from percentage values.

had a slightly lower mobility on TLC plates than *cis,trans* isomers. Thus, the sample used in this separation already may have been enriched in *trans,trans* 13-LOOH. Although soybean lipoxygenase usually does not produce *trans, trans* diene, this isomerization may occur to a minor extent.

Even though it is possible to obtain 98% 13-LOOH from an equal mixture of positional isomers, it is more advantageous to start with a mixture enriched in 13-LOOH to achieve better yields and a purer sample.

Isolation of 97% 9-LOOH

Preliminary to column chromatography, an LOOH mixture enriched with 9-LOOH (91%) was prepared by corn germ lipoxygenase oxidation of linoleic acid. Column chromatography partially separated 9- from 13-LOOH (Table III). A sample with 97% pure 9-LOOH in 20% yield resulted from the total LOOH. As can be seen from Table III, 13-LOOH was trailing into the 9-LOOH fractions in a fashion similar to that observed in Table II. Presumably, the *trans,trans* isomer of 13-LOOH may have been interfering with a clean separation.

Effect of Column Load

Increased sample sizes resulted in smaller solvent volumes necessary to elute LOOH as expected. As noted in Tables I-III, the fraction number in which LOOH was first detected depended upon the amount of LOOH present. Nevertheless, the sample sizes used in this study did not prevent the desired result, the isolation of pure 13-LOOH and 97% pure 9-LOOH. It is evident that silicic acid is a suitable support for separating isomeric LOOH in quantities sufficient for research with these compounds. This work indicates that high performance liquid chromatography (HPLC), using a silicic acid packing with greater efficiency, may result in complete analytical separations of these isomers and perhaps even preparative separation. Chan and Prescott (16) recently have used HPLC to separate the methyl ester of 13-LOOH from the methyl ester of 9-LOOH but did not apply the technique to the free acids.

Although geometrical and optical isomers were not determined, presumably the isolated 13-LOOH and 9-LOOH were predominantly 13-L-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic and 9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acids based upon previous work (3,6,17) with corn and soybean lipoxygenase.

ACKNOWLEDGMENTS

R. Kleiman supplied the mass spectral data, and W.F. Kwolek, biometrician for the North Central Region, ARS, USDA, stationed at the Northern Laboratory, provided the statistical analyses.

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Electron Spin Resonance Study of Serum Lipoproteins of Salmon *(Oncorhynchus kisutch)*: Structural Alterations Produced in High Density Lipoproteins by Mercury and Cadmium

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ABSTRACT

High denisty lipoproteins from the serum of salmon (*Oncorhynchus kisutch*), containing a maleimide spin label (4-maleimido-2,2,6,6-tetramethylpiperidinooxyl), were exposed to $HgCl_2$, $CdCl_2$, and CH_3HgCl under physiological conditions. Electron spin resonance spectrometry revealed that $HgCl_2$ and $CdCl_2$ produce significant alterations at sites representing weakly and strongly immobilized label. The observed changes appear to be taking place primarily in the surface architecture. No alterations were found in the normal spectra of high density lipoproteins exposed to CH_3H_gCl under similar conditions. Each metal species binds strongly with the apo-high density lipoproteins; however, marked differences exist in the effects produced upon high density lipoprotein structure by the inorganic ions and CH_3H_gCl.

INTRODUCTION

We showed recently that inorganic and organic mercurials are sequestered readily by the serum lipoproteins of salmon (*Oncorhynchus nerka*) (1). In studies with $^{203}HgCl_2$ and $CH_3{}^{203}HgCl$, the radioactivity in the lipoproteins was associated with protein and lipid components (1). These findings raise the question whether the interactions with toxic metals result in significant alterations in the architectural properties of the lipoproteins.

Recent studies employing electron spin resonance spectrometry (ESR) revealed that certain cations, e.g. Ca^{++}, Mg^{++}, and La^{+++}, alter the surface structures of cytoplasmic membranes of *Bacillus subtilis* (2). In the present work using ESR, we found that significant alterations occur in the architectural properties of high density lipoproteins (HDL) labeled with 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MalSL) on exposure to $HgCl_2$ and $CdCl_2$. No evidence was found to suggest that CH_3HgCl produced such alterations under similar conditions. Thus, distinct differences exist between the effects produced upon the architecture of the HDL by the inorganic and organic forms of the toxic metals.

EXPERIMENTAL PROCEDURES

Blood was obtained from the caudal vein of coho salmon (*O. kisutch*) 3-4 hr after feeding. The blood was allowed to clot, and serum was isolated by centrifugation (1). The HDL, obtained by ultracentrifugation as previously described (3), was labeled with MalSL according to the procedure of Gotto, et al. (4). The labeled HDL was passed through a Sephadex G-75 column containing 0.01 M Tris buffer (pH = 7.0), 0.15 M NaCl, and 0.005 M NaN_3 to remove free label. The labeled HDL solution was concentrated using an Amicon Minicon-A25 concentrator. The resulting HDL fraction, comprising 10.0 mg protein/ml (5) and 11.3 mg lipid/ml (6), was exposed to concentrations of $HgCl_2$, $CdCl_2$, and CH_3HgCl ranging from 0.01-1.0 mole of metal/mg protein in the Tris buffer solution. ESR spectra were obtained immediately after exposure to the metal ions and at periods of up to 24 hr thereafter.

Spectra were measured at 20 C using a Varian E-3 spectrometer. The samples were pipetted into Kimax 51 capillaries (1.6-1.8 mm diameter) and capped with parafilm. Accurate measurements were made of peaks D and E (strongly immobilized label) at high gain and slow sweep time (Fig. 1). In addition to determination of $2T_{\|}$, ratios of peaks C/B

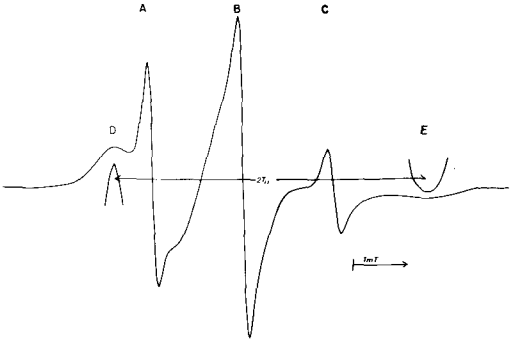

FIG. 1. Electron spin resonance spectrum of HDL from *Oncorhynchus kisutch* labeled with 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MalSL). (1 mT = 10 gauss). See text for details.

TABLE I

Alterations Produced in Electron Spin Resonance Spectra by Toxic Metal Salts: Ratios of Peaks Representing Weakly Immobilized Sites[a]

μmole Metal/mg protein	Peak ratios (C/B)[b]		
	$HgCl_2$	$CdCl_2$	CH_3H_gCl
0.01	0.87	0.86	0.98
0.10	0.82	0.80	0.99
1.00	0.61		

[a]See Figure 1 and text.

[b]The ratio of C/B was normalized so that metal-free maleimide labeled high density lipoprotein values were 1.00.

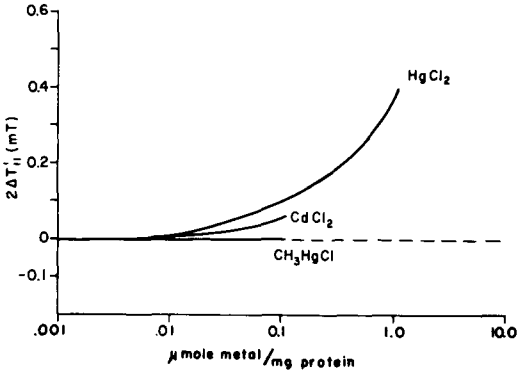

FIG. 2. Variations in hyperfine splitting shift ($2\Delta T_{\parallel}^{1}$) of high density lipoproteins from *Oncorhynchus kisutch* labeled with 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MalSL) after 24 hr exposure to toxic metal salts. See text for details.

(weakly immobilized label) were obtained. Duplicate experiments were performed with no significant differences in results.

RESULTS

In studying possible alterations in the architectural properties of serum lipoproteins exposed to $HgCl_2$, $CdCl_2$, and CH_3HgCl, the HDL was chosen because the MalSL, which reacts with -SH and -amine groups, was incorporated readily into this protein-rich density class (protein: lipid = 47.53).

The ESR spectra (Fig. 1) of labeled coho HDL exhibited characteristics of a covalently bound label. Two distinct binding sites were evident for the maleimide probe. One site (peaks A and C) showed partial immobilization (rotational correlation times <4.0 nsec) and the other site (D and E) almost complete immobilization (20 nsec $<$ rotational correlation times <115 nsec) of label. Peak B represents a composite of partially and completely immobilized sites (Fig. 1). Such findings are in agreement with results obtained with maleimide-labeled HDL from human serum (7).

The spin-labeled lipoproteins were allowed to react with ascorbic acid at varying concentrations. All peaks diminished in intensity at ca. equal rates implying that the accessibility of the labeled sites to metal ions was comparable. A strong ascorbic acid solution caused an immediate disappearance of signal, suggesting that the labeled sites are essentially on the surface of the HDL.

Perturbations in the spectra produced by the inorganic ions were readily apparent from the ratios of C/B (Table I). Both $CdCl_2$ and $HgCl_2$ had an immediate effect upon rotational reorientation, and the correlation times increased with increasing concentration of metal, as reflected by the decreasing ratios of C/B. The spectral changes were evident within the range of 0.01-1.0 μmole of metal/mg protein. Generally, spectral alterations could not be measured with reliability at levels of less than 0.01 μmole of metal/mg protein. On the basis of the present data, a precise statement of rotational correlation times could not be made from the spectra of the HDL. In contrast to the above findings, CH_3HgCl showed a negligible effect.

The outermost wings (D, E) of the spectra of HDL exposed to $HgCl_2$ and $CdCl_2$ showed a change in $2T_{\parallel}^{1}$ with time (Fig. 1). Initially there was no detectable change ($2\Delta T_{\parallel}^{1} \simeq 0$); however, after a 24 hr period, the inorganic ions (0.1 μmole/mg protein) caused a significant increase in the rigidity of the HDL structure (Fig. 2). CH_3HgCl gave no such effect at concentrations approaching the limits of solubility of this mercurial in the buffer solution. Solubility limitations and other factors deterred higher concentrations of $CdCl_2$ and CH_3HgCl from being employed.

HDL exposed to $HgCl_2$ and $CdCl_2$ were passed through a Sephadex G-75 column to remove free and loosely bound metal ions from the metallolipoprotein solution. The ratios of C/B did not return to their initial values. Thus,

it appears that alterations in properties of HDL wrought by inorganic metal ions were not readily reversible. Previous work (1) has shown that a significant fraction of the metal sequestered by the serum HDL is bound tightly.

DISCUSSION

The present studies demonstrate that the interactions of $HgCl_2$ and $CdCl_2$ with the serum HDL result in significant alterations in the architectural properties of this lipoprotein fraction. The changes in the correlation times at the weakly immobilized sites are suggestive of disordering of the protein matrices of the HDL. The increase in the hyperfine splitting at the strongly immobilized sites suggests the occurrence of a more rigid surface protein architecture, and changes in lipid-protein interactions are likely. The observation that the binding of the Hg and Cd is not completely reversible is consistant with the fact that Ca^{++} and Mg^{++} show similar behavior toward cytoplasmic membranes (2).

Although CH_3HgCl binds strongly with the apoHDL of *Oncorhynchus* serum (1), this mercurial produced no observable alterations in the architecture of the maleimide-labeled HDL. Accordingly, it appears that substantial differences exist in the nature of the interactions taking place between the inorganic and organic forms of Hg and the protein structure. A possible explanation for this difference may be that CH_3HgCl is strongly lipophillic. Such a property may well account for the observed differences in interactions between this mercurial and the inorganic form of mercury.

Further studies are indicated to determine whether the observed alterations in the HDL structure wrought by inorganic Hg and Cd produce deleterious changes in the functional properties of the serum lipoproteins, such as the transport of lipids and other substances to key tissue sites.

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Increase of Gangliosides in Atherosclerotic Human Aortas[1]

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ABSTRACT

Preparations of human thoracic aortas containing intima and media were obtained post-mortem, and gangliosides were isolated by standard techniques. The quantity of ganglioside sialic acid, as assayed by gas liquid chromatography, was lowest in normal aortas (33 ± 9 nmoles N-acetyl neuraminic acid/g wet tissue) and progressively increased in aortas containing predominantly fatty streaks (54 ± 1 nmoles N-acetyl neuraminic acid/g wet tissue), raised yellow plaques (88 ± 23 nmoles N-acetyl neuraminic acid/g wet tissue), and ulcerated lesions (270 ± 44 nmoles N-acetyl neuraminic acid/h wet tissues). Both thin layer chromatography of the gangliosides and gas liquid chromatography of the constituent sugars demonstrated the presence of a ganglioside with properties similar to a monosialyl lactosyl ceramide (GM_3) as the major ganglioside. A ganglioside with similar chemical characteristics was isolated from plasma and low density lipoproteins.

[1]Presented in part at the Canadian Federation of Biological Societies, McMaster University, Hamilton, Ontario, Canada, June 1974.

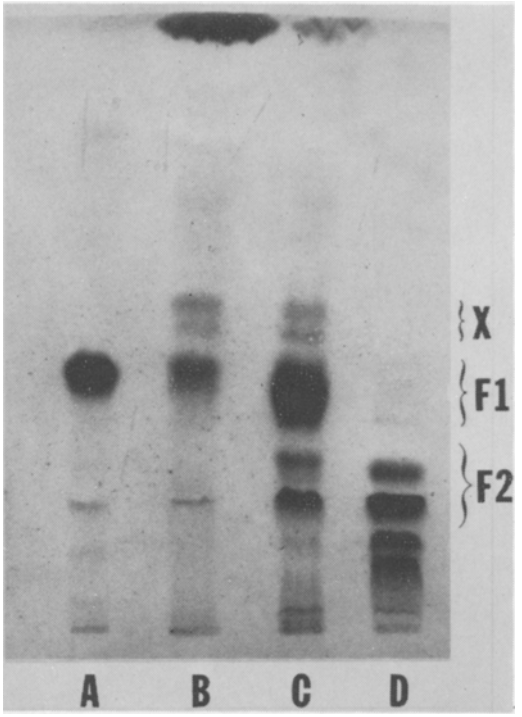

FIG. 1. Thin layer chromatography of gangliosides. Conditions as given in the text. F1 and F2 refer to fractions analyzed in Table III. Fraction X has resorcinol negative components. A = GM_3 from dog erythrocytes, B = gangliosides from a normal human aorta, C = gangliosides from a very diseased aorta containing raised yellow plaques and fatty streaks, and D = reference brain gangliosides.

INTRODUCTION

The smooth muscle cell of the aorta wall is considered important in the development of atherosclerosis (1). With thickening of the intima, cells with characteristics similar to the media smooth muscle cell appear in the subendothelial region (1,2). This change prompted the suggestion that there is an inward migration and proliferation of medical smooth muscle cells. The reasons for the loss of normal control of smooth muscle cell proliferation and migration are unknown, but there might be an analogy to the loss of contact inhibition by tumor cells. Contact inhibition is a phenomenon where normal cells growing in a monolayer cease to divide when they come in contact with each other (3). Among other alterations, transformed cells show changes in the cell surface gangliosides (4) and glycoproteins (5); and these components have been implicated in the processes of intercellular communication, such as contact inhibition. Since the migration and proliferation of the aortic smooth muscle cell is a distinct feature in atherosclerosis, an investigation has been undertaken to determine ganglioside content of intima media preparations of normal and atherosclerotic aorta.

EXPERIMENTAL PROCEDURES

Sixteen human thoracic aortas from male subjects, who were 30-70 years of age and had died of various diseases or accidents, were obtained at autopsy and were frozen until analysis. Upon thawing, the adventitia along with the external parts of media were stripped

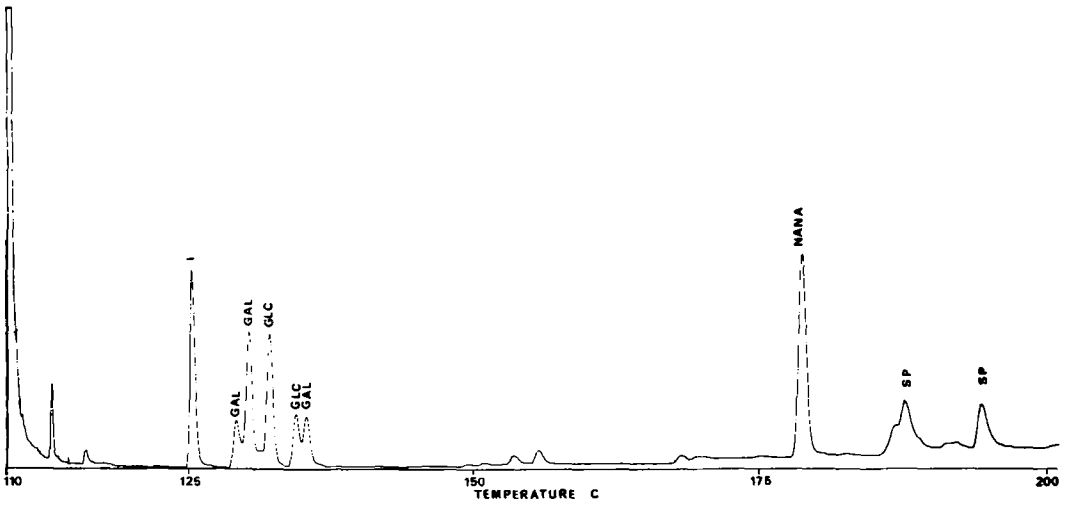

FIG. 2. Gas liquid chromatography of sugars from gangliosides. Conditions as given in text. I = inositol, GAL = galactose, MAN = mannose, NANA = N-acetyl neuraminic acid, and SP = sphingosine bases.

away. The aorta was washed and then graded as being essentially normal in appearance or containing varying quantities of fatty streaks, raised yellow fatty plaques, or extensive ulcerated lesions. The entire segment (5-10 g wet wt) was homogenized in saline with a Brinkman Polytron for 1 min. The material was rinsed off the instrument with methanol, and then chloroform was added progressively to give ratios of 1:2, 1:1, and finally 2:1 chloroform methanol for a total of 20 volumes of the solvent mixture/volume of saline homogenate. The mixture was allowed to stand for 30-60 min after each addition. The insoluble material was filtered off, and the organic phase was partitioned with: (A) 0.1 M KCl containing 0.005 M Na_2 ethylenediamine tetraacetic acid to prevent formation of calcium salts of gangliosides (6); (B) 0.2 volume of chloroform:methanol: 0.1 M KCl (3:48:47, v/v/v); and (C) 0.2 volume chloroform:methanol:water (3:48:47, v/v/v). The lower phase was evaporated, and aliquots were taken for cholesterol determinations using the autoanalyzer AA-II (7). The upper phases were combined and evaporated. The residue was taken up in a minimum volume of water and dialyzed against water for 24 hr and then lyophilized. Analysis of the upper phase ganglioside sialic acid was carried out by gas liquid chromatography (GLC) after methanolysis and trifluoroacetylation as described elsewhere (8). The fatty acid methyl esters were removed by extraction of the methanolic HCl with petroleum ether. The gangliosides also were resolved by thin layer chromatography (TLC) on Silica Gel G using a developing solvent (9) of chloroform:methanol: 2.5 N ammonia (60:35:9, v/v/v). They were detected with resorcinol for qualitative assessment and 2'6' dichlorofluorescein for preparative isolation. They were recovered from the silica gel using chloroform:methanol:water (1:1:0.2, v/v/v).

RESULTS

Thin layer chromatograms (Fig. 1) indicated the presence of resorcinol positive components in crude ganglioside preparations from normal and atherosclerotic aortas. For comparative purposes, the pattern of gangliosides of bovine brain and GM_3 isolated from dog erythrocytes also is shown. It can be seen that the major component is aorta is a rapidly migrating component with an R_f similar to the ganglioside GM_3. It has a partially resolved double pattern which is probably a reflection of fatty acid or sphingosine base composition. In diseased tissue, there were minor components present in the region of mono and disialo gangliosides and some resorcinol positive material remaining at the origin. GLC analyses (Fig. 2) of the sugars of the crude ganglioside fraction indicated the presence of four monosaccharides. The major components were galactose, glucose, and N-acetyl neuraminic acid (NANA) in ca. equal molar quantities. In addition, there was a small quantity of N-acetyl glucosamine. Using GLC and inositol as an internal standard, the ganglioside NANA was quantitated. There was no endogenous inositol in the ganglioside preprarations.

Aortas were classified as essentially normal in appearance or containing varying degrees of fatty streaks, raised yellow fatty plaques, or

TABLE I

Selected Lipid Components of Thoracic Aorta

Aorta description or source of material	N[a]	Tissue component: Ganglioside NANA[b] nmoles/g tissue	Tissue component: Cholesterol mg/g tissue
Normal <10% fatty streaks	(4)	33 ± 9 (20 – 42)	3.7 ± 0.4 (3.2 – 4.8)
20-70% Fatty streaks <10% raised yellow plaques	(3)	54 ± 1 (53 – 55)	8.5 ± 3.0 (6.4 – 11.9)
20-70% Raised yellow plaques <10% ulceration	(7)	88 ± 23 (68 – 133)	16.2 ± 2.7 (12.7 – 20.2)
30-80% Ulceration	(2)	320 ± 156 (226 – 315)	32.0 ± 2.0 (30.3 – 33.8)
Human plasma	(1)	4.4	2.0

[a]Values in parentheses indicate range of values.

[b]NANA = N-acetyl neuraminic acid.

TABLE II

Quantitation of Gangliosides in Thoracic Aorta of Type IV HLP[a]

Description of isolated area	Tissue component: Ganglioside NANA[b] nmoles/g tissue	Tissue component: Cholesterol mg/g tissue
Smooth	46	5.2
Largely normal with some fatty streaks and small raised fatty plaque	65	---
Isolated raised fatty plaque	72	7.1
Extensive ulceration	335	21.2

[a]Type IV hyperlipoproteinemic (HLP) patient (NL 45) who died of a myocardial infarction.

[b]NANA = N-acetyl neuraminic acid.

ulcerated lesions. The description is a general one, indicating the predominant state of the aorta, since normal aortas usually had occasional fatty streaks, while those possessing extensive fatty streaks also contained occasional yellow plaques. A small ulcerated lesion also was found sometimes in aortas showing extensive involvement in raised yellow plaques.

The normal appearing aortas contained the lowest quantities (Table I) of ganglioside NANA (20-42 nmoles/wet wt), while aortas containing predominantly fatty streaks had somewhat higher concentrations. Those containing extensive areas with raised yellow fatty plaques had ca. three times that found in the normal aortas, while aortas containing large areas of ulcerated lesions contained much higher quantities. In all cases, the TLC indicated a progressive increase in the component resembling sialyl lactosyl ceramide constituent (Fig. 1). The ganglioside content appeared to increase essentially in parallel to cholesterol but was not a direct reflection of the relative levels of ganglioside and cholesterol in plasma (Table I). In ulcerated tissue, the relative increase in gangliosides was much greater than cholesterol. The results indicate that there is an accumulation of ganglioside in the aorta during atherogenesis.

To study isolated plaques, a thoracic aorta was obtained from a deceased patient with Type IV hyperlipoproteinemia (male, age 45). As shown in Table II, areas were obtained which were normal in appearance or contained individual plaques. Again, the levels were lowest in the normal area with increasing values in more advanced lesions. However, even the area containing no obvious lesions had values considerably higher than those found in aortas whose overall appearance was normal.

The major ganglioside component from plasma and aorta was isolated by preparative TLC and analyzed for carbohydrates (Table III). The molar ratios are the average of two-three determinations. It can be seen that there are ca. equal molar ratios of N-acetyl neuraminic acid,

glucose, and galactose. The thin layer mobility in comparison to GM_3 of dog erythrocytes and carbohydrate composition indicates a sialyl lactosyl ceramide, but further studies would be required to determine the complete structure. The ganglioside isolated from plasma was associated largely with the low density lipoproteins.

DISCUSSION

The present results are the first report, to our knowledge, of gangliosides in the intima-media layers of the aorta wall. They also indicate that there is a progressive increase in the quantity of gangliosides with increasing severity of atherosclerosis in the aorta. Other studies have found that the concentration of cerebrosides increased with increasing severity of atherosclerosis (10). Since a similar ganglioside is found in plasma low density lipoprotein, it could be suggested that the accumulation is due to the infiltration of serum lipoproteins, as has been postulated for cholesterol (11). However, the ganglioside level in the normal aorta wall is ca. eightfold greater than in plasma on a wet wt basis (Table I), and the increase in ganglioside does not parallel the increases based upon a simple infusion and binding of plasma lipoproteins. The binding would have to be highly specific, even more so than for cholesterol.

A contribution to aortic ganglioside from blood cells is also possible on the basis of the thrombogenic theory of atherogenesis (12). Gangliosides are present in erythrocytes (13), but the major component is a sialyl tetrahexose. Although this component may be part of the F2 group (Table III), it would be present in minor quantities in the aorta preparations. The major ganglioside claimed to be present in platelets (14) was hematoside (GM_3) and, thus, similar in composition to the major ganglioside isolated in the present study. Initial analyses in our laboratory indicate very low concentrations of this ganglioside in paltelets. Finally, the increased amounts of the ganglioside may simply reflect increased synthesis by the aortic smooth muscle cells. The quantity of gangliosides is still small in comparison to the total lipids but their levels in tissue other than brain are usually not more than 1% of the lipid.

The biochemical effects of changes in the amount of cellular gangliosides and the physiochemical aspects of ganglioside binding to cells remain largely unexplored but might be important in early stages of atherogenesis. Since gangliosides may be involved in cell to cell interactions which may control proliferation of cells, the increased ganglioside levels might be involved in migration and proliferation of smooth muscle cells observed in atherosclerotic lesions (1).

TABLE III

Monosaccharide Composition of Isolated Gangliosides

Fraction[a]	Source[b]	Molar ratio of monosaccharide[c]			
		Gal	Glc	NANA	GlcNAc
F_1	A	1.00	0.85	0.90	
	P	1.00	0.90	0.90	
F_2	A	1.00	0.87	0.98	0.40

[a]Fractions isolated from thin layer chromatography as defined in Figure 1.

[b]A = Aorta and P = plasma.

[c]Gal = galactose, NANA = N-acetyl neuraminic acid, and GlcNAc = N-acetyl glucosamine.

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SHORT COMMUNICATIONS

Identification of Wax Esters in *Tetrahymena pyriformis*

ABSTRACT

Wax esters, isolated from *Tetrahymena pyriformis*, have been found to contain 45% branched chain alcohols and 76% branched chain fatty acids. No esters of tetrahymanol or of sterols were found.

INTRODUCTION

In lipid extracts from *Tetrahymena pyriformis*, many workers have noted the presence of a component that resembled cholesteryl oleate by thin layer chromatography (TLC). However, no rigorous attempts have been made to characterize it. Shorb (1) reported that, when *Tetrahymena* was grown on a medium supplemented with acetate, 17% of the neutral lipids obtained were "cholesteryl esters." Holmlund (2) noted that *Tetrahymena* incorporated [2-^{14}C] sodium acetate into squalene and a component with TLC properties like cholesteryl oleate. Since *T. pyriformis* has not been demonstrated to produce sterols, but does produce one or more triterpenoid alchols, it was considered that the component might consist of one or a series of triterpenoid esters of fatty acids.

Kapoulas and Thompson (3) have observed incorporation of label from hexadecanol-1-^{14}C by a cell-free preparation from *T. pyriformis* into a fraction termed "triglycerides and waxes." Identification was based only by TLC of the fraction with one solvent system. More recently, Jonah and Erwin (4) tentatively identified "tetrahymanol ester" and tetrahymanol as the major neutral lipids of both cilia and mitochondria derived from *Tetrahymena*. "Tetrahymanol ester" was saponified to products that appeared to be tetrahymanol and free fatty acids by TLC.

The present study has been directed to the characterization of the fraction with TLC properties of cholesteryl oleate. This fraction has been found not to consist of esters of tetrahymanol or of sterols but of a mixture of wax esters. A preliminary report of these findings has been presented elsewhere (5).

MATERIALS AND METHODS

T. pyriformis, mating type II, variety 1, was grown in 5 Fernbach flasks, each containing 1 liter of chemically defined medium (6). The cultures were shaken (50 rpm) at 28 C for 4 days in a Psychrotherm incubator (New Brunswick Scientific Co., New Brunswick. N.J.). Cells were collected by centrifugation for 10 min at 16,000 x G and were washed with 600 ml 0.9% NaCl solution. Cells were extracted first with three 200 ml portions of acetone-ether 1:1 to provide 1.08 g of lipid and then with 200 ml $CHCl_3$-CH_3OH to yield 0.212 g additional lipid. Since TLC revealed the presence of wax esters only in the first extract, it was dried with anhydrous Na_2SO_4, concentrated in vacuo, and applied as a solution in Skellysolve B to a 30 g Biosil A (100-200 mesh, Calbiochem, Los Angeles, Calif.) column. After elution of hydrocarbons with 200 ml Skellysolve B, fractions containing wax esters and more polar products (8.7 mg) were eluted with Skellysolve B-diethyl ether, 99.5:0.5. Preparative TLC with Skellysolve B-diethyl ether-glacial acetic acid, 90:10:1, provided purified wax esters (<2 mg, estimated).

TLC was conducted with 20 x 20 cm plates precoated with 0.25 mm Silica Gel F-254 on aluminum or glass (EM Reagent Division, Brinkman Instruments, Westbury, N.Y.). Wax esters comigrated with cholesteryl oleate.

Gas liquid chromatography (GLC) was performed with a Hewlett-Packard model 5750B gas chromatograph equipped with a flame ionization detector. Two columns were employed: a 10 ft, 1/4 in. inside diameter stainless steel column packed with Gas Chrom P (80-100) which was impregnated with 15% diethylene glycol succinate (DEGS), and a 6 ft, 1/4 in. inside diameter stainless steel column packed with Anakrom ABS (90-100), impregnated with 3% OV-1. The carrier gas (nitrogen) flow rate was 25 and 27 ml/min, respectively. One analysis was performed with a 6 ft, 1/8 in. inside diameter glass column, packed with Gas Chrom Q 100-120 containing 3% SE 30. This column was mounted in a Perkin Elmer 900 gas liquid chromatograph, and the carrier gas was

TABLE I

Composition of Wax Esters from *Tetrahymena pyriformis*[a]

Fatty acids			Alcohols		
Carbon no.	Tentative identity[b]	Relative amount (%)	Carbon no.	Tentative identity[b]	Relative amount (%)
14.0	$14^{:0}$	2.9	15.1	$15^{:0}$	1.6
14.5	15^{Br}	2.4	15.6	16^{Br}	2.3
15.0	$15^{:0}$	Trace	16.0	$16^{:0}$	28.0
15.5	16^{Br}	1.0	16.45	17^{Br}	19.0
16.0	$16^{:0}$	7.3	17.0	$17^{:0}$	1.8
16.5	17^{Br}	10.3	17.5	18^{Br}	8.2
17.0	$17^{:0}$	Trace	18.0	$18^{:0}$	23.2
17.5	18^{Br}	2.6	18.5	19^{Br}	15.8
18.0	$18^{:0}$	9.1			
18.5	19^{Br}	43.6			
19.0	$19^{:0}$	Trace			
19.5	20^{Br}	2.4			
20.0	$20^{:0}$	4.7			
20.5	21^{Br}	13.6			

[a]Gas liquid chromatography of the alcohol and fatty acid methyl ester fractions obtained by transesterification of the wax esters was conducted on a 15% diethylene glycol succinate column at 190 C.

[b]Fatty acyl and fatty alcohol moieties of the wax esters are designated by the number of carbon atoms in the molecule. Numerical superscripts refer to the number of double bonds, whereas the superscript "Br" indicates that the molecule is branched rather than straight chain.

nitrogen at 20 ml/min.

Hydrogenation of the alcohol and fatty acid methyl ester fractions was accomplished by mixing solutions, CH_2Cl_2-CH_3OH, 1:1, of the samples in the presence of PtO_2 for 1-1/2 hr under hydrogen at 200 psi.

Reference samples of alcohols and fatty acid methyl esters were obtained from Applied Science Laboratories, State College, Pa.

RESULTS AND DISCUSSION

The isolated wax ester preparation was transesterified in 0.2 N sodium methoxide in methanol. Products were resolved by TLC in system I, and the resulting methyl esters and free alcohols were eluted from the plate with diethyl ether and, after concentration under nitrogen, were analyzed by GLC. An aliquot of the alcohol fraction first was analyzed by GLC with a 3% SE 30 column maintained at 250 C. Reference cholesterol emerged at 6.6 min and tetrahymanol at 15.9 min. The alcohol preparation was monitored for 20 min, but no peak was noted after 5 min. It is, therefore, concluded that no detectable esters of tetrahymanol or sterols were present in the wax ester fraction from *T. pyriformis.*

Composition of the alcohol and fatty acid moieties derived from the wax esters is shown in Table I. Identification is based upon estimated carbon number obtained from plots of log retention time vs. carbon number of reference standards with two different conditions of GLC; 15% DEGS at 190 C and 3% OV-1 at 150 and 160 C for the alcohols, and at 160 and 180 C for the fatty acid methyl esters. Surprisingly, no unsaturated alcohols or fatty acids were observed. Further evidence for the lack of unsaturated compounds is provided by the fact that no qualitative or quantitative changes were noted from chromatography on 15% DEGS after hydrogenation of the alcohol and methyl ester fractions. Moreover, with one exception, GLC on 3% OV-1 yielded the same number of peaks in ca. the same relative proportions as was observed from the 15% DEGS column with the alcohol and methyl ester preparations. The only exception lies in observations with the alcohol preparation. Analysis on 15% DEGS (Table I) indicated that ca. 31% of the alcohol mixture is represented by $18^{:0}$ and $18^{:Br}$ alcohols. On 3% OV-1, ca. 34% of the total alcohol fraction is comprised of 3 poorly resolved peaks with carbon numbers equal to 17.6, 17.8, and 18.1.

The high proportion of branched moieties in the wax esters from *T. pyriformis* (45.3% branched alcohols and 76% branched fatty acids) appears to be rare among naturally occurring wax esters. Wax esters from plants (7) and marine organisms (8) are low in their content of branched chain moieties, but branched fatty acids are abundant in the wax esters of vernex caseosa, the greasy material on the skin of the newborn (9), from the uropygial glands of birds (10,11), and from the acoustic tissues of the propoise (12). The latter two sources (11,12)

also contain a high content of branched fatty alcohol moieties.

Although a very low proportion of the total fatty acids from *T. pyriformis* mating type II, variety 1, are branched chain compounds (13), it is clear that wax esters which represent <0.15% of the total lipids could contain a high proportion of branched fatty acids without markedly contributing to the total cellular content. The function of these unusual wax esters in *T. pyriformis* remains to be determined. Since they are present in very low amounts, use as an energy reserve would seem to be precluded.

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[Received September 12, 1974]

In Vitro Studies on Interaction of Rat Pancreatic Lipase and Colipase with Biliary Lipids

ABSTRACT

Lipase and colipase were prepared separately from rat pancreatic juice, and their respective interaction with biliary lipids was investigated by gel filtration on agarose in the presence of a micellar solution of sodium taurocholate. It was found that the cofactor can associate with the biliary lipids, whereas the enzyme forms a high mol wt complex only in the presence of colipase. It is suggested that biliary phospholipids might participate in the in vivo formation of the enzyme-cofactor substrate complex at the triglyceride-water interface in the presence of bile salts.

INTRODUCTION

Pancreatic lipase catalyzes the intraluminar hydrolysis of water-insoluble dietary triglycerides in the presence of high concentrations of bile salts. Enzymatic hydrolysis of triglycerides requires the presence of a protein cofactor (colipase) which is present in the pancreatic secretion (1-3).

It is well documented that lipase not only binds to the lipid-water interface of insoluble substrates but also forms water-soluble high mol wt aggregates with polar lipids which have been characterized by exclusion from Sephadex G 200 upon gel filtration (4,5). Erlanson and Borgström (6) have reported that human pancreatic lipase occurs in a macromolecular form in the intestinal content, and studies by Kimura, et al., (7) have shown that human pancreatic lipase is modified in the duodenum in the presence of bile and is converted to a high mol wt form. Kimura, et al., (7) suggested that the high mol wt lipase might result from its interaction with the biliary phospholipids in the presence of a cofactor of pancreatic juice that

also contain a high content of branched fatty alcohol moieties.

Although a very low proportion of the total fatty acids from *T. pyriformis* mating type II, variety 1, are branched chain compounds (13), it is clear that wax esters which represent <0.15% of the total lipids could contain a high proportion of branched fatty acids without markedly contributing to the total cellular content. The function of these unusual wax esters in *T. pyriformis* remains to be determined. Since they are present in very low amounts, use as an energy reserve would seem to be precluded.

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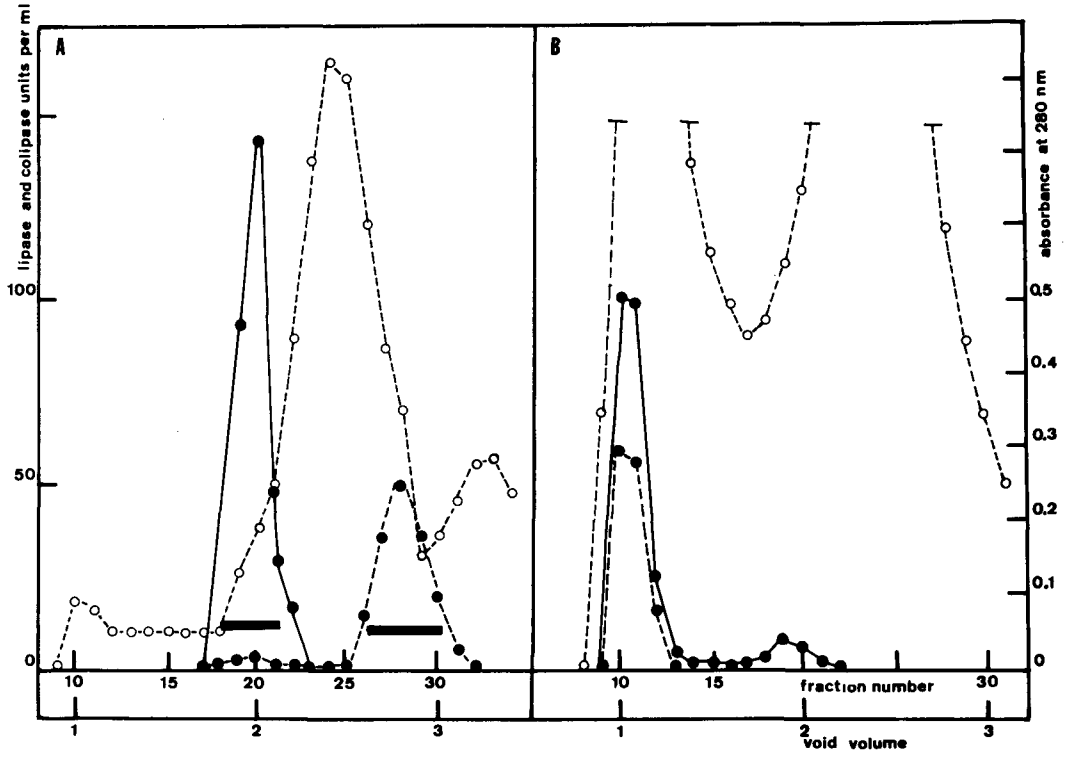

FIG. 1. Sephadex G 100 gel filtration of (A) rat pancreatic juice and (B) centrifuged rat pancreas homogenate. Volume of each fraction, 12 ml. (A) The column was loaded with a sample of juice (5 ml) containing 4400 lipase and 2250 colipase units. Fractions with lipase and colipase activity were pooled separately as indicated by the bars. (B) The column was loaded with 3700 lipase and 2500 colipase units in 8 ml. •——• = Lipase activity, •- - -• = colipase activity, and o- - -o = absorbance at 280 nm.

could be colipase. During recent investigations (8) on the behavior of biliary phospholipids during *in vivo fat digestion*, we observed that these lipids partly are associated with the emulsified phase of the intestinal content in which lipase and colipase were found. In this communication, we present the results of in vitro studies on the interaction of lipase with biliary lipids in the absence and in the presence of colipase. The results confirm the idea that colipase is required for the attachment and function of lipase at interfaces and suggest that biliary phospholipids might participate in the in vivo formation of an active complex of the enzyme at the water-substrate interface.

EXPERIMENTAL PROCEDURES

Rat pancreatic juice and rat bile were collected separately by cannulation, as previously described (8). Pancreas obtained from two rats were homogenized for 2 min in 10 times their wt of ice-cold water added with 1 mM benzamidine. The homogenate was centrifuged at 20,000 x g for 20 min, and the supernatant was collected. Gel filtration of rat pancreatic juice and centrifuged pancreas homogenate was carried out on a column of Sephadex G 100 (80 x 2.5 cm) equilibrate in 0.4 M NaCl containing 2.5 mM $CaCl_2$ and 1 mM benzamidine. Elution was performed at 4 C with the same solvent at the constant flow rate of 15 ml/hr. The lipase fractions were pooled, dialyzed against water, and lyophilized. The colipase fractions were frozen.

The following mixtures were prepared. (A) Rat bile (1.0 ml, containing 3 mg phospholipids/ml) was mixed with 0.1 ml lipase fraction (300 enzyme units). (B) Rat bile (1.0 ml) was mixed with 1.2 ml colipase fraction (445 cofactor units). (C) Rat bile (1.0 ml) was mixed with 0.1 ml lipase and 1.0 ml colipase. The mixtures were kept for 2 hr under gentle stirring. Gel filtration on agarose (Bio-Gel A-5m, 100-200 mesh) was carried out at 4 C on a column (8.0 x 1.5 cm, void volume, 59 ml) equilibrated in 0.1 M NaCl containing 6 mM sodium taurocholate and eluted with the same solvent at a constant flow rate of 10 ml/hr. In

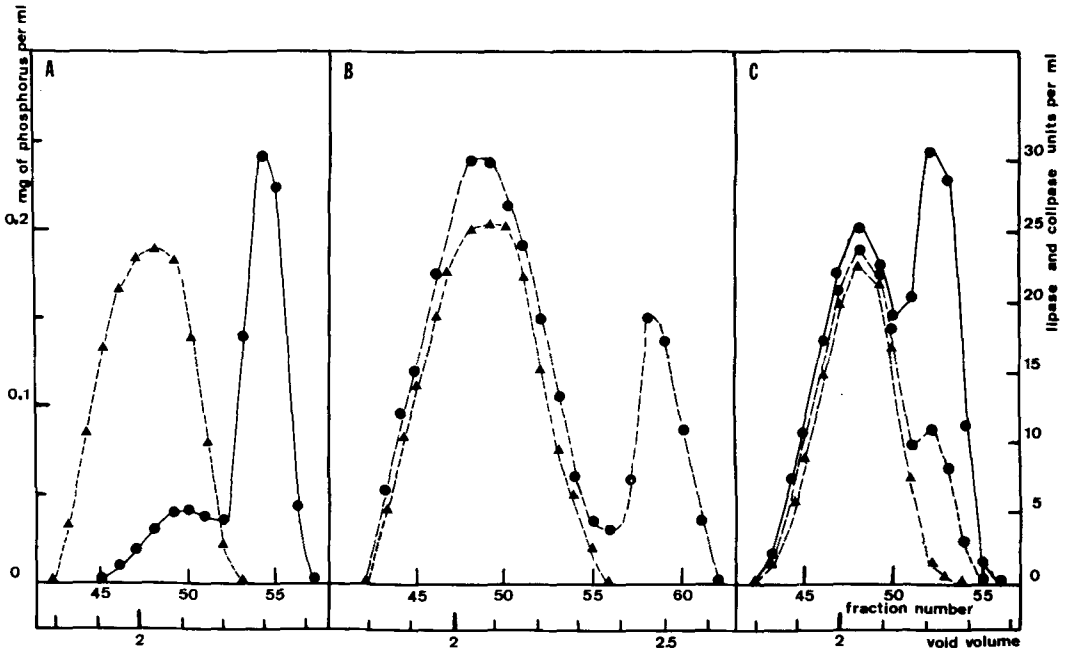

FIG. 2. Gel Filtration on agarose of rat bile added with (A) rat lipase, (B) rat colipase, and (C) lipase and colipase. Volume of each fraction, 2.5 ml. ●——● = Lipase activity, ●- - -● = colipase activity, and ▲- - -▲ = phospholipids.

these conditions, the biliary lipids were maintained in a dispersed state.

Lipase activity was determined at pH 9.0 and 25 C with the previously described potentiometric technique (9). Since the colipase-free lipase is inhibited strongly by micellar solutions of conjugated bile salts (3), sufficient amount of colipase was added to the assay system to determine the actual amount of lipase (maximum enzyme activity). One lipase unit corresponds to the liberation of one microequivalent fatty acid/min in standard conditions. Advantage was taken of the activating effect of the cofactor toward purified lipase to estimate the colipase activity in the same conditions.

Bile phospholipis were extracted with 20 volumes of chloroform-methanol (2:1, v/v), and the phosphorus content was estimated after mineralization (10).

RESULTS AND DISCUSSION

As presented in Figure 1A, full separation of lipase from its cofactor was achieved by gel filtration of rat pancreatic juice on Sephadex G 100. The removal of colipase was confirmed by the fact that the enzyme activity of the lipase fractions was as low as 2% when measured in the absence of added cofactor. Aliquots of the lipase and colipase fractions were mixed separately with rat bile, and both mixtures were passed through a column of agarose. The elution profiles are shown in Figures 2A and 2B. When lipase was mixed with bile, the major part of the enzyme was eluted at 2.3 void volumes, as in the absence of bile, and very little activity was associated with the biliary phospholipids which emerged at ca. 2.0 void volumes. In the same conditions, it could be observed that a large proportion of colipase was associated with the phospholipids, indicating that the binding capacity of colipase to the biliary lipids is independent from the presence of lipase. It is noteworthy that the apparent mol wt of the colipase fraction not bound to phospholipids is ca. 20,000-25,000 daltons. This fraction most probably represents the dimer form of the cofactor found in gel filtration (3) and in ultracentrifugational studies (2) both performed in the presence of 4 mM sodium taurodeoxycholate.

Figure 2C shows the elution diagram of rat bile mixed with lipase and colipase. In contrast with the experiment of Figure 2A, the major part of the enzyme activity now is associated with the biliary phospholipids in the form of a high mol wt complex which includes colipase. Very similar results were obtained when rat bile was mixed with an aliquot of rat pancreatic juice.

The supernatant fraction of an homogenate of rat pancreas then was filtered through Sephadex G 100 in the same conditions as pancreatic juice. It can be observed from the diagram of Figure 1B that the major part of the lipase is in an active high mol wt form eluted with the void volume of the column. This fast moving form of lipase contains almost the same amount of colipase as the sample placed on the column. The general conclusion can be made that colipase is present in the water-soluble high mol wt complex formed by lipase either with biliary lipids or with lipids from pancreas in the absence of bile salts. It is then likely that colipase is essential for the formation of a high mol wt form of the enzyme. It is suggested that biliary phospholipids, found in the intraluminar emulsified phase, might participate in vivo in the formation of an active lipase-colipase-substrate complex at the triglyceride-water interface.

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Rate of Restoration of Cardiolipin and Other Major Phospholipids during Liver Regeneration in Rat

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ABSTRACT

The rate of restoration of liver phospholipids, especially cardiolipin, during liver regeneration after two-thirds partial hepatectomy, was studied. Preoperative content of cardiolipin was not restored during the first week of regeneration, while this was the case for the other major liver phospholipids. The observations are discussed in relation to the morphogenesis of mitochondria during liver regeneration.

INTRODUCTION

After partial hepatectomy in the rat, there is a rapid increase in wt of the residual liver, which, in the course of 1 week, increases about 3 times (1). The amount of phospholipid in the liver keeps pace with this rapid growth (2,3), and, since the phospholipids occur only in the membranes of the cells (4), this means that the total amount of subcellular membranes in the cells keeps pace with the increase in mass. However, the different subcellular membranes do not increase in parallel during regeneration. Gear (5) has shown that liver mass increases more rapidly than the amount of mitochondrial protein and the total activity of a number of enzymes, and Allard, et al., (6) showed that the number of mitochondria/liver cell stays low long after the restoration of liver mass has been achieved. Another way to study the "regeneration" of mitochondria or, more specifically, of mitochondrial membranes has been made possible by the demonstration of the mitochondria as the only organelle which contains (7) (and synthesizes [8]) cardiolipin. As the cardiolipin concentration of purified mitochondria does not vary much with age in rats (9), changes in the content of cardiolipin during regeneration probably reflect the amount of mitochondrial membranes in the liver (9). In the present work, the amount of cardiolipin and, for the sake of comparison, the amounts of the other major phospholipids in the liver have been followed for the first 14 days of liver regeneration in adult rats. Laparotomized rats were used as controls. The data show that preoperative cardiolipin content is restored rapidly during regeneration, although the rate is slower than for the other major phospholipids.

MATERIALS AND METHODS

Male Sprague-Dawley rats (AB Anticimex, Stockholm, Sweden) fed laboratory chow (AB EWOS, Södertälje, Sweden) and weighing ca. 200 g (191-220 g) at the start of the experiment were used. They were kept in an artificially lighted room with lights on from 8 a.m.-8 p.m. Partial hepatectomy (10) or laparotomy were performed under ether anaesthesia in the morning between 8-11 a.m. without prior fasting of the rats. In our hands, the former overation removed 70.0 ± 0.3% (mean ± standard error, 20 rats) of the liver parenchyma. After the operation, the rats were fasted with free access to water for 48 hr and were then given food and water ad libitum. With this feeding procedure, differences in the nutritional state of the animals due to the high food consumption of the control rats (11) could be avoided. A number of rats was killed without operation at the start of the experiment. Their livers were used to assess preoperative values for liver wt and liver phospholipid content. All rats were killed by exsanguination via the abdominal aorta. The livers were excised quickly, washed, blotted dry, weighed, and homogenized in 20 volumes chloroform: methanol, 2:1 (v/v). Lipid extracts then were prepared according to Folch, et al. (12).

Liver phospholipids, except cardiolipin, were isolated by thin layer chromatography (TLC) according to Skipski, et al., (13). For the isolation of cardiolipin, a two stage TLC technique was used (14). The plates were sprayed with 1% (w/v) iodine in methanol, the spots corresponding to the different phospholipids were outlined and scraped off, and the lipids eluted according to Arvidson (15). The recovery from the plates was always 90% or better as judged from determinations of lipid phosphorous (16). Student's t-test was used for statistical computations, and the level of significance was set at 5%.

RESULTS

In the regenerating liver, the content of the individual phospholipids (Fig. 1) increased rapdily, and the preoperative content of all major phospholipids, except cardiolipin, was restored in 7 days. Preoperative cardiolipin content was not restored in the first 7 days after the operation. Initially, cardiolipin and lecithin

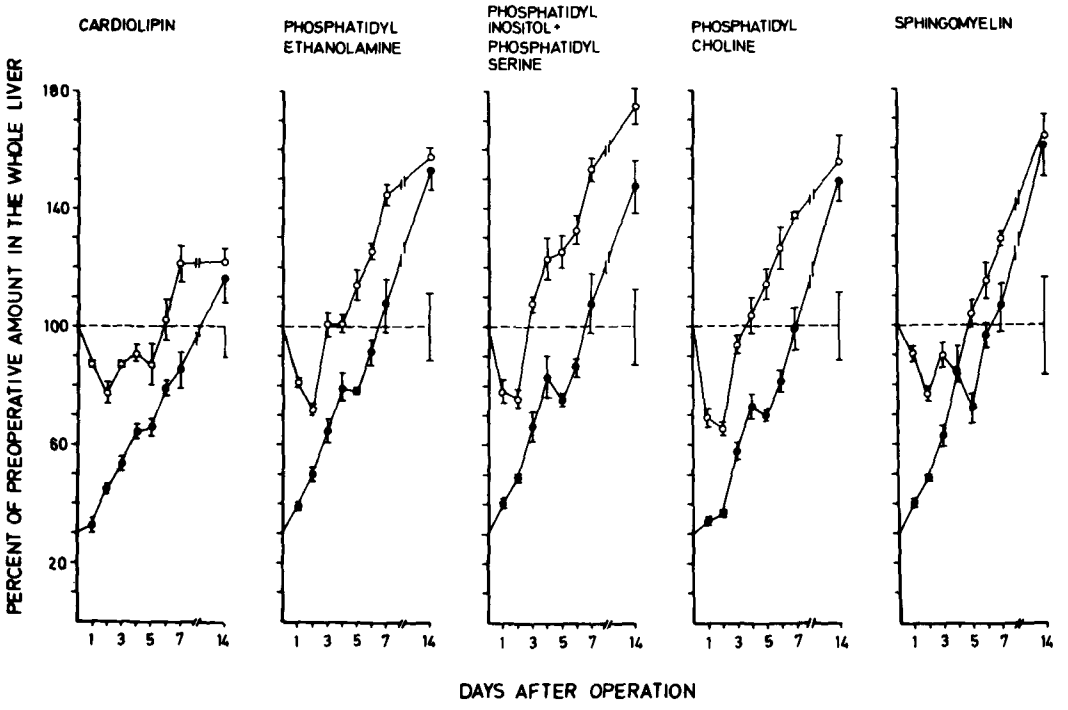

FIG. 1. Content of individual liver phospholipids in the whole liver as a function of time after partial hepatectomy ●———● or laparotomy ○———○ expressed as percent of preoperative content. Each plotted value is M ± standard deviation of 6 partially hepatectomized rats or 3 laparotomized rats or 6 unoperated zero-time (100%) control rats. The preoperative content for the different phospholipids were (expressed as μmoles ± standard deviation): cardiolipin, 9.45 ± 0.98; phosphatidyl ethanolamine, 83.84 ± 10.34; phosphatidylinositol + phosphatidylserine, 29.82 ± 3.82; lecithin, 175.32 ± 20.18; and sphingomyelin, 14.08 ± 2.34. The straight lines fitted to the data of the regenerating rats were $Y = 26.9 + 8.45x$ ($r = 0.99$); $Y = 29.9 + 10.72x$ ($r = 0.99$); $Y = 31.76 + 10.16x$ ($r = 0.96$); $Y = 22.63 + 10.5x$ ($r = 0.97$); and $Y = 30.10 + 10.7x$ ($r = 0.96$) for the different phospholipids in the same order as above.

content increased slower than the other major phospholipids but, from the third day and on, the rate of increase in lecithin was as rapid as for the other major phospholipids. The rate of increase in cardiolipin content tended to be slower than the other phospholipids at all times.

In the controls, the content of all phospholipids decreased during the first 2 days and then increased again. The rate of restoration followed the same general pattern as in the regenerating livers. Cardiolipin content did not return to the preoperative level until on the fourth day while all other phospholipids restored their preoperative content on the third day.

In both groups, the phospholipid concentration expressed as μmoles/g wet liver wt was ca. constant throughout the experiment.

Straight lines were fitted to the data from the first 7 days of regeneration (Fig. 1) by the method of least squares. Correlation coefficients of 0.96-0.99 were obtained indicating a high degree of linear relationship. The regression coefficient was 8.45 for cardiolipin and 10.16-10.72 for the other major phospholipids. The intercepts were close to 30 for all phospholipids, except cardiolipin and lecithin.

DISCUSSION

The content of all major phospholipids, except cardiolipin, was restored to the preoperative level within 7 days (Fig. 1). Cardiolipin is, however, different from the other major phospholipids also in the sense that its synthesis probably occurs only in the mitochondria (8,9), while the synthesis of the other phospholipids mainly occurs in the endoplasmic reticulum (17). Thus, it is possible that the turnover of cardiolipin is regulated in a different way than the turnover of the other major phospholipids and that this was why cardiolipin content increased at a slower rate. Since cardiolipin probably occurs only in the mitochondrial membrane (7), changes in the cardiolipin content of the liver has been taken as a measure of changes in the amount of mitochondrial membranes in

the liver (9). If this were also true for the regenerating liver, it is clear from Figure 1 that the preoperative amount of mitochondrial membrane was restored within 14 days of regeneration, in fact, probably already after 8-9 days. This is more rapid than has been shown for the restoration of preoperative amount of total mitochondrial protein (5). However, as shown by Gear (5), the inner and outer mitochondrial membranes may be restored asynchronously during liver regeneration which may explain the different rates of restoration of cardiolipin content and total mitochondrial protein.

The average daily net synthesis of the different liver phospholipids during the first week of regeneration (calculated from the slopes of the straight lines) was, in relative terms, remarkably similar for all major phospholipids, except cardiolipin. Thus, on the average, the net increase in phospholipid content of the liver remnant occurred with ca. constant proportions between the major phospholipids in accord with previous investigations (2,3,18). The average net synthesis of cardiolipin during the first 7 days of regeneration was 0.8 μmoles daily. Using the figures given by Gear (5) on the content of mitochondrial protein in regenerating and control livers, rates of net cardiolipin synthesis of 0.51-0.29 nmoles x mg^{-1} x h^{-1} and 0.11 nmoles x mg^{-1} x h^{-1} can be calculated for regenerating and control livers (second and sixth days after operation). These figures are, although minimum values (since turnover of cardiolipin probably occurs under the conditions of the experiment [G. Fex, unpublished data]), 10-60-fold greater than the rate of cardiolipin synthesis in in vitro systems (8).

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Effects of Diet and Type IIa Hyperlipoproteinemia upon Structure of Triacylglycerols and Phosphatidyl Cholines from Human Plasma Lipoproteins[1]

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ABSTRACT

Four normal and two individuals with Type IIa hyperlipoproteinemia were placed on the National Heart and Lung Institute Type IIa diet (low cholesterol, <300 mg/day, high polyunsaturated, low saturated fat diet) for 1 week and on a normal diet the following week. Plasma samples were obtained and the triacylglycerols, phospholipids, and cholesterol contents of plasma and of very low density lipoproteins, low density lipoproteins, and high density lipoproteins determined. Triacylglycerol fatty acid composition was determined and stereospecific analyses of triaclglycerols and phosphatidyl cholines performed. Structural determinations were limited to one normal and one Type IIa individual. In normal and Type IIa individuals, chylomicrons contained twice the amount of 18:0 as did the very low density lipoproteins, low density lipoproteins, or high density lipoproteins. The structure of the triacylglycerols from the very low density lipoproteins and low density lipoproteins was asymmetric with at least 50M% 16:0 in the *sn*-1 position and mostly 18:1 in positions *sn*-2 and 3. There was a marked difference in the distribution of 18:2 in low density lipoproteins of the normal and Type IIa individuals. The control contained equal amounts of 18:2 in the *sn*-1 and *sn*-3 positions, whereas IIa low density lipoprotein was asymmetric with 26% of the 18:2 in position *sn*-1 and 3% in the *sn*-3 position. Very low density lipoprotein was asymmetric with regard to 18:2 in control and IIa samples with an average of 5% of the 18:2 in position *sn*-1 and 40% in position *sn*-3. The phosphatidyl cholines contained predominantly 16:0 and 18:0 in position *sn*-1, whereas the acids in position *sn*-2 were unsaturated with very little difference between lipoprotein classes. Neither the short dietary periods nor source of plasma affected the structure of the phosphatidyl cholines.

INTRODUCTION

Only recently has the fatty acid composition of plasma triacylglycerols (TGs), phospholipids (PLs), and cholesterol esters (CEs) from patients with the different types of primary hyperlipoproteinemia and coronary patients been compared to those from healthy subjects (1,2).

The fatty acid compositions of these same lipids within the several plasma lipoproteins (LPs) from hyperlipoproteinemic patients as compared to healthy individuals apparently has not been reported, nor has the structure of the TGs and phosphatidyl cholines (PCs). Structure is defined here as the identity of the fatty acids in positions *sn*-1, 2, and 3 of the TGs and *sn*-1 and 2 of the PCs.

We hypothesized that the increased half-life of low density lipoproteins (LDLs) from Type II patients as compared to LDL from normal individuals, noted by Langer, et al., (3) might be due to differences in structure of the TGs and PCs in the LDLs from the two groups. We further postulated that the reduction of plasma cholesterol (C), often caused by consumption of fats containing 18:2, also would alter the structure of PC and TG.

In this paper, we present the fatty acid compositions of the TG and PC from plasma LPs obtained from healthy subjects and from patients with Type IIa hyperlipoproteinemia, both as affected by normal and therapeutic diets. Also given are structural data for the TGs and PCs.

MATERIALS AND METHODS

Volunteer Selection

In a preliminary screening, 69 male volunteers from the University of Connecticut faculty and staff were checked for hyperlipoproteinemia by determination of plasma C and TG contents. The plasma samples were analyzed

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TABLE I

Effects of Type II and Normal Diets upon Human Plasma Lipoprotein-Lipid Content (mg/100 ml plasma)[a]

Lipids and lipoproteins	Preliminary screening		Start of dietary periods[b]		End Type II dietary period I[c]		End normal dietary period II[d]	
	Control[e]	IIa[e]	Control	IIa	Control	IIa	Control	IIa
Cholesterol								
Plasma	207 ± 50[f]	369 ± 22	210 ± 30	423 ± 36	194 ± 42	362 ± 27	189 ± 40	363 ± 33
VLDL d < 1.006	--	--	17 ± 7	14 ± 2	9 ± 4	19 ± 3	14 ± 7	28 ± 8
LDL d 1.006-1.063	--	--	149 ± 31	341 ± 22	143 ± 36	312 ± 18	130 ± 37	287 ± 16
HDL d 1.063-1.210	--	--	45 ± 2	54 ± 7	42 ± 7	43 ± 1	47 ± 12	42 ± 0
Triacylglycerol								
Plasma	112 ± 41	163 ± 12	96 ± 35	106 ± 12	78 ± 41	109 ± 41	94 ± 41	145 ± 19
VLDL d < 1.006	--	--	59 ± 22	65[g]	42 ± 30	51 ± 6	52 ± 26	69 ± 21
LDL d 1.006-1.063	--	--	20 ± 8	50[g]	24 ± 6	49 ± 4	31 ± 16	65 ± 8
HDL d 1.063-1.210	--	--	10 ± 5	4[g]	10 ± 6	9 ± 0	11 ± 3	13 ± 6
Phospholipids								
Plasma	--	--	190 ± 22	301 ± 20	164 ± 39	258 ± 21	168 ± 31	260 ± 10
VLDL d < 1.006	--	--	20 ± 8	16 ± 4	14 ± 10	19 ± 4	20 ± 10	25 ± 5
LDL d 1.006-1.063	--	--	89 ± 9	190 ± 4	92 ± 32	175 ± 5	86 ± 40	185 ± 18
HDL d 1.063-1.210	--	--	81 ± 12	96 ± 17	63 ± 21	56 ± 5	70 ± 12	62 ± 1

[a]VLDL = very low density lipoproteins, LDL = low density lipoproteins, and HDL = high density lipoproteins.

[b]Also represent values of secondary screening.

[c]After 7 days of Type II diet and overnight fast; prior to start of normal diet.

[d]After 7 days of normal diet and overnight fast.

[e]Control, n = 4; Type IIa, n = 2.

[f]Mean ± standard error of mean.

[g]Observation on one Type IIa.

TABLE II

Protocol for Blood Samples Drawn for Lipoprotein and Lipid Analysis

Day	Time	Approximate blood volume (ml)	Purpose[b]
0[a]	After overnight fast	5	Initial screening, lipoprotein pattern
1	7:30 a.m., after 14 hr overnight fast	20	Secondary screening, Lipoprotein profile
7	5:30 p.m., 45 min after start of meal consumption	75	Isolate chylomicrons and exogenous VLDL
8	7:30 a.m., after 14 hr overnight fast	250	Lipoprotein profile, isolate endogenous VLDL, LDL, and HDL
14	5:30 p.m., 45 min after start of meal consumption	75	Isolate chylomicrons and exogenous VLDL
15	7:30 a.m., after 14 hr overnight fast	250	Lipoprotein profile, isolate endogenous VLDL, LDL, and HDL

[a]Initial screening, 5 weeks prior to dietary period I (Day 1).

[b]VLDL = very low density lipoproteins, LDL = low density lipoproteins, and HDL = high density lipoproteins.

TABLE III

Average Daily Calculated Nutritive Composition and Cholesterol Levels in Diets

Dietary period	Calories Kcal	Protein (%)	Carbohydrate (%)	Fat (%)	Cholesterol mg
Dietary period I[a] day 1-day 7	1952	22	45	31	271
Dietary period II[b] day 8-day 14	2770	17	42	39	591

[a]Therapeutic National Heart and Lung Institute diet, see "Material and Methods."

[b]Normal diet; see "Material and Methods."

automatically (4) in the laboratory of Robert P. Noble, The Sharon Research Institute, Sharon, Conn. Agarose gel electrophoresis was used to ascertain LP patterns on all plasma samples.

As a result of the preliminary screening above, six individuals, four controls, and two tentative Type IIs volunteered to participate. The identification of types was confirmed 5 weeks later by: (A) determination of total plasma C and TG levels, (B) determination of plasma LP by agarose gel electrophoresis, and (C) quantification of plasma LP lipids by ultracentrifugation. Plasma lipid levels obtained in the primary and secondary screening tests are presented in Table I. The Type II individuals were classified further as Type IIa based upon their plasma TG/C ratio (5).

Informed consent was obtained; a waiver signed by each voluneer; and permission to conduct the experiment was obtained from the University of Connecituct Human Use Committee. There were no obvious thyroid, renal, or hepatic abnormalities upon examination by physicians of the University of Connecitut Health Center; nor were any volunteers using drugs known to affect plasma lipids. Blood samples were taken in containers of appropriate size containing Na_2 ethylenediaminetetraacetic acid (EDTA) to have a final concentration of 0.1%. Plasma was obtained by centrifugation at 2000 x g for 15 min and 4 C.

Experimental Plan and Diets

The experimental plan, presented in Table II, consisted of preliminary and secondary screenings, and 2 consecutive 1 week periods of dietary intervention. During the first week, the diet designated as therapeutic followed the recommendation of the National Heart and Lung Institute handbook for physicians (6). For the second week, a normal house diet was used. The diets were prepared by a dietitian,

and the individual meals, as similar as possible in wt and composition, were obtained from the nearby Windham Community Memorial Hospital, Willimantic, Conn. All meals were consumed in the Department of Nutritional Sciences building; and, although the participants were free living, enthusiasm was high, and we believe that no cheating occurred. The average daily consumption of the diets is given in Table III.

Analytical Determinations

Total plasma C, PL, and TG were determined manually on isopropanol extracts (4) of plasma and LP fractions as described by Abell, et al., (7), Zilversmit and Davis (8), and Van Handel and Zilversmit (9), respectively. C recrystallized from acetone and purified triolein (10), clean by thin layer chromatography (TLC), were used as standards.

LP Profiles

Agarose gel electrophoresis was accomplished with a Bio-Gram A Lipoprotein Profile system (Bio-Rad Laboratories, Richmond, Calif. [11]). For better visualization of migrated LP bands, the following modified staining procedure, recommended by R.P. Noble (personal communication), was employed: a 3:2 mixture of Sudan Black B solution and water was layered over the developed slides on a flat surface. After 15 min, the slides were rinsed with water. The Sudan Black B solution was prepared by dissolving 75 mg dye in 100 ml ethanol and then adding 0.5 ml Triton X-100.

LP lipid determinations were made on 4 ml plasma samples. After layering with 2 ml 1.006 g/ml NaCl solution, the samples were centrifuged at 114,000 x g (Beckman type 40.3 rotor) for 16 hr at 4 C. All high speed centrifugations were done in a Beckman model L3-50 preparative ultracentrifuge (12). The floated very low density lipoproteins (VLDL) were removed and C, TG, and PL concentrations determined; the same analyses were performed on an aliquot of the infranatant fraction. After precipitation of the LDL with $MnCl_2$ and sodium heparin (13,14), the material was centrifuged (2000 x g for 15 min at 4 C) and the supernatant analyzed for C, TG, and PL. The concentration of lipids in the LDL were obtained indirectly from the relationship (high density lipoprotein [HDL] + LDL) − (HDL) = (LDL).

Preparative Isolation of LPs

Chylomicrons and exogenous VLDL were obtained from blood samples drawn 45 min after the consumption of a meal. Chylomicrons were removed from the plasma by flotation through 2 cm 1.006 g/ml NaCl solution at 10,000 x g for 30 min (Beckman type 50.1 rotor), cooled to 4 C, and allowed to run at ambient temperature. These alimentary particles were washed 3 x through 1 cm 1.006 g/ml NaCl solution for 30 min at 8000 x g at room temperature. The final particles, when checked by agarose gel electrophoresis, appeared free of any VLDL. Plasma cleared of chylomicrons was centrifuged 2 x at 10,000 x g for 30 min at 4 C. The resultant bottom fraction was layered with 1.006 g/ml NaCl solution and centrifuged for 18 hr at 114,000 x g and 4 C to obtain exogenous VLDL.

Endogenous particles were obtained from plasma drawn after an overnight fast at the end of each dietary period (Table II). LP classes were isolated by flotation, sequentially adjusting the density of the infranatant and isolation times as follows: VLDL, 1.006 g/ml, 18 hr; LDL, 1.063 g/ml, 24 hr; and HDL, 1.21 g/ml, 48 hr. A type 50.1 rotor (Beckman) was operated at 4 C. Density adjustments were made with a 1.478 g/ml NaCl-NaBr solution (12). Material of density $>$ 1.21 g/ml was discarded. LP homogeneity was checked by agarose gel electrophoresis.

Extraction and Isolation of Lipid Classes

Lipids were extracted from the LP by the procedure of Folch, et al., (15) and separated into neutral and polar fractions on a Unisil column (Clarkson Chemical Co., Williamsport, Pa.) by the method of Rouser, et al., (16).

TGs were isolated from the neutral lipid fraction by preparative TLC on 20 x 20 cm plates coated with 0.5 mm Silica Gel G in a solvent system of hexane/ethyl ether/glacial acetic acid, 90/30/2. Silica Gel H plates of the same dimensions and gel thickness were used to resolve PC in the polar fraction. The solvent system was chloroform/methanol/water, 65/25/4.

Stereospecific Analysis of TG and PC

These analyses were done by the methods of Brockerhoff (17,18), as modified by Christie and Moore (19). Digestion of the TG phenyl phosphatide derivatives and the PCs were both done with phospholipase 2 (formerly A_2, Brockerhoff and Jensen, [20]) *Crotalus atrox*, (Ross Allen Reptile Institute, Silver Springs, Fla.) as described by Sampugna and Jensen (21). Because the free fatty acids (FFA) from the TG digestion could not be separated from the unreacted diacylglycerol by this TLC system (21), these compounds required an additional separation on a 0.5 mm Silica Gel G plate

TABLE IV

Fatty Acid Composition (M%) of Triacylglycerols from Lipoproteins from Normal and Type IIa Subjects on a Type II Diet[a]

Lipoprotein	n	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:4	Miscellaneous
Chylomicron[b]												
Day 7												
Type IIa	2	0.6 ± 0.1[c]	0.4	28.9 ± 2.9	1.2 ± 0.1	0.3	10.5 ± 1.5	37.1 ± 3.1	20.1 ± 1.1	0.7		
Control	3	1.1 ± 0.8	0.4 ± 0.2	26.8 ± 7.1	1.9 ± 1.9	0.2	11.9 ± 0.9	38.4 ± 8.7	17.9 ± 3.5	1.0 ± 0.4	0.4 ± 0.3	
Exogenous VLDL[b]												
Day 7												
Type IIa	2	1.6 ± 0.3	0.2 ± 0.1	27.9 ± 2.5	4.7 ± 0.1	0.6	4.5 ± 0.1	42.3 ± 4.0	16.2 ± 3.0	2.0 ± 1.1		
Control	3	1.2 ± 0.9	0.3 ± 0.3	29.9 ± 1.5	3.8 ± 3.1	0.1	3.3 ± 1.2	39.5 ± 5.3	20.5 ± 3.9	0.7 ± 0.3	0.7 ± 0.5	
Endogenous VLDL												
Day 8												
Type IIa	2	1.1 ± 0.2	0.6 ± 0.3	26.9 ± 0.9	4.6 ± 0.6	1.0 ± 0.7	3.1 ± 0.4	46.6 ± 0.2	14.0 ± 2.1	0.8 ± 0.5	0.6 ± 0.4	0.7
Control	3	0.5 ± 0.4	0.2 ± 0.0	29.6 ± 3.3	3.0 ± 1.0	0.6	1.5 ± 0.6	45.6 ± 3.4	17.1 ± 3.1	1.1 ± 0.9	0.8 ± 0.5	
Endogenous LDL[d]												
Day 8												
Type IIa	2	1.4 ± 0.2	0.2	28.4 ± 1.6	5.6 ± 0.3	1.1 ± 0.9	4.2 ± 0.8	48.0 ± 1.7	10.7 ± 5.9	0.4 ± 0.2		
Control	4	0.8 ± 0.2	0.2 ± 0.1	28.8 ± 2.9	3.1 ± 1.4	0.2 ± 0.0	2.3 ± 1.3	45.8 ± 2.2	17.4 ± 1.8	0.7 ± 0.2	0.9 ± 0.7	
Endogenous HDL[d]												
Day 8												
Type IIa	2	1.0 ± 0.5	0.3 ± 0.1	24.0 ± 0.9	5.1 ± 1.5	0.3 ± 0.2	2.8	47.5 ± 1.7	16.8 ± 0.1	0.6 ± 0.1	1.3 ± 0.8	0.3
Control	3	1.0 ± 0.2	0.3 ± 0.1	31.6 ± 3.3	3.1 ± 0.8	0.6	3.5 ± 0.6	44.5 ± 2.9	13.9 ± 4.9	0.5 ± 0.1	0.9 ± 0.5	0.1

[a]See Table III for caloric and cholesterol levels; Period I.

[b]Exogenous, obtained 45 min after start of meal consumption. VLDL = very low density lipoprotein.

[c]Mean ± standard error of mean.

[d]Endogenous, obtained after 14 hr overnight fast. LDL = low density lipoprotein and HDL = high density lipoproteins.

TABLE V

Fatty Acid Composition (M%) of Triacylglycerols from Lipoproteins from Normal and Type IIa Subjects on a Normal Diet[a]

Lipoprotein	n	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:4	Miscellaneous
Chylomicron[b]												
Day 14												
Type IIa	2	5.6 ± 2.4[c]	1.2 ± 0.8	30.8 ± 4.3	3.4 ± 1.7	0.2	14.0 ± 0.3	40.5 ± 8.3	3.6 ± 0.4	0.5 ± 0.3	0.2	
Control	3	2.2 ± 0.4	0.4 ± 0.1	31.4 ± 1.2	1.8 ± 0.4	0.5	13.2 ± 5.5	44.1 ± 3.5	5.7 ± 0.9	0.3 ± 0.3	0.4 ± 0.4	
Exogenous VLDL[b]												
Day 14												
Type IIa	2	1.5 ± 0.4	0.4 ± 0.1	33.0 ± 3.1	2.2 ± 0.4	0.9 ± 0.2	5.4 ± 0.5	44.6 ± 0.8	10.1 ± 2.7	1.1 ± 0.4	0.7 ± 0.2	0.2 ± 0.1
Control	4	1.2 ± 0.8	0.4 ± 0.4	31.0 ± 2.4	2.6 ± 1.2	0.4 ± 0.4	4.8 ± 3.1	46.2 ± 2.1	11.9 ± 3.5	0.6 ± 0.3	0.7 ± 0.3	0.2 ± 0.1
Endogenous VLDL[d]												
Day 15												
Type IIa	2	1.8 ± 0.8	0.5 ± 0.1	28.7 ± 1.4	4.8 ± 2.1	1.2 ± 0.9	3.0 ± 3.0	47.6 ± 3.0	9.2 ± 1.0	0.9 ± 0.2	2.3 ± 1.8	
Control	4	1.0 ± 0.6	0.4 ± 0.3	31.8 ± 4.7	3.5 ± 1.2	0.2 ± 0.2	2.7 ± 1.7	46.5 ± 1.7	12.7 ± 3.2	0.5 ± 0.1	0.5 ± 0.2	0.2
Endogenous LDL[d]												
Day 15												
Type IIa	2	1.9 ± 1.3	0.5 ± 0.3	27.2 ± 0.2	4.0 ± 1.6	1.0 ± 0.3	4.0 ± 0.3	48.6 ± 1.5	11.2 ± 2.1	1.0 ± 0.4	0.5 ± 0.2	0.1
Control	4	1.0 ± 0.2	0.3 ± 0.1	30.4 ± 5.9	3.8 ± 0.6	0.5 ± 0.4	3.5 ± 0.8	48.0 ± 2.2	11.0 ± 3.2	0.4 ± 0.1	1.1 ± 0.2	
Endogenous HDL[c]												
Day 15												
Type IIa	2	1.8 ± 1.4	0.5 ± 0.3	28.6 ± 2.1	4.6 ± 2.0	0.6 ± 0.2	4.1 ± 0.8	51.9 ± 6.1	6.7 ± 0.4	0.7 ± 0.1	0.5	
Control	4	1.5 ± 0.5	0.4 ± 0.4	29.4 ± 2.8	4.8 ± 2.3	0.4 ± 0.2	3.5 ± 0.5	47.1 ± 4.2	11.9 ± 1.8	0.2 ± 0.2	0.7 ± 0.9	0.1

[a]See Table III for caloric and cholesterol levels; Period II.

[b]Exogenous, obtained 45 min after start of meal consumption. VLDL = very low density lipoproteins.

[c]Mean ± standard error of mean.

[d]Endogenous, obtained after 14 hr overnight fast. LDL = low density lipoproteins and HDL = high density lipoproteins.

(20 x 20 cm) developed in hexane/ethyl ether/glacial acetic acid, 90/30/2.

Fatty acids and acylglycerol products resulting from stereospecific analysis were converted to their methyl esters by 15% methanolic-HCl and ca 2N $NaOCH_3$ (22,23), respectively, for determination by gas liquid chromatography (GLC). Methyl esters were resolved on 3.12 m stainless steel columns containing 80-100 mesh Chromosorb W-HP (Johns-Manville Products, Denver, Colo.) coated with 18% DEGS. Methyl arachidate was used as an internal standard to verify by GLC that complete enzymatic digestion of PCs and phenyl phosphatides occurred as shown by an equality of mole units among the products.

RESULTS AND DISCUSSION

LP Lipid Composition

The effects of both Type II and normal diets upon the C, TG, and PL contents of total plasma, VLDL, LDL, and HDL from control and Type IIa individuals are presented in Table I. The Type II diet caused appreciable decreases in both plasma and LDL-C in the IIa subjects, but the normal diet did not affect an increase back to the original levels, and LDL-C decreased even further. This was undoubtedly due to a carry over effect from the Type II diet and the stabilized dietary regimen of the subjects.

Plasma TG levels in the IIa's responded differently than the controls. While the diet of the first week reduced plasma TG levels by ca. 19% in controls, these levels remained the same in the IIa's. After the normal diet, TG levels increased 25% in the IIa's as compared to 17% in the controls. The changes observed in total plasma TG levels also were reflected in VLDL-TG and LDL-TG concentrations. Since five out of six subjects gained wt during the normal diet period, the increased TG contents possibly reflected the greater fat and caloric content of the diet.

Changes in plasma PC concentrations paralleled those observed for plasma C, but neither these nor the changes in VLDL, LDL, and HDL were large.

The fatty acid compositions of LP-TG were found to be similar to those observed in other studies (24-27) and to the TG obtained from several human body locations (28,29). Three fatty acids accounted for 80-90% of the TG: 16:0, 25-30M%; 18:1, 45-50M%; and 18:2, 5-15M%. Comparing the mean M% for each acid between Type IIa and controls (Tables IV and V), no one acid was consistently different in any LP-TG. This was observed after each dietary period. With the mixed but regulated diets employed, the TG fatty acid compositions of the major LP are similar. There appear to be no significant differences in TG fatty acid composition and levels in individuals with normal and Type IIa lipoprotein profiles, except for a higher content of 18:0 in the chylomicrons than in any of the other LP, reflecting the composition of the diet. The only other dietary effect was the higher content of 18:2 (16.5M%) in the first week over the second (9.4M%). This effect of feeding relatively high levels of fats containing 18:2 has been observed in LP-TG (25) and plasma TG (30). In the latter instance, the 18:2 content was 30-55M%.

Allard, et al., (1) noted statistically significant slightly increased levels of 18:2 in all plasma lipid classes of Type IIa individuals as compared to normal subjects; differences not seen in our data. The analyses of Allard, et al., (1) were not done on individual LP classes but did involve 28 healthy and 25 IIa individuals as compared to the 4 and 2 each we studied.

The TG fatty acid compositions of chylomicrons and exogenous VLDL of the two groups after both dietary periods were not different, except for 18:0, and generally reflect the nature of the dietary lipids (31,32), medium and short chain acids not included.

Fatty Acid Distribution in TGs from LPs

The fatty acid distributions of all TGs analyzed stereospecifically are presented in Tables VI-IX. The fatty acid compositions of the 2 positions are the average of direct and indirect observations. The composition of the 2 position was determined indirectly by subtracting the fatty acid composition of the 1,3-diacylglycerol (DG) obtained after ethylmagnesium bromide hydrolysis, from the total TG fatty acids. The direct method for determining the composition of the 2 position was by analysis of the acid liberated from the *sn*-1,2-diacyl-3-phenylphosphate with phospholipase 2. Values of the exogenous LDL-TG for the 2 position (Table VIII—Control-Type II diet) from the control subjects, employing these two methods, showed the greatest variation. The M% difference between these two methods for this TG 2 position were: 14:0, 0.3%; 15:0, 0.3%; 16:0, 4.3%; 16:1, 1.3%; 17:0, 3.8%; 18:0, 4.2%; 18:1, 6.6%; 18:2, 5.2%; 18:3, 1.8%; and 20:4, 0.2%. The discrepancy between the two values is the result of acyl migration during ethylmagnesium bromide hydrolysis (29) and subsequent handling of reaction intermediates.

The average value of the 2 position plus the fatty acid composition of the *sn*-1 position obtained from the *sn*-1-monoacyl phenylphos-

TABLE VI

Effects of Diets upon Fatty Acid Distribution (M%) of Triacylglycerols (TG) from Control Subject's[a] Very Low Density Lipoproteins (VLDL)

VLDL source	*sn*-Position	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:4
Exogenous[b]-day 7	TG	1.9	0.6	29.5	4.2	0.3	4.0	42.6	16.1	0.7	0.1
Dietary period I	1	2.9	-	58.9	3.1	-	11.0	15.8	8.3	-	-
	2	1.1	-	11.3	4.4	0.9	0.3	60.0	21.1	0.9	-
	3	1.7	-	18.3	5.1	-	0.7	52.0	18.9	-	-
Endogenous[c]-day 8	TG	0.3	0.2	33.1	1.9	0.1	1.1	48.7	13.3	0.6	0.7
Dietary period I	1	0.3	-	66.5	0.9	-	1.5	26.6	3.5	-	0.7
	2	0.5	-	7.7	3.9	-	0.3	60.6	25.5	0.1	1.4
	3	0.1	-	25.1	0.9	-	1.5	58.9	10.9	-	-
Exogenous-day 14	TG	2.0	0.9	33.4	3.0	0.6	7.4	44.0	7.7	0.5	0.4
Dietary period II	1	2.0	0.3	58.2	2.1	0.2	19.5	14.7	2.8	-	0.2
	2	1.3	2.3	8.3	6.5	1.5	-	62.5	15.7	1.1	0.8
	3	2.7	0.1	33.7	0.4	0.1	2.7	54.8	4.6	-	0.2
Endogenous-day 15	TG	1.0	0.7	33.2	3.8	0.8	2.0	44.5	13.6	0.8	0.5
Dietary period II	1	3.4	0.8	65.1	7.9	-	3.1	19.7	-	-	-
	2	1.8	0.9	18.6	7.4	-	1.0	46.7	21.2	0.9	1.5
	3	2.2	0.4	12.9	-3.9	-	1.9	67.1	19.6	-	-

[a]Age of subject, 31 years; ht, 178 cm; wt at beginning 96.0 kg; at end 90.3 kg.

[b]Exogenous, obtained 45 min after start of meal consumption.

[c]Endogenous, obtained after 14 hr overnight fast.

TABLE VII

Effects of Diets upon Fatty Acid Distribution (M%) of Triacylglycerols (TG) from Type IIa Subject's[a] Very Low Density Lipoproteins (VLDL)

VLDL source	*sn*-Position	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:4
Exogenous[b]-day 7	TG	2.0	0.2	30.4	4.7	-	4.3	38.3	19.2	0.9	-
Dietary period I	1	4.5	0.5	64.7	2.7	-	6.7	16.6	4.3	-	-
	2	1.2	-	8.6	2.9	-	2.3	58.3	25.4	1.3	-
	3	0.3	0.1	17.9	8.5	-	3.9	40.0	27.9	1.4	-
Endogenous[c]-day 8	TG	0.9	0.9	26.0	4.0	0.3	2.6	46.4	16.1	0.3	1.0
Dietary period I	1	3.9	0.4	75.7	2.9	-	6.9	7.8	1.4	0.3	0.7
	2	1.5	1.9	10.6	3.2	-	-	55.0	25.9	-	1.9
	3	-2.7	0.4	-8.3	5.9	0.9	0.9	76.4	21.0	0.6	0.4
Exogenous-day 14	TG	1.8	0.5	29.9	2.7	1.1	4.9	43.8	12.8	1.5	0.9
Dietary period II	1	4.6	-	64.5	0.5	-	8.5	12.1	9.7	-	0.1
	2	0.2	0.7	7.5	7.5	1.8	3.5	50.9	24.2	3.5	0.2
	3	0.6	0.8	17.7	0.1	1.5	2.7	68.4	4.5	-	-
Endogenous-day 15	TG	0.9	0.4	30.1	2.8	0.2	1.9	50.7	8.2	0.7	4.1
Dietary period II	1	1.3	0.8	72.5	4.0	-	7.0	10.0	2.6	-	1.8
	2	0.8	0.8	14.6	3.6	-	-	59.9	10.0	2.0	8.3
	3	0.6	-0.4	3.2	0.8	-	-1.3	82.2	12.0	-	2.2

[a]Age of subject, 43 years; ht, 172 cm; wt at beginning of period 77 kg; at end 75.4 kg.

[b]Exogenous, obtained 45 min after start of meal consumption.

[c]Endogenous, obtained after 14 hr overnight fast.

phate subtracted from the TG fatty acid composition gave an indirect value for the *sn*-3 position. Negative values reported in the *sn*-3 position are a result of this indirect method of determination and method used in calculating the 2 position.

The distributions of TG fatty acids in all VLDL and LDL were asymmetric. The asymmetry and the specific fatty acid composition of each glycerol position appear similar to previous reports from analyses of other human tissues (28,29) and to plasma TGs (2). However, the degree of asymmetry observed in circulating LP-TG is greater.

Parijs, et al., (2) carried out stereospecific analyses on the plasma TGs from normal and hyperlipemic (Type IV) individuals. He observed essentially the same asymmetry as seen in our data and further noted statistically significant differences between both groups in the composition of the *sn*-2 and 3 positions.

Palmitic acid was esterified principally (59M%) to the *sn*-1 position of the TG molecules in the exogenous VLDL particles obtained from the control (Table VI). The 2 position acids were unsaturated, containing mainly 18:1 and 18:2; the quantities of these acids and 16:0 fluctuated, not conforming to any pattern. Higher total 18:2 levels generally were reflected by higher levels of this acid in the 2 position. Oleic acid (18:1) accounted for 52-67M% of the acids at the *sn*-3 position. Slightly higher, but similar values, were observed in endogenous particles after both dietary periods.

An even greater asymmetry was observed in the *sn*-1 and *sn*-3 position of the VLDL-TG in the Type IIa individual (Table VII). There was a 6M% increase in the 16:0 content of the *sn*-1 position over the control in all similar VLDL particles. Again, the 2 position acids were mainly unsaturated. The 3 position contained ca. 15M% more 18:1 than the control in the endogenous VLDL particles obtained after both dietary periods. As in the control, the 18:1 content of the *sn*-3 position increased after an overnight fast. In all VLDL-TG from the Type IIa individuals, the 16:0 content was reduced substantially from that observed in the *sn*-3 position of the control.

While the fatty acid distributions of the endogenous LDL-TG from both subjects were nearly identical (Table VIII), the degree of asymmetry was slightly different than observed in VLDL-TG. The *sn*-1 position contained 60-64M% 16:0, similar to the amount observed in the control. The 2 position acids were unsaturated, but increased 18:2 content was evident in the LP obtained after the first dietary period for both individuals. A slight

TABLE VIII

Effects of Diet upon Fatty Acid Distribution (M%) of Triacylglycerols (TG) from Control and Type IIa Subject's Endogenous Low Density Lipoproteins (LDL)

LDL source	*sn*-Position	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:4
Control	TG	0.6	0.2	31.9	1.8	0.3	1.6	43.8	19.1	0.6	0.1
Day 8	1	1.2	0.9	61.3	2.9	1.3	6.3	13.0	13.1	-	-
Dietary period I	2	1.0	0.3	16.3	4.6	0.8	-	45.9	30.3	0.5	0.3
	3	-0.4	-0.6	18.1	-2.1	-1.2	-1.5	72.5	13.9	-	-
Control	TG	1.0	0.3	28.1	4.0	1.0	3.0	49.3	11.8	0.4	1.1
Day 15	1	0.9	1.9	60.8	5.3	-	7.3	15.0	8.8	-	-
Dietary period II	2	1.8	0.3	12.7	9.6	2.2	0.6	49.9	20.5	0.3	2.1
	3	0.3	-1.3	10.8	-2.9	0.8	1.1	83.0	6.1	-	1.2
Type IIa	TG	1.1	0.2	26.8	5.2	0.2	3.3	46.3	16.3	0.2	-
Day 8	1	1.7	-	64.4	6.0	-	5.4	9.5	13.0	-	-
Dietary period I	2	0.7	0.2	11.4	8.4	0.2	0.1	44.7	33.7	0.6	-
	3	0.9	0.4	4.6	1.2	0.4	4.4	84.7	3.4	-	-
Type IIa	TG	0.6	0.1	27.1	2.4	0.7	3.6	50.2	13.2	1.4	0.7
Day 15	1	0.3	2.4	59.1	7.6	-	9.6	10.0	10.6	-	0.3
Dietary period II	2	-	-	8.8	1.4	1.6	2.4	53.1	28.8	2.3	1.6
	3	1.5	-2.1	13.4	-1.8	0.5	-1.2	87.4	0.2	-	0.2

TABLE IX

Relative Contents (wt %) of 16:0 and 18:2 in *sn*-Positions 1, 2, and 3 of Very Low Density Lipoproteins (VLDL) and Low Density Lipoproteins (LDL) from Normal and Type IIa Hyperlipoproteinemic Individual

		Normal				IIa			
		VLDL		LDL		VLDL		LDL	
sn-Positions		16:0	18:2	16:0	18:2	16:0	18:2	16:0	18:2
8[a]	1	66.9	8.8	64.0	22.9	87.7	2.4	80.1	25.9
	2	7.8	63.9	17.0	52.9	12.3	44.4	15.2	67.3
	3	25.3	27.3	18.9	24.3	---	36.0	5.7	6.8
15[b]	1	67.4	---	72.1	24.9	80.3	10.6	72.7	26.8
	2	19.2	52.5	15.1	57.9	16.2	40.6	10.8	72.7
	3	13.4	47.5	12.8	17.2	3.5	48.8	16.5	0.5

[a]Day 8, endogenous, obtained after 14 hr overnight fast following therapeutic diet.
[b]Day 15, endogenous, obtained after 14 hr overnight fast following normal diet.

TABLE X

Effects of Diet upon Fatty Acid Distribution (M%) of Phosphatidyl Cholines from Normal Subject's Plasma Lipoproteins[a]

Lipoprotein	*sn*-Position	14:0	15:0	16:0	16:1	18:0	18:1	18:2	20:4
Exogenous[b] VLDL	1	2.4	0.9	59.3	1.2	26.0	6.8	3.4	---
Day 7	2	---	---	2.7	1.7	2.0	15.2	62.1	15.9
Dietary period I	Total[c]	1.2	0.5	31.0	1.5	14.0	11.0	32.8	8.0
Endogenous[d] VLDL	1	0.2	---	61.7	0.6	26.0	9.6	1.9	---
Day 8	2	2.0	---	4.4	0.5	2.4	20.6	56.4	13.7
Dietary period I	Total	1.1	---	33.1	0.6	14.2	15.1	29.2	6.9
Endogenous LDL	1	1.7	0.2	58.9	0.6	28.0	7.3	3.1	0.2
Day 8	2	0.7	0.5	3.3	0.6	1.3	21.1	54.3	18.2
Dietary period I	Total	1.2	0.4	31.1	0.6	14.7	19.2	23.7	9.2
Endogenous HDL	1	2.1	0.3	63.5	1.3	21.0	7.0	4.7	0.1
Day 8	2	0.3	0.3	3.0	2.0	1.9	20.0	52.4	20.1
Dietary period I	Total	1.2	0.3	33.3	1.7	11.5	13.5	28.6	10.1
Exogenous VLDL	1	3.2	0.5	62.3	0.5	25.5	4.4	3.1	---
Day 14	2	0.8	0.8	3.5	0.8	3.0	20.8	52.1	17.5
Dietary period II	Total	2.0	0.7	32.9	0.7	14.3	12.6	27.6	8.8
Endogenous VLDL	1	2.2	0.4	56.8	0.9	29.8	5.9	3.3	---
Day 15	2	0.5	0.2	4.0	0.1	2.2	28.2	51.0	13.8
Dietary period II	Total	1.4	0.3	30.4	0.5	16.0	17.1	27.8	6.9
Endogenous LDL	1	1.9	0.2	62.1	0.4	28.5	4.9	2.0	---
Day 15	2	0.4	---	2.9	0.6	2.4	25.0	51.2	17.5
Dietary period II	Total	1.2	0.1	32.5	0.5	15.5	15.0	26.6	8.8
Endogenous HDL	1	1.7	0.5	63.8	0.4	24.3	5.0	3.7	0.2
Day 15	2	0.5	0.4	3.1	0.5	1.9	15.7	62.9	14.7
Dietary period II	Total	1.1	0.5	33.5	0.5	13.1	10.4	33.3	7.5

[a]VLDL = very low density lipoproteins, LDL = low density lipoproteins, and HDL = high density lipoproteins.
[b]Exogenous, obtained 45 min after start of meal consumption.
[c]Recalculated total: (1 + 2) ÷ 2.
[d]Endogenous, obtained after 14 hr overnight fast.

reciprocal relationship was observed in the *sn*-3 position compared to the *sn*-1 position of the VLDL-TG of the Type IIa. The level of 18:1 increased in the LDL-TG to 73-87M% for both individuals.

The differences in structure between normal and IIa LP TGs are emphasized in Table IX. These recalculated data are presented to show the amount of a particular fatty acid in each of the three positions of the TG.

On day 8, the *sn*-1 position of the endogenous LDL and VLDL of control subjects

TABLE XI

Effects of Diets upon Fatty Acid Distribution (M%) of Phosphatidyl Cholines from Type IIa Subject's Plasma Lipoproteins[a]

Lipoprotein	*sn*-Position	14:0	15:0	16:0	16:1	18:0	18:1	18:2	20:4
Exogenous[b] VLDL	1	3.0	0.9	63.4	1.1	21.1	9.1	1.1	0.3
Day 7	2	1.8	2.2	2.7	0.9	2.1	17.3	56.7	16.3
Dietary period I	Total[c]	2.4	1.6	33.1	1.0	11.6	13.2	28.9	8.3
Endogenous[d] VLDL	1	1.5	0.4	63.4	0.7	22.9	9.1	1.8	0.2
Day 8	2	0.5	0.5	1.3	0.5	1.3	14.8	65.8	15.3
Dietary period I	Total	1.0	0.5	32.4	0.6	12.1	12.0	33.8	7.8
Endogenous LDL	1	1.9	1.2	60.9	0.9	25.2	8.1	1.7	0.1
Day 8	2	0.3	0.2	1.9	0.9	2.9	13.6	60.8	19.4
Dietary period I	Total	1.1	0.7	31.4	0.9	14.1	10.9	31.3	9.8
Endogenous HDL	1	1.7	—	63.1	1.3	22.3	9.3	2.2	0.1
Day 8	2	0.2	0.4	2.2	0.6	2.8	12.9	62.8	18.1
Dietary period I	Total	1.0	0.2	32.7	1.0	12.6	11.1	32.5	9.1
Exogenous VLDL	1	4.0	0.5	66.6	0.3	20.7	6.6	1.3	—
Day 14	2	7.4	1.1	3.7	0.9	2.1	19.0	54.0	11.8
Dietary period II	Total	5.7	0.8	35.2	0.6	11.4	12.8	27.7	5.9
Endogenous VLDL	1	3.2	0.2	62.3	0.6	22.5	7.2	4.0	—
Day 15	2	0.9	0.9	2.6	0.8	2.2	18.6	58.3	15.7
Dietary period II	Total	2.1	0.6	32.5	0.7	12.4	12.9	31.2	7.9
Endogenous LDL	1	2.1	0.1	59.3	0.9	25.5	8.8	3.2	0.1
Day 15	2	0.6	0.3	3.6	0.4	3.4	14.6	61.2	15.9
Dietary period II	Total	1.4	0.2	31.5	0.7	14.5	11.7	32.2	8.0
Endogenous HDL	1	2.3	—	58.8	1.0	26.3	9.4	2.1	0.1
Day 15	2	0.2	0.2	4.0	2.3	2.7	13.7	56.8	20.1
Dietary period II	Total	1.3	0.1	31.4	1.7	14.5	11.6	29.5	10.1

[a]VLDL = very low density lipoproteins, LDL = low density lipoproteins, and HDL = high density lipoproteins.

[b]Exogenous, obtained 45 min after start of meal consumption.

[c]Recalculated total: (1 + 2) ÷ 2.

[d]Endogenous, obtained after 14 hr overnight fast.

contained 64.1 and 67.0% (Table IX) of the 16:0, respectively, whereas the IIa VLDL and LDL contained 87.7 and 80.1%, respectively. The reverse is noted with respect to the *sn*-3 position in that the control VLDL and LDL contained 25.3 and 18.9% of the 16:0, while, in the type IIa, the former contained 0 and the latter 5.7%. These differences were, however, not noted at day 15, following the normal dietary period.

The data in Table IX indicate that the VLDL in controls and Type IIa are similar in distribution of 18:2, i.e. the 18:2 is distributed asymmetrically with the least amount in the *sn*-1 position. There is, however, a difference between the day 15 normal and Type IIa VLDL in that no 18:2 was found in the *sn*-1 position of the latter. In comparing the LDL from the normal and Type IIa individuals, a striking difference in positioning is evident. The control LDL contains ca. equal amounts of 18:2 in the *sn*-1 and 3 positions, whereas the Type IIa LDL is markedly asymmetric, having small amounts of 18:2 in the *sn*-3 position. It is also noteworthy that the VLDL from controls and Type IIa individuals have a reverse distribution of 18:2 compared to the Type IIa LDL, the former containing small amounts in the *sn*-1 position, while the latter has the least in the *sn*-3 position.

Fatty Acid Distribution of PCs in LPs

The fatty acid distributions in the PC of the exogenous and endogenous VLDL and endogenous LP after each dietary period for one control and one Type IIa individual are presented in Table X and Table XI. The PC and TG were from the same LP. Generally, the fatty acid composition of PC (recalculated total; [1 + 2 ÷ 2]) of all endogenous LP of each individual after both dietary periods was similar. However, there appears to be a small difference between individual endogenous VLDL, LDL, and HDL. In the control subject (Table X), the 18:2 content of the PC is reduced, while 18:0 and 18:1 levels are increased, compared to the Type IIa (Table XI).

In all, LP-PC analyzed, the *sn*-1 position was mainly saturated, containing 60-66M% 16:0 and 20-30M% 18:0. Unsaturated fatty acids

occupied the 2 position: 13-20M% 18:1, 51-66M% 18:2, and 6-20M% 20:4. Worth noting are the relatively larger quantities of 20:4 in the PC as compared to the TG.

The high content of 16:0 in the *sn*-1 position of TG and PC is most likely due to the specificity of the enzyme, glycerolphosphate acyltransferase. Kinsella and Gross (33) have indicated that palmityl-CoA is the preferred substrate for the initial acylation of glycerol phosphate by this enzyme in bovine mammary microsomes. The rate of acylation also was accelerated highly with 16:0-CoA as compared to 14:0-, 18:0, and 18:1-CoA. This example of regulation may be more easily investigated and relevant in Type IV hyperlipoproteinemia.

The slight differences observed between both the normal and Type IIa individuals in the distribution of fatty acids in exogenous VLDL vs endogenous VLDL is believed due to the increased influx of varied fatty acids reaching the lipid during alimentary lipemia.

The varied composition, primarily unsaturated, of the 2 position in the VLDL and LDL-TG probably reflects the mixed FFA mobilized from adipose tissue and transported to the liver. Privett, et al., (34) estimated it may take 6 months of specific dietary feeding to turnover all the lipids in the rat and thus show a new fatty acid composition. The TGs synthesized during this experiment probably were influenced by both the dietary lipids and the fatty acids deposited over many months in the adipose tissue of the experimental subjects.

The composition of the *sn*-3 position in the VLDL- and LDL-TG in both the control (Table VI and VIII) and the Type IIa individual (Tables VII and VIII) indicates some type of selectivity in the placement of the third acyl group in the *sn*-1,2-DG during biosynthesis of TGs. This specificity may be due to substrate requirements for the enzyme diacylglycerolphosphate acyltransferase or the availability of fatty acid pools. This is evident by the change in 18:1 content of the *sn*-3 position in exogenous and endogenous VLDL-TG and the observed difference between dietary periods. Comparing the *sn*-3 position of any VLDL- and the LDL-TG obtained at the same time, the Type IIa individual has a higher level of 18:1. Again, substrate requirements for this final enzymatic step or specific fatty acid levels may be different between these two individuals. It has been shown that the synthesis of TG from *sn*-1,2-DG by rat liver is independent of the fatty acid composition of the diacylglycerol (35).

The differences in positioning of fatty acids in VLDL and LDL-TG (Table IX) lead to the question: what were the sources of TGs of such greatly differing structure? It has been postulated that LDL are fragments left from the action of LP lipase on chylomicrons and VLDL (36). Yet, neither the sterospecificity of the enzyme for the *sn*-1 position observed by Morley, et al., (37) and Paltauf, et al., (38) nor the lack of it noted by Assmann, et al., (39) explain the differences in TG structure between VLDL and LDL. It is our opinion that the LDL could have been derived only from the chylomicrons. Our data also indicate that there was probably no interchange of TGs between VLDL and LDL.

The differences between the *sn*-1,2-DG distribution of the LP-PC from the control (Table X) and Type IIa individual (Table XI) are small. The diet was only moderately effective in reducing 18:2 and 20:4 levels after the second period in the exogenous VLDL. That this reduction was not as great as seen in the TG would indicate a higher degree of fatty acid selection in PC biosynthesis. Again, the diets were not that different between periods, and it has been estimated that 10-14 days may be required to change the fatty acid composition of LP-PC (40) and then under highly specific fat intake. The general distribution of LP-PC reported compares well with those determined as total plasma PC in man by Marai and Kuksis (41).

The differences in TG structure between the normal and IIa individuals remain unexplained and should be confirmed. Whether or not these contribute to the delayed degradation of Type II lipoproteins is not known.

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Effect of Dietary Safflower Oil upon Lipogenesis in Neonatal Lamb

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ABSTRACT

The effect of dietary safflower oil upon lipogenesis has been investigated in neonatal lambs. Preliminary experiments with lambs suckled by their mothers showed that there was a 10-fold increase in the rate of incorporation of [^{14}C] from acetate into fatty acids in adipose tissue slices during the first 10 days post partum. Barely detectable rates of [^{14}C] acetate incorporation into fatty acids were found in liver slices from lambs during the same period. In lambs given cows' milk from birth until 11 days of age, there was also a 10-fold increase in the rate of lipogenesis in adipose tissue slices. Supplementing the diet of cows' milk with safflower oil (5 ml/lamb/day) resulted in significantly lower rates of lipogenesis in adipose tissue slices from 11 day old lambs. Administration of safflower oil had no effect upon the concentration of unesterified fatty acids, including linoleic acid, in the lamb adipose tissue slices. The data show that lipogenesis in ovine adipose tissue, like that in rodent liver and adipose tissue, is sensitive to dietary polyunsaturated fatty acids, and that, for the neonatal lamb, the effect of polyunsaturated fatty acids upon lipogenesis is not dependent upon an increase in the tissue concentration of polyunsaturated fatty acids.

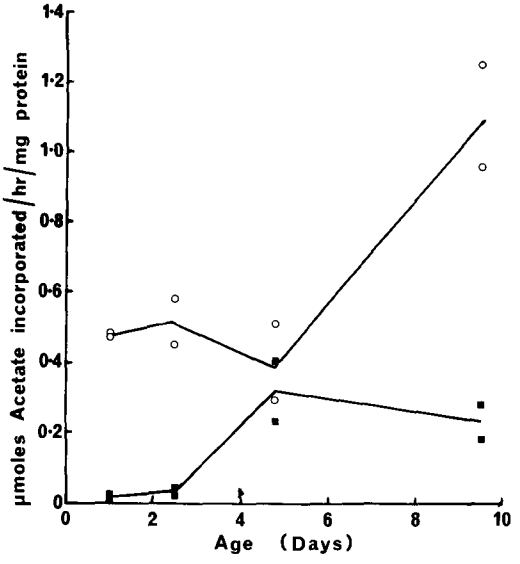

FIG. 1. Rate of incorporation of [^{14}C] from [1-^{14}C] acetate into fatty acids (■) and CO_2 (○) in adipose tissue slices from lambs of various ages suckled by their mothers.

INTRODUCTION

The supposed relationship between dietary saturated fats and cardiovascular degeneration in man has stimulated an interest in the possibility of increasing the levels of polyunsaturated fatty acids, in particular linoleic acid, in ruminant meat and milk (1-5). Little is known, however, about the effects of such increased levels of linoleic acid upon the metabolism of ruminant tissues.

It is well known that high fat diets reduce the rate of mammalian lipogenesis. Studies with rodents also have shown that diets supplemented with linoleic acid (usually given as safflower oil) resulted in a significantly greater inhibition of the rate of lipogenesis in adipose tissue and liver slices than diets containing equivalent amounts of saturated fatty acids (6-14). The mechanism by which linoleic acid exerts its extra-inhibitory effect upon the rate of lipogenesis is unknown.

Possible effects of dietary polyunsaturated fatty acids upon the metabolism of ruminant tissues normally are not observed, because these acids are subjected to biohydrogenation by the rumen microflora (15-17). However, these effects should be apparent in suckling animals before the rumen and its microflora have developed.

The present study shows that administration of safflower oil to suckling lambs reduced the rate of lipogenesis in adipose tissue slices. This effect was not associated with a change in the concentration of unesterified linoleic acid in the adipose tissue.

MATERIALS AND METHODS

Animals

Lambs were obtained from a flock of pure bred Cheviot ewes. Some lambs were allowed to suckle naturally and were killed at various ages up to 10 days after birth. Others were removed from their mothers at birth and were divided

TABLE I

Concentration of Unesterified Fatty Acids, Total Lipid, and 105,000 x g Supernatant Protein in Adipose Tissue Slices from Lambs of Various Ages

	Age				
	1-Day		2-3 Days		5-10 Days
Unesterified fatty acid concentration (μg/g wet wt)[a]					
Palmitic	590[b]	772[b]	272[b]	320[b]	333 ± 61[c]
Stearic	538	608	301	367	368 ± 91
Oleic	1289	1869	478	599	455 ± 136
Linoleic	31	56	32	35	41 ± 15
Linolenic	17	52	17	25	17 ± 5
Total	2465	3357	1156	1290	1213 ± 302
Total lipid concentration (mg/g wet wt)	360	394	496	547	563 ± 42
105,000 x g Supernatant protein concentration (mg/g wet wt)	19.6	21.2	11.1	20.2	5.7 ± 1.6
Ratio of total lipid to 105,000 x g supernatant protein concentration (mg/g : mg/g)	18.4	18.6	24.6	49.3	126.5 ± 35

[a]The concentration of unesterified fatty acids was determined in adipose tissue slices before and after incubation for 2 hr at 37 C as described in the text; pooled values are reported.

[b]Individual values.

[c]Mean ± standard error of mean of four results.

into two groups, both of which were fed cows' milk to appetite at 6 hourly intervals. One group of lambs received orally 5 ml safflower oil with the 6 am feed each day. All lambs received 2 ml multivitamin preparation (Crook's Laboratories, London, England) at 2 days of age. Blood samples were obtained from the jugular vein of each lamb immediately after birth and before the animal had access to food; further blood samples were taken 5 and 8 days after birth. The lambs of both groups were killed with a Humane Killer between 10-11 am at 11 days of age.

Tissue Incubations

Tissues were excised from the lambs immediately after death. Perirenal adipose tissue was kept in isotonic saline at 37 C; liver samples were kept in ice-cold isotonic saline. Tissue slices were prepared free-hand. Ca. 100 mg samples of tissue slices were incubated in 2.5 ml Krebs-Ringer bicarbonate buffer with half the original calcium concentration (1.27 mM) containing 8 mM [1-^{14}C] acetate, 0.1 μCi/ml, 5 mM glucose, and 0.1 international units/ml insulin. Tissue slices were incubated for 2 hr at 37 C in an atmosphere of O_2:CO_2 (95:5 v/v).

Incorporation of [^{14}C] into Fatty Acids

Incubations were terminated by the addition of 3 ml chloroform-methanol (1:1 v/v). The tissue slices were homogenized and the lipids extracted by the procedure of Folch, et al. (18). Samples of extracted lipid were transesterified using methanolic HCl (19); the fatty acid methyl esters were extracted with hexane, and the [^{14}C] content of the hexane extract was measured in a Packard liquid scintillation counter using a toluene-methanol based scintillation fluid (20). The counting efficiency for [^{14}C] was 85%.

Samples of lipid also were separated into the major lipid classes by thin layer chromatography (TLC) on Silica Gel G (19). The appropriate bands of silica gel were transferred to scintillation vials, and a gel was formed following addition of 4 ml H_2O and 10 ml Unisolve I. The [^{14}C] radioactivity was counted with an efficiency of 70%.

Lipid Analyses

The total lipid content of samples of lipid extracted from tissue slices, as described above, was determined gravimetrically (19). Further samples of lipid were used for the determination of the unesterified fatty acid content of the tissue slices. After the addition of an internal standard (pentadecanoic acid), the unesterified fatty acids were isolated by TLC on Silica Gel G (19) and methylated using BF_3-methanol (21). The methyl esters of the fatty acids were purified further by TLC on Silica Gel G with hexane-diethyl ether (9:1 v/v) as the solvent for development. The concentration of the fatty acid methyl esters finally was determined by gas liquid chromatography (GLC) using

TABLE II

Effect of Dieatry Safflower Oil upon Lamb Plasma Unesterified Fatty Acid Concentration and Composition[a]

	Newborn	Cows' milk		Cows' milk + safflower oil	
		5 days	8 days	5 days	8 days
	Plasma unesterified fatty acid concentration (μg/ml plasma)				
Linoleic acid	7 ± 3[b]	3 ± 1	6 ± 2	6 ± 2	10 ± 1
Total plasma unesterified fatty acids	122 ± 49	31 ± 5	70 ± 18	31 ± 10	45 ± 7
Number of observations	3	3	4	3	3
	Wt percentage composition of plasma unesterified fatty acids				
Palmitic	22.4 ± 3.4	38.5 ± 1.1	34.1 ± 1.1	39.8 ± 1.6	35.0 ± 1.9
Palmitoleic	2.7 ± 0.1	2.6 ± 0.1	2.6 ± 0.2	2.5 ± 0.3	2.3 ± 0.4
Stearic	9.6 ± 0.5	13.8 ± 0.4	16.8 ± 1.0	12.0 ± 1.2	15.6 ± 0.6
Oleic	57.4 ± 3.4	33.7 ± 1.0	34.6 ± 1.8	27.2 ± 1.4[c]	23.8 ± 1.5[c]
Linoleic	6.0 ± 0.8	9.1 ± 0.3	8.9 ± 2.2	16.7 ± 0.7[c]	21.5 ± 0.3[c]
Linolenic	1.8 ± 0.4	2.2 ± 0.3	2.6 ± 0.2	1.6 ± 0.1[c]	1.8 ± 0.2[c]
Arachidonic	0.1 ± 0.1	0.1 ± 0.1	0.5 ± 0.2	0.1 ± 0.1	0.3 ± 0.1
Number of observations	3	3	4	3	3

[a]Lambs were fed either cows' milk or cows' milk plus safflower oil from birth.

[b]Results are mean ± standard error of mean.

[c]Difference between the value and that for cows' milk fed lambs is statistically significant ($P<0.05$).

columns of 15% ethylene glycol succinate methyl silicone polmyer (EGSS-X) on Gas-Chrom P (100-120 mesh; Applied Science Laboratories, State College, Pa.) at 180 C in a Pye 104 chromatograph. Each individual value for the concentration of tissue slice unesterified fatty acids is the mean of determinations on 6 separate sets of tissue slices; 3 of these sets previously were incubated for 2 hr, as described above, and 3 sets were nonincubated controls.

Oxidation of Acetate to CO_2

Tissue slices were incubated with [1-^{14}C] acetate for 2 hr as described above; the CO_2 produced was trapped, and its radioactivity was determined by the method of Lin and Fritz (20).

Treatment of Blood Samples

Blood samples were collected in heparinized tubes, and plasma was obtained by centrifugation for 15 min at 2000 x g at 5 C. Plasma lipids were extracted by the method of Nelson and Freeman (22), and the concentration and composition of the unesterified fatty acid fraction were determined as described above, except that the fatty acid methyl esters were not purified further.

Protein Determination

Tissue slices were homogenized in 0.15 M KCl to give a 2.5% homogenate (w/v) using an all glass homogenizer. Homogenates were centrifuged at 105,000 x g for 60 min, and the protein concentration of the supernatant was determined by the Lowry method (23).

Presentation of Results

Results are expressed as mean ± standard error of the mean. Significance was calculated on the basis of the Mann-Whitney U test (24).

Materials

Safflower oil was purchased from Alfonal, Byfleet, England; bovine insulin, ca. 24 international units/mg, and pentadecanoic acid from Sigma Chemical Co., London, England; [1-^{14}C] acetic acid, 59 mCi/mmol, from the Radiochemical Centre, Amersham England; Silica Gel G from E. Merck, Darmstadt, Germany; boron trifluoride, 14% solution in methanol, from British Drug Houses; and Unisolve I from Koch-Light, Colnbrook, England. Solvents were either Analar grade or were redistilled before use; all other chemicals were Analar grade.

RESULTS

Preliminary experiments were performed to investigate the development of ovine lipogenesis in lambs suckled by their mothers.

Following a lag period of 2-3 days, the rate of incorporation of [1-^{14}C] acetate into fatty acids in adipose tissue slices increased ca. 10-fold during the period of study (Fig. 1). The rate of incorporation of acetate carbon into CO_2 was always greater than the rate of incorporation of acetate into fatty acids (Fig. 1), indicating that

TABLE III

Effect of Dietary Safflower Oil on the Rate of Lipogenesis in Lamb Adipose Tissue Slices[a]

	Diet	
	Cows' milk	Cows' milk + safflower oil
Rate of [1-^{14}C] acetate incorporation into:		
Fatty acids (nmoles/hr/mg protein)	236.8 ± 88.6 (4)[b]	99.2 ± 21.9 (3)[c]
Fatty acids (nmoles/hr/g wet wt)	1,730 ± 930 (4)	990 ± 230 (3)
CO_2 (μ moles/hr/mg protein)	1.99 ± 0.39 (4)	2.25 ± 0.41 (3)
CO_2 (μ moles/hr/g wet wt)	13.40 ± 4.80 (4)	25.50 ± 4.30 (3)
Ratio of the rate of incorporation of [1-^{14}C] acetate into fatty acids and into CO_2 (nmoles/hr : nmoles/hr)	0.111 ± 0.019 (4)	0.044 ± 0.004 (3)[c]

[a]Lambs were fed either cows' milk or cows' milk plus safflower oil from birth until 11 days of age.

[b]Results are means ± standard error of mean with the number of observations in parenthesis.

[c]Difference between the value and that for cows' milk fed lambs is statistically significant ($P<0.05$).

the rate of uptake of acetate was not limiting the rate of lipogenesis.

Analogous studies with liver slices showed a rate of hepatic lipogenesis of only 0.4-1.0 nmole acetate incorporated/hr/mg protein at 1 day of age, and this decreased to 0.00-0.03 nmole/hr/mg by 9-10 days after birth. The rate of incorporation of acetate carbon into CO_2 in the liver slices ranged from 20-50 nmoles/hr/mg protein during this period.

Other characteristics of adipose tissue slices from neonatal lambs are summarized in Table I. A number of changes occurred during the first 2-3 days after birth, including a marked decrease in the unesterified fatty acid concentration, and a decrease in the 105,000 x g cytosol protein concentration. No further changes were detected between 5-10 days of age, so values were pooled. The ratio of the total lipid to cytosol protein concentration increased with age, probably reflecting an increase in the lipid content/cell (14).

Effect of Safflower Oil Supplementation

Plasma total unesterified fatty acid concentration was very variable, as indicated by the standard error of the means (Table II). Administration of safflower oil had no statistically significant effect upon the plasma total unesterified fatty acid concentration or linoleic acid concentration, but it did result in a highly significant ($P<0.01$) increase in the proportion of linoleic acid in the plasma unesterified fatty acid fraction. There was also a significant decrease ($P<0.05$) in the proportion of oleic acid following safflower oil administration.

Administration of safflower oil-supplemented cows' milk for 10 days resulted in a significant ($P<0.05$) decrease in the rate of incorporation of [1-^{14}C] acetate into fatty acids in adipose tissue slices when expressed on a mg protein basis (Table III). A decrease in the rate of lipogenesis also was observed when the results were expressed/g wet wt of tissue, but this was not statistically significant. The rate of incorporation of acetate carbon into CO_2, however, was not altered significantly by dietary safflower oil when expressed on either basis. Dietary safflower oil also resulted in a significant ($P<0.05$) decrease in the ratio of the rate of incorporation of [^{14}C] from acetate into fatty acids and into CO_2.

Ninety-eight percent of the acetate incorporated into fatty acids was esterified; 15% of the label was found in the phospholipid fraction, 34% in diglycerides and 49% in triglycerides. Administration of safflower oil had no effect upon the percentage distribution of [^{14}C] among the various lipid classes.

The concentration of unesterified fatty acids of adipose tissue slices was measured before and after incubation; there being no difference, mean values are reported. Table IV clearly shows that administration of safflower oil had no effect upon the concentration of any of the unesterified fatty acids, including linoleic acid.

The concentration of 105,000 x g cytosol protein was increased significantly ($P<0.05$) by feeding safflower oil; there was also a significant decrease in the ratio of total lipid to cytosol protein concentration ($P<0.05$, Table IV).

Total milk intake over the experimental period, 13.5 kg, was the same for lambs receiving either cows' milk or cows' milk plus saf-

TABLE IV

Effect of Dietary Safflower Oil upon Unesterified Fatty Acid, Total Lipid, and 105,000 x g Supernatant Protein Concentration of Lamb Adipose Tissue Slices[a]

	Diet	
	Cows' milk	Cows' milk + safflower oil
Unesterified fatty acid concentration (μg/g wet wt)		
Palmitic	292 ± 23 (4)[b]	270 ± 33 (3)
Stearic	204 ±20	186 ± 9
Oleic	212 ± 15	208 ± 7
Linoleic	60 ± 12	71 ± 12
Linolenic	10 ± 1	9 ± 1
Total	778 ± 46	754 ± 44
Total lipid concentration (mg/g wet wt)	618 ± 23 (4)	556 ± 20 (3)
105,000 x g Supernatant protein concentration (mg/g wet wt)	6.3 ± 1.0 (4)	10.0 ± 0.1 (3)[c]
Ratio of total lipid to 105,000 x g supernatant protein concentration (mg/g : mg/g)	105.4 ± 13.9 (4)	55.9 ± 2.6 (3)[c]

[a]Lambs were fed either cows' milk or cows' milk plus safflower oil from birth until 11 days of age.

[b]Results are means ± standard error of mean with the number of observations in parenthesis.

[c]Difference between the value and that for cows' milk fed lambs is statistically significant ($P<0.05$).

flower oil; wt gain during the period was 30.4 ± 3.2% and 27 ± 3.8% of the initial body wt in the control and safflower oil-supplemented groups, respectively; the difference is not statistically significant.

DISCUSSION

Investigation of the effect of dietary fat upon adipose tissue metabolism can present the problem of choosing suitable bases for the expression of results (14). This problem is exacerbated when using neonatal animals, as animals of the same chronological age can show varying degrees of development. Microscopic examination of sections of adipose tissue, fixed in 10% formol saline, from 11 day old lambs fed cows' milk showed a marked range of adipocyte size between animals and, hence, the number of adipocytes/g tissue (results not shown). This probably accounts for the variation in supernatant protein concentration. Thus, metabolic rates in this study are probably best compared on a unit protein basis; such a basis results in relatively smaller standard error of means than a g wet wt of tissue basis (Table III). However, interpretation of the effect of dietary safflower oil upon adipose tissue lipogenesis then is complicated by the fact that there is also a change in the tissue supernatant protein concentration, the significance of which is uncertain. For this reason, it is pertinent to compare the relative rates of incorporation of acetate into fatty acids and CO_2, which clearly confirm that dietary safflower oil reduces the rate of adipose tissue lipogenesis in the neonatal lamb.

The effect of dietary safflower oil was observed, despite the fact that the lambs were receiving a fat-rich diet. The cows' milk used contained 41 g fat/liter, so that the mean daily fat consumption in the control group was 50 g fat. The 5 ml safflower oil given daily, thus, represents less than 10% of the fat intake, and, as safflower oil comprises only 70% of linoleic acid (12), the linoleic acid content of the diet of the safflower oil-supplemented animals was only 7% of the total fatty acid intake. Of course, the possibility exists that some other component of the safflower oil is the effective agent. Studies with rodents, however, using diets supplemented with fatty acid methyl or ethyl esters or trilinolein have led to the conclusion that, for rodents, at least, the effects of safflower oil are due to its linoleic acid content (6,7,10,25).

The negligible rate of lipogenesis observed in lamb liver slices during the preliminary studies precluded an examination of the effect of dietary safflower oil upon ovine hepatic lipogenesis. Very low rates of hepatic lipogenesis have been reported in adult sheep (26,27).

The mechanism by which linoleic acid exerts its extra-inhibitory effect upon lipogenesis (relative to saturated fatty acids) is unknown. Several hypotheses have been suggested (25). The report of an inverse correlation between the rate of lipogenesis and the tissue concentration of unesterified linoleic acid (28) suggests that linoleic acid, or linoleyl Coenzyme A (CoA), may be a more potent inhibitor of the activity of one or more of the enzymes involved in lipogenesis than is palmitic acid or palmityl CoA (29,30). This hypothesis has been challenged by several authors (10-12) but cannot be dismissed completely on the basis of data available to date. The present study shows that, for the suckling lamb, at least, dietary safflower oil can reduce the rate of lipogenesis without a concomittant change in the concentration of unesterified polyunsaturated fatty acids, clearly eliminating the above hypothesis as a possible explanation for the effect of safflower oil upon ovine lipogenesis.

Gozukara, et al. (31) suggested that dietary linoleic acid exerts its effect upon lipogenesis in rodents by reducing appetite and, hence, carbohydrate intake. Again this hypothesis is excluded for the present study, as food intake was the same in both the control and safflower oil-supplemented groups of lambs. Recently, Musch, et al., (25) showed that the effects of dietary polyunsaturated fatty acids upon rat hepatic lipogenesis are not dependent upon reduced food intake.

The effect of dietary safflower oil upon ovine metabolism appears to be selective, neither the rate of acetate oxidation to CO_2 nor the distribution of labeled fatty acids, synthesized from acetate, among the various lipid classes being altered. Dietary safflower oil did, however, increase the cytosol protein concentration, the value being similar to that of 2-3 day old lambs. This suggests that the normal development of the adipose tissue might be retarded by dietary safflower oil, thus accounting for the reduced rate of lipogenesis. At birth, lamb perirenal adipose tissue has the morphology characteristic of brown adipose tissue; this is lost rapidly, and, by 10 days of age, the tissue has the morphology of white adipose tissue (32,33). The change from brown to white adipose tissue can be retarded by maintaining lambs at reduced temperature (33). Clearly, the possible effects of safflower oil upon lamb adipose tissue development warrant further investigation.

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Brain Free Fatty Acid Levels in Rats Sacrificed by Decapitation Versus Focused Microwave Irradiation

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ABSTRACT

Values are presented for whole brain free fatty acid levels of rats sacrificed by decapitation vs focused microwave irradiation. Free fatty acids were quantitated by specific colorimetric analysis. Within ca. 1 min of sacrifice by either decapitation or microwave, rat whole brain free fatty acid concentrations ranged from ca. 80-100 μg/g fresh tissue. If the brain remained in the head for a total of 5 min after decapitation, free fatty acid levels increased by over 100%. The free fatty acids at this time were enriched with arachidonic acid. The increase in free fatty acid levels following decapitation was completely absent in rats sacrificed by the microwave irradiation. This microwave technique could be a valuable tool in determining free fatty acid and other heat stable compounds in brain tissue.

INTRODUCTION

Interest in cerebral free fatty acids (FFA) stems partially from the recognition that FFA can influence markedly enzymatic processes of the brain; for example, brain cerebrosidase activity is activated greatly by FFA (1,2). Also, recent interest in cerebral prostaglandin synthesis has drawn attention to the concentration and composition of brain FFA (3,4). The reported concentrations of rat brain FFA widely differ. Galli and ReCecconi (5) found amounts corresponding to 2-3 mg FFA/g whole brain; Lunt and Rowe (6), ca. 150 μg/g; and, more recently, Bazán, et al., (7) reported only 30-40 μg/g in brain excised and frozen or homogenized within 30 sec of decapitation. Bazán and coworkers (7) emphasize that the discrepancy between the various reports could be explained by a rapid increase in brain FFA content seen by them to occur during the first few min after sacrifice. This increase was presumed due to normal enzymatic activities rather than to tissue autolysis. The present studies are directed at resolving the controversy over brain FFA levels. A specific colorimetric test and the "focused microwave irradiation" technique are used to measure rat whole brain FFA concentrations.

MATERIAL AND METHODS

Experimental animals: Male Sprague-Dawley rats of 250-300 g were used for the studies involving sacrifice by decapitation and rats of 150-200 g for sacrifice by focused microwave irradiation. The rats were given free access to standard rat chow and water until shortly before sacrifice.

Sacrifice and Removal of Brain Samples

Decapitation: Following decapitation by a guillotine, the brains either were removed immediately or permitted to remain in situ at room temperature for 5 min. After removal, the brains were washed in cold isotonic saline, quickly blotted on filter paper, and dropped into liquid N_2. Between 30-40 sec were required for removal of the brains. Ca. 15-20 additional sec lapsed until the brains could be placed in the liquid N_2. Thus, the brains were frozen within 45-60 sec after decapitation.

Microwave: Rats were sacrificed by 3 sec exposure of the head to a focused microwave

TABLE I

Free Fatty Acid Content of Whole Brain as Function of Time after Sacrifice by Decapitation vs Microwave Irradiation

Method of sacrifice	1 Min postmortem[a]		6 Min postmortem[a]		6/1
	Rats (n)	μM/g[b]	Rats (n)	μM/g[b]	
Decapitation	9	0.339 ± 0.033	9	0.781 ± 0.069[c]	2.30
Microwave	9	0.279 ± 0.054	8	0.278 ± 0.039	1.00

[a]Time between sacrifice by decapitation or microwave and placing the brains in liquid nitrogen.

[b]Free fatty acid concentration is expressed as μM/g of brain (wet wt) relative to stearic acid as standard. The values are average ± standard error.

[c]P(t) of the difference between the values at 1 and 6 min is less than 0.001 (Student's t-test).

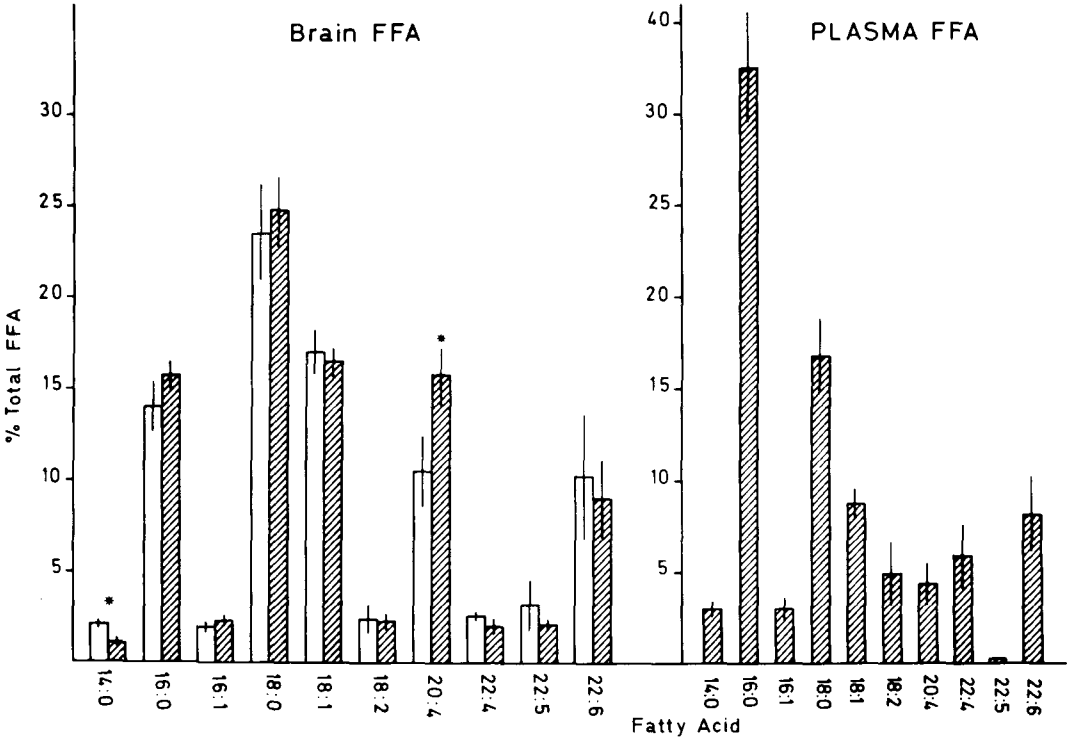

FIG. 1. Relative fatty acid composition of rat whole brain free fatty acids (FFA) and plasma FFA. The values are average ± standard error (bars) of 4 or 5 rats for the brain FFA and of 3 rats for the plasma FFA. *P (t) of the difference between 1 and 6 min is ca. equal to or less than 0.05. □ = 1 min and ▨ = 6 min.

beam in the system described by Guidotti, et al. (8). Due to space limitations, rats of less than 200g had to be used. As with the decapitation studies, the brains were removed immediately or allowed to remain in the head for 5 min. Due to the high temperature of the animal surface (ca. 70 C), the rat head was immersed in liquid N_2 for ca. 3 sec prior to excising the brain. As before, ca. 45-60 sec lapsed from the moment of sacrifice of the rats until placing the brains in liquid N_2.

Extraction and recovery of lipids: The frozen brains were weighed (1.3-2.1 g) and directly homogenized in 20 volumes of chloroform-methanol (2:1) using a Polytron homogenizer. With all samples, a known amount (160,000 dpm or less) of 1-^{14}C-stearic acid (46.8 μCi/μM, New England Nuclear Corp., Boston, Mass.) was added in 0.1 ml n-heptane to the brain homogenates to permit correction for loss of FFA in the extraction, chromatography, and recovery procedures. The purity of the ^{14}C-stearate was confirmed by radiochromatography. Less than 0.002 μM FFA was, therefore, added to each sample as 1-^{14}C-stearic acid. The homogenate was filtered through a sintered glass funnel and the residue recovered and rehomogenized in 10 volumes of 2:1 chloroform-methanol. The combined filtrates were extracted with 0.2 volumes of 0.88% KCl. The chloroform phase was recovered and concentrated (at <40 C) to ca. 1 ml under negative pressure and a stream of nitrogen. The extracts of adult whole brain contained between 100-180 mg total lipid.

Chromatography and recovery of FFA: The toal lipid extract was applied quantitatively to thin layer chromatography (TLC) plates (Silica Gel H, 1000 μ) to form a band ca. 1.5 cm wide x 15 cm long. The plates were developed in n-hexane-diethylether-glacial acetic acid (73:30:2), subsequently dried, and briefly exposed to iodine vapor, and the FFA band was located by reference to an FFA standard. After waiting ca. 5 min for the iodine to sublimate (in air), the FFA band was recovered, transferred to a glass-stoppered centrifuge tube, and extracted with 10 ml $CHCl_3$. The tubes were centrifuged, the chloroform recovered, and the extraction repeated. The pooled chloroform extracts were brought to 20.0 ml, and a 1.0 ml aliquot was transferred to a scintillation counting vial and evaporated to dryness, and the ^{14}C content was measured with a Packard Tri-Carb liquid scintillation spectrometer. An overall

average of 62 ± 2% of the added 1-^{14}C-stearate was recovered with this procedure.

Test for generation of FFA from phospholipid: The possible generation of FFA from breakdown of phospholipid during the extraction and chromatographic procedure was examined in several experiments. A known amount of 1-^{14}-C-dioleoylphosphatidyl choline, 859,000 dpm (55 mCi/mM, Applied Science Laboratories, State College, Pa.), was added to the chloroform-methanol brain homogenate or was chromatographed directly on the TLC plates. Brain total lipids were extracted, recovered, and chromatographed as before. The FFA band or, in the case of the direct chromatography of the labeled phospholipid, the area of the plate corresponding to the FFA standard was recovered and assayed for ^{14}C content. Upon direct chromatography of the ^{14}C-dioleoylphosphatidyl choline, an average 16,100 dpm (out of a total 859,000 dpm) was measured in the FFA area. However, essentially no radioactivity was detected in the FFA area when the phospholipid was recovered from the origin and rechromatographed. This shows that the measured ^{14}C-labeled FFA was present with the radioactive phospholipid as received and not generated during the chromatographic procedure. Thus, of the 859,000 dpm of ^{14}C-labeled phosphatidyl choline added to the brain homogenates, ca. 16,100 dpm was present as ^{14}C-labeled FFA at the time of addition. After carrying this amount of ^{14}C-dioleoylphosphatidyl choline through the extraction, recovery, and chromatographic procedures, 11,500 dpm, ± 120 dpm of ^{14}C (average of 3 experiments), was measured in the FFA fraction. Since only 60-70% of the FFA in the brain homogenate is recovered with our isolation procedures (based upon recovery of 1-^{14}C-stearate added to the brain homogenate), this 11,500 dpm could soley represent the 16,100 dpm of ^{14}C-FFA which already was present in the ^{14}C-labeled phospholipid when it was added to the brain homogenates. Therefore, no significant FFA appears to be generated from phosphatidyl choline and presumably other phospholipids during the extraction and chromatographic procedures.

Quantification of FFA: The chloroform-FFA-extract of the TLC plates was concentrated to 10.0 ml, and 5.0 ml was taken for measurement of FFA by the colorimetric method of Duncombe (9). The procedure involves formation of the FFA-copper salts followed by measurement of the bound copper. For these studies, zinc-dibenzyldithiocarbamate rather than sodium-diethyldithiocarbamate was used as the color developer. Standard curves were obtained with each analysis using chloroform solutions of stearic acid at concentrations ranging from 0.01-0.10 μM/ml. After correcting for FFA recovery and sample size, the concentration of FFA in the brain samples was expressed as μM FFA/g wet brain relative to stearic acid. Since different fatty acids give slightly different calibration curves, some loss of accuracy will result from using any given fatty acid as a standard. However, the loss should be slight, since the calibration curves for different long chained fatty acids lie close together (9). We observed only ca. 10% difference in the intensity of color produced by equal molar solutions of either 16:0, 18:0, 18:1, or 20:4. Stearic acid was chosen as the standard, since it was reported by Bazán (10), and confirmed by us, to be the major FFA present in rat brain.

Gas liquid chromatography (GLC) of FFA: Aliquots of the chloroform FFA extracts were evaporated to dryness and the FFA methyl esters prepared by reaction with diazomethane. The fatty acid-methyl esters were purified by TLC, recovered, and the ester mixture was analyzed by GLC as described by Galli, et al. (11).

RESULTS

Adult rat brains removed from the head and placed in liquid nitrogen within 1 min of sacrifice by either decapitation or focused microwave irradiation were found to contain ca. 0.3 μM FFA/g wet wt (Table I). This would correspond to ca. 90 μg FFA/g based upon an estimated mean mol wt of 290 for rat brain FFA (calculated from the data presented in Fig. 1).

Brains removed at 5 min after sacrifice by decapitation contained over 100% more FFA than those removed immediately (Table I). This postmortem increase was completely absent in animals sacrificed by focused microwave irradiation.

The relative composition of FFA measured in brains removed and frozen within 1 min vs 6 min after decapitation is similar with one major exception (Fig. 1). At both 1 and 6 min, 18:0 comprised ca. 25% of the total FFA, 16:0 and 18:1 each accounted for 15-17%, and 22:6, ca. 10% of the total FFA. The major compositional change of brain FFA with time after decapitation was a 50-60% increase in the relative amount of arachidonic acid, increasing from ca. 10-16% of the total during this 5 min interval. The marked difference between the composition of plasma FFA and that of brain (Fig. 1) substantiates the fact that the FFA recovered

from the brain samples is of neural origin and not due to residual blood present in the brain.

DISCUSSION

The reported values for brain FFA basal concentrations vary widely (5-7). This is not surprising in view of the notorious difficulties encountered in determining tissue FFA levels. The values presented by Bazán, et al., (7), ca. 40 μg or 0.14 μM/g brain, were the lowest reported and probably the most accurate. Their measurements were based upon charring developed TLC plates followed by estimating FFA concentrations by photodensitometry. However, this method can result in low values due to possible loss of fatty acids during the charring. In the present study, basal levels of brain FFA were quantitated in brains frozen in liquid nitrogen with 45-60 sec of sacrifice by decapitation or focused microwave irradiation. With both techniques, brain FFA concentrations (quantitated by a colorimetric test specific for FFA) were ca. twice as high as those reported by Bazán, et al., (7) for rat brains frozen within 30 sec of decapitation.

Bazán, et al., (7) demonstrated that the FFA content of rat brain increases sharply during the first 5-10 min after decapitation. We searched for the post-decapitation rise in rat whole brain FFA levels and, like Bazán, observed a marked increase in the FFA content of the brain at 6 min vs 1 min after decapitation. Although this change involved an increase in all the major FFA present in brain, it represented a disproportionte increase in the free arachidonic acid content, an observation also reported by Bazán (10). Of special interest, the postmortem increase in brain FFA levels did not occur when rats were sacrificed by focused microwave irradiation.

Focused microwave irradiation appears to be presently the superior method for quantitating heat stable components of brain which are generated rapidly or catabolized during postmortem processes. Total inactivation of rat brain enzymes, such as adenylcyclase, phosphodiesterase, and choline acetyl transferase occurs within 3 sec of exposing the head to a focused microwave beam (8), and sacrificing rats by microwave prevents the dramatic rise in brain cAMP levels which occurs after decapitation (12). Our observation that sacrifice by microwave completely prevents the postmortem rise in brain FFA supports the hypothesis (7,13) that the rapid production of FFA in adult rat brain reflects enzymatic activities, probably phospholipase activities. The physiological significance of this FFA production might relate to cerebral prostaglandin synthesis, since free arachidonic acid is produced selectively, and others recently have reported synthesis of PGE_2 and $PGF_{2\alpha}$ from endogenous substrate in rat brain slices (3,4).

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Reaction of Anti-Phosphatidyl Inositol Antisera with Neural Membranes[1]

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ABSTRACT

Ca. 15% of the phosphatidyl inositol in myelin and microsomal membranes from rat brain was detectable by anti-phosphatidyl inositol antibody. Antibody-detectable phosphatidyl inositol in myelin and microsomes readily increased when the membranes were incubated at 45 C with the antiserum. Synaptic membranes also had only a limited capacity to adsorb antibody. Quantitative binding studies with synaptic membranes and mitochondria were limited, because these membranes contain cardiolipin, which cross reacts with phosphatidyl inositol antisera. Moreover, highly purified synaptic and mitochondrial membranes contain appreciable amounts of other membrane fractions.

INTRODUCTION

The turnover of phosphatidyl inositol in cerebral cortex and sympathetic ganglia increases rapidly when electrical or pharmacological agents stimulate the tissue. This "phosphatidyl inositol effect," first described by Hokin and Hokin in 1953, has been described in a variety of tissue and has led to considerable speculation concerning the structural and functional role of phosphatidyl inositol in membranes (1). The studies of Hakomori and Rapport and their co-workers on the reaction of antiglycolipid antibodies with membrane glycolipids (2) suggested to us that antiphospholipid antibodies may be useful tools to study the location of membrane phospholipids. In previous studies, we used anticardiolipin antisera to study the location of cardiolipin in mitochondria (3). The subject of the present report is the reaction of antiphosphatidyl inositol antisera with membrane fractions isolated from brain.

METHODS

Materials

Sprague-Dawley rats (Charles River Laboratories, Boston, Mass.), 30-40 days old, were used to prepare membrane fractions. Lipids were purchased from Supelco (Bellefonte, Pa.). Silica Gel H was obtained from Brinkman (Westbury, N.Y.). The silica gel was washed before use with methanol:chloroform:formic acid, 2:1:1 (v/v), and water according to Parker and Peterson (4). Sheep erythrocytes, complement, and hemolysin were obtained from Cordis (Miami, Fla.). Enzyme substrates used to measure marker enzyme activities were purchased from Sigma (St. Louis, Mo.) or Calbiochem (San Diego, Calif.).

Membrane Preparations

Myelin was prepared as described by Norton (5). Mitochondria were isolated as described by

[1] Presented in part at the 8th FEBS Meeting, Amsterdam, August 1972.

TABLE I

Phospholipids of Brain Membranes

Membrane[a]	Phospholipid phosphorus nmoles/mg protein	Percent phospholipid distribution[b] Sph	PC	PI	PS	EP	PA+DPG
Myelin	1300	12	25	4	12	46	1
Mitochondrial	420	3	40	4	2	39	12
Synaptic	640	2	33	8	8	42	8
Microsomal	350	1	40	5	12	40	1

[a] Myelin was prepared as described by Norton (5); mitochondria were prepared as described by Clark and Nicklas (6); synaptic membranes, fraction 2, were prepared as described by Cotman and Matthews (7).

[b] Three preparations of each membrane were extracted. Each extract was analyzed in triplicate. Results are expressed as the average of 8-9 phosphorus determinations. Individual deviations from the average were less than 8%. Sph = sphingomyelin, PC = phosphatidyl choline, PI = phosphatidyl inositol, PS = phosphatidyl serine, EP = ethanolamine phosphatide, PA = phosphatidic acid, and DPG = diphosphatidyl glycerol (cardiolipin).

Clark and Nicklas (6); synaptic membranes, fraction 2, were isolated by the method of Cotman and Matthews (7). For reference studies, myelin, synaptosomal, and mitochondrial fractions were prepared as described by Eichberg, et al. (8). Microsomal membranes were isolated from the supernatant solution of a 27,000 x g for 40 min centrifugation of a 10% rat brain homogenate (w/v) in 0.25 M sucrose. The supernatant solution was centrifuged for 1 hr at 100,000 x g. The resultant microsomal pellet was washed by resuspension in 0.25 M sucrose and centrifugation at 100,000 x g.

Antibody and Enzyme Tests

Antisera to phosphatidyl inositol were prepared in rabbits. The animals were injected every other day (a total of 11-12 injections) with a suspension containing 0.3 mg hapten and the adjuvants, phosphatidyl choline and cholesterol (9). Antisera having little or no capacity to react with cardiolipin antigen were selected for binding studies. The amount of antibody absorbed to membranes was calculated by allowing graded amounts of membrane to react with antiserum. The membrane and antibody bound to it were sedimented by centrifugation. The antibody remaining in the supernatant solution and the initial concentration of the antibody were determined by a standard microflocculation assay. The dilutions of antisera that would induce flocculation of an antigen suspension containing 6 μg phosphatidyl inositol were measured (3). Alternately, the reaction of antibodies with membranes was measured by a complement fixation test. Serial dilutions of the antisera were mixed with dilutions of a membrane suspension and complement. Sensitized erythrocytes were added to the "box" titrations. Antibody reactions were determined by measuring erythrocyte lysis induced by unfixed complement. The results were expressed as isofixation curves as described by Rapport and Graf (10).

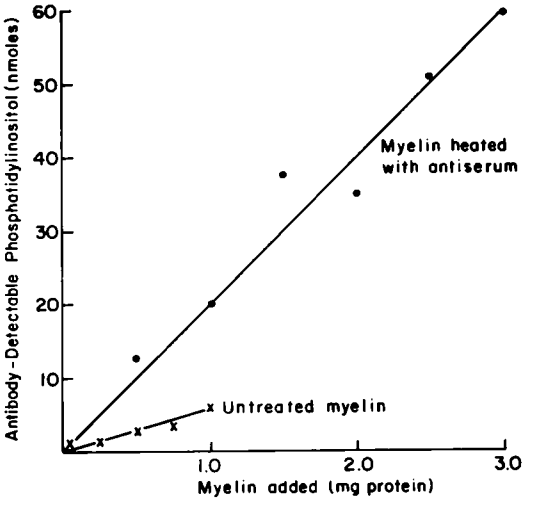

FIG. 1. Linear reaction of myelin with antiphosphatidyl inositol antisera. Conditions for the test are described in Table II.

Oxygen uptake, succinate dehydrogenase, and lactic dehydrogenase activities were used to estimate the purity of the mitochondria prepared by the Clark and Nicklas method (6). Cytochrome oxidase and monoamine oxidase were used as marker enzymes to estimate the purity of mitochondrial and synaptic membranes (11). Acid phosphatase, 5′ nucleotidase, and (Na^+-K^+) - ATPase activities were used to

TABLE II

Antibody-Detectable Phosphatidyl Inositol (PI) in Purified Neural Membranes[a]

Membrane	Range tested (mg protein)	Total PI (nmoles phospholipid phosphorus/mg protein)	Percent antibody bound Fresh	Heated
Myelin	0.1-1	52	12 ± 2	40 ± 10
Microsomal	1-10	18	12 ± 15	50 ± 15
Synaptic membranes	1- 4	51	15 ± 7	25 ± 5
Mitochondrial	1- 4	17	32 ± 6	40 ± 15

[a]Myelin was isolated by the method of Norton (5); mitochondria by the method of Clark and Nicklas (6); and synaptic membranes, fraction 2, by the method of Cotman and Matthews (7). The phospholipid composition of these membranes is listed in Table I. Myelin (5), mitochondria (6), synaptic membranes (7) and microsomes suspended in 0.15 M sodium chloride were mixed with graded amounts of antisera as previously described (3). The percent antibody bound in heated fractions was determined after the antisera-membrane solution was allowed to stand at 45 C for 30 min. Control reactions use chloroform:methanol washed brain tissue to monitor antisera inactivation at 45 C and nonspecific adsorption at 22 C which was virtually undetectable. The results are expressed as the range of 12 assays/test ± standard deviation. The method for calculating a test result has been described previously in detail (3).

TABLE III

Adsorption of Phosphatidyl Inositol (PI) Antibody by Membrane Fractions Prepared from Rat Brain

Membrane fraction	Range tested (mg protein)	Total PI (nmoles phospholipid phosphorus/mg protein)	Percent antibody bound	
			Fresh	Heated
Large myelin	0.5-4.0	29	15 ± 5	30 ± 10
Nerve ending particles	0.5-4.0	12	8 ± 2	16 ± 5
Mitochondria	0.5-4.0	11	30 ± 10	30 ± 10

[a]Membranes were isolated as described by Eichberg, et al. (8). Results are expressed as the average of 12 assays/test ± standard deviation.

monitor the purity of the synaptic membrane fraction (7). Protein was measured by the Lowry method (12).

Lipid Assays

Lipid extracts of membranes were prepared by the Folch 2:1 chloroform-methanol technique (13). The extracts were separated into phospholipid fractions by thin layer chromatography (TLC). The amount of phosphorus in the silica gel zones containing the phospholipid fractions was measured as described by Parker and Peterson (4). TLC was carried out on 0.5 mm Silica Gel H plates prepared by blending 45 g washed gel with 87 ml 1 mM sodium carbonate solution. Plates were activated at 120 C for 1 hr. Lipids were applied in a nitrogen atmosphere. The plates were developed in the solvent system chloroform:methanol, 100:2 (v/v), then redeveloped in the same dimension in the system chloroform:methanol: acetic acid:water, 100:60:16:7 (v/v). The major phospholipids had the following Rf values: sphingomyelin, 0.13; phosphatidyl choline, 0.25; phosphatidyl inositol, 0.46; phosphatidyl serine, 0.63; ethanolamine phosphatides, 0.72; and cardiolipin, 1.0.

RESULTS

Phospholipid Composition of Membranes

The phospholipid composition of highly purified neural membranes is listed in Table I. Previous studies have demonstrated that cardiolipin and phosphatidyl inositol antigens have an appreciable capacity to cross react with phospholipid antisera (9). These results limit the quantitative application of binding studies with phospholipid antisera. As shown in Table I, cardiolipin comprises less than 1% of the myelin and microsomal phospholipids. Mitochondrial and synaptic membranes contain appreciable amounts of both acidic phospholipids. Quantitative studies on the capacity of membranes to adsorb antiphosphatidyl inositol antibody was possible, therefore, only with myelin and microsomal membranes.

Reaction of Membranes with Antisera

The linear adsorption of phosphatidyl inositol antisera to untreated myelin and to myelin heated at 45 C for 30 min in the presence of antisera is shown in Figure 1. Untreated myelin or myelin which was heated, cooled, then mixed with antiserum contained ca. 6 nmoles (equivalent to 12% of the total phosphatidyl inositol) of antibody-detectable phospholipid/mg protein. When myelin was heated at 45 C for 30 min in the presence of antisera, the amount of antibody-detectable phosphatidyl inositol increased three-fourfold. Myelin adsorbed additional increments of antibody when the incubations were continued to 60 min or conducted at 55 C. However, control experiments with lipid-depleted (chloroform-methanol extracted) brain tissue indicated that the heat lability of the antisera and nonspecific antibody adsorption accelerated during prolonged incubations or at 55 C.

The amount of antibody-detectable phosphatidyl inositol in untreated myelin and microsomes is described in Table II. For reference, the capacity of purified mitochondrial and synaptic membranes to adsorb antibody also is described. Ca. 12% of the total phosphatidyl inositol in the untreated membranes react with antibody. Microsomes, similar to myelin, adsorbed increased amounts of antibody following annealing experiments, i.e. experiments wereby the antisera and membrane were heated and cooled together.

The phsophatidyl inositol content of myelin and synaptic membranes was similar, and the untreated membranes had ca. the same capacity to adsorb antibody (Table II). In annealing experiments, however, the amount of antibody-detectable phospholipid increased only slightly in synaptic membranes. These results suggest that membrane components in synaptic membranes shield phosphatidyl inositol from its

antibody more efficiently compared to microsomal and myelin membranes.

The results of the previous experiments demonstrate that the major portion of phosphatidyl inositol in purified neural membranes is not accessible to its antibody. The results in Table III demonstrate that the failure of the membranes to bind antibody was not related to the membrane preparation. Myelin, mitochondria, and synaptosomes, or nerve ending particles, were prepared by a Whittaker fractionation scheme. The phosphatidyl inositol contents of the membrane fractions were quite different compared to the purified membranes. However, the antibody-detectable phospholipid of the comparable fractions was remarkably similar.

Because of the potential cross reactions between cardiolipin and phosphatidyl inositol antigens, the specificity of the reaction of antiphosphatidyl inositol antiserum with mitochondrial and synaptic membranes, both of which contain cardiolipin and phosphatidyl inositol, remains uncertain. It is of interest to note, however, that purified synaptic membrane contains threefold more phosphatidyl inositol/mg protein compared to mitochondria, yet binds less antibody as determined by microflocculation tests. Complement fixation measurements of antibody adsorption confirm these findings. As shown in Figure 2, when measured by complement fixation, mitochondria have a 10-fold greater capacity to adsorb antiphosphatidyl inositol antibody. The striking difference between the reaction of the antisera with the membranes, which have a similar phospholipid composition, indicates that antibody adsorption relates directly to the accessibility of the membrane-bound antigen.

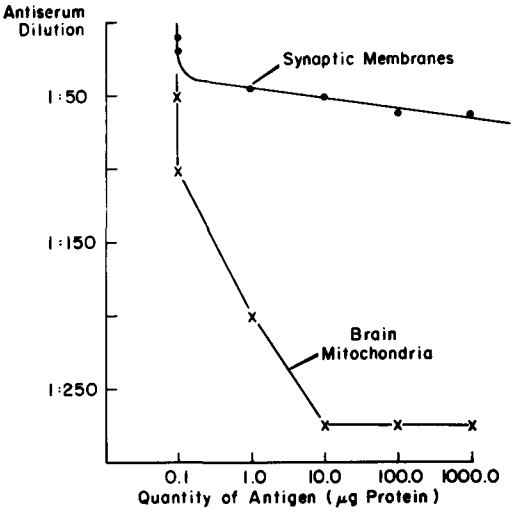

FIG. 2. Isofixation curves for the reaction of phosphatidyl inositol antiserum with rat brain mitochondria and synaptic membranes.

DISCUSSION

The major portion of phosphatidyl inositol in neural membranes was inaccessible to its antibody. The antibody-detectable phospholipid in myelin and microsomes increased to 50% or more of the total membrane phosphatidyl inositol when the membranes were heated in the presence of the antisera. Similar annealing tests with synaptic membranes detected ca. 25% of the membrane phosphatidyl inositol. These results suggested that the steric, ionic, or hydrophobic interactions which together or alone inhibited the reaction of membrane-bound phosphatidyl inositol with its antibody, were most potent in the synaptic membrane fraction. However, the interpretation of such results is limited. Our previous studies with anticardiolipin antibody and our present work indicate that antiphospholipid antibodies, in general, have little capacity to react with the acidic membrane phospholipids. Thus, a variety of factors could inhibit the reaction of the membrane-bound antigen with its antibody. The purity of the membranes used for the binding studies is a second issue. The possibilities to judge the purity of myelin through marker enzymes are few. The microsomes and mitochondria represent a collection of membrane vesicles from at least three cell types. The mitochondrial and synaptic membrane fractions had enzymic activities exactly as described (6,7). However, the synaptic membranes contained appreciable monoamine oxidase activity indicating the fraction was contaminated by mitochondrial outer membranes (11). The mitochondrial, microsomal, and synaptic membrane fractions contained 5-10% the amount of basic protein found in an equivalent wt of myelin (14).

Various functions have been proposed for membrane phosphatidyl inositol. The polar head of the molecule may act as a receptor, or it may interact with membrane proteins, such as proteolipid (15) or basic protein (16). The results of the present study favor the idea that phosphatidyl inositol interacts with membrane components. Studies with liver mitochondria and microsomes have shown that the adsorption of antiphosphatidyl inositol antiserum was enhanced following proteolytic digestion of the membrane (17). The latter studies, however, do not rule out the possibility that membrane neutral lipids, glycolipids, or other phospho-

lipids inhibit the interaction of membrane-bound phosphatidyl inositol with its antibody.

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Fatty Acid and Aldehyde Composition of Major Phospholipids in Salt Gland of Marine Birds and Spiny Dogfish

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ABSTRACT

The lipophilic components of choline phosphoglycerides and ethanolamine phosphoglycerides obtained from the salt gland of herring gull and eider duck and from the rectal gland of spiny dogfish were investigated by means of thin-layer chromatography, gas chromatography, and gas chromatography-mass spectrometry. All phospholipids analyzed were shown to contain small amounts of plasmalogens, and mainly C16, C18, and C18:1 aldehyde was detected. The fatty acids were composed of saturated, unsaturated, straight chain, and branched chain types, ranging between 14-22 carbon atoms. The lipophilic composition of the rectal gland phospholipids showed a higher degree of unsaturation and the presence of more branched chain fatty acids than that of the birds, possibly related to body temperature.

INTRODUCTION

The salt gland of marine birds (1) and the rectal gland of cartilaginous fish (2), with a specialized and elevated function of sodium chloride excretion, are suitable objects for studies of some aspects of sodium ion translocation. In earlier works (3-5), the amount of sulphatides was found correlated with the activity of Na^+-K^+-ATPase, thought to be an obligate part of a transport unit (6). However, as shown in Table I, sulphatides are only minor membrane lipid components of these organs and are, therefore, expected to contribute little to the physicochemical properties of the membranes. To add data for future studies of the lipid phase behavior in relation to enzyme activities and transport capabilities, at present of great membrane research interest (7), the lipophilic components of the major phospholipids of the salt gland of eider duck and herring gull and of the rectal gland of spiny dogfish were investigated.

In this paper, the International Union of Pure and Applied Chemistry-International Union of Biochemistry recommendations (8) for assignment of double bond positions are used here for methyl branch positions. Thus, *n* -4 means position 4 from the methyl end. In the shorthand designations for fatty acids *br* means a methyl branched chain; the number before the colon, chain length; and the number after the colon, number of double bonds. Thus, *br(n* -4) 18:0 means 14-methyl-heptadecanoic acid.

MATERIALS AND METHODS

The preparation and identity of ethanolamine phosphoglycerides and choline phosphoglycerides from the salt gland of herring gull

TABLE I

Lipid Composition[a] of Salt Glands of Herring Gull (5), Eider Duck (5), and Domestic Duck (3) and Rectal Gland of Spiny Dogfish (4)

Compound	Herring gull	Eider duck	Domestic duck	Spiny dogfish
Cholesterol	56	53	33	45
Ethanolamine phosphoglycerides	59	60	24	52
Choline phosphoglycerides	60	53	34	65
Serine phosphoglycerides	6.7	6.1	4.1	7.4
Inositol phosphoglycerides	5.3	5.1	2.2	3.8
Diphosphatidylglycerols	7.8	11.3	4.1	9.0
Sphingomyelins	22	25	12	6.7
Sulphatides	5.7	7.2	1.7	2.8
Glucosylceramides	0.11	0.63	0.55	0.09
Galactosylceramides	0.16	0.85	0.27	0.21
Xylosylceramides	0.18	---	---	---
Diglycosylceramides	0.02	0.01	---	---
Gangliosides	---	---	---	1.8

[a]Values are expressed as μmol/g dry tissue wt, except for gangliosides which are given as mg/g dry tissue wt.

TABLE II

Fatty Acid and Aldehyde Composition[a] of Phosphoglycerides from Salt Glands of Eider Duck and Herring Gull and from Rectal Gland of Spiny Dogfish

Compound	Herring gull		Eider duck		Spiny dogfish	
	Ethanolamine phosphoglyceride[b]	Choline phosphoglyceride	Ethanolamine phosphoglyceride[b]	Choline phosphoglyceride	Ethanolamine phosphoglyceride[b]	Choline phosphoglyceride
Fatty acid						
14:0	trace	trace	trace	trace	trace	2
15:0	trace	trace	trace	trace	trace	1
16:0	8	38	5	37	16	39
br 17:0	trace	trace	trace	trace	2[c]	4[d]
17:0	trace	trace	1	trace	1	trace
br(*n* -4) 18:0	---	---	---	---	1	1
18:0	23	8	29	8	11	2
18:1	12	35	7	30	10	28
18:2	5	10	trace	trace	trace	3
19:0	---	---	trace	---	trace	---
20:1	---	---	---	---	2	4
20:4	45	9	51	21	15	4
20:5	5	---	4	---	13	10
22:6	---	---	2	---	24	2
Aldehyde[e]						
16:0	25	34	25	54	10	13
17:0	---	---	trace	---	trace	---
18:0	75	55	55	28	7	6
18:1	---	---	9	trace	73	77
Unidentified	---	11	11	18	10	4

[a]The figures are given in percent of total fatty acids and percent of total aldehydes, respectively. Trace means less than 0.5%.

[b]All major fatty acids in the ethanolamine phosphoglyceride fractions were identified by mass spectrometry except 20:5. This fatty acid was identified on basis of its retention time (14) and its disappearance in the 20:0 peak upon hydrogenation. Branched chain fatty acids were identified conclusively by mass spectrometry of the corresponding methyl ethers in the ethanolamine phosphoglyceride fraction from spiny dogfish. Small amounts 18:0 and 16:0 saturated aldehydes were identified by combined gas chromatography-mass spectrometry (15) in the methyl ester fractions from ethanolamine phosphoglycerides of herring gull and eider duck. These were probably artefacts formed during methylation of plasmalogen aldehydes and are suggested to be methyl vinyl ethers (16). However, reinterpretation of data (15,16,18) clearly shows the aldehyde nature of the compounds.

[c]This figure includes *br*(*n*−2)17:0, *br*(*n*−3)17:0 and *br*(*n*−10)17:0.

[d]This figure includes 16:1.

[e]The total amount of aldehydes was estimated to be $\leq 2\%$ of the lipophilic components of ethanolamine phosphoglycerides and $\leq 0.5\%$ of choline phosphoglycerides.

and eider duck and the rectal gland of spiny dogfish were described in detail elsewhere (4,5). Before analysis of lipophilic components, the lipids were stored in chloroform-methanol, 2:1 (by volume) at -20 C under N_2 for ca. 1 year. To ascertain that no degradation had taken place during storage, the lipid fractions were analyzed by thin layer chromatography (TLC) (4,5) before methanolysis.

Preparation of Methyl Esters and Dimethyl Acetals

Lipid (1-10 mg) was methanolyzed in 9% H_2SO_4 in dry methanol at 65 C overnight (9). After addition of one volume of water, the fatty acid methyl esters and dimethyl acetals were extracted immediately with three volumes of heptane (9). The heptane phases were evaporated gently under N_2, redissolved in a small volume of heptane, and stored at -20 C under N_2 until used.

Separation and Characterization of Methyl Esters and Dimethyl Acetals by TLC

The methanolysis products were characterized by TLC (3,10) using xylene as solvent (11) and the copper acetate reagent for detection (12). For preparative TLC, water was used for the detection, and the bands were scraped off and transferred to small columns of silicic acid. The methyl esters and dimethyl acetals were eluted with chloroform-methanol, 2:1 (by volume). After gentle evaporation under N_2, the fractions obtained were redissolved in heptane and stored at -20 C under N_2. The completeness of the separation and the absence of degradation products were controlled by analytical TLC.

Hydrogenation

Fatty acid methyl esters from ethanolamine phosphoglycerides of spiny dogfish were hydrogenated with H_2 under atmospheric pressure with PtO_2 as catalyst and ethanol as solvent.

Gas Chromatography

A Hewlett-Packard model F&M 402 apparatus (Hewlett-Packard Instruments Co., Avondale, Pa.) and a Perkin-Elmer 900 apparatus (Perkin Elmer, Norwalk, Conn.) were used, both equipped with flame ionization detectors. Argon was used as carrier gas and the column temperature was 170-190 C. Glass columns of 1.8-2 m length and 3 mm inner diameter were used, packed with the following materials: 10% EGSS-X on 100-120 Gas Chrom P for methyl esters and 3% OV-1 on 100-120 Gas Chrom Q for dimethylacetals, both from Applied Science Laboratories, State College, Pa. Quantitation of peaks was done with an integrator (Hewlett-Packard Instruments Co., model 3370 B).

Mass Spectrometry

Combined gas liquid chromatography and mass spectrometry (GLC-MS) were done on an LKB-9000 instrument (LKB Produkter, Solna, Sweden). Glass columns of 2 m length and 3 mm inner diameter were used, packed with 1% OV-1 on 100-120 Gas Chrom Q (Applied Science) and operated at a temperature of 180-220 C.

Analysis of Branched Paraffin Chains

Methyl esters of saturated fatty acids were converted to their corresponding alcohols by reduction with $LiAlH_4$ (13). Methyl ethers were prepared by refluxing alcohols in CH_3I together with Ag_2O (13). For the GLC and GLC-MS analyses, columns packed with 3% XE-60 on 80-100 or 100-120 mesh Gas Chrom Q (Applied Science) were used (13).

RESULTS AND DISCUSSION

The results of the fatty acid and aldehyde analysis are presented in Table II. The identification of fatty acid methyl esters was based upon GLC retention times on two columns (polar and nonpolar) in comparison with known standards and literature data (14). Native fatty acid methyl ester fractions and hydrogenated ones also were analyzed by combined GLC-MS. Acids with branched paraffin chains were identified as their corresponding methyl ethers by combined GLC-MS, as described before (13). The identification of dimethyl acetals was based upon combined GLC-MS (18).

The lipophilic components of choline and ethanolamine phosphoglycerides of the two birds show a very similar pattern. However, when comparing the two phospholipid classes, ethanolamine phosphoglycerides contain a larger proportion of longer and more unsaturated fatty acids. This was also true in spiny dogfish. Compared with the birds, however, the composition of the two phospholipid classes, including aldehydes of the spiny dogfish, shows a higher degree of unsaturation and the presence of more branched hydrocarbon chains. The spiny dogfish, a poikilothermic animal, has a much lower body temperature, 4-15 C (19) than the birds studied, 40 C (20). An adaptation of membrane fluidity to growth temperature has been shown for several microorganisms (21) possibly needed for optimal membrane functions. The hydrocarbon fluidity is regulated by chain length, unsaturation, chain branching,

and chain substitutions of different kinds (21) A relationship between the degree of lipid unsaturation and mean environmental temperature has long been known in poikilotherms (22). The relatively higher degree of unsaturation found in the rectal gland phospholipids could, therefore, reflect a lower environmental and body temperature.

The finding of more branched hydrocarbon chains in phosphoglycerides of spiny dogfish compared with the birds is also true for sphingolipids (4,5). Except for *br(n* -10), the same type of branching, *br(n* -2), *br(n* -3), and *br(n* -4), has been found in the long chain base moiety of sulphatides in the rectal gland of spiny dogfish (23). So far, nothing is known with certainty about the biosynthesis of long chain bases with branched paraffin chains. However, if these branched chain bases described (23) are biosynthesized endogenously (15,24), one might postulate branched chain fatty acid precursors in analogy to the biosynthesis of straight chain isomers (17). Thus, such fatty acids seem to be present in the phosphoglycerides. There is a review on the biosynthesis of branched chain fatty acids elsewhere (25).

When comparing the lipophilic composition with other organs, i.e. liver, brain grey matter, etc., of different origin (16), there seems to be a fairly large variation in composition. However, the lipophilic components of the rectal gland phospholipids show a greater complexity with the presence of branched hydrocarbon chains and quantitatively and qualitatively more unsaturated fatty acids.

The relatively high amounts of cholesterol present in these organs (Table I and ref. 5), together with lipophilic components of the major phospholipids, suggest a liquid-crystalline state of the membranes. A detailed discussion on the influence of fatty acyl chain fluidity on enzyme function and especially Na^+-K^+-ATPase function recently appeared (7).

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Incorporation of Oxygen-18 into Secondary Alcohols of Grasshopper *Melanoplus sanguinipes* [1]

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ABSTRACT

Incorporation of $^{18}O_2$ and $H_2{}^{18}O$ into the secondary alcohol moiety of wax esters in the grasshopper *Melanoplus sanguinipes* was determined by mass spectrometry. Results of this study show that oxygen-18 from $^{18}O_2$ was incorporated into the secondary alcohol, whereas no incorporation of oxygen-18 from $H_2{}^{18}O$ was detected. The data suggest that the reaction, which hydroxylates n-alkanes at or near the center of the carbon chain, involves a mixed function oxidase type enzyme.

INTRODUCTION

Wax esters of aliphatic secondary alcohols constitute 26-31% of the cuticular lipids of the grasshopper *Melanoplus sanguinipes* (1). The alcohols range in chain length from 21-27 carbons, with the C_{23} homolgoue predominating. Labeled n-alkanes included in the diet or administered to the surface of the insect are metabolized to secondary alcohols and esterified (2,3). Chain length specificity is evident, with the shorter chain C_{21}, C_{23}, and C_{25} n-alkanes metabolized at a faster rate than the longer chain C_{27} and C_{29} compounds. In plants, Kolattukudy and coworkers (4-6) have demonstrated the conversion of n-nonacosane to nonacosan-14-ol and nonacosan-15-ol. In some plants, nonacosan-15-ol then is oxidized to the corresponding ketone (6).

The nature of the reaction that introduces the oxygen into long chain alkanes is not well understood. At least two possibilities have been suggested (6). One route could involve a dehydrogenation of the type observed with lower homologues in bacteria (7) followed by hydration to give a secondary alcohol. Alternatively, a mixed function oxidase type enzyme could incorporate one of the atoms from molecular oxygen into the center of the carbon chain. Experimental evidence in plants for this hypothesis includes the incorporation of low levels of oxygen-18 from molecular oxygen into ketones and inhibition of hydroxylation by iron chelators, such as phenanthroline, which was at least partially reversed by adding exogenous Fe^{2+} (6).

In the present paper, the source of the hydroxyl oxygen has been investigated by comparing the incorporation of $H_2{}^{18}O$ and $^{18}O_2$ into the secondary alcohols of *M. sanguinipes*.

EXPERIMENTAL PROCEDURES

n-Tricosane was prepared from a modified Wolff-Kishner reduction of tricosan-11-one as described earlier (11). Molecular oxygen (50 atoms percent $^{18}O_2$) and water (20 atoms percent $H_2{}^{18}O$) were obtained from Bio-Rad Laboratories, Richmond, Calif.

M. sanguinipes eggs were obtained from the USDA Grasshopper Laboratory, Bozeman, Mont. The insects were raised in wire cages at 27 C on a diet of lettuce leaves and bran fed ad libitum. The insects were used 4-7 days after ecdysis to adults. Twenty insects, 10 males and 10 females, from the same population and of equal age were used in each experimental and control group. This number was limited by available quantities of labeled oxygen.

n-Tricosane (1 mg) in 10 μliter diethylether was layered on the abdomens of each insect. For the $^{18}O_2$ study, the insects were placed in a 300 ml glass apparatus connected to a 100 ml bulb containing 1 atm $^{18}O_2$. The incubation apparatus was flushed with nitrogen for 2 min to remove atmospheric oxygen and then sealed. Preliminary studies demonstrated that this insect can withstand anoxia for at least 3 min with no apparent adverse effects. To initiate the experiment, the seal on the bulb containing $^{18}O_2$ was broken. The diffusion of $^{18}O_2$ into the incubation apparatus was hastened by alternatively cooling the 100 ml bulb with ice water and heating with hot tap water to minimize the amount of time the insects were deprived of oxygen. The mole fraction of oxygen was initially 25%. The insects were incubated at 30 C for ca. 10 hr until they expired, presumably due to exhaustion of the oxygen supply. A control group received 1 mg n-tricosane/insect and was exposed to room air at 30 C for the same period of time. For the $H_2{}^{18}O$ study, 10 male and 10 female insects

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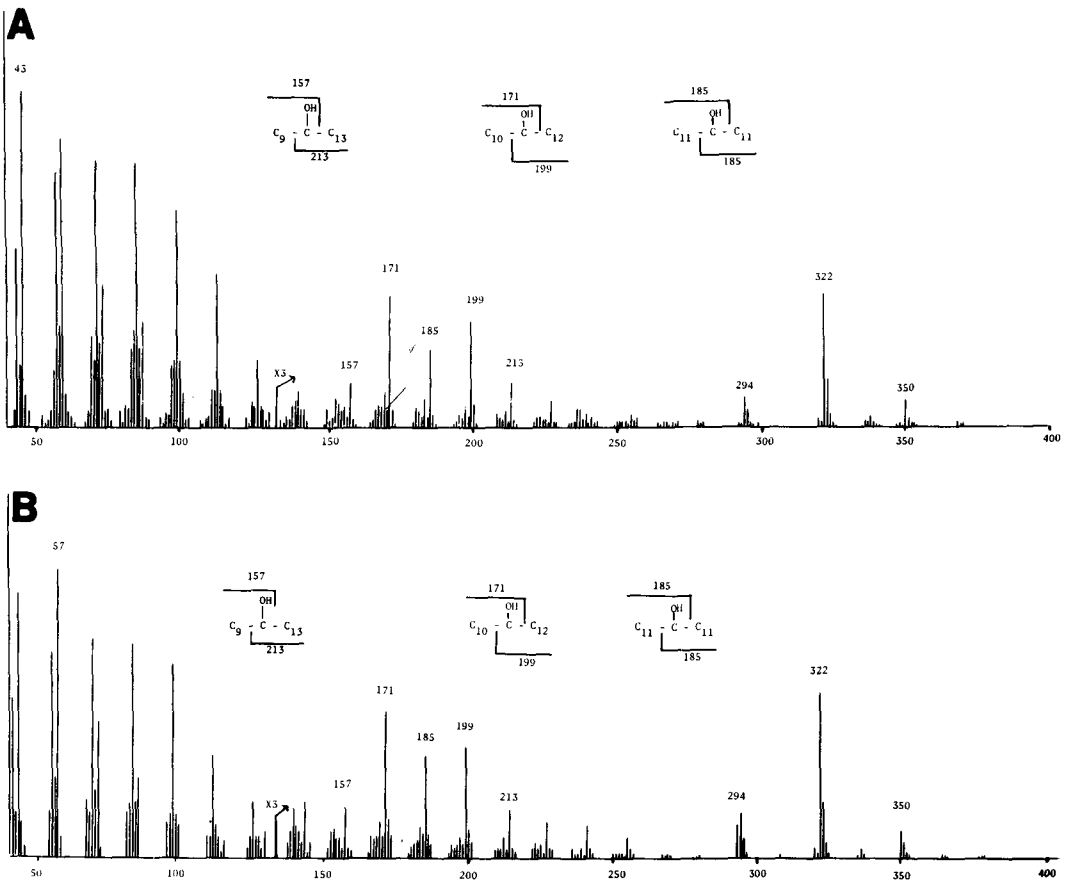

FIG. 1. Mass spectra of secondary alcohols from control (A) (top) and $^{18}O_2$ incubated insects (B) (bottom).

each were injected with 10 μliter $H_2{}^{18}O$ just beneath the cuticle between the second and third abdominal segments. The insects were allowed to metabolize for 24 hr.

At appropriate times, the insects were sacrificed and the cuticular lipids from each group extracted by immersion in hexane for 10 min. The extracts were transferred to vials and evaporated to dryness under nitrogen. The secondary alcohol wax ester was isolated by preparative thin layer chromatography (TLC) as described earlier (11) and reduced using $LiAlH_4$. This reduction leaves the C-O bond of the secondary alcohol intact (8). The secondary alcohol then was isolated by preparative TLC as described earlier (1).

The secondary alcohol fraction was gas chromatographed on a 6 ft x 1/8 in. 3% SP2250 column programed from 175-275 C in 10 min. Integration was obtained with the use of a disc integrator.

Mass spectra were obtained with a Varian CH5 mass spectrometer operating at an ionizing potential of 70 eV, current 100 μA. The samples were inserted into the ion source from gold crucibles.

RESULTS

Analytical gas liquid chromatography (GLC) of the secondary alcohol fractions showed that the C_{23} homologue comprised 73, 70, and 66% of this fraction from controls, $^{18}O_2$ incubated insects, and insects injected with $H_2{}^{18}O$.

Mass spectra of the secondary alcohols from male control (A) and $^{18}O_2$ incubated insects (B) are shown in Figure 1. The parent peaks are very small or absent, while the usual M-18 peak observed with alcohols is predominant. The peaks at 322, 294, and 350 in each spectrum are due to loss of water from the C_{23}, C_{21}, and C_{25} secondary alcohols, respectively, with the C_{23} homologue comprising the major part of the sample. The peaks at 157 and 213, 171 and 199, and 185 arise principally from cleavage on each side of the hydroxyl group of tricosan-10-ol, tricosan-11-ol, and tricosan-12-ol, with the charge remaining on the fragment containing

TABLE I

Mass Spectra Data of Secondary Alcohols from Oxygen-18 Incubated Insects and Controls

Experimental group	Intensity ratios[a]					
	324/322	215/213	201/199	187/185	173/171	159/157
$^{18}O_2$	0.15 ± 0.03	0.17 ± 0.04	0.16 ± 0.04	0.13 ± 0.02	0.15 ± 0.02	0.16 ± 0.05
Control	0.14 ± 0.03	0.10 ± 0.04	0.06 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.06 ± 0.02
$H_2{}^{18}O$	0.13 ± 0.05	0.06 ± 0.04	0.05 ± 0.03	0.04 ± 0.03	0.04 ± 0.02	0.06 ± 0.03

[a]This ratio is calculated by dividing the intensity of the fragment peak (F) into the intensity of the F + 2 peak.

the oxygen atom. The presence of oxygen-18 in secondary alcohols from $^{18}O_2$ incubated insect is clearly evident, as shown by the larger peaks at 159, 173, 187, 201, and 215 relative to controls.

For fragments believed to possess oxygen and for the M-18 peak of the C_{23} secondary alcohol, which does not contain oxygen, intensity ratios were calculated. For a particular fragment (F), this ratio was the intensity of the F + 2 peak divided by the intensity of the F peak. The difference between the F + 2 peaks from insects incubated with oxygen-18 and control insects arises from oxygen-18 incorporated into the secondary alcohol. Only fragments which exhibited a fairly intense peak were considered, so that the F + 2 peaks were large enough for reasonably accurate measurements. In each case, data from 6-8 spectra were averaged. Error limits are plus or minus one standard deviation.

The data in Table I show the intensity ratio for insects incubated with oxygen-18 compared to controls and insects injected with $H_2{}^{18}O$. The intensity ratios of the 324 (F + 2) to the 322 (F) peaks are similar for the control, $^{18}O_2$ and $H_2{}^{18}O$ incubated insects. The intensity ratios of the peaks from fragments which do contain oxygen, 159/157, 173/171, 187/185, 201/199, and 215/213, are from 2-4 times greater in the secondary alcohols from insects incubated with $^{18}O_2$ than controls or insects injected with $H_2{}^{18}O$. The greatest difference in intensity ratios between controls and $^{18}O_2$ incubated insects occurs in the 201/199 and 173/171 peaks, with a difference of 0.10 and 0.11. Data for control and $^{18}O_2$ incubated insects for the 201/199, 187/185, and 173/171 peaks all are different at greater than 99% confidence levels. The intensity ratios of control and insects injected with $H_2{}^{18}O$ is very similar, with small differences noted in the ratios of the 215/213 and 159/157 peaks. A comparison of the data from male and female insects showed no significant differences, and the data presented include both.

DISCUSSION

The mass spectra analyzed were from the total secondary alcohol sample, which accounts for the M-18 peaks at 294 and 350 in addition to the major M-18 peak at 322. These arise from the C_{21} and C_{25} secondary alcohols. The M-18 peaks from the C_{22}, C_{24}, C_{26}, and C_{27} secondary alcohols, each of which make up 1-2% of the secondary alcohol fraction (1), were very small or not detectable. Since the C_{23} secondary alcohol comprises 59% of the secondary alcohols occurring naturally in the insects (1), and makes up an even larger percentage after administration of exogenous n-tricosane, the spectra were interpreted as arising primarily from this homologue. Incorporation of small amounts of oxygen-18 into the other homologues would result in the same oxygen containing fragments as analyzed in Table I, plus peaks at 143 and 227. However, they probably contribute very little to the oxygen-18 (F + 2) peaks because of the small quantities of endogenous C_{21} and C_{25} n-alkane present (9).

From this data, the percentage of molecules which contained oxygen-18 cannot be calculated accurately, but it is probably low, in the order of 2-10%. This amount is not surprising, for the amount of secondary alcohol wax esters in the insect prior to incubation with $^{18}O_2$ is quite large, in the order of .12-.15 mg/insect, which would dilute the isotopically labeled material, and the incubation times were relatively short. However, the incorporation of $^{18}O_2$ into secondary alcohols is demonstrated clearly.

Our detection methods are less sensitive for measuring the incorporation of $H_2{}^{18}O$ mainly due to the high dilution effect from endogenous unlabeled water. We feel, however, that our negative results indicate that large scale $H_2{}^{18}O$ incorporation is absent, and, of course, these negative results are entirely consistent with our observations that the oxygen in the secondary alcohol is derived from molecular oxygen.

The incorporation of molecular oxygen and lack of incorporation of water into the secondary alcohols of *M. sanguinipes* suggest that a mixed function oxidase type enzyme is involved in secondary alcohol biosynthesis.

Mixed function oxidases play a major role in the metabolism of xenobiotics in insects (10,11). They have been shown to metabolize most types of insecticides, including 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; cyclodienes; carbamates; organophosphates; and others (11). Relatively little attention, however, has been given to endogenous compounds metabolized by this enzyme system in insects, although a number of lipophillic substrates have been shown to be metabolized by mixed function oxidases in other organisms (12,13). In *M. sanguinipes*, we have demonstrated the metabolism of n-alkanes by what appears to be a mixed function oxidase enzyme.

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SHORT COMMUNICATIONS

Fluorescent Pigments from Uterus of Vitamin E-Deficient Rats

ABSTRACT

Spectrophotofluorometric analyses of the extracts of uterus from sterile rats maintained on a vitamin E-deficient diet for a prolonged period of time indicated that the brown ceroid-like pigments present were highly fluorescent, having characteristics typical of products formed during lipid peroxidation in vivo. The aqueous-methanol layer of the uteral extract from vitamin E-deficient animals had higher fluorescence than the corresponding chloroform layer, indicating the presence of more polar water-soluble pigments than those found in most other tissues so far examined.

INTRODUCTION

In spite of the knowledge gained since the discovery by Evans and Bishop (1) that dietary vitamin E deficiency caused malformation and fetal resorption in pregnant female rats, it was demonstrated only recently by Raychaudhuri and Desai (2) that depriving animals of vitamin E for an extended period of time, starting prior to birth, causes irreversible sterility in female rats. The reproductive failure in these animals has been found to be associated with the appearance of a brown colored ceroid-like pigment in the uterus and the fallopian tubes of the female rats.

Histological studies indicate that ceroid-type pigments do accumulate in varying amount in a variety of tissues of man and animals during aging and that the accumulation depends upon the status of the dietary factors, such as vitamin E and polyunsaturated fatty acids (3-9).

The present study was undertaken to measure the accumulation of ceroid-like pigments(s) in the uterus of female rats subjected to prolonged vitamin E deficiency and to characterize the biochemical nature of these pigments using spectrophotofluorometric techniques.

EXPERIMENTAL PROCEDURES

One group of 4-5 day pregnant rats of the Wistar strain was placed on a vitamin E-deficient diet of Draper, et al., (10). Another group of similar rats was placed on a control diet similar in composition to the above diet but supplemented with 2.5 g dl-α-tocopheryl acetate (2500 international units)/kg diet.

After delivery, the mothers and newborn littermates from experimental and control groups were caged individually and fed their respective diets for 21-23 days. At this time, the young female rats were separated into individual cages, and their respective diets were continued until termination of the experiment. All animals received food and water ad libitum, and they were maintained in a thermostatically regulated (70 F) animal room.

All animals were sacrificed at the age of 195 days, and their uteri were removed, weighed, and carefully examined for visible signs of ceroid pigmentation before they were frozen. Samples of renal adipose tissues also were removed and immediately frozen for biochemical examination along with the uteri. All tissues were stored frozen until fluorescence analyses were performed.

Fluroescence Analyses

For fluorescence analyses, chloroform and aqueous-methanol extracts of renal adipose and uteral tissues were prepared according to the method described by Fletcher, et al., (11). In this study, extractions were carried out at 45 C with homogenization times of 1 and 1.5 min for uteral and adipose tissues, respectively. Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer, and values herein are expressed relative to a standard quinine sulfate solution (1 μg/ml in 0.1 N sulfuric acid) that had a relative fluorescence intensity of 21.

RESULTS

A general examination of the vitamin E-deficient rats revealed distinct differences from control animals, characterized by lower body wt, increased abdominal fat, and visible pigmentation spread throughout the uterus and the

TABLE I

Fluorescence of Extracts from Uterus of Rats Fed Vitamin E-Deficient and Supplemented Diets

	Fluorescence value[a]			
	Chloroform extract		Aqueous-methanol extract	
Number	−E	+E	−E	+E
1	3.90	0.28	7.50	0.00
2	4.80	0.20	---	0.00
3	2.30	0.29	7.00	0.00
4	4.90	0.00	5.00	0.00
5	5.70	0.27	8.60	0.00
6	1.30	0.30	3.30	0.00
7	2.00	0.22	5.00	0.00
Mean ± standard deviation	3.60[b] ± 1.70	0.26 ± 0.04	6.10[b] ± 2.00	0.00

[a]Relative to a standard solution of quinine sulfate (1 μg/ml of 0.1 N H_2SO_4) with a fluorescence intensity of 21.

[b]Difference between +E and −E values was statistically significant at $p<0.001$.

fallopian tubes. The pigmentation appeared to be of the ceroid-type, and it persisted even after resupplementation of the animals with vitamin E for up to 60 days.

The biochemical examination included recording of the fluorescence characteristics of the extracts of renal adipose and uteral tissues of vitamin E-deficient and vitamin E-supplemented rats. Extracts of the uteral tissues were exposed to high intensity UV light for 30-90 sec to oxidize any interfering retinol fluorescence. Some uteral water-methanol extracts increased in fluorescence (15-25%) upon exposure to high intensity UV light. The chloroform layer had spectral characteristics with an excitation maximum at 370 nm and a fluorescence maximum at 440-450 nm. The excitation and fluorescence maxima for the water-methanol layer were observed at 375 nm and 450-460 nm, respectively. The fluorescence value for each sample was calculated from the maximum intensity of the fluorescence peak. A comparison of the fluorescence values of extracts from uterus of rats fed vitamin E-deficient and vitamin E-supplemented diets is presented in Table I. The fluorescence values of chloroform and water-methanol extracts of uterus from vitamin E-deficient rats were significantly higher than those of vitamin E-supplemented rats. Furthermore, the water-methanol extracts showed higher fluorescence intensity than the corresponding chloroform extracts of the uterus from vitamin E-deficient rats. In the vitamin E-supplemented group, there was some fluorescence in the chloroform extract of uterus, but none in the water-methanol extract. The mean fluorescence values of renal adipose tissue in vitamin E-deficient and control groups were 0.90 ± 0.66 and 0.92 ± 0.38, respectively, indicating no significant difference between groups.

DISCUSSION

The ceroid pigment deposition in the uterus and other tissues of animals and man is now well recognized as a sign of tissue damage, which may be associated with a wide variety of abnormal conditions (7,8). In most cases, the nutritional status of the animal, as influenced by the dietary intake of polyunsaturated fat and vitamin E, determines the formation of ceroid-like pigments in various organs of the body. The abundance of ceroid pigment formation in the uterus of female rats fed a vitamin E-deficient diet for a prolonged time period, as observed in our studies, indicates irreversible peroxidative damage to the uteral tissues, which caused complete reproductive failure in these animals.

The findings from the fluorescence measurements (Table I) are quite significant, and they showed that the ceroid pigments of the uterus of sterile vitamin E-deficient animals have high fluorescence of the type found in some other tissues, such as adipose fat, bone-marrow, heart, and spleen of rats fed diets low in vitamin E and high in polyunsaturated fat (12). On the other hand, the quantitative fluorescence of renal adipose tissues of the female rats with prolonged vitamin E deficiency was not different from that of control rats that received vitamin E supplementation. Aqueous-methanol uteral extracts appear to contain more ceroid-type pigment than the chloroform-soluble extracts which indicates that uteral fluorescent products possess more polar components than those found in most other tissues.

During peroxidation of polyunsaturated fats, malonaldehyde is formed, which then can react with amino-phospholipids to form conjugated Schiff-base products with specific fluorescence characteristics (13). It appears clear from this background knowledge that the fluorescent pigments found in tissues of vitamin E-deficient animals result from the peroxidation of polyunsaturated fats, as shown in this study and as shown previously (11,12,14-16). It appears, therefore, that the ceroid-type pigments of the uterus of sterile vitamin E-deficient rats are products of in vivo lipid peroxidation, as proposed by earlier investigators (2,7).

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Novel Interference in Thiobarbituric Acid Assay for Lipid Peroxidation

ABSTRACT

The thiobarbituric acid test for lipid peroxidation, when applied to a mixture of acetaldehyde and sucrose, produces a 532 nm aborbing chromogen which is indistinguishable from that formed by malonaldehyde and thiobarbituric acid. Unless special procedures are adopted to correct for this effect, the combined action of acetaldehyde and sucrose interferes seriously with the assay of lipid peroxidation reactions, notably those implicated in alcohol-induced liver injuries. However, this unusual thiobarbituric acid effect also can be used as a sensitive method for the detection of acetaldehyde.

INTRODUCTION

The thiobarbituric acid (TBA) assay for lipid peroxidation is based upon a 532 nm absorbing chromogen which is formed by a reaction between TBA and several products of lipid peroxidation, such as malonaldehyde; α,β-unsaturated aldehydes; and several unidentified, nonvolatile precursors of these substances (1-4).

During peroxidation of polyunsaturated fats, malonaldehyde is formed, which then can react with amino-phospholipids to form conjugated Schiff-base products with specific fluorescence characteristics (13). It appears clear from this background knowledge that the fluorescent pigments found in tissues of vitamin E-deficient animals result from the peroxidation of polyunsaturated fats, as shown in this study and as shown previously (11,12,14-16). It appears, therefore, that the ceroid-type pigments of the uterus of sterile vitamin E-deficient rats are products of in vivo lipid peroxidation, as proposed by earlier investigators (2,7).

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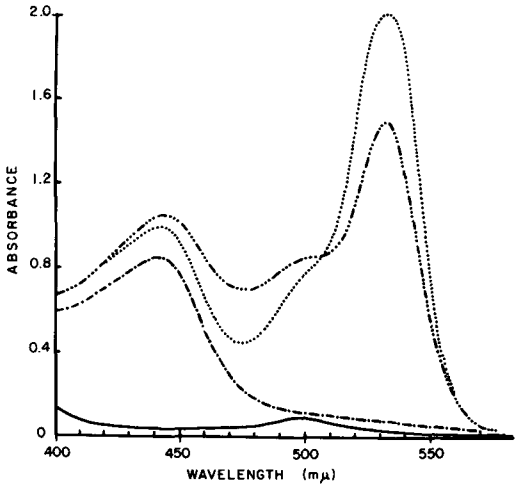

FIG. 1. Absorption specta of products formed by the reactions between thiobarbituric acid (TBA) reagent, trichloroacetic acid, and the following additives: ——acetaldehyde (4 μmoles), –●– sucrose (90 μmoles), –●●●– sucrose (90 μmoles) and acetaldehyde (4 μmoles), and ●●●● sucrose (90 μmoles) and malonaldehyde (.06 μmoles).

When applied to biological systems, the test may be complicated by the presence of interfering substances, such as aldehydes (5-7), sucrose (8), trace metals (9,10), and by the metabolism of some of the lipid peroxidation products (11-14). It appears that all of the interfering chromogens reported to date have absorption maxima which differ. from the characteristic 532 nm chromogen formed by lipid peroxidation products. The present report presents evidence for the formation of a 532 nm chromogen in the total absence of lipid peroxidation by a novel reaction between acetaldehyde, sucrose, and TBA reagent. This interference with the TBA assay appears to be of relevance to studies of ethanol metabolism.

PROCEDURES

Unless otherwise indicated, TBA assays were performed at 100 C for 15 min with 0.8 ml 20% trichloroacetic acid and 1.2 ml 0.5% 2-thiobarbituric acid in a final volume of 3.0 ml. Malonaldehyde standards were prepared by the 0.1 N HCl hydrolysis of malonaldehyde bis (dimethyl acetal). Vacuum distillations were carried out in Thunberg tubes (4). UV absorption spectra were obtained with an Aminco DW-2 spectrophotometer.

RESULTS AND DISCUSSION

Figure 1 shows the results of experiments in which TBA and trichloroacetic acid are reacted with one of the following: 4 μmoles acetaldehyde, 90 μmoles sucrose, and a mixture of acetaldehyde and sucrose. The amount of sucrose used corresponds to that present when the TBA assay is performed on a tissue homogenate prepared in 0.25 M sucrose. Surprisingly, the spectrum of the mixture is not the sum of the spectra of the individual components; instead, a new, intense peak is formed at 532 nm which corresponds in every detail to that produced by malonaldehyde in the TBA assay.

The reaction conditions of the TBA assay were changed in an attempt to establish the mechansim for the formation of the new 532 nm chromogen. At 37 C and a reaction time of 1 hr, a mixture of acetaldehyde and sucrose gave only the weak 498 nm peak due to acetaldehyde, while the malonaldehyde standard formed the usual 532 nm chromogen. Neither reaction mixture gave the 440 nm peak due to hydroxymethylfurfural, one of the products of the acid pyrolysis of sucrose (15). It appears from this that a product of the pyrolysis of sucrose reacts with acetaldehyde and TBA to form the 532 nm chromogen. This was confirmed in an experiment in which the 532 nm chromogen was formed at 37 C by a reaction between TBA, acetaldehyde, and a solution of sucrose which previously had been heated to 100 C in the presence of trichloroacetic acid.

A possible mechanism for the artifact in the TBA assay is one in which acetaldehyde reacts with a pyrolysis product of sucrose to form malonaldehyde. This possibility was investigated as follows: the acid-pyrolysis of sucrose was carried out at 100 C in the presence of acetaldehyde. The reaction products then were vacuum distilled and tested for malonaldehyde by: (A) the characteristic UV spectra of malonaldehyde (4) at pH 2 (λ_{max} = 245 nm) and pH 10 (λ_{max} = 265 nm) and (B) the formation of the 532 nm chromogen with the TBA reagent. Hydrochloric acid was used in these experiments in place of trichloroacetic acid, since the former does not absorb in the UV region. It was shown in separate experiments that the unusual TBA reaction with acetaldehyde and sucrose occurs equally well with both acids. In the control reaction, acetaldehyde was replaced by malonaldehyde in the reaction mixture.

These experiments showed that a volatile product(s) was formed in the acid-pyrolysis reaction but that this did not include malonaldehyde, since the distillate failed to form the 532 nm peak with TBA and absorbed maximally at 225 nm under both acidic and alkaline conditions. Even though the distillate was not TBA-reactive, distillation caused a reduction in

the TBA reactivity of the residue in proportion to the volume distilled. The decrease in TBA reactivity could be caused either by loss from the residue of a UV-invisible substance, such as acetaldehyde, or by the loss of the newly formed, 225 nm absorbing substance(s). From these experiments, we may deduce that a relatively nonvolatile product of sucrose pyrolysis reacts with acetaldehyde or with a volatile 225 nm absorbing substance to form a 532 nm chromogen other than malonaldehyde but this only in the presence of TBA.

In other experiments, it was shown that ethylenediaminetetraacetic acid (EDTA) had no effect upon the magnitude of the 532 nm peak, indicating that trace metal contaminants in sucrose are not a factor in the reaction (9,10). In addition to sucrose, fructose, but not glucose, formed the 532 nm peak with acetaldehyde. This fact may be due to differences in the pyrolysis reactions of these sugars (8). Other aldehydes, such as pyruvaldehyde, citral, propionaldehyde, methacryl aldehyde, and glyoxylic acid, did not produce the 532 nm chromogen in the presence of sucrose, though considerable spectral changes did occur.

The TBA reaction with sucrose and acetaldehyde also can be used as a sensitive and quantitative assay for acetaldehyde, even in tissue homogenates that contain peroxidized lipids. The method consists simply of performing TBA assays on tissue homogenates in the absence of sucrose to establish the amount of TBA-reactive material formed by lipid peroxidation processes (tissue blank). Then the assay is repeated with addition of excess sucrose. Sucrose must be present in considerable excess; under the present assay conditions, 90 μmoles sucrose form sufficient pyrolysis product to react stoichiometrically with 4 μmoles acetaldehyde. A linear relationship then is obtained between acetaldehyde concentration and net absorbance.

The present findings bear on studies of lipid peroxidation processes in systems containing both ethanol and alcohol dehydrogenase activity. We have found that, under certain specific conditions, considerable quantities of acetaldehyde may accumulate in liver homogenates to which an ethanolic solution of α-tocopherol had been added (Baumgartner, unpublished data). Our data also suggest that the acetaldehyde artifact can give rise to an erroneous impression that lipid peroxidation is occurring when, in fact, what is occurring is the conversion of ethanol to acetaldehyde. This phenomenon may be responsible for some of the controversy surrounding the hepatotoxic mechanisms of alcohols, particularly in the case where the application of different assay techniques (TBA and diene conjugation) has led to conflicting views on the participation of radical mechanisms (16-18).

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Use of Internal Vial for Determining Quench Correction in Scintillation Counting of Heterogeneous Media

ABSTRACT

An insert vial situated at the center of the counting medium and containing a standard count has been used for quench correction in the liquid scintillation counting of gels containing suspensions of silicic acid scrapings from thin layer chromatoplates. The method has provided a simple and direct means of routine count correction for a range of ^{14}C-labeled lipid classes following separation on thin layer chromatoplates. The use of insert vial provides several advantages over other methods for the count correction of these suspensions.

INTRODUCTION

The broadening of liquid scintillation techniques has seen the development of methods for counting various heterogeneous systems, including gels, emulsions, and suspensions. In work involving lipids where thin layer chromatography (TLC) plays such an important role in the detection, identification, and quantification of the various subclasses, the counting of material associated with fine particulate substances, such as silicic acid, by a suitable geling technique has provided the investigator with a quick and convenient method for the determination of activity (1). However, in spite of the relative ease of counting, the conversion of such data to meaningful and accurate results has proved to be far less satisfactory due to the many special problems presented by the opacity and heterogeneity of the medium; particular care has to be taken with the choice and application of the method for count correction (2). In this laboratory, an important part of the work involves investigation into aspects of the lipid metabolism of the rumen, and, thus, additional difficulties due to the presence of high concentrations of colored components have, therefore, been encountered with gel counting.

The present paper describes the use of an insert vial situated at the center of the counting medium and containing a "standard" count to overcome some of the problems encountered in the quench correction of such samples. Although an isolated internal standard for count correction has been tried with homogeneous systems (3,4), its use has found little favor, as, under such circumstances, it presents no real advantage over other methods available for quench correction. However, heterogeneous counting and, in particular, counting of colored suspended materials in gels present many problems with regard to count correction which may not be corrected easily for by the more popular methods of quench correction. Under these circumstances, the use of an isolated internal standard has been found to be of particular value.

EXPERIMENTAL PROCEDURES

The vial and the insert vial are shown in Figure 1. The vial was a standard Packard (Packard Instrument Co., Des Plaines, Ill.) glass vial with a 22 mm screw cap lined with cork and tin foil. The insert vial, approximate total volume 1.5 ml (length, 58 mm; outside diameter 6.5 mm; inside diameter, 5.5 mm), also was made of glass and was positioned centrally in the counting vial by insertion through a polyethylene disc held in position by the screw cap. The polyethylene disc was cut to seal the mouth of the counting vial when the screw cap was applied, thereby completely separating the area of the vial used for the sample from that of the "standard count" in the inserted vial.

The following compounds were used as the "sample" test materials: ^{14}C-cholesterol, ^{14}C-palmitic acid, and ^{14}C-glyceryl tripalmitate. To these compounds were added concentrated solutions of grass extracts, rumen liquor extracts, and a red dye to provide a range of standards containing graded concentrations of color. These standards then were applied as bands to chromatoplates coated with Kieselgel G (E. Merck, Darmstadt, Germany), and the bands, containing graded amounts of silicic acid, were scraped into counting vials. A mixture of 10 ml Unisolve 1 (Koch-Light Laboratories, Colnbrook, England) + 4 ml water then was added as the combined geling agent and scintillation medium. A solution of ^{14}C-hexadecane in 5.5 g 91% 2,5-diphenyloxazole and 9% 1,4-tris-2-(4-methyl-5-phenyloxazolyl)-benzene/liter toluene (activity 12,000 dpm/ml) was used as the standard for the insert vial. All counting was carried out in a Packard 2425 scintillation spectrometer.

The counting procedure was as follows. The samples in suitable gel form were counted first with the insert vial present, but containing no standard, to yield the sample count. Following the addition to the insert vial of 1 ml hexa-

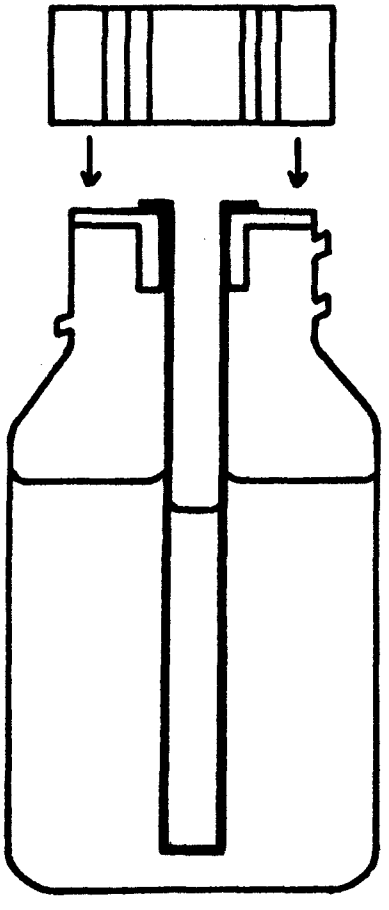

FIG. 1. Counting vial plus insert.

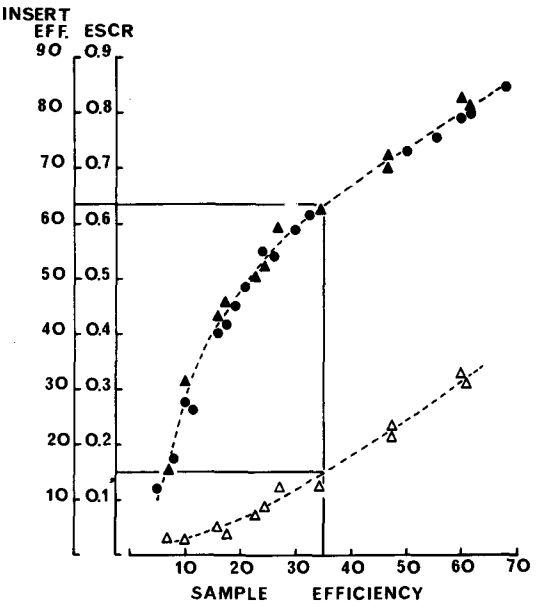

FIG. 2. Efficiency of sample count vs efficiency of insert vial standard. Sample: ^{14}C-cholesterol. ESCR = efficiency of sample count ratio. ●—● = Graded concentrations of red dye + 80-250 mg silicic acid. ▲—▲ = Graded concentrations of grass extract + 80-250 mg silicic acid. Efficiency of sample count vs external standard (^{226}Ra) channels ratio. Sample: ^{14}C-cholesterol. △—△ = Graded concentrations of grass extract + 80-250 mg silicic acid.

decane standard (the level of the standard was not allowed to exceed that of the sample), the vial was counted again to yield the sample + standard count. Simple subtraction yielded the standard insert vial count. Shaking the vial to yield a suitable gel for counting was carried out preferably with the insert vial in position. However, placement of the insert vial was possible even after the formation of the gel.

RESULTS AND DISCUSSION

A plot of insert vial standard efficiency vs sample counting efficiency is shown in Figure 2. This was obtained from a range of color quenched suspensions of silica gel (for details see Figure 2) chosen to cover sample efficiencies ranging from 70% (maximum efficiency obtainable with the system used) to less than 10%. All results obtained were found to fit the same curve, in spite of the diverse nature of the suspension mixtures used, i.e. nature of color quenching agent and sample test material; the curve for the relationship between the insert vial efficiency (Y) and sample efficiency (X) could be described by the equation:

$$Y = 0.08936X + 26.99 \log_e X - 35.87$$

and accounted for 98.8% of the total variance.

A particular advantage of the curve of the insert vial standard efficiency for the correction of sample counting becomes evident when it is compared with the curve normally derived for the external standard channels ratio technique for count correction. Although a diversity of opinion exists with regard to the validity of the external standard channels ratio method of quench correction for colored suspensions, providing that the necessary constant checks are carried out, the method continues to be used widely in view of its convenience. The curve of the external standard (^{226}Ra) channels ratio vs sample efficiency obtained for the samples containing graded concentrations of the grass extract is shown in Figure 2. It can be seen that, due to the low efficiencies with which the suspensions were counted, only small changes in the external standard channels ratio accompanied the relatively large decrease in the sample counting efficiency. Using the insert vial

standard, the same decrease in the efficiency of sample counting was reflected by relatively large changes in the efficiency of counting of the standard. The blocked-in areas on Figure 2 enable some comparison to be made of the relative merits of the two curves for count correction.

In the present series of investigations, the presence of an empty or full insert vial had no apparent effect upon sample counting efficiency. It must be remembered, however, that, although the presence of a standard in an insert vial at the center of the counting medium possesses the advantage that the standard will generate counts highly efficient, this efficiency will be entirely independent of the extent of chemical quenching in the sample medium. Therefore, care must be taken to ensure that any possible changes in the extent of chemical quenching in the sample medium are kept to a minimum.

Present investigations in this laboratory are concerned with highly colored lipid extracts, and considerable problems have been encountered with the routine counting and subsequent correction of several of several lipid classes following separation on thin layer chromatoplates and gel counting of the separated bands of silica gel. The use of the isolated internal standard, as outlined above, has enabled gel counting to be used under these conditions and the activity in these bands to be determined relatively accureately.

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Oxidative Desaturation of α-Linolenic, Linoleic, and Stearic Acids by Human Liver Microsomes

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ABSTRACT

The desaturation of stearic, linoleic, and α-linolenic acids by human liver microsomes were studied. The microsomes were isolated from liver biopsies obtained during operations. It was shown that human liver microsomes are able to desaturate 1-^{14}C-α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid; 1-^{14}C-linoleic acid to γ-linolenic acid; and 1-^{14}C-stearic acid to oleic acid in the same system described in the rat. However, the desaturation activity obtained was low compared to other mammals. This effect was attributed to fasting, premedication, or the anaesthesia.

INTRODUCTION

It is well established that stearate, linoleate, and α-linolenate are converted to oleate, γ-linolenate, and octadeca-6,9,12,15-tetraenoate, respectively, by animal liver microsomes in a reaction requiring oxygen and nicotinamide adenine dinucleotide, reduced form (NADH) or nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (1-3).

The existence of similar enzymatic systems in human liver is assumed. However, as far as we know, the microsomal desaturation activity of human liver has not been measured directly until now. Therefore, it was considered important to determine these enzymatic activities in fresh human liver.

The present paper reports that human liver biopsies provide enough material to measure microsomal fatty acid desaturation activities. From these biopsies, stearic, linoleic, and α-linolenic acid microsomal desaturation activities were determined.

MATERIALS AND METHODS

Chemicals

1-^{14}C-stearic acid (56 mCi/mmole, 99% radiochemically pure) and 1-^{14}C-linoleic acid (56.2 mCi/mmole, 99% radiochemically pure) were purchased from New England Nuclear, Boston, Mass. 1-^{14}C-α-linolenic acid (41.5 mCi/mmole, 99% radiochemically pure) was obtained from The Radiochemical Centre, Amersham, England.

Subjects

The patients in this investigation were between the age of 27-52, males and females. There was no clinical or laboratory evidence of liver diseases. Patients designated RN, YA, AS, and MR suffered from cholelithiasis and the others from tumors of the digestive system. Before their operations, the patients were premedicated with atropine and meperidine hydrochloride. The general anaesthesia consisted of thiopental sodium, halothane and methoxyfluorane or thipental sodium, halothane, and succinylcholine and was initated a few min before the operation.

Liver biopsies (3-5 g) were taken within 15 min after the abdomen had been opened and were immersed immediately in cold homogenizing medium. The liver samples were homogenized in a cold solution (3:1, v/w), consisting of 0.15 M KCl, 0.005 M $MgCl_2$, 0.004 M ethylenediaminetetraacetic acid (EDTA), 0.004 M N-acethyl-cysteine, 0.05 M phosphate buffer (pH 7), and 0.25 M sucrose. The crude homogenate was centrifuged at 20,000 x g for 10 min. The microsomal fraction was obtained by centrifugation of the 20,000 x g supernatant at 140,000 x g for 60 min in a Spinco model L2 centrifuge. The pellets were resuspended in 0.5 ml homogenizing medium. Microsomal protein (10-50 mg) was obtained from the liver biopsies.

Assay for Oxidative Desaturation of Fatty Acids

The desaturation of the fatty acids by human liver microsomes was measured by estimation of the percentage conversion of 1-^{14}C-stearic to oleic acid; 1-^{14}C-linoleic to γ-linolenic acid; and 1-^{14}C-α-linolenic to octadeca-6,9,12,15-tetraenoic acid.

Labeled acids (10-50 nmoles) were incubated with 1-5 mg microsomal protein in a Dubnoff shaker at 35 C in a total volume of 1.5 ml 0.15 M KCl, 0.25 M sucrose solution. The time period tested was 20 min. The solution contained 4 μmoles adenosine 5'-triphosphate (ATP), 0.1 μmole CoA, 1.25 μmoles NADH, 5 μmoles $MgCl_2$, 2.25 μmoles glutathione, 62.5

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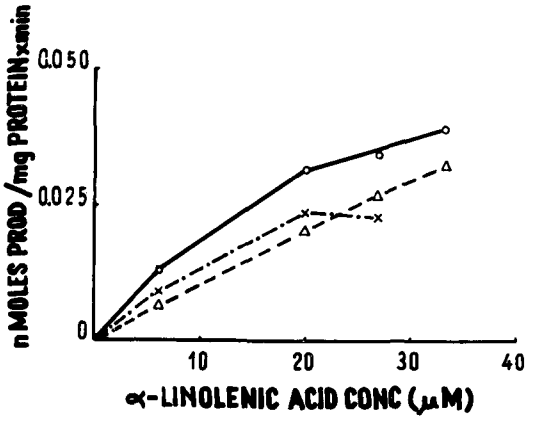

FIG. 1. Effect of the α-linolenic acid concentration upon the specific desaturation activity of human liver microsomes. Patients designated: RN (•————•), AP (x———•———x), and YA (Δ----Δ). 1, 2.5, or 5 mg microsomal protein was incubated at 35 C for 20 min under the conditions described in the text.

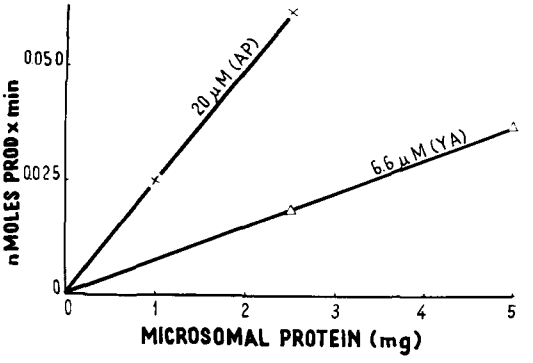

FIG. 2. Effect of the amount of microsomal protein upon the oxidative desaturation of 1-^{14}C-α-linolenic acid. Patients designated: AP (x————x), α-linolenic acid concentration, 20 μM; and patient YA (Δ——Δ), α-linolenic acid concentration, 6.6 μM. Incubation time 20 min at 35 C. Technical details described in the text.

μmoles KF, 0.5 μmole nicotinamide, and 62.5 μmoles phosphate buffer (pH 7). The reaction was stopped by addition of 2 ml 10% KOH in methanol. The fatty acids were recovered by saponification of the incubation mixture (40 min at 85 C) and extraction with petroleum ether (bp 30 C). The acids were esterified with methanolic 3 M HCl (3 hr at 68 C), and the distribution of radioactivity between substrate and product was determined by gas liquid radiochromatography in an apparatus equipped with a Packard proportional counter (4). The samples were analyzed using a column packed with 10% diethylene glycol succinate on Chromosorb W (80-100 mesh) at 180 C (3). The specific enzymatic activity expressed as μmoles product/mg microsomal protein/min was calculated from these data. The methyl esters of fatty acids were identified by equivalent chain length determination and by comparison with authentic standards. The protein content of the microsomal fraction was determined by the biuret method of Gornall, et al., (5) using crystalline bovine serum albumin as a standard.

TABLE I

Linoleic Acid Desaturation Activity in Human Liver

Subjects[a]	Substrate (μM)	Desaturation activity[b] (nmoles product/mg protein x min)
JR	6.7	0.007
	33.3	0.060
MR	20.0	0.037
	33.3	0.052

[a]Initials designate different patients.

[b]Microsomal protein (5 mg) incubated at 35 C for 20 min under the conditions described in the text.

TABLE II

Stearic Acid Desaturation Activity in Human Liver

Subjects[a]	Substrate (μM)	Desaturation activity[b] (nmoles product/mg protein x min)
AP	6.7	0.008
	20.0	0.025
AS	20.0	0.015
	33.3	0.018

[a]Initials designate different patients.

[b]Microsomal protein (5 mg) incubated at 35 C for 20 min under the conditions described in the text.

RESULTS AND DISCUSSION

The determination of the fatty acid desaturation activity in human liver microsomes is difficult because of different factors. The main problem is to obtain enough liver tissue to isolate ca. 1 mg microsomal protein. The second problem, probably as important as the first one, is to get this sample of the tissue with absolutely unaltered enzymes. Since the enzymatic desaturation system is rather unstable, this means that the sample must be obtained directly from living tissue and immediately cooled to 0-4 C. Under this condition, the desaturase activity is stable for at least 1 hr. For this reason, two procedures were tested. The first one was to obtain the sample by direct puncture of the liver. However, the amount obtained by this procedure was so small that it was impossible to isolate the microsomes with

our technique. The second procedure tested was the use of biopsies. This technique requires an operation. Therefore, it only could be used in operations where the liver was exposed and where biopsies were obtained by the surgeon. In these cases, enough tissue was obtained to separate microsomes and measure the fatty acid desaturation activity.

Figure 1 shows that the microsomes of human liver biopsies desaturate α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid. Substrate saturation curves were obtained. Desaturation enzymatic activities were calculated, since a linear relationship was found between the amount of product formed and the mg microsomes incubated (Fig. 2). The Δ6-desaturation activity for α-linolenic acid was rather low compared to other mammals (6,7).

Tables I and II show, respectively, that human liver microsomes also desaturate linoleic acid to γ-linolenic acid and stearic acid to oleic acid. However, in some patients, the linoleic acid desaturation activity was not measurable under our experimental conditions. The number of analyses was not enough to build a saturation substrate curve. The Δ6-desaturation activity for linoleic acid and Δ9-desaturation activity for stearic acid are low compared to other mammals (6,7).

Since adult subjects were analyzed, the low fatty acid desaturations may be a consequence of the age. An age effect has been found in rats (6). However, the low desaturation values also may be due to the 12 hr fasting prior to the operation, since fasting has been shown to decrease the Δ6 and Δ9-desaturation activity of rat liver microsomes (8,9). Besides, interpretation of data from most human studies is complicated by the additional influences of drugs used for premedication and induction and maintenance of anaesthesia. Ether, halothane, and barbiturates also affect enzymes involved in glucose homeostasis, and they also may alter the enzymes of lipid metabolism.

Nevertheless, the data obtained show that it is possible to measure fatty acid desaturation activity in human biopsies and that stearic, linoleic, and α-linolenic acids are desaturated by the same system described in the rat.

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Effect of Acute Dietary Alteration upon Intestinal Lipid Synthesis

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ABSTRACT

The specific activities of three enzymes engaged in complex lipid synthesis, diglyceride acyltransferase, cholinephosphotransferase, and lysolecithin acyltransferase were studied in intestinal mucosa of hamsters fed either saline, hydrolyzed casein, or corn oil for 9½ hr. In the most proximal intestine, saline feeding was associated with a reduced specific activity in villous tips with all three enzymes studied when compared with the two caloric supplemented groups. In the most distal intestine, oil feeding increased the activity of lysolecithin acyltransferase and choline phosphotransferase twofold as compared to casein fed hamsters; diglyceride acyltransferase was increased one- and one-half-fold. The response of lysolecithin acyltransferase and diglyceride acyltransferase to fat feeding was incomplete when compared to hamsters fed a fat supplemented diet for 7 days, suggesting that their pattern of response to dietary substrate was similar to the disaccharidases. By contrast, the response of cholinephosphotransferase to fat feeding was complete at 24 hr, suggesting that it responds in a manner similar to the glycolytic enzymes.

INTRODUCTION

The administration of several dietary substrates has been shown to increase the specific activity of intestinal enzymes related to the metabolism of the specific substrate administered. The response of the specific enzymes has been shown to be on the order of a day for the glycolytic enzymes (1) and 2-5 days for disaccharidases (2). As regards lipid metabolism, monoglyceride acyltransferase (acyl-CoA: monoglyceride acyltransferase) and fatty acid: CoA ligase (EC 6.2.1.3) both have been shown to increase their specific activities in intestinal microsomes of rats in response to increased dietary lipid loads (3). In those studies, the animals were fed for a period of 3 weeks, clearly longer than the intestinal mucosal cell turnover time (4). In the present experiments, hamsters were fed either corn oil, casein, or saline for 9½ hr to evaluate the effect of short term feeding upon complex lipid synthesis in the intestine. Three enzymes engaged in complex lipid synthesis were evaluated, cholinephosphotransferase (CDP choline: 1,2 diglyceride cholinephosphotransferase, EC 2.7.8.2), lysolecithin acyltransferase (acyl-CoA: 1-acyl-sn-glycerol-3-phosphorylcholine acyltransferase, EC 2.3.1.20), and diglyceride acyltransferase (acyl-CoA: 1,2-diglyceride-0-acyltransferase). The reason for choosing these enzymes for study have been previously described (5).

MATERIALS AND METHODS

Experimental design: Male golden Syrian hamsters (Eagle Laboratory Animals, Farmerburg, Ind.) weighing 100-120 g were fed Purina Lab Chow for at least a week prior to use. The animals were allowed free access to food and water until 8 a.m. on the day prior to study. At that time, food was removed from the cages and the animals fed one of three diets. Each group was fed its respective diet (corn oil, 0.15 M NaCl or hydrolyzed casein) at 11 p.m. and 4:30 a.m. in an attempt to mimic the nocturnal eating habits of hamsters. Corn oil (.25 ml) and 0.15 M NaCl (.25 ml) were given by gavage without anesthesia at each feeding. Hydrolyzed casein (0.615 g mixed with 0.6 ml water to form a paste) was delivered into the cheek pouch of the hamster using a 1 ml syringe. Observation of the hamsters showed that none of the diets administered was regurgitated. The animals were sacrificed at 8:30 a.m. by dislocation of the cervical vertebrae. The small intestine was removed, flushed with cold (2 C) 0.15 M NaCl and placed on an iced glass plate. The entire small intestine of the group fed fat had a white appearance and oil droplets could be seen oozing from the intestinal lumen of the most distal intestine. The intestine was divided into four equal segments, pylorus to cecum, and the mucosa of each segment scraped to yield a villous tip and crypt preparations, as previously described (5), from which low speed supernatant and microsomal fractions were derived. Four animals fed the same diet were sacrificed on the day of each experiment and the mucosal scrapings of the respective fractions appropriately combined. Six groups of animals were used for each dietary regimen.

Enzyme assays: Sucrase (EC 3.2.1.20), diglyceride acyltransferase, cholinephosphotrans-

ferase, and lysolecithin acyltransferase were assayed as previously described (5). Sucrose, (^{14}C) palmitoyl CoA, CDP (^{14}C) choline, and (^{14}C) oleoyl CoA were the respective substrates which were followed to end-product formation. Each assay was performed using optimal concentrations of substrates as previously defined (5). The microsomal protein concentrations and incubation times employed were calculated such that the product formed was directly proprotional to enzyme activity (5,6).

Analytical procedures: Thin layer chromatography (TLC) was performed on Silica Gel G layers ca. 500 μm thick, as previously described (5), to separate the lipid species. Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer using the solvent and scintillators described previously (5). Quenching was essentially similar in each sample (5,6).

Protein was determined by the method of Lowry, et al., (7) using dry 0.01-.04 mg crystallized albumin (Sigma Chemical Co., St. Louis, Mo.) as standards. An IBM 1130 computer was used to compare data by multivariate analysis.

Materials: These were obtained or synthesized (diglyceride, lysolecithin [1-^{14}C] oleoyl CoA) as previously described (5).

RESULTS

Sucrase: Sucrase activity was used to ensure the adequate separation of villous tips from their respective crypts. In each instance, sucrase activity in crypts was 20% or less of the activity found in their respective villous tips.

Diglyceride acyltransferase: Figure 1 relates to diglyceride acyltransferase specific activity in villous tips (upper panel) and crypts (lower panel). In villous tips, the specific activity of all three feeding groups was greatest in the most proximal intestine and decreased more distally (multivariate analysis: P = oil, .003; casein, .0019; and saline, .0202). Oil feeding resulted in greater specific activity than the saline group in the proximal three-quarter gut and was greater than the casein fed group in the distal three-quarter gut. Casein feeding increased enzyme specific activity in the most proximal gut, as compared to both other groups. Crypt alterations in activity were limited to the second gut segment where the group fed oil had greater activity than the group fed saline. Although, in the first and fourth gut segment, significant changes were noted between groups, it is not possible to state that these individual significant differences, in fact, constitute a separate population, since the multivariate analysis of the groups were insignificant.

Cholinephosphotransferase: The data relating to cholinephosphotransferase is depicted in Figure 2. The group fed saline and corn oil had similar activity in the proximal as compared to the distal intestine (multivariate analysis: P = oil, .1109; saline, .3081). However, the group fed casein showed a progressive decrement in specific activity in the distal as compared to the proximal intestine (multivariate analysis: P = .0313). Oil or casein feeding increased the specific activity of the enzyme as compared to saline-fed hamsters in the most

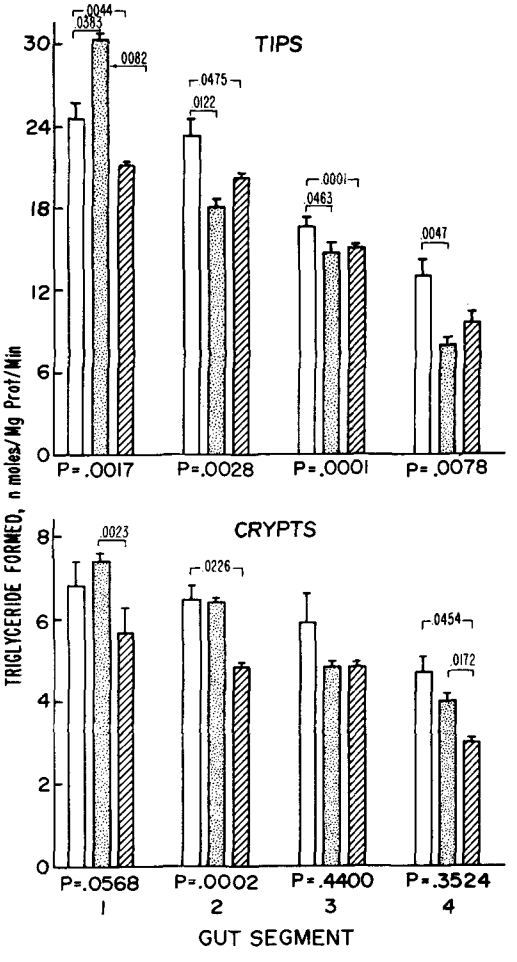

FIG. 1. Diglyceride acyltransferase in hamster intestinal microsomes. Incubation conditions are described in "Materials and Methods." The vertical lines above the bars indicate 1 standard error. The numbers above the bars indicate the univariate P values. The pairs between which significant differences were found are indicated by the brackets associated with the P values. The P values located below the ordinate of each graph indicate the multivariate analysis of each group. These multivariate P values assess the null hypothesis that enzyme activity of each group is derived from a single population. □ = Corn oil, 0.5 ml; ▨ = casein, 1.23 g; and ▩ = 0.15 M NaCl, 0.5 ml.

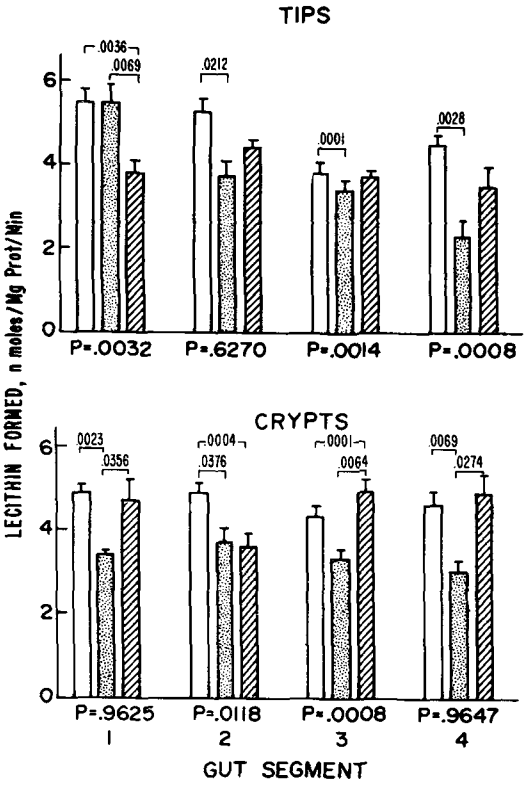

FIG. 2. Cholinephosphotransferase in hamster intestinal microsomes. Incubation conditions are described in "Materials and Methods." The vertical lines above the bars indicated 1 standard error. The numbers above the bars and the P values below the ordinate relate to the significance of the differences within groups as in Figure 1. □ = Corn oil, 0.5 ml; ▨ = casein, 1.23 g; and ▨ = 0.15 M NaCl, 0.5 ml.

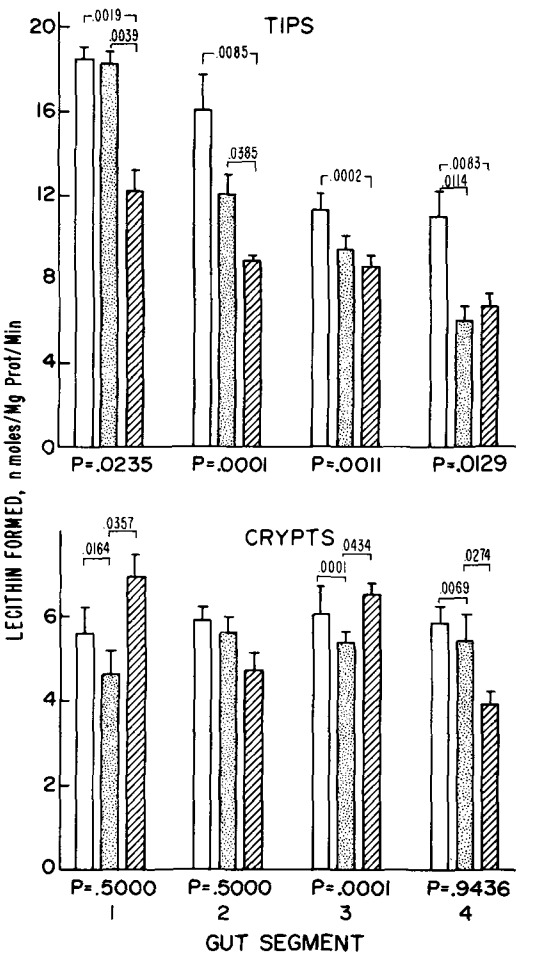

FIG. 3. Lysolecithin acyltransferase in hamster intestinal microsomes. Incubation conditions are described in "Materials and Methods." The vertical lines above the bars indicate 1 standard error. The numbers associated with the brackets above the bars and the P values below the ordinate relate to the significance of differences within groups as in Figure 1. □ = Corn oil, 0.5 ml; ▨ = casein, 1.23 g; and ▨ = 0.15 M NaCl, 0.5 ml.

proximal gut in villous tips. Oil-fed hamsters had greater specific activity than those fed casein in the distal one-half gut. Isolated changes were observed in crypts.

Lysolecithin acyltransferase: In Figure 3 are the data relating to lysolecithin acyltransferase. In villous tips, shown in the upper panel, only the group fed casein was more active in proximal as compared to the more distal intestine (multivariate analysis: P = .0002). In villous tips, oil feeding resulted in increased enzyme activity in each gut segment as compared to saline feeding and, in addition, as compared to casein feeding in the most distal segment. Casein feeding was associated with increased enzyme activity as compared to saline feeding in the proximal one-half gut. Isolated changes were observed in crypts.

DISCUSSION

Previous work has shown that there are two separate types of responses in the intestine to feeding dietary substrate. The first is that of sucrase in which it has been shown that 2-5 days of sucrose feeding were necessary to reach a peak responsiveness of the enzyme (2). The second pattern of response is demonstrated by the glycolytic enzymes in which exposure of the specific dietary substrate evokes maximum enzyme response in 24 hr and perhaps as early as 6 hr (1). It has been postulated that the maximum response of sucrase is coincident with the appearance of crypts cells on the villous tips which had been exposed 2-5 days beforehand to the specific dietary stimulus (2).

The more rapid response to the glycolytic enzymes is presumably due to other causes.

It recently has been demonstrated (8) that feeding a fat supplemented diet for 7 days increases the specific activity of lysolecithin acyltransferase fourfold, diglyceride acyltransferase threefold, and cholinephosphotransferase twofold in the distal intestine when compared to hamsters fed a chow (low fat) diet. The present experiments were designed to determine whether these three complex lipid synthesizing enzymes behaved in a manner similar to sucrase or the glycolytic enzymes as regards the time course of their response to lipid feeding. Two of the enzymes studied in the present report, lysolecithin acyltransferase and diglyceride acyltransferase, were increased two- and one and one-half-fold, respectively, in the distal intestine in oil fed as compared to casein fed hamsters. Since this response is ca. one-half that noted when the fat supplemented diet was fed for a week, it is presumed that these two enzymes respond in a manner similar to sucrase. On the other hand, cholinephosphotransferase increased two-fold in the distal intestine in the oil fed as compared to the casein fed hamsters. The increase in specific activity was similar to that found when a fat supplemented diet was fed for 7 days, suggesting that this enzyme follows a pattern of response to dietary substrate similar to the glycolytic enzymes. Furthermore, these studies provide additional information to that already presented (5,8) that cholinephosphotransferase and lysolecithin acyltransferase respond differently to physiological stimuli and might subserve different lecithin requirements of intestine (5,8).

In the present experiments, casein was chosen as the nonlipid caloric source in preference to glucose because of the previous demonstration that protein formation in villous tips is predominantly from luminal amino acids rather than endogenous sources (9). The importance of dietary protein as a major contributor to the luminal amino acid pool has been emphasized recently (10). Although it is not proven that the complex lipid synthesizing enzymes under study are synthesized in or on the villous tips, it is clear that the specific activities of two of these enzymes, diglyceride acyltransferase and lysolecithin acyltransferase, are increased significantly in the villous tips as compared to their respective crypts (5). Saline provided observations under noncaloric feeding conditions.

Saline feeding was associated with a reduced specific activity in villous tips of the most proximal intestine of all three enzymes studied in comparison with caloric supplemented animals. These results were similar to those obtained with acyl-CoA: monoglyceride acyltransferase in the jejunum of rats studied after a 24 hr fast and compared to ad libitum fed animals (11).

Although it is reported that rats do not empty their stomach of a lipid load any faster than they can absorb it (12), it was evident in hamsters that the entire intestine was involved in lipid absorption. Lipid droplets were easily demonstrable in the luminal fluid of even the most distal gut segment. Further, each gut segment had a whiter appearance than its fasted or casein fed counterpart, presumably indicating active lipid absorption. Therefore, in these animals, the most distal intestine was being perfused with this dietary substrate, 9½ hr after the first and 4 hr after the second feeding.

The statistical treatment of the data reported in these studies was by multivariate analysis combined with univariate P values. There are several instances where the univariate P values indicate a significant difference between the means of two groups of data. Nevertheless, this univariate significance is not valid when the multivariate analysis of the entire group of data, i.e. the three paired feedings, is not also significant. When more than two sets of data are being compared, a multivariate analysis of the results is the only way to ensure that individual univariate P values, in fact, represent true deviations from the group (13).

ACKNOWLEDGMENTS

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Purification and Properties of Aortic Cholesteryl Ester Hydrolase

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ABSTRACT

The enzyme(s) present in acetone-dried powder of rat and rabbit aortas, which catalyzes the synthesis and hydrolysis of cholesteryl ester, was purified partially by acid precipitation, acetone fractionation, 0-(diethylaminoethyl) cellulose chromatography, and Sephadex G-100 filtration. The synthetic activity was purified by 120-fold (rat) and 140-fold (rabbit). Purification of hydrolytic activity was 90-fold (rat) and 103-fold (rabbit). Cholesteryl ester hydrolase activity was separated from nonspecific esterase by column chromatography. Both synthetic and the hydrolytic activities are apparently the functions of one enzyme. The mol wt of the enzyme was estimated to be 140,000 dalton as determined by Sephadex G-200 gel filtration. The extracts of the acetone-dried powders of aortas of both species contained an inhibitor of synthetic activity. The inhibitor was nondialyzable and was precipitated at pH 5.7. Both activities were found to be fairly nonspecific with regard to sterol and fatty acids. With oleic acid, the relative rates of sterol ester synthesis were: cholesterol, 100; cholestanol, 94; desmosterol, 35; coprostanol, 24; ergosterol, 20; and β-sitosterol, 19. Epicholesterol was not esterified. Oleic acid was most active in cholesteryl ester synthesis, the relative rates being: oleic>linoleic>arachidonic>palmitic>stearic>butyric. The rate of hydrolysis was maximum with cholesteryl linoleate followed by oleate, linolenate, palmitate, stearate, and laurate in decreasing order.

INTRODUCTION

The presence of cholesteryl ester synthesizing and hydrolyzing enzyme activities in aortas of various species has been demonstrated amply (1-4). These activities have been shown to change under conditions of atherosclerosis: the synthesizing activities are increased greatly and the hydrolytic activities generally are decreased. However, very little is known about the nature and properties of these enzymes in aorta. Most tissues contain complex mixtures of esterase with overlapping substrate specificity. Reports of optimal conditions of these enzymes in aorta have differed between various laboratories, suggesting that different enzymes may have been tested (5-7). Purification and characterization of these enzymes, therefore, must precede any evaluation of their contribution to arterial cholesteryl ester metabolism.

Purification of cholesteryl ester hydrolase from various tissues has been described. Rat pancreatic enzyme has been purified 600-fold and to a high level of homogeneity as determined by polyacrylamide disc electrophoresis (8). Among other properties, the specificity of this enzyme also has been reported (9-12). Deykin and Goodman (13) have described a purification procedure for rat liver cholesteryl ester hydrolase by which 68-fold purification of the enzyme was achieved. Chen and Morin (14) have shown a low, but significant, activity of this enzyme in human placenta. The enzyme was purified partially and shown to have properties similar to pancreatic enzyme (14). This study reports the partial purification of and an evaluation of the specificity of cholesteryl ester hydrolase of rat and rabbit aortas.

EXPERIMENT PROCEDURES

Materials

Cholesterol and cholesteryl oleate were obtained from Sigma Chemical Co., St. Louis, Mo. Cholesterol and cholesteryl oleate (after saponification) were found to be pure by gas liquid chromatography (GLC). Cholesteryl esters of other fatty acids were obtained from Applied Science Laboratories, State College, Pa., and checked for purity by thin layer chromatography (TLC) using silicic acid impregnated by silver nitrate (5% w/w), according to the method of Morris (15). Cholestanol, stigmasterol, β-sitosterol, coprostanol, ergosterol, and epicholesterol were purchased from Steraloids, Pawling, N.Y. Desmosterol was a gift. Fatty acids (purity 99%) were purchased from The Hormel Institute, Austin, Minn., and were checked for purity by GLC. Egg lecithin was obtained from Supelco, Bellefonte, Pa., and used without further purification. [4-^{14}C] Cholesterol (specific activity, 58.5 μCi/μmole)

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and [1-^{14}C] oleic acid (specific activity, 53.5 μCi/μmole) were purchased from New England Nuclear Corp., Boston, Mass. [4-^{14}C] Cholesteryl oleate, as well as other [4-^{14}C] cholesteryl esters, was prepared and purified by the method described by Kothari, et al., (16).

Grossly normal aortas of young male rats and rabbits were used throughout the study. Aortas of rats (Wistar) and rabbits (New Zealand White) were obtained from Rockland, Gilbertsville, Pa. Animals had been killed by decapitation; and aortas dissected from the heart, immediately frozen, and shipped to our laboratory in dry ice. When received, the frozen specimens were freed from fat and connective tissues, rinsed with ice-cold saline, blotted dry and stored below -15 C until used. The acetone-dried powder of the pooled aortas was prepared as described previously (4).

Enzyme Assay

The substrate for synthetic activity was prepared as described previously (4). For routine assay, the emulsion had the following composition: 2.05 μmoles [4-^{14}C] cholesterol, 6.22 μmoles oleic acid, and 4.10 μmoles sodium taurocholate in 0.2 ml 0.1 M phosphate buffer, pH 6.1. The final volumes of the incubation mixture was made up to 1.0 ml. For one determination of sterol specificity, cholesterol in the above emulsified substrate was replaced with equimolar amounts of other sterols, and [1-^{14}C] oleic acid was used to synthesize esters labeled in the fatty acid moiety. Similarly, the emulsified substrate was modified for fatty acid specificity study, in which oleic acid was replaced with equimolar quantities of various fatty acids. In all cases, the amount of sodium taurocholate, ionic concentrations, and pH remained the same as described for the usual substrate.

The method of preparation of micellar substrate for hydrolytic acitivity was as described previous (4). The micelles contained 1.30 μmoles [4-^{14}C] cholesterol oleate, 2.58 μmoles sodium taurocholate, and 1.5 mg lecithin in phosphate buffer, pH 6.6, in a total volume of 1.0 ml. Since the critical micellar concentrations for various steryl esters with lecithin and bile salts were not known, an albumin dispersion of the ester was used as the substrate for determination of specificities and always compared with albumin dispersion of the oleate.

For determination of both activities, incubations were carried out by shaking for 1-4 hr at 37 C in a Dubnoff metabolic shaker. At the end of the incubation period, the reaction was stopped by adding 50 μliter incubation mixture to

TABLE I

Summary of Purification of Cholesteryl Ester Synthesizing and Hydrolyzing Activities from Acetone-Dried Powder of Rat and Rabbit Aortas[a]

Purification sequence	Total protein (mg)	Synthetic activity			Hydrolytic activity		
		Total units	Specific Activity	Purification	Total units	Specific activity	Purification
Rat enzyme							
Whole extract	128.0	664.0	5.2	1.0	960.0	7.5	1.0
pH 5.7 Supernatant	72.5	723.9	10.1	1.9	889.4	12.5	1.6
40% Acetone precipitate	9.8	616.2	63.0	12.1	699.4	71.4	9.5
DEAE-cellulose chromatography[b]	2.6	478.5	191.3	36.0	548.7	219.3	29.0
Sephadex G-100 chromatography	0.66	411.0	632.2	120.0	475.5	731.1	98.6
Rabbit enzyme							
Whole extract	140.5	478.1	3.4	1.0	434.0	3.1	1.0
pH 5.7 Supernatant	86.2	559.9	6.5	1.6	391.2	4.6	1.5
40% Acetone precipitate	8.5	461.5	59.8	17.6	317.8	38.5	12.4
DEAE-cellulose chromatography	2.8	401.5	146.8	52.4	285.7	106.6	41.4
Sephadex G-100 chromatography	0.74	361.4	487.2	143.2	240.8	324.8	104.7

[a]The procedure is described under "Experimental Procedures."
[b]DEAE = 0-(diethylaminoethyl).

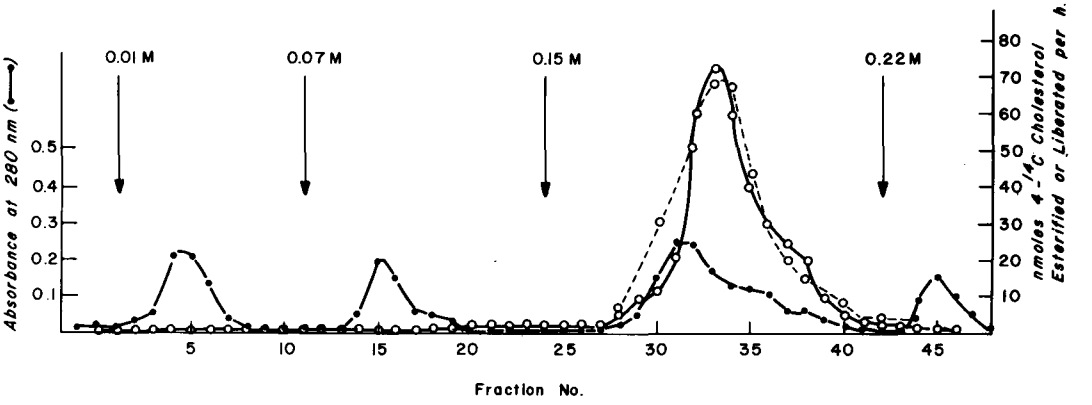

FIG. 1. 0-(diethylaminoethyl) (DEAE)-cellulose column chromatography of the 40% acetone precipitates from the whole extract of acetone powder of rat aorta. The column of DEAE-cellulose was equilibrated with 0.001 M phosphate buffer, pH 6.6 Acetone precipitates (40%) dissolved in water were applied. The column was eluted discontinuously with 0.01, 0.07, 0.15, and 0.22 M phosphate buffers, pH 6.6. Fractions of 5 ml were collected at a flow rate of 30 ml/hr. The elution of the protein was followed by measuring absorbance at 280 nm, and the elution of the enzymes was determined by the usual assay procedure. •——• = Protein E_{280}, o——o = synthetic activity, and o- - -o = hydrolytic activity.

50 μliter acetone-ethanol (1:1, v/v). This mixture was shaken and placed in a boiling water bath. Analyses were carried out on this extract. Free and ester cholesterol were separated by TLC and were estimated quantitatively in terms of radioactivity as described previously (4).

Unit of Activity

One unit represents the amount of enzyme catalyzing the esterification of, or liberation by hydrolysis of, 1 nmole free cholesterol/hr.

Assay of Nonspecific Esterase 3.1.1-6

The nonspecific esterase activity was measured by the rate of hydrolysis of 0-nitrophenyl acetate as described by Bowers, et al. (17).

Assay of Lipase

Lipase activity was assayed by following the hydrolysis of triolein as described by Mahadevan and Tappel (18).

Determination of Protein

For most of the studies, protein was determined according to the method of Lowry, et al. (19). During ion exchange and gel filtration chromatography, the protein peaks were located by absorbance at 280 nm using a Zeiss @ II spectrophotometer.

Enzyme Purification

A typical procedure for the enzyme purification consisted of five steps and was carried out as follows.

Step I–extraction: The soluble proteins were extracted from acetone-dried powder with 0.1 M phosphate buffer, pH 7.4, containing 0.05 M NH_4Cl. Usually 3-4 g powder was used from which 200-250 mg protein was extracted.

Step II–acid precipitation: The pH of the extract was lowered to 5.7 with 1 M NaH_2PO_4 solution. Stirring was continued for 5 min followed by centrifugation at 12,000 x g for 20 min. The resulting supernatant was dialyzed for 2 hr against 50 volumes of 0.005 M phosphate buffer, pH 7.4.

Step III–acetone fractionation: To the dialyzed supernatant from step II, ice cold acetone was added drop by drop until 40% concentration was reached. The precipitate was allowed to develop for 30 min and then sedimented at 14,000 x g for 10 min. The supernatant was discarded, and the precipitate was dissolved in 5 ml distilled water (pH 7.0). Occasionally, an insoluble precipitate was formed, which was removed by centrifugation at 10,000 x g for 10 min.

Step IV–0-(Diethylaminoethyl) (DEAE) cellulose chromatography: The enzyme preparation from step III was purified further by column chromatography as follows: DEAE-cellulose (cellex-D, standard capacity, Bio-Rad Laboratories, Richmond, Calif.) was washed 3 times with 0.001 M NaCl solution. It then was equilibrated for 24 hr in the cold (2-5 C) with 25 times its volume of the same solution. The suspension of the absorbant was poured gently into a column (1.5 x 50 cm) fitted at the bottom with a coarse fritted disk. After some of the suspension had settled by gravity, pressure was applied gradually to a maximum of 2 lb/sq in. from a nitrogen gas tank and was maintained until the column ht of 16 cm was achieved. The enzyme preparation from step III

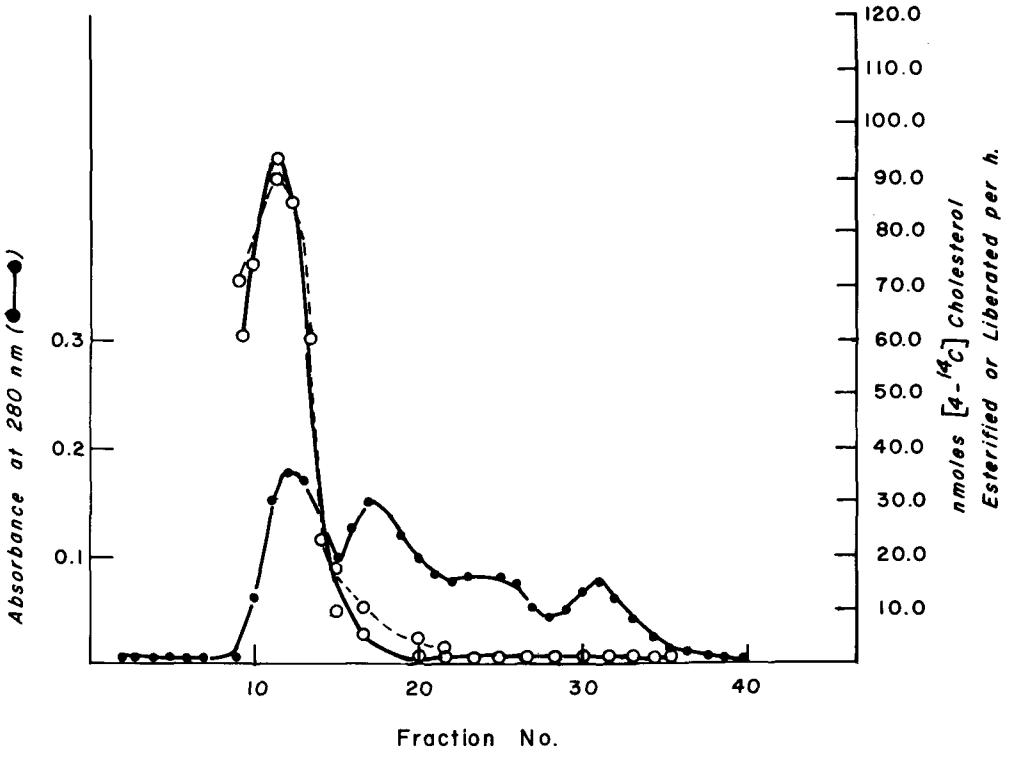

FIG. 2. Sephadex G-100 chromatography of the active fractions from 0-(diethylaminoethyl)-cellulose chromatography. The proteins were filtered with 0.05 M NH_4Cl solution at a flow rate of 14 ml/hr, and 5.0 ml fractions were collected. Details of the column and its operation are given under "Experimental Procedures." The protein content of the effluent was determined by absorbance at 280 nm, and the enzyme activities were located by assay of the fractions. ●—● = Protein E_{280}, o—o = synthetic activity, o- - -o = hydrolytic activity.

was applied to this column. The protein was eluted with a linear gradient of NaCl solution generated from 0.005 M and 0.25 M NaCl solutions (pH 7.4). Fractions of 5 ml each were collected at a flow rate of 30 ml/hr. The fractions within the peak of activity were pooled, dialyzed against double distilled water, and concentrated by lyophilization to 5 ml.

Step V–gel filtration with Sephadex G-100: The concentrated enzyme preparation from DEAE-cellulose column was applied to the Sephadex G-100 column (1.5 x 40 cm), and the elution was carried out with 0.05 NH_4Cl (pH 7.4) solution at a flow rate of 25 ml/hr. Five ml fractions were collected by an automatic fraction collector. The enzyme preparation obtained from the pooled fraction was concentrated by dehydration by polyethylene glycol treatment.

Estimation of Mol Wt

Mol wt of the partially purified enzyme protein was estimated by the method of Andrews (20). Briefly, Sephadex G-200 (particle size 40-120 μ) was allowed to swell in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.025 H NH_4Cl. The deaerated suspension was packed under gravity in a column (2.4 x 50 cm). The partially purified (specific activity, 600), dialyzed and concentrated enzyme preparation, containing 40 units of the enzyme, was mixed with a number of proteins of known mol wt and applied to the gel as a solution in 2 ml same buffer. The elution rate was 14 ml/hr, and 2.5 ml fractions were collected at 4 C. Blue dextran 2000 (1 mg) was used to indicate the void volume, and the following proteins were used as standard: aldolase, 1 mg (mol wt 140,000-150,000); bovine serum albumin, 4 mg (mol wt 65,000-70,000); and Cytochrome C, 0.8 mg (horse heart, mol wt 12,400). The position of the elution peaks of cholesteryl ester hydrolase, blue dextran, and standard proteins was determined either by direct absorption at 280 nm (bovine serum albumin), 412 nm (Cytochrome C), and 625 nm (blue dextran) or the appropriate enzyme assay.

RESULTS AND DISCUSSION

The results of a typical purification sequence

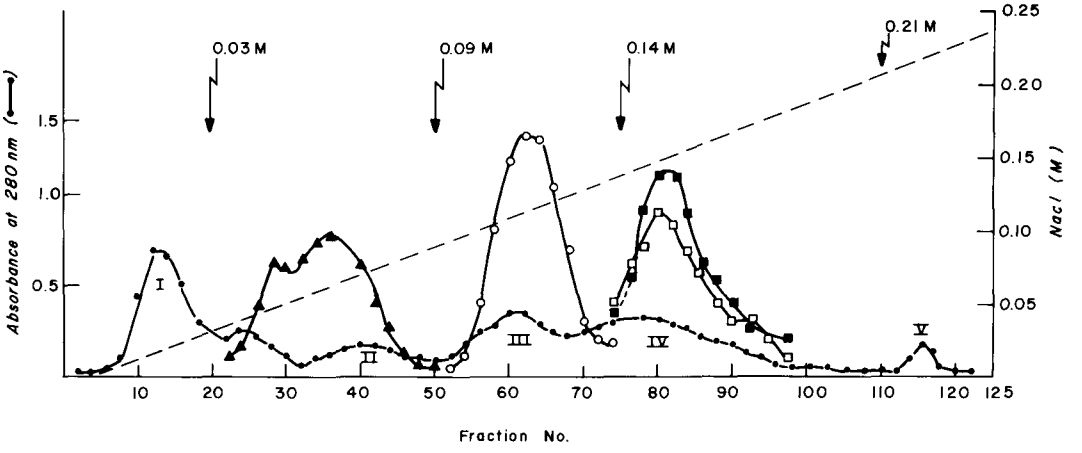

FIG. 3. 0-(diethylaminoethyl) (DEAE)-cellulose column chromatography of the whole extract from acetone-dried powder of rabbit aorta. The DEAE-cellulose column was equilibrated with 0.001 M NaCl solution. The whole extract (56.0 mg protein) was applied. The column was eluted with a linear gradient (0.005-0.250 M) of NaCl. Fractions, each containing 5 ml were collected at a flow rate of 30 ml/hr. The elution of the protein was followed by measuring absorbance at 280 nm, and the elution of the enzymes was determined by the usual assay procedure. Lipase and nonspecific esterase were determined as described under "Experimental Procedures." Cholesteryl ester synthesizing and hydrolyzing activities were eluted with peak IV, and the recovery was 89%. ●—● = Protein E_{280}, ▲—▲ = nonspecific esterase, ○—○ = lipase, ■—■ = cholesteryl ester synthesizing activity, and □—□ = cholesteryl ester hydrolyzing activity.

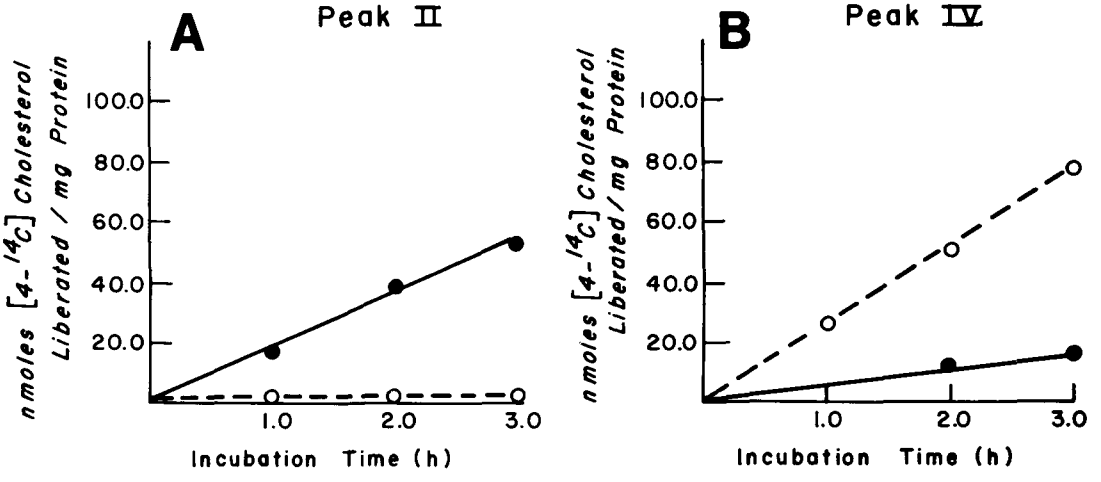

FIG. 4. The hydrolysis of [4-^{14}C] cholesteryl oleate by the enzymes eluted under peaks II and IV of Figure 3. The active fractions under peak II and Peak IV were pooled separately, dialyzed, and concentrated to 5 ml. The rate of hydrolysis was determined with the substrates having albumin dispersed [4-^{14}C] cholesteryl oleate at pH 7.4 and micellar [4-^{14}C] cholesteryl oleate, pH 6.6. Enzyme equivalent to 1.0 mg protein was used for each determination. Incubations were at 37 C for 1, 2, and 3 hr. ●—● = Albumin dispersed substrate, pH 7.4 and ○- - -○ = micelles, pH 6.6.

are given in Table I. The specific activities of the final product were between 100 and 140 times higher than that of the original material. DEAE-cellulose chromatography of the partially purified enzyme preparation (40% acetone precipitate) gave results as shown in Figure 1. As it may be seen, the synthesizing and hydrolyzing activities were eluted together with 0.15 M buffer. Similarly, both activities were eluted by gel filtration on Sephadex G-100 as shown in Figure 2. The evidence strongly suggests that the cholesteryl ester synthesizing and hydrolyzing activities are associated with the same protein.

DEAE-cellulose column chromatography of the whole extracts gave elution profiles of pro-

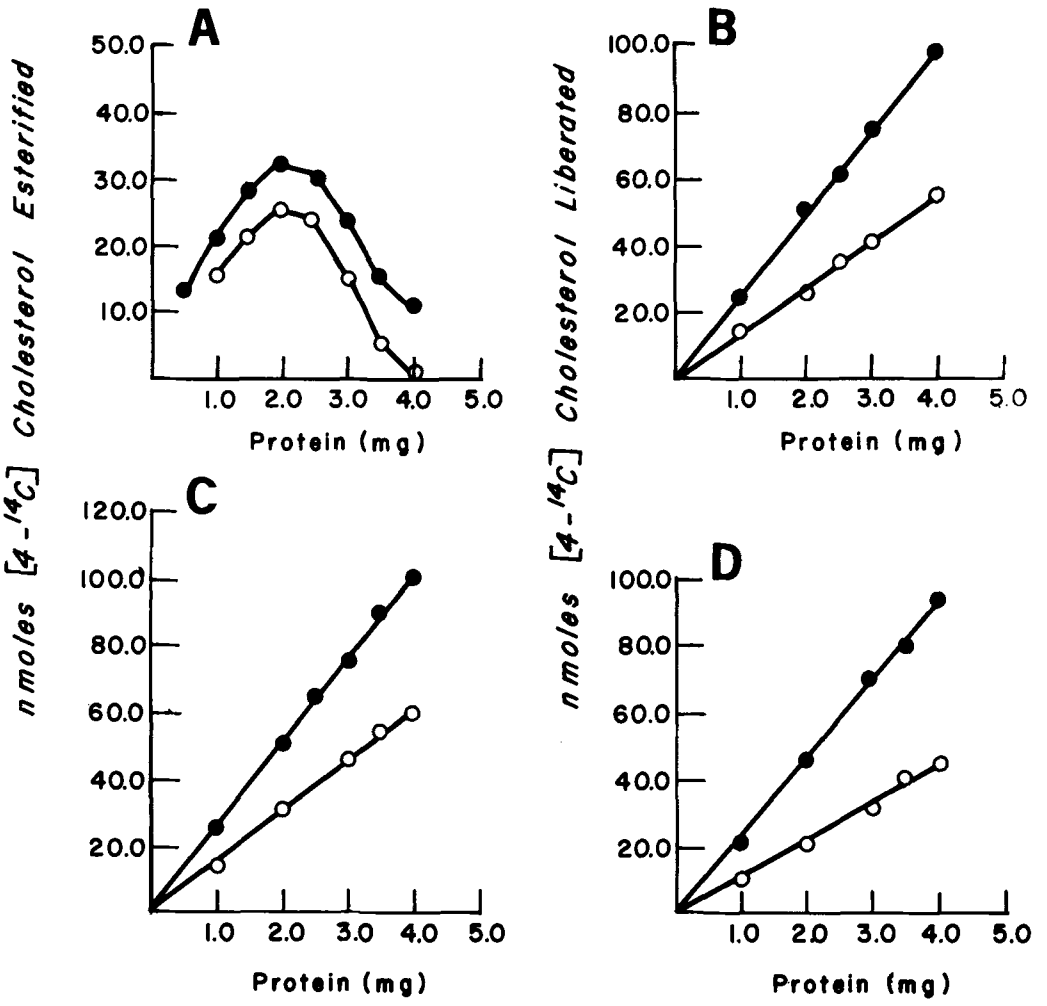

FIG. 5. The effect of protein concentration of the whole extract upon the synthesizing and hydrolyzing activities of rat and rabbit aortas. Incubations were carried out with the usual emulsified substrate (synthesis) and micellar substrate (hydrolysis). Incubations were at 37 C for 4 hr. A and B The whole extract equivalent to 1-5 mg protein was used for the assay. C and D Supernatant, pH 5.7, was supplemented with bovine serum albumin equal to the amount of protein precipitated on the adjustment of the pH. Aliquots of the preparation containing 1-5 mg protein were used for the assay. ●——● = Rat and ○——○ = rabbit.

tein and the enzymes as shown in Figure 3. Protein was eluted as 5 major peaks with a recovery of 80-90%. Cholesteryl ester synthesizing and hydrolyzing activities were eluted simultaneously at NaCl concentrations between 0.14-0.175 M. Under this peak IV, ca. 15% of the total protein was eluted with 80-85% recovery of the enzyme activities. In these experiments, lipase was eluted at NaCl concentrations between 0.09-0.14 M (peak III), and nonspecific esterase was eluted between 0.3-0.08 M NaCl concentrations (peak II). The protein of the peak II was found to catalyze the hydrolysis of 0-nitrophenyl acetate and albumin-dispersed cholesteryl oleate at pH 7.4. However, it was inactive toward the micellar cholesteryl oleate at pH 6.6. On the other hand, the protein of peak IV was most active in hydrolyzing cholesteryl oleate at pH 6.6 and did not hydrolyze 0-nitrophenyl acetate. A comparison of hydrolysis of material eluted in peaks II and IV is given in Figure 4. The results of these experiments suggest that the enzyme acting on micellar substrate is distinctly different from the enzyme hydrolyzing albumin dispersed substrate. The enzyme eluted under peak II seems to be nonspecific and hydrolyzes cholesteryl oleate. The requirements for optimum activity of this enzyme are comparable to the assay condition for aortic cholesteryl ester hydrolase re-

TABLE II

Specific Activities of Whole Aorta Extract Treated in Various Ways[a]

Treatment	Specific activity			
	Rat		Rabbit	
	Synthetic	Hydrolytic	Synthetic	Hydrolytic
None	5.2	7.5	3.4	3.1
Dialysis (20 hr)	5.5	6.8	3.8	3.0
Heating (50 C, 10 min)	0.5	1.1	0.0	0.5
Sephadex G-25 filtration	5.9	7.7	4.2	3.9
pH adjustment (5.7)	10.1	12.5	6.6	4.6

[a]The enzyme activities were determined with the usual substrates. The whole extract was treated as described in the text. Incubations were at 37 C for 4 hr. Enzyme preparation equivalent to 2.0 mg protein was used in each case. The values represent specific activity which is defined as the nmoles [4-^{14}C] cholesterol esterified or liberated/mg protein/hr.

TABLE III

Rate of Esterification of [4-^{14}C] Cholesterol (nmoles/mg protein/hr) with Various Fatty Acids by Rat and Rabbit Aorta Enzyme[a]

Fatty acid	Rate	
	Rat	Rabbit
Oleic (18:1)	49.0	37.8
Linoleic (18:2)	41.5	29.5
Linolenic (18:3)	27.5	24.7
Arachidonic (20:4)	22.6	19.0
Palmitic (16:0)	19.6	13.6
Stearic (18:0)	13.8	7.6
Butyric (4:0)	0.0	0.0

[a]The substrates were prepared as described in the text. All assay mixtures contained 2.05 μmoles [4-^{14}C] cholesterol, 6.22 μmoles free fatty acids, 4.10 μmoles sodium taurocholate, and 0.05 M phosphate buffer, pH 6.1. Partially purified enzyme (1 mg protein) was used for all determinations. Incubations were at 37 C, and the amount of esterified [4-^{14}C] cholesterol was determined initially and at intervals of 1, 2, and 3 hr. The values given are the mean of duplicate experiments. Taking the esterification with oleic acid as 100%, the relative percent values for other acids were calculated.

ported by other laboratories (1,2).

Inhibition of Synthetic Activity

It was observed that assay with increasing amounts of crude enzyme preparation resulted in increasing inhibition of the synthetic activity; at a protein concentration of 4.0 mg, 100% (rabbit) and 66% (rat) inhibition was observed (Fig. 5A). No such inhibition of hydrolytic activity was observed at higher protein concentration (Fig. 5B). Subsequently, in efforts to remove the inhibitory effect, the crude extract was dialyzed against 100 volumes distilled water for 12 hr, changing water every 4 hr, or heat treated (60 C for 10 min), or filtered through Sephadex G-25. None of the above treatments increased specific activity for synthesis. The results (Table II) suggested that the inhibitory effect was not due to the presence of a small molecular substance or the association of the enzyme with other macromolecules. However, when the crude extract was acidified (adjusted to pH 5.7) and the resultant precipitate removed by centrifugation, the supernatant was found to have higher specific activity for synthesis (Fig. 5C). It was observed further that, if the precipitate was allowed to develop for 30 min, the inhibitor was removed and the assay with the supernatant was linear with protein concentration (Fig. 5D). The precipitate (as a solution in 0.05 M NaCl), on addition to the assay with the supernatant, restored the inhibitory effect. In the case of rat enzyme, with 1.0 mg supernatant protein, the precipitate equivalent to 1, 2, and 3 mg protein produced 12, 43, and 64% inhibition, respectively. With rabbit enzyme preparation, it was 33, 78, and 100%, respectively.

The modification of an enzyme activity by a macromolecule rather than by a small molecule represents a somewhat unique method of regulation. It seems rather strange that the inhibitor did not affect the hydrolytic activity, since both synthetic and hydrolytic activities are possibly the functions of the same enzyme. To account for this, two possibilities are suggested. The enzyme has two active sites, one for each function; the inhibitor affects only the synthetic site and not the other. Alternatively and more likely, the inhibitor is an effector for the substrate, rather than that of the enzyme. In the synthetic reaction, the inhibitor may bind the free fatty acid and make it unavailable for the enzyme action.

Estimation of Mol Wt of Enzyme

A gel filtration pattern of the partially purified enzyme protein along with a protein of known mol wt was carried out. The estimate of

the mol wt of the other protein came very close to their known mol wt. The mol wt of the purified protein estimated from the elution volume in one gel filtration was ca. 140,000. This was a good approximation, since the protein was eluted very close to aldolase which is known to have a mol wt of 144,000. The estimated mol wt of pancreas enzyme is 65,000 as reported by Hyun, et al. (9). The pancreatic enzyme is shown to form a dimer (mol wt 135,000) on treatment with acetone. Thus, the estimated mol wt of aorta enzyme extracted from acetone-dried powder is close to that reported for the dimer of pancreatic cholesteryl ester hydrolyase. In the present study, no attempts were made to identify the monomeric and dimeric forms of the enzyme.

Specificity of Enzymes

Esterification of [4-^{14}C] cholesterol with oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), palmitic acid (16:0), stearic acid (18:0), and butyric acid (4:0) was measured (Table III). As may be seen in Table III, oleic acid was most effective in esterification of cholesterol; with increasing unsaturation, progressively less esterification was observed. Arachidonic acid was found to be half as effective as oleic acid. The saturated fatty acids were even less effective. Palmitic acid was ca. 40% as effective as oleic acid; stearic acid esterified poorly, and butyric acid was not esterified at all. The results of esterification of a number of sterols with [1-^{14}C] oleic acid are given in Table IV. It is evident that cholesterol and cholestanol are esterified equally well with oleic acid. Epicholesterol, with the 3(OH) in the α-position, is practically not esterified at all. Other sterols, differing from cholesterol in the ring structure or, with modified side chain, esterified to an extent 25-35% that of cholesterol.

The configuration of the A/B ring juncture is of great importance, the 5βH sterol being esterified to only 21-26% of the extent of the 5αH sterol. Side chain substitution or additional unsaturation reduces esterification by 65-81%.

The substrate specificity of hydrolyzing activity was studied by comparing the rate of hydrolysis of cholesteryl esters of various fatty acids. Since the critical micellar concentrations of most of the cholesteryl esters with sodium taurocholate and lecithin were not known, albumin dispersed substrates (pH 6.6) were prepared and used as described under "Experimental Procedures." Also, since radioactive esters of cholesterol with various fatty acids were not commercially available, unlabeled esters were used, and the free and the ester cholesterol were determined chemically by the method of Sperry and Webb (21). Even though the assay with albumin-dispersed cholesteryl oleate did not give maximum activity, the comparison of the rate of hydrolysis of various esters under these conditions may be consid-

TABLE IV

Rate of Esterification of Various Sterols with [1-^{14}C] Oleic Acid by Rat and Rabbit Aorta Enzyme[a]

Sterol	Sterol esterified (nmoles/mg protein/hr) Rat	Rabbit
Cholesterol	47.7	34.5
Epicholesterol	2.2	0.0
Cholestanol	44.5	36.0
Corpostanol	11.8	7.6
Desmosterol	16.5	12.0
β-Sitosterol	9.6	8.0
Ergosterol	10.0	10.5

[a]The substrates were prepared as described in the text. Assay mixtures contained 2.05 μmoles sterol, 6.22 μmoles [1-^{14}C] oleic acid, 4.10 μmoles sodium taurocholate, and 0.05 M phosphate buffer, pH 6.1. Partially purified enzyme (40% acetone precipitate) was used for all determinations. Incubations were carried out at 37 C, and the amount of steryl-[1-^{14}C] oleate was determined initially and at intervals of 1, 2, and 3 hr. The values given are the mean of duplicate experiments. Taking the esterification of cholesterol as 100%, the relative percent values for other sterols were calculated.

TABLE V

Rate of Hydrolysis of Various Cholesteryl Esters by Rat and Rabbit Aorta Enzymes[a]

Cholesteryl ester	Cholesterol liberated (nmoles/mg protein/hr) Rat	Rabbit
Oleate	16.2	13.0
Linoleate	20.7	14.4
Linolenate	14.0	9.6
Palmitate	13.5	9.5
Stearate	12.5	8.8
Laurate	10.0	8.0
Acetate	12.2	10.2

[a]Albumin-dispersed substrates were prepared as described in "Experimental Procedures." The assay mixture contained 1.30 μmoles cholesteryl ester, 2.58 μmoles sodium taurocholate, 0.5 mg bovine serum albumin, and 0.075 M phosphate buffer, pH 6.6. Partially purified enzyme (40% acetone precipitate) was used for all determinations. Incubations were carried out at 37 C, and the activity was measured by determining free and esterified cholesterol at 2, 4, and 6 hr of incubation. The values expressed are the mean of duplicate experiments. Taking the hydrolysis of cholesteryl ester as 100%, the relative percent of hydrolysis for other esters were calculated.

ered valid. The results are given in Table V. As shown in the table, the rate of hydrolysis was highest with linoleate, followed by oleate, linolenate, palmitate, acetate, stearate, and laurate.

The enzyme shows little specificity for either (with regard to the sterol or fatty acid). All six of the β(OH) sterols tested in this study were able to serve as substrates for the synthetic activity. Similarly, although maximum esterification was observed with oleic acid and cholesteryl linoleate was hydrolyzed to the greatest extent, the exzyme seems to be fairly nonspecific, functions with several long chain fatty acids for synthesis, and can hydrolyze cholesteryl esters differing in fatty acid moiety. This is not surprising; since the interconversions of cholesterol and cholesteryl esters are involved mainly in the process of absorption and transport of cholesterol and perhaps fatty acids, it may be advantageous for the organ to be able to form the esters from whichever fatty acid happens to be present in excess and hydrolyze whichever ester is available.

ACKNOWLEDGMENTS

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Effect of Temperature upon Linolenic Acid Level in Wheat and Rye Seedlings

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ABSTRACT

The fatty acid composition of leaves of nine wheat and one rye species was studied in relation to temperature both under laboratory and field conditions. Seedlings exposed to cold (2 C) either in laboratory or on field had higher levels of linolenic acid in their lipids than their greenhouse (22 C) germinated controls. The increase of the level of linolenic acid was accompanied by a decrease in the level of linoleic acid in field grown species. A relationship seems to exist between sensitivity to cold and accumulation of linolenic acid; those species resistant to cold displayed greater increase in their linolenic acids than those more sensitive to chilling temperatures. This response in cold resistant species was quite rapid, two days of cold exposure resulted in a significant increase of linolenic acid. The possible mechanisms responsible for the observed changes are discussed.

INTRODUCTION

It repeatedly has been observed that different organisms, ranging from bacteria to vertebrates, adjust the composition of their fats to the environmental temperature (1-9). Ability to control the unsaturation of fats may be important to tolerate some effects of low temperatures. It can be hypothesized that species unable to increase the unsaturation of their fats are handicapped when the environment becomes cold. This is suggested also by the observations of De La Roche, et al., (10) and Thomson, et al. (11) showing that cold hardiness may be connected with increased production of linolenic acid in *Secale cereale var. Sangeste* and *Triticum aestivum L. var. Rideau,* respectively. On the other hand, Lyons, et al., (12) demonstrated high amounts of linolenic acids in mitochondrial lipids of chilling resistant plants and the presence of a break in the slope of Arrhenius plots of some mitochondrial enzyme activities of certain chilling sensitive plants. In this paper, an attempt is made to find correlation between the linolenic acid production and cold hardiness in members of genus Triticum and some of their related species. Members of this genus were selected for this investigation not only because these plants exhibit a wide range of cold adaptiveness (from extreme resistance to extreme sensitivity) but also because of their great nutritional importance.

MATERIALS AND METHODS

The investigations were carried out on species listed in Table I. The sowing on the experimental field took place on October 10, 1973. The temperature decreased to 14-16 C below zero in early November, and the seedlings, having 2-3 leaves at that time, were exposed to that cold without snow cover for ca. 10-12 days. The temperature increased subsequently to ca. 0 C and remained nearly constant for another 3 months. The plant samples

TABLE I

List of Investigated Species[a]

Genus	Species	Varietas	Convarietas	Ploid level
Secale	cereale	-	Kisvárdai	Diploid
Triticum	monococcum	-	-	Diploid
Triticum	timopheevi	typica	-	Tetraploid
Triticum	turgidum ssp. carthlicum	-	-	Tetraploid
Triticum	turgidum ssp. dicoccum	-	-	Tetraploid
Triticum	turgidum	gentile	-	Tetraploid
Triticum	turgidum	buccale	-	Tetraploid
Triticum	aestivum	-	Kavkaz	Hexaploid
Triticum	aestivum ssp. spelta	album	-	Hexaploid
Triticale	-	-	No. 364	Hexaploid

[a]Seed samples of Secale and Triticum species were obtained from Institute of Genetics, Biological Research Center, Szeged, Hungary. Seed of Triticale were obtained from A. Kiss of Vegetable Crops Research Institute, Kecskemét, Hungary.

were taken for fatty acid analyses in late January or early February. The controls were germinated in a greenhouse at 22 C in early January, 1974. Usually 50 seeds were placed into filter paper, wet with 5 ml water, in Petri dishes of 20 cm in diameter. When the second or third leaves appeared, the seedlings were sampled for the determination of their fatty acid compositions. In some other investigations, the seeds were germinated in a dark room illuminated by a 100 W bulb (11,000 lux).

After the appearance of the third leaves, the seedlings were divided into two groups: one group was placed into a refrigerator set to +2 ± 1 C and illuminated by a 100 W bulb for 2 days, while the other remained at room temperature.

The leaves were cut into small pieces and homogenized in an all glass Potter homogenizer, and the fats were extracted and purified according to Folch, et al., (13). The total fats were transmethylated in sealed ampoules under CO_2 in the presence of 5% hydrochloric acid in absolute methanol at 80 C. Methyl esters were extracted quantitatively with hexane and weighed.

The gas chromatographic analyses were performed on a JGC-1100 instrument equipped with flame ionization detector. The column (6 ft, stainless steel) was packed with 15% ethylene glycol succinate on 100-120 mesh Gas Chrom P (Applied Science Laboratories, State College, Pa.). Identification of peaks was accomplished using standard mixtures of Applied Science Laboratories no. K-108 and branched chain mix-L. Quantitation was performed by triangulation technique. The accuracy of determinations was better than 2.5% in the case of major fatty acids and ca. 20% in the case of minor components.

RESULTS

Fatty Acid Compositions of Leaf Lipids of Wheat Seedlings

Linolenic, linoleic, and palmitic acids, in that order, were the major fatty acids in all species investigated. Stearic, oleic, and palmitoleic acids were only minor components. Fatty acids appearing before palmitic acids were not included in the calculations.

Linolenic, linoleic, and palmitic acids were the major fatty acids also in winter wheat seedlings (*T. aestivum*) and in rye seedings (*S. cereale*), investigated by De La Roche, et al., (10) and Thomson, et al., (11), respectively.

Effect of Temperature upon the Fatty Acid Compositions of Seedlings

Table II compares the fatty acid compositions of species grown either in a greenhouse (22 C) or on the experimental field. The fats of species germinated and grown on the experimental field were, in the most cases, considerably richer in linolenic acid than those grown in a greenhouse. On the other hand, there was a substantial decrease in the level of palmitic, oleic and linoleic acids. Palmitoleic and stearic acids displayed a less consistent behavior.

The increase in the level of linolenic acid, as compared to the greenhouse controls, varied between 10-90%. The highest values were observed in species which are most resistant to cold *(S. cereale, T. aestivum ssp. spelta, T. dicoccum, T. timopheevi)*, while those more sensitive to chilling temperatures *(Triticale, T. turgidum var. gentile)* gave a less pronounced response.

Effect of Cold upon Fatty Acid Compositions of Seedlings under Laboratory Conditions

The seedlings on the experimental field were exposed to cold for a long time before the analyses were carried out. To determine whether the accumulation of unsaturated fatty acids takes place within an even shorter period of time, warm germinated seedlings were exposed to a temperature of +2 ± 1 C for only 2 days. Cold resistant (*T. aestivum ssp. spelta* and *S. cereale*) and cold sensitive (*T. turgidum var. gentile* and *T. turgidum ssp. carthlicum)* species were selected for this investigation to detect possible differences in the response.

From Table III, it is evident that the effect of exposure to cold on the fatty acid compositions of cold resistant species is apparent already after two days.

These species were capable of increasing the level of linolenic acid and, in addition, of oleic and linoleic acid in their fats by ca. 12-15% during this time. On the other hand, changes in the fatty acid compositions of cold sensitive species were only negligible.

DISCUSSION

This paper reports the ability of a number of wheat species to increase the level of octadecapolyenoic acids in their leaf lipids in response to decreasing temperature. Similar results were obtained on *Cynidum caldarium* (14), alfalfa roots (15,16), *S. cereale* (11) and *T. aestivum* (10,17). Moreover, we have shown that the response of the seedlings to cold is quite rapid; 2 days of exposure to +2 C of warm germinated

species were sufficient to cause a considerable increase in the level of these fatty acids. The magnitude of the response seems to be correlated with the ability of plants to withstand low temperatures. Species which are known to survive extreme low temperatures (*T. aestivum sp. spelta, S. cereale, T. dicoccum, T. timopheevi*) had the highest increase in their linolenic acid levels, compared to greenhouse grown controls, while those more sensitive to cold (*T. turgidum var. gentile, T. turgidum ssp. carthlicum*) gave less pronounced response to the decreasing temperature, both under field and laboratory conditions. As there seems to be no correlation between linolenic acid level of the greenhouse grown species and sensitivity to cold, as it would be expected on the basis of investigations of Lyons, et al., (12), it may be inferred that differences in the ability to accumulate linolenic acid in response to cold may be an important factor in determining cold hardiness of these plants.

The exact mechanism by which the seedlings increase the proportion of linolenic acid in their lipids is not clear as yet. We did not separate the total lipids extracted from the plants into different lipid classes. Published data show that monogalactosyl diglycerides (MGDG) and phosphatidyl cholines (PC) are usually rich in linolenic acid, while digalactosyl diglycerides (DGDG) and phosphatidyl ethanolamines (PE) not (14,16). Consequently, an increase in the proportion of the former compounds could have evoked the observed changes in cold exposed plants. This, however, does not seem to be probable in the light of investigations of Kuiper (16) and De La Roche and Andrews (17). These authors have demonstrated that, not only the amount of the MGDG and PC, but also of PE and DGDG have increased in cold exposed alfalfa and Rideau wheat, respectively. It also has been demonstrated that the seedlings of *T. aestivum*, grown either at 2 C or at 24 C, have identical phospholipid compositions. The lipid content of the seedlings investigated in this study was not determined, and, therefore, we do not know what the actual concentration of the different fatty acids was. On the basis of the literature (10), however, a net accumulation of linolenic acid is expected.

Linolenic acid generally is considered to be formed by stepwise desaturation of stearic acid (18-20). Another possibility which attributes its formation from dodecatrienoic acid by chain elongation (21,22) must also, however, be considered. In this case, the plants produce dodecanoic acid first then desaturate it to dodecatrienoic acid. This system does not synthesize oleic or linoleic acids.

TABLE II

Fatty Acid Composition (wt %) of Wheat and Rye Seedlings Exposed to Cold in Their Natural Habitats[a]

	Secale cereale		*T. timopheevi*		*T. turgidum ssp. dicoccum*		*T. aestivum ssp. spelta*		*T. aestivum conv. Kavkaz*		*T. monococcum*		*Triticale*		*T. turgidum v. buccale*		*T. turgidum v. gentile*	
Fatty acid	Green-house	Field	Green-house	Field	Green-house	Field	Green-house	Field	Green-house	Field	Green-house	Field	Green-house	Field	Green-house	Field	Green-house	Field
16:0	17.7	13.3	18.3	11.1	22.6	12.0	14.5	12.5	18.9	14.0	21.0	12.7	19.7	13.5	25.4	11.6	15.9	10.0
16:1	3.2	2.7	1.4	2.5	1.0	2.3	2.4	0.4	1.9	0.4	0.5	2.4	2.0	2.9	2.5	0.3	2.9	0.7
X_0	-	-	-	-	-	-	-	1.7	-	-	-	-	-	-	-	1.5	-	1.3
X_1	3.6	-	11.4	1.1	-	0.6	8.5	0.5	3.7	1.4	-	0.6	-	0.6	0.9	9.8	1.7	7.1
18:0	2.1	1.6	1.5	2.5	1.6	2.5	2.4	0.7	2.5	1.7	3.6	3.6	2.0	2.5	2.0	7.0	2.4	19.5
18:1	5.8	1.6	3.9	2.5	5.7	2.3	5.2	1.6	6.2	1.7	5.0	3.0	7.3	2.7	6.5	1.4	6.4	5.8
X_2	4.4	-	4.5	-	-	-	2.7	3.5	7.6	3.7	-	-	-	-	-	5.6	4.9	4.9
18:2	23.7	8.9	18.5	8.1	26.5	7.2	25.3	15.1	24.5	13.6	27.6	7.6	21.1	8.7	26.3	11.4	29.4	8.4
18:3	41.1	72.0	42.1	72.0	43.2	73.5	38.5	63.0	37.6	63.3	45.3	71.0	47.5	69.1	36.3	51.3	38.4	42.2
Percent increase in linolenic acid	77.6		73.4		70.0		63.6		68.2		56.7		45.5		41.3		9.8	

[a]The temperature in the greenhouse was 22 C. Species germinated on the experimental field were exposed to -14 and -16 C for 10-12 days and subsequently to 0 ± 2 C for 3 months. Carbon numbers: $X_0 = 16.2$, $X_1 = 17.1$, and $X_2 = 18.8$.

TABLE III

Effect of Cold Exposure on the Fatty Acid Compositions (wt %) of Wheat and Rye Seedlings[a]

	T. aestivum ssp. spelta		*Secale cereale*		*T. turgidum v. gentile*		*T. turgidum ssp. carthlicum*	
Fatty acid	+22 C	+2±1 C	+22 C	+2±1 C	+22 C	+2±1 C	+22 C	+2±1 C
16:0	13.4	16.7	23.5	14.0	14.2	18.9	17.7	18.1
16:1	3.4	2.6	1.8	2.3	6.2	2.7	3.3	1.9
X_1	13.7	1.2	0.2	0.6	4.3	2.5	-	-
18:0	2.9	2.8	0.8	3.1	3.9	2.6	2.6	1.8
18:1	5.3	7.1	6.3	7.2	6.6	6.1	4.6	5.7
18:2	20.2	23.5	26.5	27.2	21.9	24.1	23.6	25.0
18:3	40.9	46.0	40.6	45.3	42.7	42.9	46.0	47.0

[a]The seedlings, germinated and kept at 22 C until the appearance of the second or third leaves, were exposed to 2±1 C for 2 days.

The observation that the increase in the level of linolenic acid in all species exceeded the decrease of the level of linoleic acid (Table II) suggests that a considerable proportion of linoleic acid might have been produced by this latter pathway. The fact that this has not been observed with the species exposed to cold only for a few days (Table III) can be explained by assuming that the contribution of these two pathways in determining linolenic acid level in the plants may be different in different stages of cold acclimatization. In early phase of cold acclimatization, the formation of linolenic acid from stearic acid can keep pace with increased demands for its production. In latter phase of cold acclimatization, the dehydrogenation of lauric acid, instead of stearic acid, may become more important in cold resistant species. The accumulation of stearic acid in cold sensitive species (*T. turgidum var. buccale, T. turgidum var. gentile*) upon prolonged cold exposure (Table II) is interpreted, so that even desaturation of this fatty acid was impaired in cold.

The enzyme, desaturase, requires, among other cofactors, the presence of oxygen (18,19,23). It has been suggested that the higher level of linolenic acid observed at lower temperatures is to be attributed to increased amounts of dissolved oxygen at a fixed desaturase activity (23). In the active photosynthetic tissue, however, oxygen cannot be expected to be rate limiting for the desaturase reaction, as it is produced continuously in the course of the photosynthesis. In our investigations, we found different linolenic acid levels in different seedlings under comparable illumination and temperature conditions. On the basis of the above considerations, we believe that differences in the level or activity of desaturases could have been responsible for the observed increase of linolenic acid in the cold exposed seedlings.

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Erucic Acid Metabolism by Rat Heart Preparations

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ABSTRACT

Rat heart preparations metabolized erucic acid at much slower rates than palmitic acid. This applied for activation reaction, for the conversion of acyl-CoA to acylcarnitine, and for the utilization of acyl group for oxidation. As compared to palmityl-CoA, erucyl-CoA exhibited a lower affinity for carnitine palmityltransferase (EC 2.3.1.23), the respective apparent Michaelis constants were 43 and 83 μM. Presence of erucyl-CoA or erucylcarnitine slowed the mitochondrial oxidation of palmityl groups apparently because of the slower oxidation of erucyl groups. However, presence of erucate did not inhibit the activation of palmitate. Heart mitochondria obtained from rats fed rapeseed oil (50 cal %) or corn oil diet for 3 days showed similar abilities for the coupled oxidation of various substrates and similar carnitine palmityltransferase activities. Thus, a suggestion of gross mitochondrial malfunction following rapeseed oil consumption was not confirmed.

INTRODUCTION

Erucic acid (*cis*-13-docosenoic acid) is a constituent of rapeseed oil, an edible oil used for production of margarine, shortenings, and salad oils (1). Use of rapeseed oil as a dietary component is of concern, because a high intake of this fat produces myocardial abnormalities in various animal species investigated (2). Feeding rapeseed oil to rats soon causes an accumulation of cardiac lipid, mostly as triglycerides, and necrotic lesions appear in the hearts after a prolonged feeding period (3). The significant factor involved in the pathogenicity of rapeseed oil appears to be the erucic acid (4). We have studied the myocardial metabolism of erucate to elucidate the mechanisms of its pathophysiological effects at the biochemical level. Some reports on the oxidation of erucic acid in heart mitochondria have appeared recently (5,6).

MATERIALS AND METHODS

Reagents

Palmitic acid and palmityl-CoA were obtained from the Hormel Institute, Austin, Minn., and P-L Biochemicals, Milwaukee, Wisc., respectively. Erucic acid, erucic anhydride, and erucylchloride were purchased from Nu-Chek-Prep, Elysian, Minn. [1-^{14}C]Palmitic acid was purchased from Amersham/Searle, Arlington Heights, Ill.; L-carnitine hydrochloride was from General Biochemicals, Chargrin Falls, Ohio; DL-carnitine (carboxyl-^{14}C) hydrochloride was from ICN Pharmaceutical, Cleveland, Ohio, and other biochemicals were from Sigma, St. Louis, Mo., or P-L Biochemicals. L-Palmitylcarnitine was synthesized as previously described (7).

Synthesis of Erucyl-S-CoA and L-Erucylcarnitine

Erucyl-S-CoA was prepared according to Stadtman (8), except that erucic anhydride was dissolved in tetrahydrofuran and the reaction was allowed to proceed at 50 C, since, at 0 C, no detectable synthesis occurred. Purification of the erucyl-CoA (precipitated by acidification of the reaction mixture to pH 1 with HCl) involved removal of tetrahydrofuran by evaporation and washing four times with ether/petroleum ether (1:1). Spectral analysis of the product (in water, pH 6) indicated the ratio of molar absorptivity at 232 nm (thiolester bond) to that at 260 nm (adenine ring) to be 0.53 which is characteristic of acyl-CoA esters. L-erucylcarnitine was synthesized from erucylchloride and L-carnitine hydrochloride based upon the procedure of Al-Arif and Blecher (9), except that the erucylcarnitine was extracted with butanol after the ether washings; it was 74% pure as determined by ester group analysis (10).

Animal and Diets

Eight week old male Wistar rats, obtained from Bio Breeding Laboratories of Canada, Ltd., were housed individually in metal cages with water and diets available ad libitum. Two groups of 4 rats were fed diets containing 50 cal % rapeseed oil or corn oil for a period of 3 days. The diets contained, as a percentage by wt, 30% casein, 35% cornstarch, 1% vitamin mixture, 4% salt mixture (11), 5% alphacel, and 25% of rapeseed or corn oil. The rapeseed oil contained 34.3% erucic acid, and it was a product of Cooperative Vegetable Oil Ltd., Altona, Canada. Corn oil and other dietary ingredients, including salt and vitamin mixtures, were obtained from Nutritional Biochemicals,

TABLE I

Activation of Erucic and Palmitic Acids by Rat Heart Homogenate[a]

Palmitate (mM)	Erucate (mM)	Hydroxamate formed (nmoles/min per mg protein)
1 to 6	---	17-19
---	0.15	1.2
---	0.20	1.9
---	0.25	1.7
---	0.50	1.0
2.0	0.15	20.3
2.0	0.20	20.7
2.0	0.25	21.0

[a]Activation of fatty acids was followed by hydroxamate formation (16). Reaction mixtures in a final volume of 500 μliter contained: 50 μmoles Tris-HCl (pH 7.4), 250 μmoles NH_2OH, 2 μmoles $MgCl_2$, 7.5 μmoles ATP, 0.24 μmole CoA, 2.5 μmoles dithiothreitol, and fatty acids as shown. Reaction was initiated by the addition of 180 μg protein from rat heart homogenate. Incubations were for 60 min at 37 C. Hydroxamate color was extracted with 0.5 ml of 1:10 diluted (in ethanol) Hill reagent (16).

Cleveland, Ohio. The vitamin mixture provided (in mg/100 g diet): vitamin A concentrate (200 units/mg), 4.5; vitamin D concentrate (400 units/mg), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine hydrochloride, 1.0; thiamine hydrochloride, 1.0; calcium pantothenate, 3.0; biotin, 0.020; folic acid, 0.090; and vitamin B-12, 0.00135.

Other Methods

Homogenate of rat heart was prepared in 0.25 M sucrose using a Polytron homogenizer. Methods for the isolation of mitochondria from heart with Nagarse and protein estimation were as previously described (7). Details of enzyme assay systems and conditions for mitochondrial oxidations are given in the legends to figures and tables. For carnitine palmityltransferase assay, we ascertained that when identical enzyme incubation conditions were employed, the spectrophotometric method (12) gave the same results as those obtained by the radioactive procedure (13).

RESULTS AND DISCUSSION

The amount of myocardial free fatty acids increases considerably in rats given dietary rapeseed oil (14). Administration of ^{14}C-erucic acid has shown accumulation of erucate in the free fatty acid fraction of rat liver (15). These observations suggested that erucic acid accumulation might be a consequence of the limiting ability for the activation of erucate, and the results of our experiments support this possibility. Thus, under optimal conditions (determined separately), homogenates of rat heart activated erucate at only 10% of the rate of palmitate activation (Table I). Because of the much slower activation of erucate, it was of interest to determine if its presence would inhibit the activation of other long chain fatty acids. This is to be expected if the same enzyme is involved in the activation of different long chain fatty acids. In line with this, our previous experiments had shown that the presence of slowly activated phytanate inhibited the activation of palmitate (17). However, no evidence for an inhibition of palmitate activation by erucate could be obtained; instead, under the conditions of hydroxamate assay (Table I), the presence of erucate and palmitate together showed an additive effect upon hydroxamate formation that was seen consistently in several different experiments.

The concentrations of erucate employed in the experiment of Table I were much less than those of palmitate, because excess of erucate itself was inhibitory for activation. Using radioactive assay (18), it was possible to examine the effect of erucate upon ^{14}C-palmitate activation

TABLE II

Effect of Erucate upon Activation of Palmitate[a]

Experiment	[1-^{14}C] Palmitate (μM)	Erucate (μM)	Palmityl-CoA formed (pmoles)
I	3	---	27
	3	3	27
	3	10	27
	3	50	18
II	1	---	21
	1	5	20
	1	20	17

[a]Activation was measured by the radioactive assay procedure (18) in a final volume of 400 μliter. Reactions were started by the addition of 2 μg protein from rat heart homogenate and terminated by adding 250 μliter 0.5 M H_2SO_4 after 5 min incubation at 37 C.

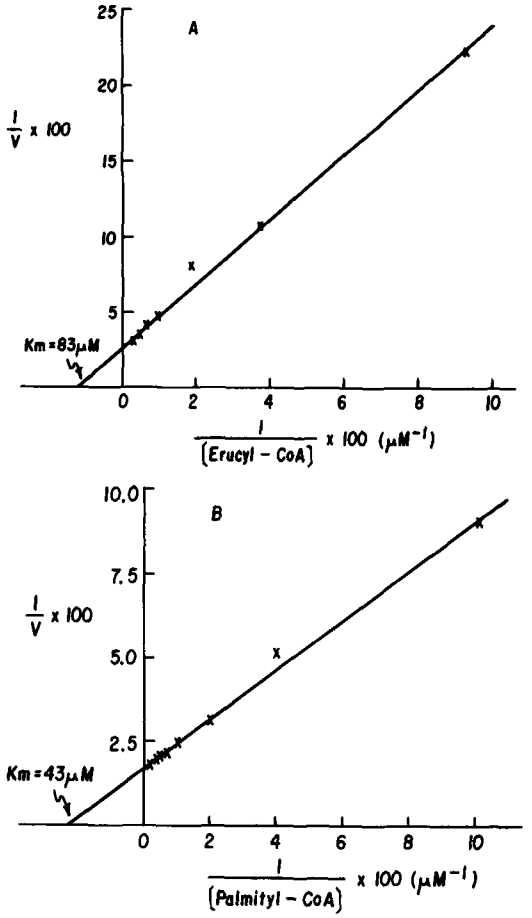

FIG. 1. Lineweaver-Burk plots of carnitine palmityltransferase activities against concentrations of (A) erucyl-CoA and (B) palmityl-CoA. The assay was according to Bieber, et al., (12) in a final volume of 200 μliter with 10 μg protein from rat heart mitochondria. L(−)-carnitine (final concentration 1.1 mM) was used to initiate the reaction at 28 C; control cuvets were without carnitine. V is expressed as nmoles of CoA formed/min per mg protein.

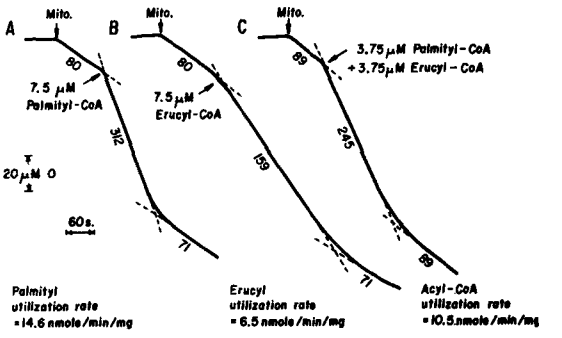

FIG. 2. Oxidation of palmityl-CoA and erucyl-CoA by rat heart mitochondria (mito.). Oxygen uptake was followed polarographically with a Clark oxygen electrode (7). To 1.7 ml medium (0.23 M mannitol, 70 mM sucrose, 20 mM Tris-HCl, 20 μM EDTA, 5 mM Pi, 2 mM L-carnitine, 5 mM ADP, 1 mM malate, pH 7.2) saturated with air at 28 C was added freshly isolated mitochondria (0.5 mg of protein) from rat heart. Other additions were made as shown. Numerals below the tracings refer to the rate of oxygen consumption in natoms/min/mg protein.

at below saturating concentrations of palmitate. Table II shows that even under these conditions a three- to fivefold molar excess of erucate did not affect palmityl-CoA formation. Some inhibition was evident at higher levels of erucic acid, but this might have been due to the known (19) unspecific inhibition of fatty acyl-CoA synthetase (EC 6.2.1.3) by an excess of long chain unsaturated fatty acids.

Following activation, utilization of fatty acids for oxidation requires conversion of fatty acyl-CoA to the acylcarnitine ester. Therefore, the ability of erucyl CoA to serve as a substrate for mitochondrial carnitine palmityltransferase (EC 2.3.1.23) was examined, and, for comparison, similar experiments were carried out with palmityl-CoA as well. Results of kinetic experiments (Fig. 1) showed that the apparent Km values with erucyl-CoA and palmityl-CoA were 83 μM and 43 μM, respectively; and the corresponding Vmax values as nmoles of CoA formed/min/mg protein were 38 and 59. Thus, compared to palmityl-CoA, erucyl-CoA was in every way a poorer substrate for the carnitine palmityltransferase of rat heart mitochondria.

Figure 2 shows that, while palmityl-CoA, in the presence of carnitine, increased the oxygen consumption rate of heart mitochondria by 290% (from 80 to 312 natoms oxygen/min/mg protein, Fig. 2, curve A), erucyl-CoA increased the oxygen consumption rate by only 100% (Fig. 2, curve B) and a mixture of both acyl-CoA esters increased oxygen uptake to a rate between those of the individual CoA esters (curve C, Fig. 2). Likewise, the utilization rate of equimolar acyl-CoA mixture was an average of the rates seen with individual CoA esters. Thus, slower oxidation of erucyl-CoA diminished the concurrent oxidation of palmityl groups, perhaps by competing for the same enzyme system. Qualitatively, our results with acyl-CoA esters are similar to those reported recently by Christophersen and Bremer (6) and Swarttouw (5), except that, under the conditions of our experiments, the stimulation of oxygen consumption rate by erucyl-CoA approached one-third of that seen with palmityl-CoA. In the experiments of Christophersen and Bremer (6), the oxygen uptake rate stimulated by erucylcarnitine was only 10% of that with palmitylcarnitine. In our experiments, carnitine

TABLE III

Effect of Dietary Erucic Acid upon Oxidative Metabolism of Rat Heart Mitochondria[a]

Substrate	Diet		Probability[b]
	Rapeseed oil	Corn oil	
	Rate of O_2 uptake[c] in the presence of ADP and malate		
Pyruvate (8 μmoles)	416 ± 30[d]	437 ± 9	NS
Glutamate (10 μmoles)	306 ± 28	358 ± 5	NS
Palmityl-CoA + carnitine	147 ± 4	162 ± 17	NS
Erucyl-CoA + carnitine	66 ± 5	71 ± 8	NS
	Carnitine palmityltransferase activity[e]		
Palmityl-CoA	49 ± 1	49 ± 1	NS
Erucyl-CoA	25 ± 1	24 ± 0	NS

[a]Rats were fed diets containing 50 cal % rapeseed oil (34.3% erucic acid content) or corn oil for 3 days. Each group had four rats.

[b]Calculated according to Student's t-test. NS = not significant ($P > 0.05$).

[c]Measured as described previously (7) and in legend to Fig. 2 (in natoms/min per mg protein).

[d]Mean ± standard error of the mean.

[e]Assayed by the radioactive method (13) at 37 C; activity expressed as nmoles of ^{14}C-acylcarnitine formed/min/mg protein.

esters of erucate and palmitate gave results indistinguishable from those seen with corresponding esters of CoA plus carnitine, and these results were not modified by the substitution of adenosine diphosphate (ADP) by 2,4-dinitrophenol (data not shown).

Houtsmuller, et al., (14) reported that rapeseed oil feeding to rats resulted in marked impairment of the ability to oxidize various substrates. Because the oxidation rates of normal mitochondria obtained by these investigators were extremely low as compared to what we observe in our experiments, the dietary experiments have been repeated to determine if the reported mitochondrial malfunction is, indeed, a true pathogenic effect of dietary erucic acid. Groups of 4 rats were fed a diet of 50 cal % rapeseed oil or a control diet of corn oil and sacrificed 3 days later when cardiac lipidosis, induced by rapeseed oil diet, is known to reach peak intensity (2,14). Hearts isolated from the rapeseed oil-fed rats were visibly pale, unlike those from the control group. A comparison of the mitochondrial oxidation rates with various substrates and carnitine palmityltransferase activities (Table III) showed no significant differences between the rapeseed and corn oil-fed rats. Respiratory control and $ADP/_O$ ratios with pyruvate or glutamate in these two groups of animals were also alike (data not shown). Thus, the suggestion (14) of a marked functional damage in heart mitochondria due to uptake of erucic acid is not supported.

From our results, it appears that activation may constitute the rate-limiting reaction in the overall metabolism of erucate in heart. Inasmuch as the presence of erucyl-CoA slowed the utilization of palmityl-CoA for oxidation, it is likely that the accumulation of activated fatty acids due to slower oxidation promotes triglyceride synthesis as suggested by Christophersen and Bremer (6). We consider it probable that triglyceride deposition in heart, following rapeseed oil consumption, results, not only from increased synthesis, but also from simultaneous inhibition of lipolysis due to the accumulation of free erucic acid. It is established that high concentrations of free fatty acids inhibit hormone-sensitive lipase in adipose tissue (20) and recent evidences suggest that such regulatory mechanisms also are present in heart (21). As is known for adipose tissue, cyclic AMP activates lipolysis in heart as well (21), and it is of interest in this connection that ingestion of rapeseed oil decreases adenyl cyclase activity in heart (22). Although a short term feeding of rapeseed oil (3 days at 50 cal %) did not alter mitochondrial function to any noticeable degree, it is possible that excess free erucic acid eventually plays a role in the development of myocardial lesions, seen in rats sustained on rapeseed oil diets, because elevated free fatty acid concentrations have been suggested (23) to be involved in myocardial necrosis.

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Identification and Quantitation of Cholanoic Acids in Hepatic and Extra-Hepatic Tissues of Rat[1]

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ABSTRACT

Tissues of rats were examined for the presence of cholanoic acids. Quantitation of extraction, deconjugation, and isolation were verified by use of radioactive standards. Identification was made by thin layer and gas liquid chromatographic comparison to standards and mass spectrometry. All tissues examined were found to contain several conjugated cholanoic acids. Liver contained primarily cholic acid and peripheral tissues primarily dihydroxy compounds, mainly hyodeoxycholic acid.

INTRODUCTION

Preliminary evidence for the presence of cholanoic acids in tissues other than liver has been presented (1). Identification and quantitation of cholanoic acids in tissues of the rat are described herein.

MATERIALS AND METHODS

Materials: All reagents used were of analytical grade. Cholesterol 1, 2-^{3}H(50 mCi/mMole) and 4-^{14}C(55 mCi/mMole) were purchased from New England Nuclear Corporation, Boston, Mass. Lithocholic-24-^{14}C (LC, 3α-monohydroxy-5β-cholanoic, 5.37 mCi/mMole); 24-^{14}C-cholic(C, 3α, 7α, 12α-trihydroxy-5β-cholanoic, 4.22 mCi/mMole); and 24-^{14}C-taurocholic sodium salt (TC, 4.11 mCi/mMole) were obtained from Mallinkrodt, St. Louis, Mo. Nonradioactive bile acid standards were obtained from Supelco, Bellefonte, Pa., as were materials for gas liquid chromatography (GLC).

Precoated silicic acid thin layer chromatography (TLC) plates were purchased from Analabs, North Haven, Conn. The plates were cleaned by running in redistilled benzene and activated by heating at 105 C for 30 min. The solvent system used was benzene:isopropanol: acetic acid (30:10:1) (2). To visualize TLC bands, the plates were sprayed with 10% phosphomolybdic acid in ethanol. Radioactivity was determined using a liquid scintillation solution (T-Cocktail) consisting of 4 g 2, 5 diphenyloxazol (PPO) and 50 mg 4-di-2(5-phenyloxazolyl)-benzene (POPOP) in 1 liter toluene.

Radiopurity of all standards and the ^{3}H-cholesterol used for rat injection was confirmed by TLC. The counting instrument was a Nuclear-Chicago Unilux II liquid scintillation system on line with an Olivetti Programma 101 computer.

Animals: Four month old female rats (CFE Strain from Carworth, Portage, Mich.) were injected intraperitoneally (IP) with 15.0 μCi of 1, 2-^{3}H cholesterol. The animals were killed by decapitation 5-10 days after the injection, and tissue samples (liver, kidney, muscle, adipose, etc.) were excised and stored at -20 C until they were analyzed. It was considered that tissue cholesterol and cholanoic acids would have incorporated a sufficient portion of the administered dose in that time to be detectable by the

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TABLE I

Recovery of Purified Standards Added to Muscle or Liver Prior to Homogenization by Extraction with Different Methods[a]

Methods: Tested compounds	HCL-EtOH extract[b] (%)	Alcoholic KOH[c] (%)	Folch[d] (%)
^{14}C-Taurocholic	82.1	12.2	5.5
^{14}C-Cholesterol	92.0	89.9	89.3
^{14}C-Cholic acid	91.6	81.0	79.4
^{14}C-Lithocholic		88.8	

[a] Means of six samples

[b] Ref. 5.

[c] Ref. 3

[d] Ref. 4.

TABLE II

Recovery after Hydrolysis of ^{14}C from ^{14}C-Carbonyl-Labeled Taurocholate and ^{14}C-Cholic Acid Added to Liver and Muscle

	^{14}C-taurocholic	^{14}C-cholic
	dpm	
In total extract	7345	4580
In non-saponifiable	18	56
In acidic extract	6710	4005
On TLC[a] band of taurocholic acid	630	
Percent deconjugation	91.35	

[a]TLC = thin layer chromatogram.

methods to be described.

Extraction: Recovery of bile acids and cholesterol by three extraction methods was checked. To each of triplicate samples of 2 g liver, skeletal muscle, and adipose, a known amount of ^{14}C-lithocholic, -cholic, -taurocholic acid, or -cholesterol was added prior to homogenization and carried through the whole extraction process. The procedures tested were an alcoholic KOH digestion modified from Abell, et al., (3), the Folch chloroform-methanol procedure (4), and the acidic ethanol method of Manes and Schneider (5).

Deconjugation: A conjugated standard, ^{14}C-taurocholate (carbonyl labeled) was added to 2 g liver, muscle, and adipose tissues before extraction. Radioactivity was counted from an aliquot of the extract prior to and after hydrolysis with 20% KOH in ethylene glycol at 220 C (5). Verification of complete deconjugation was noted by the appearance and disappearance of cholanoic acid bands on TLC plates and by recovery of ^{14}C-cholic acid from the acidic extract.

Isolation: Since the acidic lipids consist of free fatty acids and cholanoic acids (fatty acids are in ca. 10^3 greater concentration than cholanoic acids), it is particularly important to separate cholanoic acids from the mixture for subsequent GLC and mass spectrometric analysis. Among several known methods, TLC was the choice for separation because of its simplicity and effectiveness. Free cholanoic acids were applied on TLC plates, followed by elution with chloroform-methanol and reisolation with repeated TLC to separate cholanoic acids from the mixture of acidic lipids.

Derivatization: The cholanoic acids prepared by TLC were methylated (6), and trifluoracetates of methyl esters were prepared (7). Since mild conditions yield partial derivatives (enol esters of 3 Keto-Δ^4bile acid) as side products, the amount of reagent, time, and temperature were slightly elevated from Sjövall's procedures for this study. Trifluoroacetates were analyzed within a few days after preparation, because signs of decomposition appeared on storage for more than 48 hr at room temperature.

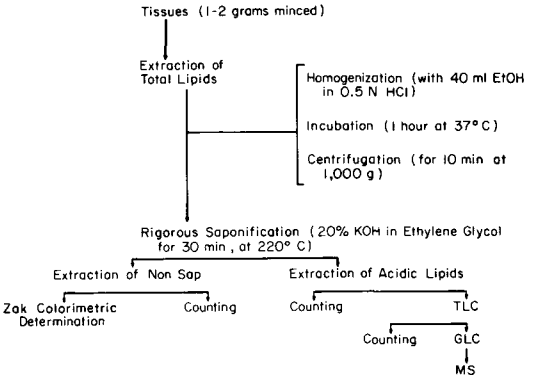

FIG. 1. Methodologic procedures for tissue cholanoic acid analysis. TLC = thin layer chromatogram, GLC = gas liquid chromatogram, and MS = mass spectrometry.

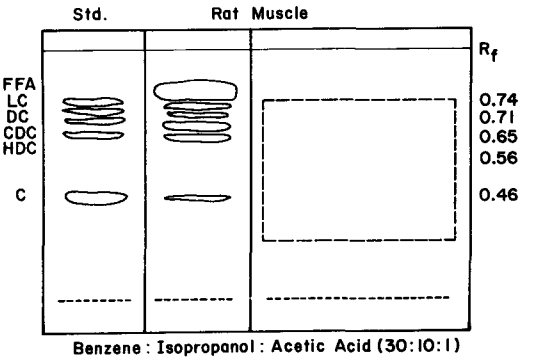

FIG. 2. Diagram of Silica Gel G thin layer chromatographic plate with left column spotted with a mixture of standard cholic (C), hyodeoxycholic (HDC), chenodeoxycholic (CDC), deoxycholic (DC), and lithocholic (LC) acids. The remainder of the plate was spotted with rat muscle extract and the left half of the plate sprayed with 10% phosphomolybic acid in ethanol. The unsprayed portion of the plate, surrounded by a dotted line, was scraped and eluted with methanol. FFA = free fatty acids and Std = standard.

TABLE III

Proportion of Individual Cholanoic Acids in Tissues as Determined by Gas Liquid Chromatography (GLC) and by Isotope Counting of Thin Layer Chromatogram (TLC) Bands of ^{3}H-Cholanoic Acids Derived from 1, 2-^{3}H-Cholesterol in Vivo[a]

Tissue	Method	Cholanoic acids					Trihydroxy acid	Unidentified
		Monohydroxy acid	Dihydroxy acids					
			Deoxy	Chenodeoxy	Hyodeoxy			
Liver	GLC	-	6.25	13.93	8.20		68.89	7.73
	TLC-^{3}H	-		(24.2)[b]	15.20		53.50	7.14
Skeletal muscle	GLC	-	4.60			(68.0)	23.20	4.20
	TLC-^{3}H	-		(38.30)	36.40		25.50	-
Adipose	GLC	-	3.80			(68.20)	16.60	11.40
	TLC-^{3}H	-		(42.50)	32.80		10.60	10.00
Kidney	GLC	3.7	17.1			(70.00)	-	8.50
	TLC-^{3}H	6.5		(33.30)	19.20		35.0	5.80

[a]Values are relative percent.

[b]Values in parentheses between two dihydroxy bile acids represent the summed quantitation of two acids which were not distinctly separated on the TLC plate.

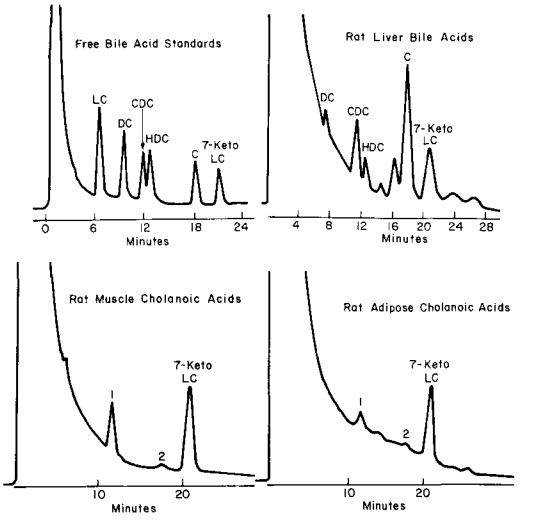

FIG. 3. Tracings of HP 402 gas liquid chromatograms of standard lithocholic (LC), deoxycholic (DC), chenodeoxycholic (CDC), hyodeoxycholic (HDC), cholic (C), and 7-keto-lithocholic acid trifluoroacetoxy methyl esters at a column temperature of 240 C and similar derivatives obtained from rat liver, adipose, and muscle with 7-keto-lithocholic acid added. Peak 1 represents dihydroxy cholanoic acid (probably hyodeoxycholic) and peak 2, cholic acid.

GLC: A glass U-tube 6 ft x 2 mm was silanized and packed with 3% OV-210 on 80-100 mesh silicone support and conditioned at 260 C for 24 hr. A Hewlett-Packard model 402 instrument equipped with dual hydrogen flame detectors and an electronic digital integrator was used. Operating conditions were: injection port 280 C, detector 275 C, and helium carrier gas 40 ml/min. Temperature of the oven was varied as described in "Results." Ethyl acetate was used to dissolve samples, and 7-keto-lithocholic acid was used as an internal standard. The area response of 7-keto-lithocholic or each individual trifluoroacetate of bile acid methyl esters was quantitative over a wide range of load (0.5-50 μg) and was related directly to the absolute wt (not mol wt) of the parent unsubstituted steroids. Quantitation in the submicrogram range was made by calculating the intensity of the output from the digital integrator compared with that of the internal standard, 7-keto-lithocholic acid.

GLC-mass spectrometry: The mass spectrometer used was an AEI, MS 12 on line with a Beckman GC 45 gas chromatograph via a molecule separator of the jet type. The instrument combination of gas chromatography and mass spectrometry provides the use of a gas chromatograph as an inlet system and a mass spectrometer as detector. A 4 ft glass U-tube

TABLE IV

Approximate Quantities of Total Cholanoic Acids in Hepatic and Nonhepatic Tissues of Rat

Tissue	μg/g Tissue
Liver	158 ± 47[a]
Skeletal muscle	8.1 ± 3.1
Adipose	6.3 ± 1.4
Kidney	25.5 ± 1.6
Pancreas	34.3
Brain	18.5

[a]Mean ± standard error of 2-3 rats.

column, otherwise the same as used in the H/P 402, was used.

Ca. 99-99.5% of the helium is removed in the molecule separator, and ca. 50-60% of the sample enters the ion source of the mass spectrometer (8). The energy of bombarding electrons was kept between 20-23 ev and the current at 40 or 60 μ amp. A scan speed of ca. 5-8 sec for the mass range 50-600 was used. The column temperature was kept at 100 C for the first 5-10 min to eliminate most materials volatile at low temperature, and then the temperature was increased to 150 C prior to the start of temperature programing. The valve into the molecule separator of the mass spectrometer was opened when the column temperature

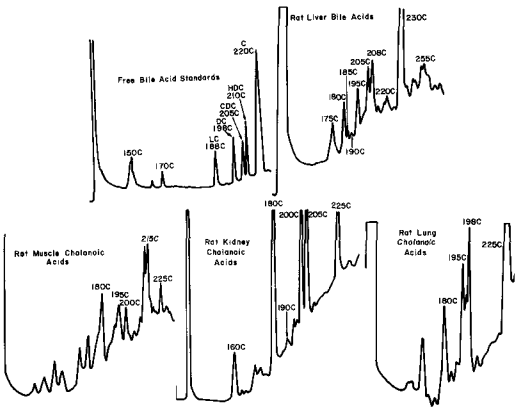

FIG. 4. Tracings of Beckman GC-45 gas liquid chromatograms of standard lithocholic (LC), deoxycholic, chenodeoxycholic, hyodeoxycholic (HDC), and cholic acid (C) trifluoroacetoxy methyl esters with temperature held at 150 C for 5 min, then programed to increase to 235 c; and similar derivatives obtained from rat liver, adipose, muscle, lung, and kidney.

reached 170-175 C. By this procedure, it was possible to eliminate all volatile contaminants and to have good GLC peaks of cholanoic acids. At the end of the sample run, the valve to the molecule separator was closed and a standard sample introduced (9) into the ion source.

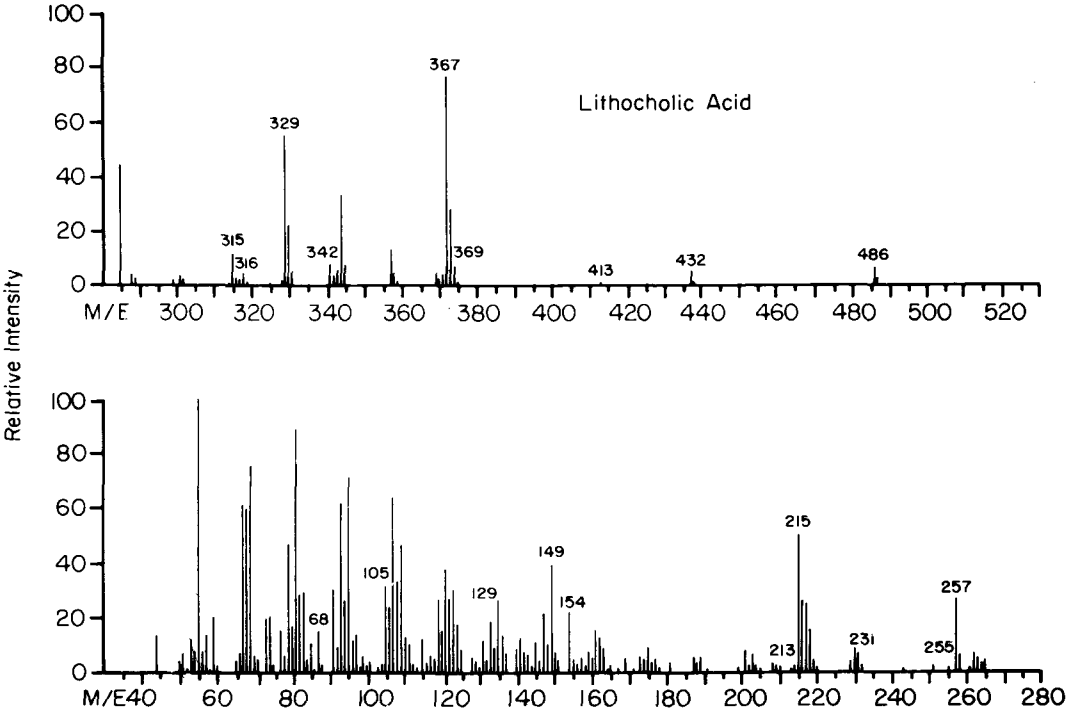

FIG. 5. Mass spectrum of the trifluoroacetoxy methyl ester derivative of standard lithocholic acid.

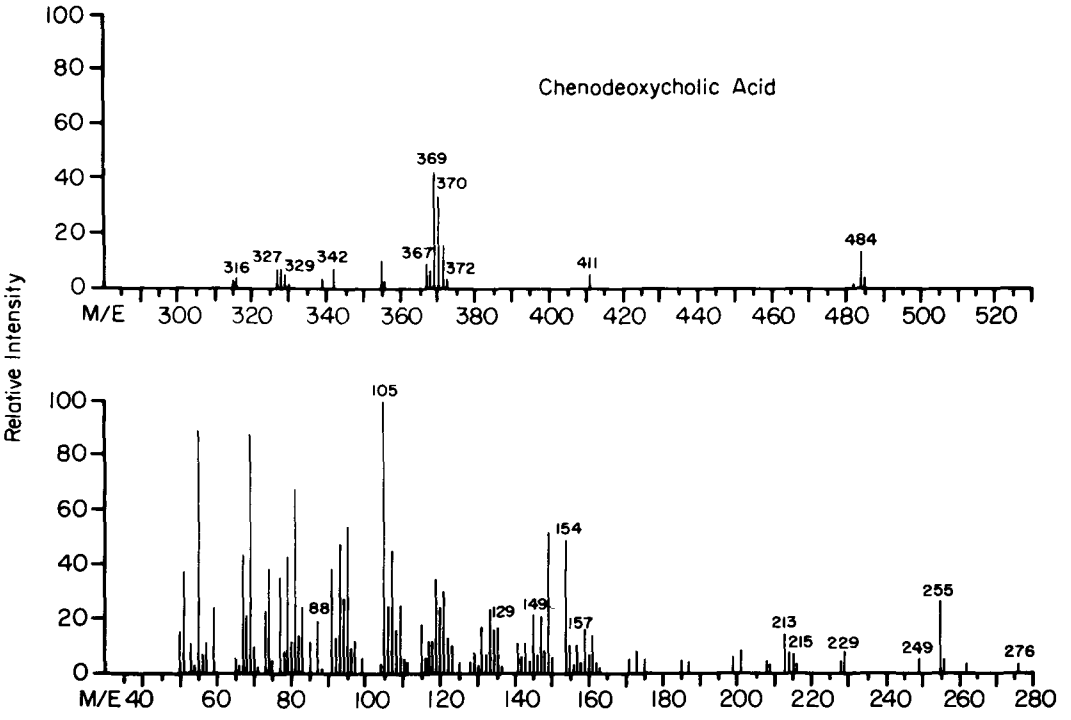

FIG. 6. Mass spectrum of the trifluoroacetoxy methyl ester derivative of standard chenodeoxycholic acid.

RESULTS

Extraction: The recovery values of the three labeled bile acids and cholesterol are given in Table I. From these results, it is apparent that the HCl-ethanolic extraction (5) was proved to be the choice method for, not only cholanoic acids, but cholesterol. The low recovery of ^{14}C-taurocholic acid by the Folch method was expected since most of the conjugated bile acids are found in the upper phase (10).

Recovery of conjugated bile acid from the alcoholic KOH digestion was very low, while free bile acid was recovered well. The protonated conjugated bile acids are sufficiently nonpolar to be extracted from an aqueous solution by n-butanol (11), but the nonaqueous solvent should be 10-20 volumes larger than the water volume. These conditions are not met by a 50% aqueous ethanol solution partitioning with 1 volume of ethyl ether as we proceeded.

Hydrolysis: Since the nature of conjugated bile acids in tissues is not known in detail, it is simpler to work with free bile acids. Some loss of bile acids, particularly with biological extracts, occurs during vigorous alkaline hydrolysis. Table II shows the satisfactory deconjugation of taurocholic acid and assured minimal loss of bile acid due to the strong hydrolysis conditions. Subsequent to these experiments, all tissues were processed as described in Figure 1.

Chromatography: Figure 2 shows a diagram of the separation patterns of muscle acidic steroids on TLC plates. Photographs of similar separations from pig tissues have been published previously (1).

Aliquots of the acidic extracts from rat tissues after administration of ^{3}H-cholesterol were applied on TLC plates. Bands on the plate corresponding to standard bile acid bands were scraped off, eluted, and counted for detection of radioactivity.

Figure 3 shows H/P 402 GLC chromatograms of free bile acid standards, with 7-ketolithocholic as an internal standard added before derivatization and chromatograms of liver, skeletal muscle, and adipose. Trihydroxy bile acid is the primary type in the liver while the dihydroxy compounds are predominant in the peripheral tissues. Table III shows the quantities of cholanoic acids found in various tissues. Table IV shows the recovery of radioactivity in TLC cholanoic acid bands and the GLC analysis of the same samples. Resolution by TLC was not as precise as GLC. The separation of chenodeoxycholic (CDC) and hyodeoxycholic (HDC) acids by GLC was acceptable if both compounds were present in substantial

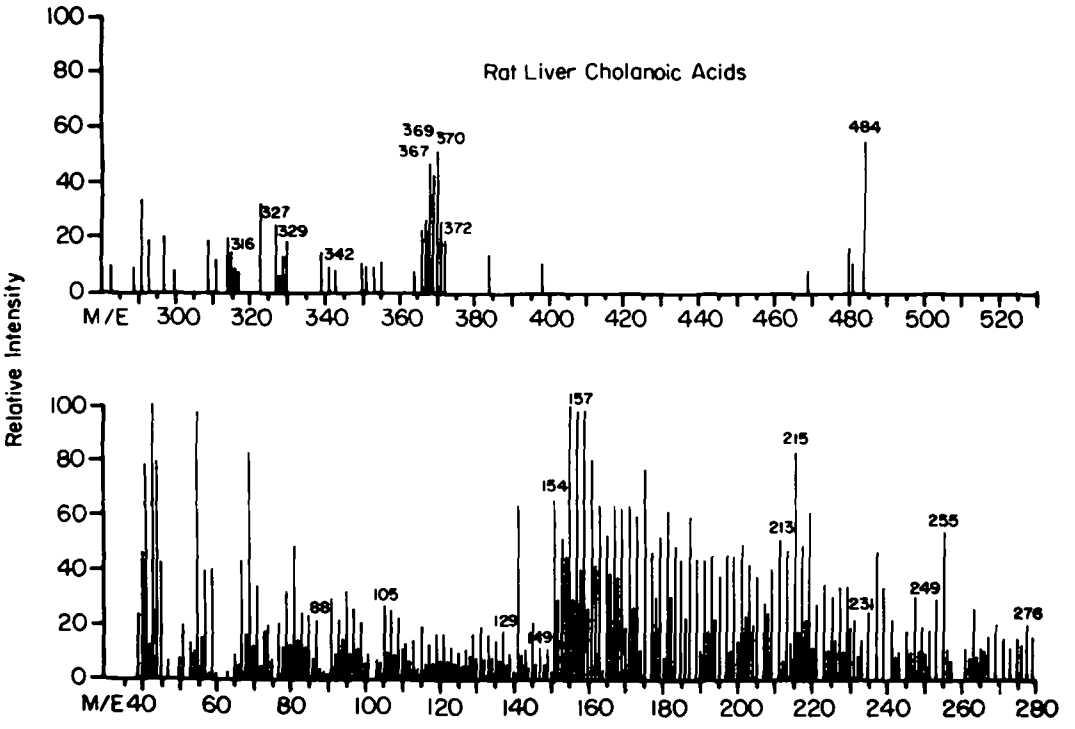

FIG. 7. Mass spectrum of trifluoroacetoxy methyl ester derivatives of a preparation from rat liver.

amounts. If only one was present, however, its identity was not absolute by retention time. The regions of TLC plates eluted for counting did not precisely coincide with standards of deoxycholic acid (DC) and CDC. Since DC is found only as the result of bacterial action in the gut lumen, it is likely that most of the radioactivity in this region is due to CDC.

GLC-mass spectrometry: Figure 4 shows Beckman GC 45 gas chromatograms of standard trifluoroacetoxy methyl cholanoates and liver, muscle, kidney, and lung. The retention times may differ from one run to the next but each bile acid derivative was resolved at a specific temperature. This technique was found very useful to get rid of impurities. The retention temperature of individual bile acid peaks was as follows: lithocholic, 188-190 C; DC, 198-200 C; CDC, 205 C; HDC, 210 C; and cholic acid, 220 C.

Figures 5 and 6 present mass spectra of standard methyl 3α-mono(trifluoroacetoxy)-cholanoate (lithocholic acid) and methyl 3α-7α-di(trifluoracetoxy)-cholanoate (CDC acid) using our instrumentation. Figure 7 shows liver and Figure 8 muscle spectra. Table V shows the occurrences of major *m/e* peaks from Sjövall (9), our standards, and various tissue cholanoic acid preparations. Comparing with mass spectra of Sjövall's pure standard bile acids and with those found in this study, tissue cholanoic acids showed most of the *m/e* peaks. Since the influence of temperature on the fragmentation patterns of bile acids has been poorly understood and the GC fragments of Sjövall's study indicated that there were many missing *m/e* peaks, some missing *m/e* peaks in our present spectra could be due to different fragmentation due to different conditions of the gas chromatography-mass spectrometry combined system from others. The present data revealed, therefore, enough clues to reason that these GLC peaks are due to cholanoic acid derivatives rather than other compounds in the tissues.

DISCUSSION

The results of this study show that cholanoic acids are present in many and probably all tissues of the rat. Apparent reasons for the fact that this has not been shown before are the very small quantitity present, the difficulty of separating cholanoic acids from a large excess of fatty acids, and the amphoteric nature of naturally occurring conjugated acids.

Cholic acid was the primary compound in liver while dihydroxy cholanoic acids (mainly HDC) are predominant in extra hepatic tissues. Okishio, et al., (12) found that cholic acid con-

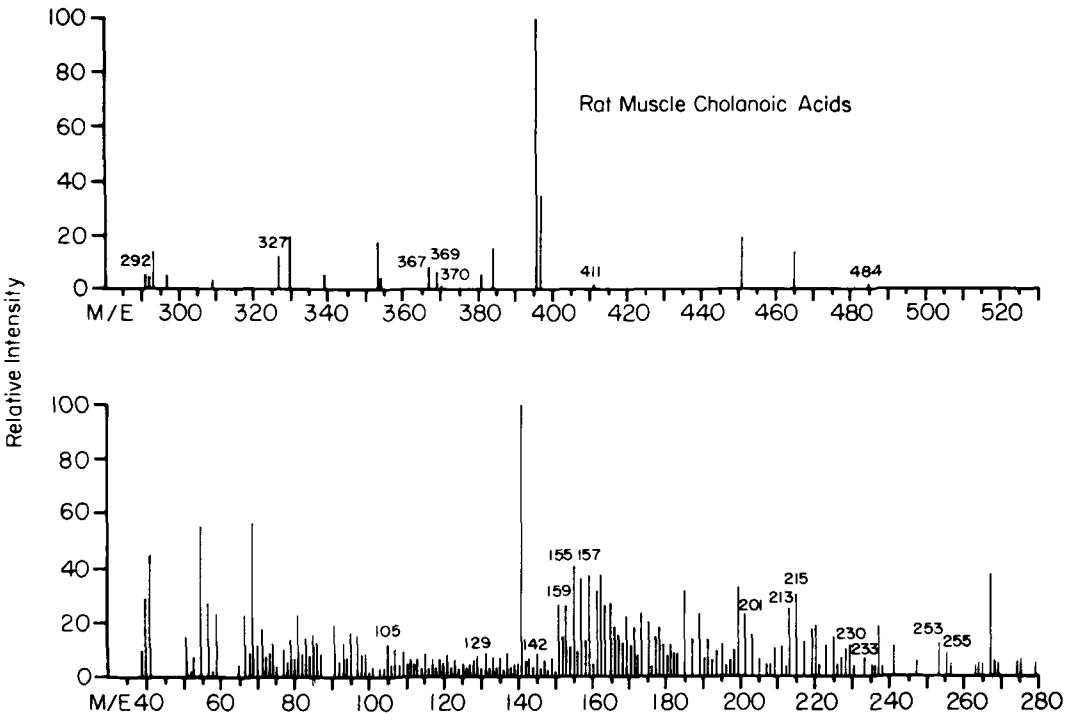

FIG. 8. Mass spectrum of trifluoroacetoxy methyl ester derivatives of a preparation from rat muscle.

TABLE V

Comparison of Major Peaks of Mass Spectra of Standards and Tissue Cholanoic Acids to Those of Sjövall (9)

	Sjövall			Our standards (mono +				
m/e	Di-OH	Tri-OH	GC[a] fragments	Di-OH)	Liver	Lung	Muscle	Kidney
		486						
484	X			X	X		X	
372	X	X	X	X	X			
370	X			X	X	X	X	
369	X	X		X	X	X	X	X
367	X	X		X	X	X	X	X
342	X	X		X				
329	X	X		X	X		X	
327	X	X		X	X	X	X	
316	X			X	X	X	X	X
276	X		X	X	X			X
257	X	X	X	X	X	X		X
255	X	X	X	X	X	X	X	X
249	X	X	X	X	X			
231		X	X	X	X			X
215	X	X	X	X	X	X	X	X
213	X	X	X	X	X	X	X	X
157	X	X		X	X	X	X	X
154	X	X	X	X	X	X	X	X
149	X	X	X	X	X	X	X	X
142	X	X		X	X	X	X	X
129	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X

[a]GC = gas chromatographic.

stitutes ca. 70% of the bile acids of liver and portal blood and 45% in peripheral blood. Hyodeoxycholic acid made up 44% of the bile acids of peripheral blood. These observations are consistent with the possibility that hyodeoxycholic acid is formed in the peripheral tissues.

The evidence indicating that radioactivity derived from ring-labeled cholesterol was found in cholanoic acids is based upon the use of 1,2 ^{3}H-cholesterol. Kritchevsky, et al., (13) reported loss of tritium from 6 α-^{3}H-cholesterol. Hepner, et al., (14) reported appearance of 40% of the tritium in body water following administration of 2,4-^{3}H-labeled bile acids. Panveliwalla, et al., (15) reported loss of 20% of the label when a mixture of the 2,4-^{3}H epimers of cholic acid was used. The 3,4 and 6,7 carbons of the ring structure are involved in various enzymatic reactions, and the stereochemistry of these reactions can account for loss of the tritium label (16). We have found that administration of 1,2-^{3}H cholesterol simultaneously with 4-^{14}C-cholesterol results in radiohomogeneity of bile acid specific activity in miniature swine for up to 16 days following simultaneous injection (unpublished). While exchange of tritium is possible, it is unlikely that the lost ^{3}H would account for labeling of bile acids isolated by TLC.

The low concentration of these compounds in extrahepatic tissues is consistent with their known detergent and toxic effects. Further studies are in progress to gain some insight into possible functions of cholanoic acids in cells.

ACKNOWLEDGMENTS

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Dietary and Hormonal Effects upon Activity of "Soluble" Protein and Particulate Fraction of Fatty Acid Desaturation System of Rat Liver Microsomes

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ABSTRACT

Rat liver microsomes were extracted with a buffered 0.15 M KCl and 0.25 M sucrose solution and fractionated by centrifugation into a particulate component and a supernatant containing a protein factor necessary for fatty acid desaturation. The Δ6 fatty acid desaturation activity of the extracted microsomes was reduced significantly, and the readdition of the supernatant restored the enzymatic activity to the original value of the whole microsomes. A protein diet or a fat-free diet increased the Δ6 desaturation activity of the whole microsomes. The activating effect was evoked upon the particulate components of the enzymatic desaturation system and not upon the protein factor present in the supernatant. Fasting, refeeding, and refeeding plus glucagon and theophylline treatments of rats also modified the Δ6 desaturation activity of whole liver microsomes. The effect also was evoked on the Δ6 desaturation system tightly bound to the microsomal membrane but not on the protein factor of the supernatant. Accordingly, the protein factor of the supernatant is considered to be different from the cyanide sensitive factor and the desaturase.

INTRODUCTION

Stearyl-CoA is converted to oleyl-CoA by rat liver microsomes in a reaction requiring both oxygen and an electron donor, such as nicotinamide adenine dinucleotide, reduced form (NADH) or nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (1). The reaction is inhibited by cyanide (1). Linoleic acid and other fatty acids also are desaturated by liver microsomes in a reaction requiring adenosine 5′-triphosphate (ATP), CoA, Mg^{++}, NADH or NADPH, and oxygen (2-6). These reactions also are inhibited by cyanide (7).

The activity of the desaturases is controlled by dietary conditions and the hormonal status of the animals (6,8-11). Moreover, it has been established that the enzyme system involved in the desaturation reaction of stearic to oleic acid has at least three integral components of the microsomal membrane: a flavoprotein, cytochrome b_5 and a terminal component called cyanide sensitive factor (1,12-14). The amount of the cyanide sensitive factor is modified by the diet and limits the overall desaturation rate (15). A similar system is considered to function with other fatty acids (16). Moreover, recent studies demonstrate that microsomal desaturation of linoleic to δ-linolenic acid; δ-linolenic to octadeca-6,9,12,15-tetraenoic acid; palmitic to palmitoleic acid; and steric to oleic acid require a protein factor loosely bound to the microsomal membrane (16,17, and A. Catalá, A.M. Nervi, and R.R. Brenner, unpublished results). This factor is detached easily from the microsome by gentle extraction with KCl or sucrose solutions of low ionic strength (16,17, and A. Catalá, A.M. Nervi, and R.R. Brenner, unpublished results). The effect is shown using either free fatty acids with CoA and ATP or the acyl-CoA thioesters (A. Catalá, A.M. Nervi, and R.R. Brenner, unpublished results).

The exact nature and specific function of this factor are still unknown. Therefore, it is important to determine whether the regulatory functions of dietary components and hormones are evoked on this protein factor or on the enzymatic system firmly bound to the microsome. The present paper reports that dietary changes and glucagon modify the enzymatic activity of the particulate system in the desaturation reaction but do not alter the activity of the extrinsic protein factor.

MATERIALS AND METHODS

Chemicals

1-^{14}C-linoleic acid (57 mCi/mmole) and 1-^{14}C-α-linolenic acid (58 mCi/mmole) were provided by the Radiochemical Centre, Amersham, England. Both acids were 99% pure. ATP disodium salt, CoA grade 1, NADH disodium salt grade II, and glutathione were purchased from Boehringer Argentina, Buenos Aires, Ar-

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gentina. Glucagon and theophylline were provided by Sigma Chemical Co., St. Louis, Mo.

Treatment of Animals

Wistar rats were used in all experiments.

Effect of Hyperproteic Diet

The procedure of Peluffo, et al., (18) was used to investigate the effect of a hyperproteic diet upon the activity of microsomal fractions. Adult male rats weighing 250-300 g and maintained on a Purina chow were divided into two groups. One group of 5 animals was force-fed a 25% suspension of casein (25 kcal/100 g body wt/24 hr) during 48 hr. The total daily food intake was administered at 6 hr intervals. The control group of 5 animals was fed the Purina chow during the same period of time. Then the animals were killed by decapitation without anesthesia. The livers of the animals of each group were pooled and microsomes separated as described elsewhere.

Effect of Fat-Free Diet

Both young and adult male rats were used. One month old rats were divided into two groups of five animals each. The first group was fed a fat-free diet ad libitum for 10 weeks. The composition of the diet was the same as described previously (19). It contained 19% defatted casein, 77% sucrose, 4% minerals, and vitamins. The second group was used as control and was maintained on the standards Purina chow. After the stated period of time, the animals were killed, livers of each group were pooled, and microsomes separated, as described below. Six month old rats maintained on a standard Purina chow were divided into two groups of five animals each. The first group was maintained on the fat-free diet, while the second group was fed the standard Purina chow. They were maintained on these diets for 8 months. As before, animals were killed, livers of different groups pooled, and microsomes separated.

Effect of Fasting, Refeeding and Glucagon Administration

Adult female rats weighing 250-300 g and maintained on a standard Purina chow were used. The rats were divided into three groups of three animals each. All rats were fasted for 48 hr. From the beginning of the refeeding period, one of these last groups was administered glucagon plus theophylline. Glucagon was administered in a dose of 200 μg/8 hr/100 g body wt by intraperitoneal injection. Theophylline also was injected intraperitoneally in a dose of 2 mg/8 hr/100 g body wt. After 8 months, the rats were killed, the livers of each group pooled, and microsomes separated.

Isolation of Microsomes

Immediately after death of the animals, livers were excised rapidly and immersed in ice-cold homogenizing solution (17). The composition of the homogenizing solution was 0.15 M KCl, 62 mM phosphate buffer (pH 7), 1.5 mM glutathione, and 0.25 M sucrose. The livers were homogenized (4:1 v/w) in the cold with this solution and centrifuged at 800 x g for 10 min. The supernatant then was centrifuged at 10,000 x g for 30 min and, after decanting, at 110,000 x g for 1 hr. to yield the microsomal precipitate. Microsomes were suspended in the homogenizing solution (2:1 v/v) and protein estimated by the Biuret reaction (20). This suspension constitutes the whole microsomes (M).

Subfractionation of Microsomes

Microsomes were subfractionated by the procedure described by A. Catalá, A.M. Nervi, and R.R. Brenner (unpublished results). Microsomes were suspended in a cold solution containing 0.15 M KCl, 0.25 M sucrose; 41.7 mM KF; 1.5 mM glutathionine; 5 mM $MgCl_2$; and 41.7 mM phosphate buffer (pH 7) (17). The ratio 5 mg microsomal protein to 3 ml extraction solution was used. The mixtures were shaken gently at 0-4 C for 15 min and then centrifuged at 110,000 x g for 1 hr. In this way, two fractions were obtained: a particulate fraction consisting of the extracted microsomes (Me), and the supernatant (Sp) containing the "soluble" protein factor that is necessary for full desaturation activity of the microsomes (16). The reconstitution of the whole microsomal system was performed when necessary by addition of 3 ml Sp to 5 mg of Me protein suspension. This was the approximate ratio present in the original microsomes (A. Catalá, A.M. Nervi, and R.R. Brenner, unpublished results).

Assay for Oxidative Desaturation of Fatty Acids

The oxidative Δ6 desaturation activity of whole microsomes (M) and microsomal subfractions was measured by estimation of the percent conversion of 1-^{14}C linoleic acid to δ-linolenic acid or 1-^{14}C α-linolenic acid conversion to octadeca-6,9,12,15-tetraenoic acid. A mixture of 3 nmoles labeled acid and 97 nmoles unlabeled acid carefully measured were incubated with 5 mg microsomal protein of either M or Me, or 5 mg of Me plus 3 ml Sp and cofactors in a total volume of 3.2 ml for 25 min at 35 C. The concentration of the cofactors in the incubation solution was 1.3 mM ATP, 0.06 mM CoA, 0.87 mM NADH, 5 mM $MgCl_2$, 1.5 mM glutathione, 41.7 mM KF, 41.7 mM

TABLE I

Behavior of Extracted Microsomes and Protein Factor Obtained from Rats Maintained on Hyperproteic Diet on Δ6 Desaturation of Linoleic Acid[a]

Fractions[b]	Linoleic acid desaturation[c] (%)
Mn	11.1
Mp	26.1
Men	3.5
Mep	12.0
Men + Spn	11.0
Mep + Spp	25.8
Men + Spp	10.8
Mep + Spn	25.4

[a]Microsomes of rats fed on Purina chow or casein were separated and extracted as specified in the text.

[b]Mn = whole microsomes from rats maintained on Purina chow, Mp = whole microsomes from rats maintained on hyperproteic diet, Men = extracted microsomes from rats maintained on Purina chow, Mep = extracted microsomes from rats maintained on hyperproteic diet, Spn = supernatant containing the protein factor from rats maintained on Purina chow, and Spp = supernatant containing the protein factor from rats maintained on hyperproteic diet.

[c]Desaturation of linoleic acid to α-linolenic acid was measured by incubation with 5 mg protein of either M or Me or 5 mg Me plus 3 ml Sp, 100 nmoles labeled linoleic acid, and cofactors at 35 C during 25 min. For other details see the text. Desaturation of Spn and Spp were not measurable.

TABLE II

Behavior of Extracted Microsomes and Protein Factor Obtained from Young and Adult Rats Maintained on Fat-Free Diet, on Δ6-Desaturation of Linoleic Acid[a]

Fractions[b]	Linoleic acid desaturation[c] Young %	Adult %
Mn	15.1	7.8
Mf	18.5	19.5
Men	6.5	6.3
Mef	10.5	12.5
Men + Spn	14.1	8.0
Mef + Spf	16.0	19.5
Men + Spf	15.8	8.6
Mef + Spn	18.0	22.0
Men young + Spn adult	16.9	
Mef young + Spf adult	19.4	
Men adult + Spn young	7.9	
Mef adult + Spf young	20.5	

[a]Microsomes of rats fed on Purina chow or fat-free diet separated and extracted as specified in the text.

[b]Abbreviations and other details as in Table 1. In addition: Mf = whole microsomes from rats fed a fat-free diet, Mef = extracted microsomes from rats fed a fat-free diet, and Spf = supernatant containing a protein factor from rats fed a fat-free diet.

[c]Desaturation of Spn and Spf not measurable.

phosphate buffer (pH 7), 0.15 M KCl, and 0.25 M sucrose.

The incubation was stopped by the addition of 2 ml 10% KOH in methanol. After saponification, the fatty acids were extracted and converted to methyl esters as described elsewhere (5). The distribution of the radioactivity between substrate and product was measured by gas liquid radiochromatography in a Pye apparatus equipped with a proprotional counter (5). The fatty acids were identified by their retention times and by comparison with authentic standards.

RESULTS AND DISCUSSION

In this work, we have taken advantage of the differences in the Δ6 desaturation activities of the microsomes of fasted rats and rats maintained on a balanced diet, on a hyperproteic diet and on a fat-free diet to detect whether controlling effects of these factors are evoked on the particulate part or on the "soluble" protein fraction of rat liver microsomes.

Effect of Hyperproteic Diet

Table I shows the effect of a hyperproteic diet upon the activity of M, Me, and (Me + Sp). Previous studies have indicated repeatedly that a hyperproteic diet increases linoleic acid desaturation activity of rat liver microsomes (11,18,20). This result is confirmed in Table I. Furthermore, Table I shows that the whole microsomes of rats fed either a balanced diet (Mn) or a hyperproteic diet (Mp) are deactivated when Sp is extracted. The readdition of the corresponding supernatants reactivates both Me practically to their original values. Particularly important is the effect of the crossed addition of the supernatants to the extracted microsomes. It is found that the addition of the supernatant from the animal maintained on a normal diet (Spn) reactivates the extracted microsomes of rats fed a hyperproteic diet (Mep) to their original value (Mp) and not to Mn. A similar thing occurs when supernatant containing the protein factor from rats maintained on hyperproteic diet (Spp) is added to extracted microsomes from rats maintained on Purina chow (Men), since Spp increases the desaturation of Men to the value of the original microsomes of normally fed rats (Mn) and not to Mp. Therefore, these results suggest that the induction effect of the hyperproteic diet is evoked on the particulate part of the desaturation system and not on the protein factor.

Effect of Age and Fat-Free Diet

Castuma, et al., (6) proved that a fat-free diet or an essential fatty acid-free diet enhances

TABLE III

Effect of Fasting, Refeeding, and Glucagon Treatment upon Δ6 Desaturation Activity of Protein Factor and Extracted Microsomes

Fractions[a]	α-Linolenic acid desaturation[b]			
	Fasted	Fasted and refed	Fasted and refed + glucagon + theophylline	
M	20.1	36.4	11.4	
Me	12.4	29.2	5.8	
Me + Sp	19.0	36.8	12.9	
Me fasted + Sp refed	21.5			
Me refed + Sp fasted	33.5			
Me refed + Sp (refed + glucagon + + theophylline)			35.5	
Me (refed + glucagon + + theophylline) + Sp refed			12.5	

[a]M = whole microsomes, Me = extracted microsomes, and Sp = supernatant containing protein factor.

[b]Desaturation of α-linolenic to octadecatetraenoic acid was measured by incubation of 5 mg protein of either M or Me or 5 mg Me plus 3 ml Sp, 100 nmoles of labeled α-linolenic acid, and cofactors at 35 C during 25 min. For more details see the text. Sp of fasted, refed, and refed + glucagon + theophylline treated animals was not measurable.

the Δ6 desaturation activity of rat liver microsomes. Table II confirms the activation effect of a fat-free diet and also shows that this activation is evoked either in young or adult animals. Moreover, liver microsomes of aged animals maintained on a balanced diet have less desaturation activity than young animals, as has been found by Peluffo, et al., (21). This experiment again demonstrates that liver microsomes of either young or adult animals, maintained on a balanced diet or one a fat-free diet are deactivated partially when they are extracted and centrifuged by the described procedure. The desaturation activities of the extracted microsomes are reactivated to the original value when the corresponding Sp is readded to the Me. Similarly to Table I, Table II shows from crossed additions of Sp to Me that any Sp, either from young or aged animals maintained on a fat-free diet or a balanced diet, activates the different Me to ca. their original values. Therefore, we reach the same conclusion as before that the activation of the Δ6 desaturation by a fat-free diet apparently is concerned with the tightly bound microsomal enzymatic system and not with the easily solubilized protein factor.

Effect of Fasting, Refeeding, and Glucagon Treatment

Earlier experiments have shown that the Δ6 desaturation activity of liver microsomes prepared from fasted rats was significantly lower than that of microsomes from animals fed continuously on a balanced diet (10,11). Refeeding restores the activity of the Δ6 desaturase to normal values (10,11). Moreover, recently de Gómez Dumm, et al., (22) found that glucagon injection during the refeeding period inhibits the reactivation of the Δ6 desaturation reaction. Therefore, these deactivation-reactivation effects also were investigated to give additional evidence to the previous results. In this case, the changes of the Δ6 desaturation activity were measured in the conversion of α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid. Table III confirms that 48 hr refeeding with a fat-free diet provokes an increase of Δ6 desaturation activity of the whole microsomes. Extracted microsomes of any type of rat showed lower enzymatic activity than whole microsomes and as happened with the experiments shown in Tables I and II, readdition of the corresponding supernatants containing the protein factor restored ca. their original levels. Crossed experiments in which the supernatants of one type of rat are added to the extracted microsomes of another type of rat, showed again that any supernatant was indiscriminately able to restore the Δ6 desaturation activity of the extracted microsomes to ca. their original values. Therefore, fasting and refeeding, as well as glucagon treatment, also evoked their deactivation-activation effects upon the microsomal desaturation system and not upon the protein factor that is not integral to the membrane. In consequence, all these results seem to be compatible with the hypothesis that the "soluble"

protein factor is not a regulatory component controlled by dietary changes. The controllable part of the Δ6 desaturation system must be one of the components firmly bound to the microsomal membrane. In the case of stearyl-CoA desaturation, Oshino and Sato (15) have shown that, of the three microsomal components: cytochrome b_5 reductase, cytochrome b_5, and the cyanide sensitive factor, the last one would be diet inductible and probably the desaturation enzyme itself (23). Since our "soluble" protein factor is necessary for both Δ6 and Δ9 desaturations (A. Catalá, A.M. Nervi, and R.R. Brenner, unpublished results), the present work adds new evidence to the postulate that this factor is not the cyanide sensitive factor or the desaturating enzyme. The specific function of the protein factor is still unknown.

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Nonsteroidal Secondary and Tertiary Amines: Inhibitors of Insect Development and Metamorphosis and Δ^{24}-Sterol Reductase System of Tobacco Hornworm

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ABSTRACT

Several new branched and straight chain secondary and tertiary amines were shown to have inhibitive effects upon development and metamorphosis and the Δ^{24}-sterol reductase system in larvae of the tobacco hornworm similar to those previously observed with a number of azasteroids. Certain of the amines which are related structurally to compounds with juvenile hormone activity in insects also blocked development and metamorphosis in three other species of insects. These compounds are lethal or inhibit development in all larval stages and thus differ in action from compounds with juvenile hormone activity where the principal effect is to block the penultimate or ultimate molt.

INTRODUCTION

Certain 25-azasteroids block the Δ^{24}-sterol reductase enzyme system(s) involved in the conversion of plant sterols to cholesterol, block other pathways of steroid metabolism in insects, and are potent inhibitors of insect growth and development (1,2). To determine the minimal structural requirements for this type of inhibitory activity, we synthesized and tested compound I (Fig. 1), which lacks the A and B rings of the steroid nucleus. Although not as potent as the more active azasteroids, this compound inhibited insect growth and development and the Δ^{24}-sterol reductase enzyme system of the tobacco hornworm, *Manduca sexta* (L.). Since compound I possessed activity similar to that of the azasteroids which appear to disrupt the ecdysone regulated processes of molting and metamorphosis by interfering with hormone biosynthesis or metabolism (2), we set out to determine whether even simpler compounds might have such inhibitory activities. In concurrent research, we also were attempting to design and synthesize compounds structurally related to chemicals with insect juvenile hormone (JH) activity that might have an action in relation to the JH-regulated processes analogous to that observed for the azasteroid inhibitors.

We now report the synthesis of a number of new straight and branched chain compounds related to chemicals with insect JH activity that block growth and development as well as steroid metabolism in certain species of insects. Most of these chemicals differ in action from compounds with insect JH activity in that they are lethal to all larval stages and are usually more active during early larval development than during the penultimate and ultimate molts.

EXPERIMENTAL PROCEDURES

Instrumentation

Mp were observed on a Kofler block, and IR spectra were obtained with a Perkin-Elmer model 221 prism-grating spectrophotometer. Gas liquid chromatographic (GLC) analyses were made on a Barber-Colman model 10 chromatograph; the GLC systems were 0.75% SE-30 and 1.0% OV-17 coated on Gas-Chrom P. NMR spectra were recorded at 60 Mc with a Varian A-60A NMR spectrometer with deuterated chloroform as the solvent and TMS as an internal standard. The mass spectra were measured by using a LKB model 9000 gas chromatograph mass spectrometer (LKB Produkter AB, Stockholm, Sweden). The samples were introduced directly into the ionization chamber, except for compounds XI and XII which were introduced through the GLC system, and the ionization energy was 70 ev.

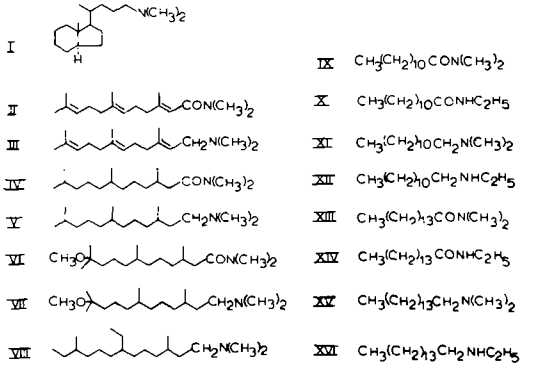

FIG. 1. Amide and amine structures.

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Materials and Chemical Synthesis

Unisil (Clarkson Chemical Co., Williamsport, Pa.) and Woelm neutral grade II alumina (Alumina Woelm, Alupharm Chemicals, New Orleans, La.) were used for column chromatographic separations. The intermediate acid used for the preparation of compound I was prepared by ozonolysis of vitamin D_2 (3,4) and chromic acid oxidation. A Wolf-Kishner reduction of the resulting keto-acid gave the intermediate β-7a-dimethyloctahydro-1-H-indene-1-acetic acid. The reaction of this acid with thionyl chloride gave the acid chloride which was immediately allowed to react with diazomethane to give the diazoketone. An Arndt-Eistert rearrangement of the diazoketone using the modified procedure of Wilds and Meader (5) gave the benzyl ester of an acid with its chain length increased by one carbon from that of the initial acid. An alkaline saponification of this ester and a repeat of Wilds and Meader's procedure yielded the acid of the desired chain length to prepare the amine I in 30% overall yield from vitamin D_2.

The intermediate farnesenic acid required for the preparations of compounds III and IV was prepared from farnesol, as previously reported (6). The 11-methoxy-3,7,11-trimethyldodecanoic acid used for the synthesis of compound VI and VII was prepared by solvomercuration-demercuration of farnesol in the presence of methanol according to the method of Brown and Rei (7) followed by hydrogenation in absolute ethanol with Raney nickel catalyst at room temperature and atmospheric pressure. The methoxy farnesol was oxidized to the acid in acetone with an 8 N solution of chromic acid in dilute sulfuric acid. The 7-ethyl-3,11-dimethyltridecanoic acid intermediate for compound VIII was prepared in a 50% overall yield by hydrogenation and hydrogenolysis of a mixture of isomers of methyl 10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate in acetic acid with platinum oxide as a catalyst. Since the resulting product showed hydroxyl absorption by IR analysis, the crude mixture was heated to 75 C in 37% hydrogen bromide-acetic acid solution, and the reaction products were rehydrogenated in ethanol with 10% palladium on charcoal as a catalyst. The saturated ester was chromatographed on activity Grade II alumina to give the purified ester, which, upon saponification and acidification, gave the acid.

Compounds I-XVI were prepared in 60-80% yield according to the general method by reaction of the appropriate acids with thionyl chloride to give the respective acid chloride. For the preparation of acid chlorides with olefinic bonds, the acids were refluxed in benzene for 2 hr with a slight excess of thionyl chloride and a stream of nitrogen passing through the solvent. The other acids were refluxed overnight without nitrogen. Reaction of the respective acid chlorides with monoethylamine or dimethylamine yielded the amide, which subsequently was reduced to the amine with lithium aluminum hydride in tetrahydrofuran. When necessary, the compounds were purified by column chromatography. The structure of intermediates and final products were confirmed by IR, NMR, and mass spectroscopy. The purity of the final products as determined by GLC and thin layer chromatography (TLC) was >98% (compound III consisted of 74 and 26% of the *E,E* and *Z,E* isomers, respectively).

Mass spectra of all compounds I-XVI showed a strong M^+ peak, except for compounds VI and VII which showed weak M^+ and a very strong peak at M-32 (CH_3OH). The spectra of the N,N-dimethyl amides II and VI showed base peaks at m/e 127 and 114, respectively. The peak at m/e 127 results from δ-cleavage to the carbonyl accompanied with transfer of hydrogen, while the base peak at m/e 114 occurring in the spectra of VI results from γ-cleavage. β-Cleavage accompanied with transfer of a hydrogen atom is also a major fission occurring in the spectra of VI as indicated by the peak at m/e 87 with an intensity nearly equal to that occurring at m/e 114. The spectra of all other amides gave base peaks at m/e 87. The spectra of all amines, except compound III, exhibited base peaks at m/e 58 that resulted from the simple fission of the carbon-carbon bond adjacent to the nitrogen atom (α-cleavage). The base peak for compound III occurs at m/e 98 (γ-cleavage). Additional physical properties are given in Table I.

Biological Test Systems

The larval test systems for the yellow fever mosquito, *Aedes aegypti* (L.), the confused flour beetle, *Tribolium confusum* Jacquelin duVal, and the house fly, *Musca domestica* L., were those previously used to assess the inhibitive effects of ecdysones and synthetic analogues upon growth and metamorphosis (8) with the following exceptions. In the tests with the yellow fever mosquito, the compounds were assayed against both first- and fourth-instar larvae, and the test insects were observed carefully during the terminal molts for JH effects, e.g. decrease in the number of adults emerging from the pupal stage, partially emerged adults unable to affect complete emergence from the pupal case, and adults that die on the water at emergence. The compounds

TABLE I

Physical Properties of Amides and Amines

Compound	Mp, C	Index of refraction[a]	NMR: N,N-Dimethyl resonances or N-methylene resonances δ	Molecular ion
I		1.4875	2.21 (s)	251
II		1.4650	3.00 (s)	263
III		1.4857	2.21 (s)	249
IV		1.4641	2.97, 3.00	269
V		1.4489	2.21 (s)	255
VI		1.4683	2.98, 3.02[b]	299
VII		1.4524	2.21 (s)[b]	285
VIII		1.4500	2.21 (s)	283
IX		1.4610	2.94, 2.99	227
X	55-56		3.0-3.6 (m)	227
XI		1.4432	2.21 (s)	213
XII		1.4463	2.4-2.9 (m)	213
XIII	33-35		2.97, 3.00	269
XIV	71-73		3.0-3.6 (m)	269
XV		1.4492	2.21 (s)	255
XVI	26-28		2.4-2.90 (m)	255

[a]Recorded at 19 C.

[b]The methoxy resonance appeared at δ 3.2.

initially were tested at 1.0, 2.5, 5.0, and 10.0 ppm on first-instar larvae and at the three highest concentrations on fourth-instar larvae. Compounds that caused 75% mortality or inhibition of development at the lowest of these concentrations were retested at lower concentrations.

The test with the confused flour beetle was essentially the same as that previously described, except that the test system consisted of 40 newly hatched larvae on 2 g diet and the compounds were tested at 0.05, 0.10, 0.25, and 0.50% (dry wt). The pupae were held on treated diet until emergence, and the insects were observed for JH effects, such as supernumerary larval molts, "giant" larvae, pupal-adult intermediates, and second pupae. The larval skin was removed from pupal-adult intermediates, and the insects were examined microscopically for morphogenetic effects, such as the presence of pupal cuticle, urogomphi, and pupal genitalia (9). In addition to the larval tests, confused flour beetle pupae collected overnight (5-22 hr old at the time of treatment) were treated topically with the test compounds. Doses of 1.0, 2.5, 5.0, and 10.0 μg/pupa in 0.1 μliter acetone solution were administered with a microapplicator. The test insects were examined microscopically and assessed for JH (morphogenetic) effects as described above.

Larvae of the tobacco hornworm used in the tests were reared on an artificial diet as previously described (10). Sitosterol, the sole added dietary sterol, was coated on the dry components of the larval diet to achieve a concentration of 0.026% wet wt (0.2% dry wt), and the test compounds were coated on the dry components of the diet at appropriate concentrations in the same manner used to coat the dietary sitosterol. The hornworms were examined for JH effects, including changes in pigmentation, "giant larvae," and inhibition of metamorphosis, and also for the specific effects previously observed with the azasteroid inhibitors, such as disruption of the early larval molts, the formation of precocious "fourth-instar prepupae," and abnormal prepupal-pupal forms (1,2, 11).

Assay of Effects of Test Compounds upon Sterol Metabolism in Tobacco Hornworm

The sterols were isolated from tobacco hornworm prepupae and analyzed by column chromatography and by TLC and GLC as previously described (10). Identification and quantitation of sterols were carried out by GLC analysis on 1.0% OV-17. The relative percentages of cholesterol and desmosterol in the sterols from treated insects indicated both the degree of dealkylation of sitosterol by the insect and inhibition of the Δ^{24}-sterol reductase enzyme.

RESULTS AND DISCUSSION

Compound I, which differs structurally from the more potent azasteroid inhibitors by the lack of the A and B rings of the steroid nucleus, was only about one-tenth as active as the most active azasteroids (2) in the mosquito test

TABLE II

Range of Concentrations of Amides and Amines in Larval Diet or Medium Required To Inhibit Development or Kill 75% of the Test Insects

Compound	Yellow fever mosquito		Confused flour beetle, %	Tobacco hornworm ppm
	First-instar ppm	Fourth-instar ppm		
I	5.0-10.0	>10	0.10-0.25	130-260
II	>10	1.0-2.5	0.10-0.25	>520
III	>10	>10	0.10-0.25	>520
IV	1.0-2.5	2.5-5.0	0.25-0.50	>520
V	1.0-2.5	2.5-5.0	0.25-0.50	33-65
VI	>10	5.0-10.0	>0.5	>520
VII	>10	>10	0.25-0.50	33-65
VIII	0.5-1.0	2.5-5.0	0.25-0.50	65-130
IX	1.0-2.5	>10	0.10-0.25	>520
X	2.5-5.0	>10	>0.5	>520
XI	2.5-5.0	>10	0.10-0.25	16-33
XII	1.0-2.5	>10	>0.5	130-260
XIII	0.25-0.50	2.5-5.0	0.25-0.50	>520
XIV	>10	>10	>0.5	>520
XV	0.10-0.25	2.5-5.0	0.10-0.25	65-130
XVI	0.10-0.25	2.5-5.0	>0.5	33-65

(Table II). When tested in the larval diet, compound I was also considerably less active against *Tribolium* larvae than the more potent azasteroids and was toxic to young larvae. This compound then differs in action from the azasteroid inhibitors for which the typical effect of the minimum lethal concentration is to block the larval to pupal molt in *Tribolium* (2). Compound I was not active against house flies at 375 ppm, the highest test concentration used in these studies. However, in the tobacco hornworm larva, I was ca. equal in inhibitory activity to 22,25-diazacholesterol, the first azasteroid tested on this insect (12), and thus is ca. one-thousandth as active as the most potent of the azasteroid inhibitors on the hornworm (2). It is also a Δ^{24}-sterol reductase inhibitor in the hornworm. At 130 ppm, it reduced the cholesterol level to less than 5% of the total tissue sterols as compared to 80-85% found in control insects, and increased the desmosterol content from the normal range of 1.0-1.5% to ca. 50% of the total sterols present in this insect (Table III). Compound I also caused the typical azasteroid effects in the hornworm; the formation of precocious "fourth-instar prepupae" and abnormal pupae that appear to be prepupal-pupal intermediates. At higher concentrations, it blocked development in the early larval stages, and the lethal effect usually occurred at the time of molt (1,2, 11).

Compound II, the N,N-dimethylamide of farnesenic acid, was the first aza-JH chemical synthesized and tested. It exhibited JH activity in the mosquito tests and the *Tribolium* assays (Table II). In the yellow fever mosquito larva, it was less active against first-instar than fourth-instar larvae, and, in the latter stage, it blocked metamorphosis and adult emergence. When it was added to the diet of the confused flour beetle, it caused JH effects, such as extending the length of time in the larval stage, supernumerary larval molts, "giant" larvae, and the formation of pupal-adult intermediates with pupal cuticle and urogomphi. When applied topically to *Tribolium* pupae, compound II caused the typical JH effects. A number of terpenoid amides structurally related to II previously have been shown to have JH activity (13). The unsaturated dimethylamine III was less active than II on fourth-instar mosquito larvae (Table II), but it still showed a low level of JH activity at the highest test concentration. Although compound III caused distinct JH effects when applied topically to *Tribolium* pupae, it did not cause the typical JH effects when tested in the *Tribolium* larval diet. Instead, it was lethal to young larvae and caused 75% mortality at concentrations of 0.10-0.25%. These results were unexpected, since we have observed that *Tribolium* larvae generally tolerate a number of the more active JH compounds in the diet at concentrations from one-thousand to ten-thousand times that required to cause JH effects and to block completely the pupal-adult molt or metamorphosis in the test insects.

Compounds IV and V, the respective saturated derivatives of II and III, were both more active against first-instar mosquito larvae than against fourth-instar larvae (Table II). The lethal effects of these compounds upon fourth-instar larvae usually occurred prior to or at the time these insects molted to pupae and resulted in larval or early pupal mortality, rather than

TABLE III

Effect of Amides and Amines Fed in Combination with Sitosterol upon Sterol Composition of Tobacco Hornworm Prepupae

Compound	Concentration ppm	Percent of total sterols[a]	
		Cholesterol	Desmosterol
I	130	4.4	50.4
II	520	80.5	1.5
III	520	19.1	39.7
IV	520	65.1	1.0
V	130	13.6	45.2
VI	520	89.0	1.5
VII	65	4.7	65.1
VIII	65	5.6	58.2
IX	520	82.8	1.4
X	520	79.4	1.6
XI	33	18.0	17.0
XII	130	69.1	4.4
XIII	520	80.2	1.2
XIV	520	75.8	3.8
XV	65	39.3	30.5
XVI	65	8.4	62.5

[a]Remainder of sterol in insect was unchanged dietary sterol.

the inhibition of metamorphosis observed for compounds with JH activity. The saturated N,N-dimethylamide IV caused JH effects when applied topically to flour beetle pupae but was not nearly as active as the unsaturated dimethylamide II. Although the saturated dimethylamine V was lethal when applied topically to flour beetle pupae, its effects were quite different from those observed for compounds with JH activity. The pupae took on a grayish-brown color in the thoracic region, particularly on the legs, and many of the treated pupae did not develop further. At lower doses, a number of the adults only partially emerged from their pupal cuticle, but, when the cuticle was removed and these insects examined microscopically, no morphogenetic effects were observed. However, these partially emerged adults had varying numbers of the segments of their legs missing, and this effect also was observed in fully emerged adults from pupae treated with lower doses of the compound. Similar effects previously had been observed for compound I when it was applied topically to *Tribolium* pupae. Neither IV nor V showed JH activity when tested in the diet of *Tribolium*; instead they were lethal to young larvae.

Since a methoxy group at C_{11} has been shown either to enhance or decrease the JH activity of certain farnesenic acid derivatives, depending upon the species of insect (14), compounds VI and VII were prepared and evaluated. The 11-methoxy-N,N-dimethylamide VI was less inhibitory than IV for both first- and fourth-instar mosquito larvae and was less active on young than on mature larvae. In the latter stage, it caused typical JH effects and blocked metamorphosis. Compound VII, the 11-methoxy derivative of V, exhibited reduced lethal activity for both first- and fourth-instar mosquito larvae (Table II). Although VI caused JH effects when applied topically to *Tribolium*, it was considerably less active than IV. Compound VII was ca. equal in activity to V when tested by topical application to *Tribolium* and caused the same type of lethal effects described for V rather than the typical JH effects observed for VI. Neither of these compounds, however, caused JH effects when tested in the diet of *Tribolium*.

The N,N-dimethylamine VIII, which has the saturated carbon skeleton of the first natural insect (Cecropia) JH to be isolated and characterized (15), was more active against first-instar yellow fever mosquito larvae than compounds I-VII (Table II). It was also more effective against young larvae than against fourth-instar larvae, and, in the latter stage, it caused larval or early pupal mortality, rather than the typical JH effects. Compound VIII did not cause JH effects in *Tribolium* either when it was added to the larval diet or when it was applied topically to pupae. Instead, in the latter test, the compound brought about lethal effects similar to those observed for compound V.

Since certain straight chain alcohols and methyl ethers have been demonstrated to have JH activity (9), a number of C_{12} and C_{15} straight chain amides and amines were synthesized and tested. All four C_{12} compounds IX, X, XI, and XII were more active against first-instar mosquito larvae than against fourth-

instar larvae (Table II). The dimethylamide IX and the monoethylamine XII were as active against the young larvae as the farnesane derivatives IV and V, and compound XII was also the most active of this series against fourth-instar mosquito larvae. The C_{15} compounds XIII, XV, and XVI were the most active of the test compounds against first-instar mosquito larvae (Table II). The 2 amines, XV and XVI, were more active than their corresponding amides, and the least active of the four C_{15} compounds, the monoethylamide XIV, caused less than 75% mortality in both first- and fourth-instar mosquito larvae at the highest test concentration. None of the straight chain C_{12} and C_{15} compounds exhibited JH activity in the mosquito tests, the *Tribolium* larval test, or in the topical test with *Tribolium* pupae.

The secondary and tertiary amines and amides generally were not very effective against the immature house fly. Only 6 of the compounds were sufficiently active in the house fly test to cause 75% or greater mortality at the maximum test concentration of 375 ppm. Of these, 2 of the branched chain compounds, VII and VIII, and 3 of the straight chain compounds, IX, XII, and XVI, were lethal at 150-375 ppm. The C_{15} straight chain dimethylamine XV, which was the most active of the series in the house fly test, was lethal at 75-150 ppm.

Although the branched and straight chain compounds were considerably less effective upon tobacco hornworm larvae than the most potent azasteroids, a number of these chemicals were quite active inhibitors of growth and development in the hornworm (Table II). Only the amines had inhibitive effects upon hornworm larval development and the most active inhibitor, the C_{12} alkyl dimethylamine XI, was lethal to 75% of the hornworm larvae at 16-33 ppm. The branched chain amines, V, VII, and VIII, and the straight chain amine, XII, caused the typical azasteroid effects in the hornworm larva, whereas the other three inhibitory straight chain amines, XI, XV, and XVI, appeared to bring about their lethal effects prior to the fourth-larval instar. None of the corresponding amides inhibited hornworm development or caused JH effects at the maximum test concentration.

The unsaturated branched chain compound III was the only amine of the series that did not inhibit development in hornworm larvae at 520 ppm (Table II). However, III did inhibit the Δ^{24}-sterol reductase of the hornworm at this concentration and caused a reduction of the tissue cholesterol level to less than 20% and an increase in the desmosterol content to nearly 40% (Table III). All of the branched and straight chain amines that inhibited development, except compound XII, were Δ^{24}-sterol reductase inhibitors in the hornworm, whereas none of the corresponding amides had an appreciable effect upon this enzyme system (Table III). Compounds VII, VIII, and XVI were particularly active Δ^{24}-sterol reductase inhibitors. At 65 ppm, these amines reduced the cholesterol content in hornworm tissues to less than 9% and increased the desmosterol to nearly 60%. These branched and straight chain amines are the first nonsteroidal acyclic compounds thus far found to have Δ^{24}-sterol reductase activity.

Certain azasteroid inhibitors disrupt steroid metabolism and the hormone regulated processes of development, molting, and metamorphosis in several species of insects (1,2). A number of the secondary and tertiary branched and straight chain amines reported in this paper exhibit activities similar to the azasteroids in the tobacco hornworm and inhibit development and metamorphosis in three other species of insects. Preliminary biochemical studies with compound XI and other straight chain amines on *Tribolium* larvae suggest that these compounds interfere with sterol metabolism and inhibit the Δ^{24}-sterol reductase activity in this insect. Whether or not the branched and straight chain amines also interfere with the pathways of metabolism of the steroid molting hormones of insects, as apparently do the azasteroids (2), remains to be determined. Other possible explanations for the effects of these inhibitory amines upon the early larval stages of insects are that they disrupt insect JH metabolism or that they function as anti-JH.

This study demonstrates the feasibility of developing nonsteroidal acyclic compounds that block steroid metabolism, molting, and metamorphosis in the tobacco hornworm and certain other insects. It also reports the design and synthesis of compounds patterned after chemicals with JH activity that are more active on the early larval stages of insects than at the penultimate or ultimate molts. This research thus provides new molecular models that should permit us to expand the kinds of compounds which may be used to disrupt the hormone regulated processes of insects and may lead to new types of chemicals for safe, selective insect control.

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The methyl 10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate was a gift from R. Bagley, HLR Sciences Inc., Nutley, N.J.

APPENDIX I
IUPAC EQUIVALENT NAMES

I = N,N,δ,7a-Tetramethyloctahydro-1-H-indene-1-butanamine
II = (*E,E*)-N,N-Dimethyl-3,7,11-trimethyl-2,6-10-dodecatrienamide
III = (*E,E*)-N,N-Dimethyl-3,7,11-trimethyl-2,6,10-dodecatrienamine
IV = N,N-Dimethyl-3,7,11-trimethyldodecanamide
V = N,N-Dimethyl-3,7,11-trimethyldodecanamine
VI = N,N-Dimethyl-11-methoxy-3,7,11-trimethyl-dodecanamide
VII = N,N-Dimethyl-11-methoxy-3,7,11-trimethyl-dodecanamine
VIII = N,N-Dimethyl-7-ethyl-3,11-dimethyltridecan-amine
IX = N,N-Dimethyldodecanamide
X = N-Ethyldodecanamide
XI = N,N-Dimethyldodecanamine
XII = N-Ethyldodecanamine
XIII = N,N-Dimethylpentadecanamide
XIV = N-Ethylpentadecanamide
XV = N,N-Dimethylpentadecanamine
XVI = N-Ethylpentadecanamine

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SHORT COMMUNICATION

Adaptive Changes in Δ9 Desaturase Activity in Rat Liver

ABSTRACT

The Δ9 desaturase activity and the ^{14}C radioactivity of the de novo synthesized fatty acids incorporated into microsomal lipids and serum triglycerides were measured under different nutritional conditions. The results obtained indicate a correlation between the values of the three parameters studied after starvation or after refeeding Purina chow or either a high carbohydrate or a high protein diet. These data suggest that liver lipogenesis and Δ9 desaturase activities respond to the same regulatory factors.

INTRODUCTION

Changes in the activity of the Δ9 desaturase under different nutritional and hormonal states have been described. The activity of this enzyme is depressed by food deprivation (1-3) and diabetes (4-6) and returns to normal values on refeeding (7-8) and insulin treatment (4,9), respectively. The highest values of Δ9 desaturase activity were elicited by high carbohydrate diets, especially when fructose or glycerol were included in those diets (10). Fructose or glycerol feeding (11,12) enhances hepatic lipogenesis, probably by increasing the hepatic concentration of α-glycerophosphate. The in vivo Δ9 desaturase activity apparently was induced by the increase in the endogenous or exogenous supply of saturated fatty acids. Dietary saturated fatty acids were able to restore the Δ9 desaturase activity depressed by the diabetic state and to increase the activity of the enzyme in normal rats (10). In this experiment, the Δ9 desaturase activity and the ^{14}C radioactivity of the de novo synthesized fatty acids incorporated into microsomal lipids and serum triglycerides were measured under different nutritional conditions.

EXPERIMENTAL PROCEDURES

1-^{14}C stearic acid (54.0mC/mmole), 99% radiochemically pure, was purchased from The Radiochemical Centre, Amersham, England. 1-^{14}C-acetic acid, sodium salt (5.0 mC/mmole) was purchased from Comision Nacional de Energia Atomica, La Plata, Argentina. Stearic acid, 99% pure, was obtained from Lipids Preparation Laboratory, Hormel Institute, Austin, Minn.

Male Wistar rats weighing 150-170 g were used throughout. The animals were maintained on a Purina chow diet and water ad libitum before use. Five groups of five animals each were used. A control group was continued on the same diet for the experimental period. The fasted group was fasted for 48 hr and allowed water ad libitum before being killed. A refed group was fasted for 48 hr and then refed with Purina chow for 24 hr. A high carbohydrate diet group was fed a diet containing 70% dextrin, 23% casein, 3% corn oil, 4% salt mixture (13), and vitamins (14) for 3 days before being killed. A high protein diet group was fed for 3 days on a diet containing 70% casein, 23% dextrin, 3% corn oil, 4% salt mixture, and vitamins. All groups of rats were injected intraperitoneally with 0.2 ml labeled solution containing 50 μC of 1-^{14}C sodium acetate. One hr after injection, the animals were killed by decapitation. Blood was collected, and the liver was separated and maintained in an ice bath until it was used for microsome isolation.

The serum lipids were extracted by the method of Folch, et al., (15), and the triglycerides were separated from the original chloroform-methanol extract by thin layer chromatography (TLC) (16) in petroleum ether-ether-acetic acid (80/20/1, v/v/v) and were extracted from the silica using chloroform-methanol (2/1, v/v). The amount of triglycerides, separated as described above, was determined by a glycerol analysis according to Snyder and Stephens (17). An aliquot of each sample was assayed for radioactivity in a Packard scintillation spectrometer.

One hepatic lobe was homogenized in a cold solution of 0.25 M sucrose (4/1, v/v). From this homogenate, washed microsomes were obtained according to Bock, et al., (18). The final pellet was resuspended in distilled water. From this suspension, lipids were extracted and assayed

for radioactivity. Protein was determined by the Lowry method (19).

Liver tissue was homogenized, and the microsomes were isolated by differential centrifugation, as previously described (20). Incubation procedure for 1-^{14}C stearic acid desaturation was performed, as previously described (12). The conversion of labeled stearic acid to oleic acid was measured by TLC of the fatty acid methyl esters on $AgNO_3$ impregnated silica gel plates (21).

RESULTS

The liver Δ9 desaturase activity measured under different nutritional conditions is given in Table I. As is clearly shown, 48 hr of starvation significantly depressed the activity of the fatty acid desaturase enzyme. However, when fasted animals were fed the chow diet, a marked stimulation of Δ9 desaturation occurred within 24 hr. When animals previously fed a chow diet subsequently were fed with a high carbohydrate diet, Δ9 desaturation was stimulated very significantly as compared with the controls. In contrast, a high protein diet did not modify significantly the activity of the enzyme when compared with the control group.

Table II shows the radioactivity of de novo synthesized fatty acids incorporated into microsomal lipids and into serum triglycerides of rats under different nutritional conditions.

The amount of radioactivity incorporated into microsomal lipids was depressed significantly during starvation and increased to ca. six times its normal level by refeeding. These results agree with those of Allmann, et al., (3), demonstrating that refeeding starved animals permits the level of fatty acid synthetic enzymes to rise far above normal. Meanwhile, the high carbohydrate diet markedly increased the radioactivity from ^{14}C acetate incorporated into fatty acids, and, as was previously described (22-24), the high protein diet did not alter the response of fatty acid synthetic enzymes.

The values of the ^{14}C radioactivity of the de novo synthesized fatty acids incorporated into serum triglycerides apparently indicate a correlation with those above described changes.

The results reported in the present paper show that liver lipogenesis activity depends upon the nutritional state of the animals. The fact that refeeding or feeding a high carbohydrate diet was associated with an increase of the liver lipogenesis and with a high activity of Δ9

TABLE I

In Vitro Oxidative Desaturation of Stearic Acid to Oleic Acid by Liver Microsomes

Group[a]	Percent conversion[b] 18:0 → 18:1
Control (5)	6.5 ± 2.1
Fasted (5)	3.0 ± 0.8 P < 0.01
Refed (5)	17.3 ± 5.6 P < 0.01
Fed high carbohydrate (5)	30.2 ± 7.6 P < 0.001
Fed high protein (5)	6.7 ± 1.4 NS

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

[b]Probability (P) values are related to controls. Data are the means ± standard deviation. NS = not significant.

TABLE II

^{14}C Radioactivity of De Novo Synthesized Fatty Acids Incorporated into Liver Microsomal Lipids and Serum Triglycerides

Group[a]	dpm/mg Microsomal protein[b]	dpm/μmol Serum triglycerides[c]
Control (5)	1,430 ± 580	7,651
Fasted (5)	577 ± 312 P < 0.02	1,604
Refed (5)	9,192 ± 1,900 P < 0.001	12,175
Fed high carbohydrate (5)	14,077 ± 3,900 P < 0.001	29,302
Fed high protein (5)	2,512 ± 675 PNS	8,707

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

[b]Probability (P) values are related to normal animals. Data are the means ± standard deviations. NS = not significant.

[c]Each value corresponds to the mean of five individual pooled samples.

desaturase suggests that both metabolic activities respond to the same regulatory factors. In the light of these results, it is interesting to note that both enzymatic activities rise far above normal on refeeding. Further work on this subject is in progress.

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LETTER TO THE EDITOR

Concerning Lipid Composition of *Sapindus mukorossi* Seed Oil

Sir: A recent paper in this journal (A. Sengupta, S.P. Basu, and S. Saha, *Lipids* 10:33 [1975]) describes a study of the triglyceride structure of *Sapindus mukorossi* (family Sapindaceae) seed oil. In their thin layer chromatographic examination of this oil, Sengupta and coworkers observed the presence of a component more polar than ordinary triglycerides, and they speculate that this may be a triglyceride which contains estolide groups. Published work from our laboratory (K.L. Mikolajczak and C.R. Smith, *Lipids* 5:182 [1970] and earlier papers cited therein) and elsewhere (M.G. Kasbekar, R.R. Talekar, and N.V. Bringi, *Indian J. Chem.* 10:244 [1972]; D. Seigler, *Phytochemistry* 13:841 [1974]) has established that previously unrecognized cyanolipids occur in considerable amounts in seed oils of many sapindaceous species, including *S. mukorossi*. Cyanolipids are not glycerides, but instead are derivatives of five-carbon hydroxynitrile moieties esterified with long chain fatty acids. On the basis of previous experimental results, we feel reasonably certain that cyanolipids are responsible for the large spots below the triglycerides in thin layer chromatograms (Figs. 2 and 3) shown by Sengupta, et al., in their paper (cited above).

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ERRATUM

An error occurred in the publication of the article, "Comparison of Lipid Composition of *Aedes aegypti* and *Aedes albopictus* Cells Obtained from Logarithmic and Stationary Phases of Growth" by E. McMeans, T.K. Yang, L.E. Anderson, and H.M. Jenkin (*Lipids* 10:99-104, 1975). The last sentence of the publication should read: "Results of studies by other investigators using Grace's *A. aegypti* cells should now be attributed to *A. eucalypti* cells."

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Essential Fatty Acid Deficiency: Metabolism of 20:3(n-9) and 22:3(n-9) of Major Phosphoglycerides in Subcellular Fractions of Developing and Mature Mouse Brain

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ABSTRACT

Essential fatty acid deficiency was initiated in young and mature mice. The metabolism of 20:3(n-9) and 22:3(n-9) in brain subcellular fractions was followed after the mice were switched from the deficient diet to a corn oil supplemented diet. After switching to the supplemented diet, the proportions of (n-9) polyunsaturated fatty acids in brain in both groups of mice decreased with time. The rate of disappearance of (n-9) polyunsaturated fatty acids was faster in the young groups than in the mature group. In the developing mice, the half-lives of the (n-9) polyunsaturated fatty acids in the total ethanolamine phosphoglycerides of brain microsomal, synaptosomal, and myelin fractions were 3, 10, and 15 days, respectively. In the mature group, the half-lives for 20:3(n-9) in diacyl-glycerophosphorylethanolamine of microsome, synaptosome, and myelin fractions were 8-10, 10, and 22 days, respectively; and the half-lives for 22:3(n-9) in alkenylacyl-glycerophosphorylethanolamine of the same subcellular fractions were 8-12, 28, and 35-40 days, respectively. In general, the rate of disappearance of 20:3(n-9) in brain was faster in the diacyl-glycerophosphorylethanolamine than in the alkenylacyl-glycerophosphorylethanolamine. These results demonstrate that the metabolism of (n-9) polyunsaturated fatty acid in brain phosphoglycerides during recovery from essential fatty acid deficiency not only varies with age, but also depends upon individual phosphoglycerides present in each subcellular fraction.

INTRODUCTION

It has been well recognized that during essential fatty acid (EFA) deficiency, there is a decrease in the proportion of 20:4(n-6) in the acyl groups of phosphoglycerides among various body tissues (1). The decrease in proportion of 20:4(n-6) usually is marked by an increase of 20:3(n-9), a new fatty acid presumably derived from 18:1(n-9) during the deficient state. Past studies on lipid changes among various body tissues during EFA deficiency have been rather extensive (2). However, in the more recent investigations, changes in brain lipids during the deficiency have been studied (3-7). Unlike other body tissues, the brain is especially rich in long chain polyunsaturated fatty acids (PUFA). Not only are specific acyl group profiles attributed to individual phosphoglycerides, they are also different in the subcellular fractions (8).

In a previous study, we have demonstrated that the increase in proportions of (n-9) PUFA in brain was greater when the deficient diet was initiated during the early developmental period (9). The ability of brain tissue to "recover" from EFA deficiency has been examined by White, et al., (10) with rats by measuring the decrease of 20:3(n-9) in brain after switching the animals to a corn oil supplemented diet. In the present investigations, we have further examined the metabolism of (n-9) PUFA in brain phosphoglycerides at a subcellular level during brain recovery from EFA deficiency. We intend to use this information to evaluate the metabolism of these acyl groups in brain phosphoglycerides during the early developmental period and after maturation.

MATERIALS AND METHODS

Animal Subjects and Induction of EFA Deficiency

Two experiments were performed with C57BL/10J mice which were purchased from Jackson Laboratory (Bar Harbor, Maine). In the first experiment, pregnant mice were obtained ca. 10 days prior to delivery. After being transferred to the laboratory, they were individually placed into plastic cages with stainless steel covers. All of the animals received water and diet ad libitum. One group of the pregnant mice was given a fat free diet and the other group was given a control diet supplemented with corn oil (General Biochemicals, Chagrin Falls, Ohio). The fatty acid-deficient diet contained the following ingredients (g/kg): casein, 240; sucrose, 710; salt mix no. 2, U.S.P. XIII (catalog no. 170870), 39; vitamin supplement

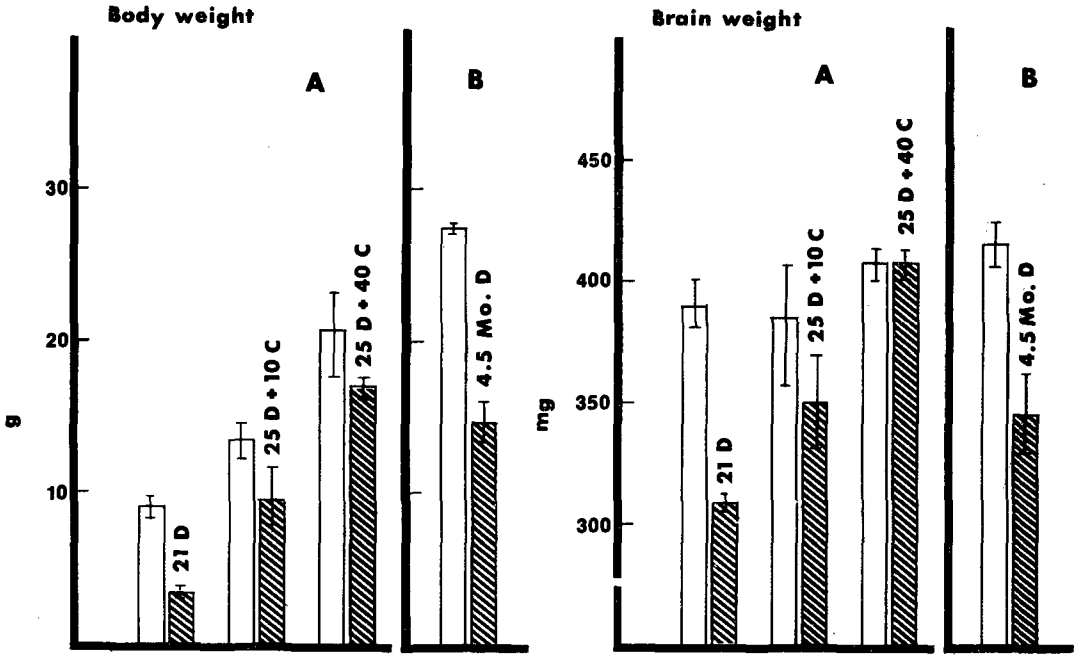

FIG. 1. (A) A comparison of the brain and body wt of control and essential fatty acid (EFA) deficient mice at 21 days of age and at 10 and 40 days after supplementing with a corn oil diet. (B) A comparison of the brain and body wt of control and EFA deficient mice at 4.5 months of age. □ = control and ▨ = deficient.

(GBI Technical Bulletin V-17), 10; and non-nutritive fiber, 9. The fatty acid-supplemented diet had the same composition as the deficient diet, except that corn oil (2%, w/w) was added. Chromatographic analysis of the fatty acid composition showed that the corn oil supplemented diet contained 16:0, 13%; 18:0, 2%; 18:1, 25%; and 18:2, 60%. After delivery, the litters were kept with the same mothers until 21 days. After this period, the litters were weaned. At 21 days, 3 young mice from each of the dietary groups were sacrificed. The brains were dissected and individually homogenized in 0.32 M sucrose. The remaining young mice in the deficient group then were switched to a control diet supplemented with corn oil. The weanlings in the control group also were given the corn oil supplemented diets at this time. At 10 and 40 days after switching to the supplemented diet, 3 mice from each dietary group were sacrificed. The brains were homogenized individually in 0.32 M sucrose, and the brain homogenates were further subjected to subcellular fractionation and lipid analysis.

In the second experiment, mice were treated similarly, except that the pregnant mice were given Purina Lab Chow instead of the synthetic diets. After weaning, the young mice also were divided into two groups, one of which was given the fatty acid deficient diet and the other the corn oil supplemented diet. These young mice were reared on this dietary scheme for a period of 3 months, after which 3 mice from each dietary group were sacrificed. The brains were dissected individually and homogenized in 0.32 M sucrose as described above. The remaining group of mice with the deficient diet then was switched to the corn oil supplemented diet. After the dietary reversal, groups of three mice were sacrificed at 1, 3, and 5 weeks, and the brain homogenates were prepared similarly.

Subcellular Fractionation of Brain Homogenates

At the end of each period, the brains were dissected and individually homogenized in 20 volumes of 0.32 M sucrose solution containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $MgCl_2$, and 15 mM Tris buffered at pH 7.4. The brain homogenates were further subjected to differential and sucrose gradient centrifugation to obtain the myelin, synaptosome-rich, and microsomal fractions. The procedure for subcellular fractionation was essentially the same as described previously (11), except that discontinuous sucrose gradient was used for isolating the synpatosome-rich fractions. The synaptosome-rich fraction was obtained by isolating the material at the 0.8 M and 1.2 M sucrose interphase after gradient centrifugation. Purity of the synaptosome-rich

TABLE I

Acyl Group Composition of Ethanolamine Phosphoglycerides in Synaptosome-Rich Fraction of Mouse Brain during Essential Fatty Acid Deficiency (21 Days of Age) and at 10 and 40 Days after Dietary Reversal[a]

	21 Days		10 Days reversal		40 Days reversal	
Fatty acids	C	D	C	D	C	D
			Percentage, wt			
16:0	7.0	5.0	5.8	8.3	5.8	6.5
18:0	22.0	23.3	22.7	23.3	23.1	23.1
18:1	11.5	10.5	12.7	13.0	15.0	14.9
20:1	1.1	1.2	2.4	1.9	3.7	3.4
20:3(n-9)	-	3.5	-	1.5	-	-
20:3(n-6)	0.7	-	0.6	-	-	-
20:4(n-6)	21.4	20.7	19.5	19.1	17.5	17.4
22:3(n-9)	-	1.6	-	0.7	-	-
22:4(n-6)	7.3	6.0	7.9	6.3	6.9	5.9
22:5(n-6)	2.8	3.2	4.5	3.8	2.8	2.7
22:6(n-3)	25.7	24.8	23.9	22.3	25.7	26.1

[a]Values are mean percentages from three brain preparations. Variations did not exceed 10% of the peak area. See text for details of experimental procedure. C = control diet, and D = deficient diet.

TABLE II

Acyl Group Composition of Ethanolamine Phosphoglycerides in Microsomal Fraction of Mouse Brain during Essential Fatty Acid Deficiency (21 Days of Age) and at 10 and 40 Days after Dietary Reversal[a]

	21 Days		10 Days reversal		40 Days reversal	
Fatty acids	C	D	C	D	C	D
			Percentage, wt			
16:0	10.2	5.8	12.9	13.5	7.4	7.7
18:0	24.7	23.4	23.1	24.7	26.4	27.5
18:1	7.1	6.8	6.5	6.5	10.2	10.0
20:1	0.7	1.1	1.1	0.7	2.6	2.2
20:3(n-9)	-	3.6	-	1.3	-	-
20:4(n-6)	17.7	17.7	15.8	17.4	14.5	14.3
22:3(n-9)	-	1.4	-	0.4	-	-
22:4(n-6)	8.3	6.1	6.1	5.4	5.4	4.8
22:5(n-6)	3.0	3.6	5.1	4.5	3.7	3.1
22:6(n-3)	28.0	28.8	29.2	27.5	30.0	30.6

[a]Values are mean percentages from three brain preparations. C = control diet, and D = deficient diet.

and purified myelin fractions has been assessed previously (11). The procedure for isolating subcellular membrane fractions was followed closely in each experiment in order to obtain good reproducibility in lipid analysis.

Analysis of Lipids from Brain Subcellular Fractions

The membranous pellet from each subcellular fraction was further suspended in 7 ml water, and total lipids were extracted by chloroform-methanol, 2:1 (v/v). A small portion of the total lipid extract was applied to a thin layer plate which was coated with Silica Gel G suspended in 0.01 M Na_2CO_3 (Brinkmann Instrument, Westbury, N.Y.). Individual phospholipids were separated by reactional two dimensional thin layer chromatography (TLC) (12) with modified solvent system for the second dimension (13). After solvent development, lipid spots were visualized by spraying the thin layer plates with 2′,7′-dichlorofluorescein in ethanol (Applied Science Laboratory, State College, Pa.). The phospholipid spots were scraped from the thin layer plates into individual test tubes. They were further subjected to alkaline methanolysis for converting the acyl groups to fatty acid methyl esters (14). Analysis of fatty acid methyl esters was

TABLE III

Acyl Group Composition of Ethanolamine Phosphoglycerides in Myelin Fraction Isolated from Mouse Brain during Essential Fatty Acid Deficiency (21 Days of Age) and at 10 and 40 Days after Dietary Reversal[a]

Fatty acids	21 Days		10 Days reversal		40 Days reversal	
	C	D	C	D	C	D
			Percentage, wt			
16:0	7.2	8.0	5.4	5.7	5.2	5.1
18:0	13.1	12.9	14.0	16.3	13.6	13.8
18:1	26.1	27.9	25.4	26.4	34.2	32.8
20:0	3.5	2.7	-	-	-	-
20:1	9.5	9.8	11.3	10.3	17.1	15.8
20:3(n-9)	-	3.8	-	1.8	-	-
20:3(n-6)	2.3	-	1.2	-	-	-
20:4(n-6)	14.0	9.3	13.9	13.3	14.1	14.7
22:3(n-9)	-	2.9	-	1.6	-	-
22:4(n-6)	11.7	7.4	14.0	11.5	7.6	8.4
22:5(n-6)	1.3	1.5	1.8	1.4	1.1	1.1
22:6(n-3)	10.5	11.2	12.1	11.8	7.2	8.4

[a]Values are mean percentages from three brain preparations. C = control diet, and D = deficient diet.

achieved by gas liquid chromatography (GLC) with dual 6 ft columns packed with ethylene glycol succinate methyl silicone copolymer on Gas Chrom-P (Applied Science Laboratory). The conditions for analysis of fatty acid methyl esters by a Hewlett Packard Research GLC were described previously (8). Integration of peak areas was done by means of a Hewlett Packard digital integrator. Repeated analysis of the same sample showed good reproducibility with variations less than 5% of the peak area. Some variations did arise due to reliability in subcellular fractionation, but such errors were always within 10% of the peak area.

RESULTS

Newborn mice from mothers fed a fat-deficient diet during the latter part of pregnancy were lower in brain and body wt when compared to controls from mothers fed a normal diet (Fig. 1). Other clinical symptoms indicating EFA deficiency, such as scaling of the skin and increased water consumption were already evident in the deficient group even at this early age. However, when the young mice were switched to a supplemented diet containing corn oil, their body growth was accelerated. Within 40 days after dietary supplement, the deficient mice had matched or exceeded slightly the control group in body wt.

When the newborns were fed a fat-deficient diet until weaning time, 5-7% of 20:3(n-9) and 22:3(n-9) was found in the ethanolamine phosphoglycerides in brain (Tables I-III). The actual proportion of the (n-9) acyl groups varied slightly with individual phosphoglycerides and subcellular fractions. In general, the ratio of 20:3(n-9) to 22:3(n-9) was ca. 2:1 (Fig. 2). When the fat-deficient diet was initiated to the weanlings and was maintained for a period of 3 months, there was 5-8% of 20:3(n-9) in the ethanolamine phosphoglycerides (Fig. 3). Further analysis of the acyl group composition of the two types of ethanolamine phosphoglycerides revealed that the percent of 20:3(n-9) was higher in alkenylacyl-glycerophosphorylethanolamine (GPE) than in diacyl-GPE (Fig. 3). On the other hand, the proportions of 20:3(n-9) in diacyl-GPE and diacyl-glycerophosphorylserine (GPS) was relatively low (less than 2% of the total) (Fig. 4). In the phosphoglycerides from this group of mice, we also have observed a general increase in the percent of oleic acid (18:1(n-9)) (Tables IV-VI).

Brain recovery from EFA deficiency by switching the animals to a diet supplemented with corn oil showed a decrease in the percent of 20:3(n-9) and 22:3(n-9) in the phosphoglycerides of brain subcellular fractions. In the group I mice, the proportions of 20:3(n-9) and 22:3(n-9) in brain subcellular fractions decreased by ca. one-half within 10 days after the dietary reversal (Tables I-III). In fact, we did not find any appreciable amount of the (n-9) PUFA fatty acids in the brain subcellular fractions when analysis was made at 40 days after dietary supplementation (Fig. 2).

There were also obvious age-related changes in acyl group composition of brain phosphoglycerides during the early developmental period. Therefore, for proper comparison of results with samples in the first group, the deficient mice were properly matched with

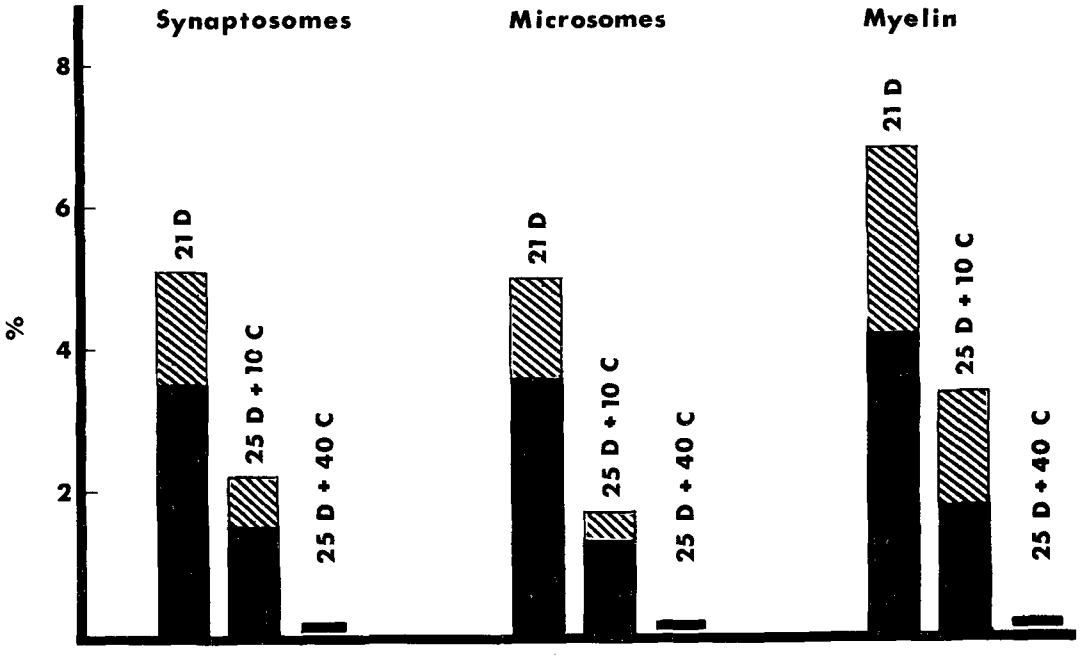

FIG. 2. The proportion of 20:3(n-9) and 22:3(n-9) in ethanolamine phosphoglycerides from synaptosome-rich, microsome, and myelin fractions during essential fatty acid deficiency and at 10 and 40 days after dietary reversal. The fatty acid deficient diet was initiated prenatally up to 21 days of age. ▨ = 22:3(n-9) and ■ = 20:3(n-9).

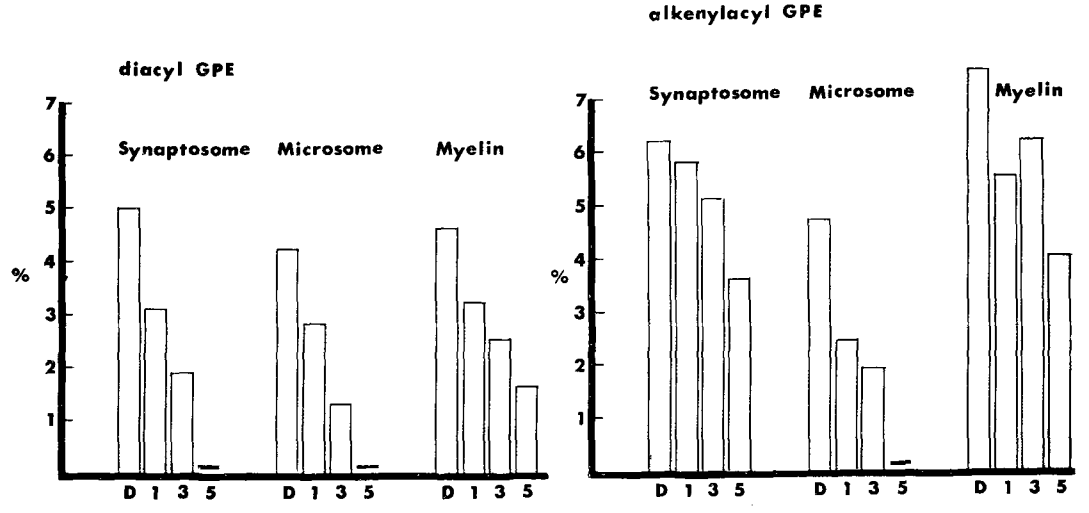

FIG. 3. The proportion of 20:3(n-9) in individual phosphoglycerides of the synaptosome-rich fraction in mature mouse brain (4 months of age) during essential fatty acid deficiency and at 1, 3, and 5 weeks after dietary reversal. Results are the mean percentages of acyl group composition from 3 samples.

controls of the same age. During development, there was an increase in the proportions of 18:1 and 20:1 and a decrease in 20:4(n-6) in most phosphoglycerides (Tables I-III). Acyl group changes during development were especially evident with the ethanolamine phosphoglycerides in the myelin fraction (Table III).

When the mature mice which had been fed a deficient diet for 3 months and were subsequently switched to a control diet supplemented with corn oil, decreasing percent of (n-9) PUFA fatty acids also was observed during the reversal. However, the rate of disappearance of 20:3(n-9) in the mature brain was

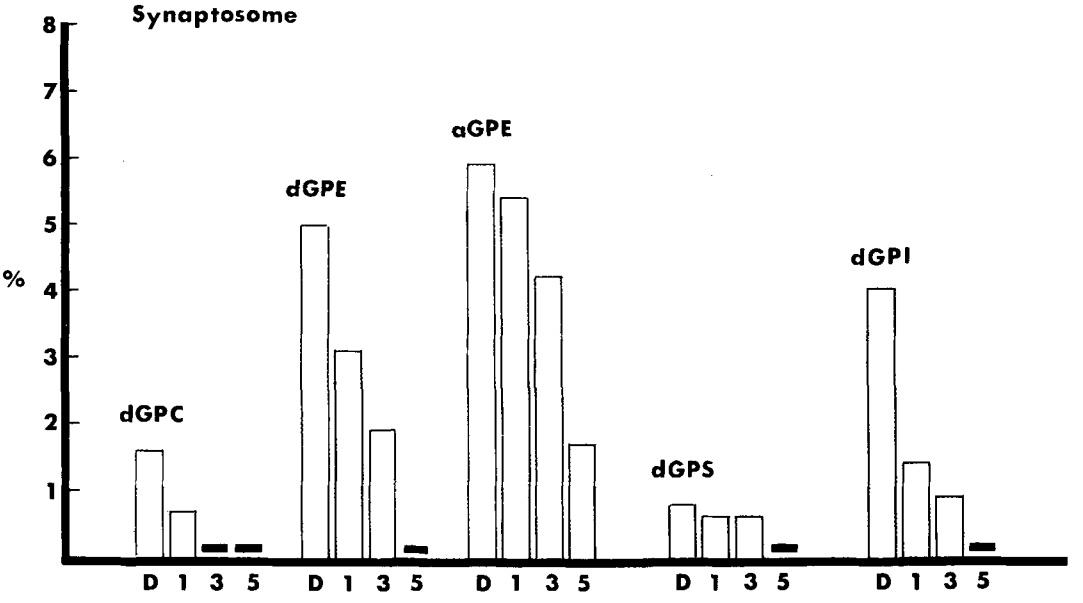

FIG. 4. The proportion of 20:3(n-9) in diacyl-glycerophosphorylethanolamine and alkenylacyl-glycerophosphorylethanolamine in different subcellular fractions during essential fatty acid deficiency and at 1, 3, and 5 weeks after dietary reversal. Results are the mean percentages of acyl group composition from 3 samples.

TABLE IV

Acyl Group Composition of Diacyl-Glycerophosphorylethanolamine (GPE) and Alkenylacyl-GPE in Myelin Fraction of Mouse Brain during Essential Fatty Acid Deficiency (4 Months of Age) and at 1, 3, and 5 Weeks during Recovery[a]

Fatty acids	Diacyl-GPE					Alkenylacyl-GPE				
	C	D	1R	3R	5R	C	D	1R	3R	5R
	Percentage, wt									
16:0	8.0	7.8	7.2	9.1	9.1	2.7	2.0	2.8	2.7	1.8
18:0	31.5	29.8	30.6	30.1	32.7	1.6	0.9	1.6	2.4	1.1
18:1	23.4	27.7	30.3	25.5	20.6	34.9	41.0	39.1	34.9	32.6
20:1	4.2	5.1	6.8	4.5	3.8	20.2	20.4	23.2	19.4	19.0
20:3(n-9)	-	4.6	3.2	2.5	1.6	-	7.5	5.5	6.7	4.0
20:4(n-6)	14.8	11.6	8.5	12.0	14.1	16.0	9.3	7.2	9.7	13.2
22:3(n-9)	-	1.3	1.6	1.3	0.8	-	3.5	4.0	4.5	3.5
22:4(n-6)	4.6	2.2	2.9	3.4	3.8	12.4	6.3	8.8	9.6	12.0
22:5(n-6)	1.4	0.7	1.1	2.0	1.6	2.0	2.2	-	1.1	1.3
22:6(n-3)	11.2	9.7	8.4	9.8	12.8	9.3	7.1	7.2	9.2	11.5

[a]Values are mean percentages from 3 samples except for the controls (C) which are means from 6-9 samples. The variation in results did not exceed 10% of the peak area. C = control diet, D = deficient diet, and R = reversal from deficient diet to control diet.

slower than that observed in the first group. The rate of disappearance of 20:3(n-9) also varied with individual phosphoglycerides and subcellular fractions. At 3 weeks after the dietary reversal, the proportion of 20:3(n-9) in diacyl-glycerophosphorylcholine (GPC) had become negligible, but a significant proportion of 20:3(n-9) was still present in the diacyl-GPE and alkenylacyl-GPE, especially in the myelin fraction (Table IV). Furthermore, the decrease in percent of 20:3(n-9) was usually faster in the diacyl-GPE than the alkenylacyl-GPE.

The diacyl-glycerophosphorylinositol (GPI) of the synaptosome-rich fraction normally contained a high proportion of 20:4(n-6). However, the decrease in percent of 20:4(n-6), as well as the increase in 20:3(n-9), in the diacyl-GPI during EFA deficiency was not as extensive as those observed with other phosphoglycerides, such as the diacyl-GPE (Fig. 4). On the other hand, we have observed a rapid decrease in the proportion of 20:3(n-9) in diacyl-GPI after dietary

TABLE V

Acyl Group Composition of Diacyl-Glycerophosphorylethanolamine (GPE) and Alkenylacyl-GPE in Synaptosome-Rich Fraction of Mouse Brain during Essential Fatty Acid Deficiency (4 Months of Age) and at 1, 3, and 5 Weeks during Recovery

	Diacyl-GPE					Alkenylacyl-GPE				
Fatty acids	C	D	1R	3R	5R	C	D	1R	3R	5R
					Percentage, wt					
16:0	9.1	9.3	8.3	9.6	9.5	5.1	3.0	3.6	4.1	6.1
18:0	35.9	33.9	34.5	33.0	37.4	3.8	2.7	3.9	1.9	2.4
18:1	11.7	11.5	14.9	12.9	12.1	14.6	16.9	18.6	15.2	14.3
20:1	-	-	-	-	-	4.7	6.2	5.8	5.1	3.6
20:3(n-9)	-	5.0	3.1	1.9	-	-	5.9	5.4	4.2	1.7
20:4(n-6)	14.2	10.8	11.8	13.7	13.4	18.6	12.4	13.2	15.3	17.6
22:3(n-9)	-	-	0.8	0.8	-	-	2.2	3.0	2.7	-
22:4(n-6)	3.8	3.5	2.6	2.9	3.0	13.3	7.7	8.7	10.9	12.2
22:5(n-6)	2.8	2.0	1.2	2.1	2.7	3.1	1.7	2.3	2.6	3.2
22:6(n-3)	22.5	25.5	22.0	23.3	21.4	36.7	41.4	35.5	37.0	38.4

[a]Values are mean percentages from 3 samples except for the controls (C) which are means from 6-9 samples. The variation in results did not exceed 10% of the peak area. C = control diet, D = deficient diet, and R = reversal from deficient diet to control diet.

TABLE VI

Acyl Group Composition of Diacyl-Glycerophosphorylethanolamine (GPE) and Alkenylacyl-GPE in Microsomal Fraction of Mouse Brain during Essential Fatty Acid Deficiency (4 Months of Age) and at 1, 3, and 5 Weeks during Recovery

	Diacyl-GPE					Alkenylacyl-GPE			
Fatty acids	C	D	1R[a]	3R	5R	C	D	1R[a]	3R
					Percentage, wt				
16:0	9.3	10.9	9.0	8.7	10.0	4.2	3.8	6.4	2.9
18:0	34.9	33.1	32.0	34.1	33.9	2.6	3.1	6.4	2.1
18:1	11.3	12.9	12.6	10.3	13.1	11.9	16.9	12.8	10.7
20:1	-	0.8	0.9	1.0	-	6.2	6.9	6.1	6.3
20:3(n-9)	-	4.2	2.8	1.3	-	-	4.7	2.4	1.9
20:4(n-6)	12.6	9.6	10.6	11.5	10.6	14.5	11.9	11.1	15.2
22:3(n-9)	-	-	0.8	-	-	-	1.7	1.7	1.6
22:4(n-6)	3.0	2.0	2.9	3.6	3.2	9.8	7.3	7.4	9.1
22:5(n-6)	2.0	1.2	2.3	2.8	3.5	2.6	1.5	3.3	3.3
22:6(n-3)	26.6	25.4	27.1	27.1	25.8	48.1	43.1	42.7	45.0

[a]Mean values were obtained from two samples only. C = control diet, D = deficient diet, and R = reversal from deficient diet to control diet.

reversal (Fig. 4).

DISCUSSION

The main object for conducting the present experiment was to make use of the 20:3(n-9) and 22:3(n-9) fatty acids synthesized during EFA deficiency as a marker for studying the metabolism of long chain PUFA in brain subcellular fractions. In previous experiments, we have studied the metabolism of phosphoglycerides in brain subcellular fractions after intracerebral injections of labeled palmitate and oleate (13,15). Results of the experiments with labeled precursors indicated an equilibration of radioactivity of the brain phosphoglycerides among the subcellular fractions. Although radioactivity was distributed rapidly among the microsomal and synaptosomal fractions, the equilibration of radioactivity in phosphoglycerides between the microsomal and myelin fractions required a longer period of 7-14 days. Due to the slow rate of equilibration of radioactivity, the turnover of myelin lipids could not be studied by the pulse labeling method.

Although the 20:3(n-9) and 22:3(n-9) fatty acids present in brain during EFA deficiency occurred in all of the subcellular fractions, a greater portion of them was found in the two major types of ethanolamine phosphoglycerides. Since the myelin membranes are rich in alkenylacyl-GPE, we also have observed a marked increase in the proportion of (n-9)

TABLE VII

Ratios of 20:3(n-9)/20:4(n-6) in Phosphoglycerides of Mouse Brain Subcellular Fractions during Essential Fatty Acid Deficiency (4 Months of Age) and at 1, 3, and 5 Weeks after Dietary Reversal[a]

Subcellular fractions	Weeks after dietary reversal	Diacyl-GPC	Alkenylacyl-GPE	Diacyl-GPE
Synaptosomes	0	0.35	0.48	0.46
	1	0.17	0.41	0.26
	3	0	0.27	0.14
	5	0	0.10	0
Microsomes	0	0.36	0.39	0.44
	1	0.12	0.22	0.26
	3	0	0.13	0.11
	5	0	0	0
Myelin	0	0.30	0.81	0.40
	1	0.17	0.76	0.38
	3	0	0.69	0.21
	5	0	0.30	0.11

[a]The ratios were derived from data in Tables IV-VI. GPE = glycerophosphorylethanolamine, GPC = glycerophosphorylcholine.

PUFA in the myelin during EFA deficiency (Table IV).

When the fat-deficient mice were switched to a control diet supplemented with corn oil, they readily recovered from the deficiency as indicated by the acceleration in body wt gain and the disappearance of the (n-9) PUFA in the phosphoglycerides among body organs. In 1971, White, et al., (10) also reported complete rehabilitation of body size of rat upon switching to the supplemented diet. In their study, brain recovery with respect to dietary reversal was not studied as a function of time. The percent of (n-9) PUFA present in the mature brain also decreased upon dietary reversal but seemingly at a slower rate than the developing brain. In the group of fat-deficient young mice given a supplemented diet after weaning, the proportions of 20:3(n-9) and 22:3(n-9) decreased by half within 10 days after dietary reversal (Fig. 2). During the rapid brain development, a measurement of the decrease of (n-9) PUFA in the myelin fraction was complicated by the amount of myelin synthesized between 21-60 days. According to the data, Norton and Poduslo obtained on the rats (16), this period may have accounted for an increase of ca. 30% of the adult myelin sheath. Therefore, we have used this factor for making a correction in determining the half-life of the myelin lipids. With this correction, the estimated half-life for the (n-9) PUFA of ethanolamine phosphoglycerides in myelin is 10-15 days. On the other hand, the (n-9) PUFA of ethanolamine phosphoglycerides in the microsomal and synaptosomal fractions showed shorter half-lives of 3 and 10 days, respectively, without accounting for the rate of synthesis.

In the second group of mice, i.e. mice were induced EFA deficiency at weaning for a period of 3 months, results indicate that the rate of disappearance of (n-9) fatty acids in the brain phosphoglycerides is slower than that observed in the young mouse brain. There is also a difference in turnover rate of acyl groups of the diacyl-GPE and alkenyl-acyl-GPE. The half-lives estimated for 20:3(n-9) of diacyl-GPE and alkenylacyl-GPE in the synaptosomal fractions are 10 and 28 days, respectively. The half-lives for diacyl-GPE and alkenylacyl-GPE in the myelin fraction are 22 and 35-40 days, respectively. In the microsomal fraction, a half-life of 8-10 days is estimated for the turnover of 20:3(n-9) in both diacyl-GPE and alkenylacyl-GPE.

In Table VII, the ratios of 20:3(n-9)/20:4(n-6) of the three major phosphoglycerides are expressed as a function of time during brain recovery from EFA deficiency. Holman (17) had initiated the use of this ratio to denote the degree of deficiency in tissue lipids during EFA deficiency. It is shown in this table that the deficient index was small in the case of diacyl-GPC. This is expected, because the diacyl-GPC in brain normally contains only a small proportion of the PUFA (14). During dietary reversal, the disappearance of 20:3(n-9) is related also to an increase in the proportion of 20:4(n-6), thus causing a rapid decrease of the ratio. Data in Table VII further demonstrates that the decrease of ratios with time was in the general order: microsomes > synaptosomes > mylein. Results from the present experiments are in good agreement with those from the intracerebral injection of labeled

fatty acid precursors (13,15). Differences in turnover of brain phosphoglycerides at a subcellular level have been reported also by injecting ^{14}C-acetate intraperitoneally into developing rats (18). In a previous study, Mead and Dhopeshwarkar (19) examined the metabolism of fatty acids in brain by feeding the rats labeled fatty acids. Their results indicated that the fatty acids are incorporated actively into the brain phosphoglycerides with considerable "turnover."

One other point of interest concerns the acyl group changes in diacyl-GPI during EFA deficiency and its recovery after dietary supplementation. In spite of the fact that the diacyl-GPI in brain is known to contain a high proportion of 20:4(n-6) (20), a relatively small proportion of 20:3(n-9) appeared in this phosphoglyceride during EFA deficiency (Fig. 4). Furthermore, the 20:3(n-9) present in diacyl-GPI during EFA deficiency disappeared more readily than that in other phosphoglycerides upon dietary reversal. The fast turnover of diacyl-GPI implies that the metabolism of this phosphoglyceride in brain must be very rapid. A similar observation has been reported by Smith and Eng (18) using labeled acetate as precursor. Probably a high proportion of 20:4(n-6) in diacyl-GPI is important for maintaining the phosphoglycerides in an active state.

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Lipid Profiles of Plasma Lipoproteins of Fasted and Fed Normal and Choline-Deficient Rats

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ABSTRACT

Three major density classes of lipoproteins and a residual protein (d>1.21) were isolated by ultracentrifugation from plasma of fasted, fed normal, and choline-deficient rats. Lipid extracts were obtained from total plasma and the various density classes of lipoproteins, and each extract was examined in detail by thin layer and gas chromatographies. The results indicated essentially identical compositions of molecular species of phosphatidyl choline, which suggested their rapid equilibration among the different plasma lipoprotein classes. In contrast, the molecular species of the triacylglycerols and cholesteryl esters showed significant differences among the chylomicrons, very low and low, and high density lipoproteins, which excluded the possibility of their ready equilibration in vivo. Omission of choline from diet resulted in a sharp and statistically significant decrease in all lipid components of the very low and low density lipoproteins within 2 days. After 10 days of choline deficiency, the lipid levels of chylomicrons and very low and low density lipoproteins were ca. one-half the levels found in the choline supplemented animals, and there were discernible distortions in their lipid composition. Reintroduction of choline led to a prompt return to normal levels and lipid composition of both chylomicron and very low and low density lipoprotein fractions. The lack of equilibration of the triacylglycerols among the lipoprotein classes under normal conditions and in choline deficiency demonstrates an as yet unrecognized source of compartmentation of plasma lipids.

INTRODUCTION

Deprivation of dietary choline has been shown to result in a significant fall in total plasma phosphatidyl choline and triacylglycerols (1), cholesteryl esters, and total phospholipids (2,3), as well as in an impairment of secretion of plasma lipoproteins by the rat liver (4-6) and intestine (7). In addition, omission of dietary choline has been observed (8) to lead to a significant increase in hepatic synthesis of fatty acids, which is manifested in the formation of triacylglycerols of increased content of palmitic acid. The fall in the total plasma phospholipids and cholesteryl esters in choline deficiency has been claimed to be due to a specific decrease in the molecular species containing arachidonic acid (9,10), which has raised the possibility that choline-deficient animals might secrete plasma lipoproteins of altered lipid composition.

The following work reports detailed analyses of the molecular species of glycerolipids in the plasma lipoproteins of fasted, fed normal, and choline deficient rats. All lipoproteins showed similar molecular species of phosphatidyl choline but differed in those of triacylglycerols and cholesteryl esters. The characteristic molecular species of triacylglycerols and cholesteryl esters in the different lipoprotein classes varied with their origin in vivo but remained unaffected by fasting or choline deficiency.

MATERIALS AND METHODS

Animals and diets: Male Wistar rats (initial wt 150-170 g) were housed in screen-bottomed cages, offered food and water ad libitum, and subjected routinely to adjustment periods of 7-10 days on commercial chow followed by 7-10 days on a choline supplemented (0.6%) hypolipotropic diet (CS group). The hypolipotropic diet (11) contained: 15% fat (hydrogenated fat; Primex, 10%; corn oil 5%; and cod liver oil concentrate with vitamin A, D and E, 0.02%); 15% protein (alcohol-extracted peanut meal, 12%; washed soya protein, 8%; and vitamin-free casein, 1%); 58.8% carbohydrate (corn starch, 10%; dextrin, 10%; and sucrose, 38.8%); salt-mixture, 3%; celluflour, 1%; sucrose-vitamin mixture, 1%; and cystine, 0.2%. For the choline-deficient group choline was omitted from the diet after this adjustment period, and the feeding continued for 2, 5, and 10 days (CD-2, CD-5 and CD-10). For the choline recovery group (CR), 10 days deficient rats were refed with choline for 5 days. The plan for starting the animals on various diets was such that the rats of all 5 groups were finally killed on the same day in the morning between 9-11 a.m. The food intake averaged 14.6 ± 0.62 and 14.7 ± 0.52 g/rat/day and the body wt gain 5.0 ± 0.48 and 4.9 ± 0.60 g/rat/

TABLE I

Choline Phospholipids of Plasma Lipoproteins and Residual Proteins[a]

Groups	Lecithins				Sphingomyelins			Lyso-lecithins	Total	
	Chylos[b]	VLDL + LDL	HDL	>1.21	Chylos	VLDL + LDL	HDL	>1.21	P	Choline phospholipids[c]
	μg P/ml plasma								μg/ml plasma	
CS	1.66	3.19	11.64	0.75	0.45	0.15	1.06	4.82	23.72	593
CD-2	1.31	2.36	10.31	0.64	0.38	0.12	0.97	5.20	21.24	532
CD-5	1.11	2.29	9.42	0.65	0.34	0.12	1.32	4.02	19.27	482
CD-10	1.00	1.81	6.19	0.59	0.34	0.09	1.11	4.13	15.26	382
CR	1.28	2.52	7.64	0.67	0.18	0.10	1.19	4.22	17.80	445

[a]Phospholipids (samples pooled from 4 rats) were separated by thin layer chromatography and phosphorus (26) was analyzed. VLDL = very low density, LDL = low density, and HDL = high density lipoproteins; CS = choline supplemented; CD = days choline deficient; and CR = choline restored.

[b]Chylomicrons.

[c]Total phospholipid = P x 25.

experimental period for the CS and CD groups, respectively.

Isolation of lipoproteins: Blood was collected into a heparinized syringe by aortic exsanguination under light ether anaesthesia. The plasma was obtained by centrifugation (Sorvall, RC2-B) at 3000 x average g for 10 min, and small aliquots were taken for estimation of protein (12) and total esterified fatty acids (13) of whole plasma. Fresh plasma (5 ml) obtained from each rat was separated into 3 lipoprotein fractions: chylomicrons (plasma at 44,680 x average g for 30 min), d< 1.006 + d = 1.006-1.063 or very low density + low density lipoproteins (VLDL + LDL) (100,500 x average g for 16 hr), d = 1.063-1.21 or high density lipoproteins (HDL) (100,500 x average g for 22 hr), and into residual proteins (d>1.21) using the Spinco (model L. rotor no. 40.3) preparative ultracentrifuge (14,15). The lipoprotein densities were adjusted by pycnometry with appropriate NaCl and NaBr solutions (16). The d<1.006 and d = 1.006-1.063 lipoprotein fractions were washed (2-3 times) by underlaying them in salt solutions of appropriate density and centrifugation for 16 hr and the d = 1.063-1.21 lipoprotein fraction was washed once by resuspending in d = 1.21 salt solutions and centrifugation for 22 hr.

Lipid analyses: Total lipids of plasma and each lipoprotein fraction were extracted and purified by the method of Folch, et al., (17). The extracts were taken to dryness under nitrogen at 40 C and made up to volume (5-7 ml) by redissolving the lipid in chloroform-methanol, 4:1. The individual lipid classes were resolved by thin layer chromatography (TLC) and the lipids recovered by elution with chloroform-methanol, 2:1, as previously described (18). Phospholipids were separated in chloroform-methanol-acetic acid-water, 150:50:2:6, which carried all neutral lipids to the solvent front as a single band. Neutral lipids then were rechromatographed in heptane-isopropyl ether-acetic acid, 60:40:3. Triacylglycerols were separated on basis of unsaturation by argentation TLC with 1-2% methanol in chloroform (15).

The fatty acids of each lipid class were quantitated by gas liquid chromatography (GLC) (19). For this purpose, the free fatty acids were methylated with diazomethane (20), as were the fatty acids liberated from cholesteryl esters by saponification (21). Other lipid ester classes were transmethylated with 5% sulfuric acid in methanol. Quantitative measurements of the neutral lipid classes were made by direct GLC as previously described (22), which also provided the profiles of the mol wt distribution of the diacylglycerols, cholesteryl esters, and triacylglycerols.

The molecular species of the phosphoglycerides of the various lipoprotein classes were assessed as previously described for total rat plasma lecithins (23). The molecular species of the triacylglycerols of the rat plasma lipoproteins were calculated from the results of the combined TLC and GLC analyses as described for human milk fat (24).

Other methods: Protein content of each lipoprotein fraction was measured by the method of Lowry, et al., (12). In the final reaction mixture for protein determination, 1 ml ether was used to remove cloudiness due to the presence of lipids (25). Lipid P of phospholipids was determined by the procedure of Dawson (26). Ester bonds of total esterified lipids

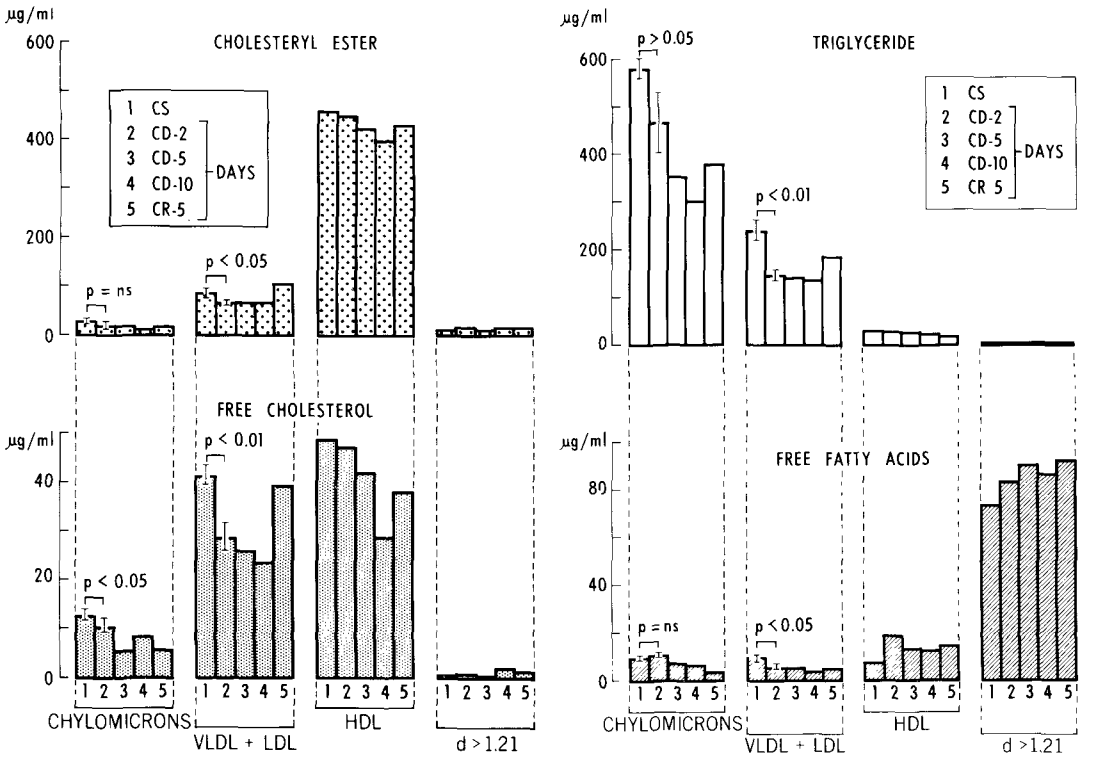

FIG. 1. Composition of neutral lipids and free fatty acids of various density classes of plasma lipoproteins of normal and choline-deficient rats. The neutral lipids were estimated by direct gas liquid chromatography (GLC) in relation to tridecanoin used as internal standard. The free fatty acids were estimated by GLC following diazomethylation. All units are expressed as μg/ml plasma. VLDL = very low density lipoprotein, LDL = low density lipoprotein, and HDL = high density lipoprotein.

and other lipids were measured using a fresh solution of alkaline hydroxylamine and ferric perchlorate as described by Skidmore and Entenman (13).

RESULTS AND DISCUSSION

Resolution of lipoproteins: Since the plasma was obtained from fed animals, extra care was necessary to separate chylomicrons from other LDL. The chylomicron fraction was, therefore, washed and recentrifuged to remove as much as possible of the VLDL and other proteins. The VLDL and LDL fractions of plasma lipoproteins were collected together.

In some instances, where a cut-off point of d = 1.040 was used (27) instead of d = 1.063 fraction (28), no significant differences were found between the VLDL + LDL and HDL fractions in the triacylglycerol, phospholipid, or cholesteryl ester composition. Since the characterization of the protein moieties of the different lipoprotein fractions was not the objective of the present study, further subfractionation, washing, and recentrifugation of these fractions were discontinued after their lipid composition had become stabilized. The recovery of triacylglycerols and phospholipids as the sum total in different lipoproteins was in the range of 86-95%. The value obtained by us for total plasma choline-phospholipids (sum of different lipoprotein fractions) was 59.3 mg/100 ml plasma (Table I), which corresponded to those, obtained by Narayan, et al., (29) and by Lomardi and Ugazio (16), of 58.2 and 61 mg/100 ml plasma, respectively. However, the value for triacylglycerols in different plasma lipoproteins obtained by Lombardi, et al., (5) after feeding a single meal of purified high-fat and high-carbohydrate diet was 48.5 mg/100 ml plasma, whereas we obtained a value of 85 mg/100 ml plasma by feeding the same diet for 10 days (Fig. 1). Over 90% of the protein was found in the residual protein fraction d>1.21, while the other three fractions made up less than 10% of the total protein but combined with ca. 90% of the total lipid. The chylomicron, VLDL + LDL, HDL, and d>1.21 fractions contained 0.03 ± 0.005, 0.17 ± 0.016, 0.61 ± 0.035, and 37.91 ± 0.73 mg protein/ml plasma, respective-

TABLE II

Fatty Acid Composition of Lecithin and Lyso-Lecithin (d>1.21 Fraction) of Plasma Lipoproteins and Residual Proteins (% of Total)

Lipoproteins[a]	Fatty acids	Animal groups[b] CS	CD-2	CD-5	CD-10	CR
Lecithin	14:0	1.1	1.1	1.0	1.0	1.0
chylomicrons	16:0	19.6	19.4	18.9	18.7	21.0
	16:1	1.3	1.2	1.1	1.4	1.2
	18:0	16.5	17.2	19.3	19.9	19.4
	18:1	22.4	20.7	20.4	20.3	19.8
	18:2	26.9	26.7	25.2	26.0	25.0
	20:4[c]	8.9	10.1	10.0	9.0	9.0
	>20:4[d]	2.2	2.2	2.3	2.3	2.4
VLDL + LDL	16:0	20:6	21.3	21.0	21.2	22.8
	16:1	1.2	1.1	1.0	1.0	1.1
	18:0	19.3	19.6	18.8	19.2	17.5
	18:1	20.1	18.9	18.9	19.8	19.7
	18:2	26.1	26.8	27.8	28.3	27.1
	20:4[c]	9.1	8.9	8.9	6.9	8.2
	>20:4[d]	2.3	2.3	2.2	2.3	2.4
HDL	16:0	18.9	20.0	19.9	20.0	21.6
	16:1	1.0	0.8	0.8	0.8	0.8
	18:0	17.9	19.0	19.7	17.9	17.4
	18:1	22.5	19.8	19.9	20.2	20.3
	18:2	27.9	27.8	26.1	28.7	27.6
	20:4[c]	8.2	9.0	9.7	9.0	8.4
	>20:4[d]	2.3	2.4	2.6	2.4	2.3
>1.21	16:0	20.5	20.1	20.7	19.0	20.0
	16:1	1.2	1.2	1.2	1.0	1.2
	18:0	18.4	18.4	18.9	18.5	17.6
	18:1	19.7	19.9	18.3	20.2	19.6
	18:2	30.2	30.2	19.9	30.1	30.9
	20:4[c]	7.9	8.0	9.1	8.3	8.6
	>20:4[d]	2.0	2.1	2.0	2.2	2.1
Lysolecithin	14:0	0.3	0.3	0.4	0.5	0.3
>1.21	16:0	41.1	40.4	37.7	36.7	40.1
	16:1	1.2	1.4	0.9	1.0	1.6
	18:0	18.7	20.1	23.7	23.1	18.0
	18:1	18.7	18.8	18.5	20.0	18.4
	18:2	17.2	16.4	15.4	16.5	17.9
	20:4[c]	1.3	1.3	1.5	1.2	1.7

[a]VLDL = very low density, LDL = low density, and HDL = high density lipoproteins.
[b]As in Table I.
[c]20:4 + 20:5 + 22:1.
[d]18:3, 20:1, 22:6.

ly, and, of these, only the VLDL + LDL fractions decreased significantly in choline deficiency as shown before (6).

Analysis of neutral lipids: The histograms in Fig. 1 show that the triacylglycerols, cholesteryl esters, free cholesterol, and free fatty acids are all decreased in the VLDL + LDL fractions in CD-2 and all these decreases are statistically significant. There was a further lowering of these lipid classes with progressing choline deficiency which was not examined statistically. Triacylglycerols were decreased, to a lesser extent, in chylomicrons due to choline deficiency. By the end of the tenth day of choline deficiency, the lipid values were ca. one-half of the starting figures. On reintroduction of choline into the diet, the lipids of these fractions returned toward the normal levels. These findings are in general agreement with those reported earlier by us (1) and also Rosenfeld and Lang (30), for total plasma triacylglycerols and phospholipids, and Ridout, et al., (2) for total plasma cholesterol esters in early choline deficiency.

Analysis of phospholipids: Table I gives the recoveries of phosphorus in the various choline containing phospholipids isolated from the plasma lipoproteins. The results from the choline supplemented groups are similar to those reported by others (16,31) for fasting rats, except for the chylomicron fraction, which is largely

absent from fasting plasma. As a result of choline omission from the diet, there was a gradual decrease in the concentration of these phospholipids in the individual lipoprotein classes, except for the lysolecithins and sphingomyelins in the d>1.21 fraction, which remained about the same throughout the experiment. After 10 days of choline deficiency, the total amount of choline containing phospholipids in the plasma was ca. 60% of the control values.

Table II gives the fatty acid composition of the lecithin and lysolecithin fractions of the rat plasma lipoproteins as recovered from the choline supplemented and choline-deficient animals. The fatty acid composition of the lecithins is closely similar in the different lipoprotein fractions and resembles that of total rat plasma lecithin analyzed by others (20,24), except for a high proportion of oleic acid and a lower proportion of arachidonic acid in the present animals. There was no significant change in the fatty acid composition of the lecithins of any of the lipoproteins with progressing choline deficiency, except for a slight decrease in the proportion of arachidonic acid in the VLDL + LDL fraction, which is consistent with the observations of Tinoco, et al., (9) and Beare-Rogers (10).

On direct GLC, the various plasma lecithins gave pyrolysis peaks (23) which corresponded closely to the mol wt distribution seen in the total plasma lecithins of a fasting rat. The composition of the molecular species of the lecithins from the individual lipoproteins was, therefore, assumed to be identical to that previously analyzed in great detail for total rat plasma lecithins (24). This interpretation of the data is in accord with the known extensive equilibration of plasma lecithins among the various lipoprotein classes in other animal species (32,33).

The fatty acid composition of the lysolecithins was assessed only for the d>1.21 fraction. Except for a small decrease in palmitic and an increase in stearic acid, there was no other change noted in this lipid class with progressing choline deficiency. It may be noted that this change was reversed upon reintroducing choline in the diet of these animals. The presence of significant amounts of linoleic acid in the lysolecithin is unusual in view of the relative absence of this acid from the 1 position of diacylglycerophosphorylcholine.

Table III gives the fatty acid composition of the lipid classes of plasma lipoproteins at different stages of choline deficiency. The fatty acids of cholesteryl esters, triacylglycerols, and free fatty acids of chylomicrons reflect the composition of dietary fat and undergo little change during the course of the experiment. The fatty acids of the cholesteryl esters in VLDL + LDL are notably different from those of the chylomicrons and exhibit an increased content of arachidonic acid, which is accommodated largely at the expense of the oleic acid. The cholesteryl esters of the HDL and d>1.21 fractions contain even larger proportions of arachidonic acid, while the HDL also may contain more linoleic acid than the d>1.21 fraction. There is a significant decrease in cholesteryl arachidonate in these fractions during choline deficiency. It has been suggested (34) that the cholesteryl esters of the VLDL + LDL fractions may be derived by synthesis in the liver, and the present data are consistent with this possibility. On the other hand, the fatty acid composition of the cholesteryl esters of the HDL fraction is consistent with their origin by vascular biosynthesis via lecithin-cholesterol acyltransferase (35). Choline deficiency had little effect upon the composition of this ester fraction. The distinct populations of the cholesteryl esters found in these lipoproteins suggest that this lipid class does not participate in an equilibration, although a net transfer of cholesteryl esters among lipoprotein classes has been demonstrated in vitro (36).

The fatty acid composition of the triacylglycerols of the VLDL + LDL fractions differs from that of chylomicrons by having a significantly higher proportion of palmitic and lower arachidonic acid content. These triacylglycerols apparently originate in the liver and do not equilibrate with those of chylomicrons, which retain their resemblance to the dietary fat. The HDL and d>1.21 fractions contain only small amounts of triacylglycerols, which also are characterized by high proportion of palmitic (HDL) and stearic (d>1.21) acids. The d>1.21 density class contains a significantly lower proportion of oleic acid than the triacylglycerols of any of the other lipoprotein classes. Obviously, the triacylglycerols of the other lipoprotein classes also were not equilibrated readily. There were no significant changes in the fatty acid composition of any of these triacylglycerols with progressing choline deficiency.

The composition of the free fatty acid fraction also showed minor differences among the various lipoprotein classes and remained relatively stable during the course of the experimentation.

Neutral lipid profiles: The GLC elution patterns of the neutral lipids (results not shown) confirmed the rather unique cholesteryl ester and triacylglycerol populations in each lipoprotein class in both control and in choline-

TABLE III

Fatty Acid Composition of Major Neutral Lipids and Free Fatty Acids of Plasma Lipoproteins and Residual Proteins[a] (% Total)

	Fatty acids	Cholesteryl esters					Triacylglycerols					Free fatty acids				
		CS	CD-2	CD-5	CD-10	CR	CS	CD-2	CD-5	CD-10	CR	CS	CD-2	CD-5	CD-10	CR
Chylomicrons	14:0	1.0	1.1	1.1	1.1	1.6	0.4	0.4	0.7	0.6	0.5	tr	tr	tr	tr	tr
	16:0	18.6	16.6	21.8	21.9	23.8	14.0	14.8	17.2	14.3	15.9	20.1	19.5	21.3	20.8	18.9
	16:1	1.1	1.0	1.3	1.3	1.4	0.4	0.4	0.4	0.4	0.5	2.5	2.7	2.1	2.3	2.2
	18:0	12.3	11.2	10.3	12.9	11.9	5.3	6.5	5.9	7.1	7.5	16.6	15.9	16.9	14.9	15.8
	18:1	30.9	29.1	27.7	27.2	26.3	44.3	44.1	44.0	42.7	43.1	35.1	32.7	33.8	36.2	36.6
	18:2	16.1	19.4	15.9	14.7	13.7	23.2	22.3	22.1	23.8	21.9	16.9	18.8	16.4	16.8	17.0
	20:3	1.1	1.1	1.2	1.2	1.1	1.7	1.5	1.7	1.5	1.5	2.0	1.8	1.3	1.1	1.5
	20:4[b]	18.9	21.5	20.7	19.8	20.1	8.9	10.4	9.2	9.8	10.0	5.4	6.9	6.5	6.0	6.0
	>20:4[c]	--	--	--	--	--	--	--	--	--	--	1.5	1.5	1.7	1.9	1.9
VLDL + LDL	14:0	0.7	0.7	0.7	0.7	0.7	2.5	1.8	1.4	1.0	1.7	tr	tr	tr	tr	tr
	16:0	16.2	17.3	16.1	16.3	15.6	25.2	24.5	22.4	21.6	21.2	23.9	22.8	22.6	22.1	24.8
	16:1	1.1	1.0	1.0	1.1	1.0	0.5	0.5	0.5	0.6	0.5	1.3	1.3	1.2	1.1	1.2
	18:0	2.0	1.9	1.0	1.6	1.8	2.7	3.8	3.7	3.8	4.7	19.5	19.4	17.7	17.5	17.9
	18:1	20.5	19.1	18.6	17.7	18.8	44.0	43.9	45.3	46.9	46.4	33.8	31.9	31.5	31.1	33.0
	18:2	22.6	26.1	27.9	29.3	25.4	21.2	20.0	20.3	20.1	20.9	10.4	10.5	11.3	11.6	10.1
	20:3	tr	tr	tr	tr	tr	1.4	1.6	1.8	1.6	1.0	1.2	1.4	1.8	1.7	1.9
	20:4[b]	36.9	34.0	34.7	33.3	36.6	2.6	4.0	4.7	4.4	3.5	8.7	11.4	12.4	13.7	10.3
	>20:4[c]	--	--	--	--	--	--	--	--	--	--	1.2	1.4	1.6	1.4	1.3
HDL	14:0	tr	tr	tr	tr	tr	3.2	3.4	3.7	3.0	3.1	tr	tr	tr	tr	tr
	16:0	10.3	12.6	11.3	11.0	10.7	22.0	25.0	23.9	23.9	22.2	24.9	20.9	24.5	25.0	23.2
	16:1	1.6	1.4	2.0	1.9	1.3	tr	tr	tr	tr	tr	1.9	1.4	1.2	1.7	1.6
	18:0	0.6	1.3	1.8	0.7	0.8	6.4	6.0	6.6	4.0	6.9	15.5	14.4	15.9	15.1	15.8
	18:1	12.4	10.7	12.1	15.8	12.1	38.2	37.9	38.1	36.9	39.4	32.7	35.4	34.1	32.1	34.4
	18:2	30.1	31.5	33.1	35.3	33.8	20.1	20.8	18.5	19.8	19.8	12.3	12.5	7.4	12.3	12.9
	20:3	tr	tr	tr	tr	tr	4.5	2.1	3.3	4.4	2.9	2.0	2.7	2.8	2.3	2.7
	20:4[b]	44.1	42.6	39.8	37.4	41.2	5.0	4.8	4.1	5.0	4.0	8.8	9.5	10.3	8.1	8.1
	>20:4[c]	--	--	--	--	--	--	--	--	--	--	2.8	3.4	3.8	3.5	1.3
>1.21	14:0	tr	tr	tr	tr	tr	2.3	2.8	2.7	1.8	1.9	0.9	1.0	1.4	0.8	0.8
	16:0	7.2	8.9	7.7	8.5	8.0	26.4	28.2	26.3	27.9	26.6	20.5	22.7	22.3	18.8	20.5
	16:1	1.0	1.2	0.8	1.2	1.2	tr	tr	tr	tr	tr	3.7	3.3	3.5	3.4	4.0
	18:0	1.4	1.8	1.3	1.1	1.2	16.9	16.2	16.3	18.1	16.9	7.3	6.1	7.9	7.8	8.6
	18:1	18.1	20.7	19.3	20.1	20.2	28.8	29.9	30.0	28.1	29.0	34.2	35.3	32.5	35.6	32.3
	18:2	24.1	25.5	26.1	26.6	26.3	19.3	18.2	19.7	20.9	19.5	23.9	21.7	23.3	23.3	24.2
	20:3	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	2.3	3.3	2.8	2.1	2.4
	20:4[b]	48.2	48.2	44.8	42.5	42.0	6.4	4.7	5.0	5.4	6.1	4.8	4.8	4.5	5.2	4.7
	>20:4[c]	--	--	--	--	--	--	--	--	--	--	1.5	1.8	1.8	2.0	1.4

[a]Animal groups as in Table I. VLDL = very low density, LDL = low density, HDL = high density lipoproteins; tr = trace.

[b]20:4 + 20:5 + 22:1.

[c]18:3, 20:1, 22:6.

TABLE IV

Mol Wt Distribution of Triacylglycerols of Chylomicrons and Very Low Density + Low Density Lipoproteins (VLDL + LDL) of Plasma and of Total Triacylglycerols of the Diet and Normal and Choline-Deficient Rat Liver

Carbon number	Dietary fat	Normal liver	CD-5 liver	CS[a]		CD-2[b]		CD-5		CD-10		CR	
				Chylos	VLDL + LDL	Chylos	VLDL + LDL	Chylos	VLDL + LDL	Chylos	VLDL + LDL	Chylos	VLDL + LDL
46			4.1										
48	0.5	2.1	24.3	4.4		2.6	1.5	2.2	4.7	3.1	2.8	7.1	0.6
50	4.8	18.7	55.5	27.2	7.2	23.1	8.7	15.9	17.7	10.2	14.3	25.5	6.7
52	13.3	42.4	12.5	49.1	61.8	53.0	53.1	28.8	55.7	28.8	49.4	43.5	57.0
54	53.8	34.5	3.4	9.1	23.3	9.3	27.0	37.1	26.8	39.8	24.4	11.0	28.7
56	5.8	2.1		8.7	6.1	10.4	7.0	8.3	8.9	9.3	6.9	9.9	6.0
58	11.0[c]			1.1	1.4	1.4	2.5	7.3	3.3	8.5	1.8	2.8	0.6

[a]CS = choline supplemented.

[b]Floating fat obtained by centrifugation of the liver homogenate of 2 days choline-deficient (CD) rat (11) contained: 46, 2.6; 48, 18.4; 50, 42.0; 52, 34.5; and 54, 2.6.

[c]This component is largely due to the addition of cod liver oil which is rich in high mol wt triacylglycerols due to the presence of 20:1 (12%), 20:5 (10%), 22:1 (10%), and 22:6 (12%) fatty acids (38).

deficient animals, as suggested above on the basis of fatty acid analysis. The apparent lack of equilibrium of esterified cholesterol among different plasma lipoproteins as demonstrated in the present study agrees with the results of previous work with radioactive markers (32,37).

Table IV gives the mol wt distribution for the triacylglycerols of the chylomicron and VLDL + LDL fractions as obtained by GLC of the other experimental samples, the dietary fat and the normal and choline-deficient rat liver. From these data and from the fatty acid composition given in Table IV, it can be seen that the triacylglycerols of all the chylomicrons are made up largely of combinations of two C_{16} and one C_{18} acid (C_{50}), two C_{18} and one C_{16} (C_{52}), as well as shorter (C_{48}) and longer (C_{54}) combinations of the common C_{16} and C_{18} acids. This triacylglycerol profile is characteristic of the bulk of the fat present in the purified diet, which these animals were receiving.

The triacylglycerols of VLDL + LDL fraction exhibited a mol wt profile characteristic of total rat liver triacylglycerols, which are made up largely of combinations of one C_{16} and two C_{18} (C_{52}), as well as two C_{16} and one C_{18} (C_{50}) and three C_{18} (C_{54}) acids. There was little change in the pattern of the triacylglycerols of plasma VLDL + LDL as a result of the omission of choline from the diet, despite dramatic changes in the pattern of total liver triacylglycerols, due largely to the large increase in the proportion of palmitic acid (8). Apparently little of the palmitic acid-rich triacylglycerols which accumulated in the liver due to choline deficiency was released into the plasma. This observation is consistent with the earlier demonstrated suppression of release of the VLDL + LDL lipoproteins by the rat liver in choline deficiency (1,4-6).

Molecular species of triacylglycerols: Table V shows the distribution of the mol wt of the triacylglycerol fractions recovered from the VLDL + LDL fractions from fasted and fed rats. Each subclass of triacylglycerols, containing 0-6 double bonds, possessed the same mass proportion and carbon number distribution in the two dietary states. This distribution of the triacylglycerols in the plasma VLDL + LDL is consistent with the composition of the molecular species of the triacylglycerols of total rat liver as reported by Slakey and Lands (39) and Akesson (40). A similar close correlation was obtained between the structure of the dietary fat and the triacylglycerols of the chylomicrons for the chow-fed animals. Since the triacylglycerol composition of the chylomicrons is very different from that of the VLDL + LDL, it

TABLE V

Molecular Species of Triacylglycerols of Very Low Density + Low Density Lipoproteins of Fasted and Fed Rats

Molecular species[a]	Metabolic state		Molecular species	Metabolic state	
	Fasted[b]	Fed		Fasted[b]	Fed
	Area %				
Monoenes (001)	18.9	13.9	Tetraenes (112)	5.6	4.3
48	4.0	3.7	48	2.0	trace
50	27.1	31.3	50	19.9	20.7
52	61.1	56.3	52	67.2	75.3
54	4.3	7.5	54	10.1	3.8
56	3.3	0.9	56	0.6	trace
Dienes (011)	28.8	22.7	Tetraenes (022)	2.9	9.8
48	0.3	0.1	48		
50	9.7	7.8	50	28.9	6.0
52	73.9	82.6	52	44.5	58.9
54	14.4	9.3	54	26.0	32.2
56	1.4	trace	56	0.3	1.6
Dienes (002)	5.2	3.8	Pentaenes (122)	6.9	11.7
48	2.3	2.3	48		
50	31.5	32.3	50	6.1	trace
52	58.2	63.1	52	35.5	37.7
54	7.8	2.1	54	50.0	62.2
56			56	8.1	trace
Trienes (012)	12.6	19.6	Polyenes (222, 024)	18.5	13.8
50	2.0	1.2	52	12.1	0.9
52	79.8	89.9	54	58.9	69.6
54	15.8	8.7	56	24.7	29.3
56	2.2		58	4.0	trace

[a]As obtained by gas liquid chromatography of the triacylglycerols of different degrees of unsaturation recovered from argentation thin layer chromatography, see text.

[b]Fasted for 18 hr.

must be concluded that these lipoprotein classes originate at different sites and that their component triacylglycerols do not equilibrate. Previous studies on the transfer of triacylglycerols among plasma lipoproteins have given equivocal results. No interchange of labeled trioleoylglycerol was found during incubation of isolated human plasma lipoproteins (41). Injection of low density lipoproteins labeled with ^{14}C-triacylglycerols into rabbits, however, led to an ca. equal specific activity in the LDL and HDL fractions after 2 hr (42). Triacylglycerol transfer to HDL also has been reported upon incubation of isolated human HDL with VLDL, egg yolk lipoprotein, or a glyceride emulsion (43). In view of the present results, it would be required that any significant mass exchanges under in vivo conditions involve like molecular species.

Significance of results: The present study confirms the relatively rapid fall in plasma triacylglycerols, lecithins, and cholesteryl esters observed previously during early choline deficiency. This fall appears to result from a specific effect of dietary choline upon the secretion or synthesis of the VLDL by the liver. The less dramatic changes in the secretion of the chylomicrons by the intestine may have resulted from an impairment of the synthesis of the LDL by the intestinal mucosa, which also depends upon lecithin biosynthesis from choline (7).

The great similarity found in the lecithins is apparently due to a rapid equilibration of these lipids among the plasma lipoproteins (33,44). In contrast, the characteristic composition of the cholesteryl esters and triacylglycerols of the chylomicrons and the plasma VLDL + LDL and HDL indicates that there is little exchange or equilibration between these lipids in vivo. In view of the lack of basic understanding of the forces involved in lipid and protein interaction, the mechanism of lipid exchange among lipoproteins must remain at a documentary stage. The present studies demonstrate an as yet unrecognized basis of compartmentation of plasma lipids.

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Studies on Chemical Nature of Lipofuscin (Age Pigment) Isolated from Normal Human Brain

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ABSTRACT

Human brain lipofuscin isolate was studied for its purity and physical and chemical properties. Purification of the impure material was achieved by gel permeation chromatography using Sephadex LH-20 and BioBeads S-X1 gels. The purified lipofuscin polymer represented ca. 12% of the starting material with the rest of the material being various mixed lipids. The mol wt of the purified lipofuscin was determined to be between 6000-7000 daltons. IR, UV-visible, NMR, and fluorometric spectra were obtained, all indicating the fundamentally lipid nature of lipofuscin. The NMR spectrum strongly resembled that of a typical long chain fatty acid. Numbers of fatty acids and several amino acids were present as a portion of the lipofuscin structure. The results obtained suggested that the brain lipofuscin employed in the present study consisted mainly of polymeric lipid and phospholipid structures along with amino acids either bound to the lipids or as included proteins.

INTRODUCTION

The terms "lipofuscin" and "ceroid" have been variously applied to a variety of partially characterized, autofluorescent lipopigments found in many animal tissues. Most often these pigments are defined simply as yellow-brown, lipid-soluble pigments having a fluorescence maximum in the region 430-470 nm (1-3).

Tappel and coworkers (2-9) have studied the fluorescent chromophore R–N=CH–CH=CH=CH–NH–R which they believe to be responsible for the spectral properties observed in these pigments. They have proposed mechanisms for the formation of this fluorophore in living tissues from various unsaturated lipids, malondialdehyde, and free amino groups from amino acids, proteins, and nucleic acids.

There have been previously only isolated attempts at characterizing these lipopigments chemically (2,7,10,11). Part of the reason for this has been, no doubt, the lack of a satisfactorily pure material suitable for analysis. Until recently, the only criteria of purity have been the solubility characteristics, color, and fluorescence properties, which do not define these pigments adequately. There are numerous reports of various lipid moieties, protein, lipoproteins, enzymes, and even free amino acids being associated with the lipopigments (8,9,12,13).

A brief review of the current status of lipopigment work has recently appeared (14). Siakotos and coworkers (15) have isolated two pigments, ceroid and lipofuscin, as apparently discrete substances based upon centrifugation and flotation techniques. In the present work, the further purification and characterization of lipofuscin isolated by the method of Siakotos, et al., (15) is carried out.

MATERIALS AND METHODS

Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Bio-Beads S-XI and BioGlas 500 were obtained from BioRad Laboratories, Richmond, Calif. Silica Gel H was purchased from Brinkmann Instruments, Des Plaines, Ill. All solvents used were reagent or spectrophotometric grade, and lipid standards were purchased from The Hormel Institute, Austin, Minn., or were prepared in our laboratory and checked for purity by gas liquid and thin layer chromatographies (GLC and TLC).

Purification of Lipofuscin

A sample of purified lipofuscin isolate (15) was monitored for the presence of contaminating lipids by TLC on 0.75 mm Silicia Gel H plates using a solvent system of 80/20 (v/v) hexane/diethyl ether and visualized by spraying with dichromate-sulfuric acid spray (16).

Subsequent purification was accomplished by gel filtration using a 3 ft long, 1/2 in. diameter, all glass column equipped with Teflon and stainless steel fittings (Chromatronix, Berkeley, Calif.). Samples were injected by means of an all Teflon sample injector. Sephadex LH-20 was allowed to swell overnight in chloroform/methanol, 2:1 v/v (C/M) before packing. The column eluent was monitored

TABLE I

Gel Filtration Data for Crude Lipofuscin on Sephadex LH-20 in Chloroform/Methanol and BioBeads S-X1 in Chloroform

Gel	Fraction	K_{av}	Percent total	Estimated average mol wt	Identity
Sephadex LH-20	A	0.00	21	> 2,000	Colored polymeric polar material
Sephadex LH-20	B	0.09	56	1,200[a]	Heterogeneous mixture
Sephadex LH-20	C	0.13	---	900	Triglyceride
Sephadex LH-20	D	0.23	8	450[a]	Cholesteryl esters
Sephadex LH-20	E	0.41	15	200[a]	Cholesterol
BioBeads[b] S-X1	A-1	0.00	12	10,000	Colored polymeric polar material
BioBeads S-X1	A-2	0.13	9	3,500	Colorless polymeric polar material

[a]See "Results and Discussion" for explanation.

[b]Further fractionation of fraction A – mol wt > 2,000.

with a Waters model 401 differential refractometer. Fractions were collected, and the solvent was removed by evaporation with dry nitrogen. The fraction having the highest mol wt, which eluted first, was then redissolved in chloroform and passed through a similarly prepared column of BioBeads S-X1 in chloroform. All fractions were evaporated and weighed. Approximate mol wt were determined using standard lipids on the columns. These calibration data are presented elsewhere (17).

The fractions were labeled as in Table I. All fractions were subjected to TLC on Silica Gel H and compared with several standard lipid classes. These results indicated the presence of cholesterol and its esters in fractions D and E (Table I). Subsequent analyses were carried out for cholesterol by the method of Searcy and Bergquist (18) on triplicate samples. The optical densities were read against a blank at 490 nm on a Spectronic 20 spectrophotometer.

Mol Wt Determinations

The fraction which excluded from the BioBeads S-X1 gel (fraction A-1) and having an apparent mol wt greater than 10,000 was injected onto a 1-1/2 ft long, 1/2 in. diameter all glass column as above which was packed with BioGlas 500 in chloroform. This packing has a mol wt range of 10,000-100,000 daltons. To prevent adsorption (see "Results and Discussion" concerning pigment adsorption to glass and silica gel) of the pigment, the BioGlas packing was silylated by being allowed to stand overnight in a mixture of chloroform and N,O-Bis-(Trimethylsilyl)-acetamide (BSA, Pierce Chemical Co., Rockford, Ill.). The silylated BioGlas was washed several times with chloroform before packing the column. The packed column also was allowed to wash for several hr with chloroform before sample introduction. The column was calibrated with polystyrene standards (ArRo Laboratories, Joliet, Ill.) over the range of 10,000-100,000 daltons.

A Mechrolab 301A vapor pressure osmometer was employed as a second method of mol wt determination. The instrument was equipped with a 37 C nonaqueous probe. The solvent, toluene, was allowed to equilibrate in the instrument overnight. A solution of fraction A-1 in toluene (22.80 mg/ml) was prepared. The standard curve was determined using a polystyrene standard of 10,300 daltons in the concentration range from 0.002-0.02 M.

For a third mol wt determination, samples of fraction A-1 in chloroform and toluene (0.5 mg/ml) were prepared for a determination of the sedimentation velocity with a Beckman model E analytical ultracentrifuge. An AN-D rotor having a double sector cell and aluminum centerpiece with sapphire windows was employed. The instrument was run at 40,000 rpm. Readouts of optical density were taken at 8 min intervals with a photoelectric scanner set at 400 nm.

Spectral Studies

Fluorescence spectra of the pigment were determined in chloroform with an Aminco-

Bowman spectrophotofluorometer standardized with quinine sulfate at a concentration of 1 μg/ml in 0.1 N sulfuric acid. The slits were 1/16 in. on slits 1, 3, 4, 6. A 1P-28 photomultiplier tube was used with sensitivity being set fully clockwise. On the 0.003 multiplier scale, the quinine sulfate had a fluorescence of 24% T. Confirmatory spectra were run in chloroform and C/M on a Hitachi MPF-2A spectrophotofluorometer. The excitation maximum for all spectra was 395 nm.

UV spectra were determined on fraction A-1 with a Beckman DB-G UV-visible spectrophotometer. Samples were prepared in chloroform and methylene chloride at concentrations of 0.041 mg/ml and 0.055 mg/ml, respectively.

IR spectra were obtained as thin film and KBr pellets with Perkin-Elmer 700 and Beckman IR-7 IR spectrophotometers. Pellets were 1% in sample concentration in KBr (IR quality, Harshaw Chemical Co., Cleveland, Ohio).

Proton NMR spectra were obtained on the fraction A-1 with Varian HR-220 (220 MHz) and HA-100 (100 MHz) NMR spectrometers using deuteriochloroform (99.5%, Bio-Rad) as the solvent.

Degradative Studies

A 5 mg sample of fraction A-1 was heated with methanol containing 4% sulfuric acid in a sealed ampoule for 10-12 hr at 80 C. The resultant mixture was extracted three times with diethyl ether and water. The collected ether extracts were washed twice with water. All aqueous portions were combined.

The corresponding lipofuscin methyl esters were injected into a Hewlett-Packard 7610A gas chromatograph equipped with a 6 ft glass column packed with 10% ethylene glycol succinate on 80-100 mesh Chromosorb W, AW. The column temperature was isothermal at 180 C. A mixture of known fatty acid methyl esters was chromatographed for comparison of carbon numbers.

The aqueous extract from the preparation of lipofuscin methyl esters was prepared for amino acid analysis by the method of Gehrke and Stalling (19) using the N-trifluoroacetyl-n-butyl ester derivative. Amino acid analyses were accomplished using a Beckman GC-5 gas chromatograph equipped with a 6 ft glass column packed with ethylene glycol adipate (Tabsorb, Regis Chemical, Morton Grove, Ill.). The column was isothermal at 80 C for 8-12 min and then programed from 80-210 C over 64 min.

Analyses for glyceride esters present in the pigment were carried out using the method of Erikson and Briggs (20). A tripalmitin standard (2 g/liter in isopropanol) was prepared according to the procedure. Absorbance at 410 nm was read against the blank with a Spectronic 20 spectrophotometer. The unknown sample, fraction A-1, was prepared in a concentration of 1.08 mg/ml.

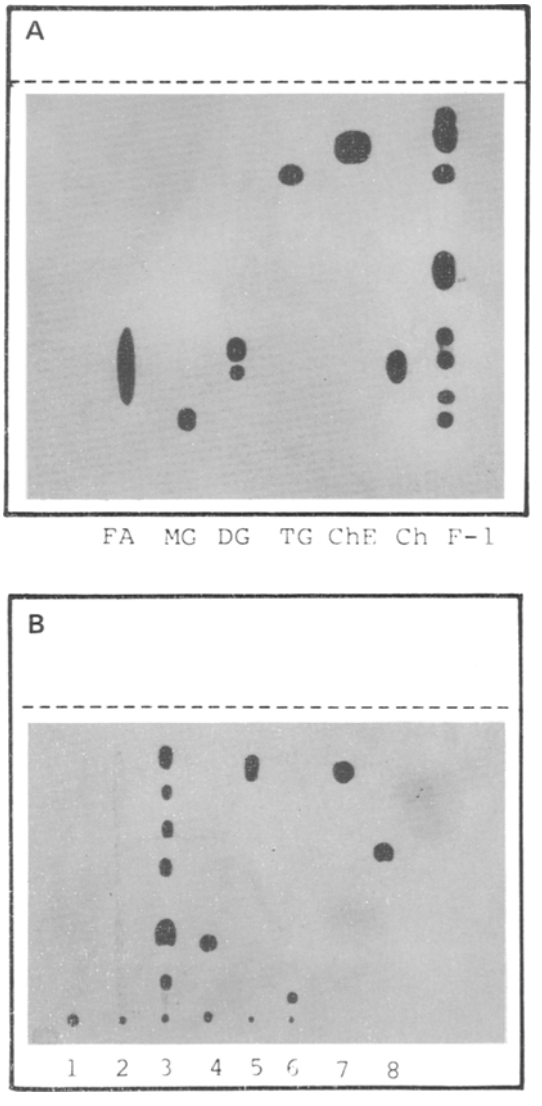

FIG. 1. A. Thin layer chromatogram of crude lipofuscin and standard lipids. FA = oleic acid, MG = monopalmitin, DG = 1,3-dipalmitin, TG = triolein, ChE = cholesteryl palmitate, Ch = cholesterol, and F-1 = crude lipofuscin. B. Thin layer chromatogram of lipofuscin fractions and standard lipids. 1 = fraction A-1, 2 = fraction A-2, 3 = fraction B, 4 = fraction C, 5 = fraction D, 6 = fraction E, 7 = cholesteryl stearate, and 8 = triolein.

RESULTS AND DISCUSSION

Purification and Mol Wt

The heterogeneous nature of the crude

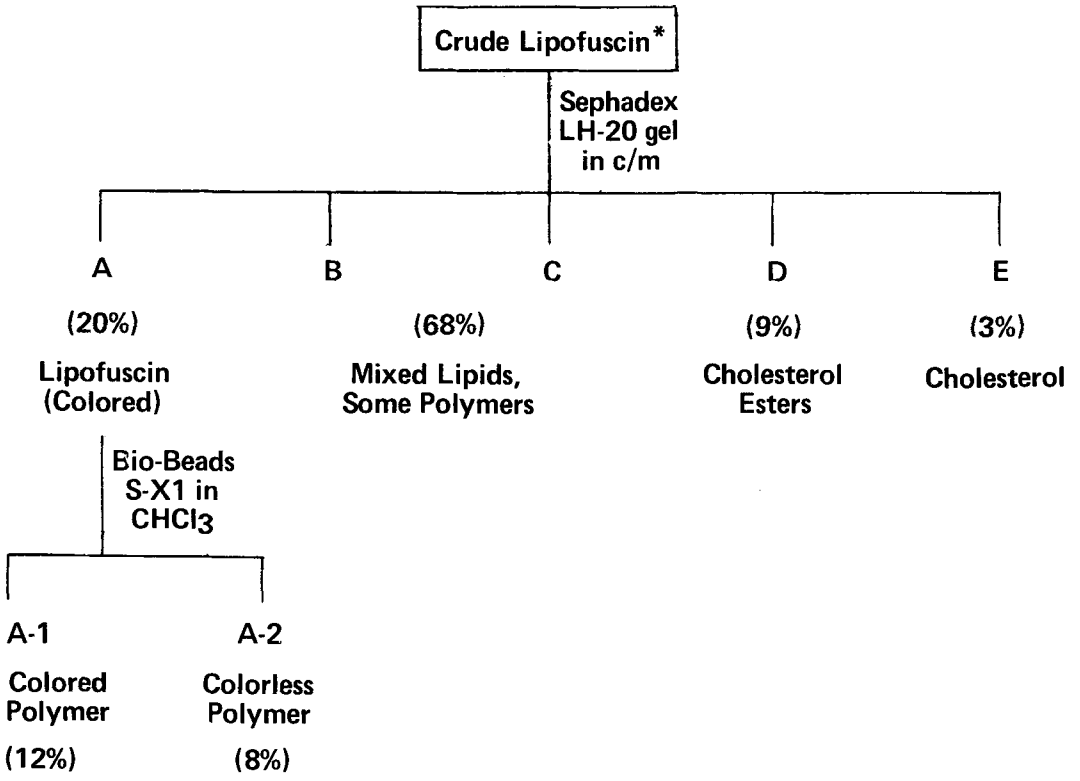

FIG. 2. Separation of lipofuscin components by gel filtration chromatography.

pigment may be seen in Figure 1A. In addition to cholesterol, cholesteryl esters, triglycerides, and monoglycerides, one prominent, highly fluorescent, unidentified component at R_f = 0.45 was present along with the colored pigment. The latter remained at the origin on the thin layer plate. Attempts to identify the component at R_f = 0.45 were unsuccessful.

The separation of lipofuscin fractions via gel chromatography yielded the fractions presented in Table I and summarized by the flow chart shown in Figure 2. The fraction labeled A was passed through the BioBeads S-X1 gel giving the fractions designated as A-1 and A-2. Fraction A-1 was brown colored; other fractions were light yellow in color when concentrated. All fractions exhibited some fluorescence under UV light. The unknown component at R_f = 0.45 appears in fraction B, giving an approximation of its mol wt as 1200. The comparison of all fractions with known standard lipids (Fig. 1B) suggested that fractions D and E contained cholesterol. The analysis for cholesterol gave 29.60 ± 0.29% (mean ± standard deviation of the mean, 3 determinations) for fraction D and 40.03 ± 1.07% cholesterol for fraction E. The lower percentage of free cholesterol in D was accounted for by the fact that it was mainly in the esterified form, whereas fraction E contained more free cholesterol.

Attempts to purify the fluorescent pigment fraction A-1 further by TLC were unsuccessful due to the tendency of the pigment to adsorb strongly to silica gel and to glass. The adsorption to glass was noted when an unsilylated BioGlas 500 column was tried. The brown pigment band stayed at the top of the column bed. Elution with 2:1 C/M removed some, but not all, of the pigment from the column. On thin layer plates, the use of polar solvents either produced a continuous streak or else carried the entire material with the solvent front.

Gel permeation chromatography was employed successfully to yield separations and mol wt estimations. However, there are limitations to the accuracy of such mol wt values. Gel

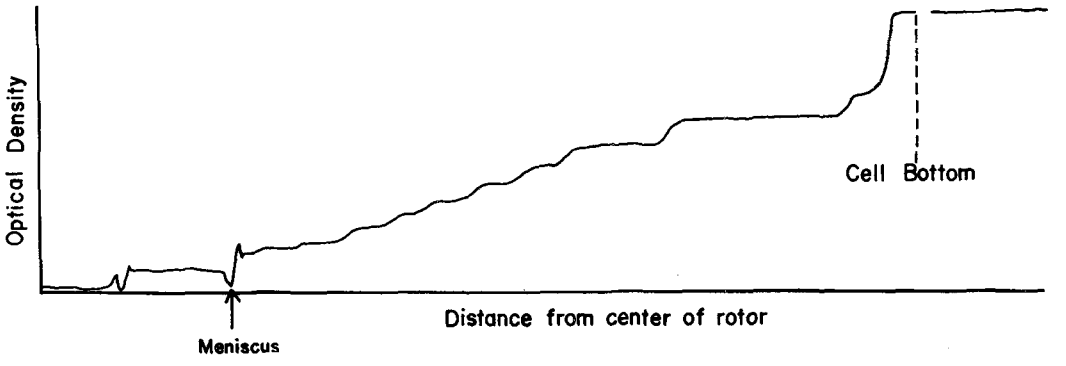

FIG. 3. Sedimentation velocity run of fraction A-1 in toluene.

filtration assumes that the calibration standards and the unknown sample are of similar molecular configuration. Since very high mol wt lipid standards are not available, the polystyrene standards had to suffice. If the structure of the pigment molecules is significantly different from these, errors will be introduced. The total exclusion of the pigment on BioBeads S-X1 and the total permeation on BioGlas 500 led to a conclusion that the mol wt of fraction A-1 was around 10,000. This conclusion was subject to uncertainties caused by the nonlinear behavior of gels near their end-limits. It must also be noted that gel permeation chromatography is not always ideal in that some sorption and solvent effects do occur. As an example of this, the LH-20 gel column, when packed in pure chloroform, failed to yield the separation of fractions D and E from fraction C. In the C/M solvent system, these fractions separated because the more polar cholesterol was retarded, as was shown previously by Nyström and Sjövall (21). This explained the low apparent mol wt of fractions D and E. The fact that these two fractions also fluoresced indicated that the cholesterol fraction and its esters fraction contained other components causing the fluorescence.

The estimated mol wt of the purified pigment (fraction A-1) as determined by vapor pressure osometry was ca. 5900. There were still difficulties with these data. The optimum concentrations for vapor pressure osmometry are 0.01-0.1 M. Lack of adequate amounts of pigment resulted in an obtainable concentration of 0.002 M. It would have been best to determine the accuracy at low concentrations by comparing values from 0.001 M and 0.1 M polystyrene solutions. However, such concentrated samples were too viscous to use. It is useful to note that a standard curve also was determined using a polystyrene standard of 3600 daltons for comparison. This curve gave the pigment mol wt as 4200. The previous value of 5900 was chosen as the more consistent with the gel column results.

Attempts to obtain information concerning the mol wt via analytical ultracentrifugation yielded the data shown in Figure 3 which depicts the optical scanner trace of a sedimentation velocity determination with toluene as the solvent. (The pigment fraction A-1 was found to float in chloroform and to sediment in toluene. This is in accord with the density values obtained by Siakotos, et al. [15].) For unknown reasons, the chloroform solution yielded no readable trace, hence the determination of $\bar{v}$ was not possible. The rather unusual pattern is most probably evidence that fraction A-1 consists of a complex multicomponent mixture.

Spectral Data

The fluorescence data obtained in the present study essentially duplicated those of earlier investigators (3,7,22) and indicated the general similarity of lipofuscin pigments. The fluorescence maximum was found to be at 465 nm when excited at 395 nm. A comparison of fluorescence intensity of fraction A-1 and the combined A-2, B, C, D, and E fractions indicated that, on a wt basis, fraction A-1 had ca. four times the fluorescence of the combined fractions. The fluorescence peak remained at 465 nm, whether the material was in pure chloroform or in C/M demonstrating the lack of a solvent effect. In the latter solvent, the Raman peak occurred at 450 nm.

The UV-visible spectra were nearly featureless. A broad rise was observed from 700 nm to the solvent cut-off. A small shoulder was observed at 244 nm in chloroform just before the solvent cut-off. A similar shoulder was observed at 232 nm in methylene chloride. Both shoulders probably indicate conjugated unsaturation. The absorbance values were 0.33

TABLE II

220 MHz Proton NMR Peaks of Fraction A-1

Chemical shift (δ), ppm	Approximate proton ratio	Identity[a]
0.91	5	Terminal CH_3-
1.25	21	Chain $-CH_2-$
1.61, 1.68 (doublet)	24	$CH_3-CH=CH-$
2.05	22	$-\underline{CH_2}-$adjacent to C–C
3.70	1	$\underline{CH_2}-O$
5.14	5	Olefinic protons

[a]Proposed.

TABLE III

Proton NMR Peak Assignments for Methyl Oleate[a,b]

Proton	δ, ppm	No. of protons
a	0.89	3
b	1.29	16
c	2.05	4
d	2.21	2
e	3.59	3
f	5.28	2

[a]The spectral data for oleic acid were obtained from the Sadtler Standard Reference NMR Spectra.

[b]CH_3-$(CH_2)_3$-CH_2-CH=CH-CH_2-$(CH_2)_5$-CH_2-C(=O)-OCH_3

a b c f f c b d e

and 0.41 for the solvents, respectively.

The amorphous nature and color density of lipofuscin did not permit totally satisfactory IR spectra to be obtained. A broad peak of moderate intensity was seen at 3400 cm^{-1}. This peak is due to hydroxyl with some small contribution from water in the sample. A triplet of peaks occurred at 2950, 2920, and 2850 cm^{-1} representing C-H stretching. These were assigned as CH_3-, asymmetric $-CH_2$-, and symmetric $-CH_2$-, respectively. Additional peaks were observed at 1740, 1725, 1660, 1460, 1390 and 1110 cm^{-1}. These were not defined clearly, but runs on several instruments confirmed these peaks. While the carbonyl peaks at 1725 and 1740 cm^{-1} are indicative of the presence of some type of ester functions, lack of additional structural information prevented unambiguous assignments.

The results of the 220 MHz proton NMR studies are shown in Table II. Small sample size gave considerable noise, making the peak integrations less accurate than normal. The tentative proton ratios and proton assignments are given. The shift values compare closely with those for methyl oleate used as a representative of a long chain ester (Table III). The peak at δ3.59 ppm representing the methoxy protons in methyl oleate probably does not correspond to the same structure resulting in the peak at δ3.70 ppm in the lipofuscin sample. The proton ratios do not coincide. This peak further indicates that some type of ester function is present in the lipofuscin sample as shown by IR spectroscopy. The methyl proton peak (0.91 ppm) in the lipofuscin spectrum appears as an incompletely split multiplet (triplet expected). Unambiguous assignment of the doublet (1.61, 1.68 ppm) is not possible. The suggestion that it is a branched methyl group is supported by the fact that such methyl groups form doublets of this shift value (23) and by the fact that free radical lipid peroxidation mechanisms can lead to chain branching (24). Signal averaging (CAT) techniques were employed, but failed to increase resolution significantly.

Degradative Studies and Analyses

The elemental analysis of fraction A-1 gave 72.38% C, 9.83% H, 3.34% N, 4.37% white ash, 0.44% S, and 0.78% P. After correcting for the ash (composition unknown), these data yielded a simplest formula of $C_{439}H_{711}N_{17}O_{40}P_2S$ by assuming the balance of the analysis to be oxygen. No metal analyses were carried out. This formula corresponded to a mol wt of 6962.

Lipofuscin fraction A-1 was converted to its corresponding methyl esters by transesterification with methanol containing 4% sulfuric acid. No color change was noted during the reaction, but an initial cloudiness disappeared with time to yield a clear solution. If formed, the methyl esters were removed by extraction with diethyl ether. The aqueous residue was examined for the presence of amino acids by spotting on filter paper, spraying with ninhydrin spray, and heating the paper to 110 C for 10 min. A deep purple spot resulted, which was not observed with the unhydrolyzed pigment. Furthermore, subsequent GLC analysis of the N-trifluoracetyl-n-butyl esters of any amino acids present in the residue from the aqueous fraction gave

several peaks which tentatively were identified as amino acid derivatives by comparison with a known mixture of amino acid standards. Due to the minute sample size available, it was not possible to conclusively identify them; however, it should be noted that these data suggested the presence of a variety of amino acids.

GLC analysis for fatty acid methyl esters in the ether extract of the transesterification mixture showed the presence of 12 discernible components plus several less well separated components. The compositional data for the 12 peaks are presented in Table IV. Methyl oleate comprises nearly half of the mixture with significant amounts of stearate and palmitate. Peaks 1 and 12 have effective carbon numbers of 11.2 and 21.9, respectively. Peak 12 may be either 20:3, 20:4, or 22:0 based upon data compiled by Hofstetter, et al., (25). This composition is typical of an oxidized lipid in which the more highly unsaturated fatty acids have been rendered nonvolatile through oxidation and polymerization.

Two additional assays, one for glycerides and one for galactose (as galactolipids), were carried out and yielded negative results. These results produced the problem of explaining the fatty acid release after methanol transesterification. It is possible that the glyceride assay used is not well suited to this type of compound. One alternative is that these fatty acids are esterified in a way other than to glycerol. The observed phosphorus percentage allows that a maximum of ca. 20% phospholipid could be present. This could account for the fatty acid release and the apparent absence of glycerides, since phospholipids will not give a color reaction with the assay used.

TABLE IV

Gas Liquid Chromatographic (GLC) Analysis of Fatty Acids from Transesterification of Fraction A-1

GLC peak number	Percent of total	Identity
1	5.20	---
2	0.45	---
3	1.	---
4	5.09	14:0
5	0.05	---
6	13.68	16:0
7,8	2.04	---
9	20.36	18:0
10	46.82	18:1
11	1.44	18:3/20:0[a]
12	3.39	---

[a]18:3 and 20:0 do not separate on this column.

Conclusions about Lipofuscin Structure

The present work has shown that previously available purification methods for lipofuscin still yielded an impure product (15). The methods described herein achieve a 5- to 10-fold purification, giving a homogeneous material of mol wt 6000-7000 daltons. Gel filtration indicated a mol wt of ca. 10,000 daltons, while vapor pressure osmometry indicated 5900 daltons. Both of these determinations were found to be in error; the error in the latter was due to the polydispersity of the system, and the errors in the former were due to the problems of calibration. Elemental analysis produced data consistent with a mol wt of 6962. Since the pigment appeared not to be a single substance, this mol wt is useful only as a general indication of mol wt. In view of the present work, it is important to note that the term "lipofuscin" should not be applied to denote a single substance but a class of substances which have the general properties described for lipofuscin.

The IR and electronic spectra are essentially those of a typical unsaturated lipid. The UV spectrum does show some, but not extensive, conjugation. The fluorescence spectrum and amino acid analysis add support to the theory of Tappel that the fluorophore is some type of schiff base structure (2). The NMR spectrum generally agrees with the fatty acid analysis. As pointed out previously, the proton ratios are aberrant. There should be only twice as many protons at 2.05 ppm as at 5.14 ppm. In view of this fact, there may be other protons under the 2.05 ppm peak. Likewise, the doublet at 1.61-1.68 ppm may contain more than one type of proton.

From the proposed empirical formula, the equation of McLafferty (26) predicts 94° of unsaturation in the whole "molecule." By comparison, phosphatidylserine (stearyl, oleyl form) has ca. 43° of unsaturation based upon a hypothetical mol wt of 6962. Thus, the pigment contains at least twice this amount of unsaturation, which comes close to explaining the observed NMR proton ratios. These unsaturated sites may be manifest as extended conjugation and as keto acids, both of which are found in autoxidized fatty acids (24).

Shortly after these studies had been completed, we were able to obtain a fourier transform proton NMR spectrum on the pigment. This revealed several additional, but incompletely resolved, peaks not previously observed, thus confirming the complex and heterogeneous nature of the pigment. In addition, exchange studies using deuterium oxide (D_2O) were carried out. No loss of any signal was observed, strongly indicating the absence of O-H or N-H protons or at least the absence of

any significant number of these. Intense fluorescence may be observed from only very small amounts of fluorophores. Thus, the lack of evidence for exchangeable protons does not exclude the possibility of the presence of a schiff base fluorophore. Certainly more evidence is needed, and, at present, no one has suggested the possibility of other fluorophores being present in peroxidized lipids.

In conclusion, it can be stated that human brain lipofuscin is a mixture of more than one polymeric lipid structure with contributions from phospholipid type and amino acid structures. Further work with larger samples may clarify some of the ambiguities and lead to a more accurate assessment of the components involved. The investigation of the source of the fatty acids from the lipofuscin isolate is important in that these data will elucidate the nature of the lipids responsible for the polymer.

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Phospholipase C Catalyzed Formation of Sphingomyelin-^{14}C from Lecithin and N-(^{14}C)-Oleoyl-Sphingosine

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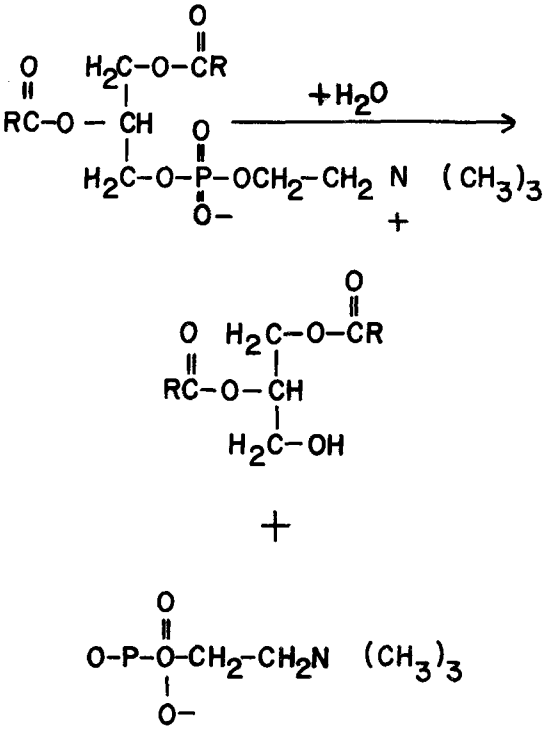
ABSTRACT

Commercial preparations of *Clostridium perfringens* were incubated with phosphatidyl choline and N-1-(^{14}C) oleoylsphingosine. A radioactive product was formed which cochromatogramed with sphingomyelin standard in three different solvent systems. Several other phospholipases and phosphatases were unable to catalyze this reaction. Neither choline, phosphoryl choline, cytidine diphosphate choline nor p-nitrophenyl phosphoryl choline were active donors. Sphingomyelin was only slightly active as a phosphoryl choline donor.

INTRODUCTION

Phospholipase C, phosphatidylcholine choline phosphohydrolase (EC 3.1.4.3), is classified as a phosphoric acid diester hydrolase. The general reaction catalyzed by this enzyme is illustrated for phosphatidyl choline (lecithin) as substrate:



The information available concerning this enzyme has been derived largely from preparations obtained from bacterial sources. The substrate specificity has been found to vary depending upon the particular species from which it has been obtained. This report shows that commercially available preparations of this enzyme have the capacity to produce radioactive sphingomyelin using phosphatidyl choline as the phosphoryl choline donor and N-1(^{14}C)-oleoyl sphingosine as the acceptor.

MATERIALS AND METHODS

The enzyme sources, batch number, and supplier employed were: *Clostridium welchii*, Sigma Chemicals (St. Louis, Mo.) type 1, no. 188-0860 and 53C6870; *C. perfringens*, Koch Light (Colnbrook, England) no. 49137; *Bacillus cereus*, Sigma Chemical, type III no. 54C-0226; phospholipase D, cabbage, Sigma Chemical Co., type III, no. 97B-0390; phospholipase A Vipera russelli, Sigma Chemical Co., no. 120C 2501; acid phosphatase, potato, Sigma Chemical no. 33C-0490; alkaline phosphatase bacterial, Worthington Biochemical Corp., (Freehold, N.J.) BAPSF9DC; and phosphodiesterase, snake venom, Worthington VPH 3BD.

Egg lecithin was obtained from Koch-Light, sphingomyelin from Supelco (Bellefonte, Pa.) and sodium deoxycholate from Schwarz/Mann (Orangeburg, N.J.). 1-(^{14}C)-Oleic acid, from New England Nuclear (Boston, Mass.), was employed for the chemical synthesis of N-1(^{14}C) oleoylsphingosine (specific activity = 1 x 10^5 cpm/nmole) as previously described (1). p-Nitrophenylphosphoryl choline was synthesized according to a published procedure (2).

The incubation tubes were prepared as follows: the various lipids routinely added were 2.5 nmoles N-1(^{14}C)oleoylsphingosine, 600 nmoles lecithin, 200 μg sodium deoxychoalte, and the solvent was removed with a stream of nitrogen. To the residue was added, for phospholipase C containing incubations, 5 μmoles Tris buffer pH 7.46 and 5 μmoles $CaCl_2$; for phospholipase D containing incubations, 20 μmoles $CaCl_2$ and 200 μmoles sodium acetate buffer at pH 5.5; for phospholipase A containing incubations, 10 μmoles $CaCl_2$ and 100

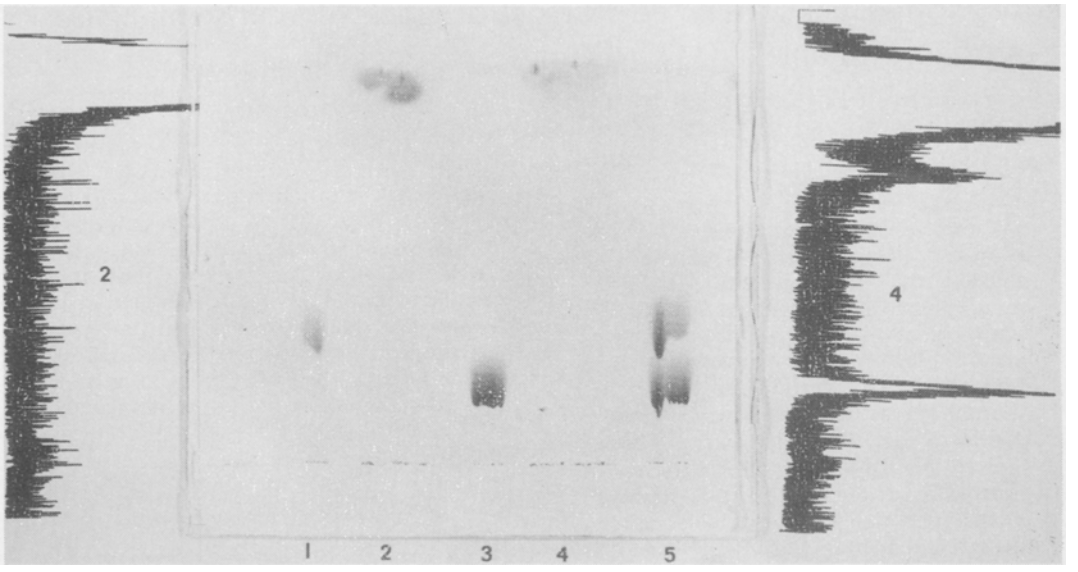


FIG. 1. Thin layer chromatogram and radioscan of product formation. Lane 1 = lecithin standard, lane 2 = chloroform column fraction, lane 3 = sphingomyelin standard, lane 4 = chloroform-methanol (9:1) column fraction, lane 5 = mixed standards. Tracing 2 is of lane 2 and tracing 4 is of lane 4.

μmoles citrate-phosphate buffer pH 6.5; for acid phosphatase containing incubations, 20 μmoles $CaCl_2$ and 200 μmoles sodium acetate buffer at 5.5; and for alkaline phosphatase and snake venom phosphodiesterase containing incubations, 6 μmole Mg acetate and 20 μmoles Tris-citrate buffer pH 8.5. All volumes were adjusted to 0.2 ml with water and the tubes placed in a bath type sonicator for 5 min. Ca. 200 μg enzyme was added and the tubes incubated at 37 C for 3 hr in air with shaking. Boiled enzyme controls were routinely carried through the identical procedures, and all values were corrected for these controls.

The incubations were terminated by the addition of 4 ml chloroform-methanol (2:1, v/v) containing 150 nmoles sphingomyelin carrier and the resulting solution processed according to the procedure of Folch, et al., (3). The final chloroform phase was transferred quantitatively to Silica Gel G plates (Analtech, Newark, Del.). These samples and suitable standards of lecithin and sphingomyelin were chromatogramed in a solvent mixture of chloroform-methanol-water (65:25:4). The plate was exposed briefly to iodine vapor and the areas corresponding to sphingomyelin and ceramide scraped into couting vials. Radioactivity was quantitated in a Packard 3380 liquid scintillation counter with Scinti-verse (Fisher Scientific, Pittsburgh, Pa.). Experimental values are expressed as that percentage of the total counts recovered in the sphingomyelin area.

The contents of five tubes, which had been incubated with phospholipase C, were pooled and concentrated to dryness. The residue was dissolved in chloroform and absorbed on a 2.5 g Unisil (Clarkson Chemical Co., Williamsport, Pa.) column (0.5 cm inside diameter). Fifty ml portions of chloroform, chloroform-methanol 9:1, acetone, and methanol were passed through the column and the subsequent treatment is detailed in the text.

RESULTS

The ability of several commercial phospholipase C preparations to catalyze the formation of a material which cochromatograms with sphingomyelin standard was examined. It was found that three different phospholipase C preparations from clostridium species catalyzed the formation of sphingomyelin. There was ca. 0.7% conversion of the ceramide added to this phosphorylated product. Visual inspection of the thin layer plates indicated that there was a significant reduction of phosphatidyl choline and a significant increase of material at the solvent front with all preparations of this enzyme. Incubation with phospholipase C of *B. cereus*, phospholipase A and D, acid phosphatase, and phosphodiesterase did not result in the formation of sphingomyelin. It was evident from the thin layer chromatography plates, however, that both phospholipases A and D hydrolyzed lecithin as anticipated.

Several compounds were examined as poten-

tial phosphoryl choline donors and were found to be ineffective. These include cytidine diphosphate (CDP)-choline, phosphoryl choline, choline and p-nitrophenylphosphoryl choline. Sphingomyelin, itself, was ca. one-fifth as effective a donor as compared to lecithin.

Fractionation of the labeled lipids contained in the incubation mixture was accomplished with a Unisil column, and a radioscan of TLC from these fractions is presented in Fig. 1. It is apparent that the chloroform effluent contained as the sole radioactive material N-1(^{14}C)-oleoylsphingosine which in this solvent migrates to the solvent front. Radioactivity corresponding chromatographically to sphingomyelin standard was found in the chloroform-methanol (9:1) eluate. The added carrier was removed in the methanol eluate. The silicic acid in this area was scraped off the plate and eluted with 50 ml chloroform-methanol (2:1). The radioactivity cochromatogramed with sphingomyelin on thin layer plates with both chloroform-methanol-glacial acetic acid-water (65:50:1:4) and chloroform-methanol 7N NH_4OH as solvents.

DISCUSSION

These preliminary experiments suggest that commercial preparations of phospholipase C from clostridium species have the capacity to carry out a "transphosphorylcholination" reaction. Phospholipase C of both *C. perfringens* (4) and *C. welchii* (5) will utilize sphingomyelin as a substrate. However, the enzyme prepared from *B. cereus* will not hydrolyze sphingomyelin (6). This is in accord with the apparent inability of the enzyme from this bacterial source to form sphingomyelin from lecithin and radioactive ceramide.

It is apparent that free phosphorylcholine is not the active species since this compound, when substituted for lecithin in the incubation mixtures, did not produce sphingomyelin. Therefore, it is reasonable to assume that there is a "phosphoryl choline-enzyme" intermediate formed which can transfer to ceramide as well as to water. Several other possible donors were found to be inactive, suggesting that lecithin is the preferred substrate. Other hydrolytic enzymes, including phospholipase A, phospholipase D, acid and alkaline phosphatase, and phosphodiesterase, did not result in sphingomyelin synthesis under these conditions.

The classical reaction for sphingomyelin biosynthesis is CDP choline + (threo) ceramide → sphingomyelin + cytidine monophosphate (CMP) (7), and this recently has been challenged. Pulse labeling of SV 40 mouse fibroblasts with ^{32}P during growth in culture suggested that the intact phosphoryl choline moiety of lecithin was transferred directly to sphinglmyelin (8,9). Cell free extracts of these cells subsequently were shown to catalyze these transfer in vitro (10,11). To explain these observations, the existence of a phosphatidyl choline: ceramide choline phosphotransferase has been postulated (11). Similar observations have been obtained with lyophilized mouse brain lysosomes (12).

In light of the results reported in this paper, it is possible that these observations with mammalian tissues are merely a reflection of phospholipase C activity of these cells and tissues. The presence of such an enzyme in mammalian tissues has been reported recently (13).

Studies on phospholipase A showed it to catalyze a transesterification reaction in addition to its usual hydrolytic activity (14). Phospholipase D has been reported to catalyze a transphosphatidylation reaction in addition to its normal hydrolytic activity (15,16). The finding that phospholipase C can carry out a hydrolytic and a transfer reaction suggests that this may be a general feature of all phospholipases.

ACKNOWLEDGMENTS

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Identification of 3, 6, 9, 12, 15-Octadecapentaenoic Acid in Laboratory-Cultured Photosynthetic Dinoflagellates[1,2]

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ABSTRACT

Polar and nonpolar chromatograms of fatty acid methyl esters derived from 11 species of photosynthetic, marine dinoflagellates cultured in modified Erd-Schrieber medium contained a component (4-23%) not identifiable by conventional graphic or arithmetic methods. Hydrogenation followed by gas liquid chromatography of the product showed the unknown component to be a straight chained 18 carbon fatty acid methyl ester. Chemical (CH_4) ionization mass spectrometry of the isolated ester gave a spectrum characteristic of methyl esters and a mol wt of 288, indicating an 18 carbon molecule with 5 double bonds, or equivalent unsaturation. The IR spectrum showed that the double bonds are nonconjugated, and all are *cis* in geometry. Electron impact mass spectrometry of the pyrrolidide derivative provided evidence that double bonds are located in the 3, 9, 15 positions and probably also in the 6 and 12 positions of the molecule. These double bond positions were confirmed by NMR spectrometry. Data obtained by quantitation of the algal methyl esters suggest the possibility that these dinoflagellates synthesize 18:5ω3 (shorthand notation for chain length: number of double bonds and position of final double bond counted from the terminal methyl group) by a 2 carbon chain shortening of 20:5ω3, rather than by the insertion of a Δ3 bond into 18:4ω3.

INTRODUCTION

In 1966, Patton, et al., (1) reported the fatty acid composition of the unicellular marine dinoflagellate, *Gonyaulax polyhedra,* harvested during a "red tide" episode in coastal waters of Southern California. Since that time Dunham, et al., (2) Ackman, et al., (3) and Harrington, et al., (4) have described the fatty acid compositions of a number of photosynthetic dinoflagellates cultured in chemically defined media under controlled conditions. The fatty acids of several dinoflagellates cultured in Erd-Schrieber medium were reported by Chuecas and Riley (5). Although unusual 18 carbon unsaturates were observed in two of these studies (1,4), none were reported to contain more than 4 double bonds.

Eleven species of photosynthetic dinoflagellates, cultured in modified Erd-Schreiber medium by the Microbiology Department of the Virginia Institute of Marine Science (VIMS), were examined for fatty acid composition by gas liquid chromatography (GLC). An analysis of polar and nonpolar chromatograms revealed the presence, in each of the dinoflagellates, of an unknown component which could not be identified by conventional mathematical or graphical techniques. This component, obtained from the species, *Prorocentrum minimum,* was identified tentatively as 3, 6, 9, 12, 15-octadecapentaenoic acid (18:5ω3) by calculations of equivalent chain length (ECL) values using capillary columns of slightly differing polarities and by identification of products arising from partial hydrazine reduction of the 18 carbon esters (6). This report describes the methods by which this tentatively identified component was identified conclusively as all-*cis*-3, 6, 9, 12, 15-octadecapentaenoic acid.

METHODS

The algae (Table I) were grown in 3 liter Fernbach flasks containing 1.5 liter of bacteria free modified Erd-Schreiber medium at 16-19 C. This medium has a base of natural estuarine water (salinity 16 o/oo) and is enriched with a soil extract and a number of salts (composition of enrichment medium available upon request). Illumination of 2700-3300 lux was provided by cool-white fluorescent tubes in a light-dark cycle of 14 hr and 10 hr, respectively. The flasks were agitated manually, twice daily, to prevent stratification of cells and concentration of metabolites. Cells were harvested by centrifugation after 14 days, while the cultures were still in the exponential growth phase.

The algal pellets were drained, and the lipids extracted with CH_3OH (10.0 ml), $CHCl_3$ (5.0 ml: 5.0 ml), and glass distilled H_2O (4.0 ml: 5.0 ml) according to the method of

[1]Presented at the AOCS Fall Meeting, Philadelphia, September 1974.

[2]Contribution number 40 from the Marine Resources Research Institute, Charleston, South Carolina 29412.

TABLE I

Major Fatty Acids of VIMS-Cultured Dinoflagellates
Wt Percent Composition

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	18:4	18:5	20:5	22:6	Σ
Group I												
Gonyaulax digensis	9.7	21.8	1.8	1.8	7.5	0.3	2.1	1.4	8.3	8.2	24.5	87.4
Gonyaulax digitale	4.1	28.3	--	1.5	7.1	0.5	--	7.0	3.8	16.2	23.8	92.3
Gonyaulax catenella	6.5	13.9	2.6	1.2	2.7	1.9	1.8	7.3	11.1	11.2	33.9	94.1
Ceratium furca	4.4	26.1	4.6	0.5	4.8	3.1	0.4	1.0	5.9	7.4	20.7	78.9
Cochlodinium heterolobatum	1.0	32.9	3.8	1.0	6.4	2.4	0.1	1.4	8.2	11.4	28.0	96.6
Prorocentrum micans	1.9	27.5	2.1	1.7	6.7	5.1	0.2	4.5	12.5	2.7	32.1	97.0
Gymnodinium splendens	1.1	28.8	--	2.7	9.8	2.8	0.1	2.1	11.0	8.0	30.3	96.7
Group II												
Gymnodinium simplex	17.5	17.6	4.4	2.9	7.6	2.1	0.4	2.4	19.4	1.1	20.4	98.2
Peridinium triquetrum	2.6	26.6	1.5	1.2	4.8	4.8	0.3	4.3	22.6	5.1	21.3	95.1
Massartia rotundata	12.1	15.5	11.1	2.4	5.6	2.2	3.9	11.6	17.7	0.3	14.1	96.5
Prorocentrum minimum	2.2	23.1	1.4	1.3	4.3	5.4	0.3	4.2	23.2	5.3	24.5	95.2

Bligh and Dyer (7). Phase separation was effected rapidly by centrifugation at low speed for 10 min. Once the $CHCl_3$ phase containing the lipids was obtained, all subsequent operations were carried out under dry N_2. The $CHCl_3$ phase was evaporated just to dryness with N_2, and the lipids were transesterified with BF_3 in CH_3OH (14% by volume) with additional CH_3OH and benzene as recommended by Morrison and Smith (8).

The fatty acid methyl esters of the 11 dinoflagellate species were separated by GLC in a Hewlett-Packard 7620 gas chromatograph equipped with dual flame ionization detectors and 1.8 m, 4 mm inside diameter glass columns packed with 5% SE-30 (Chromosorb W-AW DMCS, 100-120 mesh) or 10% diethyleneglycol succinate (DEGS) (Gas Chrom Q, 100-120 mesh) which were operated isothermally at 180 C (SE-30) and 170 C (DEGS). Helium was used as the carrier gas at 33 psig. The components of the fatty acid ester mixtures were identified provisionally by comparison of the relative retention times ($r_{18:0}$) of the peaks with those of known esters analyzed under the same chromatographic conditions. Methyl esters of cod liver oil were prepared and used as a secondary standard (9). Semilog plots of $r_{18:0}$ against the chain length were constructed for the algal extracts and for the standards (10), and Type I, II, and III separation factors (11) were calculated for all. The chromatograms were quantitated by the method of Carroll (12).

Since *P. minimum* lipids contained a greater percentage of the unknown component than the other species, cultures of this dinoflagellate were established at the Marine Resources Research Institute (MRRI) of South Carolina. River water was imported from the York River, Va., but soil for the soil extract was obtained locally. Otherwise, culture conditions were identical with those described for the VIMS cultures. Extraction and transesterification procedures were also identical. Saponification of the lipids, prior to esterification and GLC analysis, had no effect upon the presence of the unknown component, and this procedure subsequently was omitted.

The methyl esters of *P. minimum* were analyzed in a Hewlett Packard 7610 gas chromatograph equipped with dual flame ionization detectors. Glass columns (1.8 m x 2 mm inside diameter) were operated isothermally and packed with 100-120 mesh Gas Chrom Q, coated with a number of liquid phases (Table II). In addition to the identification procedures utilized for the VIMS cultures, ECLs were determined for all chromatographic peaks.

TABLE II

Equivalent Chain Lengths of Methyl Esters from *Prorocentrum minimum* on Different Liquid Phases[a]

Liquid phase	EGSS-X[b]	EGSS-Y[b]	EGSP-Z[b]	SE-30[b]	BDS[c]
Percent coating	10	10	3	3	--
Column temperature (C)	175	175	160	172	170
FAME[d]			ECL[e]		
16:0	16.00	16.00	16.00	16.00	16.00
16:1ω7[f]	16.63	16.55	16.21	15.78	16.36
18:0	18.00	18.00	18.00	18.00	18.00
18:1ω9[f]	18.58	18.50	18.25		18.26
18:2ω6	19.44	19.14	18.72	17.60	18.84
18:3ω3	20.50	19.45	19.38		19.58
18:4ω3	21.12	20.39	19.65	17.41	19.96
18:5ω3	21.98	21.00	20.02		20.52
20:5ω3	23.52	22.28	21.85	19.60	22.11
22:6ω3	26.08	25.12	24.05	20.88	24.38

[a]Data for 18 carbon esters on Silar-5CP and Apiezon-L have been published elsewhere (6).

[b]1.8 m packed column. EGSS-X = ethyleneglycol succinate methylsilicone copolymer (high polarity), EGSS-Y = ethyleneglycol succinate methylsilicone copolymer (medium polarity), EGSP-Z = ethyleneglycol succinate methylsilicone copolymer (low polarity), and SE-30 = methyl silicone.

[c]46 m wall coated open tubular column. BDS = butanediol succinate.

[d]FAME = fatty acid methyl esters.

[e]ECL = equivalent chain length.

Hydrogenation of the methyl esters followed the method of Appelqvist (13), except that micro reaction vials fitted with Mininert Teflon valves (Supelco, Bellefonte, Pa.) were substituted for the rubber capped vessels specified.

Triglycerides and phospholipids were obtained from an aliquot of *P. minimum* total lipids which was chromatographed on Prekote plates (Absorbosil 5, Applied Science Laboratories, State College, Pa.), using a solvent system of hexane: diethyl ether: acetic acid, 85:15:1, and detected with 2, 7-dichlorofluorescein. The triglyceride and phospholipid classes, eluted with $CHCl_3$: CH_3OH, 2:1, as well as an aliquot of the total lipids, were transesterified with BF_3 in CH_3OH (14% by volume) and the resulting methyl esters identified and quantitated by capillary GLC. The column used was a butanediol succinate (BDS) wall coated open tubular column of stainless steel, 46 m by 0.25 mm (Perkin-Elmer Corp., Norwalk, Conn.), operated in a Perkin Elmer 990 gas chromatograph at 170 C using helium as carrier gas at 50 psig. The electrometer output was recorded with a Honeywell model 16 Electronik 1 mv recorder.

The unknown methyl ester was isolated by preparative GLC, using a 1.8 m x 4 mm inside diameter glass column packed with 10% ethyleneglycol succinate methyl silicone polymer (EGSS-X) (100-120 mesh, Gas Chrom Q), and a few μg were submitted to chemical ionization mass spectrometry-gas chromatography, for determination of mol wt. The instrument, utilizing CH_4 as the reactant gas, was a Finnegan 1015 D mass spectrometer mated with a Finnegan 9500 gas chromatograph and 6000 data system. Operating parameters were as follows: electron energy 135 V, source pressure 750μ, and ambient source temperature. The OV-17 glass column, 1.8 m x 2 mm inside diameter, was programed from an initial temperature of 200 C, at 15 C/min, to a final temperature of 250 C.

An IR spectrum was obtained on 200 μg isolated methyl ester with a Perkin Elmer IR spectrophotometer, model 21, equipped with a Barnes .5 mm cavity cell. The sample was analyzed as a 2% solution in CS_2 (4000-2400 cm^{-1}, 2000-1650 cm^{-1} and 1400-650 cm^{-1}) and in C_2Cl_4 (2400-2000 cm^{-1} and 1650-1400 cm^{-1}).

The isolated methyl ester (1 mg) and a sample of methyl γ-linolenate were derivatized to yield pyrrolidide derivatives by the method of Andersson and Holman (14). Mass spectra were recorded with an LKB 9000 mass spectrometer-gas chromatograph at an ionization potential of 70 V. The source temperature and separator were maintained at 250 C. The 2% OV-17 glass column (1.8 m x 2 mm inside diameter) was programed from 200-250 C at 10 C/min.

A proton magnetic resonance spectrum was

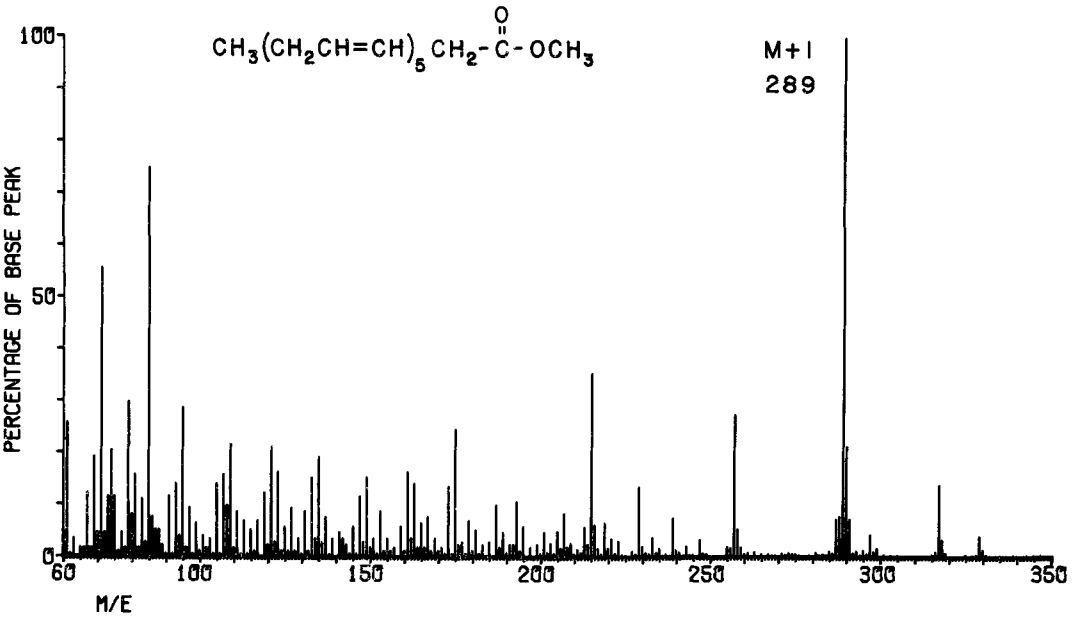


FIG. 1. Chemical (CH_4) ionization mass spectrum of methyl 3, 6, 9, 12, 15-octadecapentaenoate isolated from methyl esters of *Prorocentrum minimum*.

recorded with a Varian XL-100 NMR spectrometer locked to D_3 at 1000 Hz on 1 mg isolated methyl ester in $CDCl_3$ which contained 1% tetramethylsilane (TMS) (δ 0.00) as an internal standard.

RESULTS AND DISCUSSION

The photosynthetic dinoflagellates listed in Table I, when cultured by the described method, synthesize lipids which are particularly rich in 16:0 and 22:6ω3. Some species also contain significant amounts of 20:5ω3, and one, *M. rotundata*, contains a substantial percentage of 18:4ω3. A peak was present in the polar chromatograms of all species, ranging from 3.8-23.2%, which tentatively was identified as 22:0 or 20:3ω6 on the basis of the $r_{18:0}$. However, when the esters were analyzed on the nonpolar SE-30 column, the unknown component had a retention time which was less than that of methyl linolenate, suggesting that the component might be an 18 carbon ester with an unusual number or arrangement of ethylenic bonds.

The retention behavior of the unknown component, derived from *P. minimum* lipids, was examined on a number of columns of varying polarities (Table II). The ECL of the component decreased with decreasing polarity of the column, as did those of the known 18 carbon unsaturates. Hydrogenation of the *P. minimum* esters followed by quantitation of SE-30 chromatograms of the products confirmed that the molecule was a straight chained 18 carbon compound with an unknown degree of unsaturation.

When CH_4 is used as the reactant gas, chemical ionization mass spectrometry is a highly useful technique for determination of mol wt of methyl esters, since the base peak in such spectra is the protonated molecular ion (15). The base peak in the spectrum obtained on an isolate of the unknown ester (Fig. 1) was m/e 289, indicating the presence in the molecule of five ethylenic bonds, or equivalent unsaturation. However, as is the case with electron impact spectra of unsaturated methyl esters (16), no information concerning ethylenic bond position can be gained from the spectrum.

The IR spectrum was characteristic of that of an unsaturated methyl ester and indicated the presence of nonconjugated double bonds by absorptions at 3020 cm^{-1}, 1650 cm^{-1}, and 1395 cm^{-1} (17). The absence of significant absorption at 965 cm^{-1} indicated that all the ethylenic bonds are *cis* in geometry, and there was no evidence of terminal unsaturation (18). No absorption was present at 2150 cm^{-1}, indicating that acetylenic unsaturation, if present, would be centrally located in the molecule (19).

Through the use of capillary column GLC, it is possible to calculate, with considerable accuracy, the ECLs of polyenoic fatty acids from fractional chain length values of the monoenoic

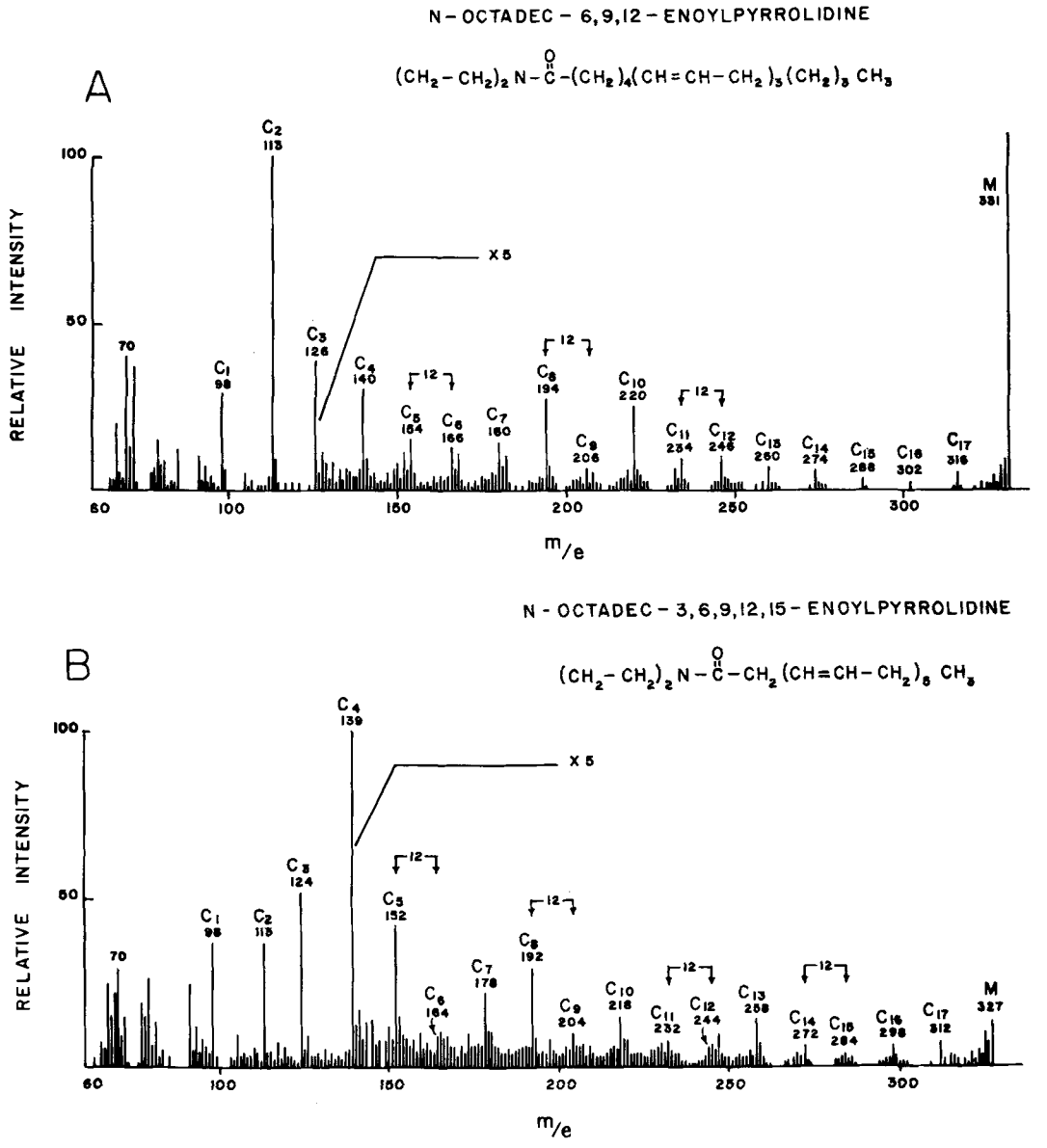


FIG. 2. (A) Mass spectrum of n-octadec-6, 9, 12-enoylpyrrolidine. (B) Mass spectrum of n-octadec-3, 6, 9, 12, 15-enoylpyrrolidine prepared from an isolate of *Prorocentrum minimum* fatty acid methyl esters.

bonds in the molecule, if one takes into account the interaction between monoenoic bonds separated by methylene groups (6,20,21). Calculations of ECLs for a hypothetical 18:5ω3 ester on BDS, Silar-5CP, and Apiezon-L capillary columns were satisfactorily close to the experimentally observed ECLs of the unknown ester. In addition, products arising from partial hydrazine reduction of the esters of *P. minimum* could be identified tentatively by similar ECL calculations. These identities were consistent with those which would be expected to arise from an 18:5ω3 ester, and it was concluded that the unknown ester was, in all probability, 18:5ω3 (6).

Andersson and Holman (14) have found that mass spectra of pyrrolidides of monounsaturated, straight chained fatty acids contain principally ions from the polar end of the molecule, which gives a simplified spectrum. Their interpretative rule for such spectra states that: "If an interval of 12 atomic mass units, instead of

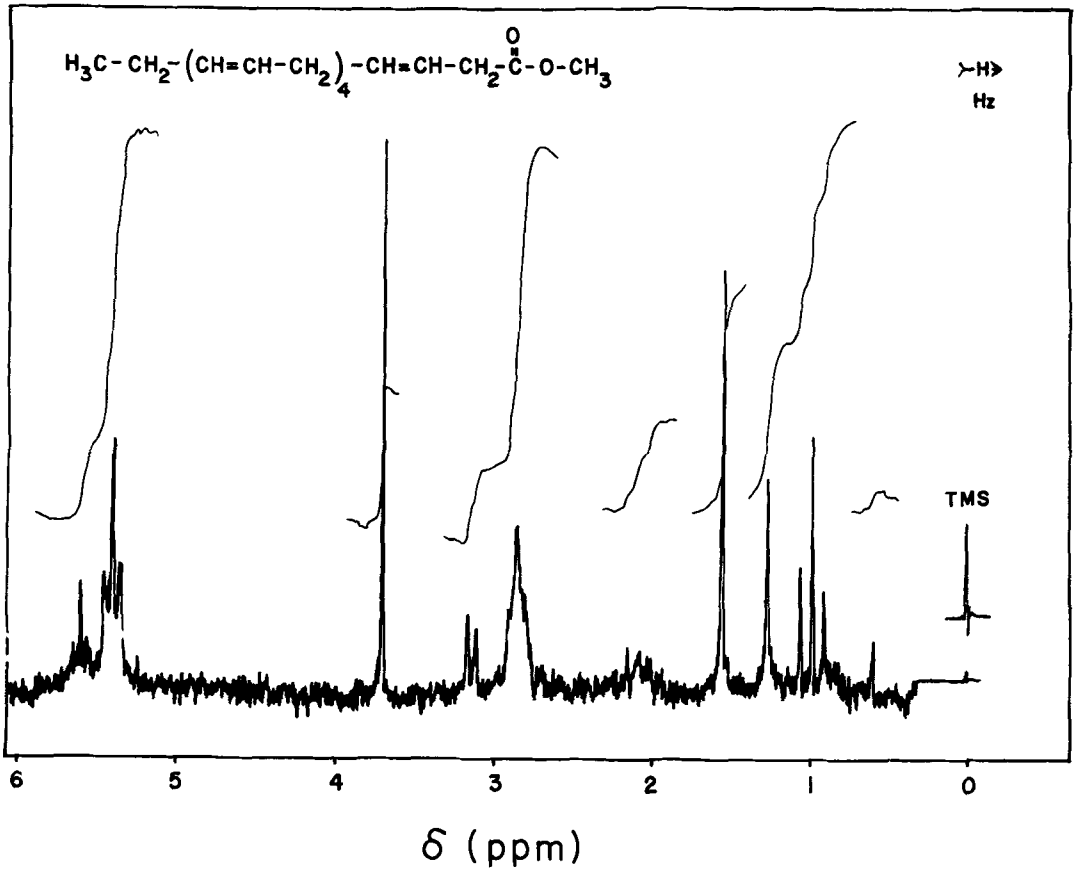


FIG. 3. Proton magnetic resonance spectrum of methyl 3, 6, 9, 12, 15-octadecapentaenoate isolated from methyl esters of *Prorocentrum minimum*. TMS = tetramethylsilane.

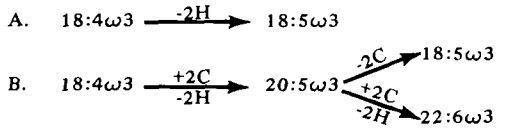
the regular 14, is observed between the most intense peaks of clusters of fragments containing n and n-1 carbon atoms of the acid moiety, the double bond occurs between carbons n and n+1 in the molecule." This rule is valid for 18:1 isomers whose ethylenic bonds lie in positions 5-15 in the molecule. The 18 carbon monoenes with unsaturation in the 4, 16, and 17 positions have unique spectra which characterize them. The spectrum of n-octadeca-6, 9, 12-enoylpyrrolidine (Fig. 2A) demonstrates clearly that Andersson and Holman's rule is applicable to polyenes, as well as to monoenes. Not only is m/e 113 the base peak of this spectrum, as was found for the monoenes studied, but also 12 atomic mass unit differences are observed between carbons 5 and 6, carbons 8 and 9, and carbons 11 and 12, thus locating double bonds in the 6, 9, and 12 positions in the molecule.

The spectrum of the pyrrolidide derivative of the tentatively identified 18:5ω3 (Fig. 2B) is less satisfactory than that of methyl γ-linolenate, in that numerous fragments of odd m/e are present. This is particularly troublesome in the region of C6 (m/e 164) and C12 (m/e 244) although both are of fractionally greater relative intensity than peaks 2 mass units greater, m/e 166 and m/e 246, respectively. With this qualification, it appears that ethylenic bonds are present in the 6, 9, 12, and 15 positions of the molecule. The unique and highly diagnostic feature of this spectrum is the base peak of m/e 139 which is the base peak in spectra of pyrrolidides having Δ3 unsaturation (B. Andersson, personal communication).

The proton magnetic resonance spectrum of the tentatively identified 18:5ω3 ester is shown in Figure 3. Interpretation of the spectrum is based upon values cited by Hopkins (22) and by Frost and Barzilay (23). The triplet at .97 δ is similar in shape and chemical shift to that observed in spectra of 18:3ω3 and marine oils rich in ω3 polyunsaturated fatty acids (24) and represents the three protons of a terminal CH_3 group which is β to an olefinic group. The multiplet at 2.08 δ has an area equivalent to

two protons and arises from the protons of one CH_2 group α to a double bond. The triplet at 5.39 δ has an area equivalent to eight protons of four olefinic groups, and the multiplet at 5.59 δ arises from the two protons of the $\Delta 3$ olefinic group (25) confirming the presence of five double bonds in the molecule. The triplet at 2.84 δ has an area equivalent to eight protons of the four CH_2 groups present between five olefinic groups, and the doublet at 3.13 δ arises from the two protons of the CH_2 group which is α to both the C=O and the $\Delta 3$ olefinic groups. The sharp singlet at 3.69 δ, with an area of three protons, arises from the $COOCH_3$ group. These signals account for all of the protons in the proposed empirical formula of the methyl ester, $C_{19}H_{28}O_2$, and confirm the tentative identification of 18:5ω3. A spectrum of the solvent established that peaks at .60 δ and 1.54 δ are solvent impurities. The signal at 1.25 δ was not present in the solvent spectrum but may arise from the polymethylene chains of methyl myristate, methyl palmitate, and methyl stearate, the major contaminants (1.3%) of the isolate, as indicated by GLC.

An ω3 18 carbon pentaene might be synthesized in one of two ways, either through dehydrogenation of 18:4ω3 (A) or possibly through the loss of an acetate unit from 20:5ω3 (B):



Data shown in Table I may be interpreted as tentative support of the second pathway. The algae of Table I were separated into two groups according to the ratio 18:5/22:6. This ratio in the algae of group I is ca. 1/3, with the exception of *Gonyaulax digitale*, in which the ratio is 1/6. In the group II algae, this ratio is almost exactly 1/1. This numerical relationship between the percentages of the two acids in the dinoflagellates is shown in Figure 4 and suggests the possibility of a biosynthetic relationship between the two acids. A similar but less obvious relationship can be shown between the amounts of 18:5ω3 and 20:5ω3, but no such relationship can be shown between amounts of 18:5ω3 and 18:4ω3.

Additional tentative support for pathway B is found in the data of Table III. This table shows the distribution of the more important fatty acids of *P. minimum* between the triglycerides and the phospholipids. The phospholipids, in which major amounts of 18:5ω3 are present, contain two 16 carbon monoenes,

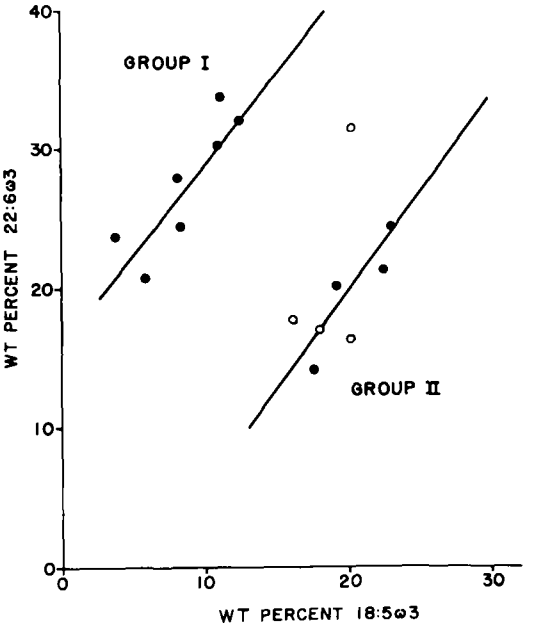


FIG. 4. The relationship between wt percentages of 18:5ω3 and 22:6ω3 in fatty acid methyl esters of the dinoflagellates of Table I (•). Cultures of *Prorocentrum minimum* from the Marine Resources Research Institute are included (○).

16:1ω9 and 16:1ω7. Since the fatty acid desaturases which produce monoenes from saturated acid precursors are generally specific for the $\Delta 9$ position in the molecule, 16:1ω7 is, therefore, usually the major 16 carbon monoene synthesized (26). On the other hand, 16:1ω9 probably arises largely from β-oxidation of 18:1ω9, the major 18 carbon monoene (6). The fact that the percentage of 16:1ω9, although low, is greater than that of 16:1ω7 in the phospholipids of *P. minimum*, suggests that chain shortening by an acetate unit may be an important biosynthetic process in dinoflagellates cultured under these particular conditions.

Fatty acids having $\Delta 3$ unsaturation have been observed in lipids of photosynthetic tissues and some seed oils, but all of these $\Delta 3$ bonds are *trans* in geometry (25,27,28), and 3, 6, 9, 12, 15-octadecapentaenoic acid appears to be unique in possessing $\Delta 3$ *cis* unsaturation.

The dinoflagellates of this study, cultured under specific conditions, synthesize substantial percentages of a fatty acid not previously reported to occur in living organisms. Previous investigations have shown the presence of unusual 18 carbon polyunsaturated acids in dinoflagellate lipids, which were identified tentatively as geometric or positional isomers of all-*cis*-18:4ω3. It now seems possible that one or more of these unusual acids may have been

TABLE III

Prorocentrum minimum Fatty Acid Methyl Esters (FAME)
Wt Percent Composition[a]

FAME[b]	Total lipid	Triglycerides	Phospholipids
14:0	4.2	6.2	4.7
16:0	29.6	34.4	24.1
16:1ω9	0.3	1.0	1.9
16:1ω7	0.5	3.4	1.5
18:0	4.1	7.6	4.5
18:1ω9	4.2	7.7	12.7
18:1ω7	1.5	0.8	1.9
18:2ω6	5.6	6.7	3.7
18:3ω3	0.4	0.5	0.4
18:4ω3	3.2	0.6	3.9
18:5ω3	18.8	4.1	19.1
20:0	2.6	3.3	0.9
20:1ω11 + 13	--	0.2	0.1
20:1ω9	--	3.9	0.1
20:5ω3	4.2	4.0	3.0
22:0	0.6	0.2	0.5
22:1ω11 + 13	--	3.7	--
22:1ω9	--	0.5	--
22:6ω3	18.0	10.4	14.2

[a]Butanediol succinate capillary column.

[b]Other esters observed in minor amounts: 16:1ω5, 18:1ω13, 20:1ω7, 20:1ω5, 20:3ω6, and 22:1ω7.

18:5ω3, but, since they were observed only in small amounts, complete structural identification would have been both laborious and time consuming. It should be noted that recent developments in mass spectrometry have greatly facilitated the structural analysis of minute amounts of unsaturated fatty acid derivatives. It also seems possible that since 18:5ω3 apparently has not been observed previously in sufficient amounts to permit certain identification, some component, or combination of components, in the modified Erd-Schrieber medium may be the stimulus for synthesis of significant amounts of this acid in these algae.

The data suggest that this acid arises from β-oxidation of 20:5ω3 rather than through the introduction of a Δ3 bond into 18:4ω3. This question can be resolved only through further investigation.

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Stereospecific Analysis of Hepatoma, Host Liver, and Normal Rat Liver Triglycerides from Animals on Chow and Fat Free Diets

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ABSTRACT

Triglycerides from normal liver, host liver, and hepatoma of rats maintained on chow and fat-free diets were subjected to stereospecific analysis. Normal and host liver triglycerides from animals on the same diet did not exhibit significant differences. Fat-free diet reduced polyunsaturated fatty acids in normal and host liver triglycerides, but had no effect upon hepatoma triglycerides. Each position of hepatoma and liver triglyceride glycerol exhibited a characteristic fatty acid composition. Palmitate concentrations were reduced dramatically and stearate levels were increased significantly at the 1 position of hepatoma triglycerides, relative to the corresponding position of liver triglycerides which were affected little by diet or tumor. Except for higher percentages of C-20 and higher fatty acids, common to all three positions, the composition of hepatoma triglycerides at the 2 position appeared normal. The 3 position of hepatoma triglycerides contained significantly higher percentages of stearate than liver. Data obtained previously for Ehrlich ascites cell triglycerides were in good agreement with this hepatoma. Data from these two neoplasms suggest that the metabolic system that regulates or controls the fatty acid composition at the 1 and 3 positions of normal tissue triglycerides does not function normally in neoplasms.

INTRODUCTION

The effects of a tumor upon the lipid metabolism of a host animal are not well understood. Likewise, it is not known how altered lipid metabolism in the host animal might affect the tumor. The literature (which contains some discrepancies) on the changes that occur in host animal lipids as a result of neoplasia has been reviewed (1,2). This is the second in a series of papers that describe the results of experiments designed to investigate the relationship between host and tumor lipid metabolism. The structure of hepatoma, host liver, and normal liver triglycerides as affected by diet are reported here.

METHODS AND MATERIALS

Normal and host animal livers and 7288CTC hepatomas were obtained from groups of animals maintained on chow and fat-free diets for 4-5 weeks; lipids were extracted and fractionated into neutral lipid and polar lipid fractions as described previously (2). Triglycerides were resolved from the other neutral lipids 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). Triglyceride bands were visualized with Rhodamine 6G and eluted from the adsorbent with chloroform-methanol (2:1, v/v). The stereospecific analysis procedure of Brockerhoff (3) scaled down for 3-5 mg quantities plus analysis of both 1, 2- and 1, 3-diglycerides, as described previously (4), was employed. The 1, 2- and 1, 3-diglycerides were resolved completely on Silica Gel G plates developed in a solvent system of chloroform-acetone, 95:5 v/v. This elimates the necessity of using boric acid in the chromatoplates and acetic acid in the developing solvent. The use of smaller sample size necessitated the purification of the diglyceride phenyl phosphates by TLC (diethylethermethanol-33% aqueous ammonium hydroxide 84:14:2, v/v/v) before enzymatic hydrolysis so that the products could be more easily located on TLC after hydrolysis. Fatty acids liberated during hydrolysis were converted to methyl esters with diazomethane (5), whereas lyso and unreacted phenyl phosphates were transesterified with methanol catalyzed by sulfuric acid (6). After the methyl esters were purified by TLC, they were analyzed by gas liquid chromatography (GLC) on ethylene glycol succinate methyl silicone polymer (EGSS-X) (10%) columns programed from 140-220 C at 2 C/min. (6). Identities of major fatty acid esters were based upon analysis before and after hydrogenation and cochromatography with standards.

Ethylmagnesium bromide was purchased from Matheson, Coleman and Bell, Norwood, Ohio. Phenyl phospherodichloridate was obtained from Eastman Organic Chemicals, Rochester, N.Y. *Crotalus atrox* venom was purchased from Ross Allen's Reptile Institute, Silver Springs, Fla. Reagents were reagent grade or better and used without further purification. The source of glass distilled solvents and stan-

TABLE I

Stereospecific Analysis of Triglycerides Derived from Normal Livers, Host Livers, and Hepatomas of Rats Maintained on Chow and Fat-Free Diets[a]

	Fatty acid percentages[b]											
Fatty acid position	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:2	20:4	22:3 + 24:0	22:4 + 24:1	>24:1
						Rat liver, normal, chow diet						
1[c]	1.5	63.2	4.3	4.6	15.6	8.0	T[d]		T			
2	0.8	9.6	3.2	1.4	41.8	42.0			0.6			
3	T	12.5	4.0	2.4	50.2	26.4	0.7		1.2			1.0
						Rat liver, normal, fat-free diet						
1	1.6	73.7	5.8	3.0	15.7							
2	1.2	16.2	11.8	2.2	68.1	T						
3	1.0	26.0	12.1	5.2	55.1	T						
						Rat liver, host, chow diet						
1	0.7	57.2	1.7	11.1	14.6	10.9			2.0			T
2	0.5	12.4	1.1	2.6	34.0	43.0	T		4.5			1.5
3		12.9	1.6	4.9	44.5	30.6	T	T	3.4			1.3
						Rat liver, host, fat-free diet						
1	1.0	64.9	3.4	7.4	21.8	1.4			T			
2	0.6	16.2	5.7	3.3	67.4	5.4			0.8			T
3	T	24.1	5.0	8.0	60.4	2.2						
						Hepatoma, host fed chow diet						
1	1.2	21.5	1.4	27.2	22.0	7.8	4.0	1.8	3.9	2.0	3.9	0.7
2	1.0	15.5	T	6.0	33.0	29.8	0.6	0.6	8.4		2.8	0.8
3	0.5	14.6	0.6	17.1	30.6	14.6	3.7	1.8	7.0	2.4	4.3	1.0
						Hepatoma, host fed fat-free diet						
1	1.1	25.3	1.1	29.6	21.2	6.2	4.1	1.2	4.6	1.6	2.8	T
2	1.1	16.2	2.0	4.8	33.8	25.0	T	T	10.5		3.2	2.5
3	0.6	14.6	2.0	16.0	32.8	12.2	3.8	1.2	8.9	1.5	4.0	1.1

[a]Analyses were performed on triglycerides isolated from a pooled sample of tissue from three-six animals in each group.

[b]Percentages represent the mean of duplicate determinations. Agreement between determinations was ±5% for major acids and ±10% for minor acids. The difference between the sum of percentages at any position and 100% represents the sum of other acid percentages not given in the table.

[c]The 1 position represents the mean of values obtained from 1, 3-diglycerides and 1, 2-diglycerides (see text).

[d]T denotes detectable quantities of less than 0.5%.

dards was Burdick and Jackson Laboratories, Muskegon, Mich., and NU-Check-Prep, Elysian, Minn.

RESULTS

The 1 position values in Table I represent the mean of 1 position percentages obtained from 1, 3-diglycerides and 1, 2-diglycerides. Generally, the 1 position values obtained from the 1, 2-diglycerides contained ca. 10% higher percentage of saturated acids, primarily palmitate, than the 1 position values obtained from the 1, 3-diglycerides, which contained higher percentages of 18:1 and 18:2 acids. The differences probably resulted from some acyl migration of the 3 position fatty acids, high in 18:1 and 18:2 acids, to the 2 position, yielding 1, 2-diglycerides. During subsequent phospholipase A hydrolysis, the 2 position fatty acids of the 1, 2-phenyl phosphates would be released, along with 1 position fatty acids from the 1, 3-diglyceride phenyl phosphates. 2, 3-Diglyceride phenyl phosphates resulting from the acyl migration of the 1 position fatty acids to the 2 position of the 1, 3-diglyceride are not hydrolyzed (3). Yurkowski and Brockerhoff (7) have reported an acyl migration of ca. 6% in 1, 3-diglycerides during analysis, whereas acyl migration in 1, 2-diglycerides was less than 1.5%. Calculated triglyceride composition percentages agreed well with the determined composition of the triglycerides when the mean values of the 1 position were used. Likewise, the calculated 2, 3-diglyceride percentages agreed well with determined percentages from the unreacted 2, 3-diglyceride phenyl phosphates. Despite the fact that the 1 position

TABLE II

Comparison of Determined and Calculated Triglyceride Carbon Number Distributions for Normal Liver, Host Liver, and Hepatoma from Rats Maintained on Chow and Fat-Free Diets

Source of triglyceride values	Carbon number percentages									
	46	48	50	52	54	56	58	60	62	64
Rat liver, normal, chow diet										
Triglyceride (deter.)[a]		2.2	14.3	52.0	19.5	10.7	1.4	T[c]		
Calculated 1-R, 2-R, 3-R dist.[b]		0.8	12.8	50.2	24.2	8.5	3.0	0.3		
Rat liver, normal, fat-free diet										
Triglyceride (deter.)	1.2	15.5	42.6	37.8	3.0					
Calculated 1-R, 2-R, 3-R dist.	0.3	9.1	38.4	45.5	6.6					
Rat liver, host, chow diet										
Triglyceride (deter.)		0.9	13.9	55.2	20.6	7.8	1.4			
Calculated 1-R, 2-R, 3-R dist.		0.4	7.4	41.6	32.0	12.4	5.0	0.9	T	
Rat liver, host, fat-free diet										
Triglyceride (deter.)		5.8	33.8	49.1	10.3	0.9				
Calculated 1-R, 2-R, 3-R dist.		1.8	18.7	53.1	24.3	1.8				
Hepatoma, host fed chow diet										
Triglyceride (deter.)		11.9	13.0	19.7	21.7	13.8	9.8	5.8	2.6	0.8
Calculated 1-R, 2-R, 3-R dist.		T	2.2	13.3	30.1	21.8	15.5	10.2	4.3	1.8
Hepatoma, host fed fat-free diet										
Triglyceride (deter.)		10.4	9.8	15.8	17.9	15.1	14.2	9.8	4.7	1.7
Calculated 1-R, 2-R, 3-R dist.		0.4	3.8	15.2	26.4	22.0	16.6	8.6	4.4	1.9

[a]Determined (deter.) triglyceride carbon number percentages were taken from earlier data (2).

[b]The 1-random-2-random-3-random distribution (dist.) was calculated from the stereospecific analysis data given in Table I.

[c]T denotes detectable quantities of less than 0.5%.

fatty acid percentages might exhibit a slightly higher level of 18:1 and 18:2 acids than the absolute value in some groups, the validity of the conclusions drawn from the data are in no way affected.

Percentages of fatty acids esterified at the 1, 2, and 3 positions of triglyceride glycerol obtained from normal liver, host liver, and hepatoma of animals maintained on chow and fat-free diets are given in Table I. Each position exhibited a characteristic fatty acid composition: the 1 position contained a high percentage of saturated fatty acids; the 2 position exhibited the most unsaturation; and the 3 position contained ca. half the level of 18:2 as the 2 position. As expected, the fat-free diet reduced the quantities of polyunsaturated fatty acids and elevated the quantities of monoenoeic acids in the triglycerides of normal and host liver. Except for slightly higher percentages of polyunsaturated fatty acids in the host liver triglycerides, normal and host liver distribution patterns of animals on the same diet were similar. Hepatoma triglycerides, unaffected by diet, exhibited completely different fatty acid distribution patterns and profiles than liver triglyceries. All hepatoma triglyceride positions contained between 15-20% C-20 and higher fatty acids, whereas host and normal liver triglycerides contained 5% or less of these acids. The 1 position of the hepatoma contained only one-third to one-half the percentage of palmitate and more than three times the percentage of stearate as liver triglycerides. Except for the higher percentage of C-20 and longer chain fatty acids, the hepatoma 2 position closely resembled the 2 position of liver triglycerides.

Percentages represent the mean of duplicate determinations. Since only minor differences were observed between control and host animals on the same diet, they may be considered as duplicate experiments for assessing reproducibility. Likewise, because the effect of diet upon the hepatoma triglycerides was minimal, the data from the hepatomas of the two groups of animals may be used to estimate variability between hepatomas.

The 1-random-2-random-3-random distributions, calculated from the data given in Table I, are compared with the determined triglyceride carbon number distributions in Table II. Generally, the determined values exhibited a higher percentage of the lower mol wt species than was obtained from calculated values based upon a 1-random-2-random-3-random distribution of the fatty acids. The lack of agreement was most

apparent in the host liver and hepatoma triglycerides.

DISCUSSION

Livers of normal rats fed the fat-free diet contained ca. 3.5 times more triglycerides than livers of normal chow-fed animals, and host liver triglyceride concentrations were ca. one-half normal liver values for each diet (2). Despite this large effect of diet and hepatoma upon liver triglyceride levels, the fatty acid composition of the 1 position remained relatively constant (Table I). 1 Position fatty acid percentages from analysis performed in other laboratories on liver triglycerides from other strains of rats maintained on different diets (8-10) agree well with the present data. The constancy of the 1 position fatty acid composition, particularly palmitate (60-70%) and stearate (4-7%), indicates the lack of fluctuation at this position due to very drastic nutritional and pathological stresses. In contrast, the 1 position of hepatoma triglycerides differed dramatically from liver (Table I). The 1 position composition of Ehrlich ascites cell triglycerides reported previously (4) also agreed very closely. The data from these two neoplasms suggest an alteration in triglyceride biosynthesis that affects the 1 position composition. Perhaps the specificity of acyl-CoA-sn-glycerol-3-phosphate acyltransferase exhibited by rat liver mitochondria (11) has been lost to some extent in neoplasms. Alternatively, the reason for the decreased palmitate-increased stearate at the 1 position of hepatoma triglycerides may lie in the fact that the dihydroxyacetone pathway (12) for phosphatidate biosynthesis probably dominates over the usual α-glycerol phosphate pathway (13) because of the characteristic decreased activity of α-glycerol phosphate dehydrogenase in most neoplasms (14). The decreased palmitate and increased stearate concentration at the 1 position contributes in part to the increased percentage of higher mol wt species of triglycerides found in neoplasms (2, 4, 15), which indicates this metabolic alteration may be common to most neoplasms.

Several laboratories (8-10) have demonstrated that the 2 position of liver triglycerides from rats on normal diets contained the highest percentage of polyunsaturated acids, primarily 18:2; this observation is confirmed by the present data. Likewise, the 2 position of hepatoma triglycerides contained the highest percentage of polyunsaturated acids. What is more important is that the hepatoma triglycerides from the fat-free fed hosts, essentially devoid of polyunsaturated acids, contained the same level of polyunsaturated acids as hepatoma triglycerides from chow-fed hosts. Perhaps the hepatoma has the ability to mobilize host phospholipids which are absorbed from the circulation, degraded, and used for triglyceride biosynthesis. Agreement between the percentages of saturated fatty acids at the 2 position of hepatoma and liver triglycerides suggests that fatty acids are esterified at the 2 position of hepatoma triglycerides in a manner similar to that of normal liver and are unaffected by diet or neoplasia.

The quantities of 18:2 esterified at the 3 position of both liver and hepatoma triglycerides suggested a relationship. Examination of 11 triglyceride determinations: hepatoma, host liver, and normal liver of the present study, Ehrlich ascites cells (4), normal rat liver (8-10), and pig liver (16) showed that the 3 position contained 55 ± 8%, as much 18:2 as the 2 position. Elimination of the two host liver determinations in the present study gave a closer correlation (54.5 ± 5%). Although the level of 18:2 at the 3 position of hepatoma triglycerides appears normal, the percentage of stearate is two to four times higher than the host or normal liver stearate percentage and previously reported stearate percentages at the 3 position of normal rat liver triglycerides (8-10). The increased percentage of stearate at the 3 position of the hepatoma triglycerides compared favorably with the previous data reported for Ehrlich ascites cell triglycerides (4). The data from these two neoplasms suggest that the specificity of diglyceride acyltransferase in neoplasms is less specific than in normal tissue. It should be kept in mind, however, that the composition of the fatty acids available for exterification of the 3 position of triglycerides in neoplasms may be different from that found in normal tissue.

The determined carbon number distribution of Ehrlich ascites cell triglycerides determined previously (4) agreed well with the calculated 1-random-2-random-3-random distribution. Such a comparison did not hold for this hepatoma or liver triglycerides (Table II). These data indicate some pairing of fatty acids occurs in the hepatoma triglycerides similar to that observed for rat liver triglycerides. Stereospecific analysis of fractionated hepatoma triglyceride fractions appears to be necessary to determine whether the preferential pairing of some fatty acids in the hepatoma is the same as in liver.

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Stimulatory Effect of Glucose upon Triglyceride Synthesis from Acetate, Decanoate, and Palmitate by Mammary Gland Slices from Lactating Mice

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ABSTRACT

Slices prepared from the mammary glands of lactating mice incorporate only small amounts of (1-^{14}C) acetate, (1-^{14}C) decanoate, or (1-^{14}C) palmitate into lipids. However, when glucose is added to the incubation medium, fatty acid incorporation is stimulated—13-fold from acetate, 17-fold from decanoate, and 2-fold from palmitate. Over 90% of the ^{14}C activity in the lipid fraction is in triglycerides. Analysis of fatty acids in the triglycerides showed that almost all of the decanoate and the palmitate were incorporated as intact molecules, while acetate yielded acids of varying chain lengths. The glucose stimulation of triglyceride synthesis is not solely due to its effect upon chain elongation but also could involve glyceride-glycerol availability, as well as other unknown factors. However, from measurements of the amounts of glycerol 3-phosphate in the tissue incubated in the presence of palmitate, it would appear that glyceride-glycerol availability is not rate limiting in triglyceride synthesis.

INTRODUCTION

As early as 1949, Folley and French (1) suggested that mammary gland slices prepared from the glands of lactating rats could synthesize copious amounts of fatty acids from glucose. It was further learned that the rate of synthesis was greatly dependent upon the physiological state of the animal. Only during lactation did the gland have this capacity (2). Slices of this tissue also could incorporate large amounts of acetate (3-6), lactate (7), and alanine (8) carbon into fatty acids provided glucose was added to the incubation medium. The mechanism of this glucose effect has been studied by many investigators (6,9,10) who generally concluded that glucose oxidation via the pentose phosphate pathway, which generates the nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) required for fatty acid synthesis, plays a major role. For some time now, we have been satisfied with this explanation as, indeed, have other workers who also have applied it to adipose tissue (11,12).

The eventual product of fatty acid synthesis by mammary glands of lactating animals is milk triglycerides. Lactating mammary glands are also capable of converting exogenous fatty

TABLE I

Utilization of Acetate, Decanoate, and Palmitate by Slices of Mammary Gland from Lactating Mice for Triglyceride Synthesis[a]

(^{14}C) Substrate			nmoles Substrate converted to:		
Compound	Conc.[b] mM	Glucose mM	CO_2	Total lipids	Triglycerides
(1-^{14}C) Acetate	2.5	0	1206 ± 156	105 ± 9	92 ± 12
(1-^{14}C) Acetate	2.5	10	833 ± 91	1387 ± 96	1290 ± 107
(1-^{14}C) Decanoate	1.25	0	91 ± 12	76 ± 5	71 ± 6
(1-^{14}C) Decanoate	1.25	10	26 ± 3	1284 ± 128	1220 ± 154
(1-^{14}C) Palmitate	0.25	0	2 ± 0	56 ± 9	45 ± 8
(1-^{14}C) Palmitate	0.25	10	1 ± 0	100 ± 9	94 ± 9

[a]Mammary gland slices (100 mg wet wt) from mice lactating for 17 days were incubated for 2 hr at 37 C in 2.0 ml Krebs-Henseleit bicarbonate buffer pH 7.4 (24) containing the sodium salts of (1-^{14}C) fatty acids at optimal concentrations indicated, defatted albumin (8 mg) in the absence or presence of glucose with 95% O_2 and 5% CO_2 as gas phase. The amount of radioactivity of each acid converted to product was determined (19,21). The results given are the means ± standard error of three experiments, each performed in duplicate with tissues from different lactating mice.

[b]Conc. = concentration.

TABLE II

Identification of Fatty Acids in Triglycerides Synthesized from Acetate, Decanoate and Palmitate by Slices of Mammary Gland from Lactating Mice[a]

Fatty acid	Glucose mM	Percent distribution of radioactivity in fatty acids: C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$
(1-^{14}C) Acetate	0	3.7	33.2	31.3	17.6	9.0	1.2	2.1	1.6
(1-^{14}C) Acetate	10	1.8	18.5	23.5	24.3	19.2	3.4	5.4	3.4
(1-^{14}C) Decanoate	0	0.5	89.6	1.8	1.8	2.7	1.9	0.9	0.5
(1-^{14}C) Decanoate	10	1.1	89.1	2.1	1.6	2.0	0.5	1.7	1.5
(1-^{14}C) Palmitate	0	1.0	1.9	1.0	1.0	64.6	19.6	5.9	4.9
(1-^{14}C) Palmitate	10	0.6	0.9	1.5	0.9	84.7	5.1	4.0	2.2

[a]Incubation conditions are given in Table I. The triglyceride fractions were isolated by preparative thin layer chromatography, and, following the saponification, fatty acids wre isolated, converted to their methyl esters, and analyzed by gas liquid chromatography (22). The values are averages of closely agreeing duplicate determinations.

acids to triglycerides (13,14). Since this latter process does not require NADPH, one would expect that the glucose stimulation of triglyceride synthesis from acids which are not elongated would be small. In these instances, glyceride synthesis could be limited by the availability of (A) glycerol 3-phosphate or other glyceride-glycerol precursors; (B) cofactors (adenosine 5′-triphosphate [ATP] and CoASH), needed for fatty acid activation, and (C) the necessary enzymes. On the other hand, one might expect that, if a short chain fatty acid is elongated prior to esterification, such a process would be greatly stimulated by glucose. Here we show that acetate and decanoate incorporation into triglycerides by slices of mammary glands from lactating mice is stimulated essentially to a similar degree by the addition of glucose to the incubation medium. However, acetate is elongated prior to triglyceride formation, whereas decanoate is not. Thus, the effect of glucose cannot be solely due to NADPH production.

MATERIALS AND METHODS

Mammary glands were removed from lactating C_3H mice which were maintained on an adequate stock diet (Wayne Lab Blox, Allied Mills, Chicago, Ill.). They had suckled 6-8 pups for 17 days at the time of sacrifice by cervical fracture. (1-^{14}C) Decanoate and (1-^{14}C) palmitate were synthesized from the appropriate Grignard reagent and $^{14}CO_2$ (15). (1-^{14}C) Acetate was obtained from New England Nuclear Corp., Boston, Mass. (U-^{14}C) Glucose was prepared photosynthetically (16,17).

The preparation of slices, incubation conditions, collection of CO_2, and assay of radioactivity have been described previously (18,19). After incubation, the tissue slices were removed from the medium and washed with water. Total lipids were extracted from the slices with a mixture of chloroform:methanol (2:1, v/v) and washed (20). The individual lipid classes were separated by thin layer chromatography (TLC) and assayed for radioactivity (21).

Triglycerides were separated by preparative TLC, saponified, and the fatty acids were extracted with ether from an acidified medium. After preparation of the methyl esters with diazomethane, the individual fatty acids were separated by gas liquid chromatography (GLC) programed at 10 C/min over the range of 80-180 C. The instrument used was a Varian aerograph (model 2740) provided with a flame ionization detector, a stream splitter and stainless steel column (6 ft x 1/8 in.) packed with 15% diethylene glycol succinate on H/P Chromosorb G. Each fatty acid fraction was collected and assayed for radioactivity (22).

Tissue contents of glycerol 3-phosphate and dihydroxyacetone phosphate were determined enzymatically in perchloric acid extracts of slices (23).

RESULTS AND DISCUSSION

In preliminary experiments, with various concentrations of each labeled substrate, the amounts of acetate, decanoate, and palmitate recorded in Table I were shown to be optimal for triglyceride synthesis. Acetate incorporation into lipids and the extent of glucose stimualtion on this process were of the same order as that observed previously with slices of mammary glands from lactating mice (18). In the presence of glucose, the conversion of decanoate to lipids was as high as that of acetate and ca. 12-fold greater than that of palmitate. The stimulatory effect of glucose on lipid synthesis from acetate and decanoate was similar and

TABLE III

Metabolism of (U-^{14}C) Glucose by Slices of Mammary Glands from Lactating Mice[a]

Addition:		nmoles Glucose converted to:			
Compound	Conc.[b] mM	CO_2	Lipids	Fatty acids	Glycerol
None		1513 ± 139	1428 ± 149	1340	89
Acetate	2.5	1104 ± 40	938 ± 117	857	81
Decanoate	1.25	1125 ± 144	694 ± 50	469	225
Palmitate	0.25	1035 ± 130	657 ± 50	606	51

[a]Experiments were carried out as described in Table I, except that unlabeled acids and (U-^{14}C) glucose (10 mM) were used. Total lipids from duplicate experiments were pooled and the triglyceride fractions were isolated. After saponification of triglycerides, analysis of radioactive free fatty acids and glycerol was carried out (19,27). The values for glucose converted to CO_2 and lipids are the means ± standard error of three experiments with different lactating mice. Values for fatty acids and glycerol are averages of two experiments each.

[b]Conc. = concentration.

TABLE IV

Glyceride-Glycerol Precursors in Mammary Gland Slices[a]

	Glycerol 3-phosphate	Dihydroxyacetone phosphate
Additions to medium	*nmoles/100 mg wet wt*	
Glucose	51.4 ± 2.7	4.3 ± 0.3
Palmitate	24.4 ± 0.9	0
Glucose plus palmitate	52.4 ± 2.0	3.7 ± 0.1

[a]Mammary gland slices (1 g wet wt) from lactating mice were incubated in 20 ml Krebs-Henseleit bicarbonate buffer (pH 7.4) containing defatted albumin (80 mg) and where indicated glucose (10 mM), palmitate (0.25 mM), or glucose (10 mM) plus palmitate (0.25 mM) for 2 hr at 37 C with continuous mechanical agitation with 95% O_2 and 5% CO_2 as gas phase. The slices were removed, washed with ice-cold water and homogenized in perchloric acid (6% w/v) (23). The content of glycerol 3-phosphate and dihydroxyacetone phosphate in the extracts were determined with glycerol 3-phosphate: nicotinamide adenine dinucleotide oxidoreductase (23). The results given are the means ± standard error of three separate experiments.

considerably greater than that from palmitate. Both, in the presence or absence of added glucose, the fatty acids were incorporated predominantly into the triglyceride fraction (Table I). Although intact rat mammary gland cells converted fatty acids mostly into triglycerides, Kinsella (25) observed that palmitate incorporation was much greater than that of decanoate. This difference in the manner in which the mammary gland cells from rats and mice utilize these fatty acids is of interest and may be related to the difference in the fatty acid composition in the milk triglycerides of the two species (26).

As observed previously (18), acetate carbon was converted to decanoate, laurate, myristate, palmitate, and some stearate with over 90% of these residing in triglycerides (Table II). On the other hand, decanoate and palmitate were incorporated mostly as intact units. Hence, while the stimulatory effect of glucose upon triglyceride synthesis from acetate could be due to the stimulation of fatty acid synthesis, that from decanoate and palmitate must be due to other factors among which is the supply of glyceride-glycerol precursors. This concept was supported by our experiments with (U-^{14}C) glucose and unlabeled fatty acids (Table III). The major lipid class synthesized from glucose was triglyceride, with radioactivity residing in both the fatty acid and glycerol moieties. The amount of glucose carbon converted to fatty acids was depressed in the presence of acetate, and to an even greater extent in the presence of decanoate or palmitate. However, the amount of glucose converted to glyceride-glycerol was 3 to 4-fold greater in the presence of decanoate than in the presence of acetate or palmitate.

Previously, we have shown that mouse mammary glands in lactating mice contain 51.8 and 3.6 nmoles/100 mg tissue of glycerol 3-phosphate (GP) and dihydroxyacetone phosphate (DHAP), respectively (28). Slices prepared from these glands, despite incubation with palmitate

for 2 hr, still contain appreciable amounts (ca. 40% of original) of GP, although they do not possess detectable amounts of DHAP (Table IV). If acyl exchange reactions with (1-^{14}C) palmitate and endogenous triglycerides did not occur, and if all the incorporated palmitate were converted to tripalmitin, only 15 nmoles GP would be required (calculated from data in Table I). Thus, there appears to be sufficient amounts of GP in the tissue to yield the observed triglyceride synthesis from palmitate. When glucose was the sole added substrate, the levels of GP and DHAP were restored to those observed in unincubated controls (Table IV). If, however, glucose and palmitate were incubated together, the levels of GP and DHAP were unchanged. Hence, the amounts of GP and DHAP in the tissue do not appear to limit triglyceride synthesis from exogenous palmitate and appear to be controlled by glycolysis.

The observations made here indicate that, besides the production of (A) NADPH via the pentose phosphate pathway and (B) glyceride-glycerol precursors, glucose also must supply other factors for the stimulation of triglyceride synthesis. Whether these involve ATP production, fatty acid uptake and activation, etc., cannot be ascertained from the present data.

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cis-5-Polyenoic Acids in *Larix leptolepis* Seed Oil

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ABSTRACT

cis-5,*cis*-9-Octadecadienoic acid (2.7%) and *cis*-5,*cis*-9,*cis*-12-octadecatrienoic acid (24.9%) are present in the seed oil of *Larix leptolepis*. The double bond positions were identified by ozonolysis and by gas chromatography-mass spectrometry of methoxy derivatives. Small quantities of branched chain acids of various chain lengths were indicated by gas chromatography. The presence of C_{17} and C_{19} branched methyl esters was confirmed by gas chromatography-mass spectrometry.

INTRODUCTION

cis-5-Unsaturation has been found previously in a large number of plant sources (1-3), among them tall oil and extracts of pine and birch (1-6). *Larix leptolepis* (Pinaceae), the Japanese larch, and a number of its relatives reportedly produce 1-5% *cis*-5-18:1 and *cis*-5,9-18:2 acids in the lipids of their needles (6). We now find that the seed oil of *L. leptolepis* contains a much larger amount (27.6%) of *cis*-5 acids, as well as small amounts of branched chain acids also similar to those in the needles (6).

EXPERIMENTAL PROCEDURES

Oil was extracted from ground seed with petroleum ether (bp 30-60 C) and analyzed by gas chromatography (GC) with an F&M 810 gas chromatograph equipped with a 3 ft x 1/8 in. stainless steel column packed with 3% OV-1 on 100-120 mesh Gas-Chrom Q. The column was temperature programed from 200-400 C at 4 C/min. The carrier gas was helium used at a flow rate of 75 ml/min.

Esters prepared from the oil by using BF_3 in methanol (7) were analyzed by GC on a Packard 7401 chromatograph equipped with both a 12 ft x 1/4 in. glass column packed with 5% LAC-2-R 446 (Resoflex) on 60-80 mesh Chromosorb W(AW-DMCS) and a 4 ft x 1/4 in. glass column packed with 5% Apiezon L on 60-80 mesh Chromosorb W(AW-DMCS). Preparative thin layer chromatography (TLC) was performed on plates spread with a 1.0 mm layer of Silica Gel G containing 20% $AgNO_3$. The developing solvent was benzene. After development, the plates were sprayed with an alcoholic solution of 2',7'-dichlorofluorescein. The bands observed under UV light were scraped from the plates, and esters were recovered from the adsorbent with ether.

Samples were introduced into the mass spectrometer through a Packard model 7401 gas chromatograph via a single stage jet-type helium separator. Spectra were taken at 70 eV with the source at 200 C. A glass column (4 ft x 1/4 in.) packed with 5% Apiezon L on 60-80 mesh Chromosorb W (AW-DMCS) was used for separation in the gas chromatograph.

Ozonolysis and GC (8) of unsaturated esters and gas chromatography-mass spectrometry (GC-MS) of their methoxy derivatives (9) were used to determine double bond positions. GC-MS analysis also was performed on the saturated esters. IR spectra were obtained with a Perkin-Elmer model 137 spectrophotometer from liquid films on sodium chloride disks. UV spectra were recorded from 0.1% solutions in absolute ethanol with a Beckman DK-2A instrument. NMR spectra were run in $DCCl_3$ solutions with a Varian HA-100 spectrometer.

RESULTS AND DISCUSSION

GC of *L. leptolepis* seed oil (19.5% oil) indicated only triglycerides ranging from C_{52}-C_{56} with the major peak at C_{54}. GC analysis of methyl esters prepared from the oil showed that almost 95% of the fatty acid composition was made up of C_{18} acids; small amounts of C_{16}, C_{17}, C_{19}, and C_{20} acids also

TABLE I

Composition of Esters from *Larix leptolepis* seed oil

Component	Percent by GLC[a]
16:0	3.3
17:0 Branch	0.3
18:0	1.7
19:0 Branch	0.2
9-18:1	20.3
11-18:1	0.6
9,12-18:2	43.9
5,9-18:2	2.7
5,9,12-18:3	24.9
19:0	0.2
20:0	0.1
11-20:1	0.6
20:2	0.4
5,11,14-20:3	0.8

[a]GLC = gas liquid chromatography.

[1]ARS, USDA.

TABLE II

Saturated Ester Fraction from *Larix leptolepis* Seed Oil

ECL[a]	Percent	M+[b]	Identity[c]
12.0	0.3		
12.2	0.7		
12.7	0.2		
13.2	0.9		
13.7	0.3		
14.0	1.0	242	14:0
14.3	0.5		
14.7	0.6		
15.0	0.8	256	15:0
15.3	1.1		
15.7	0.5		
16.0	42.7	270	16:0
16.2	0.8	284	17:0 Branch
16.7	6.6	284	17:0 Branch
17.0	1.8	284	17:0
17.3	1.5		
17.7	1.5	296	18:1
18.0	23.0	298	18:0
18.3	0.9		
18.5	0.6		
18.7	1.9	312	19:0 Branch
19.0	1.1	312	19:0
19.3	1.3		
19.6	1.1	324	20:1
20.0	3.1	326	20:0
21.3	1.6		
22.0	1.6		

[a]ECL = equivalent chain length (10). From Apiezon L column.

[b]Molecular ion from gas chromatography-mass spectrometry (GC-MS).

[c]Some unidentified components appear to be hydrocarbons.

were present (Table I). IR analysis of the oil and esters showed no *trans* unsaturation, and UV spectra between 210-360 nm showed no chromophores.

Identification of Fatty Acid Esters

Separation of the mixed methyl esters by preparative TLC on $AgNO_3$-impregnated plates yielded five fractions varying in degree and type of unsaturation from zero to three double bonds/molecule. IR spectra of the unsaturated fractions confirmed the absence of *trans* unsaturation. GC analysis showed that the saturated fraction contained mostly normal C_{16} and C_{18} esters. A small amount of 18:1 and 20:1 contaminated this fraction. The presence of small amounts of branched chain esters also was indicated in this fraction.

A homologous series of peaks was observed with equivalent chain lengths (ECLs) (10) 0.3 and 0.7 units greater than normal saturated esters on the Apiezon L column (Table II). A similar set of peaks, 0.4 and 0.7 ECL units greater than normal saturated esters, also was noted in the data from the Resoflex column. The two series tentatively were identified as branched esters on the basis of the ECLs. Mass spectrometry showed that the components with ECLs of 16.2, 16.7, and 18.7 (Apiezon L) had spectra typical of saturated fatty acid methyl esters with molecular ions of 284, 284, and 312, the same as those of the normal C_{17} and C_{19} esters (Table II). Therefore, these components are methyl-substituted C_{16} (17:0 branch) and C_{18} (19:0 branch) esters. The presence of trace amounts of C_{23}-C_{28} saturated methyl esters also was observed by GC-MS. However, the components with ECLs 12.2, 12.7, 13.2, 13.7, 14.3, 14.7, 15.3, and 15.7 (Table II) had mass spectra characteristic of saturated hydrocarbons. Molecular ions were obtained for C_{16}, C_{17}, C_{18}, C_{19}, C_{20}, and C_{21} hydrocarbons. Traces of other longer chain hydrocarbons were indicated by the mass spectral fragmentation patterns; however, there was not enough material present to detect molecular ions. The presence of these hydrocarbons in the saturated ester fraction also was indicated by TLC.

The monoene fraction was 95% C_{18} and 2.5% C_{20} with other trace components. The predominant aldehyde-ester (AE) and aldehyde (A) fragments produced by ozonolysis of this fraction were 9 AE and 9 A. They accounted for ca. 91 and 98% of the aldehyde-ester and aldehyde produced, respectively. This evidence

TABLE III

Major Fragments from Methoxy Derivatives of Unsaturated Esters from *Larix leptolepis* Seed Oil

Compound	Major ions[a] A (A')	B (B')	C (C')	D (D')	E (E')	F (F')
9-18:1	201 (215)	171 (157)	---	---	---	---
11-18:1	229 (243)	143 (129)	---	---	---	---
11-20:1	229 (243)	171 (157)	---	---	---	---
5,9-18:2	145 (159)	257 (243)	231 (245)	171 (157)	---	---
5,9,12-18:3	145 (159)	287 (273)	231 (245)	201 (187)	327 (341)	129 (115)
5,11,14-20:3	145 (159)	315 (301)	259 (273)	201 (187)	355 (369)	129 (115)

[a]See Scheme 1 for identification of fragment ions.

indicates that double bond position is Δ9 in the major C_{18} ester. The other fragments present were 11 AE and 7 A, and the amounts present indicate that 3% of the 18:1 is Δ11. The presence of only 7 A, 9 A, 9 AE, and 11 AE fragments shows that the major double bond position in the C_{20} ester is in the 11 position (9 A + 11 AE).

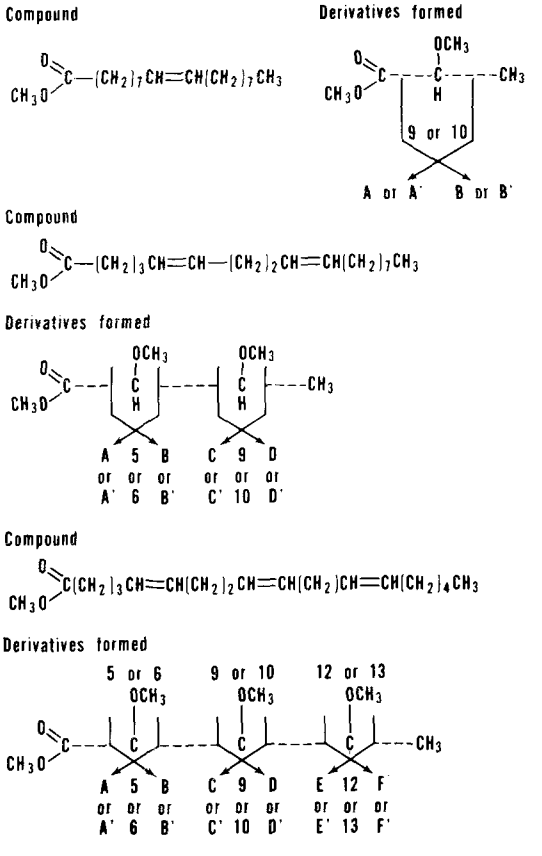
Methoxy derivatives of unsaturated fatty acids are formed as mixtures, with the methoxyl group added to either of the carbon atoms of the double bond (9). Since these positional isomers are not resolved by GC, a mass spectrum of these derivatives contains fragments contributed by all the positional isomers. Identification of the methoxy derivatives from monoenes is straightforward and well documented (9). The positions of unsaturation in the C_{18} and C_{20} monoenoic esters were confirmed by GC-MS of the methoxy derivatives of these esters (Table III).

The third fraction contained 98% C_{18} dienes. Fragments obtained from ozonolysis of this fraction (6 A, 9:1 A, 9 AE, and 12:1 AE) and GC-MS of its methoxy derivatives identify the major component to be methyl linoleate.

Interpretation of mass spectra of methoxy derivatives from more unsaturated molecules becomes more difficult because of the number of isomers formed and the resulting complexity of their mass spectra. Major ion fragments used to identify components of *L. leptolepis* seed oil are identified in Scheme 1 and tabulated in Table III. Abundant fragments showing the loss of 32 mass units (CH_3OH) accompanied most ion fragments containing methoxyl groups.

Fraction 4 contained a mixture of C_{18} esters. The predominant one (60%) had ECLs of 18.6 on Resoflex and 17.2 on Apiezon L and the other (40%) had ECLs corresponding to methyl linoleate. The unknown component was identified by GC-MS of its methoxy derivative as methyl 5,9-octadecadienoate (Table III). Since IR shows no *trans* unsaturation, this compound must be the *cis*-5,*cis*-9 isomer. Its NMR spectrum was the same as that reported by Lehtinen, et al., (11) for the *cis*-5,*cis*-9 component in tall oil.

The fifth fraction contained C_{18} (95%) and C_{20} (1.5%) triene esters and *cis*-5,*cis*-9 C_{18} diene (3.5%). IR analysis of fraction 5, the major fragments from ozonolysis (6 A, 9:1 A, 5 AE, 9:1 AE, and C_4 dialdehyde), and GC-MS of the methoxy derivatives identified the major component as methyl *cis*-5,*cis*-9,*cis*-12-octadecatrienoate. Double bonds of the C_{20} ester were at the 5, 11, and 14 positions (Table III). The NMR spectrum of the triene fraction was identical to that obtained by Smith, et al., (1)



Scheme 1

Major fragments from methoxy derivatives of unsaturated esters from *Larix leptolepis* seed oil.

for the all-*cis*-5,9,12-18:3 from *Teucrium depressum* seed oil and Powell, et al., (2) for all-*cis*-5,9,12-18:3 from *Xeranthemum annum* seed oil.

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The Incorporation of Orally Fed Radioactive γ-Linolenic Acid and Linoleic Acid into the Liver and Brain Lipids of Suckling Rats

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ABSTRACT

The incorporation of radioactivity from orally administered γ-linolenic acid-1-^{14}C and linoleic acid-^{3}H into the liver, plasma, and brain lipids of suckling rats was studied. Significantly more radioactivity from the former compound was incorporated into the liver and brain lipids 22 hr after dosing. The distribution of the radioactivity in the fatty acids of the liver and brain lipids was different for each isotope. Most of the ^{3}H was still associated with linoleic acid, whereas most of the ^{14}C was in the 20:3 and 20:4ω6 fractions. These results suggest that the desaturation of linoleic to γ-linolenic acid in vivo is a rate-limiting step in the conversion of linoleic to arachidonic acid.

INTRODUCTION

In a previous paper we compared the incorporation of radioactivity from orally administered 18:2-1-^{14}C with 20:4-$^{3}H_8$ into the liver and brain 20:4 of suckling rats (1) and found that the majority of the radioactivity in tissue 20:4 was derived from the exogenously administered 20:4-$^{3}H_8$. The pathway of synthesis of arachidonic acid from linoleic acid involves an initial desaturation of linoleic to γ-linolenic acid (18:3ω6). This reaction has been demonstrated to be rate-limiting in vitro (2).

To test whether the slow endogenous synthesis of arachidonic acid in the above experiment was due, in part, to this rate-limiting step, we have compared the incorporation of radioactivity from 18:2-^{3}H and γ-18:3-1-^{14}C into the liver, brain, and plasma lipids of suckling rats.

METHODS

Suckling rats, between 15 and 16 days old, were used in these experiments. They were bred from female rats maintained on a semisynthetic diet (3).

In the first experiment, four pups were dosed with a mixture of 18:2-9,10,12,13-^{3}H methyl ester (85 mCi/m mole, radiopurity 99%) and γ-18:3-1-^{14}C (20 mCi/m mole, radiopurity 99%, Daiichi Pure Chemical Co., Tokyo, Japan). Ca. 3 μCi of each isotope (^{14}C/^{3}H dose ratio = 1.18) was added to 0.25 ml olive oil, and the mixture was administered orally to 15 day old pups. The pups were killed 22 hr later. This time interval was chosen so that a comparison could be made between the present results and our previous work (1).

In the second experiment, three pups were dosed with ca. 1.5 μCi of each of 18:2-^{3}H and 18:2-1-^{14}C (61 mCi/m mole, The Radiochemical Center, Amersham, U.K.). The isotopes (^{14}C/^{3}H dose ratio = 1.01) were added to 0.25 ml olive oil, and the mixture was administered orally to 16 day old pups. The pups were killed 22 hr later.

Lipids were extracted from the tissue as previously described (1). Blood was collected into citrated tubes, and plasma was extracted in chloroform:methanol (1:1 v/v). Aliquots of the total lipids were assayed for radioactivity by liquid scintillation counting using an Intertechnique SL 30 scintillation spectrometer. The efficiency of counting was determined by the use of internal standards of n-hexadecane-1-^{14}C and n-hexadecane-1,2-^{3}H (The Radiochemical Center, Amersham, U.K.).

Tissue lipids were separated by thin layer chromatography (1); the cholesteryl esters (CE), triglycerides (TG), free fatty acids, cholesterol (Chol), and phospholipids (PL) were scraped quantitatively into scintillation vials and 1 ml water was added to each vial, followed by 10 ml Unisolve 1 (Koch Light Laboratories, Bucks, U.K.). In this system, the efficiency of counting, determined by the use of internal standards (above) was 47% for ^{14}C in the B channel and 22 and 38% for ^{14}C and ^{3}H, respectively, in the A channel. Using this method, the recovery of radioactivity from the plates was greater than 95% for each isotope.

The distribution of radioactivity in the fatty acids of TG and PL was determined by separation and fraction collection of the methyl esters using a preparative gas liquid chromatograph (4). The methyl esters were prepared as previously described (1), and the preparative gas liquid chromatography was carried out in a glass column 2.1 m in length x 7 mm inside diameter packed with 10% polyethylene glycol adipate (PEGA) on Diatomite C-AW 60-70 mesh at 177 C. Methyl-γ-linolenate (nonradioactive) was added to all samples prior to

TABLE I

Incorporation of Radioactivity from γ-18:3-1-^{14}C and 18:2-^{3}H into Liver and Brain Lipids of Suckling Rats

Tissue	Percent of dose		
	18:2-^{3}H	γ-18:3-1-^{14}C	p[a]
Liver lipids	3.94 ± 0.43[a]	6.05 ± 0.48	<0.0025
Brain lipids	0.27 ± 0.003	0.41 ± 0.003	<0.0005

[a]P>0.05 = no significant difference.

[b]Mean ± standard error of the mean from four pups which received a mixture of the two labeled fatty acids. The ^{14}C/^{3}H dose ratio was 1.18:1.

TABLE II

Percentage Distribution of Isotopes in Liver, Plasma, and Brain Lipid Fractions

Tissue fraction[a]		Percent distribution	
		γ-18:3-1-^{14}C	18:2-^{3}H
Liver	PL	54 ± 1[c]	28 ± 1
	TG	43 ± 1	68 ± 0.2
Plasma	PL	35[d]	45
	TG	21	17
	CE	41	34
Brain	PL	83 ± 0.01	95 ± 0.02
	Chol[e]	12 ± 0.02	1 ± 0.04

[a]PL = phospholipids, TG = triglycerides, CE = cholesteryl esters, and Chol = cholesterol.

[b]Percentage distribution of isotope in lipid fractions of each tissue (see "Methods").

[c]Mean ± standard error of the mean from four animals.

[d]Pooled analysis from four animals.

[e]Cholesterol counts were not contaminated by radioactive diglycerides.

chromatography to determine the exact position of this compound. The retention times of the methyl esters of 18:2ω6 and γ-18:3ω6 relative to 18:0 were 1.44 and 1.72, respectively.

Fatty acid fractions were decarboxylated by the Schmidt procedure as described by Goldfine and Bloch (5).

Analyses for changes were performed on the data using the paired Student t-test (6).

RESULTS

Radioactivity from both γ-18:3-1-^{14}C and 18:2-^{3}H was incorporated into the liver, plasma, and brain of the suckling rats (Table I). In the liver, the radioactivity from γ-18:3-1-^{14}C was distributed evenly between the TG and PL fractions, whereas the radioactivity from 18:2-^{3}H was incorporated preferentially into the TG fraction (Table II). In the plasma, the major lipid fractions labeled with both isotopes

TABLE III

Percentage Distribution[a] of Radioactivity in the Fatty Acids of Liver Triglycerides (TG), Phospholipids (PL), and Brain PL

Fatty acid fraction[b]	Liver TG		Liver PL		Brain PL	
	18:2-^{3}H	γ-18:3-1-^{14}C	18:2-^{3}H	γ-18:3-1-^{14}C	18:2-^{3}H	γ-18:3-1-^{14}C
16:0 + 16:1	---[c]	---	---	---	1.9 ± 0.4	18 ± 0.3
18:0 + 18:1	---	---	---	---	2.3 ± 0.3	10 ± 0.2
18:2ω6	67 ± 3.2[d]	---	67 ± 2.4	---	39 ± 1.7	---
18:3ω6	14 ± 1.0	31 ± 0.4	13 ± 1.1	4.3 ± 0.6	8.3 ± 0.5	5.6 ± 0.2
20:0-20:2	8.1 ± 1.9	4.8 ± 0.7	7.4 ± 0.5	---	5.0 ± 0.6	---
20:3ω6	3.5 ± 0.3	24 ± 0.9	2.8 ± 0.6	12 ± 1.0	6.8 ± 0.2	9.9 ± 0.1
20:4ω6	3.4 ± 0.5	20 ± 0.5	5.8 ± 0.6	56 ± 0.3	25 ± 0.8	43 ± 1.1
20:5	3.0 ± 0.4	11.0 ± 0.7	2.4 ± 0.1	13.0 ± 0.7	4.0 ± 0.8	5.0 ± 0.8
22:4 + 22:5ω6	---	6.3 ± 0.2	1.3 ± 0.3	9.7 ± 0.3	5.0 ± 0.2	5.4 ± 0.3

[a]Percentage of radioactivity in a fraction relative to total radioactivity collected. Samples were collected continuously from the solvent front to after the 22:6ω3 fraction. Values above 10% are underlined.

[b]The 18:3ω6 fraction includes 18:3ω3; the 20:0-20:2 fraction includes 20:1.

[c]Less than 1%.

[d]Mean ± standard error of the mean from four animals.

were PL, TG, and CE (Table II). In the brain lipids, most of the radioactivity was associated with the PL fraction.

The distribution of the radioactivity in the fatty acids of liver TG and PL in the 18:2-^{3}H experiment showed that most of the ^{3}H was still associated with the 18:2ω6 fraction and little with the 20:4ω6 fraction (Table III). In the brain PL, some 25% of the ^{3}H was in the 20:4ω6 fraction. On the other hand, in the γ-18:3-1-^{14}C experiment, most of the radioactivity in the polyunsaturated fatty acids of liver TG, PL, and brain PL was associated with 20:3ω6 and 20:4ω6—the longer chain metabolites of the ω6 series. The distributions of the radioactivity in the fatty acid fractions of the plasma TG, CE, and PL were similar to the respective distributions (^{14}C or ^{3}H) obtained in the liver lipids (results not shown).

Decarboxylation of the 16:0 + 16:1 and 18:0 + 18:1 fractions of the brain lipids showed that the carbon 14 in these acids was derived from a combination of de novo synthesis and chain elongation from acetate-^{14}C (Table IV). The presence of significant amounts of ^{14}C in these fatty acids and in brain cholesterol has been commented on previously (1). The very low carboxyl carbon values in the 20:3ω6 and 20:4ω6 fraction of the liver and brain showed that the radioactivity was derived directly from the fed γ-18:3-1-^{14}C.

The total incorporation of ^{14}C into the brain lipids exceeded that of the ^{3}H incorporation (Table I), but it was calculated, from Tables II and III, that, of the total radioactivity in the brain lipids, ca. 30 and 5% of the ^{14}C and ^{3}H, respectively, were associated with the brain cholesterol together with the saturated and monounsaturated fatty acids. In previous experiments in which pups were orally fed 18:2-1-^{14}C, α-18:3-1-^{14}C, and 20:4-1-^{14}C, it was shown that a substantial amount of the radioactivity in the brain lipids was associated with cholesterol and the saturated and monounsaturated fatty acids (1). It was suggested that this incorporation was a property of the type of label in the fed acid (^{14}C or ^{3}H) rather than a property of the fatty acid itself. To confirm this, 18:2-1-^{14}C and 18:2-^{3}H were fed simultaneously to 16 day old pups. The ^{14}C/^{3}H ratios in the liver and plasma lipids were similar to the dose ratio. However, in the brain lipids, the ^{14}C/^{3}H ratio was significantly greater than the dose ratio (Table V). The excess of ^{14}C over ^{3}H in the brain lipids was accounted for by the high ^{14}C/^{3}H ratio in the brain cholesterol and the saturated and monounsaturated fatty acids. As in the previous experiment (γ-18:3-1-^{14}C plus 18:2-^{3}H), it was calculated that there was ca. six times as much ^{14}C as ^{3}H in these compounds.

TABLE IV

Decarboxylation Studies on Fatty Acids Collected by Preparative Gas Liquid Chromatography

Fatty acid fraction	Relative carboxyl activity[a]		
	Liver TG	Liver PL	Brain PL
16:0 + 16:1	ND[b]	ND	0.15
18:0 + 18:1	ND	ND	0.17
18:3ω6	0.89	0.90	0.85
20:3 + 20:4ω6	0.06	0.01	0.03
22:4 + 22:5ω6	0.31	0.04	0.08

[a] ^{14}C in the -COOH group/^{14}C in total fatty acid. The fatty acid fractions from four experiments were pooled prior to decarboxylation, and the analyses were done in duplicate. The original γ-18:3-1-^{14}C had a relative carboxyl activity of 0.87 ± 0.01 (mean ± standard error of the mean for six determinations).

[b] ND = not determined.

TABLE V

The Incorporation of Radioactivity from 18:2-1-^{14}C and 18:2-^{3}H into Tissue Lipids and Fatty Acids of Suckling Rats

Tissue fraction	^{14}C/^{3}H ratio[a]
Liver lipids	1.2 ± 0.02[b]
Plasma lipids	1.0 ± 0.01
Brain lipids	2.4 ± 0.002[c]
Brain cholesterol	13 ± 1.4[d]
Brain 16:0 + 16:1	27 ± 4.2[e]
Brain 18:0 + 18:1	16 ± 1.0[d]

[a] Ratio = ^{14}C/^{3}H in fraction ÷ ^{14}C/^{3}H in dose. The dose ratio was 1.01:1.

[b] Mean ± standard error of the mean for 3 animals.

[c] The ratio differed significantly from the dose ratio, (P) <0.0025.

[d] P<0.025.

[e] P<0.05.

DISCUSSION

The 18:2-1-^{14}C–18:2-^{3}H experiment (Table V) confirms our earlier suggestion that the labeling of the cholesterol, saturated and monounsaturated fatty acids in the brain is due to the position of the label on the fatty acid and its subsequent loss, depending upon the metabolism, rather than the nature of the fatty acid itself (1). The results show that care must be taken when comparing the incorporation of radioactivity from fatty acids labeled with carbon 14 at the carboxyl carbon with more uniformly ^{3}H labeled acids into brain lipids in suckling rats.

The liver is the major site for the desaturation and chain elongation of fatty acids (7). It

commonly is assumed that linoleic acid is converted to arachidonic acid without difficulty (8). However, in vitro studies (2) have shown that the initial desaturation of 18:2ω6 to 18:3ω6 is a rate-limiting step. The amount of radioactivity found in arachidonate may be dictated, not only by the rate of conversion of linoleate or γ-linolenate to arachidonate, but also by the size of the metabolic pool of linoleate and γ-linolenate. In the present experiment, the total liver content of linoleate is much greater than that of γ-linolenate, and this is consistent with the results obtained when rats are fed up to 6.4% of their calories as either linoleate or γ-linolenate (9). That is, when γ-linolenate is fed to essential fatty acid-deficient rats there is an increase in the amount of this compound in the liver. However, rather than a large expansion of the γ-linolenate pool, the γ-linolenate is metabolized to, and results in, an increase in the liver content of 20:3 and 20:4ω6. On the other hand, when linoleate is fed to the essential fatty acid-deficient rats, there is a considerable increase in the total liver content of linoleate and a more modest and slower increase in the content of 20:3 and 20:4ω6 in the liver (9). In the 18:2-^{3}H–γ-18:3-1-^{14}C experiment, most of the radioactivity in the liver PL from orally fed 18:2-^{3}H was in the 18:2ω6 fraction, whereas most of the ^{14}C (from γ-18:3-1-^{14}C) was in the 20:4ω6 fraction and less than 5% in the γ-18:3 fraction.

Clearly, care must be taken in extrapolating from results based upon a single time point. Nevertheless, the results of this experiment demonstrate that one day after dosing the desaturation of 18:2ω6 to γ-18:3 is rate-limiting in vivo as it is in vitro (2). Further work is planned to establish if this rate-limiting step remains of importance in the long term.

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Phosphatidylkojibiosyl Diglyceride: Metabolism and Function as an Anchor in Bacterial Cell Membranes[1]

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ABSTRACT

The recently discovered phosphoglycolipid, phosphatidylkojibiosyl diglyceride (PKD), was first observed as a biosynthetic by-product of glucosyl diglyceride metabolism in *Streptococcus faecalis (faecium)* ATCC 9790. Its structure is 1,2-diacyl-3-O(2′-O-α-D-glucopyranosyl-6′-O-phosphoryl- [1″,2″-diacyl-3″-O-*sn*-glycerol]-α-D-glucopyranosyl)-*sn*-glycerol. The biosynthesis of phosphatidylkojibiosyl diglyceride occurs by a novel transphosphatidylation reaction in which a phosphatidyl group is transferred from diphosphatidyl glycerol to the primary alcohol function at the 6 position of the internal glucose of kojibiosyl diglyceride. The reaction is catalyzed by a membrane-derived enzyme. Phosphatidylkojibiosyl diglyceride is bound covalently through a phosphodiester bond to the polyglycerol phosphate moiety of membrane lipoteichoic acid from *S. faecalis*. Phosphatidylkojibosyl diglyceride has four nonpolar long chain fatty acyl groups and appears to have the necessary physico-chemical properties to anchor the long hydrophilic glycerol phosphate polymer of lipoteichoic acid to the hydrophobic environment of the membrane of *S. faecalis* and probably other gram-positive bacteria as well.

[1]One of six papers presented in the symposium, "Recent Developments in Glycolipid Isolation and Analysis," at the AOCS Fall Meeting, Philadelphia, September 1974.

INTRODUCTION

In 1961, MacFarlane first reported the presence of glycosyl diglycerides in bacteria (1). These lipids now are known to be ubiquitous among the gram-positive bacteria (2-4). They also are present in several species of fermentative *Acholeplasma* (5-8), in certain species of *Pseudomonas*, and possibly in other gram-negative bacteria (9-13).

The structurally related phosphoglycosyl diglycerides, discovered more recently, have been isolated in several different forms. The first phosphoglycosyl diglyceride discovered was isolated from *Acholeplasma laidlawaii* and originally was characterized as a "phosphatidyl glucose" by Smith and Hendrikson (14). However, this structure was revised later and proven to be glycerylphosphoryldiglucosyl diglyceride (15) (Table I). Similar compounds were detected in several varieties of *Streptococci* by Fisher and coworkers (16,17) and in *Staphylococcus epidermis* by Kates, et al., (18). The

TABLE I

Occurrence of Phosphoglycolipids in Microorganisms

Organism	Lipid	Reference
Achleplasma laidlawaii	*sn*-Glycerol-1-phosphoryldiglucosyl diglyceride	15
Streptococcus faecalis var. *zymogenes*, *S. faecalis* var. *faecalis* 7064, *S S. lactis*	*sn*-Glycerol-1-phosphoryldiglucosyl diglyceride and phosphatidyl-kojibiosyl diglyceride	16,17
Staphylococcus epimidermis	*sn*-Glycerol-1-phosphoryldiglucosyl diglyceride	18
Pseudomonas diminuta	Phosphatidylmonoglucosyl diglyceride	20
Bifidobacterium bifido var. *pennsylvanicus*	*sn*-Glycerol-1-phosphorylgalacto-furanosyl diglyceride	19
Streptococcus faecalis (faecium) ATCC 9790	Phosphatidylkojibiosyl diglyceride	21,22
Bacillus magaterium, *Pseudomonas ovalis*	Glucosaminylphosphatidylglycerol	26 27
Halophilic, halotolerant Gram-negative bacterium	Glucosylphosphatidylglycerol	28

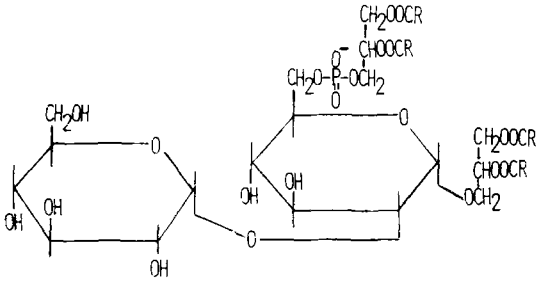

FIG. 1. Phosphatidylkojibiosyl diglyceride.

stereoconfiguration of the glycerol phosphate moiety of the bacterial glycerylphosphoryldiglucosyl diglyceride is *sn*-1, rather than the more usual *sn*-3 structure (16-18). A second variety of phosphoglycolipid is a glycerol-*sn*-1-phosphorylgalactofuranosyl diglyceride isolated recently from a *Bifidobacterium* (19). The phosphatidyl*mono*glucosyl diglyceride of *Pseudomonas diminuta* (20) is a third type of phosphoglycosyl diglyceride, and, as yet, only has been found in this one species of bacteria (Table I).

The fourth form, phosphatidyldiglucosyl diglyceride, which is more correctly named phosphatidylkojibiosyl diglyceride (PKD) (PKD more correctly describes the structure of phosphatidyldiglucosyl diglyceride; kojibiosyl is O-α-D-glucopyranosyl-[1→2]-D-glucopyranose), first was detected as a by-product of kojibiosyl diglyceride (KD) metabolism in *Streptococcus faecalis* (*faecium*) ATCC 9790 by Pieringer (21,22). Relatively crude membrane-derived enzyme preparations, apparently containing endogenous phosphatidyl donors, converted KD to an anionic lipid which subsequently was determined by Ambron and Pieringer (22) to have the structure shown in Figure 1. By using specific radioactive labeling techniques in which either the glucose, glycerol, or phosphorous moieties were labeled, the characterization could be carried out on nmole quantities of lipid (22,23). Fischer and coworkers also found this compound in *S. faecalis* var. *zymogenes* 20672, *S. faecalis* var. *faecalis* 7064, and in *Streptococcus lactis* (16,17,24). Unlike *S. faecalis* var. *zymogenes* and *S. faecalis* var. *faecalis*, which contain both PKD and glycerylphosphoryldiglucosyl diglyceride (16,17), only PKD was detected in *S. faecalis* (*faecium*) ATCC 9790 (25).

A fifth class of phosphoglycolipid discovered in microorganisms is the glycosyl derivative of phosphatidyl glycerol (PG) (Table I). Op den Kamp, et al., (26) and Phizackerley and MacDougall (27) have isolated glucosaminyl phosphatidylglycerols from *Bacillus magaterium* and *Pseudomonas ovalis*. Peleg and Tietz (28) characterized a lipid from a halophilic, halotolerant gram-negative bacterium as a glucosyl PG. This fifth variety of phosphoglycolipid is probably, in a structural and biosynthetic sense, more distantly related to any of the other types discussed above in that they are glycosyl derivatives of PG and the four other types are glycerol phosphoryl or phosphatidyl derivatives of a glycosyldiglyceride.

BIOSYNTHESIS OF PKD

As yet the only known data on the biosynthesis of phosphoglycolipids are those concerned with the formation of PKD in *S. faecalis* (*faecium*) ATCC 9790 (22,30). In the earlier studies, from our laboratory (21,22), KD was converted enzymatically to a phosphoglycolipid by the addition of a phosphatidyl group derived from an endogenous substrate. At the time of these earlier investigations, the only known substrate capable of donating a phosphatidyl moiety was CDP-diglyceride (29). However, extensive attempts to demonstrate a substrate function for CDP-diglyceride (either labeled with ^{14}C or unlabeled) in the synthesis of PKD failed (30). While these studies were being carried out, Hirschberg and Kennedy (31) reported broken cell preparations of *Escherichia coli* catalyzed the transfer of a phosphatidyl group from one PG to a second PG. Diphosphatidyl glycerol (DPG) and glycerol were the products.

When PG was tested as a possible substrate in the synthesis of PKD, radioactivity from ^{14}C-glycerol labeled PG was indeed transferred to KD to form ^{14}C-PKD. However, DPG also was synthesized simultaneously rather extensively from PG by the *S. faecalis* enzyme (30). Further investigation on other possible substrates revealed that (^{14}C-glycerol)-DPG functioned better in the synthesis of PKD than PG (Table II) (30 and unpublished observations):

$$DPG + KD \rightarrow PKD + PG$$

This novel reaction catalyzed by a membrane-derived enzyme of *S. faecalis* appears to be the first example in which DPG participates as a direct donor of a phosphatidyl group. Because the relatively crude enzyme preparations also readily catalyze the synthesis of DPG from PG, it has not been possible as yet to establish if PG is functioning as a direct or indirect (through DPG) donor of the phosphatidyl group. This interfering side reaction (PD → DPG) also prevents the isolation of stoichiometric amounts of PG product in the synthesis of PKD

TABLE II

Biosynthesis of Phosphatidylkojibiosyl Diglyceride (PKD)[a]

Substrates (14C-glycerol labeled)	Lipid products		
	PKD	PG	DPG
	(cpm on electropherogram)		
KD	624	0	0
PG	157	---	1255
DPG	0	0	---
KD + PG	1093	---	1551
KD + DPG	2163	249	---

[a]KD = kojibiosyl diglyceride, PG = phosphatidyl glycerol, DPG = diphosphatidyl glycerol. 16,392 cpm ^{14}C-KD, 8,680 cpm ^{14}C-DPG, and 35,226 cpm ^{14}C-PG (all labeled in the glycerol moieties and having an estimated specific activity of 70 cpm/nmole glycerol) were added in benzene solutions to 5 mg lyophilized 30,000 x g particulate preparation of *Streptococcus faecalis* (containing 1.4 mg protein) in the combinations indicated. After evaporation of the benzene, 0.02 ml 0.1 M $MgSO_4$ and 0.2 ml 0.1M $NaPO_4$ pH 7.3 mixed with the (^{14}C)-lipid-enzyme complex. The mixtures were incubated for 1 hr at 37 C. Controls containing no enzyme also were incubated and produced no phosphatidylkojibiosyl diglyceride (PKD). The reaction stopped with 1 ml methanol, heated for 1/2 min at 100 C, chilled, and diluted with 2 ml $CHCl_3$ and 1 ml H_2O. The lipids in the $CHCl_3$ phase were deacylated with mild base and the resulting water-soluble products separated by electrophoresis. The radioactivity at each separated product (detected by a strip scanner) was determined in a Geiger counter and is expressed as the sum of the counts on both sides of the paper. (Data taken from reference 30).

from DPG and KD. Work is in progress in our laboratory to separate these two enzymatic activities.

MEMBRANE LIPOTEICHOIC ACIDS

Membrane lipoteichoic acids are found throughout the gram-positive species of bacteria and have been characterized as polymers of glycerol phosphate in which phosphodiester bonds link the 1 and 3 positions of adjacent glycerol moieties (32). The polymer, consisting of up to ca. 35 residues of glycerol phosphate, contains a variable number of disaccharide and alanine moieties bonded to the secondary alcohol functions of the glycerol phosphate units. In *S. faecalis* N.C.I.B. 8191, the disaccharide is kojibiose (32). This is the same disaccharide that occurs in the glycolipids and phosphoglycolipids of *S. faecalis*. The possibility of a structural relationship between phosphoglycolipid and teichoic acids was suggested by Ishizuka and Yamakawa (33), who noted the striking structural similarity between the saccharide moieties of the glycolipid and the membrane teichoic acids of a given bacteria. The first indication of a covalent linkage between glycolipid and teichoic acid came from work of Wicken and Knox (34), who found that teichoic acid isolated by a phenol extraction procedure contained lipid. The lipid could not be removed by extensive chloroform-methanol washing but was removed on exposure to trichloroacetic acid. Trichloroacetic acid, when used as an extractant, produces a lipid-free teichoic acid (34). The higher acidity of the trichloroacetic acid appears to be responsible for the more extensive degradation of the product.

The phenol extracted lipoteichoic acid also is considerably more immunogenic than the trichloroacetic acid extracted material (35). Using antilipoteichoic acid antibodies coupled to ferritin, Van Driel, et al., (35) showed, in very convincing electron-microscope studies, that lipoteichoic acid is a constituent of the cell membrane and not of the wall. The antilipoteichoic acid antibody (reactive specifically to the polyglycerol phosphate portion of lipoteichoic acids found in gram-positive bacteria), when exposed to mixtures of wall and membrane fragments, complexes with the membrane but not the wall fragments. Protoplasts, derived by enzymatically removing the cell wall, interact with the antibody to a much greater extent than the whole organism. The degree to which different gram-positive bacteria complex with antilipoteichoic acid antibody correlates well with the ability of the organism as an intact cell to induce antibodies against the membrane lipoteichoic acid (35). These differences probably are caused by a variable penetration of the membrane lipoteichoic acid through the wall of the different gram-positive bacteria (35). The glycerol phosphate polymer of lipoteichoic acid is visualized as extending from the membrane into the cell wall. The polymer is believed to be secured to the membrane by a covalent bond to

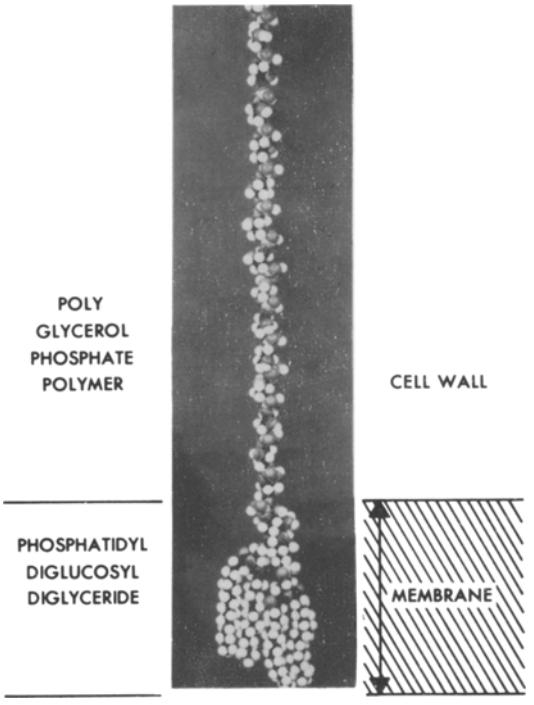

FIG. 2. Space-filling model of phosphatidylkojibiosyl (diglucosyl) diglyceride anchoring the hydrophilic glycerol phosphate polymer of lipoteichoic acid to the hydrophobic membrane of *Streptococcus faecalis*.

a lipid (29,35). Considering the highly hydrophilic nature of the long glycerol phosphate polymer and the hydrophobic environment of the cell membrane, which contains all of the lipid of gram-positive bacteria (36), it is reasonable that the polymer should be anchored to the membrane by a lipid. The assignment of an anchor function to a lipid is a relatively new role for a lipid.

LIPID OF MEMBRANE LIPOTEICHOIC ACID

The lipid component of lipoteichoic acid presumably functioning as an anchor has been identified in at least one bacterium (25). In earlier studies on *Lactobacillus fermenti*, Wicken and Knox (34) suggested that the lipid constituent of lipoteichoic acid might be a glycolipid. Their suggestion was based upon finding glucosyl-*sn*-1-glycerol as a degradation product of lipoteichoic acid (34). This fragment most probably originated from the lipid portion rather than the polyglycerol phosphate section of the molecule. Adjacent glycerol phosphate moieties of the polymer are linked at the *sn*-1 and *sn*-3 positions by a phosphodiester bond. Therefore, the polymer portion of the molecule would not be likely to produce glucosyl-*sn*-1-glycerol. Tentative evidence also suggested the presence of a phospholipid component of lipoteichoic acid (34).

The observation by Wicken and Knox (34) that lipid was associated with phenol-extracted but not with the more acidic trichloroacetic acid-extracted teichoic acid suggested to us (25) that careful differential acid hydrolysis might be a feasible approach to removing the lipid from the lipoteichoic acid of *S. faecalis* (*faecium*) ATCC 9790. *S. faecalis*, grown in the presence of (^{14}C)-glycerol, incorporate ^{14}C-label only into glycerol moieties of glycerol-containing compounds (23). (Glycerol-^{14}C)-lipoteichoic acid, isolated by phenol extraction from *S. faecalis* grown under these conditions, was purified on a column of Sepharose 6B (25). The water-soluble (glycerol-^{14}C)-lipoteichoic acid was treated with 2.3 ml $CHCl_3$-CH_3OH-3.8 N HCl (1:1:0.3 v/v) for 1 hr at 30 C (25). The percentage of 1.6 total radioactivity of the water-soluble ^{14}C-lipoteichoic acid was released into the chloroform phase of an aqueous-chloroform partition system. Lipid-soluble radioactivity (92%) was found in a very polar lipid, which, on chromatography, was more polar than any of the common naturally occurring phospho- and glycolipids. Further hydrolysis of the polar lipid with 2.3 ml $CHCl_3$-CH_3OH-3.8 N HCl (1:1:3 v/v) for 1 hr at 34 C released only one lipid, and this lipid was identified as PKD (25).

More stringent conditions of acid hydrolysis of (^{14}C)-lipoteichoic acid produced more chloroform-soluble radioactivity (up to 8%) (25). However, the ratio of polar lipid to "other" lipids was considerably lower than when less stringent conditions of acid hydrolysis were used. The "other" lipids were all structurally simpler, degradation products (monoglyceride, diglyceride, KD, monoglucosyl diglyceride, and phosphatidyl monoglucosyl diglyceride) of PKD. No additional lipids (or degradation products, thereof), such as PG, aminoacyl phosphatidyl glycerol, or DPG, which together account for a majority of free lipid of *S. faecalis* (*faecium*) ATCC 9790, have ever been detected as a part of the lipoteichoic acid (25). The absence of these major lipids not only demonstrates a specificity for PKD but also supports the idea that the release of lipid from lipoteichoic acid requires the breaking of a covalent bond. Other types of bonds, such as polar or ionic bonds usually are associated with less specific interactions between molecules.

The nature of the covalent bond between glycerol phosphate polymer and the lipid PKD

has been established as a phosphodiester bond (25,37). Treatment of the purified polar (^{14}C-glycerol)-lipid (derived from water-soluble ^{14}C-lipoteichoic acid) with 2.3 ml $CHCl_3$-CH_3OH-3.8 N HCl (1:1:0.3 v/v) for 1 hr at 34 C released several (^{14}C)-glycerol labeled, water-soluble products plus a water-soluble nonradioactive compound (25). The nonradioactive material was periodate oxidizable and chromatographed with standard glucose phosphate. It also released free glucose on exposure to alkaline phosphatase (25).

The glucose phosphate could not have come from the glycerol phosphate polymer side chain of the lipoteichoic acid, since the glucose in this part of the molecule is never bonded to phosphorus (32). Further, the glucose phosphate could not have been derived from the phosphodiester bond between the phosphatidyl and the kojibiose moieties of PKD, because the phosphatidyl moiety is linked through a phosphodiester bond to the *sixth* carbon of the internal glucose. The lack of an adjacent hydroxyl group on the glucose causes the phosphodiester bond of the phosphoglucolipid to break on the glucose side of the bond to yield only glycerol phosphate and free glucose. At no time have we ever obtained glucose-6-phosphate from PKD degradation (22,25). For the same reason, it is unlikely that the sixth position of the external glucose of the phosphoglycolipid participates in a phosphodiester bond. Preliminary evidence indicates that glucose (^{32}P)-phosphate can be derived from the polar ^{32}P-lipid treated extensively with periodate and then hydrolyzed with 0.1N HCl at 100 C for 30 min (25). These data suggest that the second and fourth carbons of the external glucose of PKD also are not involved in the phosphodiester bond between lipid and polymer moieties of the lipoteichoic acid. The phosphate group of the polyglycerol phosphate side chain is, therefore, probably attached to the third carbon of the external glucose or to the third or fourth carbon of the internal glucose of the lipid. The finding of a glucose phosphate fragment also indicates that the lipid is indeed linked to the glycerol phosphate polymer of the molecule through a phosphodiester bond.

The above results are in agreement with those of Toon, et al., (37). In an independently initiated study, these workers conclusively proved the presence of a phosphodiester bond between the polymer and lipid portions of *S. faecalis* N.C.I.B. 8191 by hydrofluoric acid (HF) degradation. This reagent, which almost exclusively attacks phosphomono- and phosphodiester groups, degraded the lipoteichoic acid to glycerol, kojibiosyl glycerol, diglyceride, KD, monoglyceride, and small amounts of fatty acids (37). The recovery of KD indicated that the lipid component had, at the minimum, the structural elements of a glycolipid. The presence of diglyceride as an HF degradation product also suggested that a phosphatidyl group might be attached to the glycolipid. However, no further clue identifying the structure of the lipid component of lipoteichoic acid was obtainable with this HF degradation technique (37).

FUNCTIONAL CONSIDERATIONS OF PKD

The immunochemical work of van Driel, et al., (35) has firmly established the association of the polyglycerol phosphate lipoteichoic acid with the membrane of the gram-positive bacteria. The relatively polar glycerol phosphate polymer of the lipoteichoic acid undoubtedly is held to the membrane, which is relatively nonpolar, via the lipid covalently bonded to the glycerol phosphate polymer. We (25) have shown that the lipid of the membrane lipoteichoic acid of *S. faecalis* (*faecium*) ATCC 9790 is PKD.

Space-filling models of the structure of PKD (Fig. 2) show that the molecule is well suited to function as an anchor. Its nonpolar "tail" consisting of four long chain fatty acids (instead of the usual two fatty acids found in most glycerides) offers a broad hydrophobic region capable of interacting with and being embedded in the nonpolar regions of the cell membrane. Its polar "head" is a strongly hydrophilic moiety due to the anionic phosphate group and the hydroxyl groups on the glucoses. In addition to interacting with the more polar molecules at or above the surface of the membrane, the hydroxyl groups provide a chemically reactive site for coupling the long glycerol phosphate polymer to the lipid anchor (Fig. 2).

ACKNOWLEDGMENTS

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Biosynthesis and Structure of Glycosyl Diglycerides, Steryl Glucosides, and Acylated Steryl Glucosides[1]

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ABSTRACT

A particulate enzyme fraction from *Mycobacterium smegmatis* catalyzed the transfer of ^{14}C-glucose from UDP-^{14}C-glucose into neutral glycolipids. The two major radioactive components were purified by column chromatography on O-diethylamino ethyl cellulose (acetate) and thin layer chromatography on silica gel in several solvents. The first product yielded a water-soluble component upon saponification, which had a hexose-glycerol ratio of 1:1 with all of the hexose being identified as glucose. The second product yielded a water-soluble component upon saponification which contained hexose and glycerol in a 2:1 ratio and, in addition to glucose, contained lesser amounts of mannose and galactose. Palmitate and oleate were the predominant fatty acids and were present in equimolar amounts. The products thus have been identified as monoglycosyldiglyceride and diglycosyldiglyceride. The diglycosyldiglyceride could also be labeled with ^{14}C-galactose when UDP-^{14}C-galactose served as the donor, but the monoglycosyldiglyceride was only slightly labeled with ^{14}C-galactose. Membrane fractions from mung bean seedlings catalyzed the transfer of ^{14}C-glucose from UDP-^{14}C-glucose into a neutral glycolipid which has been purified by thin layer chromatography and analyzed by combined gas liquid chromatography-mass spectrometry. It was determined to be a steryl glucoside with the two major sterol components being β-sitosterol and stigmasterol linked to β-D-glucose. Particulate fractions from developing cotton fibers also catalyzed the formation of steryl glucosides and, in addition, they catalyzed the esterification of steryl glucosides at the 6 position of glucose with fatty acids (primarily palmitate and oleate) from an endogenous acyl donor. Both the glucosyl transferase and the acyltransferase have been solubilized with Triton X-100 and partially purified by chromatography on Sephadex G-200. The acyltransferase activity was reconstituted by the addition of the steryl glucoside and a phospholipid acyl donor.

INTRODUCTION

Cell membranes contain various types of neutral glycolipids, including cerebrosides, glycosyl diglycerides, glycosyl glycosyl diglycerides, steryl glucosides (SG), acylated steryl glucosides (ESG), etc. While the functions of these lipids are not known at this time, there is considerable interest in their metabolism because of their possible involvement in membrane function.

We have examined the biosynthesis of several different glycolipids whose structures are shown in Figure 1. Synthesis of the glycosyl diglycerides is catalyzed by membrane fractions of *Mycobacterium smegmatis* from either UDP-^{14}C-glucose or UDP-^{14}C-galactose (1). Particulate extracts of cotton fibers catalyze the transfer of glucose from UDP-^{14}C-glucose to β-sitosterol to form SG. This same extract also catalyzes the acylation of the 6 position of the glucose moiety of ESG using an endogenous acyl donor (2,3). Studies on the biosynthesis and characterization of these various lipids will be discussed.

EXPERIMENTAL PROCEDURE

Materials: UDP-^{14}C-glucose, UDP-^{3}H-glucose and other radioactive sugar nucleotides were obtained from New England Nuclear Co., Boston, Mass. Triton X-100, L-α-lecithin, and β-sitosterol were purchased from Sigma Chemical Co., St. Louis, Mo. Phospholipids were from Supelco Co., Bellefonte, Pa., and silica gel plates were from Brinkmann Instruments Co., Westbury, N.Y.

Chromatographic procedures: Thin layer plates were run in the following solvents: (A) chloroform-methanol (C:M)-water (65:25:4, v/v), (B) chloroform-methanol-water

[1]One of six papers presented in the symposium, "Recent Developments in Glycolipid Isolation and Analysis," at the AOCS Fall Meeting, Philadelphia, September 1974.

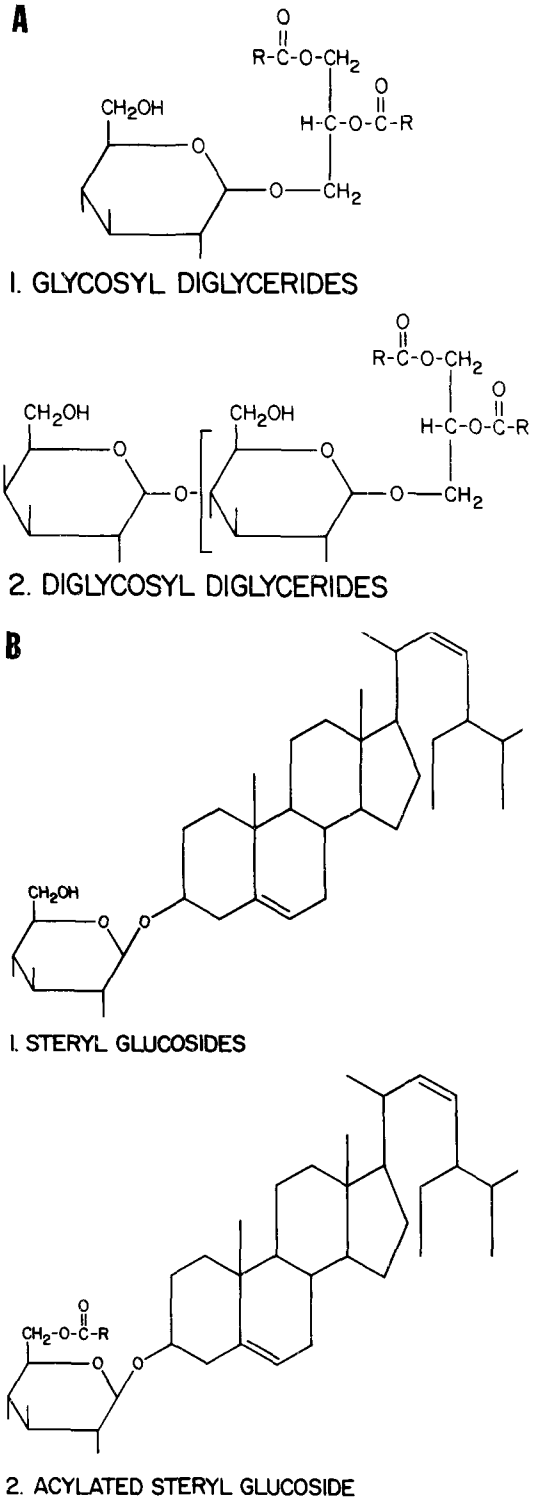


FIG. 1. Chemical structures of glycosyl diglycerides, diglycosyl diglycerides, steryl glucosides, and esterified steryl glucosides.

(85:15:0.5, v/v), (C) chloroform-methanol-water (95:5:0.2, v/v), (D) chloroform-methanol-acetic acid-water (120:80:6:10), (E) chloroform-methanol-7N ammonium hydroxide (12:60:10), and (F) dibutyl ketone-acetic acid-water (160:120:16).

Lipid bands were located by iodine vapor absorption, and radioactivity was determined by scraping sections of the plate and counting in toluene scintillator.

Gas liquid chromatography (GLC) and mass spectrometry (MS): GLC was done on a Barber-Coleman Series 500 instrument with U-shaped columns and hydrogen flame ionization detectors. Liquid phases used were SE-30, diethylene glycol succinate (Supelco) on a support of Chromosorb W and 3% ECNSS-M (Applied Science Laboratories, State College, Pa.) on Gas Chrom Q.

Trimethylsilyl ethers of the methyl glycosides were prepared by the addition of equal amonts of acetonitrile and bistrimethylsilyltrifluoroacetamide and were allowed to react for 30 min at 110 C in Teflon stoppered tubes. The trimethylsilyl ethers of intact glycolipids were prepared in the same way, except that the reaction time was increased to 60 min.

Permethyl ethers of glycolipids and sugars were prepared as described by Hakamori (4) as more fully detailed by Sanford and Conrad (5). For isolation of the methylated sugars, the reaction was terminated by the addition of 2 ml of H_2O, and methylated sugars were extracted into chloroform, washed with H_2O and dried in vacuo. The product was redissolved in 3 ml 50% diethyl ether-petroleum ether, washed with H_2O, and dried under nitrogen. Since fully methylated polysaccharides are resistent to hydrolysis (6), samples were first subjected to formolysis in 90% formic acid for 3 hr at 100 C before hydrolyzing in 1 N HCl for 2 hr at 100 C. Unhydrolyzed product was removed by chloroform extraction, discarded, and the aqueous phase was dried. Samples were dissolved in 1 ml water and reduced by the addition of 20 mg $NaBH_4$ under slightly alkaline conditions. Excess $NaBH_4$ was destroyed by the addition of acetic acid and Na^+ was removed with Dowex 50 (H^+) resin. Borate was removed by repeated evaporation with methanol. The methylated sugar alditols were acylated overnight in 1.5 ml pyridine and 1.5 ml acetic anhydride. Two ml H_2O was added, and methylated alditol acetates were extracted into chloroform and anlyzed on the gas chromatograph.

Mass spectra were recorded on an LKB 9000 GLC-MS. GLC columns were 1 or 3 m long and used a 1% SE-30 liquid phase on Chromo-

sorb W. The ionizing energy was 70 eV, except as stated otherwise. Mass spectra were scanned in 3-5 sec on the apex of the peak represented on the total ionization monitor. Temperatures were as follows: ion source 250 and 270 C; carrier gas was helium; carrier gas separation 250 C; and GLC column, 220-280 C.

Other procedures: Methanolysis of lipids was accomplished with 1 N methanolic HCl at 80 C for 4 hr (7). Water and hexane were added to the methanolysate. After mixing, the upper hexane layer was removed. This procedure was repeated and the combined hexane fractions were dried under a stream of nitrogen and redissolved in hexane for injection into the gas chromatograph. The lower, or methanolic-HCl layer, contained the sugar moiety. Silver acetate was added to remove chloride and the precipitate was removed by centrifugation. The supernatant was dried and sugars were converted to their trimethylsilyl derivatives.

Periodate oxidations were done as described previously, except that 1% Triton X-100 was added to solubilize SG and ESG each in 2 ml 0.05 M $NaIO_4$. After 70 hr at 4 C, samples were reduced with $NaBH_4$, hydrolyzed in 2 N HCl at 100 C for 2 hr, and extracted with chloroform-methanol (2:1) (C:M) to remove lipids. The aqueous phase was dried several times to remove HCl and was passed through Dowex-50 (H^+) to remove Na^+. Borate was removed by repeated evaporation with methanol. The samples then were treated with mixed bed resin, and radioactive products were isolated and identified by paper chromatography.

Alkaline hydrolysis: Glycosyl diglycerides were taken up in 1.5 ml CH_3OH, and 1 ml KOH and 7.5 ml water were added. The mixture was heated at 70 C for 1.5 hr and then allowed to stand overnight. The solution was chilled, adjusted to pH 2.5 with HCl, and extracted 4 times with 10 ml each of ethyl ether. After removal of ether, the solution was deionized with mixed bed resin, and the glycosyl glycerols were isolated by paper chromatography.

ESG was deacylated by placing the sample in chloroform-methanol (1:4) and adding NaOH to a final concentration of 0.1 N. Following incubation at 37 C for 30 min, samples were neutralized with acetic acid and glycolipids were partitioned into the C:M phase.

Enzyme assays: UDP-glucose:β-sitosterol glucosyl-transferase activity was determined by following the incorporation of radioactivity from UDP-[^{14}C]-glucose into $CHCl_3$:CH_3OH-soluble products. Reaction mixtures contained 50 mM Tris, pH 8.5, 0.1 mM UDP-[^{14}C]-glucose (25,500 cpm), 0.75 mM β-sitosterol, and enzyme in a final volume of 0.25 ml (2,3).

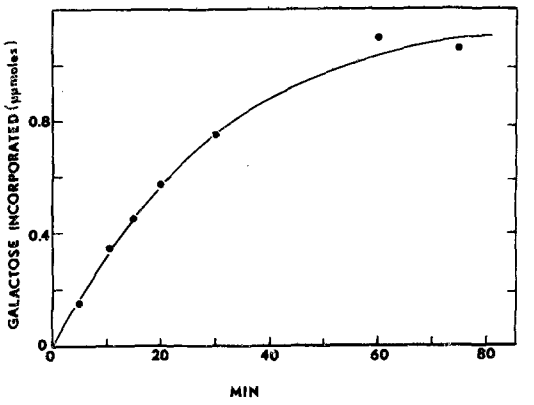


FIG. 2. Effect of time of incubation upon the incorporation of [^{14}C]-galactose from UDP-[^{14}C]-galactose into lipid. At the indicated times, samples were removed and lipids were partitioned into chloroform-methanol. Radioactivity in lipid was determined. The reaction mixtures contained 30 μliters of enzyme (3 mg protein).

TABLE I

Thin Layer Chromatographic Mobilities of Two Radioactive Glucolipids Synthesized from UDP-[^{14}C]-glc in *Mycogacterium smegmatis*

	Rf			
	Radioactive band		Standard[a]	
Solvent[b]	Slow	Fast	MGDG	DGDG
A	0.43	0.72	0.72	0.46
B	0.28	0.67		
E	0.73			
F	0.56			
D	0.87			

[a]MGDG = monogalactosyldiglyceride and DGDG = digalactosyldiglyceride.

[b]Solvents are as described in the text.

Reactions were stopped by the addition of C:M and the organic phase was separated, washed, and counted to determine its radioactive content.

For assay of acyl transferase, reaction mixtures contained ^{14}C-SG (20,000 cpm), 0.7 μmoles lecithin, 10 μmoles Tris buffer, pH 7.0, and enzyme in a final volume of 0.3 ml. After incubation for an appropriate time, reaction mixtures were extracted with C:M, and the C:M layer was dried under a stream of nitrogen. The residue was taken up in a small volume of C:M, streaked on thin layer plates and chromatographed in solvent B. ESG areas of the plates were scraped and counted.

Incubation mixtures for assay of glycosyl and diglycosyl diglyceride formation contained the following components in a final volume of

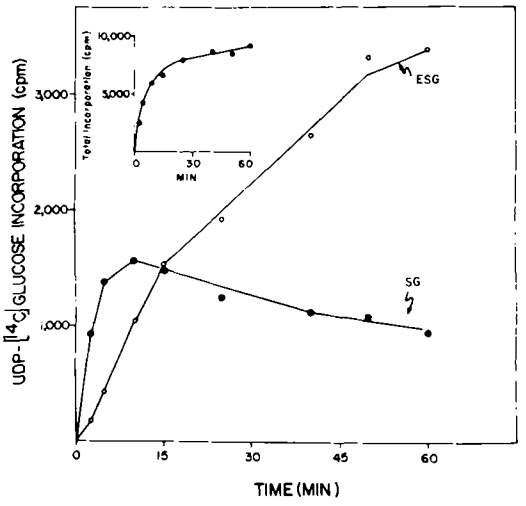


FIG. 3. Incorporation of [^{14}C]-glucose from UDP-[^{14}C]-glucose into steryl glucosides (SG) and acylated steryl glucosides (ESG) endogenous glycolipids by a particulate enzyme fraction from cotton fibers. The reaction mixture contained 0.5 nmoles UDP-[^{14}C]-glucose (100,000 cpm) in 1 ml enzyme preparation, and the incorporation was virtually completed in 30 min. Aliquots (0.1 ml) were withdrawn at indicated intervals, and aliquots of the chloroform-methanol extractable material were counted for total incorporation (inset). The remainder was separated into the SG and ESG components by chromatographing on silica gel plates in chloroform-methanol-H_2O (85:15:0.5).

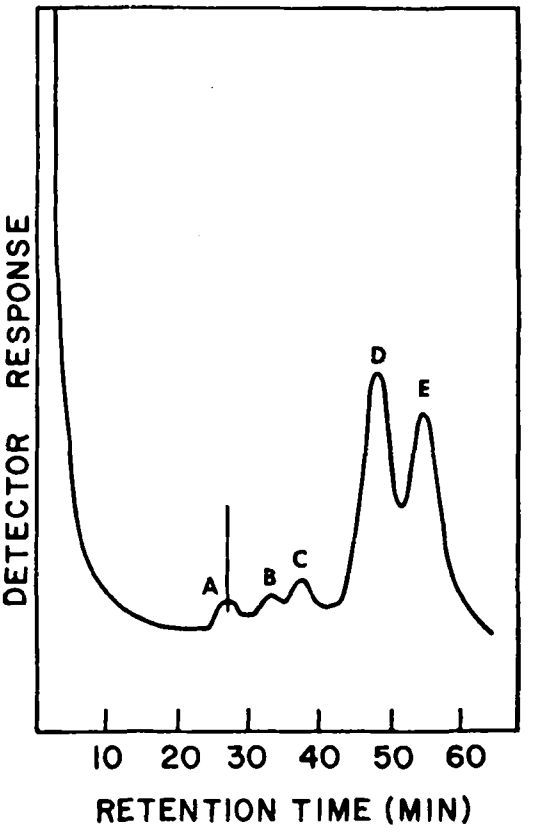


FIG. 4. Gas liquid chromatogram of the trimethylsilyl derivatives of the intact purified glycolipid mixture. Gas liquid chromatography was performed isothermally on a 0.3 M x 4 mm$_2$ column of SE-30 at 265 C and flow rate of 60 cm^3/min. The instrument was a Barber-Colman model 500 equipped with a flame ionization detector and radioactivity monitoring system. Peaks were labeled A through E.

0.3 ml: UDP-^{14}C-galactose, 0.26 nmoles (244,000 cpm/nmole) or UDP-^{14}C-glucose, 0.22 nmoles (380,000 cmp/nmole); 2 μmoles $MnCl_2$; 50 μmoles Tris buffer, pH 7.5; and an appropriate amount of particulate enzyme (1). After incubation, reaction mixtures were stopped by the addition of C:M, and the radioactivity in the C:M phase was counted.

RESULTS

Glycosyl and Diglycosyl Diglycerides

When cell-free particulate extracts of *Mycobacterium smegmatis* are incubated with UDP-[^{14}C]-glucose or UDP-[^{14}C]-galactose, radioactivity is incorporated into C:M as shown in Figure 2. This incorporation of radioactivity is fairly linear with time for ca. 25-30 min and then levels off. Incorporation of both glucose (from UDP-glu) and galactose (from UDP-gal) is stimulated by Mn^{++} or Mg^{++} and shows a pH optimum of ca. 7.0. In large scale incubations, ca. 1% of the radioactivity from UDP-[^{14}C]-glu or UDP-[^{14}C]-gal is incorporated into the C:M fraction. This radioactivity was passed through columns of O-(diethylamino ethyl) (DEAE)-cellulose (acetate) to separate the charged from the neutral lipids. Over 90% of the radioactivity originally present in the C:M fraction emerged in the wash, indicating that the lipids formed from these sugar nucleotides were neutral.

The neutral glycolipids were purified further by repeated TLC on silica gel plates. A number of iodine staining bands were observed on these plates, but only 2 of these bands contained radioactivity. The faster moving ^{14}C-band corresponded in mobility to monoglycosyl diglyceride (using monogalactosyl diglyceride as standard), whereas the slower band migrated with diglycosyl diglyceride (using digalactosyl diglyceride was standard). Each of these radioactive areas was eluted with C:M and rechromatographed on additional thin layer plates until homogeneous. As shown in Table I, the faster moving band formed in the incubations with UDP-[^{14}C]-glu was similar in mobility to monogalactosyl diglyceride, whereas the slower

band corresponded to diglycosyl diglyceride. In the case of incubation mixtures with UDP-[^{14}C]-gal, the majority of the radioactivity was in the band which corresponded to the diglycosyl diglyceride, and only small amounts of radioactivity were in the monoglycosyl diglyceride.

The purified radioactive products were deacetylated in methanolic KOH, and the aqueous and organic phases were separated and counted. Ca. 95-98% of the radioactivity originally present in the C:M phase was rendered water soluble by this treatment. Paper chromatography of the water-soluble products indicated that the monoglycosyl diglyceride gave a product which migrated like authentic samples of glycosylglycerol in two solvents, whereas the product formed from the diglycosyl diglyceride migrated with authentic diglyco-

Complete acid hydrolysis of the purified, radioactive glycolipids indicated that all of the label in the lipids formed from UDP-glu was in the glucose, while all of the label in the lipids formed from UDP-gal was in galactose. However, when the sugar composition of these lipids was analyzed by GLC of the alditol acetate derivatives, each of the lipids showed the presence of mannose, galactose, and glucose. This suggested the presence of a family of glycosyl diglycerides containing different sugars. The ratios of these sugars in the glycosyl diglycerides and diglycosyl diglycerides indicated that glucose was the predominant sugar with smaller amounts of mannose and galactose.

The fatty acids of the various glycosyl diglycerides were obtained by alkaline hydrolysis and were analyzed by gas chromatography of their methyl esters. The results of these studies showed that palmitate and oleate were the predominant fatty acids and were present in ca. equal amounts in all of the radioactive glycolipids.

Steryl Glucosides and Acylated Steryl Glucosides

The particulate enzyme fraction from mung bean seedlings and cotton fibers catalyzes the incorporation of ^{14}C-glucose from UDP-[^{14}C]-glucose into a glycolipid which was characterized as a SG as indicated below. In addition, cotton fiber membranes also catalyze the formation of a second lipid which was identified as an acylated or esterified steryl glucoside (ESG). The incorporation of radioactivity into SG and ESG by cotton fiber extracts as a function of time is shown in Figure 3. In this experiment, incubation mixtures were extracted with C:M to remove the lipids, and SG and ESG were separated on Silica Gel F plates. As shown in the inset of Figure 3, incorporation of radioactivity into the total glycolipids was almost complete within 25 min. During the last 35 min of incubation, there was a decrease in radioactivity in SG, whereas radioactivity in ESG continued to increase. This suggested an initial incorporation of ^{14}C-glucose into SG and subsequent conversion to ESG. The discrepancy between radioactivity shown in the inset and that shown in individual SG and ESG is due to a 50% decrease in counting efficiency on silica gel plates.

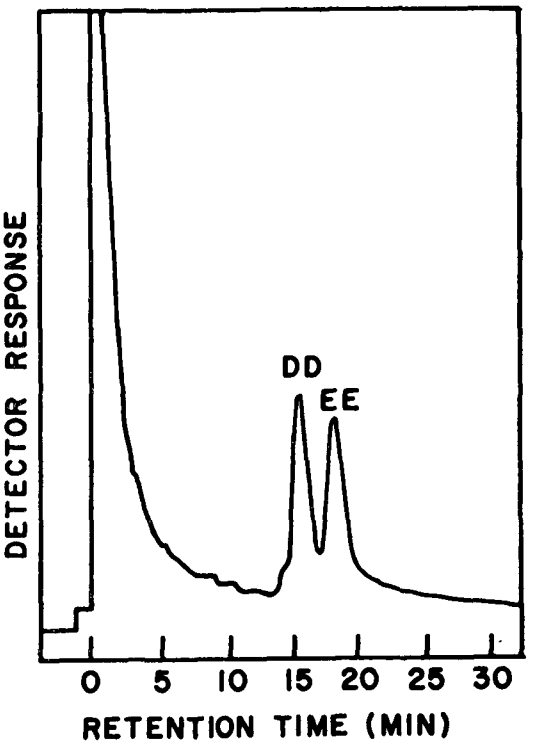


FIG. 5. Gas liquid chromatogram of the hexane fraction from a methanolysis of purified glycolipid. The aglycon peaks eluted at 220 C in 15 and 17.5 min, respectively. Other conditions were as in Figure 4. Peaks were labeled DD and EE.

To characterize the products formed in these reactions, several large scale incubations were prepared using UDP-[^{14}C]-glucose as substrate. The lipid products then were isolated by extraction with C:M. In the case of both mung bean and cotton, essentially all of the radioactivity was judged to be in neutral lipids by virtue of the fact that it was not retained on DEAE-cellulose (acetate). The lipids then were purified by repeated TLC on silica gel plates in a number of solvents. As indicated above, two lipids, which were designated as SG and ESG, were formed from cotton fibers, whereas only SG was formed by mung bean extracts. In both cases, the only sugar detected in these

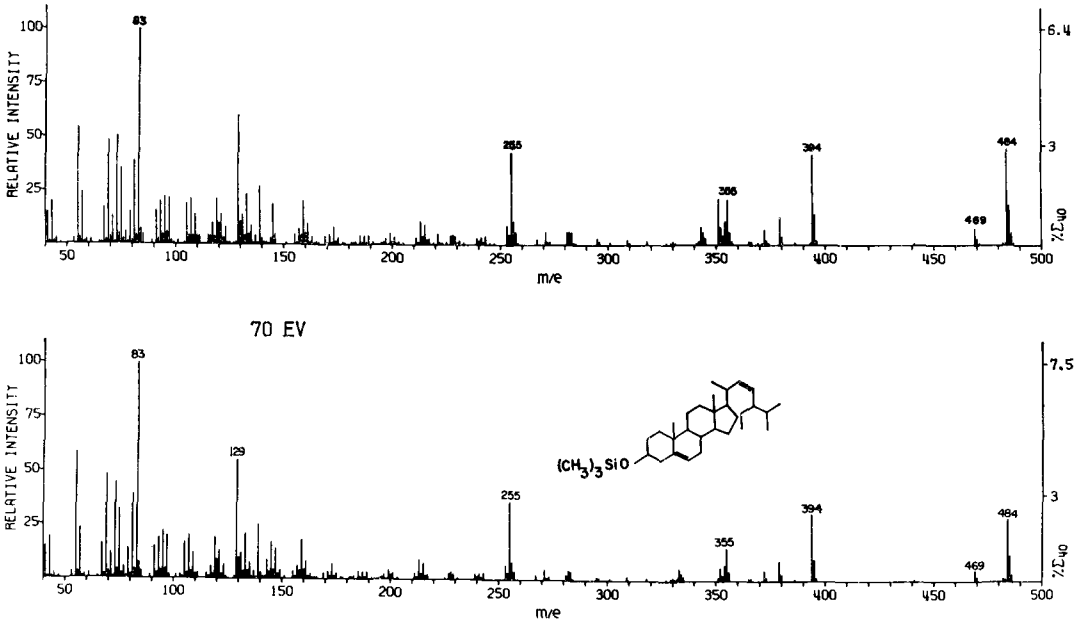


FIG. 6. The mass spectrum of the trimethylsilyl ether of the aglycon component DD from Figure 5 (top), as compared with the mass spectrum of the trimethylsilyl ether of an authentic sample of stigmasterol (lower figure). Mass spectra were recorded on an LKB-9000 combined gas liquid chromatograph-mass spectrometer. Columns were 3 m long and used a 1% liquid phase of SE-30. The ionizing electron energy was 70 eV. Spectra were scanned in 3-5 sec on the apex of the gas liquid chromatographic peak represented on a total ionization monitor. Temperatures were as follows: ion source, 250 C, helium separator, 250 C, and gas liquid chromatographic column, 220-280 C.

compounds was glucose, and this sugar contained all of the radioactivity.

The purified mung bean glycolipid was chromatographed intact as the trimethylsilyl derivative on 3% SE-30 at 265 C as shown in Figure 4. This procedure gave rise to 2 major and 3 minor peaks with the major peaks D and E eluting at 50 and 55 min. Permethyl derivatives of the intact glycolipid gave 2 peaks which eluted at 29 and 31 min using the same conditions. These data suggested that the purified fraction contained several glycolipids having mol wt of 500-600. To examine the aglycon portion of the molecule, the glycolipid was subjected to acid methanolysis, and the aglycon was extracted with hexane. Figure 5 shows the results of GLC of the hexane fraction, indicating the presence of at least two aglycon moieties, Mass spectra of these two components indicated that component DD had a molecular ion at m/e 412, whereas the EE molecular ion was 414. These mol wt suggested plant sterols. Mass spectra then were taken of the trimethylsilyl derivatives of the aglycon portion, and these were compared to the known plant sterols, β-sitosterol and stigmasterol. As shown in Figure 6, component DD was identical to stigmasterol. The molecular ion of the trimethylsilyl derivative of DD had increased 72 mass units (from 412 to 484), indicating one replaceable H, and the ion at m/e 129 indicative of Δ5-3-01 steroids. Likewise, component EE was identical to sitosterol and also showed an increase in molecular ion from 414 to 486, as well as a m/e at 129.

Because of the reported spectra for glycosides and steroids, the spectra of the trimethylsilyl ether of the intact steryl glucosides were recorded as shown in Figure 7. This is the spectra for the intact component D and shows characteristic peaks for hexose and sterol. Although the molecular ion was not observed, M-90-15 (corresponding to M-trimethylsilanol-CH_3) at m/e 757 was seen as was M-90-90-15 (at m/e 667). These ions were used for mol wt determinations. Loss of the sugar (tetratrimethylsilyl) with charge retention on the sugar portion and the cleavage of the glucose C-O glycosidic bond produces an ion at mass 451 which could lose trimethylsilanol (=90), producing an ion of mass 361, both of which are prominent in this spectrum. Cleavage of the sterol-3-(C-O) bond with charge retention on the steroid would produce an ion in this case of m/e 395 which is apparent in the spectrum. Characteristic ions for hexoses at m/e 147, 204, 217, 305, 361,

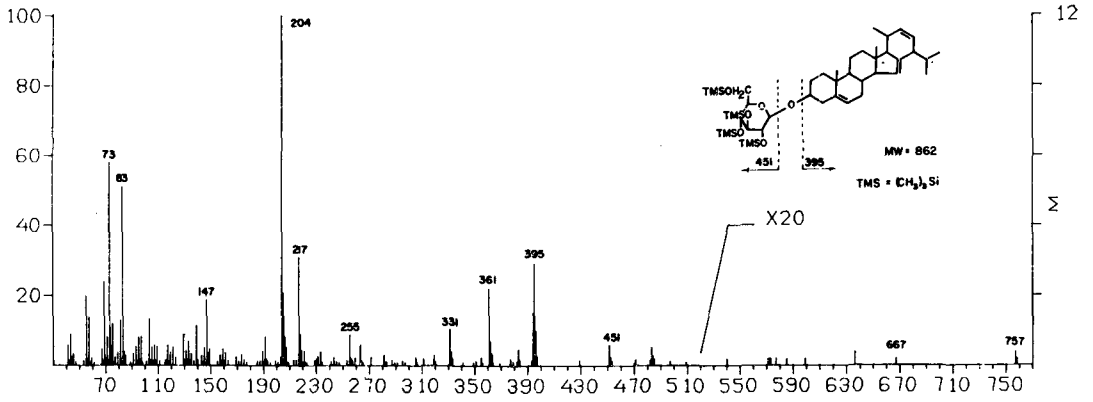


FIG. 7. The mass spectrum of the intact steryl glucoside as its trimethylsilyl ether. The spectrum is taken on component D from Figure 4 and represents the trimethylsilyl ether of stigmasteryl-glucoside. Recording conditions were the same as that for Figure 6, except that a 1 M column of SE-30 was used for gas liquid chromatography to facilitate the elution of higher mol wt components.

TABLE II

Gas Liquid Chromatography Analysis of Esterified Steryl Glucoside[a]

Sterol	Percent	Sugar	Percent	Fatty acid	Percent
β-Sitosterol	75.6	Glucose	100	Palmitic	60
Campesterol	4.3			Oleic	37
Stigmasterol	6.0			Other	3
Others	14.3				
Molar ratio	1.02		1.19		1.00

[a]The trimethylsilyl-sterol ethers and the fatty acid methyl esters of the acylated steryl glucoside (ESG) fraction were run on a 2 m x 3 mm column of 3% SE-30 on Gas Chrom Q (Analabs, North Haven, Conn.) 100-120 mesh, and the spectrum was recorded on an LKB *9000 combined gas liquid chromatograph-mass spectrometer.* The column temperature was programed from 170-280 C at 5 C/min; the separator temperature was 280 C; the ion source temperature was 290 C; and ionizing electron energy was 70 eV. The sterols were quantified using the internal standard cholestane and the fatty acid peaks using heptadecanoate as an internal standard. The sugars released from this ESG fraction by methanolysis were converted to their trimethylsilyl ethers and also quantified by gas liquid chromatography using mannitol as the internal standard.

and 451 are prominent and m/e 204 is the base peak, an indication of hexose pyranoside configuration. A similar spectrum was observed for intact component E, but m/e 759 and 669 indicated one less double bond.

After purification of the SG and ESG of cotton fibers by TLC, these two compounds were subjected to acid-catalyzed methanolysis. The agylcones then were extracted with hexane, and the trimethylsilyl derivatives were analyzed by GLC and MS. Both SG and ESG had similar sterol composition with β-sitosterol comprising over 75% of the sterols, while cholesterol, campesterol, and stigmasterol were present in amounts ranging from 3-14% each. The only sugar present in SG or ESG was glucose. The components of the ESG are listed in Table II and indicate a ratio of 1:1:1 for the glucose, sterol, and fatty acid moieties in the intact molecule. Palmitic and oleic acids were the dominant fatty acids while several others were present in smaller amounts.

To determine the position of the sugar-acyl linkage, 50,000 cpm of both SG and ESG were subjected to periodate oxidation to cleave the sugar moiety. Since there was a possibility that the ESG might be de-esterified to the SG during periodate treatment, aliquots of both SG and ESG, before and after cleavage, were chromatographed on Silica gel plates. In all cases, the radioactivity migrated as a single band. It can be seen in Table III that the acyl moiety was still intact after periodate, since, if it had been removed, ESG would have shown the same mobility as SG. Glycerol was the only radioactive product formed by periodate cleavage followed by $NaBH_4$ reduction of ESG, indicating either a 2 or 6 position as the site of acyla-

TABLE III

Cleavage Products Following Periodate Oxidation of the Steryl Glucoside (SG) and Acylated Steryl Glucoside (ESG)

	cpm in original sample	cpm in[a]			Rf[b]	
		Glycerol	Erythritol	Sorbitol	Before cleavage	After cleavage
SG	52,000	21,100	0	0	0.08	0.23
ESG	53,000	23,200	0	0	0.19	0.54

[a]The remainder of the samples were reduced, hydrolyzed, and chromatographed on paper in 1-butanol/pyridine/0.1 N HCl (5:3:2), and the areas corresponding to glycerol, erythritol, and sorbitol were cut out and counted in a liquid scintillation spectrometer.

[b]Following periodate treatment, aliquots were chromatographed in chloroform-methanol-H_2O (95:5:0.2) on Silica Gel G plates, and the positions of the radioactive bands were determined by counting silica gel sections in a liquid scintillation spectrometer.

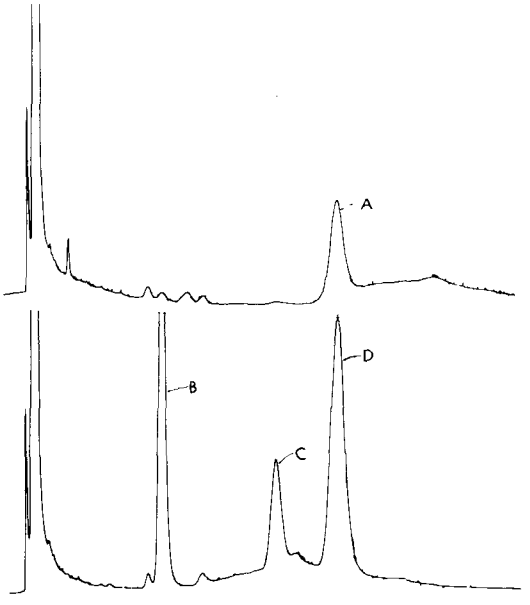


FIG. 8. Gas liquid chromatogram of the permethylated alditol acetates. The upper tracing represents the methylated glucose derived from the acylated steryl glucosides (peak A). The lower tracing represents a mixture of four permethylated glucose standards composed of the 3,4,6-trimethyl derivative from sophorose (peak C), the 2,3,6-trimethyl derivative from cellobiose (peak D), and 2,3,4-trimethyl derivative from gentiobiose (also peak D), and the 2,3,4,6-tetramethyl derivative derived from the nonreducing end of all 3 disaccharides (peak B). Samples were run on a Barber-Colman series 5000 instrument equipped with a 2 m column of 3% ESNSS-M coated on Gas-Chrom Q 100-200 mesh (Applied Science, State College, Pa.) at 190 C.

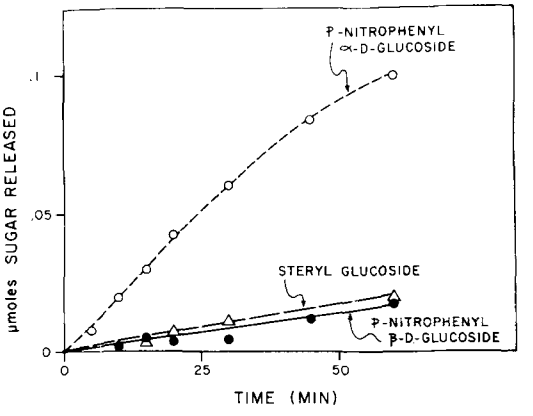


FIG. 9. Hydrolysis rate curve of steryl glucosides (SG). Two μmoles each of the SG, ρ-nitrophenyl-α-D-glucoside, and ρ-nitrophenyl-β-D-glucoside were hydrolyzed in 2 ml 50% propanol containing 0.35 N HCl at 100 C. Aliquots (0.2 ml) were withdrawn at the indicated intervals, and the amount of reducing sugar was determined.

tion. Since the yield of radioactivity in glycerol approached 50% rather than 100% the data are consistent with the formation of 1 mole glycerol which would indicate a linkage at the 6 position. Confirmation of this assignment of the acyl group at position 6 of glucose was obtained by permethylation of ESG as shown in Figure 8. A gas chromatographic profile of the methylated alditol acetate derived from ESG showed the absence of a peak corresponding to the alditol acetate of 3,4,6 tri-o-methyl glucose, eliminating the possibility of an acyl linkage at the 2 position.

To determine whether glucose was linked to the sterol moiety in the α or β configuration, the SG was hydrolyzed in 0.35 N HCl at 100 C. Aliquots were withdrawn at intervals and the amount of reducing sugar was determined. The rate of hydrolysis of the SG under these conditions is shown in Figure 9. Also shown for comparison are the rates of hydrolysis of p-nitrophenyl-α-D-glucoside and p-nitrophenyl-β-glucoside. The similarity in the rates of hydrolysis of SG and p-nitrophenyl-β-D-glucoside suggests

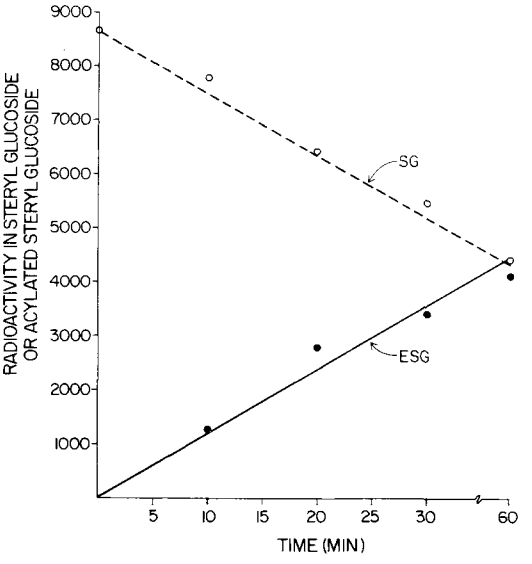


FIG. 10. Conversion of steryl glucoside (SG) to acylated steryl glucoside (ESG) by a particulate enzyme fraction from cotton fibers containing an endogenous acyl donor. Radioactive SG was dissolved in methanol, and 15 μliters (10,000 cpm) were added to enzyme in a total volume of 0.3 ml and incubated at 37 C. ^{14}C-SG and ESG were extracted with chloroform-methanol (2:1) and separated by chromatography on Silica Gel F in solvent B.

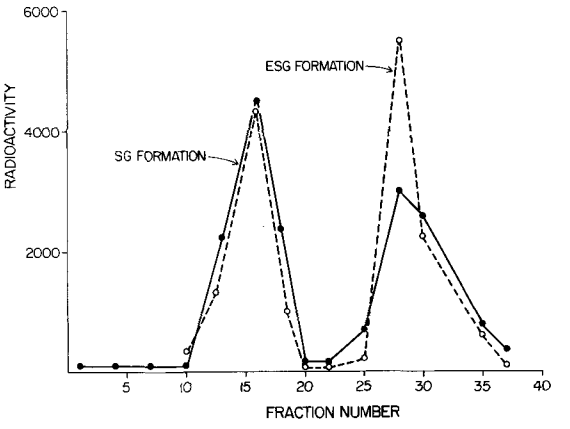


FIG. 11. Gel filtration of solubilized glucosyltransferase and acyltransferase on Sephadex G-200 (2.5 x 40 cm column). Fractions (4.5 ml) were collected and assayed for glucosyltransferase activity (steryl glucoside formation) and acyltransferase activity (acylated steryl glucoside formation).

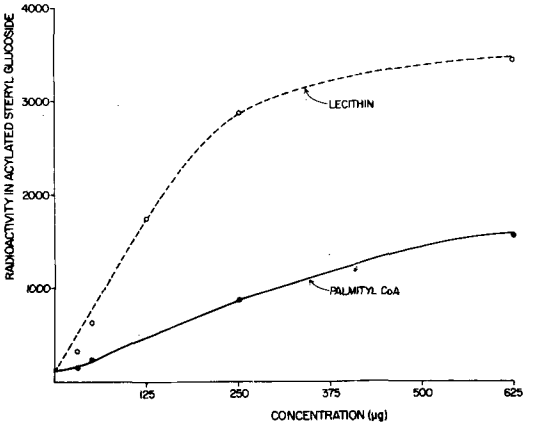


FIG. 12. Conversion of steryl glucoside (SG) to acylated steryl glucoside (ESG) by the solubilized acyltransferase which had been chromatographed on Sephadex G-200. Enzyme, SG (100,000 cpm), and crude soybean lecithin or palmityl CoA (as indicated) were incubated and assayed for the formation of ^{14}C-ESG as described in the Experimental Procedure.

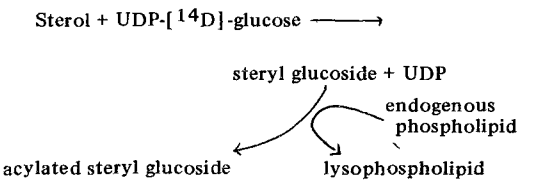
that the glucose moiety of SG is in a β-configuration.

The acylation of SG was studied using membrane preparations of cotton fibers. As shown in Figure 10, when ^{14}C-SG was incubated with these particulate extracts, radioactivity in SG declined with time, whereas radioactivity in ESG appeared in stoichiometric amounts. In this experiment, the C:M-soluble lipids were separated on thin layer plates, and the radioactivity in SG and ESG was determined. Since the acylation by the particulate enzyme proceeded for an extended period of time (Figures 3 and 10), it did not seem likely that the acyl donor could be acyl CoA. Since the likely acyl donor would then be a membrane phospholipid, we decided to determine the effect of phospholipase treatment upon the enzyme preparation. Thus, particulate enzyme was preincubated with bee venom (phospholipase) or snake venom (phospholipase) for 15 to 30 min, and the particules were reisolated by centrifugation and washed. Particles treated with bee venom lost ca. 50% of their activity, and this could be restored by the addition of lecithin to incubation mixtures.

We also have been able to solubilize both the UDP-glucose:β-sitosterol glucosyl transferase and the acyl transferase activities by treatment of the membrane particles with 0.25% Trition X-100. Both of these activities were purified partially on DEAE-cellulose and then were fractionated further on Sephadex G-200. Figure 11 shows an elution profile of the Sephadex G-200 column. Both the glucosyl transferase and the acyl transferase activities eluted in two peaks, one of which was excluded from the column, while the second peak was included in the column. The excluded peak probably represents small pieces of membrane, but the included peak probably represents truly solubilized enzyme. This enzyme fraction (peak 2) showed

no acyl transferase activity unless SG and lecithin (or some other appropriate phospholipid) were present in the incubation mixtures. Palmitoyl CoA was also somewhat effective as an acyl donor but much less so than was lecithin. These results are shown in Figure 12 which compares substrate concentration curves of lecithin and palmityl CoA as a function of ESG formation. It can be seen that lecithin was a far better acyl donor than was palmitoyl CoA.

To show that lecithin was acting as an acyl donor rather than as an activator, incubations were prepared using ^{3}H-SG (labeled in the glucose) and ^{14}C-lecithin (labeled in the fatty acids). After incubation, the various radioactive compounds were separated by TLC to isolate ESG. The isolated ESG contained both ^{14}C and ^{3}H. The ESG was chromatographed in several additional solvents, and, in each case, a radioactive band containing both ^{14}C and ^{3}H was observed which migrated with authentic ESG. When this band was subjected to alkaline hydrolysis, the tritium was separated from the ^{14}C on thin layer plates. The ^{3}H, in this case, migrated with authentic SG whereas the ^{14}C corresponded to palmitic acid. Thus, the steps in formation of ESG by these cotton fibers apparently proceed by the following series of reactions:



ACKNOWLEDGMENT

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SHORT COMMUNICATION

Inhibition of Diplopterol Synthesis in *Tetrahymena Pyriformis* by a Hypocholesteremic Compound

ABSTRACT

The hypocholesteremic compound, 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one was earlier shown to inhibit the synthesis of tetrahymanol and two unidentified lipids. It now has been demonstrated that one of the unidentified compounds is diplopterol.

INTRODUCTION

Many hypoclolesteremic compounds have been reported to suppress the growth rate of *Tetrahymena pyriformis* (1-5). The compound, 3β-(β-dimethyl-aminoethoxy)-androst-5-en-17-one (DMAE-DHA), was observed (6) to inhibit the synthesis of tetrahymanol and two unidentified lipids from sodium acetate-2-^{14}C, and to cause the accumulation of squalene before any other aspects of metabolism were affected significantly. Identification of the unknown metabolites may be of importance in accounting for the growth inhibitory properties of DMAE-DHA. It is the purpose of this communication to provide evidence that one of the higerto unidentified lipids is diplopterol.

MATERIAL AND METHODS

A culture medium (40 liters) consisting of 400 g peptone, 80 g yeast extract, 40 g KH_2PO_4, 24 g $MgSO_4 \cdot 7H_2$ 40 g sodium acetate, and 400 g glucose was adjusted to a pH of 6.8 and autoclaved for 1 hr 20 min at 15 psi. The glucose was autoclaved separately. The medium then was inoculated with 500 ml of a 2 day culture of *T. pyriformis*, mating type II, variety 1. The fermentation was conducted at 28 C in a 75 liter fermentor (Fermetron, New Brunswick Scientific Co., New Brunswick, N.J.). Air was supplied at 1 v/v/min, and agitation was at 100 RPM. Microscopic examination of the culture after 3 days incubation revealed no indication of contamination. Tetrahymena cells were collected with a model KDD-605 Westfalia separator (Centrico, Englewood, N.J.) and were washed with 3 liter of Ringer's phosphate buffer, pH 7.3 (7). The resulting heavy cell suspension (1200 ml) was disrupted in 200 ml aliquots with a Sorvall omnimixer for 30 sec at a setting of 7. The disrupted cell suspension was centrifuged for 30 min at 26,000 x g in a Sorvall RC2B centrifuge at 4 C. The supernatant was centrifuged again under the same conditions, and the two pellets of cell debris were combined and extracted and further dealt with as shown in Figure 1.

Silicic acid chromatography was performed with Biosil A, 100-200 mesh (Calbiochem, Los Angeles, Calif.) Columns were prepared by slurrying the Biosil A in the least polar solvent to be used during development, and the samples were added to the column in the same solvent. For the first Biosil A column, this was Skellysolve B-benzene 3:2, and for the second Biosil A column it was Skellysolve B-benzene 2:3. Thin layer chromatography (TLC) was conducted with 20 x 20 cm plates precoated with 0.25 mm Silica Gel F-254 on aluminum or glass (EM Reagent Division, Brinkmann Instruments, Westbury, N.Y.). Diplopterol is not visualized by exposure to iodine but can be detected by spraying with concentrated H_2SO_4 and heating 5-10 min at 110 C. A pink color develops before the compound chars. TLC systems used for following the isolation of diplopterol were: benzene-ethyl acetate, 95:5 (System I), and Skellysolve B-diethyl ether, 9:1 in an unsaturated tank (System II). In these systems, diplopterol migrates with $R_{lanosterol}$ values of 1.41 and 1.64, respectively. Tetrahymanol migrates with R_f values identical to lanosterol in both systems.

Gas liquid chromatography (GLC) was performed with a Hewlett-Packard model 5750B gas chromatograph equipped with a flame ionization detector and containing a 1/8 in. inside diameter 6 ft stainless steel column packed with Anakrom ABS 90-100 mesh coated with 1% SE-30. The column temperature was 205 C or 230 C as indicated; the injection temperature, 280 C and the detection

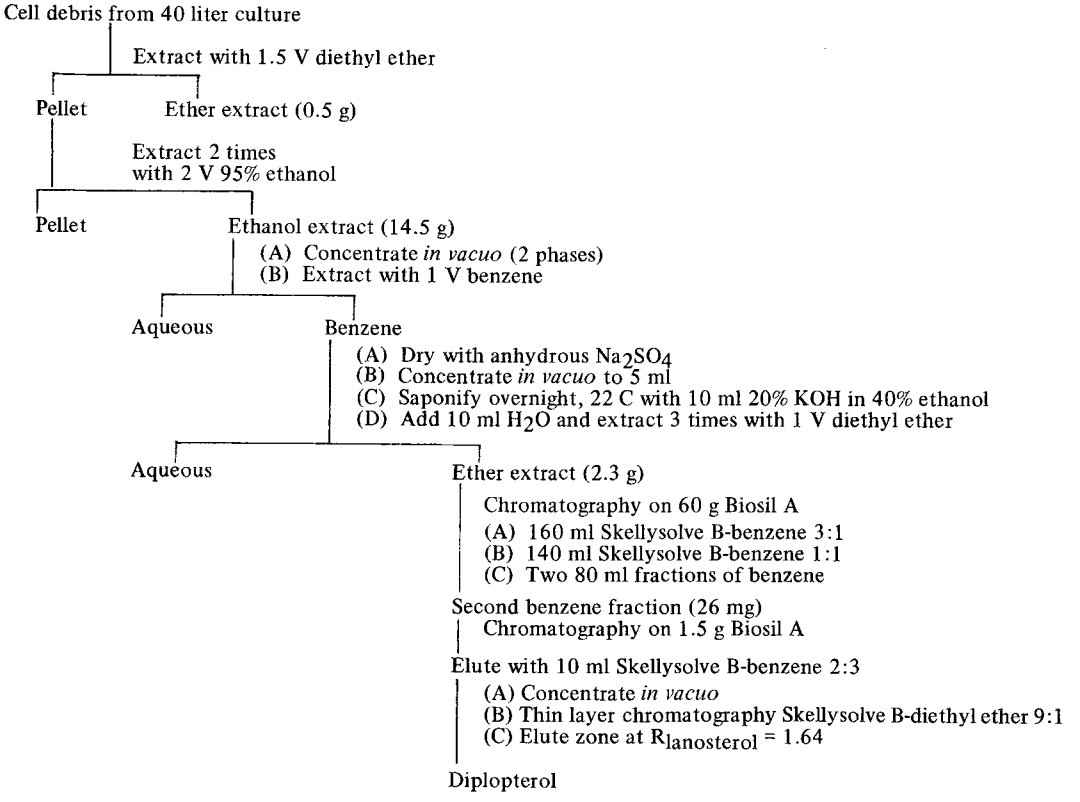

FIG. 1. Isolation of diplopterol from *Tetrahymena pyriformis*.

temperature, 290 C. The carrier gas was N_2 at 30 ml/min.

Mass spectra were obtained with a DuPont 21-492 mass spectrometer at an ionizing voltage of 70 eV. The temperature of the direct insertion probe was 150 C, and the source was 180 C. All solvents were redistilled before use. DMAE-DHA was provided, and lanosterol was purchased from Applied Science Laboratories, State College, Pa.

RESULTS AND DISCUSSION

Mallory, et al., (8) reported the isolation of a small amount of the pentacyclic triterpenoid, diplopterol, from a culture of *T. pyriformis*. Since DMAE-DHA has been shown to inhibit tetrahymanol synthesis in this organism (6), it appeared reasonable to consider that one of the unidentified lipids, whose synthesis also was inhibited by DMAE-DHA, might be diplopterol. Therefore, the labeling experiment was repeated (6). Five ml cultures of *T. pyriformis* were incubated with 25 μc sodium acetate-2-^{14}C for 3 hr in the presence and absence of 10 μm DMAE-DHA. TLC in system I of the nonsaponifiable fraction of the lipid extract revealed a labeled component slightly less polar than tetrahymanol, whose synthesis was impaired by DMAE-DHA. This component, after elution from the chromatogram with diethyl ether, was purified further by TLC on system II (Fig. 2). Complete resolution from tetrahymanol was achieved, and analysis of the eluted sample revealed that inhibition of this substance by DMAE-DHA amounted to 70%. Moreover, the labeled metabolite migrated on system II like reference diplopterol. To establish whether or not this metabolite was truly diplopterol, a larger scale culture was grown and processed as shown in Figure 1. The initial ether extract contained tetrahymanol, but little or no diplopterol. Fractions whose TLC properties on system II were identical to the unknown labeled metabolite were combined and purified as indicated in Figure 1. A small amount of crystals was obtained from the diethyl ether eluate from preparative TLC with system II. TLC characteristics of the crystalline preparation and the mother liquor were identical to those of the labeled metabolite and to reference diplopterol with 4 solvent systems: systems I, II, benzene-methanol 95:5 ($R_{lanosterol}$ = 1.13), and Skellysolve B-$CHCl_3$-CH_3OH

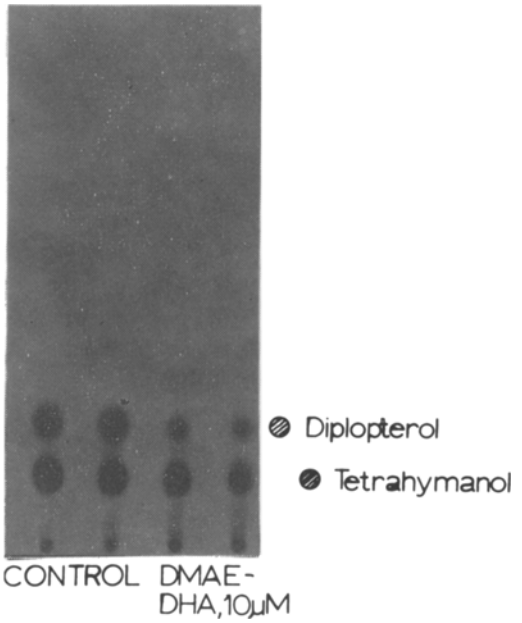

FIG. 2. Inhibition of diplopterol synthesis by DMAE-DHA. *Tetrahymena pyriformis* was incubated with [2-^{14}C]sodium acetate in the presence and absence of 10 μM 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one (DMAE-DHA) Nonsaponifiable lipids were chromatographed with system I, and compounds in a zone emcompassing tetrahymanol and a less polar product were eluted and rechromatographed in system II.

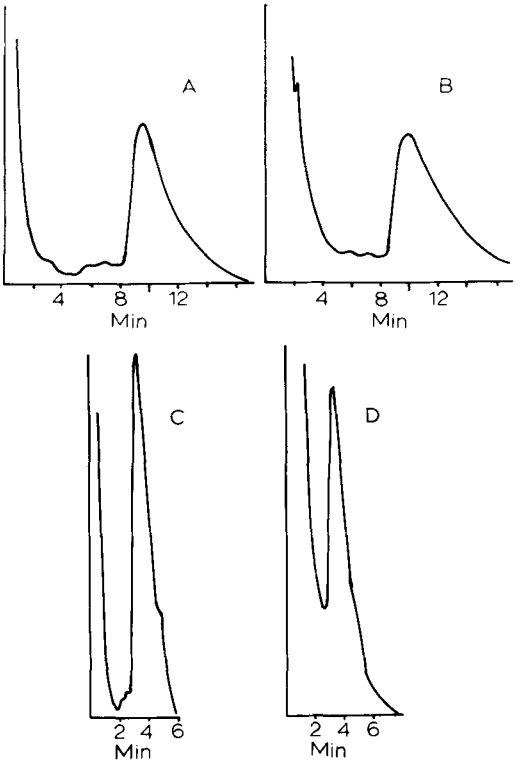

FIG. 3. Gas liquid chromatography of reference diplopterol (A and C) and a preparation isolated from *Tetrahymena pyriformis* (B and D). Curves A and B were obtained at a column temperature of 205 C and curves C and D at 230 C.

70:20:10 ($R_{lanosterol}$ = 1.22). Mass spectral data were obtained from the crystalline preparation and reference diplopterol. Both exhibited a molecular ion peak at m/e 428 and additional significant m/e peaks at 413, 410, 395, 370, 369, 367, 341, and 191. The latter m/e value was the base peak in both spectra. These values agree well with those reported by Mallory, et al., (8).

Diplopterol was first isolated from a fern by Kariyone and Ageta (9) and later shown to be identical to hydroxyhopane (10), a chemical derivative of hydroxyhopanone which was obtained from cats eye dammar (11,12). Diplopterol can undergo dehydration to form a mixture of products (10) which may account for difficulty in utilizing GLC as a means of demonstrating purity of preparations (R.L. Conner, personal communication). GLC data of reference and isolated preparations of diplopterol are shown in Figure 3, where it is evident that both samples reveal similar GLC patterns when chromatographed on 1% SE-30 at either 205 or 230 C. The occurrence of minor peaks and the altered retention times relative to cholesterol for the major peaks (1.25 at 205 C and 1.12 at 230 C) suggest that degradation of diplopterol has proceeded to a different extent at the two temperatures. The fact that both preparations behaved similarly further attests to their common identity. It is, therefore, concluded that DMAE-DHA inhibits the synthesis of diplopterol and tetrahymanol. It has been established that cyclization of squalene to tetrahymanol is inhibited by DMAE-DHA (5). Diplopterol synthesis may be inhibited at the same step.

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Fractionation and Analysis of Fluorescent Products of Lipid Peroxidation

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ABSTRACT

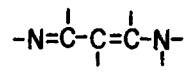
The fluorescence excitation spectrum of model conjugated Schiff base compounds that arise from the reaction of malonaldehyde with amino acids was shown to contain a maximum at 260-280 nm in addition to the previously observed maximum at 350-390 nm. Excitation at either maximum results in emission at a single maximum at 440-480 nm. The excitation and emission maxima of the model fluorescent compounds, together with the characteristic reductions in fluorescence intensity caused by alkaline pH or heavy metal coordination, provide criteria with which to examine lipid peroxidation products for the presence of the conjugated Schiff base fluorophore. Silicic acid column chromatography and silica gel thin layer chromatography were employed to fractionate the fluorescent products of model lipid peroxidation systems and of rat testicular lipid soluble extracts. These products contained large families of compounds whose fluorescence characteristics were the same as those of the Schiff base fluorophores. The fractionation methods used enabled more thorough fluorescence characterization of many of the products of lipid peroxidation, but the fluorescence criteria available do not provide definitive proof of structure.

INTRODUCTION

Lipid peroxidation has been identified as a basic deteriorative process in cellular membranes (1,2). Subcellular organelles, with their high content of polyunsaturated fatty acids (3), are especially labile to lipid peroxidation, which yields a mixture of reactive carbonyl compounds via the chain reactions of free radical intermediates (4). Among these carbonyl products is malonaldehyde (5), measurable by means of a sensitive colorimetric assay that can be used to gauge the relative extent of peroxidation of lipid systems in vitro (6,7).

Lipofuscins, or age pigments, which appear as granular deposits in the cells of various mammalian tissues, contain indigestible breakdown products of lipid peroxidation (8). The luminescent properties of age pigments have long been known (9). Much of the fluorescence is extractible with lipid solvents (10,11), and its characteristic excitation and emission maxima, at 340-375 nm and 420-490 nm, respectively, are comparable with spectra of fluorescent compounds extracted from peroxidized subcellular organelles (12). Dillard and Tappel (13) found that lipid extractible fluorescence was 10-100 times more sensitive a measure of lipid peroxidative damage than the colorimetric malonaldehyde assay.

The major fluorescent species arising from lipid peroxidation appear to be conjugated Schiff base fluorophores with the basic structure:



which is formed as the product of the crosslinking of two primary amines with malonaldehyde (14). Products of reaction of primary amino phospholipids with malonaldehyde and with peroxidizing polyunsaturated fatty acids have fluorescence maxima similar to those of the conjugated Schiff base fluorophore (15,16).

The fluorescence of the conjugated Schiff base structure is decreased reversibly by alkaline pH in either aqueous or chloroform-methanol solution and is decreased by coordination of the chromophoric nitrogens with a heavy metal chelating compound (17). There is evidence that these fluorophores have an excitation maximum in the near UV region at 260-280 nm in addition to the maximum at 340-390 nm (14); these excitation maxima have a common emission maximum at 430-490 nm.

The purposes of the research presented here were twofold. The first was the verification of the 260-280 nm excitation maximum for the conjugated Schiff base fluorophores and examination of the effect of alkaline pH upon fluorescence intensity when excited in this region, and the second was the fractionation and purification of the products of lipid peroxidation to determine the major species with characteristics of conjugated Schiff base fluorophores.

EXPERIMENTAL PROCEDURE

Materials

Fluorescence standards N,N-dileucinyl-1-

TABLE I

Fluorescence Characteristics of Amino Acid Standards in Chloroform-Methanol

Standard	Percentage aqueous fluorescence	Excitation maxima, nm		Emission maximum, nm	Percentage initial fluorescence				
					Excitation 270 nm region		Excitation 380 nm region		
					base[a]	acid[b]	base[a]	acid[b]	chelator[c]
Leu_2MA	57	270	375	450	69	80	60	72	83
Val_2MA	58	275	375	445	52	76	51	75	81
Gly_2MA	51	265	390	470	77	89	77	86	70

[a]10 μliter 4 N sodium methoxide were added to 2 ml chloroform-methanol solution.

[b]Reneutralized by addition of 10 μliter 4 N acetic acid in methanol.

[c]Final concentration of $Eu(thd)_3$ was 6 x 10^{-5} M.

amino-3-iminopropene (Leu_2MA), N,N-divalinyl-1-amino-3-iminopropene (Val_2MA), and N,N-diglycinyl-1-amino-3-iminopropene (Gly_2MA) were synthesized and purified by Chio and Tappel (14) and stored dessicated at -20 C. Quinine sulfate was purchased from Mallinckrodt Chemical Works, St. Louis, Mo.; synthetic dipalmityl phosphatidyl ethanolamine from Schwarz/Mann, Orangeburg, N.Y.; phosphatidyl choline and phosphatidyl serine from Calbiochem, San Diego, Calif., and Schwarz/Mann, respectively; arachidonic acid from Sigma Chemical Co., St. Louis, Mo.; and malonaldehyde bis(diethylacetal) from J.T. Baker Chemical Co., Phillipsburg, N.J. The purity of the phospholipid standards was verified by thin layer chromatography (TLC). Rat testes were obtained from 10-14 month old Sprague-Dawley rats maintained since weaning on a basal rat chow diet (Purina). Europium (Tric [2,2,6,6-tetramethyl-3,5-heptanedionate]), hereafter referred to as $Eu(thd)_3$, was obtained from Varian Instrument Corp., Palo Alto, Calif. Silicic acid (Bio-SiL A) for column chromatography was obtained from Bio-Rad Laboratories, Richmond, Calif. Molybdenum blue reagent for the detection of phospholipids on TLC was prepared as described by Dittmer and Lester (18). Precoated Silica Gel G TLC plates were obtained from Quantum Industries, Fairfield, N.J. All solvents used were of spectral grade.

Synthetic Phospholipid Reaction Systems

Synthetic dipalmityl phosphatidyl ethanolamine (PE) was reacted with malonaldehyde (MA) prepared by the method of Kwon and Watts (19). MA (75 μmoles) were combined with 150 μmoles of PE in 25 ml chloroform-methanol 4:1. PE and arachidonic acid were similarly reacted, except that the mixture was irradiated with UV light (maximum emission 360 nm) for 20 min at 8 hr intervals to induce free radical formation from arachidonate and to initiate peroxidation. The mixtures were allowed to react with stirring at room temperature for 72 hr. Single reactant blanks of PE, MA, and arachidonic acid were similarly treated. The fluorescence excitation and emission spectra of the products of each reaction system were recorded.

Preparation of Tissue Extracts

Fluorescent products of rat testicular tissue were extracted as described by Fletcher, et al. (11). The testes of five rats were combined for each extraction. The fluorescence spectra of the extracts were recorded, and each extract was dried in vacuo.

Fractionation of Fluorescent Products

The products of the synthetic phospholipid reaction systems were chromatographed on a silicic acid column (2.3 x 45 cm) with a stepwise gradient of chloroform with increasing concentrations of methanol. The lipid extracts of rat testes were similarly fractionated on a silicic acid column (1 x 8 cm). The contents of tubes that composed distinct fluorescent fractions were combined and dried in vacuo.

The fluorescent components recovered from silicic acid column chromatography were applied to Silica Gel G TLC plates for further fractionation. Solvents used for development were: cyclohexane-chloroform-methanol, 70:30:3; chloroform-methanol-acetic acid-water, 80:20:1:1; and chloroform-methanol-acetic acid-water, 60:40:2:1.

Analytical Methods

Excitation and emission spectra of samples dissolved in chloroform-methanol, 2:1, were measured with an Aminco-Bowman spectrophotofluorometer linked to a ratio photometer (American Instrument Co., Silver Spring, Md.) and were recorded on an X-Y recorder (model

2000, Houston Instrument Co., Bellaire, Tex.). A slit arrangement of 3, 1, and 3 mm was used for the 3, 4, and 6 slit positions, respectively. The quinine sulfate fluorescence standard (1 μg/ml [1.28 x 10^{-6} M] in 0.1 N H_2SO_4) had a fluorescence intensity of 50 with the sensitivity multiplier set at 10, and the sensitivity vernier set at 40.

The effects of alkaline pH and metal chelation on the fluorescence of components recovered from silicic acid chromatography were measured as described previously (17). Absorption by the chelating agent, $Eu(thd)_3$, between 200-300 nm prevented the observation of the quenching of fluorescence when fluorophores were excited with radiation in this region.

Compounds isolated on TLC were detected first by fluorescence under UV light with a maximum emission of 365 nm, then with iodine vapor, and finally with molybdenum blue phospholipid detection reagent.

RESULTS

The fluorescence characteristics of the three amino acid conjugated Schiff base flourescence standards, Leu_2MA, Val_2MA, and Gly_2MA, in chloroform-methanol, 2:1, were similar (Table I). Each showed two excitation maxima in the near UV region with a single emission maximum in the blue region (Fig. 1). Adjustment of the solutions to pH 10-11 by the addition of base caused a significant decrease in fluorescence intensity, which was partially reversible by reneutralization with acetic acid. This effect was noted when the standards were excited at either of the two excitation maxima. Coordination with the heavy metal compound $Eu(thd)_3$ significantly decreased fluorescence when the standards were excited at their higher wave length maxima. The fluorescence intensity of the Schiff base standards in chloroform-methanol was only 51-58% of that observed in aqueous solution.

The products of the synthetic reaction systems that contained PE and either MA or arachidonic acid were fluorescent with excitation maxima at 260 mn and 365 nm and an emission maximum at 430 nm. These products were separated by silicic acid column chromatography into four major fluorescent component fractions (Fig. 2). The spectral characteristics of each fraction conformed to those noted for the conjugated Schiff base fluorescence standards, including excitation and emission maxima and the effects of alkaline pH and $Eu(thd)_3$ coordination upon fluorescence intensity (Table II). Fraction 4 from each synthetic system was eluted with chloroform-methanol,

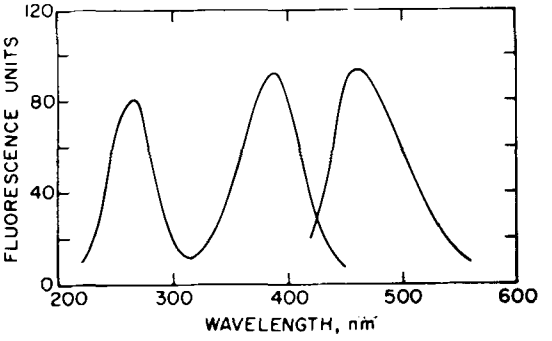


FIG. 1. Corrected fluorescence excitation and emission spectra of the conjugated Schiff base standard, Gly_2MA, in chloroform-methanol, 2:1. Excitation maxima are recorded at 265 nm and at 390 nm with the emission maximum at 470 nm.

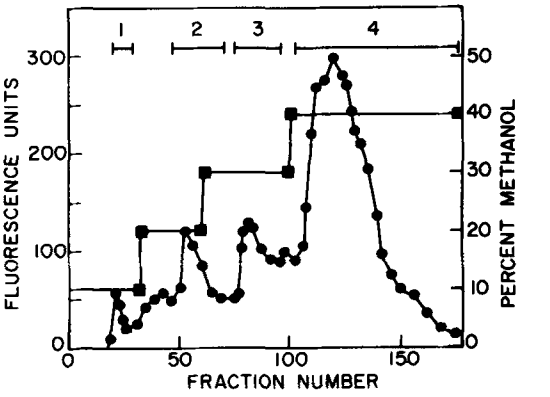


FIG. 2. Elution pattern of fluorescent products of phosphatidyl ethanolamine and malonaldehyde fractionated on a silicic acid column by chloroform solutions with increasing concentrations of methanol (■). The fluorescence intensity (●) was measured with the excitation monochrometer set at the fraction's higher wave length excitation maximum, and the emission monochrometer was set at the fraction's emission maximum. The bars beneath the component numbers indicate the fractions pooled.

60:40, and contained ca. two-thirds of the fluorescence units recovered from column chromatography.

Figure 3 is representative of the TLC of the four column fractions of the PE/arachidonic acid synthetic reaction system. Chromatograms of the corresponding fractions of the PE/MA reaction system were qualitatively identical. Fraction 4 contained four chromatographically discernible fluorescent species, two of which contained phosphate. Among the other three component fractions there were three phosphate-containing fluorescent species. The faintly fluorescent spots observed in the MA and arachidonic acid reactant blanks and the corres-

TABLE II

Fluorescence Characteristics of the Products of Synthetic Phospholipid Reaction Systems

Reaction system and column fraction numbers	Percentage recovered fluorescence	Excitation maxima, nm		Emission maximum, nm	Percentage initial fluorescence				
					Excitation 270 nm region		Excitation 370 nm region		
					base[a]	acid[b]	base[a]	acid[b]	chelator[c]
Malonaldehyde									
1	2	265	340	415	63	82	67	89	58
2	11	260	385	450	75	96	79	96	67
3	13	260	385	450	80	95	89	97	72
4	64	260	365	430	60	79	56	76	20
Arachidonic acid									
1	5	265	355	435	71	95	74	92	86
2	10	260	370	435	76	84	78	90	60
3	14	255	370	435	65	92	66	85	78
4	60	255	365	430	72	93	83	95	44

[a]10 μliter 4 N sodium methoxide were added to 2 ml chloroform-methanol, 2:1, solution.

[b]Reneutralized by addition of 10 μliter 4 N acetic acid in methanol.

[c]Final concentration of $Eu(thd)_3$ was 5×10^{-5} M.

TABLE III

Fluorescence Yield of Rat Testes Extracts and Recovery of Fluorescence from Silicic Acid Column Chromatography

Sample number	Tissue wet wt, g	Fluorescence yield[a]	Column recovery,[b] %
I	20.5	2.7	85
II	18.9	1.4	79
III	19.1	1.8	78

[a]Nanoequivalents of Gly_2MA/g tissue.

[b]Silicic acid column, 1 x 8 cm.

ponding spot in fraction 1 contained polymerized malonaldehyde, which had yellow fluorescence. All other fluorescent species were blue to violet under UV irradiation. The PE reactant blank showed two faint phosphate-bearing spots in addition to the predominant PE spot at R_f 0.60. These were not detected earlier during TLC examination for purity, and they may represent small amounts of methanolysis products of the PE.

Table III shows the fluorescence yield from the rat testes extracts. The mean yield of three extractions was 2.0 molar nanoequivalents of Gly_2MA/g wet tissue. The gross extracts were fluorescent with excitation maxima at 260-270 nm and 345-350 nm and with one emission maximum at 415-430 nm.

Silicic acid column fractionation of the extracts produced three distinct fluorescent fractions. Fraction 1 was eluted by 100% chloroform, fraction 2 by chloroform-methanol, 90:10, and fraction 3 by chloroform-methanol, 80:20. Table IV shows the fluorescence characteristics of each fraction. Fractions 2 and 3 each showed the fluorescence characteristics of the standard conjugated Schiff base fluorescent compounds. Fraction 1 had a single excitation maximum at 305-310 nm with emission at 375-400 nm. Furthermore, the mean reduction in fluorescence intensity caused by alkaline pH was significantly less pronounced for fraction 1 than for fraction 2 or 3 ($p<0.0025$).

Figure 4 shows the distribution of column chromatographed lipid-soluble compounds of rat testes obtained by TLC. The less polar solvent A was used for fraction 1, which contained a single yellow fluorescent spot. Several fluorescent species were observed in fractions 2 and 3, and all but one contained phosphate. The spots that corresponded with the PE standard were only faintly fluorescent, while the intensity of blue color produced by the molybdenum blue reagent spray indicated large amounts of phospholipid. This work is detailed more thoroughly elsewhere (20).

DISCUSSION

The characteristic fluorescence of conjugated Schiff base fluorophores at 430-490 nm with excitation at 350-390 nm is a highly sensitive means of detecting these lipid peroxidation products. It is well known that the fluorescence of conjugated Schiff bases when excited at 350-390 nm is decreased by alkaline pH or by heavy metal chelation. Observation of a second excitation maximum at 260-280 nm for each of the three fluorescent standards, Leu_2MA, Val_2MA, and Gly_2MA, and the quenching effect of alkaline pH upon fluorescence excited in this region provide additional criteria to test for

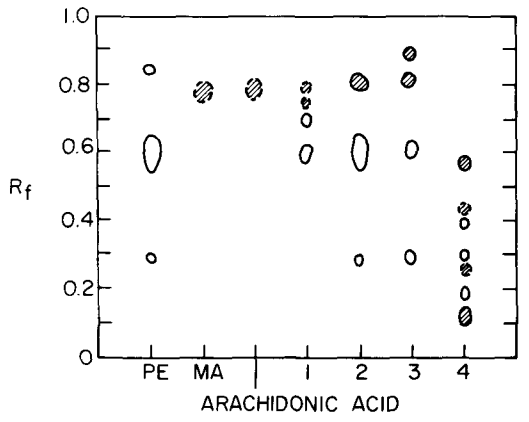


FIG. 3. Thin layer chromatogram of the reaction products of phosphatidyl ethanolamine (PE) and arachidonic acid. Silicic acid column fractions 1, 2, 3, and 4 are shown along with the reactant blanks PE, malonaldehyde, and arachidonic acid. Hatched spots were detected by fluorescence; solid circles were detected with molybdenum blue reagent; and dashed circles were detected with iodine vapors but not with molybdenum blue. Used for development was chloroform-methanol-acetic acid-water, 60:40:2:1.

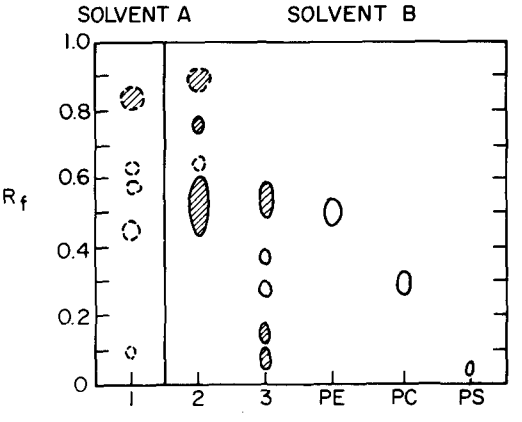


FIG. 4. Thin layer chromatogram of the lipid-soluble components of rat testes. Silicic acid column fractions 1, 2, and 3 are shown along with the phospholipid standards phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), and phosphatidyl serine (PS). Spots are designated as in Figure 3. Solvent A consisted of cyclohexane-chloroform-methanol, 70:30:3. Solvent B consisted of chloroform-methanol-acetic acid-water, 80:20:1:1.

the presence of conjugated Schiff base compounds among the fluorescent products of lipid peroxidation.

The electronic transition induced by excitation at 260-280 nm is from the singlet ground state S_0 to the second excited singlet state S_2^*. The emission observed upon decay of this excited state is that of the S_1^* to S_0 transition also characteristic of the decay of the excited state produced by excitation at 350-390 nm. The lowered fluorescence intensity of the standard compounds in chloroform-methanol solution is probably the effect of quenching by the heavy atom solvent component, chloroform, which facilitates electron spin reversal to form the excited triplet state T_1^*, which, in solution, decays nonradiatively (21).

The products of the synthetic lipid peroxidation systems contained a large family of compounds with fluorescence characteristics of conjugated Schiff base fluorophores. The single fluorescent species in greatest concentration among the products appeared to be that of R_f 0.12 found in fraction 4 recovered from silicic acid column chromatography. This species was the only fluorescent spot on TLC, other than those corresponding with polymerized MA, that

TABLE IV

Fluorescence Characteristics of Lipid-Soluble Components Extracted from Rat Testes

Sample and column fraction numbers	Percentage recovered fluorescence	Excitation maxima, nm		Emission maximum, nm	Percentage initial fluorescence				
					Excitation 270 nm region		Excitation 350 nm region		
					base[a]	acid[b]	base[a]	acid[b]	chelator[c]
I-1	15	310		400	88	93			
II-1	40	305		395	88	99			
III-1	30	305		375	88	98			
I-2	55	265	350	425	66	86	73	100	76
II-2	41	260	350	425	65	92	68	95	82
III-2	30	270	360	435	73	91	30	80	79
I-3	30	265	345	420	70	90	64	90	78
II-3	19	270	350	430	60	90	50	88	80
III-3	40	270	355	435	65	87	40	76	76

[a] 10 μliter 4 N sodium methoxide were added to 2 ml chloroform-methanol solution.

[b] Reneutralized by addition of 10 μliter 4 N acetic acid in methanol.

[c] Final concentration of $Eu(thd)_3$ was 5×10^{-5} M.

was detectable with the less sensitive means of iodine vapor. This spot, containing phosphate and fluorescing an intense turquoise blue, was probably the crosslinked PE conjugated Schiff base compound sought in this investigation.

Although the composition of the products of these simple synthetic reaction systems was complex, it was relatively constant, regardless of whether MA was added directly to the system or generated in situ via lipid peroxidation. Furthermore, virtually all of the fluorescent species produced showed specific fluorescence characteristics of the conjugated Schiff base structure.

The mean level of fluorescence extracted from the testes of middle-aged rats was comparable to that of the fluorescence extracted from mice of the same age (22). Column fractionation showed that 15-40% of the recovered fluorescence, that contained within fraction 1, could not be attributed to the Schiff base fluorophore. The coincidence of most of the lipid-soluble Schiff base-like fluorescence in rat testes with detectable phosphate supports the thesis of the involvement of phospholipid with in vivo lipid peroxidation. The testicular fluorescence corresponding to the PE standard was very faint considering the large amount of phosphate detected, and it tended to be concentrated in the forward portion of the spot. This fluorescence was probably due to lower R_f species displaced by PE during development (23).

TLC fractionation of the rat testis fluorescent products with more polar developing solvent revealed no discernible correlation with products of the synthetic peroxidation systems.

The column and TLC methods employed were of great value in extending the fluorescence analysis of lipid peroxidation products to the level of the individual fluorescent compounds. Silicic acid column chromatography proved to be a valuable preparative step for fluorescence analysis, although frequent and considerable overlap of fluorescent species between fractions showed this method to be inconcise in its fractionation. Silica gel TLC was useful for separation and identification of individual fluorescent compounds, but preparative TLC for the analysis of their fluorescence characteristics was hampered by solubilized silica, which amplified solvent Raman scatter and overshadowed measurable fluorescence.

Definitive analysis of purified fluorescent products of lipid peroxidation cannot be accomplished by means of fluorescence characterization alone. Preparative TLC of the lipid-soluble fluorescent components for IR spectrometric or gas chromatographic-mass spectrometric analysis could provide structural definition of the major fluorescent species resulting from lipid peroxidative damage. These methods previously have been used in the analysis of various model conjugated Schiff base compounds (14,24).

The accumulation of fluorescent lipofuscin pigments with age has been observed in many different mammalian tissues (25). Hendley, et al., (26) performed column and thin layer fractionations of human cardiac age pigment and found a variety of compounds with similar fluorescence emission maxima. Direct qualitative and quantitative comparison of lipid-soluble fluorescent aging products accumulating in different tissues, as well as in different organisms, would add significantly to the knowledge of the specific damaging events of aging and the cell's abilities to deal with them.

The accumulation of lipofuscin pigments is observed in connection with numerous physiological and pathological disorders, including atherosclerosis (27), liver cirrhosis (28), and neuronal lipofuscinosis (Batten's disease) (29). Analysis of the lipid-soluble fluorescent products associated with such metabolic disorders could provide a more thorough understanding of their biochemical and physiological bases.

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Substitution Reactions of Linoleic Acid Hydroperoxide Isomerase[1]

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ABSTRACT

Linoleic acid hydroperoxide isomerase was extracted from corn germ and partially purified by differential centrifugation. This enzyme catalyzed the isomerization of linoleic acid hydroperoxide (R-CHOOH-CH$\underset{trans}{=}$CH-CH$\underset{cis}{=}$CH-R_1) to the expected α-ketol (R-CHOH-CO-CH_2-CH$\underset{cis}{=}$CH-R_1) and γ-ketol (R-CH_2-CO-CH$\underset{trans}{=}$CH-CHOH-R_1). Isomerase also catalyzed the substitution of various reagents at the carbon bearing the hydroperoxide group. These fatty acid products had the following functional groupings: R-CHX-CO-CH_2-CH$\underset{cis}{=}$CH-R_1 where X is either oleoyloxy, ethylthio, or methoxy resulting from the presence of oleic acid, ethanethiol, or methanol, respectively. A crude wheat germ extract containing both lipoxygenase and isomerase enzymes reacted with linoleic acid to yield α-ketols, γ-ketols, and a substitution product, the linoleoyloxy ester of α-ketol. Characterization of these products from wheat germ enzymes showed that the substitution reaction was not unique to corn germ. Because anions of the reagents tested are typical nucleophiles, the substitution reactions may proceed by a nucleophilic mechanism as mediated by the isomerase enzyme.

INTRODUCTION

Zimmerman (1) has shown that flaxseed contains an enzyme that catalyzes the isomerization of linoleic acid hydroperoxides to monounsaturated α-ketols. When ^{18}O-labeled 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid was the substrate for this flaxseed enzyme, only one oxygen atom of the 13-hydroperoxy function transfers to the 12-oxo group, the other is not retained in the product (2). This transfer indicates that a cyclic intermediate, cyclic peroxide, or epiperoxide is involved in the reaction and that the hydroxyl group originates from elsewhere, presumably from a solvent.

Gardner (3) isolated three products from the isomerization of linoleic acid hydroperoxide in the presence of linoleic acid and corn germ isomerase. The product fatty acids were 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid (α-ketol), 13-hydroxy-10-oxo-*trans*-11-octadecenoic acid (γ-ketol), and the linoleoyloxy ester of the α-ketol, 9-(*cis*-9,*cis*-12-octadecadienoyloxy)-10-oxo-*cis*-12-octadecenoic acid (B). He postulated that acylation occurred during formation of B and was catalyzed by another enzyme; however, the involvement of an acylase was not proved. Gardner also showed that B would not form when the α-ketol was incubated with linoleic acid in the presence of the corn germ enzyme preparation, but that formation of B only occurred when linoleic acid hydroperoxide and linoleic acid were incubated with the enzyme.

Upon further examination of the enzyme reaction, we found that other fatty acids and other nucleophiles will substitute for the hydroperoxy group in the presence of corn germ isomerase. We also describe a wheat germ isomerase that in the presence of linoleic acid produces the same products as those obtained with corn germ isomerase.

EXPERIMENTAL PROCEDURES

Materials

(B37TMS X H84) (Oh43RF X A619) hybrid corn, *Zea mays*, came from the University of Illinois, Urbana. After harvest, the corn was dried with ambient air and stored at 0 F. Germ was prepared by a laboratory dry-milling operation. Germ dry milled from a soft red winter wheat, *Triticum aestivum*, was supplied by Mennel Milling Co., Fostoria, Ohio.

Lipoxygenase (EC 1.13.1.13) was obtained from Sigma Chemical Corp., St. Louis, Mo., (activity = 130,000 units/mg). The Hormel Institute, Austin, Minn., supplied linoleic acid (purity>99%). Oleic acid wwas purchased from Applied Science Laboratories, State College, Pa.

Hydroperoxide Isomerase

Isomerase was isolated by stirring hexane-defatted corn germ flour for 15 min in 0.1 M

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[1]Presented at the AOCS Spring Meeting, Dallas, April 1975.

[2]ARS, USDA.

phosphate buffer (1 g germ/10 ml) and by centrifuging at 8000 x g for 20 min to remove cellular debris. Further isomerase purification was achieved by recentrifuging the clarified supernatant at 78,400 x g for 1 hr to collect the particulate matter containing isomerase. The pellet, when resuspended in 0.2 M phosphate buffer at pH 6.9, served as the enzyme source for isomerization and substitution reactions.

Specific activity of the resuspended pellet in 50 ml buffer (equivalent to 4 g defatted corn germ) was 15.6 μmoles/min•mg protein. Isomerase activity was measured by the initial rate of decrease in conjugated diene absorption at 234 nm (3). The isomerase enzyme could be stored at -20 C in buffer for several days without substantial loss of activity. Protein content of the enzyme preparation was determined by the Folin-Wu procedure (4).

In the wheat germ study, linoleic acid was oxidized with an extract prepared from wheat germ. Full fat wheat germ (15 g) was extracted with 150 ml 0.1 M phosphate buffer (pH 6.9) and centrifuged at 10,000 x g for 20 min. The supernatant (100 ml) was mixed directly with 500 μliter linoleic acid (K salt) and 500 μliter Tween in 70 ml water under bubbling oxygen for 50 min. Reaction was stopped by acidification to pH 4 with 1 N HCl. The products were extracted with $CHCl_3$-CH_3OH 2:1 (v/v) and separated by silicic acid column chromatography (3) with an elution gradient similar to that used for product separation obtained from corn germ sequential reaction.

Substitution Reactions

Reactions were conducted with column-isolated hydroperoxide prepared by reaction of oxygen with linoleic acid in the presence of soy lipoxygenase. The method of oxidation, including the concentration of enzyme (units/ml), and the isolation of hydroperoxide were the same as used previously (5). The hydroperoxide was taken up in water in the presence of Tween, the reagent added and an equal volume of isomerase in 0.2 M phosphate buffer mixed for 1 hr.

Oleic acid, ethanethiol, and methanol were the reagents used for the corn germ isomerase reactions. The oleic acid reaction mixture contained 213 mg hydroperoxide (3.6 mM), 0.927 ml Tween, 1.854 g oleic acid (0.034 M) and 150 μmoles/min isomerase activity (equivalent to 6 g defatted germ) in a total volume of 188 ml. The ethanethiol reaction contained 213 mg hydroperoxide (3.4 mM), 0.2 ml Tween, 2 ml ethanethiol (0.14 M) and 150 μmoles/min isomerase activity (equivalent to 6 g defatted germ) in a total volume of 198 ml. Methanol reaction contained 353 mg hydroperoxide (3.7 mM), 0.175 ml Tween, 61.2 ml methanol (20% by volume) and 250 μmoles/min isomerase activity (equivalent to 10 g defatted germ) in a total volume of 301 ml.

The product mixture was acidified to pH 4 with 1 N HCl and extracted with $CHCl_3$-CH_3OH, 2:1 (v/v). The organic phase containing fatty acid products was washed twice with water. The product mix was stored in ether at -20 C.

Chromatography

Fatty acid products from the substitution reactions were separated by silicic acid column chromatography essentially under conditions described previously (3). Compounds were applied to the column in a slurry of 2 g silicic acid in hexane and eluted with a modified step-wise gradient; 350 ml 10% ether, 350 ml 20% ether, 350 ml 30% ether, 200 ml 40% ether, and 300 ml 50% ether in hexane. Fractions were monitored by Silica Gel G thin layer chromatography (TLC) using isooctane-ether-acetic acid, 70:30:1 (v/v/v) solvent system (double development). Products were detected on plates by spraying with 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl or by charring with 50% H_2SO_4. Separated compounds were combined, evaporated to dryness under nitrogen, and stored in ether at -20 C for spectral analysis. For certain spectral analyses, the methyl ester of the products were prepared with diazomethane (6).

Structural Characterization of Products

NMR, IR, and mass spectra (MS) were used to characterize the structures of the products by methods described previously (7), with one exception: the MS of 13-oleoyloxy-12-oxo-*cis*-9-octadecenoic acid (methyl ester) was obtained from a probe sample rather than by gas liquid chromatography (GLC) elution.

RESULTS

Identification of Substitution Products

Corn germ hydroperoxide isomerase action on substrate hydroperoxides in the presence of various reagents gave the usual products (α- and γ-ketols) plus products resulting from substitution of the reagent anion with the hydroperoxy group. One was a fatty acid with the following functional moiety = R-CHX-CO-CH_2-CH=CH-R_1 where X is the anion of the reagent added. Since soybean lipoxygenase was used to produce the hydroperoxide, derivatives would be predominantly from 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic acid (79%). One

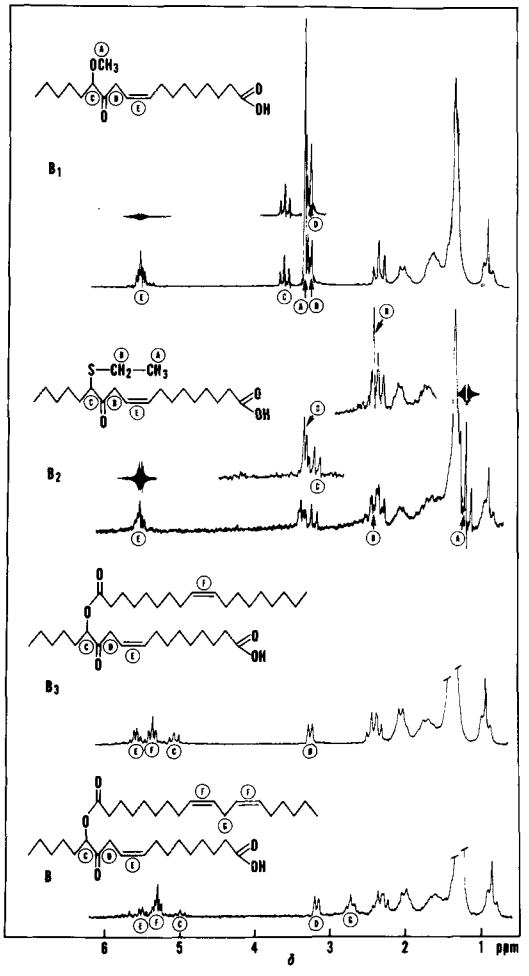

FIG. 1. NMR spectra of B_1, 13-methoxy-12-oxo-*cis*-9-octadecenoic acid; B_2, 13-ethylthio-12-oxo-*cis*-9-octadecenoic acid; B_3, 13-oleoyloxy-12-oxo-*cis*-9-octadecenoic acid; and B, 13-linoleoyloxy-12-oxo-*cis*-9-octadecenoic acid.

major isomer of the substitution product was obtained with each reagent tested. Those substitution compounds derived from the 13-hydroperoxide were identified as: 13-methoxy-12-oxo-*cis*-9-octadecenoic acid (B_1) from reaction with methanol, 13-ethylthio-12-oxo-*cis*-9-octadecenoic acid (B_2) from ethanethiol, 13-oleoyloxy-12-oxo-*cis*-9-octadecenoic acid (B_3) from oleic acid. Since minor amounts of the 9-hydroperoxide (21%) were present in the substrate, the corresponding isomer of B_1, B_2 and B_3 also was observed as detected by characteristic fragment ions in MS, although in some samples the corresponding fragment ions for this isomer were barely detectable. Elution volumes of B_1, B_2, and B_3 upon chromatography by the silicic acid column regime outlined in Experimental Procedures were in ranges of 740-820 ml for B_1 540-560 ml for B_2 and 480-550 ml for B_3. For the purposes of this paper, we need not discuss the minor isomer of these three compounds.

The substitution products were identified by their spectral properties as outlined.

Product B_1: Assignment of resonances in NMR spectrum (Fig. 1) are as follows: the methoxyl protons absorb at δ 3.34 (S, 3H). The doublet at 3.24 δ is the methylene between the double bond and the oxo-group. Double irradiation of the olefinic proton centered at δ 5.54 (m, 2H) decoupled the absorption at 3.24 δ and thus confirmed the position of the olefin as α to the C-11 methylene.

Characteristic IR absorptions (B_1 methyl ester) were 1100 cm^{-1}, methoxy C-O stretch; 1720 cm^{-1}, keto carbonyl; and 1740 cm^{-1}, ester carbonyl.

MS of product B_1 clearly supported the proposed structure. Replicate MS taken over the GLC peak showed *m/e* 115 ion with greatest intensity (CH_3-$[CH_2]_4$-$\overset{H}{\underset{\oplus}{C}}$-$OCH_3$) and *m/e* 115-31. Both M and M - 31 were absent.

Product B_2: Assignments of resonances in the NMR spectrum of product B_2 (Fig. 1) are as follows: methyl protons of ethylthio at δ 1.16 (t, 3H); methylene protons of ethylthio centered around δ 2.38. Irradiation of the absorption at δ 1.16 confirmed the position of the methyl α to the methylene of the ethylthio group. Irradiation of the olefinic protons centered at δ 5.52 (m, 2H) confirmed its position α to the C-11 methylene.

Characteristic IR absorptions (B_2 methyl ester) were 1720 cm^{-1}, keto carbonyl; 1740 cm^{-1}, ester carbonyl; and 730 cm^{-1}, sulfide absorption (8).

MS of product B_2 gave greatest intensity of *m/e* 145 ion (CH_3-$[CH_2]_4$-$\overset{H}{\underset{\oplus}{C}}$-S-$CH_2$-$CH_3$). M (*m/e* 370), M - 31, and M - 61 (ethylthio) were evident.

Another compound, produced along with B_2 in the presence of ethanethiol, was hydroxy-octadecadienoic acid, the reduction product of the linoleic acid hydroperoxide. This compound was eluted from the silicic acid column in a range of 1040-1100 ml using the solvent series described in Experimental Procedures. IR absorptions of its methyl ester were 3620, 950, and 985 cm^{-1}, which is characteristic of a *cis,trans* conjugated dienol. UV absorption at λ_{max} 233 also indicated a conjugated dienol. The R_f of this compound (methyl ester) by TLC was less than the R_f of the methyl ester of linoleic acid

TABLE I

Yields[a] of Fatty Acids Produced from Linoleic Acid Hydroperoxide in Presence of Isomerase and Various Reagents

Product[b]	Reagent			
	Methanol wt, mg	Ethanethiol wt, mg	Oleic acid wt, mg	Linoleic acid[c] wt, mg
Substitution compounds	66.7	14.5	96.0	24.1
α-Ketol	53.0	19.2	31.0	72.7
γ-Ketol	14.7	--[d]	16.6	--[d]
Excess hydroperoxide	45.9	Trace	68.4	39.9
Unknowns	14.2	8.5	12.8	16.0
Hydroxyoctadecadienoic	--[d]	48.6	--[d]	--[d]

[a]Wt of combined peak fractions as eluted from a silica acid column. Total amount of hydroperoxide used in the reactions was 353 mg for the methanol, 213 mg for ethanethiol and oleic acid. Total amount of linoleic acid used for the sequential wheat germ reaction was 452 mg.

[b]Substitution compounds: 13-X-12-oxo-*cis*-9-octadecenoic acid where X is, methoxy, ethylthio, oleoyloxy, or linoleoyloxy, according to the respective reagent used as listed in the box head: α-ketol: 13-hydroxy-12-oxo-*cis*-9-octadecenoic acid; and γ-ketol: 9-hydroxy-12-oxo-*trans*-10-octadecenoic acid.

[c]Linoleic acid enzyme reaction obtained with a sequential wheat germ reaction.

[d]Not determined.

hydroperoxide, and, since the compound did not react with a ferrous thiocyanate spray, it was not a hydroperoxide. The compound had an identical R_f to an authentic standard, which was synthesized from linoleic hydroperoxide by sodium borohydride reduction and esterification.

Product B_3: NMR spectrum of the oleoyloxy ester (B_3) of the α-ketol (Fig. 1) compares closely with the NMR spectrum of the linoleoyloxy ester (B) of the α-ketol from the wheat germ sequential enzyme reaction. The NMR spectrum of B was identical with the spectrum of the linoleoyloxy ester of the α-ketol produced from corn germ sequential enzyme reaction previously (3). One difference in the B_3 spectrum compared to that of B is the lack of absorption due to the methylene between the double bonds of the linoleoyloxy ester at δ 2.73 (m, 2H). The other difference in the B_3 spectrum is that the olefin absorption of B_3 at 5.34 δ integrates for 2H instead of 4H for B.

The IR spectrum of the free acid showed clearly that the ester carbonyl absorbed at 1730 cm^{-1} and no hydroxyl absorbed at 1070 cm^{-1} (secondary hydroxyl) or at 3460 cm^{-1}.

The most intense ion in the MS was at *m/e* 265 ($CH_3[CH_2]_7CH{=}CH{-}[CH_2]_7{-}C{\equiv}O^{\oplus}$). Less intense ions were observed at *m/e* 309 ($CH_3{-}[CH_2]_4{-}\overset{\oplus}{C}(H){-}C(H)(O){-}CH_2{-}C(H){=}C(H){-}[CH_2]_7{-}C{\equiv}O$ OCH_3) and *m/e* 309 + 16 (O at C-13). Also, M (*m/e* 590 ion) and M - 31 were detected.

Reaction Conditions

Substitution reactions were first conducted on a small scale to determine concentrations necessary for maximum yields of B_1, B_2 and B_3. A concentration series of methanol (10, 20, and 30% by volume), ethanethiol (0.01-0.225 M), and oleic acid (0.002-0.034 M) was mixed with linoleic acid hydroperoxide prepared from soy lipoxygenase and incubated 30 min with the isomerase enzyme. Yields of products were assessed visually after TLC separation and charring the plates. All three reagents can substitute in the formation of R-CHX-CO-CH_2-CH=$\underset{cis}{=}$CH-R_1. Respective R_f values of B_1, B_2, and B_3 were 0.47, 0.56, and 0.57. Optimum yields of either B_1, B_2, or B_3 appeared to be either with 20% methanol, 0.034 M oleic acid, or 0.14 M ethanethiol in the reaction mixture. Ethanethiol at concentrations higher than 0.14 M shifts products to other compounds presumably because of non-enzymic reactions.

The TLC method used in this study could separate the two isomers of the α-ketol, 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid (R_f = 0.25), and 13-hydroxy-12-oxo-*cis*-9-octadecanoic acid (R_f = 0.29).

Yields

The enzyme reactions with methanol, ethanethiol, and oleic acid were scaled-up to separate the substitution products, α-ketol and γ-ketol, by column chromatography. Yields of the separated products after column chroma-

TABLE II

Centrifugation of Linoleic Acid Hydroperoxide Isomerase Activity from Corn Germ Extracts

Fraction	Percent of total activity	Specific activity μmole/min·mg protein
Whole germ extract[a]	100	2.1
Supernatant	20	0.68
Pellet[b]	56	8.2
Liposome	29	--
Defatted germ extract[a]	100	2.0
$(NH_4)_2SO_4$ pellet[c]	30	8.1
Straight pellet	35	15.6

[a]Cellular debris removed by preliminary centrifugation at 8000 x g for 10 min.

[b]Centrifuged at 159,000 x g for 1 hr. The pellet is not washed.

[c]A separate portion of the defatted germ extract was made to 40% $(NH_4)_2SO_4$ and centrifuged at 78,400 x g for 1 hr to obtain the $(NH_4)_2SO_4$ pellet.

TABLE III

Mole Ratios of Substitution Compounds (B_n) vs α-Ketol (A) Produced by Isomerase and Effect of Concentration of Reagent

Reagent	Product	Molarity of reagent	Molarity of water	Mole ratio, B_n/A	B_n/C_n: $A/C^\circ_{H_2O}$[a]
Methanol	B_1	5.0	45	1.2	11
Ethanethiol	B_2	0.14	55	0.66	260
Oleic acid	B_3	0.034	54	1.7	2658

[a]Initial molar concentration of reagent, C°_n and of H_2O, $C^\circ_{H_2O}$.

tography are shown in Table I. (See Experimental Procedures for details of reaction.)

Yields of the substitution product with ethanethiol were lower because reduction of the hydroperoxide to the hydroxyl group apparently become a competing reaction. As expected in experiments where isomerase activity was greater, the reduction product was diminished greatly.

Purification of Corn Germ Isomerase

Zimmerman, et al., (9) has shown that isomerase is associated with mitochondria (cytochrome C oxidase as marker) in sunflower cotyledons and seeds. A partial purification of isomerase from crude corn germ extracts could be achieved by differential centrifugation (Table II). The "straight pellet" from defatted corn germ served as the hydroperoxide isomerase source.

Substitution Reaction of Wheat Germ Isomerase

Graveland (10) identified products in a wheat flour-water suspension that were presumed to be produced by lipoxygenase and linoleic acid hydroperoxide isomerase on linoleic acid. Graveland did not observe any substitution products in the wheat extracts like those observed in corn germ extracts by Gardner (3). Possibly wheat germ isomerase activity is reduced in flour suspensions. We noticed that when linoleic acid was added to a crude wheat germ extract and flushed with oxygen, the three products, B, α-ketol, and γ-ketol, were synthesized. In this reaction, the sequence of events (3) was previously shown to be: (A.) Linoleic acid is oxidized by lipoxygenase to linoleic acid hydroperoxide, (B.) linoleic acid hydroperoxide is acted upon by isomerase to form α-ketol and in the presence of residual linoleic acid the linoleoyloxy ester (B) of the α-ketol is formed, and (C.) linoleic acid hydroperoxide is acted upon by isomerase to form the γ-ketol. Figure 1 shows an NMR spectrum for B identical in properties with that of the B reported by Gardner (3).

DISCUSSION

We demonstrated that with 13-hydroperoxy-octadecadienoic acid as substrate, linoleic acid hydroperoxide isomerase catalyzed formation of substitution products from methanol, ethanethiol, or oleic acid which substituted at carbon-13 as the methoxy, ethylthio, or oleoyloxy group, respectively. Since substitution products are formed with each of the reagents, acylation is not catalyzed by an acylase enzyme

as postulated previously (3). Rather, formation of substitution products is an inherent part of the isomerase catalysis. The nature of the substitution is reminiscent of a nucleophilic reaction; each reagent being a typical nucleophile. In the absence of these reagents, the solvent, water, is the only nucleophile readily available and, thus, the usual α- and γ-ketols are the only products observed. Previously, Veldink, et al., (2) showed that the 13-hydroxyl of the α-ketol is not derived from the 13-hydroperoxy group, and, presumably, was derived from the solvent. In this study we show that other reagents, all of which are known nucleophiles, substitute even more readily than water.

The mole ratio of the substitution products to α-ketol found in each product mixture (Table III) could be considered to be a measure of the competition of the solvent, water, with the reagent. According to these ratios and molar concentrations of the reagents, it is possible to list, at least in a semiquantitative manner, an order of decreasing reactivity as follows: oleic acid>ethane thiol>methanol>water. Since reaction rates are concentration dependent and each of the nucleophiles tested was in considerable excess (approached C° at completion of the reaction), the ability of each reagent to substitute in preference to H_2O can be emphasized by including the effect of concentration in the mole ratio calculation as follows: $B_n/C_n^{\circ}:A/C_{H_2O}^{\circ}$, where B_n is moles of the substitution compound produced; C_n° is the initial molar concentration of the reagent; A is moles of α-ketol; and $C_{H_2O}^{\circ}$ is the initial molar concentration of H_2O (Table III). As can be seen from these calculated values in Table III, the order of reactivity is accentuated. In other experiments when oleic acid, ethanethiol and methanol each was reacted in equimolar concentrations (0.034 M), the mole ratio of B_2 to A was even lower (0.30) for the ethanethiol reaction than given in Table III; no B_1 product could be detected with the methanol reaction. In this manner, the relative reactivity of the three reagents was confirmed.

All the isomerase reactions occurred largely without problems, except those catalyzed in the presence of ethanethiol. Use of ethanethiol leads to significant (presumably nonenzymatic) reduction of linoleic acid hydroperoxide to the corresponding hydroxyoctadecadienoic acid, but since this side reaction is parallel to the substitution reactions of interest and the reagent is present in considerable excess, the desired comparison of the reactivity of the various nucleophiles in the course of the isomerase reaction is not compromised seriously.

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24-Methylenelanost-9(11)-en-3β-ol, New Triterpene Alcohol from Shea Butter

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ABSTRACT

A new triterpene alcohol was isolated from shea butter and its structure was shown to be 24-methylenelanost-9(11)-en-3β-ol. Gas chromatographic correlations between this triterpene alcohol and other related compounds are discussed.

INTRODUCTION

The presence of lanosta-9(11),24-dien-3β-ol (parkeol, Fig. 1, II; R_1 = OH, R_2 = iii), a biogenetically interesting triterpene alcohol, in shea butter from the kernels of *Butyrospermum parkii (Sapotaceae)* has already been known (1,2). α-Amyrin (2), β-amyrin (2,3), lupeol (2,3) and butyrospermol (2,4,5) also have been isolated from shea butter. We report here the isolation and structure of a new $\Delta^{9(11)}$-triterpene alcohol (II; R_1 = OH, R_2 = iv) from shea butter. Gas chromatographic correlations between this and related triterpene alcohols also are discussed.

EXPERIMENTAL PROCEDURES

Melting points were determined with a Micro mp apparatus (Yanagimoto Seisakusho Ltd., Kyoto, Japan) and uncorrected. All recrystallizations were performed in acetone-methanol. IR spectra (KBr) were obtained with a Type IRA-2, IR spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan). Optical rotations were measured in $CHCl_3$ using an Automatic Polarimeter, Model MP-1T (Applied Electric Lab. Ltd., Tokyo, Japan) or a Carl Zeiss Circle Polarimeter 0.01° (Carl Zeiss, Oberkochen, Germany). Concentrations used are indicated in parentheses as g/100 ml. NMR spectra in $CDCl_3$ were recorded on a JNM-C-60-HL (60 MHz, Japan Electron Optics Laboratory Co., Tokyo, Japan) and calibrated against internal tetramethylsilane as 0 ppm. Mass spectra were recorded on a Hitachi RMU-7M mass spectrometer (Hitachi Ltd., Tokyo, Japan) by probe injection, or on a Shimadzu LKB-9000 gas chromatograph-mass spectrometer (Shimadzu Seisakusho Ltd., Kyoto, Japan).

Preparative argentation thin layer chromatography (TLC) for the fractionation of triterpene acetates was carried out on the plates of Wakogel B-10 (Wako Pure Chemical Industries Ltd., Osaka, Japan) impregnated with 10% silver nitrate, using a Toyo continuous flow development preparative TLC (Toyo Roshi Kaisha Ltd., Tokyo, Japan). Separated zones were observed under UV light (3600 A) after spraying a rhodamine-6G solution in ethanol on the developed plate, and were cut off and quantitatively extracted with ether. The alumina (about 300 mesh) for column chromatography was purchased from Wako Pure Chemical Industries Ltd. Gas liquid chromatography (GLC) was performed on a Shimadzu GC-5A gas chromatograph (Shimadzu Seisakusho Ltd.) equipped with a flame ionization detector and a 2 m x 3 mm internal diameter (ID) glass column packed with 3% OV-17 on Gas Chrom-Z, 80-100 mesh. The column was operated generally at 255 C with nitrogen at 50 ml/min as carrier gas. Detector temperature was 280 C. Relative retention time (RRT) was expressed by the ratio of the retention time for the substance under examination to the retention time (30 min) for β-sitosterol. Hydrolysis of triterpene acetates was performed by refluxing for 2 hr with an alcoholic 0.5 N potassium hydroxide, and then the reaction mixture was diluted with an excess of water and extracted several times with ether. The ether extract was washed 3 times with water and dried over anhydrous sodium sulfate. The solvent was removed on a rotary evaporator.

Isolation of 24-Methylenelanost-9(11)-en-3β-ol from Shea Butter: Unsaponifiable material (69.6 g) was separated from shea butter (1.4 kg) in the same manner as described previously (2). It was treated with acetone and the acetone soluble portion (55 g) was acetylated by refluxing for 3 hr with acetic anhydride (300 ml). The reaction mixture then was poured onto 1.5 liter of ice cooled water. The brown solid mass obtained (50 g) was fractionally crystallized from acetone-methanol to remove the bulk of the less soluble acetates of α-amyrin, lupeol and butyrospermol. The final filtrate contained a viscous oily material (13.8 g). A portion (6 g) of this material was subjected to preparative argentation TLC (hexane:benzene [7:3]) for 60 min to give three principal zones. The zone closest to the starting line (1109 mg) was further fractionated by TLC (hexane:benzene [3:2]) for 70 min to give eventually a fraction (52 mg) which con-

sisted of 24-methylenelanost-9(11)-en-3β-yl acetate (RRT = 1.62; GLC purity = 95%).

24-Methylenelanost-9(11)-en-3β-yl acetate (II; R_1 = OAc, R_2 = iv), its free alcohol (II, R_1 = OH, R_2 = iv), and its 3-keto derivative (II; R_1 = O, R_2 = iv): The acetate crystallized as plates. The mp was 159.5-160.5 C, $[\alpha]_D^{30}$ + 78° (c, 1.00); the IR spectrum was 1723, 1642, 1390, 1372, 1250, 890, 813, 800, and 792 cm^{-1}; and the NMR spectrum was 0.65, 0.75, 0.87, 0.89, 0.98, 1.09, 2.05, 4.54, 4.69, and 5.21 ppm. Hydrolysis of the acetate gave free alcohol with RRT = 1.35; mp = 178.5-179.5 C (fine needles); $[\alpha]_D^{20}$ +60° (c, 0.92); NMR = 0.66, 0.75, 0.83, 0.99, 1.06, 1.08, 3.28, 4.70, and 5.25 ppm; and mass spectrum (MS) = m/e 440.4021 (molecular ion [M^+]), 425.3802, 407.3712, 397.3449, 356.3078, 341.2820, 323.2727, 313.2538, 273.2249, 259.2057, and 255.2114. The new alcohol was oxidized with CrO_3 as follows (6). A solution of 24-methylenelanost-9(11)-enol (50 mg) in pyridine (1 ml) was added to CrO_3 (55 mg) in pyridine (1 ml), and the mixture was allowed to stand at room temperature for 16 hr. Water (30 ml) was added and the product was extracted with ether. The ether extract was worked up in the usual way. The final product obtained was dissolved in hexane and chromatographed on alumina (6 g). The fractions eluted with 50 ml hexane:benzene (1:1) gave 24-methylenelanost-9(11)-en-3-one (29 mg) with RRT = 1.27; mp = 142-143 C (needles); MS = m/e 438 (M^+), 395, 354, 339, 311, 271, and 257; IR = 1703, 1382, 1373, 895, 886, 810, and 790 cm^{-1}; and NMR = 0.70, 0.77, 0.99, 1.09, 1.25, 4.70, and 5.30 ppm.

24-Methyllanost-9(11)-en-3β-yl acetate (II; R_1 = OAc, R_2 = ii) and its free alcohol (II, R_1 = OH, R_2 = ii): A solution of 24-methylenelanost-9(11)-enyl acetate (50 mg) in 20 ml ether was hydrogenated in the presence of platinum oxide (50 ml) for 3 hr at room temperature. The catalyst then was removed by filtration, and 24-methyllanost-9(11)-enyl acetate (RRT = 1.57) was almost quantitatively recovered (46 mg) with ether. The acetate crystallized as fine needles with a mp = 171-172.5 C; $[\alpha]_D^{20}$ +87° (c, 0.74); IR = 1720, 1388, 1373, 1260, 811, and 790 cm^{-1}; and NMR = 0.66, 0.76, 0.82, 0.85, 0.88, 0.90, 0.91, 1.11, 2.06, 4.51, and 5.21 ppm. Hydrolysis of the acetate gave free alcohol (RRT = 1.31) with a mp = 188.5-189 C (fine needles); $[\alpha]_D^{22}$ +62° (c, 0.34); MS = m/e 442.4178 (M^+), 427.3927, 409.3865, and 273.2263; and NMR = 0.66, 0.75, 0.82, 0.91, 1.00, 1.05, 3.25 and 5.24 ppm.

Synthesis of 24-Methyllanost-9(11)-en-3β-ol from 24-Methylcycloartanol by HCl Isomerization

Isolation of 24-methylenecycloartanyl acetate from the acetylated unsaponifiables of rice bran oil: Repeated recrystallization (11) of the acetylated unsaponifiables (37 g) of rice bran oil from acetone-methanol gave a triterpene acetate fraction (4.3 g) containing the acetates of cycloartenol (RRT = 1.52, ca. 32%), 24-methylenecycloartanol (RRT = 1.69, ca. 68%) and a trace amount of cycloartanol (RRT = 1.26). Preparative argentation TLC (hexane:benzene [7:3]) of the acetate fraction (3 g) for 40 min afforded three fractions, 1-3. Fraction 1 (58 mg) from the zone closest to the solvent front was rich in cycloartanyl acetate. Fraction 2 (868 mg) from the medium zone was cycloartenyl acetate. Fraction 3 (2010 mg) from the zone closest to the starting line gave 24-methylenecycloartanyl acetate.

24-Methylenecycloartanyl acetate (I; R_1 = OAc, R_2 = iv) and its free alcohol (I; R_1 = OH, R_2 = iv): 24-Methylenecycloartanyl acetate (RRT = 1.69, 96% pure by GLC) crystallized as needles with mp = 116-117 C; $[\alpha]_D^{30}$ +52° (c, 1.00). Hydrolysis of the acetate gave 24-methylenecycloartanol (RRT = 1.37) with mp = 121-122 C (plates); IR = 3400, 3080, 1640, 1375, 1020, and 885 cm^{-1}; and MS = m/e 440 (M^+), 425, 422, 407, 378, 353, and 300.

24-Methylcycloartanyl acetate and its isomerization: A solution of 24-methylenecycloartanyl acetate (1500 mg) in ether (50 ml) was hydrogenated in the presence of platinum oxide (110 mg) for 4 hr at room temperature. After removal of the catalyst, 24-methylcycloartanyl acetate (1480 mg, RRT = 1.64) was recovered from the ether solution. It showed, on recrystallization, an mp = 125-126 C (long needles, >99% GLC purity); $[\alpha]_D^{30}$ +53° (c, 1.25); and IR = 1730, 1370, 1248, and 1022 cm^{-1}. Hydrolysis of the acetate gave free alcohol (RRT = 1.33) with a mp = 138-139 C (fine needles). A solution of 24-methylcycloartanyl acetate (1073 mg) in $CHCl_3$ (30 ml) was treated with a stream of dry HCl at -30 C for 4 hr. The $CHCl_3$ was evaporated and the residue was taken up in ether. The ether solution was washed first with sodium carbonate aqueous solution and then with water, after which the ether layer was dried over anhydrous sodium sulfate and the ether was evaporated. Preparative argentation TLC (hexane:benzene [7:3]) of the isomerized product (1045 mg) for 50 min gave three fractions, 1-3.

24-Methyllanost-9(11)-en-3β-yl acetate (II; R_1 = OAc, R_2 = ii), its free alcohol (II; R_1 = OH, R_2 = ii), and its 3-keto derivative (II; R_1 =

O, R_2 = ii): Fraction 3 (545 mg) from the zone closest to the starting line gave 24-methyllanost-9(11)-enyl acetate (RRT = 1.57) with mp = 169.5-171.5 C (fine needles); $[\alpha]_D^{30}$ +89° (c, 1.00); IR = 1724, 1390, 1372, 1260, 812, and 791 cm^{-1}; and NMR = 0.66, 0.76, 0.82, 0.85, 0.89, 0.91, 1.10, 2.06, 4.50, and 5.22 ppm. The acetate (200 mg) was hydrolyzed to give free alcohol (RRT = 1.31, 174 mg) with mp = 187-188.5 C (fine needles) and MS = m/e 442.4209 (M^+), 427.3935, 409.3832, 259,2036, and 255.2092. A solution of 24-methyllanost-9(11)-enol (97 mg) in pyridine (2 ml) was added to CrO_3 (100 mg) in pyridine (2 ml) and the mixture was allowed to stand at room temperature for 16 hr. The product was added with water (60 ml) and extracted with ether. The ether extract was worked up in the usual way to give the oxidation product which was then chromatographed on alumina (10 g). The fractions eluted with 100 ml hexane: benzene (1:1) gave 24-methyllanost-9(11)-en-3-one (57 mg, RRT = 1.23) with mp = 138-139 C (fine needles); $[\alpha]_D^{30}$ +59° (c, 0.82); IR = 1709, 1380, 810, and 790 cm^{-1}; NMR = 0.70, 0.78, 0.82, 0.93, 1.11, 1.26, and 5.29 ppm; and MS = m/e 440.4057 (M^+), 425.3808, 313.2553, 257.1955, 245.1943, and 231.1745.

24-Methyllanost-8-en-3β-yl acetate (III, R_1 = OAc, R_2 =ii) and its free alcohol (III; R_1 = OH, R_2 = ii): The fraction 1 from the zone closest to the solvent front on the argentation TLC gave 24-methyllanost-8-enyl acetate (40 mg, RRT = 1.39, >99% GLC purity) with a mp = 133-134 C (fine needles); $[\alpha]_D^{30}$ +42° (c, 0.91); IR = 1720, 1385, 1371, and 1256 cm^{-1}. Hydrolysis of the acetate gave free alcohol (RRT = 1.15) with a mp = 155-156 C (fine needles), and MS = m/e 442 (M^+).

24-Methyllanost-7-en-3β-yl acetate (IV; R_1 = OAc, R_2 = ii) and its free alcohol (IV; R_1 = OH, R_2 = ii): The fraction 2 (208 mg), from the medium zone on the argentation TLC described above, gave a mixture of 24-methyllanost-8-enyl acetate (RRT = 1.39) and 24-methyllanost-7-enyl acetate (RRT = 1.67). The Δ^7-isomer was isolated from the mixture by treatment with CrO_3 in acetic acid (7) in the following way. The mixture (50 mg) in acetic acid (5 ml) was treated with CrO_3 (30 mg) in 90% acetic acid (1 ml) at 80 C for 10 min. The reaction product was added with water (30 ml) and extracted with ether. The ether extract was worked up in the usual way. The resulting material in hexane was chromatographed on alumina (6 g). The fractions eluted with 50 ml hexane:benzene (1:1) gave the unreacted Δ^7-isomer, 24-methyllanost-7-enyl acetate (29 mg, RRT = 1.67, >99% GLC purity) with mp = 149-150 C (needles); $[\alpha]_D^{30}$ +18° (c, 1.20); IR = 1730, 1394, 1380, 1364, 1244, 835, and 825 cm^{-1}; and NMR = 0.66, 0.83, 0.89, 0.93, 1.00, 2.06, 4.52, and 5.20 ppm. Hydrolysis of the acetate gave free alcohol (RRT = 1.38) and MS = m/e 442 (M^+).

Triterpene Alcohols with C_8-Side Chain

Cycloartenyl acetate (I; R_1 = OAc, R_2 = iii) and cycloartenol (I; R_1 = OH, R_2 = iii): Cycloartenyl acetate (868 mg, RRT = 1.52, 95% pure by GLC), isolated from the triterpene acetate mixture of rice bran oil described above, showed mp = 122-123 C (plates). Hydrolysis of the acetate gave cycloartenol (RRT = 1.23); IR = 3360, 1376, 1023, 820, and 800 cm^{-1}; and MS = m/e 426 (M^+).

Cycloartanyl acetate (I; R_1 = OAc, R_2 = i) and cycloartanol (I; R_1 = OH, R_2 = i): Hydrogenation of cycloartenyl acetate, in the same manner described above, gave cycloartanyl acetate (RRT = 1.26) with mp = 133-134 C (plates). Hydrolysis of the acetate gave cycloartanol (RRT = 1.02) with mp = 104.5-105 C (fine needles); IR = 3360, 1385, 1377, and 1021 cm^{-1}; and MS = m/e 428 (M^+).

Lanosta-9(11),24-dien-3β-yl acetate (II; R_1 = OAc, R_2 = iii; parkeyl acetate) and its free alcohol (III; R_1 = OH, R_2 = iii): Isolation of the acetate (RRT = 1.46, mp = 170-171 C, plates) from shea butter was described in the previous article (2). The NMR = 0.66, 0.76, 0.89, 0.90, 1.10, 1.62, 1.70, 2.06, 4.51, 5.10, and 5.20 ppm. Hydrolysis of the acetate gave free alcohol (RRT = 1.22) with mp = 157.5-158.5 C (fine needles); and MS = m/e 426 (M^+).

Lanost-9(11)-en-3β-yl acetate (II; R_1 = OAc, R_2 = i) and its free alcohol (II: R_1 = OH, R_2 = i): Partial hydrogenation of lanosta-9(11),24-dienyl acetate in ether over platinum oxide at room temperature gave lanost-9(11)-enyl acetate (RRT = 1.21) with mp = 174-176 C (plates); NMR = 0.66, 0.75, 0.83, 0.88, 0.90, 0.92, 1.09, 4.54, and 5.20 ppm. Hydrolysis of the acetate afforded free alcohol (RRT = 1.01) and MS = m/e 428 (M^+).

Lanosta-8,24-dien-3β-yl acetate (III; R_1 =OAc, R_2 = iii, lanosteryl acetate) and its free alcohol (III; R_1 = OH, R_2 = iii): A mixture of lanosta-8,24-dienol and lanost-8-enol purchased from L. Light & Co. (Colnbrook, England) was acetylated and separated into two fractions by preparative argentation TLC. Fraction 2 from the zone closer to the starting line afforded lanosta-8,24-dienyl acetate (RRT = 1.28, >99% GLC purity) with mp = 133-135 C (needles). Hydrolysis of the acetate gave free alcohol (RRT = 1.07) and MS = m/e 426 (M^+).

Lanost-8-en-3β-yl acetate (III, R_1 = OAc,

R_2 = i) and its free alcohol (III; R_1 = OH, R_2 = i): Fraction 1 from the zone closer to the solvent front on the TLC gave lanost-8-enyl acetate (RRT = 1.07) with mp = 119-121 C (plates). Hydrolysis of the acetate gave free alcohol (RRT = 0.89) and MS = m/e 428 (M^+).

Lanost-7-en-3β-yl acetate (IV; R_1 = OAc, R_2 = i) and its free alcohol (IV; R_1 = OH, R_2 = i): Lanost-7-enol was prepared according to the procedure given by Marker, et al., (7): A solution of lanost-8-enyl acetate in $CHCl_3$ was treated with a stream of HCl at 0 C for 3 hr to give a mixture of partial isomerization products consisting of lanost-7-enyl acetate (ca. 35%) and unreacted Δ^8-compound (ca. 65%). The mixture was then treated with CrO_3 for the removal of the Δ^8-isomer in the same manner as described above for the preparation of 24-methyllanost-7-enyl acetate. Lanost-7-enyl acetate thus obtained showed RRT = 1.28 (>99% GLC purity); IR = 1722, 1390, 1376, 1370, 1240, 834, and 823 cm^{-1}; and NMR = 0.66, 0.83, 0.88, 0.92, 0.98, 2.05, 4.53, and 5.20 ppm. Hydrolysis of the acetate gave lanost-7-enol (RRT = 1.06) with MS = m/e 428 (M^+).

RESULTS AND DISCUSSION

The new triterpene acetate (Fig. 1, II; R_1 = OAc, R_2 = iv) showed IR maxima at 1642 and 890 (>C = CH_2), and 813, 800, and 792 (>C = CH-) cm^{-1}. The free alcohol (Fig. 1, II; R_1 = OH, R_2 = iv) prepared by hydrolysis of the acetate gave MS with a M^+ at m/e 440.4021 ($C_{31}H_{52}O$, required 440.4014) and other principal ions at m/e 425.3802 (M - CH_3, required 425.3781), 407.3712 (M - CH_3 - H_2O, required 407.3676), 356.3078 (M - C_6H_{12} [part of side chain], required 356.3077), 313.2538 (M - C_9H_{17} [side chain] - 2H, required 313.2529) and 259.2057 (M - C_9H_{17} - C_3H_6 [part of ring D] - CH_2 required 259.2060) (8), indicating a C_9-side chain possessing 24-methylene group (the fragments m/e 313.2538 and 356.3078) (9) and a skeleton possessing three methyl substituents (4α, 4β and 14-) and a double bond. The NMR signals at 0.65 (singlet, 18-methyl), 0.75 (singlet, C-14α-methyl), 0.87 and 0.89 (singlets, C-4,4-dimethyl), 1.09 (singlet, 19-methyl), 2.05 (singlet, 3β-acetoxyl), 4.54 (multiplet, 3α-proton) and 5.21 (multiplet, C-11-proton) ppm of the acetate were basically identical with the corresponding signals in their chemical shifts observed on lanosta-9(11),24-dienyl acetate (parkeyl acetate, Fig. 1, H; R_1 = OAc, R_2 = iii). These results may admit to interpret the skeletal structure of the new alcohol as identical with that of parkeol. On the other hand, the chemical shift of the doublet at 1.04 ppm (*J* 6.6 Hz) attributable to the 26,27-geminal dimethyl protons of the acetate was almost identical with that observed on 24-methylene compounds such as 24-methylenecycloartanol (24-methylene-9β,19-cyclolanostanol) (10), cycloeucalenol (4α,14α-dimethyl-24-methylene-9β,19-cyclocholestanol) (11) and gramisterol (4α-methyl-24-methylene-cholest-7-enol) (12); however, it differed considerably from that recognized on the sterols with a saturated side chain such as cholesterol (i.e., doublet, 0.86 ppm, *J* 4.8 Hz) (T. Iida, T. Tamura and T. Matsumoto, unpublished data). This is explicable by the presence of a 24-methylene group in the side chain of the new alcohol. From these spectral evidences, the structure (Fig. 1, II; R_1 = OH, R_2 = iv) is assigned to the new alcohol.

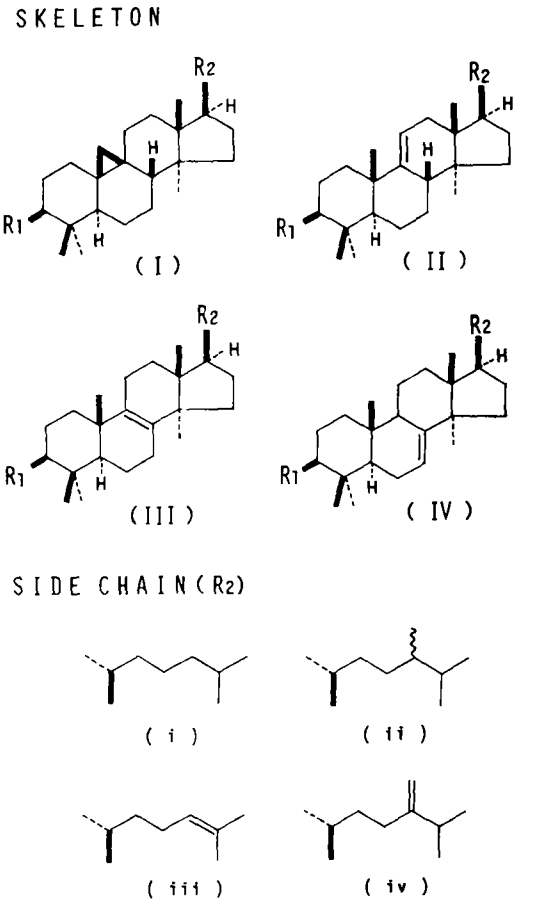

FIG. 1. Diagram of the skeletons and side chains (R_2) of lanostane series compounds. Skeleton I = 9β,19-cyclo; II = $\Delta^9(11)$; III = Δ^8; and IV = Δ^7. Side chain (R_2) i = 24-unsubstituted; ii = 24ξ-methyl; iii = Δ^{24}; and iv = 24-methylene.

TABLE I

Relative Retention Time (RRT) of the Triterpene Alcohols and ΔR_{Ac}-Values on 3% OV-17 Column

Skeletal substituent	Compound	Side chain R_2	RRT[a] R_1 = OAc	RRT[a] R_1 = OH	ΔR_{Ac}[b]
9β,19-cyclo (I)[c]	24-Methylenecycloartanol	iv	1.69	1.37	1.23
	24-Methylcycloartanol	ii	1.64	1.33	1.23
	Cycloartenol	iii	1.52	1.23	1.24
	Cycolartanol	i	1.26	1.02	1.24
$\Delta^{9(11)}$ (II)	24-Methylenelanost-9(11)-enol	iv	1.62	1.35	1.20
	24-Methyllanost-9(11)-enol	ii	1.57	1.31	1.20
	Lanosta-9(11),24-dienol (Parkeol)	iii	1.46	1.22	1.20
	Lanost-9(11)-enol	i	1.21	1.01	1.20
Δ^{8} (III)	24-Methyllanost-8-enol	ii	1.39	1.15	1.21
	Lanosta-8,24-dienol (Lanosterol)	iii	1.28	1.07	1.20
	Lanost-8-enol	i	1.07	0.89	1.20
Δ^{7} (IV)	24-Methyllanost-7-enol	ii	1.67	1.38	1.21
	Lanost-7-enol	i	1.28	1.06	1.21

[a]Retention time for β-sitosterol (30 min) is taken as 1.00.

[b]ΔR_{Ac}-value is expressed by the ratio of RRT of the acetate to RRT of the free alcohol.

[c]See Fig. 1 for diagram.

Oxidation of the free alcohol (Fig. 1, II; R_1 = OH, R_2 =iv) with CrO_3 (6) gave 24-methylenelanost-9(11)-en-3-one which showed IR maximum at 1703 (>C = 0) cm^{-1} and M^+ at m/e 438 corresponding to the formula of $C_{31}H_{50}O$. In its NMR, a relatively large lower field shift of the signals of the 19-methyl and C-4,4-dimethyl protons was observed; i.e., 1.25 (singlet, 19-methyl) and 1.09 (singlets, coincided, 4,4-dimethyl) ppm, respectively, while the resonances of the 18- and the C-14α-methyl groups actually remained in their original positions (18-methyl, 0.70 ppm; and C-14α-methyl, 0.77 ppm). Such lower field shift of the signals arisen from the protons in the neighborhood of the C-3 functional group due to the introduction of a 3-keto group in the molecule had already been reported (13,14).

Furthermore, the dihydro compound (Fig. 1, II; R_2 = ii) prepared from the new alcohol is found to be identical with 24-methyllanost-9(11)-enol prepared from 24-methylenecycloartanol, because both compounds agree with each other in their spectral data and physical constants. The dihydroacetate (Fig. 1, II; R_1 = OAc, R_2 = ii) prepared by hydrogenation of the new triterpene acetate showed IR still exhibiting the trisubstituted olefinic absorbances at 811 and 790 cm^{-1}. The MS of the free alcohol showed M^+ at m/e 442.4178 ($C_{31}H_{54}O$, required 442.4171) with other principal ions at m/e 427.3927 (M - CH_3, required 427.3936), 409.3865 (M - CH_3 - H_2O, required 409.3832) and 273.2263 (M - C_9H_{19} [side chain] - C_3H_6, required 273.2217), and indicated the presence of a C_9-saturated side chain and a skeletal double bond in the molecule. In the NMR of the dihydro-acetate a doublet attributable to the 26,27-dimethyl protons was observed at 0.87 (J 5.4 Hz) ppm indicating the same chemical shift as the case with cholesterol, while the chemical shifts of the other signals were pracially identical with those of the new triterpene acetate. Thus, it is apparent from these spectral evidences that in the hydrogenation of the new triterpene acetate the 24-methylene group is converted to 24-methyl group.

It is known that 9β,19-cyclo triterpene alcohols give a mixture of $\Delta^{9(11)}$-, Δ^{8}-, and Δ^{7}-isomers by HCl isomerization (15). To prepare an authentic specimen of 24-methyllanost-9(11)-enol, 24-methylcycloartanyl acetate obtained by hydrogenation of 24-methylenecycloartanyl acetate was treated with HCl in $CHCl_3$. GLC of this isomerized product indicated the presence of three components, the isomers above mentioned.

24-Methyllanost-9(11)-enyl acetate (Fig. 1, II; R_1 = OAc, R_2 = ii) separated from other two isomers by preparative argentation TLC showed IR bands attributable to the trisubstituted olefine at 812 and 791 cm^{-1}. The MS of the free alcohol therefrom exhibited M^+ at m/e 442.4209 ($C_{31}H_{54}O$) with the principal fragments corresponding to M - CH_3, M - CH_3 - H_2O, M - side chain - C_3H_6 - CH_2, and M - side chain - C_3H_6 - H_2O. These spectral data as well as NMR and GLC behaviors of 24-methyllanost-9(11)-enol and its acetate were essentially identical with those of the dihydro-compounds (Fig. 1, II; R_1 = OH, and OAc, R_2 = ii). Hence, the new triterpene alcohol from shea butter is characterized with reason-

TABLE II

Skeletal Separation Factors of Triterpene Alcohols on 3% OV-17 Column

Comparison of skeleton	Side chain R_2	Separation factors R_1 = OAc	Separation factors R_1 = OH
I/II (9β,19-cyclo/Δ^9[11])	iv	1.04	1.02
	ii	1.04	1.02
	iii	1.04	1.01
	i	1.04	1.01
III/II (Δ^8/Δ^9[11])	ii	0.89	0.88
	iii	0.88	0.88
	i	0.88	0.88
IV/II (Δ^7/Δ^9[11])	ii	1.06	1.05
	i	1.06	1.05

TABLE III

Side Chain Separation Factors of Triterpene Alcohols on 3% OV-17 Column

Comparison of side chain	Skeleton	Separation factors R_1 = OA_c	Separation factors R_1 = OH
ii/i	I	1.30	1.30
	II	1.30	1.30
	III	1.30	1.29
	IV	1.30	1.30
iii/i	I	1.21	1.21
	II	1.21	1.21
	III	1.20	1.20
iv/i	I	1.34	1.34
	II	1.34	1.34

able certainty as 24-methylenelanost-9(11)-en-3β-ol (Fig. 1, II; R_1 = OH, R_2 = iv).

Oxidation of 24-methyllanost-9(11)-enol with CrO_3 (6) gave a 3-keto compound which showed IR maximum at 1709 (>C = O) cm^{-1} and M^+ at m/e 440.4057 ($C_{31}H_{52}O$). The NMR pattern of this ketone was the same as that obtained with the new triterpene ketone (II; R_1 = O, R_2 = iv) except that the doublet arisen from the 26,27-dimethyl protons appeared at 0.88 (*J* 6.6 Hz) ppm for the former, and at 1.04 (*J* 6.6 Hz) ppm for the latter.

To establish the correlation of the gas chromatographic characteristics between skeletal substituent isomers of lanostane series triterpene alcohols, several members of this series with four different types of isomers, i.e., 9β,19-cyclo (I), $\Delta^{9(11)}$ (II), Δ^8 (III), and Δ^7 (IV) compounds, were gas chromatographed on 3% OV-17 column with the results shown in Table I. The correlation of ΔR_{Ac}-value, the ratio of RRT of the acetate to the RRT of the corresponding free alcohol, was found basically similar to that determined on 1.5% OV-17 column (16), indicating that the members of the 9β,19-cyclo type had somewhat larger ΔR_{Ac}-values than the other members possessing a skeletal double bond. Table II presents skeletal separation factors calculated from that data in Table I; and Table III presents side chain separation factors calculated from the data in Table I. An inspection of the data shown in Tables I, II, and III may justify the structure (Fig. 1, skeleton II: $\Delta^{9(11)}$; side chain iv: possessing 24-methylene group) given for the new triterpene alcohol in this study.

The presence of the new 24-methylenesterol in the kernels of *Butyrospermum parkii* may reflect an alternative to the apparently more common biosynthetic route through 24-methylenecycloartanol as already discussed in part by Goodwin (17).

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Dietary Fats and Properties of Endoplasmic Reticulum: I. Dietary Lipid Induced Changes in Composition of Microsomal Membranes in Liver and Gastroduodenal Mucosa of Rat

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ABSTRACT

Rats were fed for four weeks with different lipid diets to determine the effects on the endoplasmic reticulum membranes of the liver and on the postmitochondrial supernatant fraction of the gastroduodenal mucosa. The diets contained cholesterol, cacao butter, olive oil, and these in combination. The results showed that dietary lipids were able to modify the composition of the hepatic endoplasmic reticulum and, to a lesser extent, that of postmitochondrial fraction of gastroduodenal mucosa. Cacao butter in the diet decreased the relative proportion of protein in hepatic microsomes. Cholesterol and olive oil were able to increase the cholesterol content of microsomes. The trypsin digestion of membranes revealed that cholesterol increased the solubility of microsomal protein and decreased the trypsin sensitive protein-lipid binding. The neutral fat diets increased the binding of proteins to the membrane, and cholesterol had no effect when it was given in combination. The low power photomicrographs revealed vacuolization of the cytoplasm of the hepatocytes when rats were fed on lipid rich diets. Also fatty degeneration was present. Cholesterol in combination with olive oil, however, did normalize the structure of the hepatocytes to a marked extent.

INTRODUCTION

The endoplasmic reticulum (ER) consists of lamellar, tubular, and cisternal structures separating spaces from the cytoplasmic compartment of the cell (1,2). However, it cannot be separated intact; but homogenization breaks the tubular structures into vesicles, called as microsomes (1). The main components of microsomes are proteins and lipids (3,4). The protein fraction has a dual function in the membrane, having both catalytic and structural properties (5,6). The microsomal phospholipids are structural and storage components, but, moreover, they have a role in the regulation of enzyme activities and in the control of membrane permeability (4, 7-9). Cholesterol, which makes up to 7% of the total lipids, probably has both metabolic and structural functions (8,10). It stabilizes the molecular structures and makes the lipid matrix compact, and also contributes to the regulation of membrane permeability (8, 11-13).

Characteristics of the liver ER is the high adaptation capacity to various physiological and experimental conditions (14-17). The ER is not a static structure, but it is being renewed at all times. It is known that alteration in the lipid portion of the diet can change the cholesterol and phospholipid fractions in the plasma (18,19). Our main problems have been to study: (A) whether the changes in the fat and cholesterol portions of the diet are reflected in the composition of microsomal membranes in the liver and gastroduodenal mucosa; (B) whether there are any differences in microsomal structure when rats are fed saturated or unsaturated lipid diets; and (C) whether there is any difference in the trypsin sensitive fraction of the microsomal membranes when rats are fed different diets.

EXPERIMENTAL PROCEDURES

Thirty adult male rats (*Rattus norvegicus*), purchased as specific pathogen free Wistar/Af/Han/Mol/(Han 67) weighing 90-140 g, were used as experimental animals. The rats were fed test or standard diets for four weeks ad libitum before they were sacrificed. The standard diet (Hankkija Ltd., Helsinki, Finland) contained 240 g/kg protein, 35 g/kg fat and 0.4 g/kg cholesterol. The composition of experimental diets is expressed in the Table I. The content of salt mixture was: 150 g/kg NaCl, 120 g/kg KCl, 310 g/kg KH_2PO_4, 140 g/kg $Ca_3(PO_4)_2$, 210 g/kg $CaCO_3$, 90 g/kg $MgSO_4$, 14.7 g/kg $FePO_4*4H_2O$, 0.20 g/kg $MnSO_4$, 0.09 g/kg $KAl(SO_4)_2*12H_2O$, 0.39 g/kg $CuSO_4*5H_2O$, 0.57 g/kg NaF, and 0.05 g/kg KI (19). The vitamin mixture (ICN, Nutritional Biochemical Corporation, Cleveland, Ohio) contained 900,000 units vitamin A, 100,000 units vitamin D, 5.0 g α-tocopherol, 45.0 g ascorbic acid, 5.0 g inositol, 75 g choline chloride, 2.25 g menadi-

TABLE I

The Composition of Experimental Diets

Component	Diet[a] (g/kg)				
	C	CB	CB+C	00	00+C
Casein	150	100	100	200	200
Sucrose	380	210	210	495	445
Salt mixture	40	40	40	40	40
Vitamin mixture	30	30	30	30	30
Sodium cholate	10	10	10	5	5
Cholesterol	50	---	50	---	50
Cacao butter	---	340	340	---	---
Olive oil	---	---	---	240	240
Cellulose	280	180	130	---	---
Water	60	90	90	---	---

[a]C = cholesterol diet, CB = cacao butter diet, CB+C = cacao butter plus cholesterol diet, 00 = olive oil diet; 00+C = olive oil plus cholesterol diet. The composition of salt and vitamin mixtures are given in "Experimental Procedures."

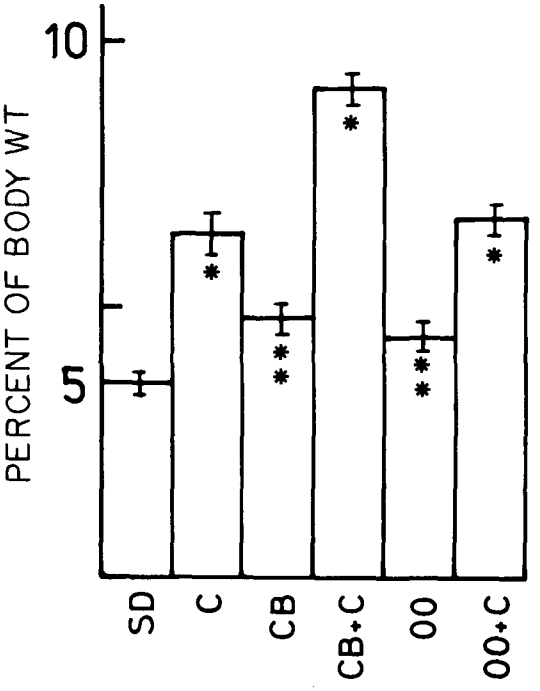

FIG. 1. The liver/body wt ratio in rats on various lipid diets. The ratio is shown as % of body wt. SD = standard, C = cholesterol, CB = cacao butter, CB+C = cacao butter plus cholesterol, OO = olive oil, OO+C = olive oil plus cholesterol. *significantly ($P<0.001$) different from standard diet. **significantly ($P<0.01$) different from standard diet.

one, 5.0 g p-aminobenzoic acid, 4.5 g niacin, 1.0 g riboflavin, 1.0 g pyridoxine hydrochloride, 1.0 g thiamine hydrochloride, 3.0 g calcium panthotenate, 20 mg biotin, 90 mg folic acid and 1.35 mg vitamin B-12. The experimental diets were prepared by a meat grinder in our laboratory.

The rats were killed by a blow on the head and bled by cutting the renal vessels. The liver, stomach, and 10 cm of the upper small intestine were immediately dissected and placed into iced 0.25 M sucrose. Part of the liver was placed in 10% formalin and microsomes were made from the rest of liver. The liver was homogenized in iced 0.25 M sucrose to give a 20% (wt/vol) suspension by a Potter-Elvehjem type glass Teflon homogenizer. The cellular debris, nuclei, and mitochondria were separated by a 10,000 x g_{av} centrifugation (Sorvall SS-1) at 4 C for 15 min, and the microsomal fraction was sedimented by 105,000 x g_{av} centrifugation (MSE Superspeed 50) for 60 min (20). The pellet was resuspended in 0.25 M sucrose to yield 1 g liver/ml. The mucosa of the stomach and small intestine were scraped off by an ampoule file and homogenized in 0.25 M sucrose by a Potter-Elvehjem type glass-Teflon homogenizer to give a 20% (wt/vol) homogenate (21). The suspension was centrifuged at 10,000 x g_{av} for 15 min at 4 C, and the supernatant was used for further studies.

The trypsin digestion of hepatic microsomes was adapted from the procedure of Hänninen and Puukka (22); 1 mg trypsin (Type III, Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, was added in volume of 1.0 ml microsomes in 0.25 M sucrose. After an incubation at 37 C for 30 minutes, the digestion was stopped by adding a respective amount of trypsin inhibitor (Type II-O, Sigma Chemical Co., St. Louis, Mo.). The unsoluble fraction was harvested by centrifugation at 105,000 x g_{av} for 60 min. The pellet was resuspended in 2 ml 0.25 M sucrose.

The microsomal protein content was determined by the biuret method using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.)

TABLE II

Protein Content, Phospholipid/Protein, and Cholesterol/Protein Ratios in Rat Liver Microsomes of Rats Fed Different Lipid Diets

Diet[a]	Protein[a] (mg/g liver)	Phospholipid/protein ratio	Cholesterol/ protein
Standard	32.6 ± 2.9[b]	0.52	0.02
Cholesterol	33.7 ± 0.4	0.42	0.04
Cacao butter	24.5 ± 1.5	0.33	0.02
Cacao butter + cholesterol	24.9 ± 0.6[a]	0.37	0.03
Olive oil	37.2 ± 4.6	0.24	0.07
Olive oil + cholesterol	28.3 ± 2.1	0.40	0.05

[a]The number of rats in each group = 5, except the cacao butter diet, where n = 4.
[b]Means and standard error of the means, given in protein contents.
[c]Statistically ($P<0.05$) different.

TABLE III

Protein Content of Hepatic Microsomes and of Trypsin-Treated Liver Microsomes of Rats Fed Different Lipid Diets

Diet[a]	Conventional microsomes[b]	Trypsin-treated microsomes[b]	% Protein released by trypsin
Standard	32.6 ± 2.9[c]	22.9 ± 1.1[d,e,f]	30
Cholesterol	33.7 ± 0.4	19.5 ± 0.8[d]	42
Cacao butter	24.5 ± 1.5	20.9 ± 1.1	15
Cacao butter + cholesterol	24.9 ± 0.6[c]	21.6 ± 1.4	13
Olive oil	37.2 ± 4.6	35.1 ± 3.0[e,g]	6
Olive oil + cholesterol	28.3 ± 2.1	26.4 ± 0.4[f,g]	7

[a]The number of rats in each group = 5, except cacao butter diet where n = 4.
[b]Means and standard error fo the means, given in protein content.
[c]Significantly ($P<0.05$) different.
[d]Significantly ($P<0.05$) different.
[e]Significantly ($P<0.01$) different.
[f]Significantly ($P<0.05$) different.
[g]Significantly ($P<0.05$) different.

as a reference (23). Membrane fractions were digested by Na-deoxycholate (5%, Fluka AG, Buchs, Switzeland) before adding biuret reagent. After measuring the samples, they were decolorized by adding KCN and the background was estimated. The amount of phospholipid was determined by measuring the inorganic phosphate after sulphuric acid hydrolysis as described by Bartlett (24), and the results were expressed as equivalents to lecithin (25). The cholesterol content of microsomes and 10,000 x g_{av} gastroduodenal supernatant were measured as described by Abell, et al., (26) as modified by Anderson and Keys (27). The liver slices fixed in 10% formalin were used to prepare micrographs for microscopic evaluation of the liver structure. The liver slices were removed from formalin into increasing concentrations of ethanol (max. 99.5%) to remove water and formalin. Hereafter the slices were embedded into paraffin and cut into 10 μm slices and fixed on microscopic glasses. The tissue preparations were visualized by staining with hematoxylin eosin. The photomicrographs were taken through a Leitz Orthoplan microscope using Agfa professional film.

RESULTS

The liver-body wt ratio was somewhat elevated in rats fed cacao butter or olive oil diets, and significant ($P<0.001$) higher (2-3 fold) in rats fed on high cholesterol diets (Fig. 1). The cacao butter diet combined with cholesterol increased the liver-body wt ratio up to 9%. The standard and cacao butter diets maintained the microsomal cholesterol-protein ratio at the lowest level (Table II). The olive oil diet was able to increase the relative cholesterol amount 3.5 fold. The high cholesterol diet and cholesterol in combination with other lipids were also able to increase the microsomal cholesterol content, but not to the same extent as the olive oil diet. The phospholipid contents in relation to protein contents in hepatic microsomes also

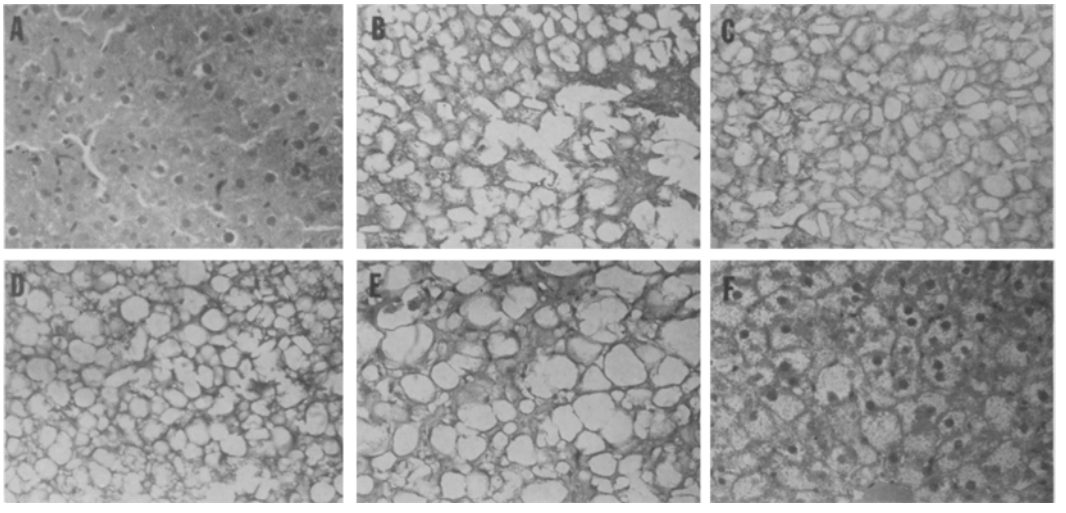

FIG. 2. The low power (250 x magnification) photomicrographs of livers from rats fed various lipid diets. The stain used was hematoxylin-eosin. A = Standard diet, B = Cholesterol diet, C = Olive oil diet, D = Cacao butter diet, E = Cacao butter plus cholesterol diet and F = Olive oil plus cholesterol diet.

showed marked variations (Table II). The rats fed olive oil diet had the lowest levels of hepatic microsomal phospholipid. They exhibited only 50% of the mean of the group fed the standard diet. The highest amount of phospholipid was found in the rats fed standard and cholesterol diets. In rats fed fat rich diets, cholesterol was also found to increase the microsomal phospholipid content (Table II). Rats fed standard, cholesterol, and olive oil diets exhibited highest protein contents in the liver. The cacao butter diet decreased microsomal protein 30% from the control level, and cholesterol in combination did not increase the protein content. In the group fed olive oil, cholesterol was found to decrease the protein level slightly (Table II).

The trypsin digestion of the hepatic microsomes revealed marked differences in the tightness of protein attachment. In the group fed the standard diet, 30% of the microsomal protein could be removed from the membranes into the soluble fraction (Table III), and in the group fed cholesterol diet, more than 40% of the protein could be solubilized. On the other hand, diets rich in neutral fats increased the trypsin sensitive protein binding in the microsomes. In the olive oil group, only 6% of the microsomal protein was solubilized. The addition of cholesterol in diets did not decrease the attachment of membrane proteins (Table III).

The low power photomicrographs showed fatty infiltration in the liver of rats on cholesterol or fat diets (Fig. 2). The high cholesterol diet (Fig. 2B) was found to cause vacuolization of the cytoplasm and partial crystallization. In livers from rats fed olive oil diet, vacuolization was also present as was possible crystallization (Fig. 2C). Fatty degeneration was found in the liver of rats fed cacao butter diet, and was also present when diet was supplemented with cholesterol (Fig. 2D and E). In the livers from rats fed olive oil plus cholesterol diet, the microscopic view was rather different from those fed olive oil or cholesterol diets alone (Fig. 2F). In this group fed olive oil containing cholesterol, cytoplasm was granular and the hepatocytes were somewhat enlarged from the controls. The fatty degeneration was not as advanced as in rats fed other lipid diets. Due to the vacuolization, nuclei were poorly visualized in hepatocytes of rats fed other diets, except the groups fed standard and olive oil plus cholesterol diets (Fig. 2A and F).

The samples of gastric and intestinal mucosa showed less variation in the contents of cholesterol, phospholipid, and protein than did the hepatic microsomes. Cholesterol, contents were highest in the groups fed cholesterol and olive oil plus cholesterol diets. The highest concentration of cholesterol was twice the control level (Table IV). The phospholipid content of the stomach was lowest in the cholesterol-fed group, and the duodenal mucosa did not show a marked variation (Table IV). The protein content of both stomach and duodenum was rather independent from the diet.

DISCUSSION

The present study was conducted to clarify

TABLE IV

Protein, Cholesterol, and Phospholipid Contents of Postmitochondrial Supernatant of The Stomach and Duodenum

Diet[a]	Protein (mg/g tissue, wet wt)[b]		Phospholipid (mg/mg protein)[b]		Cholesterol (mg/mg protein)[b]	
	Stomach	Duodenum	Stomach	Duodenum	Stomach	Duodenum
Standard	47	66	1.19	0.79	0.09	0.07
Cholesterol (C)	37	71	0.64	0.84	0.11	0.11
Cacao butter	42	59	0.98	1.23	0.06	0.06
Cacao butter + C	44	64	1.20	0.89	0.07	0.09
Olive oil	42	57	0.82	1.09	0.06	0.08
Olive oil + C	45	64	1.02	1.13	0.15	0.18

[a]The number of rats in each group = 5, except cacao butter diet, where n = 4.

[b]Expressed as mean value determined from pooled samples of each group.

whether dietary lipids play any active role in determining the composition of hepatic ER and gastroduodenal mucosa. The cacao butter and olive oil were selected as test neutral fats, because the former contains saturated fatty acids and the latter contains unsaturated fatty acids, primarily monounsaturated fatty acids. The standard diet also contained a rather high proportion of fatty acids in the unsaturated form, but the total amount of lipids was much lower than in the specific diets. The cholesterol was selected as a test diet component because it is known to have a significant membrane stabilizing effect, thus, possible effects on the protein-lipid interactions (8, 11, 12, 28). It has been shown earlier that dietary fats markedly increased cholesterol and phospholipid contents of the plasma (28). Dietary lipids change the composition of plasma after 12 weeks, but in 3-4 weeks plasma changes are distinct. For this reason, 4 weeks was selected to be the duration of the test period.

The present study revealed that dietary lipids are able to modify the composition of hepatic ER, and, to a lesser extent, the gastroduodenal mucosa. It appeared that saturated neutral fat in the diet decreased the relative proportion of hepatic microsomal protein. The diet containing unsaturated fatty acids was able to increase the proportion of membrane cholesterol. This might be due to endogenous cholesterol synthesis. The cholesterol in the diet was shown to increase hepatic cholesterol content, and it is suggested that this was because of direct cholesterol incorporation into the liver. This idea is supported also by studies of Tsai and Dyer (29), who determined that cholesterol feeding increased hepatic cholesterol content, but depressed cholesterol synthesis. It was evident from the photomicrographs that lipids caused marked fatty degeneration of the hepatocytes independent of the degree of saturation of fatty acids in the diet. However, it was interesting to note that cholesterol, when combined with olive oil, was able to prevent degeneration of hepatocytes to a great extent, although no such effect was present when cholesterol was added to the cacao butter diet.

Trypsin digestion of hepatic microsomes showed that diets containing both cholesterol and different types of neutral fats have a considerable role in the binding of trypsin sensitive proteins to the membranes. It was shown that cholesterol increased the solubility of microsomal proteins and decreased the tightness of proteins in the membrane. This finding is in good accordance with a recent study (8), where it was shown that cholesterol inhibited the penetration of proteins into phospholipid monolayers. Moreover, it was observed that neutral fats in the diet increased the binding of proteins to the membrane, so that even cholesterol was not able to loosen the binding between proteins and lipids.

The present study showed that dietary lipids are of great importance in the regulation of the composition of the hepatic ER, and in the control of the protein-lipid ratio and their interaction. The composition of the gastroduodenal mucosa is also, to some extent, adaptive to dietary modifications.

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Dietary Fats and Properties of Endoplasmic Reticulum: II. Dietary Lipid Induced Changes in Activities of Drug Metabolizing Enzymes in Liver and Duodenum of Rat

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ABSTRACT

Rats were fed cholesterol, cacao butter, or olive oil diets to determine the effect of dietary lipids on the rate of drug biotransformation in the liver and duodenum. The cholesterol rich diet maintained the hepatic aryl hydrocarbon hydroxylase activity at the same level as did the standard diet. Rats fed olive oil and cacao butter diets showed lower hepatic aryl hydrocarbon hydrorylase activity. The p-nitroanisole 0-demethylase activity was doubled in hepatic microsomes of rats fed the high cholesterol diet when compared to rats fed the standard diet. The hepatic uridine diphosphate glucuronosyltransferase activity showed different patterns depending on the in vitro treatment of the microsomal membranes. If the enzyme activity was assayed from the native, untreated microsomes, an increase in the measurable uridine diphosphate glucuronosyl transferase activity was found in rats having cholesterol rich diet. After the in vitro activation of membrane-bound uridine diphosphate glucuronosyltransferase by trypsin, the increase in measurable activity was 10 fold in the group fed the standard diet, 6 fold in group fed cholesterol, 4 fold in group fed cacao butter, and 3 fold in group fed olive oil. Trypsin digestion of microsomes increased the measurable uridine diphosphate glucuronosyltransferase activity less in rats fed diets rich in neutral fats than those fed the standard diet. In the duodenal mucosa, lipid diets decreased the activities of drug hydroxylation and glucuronidation.

INTRODUCTION

Phospholipids and cholesterol, in addition to proteins, are important structural and functional components of microsomal membranes (1-3). In previous studies it has been shown that crude dietary modifications, and changes in the chyme passage, are able to change hepatic and intestinal enzyme activities (4-8). It has been shown that diets used to feed other species change the rate of intestinal and hepatic drug metabolism in rats (7). Severe protein deficiency may result in decreased activity of drug hydroxylation and increased uridine diphosphate (UDP) glucuronosyltransferase activity (8-10). Drug hydroxylation and glucuronidation take place in the endoplasmic reticulum (ER), where an enzyme complex oxidizes drugs to their more polar metabolites, which, then are glucuronidated to more water soluble derivatives (11-13). The drug oxidizing enzymes are located on the cytoplasmic surface of the ER, whereas, UDP glucuronosyltransferase, which catalyzes the conjugation step, is located deeper in the microsomal membrane (13-17). Phospholipids exhibit an important role in drug hydroxylation, and they contribute to the control of activity of UDP glucuronosyltransferase (18,19,20). Cholesterol has a membrane stabilizing effect (2). It appears evident that the activities of drug metabolizing enzymes are dependent on the structure of the ER.

In a previous study (3) we have shown that lipid diets are able to modify the composition of hepatic and gastroduodenal ER. The present report is concerned with the regulation of the activity of drug metabolizing enzymes. An effort is made to interpret the changes in drug biotransformation as a result of compositional changes of microsomal membranes after feeding fat rich diets. In this study we have investigated the aryl hydrocarbon hydroxylase (EC. 1.14.14.2), p-nitroanisole 0-demethylase and UDP glucuronosyltransferase (EC. 2.4.1.17) in the liver and duodenal mucosa of rats.

EXPERIMENTAL PROCEDURES

Thirty adult male rats (*Rattus norvegicus*, purchased as specific pathogen free Wistar/Af/Han/Mol/[Han 67], outbred by the rotational mating system) 1.5 months of age, and weighing 90-140 g, were used. The rats were fed various neutral fat, cholesterol, and standard diets as described previously (3). After feeding rats for 4 weeks ad libitum, they were stunned by a blow on the head and were bled by cutting the renal vessels. The hepatic microsomes and postmitochondrial supernatant of duodenal mucosa were prepared as described earlier (3). The

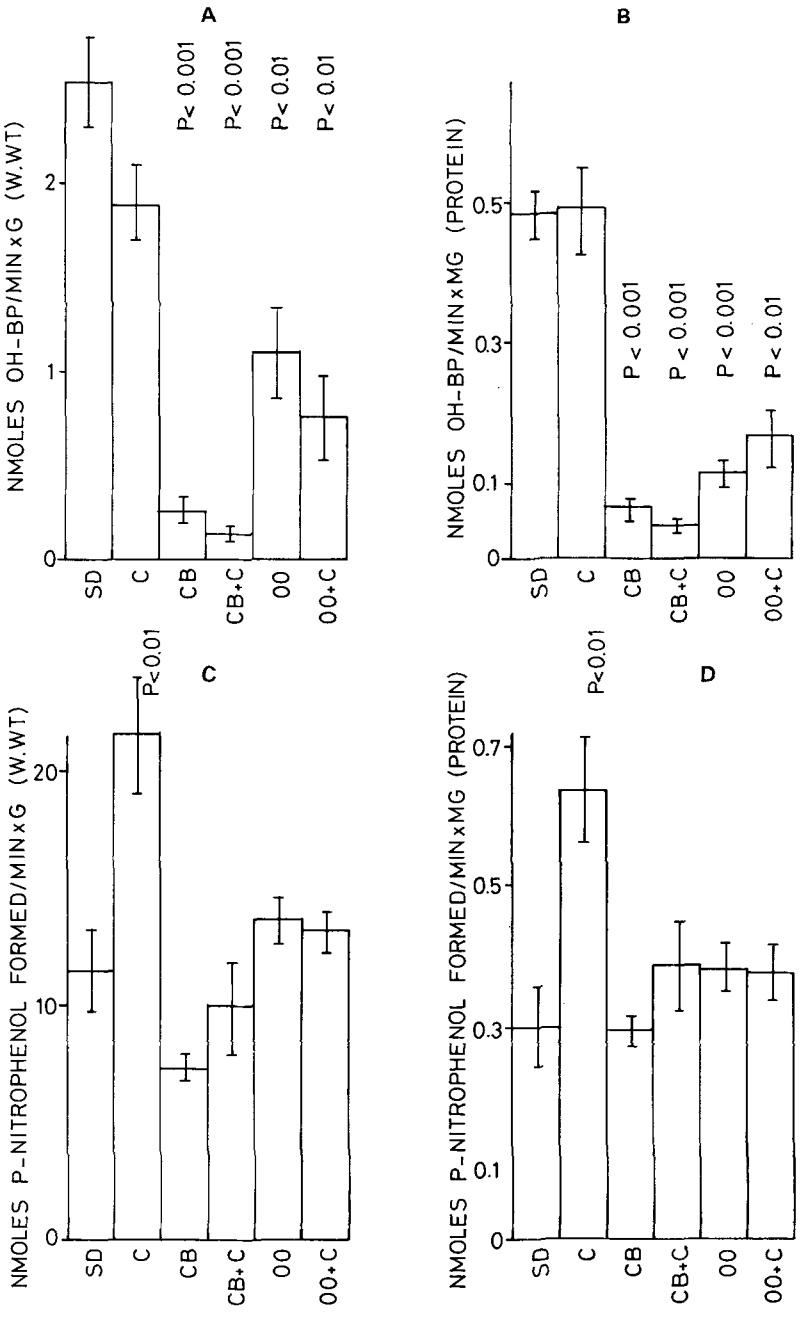

FIG. 1. The activities of aryl hydrocarbon hydroxylase (benzpyrene hydroxylase [OH-BP]) and p-nitroanisole 0-demethylase in the hepatic microsomes of rats fed different lipid diets. The data are reported as means and standard errors of the means (vertical bars). Statistical analysis, if not otherwise stated, was run by Student's t-test comparing experimental groups to the group on standard diet. SD = standard, C = cholesterol, CB = cacao butter, OO = olive oil, CB+C = cacao butter plus cholesterol and OO+C = olive oil plus cholesterol. (A) Represents the activity of aryl hydrocarbon hydroxylase/g liver (wet wt), and (B) aryl hydrocarbon hydroxylase activity/mg microsomal protein. (C) Represents the activity of p-nitroanisole 0-demethylase/g liver (wet wt) and (D) p-nitroanisole 0-demethylase activity/mg microsomal protein. The number of rats in each group = 5, except the cacao butter, where n = 4.

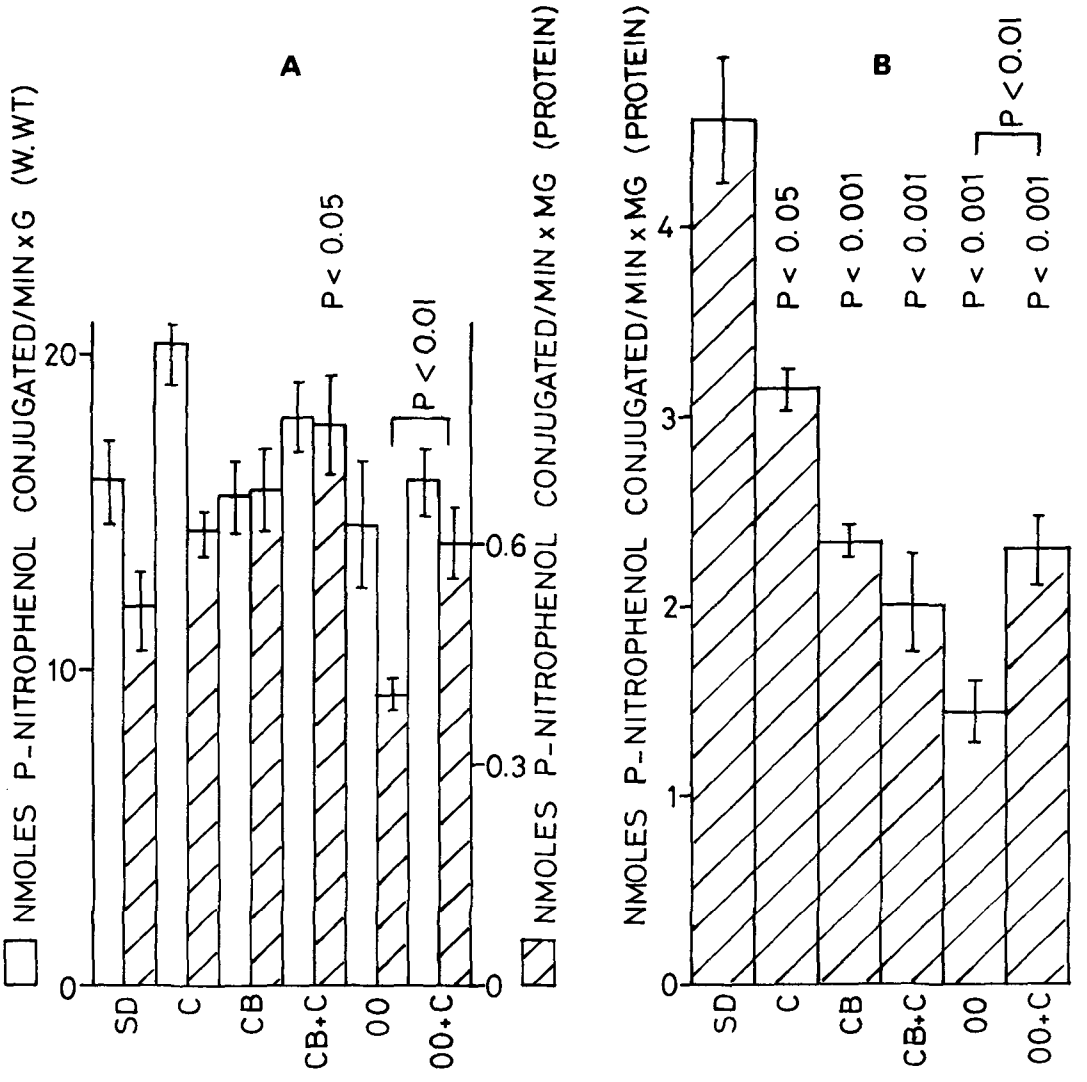

FIG. 2. The uridine diphosphate glucuronosyltransferase activity in hepatic microsomes from rats fed various lipid diets, expressed per g liver (wet wt) □ or per mg microsomal protein ▨. The nontreated microsomes are presented in (A) and trypsin treated in (B). The data are reported as means and standard errors of the means (vertical bars). SD = standard diets; C = cholesterol; CB = cacao butter; OO = olive oil; CB+C = cacao butter plus cholesterol; OO+C = olive oil plus cholesterol. For other explanations, see Figure 1.

protein content of tissue preparations was measured by the biuret reaction (3, 21).

The aryl hydrocarbon hydroxylase activity was determined using 3,4-benzpyrene (Sigma Chemical Co., St. Louis, Mo.) 0.16 mM as a substrate in the presence of 50 mM Tris-HCl buffer, pH 7.4, 1 mM $NADP^+$ (Sigma Chemical Co., St. Louis, Mo.), 5 mM $MgCl_2$, 0.005 mM $MnCl_2$, 2.5 mM Na_3-isocitrate (Fluka Ag, Buchs, Switzerland) and 0.5 International units (IU) pig heart isocitrate dehydrogenase (Boehringer, Mannheim, Germany), and 0.1-0.2 mg microsomal protein in a final volume of 0.5 ml. After 20 min incubation at 37 C the reaction was stopped by 1 ml cold acetone. The amount of hydroxylated 3,4-benzpyrene was measured by Perkin-Elmer MPF-2A spectrophotofluorometer as described by Wattenberg, et al., (22) and modified by Nebert and Gelboin (23). p-Nitroanisole 0-demethylase activity was determined by measuring the rate of formation of p-nitrophenol at 420 nm in a Perkin-Elmer 402 spectrophotometer (24,25). The incubation mixture contained 0.07 M Na-phosphate buffer, pH 7.8, 1.1 mM p-nitroanisole (Fluka Ag, Buchs, Switzerland), and the same NADPH

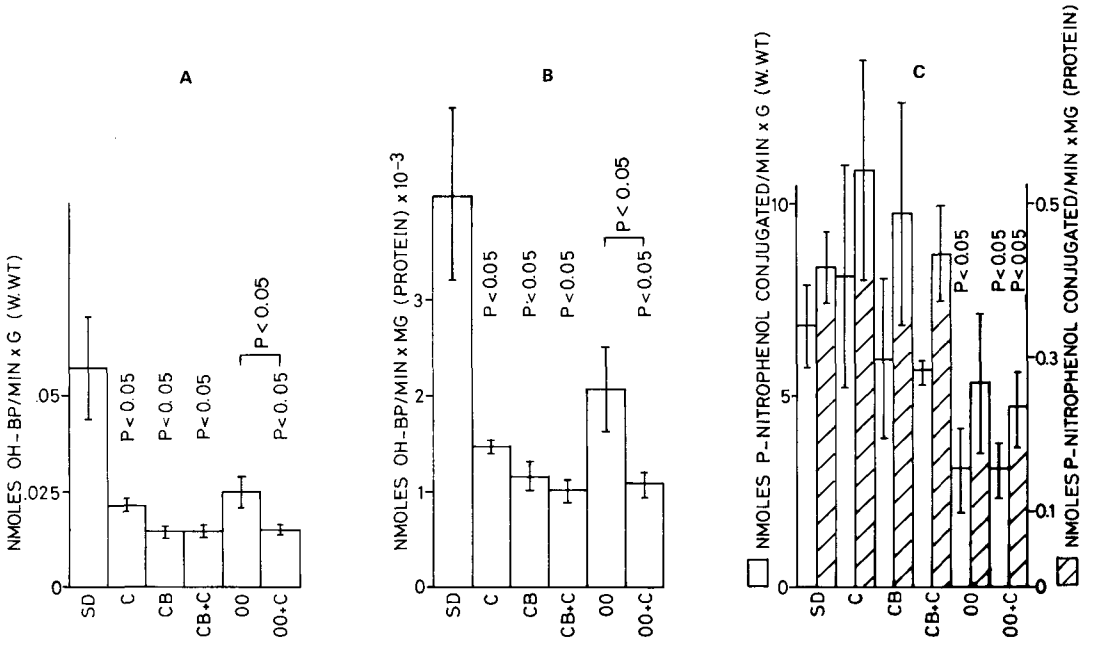

FIG. 3. The activities of aryl hydrocarbon hydroxylase and uridine diphosphate glucoronosyltransferase in duodenal mucosa of rats fed various lipid diets. The data are reported as means and standard errors of the means (vertical bars). (A) Represents the activity aryl hydrocarbon hydroxylase per g liver (wet wt), and (B) per mg microsomal protein. (C) Represents the activity of UDP glucuronosyltransferase/g liver (wet wt) □ or per mg microsomal protein ■. SD = standard diet; C = cholesterol; CB = cacao butter; OO = olive oil; CB+C = cacao butter plus cholesterol; OO+C = olive oil plus cholesterol. For other explanations, see Figure 1.

generating system as described above. The amount of microsomal protein was 1 mg in a final volume of 1 ml. The UDP glucuronosyltransferase activity was measured using uridine diphosphoglucuronic acid (ammonium salt, Sigma Chemical Co., St. Louis, Mo.), concentration of 4.5 mM, and p-nitrophenol (0.35 mM) as an aglycone (26,27). The Student's t-test was used to calculate statistical significances of the results.

RESULTS

The aryl hydrocarbon hydroxylase activity was highest in the hepatic microsomes of rats fed standard or cholesterol diets, and lowest (only 10-15% of the standard) in the rats fed cacao butter on cacao butter plus cholesterol diets (Fig. 1A,B). The aryl hydrocarbon hydroxylase activity in the group fed olive oil plus cholesterol was twice as high as the activity in these two groups whose diets were high in cacao butter or cacao butter plus cholesterol. In the groups fed olive oil or olive oil plus cholesterol, the rats fed the olive oil diet showed higher aryl hydrocarbon hydroxylase activity when calculated on the wet wt basis (Fig. 1A). On the contrary, the activity was higher in the group fed olive oil plus cholesterol diet, when calculated on the protein basis, (Fig. 1B). The p-nitroanisole 0-demethylase activity was highest in hepatic microsomes isolated from the rats fed cholesterol diet (Fig. 1 C,D). Other diets containing saturated (cacao butter) or unsaturated (olive oil) neutral fats, with or without cholesterol (including the standard diet), exhibited about 50% lower p-nitroanisole 0-demethylase activity than did the cholesterol-fed group. However, in the group fed cacao butter, cholesterol increased the activity slightly, i.e., to the same level as in rats fed diets rich in olive oil.

The hepatic UDP glucuronosyltransferase activity was measured in nonactivated microsomes and microsomes pretreated in vitro with trypsin. Trypsin treatment is known to increase the measurable UDP glucuronosyltransferase activity (17,24). In the present study, trypsin increased the measurable activity in the standard diet group ca. 10 fold. In groups of rats fed various lipid diets, the extent of activation was smaller, i.e., in the cholesterol-fed group, 6 fold; in the cacao butter-fed group, 4 fold; and in the olive oil plus cholesterol-fed group, and in olive oil fed-group, 3 fold (Fig. 2). The rats fed olive oil rich diet showed the lowest UDP glucuronosyltransferase activity when measured

with and without trypsin digestion (Fig. 2). The groups fed olive oil and cacao butter diets combined with cholesterol exhibited higher transferase activities than those groups fed olive oil and cacao butter diets without cholesterol.

The intestinal aryl hydrocarbon hydroxylase activity was highest in the rats fed standard diets, whereas, other groups exhibited activities 30-50% of that (Fig. 3 A,B). The intestinal UDP glucuronosyltransferase activity was highest in the standard diet and cholesterol groups (Fig. 3C). The rats fed neutral fat diets showed 50-80% of the activity found in the group fed standard diet (Fig. 3C). Also, the UDP glucuronosyltransferase activity was lowest in the group fed olive oil.

DISCUSSION

Paine and McLean (28) detected that 10% herring oil or olive oil had no effect on the hepatic aryl hydrocarbon hydroxylase activity, whereas, 5% olive oil enhanced it. On another study (29), it was found that 6-10% olive oil enhanced the hepatic mono-oxygenase system, compared with the data from the livers of rats fed fat free diet. Paine and McLean (28) found that a respective amount of herring oil and cacao butter enhanced renal aryl hydrocarbon hydroxylase activity in the kidneys. It might be that variations exist in the response of tissue to fatty diets. In the present study, the amount of fat in the diet was much higher (24-34% of the total wt of the diet) than in earlier studies (29,30). This increased amount of fat in the diet was found to lower the hepatic and intestinal mixed function oxidases independent of the degree of saturation of fatty acids in the diet. The response of UDP glucuronosyltransferase activity was somewhat different from the aryl hydrocarbon hydroxylase and p-nitroanisole 0-demethylase activities. It might be that lipid rich diets decreased the latency of UDP glucuronosyltransferase. Trypsin treatment did not enhance the activity as much in microsomes isolated from the lipid fed rats as from the controls. This may reflect changes in the membrane structure.

A clearcut difference was observed between the two hydroxylation reactions measured. Aryl hydrocarbon hydroxylase activity was lower in rats fed lipids diets, whereas, p-nitroanisole 0-demethylase activity exhibited a high level in rats fed cholesterol diet. The difference may be due to the different P-450 hemoproteins. Cytochrome P-450 is responsible for catalysis of p-nitroanisole 0-demethylase, and another hemoprotein, cytochrome P_1-450 or P-448, is needed for the catalysis of aryl hydrocarbon hydroxylase (30-32). It might be suggested that the enhancement of p-nitroanisole 0-demethylase activity by cholesterol is mediated through induction of cytochrome P-450, because the catabolism of cholesterol is mediated through this hemoprotein (33). The data obtained indicate that cholesterol has an important role in maintaining drug hydroxylation activity in the liver, although its role is less pronounced in the gastrointestinal tract. It is evident that the cholesterol diet enhanced p-nitroanisole 0-demethylase activity. However, no such effect was found when cholesterol was combined with fat diets.

In the preceeding paper (3), it became evident that lipid rich diets profoundly changed the composition of hepatic and intestinal microsomes. Moreover, they caused very marked microscopic changes in the liver structure by increasing the connective tissue components and by inducing hepatic cirrhosis. Extremely high proportion of lipids in the diet may cause hazardous effects on the hepatic structure and hepatic and intestinal function. Possibly, respective changes are the postulated atherogenic changes in cardiovascular system produced by lipids. Although the human diet rarely contains as high a proportion of lipids as in the present study in rats, it might be concluded from the present study and from the earlier reports (28,29) that excessive lipids in the diet may interfere with the hepatic and intestinal capacity to detoxify drugs. Thus, there might be harmful consequences in drug therapy if the possible exceptional diet of the patient has not been taken into account.

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Effect of Glucose Administration on Cholesterol and Bile Acid Metabolism in Rats

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ABSTRACT

Glucose administered to fasted rats caused a marked stimulation in hepatic cholesterogenesis and cholesterol 7α-hydroxylation, and an increase in biliary excretion of cholesterol and total bile acids. The excretion of cholic acid was not influenced during the first few hr after glucose administration, but was significantly increased after 5 hr. Chenodeoxycholic acid showed a similar change, but the increase was only ca. one tenth of that of cholic acid. The excretion of deoxycholic acid was markedly increased by 1 hr, but gradually decreased thereafter. Pretreatment with neomycin abolished the increase in deoxycholic acid by fasting and glucose administration. Other bile acid components showed no significant change. It thus was presumed that cholesterol endogenously synthesized in the liver was metabolized mainly to cholic acid. In contrast, exogenous cholesterol was metabolized mainly to chenodeoxycholic acid. During the period of the acute enhancement of cholic acid formation from the endogenous cholesterol, biliary excretion of deoxycholic acid was increased. This probably occurred through the depression of 7α-rehydroxylation of deoxycholic acid, or through the enhancement of microbial formation of deoxycholic acid in the lumen, and through the increase of intestinal absorption.

INTRODUCTION

Recently, it has been demonstrated that hepatic 7α-hydroxylation is well correlated with hepatic cholesterol synthesis (1-3). Because the reaction of cholesterol 7α-hydroxylation is regarded as a rate limiting step in bile acid synthesis in the liver (4-7), the enhancement of 7α-hydroxylation is considered to accelerate the subsequent reaction steps of bile acid synthesis. The pathway for bile acid formation from 7α-hydroxycholesterol in the liver branches off into two main routes, leading either to cholic acid or chenodeoxycholic acid (8). Usually, cholic acid is formed predominantly, but in some instances the formation of chenodeoxycholic acid increases. The ratio of cholic acid to chenodeoxycholic acid in the bile is known to vary under various conditions, by treatment with hormones (9-11) and drugs (12,13), in certain diseases (14), or according to race (15).

In the present experiment, hepatic cholesterogenesis, cholesterol 7α-hydroxylase activity, bile secretion, biliary excretion rates of cholesterol and bile acids, and their compositions were investigated in rats given an oral glucose administration after fasting for 36-40 hr.

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TABLE I

Effect of Glucose Administration on Hepatic Cholesterogenesis, Cholesterol 7α-Hydroxylation, and Biliary Excretion of Total Bile Acids and Cholesterol in Fasted Rats[a]

	Cholesterogenesis (nmoles/hr/g tissue)	Cholesterol 7α-hydroxylation (nmoles/hr/mg protein)	Bile volume (ml/hr/100 g body wt)	Biliary total bile acid (mg/hr/100 g body wt)
Control	5.52 ± 1.02[b] (14)[c]	0.36 ± 0.02[b] (14)	0.26 ± 0.02[b] (6)	2.73 ± 0.41[b] (6)
Glucose	14.88 ± 1.60[d] (14)	0.93 ± 0.14[d] (14)	0.26 ± 0.02 (8)	4.08 ± 0.33[d] (7)

[a] Two ml of 50% glucose solution/100 g body wt was administered orally after fasting for 36-40 hr. Rats were sacrificed 3 hr after glucose administration.

[b] Mean ± S.E.

[c] ( ) = No. of rats.

[d] Significantly ($P<0.05$) different from control level.

TABLE II

Hourly Changes in Bile Volume and Biliary Excretion of Total Bile Acids and Cholesterol after Glucose Administration in Fasted Rats[a]

	Hr after glucose administration				
	0	1	2	3	5
No. of rats	10	3	8	9	10
Bile volume (ml/hr/100 g body wt)	0.37 ± 0.02[b]	0.32 ± 0.05[b]	0.33 ± 0.03[b]	0.36 ± 0.02[b]	0.39 ± 0.01[b]
Total bile acids (mg/hr/100 g body wt)	3.20 ± 0.26	3.31 ± 0.58	4.00 ± 0.50	3.70 ± 0.47	5.16 ± 0.40[c]
Cholesterol (μg/hr/100 g body wt)	97 ± 4.3	85 ± 11.8	118 ± 9.0	119 ± 7.2[c]	128 ± 5.6[c]

[a]Two ml of 50% glucose solution/100 g body wt was administered orally after fasting for 36-40 hr. Bile was collected for 30-40 min after the hr indicated.

[b]Mean ± S.E.

[c]Significantly ($P<0.05$) different from values at 0 hr.

MATERIALS AND METHODS

Wistar strain male rats weighing ca. 200 g were used. They were kept in an air conditioned room (25 ± 1 C, 50-60% humidity), and maintained on a balanced commercial stock diet (Japan CLEA CA-1, Tokyo, Japan).

After fasting for 36-40 hr, 2 ml 50% glucose solution/100 g body wt was administered by stomach tube. Paired control rats were given water. Neomycin sulfate (Sigma Chemical Co. Ltd., St. Louis, Mo.) was dissolved in saline and administered orally at a dose of 25 mg/100 g body wt once a day for 3 consecutive days (including the day of experiment) before glucose administration.

Three hr after glucose administration, the rats were decapitated and the livers were perfused with 10 ml saline and remove immediately. Cholesterol synthesis activity in the liver was determined in vitro as reported previously (3). 7α-Hydroxylase assay was performed by the isotope incorporation method (3) based upon the isotope derivative method of Mitropoulos and Balasubramaniam (16). For bile acid analysis, PE-10 polyethylene tube was inserted into the common bile duct 1, 2, 3, or 5 hr after glucose administration. Bile was collected for 30-40 min under sodium pentobarbital anesthesia. Fasted, control rats served as 0 hr group without glucose administration. After hydrolysis of the bile samples with 1.25 N NaOH at 120 C, 15 psi for 6 hr, the sterol fraction was extracted with petroleum ether and cholesterol content was determined by colorimetry (17). The bile acid fraction, obtained by extracting with ethyl ether, after acidifing the hydrolysate with 2 N HCl, was analyzed by gas liquid chromatography as described previously (11). Statistical significance was estimated by the Student's t-test.

RESULTS

Table I shows in vitro hepatic cholesterol synthesis, cholesterol 7α-hydroxylation, bile volume, and biliary excretion of total bile acids 3 hr after oral administration of glucose to fasted rats. Hepatic cholesterogenesis and hydroxylation increased to as much as 3 times the control level. Although bile volume was not influenced by the administration of glucose, biliary excretion of total bile acids increased significantly ($P<0.05$).

Table II shows the excretion rates of biliary cholesterol and total bile acids at various times after glucose administration. Excretion of cholesterol was enhanced after 3 hr and remained high thereafter. Excretion of total bile acids gradually increased up to 5 hr, the longest time period examined.

Figure 1 shows two typical gas chromatograms of bile acid fractions from fasted control and glucose treated rats. As shown in this figure, the main constituent of biliary bile acid was cholic acid, which comprised 42% in fasted control rats and 38% in glucose treated rats. Deoxycholic acid was minor in control rats, but was markedly increased to comprise about 30% in glucose treated rats. The other components were not significantly altered.

The values in glucose treated rats, however, varied with time after ingestion. Table III shows the biliary excretion rates of individual bile acid fractions at various times after the treatment. The excretion of deoxycholic acid in nonfasted rats was as low as 0.32 mg/hr/100 g body wt, but was increased almost twice by fasting and

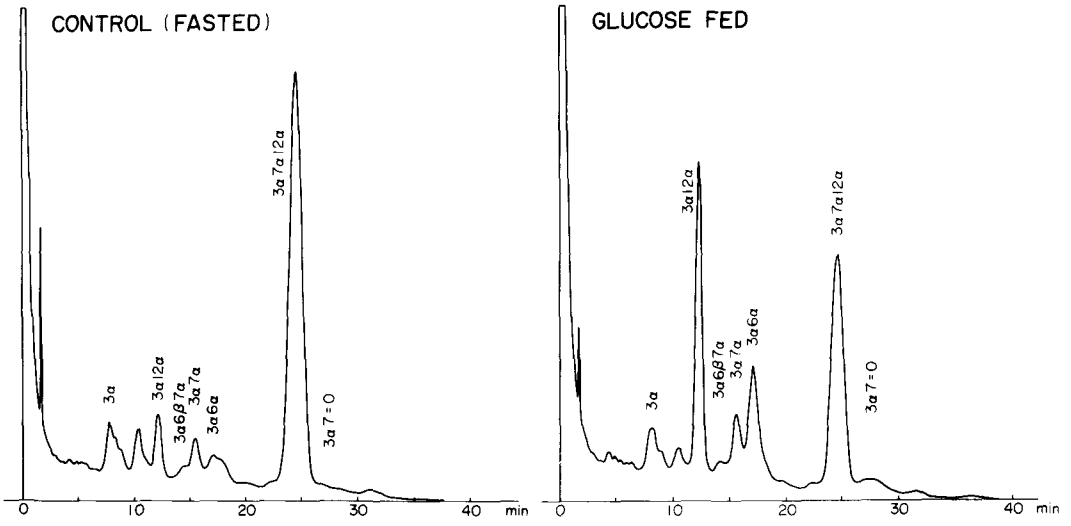

FIG. 1. Typical gas chromatogram patterns of bile acid fractions in the bile from fasted control and glucose treated rats. After fasting for 36-40 hr, rats were administered 2 ml of 50% glucose solution/100 g body wt by stomach tube, and then sacrificed 3 hr after the glucose administration.

TABLE III

Hourly Changes in Biliary Excretion of Individual Bile Acids after Glucose Administration in Fasted Rats[a]

	Hr after glucose administration				
	0	1	2	3	5
No. of rats	10	3	8	9	10
Bile acid excretion (mg/hr/100g body wt)					
Lithocholic	0.18 ± 0.03[b]	0.10 ± 0.03[b]	0.10 ± 0.03[b]	0.13 ± 0.03[b]	0.23 ± 0.03[b]
Deoxycholic	0.69 ± 0.06	1.19 ± 0.02[d]	1.18 ± 0.14[d]	1.03 ± 0.13	0.83 ± 0.07
Chenodeoxycholic	0.14 ± 0.02	0.11 ± 0.04	0.24 ± 0.04	0.28 ± 0.05	0.27 ± 0.03[d]
Hyodeoxycholic	0.33 ± 0.04	0.57 ± 0.10	0.61 ± 0.08	0.43 ± 0.10	0.64 ± 0.15
Cholic	1.35 ± 0.13	1.01 ± 0.45	1.55 ± 0.21	1.44 ± 0.24	2.57 ± 0.20[d]
Others[c]	0.52 ± 0.06	0.31 ± 0.09	0.33 ± 0.07	0.38 ± 0.06	0.63± 0.07

[a]Two ml of 50% glucose solution/100 g body wt was administered orally after fasting for 36-40 hr. Bile was collected for 30-40 min after the hr indicated.

[b]Mean ± S.E.

[c]Other bile acids comprise α-muricholic acid and keto bile acids.

[d]Significantly ($P<0.05$) different from value at 0 hr.

further by glucose administration. The highest value was obtained at 1-2 hr, then it decreased gradually over several hr. On the other hand, the excretion of cholic acid was somewhat decreased at 1 hr, but gradually increased thereafter and attained the highest level 5 hr after glucose administration. Chenodeoxycholic acid also increased later, but the amount of chenodeoxycholic acid was only ca. one tenth of cholic acid. Excretion of hyodeoxycholic acid was slightly enhanced at 2 and 5 hr after glucose ingestion. Lithocholic acid was not influenced significantly.

The increase of deoxycholic acid after glucose administration, however, was prevented by the treatment with neomycin. Table IV shows the effect of neomycin on the composition ratios of deoxycholic and cholic acids in fasted control and glucose treated rats. Fasting alone increased deoxycholic acid content, and it was further increased by glucose administration, as shown in Table III and Fig. 1. As shown in Table IV, pretreatment with neomycin prevented the increase of deoxycholic acid by fasting or glucose administration, and brought about a reciprocal increase in cholic acid.

TABLE IV

Effect of Neomycin on Composition Ratios of Deoxycholic Acid and Cholic Acid in Fasted Control and Glucose Fed Rats[a]

	Control		Neomycin[b]	
	Deoxycholic (%)	Cholic (%)	Deoxycholic (%)	Cholic (%)
Fasted control	22.0 ± 1.44[c] (10)[d]	42.1 ± 1.91[c] (10)	2.8 ± 0.53[c] (5)	72.1 ± 1.16[c] (5)
Fasted glucose	29.4 ± 2.74 (9)	37.8 ± 3.09 (9)	3.0 ± 0.43 (5)	71.5 ± 2.48 (5)

[a]Two ml of 50% glucose solution/100 g body wt was administered orally after fasting for 36-40 hr. Rats were sacrificed 3 hr after glucose administration.

[b]25 mg/100 g body wt/day was administered orally for 3 days before glucose administration.

[c]Mean ± S.E.

[d]( ) = No. of rats.

DISCUSSION

It is thought that cholesterol synthetic enzymes in the liver collaborate with cholesterol 7α-hydroxylase, which is the rate limiting enzyme for bile acid synthesis (1-3). Bile acid removal by biliary drainage or cholestyramine administration enhances cholesterol synthesis as well as cholesterol 7α-hydroxylation in rats (4-6). The data presented here clearly demonstrate that the administration of glucose to fasted rats increased biliary excretion of cholesterol and total bile acids, as well as enhancing both cholesterol synthesis and 7α-hydroxylation in the liver. Consequently, it is concluded that increased cholesterol 7α-hydroxylase activity brings about a stimulation of the subsequent reaction steps of bile acid formation. Myant and Eder (18) also suggested that an increase in cholesterol synthesis accelerates biliary excretion of bile acid, while the inhibition of hepatic cholesterol synthesis reduces bile acid excretion (12,13,19). Recently, Mitropoulos, et al., (20) demonstrated that bile acid excretion from bile fistula of rats has a diurnal variation, and that fluctuation in bile acid excretion is well synchronized with the change of β-hydroxy β-methylglutaryl (HMG) CoA reductase and cholesterol 7α-hydroxylase activity.

Although biliary excretion of cholesterol is increased by the stimulation of hepatic cholesterol synthesis (21), the increment of bile acids is much larger than that of cholesterol. In the present experiment, bile acid excretion at 5 hr after glucose administration was 2.04 mg/hr/100 g body wt over the control value, while the increase of cholesterol excretion was 43.4 μg/hr/100 g body wt. The increase of bile acid was about 50 times that of cholesterol when compared on the same molar basis. Thus, it can be concluded that the increase in bile acid excretion has more significance for sterol degradation following enhanced hepatic cholesterogenesis than cholesterol excretion does.

Biliary excretion of deoxycholic acid was markedly increased as early as 1 hr after the treatment with glucose. The level thereafter remained high for several hr with only a gradual decrease. With regard to the increase of deoxycholic acid excretion, the following are conceivable: (A) an inhibition of 7α-rehydroxylation of deoxycholic acid by the liver; (B) an increase in the formation of deoxycholic acid by intestinal microflora; and (C) an increase in the absorption of the bile acid through the intestinal wall. The increase of deoxycholic acid 1 hr after glucose administration is, however, slightly faster than the time of one enterohepatic circulation of bile acid, which is reported to be 10-12 times/day (22,23).

Neomycin administration completely suppressed the increase of biliary deoxycholic acid by glucose administration and brought a reciprocal increase in cholic acid. Samuel, et al., (24) reported that in vitro formation of deoxycholic acid from cholic acid by human fecal microflora was inhibited by previous oral administration of neomycin. However, another explanation for the effect of neomycin is that it combined with bile acids to prevent their intestinal absorption, thereby, increasing fecal bile acid excretion and, consequently, decreasing biliary excretion of deoxycholic acid (25,26).

Although it is known that deoxycholic acid reabsorbed from the intestinal tract is rehydroxylated to cholic acid in rats (27), the increase of biliary cholic acid excretion observed at a later stage is derived from hepatic cholesterol. This increase results from the stimulation of hepatic cholesterol synthesis, because the increase of cholic acid is far larger than the decrease of deoxycholic acid (Table

III). This increase of cholic acid formation after glucose feeding is in contrast to the increase of chenodeoxycholic acid formation which is observed after cholesterol feeding (11,28). Thus, it is conceivable that endogenously synthesized cholesterol is metabolized to bile acid, mainly through the cholic acid pathway, and that exogenous cholesterol is degraded through the chenodeoxycholic acid pathway. Recently, Mitropoulos, et al., (29) have indicated that newly synthesized cholesterol in the liver is a preferred substrate for the formation of cholic acid in rats. Our conclusions correspond well with this finding.

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Occurrence of 7-Methyl-7-Hexadecenoic Acid, the Corresponding Alcohol, 7-Methyl-6-Hexadecenoic Acid, and 5-Methyl-4-Hexadecenoic Acid in Sperm Whale Oils

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ABSTRACT

Two sperm whale oils from the northern hemisphere and two from the southern hemisphere were fractionated. Triglyceride and wax esters were examined for fatty acids and alcohols with monoethylenic unsaturation bearing a methyl branch on an ethylenic carbon. The 7-methyl-7-hexadecenoic acid (0.37-1.37%) was accompanied by the corresponding alcohol (0.28-0.72%), but these materials were not accompanied by shorter chain homologues. The 7-methyl-6-hexadecenoic acid was relatively less important (0.23-0.68%), but was accompanied by 5-methyl-4-hexadecenoic acid (0.10-0.39%), and a partially identified C_{13} compound. Chromatographic properties on silver nitrate impregnated silicic acid TLC and on three GLC liquid phases are reported.

INTRODUCTION

The positive identification of 7-methyl-7-hexadecenoic acid in the liver oil from the ocean sunfish (*Mola mola*) (1) created a problem because 7-methyl-6-hexadecenoic acid had been conclusively demonstrated as a component in the oils of fin whales (*Balaenoptera physalus*) and sperm whales (*Physeter catodon*) (2). The respective isolations appeared to be free of other isomeric components, but two such isomers occurring in a pure form in marine oils seemed unusual. We have examined four sperm whale oils for components of this type, including both fatty acids and alcohols, and have found the two isomeric C_{17} acids to be present in varying proportions in the different oils. Only the 7-methyl-6-hexadecenoic acid was reflected in the occurrence of 5-methyl-4-tetradecenoic acid, although it is usually the minor C_{17} isomer. A C_{13} methyl branched acid is also present. The fatty alcohols were also examined and found to contain 7-methyl-7-hexadecanol with virtually no trace of the other isomer or lower homologues.

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EXPERIMENTAL PROCEDURES

Four commercial sperm whale oils were used for this study. Two originated in Canada near Halifax, Nova Scotia, and St. John's Newfoundland, one near Albany, Australia, and one from Antarctica. The sperm whale oils were resolved into triglycerides and wax esters by TLC on Prekote (Adsorbosil-5) plates (Applied Science Laboratories, State College, Pa.) developed in petroleum ether:diethyl ether:acetic acid (85:15:1).

The basic isolation techniques differed slightly in parts from those outlined previously (1). Triglycerides were saponified, and the liberated fatty acids recovered. AOCS method Ca-6b-53 was used to separate and recover fatty acids and fatty alcohols from wax esters. The fatty acids were warmed with urea (5 parts) and methanol (10 parts), and the solution was cooled slowly in a domestic refrigerator. The non-urea-complexing fatty acids (20-30% of total) were recovered and converted to methyl esters by refluxing for 10 minutes with 7% BF_3 in methanol.

Argentation TLC of methyl esters of non-urea-complexing acids on silica gel plates impregnated with silver nitrate (Supelcosil-12D, Supelco, Inc., Bellefonte, Pa.) was carried out in two stages. The first development of streaked methyl esters with benzene:n-hexane (1:1) gave a broad band of R_f = 0.32-0.40, above the normal monoethylenic ester band and below the saturated ester band. This band was extracted with chloroform:diethyl ether (1:1) and re-run on similar plates with three successive developments in benzene:n-hexane (3:7). Three bands recovered for analytical and preparation GLC were observed. Basically, the same technique was followed for the isolation of fatty alcohols as acetates.

Oxidative fission of isolated compounds was carried out on methyl esters or alcohol acetates with oxidative workup (3). The acidic products of methyl esters were esterified with BF_3-MeOH and those of alcohol acetates with diazomethane.

NMR spectra of isolates in deuterated chloroform were taken with a Varian A-60 spectrometer. Mass spectra were done with a DuPont CEC 21-110B mass spectrometer

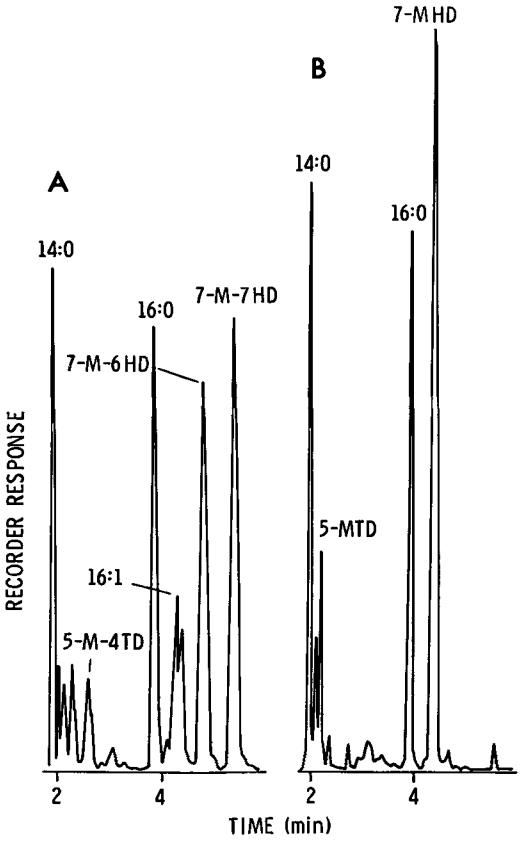

FIG. 1. Concentrate of methyl branched monoethylenic fatty acid methyl esters from exploratory, single stage $AgNO_3$-TLC separation of total fatty acids of St. John's sperm whale oil. Analyses on butanediol succinate (BDS) column at 170 C: A = before hydrogenation; B = after hydrogenation. Note disappearance of 7-methyl-7-hexadecenoate (7-M-7HD) and 7-methyl-6-hexadecanoate (7-M-6HD) from A, and reappearance as 7-methylhexadecanoate (7-MHD) in B. Materials labeled 16:1 disappear and augment 16:0. Similarly, 14:1 isomers (not identified) augment 14:0 in B. The 5-methyl-4-tetradecenoate (5-M-4TD) becomes 5-methyltetradecanoate (5-MTD), possibly augmenting some pre-existing material in this position. Saturated acids originated as part of corresponding $AgNO_3$-TLC band.

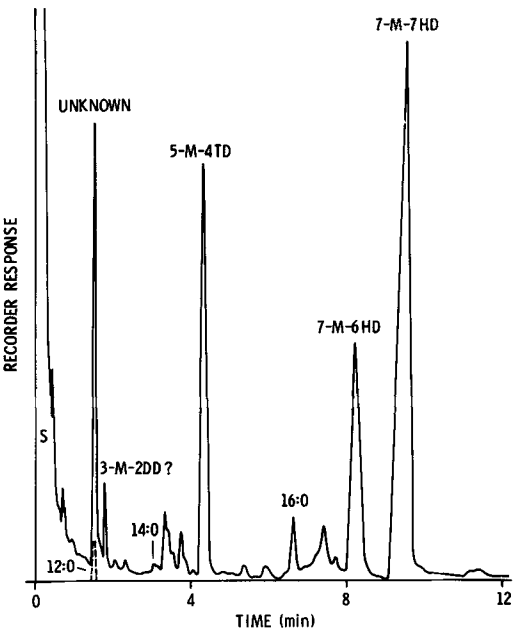

FIG. 2. Concentrate of mixture of methyl branched monoethylenic fatty acid methyl esters from methyl esters of wax ester fatty acids from Antarctic sperm whale oil. In the broad band, R_f = 0.32-0.40 after primary development on $AgNO_3$-TLC with benzene:n-hexane (1:1). Note precise coincidence of 12:0 and unknown for butanediol succinate (BDS) analysis. Conditions were similar to those of Figure 1, but run on a different column.

operated at 70 eV with a variable temperature probe used as ca. 30 C.

Analytical GLC was carried out with Perkin-Elmer Model 226 or Model 900 gas chromatographs. Columns were stainless steel, open tubular, wall coated, 150 ft (46 m) in length and 0.01 in. (0.25 mm) internal diameter (ID). When coated with butanediol succinate (BDS) they were operated at 170 C and 50 psig He for higher methyl esters, 150 C or 120 C for shorter chain lengths. Oxidative fission degradation products (e.g., 2-undecanone, methyl nonanoate) were examined at 120 C. Columns coated with SILAR-5CP and Apiezon-L were operated at 175 C and 190 C, respectively. Preparative GLC was affected with a stainless steel column 6 ft (2 m) in length and ¼ in. (6.4 mm) outside diameter (OD), packed with 5% SE-30 on Chromosorb Q, 80-100 mesh, operated at 190 C. Collection was in glass tubes surrounded by a reheat coil to reduce fogging losses.

RESULTS

The unusual fatty acids isolated from sperm whale oil included an isomer of 7-methyl-7-hexadecenoic acid (Fig. 1) and unexpectedly, a shorter chain fatty acid of similar physicochemical behavior (Fig. 2, Table I).

The identification of an isolate from one of the sperm whale oils as methyl 7-methyl-6-hexadecenoate was based partially on a mol wt of 282 obtained by mass spectrometry on an isolate with purity >90%. Major fragments of 29, 74, 115, 138, 155, etc., were similar to those reported by Sano for this isomer (2), although these were found with 7-methyl-7-hexadecenoate, and for reasons discussed elsewhere, (1), were not conclusive proof of struc-

TABLE I

	R_f (by silver nitrate silica gel TLC)		Equivalent Chain Length (by GLC)					
	Multi-development		BDS (170 C)[a]		Silar-5CP (175 C)[a]		AP-L (190 C)[a]	
	Acid methyl ester[b]	Alcohol acetate	Acid methyl ester[c]	Alcohol acetate	Acid methyl ester[c]	Alcohol acetate	Acid methyl ester[c]	Alcohol acetate[d]
7-methyl-7-hexadecenoate	0.42	0.39	16.90	16.80	16.92	16.90	16.62	16.60
7-methyl-6-hexadecenoate	0.48	--	16.59	--	16.62	--	16.20	--
5-methyl-4-tetradecenoate	0.53	--	14.88	--	14.90	--	14.50	--
Unknown	0.53	--	~12.00[e]	--	~12.00	--	~12.00	--
7-methylhexadecenoate	--	--	16.30	--	16.30	--	16.28	--
5-methyltetradecanoate	--	--	14.28	--	14.28	--	14.30	--
3-methyldodecanoate (authentic)	--	--	~12.13[e]	--	12.07	--	12.36	--

[a]BDS = butanediol succinate; Silar-5CP = 50 phenyl 50 cyanopropyl; AP-L = Apiezon-L.
[b]R_f of 12:0 = 0.7.
[c]Reference standard methyl esters.
[d]Reference standard alcohol acetates.
[e]At 120 C on butanediol succinate (BDS) the equivalent chain length (ECL) value was 12.12 for the unhydrogenated compound, ca. 12.0 for the hydrogenated compound.

ture. Hydrogenation gave a product of m/e 284 with only one peak at equivalent chain length (ECL) 16.30 on open tubular GLC on BDS. This corresponded precisely to that obtained by hydrogenation of 7-methyl-7-hexadecenoate, and gave the same pattern on mass spectroscopy as 7-methylhexadecanoate (1). The ECL value was useful but nonspecific for identification (4). Oxidative fission of the methyl ester of the isolated acid yielded 2-undecanone as the only important product other than dimethyl adipate. NMR showed absorption centered at $\delta = 5.38$, with a shoulder at 5.40.

The 5-methyl-4-tetradecenoate had a mol wt of 254, and, on hydrogenation, the product gave one GLC peak with an ECL of 14.28 on BDS. This was also nonspecific for identification purposes, but among other possibilities (4), was attributable to a 5-methyl branch. The hydrogenated product of m/e 256 had a mass spectrum corresponding to 5-methyltetradecanoate (5). The NMR spectrum of the original isolate indicated through the signal at $\delta = 5.33$, which showed a single olefinic proton, that the methyl branch was attached at an ethylenic bond. A major ozonolysis product was again 2-undecanone, accompanied by some dimethyl succinate. The products examined by GLC did not include methyl nonanoate, a potential product had 5-methyl-5-tetradecenoate been present.

A third, minor, unknown material presented difficulties in collection and purification. One isolate had a mol wt of 226 by mass spectrometry, indicating that a methyl branched monounsaturated fatty acid structure homologous with the above was entirely possible.

The details of the mass spectrum differed from those of authentic methyl-3-methyldodec-2-enoate, and the GLC retention data (Table I) also differed from that of authentic reference acids or data calculable from phytenic acid isomers (6). We found that authentic 3-methyl-*trans*-2-dodecenoic acid was not isomerized by esterification with 7% BF_3 in methanol (sealed tube, 20 min at 100 C), although vigorous saponification reportedly causes such reactions (6,7). An artifact 3-methyldodecenoic acid is suggested as a possibility by GLC data for phytenic acids (6). The GLC porperties of the unknown compound and hydrogenation product (Table I) were quite unusual. At high temperatures (170 C) the position of the unknown compound was extremely close to the position of 12:0 on the polar liquid phases; but on reducing the temperature to 120 C, the ECL value on BDS became 12.12. As the ECL of the hydrogenation product was 12.36 at 190 C on Apiezon L, these values

clearly supported the possibility that the methyl branch was on the third carbon from the carboxyl group (4,8). This was verified by mass spectral examination of the hydrogenated product which gave fragments of m/e 74 (=73+1), 101, absence of M-29, etc., expected for this structure (5,9). At an intermediate stage of purification (Fig. 2), a minor peak appeared in ca. the correct position for methyl 3-methyl-2-dodecenoate, but it was not isolated.

Only one alcohol with methyl branched ethylenic unsaturation, 7-methyl 7-hexadecen-1-ol, was found in amounts readily detectable in the four sperm whale oils (Table II). Identification was restricted to the isolation techniques, hydrogenation, and the results of oxidative fission, which gave methyl nonanoate and a C_8 keto alcohol as the principal products. The location of an appropriate alcohol-acid product in GLC analyses can be predicted ca. by GLC of comparison standards obtained by oxidative ozonolysis of the alcohols produced from rapeseed oil fatty acids. This approach gave reference C_9, C_{11}, and C_{13} alcohol-acids. On this basis the alternative 7-methyl-6-hexadecen-1-ol appeared to be absent. The keto alcohol was identified only by calculation of its ECL value as previously carried out for the keto acid (1).

DISCUSSION

The chromatographic properties of the methyl esters of the various methyl branched monounsaturated acids are given in Table I. The greater mobility on argentation TLC of the 5-methyl-4-tetradecenoate, relative to the 7-methyl hexadecenoates, is in accord with the variation in R_f observed for monoethylenic 18:1 isomers (10). However it has also been our experience that a lesser proportion of methylene carbons to ordinary ethylenic unsaturation reduces mobility (11). The moderately sharp distinction between the argentation TLC mobilities of the 7-methyl-7-hexadecenoate and 7-methyl-6-hexadecenoate was unexpected, and can perhaps be explained by an inductive effect linking the carbonyl group and the ethylenic unsaturation in the 6 position. This would be a common attribute, shared more emphatically by the 5-methyl-4-tetradecenoate and by any 3-methyl-2-dodecenoate, and accounting for their greater mobility. Of the structurally similar phytenic acids 3 show a high mobility, e.g., R_f =0.62 for the *cis*-Δ^2isomer, and by coincidence, R_f = 0.53 for the *cis*-Δ^3isomer (6). The 7-methyl-7-hexadecenoate unsaturation would act much like an unsubstituted ethylenic bond and interact more strongly with the silver ions.

TABLE II

Wt Percentages of Methyl Branched Monounsaturated Acids and a Related Alcohol in Fractions of Sperm Whale Oils

	Southern hemisphere						Northern hemisphere					
	Antarctic			Albany			Halifax			St. John's		
	TG[a]	Wax ester		TG	Wax ester		TG	Wax ester		TG	Wax ester	
	Acids	Acids	Alcohols	Acids	Acids	Alcohols	Acids	Acids	Alcohols	Acids	Acids	Alcohols
7-methyl-7-hexadecenoic (enol)	1.07	0.96	0.28	1.08	0.77	0.34	1.37	1.02	0.72	0.37	0.37	0.34
7-methyl-6-hexadecenoic	0.46	0.35	--	0.68	0.23	--	0.35	0.58	--	0.35	0.27	--
5-methyl-4-tetradecenoic	0.23	0.35	--	0.20	0.38	--	0.14	0.39	--	0.11	0.10	--
Unknown	0.38	0.10	--	0.24	0.92	--	0.15	0.94	--	0.11	0.19	--

[a]TG = triglyceride.

The GLC properties of the methyl esters of the various unsaturated isomeric acids show disparities also probably related to the position of the methyl substituted ethylenic unsaturation. There is otherwise no reason to expect a large retention time difference between the two C_{17} isomers on the polar liquid phases. The 3-methyl branch has little effect on retention time of polar liquid phases, compared to other methyl branches (4), resulting in a low fractional chain length (FCL) value (0.00-0.13). This element may be partly responsible for the low ECL value of the unknown.

The occurrence of only 1 significant methyl branched unsaturated alcohol (Table I) was unexpected. The proportions of this alcohol, although less than for the corresponding wax esters acids in 3 out of 4 oils, were relatively large enough to suggest that 3 other compounds detected as acids, if present at all, must be $\leqslant$0.05% of total fatty alcohols.

It is not known if these fatty acids and this fatty alcohol are of dietary origin or endogenous to sperm whales. The purity of the 7-methyl-7-hexadecenoic acid from the liver oil of the ocean sunfish (*Mola mola*), in which alcohols are not important (13), suggests that this acid could be the original animal biochemical product. Several other saturated monomethyl C_{17} isomers have been found in a whale oil, but 7-methyl substitution dominated the mixture despite the observation that $\Delta^{6,7}$ ethylenic unsaturation was very minor in the 16:1 acids (14). In the sperm whale, a part of this production could be diverted into fatty alcohol form as it was produced. The less important 7-methyl-6-hexadecenoic acid may be a secondary product. It and the lower homologous acid are mutually related, and evidently participate freely in chain length changes in the general saturated, monoethylenic fatty acid pool. Presumably, they may also be involved with, or are part of, the presence of an optically active 3-methyldodecenoic acid isolated from sperm whale oil (9). and indicated as a methyl branched C_{12} acid in other analysis of sperm whale oil (14). Because the C_{17} acid and alcohol with $\Delta^{7,8}$ unsaturation do not participate in chain shortening in the acid or alcohol pools, they must have a different biochemical significance.

Our results show that these acids and the alcohol are of world-wide distribution. The blood of the Atlantic bottlenose dolphin (*Tursiops truncatus*) also has a component tentatively identified as 7-methyl-7-hexadecenoate (16). Although this animal is also a toothed whale, the finding of Sano (2) that 7-methyl-6-hexadecenoic acid occurred in fin whale blubber oil indicates that this is not a peculiarity of the toothed wales. The lower homologue, 5-methyl-4-tetradecenoate, may also have been present in the bone oil from the blue whale (*Balaenoptera musculus*) (17) as well as in fin whale oil (18). A branched chain fatty alcohol, probably 7-methyl-7-hexadecenol, was obtained in a concentrate of alcohols from sperm whale oil by Sano (19), but not characterized. Although the origin and significance of this group of acids remains obscure, it does appear that they may be fairly widespread in some aquatic lipids, and that 7-methyl-6-hexadecenoic acid and its lower homologues may be concentrated into depot fats high in 12:0, 14:0, 16:0, and 16:1, simply because of a presumption of biochemical inertness.

ACKNOWLEDGMENTS

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Role of Cytidine Triphosphate and Cytidine Diphosphate in Promoting Inositol Entry into Microsomal Phosphatidylinositol[1]

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ABSTRACT

The Mn^{2+} activated incorporation of myo-inositol-^{3}H into subfractions of phosphatidylinositol in rat liver microsomes was studied in the presence and absence of cytidine triphosphate or cytidine diphosphate choline using phosphate buffer. The distribution of labeled inositol among molecular species of microsomal phosphatidylinositol was also investigated in vivo. In other experiments, the release of radioactivity from microsomes labeled with inositol-^{3}H in the phospholipid was measured after the addition of Mn^{2+}, unlabeled inositol, and cytidine nucleotide. Similar chase experiments were conducted with microsomes containing phosphatidylcholine-^{14}C or phosphatidylethanolamine-^{14}C. The addition of cytidine triphosphate or cytidine diphosphate choline stimulated the rate of inositol-^{3}H entry into microsomal phosphatidylinositol by 3.5 to 4-fold and the monoenoic plus dienoic, trienoic, tetraenoic, and polyenoic species contained 6-7, 6, 78-81, and 7-9%, of the radioactivity, respectively. These latter patterns were very similar to those observed among the corresponding molecular species when the Mn^{2+} stimulated entry of free inositol into phospholipid was studied in the absence of added cytidine nucleotide. In chase experiments, the release of radioactivity from phospholipid in the presence of cytidine triphosphate or cytidine diphosphate choline was greatly enhanced by the addition of free inositol when microsomes containing phosphatidylinositol-^{3}H, but not phosphatidylcholine-^{14}C or phosphatidylethanolamine-^{14}C, were employed. Therefore, under the present conditions, cytidine triphosphate and cytidine diphosphate choline appear to stimulate the entry of inositol into phosphatidylinositol by enhancing the Mn^{2+} activated exchange reaction in rat liver microsomes. The results suggest further that phosphatidylinositol is the preferred substrate when this reaction is stimulated by cytidine nucleotide.

INTRODUCTION

It is well established that free inositol can be incorporated into phosphatidylinositol either by reacting with cytidine diphosphate diglycerides (CDP-diglycerides) derived from cytidine triphosphate (CTP) and phosphatidic acid (1-5) or by a microsomal exchange reaction (1,6). The former pathway provides for the de novo biosynthesis of phosphatidylinositol (PI), while the latter pathway would give rise to no net synthesis of this phospholipid if microsomal PI was the endogenous substrate, as has been suggested (1). The phospholipid substrate(s) for this exchange reaction have not been established (7,8).

Recent investigations (9) have shown that the distributions of radioactivity among the various molecular species of PI due to the Mn^{2+} dependent entry of free inositol-^{3}H in rat liver microsomes were markedly different from those observed soon after the in vivo administration of inositol-^{3}H. The products of the Mn^{2+} stimulated exchange reaction were largely tetraenoic species of PI (9), whereas the CDP-diglyceride pathway apparently produced mainly monoenoic plus dienoic and tetraenoic molecules (9-11). These results suggested that, under physiological conditions, the major proportion of free inositol entering the various phosphatidylinositols in rat liver microsomes apparently does so by reaction with CDP-diglycerides, and not by the Mn^{2+} stimulated exchange reaction (9).

There have been numerous reports in the literature (1,2,5,7) indicating that the entry of free inositol into microsomal PI in the presence of divalent cations can be greatly enhanced by the addition of CTP or even CDP-choline in the absence of added phosphatidic acid. However, there is considerable uncertainty as to the mechanism responsible for the stimulatory effect of CTP under such in vitro conditions. Some investigators have suggested that the added nucleotide may have been required for the in situ synthesis of CDP-diglyceride from endogenous phosphatidic acid (5), which could contribute to de novo biosynthesis of PI from

[1]Presented in part at the AOCS Fall Meeting, Philadelphia, October 1974.

TABLE I

Distribution of Inositol-^{3}H among Molecular Species of Microsomal Phosphatidylinositol in Vivo and in Vitro[a]

Chemical classes	Mole-% of total	Distribution of radioactivity (%)		Relative specific activity[c]	
		In vivo	In vitro[b]	In vivo	In vitro[b]
Monoenes + Dienes	8.7 ± 1.0	35.5 ± 3.1	3.9 ± 0.3	4.06 ± 0.36	0.44 ± 0.03
Trienes	9.8 ± 1.0	3.8 ± 0.1	6.9 ± 0.5	0.38 ± 0.01	0.70 ± 0.05
Tetraenes	67.5 ± 1.7	47.8 ± 4.4	83.0 ± 0.7	0.71 ± 0.07	1.23 ± 0.01
Polyenes	14.0 ± 0.7	13.0 ± 1.5	6.2 ± 0.9	0.93 ± 0.11	0.44 ± 0.07

[a]All values are given as means ± S.E. The compositional data are from 7 independent analyses, while the isotopic results are from 4 (in vivo) or 6 (in vitro) determinations.

[b]Incubations were conducted for 20-60 min in the presence of $MnCl_2$ (2 mM), inositol-^{3}H (0.67-6.7 mM), and 6.2 mg microsomal protein as described in Materials and Methods.

[c]Relative specific activity is defined as the % of total radioactivity in the class, divided by the mole-% contribution of the class to the total mixture.

free inositol. Alternatively, it has been suggested that the nucleotides may stimulate inositol entry into microsomal PI by enhancing the exchange reaction (1,2). The purpose of the work described herein was to determine the likely mechanism by which cytidine nucleotides enhance the entry of inositol into PI in rat liver microsomes. This question was approached in part by comparing the molecular species of PI formed from free inositol in the presence or absence of nucleotide coenzymes. Furthermore, experiments were conducted to define the nature of the phospholipid substrate for the microsomal exchange reaction.

MATERIALS AND METHODS

Materials

Myo-inositol-2-^{3}H (2800 mc/mmole), myo-inositol-U-^{14}C (215 mc/mmole), choline-1,2-^{14}C chloride (21 mc/mmole), and ethanolamine-1, 2-^{14}C hydrochloride (6.3 mc/mmole) were purchased from the New England Nuclear Corp., Boston, Mass. For the in vitro studies, the radioactive inositol was diluted with unlabeled carrier myo-inositol (Calbiochem, La Jolla, Calif.). CTP and CDP-choline were obtained from Calbiochem. Lipid standards for TLC were purchased from Serdary Research Laboratories (London, Ontario, Canada) and Applied Science Laboratories (State College, Pa.). All chemicals and solvents were of analytical grade.

Animals and Incubation Procedures

The animals used in these studies were male Wistar rats weighing 150-170 g. They were maintained as described (9) until sacrificed. For the in vivo experiments, the animals were injected intraperitoneally with 50 μc of a tritiated inositol solution (0.3 ml) and killed by cervical fracture after 45 min. When choline-^{14}C or ethanolamine-^{14}C was used, 25 and 50 μc, respectively, were administered 60 min prior to time of sacrifice. All animals were sacrificed after fasting for 17 hr and microsomal fractions were prepared as described elsewhere (9).

The incubation medium used to study the microsomal incorporation of myo-inositol into phosphatidylinositol in the presence of cytidine nucleotides was as follows unless described otherwise in the text. The medium contained 50 mM phosphate buffer (pH 7.4), 2 mM $MnCl_2$, 0.67-1.67 mM myo-inositol-^{3}H (2-7 x 10^5 cpm) or 0.67-1.67 mM myo-inositol-^{14}C, 2 mM CTP or 2 mM CDP-choline, and 1.3-6.2 mg microsomal protein in a total volume of 1.5 ml. The reaction was started by the addition of enzyme, and usually was conducted for 60 min at 37 C.

Lipid Analyses

At the end of the incubation, the lipids were extracted immediately, and the individual phospholipids were isolated using methods similar to those outlined previously (9). Aliquots of the purified lipid extract, as well as the upper aqueous phase, also were taken for measurement of radioactivity (9) in Aquasol (New England Nuclear Corp.). The molecular species of PI were separated by means of argenation TLC into monoenoic plus dienoic, trienoic, tetraenoic, and polyenoic (> tetraenoic) subclasses (10). The various fractions were visualized under ultraviolet light, eluted from the silica gel, and the associated radioactivity was determined by scintillation counting (9-11). In other experiments, PI from unincubated microsomes was separated into its respective species and the fatty acid composition of the total PI plus the individual fractions were determined by

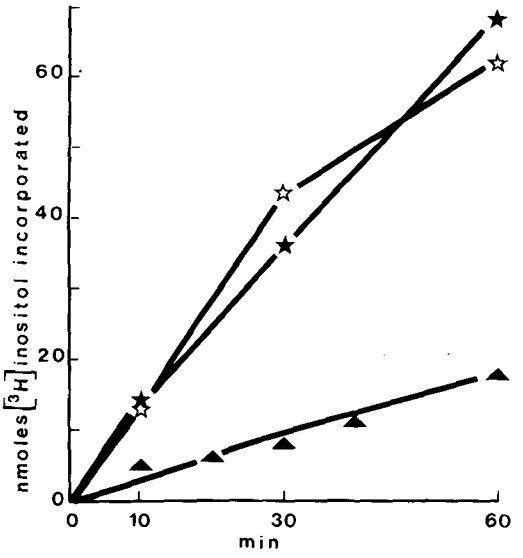

FIG. 1. The effect of cytidine triphosphate (CTP) and cytidine diphosphate choline (CDP-choline) on the incorporation of inositol into phosphatidylinositol. Incubation mixtures contained $MnCl_2$ (2 mM), inositol-^{3}H (1.67 mM), and 6.2 mg microsomal protein. ▲ = No added cytidine nucleotide; ★ = CTP added; ☆ = CDP-choline added.

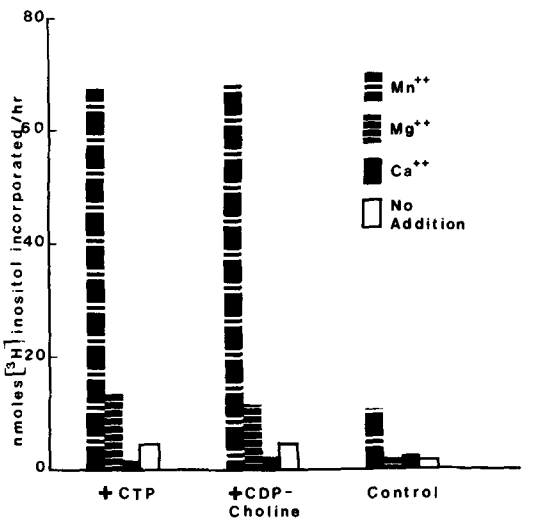

FIG. 2. The effect of various metal ions on the incorporation of inositol into phosphatidylinositol in the presence or absence of cytidine nucleotides. $MnCl_2$, $MgCl_2$, or $CaCl_2$ was added at 2 mM. Incubation mixtures also contained inositol-^{3}H (0.67 mM) and 6.2 mg microsomal protein. Incubation time was 60 min.

quantitative GLC (9) after transmethylation of the eluted phosphatides with 6% H_2SO_4 (by vol) in methanol at 75 C in the presence of known amounts of pentadecanoic acid as internal standard. Statistical analyses were conducted using Student's t-test (12).

In some experiments, aliquots of the upper phase of total lipid extracts were concentrated, a suitable carrier added (free inositol, glycerophosphorylinositol, etc.), and free inositol was separated by means of TLC on Silica Gel G using double development in chloroform: methanol:acetic acid:water (50:25:7:3) as the solvent (13). Staining of appropriate markers applied to the outer edges of each chromatogram was affected by spraying with silver nitrate solution (14) after removing corresponding regions, which contained the radioactive samples, for scintillation counting.

RESULTS

Table I gives the quantitative contribution of the various chemical classes to the total PI isolated from rat liver microsomes. The monoenes plus dienes, trienes, tetraenes, and polyenes represented 9, 10, 67, and 14%, respectively, of the total PI. The proportions of the four subclasses and their fatty acid compositions were in excellent agreement with other work (9). Table I also gives the percentage distribution of radioactive inositol among the various molecular species of microsomal PI of rat liver both in vivo and in vitro. In confirmation of earlier in vivo studies (9,10), the early entry of labeled inositol into microsomal PI resulted in most of the total radioactivity being associated with monoenoic plus dienoic (36%) and tetraenoic (48%) molecules, with lesser amounts in trienoic (4%) and polyenoic species (13%). In contrast, 83% of the total radioactivity was associated with tetraenoic PI when inositol-^{3}H was incubated with rat liver microsomes in the presence of $MnCl_2$ using phosphate buffer. These latter distributions were in close agreement with those observed when Tris-HCl buffer (50 mM, pH 7.4) was used under otherwise similar conditions (9). The relative specific activity of the tetraenoic species labeled by the Mn^{2+} stimulated entry of inositol-^{3}H in vitro was 2-3 times that for all other subfractions, while the in vivo experiments produced monoenoic plus dienoic molecules with a specific activity 4-11 times all other fractions.

The effect of added CTP or CDP-choline on the Mn^{2+} stimulated entry of free inositol into PI as a function of incubation time is plotted in Fig. 1. The presence of either cytidine nucleotide at 2 mM in phosphate buffer caused a 3.5-4-fold increase in the rate of inositol incorporation into PI. A similar stimulatory effect of these compounds has been reported with no added phosphatidate using microsomal systems

TABLE II

Distribution of Inositol-^{3}H among Molecular Species of Microsomal Phosphatidylinositol Following Incubation with CTP or CDP-Choline[a]

Chemical classes	Additions to incubation medium		
	None	CTP[b]	CDP-choline[b]
Monoenes + Dienes	3.9 ± 0.3	7.2 ± 1.5	6.0 ± 1.9
Trienes	6.9 ± 0.5	6.3 ± 0.3	6.6 ± 1.3
Tetraenes	83.0 ± 0.7	77.7 ± 1.5[c]	80.5 ± 2.7
Polyenes	6.2 ± 0.9	8.8 ± 0.7	6.9 ± 0.7

[a]Conditions as described in footnote to Table I and Materials and Methods. Results are given as means ± S.E. for 6, 6, and 5 experiments in the case of no addition, CTP, or CDP-choline treatments, respectively.

[b]CTP = cytidine triphosphate; CDP-choline = cytidine diphosphate choline.

[c]Significantly different ($P<0.05$) from value in corresponding chemical classes obtained under conditions where no cytidine nucleotide was added to the incubation medium.

from various mammalian tissues (1,2,5,8). Paulus and Kennedy (1) observed a 4-5 fold stimulation by CTP and CDP-choline using phosphate buffer, while only a 15% enhancement was found with added CTP in Tris buffer.

Figure 2 illustrates the effect of adding various metals on inositol incorporation into PI in the presence or absence of cytidine nucleotides. In the presence of CTP or CDP-choline, the rates observed with added Mn^{2+} were 15 times greater than those obtained when this metal was omitted. Mg^{2+} was found to be only 11-14% as efficient as Mn^{2+} in stimulating inositol entry into PI when incubated with cytidine nucleotides, while Ca^{2+} was essentially ineffective. The preferential enhancement of inositol incorporation by Mn^{2+} in the absence of cytidine nucleotides has been observed previously (6).

Table II gives the percentage distribution of radioactive inositol among the various molecular species of PI when rat liver microsomes were incubated in the absence or in the presence of CTP or CDP-choline. Over 96% of the radioactivity incorporated into microsomal lipid was associated with PI as revealed by TLC. The distributions observed in the presence of either cytidine nucleotide were very similar to the distributions observed when these nucleotides were omitted from the incubation medium. Statistical analysis revealed no significant ($P>0.05$) differences from control incubations (no added nucleotide) in all but one case. In this latter case, the difference was not strongly significant ($P = 0.01$).

To provide further insight into the mechanism by which CTP and CDP-choline enhanced the entry of inositol into microsomal PI, chase experiments were conducted by incubating microsomes which contained inositol-^{3}H in the PI with unlabeled inositol plus Mn^{2+} and either of the two nucleotides. The results from such experiments are reported in Fig. 3. There was a steady increase in the amount of radioactivity appearing in the upper aqueous phases of lipid extracts with increasing incubation times up to 60 min. These results at all times agreed very closely (within 25%, $n = 10$) with those obtained by measuring the disappearance of inositol-^{3}H in PI from the lower chloroform phase of total lipid extracts. Of the radioactivity present in the control samples (unincubated) 103.4 ± 0.7% (mean ± S.E., $n = 10$) was recovered in the lower plus upper phases after incubation for various times. TLC analyses showed that an average of 78% or more of the water soluble radioactivity released into the upper phase of lipid extracts migrated with free inositol ($R_f = 0.28$). Previous work with radioactive microsomes from the pig thyroid gland (15) or rat brain (16), which contained PI labeled with ^{32}P and inositol-^{3}H, revealed that incubation of such preparations with nucleotide coenzymes and unlabeled inositol resulted in a large loss of inositol-^{3}H, but not ^{32}P, from the microsomal phospholipid.

Table III gives the effect of various cofactors on the loss of radioactivity from microsomal PI labeled with inositol-^{3}H. Maximal release of radioactivity from this phospholipid occurred in the presence of inositol plus Mn^{2+} plus CTP or CDP-choline. When any one of these four cofactors was omitted, the total radioactivity released was only 10-30% of that observed when inositol plus Mn^{2+} plus the nucleotide coenzyme were present. Jungalwala (16) observed a loss of radioactive inositol, but not phosphate, from PI in rat brain microsomes

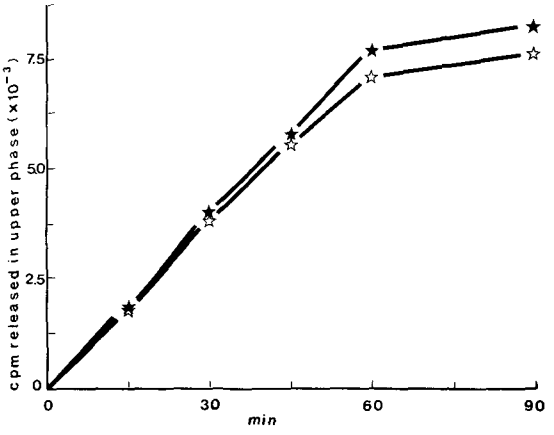

FIG. 3. The release of radioactivity from microsomal phosphatidylinositol-^{3}H in relation to time in the presence of cytidine nucleotides. Incubation mixtures contained $MnCl_2$ (2 mM), unlabeled inositol (0.67 mM), cytidine nucleotide (2 mM), and microsomes (4.2 mg protein) labeled with phosphatidylinositol-^{3}H (9310 cpm/mg protein). Incubation time was 60 min. ★ = CTP added; ☆ = CDP-choline added.

when nucleotide coenzymes plus inositol were added to the incubation medium. Mn^{2+} and phosphate buffer were employed in the present investigation; Jungalwala (16) used Mg^{2+} and Tris buffer.

Chase experiments similar to those given in Table III were also conducted using labeled microsomes containing choline-^{14}C in phosphatidylcholine or ethanolamine-^{14}C in phosphatidylethanolamine. Table IV gives the results from a series of experiments using these latter microsomal preparations. In all experiments, near maximal release of radioactivity from phospholipid labeled with choline-^{14}C or ethanolamine-^{14}C was consistently obtained when Mn^{2+} plus cytidine nucleotide were added to the incubation medium. Omission of Mn^{2+}, CTP, or CDP-choline decreased the amount of total radioactivity released from microsomal phospholipid by 43-73, 51-76, and 14-54%, respectively. In the case of microsomal phospholipid labeled with choline-^{14}C, the omission of free inositol from incubation mixtures did not significantly affect the amount of radioactivity released in the presence of Mn^{2+} plus CTP or CDP-choline. The addition of free choline in certain experiments did not increase the radioactivity that was lost, but actually caused a 25% inhibition as compared to controls. In experiments similar to those in Fig. 1, the rates of inositol entry into PI with the microsomal preparations of Table IV were found not be significantly different from other results (Fig. 1) and were not affected by the presence of free choline. When microsomes containing

TABLE III

Requirements for Release of Radioactivity from Microsomal Phosphatidylinositol-^{3}H[a]

Additions	% of lipid radioactivity released
$MnCl_2$+inositol+CTP[b]	24.9
$MnCl_2$+CTP	8.4
Inositol+CTP	2.6
$MnCl_2$+inositol	4.8
$MnCl_2$+inositol+CDP-choline[b]	22.0
$MnCl_2$+CDP-choline	3.2
Inositol+CDP-choline	3.5

[a]Incubations were conducted for 60 min in the presence of $MnCl_2$ (2 mM), unlabeled inositiol (0.67 mM), and 4.2 mg microsomal protein (47.1 nmoles PI/mg protein) containing 39,100 cpm. The specific activity of the total, monoenoic + dienoic, trienoic, tetraenoic, and polyenoic PI was 198, 804, 75, 141, and 184 cpm/nmole, respectively. Incubation of these microsomal preparations with inositol-^{14}C rather than unlabled inositol under otherwise identical conditions gave rates of inositol entry into PI of 11.9 and 9.5 nmoles/mg protein/hr in the presence of CTP and CDP-choline, respectively.

[b]CTP = cytidine triphosphate; CDP-choline = cytidine diphosphate choline.

phosphatidylethanolamine-^{14}C were used, a slight enhancement in the amount of radioactivity lost (up to 13%) resulted when free inositol was added to the incubation mixtures containing Mn^{2+} and cytidine nucleotides.

DISCUSSION

There have been a number of reports in the literature (1,2,5) indicating that added CTP and CDP-choline can greatly stimulate the entry of free inositol into PI, even though phosphatidate is omitted, when microsomes from various tissues are incubated in the presence of divalent cations such as Mn^{2+} or Mg^{2+}. It has been speculated that these effects of nucleotide coenzymes may reflect the de novo synthesis of PI from endogenous CDP-diglyceride, which, in turn, could be derived from endogenous phosphatidic acid (5), or from an enhancement of a microsomal exchange reaction between free inositol and inositol in phospholipid, which would not provide for the net synthesis of PI (1). In the past, a distinction between these two alternatives has been difficult. The investigation described herein was partly initiated to understand more clearly the action of CTP and CDP-choline using a microsomal preparation from rat liver.

The present results indicate that the Mn^{2+} stimulated entry of free inositol-^{3}H in rat liver

TABLE IV

Release of Radioactivity from Microsomes Containing Phosphatidylcholine-^{14}C or Phosphatidylethanolamine-^{14}C[a]

Additions	% Lipid radioactivity released from microsomes[b]			
	Choline-^{14}C		Ethanolamine-^{14}C	
	1[c]	2	1	2
$MnCl_2$+inositol+CTP	8.0		5.3	6.6
$MnCl_2$+CTP	8.5		4.7	6.4
Inositol+CTP	3.5		2.2	
$MnCl_2$+inositol	2.1	3.0	2.6	
$MnCl_2$+inositol+CDP-choline	3.7	3.5	5.6	7.0
$MnCl_2$+CDP-choline	3.4	3.8	5.0	6.4
Inositol+CDP-choline	2.1		1.5	
$MnCl_2$+inositol+CDP-choline+choline	2.8			
$MnCl_2$+inositol+choline		2.3		

[a]The conditions in experiment 1 were identical to those described in footnote to Table III, except that microsomes contained choline-^{14}C or ethanolamine-^{14}C rather than inositol-^{3}H. In experiment 2, 1.3 mg microsomal protein and 13.3 mM inositol (choline-^{14}C) or 5.3 mg microsomal protein and 1.33 mM inositol (ethanolamine-^{14}C) were employed. Free choline was added at 0.67 mM.

[b]These preparations contained 17,970 and 11,340 cpm/mg protein of choline-^{14}C and ethanolamine-^{14}C, respectively. Of the microsomal choline-^{14}C and ethanolamine-^{14}C, 93 and 89% were associated with phosphatidylcholine and phosphatidylethanolamine, respectively.

[c]Experiment number.

microsomes, as studied in the absence of cytidine nucleotides, produces labeled PI in which most of the radioactivity (83%) is associated with tetraenoic species. However, most of the radioactivity appearing in microsomal PI soon after the administration of inositol-^{3}H in vivo was located in monoenoic plus dienoic (36%) and tetraenoic (48%) species. These and other in vivo experiments with radioactive inositol, glycerol, and orthophosphate (10,11) confirm and extend an earlier claim (9) that a major portion of the free inositol entering PI in vivo apparently does so by way of de novo synthesis according to established schemes (1,2), rather than by the Mn^{2+} stimulated exchange in rat liver microsomes. Furthermore, the present results show that the distributions of radioactivity among the molecular species of PI, after incubating microsomal preparations with inositol-^{3}H and CTP or CDP-choline, were almost identical to those produced by the Mn^{2+} stimulated exchange reaction, even though these nucleotides greatly enhanced the rate of inositol entry into PI. Whereas inositol was incorporated predominantly into arachidonoyl molecules by the exchange reaction, the de novo synthesis of PI in rat liver apparently gives rise to a mixture of monoenoic plus dienoic, as well as tetraenoic species, based on the present and earlier findings (11,17). Under the present conditions, therefore, CTP and CDP-choline appear to enhance inositol incorporation into liver phospholipid by stimulation of the exchange reaction, as has been suggested (1). Prottey and Hawthorne (5) found no requirement for added phosphatidic acid for the incorporation of inositol into PI in the presence of CTP using microsomes from guinea pig pancreas. It was suggested that the endogenous concentration of phosphatidic acid in the microsomes may have been suitable for enzyme activity (5). It is unlikely that such a mechanism is of major significance under the present conditions when CTP or CDP-choline were added because arachidonoyl species represent ca. 14% of the total phosphatidic acids in rat liver (18,19), whereas tetraenoic species contained 78-81% of the radioactive inositol entering PI. Furthermore, a highly specific entry of phosphatidic acid molecules containing arachidonic acid into hepatic PI does not appear to be supported by the results of Akino and Shimojo (17). In addition, CTP and CDP-choline were almost equally effective in stimulating inositol incorporation (Fig. 1) while CTP has been found to be much more effective than CDP-choline in promoting the conversion of glycerol-3-phosphate into PI in liver (1) or phosphatidic acid into PI in brain (2).

The chase experiments with microsomes containing PI-inositol-^{3}H gave results analagous to those for inositol entry into PI. The dependency on Mn^{2+}, cytidine nucleotide, and inositol for maximum release of radioactivity

from PI and the relative response to CTP or CDP-choline (Table III) indicates a close similarity to the incorporation results in Figs. 1 and 2. It should be pointed out, however, that the small amounts of radioactivity lost from microsomal PI in the absence of added inositol may be due to the partial degradation of CTP and CDP-choline to cytidine monophosphate (CMP) by these enzyme preparations. This degradation could cause a reversal of the pathway leading to the de novo biosynthesis of PI as follows:

$$\text{CMP} + \text{phosphatidylinositol} \rightleftharpoons \text{CDP-diglyceride} + \text{inositol}$$

However, the optimal loss of radioactive inositol from PI in the presence of Mn^{2+} and cytidine nucleotide, or its degradation products, is dependent upon added inositol. This suggests the operation of the following overall reaction:

$$\text{Inositol} + \text{phosphatidylinositol-}^{3}\text{H} \rightleftharpoons \text{inositol-}^{3}\text{H} + \textit{phosphatidylinositol}$$

The possibility that membrane bound phosphatidic acid or CDP-diglyceride derived from PI could serve as intermediates in this exchange (15) cannot be excluded at present. The chase experiments reported herein are somewhat comparable to those using microsomes from the thyroid gland of the pig (15) and from rat brain (16). In the latter studies, microsomal phospholipids labeled with inositol-^{3}H and ^{32}P-phosphate were found to release only the inositol-^{3}H label upon incubation with CTP and nonradioactive inositol. However, Mg^{2+}, instead of Mn^{2+}, and Tris-HCl, rather than phosphate buffer, were used in these experiments (15,16). These different conditions render a direct comparison more difficult.

The results reported in Table IV indicate no enhancement of choline-^{14}C release, and only slight stimulation of ethanolamine-^{14}C release from microsomal phospholipid due to the addition of free inositol, in the presence of Mn^{2+} and cytidine nucleotide. In contrast, the release of inositol-^{3}H from microsomal PI was markedly stimulated under identical incubation conditions (Table III). These findings strongly suggest, therefore, that PI, rather than phosphatidylcholine or phosphatidylethanolamine, is the preferred substrate for the Mn^{2+} stimulated entry of inositol into PI in the presence of CTP or CDP-choline. The radioactivity lost from phosphatidylcholine-^{14}C and phosphatidylethanolamine-^{14}C in the presence of Mn^{2+} and cytidine nucleotides or their degradation products may reflect the back reaction of the CDP-choline:1,2-diacylglycerol cholinephosphotransferase and CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase respectively (20).

The data in Table III permit a comparison of the amount of inositol incorporated into PI with that released during incubation of microsomes with free inositol plus Mn^{2+} plus CTP or CDP-choline. If the amount of inositol lost from the various molecular fractions of PI is proportional to the percent distribution of newly incorporated inositol among the corresponding fractions following incubation, the following holds true:

Sum of cpm lost from 4 subfractions/mg protein/hr = 2,318 (for CTP)

Cpm lost from each subfraction/mg protein/hr = $\frac{x}{100} \cdot y \cdot z$, where

x = % of total radioactivity in fraction (in vitro data, Table II),

y = specific activity of fraction in cpm/nmole (Table III),

z = nmoles of inositol released from total PI/mg protein/hr.

Thus, for the incubations containing Mn^{2+} + inositol + CTP (Table III),

$$\frac{7.2(804) + 6.3(75) + 77.7(141) + 8.8(184)}{100} z = 2,318.$$

Solution of the above gives z = 12.3, while for CDP-choline, z = 11.4 nmoles/mg protein/hr. These results are in agreement with the incorporation studies which gave values of 11.9 and 9.5 nmoles/mg protein/hr for inositol entry in the presence of CTP and CDP-choline, respectively. These calculations add support to the concept that the entry of free inositol into microsomal PI in the presence of Mn^{2+} and CTP or CDP-choline as studied herein is mainly via an exchange reaction with inositol bound to phospholipid.

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Response of Lipogenesis and Fatty Acid Synthetase to Physical Training and Exhaustive Exercise in Rats[1]

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ABSTRACT

The effect of physical training and exhaustive exercise on fatty acid synthesis in rat liver and adipose tissue has been investigated. Exercise training (treadmill running) significantly ($P<0.05$) decreased body wt, epididymal fat pad wt, adipocyte size, and hepatic fatty acid synthetase activity. Training did not significantly affect adipose tissue cell number, lipogenesis from glucose-U-^{14}C, or fatty acid synthetase. Exercise to exhaustion immediately prior to sacrifice significantly ($P<0.05$) decreased lipogenesis from glucose-U-^{14}C and fatty acid synthetase in adipose tissue from trained but not untrained rats. Liver fatty acid synthetase was not significantly influenced by exhaustive exercise. The results of this study indicate that rats may adapt to physical training by decreasing adipose tissue lipogenesis during exhaustive exercise. This adaptation in energy metabolism may facilitate physically trained animals in conserving blood glucose during exhaustive exercise, thereby prolonging endurance.

INTRODUCTION

During prolonged nonexhaustive exercise, physically trained individuals derive a greater proportion of their energy from the oxidation of fat than do sedentary individuals performing at similar workloads (1). The enhanced ability to oxidize fatty acids is believed to be a biochemical adaptation to physical training that permits an exercising animal to conserve crucial carbohydrate stores. Fatty acids oxidized during exercise can be of either dietary or de novo origin. Regardless of source, the majority of these fatty acids are provided by mobilization from adipose tissue depots (2). Previous investigations have indicated that the increased energetic demands imposed upon adipose tissue by daily exercise training increases the turnover rate of adipose tissue fatty acids almost 2-fold (3). The increased turnover rate of adipose tissue fatty acids in also reflected by an increased mobilization (4-6) and esterification (7) capacity of adipose tissue from physically trained subjects.

While the effect of exercise and training on the degradation of lipids is well documented (1), the effect of exercise and training on fatty acid synthesis has received less attention. Simko, et al., (8) reported that rats physically trained by swimming appeared to divert acetate from hepatic fatty acid synthesis to cholesterol synthesis. Experimental evidence documenting the effect of exercise on adipose tissue lipogenesis is lacking. However, several reports on other types of stress, including whole body centrifugation (9) and cold exposure (10), have been published. Feller, et al., (9) found that rats stressed by centrifugation had an increased rate of adipose tissue lipogenesis, and suggested that when adipose tissue is yielding lipids into the blood, it is also synthesizing greater amounts of fatty acids. This concept is further supported by studies demonstrating that cold acclimated rats utilizing large quantities of fatty acids for thermogenesis have an accelerated rate of adipose lipogenesis from acetate (10).

Because definitive experimental results concerning the effect of exercise on fatty acid synthesis by adipose tissue and liver are lacking, the present study was conducted to test the effect of long term physical training and exhaustive exercise on fatty acid synthesis in the rat. Lipogenesis from glucose was assayed as a metabolic indicator of the overall pathway of fatty acid synthesis. Fatty acid synthetase was assayed as an index enzyme, because it has been shown to respond to diet and starvation (11,12), two conditions known to affect fatty acid synthesis.

MATERIALS AND METHODS

The data presented in this paper are from two experiments, designated I and II. Experiment I tested the effect of exercise training (chronic exercise of long duration) and exhaustion (a single occurrence of exhaustive exercise) on the total lipid synthetic capacity of adipose tissue slices from glucose. Experiment II tested the effect of training and exhaustion on the activity of hepatic and adipose tissue fatty acid synthetase.

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Experiment I. Male Carworth CFN rats, 5 weeks of age, weighing 85-105 g, were divided initially into two groups, designated trained and untrained. Trained rats were exercised five days per week on a motor driven treadmill (Quinton Instrument Co., Seattle, WA) for 13 weeks prior to sacrifice. The duration and intensity of the exercise were progressively increased throughout the experimental period until the rats were running 120 min/day at 29.5 m/min, at an 8% grade, with a 30 sec sprint (53.6 m/min) interposed every 10 min. Untrained rats remained sedentary in their cages for the duration of the experiment, except for a brief period of familiarization running (5 min/day) on the treadmill during the week prior to sacrifice. Commercial lab chow (Wayne Lab Blox, Allied Mills, Chicago, IL) and water were supplied ad libitum throughout the experiment. Throughout the training period, lighting was regulated to provide 12 hr of light and 12 hr of darkness. Room temperature was maintained at 20 C.

One-half of the rats in each group were sacrificed by decapitation immediately after running to exhaustion on the treadmill, and the remainder were sacrificed at the same time in the rested state (48 hr since last exposure to exercise). Trained rats were exhausted at 43.9 m/min and untrained rats at 20.1 m/min. Trained rats could run ca. 200 min and untrained rats ca. 80 min before exhaustion occurred at these workloads. Rats had to be exhausted at different workloads because trained rats would not exhaust readily at 20.1 m/min, and untrained rats would not run at 43.9 m/min. Rats were judged to be exhausted when they refused to run following electrical stimulation and exhibited deep body rectal temperatures of ca. 41 C.

Immediately following sacrifice, both epididymal fat pads were removed and rinsed in 0.15 M KCl. One fat pad was utilized for the determination of adipocyte cell numbers and cell size, while the remaining fat pad was used for lipogenesis studies. A portion of one fat pad was homogenized in 4 volumes of 0.15 M KCl and centrifuged 600 g for 10 min. The resulting infranatant was filtered through 2 layers of cheesecloth and analyzed for protein by an automated Lowry procedure (13). Fat cells were isolated following collagenase digestion as described by Therriault, et al., (14). Cell numbers were estimated by measuring the DNA content of the isolated washed fat cells, as described by Novak and Monkus (15). The triglyceride content of the isolated fat cells was determined by a semiautomated fluorometric procedure (Technicon auto analyzer method file N-78 a I/II, Technicon Instruments Corp., Tarrytown, N.Y.). Cell size is expressed as ηg triglyceride/cell.

Fat pads used in the lipogenesis incubations were sectioned to obtain representative samples (100-200 mg/sample), and incubated in triplicate in siliconized 25 ml glass vials. Each vial contained bovine insulin (0.1 International Unit [IU]/ml), penicillin G (50 IU/ml), streptomycin (0.5 mg/ml), and β-D glucose (5 mM containing 0.3 μCi/ml of β-D glucose-U-^{14}C [New England Nuclear, Boston, MA, 183 mCi/mM] in 3.0 ml Ca^{++}-free Krebs Ringer bicarbonate buffer (pH 7.4). The vials containing the tissue slices were gassed with 95% O_2:5% CO_2, stoppered and incubated in a metabolic shaker at 90 cycles/min for 2 hr, 38 C. The reaction was terminated by quick freezing the reactants in a dry ice-acetone bath. The fat pads then were extracted by a Folch wash procedure (16) and the lipid extract was counted for ^{14}C in a liquid scintillation counter with external standardization to correct for quenching. Results are expressed as ηmoles glucose-U-^{14}C converted to lipid per 2 hr per 10^6 cells, per mg protein, or per fat pad per unit metabolic body size (MBS = body wt in $Kg^{0.734}$).

The results were analyzed for significant differences by the Neuman-Keul's procedure (17). The level of significance chosen was $P<0.05$. All values are shown as the mean ± the standard error of the mean ($\bar{x}$ ± SEM).

Experiment II. Male Sprague Dawley rats weighing an average 220 g were fed a commercial lab chow (Wayne Lab Blox, Allied Mills, Chicago, IL) ad libitum and subjected to 6 weeks of intensive treadmill running. At the end of 6 weeks of training, rats were running 35 m/min for 60 min/day, 5 days/week, at a 7.5% grade. Untrained rats were exhausted at 15 m/min, 0% grade, while trained rats were exhausted by running at 35 m/min, 7.5% grade for 2 hr, then increasing the grade to 12% until exhaustion occurred. At these workloads, untrained rats could run ca. 50 min and trained rats 140 min before exhaustion occurred.

Rats were sacrificed as described in Experiment I, and liver and epididymal adipose tissue removed and homogenized for 30 seconds with a VirTis homogenizer (The VirTis Co., Gardiner, N.Y.) in 3 volumes of 0.05 M potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and 1 mM EDTA. The homogenate was centrifuged 105,000 x g for 60 min. The 105,000 x g supernatant was filtered through glass wool and assayed for fatty acid synthetase (FAS) by the method of Plate, et al., (18). FAS was measured spectrophotometrically by following the decrease in absorbance at 340 ηm

TABLE I

Effect of Physical Training and Exhaustive Exercise on Body Wt, Epididymal Adipose Tissue Wt, Cell Size, Cell Number, Protein Content, and Lipogenesis, Experiment I

Measurement	Physical condition[a]			
	Untrained		Trained	
	Rested	Exhausted	Rested	Exhausted
Body wt, g	404 ± 9[1] (16)	404 ± 8[1] (16)	286 ± 9[2] (16)	303 ± 9[2] (16)
Fat pad wt, g	3.9 ± 0.2[1] (16)	3.7 ± 0.2[1] (16)	1.5 ± 0.1[2] (16)	1.8 ± 0.2[2] (16)
Adipocyte size, ηg triglyceride/cell	199 ± 50[1] (16)	210 ± 53[1] (16)	86 ± 12[2] (16)	83 ± 12[2] (16)
Adipose tissue cell numbers, adipocytes/fat pad x 10^{-6}	15.9 ± 2.0[1] (16)	14.7 ± 1.8[1] (16)	12.7 ± 1.9[1] (16)	12.8 ± 1.7[1] (15)
Adipose tissue 600 x g protein, mg/fat pad	13.0 ± 1.4[1] (15)	11.6 ± 1.4[1,2] (15)	7.1 ± 0.7[3] (16)	8.6 ± 1.0[2,3] (16)
ηMoles glucose-U-^{14}C converted to lipid/2 hr/mg protein	183 ± 13[1] (15)	241 ± 40[1] (15)1	209 ± 26[1] (15)	103 ± 23[2] (16)
ηMoles glucose-U-^{14}C converted to lipid/2 hr/10^6 cells	139 ± 18[1,2] (14)	147 ± 22[1,2] (13)	177 ± 36[1] (15)	68 ± 21[2] (14)
ηMoles glucose-U-^{14}C converted to lipid/2 hr/fat pad/MBS[b]	4237 ± 276[1] (16)	4358 ± 325[1] (16)	3780 ± 318[1] (15)	1892 ± 390[2] (16)

[a]Values shown are mean ± SEM; number of animals is in parenthesis. Rats were trained and exercised to exhaustion and assays conducted as described under Materials and Methods. Means not sharing a common superscript are significantly different, $P<0.05$.

[b]MBS = metabolic body size.

resulting from nicotinamide adenine dinucleotide phosphate (NADPH) oxidation during fatty acid synthesis. The reaction mixture contained 75 mM potassium phosphate, pH 6.5, 10 mM dithiothreitol, 0.14 mM NADPH, 0.075 mM acetyl-CoA, and 0.225 mM malonyl-CoA with varying concentrations of 105,000 x g supernatant. FAS activity is expressed as η moles NADPH oxidized per min per mg protein, per g tissue, or per tissue per unit metabolic body size. Differences between the four treatment groups (trained rested, trained exhausted, untrained rested, untrained exhausted) were tested by the Neuman-Keul's procedure (17).

RESULTS

The results of Experiment I are given in Table I. Trained rats weighed less than untrained rats and possessed smaller epididymal fat pads. Adipose tissue cell numbers were not significantly affected by training or exhaustion. Glucose-U-^{14}C incorporation into lipid on a per mg protein, per 10^6 cells, or per fat pad per MBS basis, indicated that lipogenesis was not significantly affected by training. Exhaustive exercise was associated with a significant ($P<0.05$) decrease in lipogenesis in trained, but not in untrained rats, with all forms of data expression.

The results of Experiment II are shown in Tables II and III. As in Experiment I, physical training decreased both body wt and fat pad wt (Table II). Liver wt (Table III) was not affected by training, but was decreased by exhaustive exercise in the trained group of rats, perhaps due to glycogen depletion and water loss. Liver (105,000 x g) protein (Table III) was not changed by training or exhaustion, but adipose (105,000 x g) protein (Table II) tended to be lower in trained rested, and higher in trained exhausted rats, compared to the other groups. The reason for these variations in protein was not apparent. Adipose tissue FAS expressed per mg protein (Table II) was not affected by training, but was significantly ($P<0.05$) decreased by exhaustive exercise in trained rats. However, if adipose tissue FAS was expressed on a per g adipose tissue or per fat pad per MBS basis, no significant exhaustion effect was evident. FAS activity in untrained rats was not statistically altered by exhaustive exercise. Liver FAS (Table III) was significantly ($P<0.05$) lower in trained rats, but was not affected by exhaustive exercise in either untrained or trained groups.

DISCUSSION

The metabolic activity of adiposue tissue has been shown in numerous studies to be influenced by both cell size and cell numbers. Under the conditions of the present study, physically trained rats tended to have less adipocytes per epididymal fat pad, but the differences were not statistically significant. These results are in contrast to a previous report (19) demonstrat-

TABLE II

Effect of Physical Training and Exhaustive Exercise on Body Wt, Fat Pad Wt, Adipose Tissue Protein, and Fatty Acid Synthetase Activity, Experiment II

Measurement	Physical condition[a]			
	Untrained		Trained	
	Rested	Exhausted	Rested	Exhausted
Body wt, g	394 ± 6[1]	383 ± 6[1]	351 ± 4[2]	345 ± 5[2]
Fat pad wt, g	4.3 ± 0.3[1]	3.4 ± 0.2[2]	2.3 ± 0.1[3]	2.3 ± 0.1[3]
Adipose tissue soluble protein, mg/g tissue	6.8 ± 0.7[1,2]	6.6 ± 0.7[1,2]	3.4 ± 0.3[1]	9.0 ± 1.6[2]
Fatty acid synthetase				
ηMoles/min/mg protein	4.6 ± 0.9[1,2]	3.6 ± 0.3[1]	5.9 ± 0.6[2]	2.8 ± 0.3[1]
ηMoles/min/g tissue	8.6 ± 0.9[1]	9.6 ± 1.5[1]	11.4 ± 1.5[1]	15.6 ± 3.8[1]
ηMoles/min/fat pad/MBS[b]	73.5 ± 10.6[1]	65.5 ± 10.3[1]	55.6 ± 6.3[1]	73.7 ± 13.9[1]

[a]Values shown are mean ± SEM; 8 animals per group. Rats were trained and exercised to exhaustion, and assays conducted as described under Materials and Methods. Means not sharing a common superscript are significantly different, $P<0.05$.

[b]MBS = metabolic body size.

ing that a program of daily exercise by swimming rats, starting at one week of age, significantly decreased fat pad cell number. Because the first 6 weeks of life are a time of rapid cell proliferation in the rat (20), it is reasonable to assume that implementation of exercise early in this hyperplastic stage of adipose tissue development might exert a greater effect on cell numbers, as shown by Oscai, et al., (19). The lack of a greater training effect on cell numbers in this study may relate to the age of the rats (5 weeks) at the start of the exercise training period. The rats in this study were probably past the stage of primary hyperplastic fat depot development, and consequently, exercise may have exerted less effect on cell number.

Fat cells from the trained rats in Experiment I were significantly ($P<0.05$) smaller than those of the untrained rats, but incorporated similar amounts of glucose-U-^{14}C to lipid. A similar lack of a training effect on adipose tissue fatty acid synthesis was indicated by the fatty acid synthetase data in Experiment II. These results were somewhat surprising in that cell size has been implicated as an important determinant of adiposue tissue metabolism (14,21,22). Glucose synthesis into triglycerides has been shown to increase with fat cell size in humans (22-24). In contrast, lipid synthesis in rat adiposia is inversely related to cell size (21,25,26). It has been demonstrated that human adiposue tissue converts glucose largely to the glyceride moiety of triglycerides (22,27,28), whereas, de novo synthesis of fatty acids from glucose is much more important in rat adiposia (29,30). Romsos and Leveille (26) have shown the decrease in triglyceride synthesis associated with increasing rat adipocyte size can be attributed to a reduction in fatty acid synthesis, with glyceride-glycerol synthesis relatively unaffected by cell size. However, it should be noted that the relationship between adipose cell size and lipogenesis can be altered by overriding nutritional and physiological manipulation (21,31,32). If one draws a parallel between cold acclimatization and exercise training, noting that both treatments increase free fatty acid (FFA) mobilization from adipose tissue, trained rats might be expected to synthesize fatty acids at an enhanced rate, similar to cold acclimated rats (10). However, exercise training did not significantly affect lipid synthesis in rats sacrificed in the rested condition. Adipose tissue lipogenesis in exhausted trained rats was markedly lower than that of exhausted untrained rats.

Liver FAS was significantly ($P<0.05$) lower in trained rested rats compared to their untrained counterparts. This agrees with the results of Simko, et al., (8) who found that trained rats incorporated significantly less injected acetate-1-^{14}C into total liver fatty acids than untrained rats. The difference between the response of adipose tissue and liver to training may be due to a change in acetate utilization by the liver. Trained rats incorporate less acetate-1-^{14}C into liver fatty acids, but, at the same time, incorporate more acetate into cholesterol (8). Because adiposue tissue is not an important organ of cholesterol synthesis, a similar diversion of acetate from lipogenesis apparently does not occur.

Exhaustive exercise significantly ($P<0.05$) decreased adipose tissue lipogenesis from glucose-U-^{14}C (Experiment I) and possibly FAS (Experiment II) in trained, but not untrained animals. Because untrained rats could not run

TABLE III

Effect of Physical Training and Exhaustive Exercise on Liver Wt, Protein, and Fatty Acid Synthetase Activity, Experiment II

Measurement	Physical condition			
	Untrained		Trained	
	Rested	Exhausted	Rested	Exhausted
Liver wt, g	12.9 ± 0.7[1]	11.3 ± 0.7[1]	12.3 ± 0.4[1]	8.7 ± 0.4[2]
Liver soluble protein, mg/g tissue	82.5 ± 4.4[1]	85.9 ± 2.5[1]	78.0 ± 3.9[1]	88.9 ± 5.2[1]
Fatty acid synthetase				
ηMoles/min/mg protein	3.2 ± 0.3[1]	2.8 ± 0.2[1,2]	2.3 ± 0.2[2]	2.0 ± 0.1[2]
ηMoles/min/liver/MBS[b]	707 ± 122[1]	644 ± 56[1]	505 ± 46[1]	516 ± 42[1]

[a]Values shown are mean ± SEM; 8 animals per group. Rats were trained and exercised to exhaustion and assays conducted as described under Materials and Methods. Means not sharing a common superscript are significantly different, $P<0.05$.

[b]MBS = metabolic body size.

as fast or as long as trained rats, it is difficult to state whether the difference in lipogenesis between exhausted trained and exhausted untrained rats is due to training induced metabolic differences or the workload performed. However, it can be stated that trained and untrained rats differed markedly in the lipogenic potential of their adipose tissue at the point of exhaustion. Because the adipose tissue in Experiment I was incubated in vitro in the presence of insulin and in the absence of other circulating hormones, it would seem unlikely that the difference in lipogenesis between trained and untrained rats was due to hormonal influences. The time required to exhaust trained rats was ca. 200 min, so it is unlikely that differences in lipogenesis were attributable to changes in enzyme content of the tissue. The incubation media was also well fortified with glucose, so that substrate was not limiting. Several plausible mechanisms may be responsible for decreasing lipogenesis in adipose tissue of trained rats at the point of exhaustion, i.e., the availability of a critical cofactor such as Coenzyme A (33), an alteration of the NADPH:$NADP^+$ ratio, or inhibition of key enzyme activity by a metabolite elevated by exhaustion. An inhibition of adipose tissue FAS in exhausted trained rats is suggested by the results of Experiment II, although the results are difficult to interpret due to the difference in adipose protein between the groups. Fatty acid synthesis is believed to be directly related to the glycolytic rate in fed animals (34). Also characteristic of states in which fatty acid synthesis is decreased is a high rate of fatty acid oxidation (34). Trained rats have an almost 2-fold increase in fatty acid oxidation (35,36), and fatty acid mobilization (6) potential compared to untrained rats. High levels of fatty acyl-CoA in adipose tissue of exhausted trained rats may also act to decrease lipogenesis. Goodridge (37) suggests that relatively small changes in acyl-CoA concentration can be amplified to effect very large changes in the rate of fatty acid synthesis, possibly by direct inhibition of acetyl-CoA carboxylase, or by inhibiting mitochondrial citrate carrier resulting in a reduced activation of acetyl-CoA carboxylase by citrate. Further support for regulation of lipogenesis by the cellular FFA level is provided by studies demonstrating that epinephrine inhibits fatty acid synthesis by adipose tissue (33). Therefore, it is conceivable that depressed adipose tissue lipogenesis may be secondary to elevated FFA levels induced by catecholamines, secreted in response to the stress of exhaustive running. This action may be more effective in trained rather than untrained rats due to a longer exposure time to high levels of fatty acids occasioned by the increased sensitivity of epinephrine sensitive lipase in adipose tissue of trained rats (6).

Irrespective of the mechanism that allows trained rats to decrease fatty acid synthesis during exhaustive exercise, such an adaptation appears useful insofar as direct oxidation of glucose would be energetically more efficient to the exercising animal than converting its energy to fat prior to oxidation. Although the quantitative significance of a shutdown of lipogenesis during exhaustive exercise is not known, it is conceivable that such an adaptation might permit a certain degree of critical blood glucose conservation, thereby prolonging endurance.

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Effects of Bacteriophage M13 Infection upon Phospholipid and Fatty Acid Compositions of *Escherichia coli*

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ABSTRACT

Escherichia coli K38 were grown and infected with wild type and amber mutants of bacteriophage M13 in the early log phase. Lipid compositions of the infected and healthy cultures, grown under identical conditions, were determined 2 hr after infection. From the results, it was observed that total lipid and total phospholipid content remained nearly constant, suggesting that the cell membrane which contained the maximum phospholipids was not damaged by the infection. Moreover, the percentage of diphosphatidylglycerol and lyso- compounds corresponding to phosphatidylethanolamine and phosphatidylglycerol increased, while phosphatidylethanolamine and phosphatidylglycerol decreased. The increase in lyso- compounds may be due to the release of phospholipase A_2 (a periplasmic enzyme) from the cell wall after damage by the infection. Bacteriophage M13 infection had no effect on the fatty acid composition of the phospholipids.

INTRODUCTION

Some major changes in phospholipid composition of growing *E. coli* occur during the transition from the exponential to the stationary phase of growth. These changes are an increase in diphosphatidylglycerol, a decrease in phosphatidylglycerol, an increase in cyclopropane fatty acids, and a decrease in unsaturated fatty acids (1-3). The first two changes were taken to be related, because phosphatidylglycerol has been proved to be the precursor of diphosphatidylglycerol (4), and the increase in diphosphatidylglycerol phosphorus was almost stoichiometric with the decrease in phosphatidylglycerol phosphorus (1,2). Similar changes in the proportions of phosphatidylglycerol and diphosphatidylglycerol also can be brought about within the exponential growth phase by addition of colicin (5), cyanide (6), or penicillin (7), and also by infecting the cells with bacteriophage T_4 (8). Production of lysophosphatidylethanolamine also has been reported under some of the above conditions (8,9). The present investigation deals with the changes in the phospholipid composition of *E. coli* in the exponential growth phase when infected with bacteriophage M13. The fundamental difference between bacteriophage T_4 and M13 is that T_4 causes cell lysis, whereas, M13 does not. It has been reported that M13 phage particles continuously 'ooze' out of the intact cells and grow simultaneously with virus synthesis (10).

MATERIALS AND METHODS

Stocks of the wild type and amber mutant phages, mutated at genes 5 and 8, were prepared by adding suspensions of wild type M13 and its amber mutants to the early log phase cultures of *E. coli* HfrC and *E. coli* K38, respectively, at multiplicity of infection 5-10. The cultures, after infection, were grown for ca. 8 hr at 37 C and 32 C for the wild type and the amber mutants, respectively. The bacteria were then inactivated by shaking with ether and removed by centrifugation. The clear supernatants were stored as the phage source.

E. coli K38 cultures were grown at 37 C under continuous aeration in a medium containing glucose as carbon source, bacto vitamin free casamino acids (Difco, Detroit, Mich.) as nitrogen source, and other salts. The pH of the medium was adjusted to 7.0 by using phosphate buffer. The bacterial cultures were infected with the wild type and the amber mutants in the early log phase. The comparison of the lipid compositions of the infected and healthy (uninfected) cultures, grown under identical conditions, were made 2 hr after infection. The cells were harvested by centrifugation and washed with distilled water.

Lipids were extracted from the cells by the procedure of Bligh and Dyer (11). The extracted lipids were dried in vacuo and analyzed for their phosphorus content (12,13). The lipids were fractionated on 100-200 mesh silicic acid columns (Mallinckrodt Chemical Works, St. Louis, Mo.) with a discontinuous gradient of methanol in chloroform (14). Final purification of the different phospholipids was performed by preparative thin layer chromatography on silica gel plates with the solvent system containing chloroform:methanol:water (65:25:4).

Preliminary identification of the phospholipids was obtained by chromatographic com-

TABLE I

Total Lipid and Phospholipid Contents of *E. coli* Uninfected and Infected with Bacteriophage M13

Culture	Total lipid (mg/gm dry cell)	Total phospholipid (μmole/gm dry cell)
Uninfected	63	65
Wild type-infected	61	64
Amber 5-infected	58	60
Amber 8-infected	65	66

parison with known phospholipids. The reference phospholipids, used were diphosphatidylglycerol, phosphatidylethanolamine, and lysophosphatidylethanolamine (Applied Science Laboratories, State College, Pa.). Phosphatidylglycerol was synthesized as described by Bonsen, et al., (15) and lysophosphatidylglycerol was obtained by the action of phospholipase A on phosphatidylglycerol (16). The chromatograms were compared both on silica gel thin layer plate using a solvent system containing chloroform:methanol:water (65:25:4) and on silica impregnated paper with diisobutylketone:acetic acid:water (40:25:5). The chromatograms were stained with A) the molybdate reagent for phosphate groups; B) ninhydrin reagent for free amino groups; and C) metaperiodate benzidine reagent for vicinal hydroxyl groups.

Mild alkaline hydrolysis of the phospholipids was done by a modification of the procedure of Dawson (17) and enzymic hydrolysis with phospholipase A by the procedure of Magee and Thomson (16). Glycerol and the number of acyl groups were determined by the procedures of Renkonen (18) and Snyder and Stephens (19), respectively.

Methyl esters of the component fatty acids were prepared directly from the phospholipids by methanolysis as described by Luddy, et al., (20). A portion of the methyl esters was catalytically hydrogenated at room temperature for two hr using palladium on charcoal as the catalyst. A portion of these hydrogenated methyl esters was subjected to more drastic hydrogenation (21). The sample was dissolved in glacial acetic acid and the hydrogenation carried out at 40 C for 8 hr using Adam's platinum oxide as the catalyst. All of the above samples were analyzed by GLC.

An F&M Model 700-R dual column analytical gas chromatograph with dual flame ionization detector was used. Chromatograms were taken on 2 6-ft x 0.25 in. columns, one of 5% diethylene glycol succinate polyester (DEGS), and the other of 5% silicone gum rubber (SE30), both on 60-80 mesh Gaschrome Z (Applied Science Laboratories, State College, Pa.). The former column was kept at 150 C, and the latter at 200 C during operation. The carrier gas was nitrogen, and the flow rate was 40 ml/min. The operating pressures for air and hydrogen for the detector were 40 and 20 psi, respectively.

The peaks of the chromatograms were identified by the following methods. A) The retention times of the component esters of the sample were compared with those of reference esters both on polar (5% DEGS) and nonpolar (5% SE30) columns. B) Carbon numbers were determined by the method of Ackman, et al., (22) before and after hydrogenation. The position of the double bond in the monounsaturated fatty acids was determined by the permanganate periodate oxidation method as adopted to microscale by Scheuerbrandt and Bloch (23), followed by the GLC identification of the resulting dicarboxylic acids. C) For the confirmation of the presence of cyclopropane ring in fatty acids, the chromatograms of the drastically hydrogenated samples were compared with those of the hydrogenated and original samples prepared under identical conditions. Usual hydrogenation does not affect the cyclic ring of the fatty acids. Here, as in the hydrogenated sample, the unsaturated ester peaks disappeared with the increase in the area of the corresponding saturated ester peaks. The cyclopropane fatty acid ester peaks also disappeared with the formation of two new peaks, due to the n-saturated and branched chain acids formed by drastic hydrogenation of cyclopropane fatty acids.

RESULTS AND DISCUSSION

In the present investigation, five different phospholipids were identified in *E. coli* from their chromatographic behavior. They were confirmed further by chromatographic identification of the products of mild alkaline hydrolysis and lipolysis by phospholipase A. In some cases the molar ratios of glycerol, phosphate, and acyl groups were also determined. The identified phospholipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, lysophosphatidylethanolamine, and lysophosphatidylglycerol. A ninhydrin positive spot, remaining at the starting point in the thin layer chromatograms, could not be identified. All these phospholipids were observed in *E. coli* by other investigators (1-3). Presence of phosphatidic acid and phosphatidylserine in trace amounts in *E. coli* lipid was reported by a

TABLE II

Phospholipid Composition (wt-%) of *E. coli* Uninfected and Infected with Bacteriophage M13

Phospholipids	Uninfected	Culture infected		
		Wild type	Amber 5	Amber 8
Diphosphatidylglycerol	8.4	10.9	10.9	11.8
Phosphatidylethanolamine	75.9	72.9	73.3	73.5
Phosphatidylglycerol	13.1	8.3	9.1	6.2
Lysophosphatidylethanolamine	1.0	3.9	3.6	3.6
Lysophosphatidylglycerol	0.5	3.4	2.8	4.5
Others	0.3	0.6	0.2	0.4

TABLE III

Fatty Acid Composition (wt-%) of *E. coli* Lipids in Cultures Uninfected and Infected with Bacteriophage M13

Fatty acids		Uninfected	Culture infected		
			Wild type	Amber 5	Amber 8
	12:0	0.3	0.1	0.1	0.1
	14:0	4.2	3.7	4.3	5.0
	15:0	0.4	0.1	0.3	—
	16:0	44.1	43.0	46.4	41.1
	16:1	23.0	23.7	23.9	23.9
Cyc[a]	17:0	5.3	5.6	5.2	5.4
	18:0	1.4	0.6	0.6	0.2
	18:1	17.9	19.7	16.1	20.6
Cyc	19:0	3.2	3.8	3.1	3.8

[a]Cyc = cyclo.

few workers (4), but these were not detected in the present study, probably due to their very low concentrations.

In the present investigation, the fatty acids corresponding to the phospholipids of *E. coli* were identified as 12:0, 14:0, 16:0, 18:0, 16:1, *cis*-vaccenic (18:1), *cis*-9,10-methylene hexadecanoic (17:0), lactobacillic (19:0) acids, and a 15-carbon, straight chain, saturated fatty acid (15:0). All these fatty acids were previously reported (1,2) to be present in *E. coli* phospholipids. Several workers reported the 15-carbon acid to be a cyclopropane fatty acid (1), but during this investigation the 15-carbon acid proved to be a straight chain, saturated fatty acid. Batchelor and Cronan (24) identified a tetradecanoic acid as a component fatty acid of phospholipids of *E. coli* and found it to comprise about 15% of the total, unsaturated fatty acids. In the present study this acid was identified, but was present only in trace amounts.

It has been reported that several changes occurred in phospholipid compositions of *E. coli* when infected with bacteriophage T_4. Bannett, et al., (8) showed that 5-40% of the cellular phospholipids of *E. coli* were degraded within 25 min after infection, i.e., within the time of lysis, with different r (rapid lysis) mutants of phage T_4. It was reported by Ray (25) that bacteriophage M13 infection damaged the cell wall structure of the host, resulting in the release of lipopolysaccharides, and that even gram-negative *E. coli* became permeable to actinomycin D. Thus, permeability of the cytoplasmic membrane might be altered somewhat, but the membrane, as such, was not damaged. Infected cells maintained their osmotic stability (26). In the present study, it was observed that no significant change in the total phospholipid content of *E. coli* due to infection by bacteriophage M13 took place (Table I). This may be accepted on the basis of the above fact that some alteration in the cell wall was caused by M13 infection in *E. coli*, but, unlike T_4 infection, the cytoplasmic membrane was not damaged.

Moreover, in the case of T_4 infection, Peterson, et al., (6) found an altered labeling pattern of the phospholipids in the host cell. Although the overall incorporation of ^{32}P was found to decrease, the relative amounts of phosphatidylglycerol and diphosphatidylglycerol were increased. In the present study, a change in the composition of the phospholipids of the host cells due to M13 infection was also observed (Table II). Here, the percentage of diphosphatidylglycerol increased, while that of

phosphatidylethanolamine and phosphatidylglycerol decreased. However, the more interesting observation was the considerable increase in the lyso- compounds derived from phosphatidylethanolamine and phosphatidylglycerol. The decrease in phosphatidylethanolamine was almost stoichiometric with the increase in the corresponding lyso- compound, but the decrease in phosphatidylglycerol was equivalent to the sum of the increase in lysophosphatidylglycerol and diphosphatidylglycerol. Similar increases in these lyso- compounds were also reported as a result of T_4 infection (8, 9, 27). This could be explained by possible damage caused by T_4 infection to the cell wall structure, resulting in the release of periplasmic enzymes located between the cell wall and the membrane (27). Phospholipase A_2, a membrane bound enzyme, could cause uncontrolled production of lyso- compounds. Alternatively, another enzyme, lysophospholipase, might be inhibited, causing an accumulation of lyso- compounds. In *E. coli* cells infected with M13, release of alkaline phosphatase, a periplasmic enzyme, has been reported (25). The present results may be explained by the damage to the cell wall of *E. coli* by the M13 infection. This may have made the cell membrane more susceptible to uncontrolled activities of the lipase, thus, producing increased amounts of lyso- compounds. The increase in diphosphatidylglycerol may have resulted in enhanced biosynthesis from phosphatidylglycerol caused by the M13 infection. The results in Table III showed that M13 infection had no significant effect on the composition of the component fatty acids of the phospholipids of *E. coli*.

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SHORT COMMUNICATIONS

Acetylenic Acids from Mosses

ABSTRACT

Two new acetylenic fatty acids, 9,12-octadecadien-6-ynoic and 11,14-eicosadien-8-ynoic, were identified from lipids of the moss, *Fontinalis antipyretica*. They resemble the previously identified 9,12,15-octadecatrien-6-ynoic acid by having a methylene interrupted unsaturated system. The C_{20} acetylenic acid shows that the capability of mosses to synthesize polyolefinic acids of this chain length applies, in certain species, also to olefinic-acetylenic acids.

INTRODUCTION

Acetylenic fatty acids have been known for a long time as lipid constituents in some seed plants and microorganisms (1-3). These acids have chain lengths of not more than 18 carbon atoms, and it is generally accepted that they are biosynthetically derived from the common C_{18} acids (1,4). However, the unsaturated system of polyunsaturated acetylenic acids normally is conjugated, and *trans* configuration of double bonds is often encountered. *cis*-9-Octadecen-12-ynoic (crepenynic, from some Compositae and Santalaceae) and all *cis*-9,12,15-octadecatrien-6-ynoic (recently found in lipids of several mosses [5]) are the only exceptions where a methylene interrupted system of unsaturated bonds is encountered. The latter has the *cis* double bonds and the $\omega 3$ structure of linolenic acid. We are reporting here two additional acetylenic acids, 9,12-octadecadien-6-ynoic and 11,14-eicosadien-8-ynoic, from the moss *Fontinalis antipyretica* with double bond structures identical to those of linoleic and *bis*-homolinoleic acids.

MATERIALS AND METHODS

The aquatic moss, *F. antipyretica*, was collected from a slow flowing river in Millersdale, Derbyshire, England. Methods for extraction, analysis, isolation, and identification of structures were essentially as described previously (5). The lipids were extracted with $CHCl_3/CH_3OH$. The acetylenic triglycerides were isolated by column adsorption chromatography, and methyl esters were obtained from them by acidic interesterification. Fatty acid methyl esters were analyzed by GLC on two phases of different polarity. They were isolated by preparative GLC using a stream splitter, 1:5, with a column of 20% diethylene glycol succinate (DEGS) on siliconized Chromosorb W, 60-80 mesh (Johns Manville, Celite Division, New York, N.Y.), at 190 C.

Mass, UV, and IR spectra of the novel esters were determined under conditions used for 9,12,15-octadecatrien-6-ynoate (5). Chemical degradations were carried out by ozonization followed by hydrogenation over Lindlar catalyst (Fluka A.G., Buchs, Switzerland). Aliquots of the products were subjected to GLC on DEGS at 100 C to analyze them for monofunctional fragments, and on ethylene glycol succinate (EGS) at 143 C, before and after esterification with diazomethane, to analyze them for difunctional fragments, aldehyde-ester and di-ester, respectively.

RESULTS

Gas liquid chromatograms of methyl esters from total lipids of *F. antipyretica* revealed two peaks, together representing more than one-third of total areas, with equivalent chain lengths (ECL) (6) which did not coincide with any of the fatty esters commonly derived from moss lipids. Their ECL values were 22.8 and 24.8 on EGS, and 21.6 and 23.7 on DEGS. Preparative fractionation of the moss lipids showed that the novel acids were in triglycerides which migrated in thin layer and column chromatography similar to the acetylenic triglycerides of *Ceratodon purpureus* (5). The new acids represented three-fourths of the acids in these triglycerides, the remainder being acids commonly found in moss lipids. The two unknown esters were isolated by GLC to identify the structures.

Aliquots of the esters were hydrogenated and GLC indicated methyl stearate and arachidate as the products. Mass spectrometry confirmed this by showing molecular ions m/e 298 and 326. The nonhydrogenated esters gave molecular ions, m/e 290 and 318, indicating for both an unsaturation equivalent to 8 H atoms.

Ozonization and subsequent procedures yielded hexanal from both the C_{18} and C_{20}

esters. Therefore, the unsaturated systems had a terminal double bond in position 12 and 14, respectively, equivalent to an ω6 structure. C_4 or higher homologs of aldehyde esters were not found. After esterification of the degradation products with diazomethane, hexanedioate was identified from the C_{18} ester and octanedioate from the C_{20} ester. Therefore, the proximal unsaturation of the systems was a triple bond (5,7) in position 6 of the C_{18} ester and position 8 of the C_{20} ester. The molecular wts indicated the presence of another double bond which had to be between the unsaturations identified in the foregoing. This double bond was in position 9 of the C_{18} and 11 of the C_{20} ester, because IR and UV spectra did not reveal conjugation or allenic double bonds. No *trans* configuration was detected.

From these results, it can be concluded that the novel compounds are *cis*-9,*cis*-12-octadecadien-6-ynoic and *cis*-11,*cis*-14-eicosadien-8-ynoic acids.

DISCUSSION

The triglycerides which contain 36% 9,12-octadecadien-6-ynoic, together with 39% 11,14-eicosadien-8-ynoic acids, migrate in adsorption chromatography much slower than the common triglycerides of *F. antipyretica*. By wt, they represent more than half of all triglycerides. The selective distribution of acids in triglycerides, the wt ratio of acetylenic and non-acetylenic triglycerides, and their analytical properties are very similar to those encountered with the two types of triglycerides of *C. purpureus* (5). However, 9,12,15-octadecatrien-6-ynoic acid, which is predominant in triglycerides of *C. purpureus*, was not detected in *F. antipyretica*.

The acetylenic acids so far identified from moss lipids exhibit much closer structural relationships to linoleic and linolenic acids than any other natural polyunsaturated acetylenic acids. Acetylenic acids of a chain length with more than 18 carbon atoms so far had not been encountered in natural lipids. However, the occurrence of a C_{20} acetylenic acid shows that a triple bond does not prevent elongation of C_{18} to C_{20} chain length or, in possibly reversed sequence, that a C_{20} chain length does not prevent desaturation to form a triple bond. Neither of these processes can be expected from seed plants which produce acetylenic fatty acids, since they do not synthesize polyunsaturated acids with more than 18 carbon atoms.

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[Received April 8, 1975]

Taxus baccata Seed Oil: A New Source of *cis*-5,*cis*-9-Octadecadienoic Acid

ABSTRACT

Methyl esters prepared from the seed oil of the conifer *Taxus baccata* L. were found by gas liquid chromatography to contain 12% of a component which, when isolated by preparative thin layer chromatography and characterized by mass spectrometry, ozonolysis and nucle-

esters. Therefore, the unsaturated systems had a terminal double bond in position 12 and 14, respectively, equivalent to an ω6 structure. C_4 or higher homologs of aldehyde esters were not found. After esterification of the degradation products with diazomethane, hexanedioate was identified from the C_{18} ester and octanedioate from the C_{20} ester. Therefore, the proximal unsaturation of the systems was a triple bond (5,7) in position 6 of the C_{18} ester and position 8 of the C_{20} ester. The molecular wts indicated the presence of another double bond which had to be between the unsaturations identified in the foregoing. This double bond was in position 9 of the C_{18} and 11 of the C_{20} ester, because IR and UV spectra did not reveal conjugation or allenic double bonds. No *trans* configuration was detected.

From these results, it can be concluded that the novel compounds are *cis*-9,*cis*-12-octadecadien-6-ynoic and *cis*-11,*cis*-14-eicosadien-8-ynoic acids.

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The triglycerides which contain 36% 9,12-octadecadien-6-ynoic, together with 39% 11,14-eicosadien-8-ynoic acids, migrate in adsorption chromatography much slower than the common triglycerides of *F. antipyretica*. By wt, they represent more than half of all triglycerides. The selective distribution of acids in triglycerides, the wt ratio of acetylenic and non-acetylenic triglycerides, and their analytical properties are very similar to those encountered with the two types of triglycerides of *C. purpureus* (5). However, 9,12,15-octadecatrien-6-ynoic acid, which is predominant in triglycerides of *C. purpureus*, was not detected in *F. antipyretica*.

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Methyl esters prepared from the seed oil of the conifer *Taxus baccata* L. were found by gas liquid chromatography to contain 12% of a component which, when isolated by preparative thin layer chromatography and characterized by mass spectrometry, ozonolysis and nucle-

TABLE I

Composition[a] of Methyl Esters from *Taxus baccata* Seed Oil

Ester	ECL[b]	Mixed esters	Fractions from preparative TLC[c]			
			1	2	3	4
8:0	8.0	Tr[d]		Tr	Tr	0.8
10:0	10.0	Tr			Tr	0.8
12:0	12.0	Tr			Tr	0.8
	12.7	Tr				
14:0	14.0	Tr			Tr	0.6
16:0	16.0	4.1			0.3	55.6
	16.4				0.2	
	17.4				0.1	
18:0	18.0	3.1		0.2		41.6
18:1 Δ^9	18.2-18.3	59.3		1.4	97.1	Tr
18:2 $\Delta^{5,9}$	18.6	12.2	82.5			
18:2 $\Delta^{9,12}$	18.8	16.8		96.8		
	19.2		0.8			
18:3 $\Delta^{9,12,15}$	19.8	1.0	6.8			
20:1 Δ^{11}	20.1-20.3	1.5			2.1	
20:2 $\Delta^{5,11}$	20.6	0.7	1.3			
20:2 $\Delta^{11,14}$	20.8			1.5		
20:3 $\Delta^{5,11,14}$	21.2	1.2	8.2			

[a]From GLC. Data expressed as area percentage.

[b]ECL = Equivalent chain length determined on LAC-2-R 446 column.

[c]Preparative TLC on 1 mm layers of Silica Gel G impregnated with 20% silver nitrate. Solvent = benzene.

[d]Tr = Trace.

ar magnetic resonance, was identified as *cis*-5,*cis*-9-octadecadienoic acid.

INTRODUCTION

Fatty acids with isolated Δ^5 double bonds, either *cis* or *trans*, have been identified as constituents of lipids from various sources. Since our 1970 review of unusual fatty acids in plants (1), at least 20 papers have been published in which these Δ^5 unsaturated acids have been identified in seed oils, microorganisms, and tall oil. Some of these acids are monoenoic while others have the Δ^5 double bond in combination with those in the familiar ω-9, ω-6,9, or ω-3,6,9 positions. *cis*-5,*cis*-9-Octadecadienoic acid has been detected previously only in the seed oils of *Teucrium depressum* (2), *Larix leptolepis* (R. D. Plattner, G. F. Spencer, and R. Kleiman, unpublished data), in 4 species of the Chenopodiaceae (3), and in tall oil (4). 5,9-Octadecadienoic acid (geometry unspecified) also has been reported in seed oils of the conifer *Pinus radiata* (5,6). In their analysis of leaf lipids of 33 species of 4 coniferous families (Pinaceae, Taxodiaceae, Cupressaceae and Taxaceae [including *Taxus baccata*]) Jamieson and Reid (7) detected 5,9-octadecadienoic acids in all 22 species of Pinaceae studied, but none were reported in any genus of the other families.

We are reporting the presence of 12% of *cis*-5,*cis*-9-octadecadienoic acid in the seed oil of *Taxus baccata*. This is the highest concentration of any 5,9-octadecadienoic acid so far found in a seed oil.

EXPERIMENTAL PROCEDURES

Ground seeds were extracted 6 hr with petroleum ether (bp 30-60 C) in a Butt-type extration apparatus (8). Transesterification of the oil with 5% anhydrous HCl-methanol gave the mixed methyl esters. The esters were analyzed by gas liquid chromatography (GLC) on an F&M Model 402 gas chromatograph equipped with Apiezon L (ApL) and LAC-2-R 446 (R 446) columns as described by Kleiman et al., (9). The common esters were identified by their equivalent chain length (ECL) (10).

Preparative thin layer chromatography (TLC) of the mixed esters was carried out with benzene as the developing solvent on 1 mm layers of Silica Gel G impregnated with 20% silver nitrate. The separated bands were located under long wave UV light after spraying with an alcoholic solution of dichlorofluorescein. Analytical TLC was conducted on 0.25 mm layers of the same adsorbents and with the same solvent system. Spots were visualized by charring with a potassium dichromate-sulfuric acid spray.

IR spectra were determined on 1% CCl_4 solutions in 1 mm NaCl cells on Perkin-Elmer

Model 137 and 337 instruments.

Nuclear magnetic resonance (NMR) spectra were recorded with a Varian HA-100 spectrometer on deuterochloroform solutions. GLC coupled mass spectrometry (GC-MS) was carried out on the apparatus described by Kleiman and Spencer (11), escept that a 6 ft x 2 mm glass column packed with 3% Silar-5CP (Applied Science Laboratories, State College, Pa.) was used. Ozonolysis and subsequent GLC analyses of the products were carried out by the method of Kleiman, et al., (12).

RESULTS AND DISCUSSION

Petroleum ether extraction of 5.00 g seed provided 1.23 g oil (24.6%). In addition to esters of the common fatty acids, GLC analyses of methyl esters from *T. baccata* seed oil (Table I) showed the presence of the unusual component with an ECL value of 18.6 (R 446). Preparative argentation TLC of the mixed esters provided the following fractions (numbered consecutively from the origin with wt percentages in parentheses): 1 (14), 2 (17), 3 (60), and 4 (9). These fractions had R_f values corresponding to trienoic, dienoic, monoenoic, and saturated esters, respectively, and these identifications of fractions 2, 3, and 4 were confirmed by GLC (Table I). Instead of the expected high linolenate content (ECL 17.7 [ApL] and 19.8 [R 446]) in fraction 1, the major component was the unusual compound with ECL 18.6 (R 446) and 17.4 (ApL). GC-MS of this major component showed a molecular ion (M^+) at m/e 294, which is the mol wt of a methyl ester of a C_{18} dienoic acid.

GLC analysis of the ozonolysis products of this diene gave peaks (area percentages in parentheses) for C_9 aldehyde (45.8), C_5 aldehyde-ester (25.4), C_4 dialdehyde (12.1), and C_9 monoenoic aldehyde-ester (8.8). These cleavage products are consistent with a structure having double bonds in the 5- and 9-positions of a normal 18-carbon chain. The IR spectrum of this fraction was similar to that observed previously for esters of other *cis*-Δ^5 acids isolated in this laboratory (13) especially in the 8-9 μm region. The spectrum did not show the absorbance at 10.3 μm characteristic of *trans* double bonds. Accordingly, both double bonds must have the *cis* configuration. In the NMR spectrum of TLC fraction 1, 34 protons were distributed as follows: δ 0.9 (distorted t, 3H, terminal methyl), δ 1.3 (s, 12H, shielded methylenes) δ 1.7 (m, 2H, methylene group β to double bond and carboxyl groups), δ 2.1 (m, 8H, methylenes α to 1 double bond), δ 2.3 (t, 2H, methylenes α to carboxyl), δ 3.6 (s, 3H, carbomethoxy group), δ 5.3 (m, 4H, vinyl protons). There was no signal at δ 2.8, the chemical shift observed for diallylic methylene groups. As noted previously (14), the multiplet centered at δ 1.7 is characteristic of Δ^5 fatty acids, although the apparent multiplicity (quartet of doublets) is misleading; apparently there is virtual coupling and second order splitting. Our NMR spectrum is in fair agreement with that of Lehtinen, et al., (4) for this 5,9-unsaturated ester. The observed cleavage products and spectral data establish the identity of the major unusual fatty acid component of *T. baccata* seed oil as *cis*-5,*cis*-9-octadecadienoic acid.

Fractionation by argentation TLC resulted in concentration of certain minor components, and enabled us to identify them tentatively as 20:2 $\Delta^{5,11}$ and 20:3 $\Delta^{5,11,14}$ from their ECL and M^+ values (Table I).

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Brassica campestris var. Span: I. Fractionation of Rapeseed Oil by Molecular Distillation and Adsorption Chromatography[1]

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ABSTRACT

Procedures for the large scale isolation of pure triglycerides and fractions rich in nontriglyceride components from Span rapeseed oil are described. Fractionation of *Brassica campestris* var. Span rapeseed oil by molecular distillation yielded 4 triglyceride fractions, all of which contained traces of sterol esters. An additional triglyceride fraction rich in free and esterified sterols and other volatile components was obtained from the oil. Separation by adsorption chromatography of Span rapeseed oil yielded three fractions: A) a pure triglyceride fraction; B) a triglyceride fraction rich in sterol esters; and C) another fraction containing free sterols and other polar components.

INTRODUCTION

Recent reports in the literature indicate that feeding rapeseed oil (RSO) to weanling male rats for about 16 weeks causes myocardial lesions, irrespective of the level of erucic (Δ^{13}-*cis*-docosenoic) acid (1-5). Some authors (6) have ascribed the cardiopathogenicity of RSO solely to erucic acid, while others (1-5) have suggested that additional factors, such as unsaponifiable components or a fatty acid imbalance, may be involved.

To establish if the long term lesions observed in male rats were caused by the triglycerides or by some nontriglyceride components of RSO, *Brassica campestris* var. Span RSO was fractionated by molecular distillation and adsorption chromatography in sufficient amounts to perform extensive long term feeding trials. In this report, the preparation of pure triglycerides and fractions rich in nontriglycerides are described. The results obtained by feeding these fractions to male rats for 16 weeks will be described in a separate communication (7).

MATERIALS AND METHODS

Molecular distillation. Span RSO processed by Western Canada Processors, Lethbridge, Alberta, was distilled by Distillation Products Industries, Rochester, N.Y., in a molecular still, Model CMS-36. For the first cut, a total of 953 kg oil was passed through the CMS-36 to yield 274 kg distillate 1. Detailed conditions are listed in Table I. Five strip cuts of each successive residue were performed to yield distillates 2-6. Distillate 7 was obtained by recycling the residue, and the distillate was collected in two separate containers labeled 7A and 7B. The residue after the 7th distillate was 41 kg. The pressure was 10 μm during the first distillation and gradually decreased to 1-2 μm during distillations 5-7. The feed rate into the molecular still rotor was different for the separate cuts, and the residue temperature varied between runs from 248-278 C (Table I).

Distillates were cooled to ca. 80 C and collected in appropriate drums; nitrogen was flushed continuously into the container during the collection. Antioxidant was added at 0.1% by wt of the oil (Tenox II formulation: 0.02% butylated hydroxyanisole, 0.006% propyl gallate, 0.004% citric acid, and 0.7% propylene glycol). The molecular distillation was monitored by TLC on Silica Gel G plates using hexane:diethyl ether:acetic acid (85:15:1) as developing solvent, and the spots were visualized by charring after spraying with H_2SO_4:ethanol (1:1).

Distillates 1 and 2 (Table I; 337 kg) were combined and then redistilled to concentrate the volatile unsaponifiable components of Span RSO, giving distillate 1′ (101 kg) and residue 1′ (231 kg). Distillate 1′ was then redistilled to give distillate 1″ (32 kg) and residue 1″ (68 kg).

Adsorption chromatography. The triglycerides were isolated from Span RSO (200 kg) by adsorption chromatography at Applied Science Laboratories Inc., State College, Pa. The column (internal diameter [ID], 76 cm; ht, 91 cm) was packed dry with Silica Gel H. Rapeseed oil (ratio of oil to adsorbent 3.5:1; w:w) was dissolved in a minimum amount of petroleum ether and passed through the column with the same solvent until no more sterol esters (F1) emerged, as determined by monitoring the eluate by TLC. Pure triglycerides (F2) were then eluted with 2% diethyl ether in petroleum ether until diglycerides emerged, as checked by TLC. This was followed by a $CHCl_3$:MeOH (1:1) wash of the adsorbent to remove the remaining triglycerides and polar lipids (F3). Solvents were removed by a solvent

[1]Contribution no. 559 Animal Research Institute.

TABLE I

Fatty Acid Composition[a] of Span Rapeseed Oil and its Fractions Obtained by Molecular Distillation

Fatty acid[b]	Span	Distillation Fractions								Distillate 1'	Residue 1'	Distillate 1''	Residue 1''
		1	2	3	4	5	6	7A	7B				
14:0	0.1	0.1	0.1	0.1	0.1	0.1	<0.1	0.1	<0.1	0.2	0.1	0.4	0.1
16:0	4.8	6.2	5.2	4.9	4.7	4.5	3.3	2.2	1.8	7.7	6.1	9.3	7.5
16:1	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.4	0.3	0.5	0.4
18:0	2.1	1.9	1.8	1.9	2.0	2.0	2.0	2.0	1.7	1.9	2.3	1.8	2.2
18:1	58.6	58.5	59.4	58.9	60.5	60.9	58.4	53.2	50.0	60.6	62.4	59.4	60.4
18:2	19.5	22.2	21.5	21.9	20.2	19.9	20.4	19.9	19.0	20.6	18.0	20.7	20.3
18:3	5.2	5.9	6.2	5.8	5.6	5.3	5.5	6.0	6.8	5.4	5.3	5.3	5.5
20:0	0.6	0.4	0.4	0.5	0.5	0.6	0.7	0.9	0.9	0.3	0.5	0.3	0.4
20:1	3.3	2.4	2.5	3.1	2.8	3.0	4.2	5.6	5.8	1.6	1.7	1.2	1.4
22:0	0.3	0.1	0.2	0.2	0.2	0.2	0.3	0.5	0.6	<0.1	0.2	<0.1	0.1
22:1	4.8	1.6	2.2	2.3	2.7	3.1	4.5	8.3	11.6	1.0	2.8	1.0	1.4
24:0	0.1	0.1	–	–	0.1	0.1	0.1	0.3	0.6	–	–	–	–
24:1	0.2	--[c]	–	0.1	0.1	0.1	0.2	0.7	1.1	--	0.1	--	–
Amounts (kg)	953	274	63	95	133	84	71	99	77	101	231	32	68
Residue temperature (C)		278	262	258	256	254	254	248	248	247		247	
Distillation rate (ml/min)		240	286	286	375	375	375	480	480	545		480	

[a]Fatty acid composition is expressed as mole-% of total fatty acid.

[b]Fatty acids are designated by no. of carbon atoms:no. of double bonds. Trace amounts of 15:0, 17:0, 20:2, and 22:2 were observed, but are not included in this table.

[c]-- = No detectable amounts of fatty acid observed.

stripper at 30 C followed by high vacuum. Antioxidant purchased from Griffith Laboratories Ltd., Scarborough, Ontario, was added at 0.1% by wt to all fractions. It contained 13.3% butylated hydroxytoluene, 3.3% propylgallate, 3.3% citric acid, 2% butylated hydroxyanisole, and the remainder monoglyceride citrate and vegetable oil. The total yield from 200 kg Span RSO was: 40, 41, and 21 kg of fractions F1, F2, and F3, respectively. A further yield of 66 kg triglycerides was obtained which contained various levels of sterol ester and diglyceride contamination. About 84% by wt of the original oil was recovered in the eluates; the remainder was left on the adsorbent.

Analyses of oils. Methyl esters were prepared by transesterifying the oils with 5% (w:w) dry HCl gas in anhydrous methanol for 45 min at 80 C (8), and purifying the resulting esters by TLC using the solvent hexane:diethyl ether (90:10). The methyl esters were analyzed by GLC using two stationary phases: 5% butanediol succinate on 80-100 mesh Chromosorb G High Performance, and 3% Silar 5CP (cyanopropylphenylsiloxane) on 80-100 mesh Gas-Chrom Q (Applied Science Laboratory Inc., State College, Pa.). Peaks were identified by cochromatography with authentic methyl esters and quantitated by use of an Infotronic 208E integrator.

Sterols were analyzed quantitatively and qualitatively by GLC. A sample of oil weighing 50-200 mg was placed in a 15 ml culture tube fitted with a screw cap and teflon liner, and 1 ml $CHCl_3$ and 5 ml anhydrous HCl:MeOH (5% by wt) was added. The oil was transesterified at 90 C for 1 hr, then reduced in volume under a stream of nitrogen and applied quantitatively onto TLC. The solvent hexane:diethyl ether (50:50) was used to isolate the sterols. The sterol band was scraped off the TLC plate and the adsorbent was eluted with $CHCl_3$:MeOH (1:1). The complete eluate was transferred to a 1 ml reaction vial and brought to dryness under a stream of nitrogen. Appropriate amounts of Tri-Sil/BSA (N,O-bis-(trimethylsilyl)-acetamide, Pierce Chemical Co., Rockford, Ill.), and cholestane dissolved in $CHCl_3$ were added to the reaction vial. Cholestane was used as internal standard for the quantitative determination of sterols by GLC. The silylated sterols were extracted with hexane (9) and analyzed by GLC using a 2 m x 4 mm ID glass column packed with 5% OV-101 on 80-90 mesh Anakrom ABS (Supelco, Inc., Bellefonte, Pa.). The chromatograph was operated isothermally at 260 C, and nitrogen was used as the carrier gas at 50 ml/min. Authentic standards were used to identify the sterols.

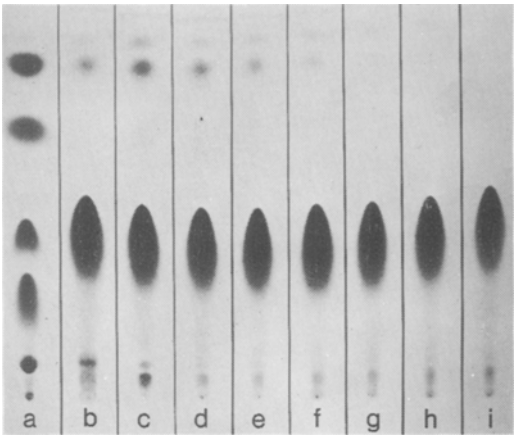

FIG. 1. Thin layer chromatograph. a = Standard mixture containing cholesterol oleate, methyl oleate, triolein, oleic acid and cholesterol, in descending order; c = distillate 1; d = distillate 2; e = distillate 3; f =distillate 4; g = distillate 5; h = distillate 6; i = distillate 7. Adsorbent; Silica Gel G. Solvent; hexane: diethyl ether:acetic acid (85:15:1). Indicator; charring after spraying with sulfuric acid ethanol solution.

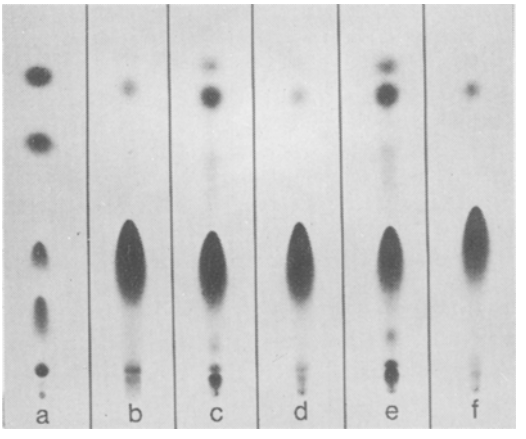

FIG. 2. Thin layer chromatography. a = Standard mixture (See Fig. 1 for details); b = Span rapeseed oil; c = distillate 1′; d = residue 1′; e = distillate 1″; f = residue 1″. Adsorbent, solvent and indicator, see Figure 1.

RESULTS

The fractions obtained from molecular distillation of 953 kg Span RSO were analyzed by TLC (Figure 1). Triglycerides were the major component in all fractions obtained. The first distillates removed the more volatile constituents present in the oil, i.e., hydrocarbons, methyl esters, free fatty acids, alcohols, sterols, mono- and diglycerides, some sterol esters and other volatiles. Free sterols were detected only in distillates 1-3 as determined by the character-

TABLE II

Sterol Content of Span Rapeseed Oil and its Fractions Obtained by Molecular Distillation and Adsorption Chromatography

Dietary oils	% Total sterol in oil	% Sterol composition[a]		
		Brassicasterol	Campesterol	β-Sitosterol
Span	0.25	6.7	32.6	60.8
MD 1[b]	0.62	6.9	34.3	58.9
MD 2	0.12	4.5	33.7	61.8
MD 3	0.09	3.4	32.5	64.1
MD 4	0.03	2.5	28.7	68.9
MD 5	0.01	1.3	20.6	78.1
MD 6	0.03	0.8	25.4	73.8
MD 7A	0.003	1.8	20.2	77.9
MD 7B	0.003	1.6	18.4	80.0
MD 1'[c]	1.95	8.0	31.6	60.2
Residue 1'	0.18	4.9	32.0	63.2
MD 1''[c]	3.68	8.1	32.7	59.1
Residue 1''	0.49	5.3	33.8	60.9
F1[d]	0.64	4.4	36.4	59.1
F2	–	–	–	–
F3	0.006	5.2	28.0	66.8

[a]The relative retention times of the sterols on this GLC column were: cholestane, 1.00; cholesterol, 2.03; brassicasterol, 2.24; campesterol, 2.59; stigmasterol, 2.77; and β-sitosterol, 3.16.

[b]MD = molecular distillate.

[c]MD 1' and MD 1'' each contained 0.3% cholesterol.

[d]F = fractions obtained from adsorption chromatography.

istic coloration during charring after spraying the chromatogram with H_2SO_4. Six separate distillates were required to yield a relatively pure triglyceride fraction low in sterol esters (Fig. 1, c-h). The remaining oil was distilled by recycling the residue to give distillates 7A and 7B.

To obtain a fraction rich in the volatile components of Span RSO, distillates 1 and 2 were combined and refractionated to yield distillate 1'; the latter distillate was redistilled once more to give distillate 1''. A TLC chromatogram of molecular distillates 1' and 1'', and their corresponding residues is shown in Figure 2. The enrichment of hydrocarbons, sterol esters, methyl esters, free fatty acids, free sterols, and mono- and diglycerides was apparent by comparing the distillates with the corresponding residues.

The fatty acid composition of all distillates is shown in Table I. A molecular distillation of triglycerides was apparent; the first distillates were higher in 16:0 and lower in the C_{20}, C_{22} and C_{24} fatty acids relative to Span RSO. The reverse fatty acid composition was found in the later distillates. The fatty acid composition of distillates 5 and 6 was similar to that of the original Span RSO, whereas, distillates 7A and 7B had lower levels of 16:0 and 18:1, and had a greater concentration of 20:1 and 22:1 compared to the original oil. Distillate 1'' was a distinctly different fraction, containing a high level of 16:0 and low levels of 20:1, 22:1, and 24:1 in addition to an enrichment of non-triglycerides as seen from Figure 2.

The sterol content of Span RSO and of its fractions obtained by molecular distillation is shown in Table II. By molecular distillation, most of the sterols were removed from distillates 5, 6, 7A, and 7B. In contrast, molecular distillate 1'' represents a 14.6-fold increase in sterols compared to the original Span RSO. Furthermore, a separation of the sterols by molecular distillation was also apparent (Table II). The relative concentration of brassicasterol and campesterol decreased in the later distillates, while the relative concentration of β-sitosterol increased proportionally. The absolute amount of brassicasterol in distillates 7A and 7B was very low, while the level of this sterol was slightly higher in distillates 5 and 6 compared to distillate 7A and 7B. The relative concentration of the three major sterols in molecular distillate 1'' was comparable to that found in the original Span RSO.

The three fractions obtained from adsorption chromatography of Span RSO were analyzed by TLC (Fig. 3). All fractions consisted mainly of triglycerides, and in addition, F1 contained an appreciable amount of sterol esters and hydrocarbons. No sterol esters or sterols were detected in F2 even when 8 mg oil was applied onto the TLC plate (Fig. 3 e), but traces of diglyceride were found. Fraction F3

contained traces of free sterols and some diglycerides.

All three fractions were quantitatively analyzed for sterols by GLC as described in Materials and Methods, and the results are given in Table II. There was a 2.6-fold enrichment of total sterols in F1 compared to Span RSO. Because all the sterols were present in the ester form in F1 (Fig. 3), and the sterol content of the free and esterified forms in the original Span RSO was ca. the same (3), F1 represented a five-fold enrichment of sterol esters. No sterols were detected in F2, and only traces of free sterols were found in F3. There were no apparent differences in the sterol composition of the free and esterified sterols between F1, F3, and Span RSO.

The fatty acid composition of the three fractions isolated by adsorption chromatography are given in Table III. Moderate changes in the fatty acid compositions of the fractions can be expected because the oil was separated by adsorption chromatography, which is known to retard polyunsaturates compared to saturates. Fraction F3 contained higher levels of 18:2 and 18:3, and generally lower levels of saturates and monounsaturates compared to F1. The level of 20:1 and 22:1 was highest in F1 and lowest in F3.

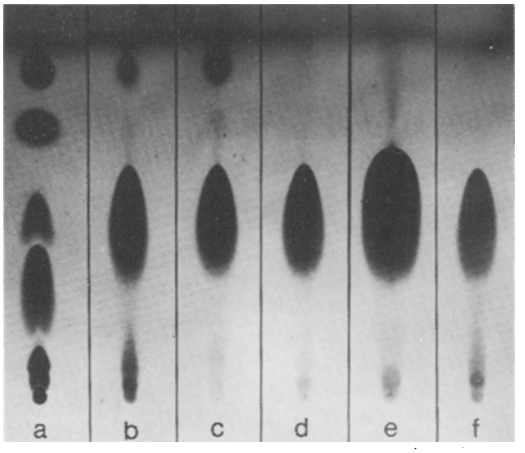

FIG. 3. Thin layer chromatograph. a = Standard mixture containing cholesterol oleate, methyl oleate, triolein, oleic acid, cholesterol, diolein and monoolein in descending order; b = Span rapeseed oil; c = adsorption chromatography fraction 1; d = 2 mg of fraction 2; e = 8 mg of fraction 2; f = fraction 3. Adsorbent, solvent and indicator, see Figure 1.

DISCUSSION

Rapeseed oils low in erucic acid have been reported to cause myocardial lesions when fed for several months to male rats (1-5). Abdellatif and Vles (6) observed myocardial lesions in male rats that had been fed diets containing synthetic triglycerides high in erucic acid for 24 weeks. Other investigators were unable to consistently produce myocardial lesions in male rats by feeding similar synthetic triglycerides high in erucic acid (2,10). These inconsistent results have been attributed to slight, but significant differences between the fatty acid composition of the synthetic triglycerides and those of rapeseed oils (2). It should be noted, however, that in addition to differences in the fatty acid composition between synthetic triglycerides and tri-

TABLE III

Fatty Acid Composition[a] of Span Rapeseed Oil and its Fractions Obtained by Adsorption Chromatography

Fatty acid[b]	Span	Fractions		
		F1	F2	F3
14:0	0.1	0.1	0.1	0.1
16:0	4.8	5.7	6.1	6.1
16:1	0.2	0.1	0.3	0.5
18:0	2.1	3.3	3.1	2.5
18:1	58.6	49.3	49.6	42.5
18:2	19.5	17.5	19.9	24.5
18:3	5.2	5.8	6.9	13.6
20:0	0.6	1.0	0.8	0.4
20:1	3.3	6.3	5.2	4.0
22:0	0.3	0.5	0.4	0.3
22:1	4.8	8.8	6.2	4.2
24:0	0.1	0.4	0.3	0.2
24:1	0.2	0.7	0.6	0.4

[a]Fatty acid composition expressed as mole-% of total fatty acids.

[b]Fatty acids are designated by no. of carbon atoms:no. of double bonds. Trace amounts of 15:0, 17:0, 20:2, and 22:2 were observed, but are not included in this table.

glycerides of natural oils, there are also differences in the position of fatty acids on the glycerol moiety, as well as differences in the position of the double bonds in fatty acids (11). Therefore, in the present study triglycerides from natural RSO were isolated.

Molecular distillation and adsorption chromatography were used to isolate triglycerides; these two methods do not isomerize fatty acids on the triglycerides molecule. *B. campestris* var. Span was fractionated since this oil was low in 22:1 and had previously been shown (3) to cause myocardial lesions in male rats. It was hoped, by using these two separation techniques, that pure triglycerides and fractions containing enriched nontriglyceride components could be isolated in sufficient amounts to feed larger numbers of male rats for 16 weeks. A comprehensive review of minor constituents in RSO has been published previously (11).

Molecular distillation has been used previously to remove free fatty acids, sterols, methyl esters, alcohols, mono- and diglycerides, hydrocarbons, and other volatile components from triglycerides (12-15). In the present study, these compounds were concentrated easily into the light distillate 1″. The isolation of pure triglycerides required the removal of the sterol esters, the volatility of which is similar to triglycerides (14). This was partially achieved by performing 6 small, successive distillation cuts. Molecular distillation gave 4 fairly pure triglyceride fractions (MD5, 6, 7A, and 7B) which contained small amounts of sterol esters with slightly different fatty acid compositions. Because brassicasterol, the characteristic sterol present in RSO, was rich in the earlier distillates, the later triglyceride fractions were relatively free of this sterol.

The principles of adsorption chromatography were employed on a large scale to achieve the separation observed here with Span RSO. The eluate of the column was monitored by TLC to obtain a highly purified triglyceride fraction. The fatty acid composition of fraction F2 was similar to Span RSO. In addition, F2 was free of sterol and sterol esters and only traces of diglycerides were detected. Fraction F1 was a triglyceride fraction rich in hydrocarbons, sterol esters, and compounds less polar than triglycerides. The final fraction F3 contained some free sterols and mono- and diglycerides. Most of the polar compounds were retained by the adsorbent, as was established by analyses of the adsorbent after chromatography.

The cardiopathogenicity to male rats of triglycerides (fractions 5, 6, 7B, and F2) and of fractions of Span RSO rich in sterol esters (F1), free sterol (F3), and free and esterified sterols plus other volatile constituents (distillate 1″) will be described in the next publication of this series (7).

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Brassica campestris var. Span: II. Cardiopathogenicity of Fractions Isolated from Span Rapeseed Oil when Fed to Male Rats[1]

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ABSTRACT

Rapeseed oils low in erucic acid caused myocardial lesions when fed to weanling male rats for 16 weeks. The cardiopathogenic properties appear to be associated with the triglycerides of the oil, and not to nontriglyceride components present in fully refined rapeseed oil. Cardiac lipid analysis confirmed that erucic acid accumulation was proportional to the concentration of this acid in the diet.

INTRODUCTION

Recent reports in the literature indicate that feeding rapeseed oil (RSO) to weanling male rats causes lipidosis in the heart within the first week and myocardial lesions after several months, even when RSO low in erucic acid is fed (1-4). It has been shown that the early lipidosis in the heart is directly related to the erucic acid concentration in the dietary oils (2, 5-8). However, at present the cause of the necrotic and fibrotic lesions which occur after feeding RSO for 16 weeks is not understood.

Myocardial necrosis has been reported in rats under such stress as catecholamine injection, dietary vitamin K deficiency, severe muscular exertion, exposure to cold and digitalis intoxication (9), or seamin, an alcohol soluble substance found in sesame oil (10). Feeding cyclopropenoid fatty acids is known to produce liver and kidney necrosis (11). The possibility may exist, therefore, that a nontriglyceride component is present in the completely refined RSO which is responsible for the necrotic and fibrotic lesions. On the other hand, the lesion producing properties of RSO may be attributable to its triglycerides.

To establish whether the long term lesions are caused by the triglycerides or some nontriglyceride component present in the oil, *Brassica campestris* var. Span RSO was fractionated by molecular distillation and adsorption chromatography, as described previously (12), to yield highly purified triglycerides and fractions rich in nontriglyceride constituents. These fractions were fed to weanling male rats for 16 weeks. Hearts of these rats were examined for myocardial lesions and fatty acid composition.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Bio-Breeding, Ottawa, Ontario) 3 weeks of age and weighing 40-50 g, were randomly selected, identified by ear markings, assigned 2 per cage, and fed the experimental diets for 16 weeks. The semisynthetic diet consisted of 20% casein, 20% sucrose, 30% cornstarch, 1% vitamin mixture, 4% U.S.P. XVII mineral mixture, 5% pure wood cellulose, and 20% test oil (w/w). The following oils were used: corn oil (St. Lawrence Starch Co. Ltd., Port Credit, Ontario); olive oil (Pastene Co. Ltd., Montreal, Quebec); safflower oil (Teklad Mills, Madison, Wisc.); *B. napus* var. Oro and *B. campestris* var. Tower (Cooperative Vegetable Oil Ltd., Altona, Manitoba); and *B. campestris* var. Span (Western Canada Processors, Lethbridge, Alberta). The fractions obtained from Span RSO by molecular distillation (MD) 5, 6, 7B, and 1″ and by adsorption chromatography F1, F2, and F3 have been described previously (12).

Experiment I consisted of 2 groups of 20 rats fed Purina Laboratory Rat Chow or semisynthetic diet containing olive oil, and 6 groups of 50 rats fed semisynthetic diet containing Oro RSO, Span RSO, or MD 5, 6, 7B and 1″, from Span RSO (12). Because of the limited supply of MD 1″, 25 of the 50 rats were killed after 12 weeks and their hearts examined histologically. The remaining 25 rats were kept the entire 16 weeks on this diet. After 16 weeks, all rats were killed and their hearts examined histologically. One quarter of the heart was removed for lipid analyses from 10 rats on each diet; the remaining portion of these hearts was prepared for histological examination.

Experiment II consisted of 4 groups of 10 rats fed chow or semisynthetic diet containing olive oil, Span RSO, or triglyceride fraction F2 obtained from Span RSO by adsorption chromatography (12). Rats were killed after 16 weeks on treatment and hearts removed for

[1]Contribution no. 575 Animal Research Institute.

TABLE I

Fatty Acid Composition of Chow and Dietary Oils

Fatty acids	Diets						
	Chow[a]	Olive	Corn	Safflower	Tower	Oro	Span
16.0	16.9	11.9	15.0	9.9	5.2	5.0	4.8
16:1	1.8	0.7	0.1	0.1	0.2	0.3	0.2
18:0	3.2	2.9	2.6	3.4	1.8	2.5	2.1
18:1	22.4	73.4	27.8	13.9	56.9	62.2	58.6
18:2	38.9	8.9	52.7	71.9	24.0	18.9	19.5
18:3	3.1	0.8	0.6	0.2	8.4	6.7	5.2
20:1	2.9	0.4	0.3	0.2	1.3	1.9	3.3
22:1	3.7	0.4	-	-	0.8	1.6	4.8
24:1	-	-	-	-	0.2	0.2	0.2

[a]Chow contained fish meal as part of its formulation. The fatty acid composition is that of the total lipids extracted from the diet with chloroform: methanol (2:1).

lipid analysis and histological examination.

Experiment III consisted of 8 groups of 24 rats fed chow or semisynthetic diet containing corn oil, safflower oil, Tower RSO, Span RSO, or fraction F1, F2, and F3 obtained from Span RSO by adsorption chromatography (12). All rats were killed after 16 weeks on these diets; 20 rats were examined histologically and 4 rats were used for lipid analyses.

For histological examination hearts were fixed in 10% neutral buffered formalin. Three sections were prepared from each heart: a) one central section extending from apex to base and including interventricular septum, atrial and ventricular walls; b) a section parallel to the first and extending through the right ventricle; and c) a third section parallel to the other two and extending through the left ventricle. The 3 sections were equidistant from each other. From these, 6 μ sections were made and stained with hematoxylin and eosin.

All experimental rats were numerically coded and not identified until the histological examination was completed. The results were reported as the number of rats affected, as well as the number of lesions per affected heart. No attempt was made to grade individual lesion as to pathological severity. Instead, groups were made to include rats which had 1-2, 3-5, 6-10, and >10 lesions per heart. Approximate Chi-square statistics, obtained following the approach of Fienberg (13) were used to examine the frequency tables (Tables II-IV) for evidence of differences in patterns of incidence and severity among the various diets.

The techniques for homogenization of rat hearts, extraction of lipids, and preparation and analysis of fatty acid methyl esters have been described (2). Analysis of variance was performed on cardiac lipids and wt gains and significant difference at the 1% level ($P < 0.01$) were determined using Duncan's Multiple Range test (14).

RESULTS

The fatty acid composition of chow, corn oil, olive oil, safflower oil, Tower RSO, Oro RSO, and Span RSO is shown in Table I. The composition of the fractions isolated from Span RSO by molecular distillation and adsorption chromatography are fully described in an accompanying publication (12). Oro and Tower RSO were fully refined rapeseed oils low in 20:1 and 22:1 with a total sterol concentration of 0.29 and 0.23%, respectively.

Growth rates of male rats fed MD 1'' were significantly lower ($P < 0.01$) compared to rats fed the diet containing olive oil (experiment I). The wt gains of rats fed Oro RSO, Span RSO, or MD 5, 6, and 7B (purified triglycerides) were slightly lower than, but not significantly different ($P < .0.01$) from the control diets (chow and olive oil), or MD 1''. Male rats fed the purified triglyceride fraction F2 obtained by adsorption chromatography (experiment II) performed as well as the control diets (chow or olive oil), whereas, feeding Span RSO gave a slightly depressed growth rate which was not significant at the 1% level. In experiment III, male rats fed safflower oil, Span RSO, and fractions F1, F2, and F3 obtained by adsorption chromatography gained significantly less ($P < 0.01$) compared to rats fed corn oil. Rats fed chow or Tower RSO gained less than those fed corn oil but more than those fed Span RSO or fractions F1, F2, or F3. However, none of these difference were significant.

The inclusion of RSO in the diet resulted in myocardial accumulation of fatty acids characteristic of these oils, such as 20:1, 22:1, and 18:3. There was considerable evidence of a

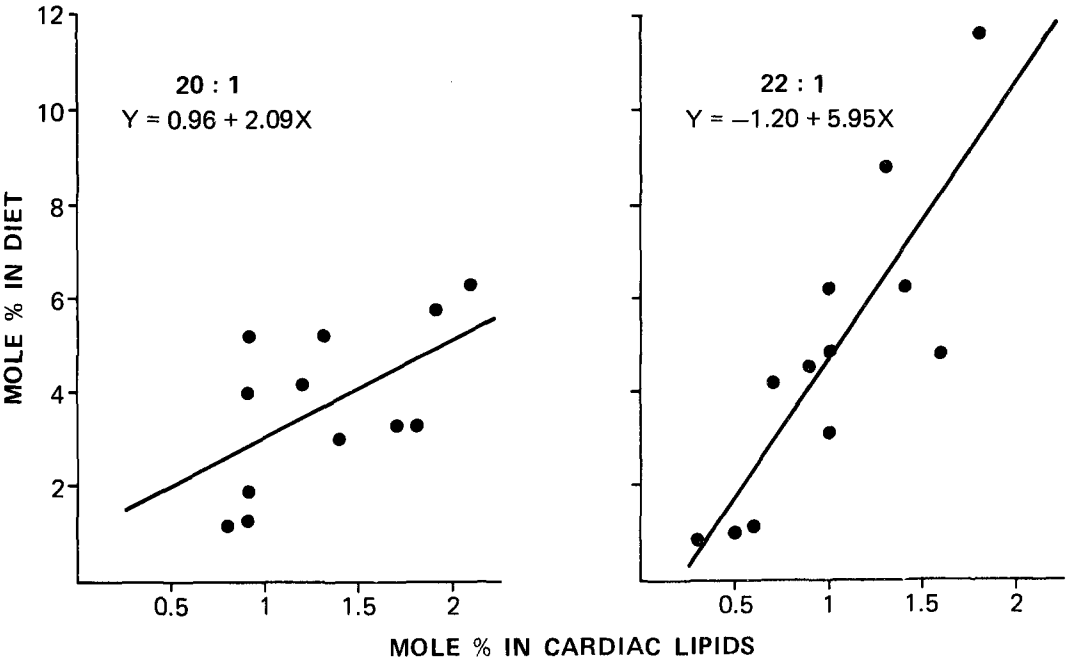

FIG. 1. Relationship of 20:1 and 22:1 in the diet compared to the relative concentration of these acids in total cardiac lipids after 16 weeks on diets containing rapeseed oil (RSO) or fractions isolated from RSO. Each point represents the mean of 4 or more rats.

linear relationship (r = 0.85) between the dietary level of 22:1 and that found in total cardiac lipids after 16 weeks (Figure 1). The linear relationship was less evident (r = 0.57) with 20:1 (Figure 1). A possible explanation for this could be that 20:1 is derived from the diet and by β-oxidation of 22:1 (15). Linolenic acid was found in the cardiac lipids of rats fed diets containing this fatty acid.

A comparison of the cardiac lipids of rats fed diets containing olive oil or RSO showed a remarkable similarity in the fatty acid profile of principal fatty acids. Diets containing corn or safflower oil, when fed to rats, gave significantly higher levels ($P < 0.01$) of 16:0 and 18:2 in the heart compared to rats fed RSO, while the level of 18:1 was lower ($P < 0.01$) and the relative abundance of 18:0 and 20:4 was not significantly different.

No major difference was observed in the principal fatty acids in the hearts of rats fed Span RSO compared to MD 5, 6, 7B, and 1″, other than 20:1 and 22:1 being lower ($P < 0.01$) when MD 1″ was fed. With few exceptions, the same was evident in experiment II and III; no major difference was observed between the cardiac lipid composition of rats fed Span RSO and rats fed fractions F1, F2, and F3 from adsorption chromatography. The exceptions were 20:1 and 22:1, which were significantly higher, ($P < 0.01$) when F1 was fed, and 18:3 which was higher when F3 was fed.

In accordance with our previous observations (2), the results in Table II show that rats fed control diets had heart lesions, but the incidence and the severity was generally less than that observed in rats fed RSO or its fractions. In this experiment, when rats fed Oro and Span RSO were compared to rats fed chow or olive oil, neither incidence nor severity were significantly different at the 5% probability level. However, it should be pointed out that the incidence of lesions approached significant difference ($P = 0.06$). As shown in Table II, fractions MD 7B and MD 1″ were highly cardiopathogenic ($P < 0.001$) compared to MD 5, MD 6, Span RSO, and Oro RSO, both in incidence and severity. Finally, the 25 rats fed the MD 1″ diet for 12 weeks showed significantly fewer lesions ($P < 0.01$) than rats fed the same diet for 16 weeks; no difference was observed in severity between these 2 groups.

The results in Table III show that the incidence of myocardial lesions was not different in the rats fed the pure triglyceride F2 and the original Span RSO. However, the incidence for both was significantly higher ($P < 0.001$) than that observed in rats fed the control diets. This observation was confirmed by the results shown in Table IV, which in addition showed no dif-

TABLE II

Myocardial Lesions in Male Rats: Experiment I

Diet	Incidence	Severity index[a] 1-2	3-5	6-10	>10
Chow	6/20	4	2	0	0
Olive	4/20	4	0	0	0
Oro RSO[b]	19/50	9	7	3	0
Span RSO	23/50	13	4	6	0
MD5[c]	31/50	17	9	4	1
MD6	24/50	8	10	3	3
MD7B	40/50	9	11	9	11
MD1″	19/25	8	5	0	6
MD1″(12 wks)	10/25	3	4	1	2

χ^2 Analysis[d]	Incidence	(d.f.)[e]	Severity	(d.f.)[e]
Control	0.53	(1)	2.38	(1)
Treated	27.25***	(5)	41.25***	(15)
Control vs. treated	14.72***	(1)	8.71*	(3)
Control vs. Oro, Span, MD5 & MD6	7.91**	(1)	5.92	(3)
Control vs. Oro and Span	3.67	(1)	4.96	(2)
MD7B and MD1″ vs. Oro, Span, MD5, and MD6	21.06***	(1)	20.26***	(3)
MD1″(12 wks vs. 16 wks)	6.82**	(1)	0.62	(3)

[a]Severity index is the number of rats with lesion scores of 1-2, 3-5, 6-10 and >10 in 2 sections per heart.

[b]RSO = Rapeseed oil.

[c]MD = molecular distillate.

[d]A comparison of rats affected to no. rats examined was used in the analysis of incidence. Only rats with heart lesions were compared in the analysis of severity. Significant difference: *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$).

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

ference in the incidence of myocardial lesions with the feeding of Tower RSO, Span RSO, F1, F2, and F3. This treated group showed a highly significant difference in incidence ($P < 0.001$) and severity ($P < 0.01$) of heart lesions when compared to the group of rats fed chow, safflower oil, or corn oil.

DISCUSSION

As indicated in our previous publication (2) and again emphasized here, male rats fed control diets, i.e., chow, corn oil, olive oil, or safflower oil, have a definite incidence of myocardial necrosis, which may in some instances approach that observed in rats fed Oro or Span RSO (Table II). This would suggest that the factor(s) responsible for myocardial lesions in male rats are not unique to RSO. In general, however, when RSO or fractions isolated from RSO were fed to male rats, the incidence of myocardial necrosis was considerably greater than that observed in rats fed control diets (Tables III and IV).

Rocquelin and Cluzan (1) were the first to suggest that a cardiotoxic factor might be present in the unsaponifiable fraction of RSO. More recently, Beare-Rogers, *et al.*, (16) claimed to have concentrated this factor in the deodorizer condensate from *B. campestris* RSO. The results of the present study deal with the question of which constituent of RSO causes myocardial necrosis in male rats.

From the present work, brassicasterol, characteristic of the Cruciferae family (17, 18), certainly could be eliminated as causing these pathogenic results. This sterol was not found in triglyceride fraction F2 obtained from adsorption chromatography and was present in only very low levels in MD 5, 6, and 7B, and yet these fractions showed as great or greater incidence of myocardial lesions than the original oil. Furthermore, male rats fed fractions F1 and MD1″ which were highly enriched with brassicasterol did not exclusively cause myocardial lesions compared to fractions which were devoid of this sterol. The glucosinolates (18) may also be ruled out, because these compounds would be retained on the adsorbent in adsorption chromatography and remain in the residue of molecular distillation. Similarly, terpenic alcohols (18) would be concentrated in the light

TABLE III

Myocardial Lesions in Male Rats: Experiment II

Diet	Incidence	Severity index[a] 1-2	3-5	6-10	>10
Chow	1/10	0	0	1	0
Olive	2/10	2	0	0	0
Span Rapeseed Oil	6/10	3	0	3	0
F2[b]	8/10	2	3	0	3
χ^2 Analysis[c]		Incidence (d.f.)[d]		Severity (d.f.)	
Controls		0.40	(1)	3.82	(2)
Treated		0.97	(1)	12.39**	(3)
Control vs. Treated		13.21***	(1)	2.97	(3)

[a]Severity index is the no. rats with lesion scores of 1-2, 3-5, 6-10, or > 10 in 3 sections per heart. Note: 4 of the rats fed the diet containing F2 had only 2 sections examined histologically.

[b]F2 = pure triglyceride fraction obtained from Span Rapeseed oil by adsorption chromatography.

[c]See Footnote d in Table II.

[d]d.f. = degrees of freedom; see footnote e in Table II. ** = ($P < 0.01$); *** = ($P < 0.001$).

TABLE IV

Myocardial Lesions in Male Rats: Experiment III

Diet	Incidence	Severity index[a] 1-2	3-5	6-10	>10
Chow	11/20	5	4	1	1
Safflower	7/20	7	0	0	0
Corn	9/20	4	3	1	1
Tower RSO	15/20	8	2	2	3
Span RSO	17/20	2	1	3	11
F1[b]	19/20	3	6	3	7
F2	16/20	10	1	2	3
F3	16/20	7	2	4	3
χ^2 Analysis[c]		Incidence (d.f.)[d]		Severity (d.f.)	
Control		1.63	(2)	9.03	(6)
Treated		3.80	(4)	23.61*	(12)
Control vs. treated		24.99***	(1)	11.55**	(3)

[a]Severity index is the no. rats with lesion scores of 1-2, 3-5, 6-10, or >10 in 3 sections per heart.

[b]F1, F2 and F3 = fractions isolated from Span RSO by adsorption chromatography.

[c]See Footnote d in Table II.

[d]d.f. = degrees of freedom; see footnote e in Table II. * = ($P < 0.05$); ** = ($P < 0.01$); (*** = $P < 0.00$).

distillate MD 1″ from molecular distillation and would be present in F3 obtained from adsorption chromatography. We conclude, therefore, because all the fractions tested caused myocardial lesions, and because triglycerides are the major constituents of all these fractions, that the triglycerides of RSO are the major components responsible for the myocardial lesions. In support of this, when RSO was tested for cardiotoxicity after various stages of commerical refinement, no decrease in cardiotoxicity was observed (19), in spite of the fact, that during deodorization volatile compounds were removed.

Nevertheless, feeding MD 1″, a fraction which contains many of the compounds present in the deodorizer condensate which was fed by Beare-Rogers, *et al.*, (16), did give a higher incidence of myocardial necrosis. Such results must be interpreted with caution because both the deodorizer condensate and MD 1″ still contained 80-90% mono-, di-, and triglycerides.

However, the possibility that a second cardiotoxic factor not related to triglycerides may be present in this fraction cannot be excluded at this time.

An early concept proposed by Abdellatif and Vles (7,20) suggests that 22:1 and 20:1 fatty acids are the primary agents responsible for the pathogenic properties of RSO, because myocardial lesions could be reproduced by feeding a 1:1 mixture of sunflower oil and crude trierucin (84%, 22:1). This view is no longer tenable because new RSO varieties very low in 20:1 and 22:1 still result in significant incidence of myocardial lesions when fed to male rats. Nevertheless, as indicated by the present work, as well as by the results of Rocquelin, *et al.*, (21), when the level of long chain monoenes exceeds an, as yet, unspecified level, it can by itself cause an increased incidence of myocardial necrosis. For that reason, a dose response of myocardial lesions to 22:1 could be expected by feeding a RSO high in 22:1, but not one low in 22:1. The results of Beare-Rogers, *et al.*, (16) appear to confirm this hypothesis.

We conclude that the primary myocardiotoxic factor of RSO is in the triglyceride fraction, but it may not be due solely to long chain monoenoic acids, i.e., 20:1, 22:1, and 24:1. As suggested in our earlier work (2), RSO triglycerides have a composition which is different from corn, safflower, olive, or soybean oil. These differences include altered saturated to unsaturated ratios with low levels of 16:0, a high concentration of 18:3, and a low 18:2/18:3 ratio. It seems possible that RSO triglycerides have a combination of properties that are responsible for cardiotoxicity in male rats. In addition to the properties listed above, other characteristics unique to RSO may include the presence of n-7 fatty acids (22) and an asymmetric distribution of 18:3 in the triglycerides (23).

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Reaction of Ergosteryl Acetate with Maleic Anhydride and Preparation of 5,7-Ergostadien-3β-ol[1,2]

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ABSTRACT

An improved, large scale synthesis of the ergosteryl acetate-maleic anhydride Diels-Alder adduct and its pyrolysis are described. The complex mixture obtained by reaction of the two constituents was refluxed with methanol to convert the succinic anhydride derivatives, formed by the "ene" reaction, to soluble half esters, leaving the insoluble Diels-Alder adduct largely unchanged. The latter was hydrogenated and pyrolyzed in vacuo to yield 5,7-ergostadienyl acetate together with lesser quantities of the acetates of 7,9(11)-ergostadien-3β-ol, 6,8(9)-ergostadien-3β-ol, and 8,14-ergostadien-3β-ol. These components were separated and purified by argentation column chromatography and crystallization.

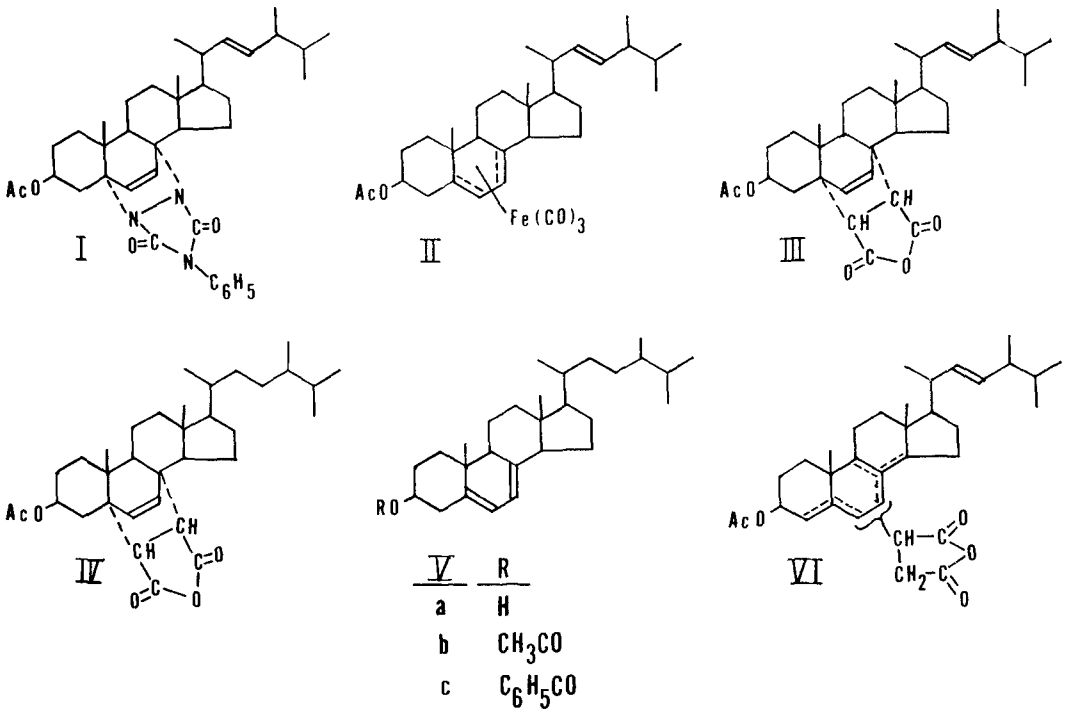
[1]Contribution No. 2395, Arizona Agricultural Experiment Station.

[2]Presented at the AOCS Meeting, Philadelphia, October, 1974.

INTRODUCTION

We required 5,7-ergostadien-3β-ol (Va; Scheme 1) for our studies on sterol utilization by species of *Drosophila*. A diene synthesis (1) applied to 22,23-dihydrobrassicasteryl acetate was considered and rejected because preparation of this sterol in a large quantity is time consuming (2,3). The hydrogenation of diene blocked derivatives of ergosterol seemed to be a better route.

At first, the triazoline dione adduct (I; Scheme 1) appeared ideal for this purpose. The derivative is obtained in good yield and can be converted readily back to ergosterol with $LiAlH_4$ (4). Hydrogenation of I (Scheme 1) over palladium, platinum or Raney nickel, however, always reduced the Δ^6 bond before the Δ^{22} was attacked (A. Wilkinson, unpublished



Scheme I. Formulas of adducts and products.

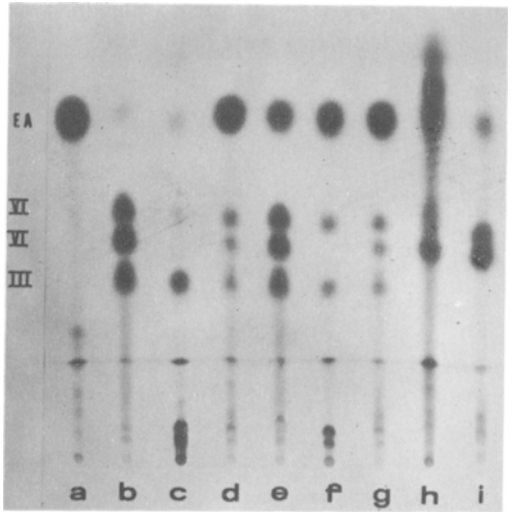


FIG. 1. TLC, System A. a = Ergosteryl acetate (EA); b = EA-maleic anhydride (MA) reaction in inert solvents; c = EA-MA reaction in polar solvents; d = EA-MA reaction, 4:1 ratio of reactants, respectively; e = EA-MA reaction in 25 ml benzene; f = EA-MA reaction with 0.025 ml triethylamine; g = EA-MA reaction, room temperature, 4 days; h = same as g plus BF_3; i = same as g plus $AlCl_3$. III,VI = see Scheme 1.

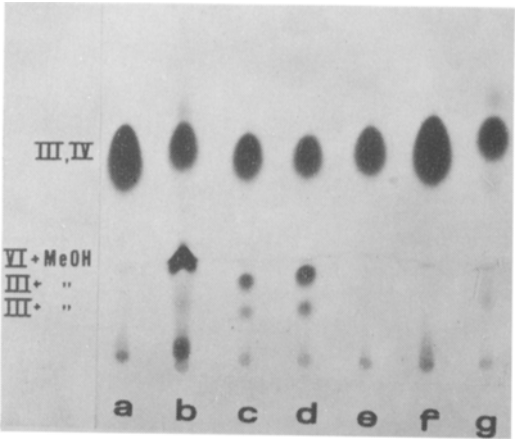


FIG. 2. TLC System A. a = Adduct III, 3 days, 170 C, with and without maleic anhydridie (MA); b = ergosteryl acetate (EA)-MA reaction products refluxed 4 hr in methanol; c = adduct III refluxed overnight in methanol; d = crude EA-MA adduct III; e = purified adduct III; f = 22,23-dihydroadduct IV; g = 6,7; 22,23-tetrahydroadduct. III,IV,V = see Scheme 1.

data). After our work was completed, similar results were reported (5).

The iron tricarbonyl complex (II; Scheme 1) was prepared next (6). It tenaciously resisted hydrogenation over palladium, even at 150 C and 14 atm. Use of Raney nickel destroyed the yellow complex at room temperature and gave 7,22- and 7-derivatives, the typical products of hydrogenation of ergosteryl acetate (EA) over nickel (A. Wilkinson, unpublished data).

We then turned to the classical synthesis of 5,7-ergostadienylacetate (Vb; Scheme 1) via the maleic anhydride (MA) adduct (III; Scheme 1) and its dihydro derivative (IV; Scheme 1) (7). This procedure has been used frequently for the preparation of 22,23-dihydroergosteryl acetate and the free sterol (8-12), but experimental details and physical constants are missing in several of these reports. A number of related studies (13-19) discussed the EA reaction with MA and described products of the general formula (VI; Scheme 1) formed by the "ene" reaction (20) between the reactants.

In this paper we report further studies on the reaction of EA with MA, a simple procedure for the large scale preparation and purification of the Diels-Alder adduct (III; Scheme 1), its hydrogenation and pyrolysis, and the isolation of Vb (Scheme 1), and three other ergostadienyl acetates from the pyrolytic reaction mixture.

EXPERIMENTAL PROCEDURES

EA was recrystallized from chloroform-methanol, MA was sublimed at atmospheric pressure, and thiophene free benzene was dried over sodium and distilled. Sealed tube reactions were run under N_2 in an oil bath. Autoclave reactions were run in the 1 liter Parr Series 4500 pressure apparatus. Melting points are corrected and were taken in vacuo in capillary tubes with a Thomas-Hoover apparatus. Systems for TLC were: A) chloroform:acetone: acetic acid (97:2:1), silica gel plates: B) chloroform:acetone (98:2), 10% $AgNO_3$-silica gel plates; 30% H_2SO_4 spray. GLC with 5% OV-101 on Anachrom ABS, 260 C; relative retention times of ergostanyl acetate derivatives (cholesteryl acetate = 1.00) were: $\Delta^{8(14)}$ = 1.26; Δ^5 = 1.27; $\Delta^{7,9(11)}$ = 1.35; $\Delta^{8,14}$ =1.36; $\Delta^{6,8(9)}$ = 1.36; $\Delta^{5,7}$ = 1.36; $\Delta^{5,7,22}$ = 1.20. UV spectra in 95% ethanol were obtained with the Perkin-Elmer 202.

Preparation of EA-MA Adduct III

A. Preliminary experiments:

1. EA and MA (200 mg, each) in 5 ml inert solvent (ethyl acetate, 1,2-dichloroethane, carbon disulfide, mesitylene [Matheson Coleman and Bell, Los Angeles, CA], Skellysolve B [Skelly Oil Company, Tulsa, OK], tetrahydrofuran, carbon tetrachloride, chloroform, acetic acid, benzene, xylene [Mallinckrodt Chemical Works, St. Louis, MO], acetonitrile, mono-, di- and triglyme, acetic anhydride [Aldrich Chemical Company, San Leandro, CA]) or no sol-

vent were heated in sealed tubes 3 hr at 180 C. The tubes were cooled, opened, and the contents assayed by TLC (Fig. 1b).

2. The same reaction (Experiment 1) was run in 5 ml polar solvents (dimethyl formamide, dimethyl acetamide, ethylene carbonate, dioxane, pyridine, collidine [Aldrich Chemical Co., San Leandro, CA]). The reaction mixtures were poured into water and the products extracted with benzene and assayed by TLC (Fig. 1c).

3. EA (200 mg) and MA (¼ to 3 moles/mole EA) were heated at 180 C, for 3 hr in benzene (Fig. 1d).

4. EA (200 mg) and MA (100 mg) were heated at 180 C for 3 hr in 5-25 ml benzene (Fig. 1e).

5. EA (200 mg) and MA (100 mg) in 5 ml benzene were heated at 180 C for 3 hr with 0-0.1 ml triethylamine (Fig. 1f).

6. EA and MA (200 mg, each) in 5 ml benzene, N_2, 25 C, 4 days (Fig. 1g).

7. Same as Experiment 6, with 0.5 ml BF_3 etherate (Fig. 1h).

8. EA and MA (200 mg, each) in 5 ml ethylene chloride, 6.6 mg sublimed $AlCl_3$ added, room temperature, 4 days (mole ratios 1 EA:4 MA:0.1 $AlCl_3$) (Fig. 1i).

9. Adduct III (50 mg) in 3 ml benzene, sealed tube, N_2 170 C, 3 days with and without 50 mg MA (Fig. 2a).

10. Products from a typical EA-MA reaction in benzene (Fig. 1b) were refluxed with methanol for 4 hr (Fig. 2b).

11. Adduct III (700 mg) in 50 ml methanol was refluxed overnight (Fig. 2c).

B. Large scale preparations: EA (100 g) and MA (50 g) in 600 ml benzene were heated under N_2 in the autoclave for 20 hr at 150 C. The yellow charge transfer complex that formed immediately between the reagents gradually disappeared as the reaction progressed. After cooling and addition of Celite (Johns-Manville, Denver, CO), the benzene solution was filtered and solvent removed in vacuo in a 2 liter flask. Methanol (850 ml) was added to the residue (Fig. 1b), and the suspension refluxed 4 hr to convert the "ene" reaction products (VI; Scheme 1) to mono-methyl esters (Fig. 2b). The resulting mixture was cooled overnight at 4 C after which the prisms of the Diels-Alder adduct III (Scheme I) were removed and dried (Fig. 2d) (mp = 215-218 C). Yield from five such runs was 132 g (21.5%). The combined products were recyrstallized from 2 liters acetone to give 127 g pure III (Scheme 1) (Fig. 2e) with a mp = 219.5-220.5 C. In the literature, the mp is reported as: 210-212 C (9); 216 C (8,14); 216-218 C (16,18); 217.5-218 C (11); and 217-220 C (19). The compound pyrolyzed in the injection port of the gas chromatograph (290 C) to give a single peak corresponding in retention time to that of EA.

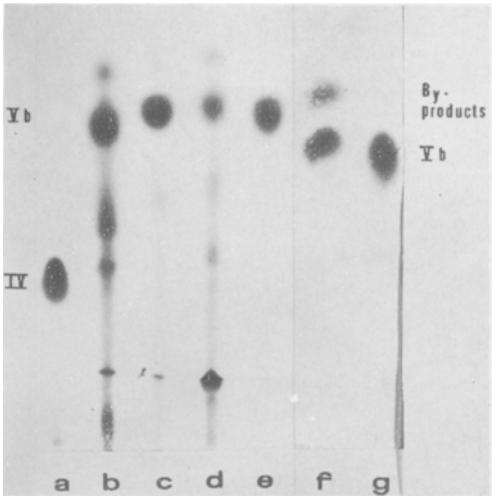


FIG. 3. TLC System A (left side). a = Dihydro-adduct IV; b = sublimate from pyrolysis of IV; c = methanol insoluble ergostadienyl acetates; d = methanol soluble products; e = crude 5,7-ergostadienyl acetate. TLC, System B (right side). f = Crude 5,7-ergostadienyl acetate; g = purified 5,7-ergostadienyl acetate. IV,V = see Scheme 1.

22,23-Dihydro adduct IV

Adduct III (50 g) in 1 liter distilled ethyl acetate was stirred overnight at room temperature and atmospheric pressure with 10% Pd on carbon (4 g) and hydrogen. Catalyst and solvent were removed to leave IV (Scheme 1) as an amorphous solid (Fig. 2f), (sinters = 172 C, melts = 203.5-205 C). The literature report sinters = 172-174 C, melts = 202-203 C (14). All attempts to crystallize IV from many solvents failed. The compound also pyrolyzed in the gas chromatograph and gave a single peak corresponding in retention time to that of 22,23-dihydroergosteryl acetate (Vb; Scheme 1).

6,7;22,23-Tetrahydro adduct

Adduct III (0.5 g) in 50 ml ethyl acetate containing five drops of 70% $HClO_4$ was stirred with PtO_2 (0.15 g) and hydrogen for 2 weeks. Catalyst was removed, the solution washed with aqueous sodium acetate and evaporated. The residue was crystallized from methanol to yield 0.2 g tetrahydro EA-MA Diels-Alder adduct (Fig. 2g) with a mp = 186-188 C. In the literature, the mp is reported to be 187-187.5 C

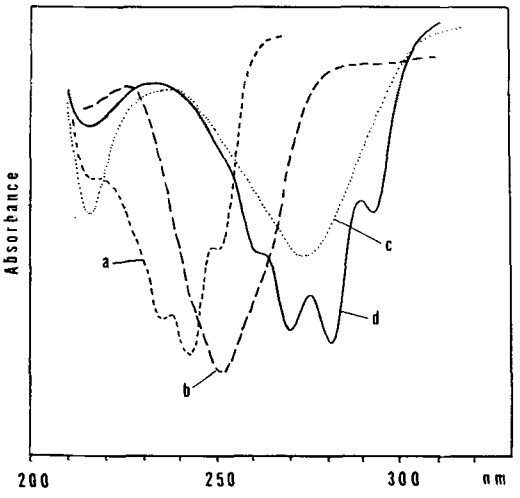


FIG. 4. UV spectra of: a = 7,9(11)-ergostadien-3β-ol; b = 8,14-ergostadien-3β-yl acetate; c = 6,8(9)-ergostadien-3β-ol; d = 5,7-ergostadien-3β-ol.

(14). The tetrahydro adduct gave no peak on GLC and was stable to pyrolysis (220 C, 6.5 hr, in vacuo).

Pyrolysis of dihydro adduct IV

A magnetic stirring bar and 10 g IV (Scheme 1) (Fig. 3a) were placed in each of 2 1-liter filter flasks which were closed with a rubber stopper and attached to a vacuum manifold. The system was evacuated several times and flushed with N_2, a water aspirator vacuum applied (32 mm), and the flasks immersed to a depth of 4 cm in 220 C oil baths on magnetic stirrer hot plates. After 5 hr, an oil pump vacuum was applied (0.2 mm) and the products of pyrolysis sublimed to the cooler walls of the flasks for 2 days. After cooling, N_2 was admitted to the system and the sublimates (Fig. 3b) removed from the unsublimated residues with benzene. Evaporation of the benzene left a solid residue (80 g) that was suspended in 800 ml methanol and refluxed 4 hr under N_2. The mixture was cooled at 4 C overnight and filtered (Fig. 3c,d). The precipitate was refluxed briefly under N_2 with 250 ml acetone, the mixture again cooled overnight, and filtered to give 40 g crude 5,7-ergostadienyl acetate (Vb; Scheme 1); mp =155-156 C; ϵ_{282} = 9,200, ca. 80% pure (Fig. 3e,f).

Purification of 5,7-ergostadienyl acetate (Vb)

Four kg 100 mesh SiO_2 (Mallinckrodt Chemical Co., St. Louis, Mo.), 800 g $AgNO_3$, and 2 kg Super-Cel (Johns-Manville, Denver, CO) were slurried with 3 liters water and dried on the steambath and at 110 C. The mixture was screened (80 mesh) and activated overnight at 110 C. Typically, 1 kg of this mixture was poured into 6 x 120 cm chromatographic tubes with 5% ether in low boiling petroleum ether (Skellysolve F, Skelly Oil Co., Tulsa, OK), the steryl acetate samples placed on the columns (10 g in 30 ml benzene), and the columns eluted with 5% ether in Skellysolve F. Fractions (500-700 ml) were collected, evaporated at atmospheric pressure under N_2. The residues were analyzed by GLC, TLC, and UV. After a run, the columns were eluted with ether, the packing dried, rescreened, reactivated, and reused.

A single passage of crude Vb through the tubes gave the fractions shown in Table Ia. Six more runs on pooled materials gave the fractions also shown in Table Ib. The two purest samples (D and E, Table I) were recrystallized from ethanol to give 3.45 g Vb (97.3% purity by UV) and 9.95 g Vb, respectively; mp = 162-163 C; ϵ_{282} = 11,400 (98.4% purity). A sample of the latter was recrystallized from acetone to yield Vb (Fig. 3g); mp =162.5-163.5 C; ϵ_{282} = 11,560 (99.7% pure). The literature report the mp as 155-157 C (11), 157-158 C (7), 160-162 C, 165-166 C (9). As a standard for UV, a chromatographically pure sample of 7-dehydrocholesteryl acetate (mp = 149-149.5 C) exhibited an ϵ_{282} = 11,600 with the spectrophotometer.

5,7-Ergostadien-3β-ol (Va)

A portion of Vb was hydrolyzed under N_2 with alcoholic KOH and the product recrystallized from acetone to give long needles of Va monohydrate, with a mp = 161.5-162.5 C, and ϵ_{282} = 11,600 (Fig. 4d). In the literature the mp= 150-151 C (9), 152-153 C (7,12), and 153-154 C (8).

5,7-Ergostadien-3β-yl benzoate (Vc)

Va (200 mg) in 5 ml pyridine were stirred under N_2 while 3 0.2-ml portions of benzoyl chloride were added over 30 min. Stirring was continued for 2 hr, after which time 50 ml methanol was added to decompose excess benzoyl chloride, solubilize the pyridinium hydrochloride, and precipitate the steryl benzoate (Vc; Scheme 1). The latter was recrystallized from acetone-benzene, with a mp = 162.5-163 C. The mp as cited in a previous study (9) is 156-157 C.

Separation of byproducts formed during pyrolysis of IV

Fraction B (13.4 g; Table Ib) was placed on a 1 kg silver nitrate column and eluted with 1% ether in Skellysolve F. Eluates (300 ml) from

TABLE I

Silver Nitrate Column Purification of Crude 5,7-Ergostadienyl Acetate (40 g)

	a		b	
Fraction	Wt (g)	Purity by UV	Wt (g)	Purity by UV
A	0.52	Byproducts	1.17	Oily impurity
B	3.55	Byproducts	13.75	Byproducts
C	11.17	60%	1.01	60-80%
D	13.25	80-90%	4.43	>93%
E	8.71	>95%	12.48	>95%
F	0.96	ca. 90%	5.48	Waxy ether eluate from columns
Recovery	38.20		38.30	

the column were analyzed by UV; all of the diene byproducts had the same retention time on GLC and the same R_f on TLC. The following crude fractions were obtained A) 1.51 g, rich in 7,9(11)-ergostadienyl acetate; B) 3.16 g, rich in 6,8(9)-ergostadienyl acetate; and C) 1.3 g, rich in 8,14-ergostadienyl acetate. The compounds were purified and identified as follows.

7,9(11)-Ergostadien-3β-ol. The impure acetate was hydrolyzed and free sterol purified on a 20% silver nitrate alumina column with 20% ether in Skellysolve F. The sterol was recrystallized from methanol, with a mp = 145.5-146.5 C, and ϵ_{236} = 14,300, ϵ_{243} = 16,200, and ϵ_{251} =10.800 (Fig. 4a). As cited in the literature, mp = 149-151 C (11), ϵ_{242} = 9,400 (11). Dorfman (21) lists ϵ_{236} = 14,200, ϵ_{243} = 15,700, and ϵ_{251} = 10,900 for 7,9(11),22-ergostatrien-3β-ol.

6,8(9)-Ergostadien-3β-ol. The impure acetate was recrystallized several times from acetone, hydrolyzed, and the free sterol recrystallized from acetone and ethanol, with a mp = 158-159 C, and ϵ_{273} =5,000 (Fig. 4c). The compound has never been reported. Its structure was deduced from the UV spectrum; ϵ_{275} = 4,700 for 6,8(9)-coprostadienol and ϵ_{275} = 5,300 for 6,8(9)-cholestadienol (21). The free sterol partially decomposed on a silver nitrate alumina column.

8,14-Ergostadien-3β-yl acetate. The impure acetate was rechromatographed on a 20% $AgNO_3$ silica gel column with 1% ether in Skellysolve F and recrystallized many times from methanol. The 8,14-acetate was enriched in the methanol soluble fractions. Eventually a chromatographically pure sample was obtained, (mp = 136-137.5 C, λmax 251 nm) (Fig. 4b). As cited in the literature, the mp = 136-138 C, λmax 251 nm (22). The 8,14-acetate was hydrogenated over Pd in ethyl acetate-acetic acid to 8(14)-ergosten-3β-yl acetate, with a mp = 109.5-111 C. As cited in the literature, mp = 110-111 C (23). The free diene also partially decomposed when purification was attempted on a silver nitrate alumina column.

DISCUSSION

The composition of the product mixture obtained by reaction of EA with MA appeared to be insensitive to the conditions of the reaction. The same ratio of products formed, III vs VI as assessed by TLC, when the reaction was run in all types of inert solvents (Fig. 1b), when 0.25-3 moles MA were used/mole EA (Fig. 1d), and when the concentration of the reagents was varied (Fig. 1e). Although the reaction was inhibited in polar solvents and amines (Fig. 1c), partial inhibition by very small amounts of triethylamine did not prevent formation of "ene" reaction products (Fig. 1f). Even at room temperature, where the reaction was very slow (13), the usual ratio of products still formed (Fig. 1g).

The Diels-Alder additions of MA to aromatic dienes and of α,β-unsaturated carbonyl compounds to butadienes are catalyzed by Lewis acids (24-26). Neither $AlCl_3$ nor BF_3 were of any value in our case, however; the rate of isomerization of EA by these reagents canceled any catalytic effect they might have had on the Diels-Alder reaction (Fig. 1h,i).

To determine whether the "ene" products (VI) were produced by rearrangement of III, the latter was heated in benzene for 3 days at 170 C with and without MA. No changes were noted (Fig. 2a), showing that once formed, III is stable to the reaction conditions. These experiences, plus those of others (14,16-18), indicate that a 20-25% yield of III is the best attainable from the reaction of EA with MA.

At first, we isolated III by crystallization

(14) or column chromatography (9), but these methods were tedius and did not readily give pure samples. By chance we noted that when the reaction mixture (Fig. 1b) was crystallized from hot methanol, a portion of the "ene" products was converted to materials having a lower R_f on TLC. When the mixture was refluxed 4 hr in methanol, virtually all of the "ene" products were converted to more polar compounds (Fig. 2b). The reaction of methanol with the mixture of monosubstituted succinic anhydrides VI to form methyl hydrogen succinates is apparently much faster than its reactions with the rigid, more hindered, disubstituted succinic anhydride III. Even when III was refluxed 18 hr with methanol, less than half of the material was converted to a mixture of two monoesters (Fig. 2c).

This led to a convenient preparation of III in large quantities without fractional crystallizations or column chromatography. EA can be heated with excess MA in a number of common solvents under N_2 until all of the sterol has reacted (Fig. 1b). The solvent is then removed, and the residue refluxed with methanol for 4 hr to react with and solubilize the "ene" products (VI). The Diels-Alder adduct (III) reacts only to a negligible extent under these conditions and remains insoluble (Fig. 2d). It is then crystallized from acetone to remove traces of impurities (Fig. 2e).

Hydrogenation of III to IV (Fig. 2f) over Pd goes smoothly; overhydrogenation is not a problem. Even if a little occurs, the tetrahydroderivative (Fig. 2g) is stable to pyrolysis. Underhydrogenation of III can be detected by GLC. Pyrolysis of III and IV in the injection port of the gas chromatograph yields EA and its 22,23-dihydroderivative, two sterols that are easily separated by this method.

Many small scale experiments on the pyrolysis of the dihydro adduct (IV) led to the method described in the previous section. Pyrolyses in solution (squalene or dibutyl phthalate), in benzene containing cyclopentadiene, at various temperatures (190-240 C) and times, and in different types of apparatus were all tried. None of the methods prevented formation of anhydride containing byproducts (Fig. 3b). These, however, could be removed readily from the mixture of ergostadienyl acetates by refluxing the pyrolyzate in methanol. The anhydrides were converted to methyl hydrogen esters and dissolved (Fig. 3d) in the alcohol, whereas, the steryl acetates remained largely insoluble (Fig. 3c). It could not be determined whether the anhydrides were derived from rearrangements of IV at 220 C or in a recombination of released MA with Vb or other dienes by the "ene" reaction.

Separation of 5,7-ergostadienyl acetate from the other diene acetates by multiple recrystallizations was uneconomical. Chromatography of the crude steryl acetate mixture (Fig. 3e,f) on silver nitrate columns led to Vb in 93-95% purity, after which it could be crystallized to 99.7% purity without excessive loss of material. Chromatography of 5,7-dienes on silver nitrate columns led to some losses (27), but these were minimized by exclusion of air, where possible, and by working with acetates (28).

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Unique Pathways of Sterol Metabolism in the Mexican Bean Beetle, A Plant-Feeding Insect[1]

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ABSTRACT

Radiolabeled sterols, ^{14}C-cholesterol, ^{14}C-cholestanol, ^{3}H-stigmasterol, ^{3}H-stigmastanol, and ^{3}H-sitosterol, were fed to larvae of the Mexican bean beetle, *Epilachna varivestis* Mulsant, by coating soybean leaves. Free sterol and sterol ester fractions from treated insects were isolated and analyzed, and in each case nearly 30% or more of total radiolabeled sterols retained by the insect were found in the sterol ester fraction after 8 days. Δ^5-Dietary sterols were readily reduced to stanols, and C_{29}-stanols thus produced were dealkylated to cholestanol. Significant amounts of labeled lathosterol were formed from Δ^5-C_{29} sterols; little, if any, radiolabeled cholesterol was detected in insects fed either of the labeled Δ^5-phytosterols, stigmasterol or sitosterol. Sterol metabolism of this insect thus differs considerably from that found for most phytophagous insects.

INTRODUCTION

The Mexican bean beetle, *Epilachna varivestis* Mulsant, reared on a natural host plant, soybean leaves, recently has been shown to have an unusual composition of sterols compared with that of other phytophagous insects (1,2). A salient difference in the sterols of this insect was the high level of saturated sterols (>50%) present in the egg, larval, and adult stages, as opposed to extremely low levels of saturated sterols found in soybean leaves. In addition, lathosterol (7-cholesten-3β-ol) was a major sterol normally found in this insect, comprised ca. 12% of the total sterols, and was accompanied by lesser quantities of Δ^7-campestenol and Δ^7-stigmastenol. There was also a surprisingly low level of cholesterol in the sterols of this bean beetle; < 5% of total sterols in each of the developmental stages examined was cholesterol.

From this information, it would appear that the Mexican bean beetle is able to reduce dietary Δ^5-phytosterols, e.g., sitosterol, stigmasterol, and campesterol, dealkylate corresponding stanols to produce cholestanol, and introduce a Δ^7-bond to produce lathosterol and other Δ^7-sterols. To obtain direct evidence for these possible pathways of sterol metabolism in this insect, Mexican bean beetle larvae were fed 5 radiolabeled sterols, ^{14}C-cholesterol, ^{14}C-cholestanol, ^{3}H-stigmasterol, ^{3}H-stigmastanol, and ^{3}H-sitosterol, coated on soybean leaves. Sterols from treated insects were isolated and analyzed to determine the fate of test compounds.

EXPERIMENTAL PROCEDURES

Newly hatched Mexican bean beetle larvae were reared on soybean leaves as described previously (1). After 14 days, groups of 20 larvae were placed on soybean leaves that had been dipped in an acetone solution of one of the radiolabeled sterols. The specific activities of labeled sterols ranged from 4000 to 5000 cpm/μg. Insects were held 8 days on treated leaves and provided with fresh leaves ad libitum. Then they were transferred to untreated leaves for a day to clear the intestinal tract of labeled dietary sterols. They were weighed and stored frozen until they were extracted and analyzed.

[4-^{14}C]-Cholesterol used in this study was purchased from Amersham/Searle Corporation (Arlington Heights, IL). ^{14}C-Cholestanol was produced in our laboratory through catalytic reduction of ^{14}C-cholesterol in the presence of PtO_2 in acetic acid, and was purified carefully to remove all cholesterol. [2,4-^{3}H]-Sitosterol and [2,4-^{3}H]-stigmasterol were prepared as described previously (3). ^{3}H-Stigmastanol was obtained through catalytic reduction of ^{3}H-stigmasterol. Radiochemical purity was >97% in each of the labeled sterols, as established by radiochromatogram scanning and trapping effluent fractions from gas liquid chromatography (GLC).

Frozen insects were homogenized in chloroform:methanol (2:1) in a Tenbroeck tissue grinder, and crude lipids were fractionated as described previously (4) by column chromatography on Unisil (Clarkson Chemical Co., Williamsport, PA) to separate sterol esters from free sterols. Fractions were monitored by thin

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TABLE I

Uptake and Incorporation of Radiolabeled Sterols into Sterol Ester and Free Sterol Fractions of Mexican Bean Beetle Larvae

Dietary sterol	Incorporation (μg/larva)[a]	Total neutral sterols[b] (%)	
		Sterol esters	Free sterols
^{14}C-Cholestanol	6.1	38.5	61.5
^{14}C-Cholesterol	7.0	37.2	62.8
^{3}H-Stigmastanol	2.4	34.0	66.0
^{3}H-Stigmasterol	1.8	32.4	67.6
^{3}H-Sitosterol	2.1	29.0	71.0

[a]Based on specific activity of dietary sterol.

[b]Combined ester and free sterol fractions (total neutral sterols) accounted for >98% of total radioactivity recovered from Unisil columns in each case.

TABLE II

Relative Percentage of Saturated and Unsaturated Radiolabeled Sterols Isolated from Sterol Ester and Free Sterol Fractions from Mexican Bean Beetle Larvae[a]

Dietary sterol	Sterol esters (%)		Free sterols (%)	
	Saturated	Unsaturated	Saturated	Unsaturated
^{14}C-Cholestanol	> 99.5	< 0.5	> 99.5	< 0.5
^{14}C-Cholesterol	77.3	22.7	47.7	52.3
^{3}H-Stigmastanol	98.7	1.3	98.8	1.2
^{3}H-Stigmasterol	52.4	47.6	44.2	55.8
^{3}H-Sitosterol	77.4	22.6	33.6	66.4

[a]Fractions obtained by $AgNO_3$-impregnated Unisil column chromatography of acetates of ester and free sterols and monitored by $AgNO_3$-thin layer chromatography.

layer chromatography (TLC) and by counting aliquots on a Packard Tricarb Scintillation Spectrometer. The fractions containing sterol esters and free sterols were saponified, and sterols were isolated and purified by column chromatography (4). Sterols from sterol ester and free sterol fractions were analyzed separately in these studies.

Sterols were acetylated, and acetates were fractionated by chromatography on 3 g columns of 20%-$AgNO_3$-impregnated Unisil as described previously (1) to separate saturated from unsaturated sterol acetates. Aliquots of column fractions were analyzed on 20% $AgNO_3$-impregnated Silica Gel H chromatoplates developed in benzene:n-hexane (1:1). The $AgNO_3$-TLC system separated sterol acetates in this study into 2 bands, one containing all Δ^0-sterol acetates, and one containing Δ^5- and Δ^7-sterol acetates. Δ^5-Sterol acetates only partially separate from lathosterol acetate on this system. Column chromatographic fractions were analyzed by GLC on a 1% OV-17 column, by UV scanning, and by radioassay of aliquots by liquid scintillation spectrometry. GLC trapping was utilized to determine relative distribution of radioactivity in various column fractions.

RESULTS AND DISCUSSION

Uptake and retention of radiolabeled sterols by Mexican bean beetle larvae are summarized in Table I. Dietary C_{27}-sterols, cholesterol and cholestanol, were incorporated into tissues to a greater extent than any dietary C_{29}-sterols. The percentage of total labeled sterols in the ester fraction in each test in this series comprised 29-39% of the total sterols, a distribution that generally agrees with that found from quantitation by GLC of free and sterol ester fractions from larval and pupal Mexican bean beetle samples in which sterol esters comprised 25-30% of total sterol mass. Interestingly, most of the lathosterol was present in the free sterol fraction.

The metabolic fate of labeled sterols administered to bean beetle larvae was determined by fractionating sterol acetates by $AgNO_3$-impregnated column and TLC, and trapping the effluent from GLC. Major changes that were found regarding saturation of Δ^5-dietary sterols or

TABLE III

Relative Percentage of C_{27}-Sterol in the Sterol Ester and Free Sterol Fractions Resulting from Dealkylation of Dietary Radiolabeled C_{29}-Sterols by Mexican Bean Beetle Larvae

Dietary sterol	Sterol esters[a] (%)		Free sterols[a] (%)	
	Saturated	Unsaturated	Saturated	Unsaturated
^{3}H-Stigmastanol	2.8	Trace	4.1	Trace
^{3}H-Stigmasterol	57.3	15.3	61.5	53.1[b]
^{3}H-Sitosterol	12.5	10.0	21.8	29.3[b]

[a]Relative percentage of C_{27}-sterol in each fraction as determined by gas liquid chromatographic trapping and radioanalysis.

[b]Major component lathosterol.

unsaturation of Δ^0-dietary sterols are presented in Table II. Of the labeling in saturated sterols from larvae fed ^{14}C-cholesterol 77.3 and 47.7% was found in the sterol ester and free sterol fractions, respectively, an indication of efficient conversion to cholestanol. In the case of C_{29}-Δ^5-sterols, ^{3}H-stigmasterol and ^{3}H-sitosterol, 52.4 and 77.4%, respectively, of the activity appeared in saturated sterol ester fractions, and 44.2 and 33.6%, respectively, appeared in saturated free sterol fractions. These values represent both stigmastanol and cholestanol in these saturated sterol fractions derived from stigmasterol and sitosterol. Thus, it is obvious that all Δ^5-sterols investigated were readily reduced by this insect.

Lathosterol, or any other Δ^7-sterol formed from labeled dietary sterols, is included in unsaturated sterol fractions (Table II). Lathosterol comprises > 50% of radiolabel in unsaturated free sterol fraction from bean beetles fed ^{14}C-cholesterol. Very little unsaturation of the 2 dietary stanols, cholestanol and stigmastanol, occurred, an indication that only a small quantity of lathosterol was formed from either saturated stanol. This indicates that these stanols did not enter as readily as Δ^5-sterols into some system that concertedly reduced and dealkylated Δ^5-sterols and then produced lathosterol. These results could also indicate that these stanols, e.g., cholestanol or stigmastanol, were not the principal precursors of lathosterol, but other pathways might be operative in lathosterol synthesis. However, results from GLC trapping tentatively indicate that some lathosterol was formed from these 2 stanols.

C_{29}-Sterols were converted to C_{27}-sterols in every case, and the extent of dealkylation is summarized in Table III. Radioanalysis of fractions trapped from GLC indicated that significant amounts of labeled cholestanol were derived from both stigmasterol and sitosterol. Lathosterol was the major component in labeled unsaturated free sterol fractions derived from dealkylated dietary sterol in these 2 cases; >30% of unsaturated free sterol fraction from ^{3}H-stigmasterol and >15% of this fraction from ^{3}H-sitosterol were identified as labeled lathosterol. Lesser amounts of labeled lathosterol were also present in unsaturates of the sterol ester fractions. A small amount of cholesterol also may have been present in these unsaturated fractions, but association of radiolabel with cholesterol was not unequivocally established in these studies.

Some cholestanol was produced from ^{3}H-stigmastanol, represented by saturated fractions in Table III, resulting from dealkylation, but considerably less than from Δ^5-dietary sterols. As in the production of lathosterol from stanols, this may indicate that the more commonly available Δ^5-dietary sterols more readily enter some complex or system of enzymes that is able to reduce sequentially the Δ^5-bond, to dealkylate the C-24-alkyl substituent of the side chain, and to incorporate the Δ^7-bond into the nucleus. Apparently, this insect less efficiently metabolizes a C_{29}-saturated dietary sterol than a C_{29}-Δ^5-dietary sterol.

Previously reported data concerning normal sterol content of Mexican bean beetles fed soybean leaves (1) taken together with data from metabolic studies with radiolabeled dietary sterols presented here, indicate that the proposed scheme shown in Figure 1 presents probable metabolic interrelationships for dietary radiolabeled sterols used in this study. Existence of other metabolic pathways, such as the conversion of sitosterol through the intermediates fucosterol and desmosterol to cholesterol (2), cannot be ruled out completely. However, the fact that labeled cholesterol could not be identified unequivocally as a metabolite of either ^{3}H-sitosterol or ^{3}H-stigmasterol in these studies, along with the fact that neither desmosterol nor fucosterol were detected in this or our previous study (1), argues against the presence of the sitosterol → fucosterol → desmoster-

ol → cholesterol pathway being a major metabolic route in this insect. In addition, there was no accumulation of desmosterol in Mexican bean beetles reared on bean leaves treated with certain azasterols that generally inhibit Δ^{24}-sterol reductase in insects (2) and would be expected to bring about an accumulation of desmosterol if the latter pathway were operative. There may be a Δ^{24}-sterol intermediate involved in the conversion of stigmastanol to cholestanol that is analogous to the intermediate desmosterol which occurs in the sterol dealkylation pathways of certain other insects (2). Studies are now underway to determine whether or not such an intermediate does occur.

The Mexican bean beetle, which can introduce the Δ^7-double bond into the sterol nucleus, differs from *Xyleborus ferrugineus* (F.) and *Drosophila pachea* Patterson and Wheeler, 2 species of insects that require a dietary source of Δ^7-sterol (6,7). This requirement for a Δ^7-sterol can best be explained by its functioning as a precursor for essential Δ^7-steroid insect molting hormones (ecdysones). A number of other insects can introduce a Δ^7-bond into the steroid nucleus (2,8), and lathosterol has been reported to be a major component of sterols of certain other invertebrates, including some species of nematodes (9,10) and mollusks (11). However, the question remains as to how this unique metabolic system evolved in a plant-feeding insect. If phytophagy arose secondarily in an insect that was originally predacious, as with most lady bug beetles, some variations from the usual routes of metabolism of phytosterols might be expected. The Mexican bean beetle is, therefore, an extremely interesting insect from the standpoint of sterol metabolism, particularly plant sterol metabolism. When a synthetic diet becomes available, it will be possible to carry out more definitive metabolic studies with sterols in this species.

Comparative biochemical studies with insects such as the tobacco hornworm, *Manduca sexta* (L.) (2), confused flour beetle, *Tribolium confusum* Jacquelin duVal (8), and Mexican bean beetle indicate that considerably more diversification exists with regard to sterol metabolism in phytophagous insects than was believed formerly. Studies of related insects

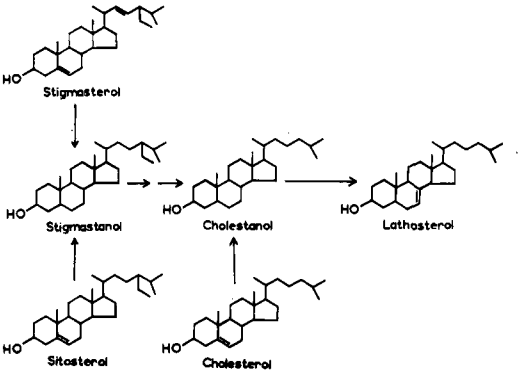

FIG. 1. Proposed metabolic scheme for radiolabeled dietary sterols in the Mexican bean beetle based on known metabolites and conversions.

with differing feeding habits, such as phytophagous and predacious species of the family Coccinellidae, could provide information to relate biochemical and physiological adaptation to phylogeny and speciation within such a group of insects.

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Elongation and Desaturation of Dietary Fatty Acids in Turbot *Scophthalmus maximus* L., and Rainbow Trout, *Salmo gairdnerii* Rich.

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ABSTRACT

Turbot and rainbow trout, which had previously received diets free of fat, were fed [1-^{14}C] fatty acids. The distribution of radioactivity in the tissue fatty acids was examined 6 days later. In rainbow trout fed [1-^{14}C] 18:3ω3, 70% of the radioactivity was present in 22:6ω3 fatty acid. In contrast, turbot fed [1-^{14}C] 18:1ω9, 18:2ω6, or 18:3ω3 converted only small amounts of labeled fatty acids (3-15%) into fatty acids of longer chain length. The major product of the limited modification found in turbot was the dietary acid elongated by 2 carbon atoms.

INTRODUCTION

Studies carried out in recent years on freshwater and euryhaline fish (1-5) indicate that the ability of these species to elongate and desaturate dietary fatty acids is similar to that of birds and mammals. Thus, when rainbow trout were fed diets containing oleic, linoleic, or linolenic acids, substantial quantities of 20:3ω9, 22:5ω6, or 22:6ω3, respectively, appeared in the lipids of different tissues (1).

In recent experiments with marine flatfish, in which both plaice (6) and turbot (7) were fed diets containing linoleic acid as the main dietary fatty acid, little 20:4ω6 or 22:5ω6 appeared in the lipids of either hepatic or extrahepatic tissues. These findings implied that marine flatfish have a more limited capacity to elongate and desaturate dietary linoleic acid than do rainbow trout.

To examine the capacity of marine flatfish to modify dietary fatty acids, pellets containing [1-^{14}C] oleic, [1-^{14}C] linoleic, or [1-^{14}C] linolenic acid were fed to turbot which had been fed fat free diets for some weeks previously. The distribution of radioactivity in tissue fatty acids of these turbot was examined 6 days later.

EXPERIMENTAL PROCEDURES

Wild turbot, about 6 months old, were weaned to a pelleted moist (35% water) artificial diet in the Institute aquarium. Then, they were given a fat free diet containing 50% crude protein (supplied by a fish protein concentrate) for 16 weeks. Hatchery reared rainbow trout, about 9 months old, were fed the same fat free diet for 6 weeks.

[1-^{14}C] Oleic acid, [1-^{14}C] linoleic acid, and [1-^{14}C] linolenic acid (The Radiochemical Centre, Amersham, England) were dissolved in chloroform to give solutions containing 1 μCi fatty acid per μl. Portions (10 μl) then were added from a microsyringe to pellets which had been freeze dried previously. The pellets then were rehydrated by serial additions of distilled water, and offered singly to tanks of fish. The fish which took the pellet then was transferred to another tank, or to the CO_2 collection apparatus.

After 6 days, fish were killed by a blow on the head, and lipids extracted by the method of Bligh and Dyer (8) as modified by Allen, et al., (9). Samples of lipid extracts were transesterified with methanolic HCl (10) for gas chromatographic analysis of methyl esters.

Radioactivity in fatty acids of the fish was measured as follows. Fatty acids were separated by gas liquid chromatography (GLC) on 1.5 mx4 mm internal diameter (ID) glass columns containing 10% EGSS-X or EGSS-Y on 100-120 mesh Gas Chrom Q. Nitrogen (50 ml/min) was used as carrier gas, and a Pye 104 gas chromatograph was equipped with flame ionization detectors. The column effluent was split 10:1 by a Pye Manual Preparative Kit with a hypodermic needle silver soldered inside the heated outlet. This modification permitted collection of fractions on cigarette filter tips impregnated with silicone oil (11). Samples of methyl esters of ca. 2000 cpm were injected, and fractions collected every min. The tips were transferred to 10 ml scintillation fluid (Instagel, Packard Instrument Co. Inc., Downers Grove, IL) in a scintillation vial. Samples were counted in a Tricarb model 3385 liquid scintillation spectrometer. Results were plotted on the mass trace from the GLC recorder. Peaks in this activity trace were integrated by addition of the cpm in individual vials comprising the peak. Recovery of [1-^{14}C] oleic acid methyl ester by this method was ca. 90%.

Identity of certain fatty acids was confirmed by hydrogenation of methyl esters. Hydro-

TABLE I

Distribution of Radioactivity of Tissue Fatty Acids of Turbot and Trout Fed Different Dietary Fatty Acids

Radioactive fatty acid fed	Turbot			Trout
	18:1ω9	18:2ω6	18:3ω3	18:3ω3
	(Total radioactivity)			
14C	0.4	0.5	1.0	--
16C	1.2	2.6	3.7	2.2
18:0	2.9	1.3	5.3	1.3[a]
18:1	90.4	1.8	--	1.3[a]
18:2	--	89.0	--	--
18:3	--	--	71.7	7.2
18:4	--	--	--	4.8
20:1	2.9	--	--	--
20:2	--	4.8	--	--
20:3	--	--	15.2	--
20:4	--	--	--	4.8
20.5	--	--	--	5.2
22.1	0.8	--	--	--
22:3	--	--	0.6	--
22:5	--	--	--	3.0
22:6	--	--	--	69.8
24:1	2.2	--	--	--

[a]Incomplete separation on activity trace.

genation was carried out in ethanol using platinum oxide on silicic acid as catalyst (12).

For measurement of expired $^{14}CO_2$ from turbot, 2 fish were placed in 4 liters filtered sea water containing 200 mg sodium benzyl penicillin and 200 mg streptomycin sulphate in a desiccator. The sea water was buffered at pH 8.0 with 10 g fish grade Trizma (Sigma Chemical Co. Ltd., London, England). Three gas absorption bottles, each containing 25 ml 1M NaOH were attached in series to the desiccator, and air, free of CO_2, was drawn constantly through the system. After 24 hr, fish were transferred to the desiccator of an identical system. The sea water in the original system then was acidified to pH 2 with 6M HCl, and air, free of CO_2, was passed through the sea water gas absorption bottles for an additional 60 min. In this manner, all expired air was collected over separate 24 hr periods. Recovery of $^{14}CO_2$ from added $Na_2{}^{14}CO_3$ was greater than 95% in this system.

RESULTS

Table I shows the distribution of radioactivity in tissue fatty acids of experimental fish. The number and species of fish given each fatty acid were 18:1 (5 turbot), 18:2 (2 turbot), and 18:3 (3 turbot and 2 trout). Fourteen and sixteen carbon acids were not resolved on the activity trace for some samples, and results are shown as totals for each chain length. Identification of acids derived from oleic acid was confirmed by chromatography of hydrogenated samples. 20:2ω6, 22:3ω3, and 22:6ω3 were identified by gas chromatography on 2 columns (EGSS-X and EGSS-Y), and by comparison with known standards. Identification of intermediates in the biosynthesis of 22:6ω3 is tentative. It is based on retention data from a single column, because clear separation of close running peaks, (e.g., 18:3ω3, 18:4ω3), was not obtained on the activity trace for the EGSS-X column.

In turbot fed each of the 3 fatty acids, a large proportion of radioactivity remained in the unchanged parent acid. The main product of longer chain length than the fatty acid fed was formed by addition of 2 carbon atoms to the chain, but with all 3 fatty acids, this product accounted for only a small proportion of the radioactivity recovered in the lipid fraction. The percentage of radioactive fatty acid elongated became greater as the degree of unsaturation increased. A small amount of radioactivity was invariably present in fatty acids of shorter chain length than that fed. Analysis of the radioactive fatty acids fed showed that these shorter chain acids were not present, and, therefore, conversion must have been carried out by the turbot.

In contrast to results obtained with turbot, most of the radioactivity occurring in tissue fatty acids of trout was present in 22:6ω3, not in the parent linolenic acid fed. Again, a small amount of radioactivity was present in acids of shorter chain length than that fed.

TABLE II

Radioactivity in Expired CO_2 from Turbot Fed 1-^{14}C Fatty Acids

Day	Fatty Acid		
	18:1ω9	18:2ω6	18:3ω3
	(total dpm x 10^{-4})		
1	32.10	37.90	43.04
2	21.42	28.12	22.05
3	3.54	11.35	6.35
4	1.41	6.06	4.82
5	0.90	6.53	4.41
6	1.20	4.47	2.35
Total	60.57	94.43	83.02
Ratio of dpm CO_2/dpm lipid	0.15	0.57	0.64

It is known that rainbow trout elongate and desaturate 18:1ω9, 18:2ω6, and 18:3ω3. Therefore, only one of these radioactively labeled acids was fed to trout to ensure that desaturation and chain elongation occurred under the experimental conditions used. It can be inferred that desaturation and chain elongation of [1-^{14}C] 18:1ω9 and [1-^{14}C] 18:2ω6 would also have occurred had either of these acids been fed.

The percentage composition of fatty acids of the fish also was calculated. Total fatty acids from experimental turbot contained 18.0% by wt of 22.6ω3, and from rainbow trout, 22.6% by wt of 22:6ω3 (average values).

Expired CO_2 from 2 turbot from each experimental group was collected for 6 days following administration of isotope. The radioactivity in expired CO_2 is given in Table II. Fatty acids were oxidized rapidly at first, with the peak of oxidation occurring within 24 hr, and decreasing rapidly to nearly 0 after 6 days. In addition to the total radioactivity collected over the 6-day period, the ratio of CO_2 expired radioactivity to incorporated radioactivity also is given. Although the same amount of isotope was added to each pellet, some loss of labeled fatty acids may have occurred during feeding. Thus, the ratio of radioactivity in expired CO_2 to radioactivity incorporated into tissue lipid is a more reliable indication of the relative amounts of each fatty acid oxidized. Expired CO_2 was not collected from rainbow trout, because they do not survive for 24 hr in static water.

DISCUSSION

Earlier studies on turbot (7) and plaice (6) indicated that these marine flatfish do not elongate and desaturate dietary fatty acids readily. The present study amply confirms these findings. In fact, over a 6-day period, no desaturation of dietary oleate, linoleate, or linolenate occurred in turbot, and chain elongation of these dietary acids was very limited. The time period elapsing between feeding of radioactive acids and analysis of turbot, during which time the fish were fed nonlabeled fat free diet, was considered sufficient to permit even a low rate of desaturation to become evident.

One possibility is that the presence of preformed long chain polyunsaturated acids in the tissues may have inhibited elongation and desaturation of 18-Carbon acids from the diet. This seems to be rather unlikely. In the parallel experiment with rainbow trout which contained a higher tissue level of 22:6ω3 than did turbot, extensive elongation and desaturation of linolenic acid occurred.

Wild turbot contain relatively large amounts of polyunsaturated fatty acids of the ω3 series. They are carnivorous, and presumably obtain a luxus of polyunsaturated fatty acids in their food, thereby negating the value of, or the need for, a pathway from 18 C acids to long chain polyunsaturated acids. The loss or absence of such a pathway in turbot is not apparently disadvantageous in the natural environment.

The culture of such fish, currently being attempted in the UK, would require a diet containing the preformed polyunsaturated acids for normal growth and freedom from pathology. This need is likely to be accentuated because of the higher rates of oxidation found for unsaturated acids. Brown and Tappel (13), studying fatty acid oxidation by isolated carp mitochondria, also found increased rates of oxidation with increasing degree of unsaturation. If this effect is physiologically significant, a diet containing a sufficiently high level of preformed polyunsaturated fatty acids would be required for normal growth of cultured turbot to prevent essential fatty acid deficiency after oxidative losses.

It has been suggested recently (1) that the adequacy of essential fatty acid intake in trout may be assessed by the ratio of 20:3ω9/22:6ω3 in the tissue phospholipids. In the light of the present results, the inability of the turbot to make 20:3ω9 from 18:1ω9 makes this index inapplicable to turbot even when 22:6ω3 is present in the diet.

The small amount of radioactivity present in shorter chain fatty acids than that fed turbot and rainbow trout may have resulted from fatty acid synthesis reutilizing radioactive acetate released during β oxidation. The increasing amounts of radioactivity in C-14 and C-16 acids with the same degree of unsaturation of acid fed (Table I) parallels increases in rates of fatty

acid oxidation (Table II), and supports this explanation. The dilution of radioactive acetyl CoA by endogenous acetyl CoA would be compensated, to some extent, by the randomization of labeled carbon throughout the fatty acid chain.

While the present work has obvious implications for the culture of turbot, the causes of the marked differences in synthetic ability of the different fish are less clear. Further work is required to elucidate the relationship between the ability of a species to modify dietary fatty acids and factors such as habitat, position in the food chain, and hormonal status.

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Methylating Activity of (Methyl-^{14}C)-S-Adenosylmethionine by Microsomes of the Insect *Ceratitis capitata*

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ABSTRACT

The methylating activity of (methyl-^{14}C)-S-adenosylmethionine by microsomes from different stages of development of the insect *Ceratitis capitata* was studied in a series of in vitro experiments. Larval and pharate adult microsomal preparations were used in the in vitro conditions, and the utilization of the methyl group of the S-adenosylmethionine for the synthesis of phospholipids was evaluated. Incorporation of radioactivity in lipids by pharate adult microsomes was significantly higher than that by larval preparations. In both cases, phosphatidyl ethanolamine showed the highest levels of radioactivity incorporation. Free bases from total lipid hydrolysates were resolved by paper chromatography, and the labeling was investigated by radioactivity scanning of paper chromatograms. Larval and pharate adult preparations showed clearly the presence of dimethylethanolamine and choline. The presence in the incubation media of phosphatidyldimethylethanolamine and deoxycholate enhanced the radioactivity incorporation into choline, whereas, the first stages of methylation were inhibited. These findings confirmed previous results using insect homogenates.

INTRODUCTION

The in vitro and in vivo methylating activity of L-(methyl-^{14}C)-methionine has been studied previously during development of the Dipterous *Ceratitis capitata* (1).

Larval and pharate adult homogenates of the insect were used in the in vitro conditions, and the utilization of the methyl group of methionine for the synthesis of different classes of phospholipids was evaluated. Incorporation of radioactivity in lipids by pharate adult homogenates was significantly higher than that by larval homogenates. In both cases phosphatidyl ethanolamine showed the highest levels of radioactivity incorporation. Monomethyl ethanolamine was the only methyl derivative that appeared in the hydrolysates of lipids synthesized by larval homogenates, whereas mono-, di-, and trimethylethanolamine were clearly detected in those synthesized by pharate adult homogenates.

Administration of L-(methyl-^{14}C)-methionine to larvae confirmed the existence of methylation reactions in the metabolic activity of the insect.

This communication is an extension of the above studies, and reports results on the methylation activity of (methyl-^{14}C)-S-adenosylmethionine in the microsomal fraction of larvae and pharate adults of *Ceratitis capitata*.

MATERIALS AND METHODS

Materials

(Methyl-^{14}C)-S-adenosylmethionine was purchased from the Radiochemical Centre (Amersham, UK). The specific activity was 56 μCi/mmol.

Rearing of Insects

Larval and pharate adult *C. capitata* (Wiedemann) were used. Diet, temperature, and humidity conditions during culturing were controlled carefully. Culturing of the insect was carried out under the conditions previously described (2).

Microsomal Preparations

Larvae were reared 2-3 days before the larval-pupal apolysis, and were starved 3-4 hr before used. Pharate adults were collected 5 days beyond the larval-pupal apolysis. Both larvae (60 g) and pharate adults (30 g) were washed carefully, and were homogenized directly with 90 ml cold homogenizing buffer (0.35 M sucrose-0.05 M tris[hydroxymethyl]aminomethane (tris)-1 mM mercaptoethanol, pH 7.6) in a Potter-Elvehjem glass homogenizer with a Teflon pestle. All subsequent preparative procedures were carried out at 2-4 C. Crude homogenates were filtered through a cheese cloth, and then freed of cellular debris, nuclei, and mitochondria by centrifuging twice at 6000 g for 5 min. Supernatant was centrifuged at 20,000 g for 10 min, and this mitochondria free supernatant was centrifuged at 105,000 g for 90 min in a SS34 rotor, L-4 Beckman Ultracentrifuge. The pellet of microsomes was washed and suspended in 0.05 M tris-HCl, pH 8.8. This suspension was used as the enzyme source for the synthesis experiments. Glucose-

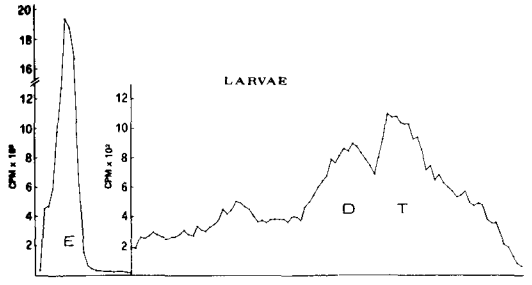

FIG. 1. In vitro incorporation of radioactivity from (methyl-14C)-S-adenosylmethionine by larval microsomal preparations of *Ceratitis capitata* in the absence of phosphatidyl dimethylethanolamine (PDE) and deoxycholate (DC). Scanning of radioactivity on paper chromatograms after separating the bases from lipid hydrolysates. E = ethanolamine, D = dimethyl ethanolamine, T = choline.

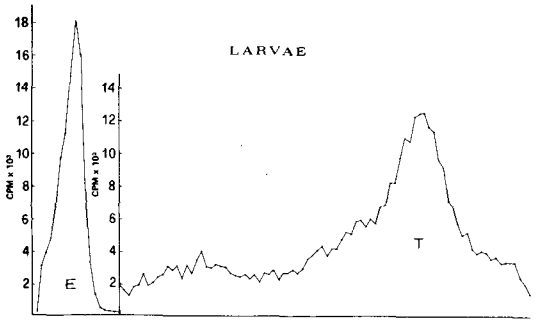

FIG. 2. In vitro incorporation of radioactivity from (methyl-14C)-S-adenosylmethionine by larval microsomal preparations of *Ceratitis capitata* in the presence of phosphatidyl dimethylethanolamine (PDE) and deoxycholate (DC). Scanning of radioactivity on paper chromatograms after separating the bases from lipid hydrolysates.

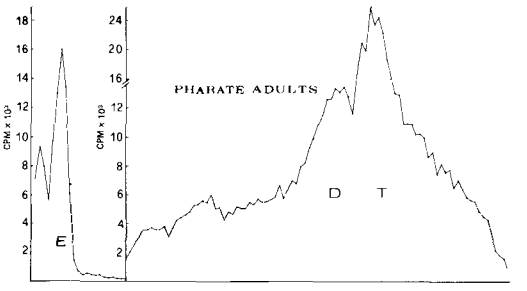

FIG. 3. In vitro incorporation of radioactivity form (methyl-14C)-S-adenosylmethionine by pharate adult microsomal preparations in the absence of phosphatidyl dimethylethanolamine (PDE) and deoxycholate (DC). Scanning of radioactivity on paper chromatograms after separating the bases from lipid hydrolysates.

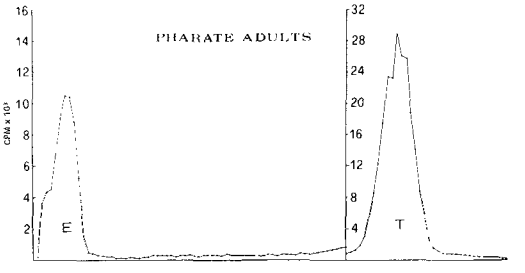

FIG. 4. In vitro incorporation of radioactivity from (methyl-14C)-S-adenosylmethionine by pharate adult microsomal preparations in the presence of phosphatidyl dimethylethanolamine (PDE) and deoxycholate (DC). Scanning of radioactivity on paper chromatograms after separating the bases from lipid hydrolysates.

6-phosphate dehydrogenase, assayed by the method of Bourre, et al., (3), could not be detected after washing the microsomal fraction.

Assay Mixtures

The microsomal fractions were suspended with a hand operated, all glass homogenizer in the 0.05 M tris-HCl buffer, pH 8.8 (0.6 ml/g larvae; 0.3 ml/g pharate adults) homogenization medium. Larval and pharate adult microsomal suspensions contained 14.7 mg and 13.6 mg protein/ml respectively. The incubation mixture, containing 5 μCi of (methyl-^{14}C)-S-adenosylmethionine, 0.24 mg sodium penicillin, 0.4 mg streptomycin sulphate, and 1 ml microsomal suspension, was shaken at 37 C for 90 min. In some cases, the homogenization mixture also contained 2 mg phosphatidyl-N,N'-dimethyl ethanolamine and 4 mg sodium desoxycholate, previously sonicated for 15 sec.

Extraction and Hydrolysis of Lipids

At the end of the incubation, the reaction was stopped by the addition of chloroform, and the total lipids were obtained according to the method of Bligh and Dyer (4). Total lipids were hydrolyzed with 1 N HCl (0.1 ml/mg lipids) at 100 C for 16 hr. After hydrolysis, the mixture was chromatographed on paper according to the method described previously (1). Radioactivity present in paper chromatograms was tested as described previously (1).

Three microsomal fractions were prepared independently and used in 3 series of incubation experiments. The incubation mixtures were separately handled, and the patterns of radioactivity of paper chromatograms were qualitatively identical. Figures 1-4 show the radioactivity scannings, results of which represent the intermediate values (SD 10%).

RESULTS AND DISCUSSION

Experiments were carried out to test the

phosphatidyl ethanolamine:adenosylmethionine methyltransferase activity in the microsomal preparations from larvae and pharate adults of the insect *C. capitata*. Incubation of microsomal preparations with (methyl-^{14}C)-S-adenosylmethionine and extraction of total lipids yielded, after hydrolysis, a mixture of bases that were separated and identified by paper chromatography.

Figures 1 to 4 show the radioactivity patterns of labeled bases from the phospholipids synthesized under different experimental conditions. Synthesis was performed by larval and pharate adult microsomal preparations in the presence or absence of phosphatidyl dimethylethanolamine (PDE) and deoxycholate (DC).

Figures 1 and 2 show the radioactivity patterns of labeled bases from the phospholipids synthesized using larval microsomal preparations of the insect. Radioactivity incorporation shown in Figures 1 and 2 was carried out in the presence or absence of both PDE and DC. Ethanolamine exhibited the highest and very similar levels of incorporation under both experimental conditions. However, the pattern of labeled bases in Figure 1 shows clearly the significant incorporation into dimethylethanolamine and choline; the presence of DC (Figure 2) inhibited the first methylating step. This finding is in agreement with results of Cooksay, et al., (5). The simultaneous presence of PDE as methyl acceptor enhanced the levels of choline as the main methylated base. These facts agree with the participation of the methionine methyl groups in the [1C] methylation reactions in the synthesis of phospholipids by the insect.

Figures 3 and 4 show the radioactivity patterns of labeled bases from the phospholipids synthesized using pharate adult microsomal preparations of the insect. As in the larval preparations, the absence of DC allowed the synthesis of methylated intermediates (Figure 3), whereas, the presence of both DC and PDE increased notably the levels of radioactivity in choline without any significant presence of mono- and dimethylethanolamine.

These results support the more active methylation capacity of the pharate adult preparations. They are in agreement with previous results obtained in in vitro experiments using whole insect homogenates. These data confirm, therefore, the participation of adenosylmethionine methyltransferase in the biosynthesis of phosphatidyl choline by the insect *C. capitata*.

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Effects of Structural Variation in β-Monoglycerides and Other Lipids on Ordering in Synthetic Membranes

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ABSTRACT

Studies of β-monoglyceride multilayers were carried out using a variety of spin probes. Effects of variables such as chain length, unsaturation, and branching on organization of acyl chains in lipids of model membranes were assessed. In addition, effects of added cholesterol on membrane order were determined. Results indicated that pure β-monolaurin yields highly ordered films, whereas, unsaturated glycerides such as β-monoolein, β-monolinolein, and analogous lecithins yield fluid films. Branched monoglycerides behaved similarly to β-monoolein, suggesting that branching in acyl chains is an effective substitute for unsaturation in maintaining membrane integrity. Multilayers of β-monoglycerides exhibited similar properties to those of more complex lipids such as phospholipids. β-Monoglycerides, by virtue of the presence of a single acyl chain, provided a relatively simple and effective alternative to the use of phospholipids in studies of membrane architecture.

INTRODUCTION

The function of membranes in living systems is closely related to structure and organization of lipids in bilayers (1-4). Accordingly, much effort has been directed toward elucidating bilayer membrane structure (5-7). Lipid bilayers (lamellae) prepared with one or more constituents of membranes, e.g., phospholipids, cholesterol, various glycerides, are often used to simulate membranes in model systems. The use of such models reduces the multitude of complexities encountered in the study of real membranes, making it possible to restrict study to one facet of membrane structure at a time. Lipid bilayers are readily prepared and experimental data are relatively easy to interpret.

The ability of lipids to form organized, ordered layers has been studied by a number of investigators (8-12); the recent review by Roubal (13) describes studies for lipid-protein organization and interaction in a variety of membrane systems. Schreier-Muccillo, et al., (12), have performed spin labeling studies on a variety of lipids to ascertain conditions necessary for the formation of ordered systems. They reported that saturated phospholipids such as dipalmitoyl lecithin formed ordered layers (bilayers), whereas, saturated monoglycerides excluded spin labels at room temperature. However, when cholesterol was added, both phospholipids and monoglycerides yielded films of increased order.

In the present study, we show, that with one exception, spin labels do intercalate with monoglycerides in the absence of cholesterol at room temperature. Moreover, addition of cholesterol induced order and changes in bilayer films formed from saturated, monounsaturated, and branched monoglycerides. Thus, effects of individual structural features of monoglycerides are evaluated, and the degree of order of the lipid matrix is attributed to these parameters. Furthermore, these results are compared with data obtained using phospholipids with the same acyl moieties to point out similarities that may exist between related lipid classes.

MATERIALS AND METHODS

Lipids

L-α-di(dodecanoyl) Lecithin (dilauroyl lecithin), L-α-di(tetradecanoyl)-lecithin (dimyristoyl lecithin), L-α-di(*cis*-9-octadecanoyl) lecithin (dioleoyl lecithin), L-α-di(*cis*, *cis*-9, 12-octadecanoyl) lecithin (dilinoleoyl lecithin), and L-α-di(*cis*, *cis*, *cis*-9, 12, 15-octadecatrienoyl) lecithin (dilinolenoyl lecithin), (purity, ca. 99%), were obtained from Serdary Research Laboratories (London, Ontario, Canada). Chromatographically pure 2-(n-dodecanoyl) glycerol (β-monolaurin) and 2-(n-octadecanoyl) glycerol (β-monostearin) were obtained from Applied Science Laboratories, Inc. (State College, PA).

2-(*cis*-9-octadecanoyl) Glycerol (β-monoolein), 2-(*cis*, *cis*-9, 12-octadecadienoyl) glycerol (β-monolinolein), β-monodocosahexaenoin, and 2-(16-methylheptadecanoyl) glycerol (β-monoisostearin) were prepared from corresponding triacylglycerols using steapsin (14).

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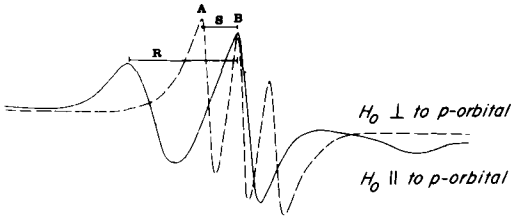

FIG. 1. Spin label spectra for oriented multilayers of lipids exhibiting perfect ordering. Line splitting distances, R and S, and peak ratio, A:B, provide necessary data needed to assess the degree of order. The solid line is for cholestane label (CSL) recorded for the p-orbital parallel to the magnetic field, indicated $H_0\parallel$. The dashed line is for CSL recorded for the field perpendicular to p-orbital of nitroxide, indicated as $H_0\perp$. This convention obtains for all of the figures in this paper where CSL is discussed. In the case of the nitroxide stearate spin labels, this order is reversed. Horizontal axes, by convention, represent field strengths (gauss). However, some spectra were photographed at slightly different magnifications and a single marker will not fit all spectra. Pertinent gauss measurements are presented in the tables.

Because β-monoglycerides undergo a partial isomerization to α-isomer, the 'pure' β-monoglycerides reported here are mixtures consisting of 80% β-isomer and 20% α-isomer. Moreover, the 'β-monoisostearin' contained 74% iso-chains, comprised of C_{17} (5.7 mole %), C_{18} (45 mole %), C_{19} (13.4 mole %), and C_{20} (9.8 mole %). Because of this, we refer to this material as β-monoisoglyceride.

Cholesterol (> 99% pure) was obtained from General Biochemicals (Chagrin Falls, OH). With the exception of β-monoisostearin, content of cholesterol in bilayer films is expressed in mole %; because of the mixed nature of the β-monoisostearin material, % by wt of cholesterol is given for iso- lipids.

Spin Labels

The following spin labels were used in this study: 17β-hydroxyl-4′, 4′-dimethylspiro-(5α-androstane-3,2′-oxazolidin)-3′-yloxyl (cholestane spin label [CSL]), 2-(3-carboxylpropyl)-4, 4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-nitroxide stearate [5NS]), 2-(10-carboxydecyl)-2-hexyl-4-, 4-dimethyl-3-oxazolidinyloxyl (12-nitroxide stearate [12NS]), and 2-(14-carboxytetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazolidinyl-oxyl (16-nitroxide stearate [16NS]). Labels were obtained from Syva Inc. (Palo Alto, CA).

The nitroxide moiety of CSL is the most polar portion of this probe, and, therefore, is directed to the membrane (multilayer) surface (15). Hence, CSL was used primarily as a probe for monitoring environment in the surface region of the multilayers. However, because CSL is a flat, rigid molecule, mobility of this probe is also influenced by the environment over the entire molecule; therefore, some caution must be exercised in interpretation of data. Nitroxide stearate labels (NS labels) were chosen to study the inner region of the membrane. Thus, 5NS with the nitroxide moiety located 5 carbon atoms from the polar carboxyl head group, reflects the environment at this position in the membrane. Similarly, 12NS and 16NS report the environment at greater depths, with 16NS probing near the middle of the bilayer membrane.

Oriented Quartz Plate - Spin Label Studies

Bilayer films were prepared by carefully depositing a chloroform solution containing 0.25 mg lipid with spin label (ratio of lipid to label, 150:1, w/w) onto the broad face of a quartz plate (0.5 cm x 2 cm x 0.1 cm).

Initially, residual solvent was removed by placing the plate in a vacuum for ca. 45 min, followed by a brief (10-15 sec) exposure of the plate to a temperature of 50-60 C. Later it was found that the same results were obtained by exposing the plate to a fast flowing stream of dry nitrogen at room temperature for 20 min. Dry plates were then inserted into the electron paramagnetic resonance (EPR) cavity of a Varian E-3 spectrometer, and positioned in the magnetic field via a quartz rod sealed to one end of the plate and held by the collet of a goniometer. EPR spectra were recorded for the face of the plate, parallel and perpendicular to the applied magnetic field. All EPR measurements were made at room temperature (ca. 22 C).

Measurement of Membrane Order

The degree of ordering is indicated by differences in spectra for 2 orientations of the quartz plate in the applied field. The greater the difference in line splittings for these 2 orientations, the greater is the ordering. Figure 1 shows the spectra for optimum ordering of the label by a parallel array of membrane lipids, e.g., phosphatidylcholine, with their long amphiphilic axes normal to the broad surface of the quartz plate. Membranes exhibiting optimum order give an R value of ca. 32 gauss and an S value of ca. 6 gauss (Fig. 1). The R value decreases and the S value increases when the environment about label becomes less ordered, e.g., the parallel array arrangement of lipids is no longer maintained; prevailing conditions are such that long amphiphilic axes of lipids undergo independent rotational and lateral motion. As the environment tends toward fluidity, movement of acyl chains becomes independent

of one another, and motion is random in nature. When an oriented (ordered) system is no longer maintained, spectra become independent of the applied magnetic field, resulting in a 3 narrow line, solution spectrum as a result of isotropy. Another parameter, the so-called order parameter, is also used to assess membrane order; this is the ratio of peaks A:B (Fig. 1), measured for oriented films showing anisotropy. For this measurement to be valid as an indicator of ordering, the plate must be aligned such that the p-orbital of the label is oriented perpendicular to the applied magnetic field (16). A ratio of unity would indicate perfect alignment in the film, while a zero value would indicate no order at all. Although R and S values are good indicators, the A:B ratio appears to describe more completely the degree of ordering.

RESULTS

Pure Monoglycerides, and Monoglycerides Containing Cholesterol

Spin label spectra of pure β-monolaurin films are shown in Figure 2A,B,C,D. A comparison of R and S values (Table 1) shows that all 4 labels exhibit considerable mobility, approaching a purely isotropic condition in the hydrophobic interior (Fig. 2D for 16NS). Addition of cholesterol greatly enhances ordering of both CSL (Fig. 2a) and 5NS (Fig. 2b), indicating an improved alignment and order at the surface and in the hydrophobic region extending at least to the depth of nitroxide in 5NS. For membranes containing a mixture of monolaurin:cholesterol (48.5:51.5 mole %), the spectra with CSL indicate almost perfect order of components (R = 23.5 gauss, S = 7.5 gauss, and A:B = 0.94; Table I). The spectra obtained with labels 12NS and 16NS are shown in Figure 2c,d. The 5NS spectra, similar to that of CSL, show anisotropies, with parameters of R = 23 gauss, S = 8 gauss, and A:B = 0.45.

The study of β-monostearin presented a rather unique situation in that this monoglyceride excludes label (17). Exclusion of label is immediately apparent because the usual 3-line resonance is replaced by a single, broad resonance, a condition arising from localized pools of label undergoing spin spin interaction (12).

Interestingly, addition of cholesterol to β-monostearin permits label intercalation into the membrane (Fig. 3A; Table I). Spectra for β-monostearin with 68.9 mole % cholesterol (CSL label) exhibits parameters of R = 25 gauss and S = 7 gauss. However, the A:B ratio is only 0.23, indicating imperfect alignment of lipid chains in the multilayer matrix. Spectra in Figure 3B and C for 5NS and 12NS indicate anisotropy at these label positions. Deep in the hydrophobic interior of the membrane, at the position of nitroxide in 16NS (Fig. 3D), the

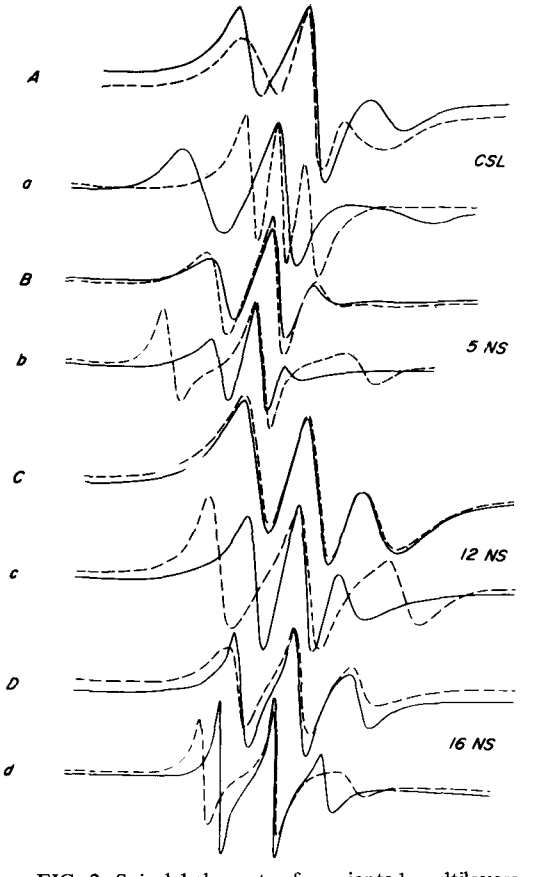

FIG. 2. Spin label spectra for oriented multilayers of β-monolaurin. Spectra A,B,C, and D are for multilayers free of cholesterol. Spectra a,b,c, and d are for multilayers containing 51.5 mole % cholesterol.

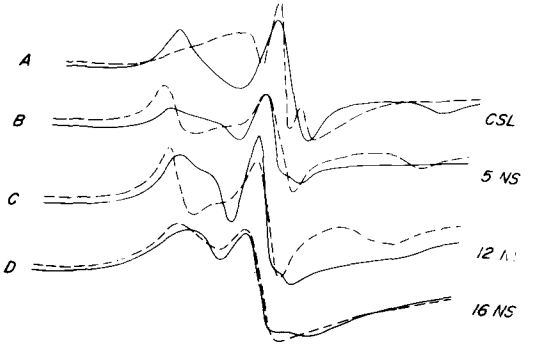

FIG. 3. Oriented multilayers of β-monostearin containing 68.9 mole % cholesterol. A = Cholestane spin label; B = 5-Nitroxide spin label; C = 12-Nitroxide spin label; D = 16-Nitroxide spin label.

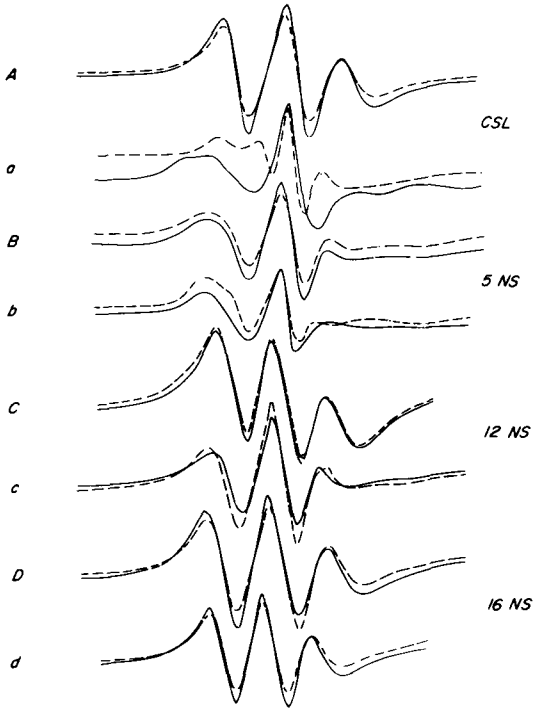

FIG. 4. Oriented films and multilayers of β-monoolein. Spectra A,B,C, and D are for labels in pure films of monoolein. Spectra a,b,c, and d are for multilayers containing 38.7 mole % cholesterol. CSL = cholestane label; NS = nitroxide stearate.

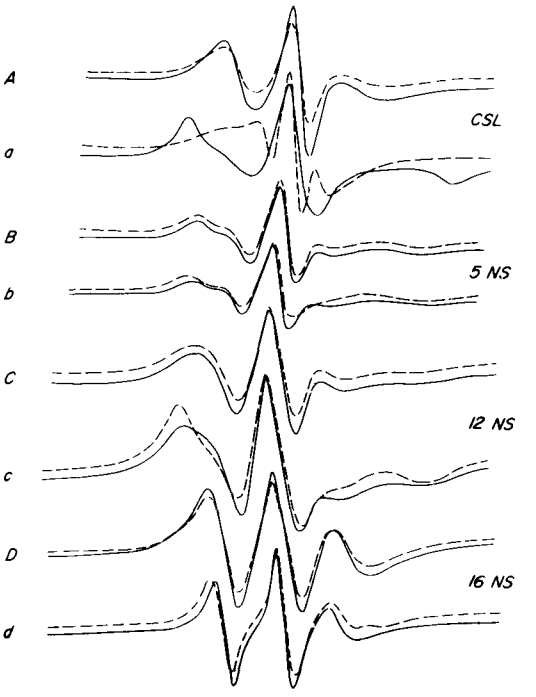

FIG. 5. Oriented multilayers of a mixture of branched-chain monoglycerides, high in β-monoisostearin. Spectra A,B,C, and D are for multilayers free of cholesterol. Spectra a,b,c, and d are for multilayers containing 50 wt% cholesterol. CSL = cholestane label; NS = nitroxide stearate.

spectra are almost identical for either orientation of the plate in the magnetic field, and the high field line vanishes.

An unsaturated β-monoglyceride, e.g., β-monoolein, was used next. This monoglyceride, which has a lower melting point than β-monostearin, does not exclude label. The spectra of pure monoolein with all 4 labels exhibited isotropy (Fig. 4A,B,C,D). Addition of 38.7 mole % cholesterol resulted in some anisotropy (Fig. 4a,b,; Table I). The ratio of A:B for CSL was 0.16. Small increases or decreases in the proportion of cholesterol produced an isotropic spectrum. In the case of 12NS and 16NS, no differences were seen in spectra for monoolein with or without cholesterol.

Films of pure monoglycerides, β-monolinolein and β-monodocosahexaenoin, gave isotropic spectra for all labels. The isotropic spectrum for β-monodocosahexaenoin was not altered by additions of cholesterol, and there was only minimal broadening of the resonance lines for β-monolinolein (CSL label) for levels of cholesterol up to 58.5 mole %.

Mixed Monoglycerides

Exclusion of spin labels by monostearin can be overcome by several methods. Use of elevated temperature (12) or addition of cholesterol can be used to perturb the film and allow label intercalation. Instead of cholesterol, however, other lipids can be used for causing the necessary perturbations. Accordingly, we have shown that CSL intercalates into membrane, at least partially, when 6.6-13.0 mole % of monolaurin is mixed with monostearin.

In contrast to β-monostearin, β-monoisoglyceride, which is also a saturated monoglyceride, does not exclude label. Because monoisostearin contains a mixture of only long chain isomers, $> C_{17}$, it appears that, like unsaturation, branching alters packing between acyl chains, providing a more open structure and, thereby, allowing label intercalation.

Addition of cholesterol to β-monoisoglyceride induced order primarily at the surface, as indicated by pronounced anisotropy in spectra for CSL probe. Thus, for a membrane comprised of 50 % by wt β-monoisoglyceride and 50 % by wt cholesterol (Fig. 5a), values for R and S are 26 and 7 gauss, respectively, with A:B = 0.27 (Table I). Some anisotropy not readily apparent in the 5NS spectra exists in the case of 12NS (Fig. 5c). The order is once again

reduced at the 16-position (Fig. 5d). Why anisotropy is more pronounced at the 12NS position and not at the 5NS position is not understood.

Phospholipids

Although dilauroyl lecithin and dimyristoyl lecithin exhibited more order than β-monolaurin in the absence of cholesterol, it was observed that phospholipids also required addition of cholesterol to promote an improved ordering of membranes (Table II). Accordingly, a 61.6 mole % cholesterol level in dilauroyl lecithin produced the highest degree of order; R,S, and A:B values of 21.6 gauss, 6.5 gauss, and 0.45, respectively, were recorded (Table II). Dimyristoyl lecithin with an optimum cholesterol content (63.7 mole %) gave values of 21 gauss, 6.5 gauss, and 0.67, respectively. Analogous results have been reported for cholesterol containing bilayers of dipalmitoyl lecithin (18).

Distearoyl lecithin, like β-monostearin, excludes label in absence of cholesterol. Addition of 50.5 mole % cholesterol results in an A:B ratio of only 0.13 (CSL probe), which increases to a maximum of 0.22 at a level of 67.1 mole % of sterol. Further addition of cholesterol reduced the A:B ratio, and at 86 mole % cholesterol, the ratio was 0.05.

Diooleoyl lecithin behaves in an analogous manner to β-monoolein. Addition of cholesterol (75.3 mole %) resulted in broadening of resonance lines, thereby indicating a small degree of anisotropy. Because there was no difference between spectra for either orientation of the quartz plate in the applied magnetic field, the data suggest that label undergoes a rapid anisotropic tumbling. Cholesterol content below 75.3 mole % did not result in any marked changes in spectra from those of pure diooleoyl lecithin itself. Dilinoleoyl lecithin and dilinolenoyl lecithin films were fluid under all conditions. In no case did it appear that lecithin cholesterol films were as perfectly ordered as those of monolaurin cholesterol.

DISCUSSION

Results of this study show that the short chain lipid, β-monolaurin, forms exceptionally well ordered films with cholesterol. Although the content of cholesterol necessary to achieve a high degree of ordering in these films is quite high, ordering observed is due to monoglyceride cholesterol interactions, and not simply due to high cholesterol content. This conclusion is supported by the fact that pure cholesterol films exhibit only broad, orientation indepen-

TABLE I

Effects of Cholesterol on Line Splittings and Order Parameters in Oriented Lipid Multilayers

Label[a]	Measured parameters[b]	Lipid																	
		β-monolaurin (mole %)				β-monostearin (mole %)				β-monoolein (mole %)					β-monoisostearin[c]				
		0	41.4	51.5	62.3	0	48.7	58.8	68.9	79.2	0	28.8	38.7	43.6	48.6	0	40	45	50	70
CSL	R	16.5	21.0	23.5	23.0	--	21.0	21.0	25.0	23.0	17.0	18.0	23.0	23.0	17.0	17.5	25.0	26.0	26.0	25.0
	S	14.5	10.0	7.5	8.5	--	5.5	5.5	7.0	6.5	17.0	17.0	8.0	8.0	16.0	17.5	8.5	8.0	7.0	19.0
	A:B	--	--	0.94	--	--	0.04	0.07	0.23	0.05	--	--	0.16	0.07	--	--	0.21	0.25	0.27	--
5NS	R	17.5	--	26.0	--	--	--	--	25.0	--	17.0	--	25.0	--	--	20.0	--	--	22.0	--
	S	15.0	--	12.0	--	--	--	--	13.0	--	17.0	--	9.0	--	--	20.0	--	--	13.0	--
	A:B	--	--	0.54	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
12NS	R	15.5	--	21.0	--	--	--	--	22.0	--	15.0	--	19.0	--	--	22.0	--	--	20.0	--
	S	14.5	--	12.0	--	--	--	--	11.5	--	14.5	--	14.5	--	--	21.0	--	--	19.0	--
16NS	R	14.5	--	17.0	--	--	--	--	16.5	--	14.5	--	18.0	--	--	17.5	--	--	15.0	--
	S	13.0	--	12.5	--	--	--	--	14.0	--	14.5	--	17.5	--	--	17.5	--	--	14.0	--

[a]CSL = Cholestane label; NS = nitroxide stearate.

[b]R and S values are given as gauss. In making measurements of spectra for tabular data the solid line in the figures indicate H_0 parallel to the p-orbital of CSL nitroxide moiety; the dashed line represents perpendicular orientation. Order is reversed for nitroxide stearates.

[c]β-Monoisostearin used here contains various long chain iso acids. Accordingly, cholesterol content in β-monostearin bilayers is given in % by wt.

TABLE II

Effects of Cholesterol on Line Splittings and Order Parameter in Oriented Multilayers of Dilauroyl Lecithin and Dimyristoyl Lecithin

Lipid	Label[a]	Measured parameter[b]	Cholesterol content (mole %)			
			0	34.9	61.6	82.8
Dilauroyl lecithin	CSL	R	20	--	21.6	21
		S	12	--	6.5	10
		A:B	0.15	--	0.45	PS[c]
	5NS	R	20	--	20.6	--
		S	16	--	16.0	--
	12NS	R	20	--	--	--
		S	16	--	--	--
	16NS	R	18.0	--	--	--
		S	16.0	--	--	--
			0	36.9	63.7	84.0
Dimyristoyl lecithin	CSL	R	20.5	21.6	21	19
		S	7.5	7.0	6.5	6.5
		A:B	0.22	0.6	0.67	0.13
	5NS	R	28	--	24	24
		S	18	--	7	17
		A:B	--	--	--	PS[c]

[a]CSL = Cholestane label; NS = nitroxide stearate.

[b]See footnote b, Table I.

[c]Powder spectrum (16).

dent spectra. For most lipid studies, cholesterol content beyond an optimum value is disruptive, that is, it causes a loss of order. Analogous to this are the studies of Schreier-Muccillo, et al., (18) who indicate that increasing levels of cholesterol in dipalmitoyl lecithin first show a condensing effect (ordering), but that beyond an optimum cholesterol content, the effect is disruptive due to weakening of strong interchain interaction between acyl chains, promoting a transition to the liquid crystal state.

The observed spectral anisotropy for CSL in β-monolaurin cholesterol films indicates a high degree of order in the membrane. This observation for CSL, however, does not provide specific information as to where this order exists, because CSL responds generally to any environmental restraint within the boundary of the entire lable. However, because the nitroxide moiety, being the most polar portion of the probe is aligned with the polar membrane surface, we may suppose that the data indicate high ordering of polar head groups. In addition, however, spectra for 5NS also exhibit anisotropy, indicating ordering in the hydrophobic region ca. 5 carbon atoms from the surface. Taken together, these data indicate that cholesterol is able to quench tumbling of the nitroxide about the hydrocarbon chain of the nitroxide stearate. Also, it appears that cholesterol is able to restrict lateral motion, side to side movement of acyl chains, at least to the depth of nitroxide in 5NS. If either of these motions predominated, 5NS spectra would be isotropic. Beyond the 5NS position of the nitroxide stearate chain, the spectra take on a different character. The acyl chains are less constrained and at carbon-12 position of the 12NS label, the spectra indicate considerable motion. Finally, at the maximum depth reported by 16NS, the spectra indicate an environment of high fluidity. Hence the ordering of the membrane by cholesterol extends from the surface to at least the position of nitroxide in 5NS. Order does not extend, however, over the entire breadth of the membrane. Although Schreier-Muccillo, et al., (12) found that well-ordered monolaurin films were achieved with 5% added cholesterol, we found the cholesterol requirements to be much higher. Because we do not know the exact nature of the material used by these workers, it is tempting to speculate that slight variations in samples or experimental techniques are responsible for observed differences. In any event, our data suggest that packing of acyl chains in the short chain lipid, monolaurin, in the absence of cholesterol, is not as tight as that originally reported (12).

Stearic acid has a unique position in this study, for of all the various parameters studied, this long chain fatty acid alone is able to exclude label. It does not matter whether stearic acid is part of a monoglyceride or lecithin; the effect is the same. Addition of cholesterol or

short chain monoglyceride to derivatives of stearic acid promotes label intercalation, and some order is induced. In contrast to stearic acid derived lipids, we found that all other compounds intercalate labels readily. However, Schreier-Muccillo, et al., (12), report that all saturated monoglycerides, e.g., monolaurin, monopalmitin, do not intercalate labels at room temperature.

Our studies indicate that in lipid multilayers, branched lipids behave like unsaturated ones, imparting fluidity to the multilayers. In this context, it is noteworthy that Silbert, et al., (19), have shown that branched chain fatty acids disrupt packing of acyl chains and are able to replace totally *cis*-unsaturation in membranes of bacteria, *Escherichia coli*. For example, hexadecenoic acid and octadecenoic acid were replaced by 12-methyltetradecanoic acid and 14-methylhexadecanoic acid, respectively. Moreover, Varanasi and Malins (20) have recently shown that monounsaturated acids commonly present in most mammalian systems are partially replaced by branched acids in phospholipids of porpoise tissues.

ACKNOWLEDGMENTS

T.K. Collier prepared many of the samples used in this study. This work was supported in part by the Office of Naval Research, Contract N-00014-72-A-0202-0001.

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Sterols of the Adult and Juvenile Forms of Ivy, *Hedera helix* L.

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ABSTRACT

Sterols have been isolated from both adult and juvenile ivy in free and esterified form. Stigmasterol or its C-24 isomer, poriferasterol, is the main component, and lesser amounts of sitosterol, α-spinasterol, 5α-stigmast-7-en-3β-ol, cholesterol, and campesterol also were detected. Flowers and fruits were found to contain a glycoside fraction in which stigmasterol, together with some sitosterol, could be detected.

INTRODUCTION

Many plants, particularly trees and bushes grown from seed, grow vegetatively for a number of years before the first appearance of sexual reproduction, i.e., flowering and fruit formation. Often there is a marked change in physical form between juvenile, nonflowering forms and adult forms, in which flowering occurs. Thus, leaf shape may be radically different between the 2 forms, e.g., species of *Juniperus*, and methods of propagation may differ greatly, as exemplified by ivy (1). Ivy (*Hedera helix* L.) exhibits the phenomenon of having both adult and juvenile growth simultaneously on the same plant, thereby providing material which is genetically homogeneous (2).

Adult material of *Hedera helix* is distinguished by a bushy habit, light colored leaves, and small umbels of flowers, born in late summer. The umbels develop into racemes during autumn, and fruits ripen the following spring. It is very difficult to propagate this form by means of cuttings. Juvenile ivy grows as long vines which exhibit a climbing habit, and adventitious roots are readily formed at each node, thereby facilitating propagation by means of cuttings. Leaves are a darker green, often variegated and pigmented with anthocyanin, and are more palmate in shape than those of adult material.

Biochemical and physiological changes associated with the transition from juvenile to adult forms in plants are not clearly understood, and we have chosen ivy as a convenient source of material to commence a study of these changes. In the animal kingdom, steroids are known to exert a great influence in growth and development of sexual reproduction (3). By analogy, we have chosen to start with a study of the steroidal compounds present in both adult and juvenile material. In this paper, we report on sterols present in leaves, flowers, and fruits.

EXPERIMENTAL PROCEDURES

Ivy

Leaf material was harvested from a single plant growing on the banks of the River Kelvin in the Garscube Estate, Glasgow, Scotland (National Grid Reference NS552703). Flowers were collected in October from several bushes in a similar location. Fruits and adult leaf material were collected in April from a bush alongside the Crinan Canal, Argylshire, Scotland (National Grid Reference NR796929). Fruits were not produced on the bushes at Garscube. All materials were stored frozen unless used immediately.

Chemicals

Hydroxyalkoxy-LH-20 lipophilic sephadex was prepared in 2 stages from Sephadex G-25 (4). Analar or other commercial grades of solvents were used for extraction. Anasil B was purchased from Analabs Inc., North Haven, CN. Derivatization reagents used were Methelute (Pierce Chemical Co., Rockford, IL), bistrimethylsilyl acetamide (BSA), (Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire, UK), trifluoroacetylimidazole, heptafluorobutytylimidazole (Pierce Chemical Co., Phase Separations Ltd.) and other reagents were obtained from standard commercial sources. Gas chromatography packings were obtained from Phase Separations Ltd.

Extraction Procedures

Whole leaf material was mascerated in chloroform:methanol (2:1) and the brei filtered via a Soxhlet thimble. The residue then was extracted for 18 hr in a Soxhlet extractor.

Isolation of Sterols

Sterols were isolated from crude extract by evaporation of solvent to a small volume followed by application of an aliquot as a strip to a thin layer chromatographic (TLC) plate (0.25 or 0.4 mm thickness) along with marker spots of cholesteryl palmitate and brassicasterol. Development was with chloroform; detection of zones was achieved by spraying with dichlorofluorescein; and materials were recovered by mechanically transferring the indicated

zones to small chromatography columns, after which sterols or esters were eluted with diethyl-ether. For larger scale isolation of sterols for structure determination, crude extract was triturated with benzene, filtered, and the solution applied to a column of hydroxy-alkoxy-LH-20 sephadex (4). Zones corresponding to sterol ester and free sterol (standard elution volumes [SEV] = 50 and 110, respectively) were eluted, sterol glycosides were recovered from the column by elution with 8% EtOH in benzene and emerged immediately after the ethanol solvent front (5).

Gas Liquid Chromatography (GLC) of Sterols

GLC was carried out using a 1.5 m, 3% OV-17 column and a 3 m, 2% SE-33 column, both at 265 C, in a Pye 104 Model 14 gas chromatograph. Comparison was made with authentic sterols and their derivatives.

GLC-Mass Spectrometry (MS) of Sterols

GLC-MS was carried out using GLC columns with an AEI MS-30 single beam mass spectrometer, ion source 200 C, membrane separator 240 C, ionization voltage 70 eV, and using helium as carrier gas.

Derivatives

Sterol derivatives were prepared using BSA for preparation of trimethylsilyl (TMSi) ethers and pyridine:acetic anhydride (1:2) for preparation of acetates. Trifluoroacetate and heptafluorobutyrate derivatives of sterol glycosides were formed in pyridine solution by reaction for 10 min with the corresponding imidazole derivative. GLC (OV-17) was performed directly on the reaction mixtures.

Sterol Esters

These were hydrolyzed by dissolving in methelute and the solution heated for 2 hr at ca. 60 C. TMSi ethers were formed by removal of the methelute by hot plate or rotary evaporator, and reaction of the residue with BSA. The crude mixture was found suitable for GLC.

TLC on Anasil B

Plates were prepared by slurrying Anasil B in water (1:2.2, w/v), spreading at 0.25 or 0.5 mm thickness, and heating at 100 C before use. Plates were developed with 1-2% ether in petroleum for 4-6 hr using a Shandon continuous development tank in which the atmosphere was saturated with the solvent system (6). Sterol acetates were located with dichlorofluorescein or 4N sulphuric acid. Typical R_s values (α-spinasterol = 1.0) for acetates were: sitosterol = 2.2; stigmasterol = 1.6; 5α-cholest-7-en-3β-ol = 1.4; cholesterol = 1.85; and ergosterol = 1.15.

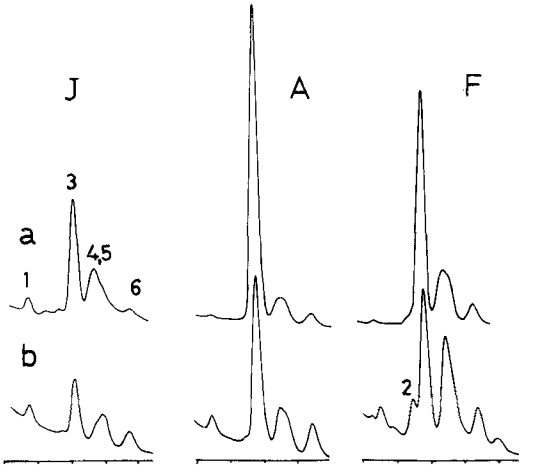

FIG. 1. Gas chromatograms of free sterol fractions a) from juvenile leaf (J), adult leaf (A) and flowers (F) of *Hedera helix* L., and b) corresponding ester fractions after hydrolysis. 1 = cholesterol; 2 = campesterol; 3 = stigmasterol; 4 = sitosterol; 5 = α-spinasterol; and 6 = 5α-stigmast-7-en-3β-ol.

RESULTS

Free and esterified sterol fractions were isolated from adult and juvenile leaves and from flowers. All 6 fractions were similar in composition, analysis of GLC and comparison with data for authentic compounds suggested stigmasterol as the main component, with lesser amounts of sitosterol, α-spinasterol (these 2 being only partially resolved), cholesterol, campesterol, and 1 other compound, possibly 5α-stigmast-7-en-3β-ol or cycloartenol (Fig. 1). Analyses by GLC-MS (7) further confirmed the assignment for stigmasterol. They were in agreement with other assignments, and suggested that the questionable compound stigmast-7-en-3β-ol rather than cycloartenol. TLC of derived acetates on Anasil B gave 3 steroid zones which were analyzed by GLC and GLC-MS. The most mobile zone contained (as acetates) sitosterol with lesser amounts of stigmasterol, campesterol, and cholesterol. The middle zone was predominantly stigmasterol, and also contained stigmast-7-en-3β-ol, as was expected from analysis of standards. The polar zone was a 1:1 mixture of 2 compounds having correct chromatographic and mass spectrometric properties for stigmasterol and α-spinasterol. Repeated chromatography yielded fractions for stigmasterol acetate and α-spinasterol acetate which were $>80\%$ pure. These compounds yielded optical rotatory dispersion (ORD) curves almost identical with those for authentic sterols. It has been

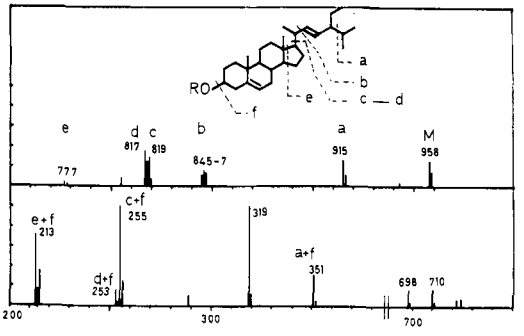

FIG. 2. Mass spectrum of stigmasteryl glycoside tetratrifluoracetate isolated from flowers. AEI MS-30, gas liquid chromatography (GLC) inlet, 70 eV. M = molecular ion.

reported without details (8) that ORD curves of Δ^{22} sterols exhibit differences between 24R and 24S alkylsubstituted isomers. In the present work, ORD curves for isolated $\Delta^{5,22}$- and $\Delta^{7,22}$-sterols resembled more closely the ORD curve for stigmasterol than that for poriferasterol, its 24β-isomer. It should be emphasized that apart from the ORD curves, no data which might distinguish compounds isomeric at C-24 have been obtained, and the presence of alternate isomers cannot be excluded absolutely. The possibility of alternative double-bond isomers to Δ^{7} compounds, e.g., $\Delta^{8(9)}$- or $\Delta^{8(14)}$- was discounted on the basis of GLC data.

Flowers also yielded a sterol glycoside fraction obtained from lipophilic sephadex which was subject to analysis by GLC and GLC-MS of trifluoroacetate and heptafluorobutyrate derivatives (5). Results were consistent with the main compound being stigmasteryl glycoside (M^+, M-15, M-43, M-side-chain, M-side chain, and ring D, plus ions at m/e 351, 255, 253, 213 as shown in Figure 2, and with sitosteryl and stigmast-7-en-3β-yl glycosides also being present. The heptafluorobutyrate also showed the ions for cleavage at m/e 394 and 395. No clear data for α-spinasteryl glycoside could be obtained. The sugar moiety has not been identified definitely.

Fruits collected in Argyleshire were analyzed by extraction with chloroform:methanol and examined for free and glycoside sterols. Results were similar to those for flowers described earlier, and sterols of leaf material from the same bush were not significantly different from those collected at Garscube.

DISCUSSION

Sterol patterns of adult and juvenile ivy leaf were essentially identical, with quantities of sterol per 100 g fresh leaf being: cholesterol (trace); campesterol, 1 mg; stigmasterol, 9 mg; sitosterol plus α-spinasterol, 2.5 mg; and stigmast-7-en-3β-ol, 0.5-0.7 mg.

It is clear also that there were only small quantitative differences between free and esterified sterols. At the present time we cannot comment on any possible involvement of sterols in the transition between juvenile and adult phases of growth in ivy. Sterols are considered to be components of membranes in eukaryotic organisms, and they occur in this species at levels which are typical for such organisms (9). If other steroidal compounds, such as mammalian sex hormones, play a role in the phase transition, it is possible that they do so only at very low levels, and it is improbable that we would observe any change in the level of sterols associated with their possible biotransformation into such hormones.

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Enzymatic Synthesis of Glucocerebroside by UDP-Glucose: Ceramide Glucosyltransferase during Ontogenesis of Chicken Retina

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ABSTRACT

The activity of uridine diphosphate-glucose:ceramide glucosyltransferase during chick retina ontogenesis was studied. This enzyme catalyzes the first step in the biosynthetic pathway leading to gangliosides. The glucosyltransferase activity was detected in 8 day old embryos, and was present in adult animals. The highest specific activity was found ca. day 10 of embryonic life. The radioactive product was identified as glucocerebroside by means of radiochromatography. The presence of phosphatidylcholine in the incubation mixtures was found to stimulate the enzyme.

INTRODUCTION

Glucocerebrosides are widely distributed in animal tissues such as kidney (1), aortic tissue (2), and spleen (3) as well as nervous tissue (4-6). Their role in the synthesis of gangliosides and other complex glycolipids is well established (7). The properties of the enzyme which catalyzes the formation of glucocerebroside from uridine diphosphate (UDP)-glucose and ceramide, the UDP-glucose:ceramide glucosyltransferase (ceramide glucosyltransferase in the further text), has been studied in brain (8,9), spleen (10), and kidney (11).

We were interested in the functional role of retinal gangliosides undergoing light stimulation (12), and, more specifically, in the metabolism of gangliosides of synapses responsible for transfer of visual information.

Recently, Hughes and La Velle (13) published new extensive data concerning the synaptogenic sequence of the chick retina. In this investigation, we studied the activity profile of ceramide glucosyltransferase in chick retina to correlate it with biochemical events accompanying synaptogenesis in the retina.

MATERIALS AND METHODS

The source of nonhydroxy fatty acid (NFA)-ceramide, gluco-, and galactocerebrosides and of UDP-Glu[^{14}C] has been given before (8,14). Lecithin (pure phosphatidyl choline) was isolated from pig brain (15).

Leghorn chicken embryos were killed after 8-21 days of incubation, and chicks were killed 1-30 days after hatching. Adult animals also were used. The retinal tissue was removed immediately after killing, and 10% (w/v) homogenates were prepared in 0.32 M sucrose containing 3×10^{-3} M Na_2HPO_4 and 10^{-3} M Na_2EDTA.

The assay conditions were based essentially on previous studies (8,9), and no attempts were made to establish optimal pH, bivalent cation and buffer concentrations. The incubation mixture contained 0.1 μmole NFA-ceramide, 0.50 mg Triton X-100, 150 μg lecithin, 1 μmole $MgCl_2$, 10 μmoles Tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.5), 20 nmoles (5 μCi/μmole) UDP[U-^{14}C]glucose, and 0.5-1.5 mg protein of homogenate in 0.25 ml final volume. Ceramide, Triton X-100, and lecithin were dissolved in chloroform:methanol (2:1) added before the other compounds, and chloroform:methanol removed by vacuum evaporation. Incubations were carried out at 37 C for 60 min in a shaking bath. The reaction was stopped by adding 2.5 ml chloroform:methanol (2:1), and separation into two phases was achieved by addition of 0.25 ml water (16). The lower phase was washed twice with 0.5 ml chloroform:methanol:water (3:48:47). Standard glucocerebrosides were added, and TLC separation of glucocerebrosides was carried out on sodium borate impregnated plates with chloroform:methanol:water (24:7:1) as development solvent (17). Glucocerebrosides were located by spraying with 2′,7′-dichlorofluorescein solution (18), and corresponding areas were transferred into counting vials containing 10 ml 0.4% Omnifluor (NEN Chemicals, Dreieichenhain, Germany) in toluene; radioactivity was measured in an Intertechnique liquid scintillation spectrometer. For identification of radioactive products, when relatively large amounts of lipid material were present, mild alkaline hydrolysis was performed before TLC (25). Synthesis in vitro of retinal glucocerebrosides was a linear function of enzyme concentration up to ca. 1 mg protein. Activity was determined at 2 different enzyme concentrations, corresponding to linear parts of the activity-enzyme concentration plots. No corrections were made for blanks in which cera-

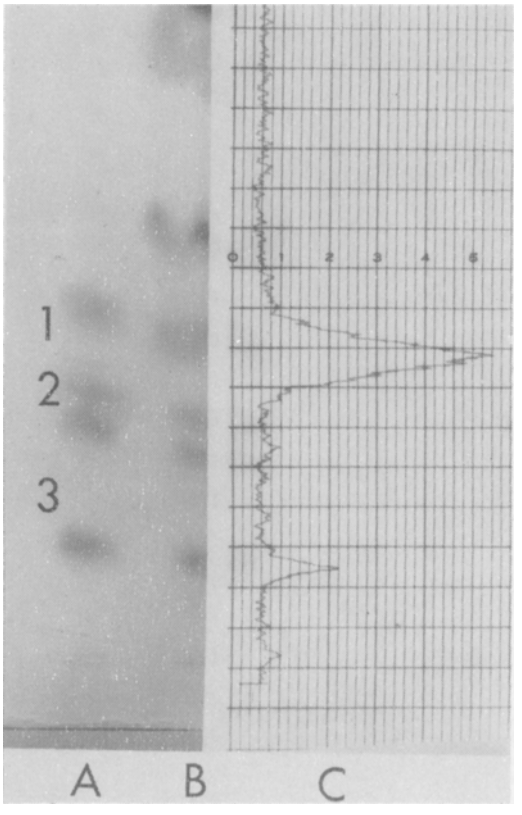

FIG. 1. Thin layer chromatogram (TLC) of radioactive glycolipids after incubation of retinal homogenate with labeled uridine diphosphate (UDP)-glucose and nonhydroxy fatty acid (NFA)-ceramide. Amounts of components were increased 10 times and incubated 2 hr; 10 mg retinal protein (1 day old chick) were used. Total lipids extracted from the incubation mixture were subjected to a mild alkaline methanolysis (25). Alkali resistant lipids were spotted on a borate impregnated plate and developed as described in Materials and Methods. Orcinol sulfuric acid reagent (26) was used for detection. (A) Standard glycolipids; (B) Alkali resistant lipids from the incubation mixture; (C) Radioactivity scanning (Thin Layer Scanner II, LB 2723, Berthold). Identification of glycolipids: (1) glucocerebrosides, (2) galactocerebrosides (2 spots), (3) lactosylceramide.

mide was omitted (<30% whole radioactivity detected in glucocerebrosides). Proteins were determined according to Lowry, et al., (19).

RESULTS

Identification of Labeled Products

Identification of labeled products was made only by cochromatography with standard glycolipids isolated from beef spleen. Because of limited amounts of retinal tissue and low synthesizing capacity of the incubation system, structural analysis of labeled products was not

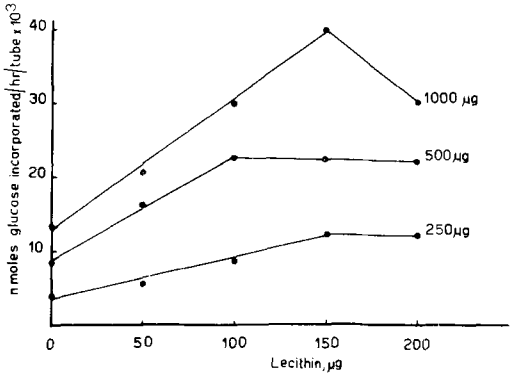

FIG. 2. Effect of lecithin on uridine diphosphate (UDP)-glucose:ceramide glucosyltransferase at different protein concentrations.

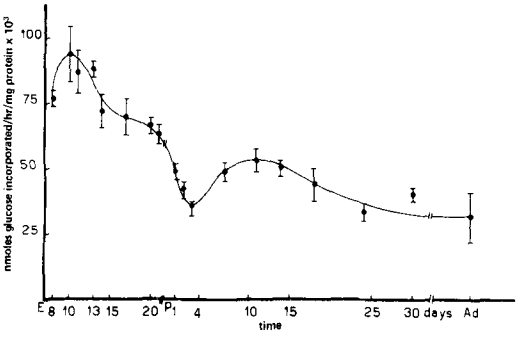

FIG. 3. Ceramide glucosyltransferase activity in chick retina as function of age. Each value is the mean of 2-4 determinations. E = Embryonic age; P = Postnatal age; P_1 = hatching day; Ad = adults.

undertaken in this work.

As shown in Figure 1, about 80% of total radioactivity was associated with peak I, which cochromatographed with authentic glucocerebrosides. The position of this main peak indicates the presence of labeled material moving less rapidly than standard glucocerebrosides from spleen, which contain only NFA. Similar observation has been made previously in brain (7,8,20) in which this phenomenon was attributed tentatively to synthesis of α-hydroxy fatty acid (HFA) HFA-glucocerebrosides. It is interesting to note a small peak migrating as standard lactosyl ceramide (ca. 20% total radioactivity). This may be explained by the presence of galactosyltransferase specific glucocerebroside in retina, as it was shown previously in brain (21,22).

Stimulation of Glucocerebroside Synthesis by Lecithin

Addition of lecithin to the incubation mixture substantially increased ceramide glucosyl-

transferase with the maximum of activation of ca. 150 μg lecithin per tube, when 0.25-1.0 mg retinal proteins were used (Fig. 2).

Development of Ceramide Glucosyltransferase Activity

Figure 3 shows the evolution profile of ceramide glucosyltransferase in chicken retina. The first maximum activity was observed at day 10 of embryonic life. It corresponds to the highest activity detectable in chicken retina. From day 10 of embryonic life to day 3 of postnatal life, there was a rather rapid decrease. The second maximum occurred on day 11 of postnatal life. This maximum was less marked and rather flat until day 14. Activity then decreased slowly, and in older animals was found to be ca. one third of maximum value observed.

DISCUSSION

Developmental changes of ceramide glucosyltransferase have been studied previously in embryonic chick brain by Basu, et al., (21). These workers investigated the period between days 7-20 of embryonic life. Evolution of ceramide glucosyltransferase activity during this period in chicken brain was found to be similar to that reported in the present work for retina, except that maximal activity of glucosyltransferase was found earlier in retina (day 10 of embryonic life) than in brain (day 13 of embryonic life). Comparison cannot be made for the postnatal period, because data on chicken brain are missing. Nevertheless, data are available for brain of other species. Our results for the postnatal chicken retina are similar to those reported by Neskovic, et al., in mouse brain (8).

The role of ceramide glucosyltransferase in the synthesis of brain gangliosides seems to be confirmed by developmental studies (22). There is good accordance between periods of rapid ganglioside accumulation and the highest enzyme activity (23). Brenkert and Radin (24) suggested that ceramide glucosyltransferase may be a limiting factor in ganglioside formation. At the present time, we could not confirm such a close time correlation between these 2 phenomena in retina. Our studies on ganglioside evolution in chicken retina during development (23) show a relatively rapid accumulation of total ganglioside concentration which remains appreciable until day 20 of embryonic life.

It is difficult at this time to make a correlation between ceramide glucosyltransferase evolution and morphological events of developing chicken retina, because these changes are extremely rapid and overlap frequently (13). However, it seems that the enzyme levels are highest during the earliest stage of synaptogenesis in chicken retina, e.g., during formation and development of inner and outer plexiform layers.

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Lipids of Cultured Hepatoma Cells: VII. Structural Analyses of Glycerolipids in Minimal Deviation Hepatoma 7288C

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ABSTRACT

Phosphatidylcholine, phosphatidylethanolamine, and triglycerides were isolated from minimal deviation hepatoma 7288C cells cultured as monolayers to confluency in roller flasks containing Swim's 77 medium supplemented with 5% fetal calf serum, plus 20%, 10%, or 5% bovine serum. Fatty acid distribution at each position of glycerol was determined for the 3 glycerolipid classes, and carbon number distributions of triglycerides and diglycerides derived from phosphatidylcholine and phosphatidylethanolamine were quantitated by high temperature gas liquid chromatography. Fatty acid composition was only marginally affected by the level of bovine serum in the culture medium. Percentage composition of fatty acids esterified at each position of the 3 glycerolipids was different, indicating a nonrandom distribution of acyl groups in triglycerides and the 2 diacyl phosphatides. The carbon number distribution of diglycerides derived from phosphatidylcholine and phosphatidylethanolamine was different, and neither carbon number distribution agreed with the calculated 1-random, 2-random diacyl distribution, thus indicating pairing of certain acids in the diglycerides derived from these phospholipid classes. The determined triglyceride carbon number distributions did not show complete agreement with those calculated, assuming a 1-random, 2-random, 3-random type of fatty acyl distribution, suggesting preferential pairing of some acids in this lipid class. The 1-, 2-diglycerides derived from phosphatidylcholine, phosphatidylethanolamine, and triglycerides differed, indicating either selectivity in utilization of diglyceride species in biosynthesis of these glycerolipids, or modification of glycerolipids after their initial synthesis.

INTRODUCTION

Wood and colleagues have examined in great detail glycerolipids of rat liver (1-4) and those obtained from a number of transplantable rat and mouse tumors (3-7). Such studies, recently reviewed (8), revealed that tumor lipids possess important qualitative and quantitative structural differences from that of normal tissue. A series of reports from this laboratory has described the qualitative and quantitative changes in individual phospholipids and neutral lipid classes from minimal deviation hepatoma 7288C cells (HTC) when cultured on a variety of media (9-12). Lack of information on glycerolipid structure in these HTC cells has prompted the present investigation. In this report, we describe positional analyses and structure of glyceride species of triglyceride (TG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) isolated from HTC cells cultured on varying levels of serum.

EXPERIMENTAL PROCEDURES

Minimal deviation hepatoma 7288C cells (HTC) were grown as monolayers in roller cultures on a modified Swim's-77 medium (Grand Island Biological Co., Grand Island, N.Y.), supplemented with varying amounts of serum, as described previously (9). Glycerolipids analyzed in the present study were from the same source of HTC lipids described in earlier reports (9-12). TG, PC, and PE of $> 99\%$ purity were isolated by preparative thin layer chromatography (TLC). Positional analysis of fatty acids of PC and PE was performed as described previously (2). PC and PE also were hydrolyzed with phospholipase C to diglycerides (5), and the diglycerides converted to diglyceride acetates (13) and hydrogenated. Carbon number distribution of hydrogenated diglyceride acetates was examined by high temperature gas liquid chromatography (GLC) as described previously (5). Similarly, TG were hydrogenated and analyzed intact by GLC as described previously (5). The stereospecific analysis of TG was performed by the Brockerhoff procedure (14), modified for 3-5 mg TG as described recently (3). The source and quality of standards, solvents, and reagents were the same as given previously (3,4).

RESULTS

Positional Analysis of PC and PE

Positional analysis of the fatty acids of PC

TABLE I

Distribution of Fatty Acids at 1- and 2-Positions of Phosphatidylcholine and Phosphatidylethanolamine Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum

Basal medium supplement	Position	Fatty acid percentage[a]							
		16:0	16:1	18:0	18:1	18:2	20:1	20:4	22:6
		Phosphatidylcholine							
20% Bovine serum + 5% Fetal calf serum	1	47.9	4.0	19.6	23.3	1.5	1.9	T[b]	T
	2	18.5	5.7	4.9	43.4	13.6	1.0	10.3	0.1
10% Bovine serum + 5% Fetal calf serum	1	50.2	2.9	17.4	23.6	1.2	2.9	T	T
	2	16.3	7.2	2.5	43.4	17.8	1.2	7.4	1.4
5% Bovine serum + 5% Fetal calf serum	1	58.0	2.3	16.5	22.2	0.1	0.2	T	T
	2	17.8	11.6	3.4	48.1	12.5	0.1	3.9	0.8
		Phosphatidylethanolamine							
20% Bovine serum + 5% Fetal calf serum	1	11.9	1.2	40.1	43.9	1.7	T	T	T
	2	9.2	1.4	15.6	49.6	6.4	0.1	16.0	0.1
10% Bovine serum + 5% Fetal calf serum	1	10.8	0.1	41.4	47.1	0.7	T	T	T
	2	5.6	2.4	11.6	41.2	10.0	1.8	19.2	1.4
5% Bovine serum + 5% Fetal calf serum	1	12.9	0.1	39.0	47.5	0.5	T	T	T
	2	8.0	4.2	13.7	45.6	9.4	0.1	14.0	0.8

[a]Difference between sum of fatty acid percentages in each row and 100% represents the sum of minor fatty acids not given in the table.

[b]T = Detectable levels, but $< 0.1\%$.

and PE is given in Table I. Both phospholipid classes have quantitatively unique positional distributions of fatty acids, suggesting a preference of certain fatty acids for 1 of the 2 acyl positions. Reduction of arachidonic acid (20:4) at 2-position of PC was found when cells were cultured on decreasing levels of bovine serum. Otherwise, only minor changes in fatty acid composition at both acyl positions were observed with decreasing levels of bovine serum in the culture medium. 1-Position fatty acids of PC are much more saturated than the corresponding position in PE. Octadecenoic acid (18:1) in 1-position of PC was one-half that of the 2-position, whereas, in PE, percentages of 18:1 at both positions were similar. Position 1 of both phosphatides contained only insignificant levels of polyunsaturates, whereas, fatty acids esterified at position 2 were primarily unsaturated, ranging from 75% to 85% of the total.

Stereospecific Analysis of Triglycerides

Table II shows the distribution of fatty acids among the acyl positions of TG isolated from HTC cells cultured on the 3 levels of serum. Although positional distribution of fatty acids of TG were not nearly so unique as that for the diacylphosphatides, each position did have a quantitatively different fatty acid composition, suggesting a nonrandom distribution of fatty acids. Only minor changes in fatty acid composition at acyl positions of TG were observed as the level of bovine serum in the culture media was reduced. Although changes in the level of 18:2 and 20:1 at the 3-position were evident, these changes were not consistent with the decrease in bovine serum concentration in the culture medium. Octadecenoic acid represented ca. 50%, 60%, and 70% of the 1-, 2-, and 3-positions, respectively. Hexadecanoic acid (16:0) did not predominate any of the acyl positions. Position 2 possessed ca. 3 times more 18:2 than the other positions, whereas, the 2-position contained the least amount of 18:0 in comparison to the other acyl positions.

Analysis of Intact Diglyceride Acetates of PC and PE

Table III shows that the carbon number distribution of diglycerides derived from PC was strikingly different from that of PE, and, except for minor changes, individual distributions were affected marginally by the level of serum in the culture medium. In general, the percentages of diglyceride species of PC possessing carbon numbers 34 and 36 were similar, and combined they constituted $> 75\%$ of the total diglyceride species. As cells were cultured on decreasing levels of bovine serum, the quantity of diglyceride carbon number 34 and 36 from PE increased. Regardless of serum level, there was a noticeable increase in higher mol wt carbon number species of PE relative to PC. Also shown in Table III are calculated carbon number percentages derived from formula based on the 1-random, 2-random fatty acid distribution

TABLE II

Distribution of Fatty Acids at 1-, 2-, and 3-Positions of Triglycerides Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum

Basal medium supplement	Position	Fatty acid percentage[a]								
		14:0	16:0	16:1	18:0	18:1	18:2	20:0	20:1	20:4
20% Bovine serum +	1	0.9	13.8	4.6	14.7	54.6	2.6	1.1	6.8	0.3
5% Fetal calf serum	2	1.5	11.4	6.3	4.5	59.9	11.2	0.2	1.6	2.2
	3	T	3.9	2.2	6.3	75.4	4.4	0.6	6.3	T
10% Bovine serum +	1	1.4	18.1	5.3	15.2	49.2	2.2	1.1	7.3	T
5% Fetal calf serum	2	1.4	13.3	6.6	4.9	57.4	10.9	0.3	2.3	1.4
	3	0.7	11.9	3.1	11.7	62.5	3.2	1.0	5.7	T
5% Bovine serum +	1	1.5	18.9	6.0	13.6	51.3	3.4	0.6	4.1	0.3
5% Fetal calf serum	2	0.6	16.0	7.2	4.8	60.4	7.7	T	1.5	1.4
	3	T	11.7	2.6	10.3	73.5	T	T	1.8	T

[a]Difference between sum of fatty acid percentages in each row and 100% represents the sum of minor fatty acids not given in the table.

[b]T = Detectable levels, but < 0.1%.

TABLE III

Distribution of Carbon Numbers in Diacyl Acetates of Phosphatidylcholine and Phosphatidylethanolamine Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum

Basal Medium supplement		Percent carbon number[a]						
		30	32	34	36	38	40	42
		Phosphatidylcholine						
20% Bovine serum +	Diacyl	0.7	6.6	36.7	40.1	14.3	1.6	-
5% Fetal calf serum	Calc.[b]	0.4	13.2	43.7	35.0	7.1	0.5	0.0
10% Bovine serum +	Diacyl	0.3	6.4	38.5	38.4	13.3	2.9	-
5% Fetal calf serum	Calc.	0.6	13.4	44.9	33.6	6.7	0.7	0.0
5% Bovine serum +	Diacyl	0.3	8.6	40.6	36.3	12.9	1.3	-
5% Fetal calf serum	Calc.	1.1	18.8	50.3	27.6	2.0	0.1	0.0
		Phosphatidylethanolamine						
20% Bovine serum +	Diacyl	0.4	0.7	8.8	54.5	33.3	2.2	-
5% Fetal calf serum	Calc.	0.0	1.9	19.4	63.8	13.8	1.0	0.0
10% Bovine serum +	Diacyl	0.4	0.9	10.3	51.9	32.2	4 2	-
5% Fetal calf serum	Calc.	0.0	1.0	13.8	59.0	21.5	4.6	0.0
5% Bovine serum +	Diacyl	0.9	0.6	15.5	57.5	22.4	3.0	-
5% Fetal calf serum	Calc.	0.1	2.1	19.5	61.8	13.5	2.9	0.0

[a]Carbon atoms of acetate are not included in carbon numbers.

[b]Values were calculated from the determined abundance of each even carbon numbered fatty acid in the 1- and 2-positions (Table I) and possible fatty acid combinations of each carbon number. A sample 1-random, 2-random distribution calculation of C-32 is given: 32 = (16 x 16) + (14 x 18) + (18 x 14) x 100. Each multiplicand and multiplier represents a combination or permutation of fatty acid chain lengths present that could give rise to C-32. The multiplicand represents the 1-position, and the multiplier, the 2-position. Determined fatty acid percentages are substituted, and the resulting sum of the products represents 1-random, 2-random distribution percentage of C-32.

hypothesis. Values calculated from the composition of the 1- and 2-positions of PC and PE shown in Table I did not agree with those determined experimentally.

Analysis of Intact Triglycerides

TG carbon number distribution based on mol wt of TG species from cells cultured on the 3 levels of serum was determined by high temperature GLC, and is given in Table IV. Three sets of theoretically derived TG distributions also were calculated for each serum level, and also are given in Table IV. Results from stereospecific analysis of TG (Table II) and diglyceride species derived from PC and PE (Table III) provided the data for calculating

TABLE IV

Comparison of Determined and Calculated Carbon Numbers of Triglycerides, Phosphatidylcholine, and Phosphatidylethanolamine Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum

Glyceride[a]	Percent carbon number[b] 48	50	52	54	56	58	60	Chi-[c] Square
	20% Bovine serum + 5% fetal calf serum							
TG (determined)[d]	0.2	9.6	26.7	45.8	13.0	3.4	1.3	
Calc. (1-, 2-, 3-random)[e]	0.7	6.2	27.7	51.9	12.3	0.9	0.0	9.9
Calc. (PC diacyl Ac + 3 pos. TG)[f]	0.4	8.1	35.1	38.5	15.4	2.4	0.1	18.7
Calc. (PE diacyl Ac + 3 pos. TG)[f]	0.0	1.2	11.1	50.3	32.9	4.2	0.2	99.3
	10% Bovine serum + 5% fetal calf serum							
TG (determined)	2.8	14.3	28.7	36.8	12.2	4.2	1.0	
Calc. (1-, 2-, 3-random)[e]	1.7	10.2	32.3	43.9	10.8	0.9	0.0	16.1
Calc. (PC diacyl Ac + 3 pos. TG)[f]	1.2	11.1	36.3	34.5	13.4	3.2	0 2	8.4
Calc. (PE diacyl Ac + 3 pos. TG)[f]	0.2	2.6	16.2	46.0	29.2	5.4	0.3	131.3
	5% Bovine serum + 5% fetal calf serum							
TG (determined)	3.0	15.3	31.9	38.1	8.9	2.1	0.7	
Calc. (1-, 2-, 3-random)[e]	1.5	11.1	36.4	45.0	5.6	0.2	0.0	28.2
Calc. (PC diacyl Ac + 3 pos. TG)[f]	1.2	13.1	39.5	33.1	11.7	1.3	0.0	6.4
Calc. (PE diacyl Ac + 3 pos. TG)[f]	0.1	2.8	21.5	52.2	20.4	2.9	0.1	159.0

[a]TG = triglyceride; PC = phosphatidylcholine; PE = phosphatidylethanolamine; AC = acetate.

[b]A carbon number represents the sum of carbon atoms in the 3 fatty acids esterified to glycerol.

[c]Chi-square values were calculated for comparing TG (determined) distribution with that of calculated triglyceride distribution.

[d]Analyzed intact by gas liquid chromatography (GLC).

[e]The 1-random, 2-random, 3-random distribution was calculated from the determined composition of the 1-, 2-, and 3-position of the triglyceride.

[f]Values were calculated from determined abundance of each diacyl even carbon number (1- and 2-position) and even carbon number distribution of fatty acids at position 3 of TG.

these TG distributions. On comparing the determined TG distribution with that of the 1-random, 2-random, 3-random distribution at a particular serum level, the most striking difference occurs at carbon number 54. At each serum level, absolute percentage of the calculated carbon number 54 is 6-7% higher than that of the determined value. The elevated value for calculated carbon number 54 could be explained by preferential association of fatty acids with 18 carbons esterified at acyl positions on the glycerol molecule. At each serum level, determined TG carbon number distribution did not show complete agreement with those calculated, assuming a 1-random, 2-random, 3-random fatty acid distribution. The other 2 calculated TG carbon number distributions at each serum level have been computed from the values of diglyceride species derived from PC and PE, plus the fatty acids experimentally determined for the 3-position of TG. Regardless of serum level or the diacyl-phosphatide examined, none of the calculated distribution percentages agreed completely with the determined TG carbon number percentages. Chi-square values derived by comparing determined TG distribution to 1-random, 2-random, 3-random distribution or PC diglycerides, plus the 3-position of TG, increased and decreased, respectively, as the level of bovine serum in the culture medium decreased. Thus, on the basis of Chi-square values, the data suggest that TG carbon number percentages calculated from PC diglycerides, plus the 3-position of TG, more closely agreed with those of determined TG percentages than did similar calculations using percentages of PE diglycerides.

DISCUSSION

Earlier reports of this series described the qualitative and quantitative changes in individual cellular and media lipid classes from HTC cells cultured on media containing varied amounts of serum and lipids (9-12). In addition to those studies, the present investigation allows one to a) examine the effect of serum level on the structure of major glycerides, b) gain insight into metabolic relationships of glycerides comprising these hepatoma cells, and c) compare glyceride structure of HTC cells with

that of hepatoma 7288CTC and compare both with normal liver and Ehrlich Ascites cells.

Comparison of Positional Analysis of PC, PE, and TG

Fatty acid composition of major glycerides of these cells was only marginally affected by the level of bovine serum in the media. Comparison of fatty acids at the 1- and 2-positions of the diacyl-phosphatides (Table I) and triglyceride (Table II) reveals no agreement in percentage compositions of fatty acids. The level of 16:0 at the 1-position of PE in these HTC cells, as well as in 7288CTC (4), was 4-5 times lower than the 1-position of PC. In both neoplasms, the reduced level of 16:0 in PE was offset by increased percentages of 18:0 at the 1-position of PE. The decreased level of 16:0 at the 1-position of hepatoma PE is reduced much from that found in normal rat liver (1,4). In general, the level of 16:0 at the 1-position of TG from normal liver represents one-half to two-thirds of total fatty acids of that position (1,3), whereas with HTC or 7288CTC (3), the level was reduced 2 to 3 fold. Reduction of 16:0 at the 1-position of PE and TG in these 2 neoplasms very likely is not the result of decreased synthesis of 16:0, because the level of 16:0 in PC was comparable to normal liver. However, these data may suggest that fatty acids of the primary position of PE and TG in neoplasms arise from the same fatty acid pool, whereas, 1-position fatty acids of normal rat liver PC and PE appear to arise from the same source, as suggested earlier (4).

We have reported earlier that these HTC cells are characterized by high levels of 18:1 fatty acids in both neutral lipid and phospholipid classes (9-12). Thus, the present study demonstrates that the high level of 18:1 occurs at all 3 acyl positions of TG (Table II), at both acyl positions of PE, as well as at the 2-position of PC (Table I). A rather striking feature of the present study was the similar levels of 18:1 (45%) at the 2-position of PC and PE. Although the level was twice that of PE in the Ehrlich ascites cells (5), it was quite comparable to that observed in PC and PE of 7288CTC (4). PC from hepatoma 27 (15) and Yoshida hepatoma (16) have been shown to contain increased levels of 18:1 at the 1-position, in comparison to normal liver. In contrast, PC of these HTC cells, 7288CTC (4), or hepatoma 5123C (16) did not exhibit such an elevation of 18:1 at the 1-position. However, 18:1 at the 2-position of PC in hepatoma 27 (15), Yoshida hepatoma (16), hepatoma 5123 (16), or 7288CTC (4) was elevated in comparison to that of normal liver. Even though these HTC cells, as well as several other neoplasms, possess elevated levels of 18:1 in several lipid classes, these levels cannot be regarded as a generalization, because major glycerolipids of only a few neoplasms have been examined in great detail. An earlier communication (12) reported that vaccenic acid represented 30-70% of the 18:1 fraction from PC, PE, and TG of these HTC cells. Subsequent studies with radiolabeled fatty acids confirmed the earlier findings, as well as demonstrated that biosynthesis of vaccenic acid occurs by elongation of palmitoleic acid (17). Because the increased level of 18:1 found in the glycerolipids of tumors by several investigators has not been established by double bond positional analysis, it is possible that vaccenic acid, and not oleic acid, accounts for increased levels of 18:1 fraction of neoplasms.

Hepatoma 7288CTC exhibits reduced levels of C-20 and C-22 fatty acids in PC and PE, as well as increased levels of those acids in TG, relative to normal liver or host liver (4). A similar trend was observed with these HTC cells which agreed with that reported earlier for Ehrlich ascites cells (5), and for the PC fraction of Yoshida hepatoma (16), and hepatoma 5123C (16). The level of 20:4 in PC and PE fractions of these HTC cells, although reduced from the level in normal liver, was comparable to 7288CTC (4). Reduced levels of C-20 and C-22 fatty acids in neoplasms may be due to the apparent decreased desaturation activities of 18:2 or 18:3, which give rise to C-20 or C-22 fatty acids as recently proposed by Chiappe, et al., (18,19). TG of these HTC cells did not possess significant quantities of 20:4 at any acyl positions, as was found in the 7288CTC (4), but rather possessed elevated levels of 20:1 at the 1- and 3-positions of TG. In a previous communication, it was shown that the 20:1 fraction of TG was comprised predominately of $\Delta 11$ and $\Delta 13$ isomers, presumably arising from elongation of oleic and vaccenic acids (12).

Comparison of Determined and Calculated PC and PE Composition

Carbon number distributions of PC diglycerides were different from PE diglycerides, and neither agreed with the calculated 1-random, 2-random fatty acid distribution. These data would suggest that pairing of certain fatty acids, or selectivity of diglyceride species possessing fatty acids of certain chain length, occurs during biosynthesis of PC or PE. However, the lack in correlation of determined versus calculated carbon number distribution could also arise through the deacylation-reacylation pathway (20). Wood and coworkers have shown that determined carbon numbers of PC di-

glycerides from Ehrlich ascites cells (5) and PC and PE diglycerides from 7288CTC (4) agreed remarkably close with calculated 1-random, 2-random distribution of fatty acids, whereas, PC and PE diglycerides from normal liver showed pairing of specific fatty acids (1,4). Even though differences exist in fatty acid composition of PC of these HTC cells and 7288CTC, determined carbon numbers of diglycerides of PC of these 2 neoplasms agreed remarkedly close. Similarly, the PE fraction isolated from these HTC cells cultured at 10% serum and PE from 7288CTC (4) showed almost complete agreement. Because respective carbon number distributions of PC and PE from HTC and hepatoma 7288CTC agree, one might question how it is possible for these HTC cells to exhibit pairing of fatty acids in PC and PE when 7288CTC seems to exhibit a 1-random, 2-random distribution of fatty acids in phosphatides. Although HTC and 7288CTC are both derived from the same tissue of origin, differences in apparent distribution of fatty acids in phospholipids could possibly be due to the environment that these cells are subjected to during growth. HTC cells are continually bathed in an optimal growth environment, whereas, the nutritional status of 7288CTC may be limited by factors such as absorption and transport of metabolites. Continued investigations of comparative routes of phospholipid biosynthesis in these 2 neoplasms, as well as in normal liver, are necessary to evaluate more fully the different types of molecular species of phosphoglycerides that characterize these cells.

Comparison of Determined and Calculated TG Composition

The level of bovine serum in the culture media resulted in only marginal changes in distribution of molecular species of TG isolated from these cells (Table IV). Comparison of determined and calculated TG carbon number distribution suggest that these HTC cells exhibit a type of fatty acid distribution other than 1-random, 2-random, 3-random, in agreement with previous data derived from TG of 7288CTC (3) and normal liver (1,3). In contrast, determined carbon number distribution of Ehrlich ascites cell TG agreed well with calculated 1-random, 2-random, 3-random distribution (5). Slakey and Lands (21) and Akesson (22) have proposed that normal rat liver TG may exhibit a 1-random, 2-random, 3-random arrangement of fatty acids. Those authors first separated the TG fraction according to degree of unsaturation, and on recovered TG species performed stereospecific analysis. The apparent type of arrangement of fatty acids in TG of normal liver as proposed by Wood and coworkers (1,4), in contrast to that proposed by other laboratories (21,22), may reside in the method of approach of analyzing TG species. In the present investigation, as well as previous reports (1,4), carbon number distribution for species of TG was determined by GLC, which quantifies TG species according to mol wt. Interpretation of the data utilizing both methods of approach may be correct. However, it appears that stereospecific analysis of TG fractionated according to degree of unsaturation and mol wt is necessary to completely resolve the composition of TG.

Differences between TG carbon number distributions calculated from relative abundance of either PC or PE diglycerides, plus fatty acids at the 3-position of TG, to that of determined TG carbon number suggest that diglyceride species possessing fatty acids of specific chain length utilized for biosynthesis of TG are different from those of PC and PE. In an earlier report, Wood and Snyder (5) showed that the TG distribution calculated from PC diglycerides and 3-position fatty acids of TG of Ehrlich ascites cells agreed remarkedly close to that of the determined TG values, whereas in normal rat liver no such agreement was observed (1).

The present investigation must be examined in light of earlier reports suggesting that cellular TG and PC of Ehrlich ascites cells are derived from randomly biosynthesized diglycerides utilized at random (5), in contrast to the apparent diglyceride selectivity for biosynthesis of TG, PC, and PE in normal rat liver (1). Because intermediate diglycerides may arise by a variety of metabolic pathways in vivo, elucidating the type of fatty acid distribution in TG of liver cells may be a difficult task. However, detailed studies of fatty acid composition and structural analysis emphasize the existence of some order in the distribution of fatty acids of glycerolipids, but fail to reveal how selective incorporation occurs. It is imperative that structural analyses of major lipid classes from more normal and neoplastic tissues, and, if possible, the tissue of origin of the neoplasm, be examined if analytical studies are to make contributions toward our knowledge of biosynthesis and metabolic interconversions of glycerolipids in neoplasia.

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Analyses of Renal Medullary Lipid Droplets from Normal, Hydronephrotic, and Indomethacin Treated Rabbits[1]

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ABSTRACT

Lipid droplets isolated from rabbit renal medullary tissue were analyzed and found to be composed of triglyceride and free fatty acids in a ratio of 2.9:1. These triglycerides were unique when compared to triglycerides of other rabbit tissues examined, in that they contained high percentages of octadecanoic acid (stearic acid, 9.8%), 5,8,11,14-eicosatetraenoic acid (arachidonic acid, 6.8%), and 7,10,13,16-docosatetraenoic acid (adrenic acid, 10%). Lipid droplet triglycerides were found to increase during experimental hydronephrosis and after administration of indomethacin, a prostaglandin synthetase and phosphodiesterase inhibitor. From gas liquid chromatography of fatty acid methyl esters of these triglycerides, it was determined that they were enriched further in their percent composition of 9,12-octadecadienoic acid (linoleic acid) and arachidonic acid, a prostaglandin precursor. The inverse relationship between lipid droplets and prostaglandin content in the inner medulla suggested a significant role of lipid droplet triglycerides as storage pools for prostaglandin precursors.

INTRODUCTION

Interest in lipids of the renal inner medulla has been aroused recently because the antihypertensive function of the inner medulla has been traced in part to its production of large quantities of prostaglandins E_2 and $F_{2\alpha}$ (1,2,3) and other neutral lipids (4). Highly structured interstitial cells of the inner medulla have been implicated as a site of prostaglandin production (5) and contain osmiophilic lipid droplets sensitive to fluctuations in salt and water balance (6-10). In addition, a diminution of lipid droplets has been observed in several different types of rat and rabbit hypertension and in human malignant hypertension (6-8). During hydronephrosis or after administration of indomethacin, a prostaglandin synthetase and phosphodiesterase inhibitor, the number of lipid droplets and their triglyceride content were observed to increase significantly (11). Extracts of inner medullas and lipid droplets, isolated by sucrose density centrifugation from rat and rabbit inner medullary homogenates, have been reported in preliminary communications to contain significant amounts of arachidonic acid, the precursor for prostaglandins E_2 and $F_{2\alpha}$ (11-14).

The present communication reports analyses of the renal inner medullary lipids and lipid droplets of normal rabbits and rabbits with increased number of lipid droplets (11) due to hydronephrosis or indomethacin treatment. Fatty acid composition of triglycerides, free fatty acids, and cholesteryl esters, which comprise the lipid droplets, are compared to fatty acid compositions of these lipid classes in other rabbit tissues.

MATERIALS AND METHODS

Male, New Zealand rabbits (2-2½ kg) were obtained from HARE, Inc. (West Milford, NJ) and were fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) ad libitum. After sacrifice, tissue samples were quickly dissected, cleaned, and frozen on dry ice. Frozen samples were weighed and homogenized in cold chloroform and methanol as described by Bligh and Dyer (15). Extracts were filtered and centrifuged. The lipid containing chloroform layer was removed and evaporated under nitrogen at room temperature, and the lipid residue was dissolved in a minimum of chloroform and stored at -20 C under nitrogen.

Lipid classes were separated by chromatography on silica gel impregnated glass fiber sheets, ITLC type SA (Gelman Instrument Co., Ann Arbor, Mich.). Lipid residues were streaked onto the chormatography media under a stream of nitrogen and developed in hexane: diethyl ether:acetic acid (90:10:0.2). A lipid standard containing cholesterol, cholesteryl oleate, triolein, oleic acid, and lecithin (TLC reference 18-5A) was obtained from Nu Chek Prep (Elysian, Minn.) and used on each chromatogram. Areas containing various lipids were identified under ultraviolet light after spray-

[1]Presented in part at the Biochemistry-Biophysics Meeting, Minneapolis, 1974, and AOCS 48th Annual Fall Meeting, Philadelphia, 1974.

TABLE I

Fatty Acid Composition of Rabbit Tissue Triglycerides

Fatty acid methyl esters	Composition (%)							
	Heart (7)[a]	Liver (7)	Adrenal (7)	Testis (7)	Adipose (7)	Renal cortex (7)	Inner medulla (7)	Renal lipid droplets (8)
14:0	4.0 ± 0.7[b]	4.2 ± 0.4	2.6 ± 0.2	2.5 ± 0.1	3.1 ± 0.2	2.3 ± 0.2	2.3 ± 0.3	1.3 ± 0.2
16:0	29.3 ± 1.6	37.9 ± 1.7	21.5 ± 1.9	23.5 ± 0.8	25.0 ± 0.6	30.9 ± 1.3	25.9 ± 0.4	20.8 ± 0.6
16:1	3.5 ± 0.6	4.0 ± 0.6	3.4 ± 1.0	9.1 ± 0.8	5.2 ± 0.9	3.9 ± 0.8	4.0 ± 0.5	2.6 ± 0.6
18:0	4.5 ± 0.5	3.7 ± 0.4	5.0 ± 0.7	3.0 ± 0.2	4.6 ± 0.2	6.2 ± 0.3	9.9 ± 1.0	9.8 ± 0.4
18:1n-9	21.4 ± 1.4	29.6 ± 1.1	21.1 ± 1.3	25 7 ± 0.8	25.4 ± 0.8	22.7 ± 1.0	20.7 ± 0.4	20.1 ± 0.5
18:2n-6	30.6 ± 2.7	12.7 ± 1.7	30.7 ± 2.7	26.2 ± 1.7	26.6 ± 1.1	24.3 ± 1.2	22.5 ± 1.2	20.9 ± 0.5
18:3n-3	5.5 ± 0.9	3.0 ± 0.4	10.8 ± 1.1	6.1 ± 0.7	7.8 ± 0.7	3.8 ± 0.3	2.8 ± 0.3	2.7 ± 0.1
20:3n-6	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	--	0.1 ± 0.0	0.2 ± 0.1	1.2 ± 0.2
20:4n-6	0.5 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	0.6 ± 0.0	0.3 ± 0.0	2.7 ± 0.6	3.3 ± 0.3	6.8 ± 0.5
22:4n-6	--	--	0.3 ± 0.1	0.2 ± 0.0	--	0.1 ± 0.0	4.3 ± 0.5	10.0 ± 0.4
22:5n-6	--	0.4 ± 0.2	--	0.4 ± 0.1	--	--	0.1 ± 0.0	0.3 ± 0.1
22:5n-3	--	--	--	--	--	0.1 ± 0.0	0.3 ± 0.1	1.8 ± 0.3
% PUFA[c]	36.7	17.4	43.1	33.8	34.7	31.1	33.5	43.7

[a]Number of determinations; 2 rabbits were used for each determination.

[b]Mean ± standard error.

[c]PUFA = polyunsaturated fatty acids.

ing the chromatogram with ethanolic 4',5'-dichlorofluorescein. Lipid classes were eluted from the sheets with chloroform:methanol (2:1), and dried under N_2 before transmethylation with borontrifluoride-methanol (14%) (Supelco, Inc., Bellefonte, Pa.) (16). Methyl esters were extracted into hexane and immediately analyzed by gas liquid chromatography (GLC) using a Hewlett-Packard Research Chromatograph (Model 7620A). The chromatograph was equipped with a hydrogen flame detector and a digital integrator (Hewlett-Packard, Model 3372A). Separation of fatty acid methyl esters was achieved on a stainless steel column (8 ft x 1/8" outer diameter [OD]) packed with 10% EGSS-X on Gas Chrom P 80/100 Mesh (Applied Science Laboratories, State College, Pa.) at an operating temperature of 186 C. Prepurified nitrogen was used as the carrier gas. Identities of unknown fatty acid methyl esters were established by a comparison of retention times and equivalent chain lengths to those of standards (17).

Quantitative GLC was performed using the following lipids as internal standards: 11,14-eicosadienoic acid, tri-11,14-eicosadienoin, and cholesteryl-11,14-eicosadienoate (Nu Chek Prep, Elysian, Minn.). Additions of 100-200 μg of each standard were made to samples prior to chloroform-methanol extraction. After GLC, correction factors were used for standard triglyceride fatty acid (0.91) and cholesterol ester fatty acid (0.46) to determine μg lipid. Recovery of triglyceride averaged 82%; that for cholesteryl ester was 86%. The data as reported were corrected for losses, and are expressed as μg lipid per g wet inner medulla. Significance was determined by using student's t-test.

Renal medullary lipid droplets were purified from fresh rabbit inner medullas by the method of Anggard, et al., (14). Before homogenization in cold 0.25 M sucrose, medullary tissue was minced using a McIlwain tissue chopper (Brinkman Instruments, Westburn, NY) at fine settings. Isolated lipid droplets were purified further by floatation through water. This was accomplished by resuspending lipid droplets in sucrose to a final concentration of 0.4 M sucrose, then overlaying with cold glass distilled water and centrifugation in a Beckman SW 27 rotor at 100,000 x g for 2 hr. This same procedure was used to isolate lipid droplets from rabbits which had been manipulated to increase lipid droplet content of the medullary interstitial cells. Either administration of indomethacin (5 mg/kg i.v., Merck, Sharp, and Dohme, Rahway, NJ) or unilateral hydronephrosis, using the contralateral kidney as an internal control, was employed as described previously (11).

TABLE II

Fatty Acid Composition of Cholesteryl Esters from Rabbit Tissues

Fatty acid methyl esters	Composition (%)					
	Liver (7)[a]	Adrenal (7)	Testis (7)	Spleen (7)	Renal cortex (7)	Renal lipid droplets (8)
14:0	2.8 ± 0.4[b]	2.6 ± 0.2	3.0 ± 0.4	3.8 ± 0.3	2.9 ± 0.5	3.3 ± 0.6
16:0	37.3 ± 1.9	14.7 ± 1.1	31.5 ± 2.0	28.6 ± 1.3	25.3 ± 1.1	23.5 ± 0.6
16:1	4.2 ± 1.1	3.0 ± 0.4	5.0 ± 1.3	4.7 ± 1.2	4.0 ± 1.0	4.7 ± 0.4
18:0	6.9 ± 0.7	5.5 ± 0.5	5.8 ± 1.1	9.2 ± 1.8	9.4 ± 1.1	9.7 ± 1.2
18:1n-9	29.6 ± 2.4	29.0 ± 1.6	27.0 ± 1.3	29.6 ± 1.2	27.7 ± 2.2	27.6 ± 0.8
18:2n-6	12.3 ± 2.3	15.0 ± 2.1	14.7 ± 2.7	18.8 ± 1.0	22.7 ± 1.7	17.2 ± 1.4
18:3n-3	1.8 ± 0.4	2.3 ± 0.5	3.0 ± 0.6	2.3 ± 0.4	3.2 ± 0.6	1.6 ± 0.2
20:3n-6	--	1.7 ± 0.7	1.2 ± 0.5	--	--	0.4 ± 0.1
20:4n-6	0.7 ± 0.1	8.1 ± 0.8	2.4 ± 0.9	--	--	5.0 ± 0.3
22:3n-6	--	--	--	--	--	3.2 ± 0.5
22:4n-6	--	12.3 ± 0.6	--	--	--	1.8 ± 0.1
22:5n-6	--	1.6 ± 0.3	--	--	--	--
22:5n-3	--	2.7 ± 0.6	--	--	--	0.8 ± 0.1
22:6n-3	--	0.2 ± 0.1	--	--	--	--
% PUFA[c]	14.8	43.9	21.3	21.1	25.9	29.2

[a]Number of determinations; 2 rabbits were used for each determination.

[b]Mean ± standard error.

[c]PUFA = polyunsaturated fatty acids.

Three groups of rabbits composed of 2 animals each received indomethacin. Hydronephrotic inner medullas of 5 rabbits were divided into 2 groups (2 animals in 1 group and 3 in the second) for isolation and analysis of lipid droplets.

Prostaglandin $F_{2\alpha}$ was measured by a radioimmunoassay technique sensitive to 5 pg of $PGF_{2\alpha}$ (11).

Protein determinations were performed according to the method of Lowry, et al., using bovine serum albumin as standard (18).

RESULTS

Fatty acid composition (mean ± SE) of control rabbit tissue triglycerides is given in Table I. In all tissues examined, except kidney, the relative abundance of stearic (18:0) and arachidonic (20:4n-6) acids in the triglycerides was low. Renal cortex, inner medulla, and isolated lipid droplets contained relatively high percentages of arachidonic acid; 2.7, 3.3, and 6.8%, respectively. Furthermore, inner medulla and lipid droplet triglycerides contained significant amounts of both stearic (9.9 and 9.8%, respectively) and adrenic acid (22:4n-6) (4.3 and 10%, respectively). The only other tissue lipid which contained a significant percentage of 22:4n-6 was the cholesteryl ester fraction from adrenals (Table II). The high percentage of polyunsaturation found in lipid droplet triglycerides (43.7%), as compared to the other tissues examined, were found to correlate with lower percentages of palmitic and linoleic acids (Table I).

Sufficient quantities of cholesteryl esters could be isolated from liver, adrenal, testis, spleen, kidney cortex, and lipid droplets for GLC analysis (Table II). Unlike rat inner medulla, no cholesteryl esters could be detected in total lipid extracts of rabbit inner medullas (12,13), and only a small percentage of lipids isolated from lipid droplets were cholesteryl esters (Table IV). Polyunsaturation of cholesteryl esters found in most tissues was low and confined to linoleic acid (18:2n-6). However, arachidonic acid was found in cholesteryl esters of testis and renal lipid droplets (2.4 and 5%, respectively). In addition, small percentages of 22:3n-6 (3.2%) and 22:4n-6 (1.8%) were observed in lipid droplets. Adrenal cholesteryl esters contained the highest percentage of polyunsaturation (43.9%) with significant amounts of 20:4n-6 and 22:4n-6 (8.1 and 12.3%, respectively). These percentages agree with previously published data on fatty acid composition of adrenal cholesteryl esters from other animals (19,20).

Unlike most subcellular vesicles, isolated renal lipid droplets contained almost undetectable amounts of phospholipids. This result agrees with ultrastructural observations which had suggested that, although these lipid vesicles arise as isolated foci, they lack a complete membrane structure (21,22). GLC analyses of other tissue phospholipids, which remained at the origin of the thin-layer chromatogram after development in neutral lipid solvent system, re-

TABLE III

Fatty Acid Composition of Rabbit Tissue Phospholipids

Fatty acid methyl esters	Composition (%)						
	Heart (7)[a]	Liver (7)	Adrenal (7)	Testis (7)	Spleen (7)	Renal cortex (7)	Renal inner medulla (7)
14:0	1.1 ± 0.4[b]	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
16:0	20.7 ± 2.0	23.4 ± 1.0	20.2 ± 1.3	28.7 ± 0.9	22.4 ± 1.6	22.0 ± 1.4	19.8 ± 0.6
16:1	0.7 ± 0.3	0.7 ± 0.2	0.5 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.4 ± 0.2
18:0	13.9 ± 0.8	18.6 ± 0.5	19.1 ± 0.7	10.6 ± 0.3	16.8 ± 0.6	15.1 ± 1.1	14.4 ± 0.9
18:1n-9	10.3 ± 0.9	13.1 ± 1.6	18.9 ± 1.0	14.0 ± 0.6	12.8 ± 0.9	13.1 ± 0.7	18.8 ± 0.6
18:2n-6	24.8 ± 0.7	31.6 ± 1.6	8.9 ± 0.5	7.8 ± 0.2	19.3 ± 2.0	25.7 ± 1.8	19.5 ± 0.3
18:3n-3	0.8 ± 0.2	2.8 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	0.6 ± 0.1
20:3n-6	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	4.5 ± 0.5	1.2 ± 0.1	1.9 ± 0.4	0.7 ± 0.0
20:4n-6	19.7 ± 1.3	5.9 ± 0.5	27.5 ± 1.1	15.0 ± 0.7	17.6 ± 1.0	13.9 ± 0.8	21.6 ± 0.6
22:4n-6	0.5 ± 0.1	0.2 ± 0.1	0.9 ± 0.2	3.3 ± 0.2	1.7 ± 0.4	1.0 ± 0.3	0.4 ± 0.0
22:5n-6	0.4 ± 0.1	0.3 ± 0.0	0.1 ± 0.1	10.8 ± 0.9	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
22:5n-3	1.9 ± 0.3	0.4 ± 0.1	0.8 ± 0.2	0.4 ± 0.1	2.0 ± 0.4	0.8 ± 0.3	0.3 ± 0.0
% PUFA[c]	48.4	41.8	39.1	42.2	43.3	44.8	43.3

[a]Number of determinations; 2 rabbits were used for each determination.

[b]Mean ± standard error.

[c]PUFA = polyunsaturated fatty acids.

TABLE IV

Quantitative GLC Analysis of Lipid Droplet Lipids

Fatty acid methyl esters	Composition (%)[a]			
	Triglycerides	Free fatty acid	Cholesteryl esters	Polar lipids
14:0	1.6	1.5	2.4	1.8
16:0	21.7	19.9	20.4	30.1
18:0	9.1	7.9	10.8	17.3
18:1n-9	20.4	22.9	26.2	21.1
18:2n-6	22.0	26.7	18.1	15.5
18:3n-3	2.8	3.9	2.9	0.7
20:3n-6	0.5	1.1	0.5	--
20:4n-6	5.4	4.9	7.8	11.3
22:3n-6	--	--	2.8	--
22:4n-6	10.4	3.7	2.7	--
Total μg lipid/g inner medulla ± S.E.M.	281 ± 59	98 ± 28	6.4 ± 2.6	4.2 ± 2.4
% Total	72.1	25.2	1.6	1.1

[a]Average of 3 experiments; 2 animals were used in each experiment.

vealed fatty acid compositions as given in Table III. Phospholipids of renal inner medulla, like phospholipids of other tissues, contained an abundance of arachidonic acid (20:4n-6) and only small percentages of adrenic acid (22:4n-6). The testis was the exception, because it contained significant percentages of 20:4n-6, 22:4n-6, and 22:5n-6 (15.0, 3.3, and 10.8%, respectively). Although material from lipid droplets recovered from the phospholipid region of the thin layer chromatogram contained a high percentage of arachidonic acid (11.3%), the fatty acid composition of this material was not comparable to phospholipids found in other rabbit tissues (Tables III and IV) and was contaminated by small amounts of mono- and diglycerides.

Except for renal lipid droplets, rabbit tissues examined contained insignificant amounts of free fatty acids. By quantitative GLC, the amount of free fatty acid found in isolated lipid croplets comprised an average of 25% (n = 3) of the lipids (Table IV), second only to the triglyceride content (72%). When compared to fatty acid composition of triglycerides, free fatty acids were slightly enriched in linoleic acid, slightly depleted in arachidonic acid, and markedly depleted in adrenic acid (Table IV).

Prostaglandin content of isolated lipid droplets was determined using a sensitive radio-

TABLE V

Quantitative GLC Analysis of Inner Medullary Lipid Droplet Triglycerides from Hydronephrotic and Indomethacin Treated Rabbits

Fatty acid methyl ester	Composition (%) 48 Hr HN[a] (2)[b]	48 Hr C[a] (2)[b]	72 Hr HN[a] (2)[b]	72 Hr C[a] (2)[b]	Indomethacin 5.0 mg/kg (3)	Controls (3)
14:0	1.2	1.7	0.5	1.9	0.4	1.6
16:0	16.7	20.4	15.7	18.7	18.0	21.7
18:0	8.3	10.4	8.9	9.4	12.7	9.4
18:1n-9	19.7	15.6	15.7	16.5	22.9	20.4
18:2n-6	32.7	27.6	32.3	29.5	24.3	22.0
18:3n-3	3.0	2.2	3.0	2.3	3.6	2.8
20:3n-6	0.4	0.9	1.1	1.0	0.4	0.5
20:4n-6	7.8	5.4	7.8	6.1	8.9	6.8
22:4n-6	2.9	4.8	5.2	6.9	3.0	10.6
22:5n-6	0.3	0.5	0.5	tr	0.3	0.3
22:5n-3	0.7	1.2	2.9	1.3	0.7	1.8
% PUFA[c]	47.8	42.6	52.8	47.1	41.2	44.8
μg/g Inner medulla	458	121	2155	381	660	292

[a]HN = hydronephrotic; C = contralateral.

[b]Number of experiments: 2 rabbits were used for each control experiment. Numbers in columns are averages of experimental results.

[c]PUFA = polyunsaturated fatty acids.

immunoassay for detection of $PFG_{2\alpha}$ (11). From 3 analyses, an average of 11.7 ± 0.04 ng $PFG_{2\alpha}$/g inner medulla was detected. This value is only 8% of the amount of prostaglandin $F_{2\alpha}$ found in inner medullary extracts (11), confirming the observation of Anggard, et al., in which 75-80% of prostaglandins of the inner medulla were found in the cytosol fraction (14).

Protein analysis by the method of Lowry, et al., (18) of lipid free lipid droplet residues revealed an average of 18 μg protein ± 4 μg (n = 7) in lipid droplets isolated from a single inner medulla (average wet wt, 0.35 g per inner medulla).

Next, analyses of lipid droplets from hydronephrotic and indomethacin treated rabbit inner medullas were performed. By TLC and quantitative GLC, it was determined that the amount of triglyceride and the amount of unsaturation were increased during hydronephrosis (Ref. 11 and Table V). In lipid droplet lipids, ca. 90 and 95% were in the form of triglycerides after 48 and 72 hr, respectively, in the hydronephrotic state. Similarly, lipid droplets derived from indomethacin treated rabbits contained 91% of lipids as triglycerides. Contralateral inner medullary lipid droplets, however, contained 66% and 63% (at 48 and 72 hr, respectively) of lipids as triglycerides, slightly less than the control value of 72% (Table IV). Fatty acid analyses of triglycerides (Table V) revealed a significant ($p<0.001$) increase in the percent of linoleic acid for hydronephrotic samples at 48 and 72 hr (32.7 and 32.3%, respectively), as compared to controls (22%). Furthermore, there was a marked but not significant increase in the percent arachidonic acid for hydronephrotic samples at 48 and 72 hr and for indomethacin treated samples (7.8, 7.8, and 8.9%, respectively), as compared to controls (6.8%). Contraleteral triglycerides were significantly increased ($p<0.001$) in their linoleic acid content (27.6% and 29.5% for 48 and 72 hr samples, respectively), as compared to normal controls (22.0%) and slightly decreased in their arachidonic acid content (5.4 and 6.1% for 48 and 72 hr samples), as compared to normal controls (6.8%). The percentage of adrenic acid (22:4n-6) was significantly depressed ($p<.001$) in all these triglycerides. However, total polyunsaturation in both the 48 and 72 hr hydronephrotic and 72 hr contralateral samples had increased, the highest being 52.8% after 72 hr in the hydronephrotic state (Table V).

DISCUSSION

Renal lipid droplets from control rabbit inner medullas were found to consist mainly of triglycerides and free fatty acids in a ratio averaging 2.9:1. In addition, small amounts of cholesteryl esters, cholesterol, phospholipids, and protein were identified. Unlike the communication of Nissen and Bojesen (13) which reported substantial amounts of cholesteryl esters in rat

inner medulla and isolated lipid droplets, no cholesteryl esters were detected in inner medullas of rabbits, and only a small percentage (1.6%) of isolated lipid droplet lipids were cholesteryl esters (Table IV). While this work was in progress, a report by Bojesen (23) corrected the earlier report in which cholesteryl esters were identified as the main lipid component of renal lipid droplets and in which 22:4n-6 was misidentified as 22:5n-6.

The results in this report extend the observations of Anggard, et al., (14) and Bojesen (23) who reported that substantial amounts of arachidonic acid (~5%) could be detected in neutral lipids of lipid droplets and polar lipids of other cell fractions of rabbit kidney. The majority of the arachidonic acid of isolated lipid droplets was found in triglycerides; an average of 6.8% of triglyceride fatty acid was arachidonic acid (Table I), a value in excellent agreement with that reported by Bojesen (6.4%) (23). In addition, triglycerides also were found to contain significant amounts of adrenic acid (10%), a fatty acid 2 carbons longer than arachidonic acid. This percentage was significantly lower than that reported by Bojesen (22.4%) for rabbits, but in good agreement with that found for rat (12.6%) and dog (11.0%). The uniqueness of these triglycerides became more apparent when their total polyunsaturation was considered; 43.7% for normal samples, and as high as 53% during prolonged hydronephrosis (72 hr), a degree of unsaturation not found in the triglycerides of other tissues examined (Tables I and V). Higher degrees of unsaturation were obtained utilizing techniques in which lipid droplet population of renal interstitial cells was increased and prostaglandin levels decreased (11). Fatty acid composition of triglycerides did not reflect dietary lipids or rabbit serum triglycerides. In the former case, no arachidonic acid was detectable. In the latter case, less than 2% of serum triglyceride was arachidonic acid, and levels of adrenic acid were undetectable (K. Comai, unpublished data, 1975).

The possible physiological significance of these unique triglycerides is apparent if several facts are considered. The immediate precursor for prostaglandins E_2 and $F_{2\alpha}$ is free arachidonic acid (24,25). However, as reported here, lipid extracts of rabbit inner medullas contain little free fatty acid. In addition, the renal inner medulla releases large quantities of prostaglandins (ca. 180 ng PGE_2/min, [rabbit, 26], and ca. 300 ng PGE_2/min [dog, 27]) which have been implicated in maintenance of resting blood flow to the kidney and intrarenal distribution of blood flow (26,28). Yet prostaglandins in these quantities are not stored in any subcellular component of the inner medulla (14).

These facts, plus the existence of an inverse relationship of inner medullary prostaglandin content and lipid droplet population of interstitial cells (11), support the likelihood that lipid droplet triglycerides are active storage vesicles for arachidonic acid, the prostaglandin precursor. Furthermore, this suggests the existence of a triglyceride lipase for release of arachidonic acid, and an integrated system for prostaglandin biosynthesis.

Because lipid droplet triglycerides did not reflect dietary lipids or serum triglycerides, the possibility exists that arachidonic and adrenic acids were chain elongated and desaturated from less unsaturated precursors by inner medullary microsomal enzyme systems before incorporation into triglycerides. However, high percentages of stearic acid (18:0) found in all isolated lipid droplet triglycerides, but not found in other rabbit triglycerides, indicate a phospholipid origin for some of the lipid droplet triglyceride. Some enzymes of phospholipid metabolism, such as CDP-choline:diacylglycerol choline phosphotransferase, are reversible (29). Such enzymes, usually of microsomal origin, give rise to diacylglycerols, a branch point in lipid metabolism leading to either phospholipids or triglycerides (30). Montfoort, et al., have shown preferential pairing of saturated and unsaturated fatty acids in phosphatidylcholine of fat and rabbit kidney (16:0 with 18:2n-6, and 18:0 with 20:4n-6) (31). Fatty acid composition and total polyunsaturation of inner medullary phospholipids are similar to those found for isolated lipid droplet triglycerides. Inner medullary phospholipids are much richer in arachidonic acid than lipid droplet triglycerides (21.6% vs 6.8%). However, these phospholipids are devoid of adrenic acid, whereas, lipid droplet triglycerides are relatively rich in this long chain fatty acid (10%) as well as other polyunsaturated fatty acids (Table I). A position analysis of these triglycerides would support the phospholipid origin hypothesis.

The significant percentage of adrenic acid in the triglyceride (10%) may represent a form of storage for arachidonic acid, or may be a substrate for prostaglandin biosynthesis (32,33). There is precedent for the former suggestion of retroconversion of long chain unsaturated fatty acids. Both rat liver and testis were found to convert 22:5n-6 to 20:4n-6 (34,35), and rat liver converted 22:4n-6 to 20:4n-6 (36). In an elegant series of experiments, Stoffel, et al., located enzymic activity for conversion of 22:4

to 20:4n-6 in the inner membrane of rat liver mitochondria, indicating a β-oxidation mechanism (36). If the process of retroconversion occurs in the inner medulla, it would be laborious route involving triglyceride hydrolysis and partial β-oxidation by mitochondrial enzymes prior to prostaglandin synthesis in the microsomes. Furthermore, biological significance of retroconversion of fatty acids has not been determined.

Because Struijk, et al., (32) and Tobias, et al., (33) have demonstrated that adrenic acid can serve directly as a substrate for prostaglandin synthetase of sheep vesicular glands, and that prostaglandin like material was formed, it is likely that the large amounts of this fatty acid found in lipid droplet triglycerides are utilized for prostaglandin production. Unfortunately, few of the biochemical and physiological properties of the 22-carbon prostaglandins have been reported.

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SHORT COMMUNICATIONS

High Levels of Pancreatic Nonspecific Lipase in Rattlesnake and Leopard Shark

ABSTRACT

Hydrolysis of synthetic triglycerides by rattlesnake and leopard shark pancreatic enzymes revealed striking differences in specificity, depending on the presence or absence of sodium taurocholate. Without added sodium taurocholate the classical specificity of pancreatic lipase was expressed. Rattlesnake enzymes, in the presence of sodium taurocholate, attacked the unsaturated oleic acid in the 2-position of racemic glycerol-1-palmitate-2-oleate-3-stearate nearly twice as fast as either outside saturated fatty acid. In this instance, over 90% of the monoglyceride which accumulated were 1-monoglyceride. These results are attributed to very high levels of bile salt activated nonspecific lipase. Eight vertebrate species were compared. With the exception of the rattlesnake and leopard shark, the other species (3 elasmobranchs and 3 mammals) all exhibited low levels of nonspecific lipase, e.g. less than 5% hydrolysis of the 2-position of racemic glycerol-1-palmitate-2-oleate-3-stearate in the presence of sodium taurocholate.

INTRODUCTION

Pancreatic lipase (glycerol ester hydrolase, EC 3.1.1.3) has been studied extensively for more than a century. Its classical specificity for the primary ester linkages of a triglyceride molecule has been the cornerstone of our understanding of the specificity of fat digestion in higher animals (1, 2, 3). This so called positional specificity now appears to be absolute (4, 5, 6), although, for years investigators often found that a small amount (1-5%) of the 2-position fatty acid of triglycerides was released during incubations with crude rat and porcine pancreatic enzymes. By selective inactivation of pancreatic lipase, Mattson and Volpenhein (7) succeeded in detecting a small amount of another lipase in rat pancreatic juice. They named this new enzyme nonspecific lipase (6) and found that it required bile salts for activity and attacked esters of primary as well as secondary alcohols. In addition they calculated that pancreatic lipase in the rat had an activity 10-60 times greater than that of nonspecific lipase (6, 7, 8, 9). This report provides evidence that in at least two vertebrates, the western rattlesnake, *Crotalus viridis*, and the leopard shark, *Triakus semifasciata*, intestinal fat digestion is mediated by comparable levels of both nonspecific lipase and pancreatic lipase.

MATERIALS AND METHODS

The synthetic triglycerides racemic glycerol-1-palmitate-2-oleate-3-stearate (*rac*-POS) and *sn*-glycerol-1,2-dioleate-3-palmitate (*sn*-OOP) were synthesized and donated by J. G. Quinn (10). A-grade sodium taurocholate (NAT) was purchased from CalBiochem (San Diego, Calif.). Acetone-ether powders of fresh pancreas were prepared (11) and their aqueous extracts used without further treatment.

The synthetic triglycerides were subjected to 15 min digestions by the various pancreatic extracts. All assays were run in duplicate at pH 8.0, at room temperature on 1.0 mg substrate in 0.5 ml enzyme and buffer (0.05M Tris-[tris(hydroxymethyl)aminomethane]-HCl, 0.1N NaCl, and 0.02M $CaCl_2$). NaT, when present, was added to give an assay concentration of 10 mM. High speed agitation was provided by an orbital finishing sander as previously described (12). The reactions were stopped with 3N HCl, and the lipid products extracted into ethyl ether and isolated by thin layer chromatography (TLC) (13, 14). Quantification of the fatty acids released and elimination of blanks was accomplished by using an internal standard fatty acid (15). Acyl migration (16) of partial glycerides was not observed under the experimental conditions used. Following isolation by TLC, the fatty acids and monoglycerides were methylated and analyzed by GLC (12).

RESULTS AND DISCUSSION

Hydrolysis of synthetic triglycerides by the rattlesnake and shark pancreatic enzymes revealed striking differences in specificity, depending on the presence or absence of bile salts (Table I). Without added NaT the classical specificity of pancreatic lipase was expressed.

TABLE I

Hydrolysis of Synthetic Triglycerides by Acetone Powders of Rattlesnake, Shark and Rat Pancreas in the Presence and Absence of Sodium Taurocholate (NaT).

Animal	Substrate[a]	Lipolytic product	NaT	Fatty acid composition of lipolytic product (wt percent) 16:0[b]	18:1	18:0	Percent hydrolysis[c] (%)
	Intact *rac*-POC			33.7	30.8	35.5	
	Intact *sn*-OOP			33.8	66.2		
Rattlesnake	*rac*-POC	Fatty Acids	-	53.8	nd	46.2	20%
		Fatty Acids	+	27.5	47.5	24.9	60%
		Monoglycerides	-	nd	100.0	nd	
		Monoglycerides	+	42.6	9.1	48.3	
	sn-OOP	Fatty Acids	-	48.5	51.5		22%
		Fatty Acids	+	19.8	80.2		34%
		Monoglycerides	-	nd	100.0		
		Monoglycerides	+	68.6	31.4		
Shark	*rac*-POS	Fatty Acids	-	50.6	nd	49.4	7%
		Fatty Acids	+	34.9	31.1	34.0	49%
		Monoglycerides	-	nd	100.0	nd	
		Monoglycerides	+	26.3	46.4	27.3	
Rat	*rac*-POS	Fatty Acids		51.3	nd	42.7	22%
		Fatty Acids	+	54.5	4.8	40.6	28%

[a]For systematic names see text.

[b]Carbon chain length:number of double bonds, 16:0 = palmitic acid (P); 18:1 = oleic acid (0) 18:0 = stearic acid (S). nd:none detected.

[c]The percent hydrolysis is based on the micrograms of fatty acid released.

With *rac*-POS addition of bile salt stimulated activity 3 and 7 times in the rattlesnake and shark, respectively, as compared to only 1.3 times in the rat. In the presence of bile salt, the unsaturated oleic acid in the 2-position of *rac*-POS was attacked nearly twice as fast as either outside saturated acid by the rattlesnake enzymes. In this instance over 90% of the monoglycerides which accumulated were 1-monoglyceride. With *sn*-OOP, ca. 70% of the monoglycerides were 1-monoblyceride. The pancreatic enzymes of the leopard shark showed a similar specificity, although not quite as pronounced as the rattlesnake. In the rat, less than 5% of the 2-position fatty acid was released from *rac*-POS in the presence of bile salt (Table I). Identical results were also obtained with acetone-ether powders of dog and fur seal (*Callorhinus ursinus*) pancreas.

Two studies of fat digestion in fish have shown the accumulation of the triglyceride 2-position fatty acid in the free fatty acids produced during lipolysis (12, 17). In both of these studies the 2-position was hydrolyzed only when the fatty acid was unsaturated. When the acid was saturated, it was not attacked to any degree. Because bile salt was present and the preparations were crude, both studies measured the physiological specificity of the two lipases together. It is noteworthy that in the dogfish, *Squalus suckleyi*, the blue shark, *Prionace glauca*, and the sting ray, *Urolophus halleri*, pancreatic nonspecific lipase levels were identical to that in the rat, e.g., less than 5% hydrolysis of the 2-position acid from *rac*-POS. Thus a high level of pancreatic nonspecific lipase is not a general feature of all fish and shark digestive systems.

Because of its quantitatively minor role in mammalian triglyceride digestion, the functional significance of nonspecific lipase has remained somewhat obscure. Some workers have suggested that sterol ester hydrolase and nonspecific lipase are the same enzyme (16, 18); however, Hyun, *et al.* (19) in their purification of sterol ester hydrolase, lost nonspecific lipase activity in the final step. Morgan, *et al.*, (18) isolated two different lipase activities in rat pancreatic juice. Their results indicated that nonspecific lipase attacks micellar lipids, while lipase prefers substrate in the emulsified form. Borgström (1) suggested that nonspecific lipase may be responsible for the activity of pancreatic juice towards the esters of vitamins A, D,

and E. Bradshaw and Rutter (20) showed that nonspecific lipase was the predominant lipolytic enzyme in the embryonic rat pancreas, while lipase dominated in the adult. It is clear, though, that in the rattlesnake and leopard shark, the overall specificity of fat digestion is determined as much, if not more, by nonspecific lipase than by pancreatic lipase. In these cases, it must be said that triglyceride digestion is mediated not primarily by one enzyme, but by two.

Primary esters of pancreatic lipase-resistant fatty acids (21) were hydrolyzed by the leopard shark and rattlesnake enzymes only in the presence of bile salt. Preliminary results indicate that although both lipases find wax ester a poor substrate, compared to triglyceride, nonspecific lipase appears to be better designed for wax ester hydrolysis. Thus, until this poorly studied enzyme can be obtained in pure form, the name nonspecific lipase of Mattson and Volpenhein (6) seems appropriate.

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KCN Inhibition of Lipoxygenase in Purple, Green, and White Eggplants

ABSTRACT

Lipoxygenase activity in three cultivars (purple, green, and white) of eggplant, *Solanum melongena*, were compared. Activity was greatest in the purple and lowest in the white variety. In contrast to reports that NaCN did not inhibit eggplant lipoxygenase, in these studies cyanide completely inhibited the enzyme in all three varieties.

INTRODUCTION

Methods for the preservation of quality of raw vegetables for a rather long storage time have largely centered around control of enzymes that affect flavor and quality. Peroxidation of lipids catalyzed by lipoxygenase (EC 1.13.1.13) is considered a major, if not the major, cause of quality deterioration in unblanched vegetables. Lipoxygenase activity has been reported in eggplants (1), and the enzyme

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INTRODUCTION

Methods for the preservation of quality of raw vegetables for a rather long storage time have largely centered around control of enzymes that affect flavor and quality. Peroxidation of lipids catalyzed by lipoxygenase (EC 1.13.1.13) is considered a major, if not the major, cause of quality deterioration in unblanched vegetables. Lipoxygenase activity has been reported in eggplants (1), and the enzyme

has been isolated and partially characterized (2). The isolated enzyme was reported to be unaffected by cyanide (2). Although not mentioned, these studies were presumably done on the purple variety.

Recently, two different cultivars (a light green, pear shaped fruit and a pure white, rounded fruit) have been appearing in Southern Louisiana. The purpose of this report is to present a comparison of lipoxygenase activities in the three eggplant cultivars, grown and analyzed under identical conditions.

EXPERIMENTAL PROCEDURES

Eggplant Homogenates

Similar size fruit were obtained from plants grown under identical conditions in the same outdoor location in Chalmette, La. They were stored in a refrigerator at 4 C for 1-2 days before peeling and homogenizing in a food blender. Peeled fruit were cut into 1 cm^3 pieces and immediately homogenized (50 g/250 ml cold deionized water) for 1 minute in the blender. The homogenates were centrifuged at 15,000 g at 9 C for 15 min. The supernatants then were carefully decanted into clean test tubes, and the tubes placed in an ice bath until assayed. Nitrogen contents of the supernatants were determined by the Kjeldahl method.

Lipoxygenase Assays

The procedure was essentially that employed for assay of lipoxygenase in peanuts (3), using 1 ml (0.83×10^{-4} M) Tween-solubilized linoleic acid as substrate, and 0.1 ml eggplant supernatant as enzyme source in 3 ml total volume. Activity at pH 6.5 was measured as change in optical density at 234 nm in a Beckman DU recording spectrophotometer for the first 5 min of the reaction. KCN was added at a final concentration of 10^{-3}M for the inhibitor tests as reported by Grossman, et al., (2). Tubes containing enzyme plus KCN were placed in an ice bath for 30 min before testing to allow for any possible effect of (CN) to take place.

RESULTS AND DISCUSSION

As seen in Fig. 1, both the amount of activity and the reaction rate for the purple eggplant were substantially greater than for the green and white varieties. The initial reaction rate for lipoxygenase in green eggplant, while slower than that for the purple variety, was faster than the rate for the white eggplant. The reason for these differences is unknown. Whether it was due to different or less active isoenzymes, or to the presence of lipoxygenase inhibitors in the newer cultivars, can be determined only by purification of the enzyme from all three eggplants. Grossman, et al., (2) obtained two peaks of activity after chromatography of eggplant extract over ecteola cellulose. Most activity was concentrated in the first peak, which had a specific activity of 230 units compared to 72 units of activity for peak 2. Purification of lipoxygenase from all three varieties, as done by Grossman, et al., (2) is necessary to determine if the lipoxygenase activities for the green and white eggplants have greater concentrations of the less active, second peak reported by Grossman, et al., (2).

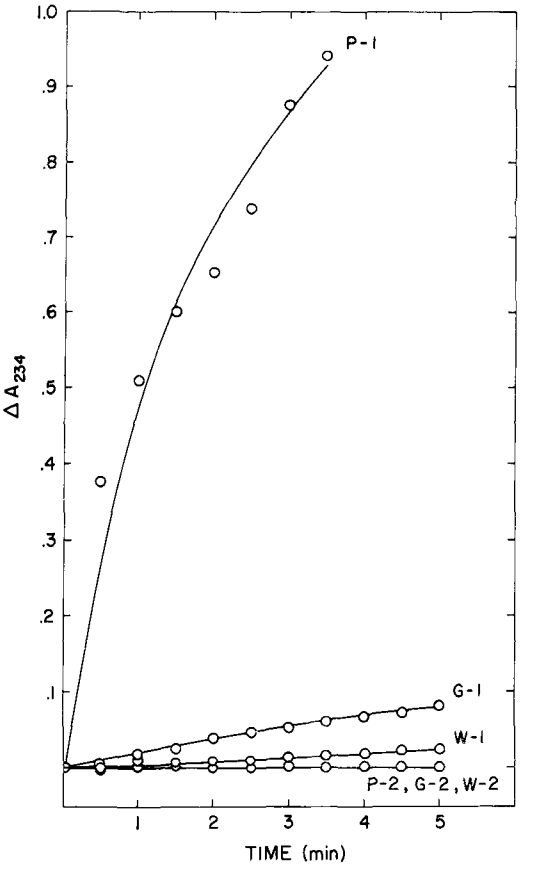

FIG. 1. Lipoxygenase activity in purple, white, and green eggplants with and without KCN. Enzyme concentration was 0.1 ml/cuvette (equivalent to 20 mg fresh tissue extract) plus 1 ml (0.32×10^{-4}M) linoleic acid substrate in a total of 3 ml. P-1, W-1, and G-1 are activity curves for purple, white, and green eggplants without added KCN. P-2, W-2, and G-2 are the curves for extracts with added KCN.

Hayano (4), in describing oxygenases in lipid metabolism, reported that fatty acid hydrolases could be stimulated by cyanide; and that no prosthetic groups or cofactors appeared to be involved in lipoxygenase action. A recent report

by Chan (5), that soybean lipoxygenase contained iron, seemed to conflict with findings of Grossman, et al., (2), who reported no significant inhibition of eggplant lipoxygenase by NaCN. Therefore, the effect of 10^{-3}M cyanide on lipoxygenase in extracts of all three eggplant cultivars was tested and is shown in Fig. 1 (curves P-2, W-2, and G-2). KCN completely inhibited the activity in all three extracts. Grossman, et al., (2) did not mention a 30 min incubation of enzyme and cyanide before testing, as was done in this work. Therefore, it is possible that some natural inhibitor(s) of the cyanide effect might have been removed during the isolation procedure by them. Further purification of the enzyme from the three eggplant varities will help clarify these issues.

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Identification of β-Sitosterol, Campesterol, and Stigmasterol in Human Serum

ABSTRACT

The presence of 3 plant sterols, β-sitosterol, campesterol, and stigmasterol, has been demonstrated in the serum from 2 patients with β-sitosterolemia and xanthomatosis.

INTRODUCTION

In a previous communication (1), the presence of high concentrations of β-sitosterol, as well as 2 other plant sterols, campesterol and stigmasterol, in the blood and tissues of 2 sisters who have developed extensive xanthomatosis since childhood, has been reported. The observed, greater than normal intestinal absorption of β-sitosterol in both of these patients suggests the cause of this disease, presently termed β-sitosterolemia and xanthomatosis. Furthermore, it has been postulated that in some, as yet, undetermined manner, plant sterols originating from the diet initiated development of xanthomatosis, and perhaps atheroma.

It is the purpose of this communication to report and confirm the presence of 3 plant sterols, β-sitosterol, campesterol, and stigmasterol, via combined gas chromatography-mass spectrometry (GC-MS), in the blood of these 2 patients.

MATERIALS AND METHODS

About 1 ml blood plasma or serum from the 2 patients was saponified following the procedure of Abell, et al., (2). The unsaponifiable portion was subjected to digitonin precipitation. Precipitated sterol concentrate was extracted with hexane, washed with water, dried over sodium sulfate, and the solvent evaporated to dryness. Purified sterol concentrate was dissolved in ca. 500 μl pyridine, and shaken with 100 μl Bis Trimethylsilyl Acetamide (BSA) (Pierce Chemical Co., Rockford, IL), and heated in an oven at 70 C for 30 min. Trimethylsilyl ether derivative of the sterol concentrate was

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subjected to GC separation. A Varian Aerograph model 500B equipped with hydrogen flame detector was used in this experiment. A stainless steel column packed with SP 2250 on 80/100 Supelcon AW-DMCS [Supelco, Inc., Bellefonte, PA) was connected to the detector through a splitter with a split ratio of 1:8. The flow of nitrogen used as carrier gas was maintained at 60 ml/min, column temperature was programmed from 150-280 C at 6°/min; the inlet and detector temperatures were maintained at 250 C and 300 C, respectively. The majority of GC effluent from one end of the splitter was allowed to pass through a fritted glass helium separator and into a RMU-6 Hitachi-Perkin-Elmer single focusing mass spectrometer, which, in turn, was interfaced with a Varian Spectro System 100 data system, and also to a high speed plotter and printer. Mass spectra were determined each 10 sec at an ionizing voltage of 22 and 70 ev, and stored on magnetic tape. Selected spectra were processed to obtain printed copies of the mass spectra of interest.

RESULTS AND DISCUSSION

The sterol concentrates isolated from human plasma were subjected to combined gas chromatography and mass spectrometry after conversion to their respective trimethylsilyl derivatives. A gas chromatogram (Fig. 1) was obtained under conditions mentioned previously using 5α-cholestane as the internal standard. In addition to cholestane, 4 more peaks were obtained. These peaks were identified as cholesterol, campesterol, stigmasterol, and β-sitosterol. The peaks had the same retention time under identical conditions as those of authentic standards. Effluent corresponding to these peaks was allowed to pass through the ion source of the mass spectrometer, and each of these peaks was separately scanned, and mass

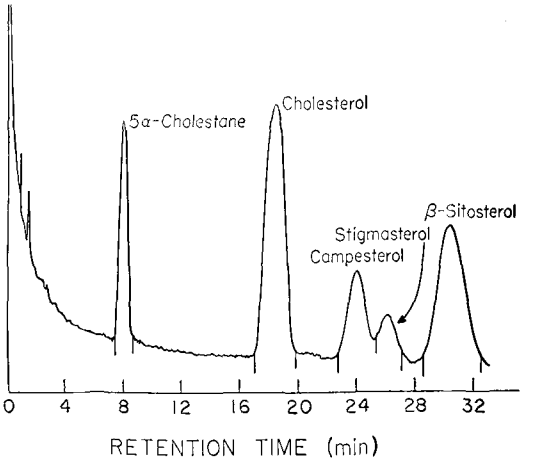


FIG. 1. Gas chromatogram of trimethylsilyloxy ether of steroids from human plasma.

spectra were recorded.

Mass Spectral Data

Mass spectra of trimethylsilyl (TMS) derivatives of cholesterol and 3 other compounds previously identified tentatively as plant sterols, campesterol, β-sitosterol, and stigmasterol, were superimposable on the authentic standards run under the same conditions. The spectrum of stigmasterol trimethylsilyl ether derivative was in good agreement with that reported by Brooks, et al., (3) and Eneroth, et al., (4). Six of the most important fragmentations in the mass spectra of the 3 plant sterols are summarized in Table I.

The TMS ethers of β-sitosterol, stigmasterol, and campesterol gave molecular ions at m/e 486 (10%), 484 (7.5%), and 472 (10%), respectively. The characteristic peak at m/e 129 of Δ^5-3β-trimethylsilyloxy steroid for all 3 sterols, which fall under the same classification, was in

TABLE I

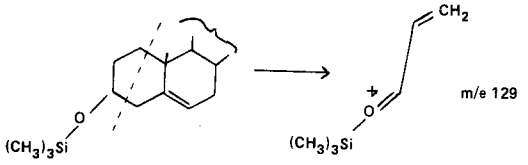
Characteristic Fragment Ions in the Mass Spectra of β-Sitosterol, Stigmasterol, and Campesterol

Compound	Mol. Ion m/e	M-TMS m/e	m/e = 129	M-129	M-(TMS[a] + Side Chain) - H -	Side Chain
β-Sitosterol	486 (10%)[b]	397 (29%)	129 (100%)	357 (43%)	275 (8.5%)	143 with rearrange H^{++}
Stigmasterol	484 (7.5%)	395 (12%)	129 (42%)	355 (40.5%)	255 (20.2%)	139 (17.6%)
Campesterol	472 (10%)	383 (29%)	129 (18.5%)	343 (45%)	255 (14.2%)	128 with rearrange H^{+} (100%)

[a]TMS = Trimethylsilyl.

[b]Relative Intensity.

agreement with the finding of Brooks, et al., (3). The peak at m/e 129 has been identified as the fragment originating from the breakdown of ring A along with the TMS moiety.



Similarly, the other characteristic fragmentation ion from Δ^5-3β-trimethylsilyloxy steroids as reported by Brooks, et al., (3) was a series of ions from M-129. These ions were also prominant at m/e 357 (43%), 355 (40.5%), and 343 (42%) in the mass spectra of the β-sitosterol, stigmasterol, and campesterol trimethylsiloxy derivatives, respectively.

Moderately intense ions observed at m/e 397 (29%), 395 (12%), and 383 (29%) correspond to the loss of a TMS moiety from β-sitosterol, stigmasterol, and campesterol, respectively.

The structural feature which distinguishes each of these 3 plant sterols is the side chain. The side chain of β-sitosterol contains a $C_{10}H_{20}$ chain, while stigmasterol has a $C_{10}H_{18}$ chain, due to the presence of a double bond at carbon 22, and campesterol has a C_9H_{19} chain. This difference is exhibited in the fragmentation pattern, and can be used to distinguish these sterols from each other. The peak at m/e 143 (14.7%) was due to fragmentation of β-sitosterol side chain with rearrangement and addition of 2 protons; m/e 128 (100%) may be attributed to fragmentation of campesterol side chain followed by rearrangement with addition of 1 proton. The ion at m/e 139 (17.5%) was attributed to a similar fragmentation of stigmasterol, with no corresponding rearrangement. Similarly, peaks at m/e 275, 255, and 255 were due to the loss of TMS and the side chain moiety from parent compounds of sitosterol, stigmasterol, and campesterol, respectively, all with a loss of 1 proton. The base peak at m/e 83 for stigmasterol is characteristic of Δ^{22} and C_{29} sterol as reported by Eneroth, et al., (4). These patterns superimpose exactly on authentic spectra of 3 sterols mentioned above. Therefore, the presence of β-sitosterol, stigmasterol, and campesterol are confirmed in human plasma obtained from these 2 patients with this newly described lipid storage disease (1).

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Aerobic Pentane Production by Soybean Lipoxygenase Isozymes[1]

ABSTRACT

The effects of oxygen on production of pentane and compounds absorbing at 234 nm and 285 nm by soybean lipoxygenase isozymes I and II were examined in a model system. Aerobic conditions increased pentane production. Differences in dienone formation (A_{285}) and diene conjugation (A_{234}) indicate the reaction sequences of the 2 isozymes are not the same.

[1]Paper No. 4551, Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh North Carolina.

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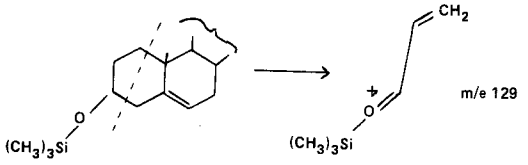

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INTRODUCTION

The role of oxygen in lipoxygenase reaction has received recent attention (1,2). Garssen, *et al.*, (2) reported that pentane and 13-oxotridecadienoic acid were formed under anaerobic conditions in a model system containing soybean lipoxygenase, linoleic acid, and 13-hydroperoxy linoleic acid. Absorption at 285 nm, indicative of a conjugated dienone chromophore, increased about the time the system became anaerobic.

Pentane and hexanal are major volatile reaction products of the peanut lipoxygenase-linoleic acid system (3,4). Pattee, *et al.*, (4) found that oxygenation of linoleic acid substrate before enzyme addition and during the reaction period increased levels of both pentane and hexanal. Singleton, *et al.*, (5) confirmed these results using raw peanut homogenates. The present investigation was undertaken to determine if soybean lipoxygenase isozymes produced pentane in an aerobic model system, and to determine the relationship between production of pentane and conjugated dienone chromophore compounds absorbing at 285 nm as reported by Garssen, *et al.*, (2).

MATERIALS AND METHODS

Materials and purification as described by Johns, *et al.*, (6) were used, except that the NaCl gradient on DEAE-Sephadex was 0.02-0.3 M. Assays were conducted in either a cuvette or an apparatus fitted with a Clark oxygen electrode and a serum stopper as described by Johns, *et al.*, (7). The reaction vessel contained 6.6 μmoles of linoleic acid and 0.08% Tween 20 in 0.1 M borate buffer, pH 7 or pH 9, and enzyme (0.003-0.1 ml in 0.1 M phosphate buffer, pH 8.5) in a total volume of 3 ml. Pentane was measured after 15 min by the method of St. Angelo, *et al.*, (3) with a Varian Aerograph model 1840 gas chromatograph equipped with a Porapak Q stainless steel column (1/4 in. x 1 ft) programmed from 60-200 C at 5 C/min. Peak areas were integrated with an Infotronics CRS-100 digital readout system, and pentane data are presented as integrator area units. Spectrophotometric measurements were made with a Coleman model 124 double beam spectrophotometer by using appropriate blanks. Measurements were made alternately at 234 nm and 285 nm throughout the course of the 15 min reaction period. Oxygen was introduced into some samples by vigorously bubbling oxygen into vessels for the times indicated. Both oxygenated and nonoxygenated assays were conducted in reaction vessels open to the atmosphere.

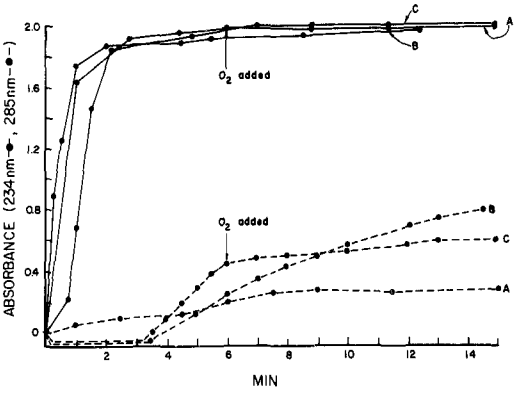

FIG. 1. Absorbance at 234 nm (●—●) and 285 nm (●--●) in (A) oxygenated, (B) nonoxygenated, and (C) delayed oxygenated (oxygen added after 6 min) linoleic acid-soybean lipoxygenase isozyme I model systems. The reaction vessel contained 6.6 μmoles linoleic acid and 0.8% Tween 20 in 0.01 M borate buffer, pH 9, and 2.4 μg protein in a total volume of 3 ml.

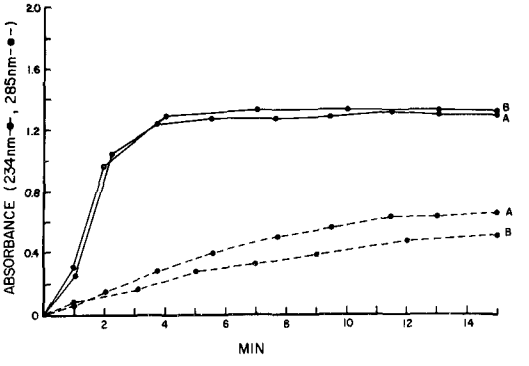

FIG. 2. Absorbance at 234 nm (●—●) and 285 nm (●--●) in (A) oxygenated and (B) nonoxygenated linoleic acid-soybean lipoxygenase isozyme II model systems. The reaction vessel contained 6.6 μmoles linoleic acid and 0.08% Tween 20 in 0.01 M borate buffer, pH 7, and 37 μg protein in a total volume of 3 ml.

RESULTS AND DISCUSSION

Pattee, *et al.*, (4) demonstrated that peanut lipoxygenase produces more pentane from linoleic acid under oxygenated than nonoxygenated conditions. In our study, soybean lipoxygenase I (.004 mg per assay, pH 9) produced 92 x 10^3 and 288 x 10^3 pentane area units, and isozyme II (.053 mg per assay, pH 7) produced 31 x 10^3 and 74 x 10^3 pentane area units under nonoxygenated and oxygenated conditions, respectively. These results extend the results of Garssen, *et al.*, (2), and indicate that soybean lipoxygenase can produce pentane under aerobic conditions. Garssen, *et al.*, (2)

indicated that under anaerobic conditions, formation of carbonyl compounds and pentane from linoleic acid hydroperoxides is dependent on the presence of both native lipoxygenase and fatty acids that are normal substrates of the enzyme. They assumed that initiation of the reaction was due to formation of radicals from the fatty acids, which also occur as intermediates in the aerobic reaction. They further indicated that in the absence of oxygen, fatty acid radicals abstract a hydrogen radical from the hydroperoxide, yielding a peroxy radical, which is then subject to rearrangements. This abstraction may also occur in the presence of oxygen. However, Huyser (8) indicated that oxygen can react with fatty acid radicals to yield a peroxy radical which is subject to rearrangements. This, of course, is one step in autoxidation, but, in the presence of lipoxygenase, it should proceed rapidly because fatty acid radical concentration should not be limiting. Pentane production may increase in the aerobic system as a result of increased peroxy radical concentration. Oxygen does, in fact, shift the reaction mechanism toward increased pentane production, and causes a change in formation, structure, or reaction of carbonyl compounds in the reaction.

Added oxygen affected dienone formation (A_{285}) and not diene conjugation (A_{234}) in the linoleic acid-soybean lipoxygenase isozyme I system (Fig. 1). Diene conjugation was rapid in both oxygenated and nonoxygenated systems, whereas, dienone formation was less in the oxygenated system than the nonoxygenated. Continuous oxygen addition 6 min after initiation of the reaction stopped the rapid increase in A_{285} (Fig. 1). Addition of oxygen not only decreased carbonyl production, but also increased pentane production. This situation poses a paradox because the 5-carbon pentane leaves a 13-C carbonyl molecule which should absorb at 285 nm. The 13-C molecule may be removed by a secondary reaction, thus, the end of the rapid increase in A_{285}.

In contrast to the isozyme I system, oxygen addition did not inhibit A_{285} in the isozyme II system (Fig. 2). The fact that oxygen did not affect A_{285} may be related to the lag period described by several workers (2,9,10). For equal activity, i.e., the amount of isozyme I and isozyme II giving equal oxygen uptake, isozyme I had a longer lag period than II. Isozymes I and II appeared to have different reaction mechanisms, or at least to produce slightly different compounds in the presence of oxygen. This is shown in the initial 3-4 min of the reaction by comparing A_{285} of nonoxygenated isozyme I and II systems. With isozyme I, A_{285} decreased below the null point for 3-4 min, while with isozyme II, it did not. This indicates that intermediates between linoleic acid and linoleic acid hydroperoxide are different, and may imply that compounds absorbing at 285 nm react differently with the isozymes.

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Bile Acid Metabolism in Mammals: IX. Conversion of Chenodeoxycholic Acid to Cholic Acid by Isolated Perfused Rat Liver

ABSTRACT

Current dogma of bile acid synthesis in mammals insists that hydroxylation of the ring structure at C-12 precedes side chain oxidation, and that chenodeoxycholic acid is not converted to cholic acid under normal conditions. This report concerns the conversion of chenodeoxycholic acid to cholic acid by isolated, perfused rat liver. Results indicate that isolated perfused rat liver has a definite, but limited, capacity for synthesis of cholic acid from chenodeoxycholic acid.

INTRODUCTION

Current concepts of bile acid formation in mammals suggest that hydroxylation at C-12 precedes side-chain oxidation (1) and that chenodeoxycholic acid (CDCA) is not hydroxylated to cholic acid (CA) in vivo (2). However, this is not the case in nonmammalian species, and the python (3), eel (4), chicken (5) and trout (6) have been found to form CA from CDCA. Recently, Samuels and Palmer reported the conversion of CDCA to CA in humans with obstructive jaundice; they suggested that 12α-hydroxylation of CDCA can be induced in man during biliary stasis (7). In studies on the metabolism of CDCA by the isolated perfused rat liver, we detected appreciable amounts of β-muricholic acid in the bile produced after the addition of CDCA to the perfusion medium (8). Although we did not detect α-muricholic acid in this bile, we did detect a small amount of another trihydroxy bile acid which had the same specific activity as added CDCA, and which appeared to be CA on the basis of thin layer chromatography (TLC) and gas liquid chromatography (GLC). We have now confirmed by mass spectroscopy (MS) and radio gas chromatography that this latter trihydroxy bile acid synthesized from CDCA by the isolated perfused rat liver is CA.

MATERIALS AND METHODS

Eighteen experiments involving perfusion of isolated rat liver were performed by methods reported previously (9-11). Wistar strain rats of both sexes, weighing 250-300 g, were maintained on Purina Rat Chow diet. After 2 hr of base line perfusion during which the bile acid pool of the system apparently was depleted (8), exogenous CDCA (Ikapharm, Ramat-Gan, Israel) was added to 100 ml perfusion medium. No contaminants were seen by GLC when large amounts of CDCA were analyzed. CDCA labeled with C^{14} in the C-24 position (Tracer Labs, Waltham, Massachusetts; specific activity, 35.8 mCi/mmole) contained a maximum impurity of 3%, which was a monohydroxy bile acid. In 12 experiments on 6 animals of each sex, a mixture of 1 μmole CDCA and 5 μCi radiolabeled CDCA was purified by TLC before addition to the perfusion medium. The other 6 experiments involved male rats and the addition of 10, 20, or 30 μmoles of CDCA to the perfusion medium. Each perfusion was continued for 3 hr following the addition of CDCA to the perfusion medium. Bile was collected in hourly aliquots and its bile acid composition analyzed

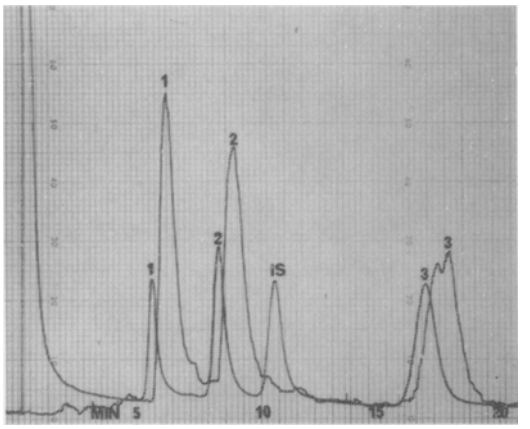

FIG. 1. Radio gas chromatography of bile acids secreted in 1 μmole chenodeoxycholic acid -C-24-14C perfusion. Upper tracing, radioactivity; lower tracing, mass. Peaks 1, 2, and 3 represent β-muricholic, cholic, and chenodeoxycholic acids, respectively. IS =internal standard, 5-β-cholanic acid. Instrument: Packard Model 1894 proportional radioactivity monitor and a copper oxide furnace. Column 120 cm x 2 mm inside diameter glass tube packed with 19:1 (w/w) mixture of 3% HI-EFF-8BP and 3% OV 210 on 100-120 mesh Gas Chrom Q, respectively. Carrier gas, helium 55 ml/min. Temperatures: Column, 225 C; injector, 255 C; detector, 240 C. Proportional Counter Conditions: Range, 3000 CPM; time constant, 10 sec; high voltage, 1650 v; quench gas, propane 5 ml/min. Combustion furnace, 750 C. Flame, ionization mass detector with 10:1 stream splitter. Sample 2 μl of 1% solution of bile acid methyl ester trimethylsilylether in silylation mixture. Total radioactivity: 12,000 dpm.

TABLE I

Biliary Bile Acid Secretion After Addition of Chenodeoxycholic Acid to Perfusion Medium

Rat liver:	Female[a]	Male[a]	Male[b]		
Chenodeoxycholic acid added (μmole)	1	1	10	20	30
Liver wt (g)	7.5 ± 0.9	8.3 ± 1.3	9.9 8.4	10.2 10.8	9.8 9.2
Added bile acid secreted into bile (%)	89 ± 1.1	91 ± 2.3	90 91	89 91	90 89
Bile acid secretion (nmole/g liver)					
Total	119 ± 17	109 ± 8	960 1080	1769 1679	2750 2880
Chenodeoxycholic acid	71 ± 12[c]	34 ± 6[c]	700 750	1222 1152	2090 2202
β-Muricholic acid	12 ± 5[d]	41 ± 7[d]	220 284	480 469	605 619
Cholic acid	35 ± 9	34 ± 4	40 46	47 58	55 59

[a]Average of 6 perfusions (mean ± standard error).

[b]Two perfusions.

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

[d]Significantly ($P < 0.001$) different.

as described previously (12). Appropriate aliquots of the bile acid methyl esters of the various perfusions were pooled, converted to trimethylsilyl ethers (13) and analyzed by a combined GLC-MS system and by radiogas chromatography (6).

RESULTS AND DISCUSSION

In control experiments in which no exogenous bile acid was added to the perfusion medium, biliary secretion of bile acids in 3 hr. was negligible (8). In those experiments in which 1 μmole CDCA was added to the perfusion medium, 91% of the bile acid was secreted in the bile produced by the livers of male rats and 89% in those of female rats. β-Muricholic acid accounted for 10% of the biliary bile acids in females, 38% in males ($P < 0.001$) (Table I). CDCA accounted for 60% of biliary bile acids in females, and 31% in males ($P < 0.001$) (Table I). In both male and female rats, trihydroxy bile acid previously identified as CA on the basis of TLC and GLC accounted for ca. 30% of the biliary bile acids secreted. There was no significant difference in specific activity of these 3 bile acids as measured by direct count, GLC quantitation of various TLC bands of methyl esters obtained by the system of Subbiah, et al., (14), or by radio gas chromatography (6) (Fig. 1).

When standards of α-muricholic acid, β-muricholic acid, and CA were subjected to GLC-MS as the trimethylsilyl ether of their methyl ester, α-muricholic acid gave a base peak at m/e 458, β-muricholic acid at m/e 285, and CA at m/e 253. Trihydroxy bile acid in the above experiments, identified tentatively as CA, gave a base peak at m/e 253, and its mass spectrum was identical to that of standard CA obtained in our laboratory and by other investigators (15).

CDCA in greater concentrations is toxic to isolated female rat liver, but not to isolated male rat liver (8). In studies using the livers of male rats and 10-30 μmoles CDCA, it was found that the amount of β-muricholic acid secreted in the bile increased progressively as the amount of CDCA added to the perfusion medium was increased (Table I). This was not the case for CA; biliary secretion of CA increased very little with the addition of larger amounts of CDCA to the perfusion medium (Table I).

These experiments have demonstrated that mammalian liver can 12α-hydroxylate the ring structure of bile acids after oxidation of their side chain. These experiments also have demonstrated that, at low perfusion medium concentrations (0.01 mmolar) CDCA, ca. 30% CDCA can be converted to CA. It appears that the capacity of isolated perfused liver of the male rat to convert CDCA to CA is limited.

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Specificity of Digestive Lipases in Hydrolysis of Wax Esters and Triglycerides Studied in Anchovy and Other Selected Fish

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ABSTRACT

The physiological specificity of fat digestion in several species of marine fish was studied by incubating a variety of synthetic and natural lipid substrates in fish intestinal fluid. Wax ester and triglyceride hydrolyses were studied in vivo and in vitro. In vivo feeding studies showed triglyceride hydrolysis and reesterification in the gut occurred 4 times faster than wax ester metabolism. In vitro comparisons of wax and triglyceride lipolysis always showed triglycerides to be hydrolyzed faster than wax esters; however, wide variation in the ratio occurred among different batches of intestinal juice. Ca. 50% of the 2-monoglycerides formed in the lipolytic sequence were hydrolyzed. Esters of lipase resistant fatty acids (20:4 and 20:5) were cleaved faster than normal fatty acid esters (18:2 and 18:3). Two of the species studied, the northern anchovy, *Engraulis mordax* and the jack mackerel, *Trachurus symmetricus*, empty lipase(s) into their gall bladders and produce phospholipid-free bile.

INTRODUCTION

Energy metabolism in marine organisms generally is focused on production and utilization of lipids. Work in the past decade has shown that wax esters are quantitatively one of the most abundant lipid classes in the marine environment (1-3). Many of the oceans most successful life forms, e.g. reef corals, copepods, and midwater fishes, have wax esters as their major reserve fuel. However, our knowledge of the biochemical machinery involved in wax metabolism in the food chain is scant. Among fishes, hydrolysis of wax occurs in the intestinal tract, and the released alcohol is oxidized to fatty acid (4,5). The enzyme responsible for the hydrolysis has not been characterized, and it is to this end that the present work was instigated.

The northern anchovy, *Engraulis mordax*, was chosen because 30-50% of its diet is calanoid copepods, which can be 70% wax ester on a dry wt basis (6,1). Morphologically unique with its many pyloric caeca and diffuse pancreas, the teleost digestive system has not lent itself easily to studies of fat digestion (7-9). Leger (10,11) partially purified a lipase from the trout, *Salmo gairdneri*, and concluded that the enzyme was specific for primary esters, but also appeared to show a preference for oleic acid esters regardless of position. Brockerhoff (7) analyzed the lipids of the intestinal contents of cod and concluded that lipolysis in fish corresponded in its action to the pancreatic lipase of mammals. Certain common marine lipids, however, such as the wax esters and esters of polyunsaturated fatty acids were very poor substrates for pancreatic lipase (12,13). Thus the purpose of this study was to compare the specificity of mammalian pancreatic lipase with the physiological specificity of lipolysis in some species of marine fish.

MATERIALS AND METHODS

Source of Materials

All chemicals used in this study were ACS Reagent Grade. The polyunsaturated methyl esters and oleyl alcohol (99% pure) were purchased from Nu Chek Prep (Elysian, MN). Crude hog pancreatic lipase was obtained from Pierce Chemical Company (Rockford, IL). Triolein(1-^{14}C), 173 mc/mM, methyl(1-^{14}C)oleate, 59 mc/mM, and (9,10-^{3}H)oleic acid, 93 mc/mM were obtained from Dhom Products, Ltd. (North Hollywood, CA). Olive oil (Pompeii) was purchased locally. Anchovy triglyceride and copepod wax ester were extracted from their natural sources and purified according to Nevenzel, et al., (14). Thin layer chromatography (TLC) of the above lipids showed them to be at least 99% pure in terms of lipid class composition and radiopurity, and in the case of the labeled lipids, gas liquid chromatography (GLC) indicated > 99% purity for fatty acid composition.

Preparation of Substrates

The synthetic triglycerides were prepared by the method of Quinn, et al., (15) and checked by TLC and GLC for purity and composition.

Oleyl(9,10-^{3}H)oleate was prepared according to the following scheme. In a 10-ml screw cap test tube with a teflon liner were placed 0.18 mMoles oleic acid (purified from a commercial product by fractional distillation of the

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methyl esters; 95% pure by GLC) containing ca. 50 μCi (9,10-^{3}H)oleic acid (Dhom Products, Ltd., North Hollywood, CA).; 93 mc/mM), 0.349 mMoles of oleyl oleate, 0.029 mMoles p-toluene sulfonic acid monohydrate, and 1.0 ml benzene. The tube was flushed with nitrogen, sealed, and heated in an oil bath at 85 C for 2 hr. After cooling, 9.0 ml petroleum ether (bp 60-70 C, redistilled) was added, and the desired wax ester was isolated by chromatography on a 1.5 cm x 20.0 cm column of Florisil, eluting oleyl(9,10-^{3}H)oleate with 2 column volumes (53 ml) of 3% diethyl ether in petroleum ether (v/v). The chemical yield was 89%.

Fish

The following fish species were used: northern anchovy, *Engraulis mordax* Girard; pink salmon, *Oncorhynchus gorbuscha* Walbaum; striped bass, *Morone saxatilis* Walbaum; jack mackerel, *Trachurus symmetricus* Ayres; and pacific mackerel, *Scomber japonicus* Houttuyn. The anchovies, bass, and mackerel were maintained in filtered seawater (14-15 C) in 20 ft circular tanks at the NMFS Southwest Fisheries Center in La Jolla, CA. Pink salmon were obtained live from commercial fisherman and maintained aboard ship in 8 ft circular tanks of fresh circulating seawater (Alert Bay, British Columbia, Canada, *Alpha Helix* British Columbia Expedition, 1974). Anchovies, the primary species studied, were obtained from the Mission Bay Bait Barge (San Diego, CA) in 500-1000 fish lots and trucked to the Southwest Fisheries Center. These fish were 1-2 days removed from the wild state. In captivity they were fed a commercially prepared fish chow (Oregon moist-formula 2, Bioproducts, Inc., Warrenton, OR) which contained 14% lipid on a wet wt basis and no wax ester.

Collection of Intestinal Fluid

The intestinal contents, including the contents of the pyloric caeca, were extruded with forceps into a beaker over ice. Recently fed fish were avoided because of their high concentration of intestinal solids and lipids. After a fish's stomach had emptied, the intestine and pyloric caeca became swollen with a clear green fluid possessing strong lipolytic activity. A post absorptive anchovy, wt, 15 gms, provided from 0.25-0.50 ml of this fluid. For a typical experiment, the intestinal juice from 20-30 individual fish was collected and pooled. The pooled contents were centrifuged at 4 C for 20 min at 27,000 X g. The supernatant solution was withdrawn with care to avoid the surface lipid and used unaltered as the digestion medium. Among the fish used, the pH of the intestinal juice ranged from 7.6-9.2, with a mean pH value of ca. 7.8. The bile salt concentration was not determined.

In Vivo Feeding Experiments

Only anchovies were used for in vivo studies. Two feeding experiments were conducted a week apart on randomly selected animals from the same batch of tank held fish. The fish were starved for 48 hr prior to each feeding study. In preliminary experiments, halves of gelatin capsules were used to administer the tracer, but regurgitation often occurred. For the work described here, the lipid was injected directly into the stomach using a 25 μl Hamilton syringe fitted with a 5.25 cm piece of tygon tubing. Fish fed tracer were removed from the main holding tank to a smaller 10 ft fiberglass tank, where they were scooped out at the appropriate time intervals.

The wax ester feeding experiment consisted fo feeding 14 anchovies of ca. the same size, 15 μl each of polyunsaturated *Calanus plumchrus* wax ester containing ca. 300,000 c.p.m. of oleyl(9,10-^{3}H)oleate. The fish were sacrificed in pairs at 0.5, 2.4, 8.5, 15.5, 25, and 32.5 hr. The entire alimentary tract from the esophagus to anus, including the contents was removed and extracted by the method of Folch, et al., (16). The remainder of the fish was extracted according to the method of Bligh and Dyer (17). Both lipid extracts were taken to dryness under vacuum and redissolved in chloroform. Duplicate aliquots of the chloroform phases were assayed for total activity. Solvent was removed by evacuation over a heated water bath, and the resulting lipid was dissolved in 10 ml scintillation fluid and counted. To determine distribution of label among specific lipid classes, a single TLC system was employed (petroleum ether:ethyl ether:acetic acid, 70:30:1). This system resolved wax esters with methyl esters, triglycerides, free fatty acids, and phospholipids. Spots were visualized by brief exposure to iodine vapor and, following sublimation of the iodine, were scraped directly into scintillation vials and counted.

The triglyceride feeding experiment was conducted as above with force feeding of 15 μl of purified anchovy triglyceride labled with 240,000 cpm methyl(1-^{14}C)oleate, 59 mc/mM. Eighteen fish were fed and sacrificed in pairs at 0.5, 1, 2, 4, 6, 8, 14.5, 25, and 31 hr.

In Vitro Assays

Small aliquots (2-3 ml) of olive oil or oleyl oleate stock solutions were labeled with either triolein(1-^{14}C) or oleyl(9,10-^{3}H)oleate (40,000 cpm/0.1 ml stock solution) prior to use. Stock solutions of substrate were made to a concentration of 12.5 mg/ml in acetone. Substrate (0.1 ml) was added to 2.5 ml vials, and the

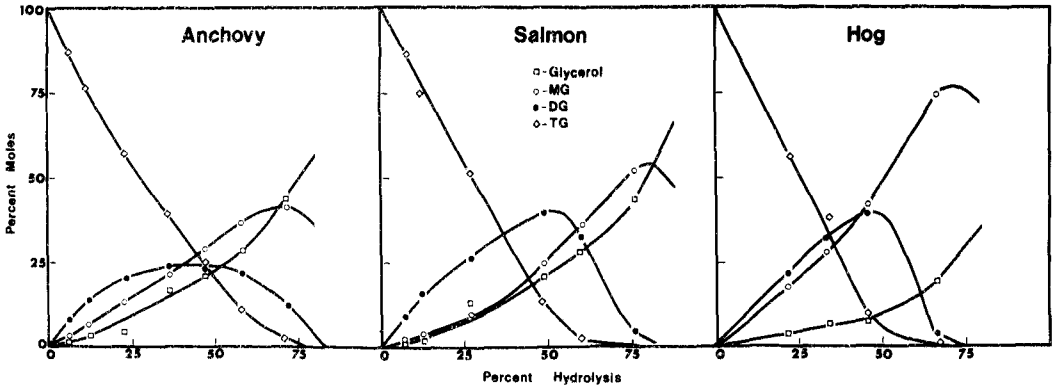

FIG. 1. Three successive steps of triglyceride lipolysis in vitro by anchovy and pink almona intestinal fluid and hog pancreatic lipase. MG = monoglyceride; DG = diglycerides; TG = triglycerides. The ordinates indicate the number of moles of each compound for 100 moles of incubated triglycerides.

solvent was removed by a stream of nitrogen. Unless indicated, all assays were conducted at room temperature on 1.25 mg substrate. Following addition of substrate and removal of solvent, 0.5 ml of intestinal juice was added, and the vial was capped with a foil lined cap. Agitation was provided by a Rockwell Orbital sander model 505, 30 cycles/sec. The reaction was stopped by adding 3 drops of 3N HCL to pH 2 or less, followed by vigorous shaking. In the nonradioactive assays, an internal standard fatty acid, heptadecanoic acid (17.0), was added in 0.2 ml methanol, and the vials were shaken again. Extraction was accomplished by adding ca. 1.0 ml ethyl ether, shaking on the sander, and then removing the solvent with nitrogen. This left the lipid on top of the aqueous phase. Addition of several drops of ethyl ether redissolved the lipid, which then was spotted directly on precoated silica gel plates (EM Laboratories, Inc., Elmsford, NY) with a Pasteur pipette.

The solvent system most often used to separate the products of lipolysis was ethyl ether:ammonium hydroxide (100:1). This system separated triglycerides, diglycerides, monoglycerides, and fatty acids, in order of decreasing R_f. To monitor any possible acyl migration, 2 other solvent systems were used. Ethyl ether:petroleum ether (40:60) was used to separate triglyceride, fatty acid, $\alpha\alpha$-diglyceride, and $\alpha\beta$-diglyceride, in order of decreasing R_f values (7). The mixture acetone:chloroform (15:85) separated triglyceride, fatty acid plus diglyceride, β-monoglyceride, and α-monoglyceride in this order (18). Bands corresponding to the desired classes of lipids were visualized by iodine vapors and, following sublimation of the iodine, were scraped into either a 10 ml counting vial or a 2.0 ml glass vial fitted with a foil lined cap.

Radioactive samples were counted on a Beckman Model LS-100C Liquid Scintillation System. The cocktail used was diphenyloxazole (PPO):Triton X-100:toluene (16.6 gm:1000 ml:2000 ml). The counting efficiencies for carbon-14 and tritium were 94% and 38%, respectively, and did not vary signficantly as long as traces of iodine and chloroform were removed.

The comparisons of wax and triglyceride hydrolysis were carried out with labeled substrate as were the sequence studies. The percentage of each reaction product formed was calculated from the sum of the radioactivities of the 4 possible products.

For determinations of fatty acid and positional specificity, the fatty acids isolated on TLC were scraped into a vial, esterified, and analyzed directly by gas liquid chromatography (GLC). The fatty acids were esterified in 1.0 ml of 5% H_2SO_4 (conc.), 5% benzene in absolute methanol. The vials were flushed with nitrogen, sealed, and heated in a boiling water bath for 5 min. The methyl esters were isolated by adding 1.5 ml water and extracting with petroleum ether.

GLC analyses were performed on a Varian Aerograph, Series 1800 Gas Liquid Chromatograph equipped with a flame ionization detector. Fatty acid methyl esters were analyzed on a 2.1 m 10% SP-222-PS on Supelcoport 100/200 mesh column and a 0.8 m 3% JXR on Gas Chrom Q-100/120 mesh column. Both columns were 3.2 mm outside diameter and 2.2 mm inside diameter stainless steel and operated with nitrogen carrier gas at 40 psi and a flow rate of 40 ml/min. The SP-222 column was temperature programmed between 180-200 C and the JXR column was used isothermally at 175 C. Quantification was done with an internal standard fatty acid according to Patton and Quinn (19).

The commercial pancreatic lipase was dis-

TABLE I

Hydrolysis of Synthetic Triglycerides by Fish Intestinal Fluid and Pancreatic Lipase[a]

Substrate[b]	Lipase(s) source	Fatty acids released (% by wt ± S.D. 16:0	18:1	18:0
Intact *rac*-POS		33.7	30.8	35.5
Intact *rac*-PSO		32.7	36.2	30.6
Intact *sn*-OOP		33.8	66.2	
rac-POS	Anchovy (wild)[c]	40.0 ± 1.7	29.1 ± 0.8	30.9 ± 0.9
	Anchovy (aquarium)	42.3 ± 2.7	29.6 ± 4.2	28.0 ± 1.7
	Striped bass	41.1 ± 0.2	28.9 ± 0.8	30.0 ± 1.0
	Pink salmon	52.1 ± 0.8	15.2 ± 1.7	32.8 ± 2.5
	Hog pancreas	48.0 ± 0.8	trace	52.0 ± 0.8
rac-PSO	Anchovy (wild)	37.0 ± 0.4	52.4 ± 4.2	10.6 ± 4.6
	Pink salmon	41.7 ± 7.8	51.2 ± 6.7	7.1 ± 1.2
	Hog pancreas	44.9 ± 1.2	55.6 ± 1.2	trace
sn-OOP	Anchovy (wild)	41.6 ± 1.5	58.4 ± 1.5	
	Hog pancreas	52.6 ± 0.9	47.4 ± 0.9	

[a]Digests at room temperature on 1.25 mg substrate for 15 minutes.

[b]*rac*-POS = racemic-glycerol-1-palmitate-2-oleate-3-stearate; *rac*-PSO = racemic-glycerol-1-palmitate-2-stearate-3-oleate; *sn*-OOP = (sn-glycerol-1,2-dioleate-3-palmitate.

[c]Wild fish were 1-2 days from open ocean capture, aquarium fish were fed commercial fish chow for at least 4 weeks.

solved in buffer (ca. 1 ml/assay) 0.05M Tris, 0.1N NaCl, 0.02M $CaCl_2$ made to pH 8. A volume of 0.5 ml was used per assay.

RESULTS

Olive Oil Lipolysis

Figure 1 presents the in vitro sequence of lipolysis of triolein in olive oil by anchovy and salmon intestinal fluid and hog pancreatic lipase. TLC analyses of the diglycerides and monoglycerides formed in reactions with hog pancreatic lipase revealed only traces of 1,3-diglyceride and 1 and 3-monoglycerides even after 90 min. Regardless of the time course, the pancreatic lipase sequence essentially stopped at ca. 66% hydrolysis with over 75% of the monoglyceride still intact. Hydrolysis of total ester bonds of olive oil by fish intestinal juice continued beyond 66%, but also stopped at ca. 80%. Longer incubations failed to release additional fatty acid. The most distinctive difference between salmon and pancreatic lipase is the hydrolysis of monoglyceride by the salmon. The anchovy also exhibited monoglyceride hydrolysis as well as a reduced accumulation of diglyceride. At 50% hydrolysis, both the salmon and pancreatic lipase assays contained ca. 40% diglyceride while the anchovy digest showed only 25%. The hydrolysis of monoglyceride by the fish intestinal juice appeared to stop when triglyceride and diglyceride were depleted.

Synthetic Triglycerides

The nomenclature used here for triglycerides is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1967). Three synthetic triglycerides were subjected to lipolysis by fish intestinal juice and pancreatic lipase to further investigate the overall positional specificity of fish fat digestion. Hydrolysis in all cases was kept between 10-30%. Results with *rac*-POS (racemic-glycerol-1-palmitate-2-oleate-3-stearate) in Table 1 show a striking difference between the fatty acid products of pancreatic lipase and fish intestinal juice. Release of the 2 position oleic acid occurred in the fish assays, but not with pancreatic lipase. Addition of pancreatic lipase to boiled fish intestinal fluid released only the 1 and 3 position acids. The bass and anchovy both demonstrated significant hydrolysis of the 2-position acid with the salmon showing a lower value. The specificity of lipolysis by wild and captive anchovies was not significantly different.

Lipolysis of *rac*-PSO (racemic-glycerol-1-palmitate-2-stearate-3-oleate) by fish showed a smaller amount of 2 position acid released than with *rac*-POS. With this substrate the 2-monoglyceride was the saturated monostearin, whereas *rac*-POS yielded the unsaturated monoolein.

The enantiomeric substrate *sn*-OOP (sn-glycerol-1,2-dioleate-3-palmitate) was employed to determine if a fish lipase has a preference for one end or the other of the glycerol portion of a triglyceride molecule. The results at the bottom of Table 1 show that anchovy lipase(s), like pancreatic lipase, is nonstereospecif-

TABLE II

Hydrolysis of Mixture of Four Polyunsaturated Methyl Esters by Fish Intestinal Fluid and Pancreatic Lipase[a]

Enzyme source	Fatty acids released (% by wt ± S.D.)			
	18:2	18:3	20:4	20:5
Intact mixture	42.0	23.7	20.51	13.8
Anchovy (wild)	29.9 ± 2.1	11.7 ± 2.7	28.4 ± 1.2	29.9 ± 3.1
Striped bass	30.9 ± 1.7	13.1 ± 0.5	27.5 ± 0.7	28.5 ± 3.5
Pink salmon	42.3 ± 4.2	22.6 ± 1.0	19.7 ± 3.2	15.4 ± 2.1
Hog pancreatic lipase	55.6 ± 1.4	34.7 ± 1.1	5.3 ± 0.1	4.2 ± 0.1

[a]Digested at room temperature in 0.5 ml intestinal juice on 1.0 mg substrate for 15 min.

ic. Again the apparent release of 2 position acid was not as dramatic as with *rac*-POS.

Polyunsaturated Methyl Esters

To determine if fish demonstrated inefficiency in hydrolyzing specific polyunsaturated fatty acid esters, a liquid mixture of 4 methyl esters was used as a substrate. This resistance phenomenon was characteristic of pancreatic lipase. The advantage of studying fatty acid specificity via analyses of methyl ester hydrolysis was that it eliminated the problem of the positioning of the fatty acid on a triglyceride molecule and the problem of acyl migration. The esters and their wt percentages in the intact mixture are presented at the top of Table II, along with the experimental results. In marked contrast to the results with pancreatic lipase, fish intestinal juice hydrolyzed the resistant arachidonic acid (20:4) and eicosapentaenoic acid (20:5) more rapidly than linoleic acid (18:2) and linolenic acid (18:3). Hydrolysis by anchovy, bass, and pancreatic lipase was between 20-30%. The salmon lipase reaction went almost to completion (90% hydrolysis).

Wax vs Triglyceride Hydrolysis, In Vitro

Separate digestions of oleyl-oleate and olive oil by fish intestinal juice are shown in Table III. The ratio in the right hand column is based on the assumption that both fatty acids and monoglycerides represent end products of lipolysis. Anchovies, both wild and captive fish, were assayed at random times, and the differences in triglyceride hydrolysis from one test group to another reflect the variability in the specific activities of the particular batch of juice collected. The results show that the ratio of triglyceride hydrolysis to wax hydrolysis was variable, but always greater than 1 and in most cases greater than 4.0. The majority of fish lipases assayed also showed a greater ability than pancreatic lipase to hydrolyze wax ester. While there was variability in the triglyceride (TG)-wax ester (WE) ratio, positional specificity, and fatty acid specificity were always as described above.

Wax vs Triglyceride Hydrolysis, In Vivo

Figure 2 shows the digestion of triglyceride as seen by the disappearance of labeled methyl oleate and the appearance of activity in intestinal tissue glycerides. Not shown is the recovery of label in phospholipids, which averaged 12.4% in the last 8 fish sacrificed. Complete hydrolysis of the initial methyl oleate and reesterification to tissue triglycerides occurred in ca. 8 hr. Thereafter, activity began to appear slowly in the body of the fish. In the 2 fish sacrificed at 30.5 hr, ca. 60% of the total label was recovered in the gut and the rest in the body.

The results of the wax ester feeding experiment are shown in Figure 3. These fish were taken from the same group of fish used above, and also starved for 48 hr prior to administration of tracer. In contrast to the results of the triglyceride feeding experiment, hydrolysis and reesterification of wax ester proceeded at a much slower rate and required over 32 hr for completion. After 32 hr, the majority of the recovered dose (ca. 88%) was still in the gut tissue in both fish. Thus, in fish with identical histories, hydrolysis and reesterification of triglyceride in the gut proceeded 4 times faster than with wax esters.

DISCUSSION

A recent study of bacterial flora in the digestive tracts of marine fish suggests that some specific species of *Vibrio* are indigenous to the digestive tracts of marine fish which have a highly developed digestive system, but fish which have an undeveloped stomach system have no indigenous bacterial flora in their digestive tracts (20).

Several nonempirical findings resulted from this study. Like Brockerhoff and coworkers (7), we were unsuccessful in our attempts to find a digestive lipase in the pyloric caeca or diffuse pancreas of various fish. Employing our techniques, homogenates and acetone powders of

TABLE III

Hydrolysis of Wax Ester and Triglyceride by Fish Intestinal Juice[a]

Fish[b]	Triglyceride products[c] (% by wt)				Wax ester products (% by wt)		Ratio[c]: MG + FA(TG) / FA(WE)
	TG	DG	MG	FA	WE	FA	
Anchovy (ocean, Feb., 74)[b]	54.5 ± 3.5	13.0 ± 1.3	6.8 ± 1.2	25.7 ± 2.4	78.2 ± 2.2	21.8 ± 2.2	1.5
Anchovy (ocean, Oct., 74)	67.4 ± 0.4	14.0 ± 0.0	3.3 ± 0.1	15.2 ± 0.3	96.3 ± 1.2	3.8 ± 1.2	4.9
Anchovy (tank held)	46.3 ± 4.9	20.3 ± 1.0	7.4 ± 1.4	25.9 ± 2.4	96.5 ± 3.0	3.5 ± 3.0	9.5
Anchovy (tank held)	42.2 ± 2.3	16.8 ± 2.1	9.7 ± 1.4	31.3 ± 3.1	89.7 ± 1.3	10.5 ± 1.3	4.0
Anchovy (tank held)							
-4 C	85.3 ± 3.4	6.0 ± 0.4	2.3 ± 0.7	6.6 ± 2.2	98.8 ± 0.0	1.2 ± 0.0	7.4
20 C	44.0 ± 2.9	18.2 ± 0.2	9.7 ± 1.9	28.9 ± 2.5	96.8 ± 0.6	3.3 ± 0.6	11.3
Pacific mackerel	65.8 ± 0.8	16.8 ± 0.8	1.3 ± 0.4	16.3 ± 1.3	96.9 ± 2.0	3.1 ± 2.0	5.7
Jack mackerel	59.2 ± 3.3	21.1 ± 0.5	3.1 ± 0.3	16.6 ± 3.0	96.4 ± 0.3	3.5 ± 0.3	5.5
Pink salmon	80.1 ± 2.7	8.7 ± 1.6	1.6 ± 0.4	9.5 ± 0.6	98.2 ± 0.5	1.8 ± 0.5	6.2
Striped bass	22.0 ± 2.4	15.0 ± 1.0	10.0 ± 0.1	52.6 ± 2.2	78.9 ± 3.2	21.1 ± 3.2	3.0
Hog pancreatic lipase	55.7 ± 0.6	15.5 ± 0.2	7.3 ± 1.1	21.5 ± 2.0	97.0 ± 0.6	3.0 ± 0.6	9.6

[a]Assays were run at room temperature, for 15 min, in duplicate on 1.25 mg substrate, either oleyl-oleate or olive oil, using 0.5 ml intestinal juice.

[b]Ocean – within 1-2 days of wild state; tank held – over 4 weeks in captivity; temperatures refer to assay conditions.

[c]TG = triglyceride; DG = diglyceride; MG = monoglyceride; FA = fatty acid; WE = wax ester.

teleost pancreatic tissue, which can, with some difficulty, be isolated, did not possess detectable lipolytic activity. Acetone powders of fish intestinal juice also were inactive. Only intact intestinal fluid, fresh or lyophilized, possessed lipolytic activity, and this usually was very marked. The inactivation of intestinal juice lipase activity by acetone extraction suggests a possible role of phospholipid in enzyme activity. In certain species, lipolytic activity also was found in the gall bladder bile. Chelsey (21) first noticed this in the jack mackerel, but suggested that his values may have been in error. Analyses of anchovy bile lipase showed it to have the same specificity as the intestinal juice. In conjunction with the presence of digestive enzymes in fish bile is the absence of biliary phospholipid. Not all species examined possessed bile lipase. Bass, salmon, and pacific mackerel did not, and the anchovy and jack mackerel did. In the species without biliary lipase, only traces of phospholipid were found in the bile.

Pancreatic lipase has been shown to have a high specificity for the outer or primary ester bonds of the triglyceride substrate (22-24). As a consequence, 2-monoglyceride is hydrolyzed at a very low rate, if at all. However, hydrolysis of the monoglyceride can take place easily after isomerization to the 1-monoglyceride. This reaction does not seem to have any biological significance (25) and could not be detected in the in vitro assays reported here. The hydrolysis of the 2 position fatty acid observed earlier (11) and in this report is probably the result of the enzyme called carboxylic ester hydrolase by Borgström and coworkers (25,26) and nonspecific lipase by Mattson and Volpenhein (22,27-29). The exact specificity and bile requirements of this enzyme are as yet unclear, and it remains to be isolated in pure form. Hyun, et al., (30) in their purification of cholesterol ester hydrolase, reported the loss of nonspecific lipase activity in the final step. Because of its quantitatively minor role in the physiological specificity of triglyceride lipolysis in mammals (22,27), nonspecific lipase has long lain in the shadow of lipase (glycerol ester hydrolase, EC 3.1.1.3), serving mainly to fuel the controversey over the absolute specificity of lipase (EC 3.1.1.3). The results of this study suggest that fish have a lipase that can attack all 3 positions of a triglyceride molecule.

A double bond near the carboxyl terminus on carbons 2 through 6 in a straight chain fatty acid esterified to glycerol at a primary hydroxyl has been found to inhibit the activity of lipase (EC 3.1.1.3) (13,31-33). Certain of these resistant acids, e.g., docosahexaenoate (22:6) and eicosapentaenoic (20:5) acids, are ubiquitous

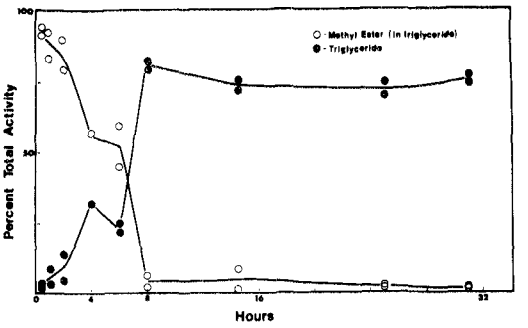

FIG. 2. In vivo digestion of triglyceride in the gut of the anchovy. Eighteen fish were force fed 15 mg purified anchovy triglycerides (TG) (+ 240,000 cpm methyl 1-C14 oleate [59 mc/mM]) and sacrificed in pairs at the times specified. The rapid loss of activity in the methyl ester marked triglyceride was followed by labeling of the intestinal triglycerides. Recovery of the administered dose averaged 81%.

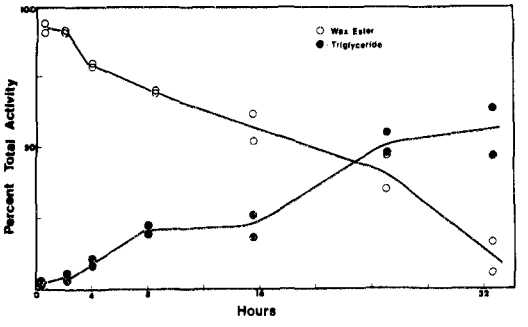

FIG. 3. In vivo digestion of wax ester in the gut of the anchovy. Fourteen fish were force fed 15 mg copepod wax ester (+ 300,000 cpm oleyl-oleate) and sacrificed at times specified. As activity disappeared from the wax, the intestinal tissue triglycerides became radioactive. Recovery of the administered label averaged 89% for the 16 fish.

components of marine lipids. Patton and Quinn (19), using a mixture of polyunsaturated methyl esters, showed that the digestive lipase of a mollusc and the pancreatic lipase of a skate were both unable to hydrolyze resistant acids. This interesting specificity of lipase (EC 3.1.1.3) may be a universal quality of 1,3 specific lipases regardless of animal origin. Bacterial and tissue lipases have yet to be analyzed for it. The results of this study with the polyunsaturated methyl esters indicate that the resistant acid phenomenon is not a prominent characteristic of the overall profile of lipolysis by fish intestinal juice. An enzyme such as nonspecific lipase may not show resistant acid specificity, and could thus account for the observed results.

From a marine lipid chemist's point of view, there is an incompatibility between the classic specificity of pancreatic lipase (EC 3.1.1.3) and

the diets of marine animals. Pancreatic lipase, as described in swine, acts 20-50 times slower on wax esters than on triglycerides (12). Large quantities of wax esters are stored by marine species (1,3,34) which represent the most abundant groups of marine animals, Annelids, Arthropods, Chaetognaths, Chordata, Coelenterata, and Mollusca. There are a few reports on absorption, metabolism, and excretion of wax esters in mammals (35,36), and only one report in fish (4). Using the freshwater gourami, *Trichgoaster cosby*, and dual labeled wax ester, Rahn and coworkers (4) found extensive intestinal hydrolysis of wax esters with part of the alcohol being reesterified, but most of it undergoing oxidation to acid which then behaved as dietary acid. A comparative study using a wide variety of marine fish indicated that fish possess a general facility for hydrolysis of wax esters followed by oxidation of the released alcohol and subsequent incorporation of the resulting fatty acid into acyl lipids (5). None of the above studies have offered any evidence for the presence of a specific wax ester lipase separate from lipase (EC 3.1.1.3).

The results of this investigation offer 2 insights into what is still a little understood area. First, it does appear that in fish, even in species that consume large quantities of wax (6), triglycerides are hydrolyzed faster than wax esters during digestion. Both in vitro and in vivo assays bear this out, but more interesting than this is the variability within the anchovy data. The differing ratios cannot be explained by the previous histories of the fish, because fish from identical habitats showed wide variability. The variability of different batches of intestinal juice could be due to different concentrations of mucous, fatty acids, monoglycerides, or bile salts. Nonenzymic physico-chemical factors may make emulsions of wax esters more or less susceptible to hydrolysis by lipase or nonspecific lipase. Thus, intestinal juice rich in monoglycerides and fatty acids may be a more suitable medium for the hydrolysis of wax esters than juice depleted in these components. The cause of this variability is currently under investigation.

In conclusion, the physiological specificity of lipolysis by fish intestinal juice is markedly different from the classified specificity of pancreatic lipase (EC 3.1.1.3). Differences in positional, fatty acid, and substrate specificity were observed. Whereas, lipase (glycerol ester hydrolase, EC 3.1.1.3) seems to play the dominant role in fat digestion in mammals, the results of this investigation suggest the presence of another enzyme that may compete effectively with lipase as a major fat digesting enzyme in fish. We are currently working towards separating different digestive lipase activities of fish by conventional protein separation techniques.

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Effect of the Nature and Amount of Dietary Energy on Lipid Composition of Rat Bone Marrow

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ABSTRACT

The effect of the nature and amount of dietary calories on the lipid composition of bone marrow of rats was studied. Male weanling rats were fed 3 isocaloric diets, containing high carbohydrate, normal protein, and high protein, and a fourth high fat diet for 49 days. Feeding of the high carbohydrate, high protein, and high fat diets caused a significant increase in the level of total lipids compared to the normal protein diet. This increase of total lipids was due primarily to the increase in the level of triglycerides. There was no significant difference in fatty acid composition of either nonpolar or polar lipids of bone marrow among rats fed high carbohydrate diet and those fed normal protein diet. A comparison of fatty acid compositions between bone marrow lipids of rats fed high protein diet and the other 2 isocaloric diets revealed that the proportion of palmitic acid was higher and the proportion of oleic acid was lower in animals fed high protein diet than in animals fed the other 2 diets. Compared to the 3 isocaloric low fat diets, dietary feeding of high fat diet caused a decrease in the proportion of palmitic and palmitoleic acids and an increase in the proportion of oleic and linoleic acids in total fatty acids of both nonpolar and polar lipids.

INTRODUCTION

Bone marrow possesses a high amount of lipids (1,2) and acts as a storage depot (3). In time of need, it contributes some of its lipids, namely linoleic and linolenic acids, to blood cells (3,4). Even though it has been suggested (5) that an active transport system exists for the preferential removal of these polyunsaturated fatty acids from marrow, their eventual fate still has not been determined. Composition of marrow lipids has been shown to be influenced by variations in age (1), species (2), hematopoietic cellularity (2,5-7) and location of the tissue (1,2). Marrow fat cells have been reported to be similar to cells of adipose tissue in terms of lipid composition (3,6,7). Fatty acid composition of marrow phospholipids also has been shown to be identical to that of erythrocytes (8). It is known that the fatty acid pattern of erythrocytes is influenced by dietary lipids (9,10). It also is well established that the amount and composition of adipose tissue fat is influenced by dietary energy containing macronutrients, fat, carbohydrate, and protein (11-18). Thus, it is reasonable to assume that fatty acid composition and amount of marrow fat will be affected by dietary manipulations in a manner similar to that observed with both erythrocytes and adipose tissue. In fact, several studies (8,16,19-21) have been reported to support this view. For example, it has been shown clearly that: a) dietary safflower oil and dietary coconut oil readily influence fatty acid composition of triglycerides in marrow (16); b) dietary linoleic acid and dietary myristic acid influence fatty acid composition of both triglycerides and phospholipids fractions of marrow (8); and c) dietary cholesterol causes accumulation of cholesterol in marrow (19-21). However, the relationship of the amount and source of dietary calories with bone marrow lipid composition is not known. Understanding the effect of dietary calories on the lipid composition of marrow is not only important from the standpoint of bone marrow biochemistry, but also from the viewpoint of the involvement of lipids in hemopoieses (22,23). The purpose of the present study was to determine the effect of the quantity and the source of dietary calories, as fat, carbohydrate, and protein, upon the lipid composition of bone marrow.

METHODS AND MATERIALS

Animals and Diets

Rats were used as experimental animals because their bone marrow fatty acid composition (7) resembles that of man (6). Male 21-day old weanling rats of the Sprague-Dawley strain were assigned to 4 dietary treatments of 30 rats each on the basis of body wt and litter mates. All animals, housed singly and randomly in raised screen wire cages in air conditioned animal quarters, had free access to water and were fed ad libitum the appropriate diets (Table

[1]To whom inquiries should be directed.

TABLE I

Composition of Experimental Diets

Ingredients[a]	Composition (%)			
	Diet 1	Diet 2	Diet 3	Diet 4
Casein (vitamin-free)	18.0	29.0	59.0	29.0
Sucrose	20.0	20.0	20.0	20.0
Cornstarch	48.0	37.0	7.0	10.0
Brewers yeast	2.0	2.0	2.0	2.0
Cottonseed oil[b]	8.0	8.0	8.0	35.0
Salt mixture[c]	4.0	4.0	4.0	4.0
Vitamin mixture[d]				
Calories as Protein (%)	17.0	28.0	57.0	21.0
Calories as CHO (sucrose) (%)	19.0	19.0	19.0	15.0
Calories as CHO (cornstarch) (%)	47.0	36.0	7.0	7.0
Calories as Fat (%)	17.0	17.0	17.0	57.0
Caloric value (Kcal/100 g)	416	416	416	551
Calories as fat (Kcal) / Calories as CHO (Kcal)	0.26	0.31	0.65	2.6

[a]Nutritional Biochemicals Corp., Cleveland, OH.

[b]Fatty acid composition: myristic, 2.5; palmitic, 25.6; stearic, 5.1; oleic, 25.2; linoleic, 41.2; linolenic, 0.4.

[c]U.S.P. XIII salt mixture no. 2.

[d]Vitamin fortification mix, 0.336 mg/100 g of diet, containing in mg/g of mixture: vitamin A concentrate (crystalline vitamin A acetate, 200,000U/g), 4.5; vitamin D concentrate (crystalline calciferol, D_2 400,000 U/g), 0.25; α-tocopherol, 5.0; ascorbin acid, 45.0 g inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine Hcl, 1.0; thiamine Hcl, 1.0; calcium pentothenate, 3.0; and, in ug/g of mixture):biotin, 20.0; folic acid, 90.0; vitamin B_{12}, 1.35.

I) for a total of 49 days. Three of the 4 diets, diets 1,2, and 3, were: a) isocaloric; b) identical in the level and source of fat; and c) different in the ratio of protein to carbohydrate calories. The other diet, diet 4, was identical with one of the first 3 diets, diet 2, in its protein content, but had a higher fat to carbohydrate ratio; hence, higher caloric value. All diets contained the same amounts of brewers yeast, vitamins, and minerals. A record was kept for food consumption and body wt gain of each animal during the experimental period.

Tissues

Femurs were used as the source of marrow because: a) lipids are distributed more uniformly in proximal, center, and distal parts of the femurs than in those of other sources of marrow (1); and b) marrow of rat femurs is very active hematopoietically (7). At the end of the experiment, animals were sacrificed by decapitation. Femurs were removed, and the bone was freed of muscle and connective tissue. The ends of the femur were cut off with a postmorten saw, and the cylindrical shaft was incised down its entire length. Marrow was removed with a small, straight spatula and collected in a small tared container, weighed, and stored at -20 C prior to lipid extraction.

Lipid Extraction

Marrow, ca. 80-100 mg of marrow per rat, was pooled from 3-5 rats to yield 6 specimens for each diet group. The wet tissue was extracted twice with chloroform:methanol (1:2, v/v) by the method of Bligh and Dyer (24). The total lipid extract was freed from nonlipid contaminants by sephadex column chromatography (25). A known aliquot of eluate from the sephadex column was evaporated to dryness in a tared flask and the wt of lipid residue determined. All solvents for lipid extraction and chromatography were of the analytical reagent grade.

Separation of Polar and Nonpolar Lipids on Silicic Acid Microcolumns

Silicic acid slurry was made as suggested by Rouser, et al., (26) and poured into a 10 x 150 mm chromatography column. Generally, 20-30 mg lipid mixture, in 1 ml chloroform, was applied to the column. Lipids were separated into nonpolar and polar fractions by successive use of chloroform and methanol according to the procedure used by Snyder and Cress (7).

Analytical Methods

Quantity of nonpolar and polar lipids was determined by measuring the wt of the residue after evaporation of an aliquot of the chloro-

form and methanol solutions in tared containers. Evaporation was done in vacuum by means of a rotary evaporator at 40-50 C, and the residue was dried over phosphoric anhydride in a vacuum desiccator.

Thin Layer Chromatography

Thin layer chromatography (TLC), with Silica Gel G as the adsorbent, was used to evaluate the qualitative and quantitative changes in the lipids of marrow. A solvent system, petroleum ether (bp, 60-70 C):diethyl ether:acetic acid (90:10:1, v/v) (27) was used for the separation of nonpolar lipids into individual components for quantitation by the charring transmission densitometric procedure of Rouser, et al., (28). Concentrated surfuric acid was used to visualize separated compounds, and a Model 520A Photovolt Densitometer was used for the measurement of lipids. Qualitative changes in polar lipids were evaluated by 2-dimensional TLC using a pair of solvent systems, chloroform:methanol:conc. ammonia (65:35:5, v/v) and chloroform:methanol:acetone:acetic acid:water (5:1:2:1:0.5, v/v) (29). Phosphomolydbate sprays (30) and α-naphthol reagent (31) were used as the visualizing agents for the detection of separated phospholipids and glycolipids, respectively. Quantitative changes in phosphatidyl choline were evaluated by 1-dimensional TLC of polar lipids in a solvent system, chloroform; methanol; acetic acid; water (200:120:25:15, v/v) (32) and measurement by charring-transmission densitometry (28).

Preparation of Methyl Esters of Fatty Acids of Lipids

Total nonpolar and total polar lipids in all cases were saponified, and the isolated fatty acids converted to methyl esters by heating for 5 min at 75 C with 14% boron trifluoride in methanol according to the method of Metcalf and Schmitz (33).

Gas Liquid Chromatography

The methyl esters of fatty acids were separated on a 6 ft x 1/4 in. column of 10% diethylene glycol succinate on Chromosorb W AW (80-100 mesh) in a Packard Model 7400 gas chromatograph equipped with flame ionization detector. Other conditions were as follows: column temperature programmed from 120 to 200 C at 10 degree increases/min; nitrogen flow rate, 40 cc/min; inlet temperature set point, 250 C; and detector temperature set point, 240 C. Assignments for each peak were obtained from a semilogarithmic plot of relative retention time versus chain length and degree of unsaturation of standard mixtures of fatty acid methyl esters. Peak areas were determined by multiplying the ht by the width at half ht. The relative percent of each fatty acid methyl ester was determined by dividing the area under individual peaks by the total peak area.

Statistical Analysis

Data were treated statistically using the Student's t test (34). The variability of the data is presented as mean ± SE. Differences at $P<0.05$ were considered significant.

RESULTS

The data on the effect of diet on growth, mortality rate, final marrow wt, feed efficiency, and calorigenic efficiency of rats is shown in Table II. When the 3 isocaloric diets, diets 1, 2, and 3), were fed, the body wt gain for diet 1 was less than that for diet 2, but almost equal to that for diet 3. The body wt gain of animals on the high fat diet, diet 4, was greater than that of the animals on diets 1 and 3, but less than that of animals on diet 2. There was no significant difference in feed efficiency between the 4 groups of animals; however, the calorigenic efficiency of diet 4 was greater than that of the other 3 diets, those 3 having about the same calorigenic efficiency. A high mortality rate was observed only among rats fed the high protein diet, diet 3. A sampling of blood chemistries of this group showed a high blood urea value, e.g., BUN=40-45 mg%. All deaths in this group occurred within 2 weeks of the start of the experiment, and it was not known whether the deaths were related to diet.

There was no significant difference in the final marrow wt between the 4 groups of animals.

Data on the effect of diet on the nature and amount of marrow lipids is shown in Table III. Triglycerides were the predominant marrow lipids in all groups. Other naturally occurring lipids, such as cholesteryl esters, free fatty acids, free cholesterol, monoglycerides, diglycerides, and various polar lipids also were present in significant amounts. Phosphatidyl choline was the predominant polar lipid in the marrow of all groups. The bone marrow of animals fed diets 1, 3, and 4 contained equal quantities of total lipids. These values were higher than the total bone marrow lipid of animals receiving the second diet, diet 2. This observed difference was due to the larger quantities of triglycerides in the lipids of marrow taken from animals which were fed diets 1, 3, and 4. Data on the effect of diet on fatty acid composition of total nonpolar and total polar lipids of bone marrow

TABLE II

Effect of Diet on Mortality Rate, Growth, Marrow Wt, Feed Efficiency, and Calorigenic Efficiency of Rats

Parameters	Diet 1 (30,30)[a]	Diet 2 (30,30)	Diet 3 (30,18)	Diet 4 (20,29)
Body wt at start, (g)	43.9 ± 0.4 [b]	39.6 ± 0.4[c]	41.8 ± 0.5[c,d]	37.9 ± 0.4[c,d,e]
Body wt at end (g)	274.3 ± 3.3	335.5 ± 4.6[c]	274.9 ± 3.6[d]	320.6 ± 4.1[c,d,e]
Body wt gain (g)	230.1 ± 3.3	295.9 ± 3.3[c]	233.1 ± 3.2[d]	282.7 ± 3.0[c,d,e]
Final wt of Femur Marrow/ 100 g body wt (g)	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ±0.01
Total feed consumption (g)	640.1 ± 5.8	783.8 ± 5.1	647.4 ± 3.7[d]	764.1 ± 6.2[c,d,e]
Total calorie consumption (Kcal)	2662.2 ± 24.2	3257.2 ± 21.5[c]	2691.5 ± 15.4[d]	4209.6 ± 34.4[c,d,e]
Feed efficiency $\frac{\text{body wt gain [g]}}{\text{feed consumed [g]}}$	0.36 ± 0.01	0.38 ± 0.01	0.36 ± 0.01	0.36 ± 0.01
Calorigenic efficiency of rats $\frac{\text{calorie consumed [Kcal]}}{\text{body wt gain [g]}}$	11.57 ± 0.3	11.01 ± 0.6	11.50 ± 0.5	14.88 ± 0.7[c,d,e]

[a]First and second numbers in parenthesis represent the number of animals at start and end of the experiment respectively.

[b]Each value is the mean ± SE of respective number of observations.

[c]Statistically ($P<0.05$) different from diet 1.

[d]Statistically ($P<0.05$) different from diet 2.

[e]Statistically ($P<0.05$) different from diet 3.

TABLE III

Effect of Diet on the Nature and Amount of Rat Marrow Lipids

Parameters	Diet 1	Diet 2	Diet 3	Diet 4
Total Lipids	9.6 ± 0.5[a]	6.8 ± 0.3[c]	10.1 ± 0.5[d]	10.1 ± 0.5[d]
Total nonpolar lipids	8.7 ± 0.4	5.8 ± 0.3[c]	9.2 ± 0.5[d]	8.8 ± 0.06[d]
Cholesterol esters	0.8 ± 0.04	0.8 ± 0.04	0.4 ± 0.3	0.7 ± 0.03
Triglycerides	6.0 ± 0.3	3.7 ± 0.2[c]	5.9 ± 0.3[d]	5.9 ± 0.3[d]
Free fatty acids	0.8 ± 0.04	0.5 ± 0.02[c]	1.4± 0.5	1.0 ± 0.05[d]
Free cholesterol	1.1 ± 0.05	0.8 ± 0.04[c]	1.3± 0.06[d]	1.2 ± 0.06[d]
Mono- & diglycerides	Trace	Trace	0.2 ± 0.1	Trace
Total polar lipids[b]	0.9 ± 0.04	1.05 ± 0.15	0.9± 0.04	1.3 ± 0.4
Phosphatidyl choline	0.7 ± 0.03	0.8 ± 0.04	0.7± 0.03	1.0 ± 0.3

[a]Each value is the mean (g/100 g wet tissue) ± SE of 6 observations.

[b]Phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, sphingomyelin, and glycolipids were the polar lipids detected.

[c]Statistically ($P<0.05$) different from diet 1.

[d]Statistically ($P<0.05$) different from diet 2.

TABLE IV

Effect of Diet on Fatty Acid Composition of Total Nonpolar Lipids of Rat Marrow

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4
14:0	0.8 ± 0.5[a]	3.8 ± 2.2	2.9 ± 1.4	2.0 ±0.6
16:0	35.8 ± 2.6	44.0 ± 2.9	57.4 ± 4.2[b]	28.4 ± 1.2[b,c,d]
16:1	5.7 ± 0.8	5.6 ± 0.8	9.0 ± 2.2	1.9 ± 0.8[b,c,d]
18:0	11.7 ± 4.1	5.6 ± 0.6	10.7 ± 1.1[c]	9.1 ± 1.1[c]
18:1	44.1 ± 3.2	38.0 ± 5.7	15.9 ± 4.2[b,c]	49.4 ± 3.6[d]
18:2	1.7 ± 0.3	3.0 ± 0.4	2.1 ± 0.6	6.0 ± 0.2[b,c,d]
18:3	0.2	Trace	Trace	2.0 + 1.1
20:0	Trace	Trace	2.0 ± 0.8	Trace
20:4	Trace	Trace	Trace	1.2 ± 0.6

[a]Each value is the mean % of total fatty acids ± SE of 6 observations.

[b]Statistically ($P<0.05$) different from diet 1.

[c]Statistically ($P<0.05$) different from diet 2.

[d]Statistically ($P<0.05$) different from diet 3.

is shown in Table IV and V, respectively. Palmitic, stearic, and oleic acids were the predominant fatty acids of both nonpolar and polar lipids in all groups. There was no significant difference in fatty acid composition of either nonpolar or polar lipids between rats fed diet 1 and those fed diet 2. However, when the third isocaloric diet, diet 3, was fed, fatty acid composition of both nonpolar and polar lipids from this group of animals was different from that of rats fed either diet 1 or diet 2. For example, when the percent fatty acid was determined for any given quantity of total fatty acids in marrow lipids, it was found that palmitic acid was higher and oleic acid was lower for animals fed diet 3 in comparison to values obtained for animals fed the other 2 isocaloric diets. Further, in comparison to the other 3 diets, the high fat diet, diet 4, influenced fatty acid composition of both nonpolar and polar lipids markedly. The percent palmitic and palmitoleic acids of total fatty acids was lower, while the percent oleic and linoleic acids of total fatty acids was higher in both nonpolar and polar lipids of the high fat group. There were no other significant alterations in marrow fatty acid composition as influenced by the dietary regime.

DISCUSSION

Triglycerides were the major lipid class in the marrow of all groups of rats. This finding supports the observation of others (6,7,35) that triglycerides are, in general, the major class of lipids in marrow of animals including human. Moreover, it shows a similarity with adipose tissue, because triglycerides are also the major lipid class in adipose tissue of animals (16,36).

Palmitic, stearic, and oleic acids are the major marrow fatty acids, and this observation is similar to those of other investigators

TABLE V

Effect of Diet on Fatty Acid Composition of Total Polar Lipids of Rat Marrow

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4
14:0	1.3 ± 0.2[a]	1.3 ± 0.2	1.6 ± 0.1	0.7 ± 0.2[d]
16:0	34.2 ± 6.2	56.4 ± 5.4	53.6 ± 4.9	25.7 ± 4.1[c,d]
16:1	6.2 ± 0.2	7.6 ± 0.4[b]	7.2 ± 0.5	2.3 ± 0.1[b,c,d]
18:0	17.5 ± 6.2	6.3 ± 3.2	11.6 ± 2.1	9.8 ± 1.1
18:1	38.6 ± 6.1	26.3 ± 6.5	24.0 ± 6.8	50.4 ± 9.8
18:2	2.2 ± 0.2	2.1 ± 0.1	2.0 ± 0.1	6.6 ± 0.1[b,c,d]
18:3	Trace	Trace	Trace	1.0
20:0	Trace	Trace	Trace	2.1
20:4	Trace	Trace	Trace	1.4

[a]Each value is the mean (as percent of total fatty acids) ± SE of 6 observations.
[b]Statistically ($P<0.05$) different from diet 1.
[c]Statistically ($P<0.05$) different from diet 2.
[d]Statistically ($P<0.05$) different from diet 3.

(6,7,16). The low level of linoleic acid in both nonpolar and polar lipids, as observed in the present study, corroborates the results of Lund, et al., (6) and Snyder and Cress (7). Bollinger (16), however, has reported a higher level of linoleic acid in total fatty acids of bone marrow triglycerides. The exact cause of this disagreement in linoleic acid content is not known. However, it is known that: a) marrow has a remarkable ability to mobilize linoleic acid and linolenic acid into blood plasma in time of needs (3,4); b) proportion of linoleic acid in total fatty acids of marrow triglycerides decreases with age (6); and c) dietary fat influences the fatty acid composition of marrow lipids (8,16,19-21).

Feeding of high carbohydrate diet, diet 1, the high protein diet, diet 3, and high fat diet, diet 4, caused accumulation, of more fat than diet 2. It is difficult to determine why diet 2 yielded values that were markedly different from the other three, because its protein content was intermediate to that of diets 1 and 3, and similar to that of diet 4. If an explanation is sought, it cannot be simply in terms of protein content alone, but must involve some other complicated interaction between protein content, carbohydrate content, fat content, and caloric density. It should be noted that a previous report (16) has indicated that dietary feeding of a high safflower oil diet produces a fatty marrow. It also is known that feeding of a high carbohydrate diet causes an accumulation of fat in the adipose tissue (11,16).

In the present study it was observed that the dietary feeding of the high cottonseed oil diet caused a 2- to 3-fold increase in the proportion of linoleic acid in total fatty acids of both the nonpolar and polar lipids of marrow. This observation corroborates that of Bollinger (16) who has shown that dietary safflower oil influences the fatty acid composition of the accumulated triglycerides in both marrow and adipose tissue by readily supplying linoleic acid to the site of increased triglycerides synthesis. Thus, this observation further supports the notion that fat cells of bone marrow are responsive to the diet and are in dynamic equilibrium with some of the components.

Even though linoleic acid was increased in marrow lipids due to feeding of the high cottonseed oil diet, this increase was not as remarkable as anticipated. It has been reported by Hirsch (36) that humans receiving 40% of calories as corn oil do not change their adipose tissue fatty acid composition in 10 weeks of feeding; but that by 20 weeks there appears the beginning of a significant rise and a final upsweep of linoleic acid. Thus, it remains to be seen whether the rise of linoleic acid in marrow lipids, as observed in the present study, is just a beginning, and that a final upsweep was forthcoming were the experiment continued beyond 7 weeks of feeding. However, it should be noted here that the fact that this experiment yielded results similar to those observed in humans does not necessarily support the data obtained in humans because of species differences.

The data presented here relating to the effect of diets on the quality and quantity of lipids and fatty acids must be supplemented with further data, including other species, before firm conclusions may be drawn.

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Fluorescent Products and Polyunsaturated Fatty Acids of Human Testes

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ABSTRACT

Lipid soluble fluorescent pigments from human testis were fractionated by silicic acid column chromatography and silica gel thin layer chromatography. Fluorescence analyses revealed a family of at least 3 compounds with similar fluorescence properties, including excitation and emission maxima, reversible fluorescence quenching by alkaline pH, and fluorescence quenching by heavy metal chelation. These fluorescence characteristics strongly indicated the presence of the conjugated Schiff base fluorophore -N=C-C=C-N-. The chromatographic separations employed enabled a more definitive fluorescence characterization of the lipid soluble pigments known to accumulate in tissues with age and as a result of lipid peroxidation. Total lipids and fatty acid composition of the total lipids were determined. Polyenoic acids constituted about 40% of the total fatty acids. Histological examination of the tissues revealed some degeneration and edema, but significant spermatogenesis and normal complement of Leydig cells.

INTRODUCTION

Lipofuscin pigments accumulate with age in several mammalian tissues including the testis (1). These pigments contain breakdown products of nonenzymatic lipid peroxidation (2). The autofluorescent properties of lipofuscins are well known, and much of the fluorescence is extractible with lipid solvents (3). Fluorescence spectra of chloroform-methanol extracts of age pigments show excitation maxima in the 340-375 nm region, with emission maxima in the 420-490 nm region. Fluorescence maxima in these regions are also characteristic of products of in vitro lipid peroxidation systems (4,5). The amount of lipid soluble fluorescence extracted from tissues is directly proportional to dietary levels of polyunsaturated fats and inversely proportional to dietary levels of the antioxidant, vitamin E (6).

It is probable that fluorescence of lipid peroxidation products is due primarily to the conjugated Schiff base fluorophore, -N=C-C=C-N-, produced as a result of crosslinking of 2 primary amines with malonaldehyde, an important product of lipid peroxidation (7). Lipid soluble fluorescent pigments are produced in the crosslinking of amino phospholipids, e.g., phosphatidyl ethanolamine and phosphatidyl serine (8).

Characterization of the fluorescence of the conjugated Schiff base chromophore includes the reversible quenching of fluorescence by alkaline pH in either aqueous or chloroform-methanol solutions, and quenching due to heavy metal chelation with the chromophoric nitrogens (9). The presence of an additional excitation maximum at 260-280 nm has been confirmed (10).

Testicular tissue of adult humans contains a significant amount of lipid, and from 30 to 40% of the fatty acids of the lipid are polyunsaturated fatty acids (11). The major lipid is phosphatide, but triglycerides and free and esterified cholesterol, as well as glycolipids, also are present. The chief phosphatide is phosphatidyl choline, but significant quantities of phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, and sphingomyelin also are present.

This paper reports fractionation of the components of lipid soluble fluorescent pigments of human testis and evaluation of their spectral characteristics, including their full fluorescence spectra and the quenching effects of elevated pH and of heavy metal coordination. In addition, the amount of extractible fluorescence is compared with tissue levels of various polyunsaturated fatty acids and with the histological state of the tissue.

EXPERIMENTAL PROCEDURE

Materials

Quinine sulfate was obtained from Mallinckrodt Chemical Works, (St. Louis, MO). The conjugated Schiff base fluorescence standard, N,N-diglycyl-1-amino-3-iminopropene

TABLE I

Fluorescence Yield of Human Testis Extracts and Recovery of Fluorescence from Silicic Acid Column Chromatography

Sample number	Age of donor	Peroxidizability[a]	Fluorescence yield[b]	Column recovery (%)[c]
I	70	1.39	10.5	84
II	51	1.29	7.7	87
III	81	1.30	11.3	75
IV	66	1.34	6.6	69
V	64	1.27	8.6	77

[a]Relative peroxidizability of testicular lipids calculated by the method of Witting (16).
[b]Nanoequivalents of Gly_2MA per gram of tissue.
[c]Silicic acid column.

(Gly_2MA) was prepared by Chio and Tappel (12). Europium (Tric[2,2,6,6-tetramethyl-3,5-heptanedionate]), hereafter referred to as $Eu(thd)_3$, was obtained from Varian Instrument Corp. (Palo Alto, CA). Silicic acid for column chromatography was obtained from Bio-Rad Laboratories (Richmond, CA). Molybdenum blue reagent for the detection of phospholipids on thin layer chromatography (TLC) was prepared as described by Dittmer and Lester (13). Precoated Silica Gel G TLC plates were obtained from Quantum Industries (Fairfield, NJ). All solvents used were of spectral grade.

Testes were obtained from the operating room at Vanderbilt Hospital, Nashville, TN. The samples were obtained at orchidectomy for prostatic cancer, but the testes were not involved. The organs were placed directly on ice by the surgeon and were taken to the lipid laboratory. A portion was immediately frozen in liquid nitrogen, packed in dry ice, and shipped by air to the laboratory in California. A small section of tissue was fixed in Bouin's solution for histological study and the remaining material was homogenized in chloroform-methanol. One sample (II) was from an autopsy case at Vanderbilt Hospital. It has been shown that the lipid and fatty acid composition of testes removed at autopsy is similar to that of testes removed at orchidectomy from humans of the same age group (14).

Preparation and Fractionation of Tissue Extracts

A glass and Teflon homogenizer was used for separate extractions of pigments from 5 individual testis samples. Each sample was finely minced, combined with chloroform:methanol (2:1, v/v) in a volume to tissue wt ratio of 20:1, and homogenized for 2 min at high speed in a 45 C water bath. An equal volume of water was added, the combination was mixed thoroughly in a vortex mixer, and the emulsion was centrifuged for 10 min at 1700 X g to aid in separation of solvent layers. The chloroform rich layer was removed and assayed fluorometrically. The extract of each testis sample was dried in vacuo.

Each dried extract was resuspended in chloroform and fractionated on a silicic acid column (1 X 8 cm) using chloroform combined with increasing concentrations of methanol. Fractions composing fluorescence peaks were pooled and dried in vacuo, then redissolved in chloroform:methanol (2:1, v:v) for fluorescence analysis.

The contents of each peak recovered from silicic acid chromatography were fractionated further on Silica Gel G TLC. Solvents used for development were: solvent A,, cyclohexane: chloroform:methanol (70:30:3); and solvent B, chloroform:methanol:acetic acid:water (80:20:1:1).

Testicular samples for lipid analyses were homogenized in Folch mixture and aliquots were used either for total lipid analysis or for gas liquid chromatographic (GLC) analysis of the total fatty acids.

Analytical Methods

Excitation and emission spectra were measured with an Aminco-Bowman spectrophotofluorometer linked to a ratio photometer (American Instrument Co., Silver Spring, MD) and were recorded on an X-Y recorder (Model 2000, Houston Instrument Co., Bellaire, TX). A slit arrangement of 3, 1, and 3 mm was used for the 3, 4, and 6 slit positions, respectively. Quinine sulfate at 1 μg/ml (1.28×10^{-6}M) in 0.1 N H_2SO_4 was used as a fluorescence standard. The standard solution had a fluorescence intensity of 50 with the sensitivity multiplier set at 10 and the sensitivity vernier set at 40.

The effects of pH and metal chelation on the fluorescence of fractions recovered from silicic

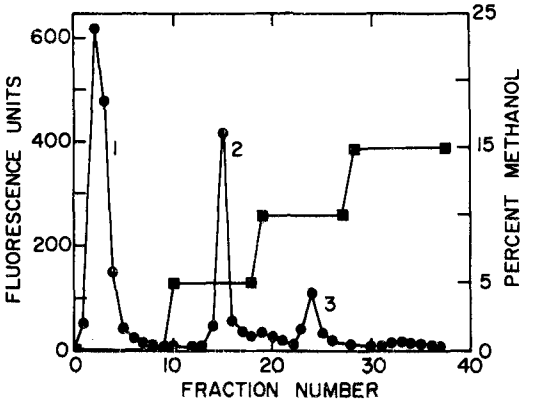

FIG. 1. Elution pattern of chloroform-methanol extracts of human testis fractionated on a silicic acid column by chloroform solutions with increasing concentrations of methanol [▪]. The fluorescence intensity [•] was measured with the excitation monochrometer set at the fractions's 340-375 nm excitation maximum and the emission monochrometer set at the fractions's emission maximum.

acid chromatography were measured as described previously (9). Absorption by the chelating agent $Eu(thd)_3$ in the 250-300 nm region prevented the observation of the quenching of fluorescence when the fluorophores were excited with radiation in this region. Compounds isolated on thin layer chromatograms were detected first by fluorescence under UV light with a maximum emission at 365 nm, then with iodine vapor, and finally with molybdenum blue phospholipid detection reagent. Total lipid analyses and gas chromatographic analyses of total fatty acids were done as reported previously (11).

RESULTS

Table 1 shows data on the fluorescence yield from the human testis sample extracts. The mean yield was 8.9 nanoequivalents of Gly_2MA per gram of tissue, more than twice the level of fluorescence similarly extracted from aged mouse testes (15). Each sample extract had fluorescence excitation maxima at 265-270 nm and 345-350 nm, and one emission maximum at 485-490 nm.

Silicic acid column fractionation of the extracted compounds yielded a reproducible elution pattern of 3 peaks of fluorescence (Fig. 1), denoted as fractions 1, 2, and 3. The average percentage of the total recovered fluorescence contained within each fraction was 66, 23, and 11%, respectively.

The corrected fluorescence spectrum of fraction 1 from testis samples had 2 excitation maxima with a single emission maximum in the

TABLE II

Fluorescence Characteristics of Fractions Separated by Silicic Acid Column Chromatography[a]

Fraction numbers	Percentage recovered fluorescence	Excitation maxima (nm)	Emission maximum (nm)	Percentage initial fluorescence				
				265-270 nm excitation		340-375 nm excitation		
				base[b]	acid[c]	base[b]	acid[c]	chelator[d]
1	66±5.2	269±1.1	343±2.7	492±2.7	63±11.6	97±3.1	54±5.6	95±1.2	88±2.5
2	23±4.8	270±0.0	355±3.5	464±6.5	61±6.6	96±3.3	66±7.3	98±1.8	76±4.5
3	11±1.5	265±0.0	356±2.2	438±2.7	63±7.1	94±6.6	53±10.6	86±7.9	70±7.0

[a]Given are mean values ± standard deviation for 5 samples.
[b]10 µl of 4 N sodium methoxide added to 2 ml chloroform:methanol (2:1) solution.
[c]Reneutralized by addition of 10 µl of 4 N acetic acid in methanol.
[d]The final concentration of $Eu(thd)_3$ was 5×10^{-5} M.

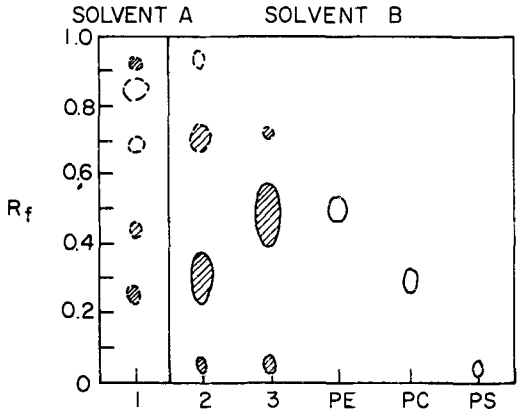

FIG. 2. Thin layer chromatograms of the lipid soluble components of human testis. Silicic acid column fractions 1, 2, and 3 are shown along the phospholipid standards phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), and phosphatidyl serine (PS). Hatched spots were detected by fluorescence; solid circles were detected with molybdenum blue reagent; dashed circles were detected with iodine vapors, but not with molybdenum blue. Solvent A consisted of cyclohexane:chloroform:methanol (70:30:3). Solvent B consisted of chloroform:methanol:acetic acid:water (80:20:1:1).

blue region, which was characteristic of each of the 3 fluorescent fractions eluted during silicic acid column chromatography (Table II).

Table II records the effects of alkaline pH and $Eu(thd)_3$ coordination on the fluorescence intensity of the 3 silicic acid column fractions from each tissue sample. Each fraction showed a significant drop in fluorescence intensity at both excitation maxima when the sample was adjusted to pH 10. Reneutralization raised the fluorescence intensity to values at or near the levels originally observed. The effect of europium chelation was consistent qualitatively, if not quantitatively. Mean reductions in fluorescence intensity recorded for fractions 1, 2, and 3 were 10, 24, and 30%, respectively.

Figure 2 shows the distribution on Silica Gel G TLC of compounds in the 3 fractions. The compounds in fraction 1 could be separated only with highly apolar solvent A and included 3 that were fluorescent, but that did not contain phosphate. When chromatographed with the more polar solvent B, one fluorescent spot with an R_f of 0.95 was observed. Fractions 2 and 3 together contained 4 fluorescent species, 3 of which corresponded in migration to the 3 standard phospholipids. Fluorescent spots corresponding in migration to phosphatidyl ethanolamine and phosphatidyl choline were consistently very faint and most concentrated in the forward portion of the spots. The R_f values of phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl serine were 0.50, 0.28, and 0.06, respectively.

In Table III are given the total lipid and fatty acid composition of the testes. Total lipid values varied between 24 and 32 mg/gm. Values for total saturated acids, mostly 14:0, 16:0, and 18:0, ranged from 35 to 39% of total fatty acids, and monoenes, 16:1 and 18:1, between 16 and 22%. The values for individual polyenes are shown in the table. Total polyenes varied only from ca. 39 to 45%, and there were no exceptional differences in individual polyenes between samples.

Histological examination revealed that, in general, spermatogenesis was active and that Leydig cells appeared normal, but there was some edema of the interstitial tissue. Samples II and III had the most active spermatogenesis, and sample I the least. Degeneration of seminiferous tubules was greatest in sample I.

Peroxidizability factors for each sample were calculated, as described by Witting (16), on the basis of fatty acid content (Table III). There were no significant correlations of fluorescence yield (Table I) with age of donor or with the product of age and peroxidizability. All linear and exponential correlations were ca. 0.7. Higher linear and exponential correlations of 0.8-0.9 ($P<0.1$) were obtained for total fluorescence in silicic acid column fraction 1 (Table II versus age and age times peroxidizability.

DISCUSSION

A primary goal of this investigation was the fractionation of the lipid extracts of human testes into the major compounds identifiable through fluorescence characterization as conjugated Schiff base fluorophores. In mammalian testicular material, age pigment accumulates in the interstitial cells where the steroid hormone testosterone is elaborated from cholesterol (17). High interstitial levels of phospholipid, of which perhaps 40% is phosphatidyl ethanolamine (18), indicate the probability that malonaldehyde crosslinked phosphatidyl ethanolamine is the major fluorescent product of lipid peroxidation. Other amine bearing lipids, such as phosphatidyl serine and sphingosine, derived from sphingomyelin, are likely to be involved as well.

The three major silicic acid column fractions had similar fluorescence characteristics, including excitation and emission maxima, reversible alkaline quenching of fluorescence, and $Eu(thd)_3$ quenching of fluorescence when excited at the higher wavelength maximum. These 3 independent fluorescence characteristics observed in concert offer strong evidence for the

presence of the conjugated Schiff base chromophore. The mean emission maximum of each column fraction was significantly different from those of the other 2 ($P<0.0005$), and the mean chelation effects on the fluorochromes in fractions 2 and 3 were significantly greater than the effect on fluorochromes in fraction 1 ($P<0.0005$). This evidence indicates the presence of different compound(s) in each fraction with fluorescence characteristics of the conjugated Schiff base fluorophore.

TLC of the lipid soluble components of the fluorescent pigments confirmed the presence of several fluorescent compounds. Contrary to expectations, less than one-third of the total lipid soluble testicular pigment was attributable to phosphate containing compounds, and these were found only in fractions 2 and 3. This observation is not inconsistent with the conjugated Schiff base chromophore structure, because phosphate in crosslinked phospholipids that accumulate in lysosomes might be recovered for the cell's phosphate pool by the action of phospholipases, and nonphosphate containing Schiff base structures could arise from sphingosine, the hydrolytic product of testicular sphingomyelin.

The coincidental migration on TLC of fluorescent compounds with the standards phosphatidyl ethanolamine and phosphatidyl choline is very likely the result of displacement by these highly concentrated phospholipids in fractions 2 and 3. Coincidental TLC migration of peroxidized microsomal fluorescent pigments with phosphatidyl ethanolamine has previously been noted (19).

The fractionations achieved with the column and thin layer chromatographic techniques employed revealed in the lipid extracts of testes a complex population of compounds with similar fluorescence characteristics. These separations allowed more critical examination of the fluorescence properties of the components of lipid pigments.

Exponential and linear correlations of fluorescence of testes extracts versus age of mice have been found (15). Chloroform-methanol extractible fluorescence can be used as a rough quantitative measure of tissue peroxidative damage. Lipid extractible fluorescence has been correlated with in vitro peroxidation damage to microsomes (5). Based upon similar calculations as used by Dillard and Tappel (5), ca. 3.5 μmoles of lipid peroxides would have been produced per gram of human testicular tissue to account for the mean level of extracted fluorescence. This estimate of in vivo free radical damage is, at best, a rough approximation.

The relationship of fluorescence accumula-

TABLE III

Total Lipid and Major Fatty Acid Composition of Total Fatty Acids of Adult Human Testes

Sample number	Total lipid (mg/g)	Major fatty acid composition (% total fatty acids)										
		Total saturated acids	Total monoenes	18:2	18:3	20:2	20:3	20:4	22:4	22:5	22:6	24:4 24:5
I	23.9	35.1	17.2	4.5	0.5	1.3	8.0	10.5	2.3	5.6	8.8	3.4
II	32.1	36.2	22.0	5.7	0.4	0.8	6.3	11.4	2.5	3.2	7.5	3.1
III	24.2	37.0	19.5	4.3	0.4	0.7	10.2	9.3	2.2	2.3	9.7	1.6
IV	28.5	35.8	16.3	5.6	0.1	0.7	5.6	12.2	2.0	3.4	9.5	2.0
V	29.0	38.8	18.3	5.4	0.3	0.6	6.0	10.5	3.1	2.5	8.4	1.7

tion to age and to free radical cellular damage is clearly based on several tissue-specific factors, including cellular levels of polyunsaturated fats, antioxidant capabilities, and the ability to eliminate indigestible damage products from the cells.

That the testis contains a major proportion of its fatty acids as polyenes has been shown by several investigators and is well illustrated in the human cases reported in this paper. The concentration of polyenes in testes of younger humans is not known as well as that in adults, but in 2 infants (autopsy cases) the polyenes represented $< 20\%$ of the total fatty acids, while in a 4-month old child (autopsy case), the concentration had risen to ca. 40% of total fatty acids (14). The total lipid of the 2 infants also was higher than values obtained for adults, but in the 4-month old child, testicular total lipid was similar to that of adults. In the rat it has been shown that the polyenes increase in concentration with sexual maturation, largely at the expense of stearic and oleic acids (20). In these studies, the highest fluorescence yields were obtained for testes that had the lowest total lipid (samples I and III). The total polyenes of all the samples were similar, 39-40% of total fatty acids, so that it is difficult to make detailed correlation of fluorescence yield with individual samples. However, it is apparent that abundant reactants, in the form of polyenoic acids, exist in the organs.

Although phosphatide analyses were not done in these particular samples, it has been shown in similar samples (11,14) that 50-60% of the total lipid is phosphatide and of this ca. 50% is phosphatidyl choline, ca. 20% is phosphatidyl ethanolamine, and there is 8 to 9% each of phosphatidyl serine, phosphatidyl inositol, and sphingomyelin, as well as small amounts of other components. It is also of interest that the sum of the lipid classes analyzed, i.e., total phospholipids, total glycerides, free fatty acids, and free and esterified cholesterol, account only for about 80% of the total lipid, as determined gravimetrically. Thus, the total lipid extracts contain lipid soluble compounds not included in the usual analyses. Part of this has been shown to be glycolipids (21), but at least a portion of the balance may be the fluorescent products under discussion.

Histological examination of the tissues was done to estimate the amount of degeneration due to age. Although some degeneration had occurred and there was some edema of interstitial tissue, there was active spermatogenesis in all testes samples. Lipid droplets were present and Leydig cells appeared to be normal in quantity. Thus, the samples appeared satisfactory for study.

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Biosynthesis of Fatty Acids from Acetate in Soybean Suspension Cultures

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ABSTRACT

Suspension cultures of finely divided soybean cells established from callus were incubated with sodium [1-^{14}C] acetate for periods up to 86 hr. Lipids and fatty acids were analyzed for radioactivity in samples harvested at logarithmic time periods. Incorporation of acetate into cell lipid was directly proportional to the logarithm of time up to 32 hr, after an initial lag of 4-6 hr. Most of the lipid radioactivity was found in the phospholipid fraction, and all common soybean fatty acids became labeled within 6 hr. The order of labeling and distribution of radioactivity with time were essentially the same as in tissues from intact growing plants. These results support the concept of sequential desaturation of oleic acid in the cells. It was concluded that valid studies of the biosynthesis of common lipids in the soybean can be carried out for extended periods of time by use of undifferentiated cells in suspension cultures.

INTRODUCTION

Studies in our laboratory of the synthesis of fatty acids and complex lipids in the soybean have involved use of seed tissue obtained 40 days after flowering, a period of rapid growth and lipid synthesis. Incorporation of labeled acetate into lipid of seed slices is rapid and directly proportional to the logarithm of time for at least 2 hr. Pulse experiments have allowed observation of lipid synthesis for periods up to 48 hr. Soybean seed lipid is primarily neutral; however, in early stages of incubation, incorporation of acetate into fatty acids and further transformation of the acids is observed only in the phospholipid fraction (1).

Callus, undifferentiated soybean cells, grown in suspension culture contain relatively little lipid and it is primarily phospholipid (2). While the gross fatty acid composition of the cultured cells differs widely from that of the soybean lipid, it is similar to the composition of the seed phospholipid fraction. We have recently reported that the proportions of fatty acids in cell cultures are altered subsequent to treatment with various mixtures of plant hormones in the growth medium (3).

Callus cells derived from higher plants are easily generated and may be propagated on agar or in suspension cultures on defined media. Cells in suspension culture are living and dividing, appear homogeneous, present a large surface area, and can be cloned (4). Protoplasts of callus cells undergo fusion or transfer of genetic information (5-8), and differentiation to plants is now possible for a number of species (9,10). Mutation of cloned cells or cell selection for desired characteristics after chemical or hormonal treatment should vastly increase the possibilities for genetic improvement in higher plants. Furthermore, the characteristics of the homogeneous cells and protoplasts render them ideal candidates for study of synthetic reactions or assay of synthetic capabilities after treatment. Confirmation that callus cells in cultures exhibit the same sequences of formation and transformation of common fatty acids as plant tissues was considered basic to use of such cells as models for plants or for development of assay procedures.

MATERIALS AND METHODS

A callus culture of soybean (*Glycine max* L., strain PI-153-245) was grown to relatively large volume in a 1 liter Erlenmeyer flask on B5 medium (11) containing 0.1 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) and 10 g/liter of Bactoagar (Difco Laboratories, Detroit, MI). The callus was transferred to a 500-ml Erlenmeyer flask containing the same medium, but without agar on a reciprocal shaker (5 cm travel each direction, 66 cycles/min) at 21-22 C and with 8 hr/day of overhead incandescent lighting (216 lx). Finely divided suspension was separated and transferred to fresh medium at 4-week intervals until 5 transfers had been made. The resulting thick homogenous stock suspension was used for experiments 4 weeks after the fifth transfer.

For determination of growth curves, 5-ml aliquots of the stock suspension were transferred to 125-ml Erlenmeyer flasks, each containing 20 ml fresh medium held on a gyrotory shaker (100 rpm) at 21-22 C under fluorescent lighting 8 hr/day (430 lx). Five replicates were harvested at 5-7 day intervals up to 45 days.

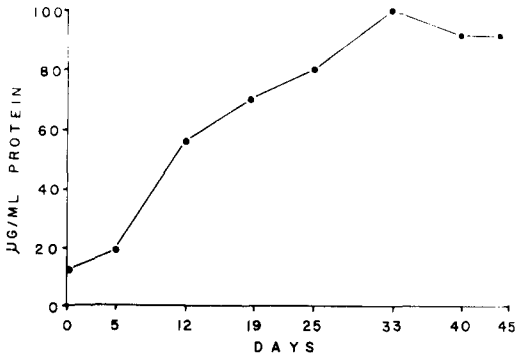

FIG. 1. Growth of soybean cells in suspension culture after transfer to fresh B5 medium. Growth measured as µg/ml of protein in cell hydrolysate.

TABLE I

Fatty Acid Compositions of Soybean Seeds and Soybean Callus Cells in Suspension Culture[a]

Fatty acids[b]	Seed lipid (%)	Seed phospholipid (%)	Suspension culture lipid (%)
16:0	10	33	37
16:1	1	<1	1
18:0	4	8	8
18:1	23	8	7
18:2	51	44	4
18:3	10	7	44

[a]Seeds harvested 40 days after flowering; cultures harvested 21 days after transfer to fresh media.

[b]Fatty acids analyzed by gas liquid chromatography as methyl esters.

Each replicate was centrifuged at 2000 x g for 15 min, and the pellet blotted to measure packed cell volume and wet wt. After drying at 60 C for 12 hr, dry cell wt was determined, and the cells were hydrolyzed in 5 ml 1N NaOH at 85 C for 100 min. The hydrolysate was filtered through Whatman No. 1 filter paper, and 1-ml aliquots were used for determination of protein by the Lowry procedure (12) using bovine serum albumin as the standard. Readings were made on a Coleman Model 14 spectrophotometer at 560 mµ.

Incorporation of acetate into lipids of cells in culture was studied using stock suspension 21 days after transfer to fresh medium. Aliquots (15 ml) of a single suspension culture were aseptically transferred to 50-ml Erlenmeyer flasks which were closed with foam plugs and placed on a reciprocating water bath shaker under conditions identical to those under which cotyledon slices were incubated, (25 C, moderate shaking rate, illumination by a single 100-watt incandescent bulb placed 25 cm above the center of the shaker). Aliquots of a sterile aqueous solution of sodium [1-^{14}C] acetate were injected through the foam plugs, 10 µCi/flask. Duplicate flasks were harvested at ca. logarithmic time periods from 5 min to 86 hr. On removal, flasks were held in a beaker of boiling water for 15 min to destroy enzymatic activity. Cells were recovered by centrifugation at 1000 x g for 15 min, were resuspended in fresh medium and recentrifuged prior to extraction. Slices of 40-day-old soybean seeds were incubated with sodium [1-^{14}C] acetate in the presence of air as described previously (13).

Extraction and fractionation of lipids, methanolysis, and fractionation of esters by thin layer chromatography (TLC) and gas liquid chromatography (GLC), and determination of radioactivity in samples and fractions were performed as described previously (13). Gravimetric determinations were performed on aliquots using a Cahn Gram electrobalance (Cahn Division of Ventron Instrument Corporation, Paramount, CA).

RESULTS AND DISCUSSION

Packed cell volume, wet wt, and dry wt of replicates of growing cell suspension cultures were erratic and irreproducible. However, protein determination after alkaline hydrolysis was reproducible and allowed construction of a growth curve (Fig. 1). A lag in growth for the first 5 days and an eventual plateau at about 33 days are characteristic of suspension cultures. Growth during the period between days 12 and 33 appeared linear with time, and the approximate midpoint, day 21 after transfer, was selected for use in subsequent studies.

Fresh tissue of soybean seeds 40 days after flowering contain 70% water and 15% lipid. Of this lipid, about 15% is phospholipid; therefore, phospholipid content on a dry wt basis is calculated to be ca. 7-8%. Cells centrifuged from suspension cultures contain 98% water and only 0.2% lipid. The lipid is almost entirely phospholipid, (98%), and on a dry basis the phospholipid content is 10%, or about the same as in seeds. Compositional data on fatty acids in seed and callus lipids are presented in Table I. While total seed lipid composition showed little resemblance to that of the callus cells, seed phospholipid composition was quite similar to that of the callus lipid. It is of interest that a reversal of the relative proportions of linoleic and linolenic acids was observed in the phospholipids of the 2 different kinds of cells.

Short term incorporation of sodium [1-^{14}C] acetate into the lipids of 40-day bean slices and 21-day suspension cells is compared in Table II.

As previously reported (1), incorporation of acetate into fatty acids of bean slices is rapid and is first observed in the phospholipid fraction during early stages of incubation. By comparison, incorporation into callus lipid appeared to be much slower, perhaps as a result of the high degree of hydration of these cells. While the pattern of incorporation in slices was apparent at 3 hr, callus cells were incubated for an additional 3 hr to allow uptake of sufficient radioactivity to discern a definite pattern.

Because the suspension cells contain primarily phospholipid, it is not surprising that the radioactive fatty acids occur larely in this form, as in the seed slices. In both preparations, phosphatidic acid and the combined phosphatidyl serine and phosphatidyl inositol fraction contain the highest proportion of label, with little occurring in phosphatidyl ethanolamine. The phospholipid designated x, previously reported by us to be associated with the appearance of labeled monoenoic acid in soybeans (13), contains an appreciable portion of the radioactivity in both types of cells. The notable difference between phospholipids of the seeds and the suspension cells is the relatively higher proportion of ^{14}C-labeled phosphatidyl choline (PC) in the former. The distribution of radioactivity in fatty acids by unsaturation indicates a higher proportion existing in polyenes of the callus cells than in seeds. This is surprising in light of our previous finding in bean slices that appearance of polyenoic acids is associated with PC and a lower relative proportion of labeled PC in the callus cells than in slices. Although incorporation of acetate into the cells appears to occur at a low rate, its incorporation into polyenoic acids apparently is not delayed.

Incorporation of acetate into lipids and fatty acids of plant preparations for extended periods, either with constant exposure or as pulse experiments, has been used to circumvent the difficulty of administration and desaturation of long chain preformed fatty acids in plant preparations maintained at low pH values. The sequence of incorporation into fatty acids or precursor-product curves resulting from such experiments lend support to the concept of sequential desaturation of oleic acid in higher plants. Callus cells in suspension culture incubated with sodium [1-^{14}C] acetate (Fig. 2) exhibit a lag in the incorporation of isotope into the total lipid, and incorporation does not approach linearity with the log of the time until ca. 4-6 hr. Treatment of developing bean slices, on the other hand, produces this relationship from the outset. Bean slices in our experiments have been unable to maintain the initial rate of acetate incorporation beyond 8-10 hr, while it

TABLE II

Incorporation of Sodium [1-^{14}C] Acetate into Lipids and Fatty Acids of Soybean Seed Slices and Soybean Callus Suspensions[a]

Fraction	Seed slices[b] (%)	Suspension cells[b] (%)
Total lipid	10.7	0.3
Total phospholipid	60	88
Phospholipid distribution[c]		
PA	32	30
X	16	19
PC	20	5
PE	4	8
PS + PI	24	27
Phospholipid fatty acids[d]		
Saturated	64	51
Monoenoic	30	29
Dienoic	4	16
Trienoic	2	4

[a]Seeds 40 days after flowering; callus cells 21 days after transfer to fresh medium; conditions of incubation and analysis in text.

[b]Slices incubated 3 hr; callus cells incubated 6 hr.

[c]PA = phosphatidic acid; X = unidentified minor phospholipid; PC = phosphtidyl choline; PE = phosphatidyl ethanolamine; PS = phosphatidyl serine; and PI = phosphatidyl inositol.

[d]Fatty acids analyzed by argentation thin layer chromatography of mixed methyl esters.

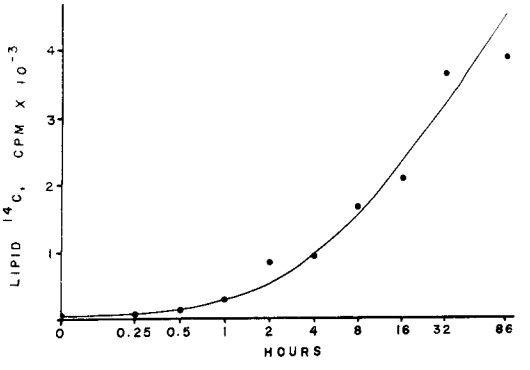

FIG. 2. Incorporation of radioactivity into total lipid of soybean suspension cultures incubated in the presence of sodium [1-^{14}C] acetate.

is evident that cultured cells continue incorporating acetate into lipid for at least 32 hr, and perhaps longer. Because these cells are in a state of active growth and metabolism, it is not surprising that experiments for extended periods of time are possible.

Analysis of the radioactivity in fatty acids according to unsaturation (Fig. 3) show that saturated and monoenoic acids do not become significantly labeled until after the first half hr, while dienoic acid required $>$ 1 hr, and trienoic

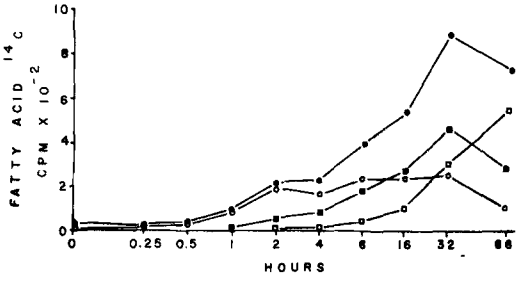

FIG. 3. Incorporation of radioactivity into fatty acids of soybean suspension cultures incubated in the presence of sodium [1-14C] acetate. Fatty acids separated as methyl esters by argentation chromatography; ● = saturated; ○ = monoene; ■ = diene; □ = triene.

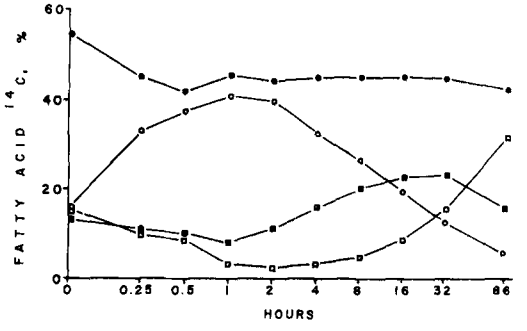

FIG. 4. Distribution of radioactivity in fatty acids of soybean suspension cultures incubated in the presence of sodium [1-14C] acetate. Fatty acids separated as methyl esters by argentation chromatography; ● = saturated; ○ = monoene; ■ = diene; □ = triene.

acid > 4 hr of incubation. The saturated activity increases through the first 32 hr, but the radioactivity of monoenoic acid appears to plateau as the activity in the dienoic acid increases, the latter eventually exceeding the former. During the period from 32 to 86 hr, radioactivity of the fatty acids decreases, except that of the trienoic fraction, which continues to rise. Soybean seed slices and homogenates previously had not been viable for a sufficient length of time to permit us to observe this extensive an increase in linolenic acid.

In terms of proportions of total activity (Fig. 4), curves are quite typical of those obtained using plant preparations in pulse labeling studies. The portion of the total activity in saturated acids is relatively constant throughout; however, the relationships between monoenoic and dienoic acids and between dienoic and trienoic acids are suggestive of sequential desaturation of oleic acid, as has been observed in pulse studies using preparations of developing soybeans (1) and other plants (14). Observation of these changes in cells remaining in the presence of acetate is interpreted to mean that turnover of fatty acids is probably occurring at a rate greater than that of acetate absorption.

Undifferentiated soybean cells in suspension culture are capable of synthesizing common saturated and unsaturated fatty acids and phospholipids from labeled acetate administered during a period of rapid growth. The same order of labeling with time and apparent conversion of radioactivity to progressively more highly unsaturated fatty acids observed in tissues from developing plants occurs in these cells as well. Although incorporation of acetate apparently takes place at a low rate in the cells and is subject to an initial lag, the same synthetic interrelationships reported for tissues of developing plants are observed in the callus cells and experiments may be carried out for longer periods of time. Treatment of cells in suspension culture with plant hormones, other chemicals, or forms of radiation and cloning of treated cells could give rise to cell lines possessing lipid characteristics not found in presently available soybean strains. Valid assay for alteration in synthetic capability in selected or modified cells by incubation with labeled acetate should be possible.

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Positional Specificity of γ-Ketol Formation from Linoleic Acid Hydroperoxides by a Corn Germ Enzyme[1]

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ABSTRACT

We have shown unequivocally that the positional specificity of γ-ketol formation by a corn germ enzyme was different from that observed previously by others with an alfalfa seedling enzyme. When the pure positional isomers of linoleic acid hydroperoxide served as substrates, the corn germ enzyme formed one of two γ-ketols: 12-oxo-9-hydroxy-*trans*-10-octadecenoic acid from 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (99+% pure) and 10-oxo-13-hydroxy-*trans*-11-octadecenoic acid from 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (96% pure). Also isolated from these reactions was one of two α-ketols commonly found as a result of catalysis by linoleic acid hydroperoxide isomerase: 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid from the 13-hydroperoxide and 10-oxo-9-hydroxy-*cis*-12-octadecenoic acid from the 9-hydroperoxide. Evidence is offered that γ-ketol formation is catalyzed by linoleic acid hydroperoxide isomerase, the same enzyme responsible for α-ketol production.

INTRODUCTION

In 1974, Esselman and Clagett (1) investigated a lipoperoxidase enzyme in alfalfa seedling extracts that catalyzed the formation of 2 γ-ketols, 12-oxo-9-hydroxy-*trans*-10-octadecenoic acid (9,12-ketol) and 10-oxo-13-hydroxy-*trans*-11-octadecenoic acid (10,13-ketol), from 9-hydroperoxyoctadeca-10,12-dienoic acid (9-LOOH) and 13-hydroperoxyoctadeca-9,11-dienoic acid (13-LOOH), respectively. This specificity of γ-ketol formation implied that possibly previous conclusions in 1970 by Gardner (2) could be erroneous. Using corn germ extracts and various substrates, none of which were pure isomeric hydroperoxides, Gardner (2) deduced that 9,12-ketol and 10,13-ketol originated from 13-LOOH and 9-LOOH, respectively, which was an entirely different specificity compared with the alfalfa enzyme. Also he (2) concluded that the γ-ketol resulted from catalysis by linoleic acid hydroperoxide isomerase, which was the enzyme responsible for the formation of α-ketols (3).

Because results of the γ-ketol studies (1,2) conflicted, we reexamined γ-ketol formation by corn germ extracts in greater detail. Using pure positional isomers of the substrates, 9-LOOH and 13-LOOH, we produced unequivocal evidence that the previous conclusion about γ-ketol formation in corn germ was correct. Further evidence is described that indicates γ-ketol formation probably occurred by action of linoleic acid hydroperoxide isomerase in a manner analogous to α-ketol formation.

METHODS

Substrate Hydroperoxides

13-LOOH (99+%) and 9-LOOH (96%) were isolated as described previously (4). In certain experiments, when the enzyme reacted with the substrate in the presence of methanol or oleic acid, the substrate was ca. 79% 13-LOOH and 21% 9-LOOH prepared as described by Gardner, et al., (5). The isomeric mixture was used in these reactions because larger quantities of substrate could be prepared more easily for the relatively large scale up required to isolate sufficient quantities of products that were minor components.

Enzyme Reaction

The enzyme was extracted from cold hexane defatted corn germ flour (corn variety [B37TMSXH84] [Oh43RFXA619]) with 0.1 M phosphate buffer (pH 6.9, 1 g germ/10 ml). The homogenate was centrifuged at 9000 g for 20 min to remove cellular debris and then purified further by recentrifugation of the supernatant at 105,000 g for 1 hr to collect the particulate matter containing the enzyme.

Concentrations present in the reactions were 3.7 mM of either 13-LOOH or 9-LOOH, 0.12% Tween 20, and 0.1 M phosphate (pH 7.0). The enzyme concentration was equivalent to 32 mg corn germ flour (originally used) per ml and was determined to be 0.24 mg protein/ml by the Folin-Wu procedure (6). Reaction conditions were 26 C for 30 min. Products were extracted with $CHCl_3$:CH_3OH (2:1) after adjusting the pH of the reaction mixture to 4.

[1]Presented at the AOCS Spring Meeting, Dallas, Texas, April, 1975.

The enzyme reactions that occurred in the presence of either methanol or oleic acid were described by Christianson and Gardner (7).

Chromatography

α- and γ-Ketols. α- and γ-Ketols were separated by column chromatography (2), except the elution sequence was modified as follows: 300 ml 30% ether, 300 ml 40% ether, 600 ml 50% ether, and 300 ml 70% ether in hexane. The sample was applied to the column as a hexane slurry with 2 g silicic acid (100 mesh, Mallinckrodt Chemical Co., St. Louis, MO, analytical reagent). Fractions from the column were monitored by applying 40 μl samples to thin layer chromatography (TLC) plates (250 μ, Silica Gel G) followed by developing the plates with isooctane:ether:acetic acid (50:50:1).

After isolation of α- and γ-ketols by column chromatography, these compounds were hydrogenated, methyl esterified, and then applied to TLC for final clean up. The TLC plates were 250 μ thick for sample sizes 6-9 mg and 0.5 mm thick for samples 20-36 mg. The α-ketol, saturated, methyl ester, was isolated after double development with hexane:ether 85:25 (R_f = ca. 0.3). The similar derivative of the γ-ketol was isolated after development with hexane:ether 1:1 (R_f = 0.26). Water was sprayed on the TLC plate to detect the product; one major band was readily detected for each product. A small portion of the plate was sprayed with 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl to visualize ketones. The only significant reaction to the reagent corresponded with the water detectable band. Charring after the band of interest had been scraped off revealed the presence of many minor components. These trace components were not investigated.

Substitution Products. Those products from substitution reactions with either oleic acid or methanol were isolated by silicic acid column chromatography by the methods of Christianson and Gardner (7). The substitution products described here were minor products compared to those described previously (7). The product, 12-oxo-13-methoxy-9-hydroxy-*trans*-10-octadecenoic acid (13-methoxy-9,12-ketol), needed no further purification; however, the oleoyl ester of the γ-ketol required additional TLC isolation. The oleoyl ester, 9-oleoyloxy-12-oxo-*trans*-10-octadecenoic acid, was methyl esterified and isolated after double development with hexane:ether 85:15. The band (R_f = 0.38) absorbing short wave ultraviolet (UV) light was scraped off.

Structure Characterizations

Methods for spectral characterization by infrared (IR) and nuclear magnetic resonance (NMR) and for making derivatives were as outlined before (5). Trimethylsilyloxy derivatives were analyzed by mass spectroscopy (MS) in tandem with gas liquid chromatography (GLC) as described by Kleiman and Spencer (8). The GLC column (6 ft X 1/4 in., glass) was packed with Gaschrom Q coated with 3% Silar 5CP (Supelco, Bellefonte, PA). The temperature was programmed from 175-230 C at 4 C/min.

Methyl 9-oleoyloxy-12-oxo-*trans*-10-octadecenoate was introduced into the mass spectrometer through a direct insertion probe. The spectrum was taken at a probe temperature of 340 C.

TABLE I

Components Separated by Column Chromatography After Enzymic Action on Two Isomers of Linoleic Acid Hydroperoxides

Substrate[a]	Fractions pooled[b]	Identity of component[c]	Wt (mg)
13-LOOH	8-19	Unknowns	7.8
	20-36	Mostly LOOH[d]	21.2
	37-41	Unknowns	4.1
	42-60	12,13-Ketol	72.6
	61-91	Unknowns	15.8
	92-116	9,12-Ketol	17.3
	117-139	Unknowns	7.2
	Total		146.0
9-LOOH	9-29	Unknowns	5.9
	30-40	LOOH	18.9
	41-46	Unknowns	2.7
	47-68	9,10-Ketol	40.0
	69-78	Unknowns	3.0
	79-110	10,13-Ketol	13.3
	111-142	Unknowns	5.5
	Total		89.3

[a]Amounts of sample applied to the column were products from reaction of 13-hydroperoxyoctadeca-9,11-dienoic acid (13-LOOH) (167 mg) and 9-hydroperoxyoctadeca-10,12-dienoic acid (9-LOOH) (124 mg) as substrate.

[b]Fraction volume was ca. 10 ml.

[c]Linoleic acid hydroperoxide (LOOH); 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid (12,13-ketol); 12-oxo-9-hydroxy-*trans*-10-octadecenoic acid (9,12-ketol); 10-oxo-9-hydroxy-*cis*-12-octadecenoic acid (9,10-ketol); and 10-oxo-13-hydroxy-*trans*-11-octadecenoic acid (10,13-ketol).

[d]Fractions 20-36 were a mixture of approximately 80% LOOH and 20% oxooctadecadienoic acid by analysis of absorptivity at 232 and 269 nm.

RESULTS

13-LOOH Substrate

When interacted with corn germ enzyme, 13-LOOH (99+% pure) was converted into α- and γ-ketols, which, along with a few other

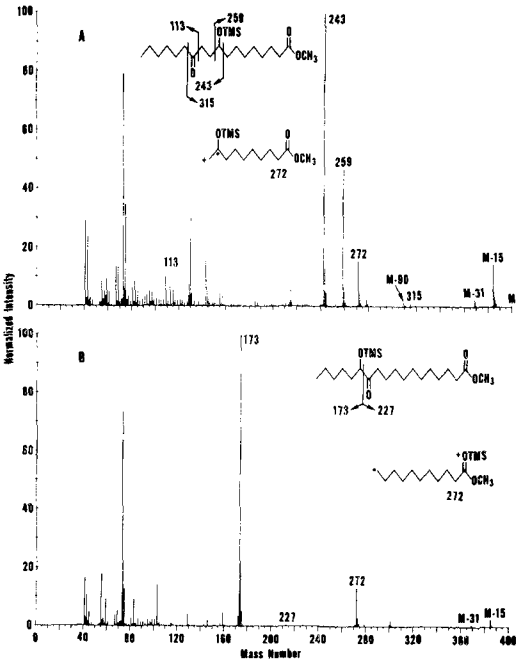


FIG. 1. Mass spectra showing specificity of γ-ketol (A) and α-ketol (B) formation from 13-hydroperoxy-*cis*-9,*trans*-11-octadecadeinoic acid by a corn germ enzyme. The ketols were hydrogenated, esterified, and treated with bis(trimethylsilyl)-trifluoroacetamide in acetonitrile before analysis. OTMS = trimethylsilyloxy.

components, were separated by column chromatography (Table I). The γ-ketol was identified as only one isomer, 9,12-ketol, while the α-ketol was found to be 12,13-ketol, exclusively. Unreacted 13-LOOH was the only other component identified, and it appeared to be mixed with a small amount of oxooctadecadienoic acid (Table I). The unreacted component was identified by TLC with authentic 13-LOOH, by its absorption at 232 nm, and by its reactivity to a ferrous thiocyanate spray. The small amount of oxooctadecadienoic acid was indicated by a smaller absorbance at 269 nm compared with that at 232 nm.

12-Oxo-9-hydroxy-trans-10-octadecenoic acid (9,12-ketol). γ-Ketol was identified tentatively by its properties on TLC, i.e., R_f value, absorption of short UV light, and reactivity to 2,4-dinitrophenylhydrazine spray (2). An NMR spectrum confirmed its identity as the γ-ketol on the basis of comparison with a previous spectrum (2). An IR spectrum of the γ-ketol methyl ester had the following absorptions: 3400 cm^{-1}, hydroxyl; 1740 cm^{-1}, ester carbonyl; 1635 cm^{-1}, olefin α,β to a carbonyl; and 980 cm^{-1}, *trans*-monoene.

The position of functional groups was established by MS after γ-ketol was converted to the methyl oxohydroxyoctadecanoate derivative, then isolated from TLC, and finally, reacted with Regisil (bis-[trimethylsilyl]-trifluoroacetamide in acetonitrile; Regis Chemical Co., Chicago, IL). As this γ-ketol derivative was being eluted by GLC, the entire peak was sampled and a mass spectrum made at 9 regular intervals. All 9 spectra were similar in all features, and a representative spectrum is reproduced in Figure 1A. MS yielded conclusive evidence that this γ-ketol was the 9,12-ketol isomer. Fragment ions were all characteristic of its structure. The 272 m/e ion was a rearrangement ion produced by cleavage between C-10 and C-11, followed by H migration from C-9 to the carbonyl oxygen and charge migration as outlined by McLafferty (9). Two spectra taken at the end of the GLC elution peak contained only one ion, 173 m/e, which was characteristic of the other isomer, 10,13-ketol. Because the relative intensity of the 173 m/e ion in these 2 spectra was only 1% and 3%, this ion was considered inconsequential.

During GLC work, it was found that methyl 12-oxo-9-hydroxyoctadecanoate reacted slowly with Regisil to yield the trimethylsilyloxy derivative. Standing overnight or longer at room temperature was required to effect nearly complete conversion. GLC-MS evidence indicated that the methyl oxohydroxyoctadecanoate was simply reacting slowly with the Regisil. Undoubtedly the slow reaction rate resulted from intramolecular hydrogen bonding between keto and hydroxyl groups.

12-Oxo-13-hydroxy-cis-9-octadecenoic acid (12,13-ketol). The 12,13-ketol isolated from column chromatography had a TLC R_f value similar to that described before (2). Reactivity to 2,4-dinitrophenylhydrazine spray was observed.

Although the NMR spectrum of the α-ketol has been reported (2,10), we can describe it more completely: methylene α to olefin, δ 2.00; C-11 methylene, broad doublet at δ 3.22 ($J_{10,11}$ = 5 Hz, $J_{9,11}$ = <1 Hz); C-13 carbinol methine, multiplet at δ 4.23; *cis* olefinic protons, centered at δ 5.54 ($J_{9,10}$ = 11). Assignments were verified by decoupling experiments. The IR spectrum of the 12,13-ketol methyl ester was essentially identical to the one reported by Zimmerman and Vick (10).

For MS, 12,13-ketol was converted to methyl 12-oxo-13-hydroxyoctadecanoate, isolated from TLC and treated with Regisil. As this derivative was eluted by GLC, a mass spectrum was taken at 12 regular intervals over the entire peak. All spectra were nearly identical in fea-

tures, and the fragment ions were indicative of its proposed structure. A representative mass spectrum is reproduced in Figure 1B. Rearrangement ions similar to the 272 ion in Figure 1 have been observed by Kleiman and Spencer (8). None of the spectra had an 259 m/e ion, the one most characteristic of the derivative from 9,10-ketol.

9-LOOH Substrate

Products resulting from enzyme action on 9-LOOH (96% pure) were separated by column chromatography (Table I). Two products were identified as 9,10-ketol and 10,13-ketol; no other ketol isomer was found. Among the many other minor components, unreacted 9-LOOH was the only one identified (Table I).

10-Oxo-13-hydroxy-trans-11-octadecenoic acid (10,13-ketol). IR and NMR spectra and TLC properties of the 10,13-ketol were identical to those described for the 9,12-ketol. However, an NMR decoupling experiment allowed a more complete description of the olefinic absorptions than reported previously (2). Decoupling the C-13 carbinol methine at δ 4.28 collapsed the C-12 olefinic doublet of doublets at δ 6.78 to a doublet. The apparent coupling constants were: $J_{12,13}$ = 5 Hz and $J_{11,12}$ = 16 Hz.

GLC-MS of a derivative of 10,13-ketol was done as described for 9,12-ketol. Eight mass spectra were sampled at regular intervals over the GLC peak, one of which is shown in Figure 2A. Fragment ions were characteristic of its structure. The 186 m/e ion was due to cleavage between C-11 and C-12, followed by rearrangement with charge migration analogous to a similar ion described for the 9,12-ketol isomer. In addition, rearrangement with charge retention occurred on the other fragment forming the 244 m/e ion by a mechanism described by McLafferty (9). Several mass spectra had ions characteristic of 9,12-ketol, but all had minor intensity. Four of the 8 had an 259 m/e ion less than 1% normalized intensity, and 5 had an 243 m/e ion of 1% normalized intensity or less, while the remaining spectra had no evidence of these ions. Therefore, it was concluded that the 9,12-ketol was largely absent.

10-Oxo-9-hydroxy-cis-12-octadecenoic acid (9,10-ketol). NMR and IR spectra and TLC properties of 9,10-ketol were essentially identical to those determined for 12,13-ketol, except that TLC mobility of 9,10-ketol was slightly less than 12,13-ketol.

A derivative of 9,10-ketol was analyzed by GLC-MS as described for 12,13-ketol. Eight mass spectra were scanned at regular intervals over the GLC peak, and a representative spectrum is shown in Figure 2B. Because the fragment ion most characteristic of the 12,13-ketol, 173 m/e, was absent from all spectra, this sample was the 9,10-ketol isomer.

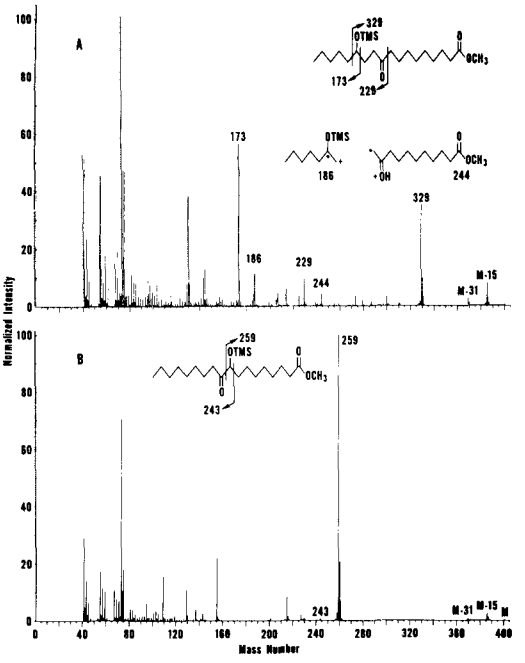


FIG. 2. Mass spectra showing specificity of γ-ketol (A) and α-ketol (B) formation from 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid by a corn germ enzyme. Derivatives were formed as in Figure 1. OTMS = trimethylsilyloxy.

Substitution Reaction with Oleic Acid

The corn germ enzyme was reacted with a mixture of hydroperoxide isomers (79% 13-LOOH and 21% 9-LOOH) in the presence of 0.034 M oleic acid. In addition to the usual products, α- and γ-ketols, substitution compounds resulted. One of these was the oleoyl ester of the α-ketol, which was a reaction product described by Christianson and Gardner (7). In our study, another substitution compound was identified as the oleoyl ester of γ-ketol. Because the substrate was an isomeric mixture, the compound was also a mixture; however, one isomer predominated because the substrate was mostly 13-LOOH. The oleoyl ester of γ-ketol, mostly 9-oleoyloxy-12-oxo-*trans*-10-octadecenoic acid, was obtained from fractions 67-75 after silicic acid chromatography, and it amounted to 6% by wt of substrate used. With a 13-LOOH substrate, the analogous reaction of the α-ketol substitutes the oleoyl group at C-13 instead of at C-9 with the γ-ketol (7). The proposed mechanism of

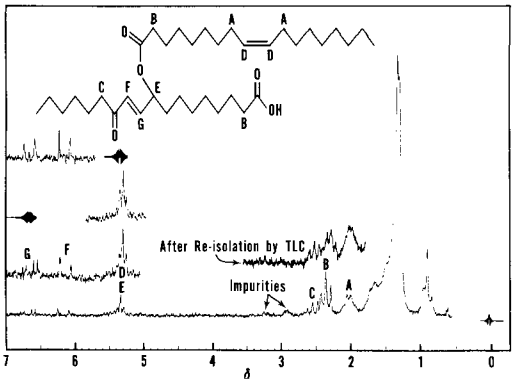
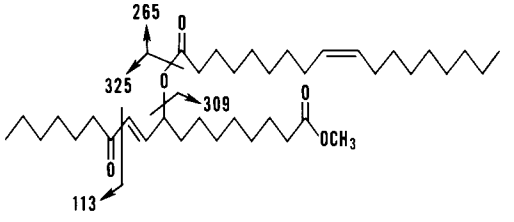
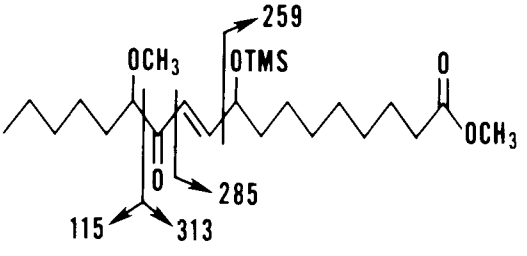


FIG. 3. Nuclear magnetic resonance spectrum of 9-oleoyloxy-12-oxo-*trans*-10-octadecenoic acid. Letters refer to the protons as indicated by the structures shown. TLC = thin layer chromatography.



Structure I. Fragment ions expected in the mass spectrum of methyl 9-oleoyloxy-12-oxo-*trans*-10-octadecenoate.



Structure II. Fragment ions observed in the mass spectrum of methyl 12-oxo-13-methoxy-9-trimethylsilyloxy-*trans*-10-octadecenoate. OTMS = trimethylsilyloxy.

these reactions is discussed later.

9-Oleoyloxy-12-oxo-trans-10-octadecenoic acid. This oleoyl ester of γ-ketol eluted from a silicic acid column immediately after the oleoyl ester of α-ketol; the latter compound was reported by Christianson and Gardner (7). Absorption at 220 nm λ_{max} ether indicated an α,β-unsaturated carbonyl.

An NMR spectrum of γ-ketol oleoyl ester indicated the structure proposed (Fig. 3). This spectrum had 2 spurious absorptions at δ 3.18 and δ 2.87 due to impurities, and because of these impurities, the pertinent absorptions integrated somwhat lower than expected. After methyl esterification and isolation from TLC, the absorptions at δ 3.18 and δ 2.87 were eliminated, and the others integrated closely to the values expected. Olefinic absorptions were similar to those reported for γ-ketol (2).

A probe MS (Structure I) yielded the following significant ions >100 m/e: 113 m/e, 16% relative intensity (RI); 149 m/e, 57% RI (possibly due to a phthalate plasticizer impurity); 265 m/e, 2% RI; 277 m/e (309-32), 6.5% RI; 309 m/e, 33% RI; 326 m/e (325 + 1), 3% RI; 559 m/e (M - 31), 0.27% RI; and 590 m/e (M), 0.36% RI. The m/e 199 ion, indicative of the other isomer, methyl 13-oleoyloxy-10-oxo-*trans*-11-octadecenoate, was not intense (1.5% RI) and was undoubtedly due to the product from the minor quantity of 9-LOOH in the substrate.

Substitution Reaction with Methanol

In the presence of 20% methanol, corn germ enzyme catalyzed the conversion of a mixture of hydroperoxide isomers, mostly 13-LOOH, to a variety of products; some of these were described by Christianson and Gardner (7). The one described here eluted from the silicic acid column in fractions 130-136 between elution of α-ketol and γ-ketol. This product, 12-oxo-13-methoxy-9-hydroxy-*trans*-10-octadecenoic acid, was 4% by wt based on the wt of substrate used.

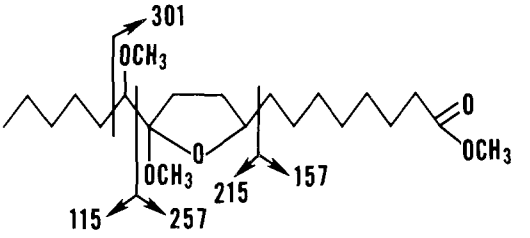
12-Oxo-13-methoxy-9-hydroxy-trans-10-octadecenoic acid (13-methoxy-9,12-ketol). The IR spectrum of 13-methoxy-9,12-ketol methyl ester had the following absorptions: 3350 cm^{-1}, hydroxyl; 1735 cm^{-1}, ester carbonyl; 1630 cm^{-1}, olefin α,β to a carbonyl; 1100 cm^{-1}, methoxy C-O; and 980 cm^{-1}, *trans*-monoene. An NMR spectrum had the following features: carbinol methine proton at C-9, δ 4.51 (m, 1H); ether methine proton at C-13, δ 3.70 (t, 1H); methoxyl methyl, δ 3.30 (s, 3H); C-11 olefinic proton α to a carbonyl centered at δ 6.61 (d, $J_{10,11}$ = 16 Hz, 1H); methylene α to fatty acid carbonyl at δ 2.32 (t, 2H); and C-10 olefinic proton centered at 6.93 δ (dd, $J_{10,11}$ = 16 Hz and $J_{9,10}$ = 6 Hz, 1H). The C-10 olefinic coupling constants were verified by experiments that decoupled the C-9 carbinol methine.

Major features observed in the mass spectrum (Structure II) indicated the proposed structure: 115 m/e, 100% RI; 115-32 m/e, 43% RI; 259 m/e, 11% RI; 285 m/e, 4% RI; 313 m/e, 13% RI; 365 m/e (M-31-32), 2% RI; M-15, 0.4% RI; and M (428), 0.4% RI. However, 2 fragment ions were observed that are characteristic of another isomer, 10-oxo-9-

methoxy-13-hydroxy-*trans*-11-octadecenoic acid (9-methoxy-10-13-ketol) as follows: 173 m/e, 6% RI; and 201 m/e, 4% RI. These ions were not so intense as the corresponding ions from 13-methoxy-9,12-ketol; therefore, 9-methoxy-10,13-ketol was considered to be a minor product, having its origin from the small amount of 9-LOOH in the substrate.

A somewhat anomalous behavior was noted during the MS-GLC of the 13-methoxy-9,12-ketol. Another major GLC peak, other than the one used to obtain the MS data reported above, was observed at later elution times. MS fragment ions and the molecular ion from this peak indicated this compound was possibly derived from the 13-methoxy-9,12-ketol by desaturation between C-9 and C-8. It is impossible now to explain how this reaction could have occurred. Because no evidence for this structure was observed in either the NMR or the IR spectra, apparently the postulated unsaturated compound formed at some stage after NMR and IR analyses.

Reduction of 13-methoxy-9,12-ketol in methanol with H_2-Pd resulted in formation of the saturated compound which appeared to have cyclized into the corresponding methoxy-hemiacetal, as determined by MS. The following MS ions were observed in Structure III. The most intense ions were m/e 115, 157, 270 (301-31), and 340 (M-32). No molecular ion was observed.



Structure III. Fragment ions expected in the mass spectrum of a hydrogenation product (CH_3OH solvent) of methyl 12-oxo-13-methoxy-9-hydroxy-*trans*-10-octadecenoate.

DISCUSSION

By using pure isomers of 9-LOOH and 13-LOOH, the positional specificity of enzymic formation of γ-ketols in corn germ was established. From 9-LOOH the 10,13-ketol was produced and from 13-LOOH, the 9,12-ketol. Although the positional specificity of α-ketol production has been established adequately by many workers (2,10,11), before our study α-ketol formation had not been investigated with pure isomeric substrates. The 12,13-ketol was derived from 13-LOOH and the 9,10-ketol from 9-LOOH as expected.

Although the positional specificity of α-ketol formation is agreed upon by all workers to date, conflicting data on γ-ketol formation was the stimulus that initiated our latest work. Esselman and Clagett (1) concluded that alfalfa seedling enzyme yielded the 9,12-ketol from 9-LOOH and 10,13-ketol from 13-LOOH. This specificity of enzyme action contradicted a previous conclusion by Gardner (2) with a corn germ enzyme. The research reported here using pure isomeric substrates confirmed the previous conclusion about γ-ketol formation by corn germ. In view of these conflicting conclusions and because Esselman and Clagett (1) used a mixture of isomers as a substrate, we believe further experimentation with the alfalfa seedling enzyme may be appropriate. It is possible, however, that the mechanism of γ-ketol formation in corn, a cereal, is entirely different in alfalfa, a legume. A comparison of the two systems indicates they are different.

Esselman and Clagett (1) noted that the products from alfalfa seedling extracts did not contain α-ketols, a condition which inferred the absence of linoleic acid hydroperoxide isomerase (LOOH-isomerase). Seemingly, alfalfa seedlings and corn germ differed in their content of LOOH-isomerase.

Using ^{18}O-labeled linoleic acid hydroperoxide, Veldink, et al., (12) showed on the one hand, that only one oxygen of the hydroperoxy group was transferred to the keto group of α-ketol during catalysis by flaxseed LOOH-isomerase. Presumably, the hydroxyl group came from the water solvent. On the other hand, Esselman and Clagett (1) showed that both oxygens of the hydroperoxy group were transferred to the keto and hydroxyl groups of γ-ketol. It seemed unlikely that LOOH-isomerase was responsible for γ-ketol formation in alfalfa seedling extracts; whereas, in corn germ it was likely that LOOH-isomerase was responsible on the basis of our evidence.

Common to both α- and γ-ketol formation by the corn germ enzyme was the production of a keto group at the carbon α to the carbon originally bearing the hydroperoxy group. Also common to both ketols was the formation of a methylene α to the keto group. Because the keto group arose from an olefinic carbon, possibly the olefinic hydrogen could have been transferred to either carbon α to it, depending on which ketol was synthesized.

As catalyzed by the corn germ enzyme, the origin of the hydroxyl group in both α- and γ-ketols may have been from the water solvent. Christianson and Gardner (7) demonstrated

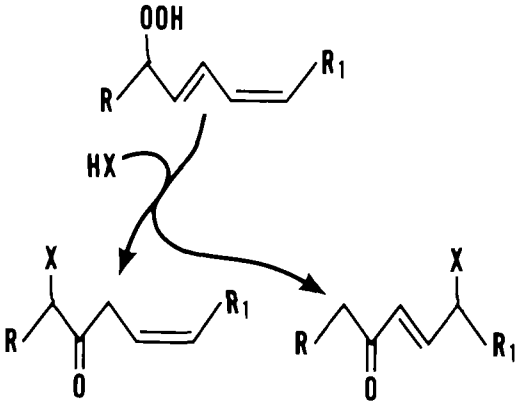


FIG. 4. Pathway of α-and γ-ketol formation by corn germ linoleic acid hydroperoxide isomerase.

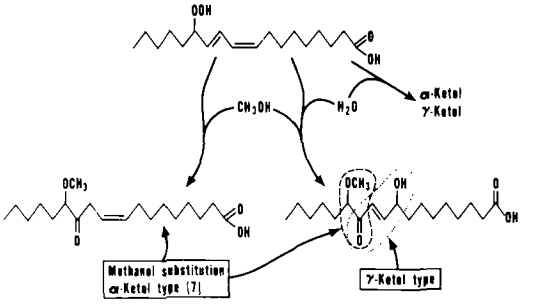


FIG. 5. Proposed pathway of formation of 12-oxo-13-methoxy-9-hydroxy-*trans*-10-octadecenoic acid and other products by linoleic acid hydroperoxide isomerase in the presence of 20% methanol.

that various reagents (HX) in the presence of the corn germ enzyme could substitute the group (X) at the carbon originally bearing the hydroperoxy group. The result was an α-ketol with an X group in place of the hydroxyl (Fig. 4). For example, in the presence of oleic acid, 13-LOOH was converted to 13-oleoyloxy-12-oxo-*cis*-9-octadecenoic acid. We showed that in the presence of oleic acid an analogous reaction also occurred with γ-ketol. However, the oleoyloxy group substituted not at the carbon originally bearing the hydroperoxy group as in the α-ketol substitution, but δ to it. From 13-LOOH, 9-oleoyloxy-12-oxo-*trans*-10-octadecenoic acid was formed. This reaction with oleic acid provided evidence that both α- and γ-ketol formation may have proceeded by a substitution mechanism and so may have been catalyzed by a common enzyme, LOOH-isomerase (Fig. 4).

The involvement of LOOH-isomerase in γ-ketol formation additionally was suggested by formation in the presence of methanol of a product that possessed structural characteristics of both α- and γ-ketols. 12-Oxo-13-methoxy-9-hydroxy-*trans*-10-octadecenoic acid was identified as being formed in 20% methanol from 13-LOOH. Christianson and Gardner (7) showed that the methoxy derivative of the α-ketol was produced in the presence of 20% methanol, i.e., 12-oxo-13-methoxy-*cis*-9-octadecenoic acid from 13-LOOH. Apparently, the usual substitution reaction of the α-ketol had occurred (12-oxo-13-methoxy) as well as γ-ketol formation (12-oxo-9-hydroxy) on the same molecule (Fig. 5). Possibly other double substitution reactions at both C-9 and C-13 could have been induced with LOOH-isomerase, but these reactions may have been so minor as to escape detection.

Presumably such divergent families of plants as corn and alfalfa would have different enzymes to act upon fatty acid hydroperoxides, and different products may have been derived from the same hydroperoxide isomer, depending upon the enzyme source. When γ-ketol formation by the corn germ enzyme was compared with that reported for the enzyme from alfalfa (1), a totally different mechanism of enzyme action and different enzymes were indicated for the two.

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Phosphatidyl Choline: Donor of 18-Carbon Unsaturated Fatty Acids for Glycerolipid Biosynthesis

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ABSTRACT

Kinetics of radiocarbon incorporation into the phosphatidyl choline of pumpkin leaf fed 1-^{14}C-acetate at low light intensity were strongly suggestive of lipid bound fatty acids acting as substrates for desaturase enzymes. After pulse labeling in direct sunlight with the same precursor, phosphatidyl choline and phosphatidyl glycerol contained up to 90% of total glycerolipid radiocarbon at the shortest sampling times. Subsequent loss of radiocarbon from phosphatidyl choline and a corresponding gain in other glycerolipids is taken to indicate a net flow of long chain fatty acids through phosphatidyl choline and into other lipids. It is proposed that there may be 2 separate synthetases in leaf tissue, one producing oleic and the other palmitic acids as their end products. Oleic acid is transferred almost exclusively to phosphatidyl choline, where it is further desaturated to linoleic and linolenic acids before being made available for the biosynthesis of other lipids. Palmitic acid is transferred mainly to phosphatidyl glycerol, where it is desaturated to *trans*-Δ^3-hexadecenoic acid.

INTRODUCTION

From an earlier study (1) on the incorporation of 1-^{14}C-acetate into pumpkin leaf lipids and the subsequent metabolism of fatty acids bound to various glycerolipids, it was suggested that the phospholipid, phosphatidyl choline (PC), played an important role in the mechanism of long chain fatty acid desaturation in leaves. It appeared possible from those results that PC bound fatty acids were, in fact, the substrates for the enzymes which desaturated oleic to linoleic and linolenic acids in leaves (2) in a manner analogous to the role of phosphatidyl ethanolamine (PE) bound oleate in the synthesis of methyl stearic acid (3) and cyclopropane fatty acids (4) in bacteria. It was further suggested from the pumpkin leaf study that the polyunsaturated fatty acids formed on PC were subsequently transferred to other glycerolipids, particularly monogalactosyl diglyceride (MDG), within the cell (1).

A specific role for PC in the desaturation of oleate to linoleate in *Chlorella* has been suggested (5), and recent evidence indicates a direct desaturation of PC bound oleate in fungal microsomes (6-9). Thus far, only the earlier work on pumpkin has indicated an apparent net flux of fatty acids through PC and into other glycerolipids.

This communication reports in more detail on the kinetics of labeling of PC in intact pumpkin leaf, and on the transfer of fatty acids from PC to other glycerolipids within the leaf. The phenomenon has been observed in leaves of other species, and when using different methods of introducing the radioactive precursor into the leaves.

METHODS

Pumpkin plants (*Cucurbiter pepo*, cultivar 'Queensland Blue') were grown in the glasshouse between September and November, or were grown and used in the field between January and March. Spinach (*Spinacea oleracea*), sorghum (*Sorghum bicolor*), and sunflower (*Helianthus anuus*) were glasshouse grown in Spring, and sunflower was also grown and used in the field in Summer.

The radioactive precursor, 1-^{14}C-acetate, was fed either by painting 1-2 ml (50-100 μc) of a 1-2 mM solution evenly over the surface of attached leaves with a camel hair brush, or via the cut petiole of detached leaves. To enable measurement of a time course of 1-^{14}C-acetate incorporation into leaf lipids, the rate of uptake of the precursor applied to the leaf surface was reduced by shading the leaf. Sampling of leaf material, extraction, separation, and counting of lipids and preparation of glycerolipid fatty acids were essentially as described previously (1,10). Nonpolar glycolipid and phospholipid classes were prepared from larger scale extractions by the silicic acid-acetone column chromatographic technique (11). Phosphatidyl choline was isolated from phospholipid fractions by semipreparative, thin layer chromatography (TLC). Fatty acid moieties of PC were isolated, hydrogenated, and degraded by the method of Harris, et al., (12).

Fatty acid methyl esters were analyzed for concentration and radioactivity using 200 x 0.4 cm columns of 15% ethylene glycol suc-

TABLE I

Time Course of ^{14}C Incorporation from 1-^{14}C-acetate into Pumpkin Leaf Phosphatidyl Choline under Low Light[a]

Time after labeling (min)	PC-^{14}C (dpm x 10^{-3}/sample)	Total fatty acid ^{14}C (%)				
		16:0	18:0	18:1	18:2	18:3
15	107	7	-	86	7	-
30	217	8	-	61	31	-
60	400	7	6	53	34	-
120	900	7	6	31	47	7

[a]50 μc of 1-^{14}C-acetate was applied to the leaf surface in a shaded area of a glasshouse. The light intensity was one-tenth of direct sunlight in the glasshouse.

TABLE II

Transfer of Radiocarbon from Phosphatidyl Choline to Other Glycerolipids after Pulse Labeling of Pumpkin Leaf with 1-^{14}C-Acetate[a]

Glycerolipid[b]	cpm x 10^{-4}/g fresh leaf		Net change
	0.5 hr	2 hr	
PC	62	48	-14
PG	11.8	12.4	+ 0.6
MGD	3.2	11.4	+ 8.2
PE	6.1	8.8	+ 2.7
PI	2.5	2.8	+ 0.3
DGD	0.3	0.8	+ 0.5
SL	>0.1	>0.1	0
Totals	86.0	84.2	

[a]100 μc (2 μmole) of 1-^{14}C-acetate in 2 ml was applied evenly over the surface of a large leaf on a field grown plant and in full sunlight. One-half of the leaf was removed 0.5 hr after and the other half 2 hr after applying the precursor. Uniformity of the application is indicated by similarity in total glycerolipid radiocarbon from each half leaf. Nonpolar lipids accounted for about 12% of the total lipid radiocarbon at both sampling times.

[b]PC = phosphatidyl choline; PG = phosphatidyl glycerol; MGD = monogalactosyl diglyceride; PE = phosphatidyl ethanolamine; PI = phosphatidyl inositol; DGD = digalactosyl diglyceride; SL = sulpholipid.

cinate on Chromasorb W in an Aerograph Model 200 gas chromatograph fitted with a thermal conductivity detector, and coupled to a Nuclear Chicago Biospan radioactivity detector. In later experiments, similar columns were employed in a Yanaco G-80 gas chromatograph fitted with flame ionization detectors and effluent stream splitters. Methyl esters were collected in 75 x 0.4 cm glass tubes moistened with xylene, and were flushed into vials for scintillation counting.

RESULTS

Shaded pumpkin leaf (light intensity one-tenth of direct sunlight in the glasshouse) incorporated 50 μmole of 1-^{14}C-acetate into PC at a linear rate for 2 hr (Table I). On radioautographs of total lipids separated by TLC, PC was the first spot to appear, and was, by far, the strongest spot at all times. Phosphatidyl glycerol and nonpolar lipids, i.e., the chromatographic front, were the next most strongly labeled lipids, but, by 2 hr PE, phosphatidyl inositol (PI) and MGD also contained detectable radioactivity. Densitometric scans of radioautographs showed that at 15 min, 80% of the radiocarbon incorporated into the total lipid was in PC, and 20% was in the nonpolar lipid. By 2 hr, this had changed to 60% in PC, 15% in PG, 15% in nonpolar lipid, and the remainder distributed among MGD, PE, and PI. Neither digalactosyl diglyceride (DGD) nor sulpholipid (SL) contained radioactivity detectable by this method.

Oleic acid was the first fatty acid of PC to incorporate label, and was, in fact, labeled almost to equilibrium by 2 hr. Linoleic acid labeling within PC initially lagged behind that of oleic acid, but then accelerated and exceeded that of oleic acid within the 2 hr (Table I). In graphical form, these data produced curves typical of a precursor-product relationship for the reaction PC-oleate → PC-linoleate, and the sum of the radioactivity in oleic plus linoleic acids increased linearly over the 2 hr. Incorporation into PC bound palmitic and stearic acids was linear with time and an order of magnitude slower than the initial rate for oleate. Significant labeling of the α-linolenate moiety of PC was not observed until after 2 hr.

Leaves of field grown pumpkin incorporated 100 μc (2 μmole) of 1-^{14}C-acetate into lipids in < 30 min in full sunlight (Table II). When the 2 halves of these leaves were examined at 0.5 and 2 hr after this pulse labeling, a clear indication was obtained of transfer of radiocarbon from PC to other lipids, especially MGD, even in this relatively short period (Table II). There was no change in the labeling of the nonpolar lipid

TABLE III

Concentrations and Specific Radioactivities of Oleic and Linoleic Acids in Nonpolar Lipids Compared with Phosphatidyl Choline of Pumpkin Leaf*

	Concentration (μg/g fresh wt leaf)	Specific radioactivity (dpm/μg fatty acid)	
		0.5 hr	2.0 hr
Nonpolar lipid			
18:1	5.4	2,800	2,540
18:2	7.0	1,460	1,915
Phosphatidyl choline			
18:1	25	15,000	3,900
18:2	100	3,000	2,800

*See Table II.

TABLE IV

Relative Specific Radioactivities of Fragments Prepared from Phosphatidyl Choline-bound Oleic and Linoleic Acids Isolated from Sunflower Leaf Labeled 20 min with 1-^{14}C-acetate[a]

Degradation product	Fatty Acids						
	12:0	13:0	14:0	15:0	16:0	17:0	18:0
Relative Specific Activity							
Phosphatidyl choline-18:1	97	86	90	88	86	106	102
Phosphatidyl choline-18:2	NM[b]	22	20	22	33	36	41

[a]PC-oleate was spiked after isolation and prior to hydrogenation and α-oxidation with 250 μg of unlabeled methyl stearate, while PC-linoleate was taken through the whole procedure (11) unfortified. The precursor was applied to the leaf surface of glasshouse grown plants in full sunlight.

[b]NM = not measured.

fractions which contained ca. 12% of the total lipid radioactivity. Specific radioactivities of the oleic and linoleic acids of the nonpolar lipids were compared with those in PC to determine whether labeling of the nonpolar lipids could have preceded labeling of PC. In spite of 5-fold and 14-fold higher concentrations of oleic and linoleic acids, respectively, PC fatty acids had 5- and 2-fold higher specific radioactivities, respectively, at the earliest sampling times (Table III). It seems unlikely, therefore, that labeling of nonpolar fatty acids preceded labeling in PC fatty acids.

The 18-carbon fatty acids of PC were formed by de novo synthesis rather than by elongation of existing 16-carbon chains. Degradation, by α-oxidation (12) of oleic and linoleic acid moieties of PC isolated from sunflower leaf 20 min after applying 1-^{14}C-acetate to the leaf surface in full sunlight, showed that oleic acid was uniformly labeled (Table IV), while linoleic acid was labeled more heavily toward the carboxyl end of the chain. This latter pattern of labeling might be expected in oleic acid at much shorter sampling times, because the first labeled product to emerge from the oleic acid synthetase after 1-^{14}C-acetate application would be labeled exclusively in the C-1 position.

Although this apparent involvement of PC in polyunsaturated fatty acid metabolism was most marked in leaves of rapidly growing pumpkin plants, experiments with attached and detached leaves of other species confirmed these findings (Table V). PC was always the most heavily labeled glycerolipid at the earliest sampling times, and a very high proportion of the radiocarbon in PC was associated with the oleic and linoleic acid moieties (Table V). The second most strongly labeled glycerolipid was PG, and this was usually, though not always, labeled in the palmitic and *trans*-Δ^3-hexadecenoic acid moieties (Table V). These 2 lipids accounted for 70-90% of the total radioactivity recovered in the leaf glycerolipids. PC radioactivity invariably declined with time relative to total lipid radioactivity after pulse feeding 1-^{14}C-acetate, whether by surface application

TABLE V

Incorporation of 1-^{14}C-acetate into Glycerolipids and their Constituent Fatty Acids of Different Leaves

Leaf	Glycerolipid[c]	Hr after feeding	Total glycerolipid-^{14}C (%)	Total fatty acid ^{14}C (%) 16:0 + 16:1	18:1 + 18:2
Spinach[a]	PC	2.5	41	13	86
	PG	2.5	30	73	27
Sorghum[a]	PC	1	45	16	84
	PG	1	25	46	54
	MGD	1	15	41	59
Sunflower[a]	PC	0.75	50	3	93
	PG	0.75	26	44	53
	MGD	0.75	11	13	86
Sunflower[a]	PC	0.33	63	14	82
	PG	0.33	20	65	33
	MGD	0.33	5	29	69
Pumpkin[b]	PC	1	67	10	89
	PG	1	20	71	29

[a]Leaf fed by petiole uptake of 1-^{14}C-acetate; spinach in subdued daylight, sorghum and sunflower in full sun.

[b]Leaf fed by surface application of the precursor in full sunlight.

[c]PC = Phosphatidyl choline; PG = phosphatidyl glycerol; MGD = monogalactosyl diglyceride.

TABLE VI

Turnover of Phosphatidyl Choline (PC) Bound and Phosphatidyl Glycerol (PG) Bound Fatty Acids after Pulse-feeding 1-^{14}C-Acetate to a Leaf of a Slowly Growing Pumpkin Plant[a]

Hr after labeling	Total fatty acid ^{14}C (%) 16:0	16:1	18:0	18:1	18:2	18:3
PC						
0.5	14	0	0	42	44	0
1	19	0	0	32	46	4
6	29	0	4	14	39	14
20	37	0	3	4	27	21
44	49	0	12	0	18	21
92	52	0	12	0	15	21
PG						
0.5	81	0	0	19	0	0
1	75	5	0	20	0	0
6	72	10	0	18	0	0
20	65	18	0	17	0	0
44	38	46	0	16	0	0
92	27	54	0	19	0	0

[a]At 0.5 hr, PC contributed 52% and PG 34% of total glycerolipid radiocarbon. After 44 hr, these figures were 27 and 33%, respectively. At 44 hr, monogalactosyl diglyceride (MGD) contained 16% of glycerolipid radiocarbon and α-linoleic acid 65% of MDG fatty acid radioactivity. The 1-^{14}C-acetate was fed by application to the leaf surface in full sunlight.

or by petiole uptake. In contrast, the proportion of radioactivity in other glycerolipids increased with time, though total lipid radiocarbon remained fairly constant.

The relative incorporation of 1-^{14}C-acetate into PC compared with PG of pumpkin leaf was not constant, but appeared to vary with growing conditions of the plants. For reasons outlined earlier (1), large leaves had been induced on small pot grown plants by repeatedly removing the growing points. It was found, however, that these leaves incorporated proportionately more 1-^{14}C-acetate into PG compared with PC than did leaves of field grown plants used in the earlier study (1). The PC/PG-^{14}C ratio was found to be 1.5-2.2 for leaves of pot grown plants labeled at midday, and a ratio of 3.3-3.7 for the same material labeled at 8 am. The ratio was consistently 5-6 in leaves on the large field grown plants labeled between 9-11 am. Because the bulk of the radiocarbon in PG was in palmitic acid, and because leaves of the slowly growing plants would be expected to contain high endogenous levels of photosynthate, these results suggest that 1-^{14}C-acetate may be biased more or less toward the synthesis of palmitic acid than oleic acid, depending on the nutritional status of the leaf. It was also apparent that when the PC/PG-^{14}C ratio was low, the 16-carbon fatty acids contained proportionally more of the total fatty acid radiocarbon within PG than when this ratio was high (Tables V and VI). In contrast, however, there were no instances when the 16-carbon fatty acids of PC contained $>16\%$ of the total PC fatty acid radiocarbon at the earlier sampling times.

A loss of 18-carbon fatty acids from PC was

reflected in the steady increase in the proportional labeling of palmitic acid in PC in a pulse chase type of experiment (Table VI). During the chase, the specific radioactivities of oleic and linoleic fell quite rapidly, while that of palmitic acid remained constant. On the other hand, the specific radioactivity of palmitic acid of PG declined steadily, while that of *trans*-Δ^3-hexadecenoic acid within PG steadily increased (Table VI). There was no further metabolism of PG bound oleic acid. This experiment also serves to illustrate the relatively specific insertion of palmitic acid into PG and oleic acid into PC.

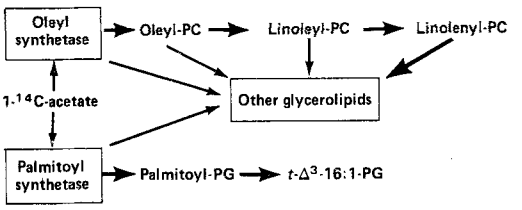

FIG. 1. Scheme to account for proposed interrelationships between long chain fatty acids and glycerolipids after feeding 1-^{14}C-acetate to intact leaves. Heavy arrows indicate major and light arrows minor flow paths of long chain fatty acids.

DISCUSSION

The kinetics of 1-^{14}C-acetate incorporation into oleic acid of PC, and the subsequent movement of the label from esterified oleic to linoleic acids strongly suggest that PC bound oleate was the direct precursor of PC bound linoleate, i.e., oleic acid was desaturated while esterified to PC. The alternatives are either that oleate was split off the phosphatide, desaturated while attached to the desaturase enzyme, and reattached to the phosphatide (5), or that the oleate was split off the phosphatide and desaturated as the Coenzyme A derivative (13) before reattachment to the phosphatide. In either case, after reattachment, the linoleate again must have been detached either for further desaturation to α-linolenate or for transfer of the acyl group to other lipids. If, as seems likely, the desaturation of oleyl-PC proceeds as far as α-linolenyl-PC, then the latter mechanisms would require 3 separate attachings and detachings of PC-acyl groups to complete the process of desaturation. It probably would be more favorable energetically to perform the desaturation on the PC bound fatty acids. Good evidence for such a direct desaturation of oleyl-PC has been obtained with yeast microsomes (7).

Recently, it has been proposed that linolenate formation in leaves proceeds by elongation of a hexadecatrienoic acid (14,15), but direct desaturation of linoleate to linolenate has been demonstrated in plants (16-18). In *Penicillium chrysogenum*, although both pathways may operate concurrently, the route of linolenate synthesis under aerobic conditions is predominantly via linoleate (19). In young maize leaves, linolenate appears to be synthesized by direct desaturation of linoleate, rather than by chain elongation of a hexadecatrienoic acid (20).

α-Linolenic acid is the predominant fatty acid of green leaves, and is the major end product of 1-^{14}C-acetate incorporation into leaves (1). Most of this α-linolenate is esterified to the chloroplast glycerolipids MGD and DGD, but in every pumpkin glycerolipid, with the exception of PG, it is the major component (1). Rates of α-linolenate biosynthesis, however, are quite slow, relative to those of oleate and linoleate, as judged by incorporation of 1-^{14}C-acetate (2). While the evidence for further metabolism of linoleate to linolenate while esterified to PC is not nearly as convincing as that for oleate to linoleate, it is a logical extension of the hypothesis that this should occur. The failure of α-linolenate to accumulate in PC could be explained by a transfer, almost immediately upon formation, of the labeled fatty acid to other lipids. It was clearly shown by long term studies of glycerolipid interrelationships in attached pumpkin leaves that the 18-carbon fatty acids were lost from PC (1), and has been amply confirmed in the present study. In every instance, the counts in PC declined with time after pulse labeling with 1-^{14}C-acetate, and the specific radioactivity of esterified palmitate remained unchanged, while that of oleate plus linoleate plus linolenate steadily declined. At the same time, the specific radioactivities of these fatty acids in other glycerolipids were increasing with time, even though the counts in total lipids did not change and all available 1-^{14}C-acetate had long since been fixed. Thus, a transfer of 18-carbon fatty acids from PC to other glycerolipids appears certain. The behavior of the fatty acid moieties of PG was in sharp contrast to that of the acyl groups of PC. At the earliest times after pulse labeling, only palmitate and oleate of PG were significantly radioactive. In some cases there was no further movement of label from oleate even after 92 hr, and the only change that took place was a decrease in labeling of palmitate accompanying an almost equivalent increase in labeling of *trans*-Δ^3-hexadecenoic acid. This is quite consistent with the suggestion, based on different data (21), that this unique fatty acid

was synthesized from precursor while esterified to PG.

This apparent specific insertion of different fatty acids into the different phosphatides, and the variable ratio of PC/PG radiocarbon in leaves growing under different conditions, prompts the suggestion that there may be 2 separate fatty acid synthetases in leaf tissue. One of these produces palmitic acid as its end product, and this is transferred almost exclusively to PG, while the other produces oleate as its end product, and this is transferred almost exlusively to PC (Fig. 1). Desaturation of the bound acyl groups then takes place. Finally, transfer of the acyl groups from PC to other newly synthesized lipids completes the picture. Different rates of synthesis from 1-^{14}C-acetate of palmitate relative to oleate (affecting the PC/PG radiocarbon ratio) could be a result of different stimulations or inhibitions of either synthetase in leaves from slowly growing compared with rapidly growing plants.

The other possibility is that oleate is formed simply by chain elongation of palmitate produced by a single synthetase, and this is followed by desaturation of the stearate (22). However, it is worth considering that in autotrophically grown *Euglena gracilis*, there are at least 2 synthetases capable of de novo synthesis of long chain fatty acids, and that the predominant products of these synthetases are palmitate and stearate, respectively (23). Also, in developing soybean cotyledons, it was concluded that oleate was not formed from palmitate or stearate, but was synthesized in parallel with these saturated fatty acids (24).

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Inhibitive Effects of Structurally Modified Azasteroids and Related Nitrogen Containing Steroids on Insect Growth and Development[1]

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ABSTRACT

A number of azasteroids and other nitrogen containing steroids with a modified nucleus or side chain were prepared and tested for their inhibitory effects on the growth and development of several species of insects. Structure-activity studies showed that compounds with a structurally related steroid nucleus and side chain were approximately equal in inhibitory activity for a particular species. The replacement of the tertiary amino group in the side chain of the 5β-steroid with other nitrogen substituents, such as nitro, cyano, acetylamino, or a quaternary ammonium salt, resulted in a considerable loss of inhibitive activity in the tobacco hornworm or the yellowfever mosquito. However, certain modifications of the azasteroid nucleus resulted in compounds that still retained high biological activity. As a result, a compound was synthesized that lacked the A and B rings of the steroid nucleus and that inhibited insect growth, molting, and metamorphosis and the Δ^{24}-sterol reductase enzyme system of the tobacco hornworm.

INTRODUCTION

Previous studies of the structure-activity relationships of a number of azasteroids showed that certain 25-azasterols were more effective in inhibiting insect growth and development than either diazasterols or monoazasterols with a secondary nitrogen at the 23 or 24 position (1). As a result, we were able to design and synthesize a number of new 25-azasteroids that were considerably more active inhibitors for several species of insects (2). These azasteroids were also potent inhibitors of the Δ^{24}-sterol reductase enzyme, caused an accumulation of desmosterol, and blocked the conversion of plant sterols to cholesterol in certain insects. Subsequently, in an effort to determine whether the tertiary nitrogen was a prerequisite for maximum biological activity and to determine the minimal structural requirement for this activity, we synthesized a number of new nitrogen containing steroids with modified and/or shortened side chains and modified steroid nuclei. In the present paper, we report the synthesis and compare the inhibitory activities of these new compounds with those of our previous 3 most active 25-azasteroids on 4 species of insects. The IUPAC equivalent names used throughout this paper are listed in Figure 1.

[1]Presented in part at AOCS Fall Meeting, Philadelphia, September, 1974.

[2]Visiting Fulbright Researcher, 1972-1973.

EXPERIMENTAL PROCEDURES

Biological Test Systems

The larval test systems for the yellowfever mosquito, *Aedes aegypti* (L.), the confused flour beetle, *Tribolium confusum* Jacquelin duVal, and the house fly, *Musca domestica* L., were those previously used to assess the inhibitive effects of ecdysone and synthetic analogs on growth and metamorphosis (3). The larval test system for the tobacco hornworm, *Manduca sexta* (L.), was as previously described for testing azasteroid inhibitors (1).

Instrumentation

Melting points were observed on a Kofler block, and infrared (IR) spectra were obtained with a Perkin-Elmer model 221 prism-grating spectrophotometer. Gas liquid chromatographic (GLC) analyses were made on a Barber-Colman model 10 chromatograph equipped with a beta ionization detector cell. GLC systems were 0.75% SE-30 and 1% OV-17 coated on Gas-Chrom P and the columns temperatures were 236 and 230 C, respectively. NMR spectra were recorded at 60 Mc with a Varian A-60A NMR spectrometer with deuterated chloroform as the solvent and trimethyl silyl (TMS) as an internal nuclear magnetic resonance (NMR) standard. The mass spectra were obtained by using an LKB model 9000 gas chromatograph mass spectrometer (LKB Produckter AB, Stockholm, Sweden). Samples were introduced directly into the ionization chamber (ionization energy 70 ev.) except for compound XLI which was introduced through the gas chromatography (GC) system.

Materials and Chemical Synthesis

Basic and neutral alumina (Woelm) were obtained from Waters Associates Inc. (Framingham, MA), and the required amount of water

XVI = 3β-Methoxy-chol-5-en-24-dimethylamine
XVII = 3β-Methoxy-24,23-dinorchol-5-en-22-dimethylamine
XVIII = 3β-Trimethylsiloxy-chol-5-en-24-dimethylamine
XIX = 5α-Cholan-24-dimethylamine
XX = 24,23-Dinor-5α-cholan-22-dimethylamine
XXI = 5α-Cholan-24-methylethylamine
XXII = 5β-Chol-2-en-24-dimethylamine and
5β-Chol-3-en-24-dimethylamine
XXIII = 5β-Chola-2,7,11-trien-24-dimethylamine
XXIV = 2,3-Seco-5β-cholan-24-dimethylamine
XXV = 3,4-Seco-5β-cholan-24-dimethylamine
XXVI = 2ξ,3ξ-Dichloro-5β-cholan-24-dimethylamine
XXVII = 3α-Hydroxy-5β-cholan-24-dimethylamine
XXVIII = 3α-Trimethylsiloxy-5β-cholan-24-dimethylamine
XXIX = 5β-Cholan-24-dimethylamine
XXX = 24-Nor-5β-cholan-23-dimethylamine
XXXI = 24,23-Dinor-5β-cholan-22-dimethylamine
XXXII = 5β-Cholan-24-trimethylammonium iodide
XXXIII = 24,23-Dinor-5β-cholan-22-aminoethanol-22-*p*-toluenesulfonate
XXXIV = 5β-Cholan-24-amine
XXXV = 24-Nitro-5β-cholane
XXXVI = 5β-Cholan-24-acetamide
XXXVII = 24-Dimethylureido-5β-cholane
XXXVIII = 24-Cyano-5β-cholane
XXXIX = 24-Nor-23-cyano-5β-cholane
XL = 24,23-Dinor-5β-cholan-22-diethylphosphate
XLI = N,N,δ-7a-Tetramethyloctahydro-1-H-indene-1-butanamine.

FIG. 1. IUPAC equivalent names.

was added to make activity Grade II alumina. Thin layer chromatographic (TLC) analyses were made on Quanta/gram precoated silica gel plates (Quantum Industries, Hanover, NJ). Cholic acid was purchased from Nutritional Biochemicals Corp. (Cleveland, OH), and 5β-cholanic acid was readily prepared via oxidation of cholic acid methyl ester to the triketone, dehydrocholic acid methyl ester, and its subsequent reduction and saponification by the Wolff-Kishner reaction. Lithocholic acid (3α-hydroxy-5β-cholanic acid) was prepared from methyl cholate by the methods of Sarel and Yanuka (4).

The intermediate acid required for the preparation of compound XLI was prepared by ozonolysis of vitamin D_2 (5,6) and chromic acid oxidation of the keto-aldehyde in acetone with an 8N solution of chromic acid in dilute sulfuric acid (7). A Wolff-Kishner reduction of the resulting keto-acid gave the intermediate β-7*a*-dimethyloctahydro-1*H*-indene-1-acetic acid. The reaction of this acid with thionyl chloride gave the acid chloride, which immediately was allowed to react with diazomethane to give the diazoketone. An Arndt-Eistert rearrangement of the diazoketone by the modified procedure of Wilds and Meader (8) gave the benzyl ester of an acid with its chain length increased by one carbon from that of the initial acid. An alkaline saponification of this ester and a repeat of Wilds procedure gave the acid of the desired chain length for preparing the amine XLI in a 30% overall purified yield from vitamin D_2.

The new azasteroids were all prepared in 60-80% yield according to the general method of synthesis via reaction of the appropriate steroidal acid with thionyl chloride to give the steroidal acid chloride and its reaction with dimethylamine, methylethylamine, or ammonia to give the amide and its subsequent reduction with lithium aluminum hydride in tetrahydrofuran to the amine (9). The dichloroazasteroid XXVI was prepared in 50% yield via chlorination of 5β-chol-2-en-24-dimethylamine in carbon tetrachloride at 5 C.

The quaternary ammonium salt XXXII was prepared in 90% yield by the reaction of the tertiary amine XXIX and methyl iodide in acetone:acetonitrile (3:1) in a sealed tube overnight at 70 C. The ammonium tosyl salt XXXIII was readily prepared in 95% yield by heating at 75 C for 1 hr a mixture of 2-dimethylaminoethanol and the 22-tosylate of 24,23-dinor-5β-cholan-22-ol, prepared from lithium aluminum hydride reduction of the methyl ester of XVb (Scheme II).

The oxidation of the primary amine XXXIV with *m*-chloroperbenzoic acid gave the nitro derivative XXXV in 45% purified yield. Compound XXXVI was prepared in nearly quantitative yield by the reaction of XXXIV in pyridine at room temperature overnight with acetic anhydride. The reaction of XXXIV with dimethyl carbamyl chloride in xylene containing 6 equivalents of triethylamine at reflux temperature for 2 hr gave XXXVII in 77% yield. The reaction of the 24-tosylate of 5β-cholan-

24-ol with sodium cyanide in a solution of dimethylsulfoxide:benzene (3:1) at room temperature overnight gave the cyano compound XXXVIII in a 72% purified yield. A similar reaction of the 23-tosylate of 24-nor-5β-cholan-23-ol with sodium cyanide gave compound XXXIX. The reaction of 24-nor-5β-cholan-23-ol at reflux temperature for 7 hr with diethyl chlorophosphite in benzene containing 2 equivalents of triethylamine gave the diethylphosphate ester XL in a 65% yield. The TMS derivatives XVIII and XXVIII were prepared in nearly quantitative yield by the reaction of 25-azacholesterol and XXVII, respectively, in pyridine at 70 C overnight with N,N-bis(trimethylsilyl) acetamide.

When necessary, the compounds were purified by column chromatography. The structures of the intermediates and final products (Fig. 2) were confirmed by IR, NMR, and mass spectroscopy. Purity of the final products as determined by GLC and TLC was > 98%.

Mass spectra of all compounds shown in Figure 2 gave strong M^+ and M-15 peaks (Table I) except for the quaternary ammonium salt XXXII and compound XXXIII. Spectra of all the N,N-dimethylamine compounds showed base peaks at m/e 58 that resulted from the simple fission of the carbon-carbon bond adjacent to the nitrogen atom (α-cleavage). A base peak at m/e 72 in the spectrum of compound XXI indicated a similar cleavage for this compound. The base peak for the primary amine XXXIV occurred at m/e 84, which was the side chain fragment resulting from fission of the 17-20 bond. In the spectra of compounds XXXV-XXXIX, the base peaks occurred at m/e 217, which was the fragment of the steroid nucleus resulting from fission of the 13-17 and 14-15 bonds of the D-ring. In the spectrum of the quaternary ammonium salt XXXII, the base peak occurred at m/e 142 which was the methyl iodide fragment; in the spectrum of XXXIII the base peak occurred at m/e 300, the 24,23-dinor-5β-cholene fragment. Additional physical properties of compounds of Figure 2 are given in Table 1.

General Procedure for the Preparation of Intermediates in Schemes I and II

Methyl 5β-chol-2-enoate (IIa, Scheme I) and methyl 5β-chol-3-enoate (IIIa, Scheme I). A mixture of 24.3 g methyl 3α-tosyl-5β-cholanoate (Ib) and 12.15 g each of lithium carbonate and lithium bromide was refluxed for 1 hr. The solution was filtered while hot, and the filtrate was cooled and poured into ice and water. The semicrystalline material was collected and then dried under vacuum to give 15 g of a ca. 1:1

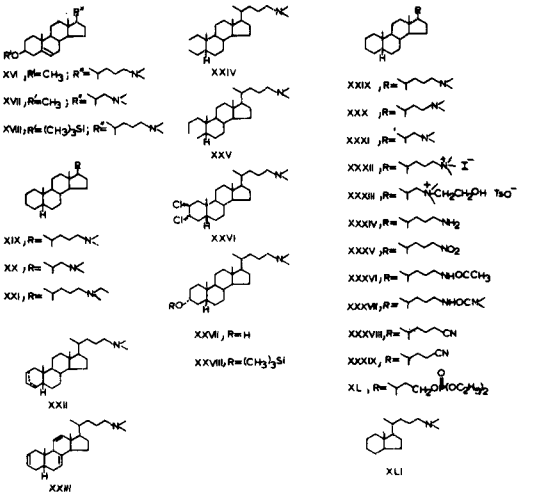


FIG. 2. Structures of nitrogen containing steroids.

mixture of IIa and IIIa as indicated by GLC analyses. The crude mixture was dissolved in hexane and filtered through 100 g hexane-washed alumina. The first 500 ml of hexane eluted 5.7 g of material, and the next 250 ml of hexane:benzene (1:1) eluted an additional 5.7 g of material. Approximately 5.7 g of the material from the hexane fraction dissolved in 30 ml hexane was placed on a 400-g column of Unisil impregnated with 20% silver nitrate in a column 5 x 37 cm tapered to 3 x 43 cm. The column was developed by gradient elution with 1 liter of benzene added dropwise into a 1-liter mixing flask filled with hexane, which was added directly to the column. Once the benzene had been added to the mixing flask, 500 ml of a mixture of benzene:hexane (6:1) also was added dropwise to the mixing flask. Approximately 850 ml of solvent was passed through the column; then 80 20-ml fractions were collected. When the fractions were monitored by GLC on an OV-17 column, fractions 30-40 contained the Δ^3-compound with > 98% purity, and fractions 55-78 contained the Δ^2-compound with > 95% purity. Intermediate fractions containing varying mixtures of the 2 compounds were combined on the basis of degrees of purity for rechromatography. From 3 chromatographic fractionations, we obtained 3.0 g of > 98% pure Δ^3-compound (IIIa, Scheme I), mp 74-76 C, NMR, δ 0.67 (s, 3, 18-methyl), 0.99 (s, 3, 19-methyl), 0.93 (d, 3, J = 6 Hz, 21-methyl), 3.68 (s, 3, $COOCH_3$), multiplet at 5.22-5.78 (olefinic protons); and 4.0 g of > 95% pure Δ^2-compound (IIa, Scheme I), mp 70-71 C, NMR, δ 0.67 (s, 3, 18-methyl), 0.97 (s, 3, 19-methyl), 0.91 (d, 3, J = 5 Hz, 21-methyl), 3.68 (s, 3, $COOCH_3$),

TABLE I

Physical Properties of Nitrogen Containing Steroids

Compound[a]	Melting point (C)	Nuclear magnetic resonance (δ)				Mass spectral data		
		Methyl resonances			N,N-Dimethyl resonances	Base peak	M^+ - first loss	Molecular ion
		18-H	19-H	21-H[b]				
XVI	133-134	0.68	1.00	0.93	2.22(s)[c]	58	386	401
XVII	115-117	0.73	1.03	1.02	2.22(s)	58	358	373
XVIII	102-104	0.68	1.00	0.91	2.22(s)	58	444	459
XIX	87-88	0.65	0.78	0.95	2.22(s)	58	358	373
XX	128-130	0.70	0.80	0.93	2.20(s)	58	330	345
XXI	78-79	0.66	0.78	0.95	2.22(s)[d]	72	372	387
XXII	235-240[e]	0.67	0.97	0.92	2.24(s)	58	356	371
XXIII	215-218[e]	0.63	0.84	0.95	2.24(s)	58	352	367
XXIV	67-68	0.66	0.92	0.91	2.22(s)	58	360	375
XXV	-	0.66	0.93	0.92	2.22(s)	58	360	375
XXVI	-	0.67	1.00	0.92	2.28(s)	58	426	441
XXVII	109-112	0.65	0.92	0.93	2.22(s)	58	374	389
XXVIII	-	0.64	0.90	0.92	2.22(s)	58	446	461
XXIX	63-65	0.66	0.94	0.93	2.22(s)	58	358	373
XXX	80-81	0.66	0.92	0.93	2.22(s)	58	344	359
XXXI	67-68	0.68	0.93	0.93	2.22(s)	58	330	345
XXXII	268-269	0.65	0.92	0.98	3.52(s)	142	373	[f]
XXXIII	206-208	0.65	0.91	1.12	3.22(s)	300	472	[f]
XXXIV	225-231[e]	0.67	0.94	0.95	-	84	330	345
XXXV	-	0.63	0.92	0.93	-	217	360	375
XXXVI	108-109	0.67	0.93	0.91	1.97[g]	217	372	387
XXXVII	156-157	0.65	0.93	0.92	2.91	217	401	416
XXXVIII	108-112	0.66	0.93	0.94	-	217	340	355
XXXIX	126-128	0.68	0.93	0.92	-	217	326	341
XL	24-26	0.63	0.91	0.95	-	155	453	468
XLI	-	0.87[h]	-	0.93[i]	2.21(s)	58	236	251

[a]See Figure 1 for IUPAC equivalent names.

[b]For the 21-methyl resonance, J = 4-6 cps.

[c](s) = singlet.

[d]The N-methylene of the N-ethyl appears as a multiplet at δ 2.0-2.6.

[e]Mp of the amine hydrogen chloride.

[f]Spectrum showed no molecular ion.

[g]N-acetyl resonance.

[h]The δ-methyl resonance of compound XLI.

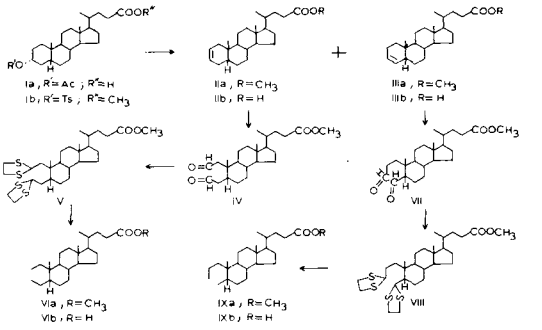
[i]The 7a-methyl resonance of compound XLI.

multiplet at 5.60 (olefinic protons).

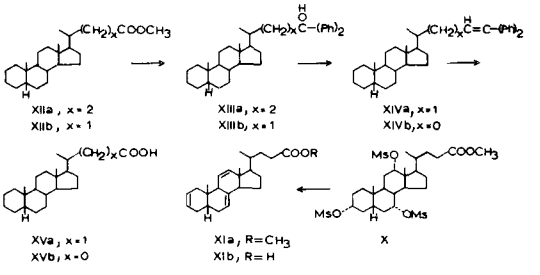
Methyl 2,3-bisethylenedithioketal-2,3-seco-5β-cholanoate (V, Scheme I) and methyl 3,4-bisethylenedithioketal-3,4-seco-5β-cholanoate (VIII, Scheme I) via the dialdehydes VI and VII (Scheme I), respectively. A solution of 2.0 g of IIa or IIIa (Scheme I) in 40 ml methylene chloride at -70 C was treated with ozone until a slight excess was present (ca. 3 hr). To the cold solution, 2.0 g zinc dust and 10 ml acetic acid were added, and the stirred mixture was allowed to come to room temperature. After stirring for 1 hr, the solution was filtered, and the filtrate was concentrated to a small volume, diluted with water, and extracted with hexane. The hexane solution was washed first with 5% sodium bicarbonate solution and then with water and dried over anhydrous sodium sulfate. Removal of the solvent under vacuum gave 2.1 g of the oil dialdehyde IV or VII (Scheme I). To 2.0 g crude dialdehyde (IV, Scheme I) in 1 ml ether and 1 ml ethanedithiol at 5 C, 1.1 ml boron trifluoride etherate was added. After 10 min of stirring the mixture with a glass rod, a thick paste was formed. The paste was triturated with hexane and filtered, and the precipitate was washed with 70% aqueous methanol. The yield of the bisethylenedithioketal derivative V (Scheme I) was 1.8 g, mp 156-158 C, NMR, δ 0.64 (s, 3, 18-methyl), 1.12 (s, 3, 19-methyl), 0.89 (d, 3, J = 4 Hz, 21-methyl), 3.28 [m, 10, -CH(-S-CH_2CH_2S-)] 3.67 (s, 3, $COOCH_3$).

The crude dialdehyde VII (Scheme I), under similar experimental conditions, yielded 1.75 g of 3,4-bisethylenedithioketal derivative VIII (Scheme I), mp 121-123 C, NMR, δ 0.65 (s, 3, 18-methyl), 0.98 (s, 3, 19-methyl), 0.89 (d, 3, J = 5 Hz, 21-methyl), 3.20 [m, 10, -CH(-SCH_2CH_2S-)], 3.67 (s, 3, $COOCH_3$).

Methyl 2,3-seco-5β-cholanoate (VIa, Scheme I) and Methyl 3,4-seco-5β-cholanoate (IXa, Scheme I). A mixture of 1.7 g bisethylenedithioketal (V, Scheme I), 40 ml dry dioxane, and 1.5 teaspoons (ca. 4.5 g) of Raney nickel catalyst (W-2) was refluxed overnight. The solution was mixed with a small quantity of Celite, filtered, and concentrated to dryness under vacuum to give 1.2 g residue. The residue was chromatographed over 30 g hexane-washed neutral alumina (activity grade II), and 100-ml fractions were collected, 2 of hexane, and 3 of hexane:benzene (1:1). On the basis of TLC analyses, the first 2 fractions of hexane:benzene mixture were combined to give, after crystallization from acetonitrile, 1.0 g VIa (Scheme I), mp 38-40 C, NMR, δ 0.66 (s, 3, 18-methyl), 0.92 (s, 3, 19-methyl), 0.89 (d, 3, J = 5 Hz, 21-methyl), 3.68 (s, 3, $COOCH_3$).



Scheme I. Synthesis of intermediates for preparation of compounds in Figure 2.



Scheme II. Synthesis of intermediates for preparation of compounds in Figure 2.

The Raney nickel catalytic reduction of 2.6 g 3,4-bisethylenedithioketal VIII (Scheme I) and work-up as in the reduction of V (Scheme I) gave 1.6 g noncrystalline IXa (Scheme I), NMR, δ 0.66 (s, 3, 18-methyl), 0.89 (d, 3, J = 5 Hz, 21-methyl), 3.68 (s, 3, $COOCH_3$).

Methyl 5β-chola-2,7,11-trienoate (XIa, Scheme II) and its carboxylic acid (XIb, Scheme II). A mixture of 7.0 g methyl 3α,7α,12α-trimethylsulfonyl-5β-cholanoate (X, Scheme I), prepared from the mesylation of cholic acid methyl ester, and 2.5 g each of lithium carbonate and lithium bromide and 60 ml dimethylformamide were refluxed for 30 min. The solution was filtered while hot; the filtrate was cooled and then poured into ice and water; and the precipitate was collected. Crude precipitate of methyl cholatrienoate (XIa, Scheme I) was saponified with 5% potassium hydroxide in 90% ethanol; the solution was diluted with water and acidified with 6N hydrochloric acid; and the precipitate was collected. Recrystallization twice from acetone:methanol gave 4.1 g XIb (Scheme II), mp 170-172 C. A sample of acid was converted to the methyl ester (XIa, Scheme II) with diazomethane. The GLC analyses of this sample of the methyl ester on an SE-30 column showed only one peak. The GLC

analyses of the final product XXIII also showed only one peak, which suggested that primarily the methyl 5β-chola-2,7,11-trienoate was formed during the demesylation.

24-Nor-5β-cholanic acid (XVa, Scheme II) and 24,23-dinor-5β-cholanic acid (XVb, Scheme II). The methyl 5β-cholanoate (XIIa, Scheme II) or the methyl 24-nor-5β-cholanoate (XIIb, Scheme II) in benzene was added to a refluxing solution of 4 equivalents of phenylmagnesium bromide in ether. After the reaction mixture had refluxed for 3 hr, an additional quantity of benzene was added; the ether was distilled off; and the mixture then was refluxed overnight. The benzene solution was poured into ice and water, and the mixture was acidified with a dilute solution of hydrochloric acid. The mixture then was extracted with benzene, and the benzene extract was washed with water, dried over sodium sulfate, and concentrated to dryness under vacuum to give the cholanyldiphenyl carbinol (XIIIa, Scheme II) or the 24-norcholanyldiphenyl carbinol (XIIIb, Scheme II). The diphenyl carbinol derivatives (15 g) in 150 ml dioxane containing 15 ml sulfuric acid, by stirring overnight at room temperature, were converted to the diphenylethylene derivatives XIVa (Scheme II), mp 117-118 C, and XIVb (Scheme II), as an oil. Ozonization of XIVa (Scheme II) in methylene chloride at -80 C and oxidation of the resultant crude aldehyde mixture with chromic acid solution in acetone (7) gave, in nearly quantitative yield, the acid XVa (Scheme II), mp 175-177 C (lit.(10), mp 177 C). A similar sequence of reactions with XIVb (Scheme II) gave the dinoracid XVb (Scheme II), mp 209-212 C (lit.(10), mp 214 C).

RESULTS AND DISCUSSION

The inhibitive ranges of concentrations of these newly prepared compounds in the yellowfever mosquito and the tobacco hornworm are presented in Table II. Compounds XVI, XIX, and XXIX, which were our most active azasteroids in previous tests (2), are included for comparison. Because the confused flour beetle and the house fly were not particularly affected by the majority of these new compounds, the results of the biological tests with these 2 insects will be summarized briefly.

In the house fly test system, only 6 of the compounds listed in Table II (XVI, XVII, XXIV, XXV, XXXI, and XXXIV) inhibited growth and development of or killed 75% of the test insects at a dietary concentration of 150-375 ppm. However, several of the other azasteroids at concentrations within this range did cause the characteristic effects previously observed for azasteroid inhibitors (2). Of the 6 active compounds, only compound XVII has the steroid nucleus similar to XVI, our most active azasteroid in this insect, though it differed in its side chain by having 2 less carbon atoms. The other 4 compounds with activity in this insect all have the A/B ring *cis* configuration; yet, they differ from each other in several respects including chain length, opened ring A, or the possession of a primary amino group.

Of the more than 80 azasteroids tested on the confused flour beetle, only compounds XVI and XVII, which possessed a Δ^5-bond and a 3β-methoxy group, were active at a dietary concentration of $<$ 100 ppm. Except for compounds XXVI and XXXVII, that were active in this insect at concentrations between 500-1000 ppm, all other compounds listed in Table II were inactive even at concentrations of 1000 ppm. The results suggest that an azasteroid such as XVI with a steroid nucleus without a functional group other than a Δ^5-bond could be quite active in the confused flour beetle.

Although the majority of the new nitrogen containing steroids were not very active in either the confused flour beetle or the house fly, a number of these compounds were active at $<$ 1 ppm in the yellowfever mosquito and the tobacco hornworm. The results of tests of the yellowfever mosquito with Δ^5-steroids showed that compounds XVI and XVII were equally active, but the TMS-derivative XVIII was only ca. one-tenth as inhibitive as XVI. Of the azasteroids with the A/B ring *trans* configuration, compound XX, with the shortened side chain, had a greater inhibitive effect than XIX. However, the substitution of an N-ethyl group for an N-methyl, as in compound XXI, resulted in no change in biological activity from that of XIX.

When we compare the inhibitive effects of the 17 compounds with an A/B ring *cis* configuration in the mosquito with the activity of compound XXIX, only compounds XXX and XXXI, which have a shorter side chain than XXIX, and compounds XXXII and XXXIV were more active. Interestingly, compound XXXIV, a steroidal primary amine, was ca. 4 times more active in the yellowfever mosquito than the steroidal tertiary amine XXIX. The A/B ring *cis* steroids XXXV-XXXIX, which are without a tertiary amino group, were inactive at a concentration of 10 ppm except for the acetyl derivative XXXVI, which was active at concentrations of 5-10 ppm in this insect. The seco compounds XXIV and XXV and compound XXII, which consists of a 1:1 mixture of the 2- and 3-ene compounds, were equal in activity to XXIX in the yellowfever mosquito larvae. The

TABLE II

Range of Concentrations of Nitrogen Containing Steroids in Larval Diet or Medium Required to Kill or Inhibit Development in 75% of the Test Insects

Compound[a]	Yellowfever mosquito (ppm)	Tobacco hornworm (ppm)
Δ^5-Azasteroids		
XVI	0.50 - 1.00	0.25 - 0.50
XVII	0.50 - 1.00	4.00 - 8.00
XVIII	5.00 -10.00	33 -65
5α-Azasteroids		
XIX	0.50 - 1.00	0.50 - 0.75
XX	0.25 - 0.50	4.00 - 8.00
XXI	0.50 - 1.00	0.50 - 0.75
Mono- and triunsaturated 5β-azasteroids		
XXII	0.50 - 1.00	0.10 - 0.25
XXIII	1.00 - 2.50	0.10 - 0.25
Seco-5β-azasteroids		
XXIV	0.50 - 1.00	0.25 - 0.50
XXV	0.50 - 1.00	0.25 - 0.50
5β-Azasteroids		
XXVI	2.50 - 5.00	0.50 - 1.00
XXVII	2.50 - 5.00	1.00 -10.0
XXVIII	>10	<16
XXIX	0.50 - 1.00	0.10 - 0.25
XXX	0.25 - 0.50	0.25 - 0.50
XXXI	0.25 - 0.50	30
XXXII	0.25 - 0.50	>130
5β-Steroids with other nitrogen substituents		
XXXIII	2.50 - 5.00	>130
XXXIV	0.10 - 0.25	16 - 33
XXXV	>10	>65
XXXVI	5.00 -10.0	>130
XXXVII	>10	>260
XXXVIII	>10	>130
XXXIX	>10	>130
5β-Steroid with phosphorus substituent		
XL	>10	>260
Nonsteroidal amine		
XLI	5.0 - 10.0	130-260

[a]See Figure 1 for IUPAC equivalent names.

dichloro derivative XXVI was active at concentrations of 2.5-5.0 ppm in this insect.

Of the compounds listed in Table II, only compounds XXII and XXIII, which have the A/B ring *cis* configuration, as does the previously most active azasteroid XXIX (2), were equal in activity to XXIX in the tobacco hornworm. Compound XXX, which has one carbon less in its side chain than XXIX, exhibited slightly less biological activity than XXIX; compound XXXI with 2 carbons less in its side chain than XXIX showed far less inhibitive activity. The presence of a nitrogen substituent other than the tertiary amino group in the side chain of the A/B ring *cis* steroids XXXII-XXXIX also resulted in a considerable loss of biological activity in the tobacco hornworm.

The Δ^5-azasteroid XVII, with a side chain containing 2 less carbon atoms than XVI, and the TMS derivative XVIII were far less inhibitive than the 3-methoxy-Δ^5-azasteroid (XVI) in the tobacco hornworm. Additionally, the A/B ring *trans* azasteroid XX, which has a shorter side chain than XIX, showed far less biological activity than XIX in this insect. However, the replacement of an N-methyl group with an N-ethyl as in XXI resulted in no change in activity in the tobacco hornworm. The phosphate ester XL was without inhibitive activity in all test systems at the highest dietary concentration examined.

In this comparative study, none of the new nitrogen containing steroids was appreciably more inhibitive than the most active azasteroids

tested previously (2). However, the results indicated that a prerequisite for an azasteroid that will inhibit insect growth and development was a side chain with a primary, secondary, or tertiary amino group, and in most instances preferably a tertiary amino group. Thus, to determine the minimal structural requirement for this type of inhibition of insect growth and development, we synthesized compound XLI, which lacked the A and B rings of the steroid nucleus. This compound inhibited growth and development in both the yellowfever mosquito and the tobacco hornworm though it was not as potent as the more active azasteroids (Table II). Also like certain azasteroids, it blocked the Δ^{24}-sterol reductase enzyme of the tobacco hornworm. At 130 ppm in the diet, it caused reduction of the cholesterol level to $< 5\%$ of the total tissue sterol as opposed to the 80-85% found in control insects, and increased the desmosterol content from the normal range of 1.0-1.5% to ca. 50% of the total sterol present in the tobacco hornworm.

Our previous results with azasteroid inhibitors (2) demonstrated the feasibility of disrupting the hormone mediated processes of insects with relatively simple nonhormonal compounds that apparently interfere with hormone biosynthesis and metabolism. As a result of the present study, we now have synthesized simple nonsteroidal amines, some of which proved to be inhibitors of insect growth, molting, and metamorphosis (11). These nonsteroidal compounds should permit us to expand the type of chemicals that block the hormone regulated processes of growth, molting, and metamorphosis in insects, and, thus, may lead to the development of new types of safe, selective insect control chemicals.

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Enzymatic Conversion of 5α-Cholesta-7,14-dien-3β-ol to 5α-Cholesta-8,14-dien-3β-ol

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ABSTRACT

5α-Cholesta-7,14-dien-3β-ol, previously shown to be efficiently converted to cholesterol upon incubation with rat liver homogenate preparations under aerobic conditions, has been studied as to its possible conversion to 5α-cholesta-8,14-dien-3β-ol. Efficient conversion was observed upon incubation in the presence of washed microsomes of rat liver under anaerobic conditions. This observation is of importance in consideration of possible metabolic pathways in the biosynthesis of cholesterol.

INTRODUCTION

The configuration of the hydrogen at carbon atoms in the various sterols mentioned in this paper is α. The designation of the configuration as 5α is omitted throughout the text to conserve space.

Cholesta-8,14-dien-3β-ol and cholesta-7,14-dien-3β-ol have been shown to serve as efficient precursors of cholesterol upon incubation with rat liver homogenate preparations incubated under aerobic conditions (1-5). In a previous study (4), we directed our attention to the possible enzymatic conversion of cholesta-8,14-dien-3β-ol to cholesta-7,14-dien-3β-ol. Microsomal preparations from rat liver have been shown to catalyze the efficient conversion of a number of Δ^8-sterols to the corresponding Δ^7-sterols (6-8). The reaction proceeds under anaerobic conditions and occurs in the absence of added cofactors (6-8). Wilton, et al., (9) and Scala, et al., (10) have reported findings which indicate the reversibility of the conversion of cholest-8-en-3β-ol to cholest-7-en-3β-ol. The enzymatic reduction of the Δ^{14}-double bond of $\Delta^{8,14}$-sterols has been reported to be dependent on the presence of reduced nicotinamide adenine dinucleotide phosphate (2,9). To investigate the possible convertibility of cholesta-8,14-dien-3β-ol to cholesta-7,14-dien-3β-ol, we incubated the former sterol, in labeled form, with preparations of washed microsomes of rat liver under anaerobic conditions in the absence of added reduced nicotinamide adenine dinucleotide phosphate. No significant conversion of the $\Delta^{8,14}$ to the $\Delta^{7,14}$-sterol could be demonstrated (4).

We now wish to report that when cholesta-7,14-dien-3β-ol was incubated with washed microsomes of rat liver under anaerobic conditions, the efficient conversion of the $\Delta^{7,14}$-sterol to cholesta-8,14-dien-3β-ol was observed.

MATERIALS AND METHODS

Procedures used for the measurement of radioactivity (3,4), colorimetric assay of steryl acetates (7), gas liquid radiochromatographic analyses (11), and the preparation of steryl acetates (12) have been described previously. Chromatographic separations of steryl acetates on columns of alumina-Super Cel-silver nitrate (3,4,7,11,12) and on columns of Silica Gel-Super Cel-silver nitrate (3,4) were carried out as previously described, except that in the latter case Silica Gel GF was used in place of Silica Gel G. Samples of cholesteryl acetate (7,12), 3β-acetoxy-cholesta-8,14-diene (3), 3β-acetoxy-cholesta-7,14-diene (4), 3β-acetoxy-cholest-8(14)-ene (7), 3β-acetoxy-cholest-8-ene (7), 3β-acetoxy-cholest-7-ene (7,12), [3α-^{3}H]-cholest-8-en-3β-ol (7), and [3α-^{3}H]-cholesta-7,14-dien-3β-ol (4) were prepared previously.

The livers (44.6 g) of 8 female Sprague-Dawley rats were homogenized in 100 ml 0.1 M potassium phosphate buffer (pH 7.4), using a loose fitting Teflon-on-glass homogenizer. Whole cells, nuclei, and mitochondria were removed by centrifugation at 10,000 x g for 30 min at 0 C. The supernatant fraction was recentrifuged at 100,000 x g for 1 hr. The isolated microsomal fraction was suspended in buffer and recentrifuged for 1 hr at 100,000 x g. The washed microsomal fraction so isolated was suspended in 100 ml phosphate buffer, and 25 ml portions were added to 3 flasks.

[3α-^{3}H]-Cholesta-7,14-dien-3β-ol (50 μg; 8 x 10^5 cpm) in 0.1 ml of propylene glycol was incubated for 3 hr at 37 C under anaerobic conditions, as described previously (7), with 25 ml washed microsomal suspension. A similar incubation of the labeled [3α-^{3}H]-cholesta-7,14-dien-3β-ol was carried out as described above with 25 ml microsomal suspension which had been heated at 80 C for 30 min prior to incubation. To establish that the washed microsomal preparation was active in the catalysis of the

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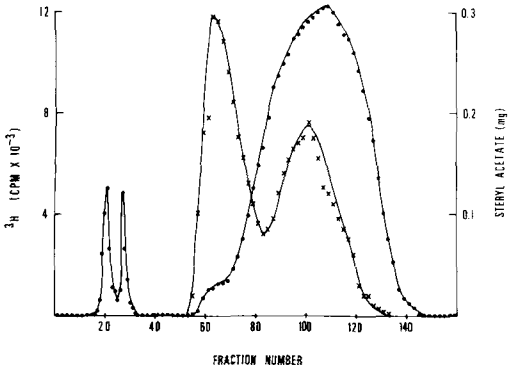

FIG. 1. Silica Gel GF-Super Cel-silver nitrate column (50 x 1 cm) chromatographic analysis of acetates of sterols recovered after incubation of [3α-3H]-cholesta-7,14-dien-3β-ol with washed rat liver microsomes under anaerobic conditions. •—• = radioactivity; x—x = steryl acetate measured colorimetrically. The first peak was due to 3β-acetoxy-cholesta-7,14-diene and the second peak was due to 3β-acetoxy cholesta-8,14-diene. The solvent (hexane:benzene, 70:30) flow rate was 0.20 ml/min. Fractions 4.0 ml in volume were collected.

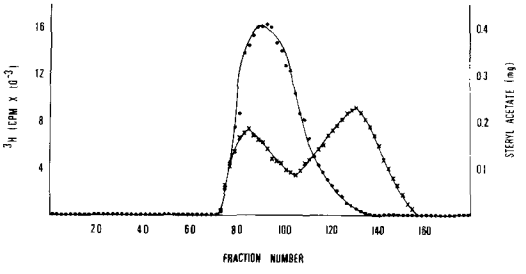

FIG. 2. Silica Gel GF-Super Cel-silver nitrate column (50 x 1 cm) chromatographic analysis of acetates of sterols recovered after incubation of [3α-3H]-cholesta-7,14-diene-3β-ol with washed rat liver microsomes under anaerobic conditions. The microsomes were heated at 80 C for 30 min prior to incubation. •—• = radioactivity; x—x = steryl acetate measured colorimetrically. The first peak was due to 3β-acetoxy-cholesta-7,14-diene, and the second peak was due to 3β-acetoxy-cholesta-8,14-diene. The solvent (hexane:benzene, 70:30) flow rate was 0.175 ml/min. Fractions 3.5 ml in volume were collected.

conversion of cholest-8-en-3β-ol to cholest-7-en-3β-ol, [3α-^{3}H]-cholest-8-en-3β-ol (∿34 μg; 8.2 x 10^5 cpm) in 0.4 ml propylene glycol was incubated with 25 ml microsomal preparation under anaerobic conditions as described above. Each of the incubation mixtures were heated under reflux for 1 hr with 25 ml 10% ethanolic KOH solution and extracted 3 times with 100 ml portions of petroleum ether. The combined extracts were washed with water and dried over anhydrous sodium sulfate. Recovered sterols were acetylated with acetic anhydride and pyridine, and aliquots of the resulting acetates, in the case of the incubations of the $\Delta^{7,14}$-sterol, were subjected to chromatography on 50 x 1 cm columns of Silica Gel GF-Super Cel-silver nitrate using hexane:benzene (70:30) as the eluting solvent. Unlabeled 3β-acetoxy-cholesta-8,14-diene and 3β-acetoxy-cholesta-7,14,diene were added as markers. Aliquots were taken for assay of radioactivity and steryl acetate content. In the case of steryl acetates derived from the incubation of the [3α-^{3}H]-cholest-8-en-3β-ol, an aliquot of the acetylated sterols was subjected to chromatography on a 50 x 1 cm alumina-Super Cel-silver nitrate column using hexane:benzene (90:10) as the eluting solvent. 3β-Acetoxy-cholest-8(14)-ene and cholesteryl acetate were added as markers. Aliquots were taken for assay of steryl acetate content and radioactivity.

RESULTS

Figure 1 shows the chromatogram obtained from the sterols recovered after incubation of [3α-^{3}H]-cholesta-7,14-dien-3β-ol with washed microsomal preparation under anaerobic conditions. The chromatographic system used permits the separation of the acetates of monounsaturated sterols, such as the acetates of the Δ^8, Δ^7, and $\Delta^{8(14)}$-C_{27} sterols, from acetates of the conjugated C_{27} dienols, such as the $\Delta^{7,14}$, $\Delta^{8,14}$, $\Delta^{5,7}$-sterols. Moreover, these conjugated dienes were separable from one another on this type of column, as shown in Figure 1 and in previous publications (4,5,13). The bulk of the radioactivity was associated chromatographically with 3β-acetoxy-cholesta-8,14-diene. A small amount of recovered radioactivity (5.7%) was recovered in fractions 16 through 33, corresponding to the mobility of the monounsaturated steryl acetates noted above. That the observed high conversion of the added $\Delta^{7,14}$-sterol to the $\Delta^{8,14}$-sterol was not due to a nonenzymatic isomerization of the double bond of the added substrate during any portion of the experiment was indicated by the results obtained upon incubation of the labeled substrate with the microsomal preparation, which had been heated for 30 min at 80 C prior to incubation. The resulting chromatogram of the sterols recovered from this incubation is shown in Figure 2, and indicates that virtually all of the radioactivity shows the chromatographic mobility of the added substrate.

That the enzyme preparation utilized in these experiments was active in the catalysis of the conversion of cholest-8-en-3β-ol to cholest-7-en-3β-ol was indicated by the results presented below. The acetates of the sterols recovered after incubation of [3α-^{3}H]-cholest-8-en-3β-ol

were chromatographed on a system which permited the separation of the acetates of cholestan-3β-ol, cholest-8(14)-en-3β-ol, cholest-8-en-3β-ol, cholest-7-en-3β-ol, and cholesterol (4,5,11). In this system, the Δ^8 and Δ^7-isomers eluted from the column between the $\Delta^{8(14)}$ and Δ^5-isomers. The contents of this region of the chromatogram (fractions 19 through 25; Figure 3) were pooled and subjected to gas liquid radiochromatographic analysis. Approximately 92% of the radioactivity was associated with 3β-acetoxy-cholest-7-ene.

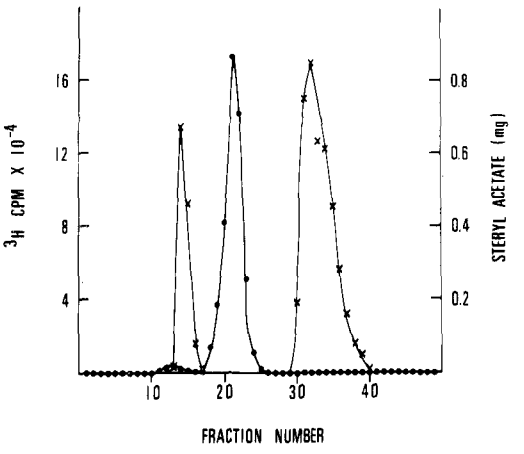

FIG. 3. Alumina-Super Cel-silver nitrate column (50 x 1 cm) chromatographic analysis of acetates of sterols recovered after incubation of [3α-^{3}H]-cholest-8-en-3β-ol with washed rat liver microsomes under anaerobic conditions. ●—● = radioactivity; x—x = steryl acetate measured colorimetrically. The first peak was due to 3β-acetoxy-cholest-8(14)-ene and the second peak was due to cholesteryl acetate. The solvent (hexane:benzene, 90:10) flow rate was 0.143 ml/min. Fractions 4.3 ml in volume were collected.

DISCUSSION

The results presented herein indicate the efficient conversion of cholesta-7,14-dien-3β-ol to cholesta-8,14-dien-3β-ol upon incubation with washed microsomes of rat liver, presumably free of significant amounts of reduced nicotinamide adenine dinucleotides required for the reduction of the Δ^{14}-double bond of these sterols, under anaerobic conditions. Under the same conditions, little or no enzyme catalyzed conversion of the $\Delta^{8,14}$-sterol to the $\Delta^{7,14}$-sterol occurs (4,5). The combined results suggest the possibility that, under the conditions employed, the enzymatic conversion of cholesta-7,14-dien-3β-ol to cholesta-8,14-dien-3β-ol may be a reversible process with the equilibrium favoring the $\Delta^{8,14}$-sterol. Such a situation would be in contrast to the proposed reversibility (9,10) of the conversion of cholest-8-en-3β-ol to cholest-7-en-3β-ol, in which the equilibrium favors that Δ^7-sterol (6-10).

The demonstration of the convertibility of the $\Delta^{7,14}$-sterol to the $\Delta^{8,14}$-sterol constitutes an extension of our knowledge concerning the metabolism of this potential intermediate in sterol biosynthesis. We have shown previously that cholesta-7,14-dien-3β-ol, upon incubation with a 10,000 x g supernatant fraction of a rat liver homogenate, was efficiently converted to cholesterol under aerobic conditions (4,5). Incubation of the diene under the same conditions under anaerobiosis yielded cholest-7-en-3β-ol as the major product (4,5). Significant amounts of cholest-8(14)-en-3β-ol also were formed under these conditions (4,5).

While significant information exists relative to the metabolism of cholesta-7,14-dien-3β-ol, no information exists relative to the occurrence of this sterol in tissues and the mode of formation of this compound. It is important to note that Alexander, et al., (14) have recently reported the formation of radioactive material which co-chromatographed on thin layer chromatography with authentic 4,4-dimethyl-cholesta-7,14-dien-3β-ol after incubation of labeled lanost-7-en-32,3β-diol with washed microsomes of rat liver in the presence of an NAD and NADPH generating system and a trap of 4,4-dimethyl-cholesta-7,14-dien-3β-ol. The Δ^7-lanosterol derivative used as a substrate was a probable intermediate in the conversion of lanosta-7,24-dien-3β-ol to cholesterol. The occurrence in skin of lanosta-7,24-dien-3β-ol and its formation from labeled precursors has been reported previously (15-18).

Further studies directed regarding the occurrence, formation, and metabolism of cholesta-7,14-dien-3β-ol are in progress.

ACKNOWLEDGMENTS

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Studies on Tocopherol Derivatives: V. Intestinal Absorption of Several d,1-3,4-$^3H_2$-α-Tocopheryl Esters in the Rat

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ABSTRACT

Twelve d,1-3,4-$^3H_2$-α-tocopheryl esters were synthesized from d,1-3,4-$^3H_2$-α-tocopherol. They were acetate, propionate, butyrate, isobutyrate, caprylate, palmitate, acid succinate, benzoate, nicotinate, o-hydroxybenzoate, o-acetoxybenzoate, and pivalate. The hydrolysis of these esters with bile-pancreatic juice and with 9,000 x g supernatant of small intestine and liver homogenates of rats was examined. When these esters were incubated in small intestine or liver supernatants, hydrolysis occurred at a similar rate. In the incubation experiments, α-tocopheryl acetate, propionate, butyrate, isobutyrate, caprylate, palmitate, and acid succinate were classified as an easily hydrolyzable group. α-Tocopheryl benzoate and nicotinate were in a moderately hydrolyzable group. o-Hydroxybenzoate and pivalate, which resisted hydrolysis, were in a scarcely hydrolyzable group. o-Acetoxybenzoate was easily hydrolyzed to the o-hydroxybenzoate. Hydrolysis on straight chain fatty acid esters of α-tocopherol easily occurred in bile-pancreatic juice. In in vivo experiments, the lymphatic absorption rate of 6 esters, acetate, palmitate, acid succinate, nicotinate, o-hydroxybenzoate, and pivalate, was measured on thoracic duct fistula rats. Easily hydrolyzable esters were recovered mostly in lymph as α-tocopherol, whereas, an ester which strongly resisted hydrolysis, such as pivalate, appeared mainly unchanged. This fact suggested that hydrolysis of α-tocopheryl esters was not necessarily a prerequisite for intestinal absorption. The percentage of absorption of slowly hydrolyzed esters in lymph was relatively lower than that of moderately or easily hydrolyzable esters.

INTRODUCTION

Vitamin E (α-tocopherol) generally has been used in the form of an ester for medication or nutrition because of its susceptibility to oxidation. α-Tocopherol (1), α-tocopheryl acetate (2), and α-tocopheryl acid succinate (3) have been used as substrates for the study of vitamin E, because the metabolic fate of these compounds is thought to be similar in all tissues. Blomstrand, et al., (4) have reported that α-tocopheryl acetate administered orally to human males was hydrolyzed prior to being absorbed as α-tocopherol into the lymph. Gallo-Torres (2) reported a similar scheme in rats using the same technique. Absorption of ^3H-labeled α-tocopheryl nicotinate was approximately 2 times higher than that of ^3H-labeled α-tocopheryl acetate (5). Recently Jayanthi Bai, et al., (6) pointed out the presence of vitamin E esterase activity in vitro on α-tocopheryl acetate in tissues of chicken. We were interested in the hydrolysis of α-tocopheryl esters, including acetate and nicotinate, and in the intestinal absorption of these esters.

In vitro and in vivo experiments were performed to determine whether or not hydrolysis of α-tocopheryl esters was necessary for intes-

TABLE I

Elemental Analysis of d,1-α-Tocopheryl Esters

d,1-α-Tocopheryl	Molecular formula	Calculated		Found	
		C(%)	H(%)	C(%)	H(%)
Propionate	$C_{32}H_{54}O_3$	78.96	11.18	78.90	11.35
Butyrate	$C_{33}H_{56}O_3$	79.14	11.27	79.46	11.25
Isobutyrate	$C_{33}H_{56}O_3$	79.14	11.27	79.39	11.20
Caprylate	$C_{37}H_{64}O_3$	79.79	11.58	79.57	11.62
Benzoate	$C_{36}H_{54}O_3$	80.85	10.18	80.81	10.18
Pivalate	$C_{34}H_{58}O_3$	79.32	11.36	79.54	11.50

TABLE II

Assignments of Nuclear Magnetic Resonance Spectra and Mass Spectra of d,1-α-Tocopheryl Esters[a]

Compound	τ Value[b]	Molecular ion m/e	Fragment ion m/e
Propionate	7.4 (2H, q, $CH_3\underline{CH_2}$-)	486	430
Butyrate	7.4 (2H, t, $CH_3CH_2\underline{CH_2}$-)	500	430
Isobutyrate	7.1 (1H, m, >$\underline{CH}$-)	500	430
Caprylate	7.4 (2H, t, -$\underline{CH_2}CO$-)	556	430
Benzoate	1.7-2.6 (5H, m, arom.$\underline{H}$)	534	430
Pivalate	8.6 (9H, s, $(\underline{CH_3})_3C$-)	514	430

[a]Only signals characteristic of esters are cited.

[b]Chemical shifts were expressed as τ value. Signals are abbreviated as follows: s, singlet; t, triplet; q, quartet; m, multiplet.

TABLE III

Rf-Values of d,1-α-Tocopherol and Derivatives on Thin Layer Chromatograms

Compound	Solvent[a] A	B	C	D	E	F	G	H
α-Tocopherol	0.50	0.21	0.25	0.41	0.76	0.77	0.42	0.75
α-Tocopheryl acetate	0.68[c]	0.23	0.32					
α-Tocopheryl propionate	0.83[c]	0.27	0.42					
α-Tocopheryl butyrate	0.89[c]	0.30	0.50					
α-Tocopheryl isobutyrate	0.90[c]	0.35	0.51					
α-Tocopheryl caprylate	0.95[c]	0.44	0.54					
α-Tocopheryl palmitate	0.95	0.51	0.67	0.84[c]				
α-Tocopheryl acid succinate[b]								0.22[c]
α-Tocopheryl benzoate	0.89[c]	0.36	0.52					
α-Tocopheryl nicotinate					0.44	0.45[c]		
α-Tocopheryl o-hydroxybenzoate	0.93	0.52	0.63	0.77[c]				
α-Tocopheryl o-acetoxybenzoate	0.43	0.12	0.20[c]					
α-Tocopheryl pivalate	0.92[c]	0.34	0.57				0.68	
α-Tocopheryl quinone	0.05	0.02	0.04	0.14	0.56	0.38	0.05	0.79
Dimers and/or trimers	0.93	0.23	0.52	0.80	0.93	0.89	0.51	0.91

[a]Solvents (v/v): A = petroleum ether:diisopropyl ether (8:2); B = hexane:benzene (1:1); C = hexane:ethyl acetate (95:5); D = hexane:ethyl acetate (9:1); E = benzene:ethyl acetate (8:2); F = chloroform:hexane (95:5); G = benzene:hexane (95:5); H = benzene:methyl alcohol (9:1).

[b]Thin layer plate contained 3.7% ethylene diamine tetra acetic acid.

[c]The solvent used for identification of radioactivity in incubation products and lymph.

tinal absorption and to examine the rate of absorption into the lymphatics of α-tocopheryl esters. Acetate, propionate, butyrate, isobutyrate, caprylate, palmitate, acid succinate, benzoate, nicotinate, o-hydroxybenzoate, o-acetoxybenzoate, and pivalate were used as substrates in the in vitro experiments. Six of these esters, acetate, palmitate, acid succinate, nicotinate, o-hydroxybenzoate, and pivalate, were examined for lymphatic absorption using thoracic duct fistula rats.

MATERIALS AND METHODS

Nuclear magnetic resonance (NMR) spectra were measured using $CDCl_3$ solution with a JNM-PS-100 (100 Mc) spectrometer (Japan Electron Optics Laboratory, Tokyo, Japan). The mass spectra were recorded on a JMS-01 SG-2 spectrometer (Japan Electron Optics Laboratory).

Acetate (7), palmitate (8), acid succinate (9), nicotinate (10), o-hydroxybenzoate (11), and o-acetoxybenzoate (11) of α-tocopherol used as carriers were prepared by the procedures described previously. Remaining esters, such as propionate, butyrate, isobutyrate, caprylate, benzoate, and pivalate, were obtained by condensing d,1-α-tocopherol with the corresponding acid in polyphosphate ester (PPE) (12), followed by purification by silica gel column chromatography. Identification of esters thus obtained was performed by elemental analysis (Table I), NMR spectra (Table II), and mass spectra (Table II). R_f values of 12 kinds of esters, α-tocopherol, α-tocopheryl quinone, and

dimers and/or trimers of α-tocopherol (13-15) on thin layer chromatography (TLC) are shown in Table III. In the case of acid succinate, the thin layer plate contained 3.7% ethylene diamine tetra acetic acid to avoid tailing of the spot (16).

Preparation of Labeled Compounds

d,l-3,4-$^3H_2$-α-Tocopherol. Isophytol (19.2 mg, 65 μmoles), 1,2-$^3H_2$-isophytol (10.6 mCi; specific activity, 4.0 mCi/mg; radiochemical purity, 87.7%) and trimethylhydroquinone (8.5 mg, 56 μmoles) were condensed under reflux for 4 hr in ethyl acetate (0.4 ml) using 0.03 ml sulfuric acid as a catalyst. After cooling, products were extracted with 30 ml hexane. The hexane layer was washed with diluted aqueous sodium hydrogen carbonate and water. The washed hexane layer was evaporated in vacuo, and the obtained residue was purified on preparative TLC in petroleum ether:diisopropyl ether (8:2, v/v). Total radioactivity of the purified compound was 4.4 mCi and specific activity was 262 μCi/mg. The radiochemical purity was examined by TLC fractionation method described in the analysis of the incubation product. More than 97% of the radioactivity was associated with α-tocopherol. No difference in radiopurity was found in previously synthesized d-5-methyl-^{14}C-α-tocopherol (17) in 2 solvent systems (hexane:ethyl acetate, 95:5; hexane:benzene, 1:1) of TLC.

d,l-3,4-$^3H_2$-α-Tocopheryl propionate. A mixture of d,1-3,4-$^3H_2$-α-tocopherol (1.08 mCi, 4.1 mg), d,l-α-tocopherol (19 mg, 44 μmoles), propionic acid (4.4 mg, 59 μmoles), and PPE (0.7 g) were heated to 80-100 C and stirred for 6 hr. After cooling, 5% aqueous sodium hydrogen carbonate was added carefully to the reaction mixture until neutralized. The product was extracted with 30 ml ethyl acetate, washed with water, and evaporated in vacuo. The oil residue obtained again was washed with water, purified on preparative TLC using petroleum ether:diisopropyl ether (8:2, v/v). The yield was 20 mg of d,1-3,4-$^3H_2$-α-tocopheryl propionate of more than 97% radiopurity. The specific activity of the propionate was 34 μCi/mg.

d,1-3,4-$^3H_2$-α-Tocopheryl butyrate, d,1-3,4-$^3H_2$-α-tocopheryl isobutyrate, d,1-3,4-$^3H_2$-α-tocopheryl caprylate, d,1-3,4-$^3H_2$-α-tocopheryl palmitate, d,1-3,4-$^3H_2$-α-tocopheryl benzoate, and d,1-3,4-$^3H_2$-α-tocopheryl o-hydroxybenzoate were synthesized and purified by the same procedure as described for the preparation of propionate. Labeled α-tocopheryl esters were identified by R_f values on TLC with nonradioactive reference compounds. The radiopurity of all the labeled esters was more than 97%.

Thin Layer Chromatography

TLC was performed on plates of Silica Gel GF_{254} (E. Merck Co., Darmstadt, Germany). The thickness of the plates was 0.75 mm for preparative work and 0.25 mm for analysis. The TLC zones were scraped and radioactive material extracted by shaking vigorously for 2 min with 15 ml scintillation fluid.

Measurement of Radioactivity

Radioactivity was measured by a liquid scintillation counter, Aloka, Model LSC-652 (Nihon Musen, Tokyo, Japan). Counting efficiency was determined by external standardization. All results were calculated as disintegrations per min, and all determinations were performed twice. Scintillation fluid consisted of 16 g 2,5-diphenyl oxazole (PPO), 0.9 g 2,2′-p-phenylene bis(4-methyl-5-phenyl oxazole) (POPOP), 400 g naphthalene, 400 g ethyl cellosolve, 600 ml toluene, and 3,000 ml dioxane.

In Vitro Experiments

Male Wistar rats weighing 270-290 g were used. The combination of the rats' bile and pancreatic juice was collected by cannulation of the common bile-pancreatic duct (18). Rats were fasted for 16 hr, then killed by decapitation, and the livers and the small intestines were removed immediately into ice cold 0.1 M phosphate buffer at pH 7.4. The first 11 cm of small intestine from the pyloric end was discarded, the next 33 cm was homogenized in 10 ml 0.1 M phosphate buffer per gm of tissue.

Incubation of d,1-3,4-$^3H_2$-α-Tocopheryl Esters in Bile-Pancreatic Juice

To 1 ml of bile-pancreatic juice of rats, 0.1 ml 1% Tween 80 solution of labeled α-tocopheryl ester (0.13 μmole, 2 μCi) was added and incubated at 37 C for 60 min. After incubation, 0.05 ml of the incubation mixture was spotted on a thin layer plate with 200 μg each of α-tocopheryl ester, α-tocopherol, α-tocopheryl quinone, and dimers and/or trimers of α-tocopherol as carriers. The plate then was developed in the solvent systems shown in Table III.

Incubation of d,1-3,4-$^3H_2$-α-Tocopheryl Esters in Liver or Small Intestine 9,000 x g Supernatant

A 10% homogenate of liver or small intestine from rats was prepared in 0.1 M phosphate buffer at pH 7.4 and centrifuged at 9,000 x g for 20 min. Six ml of supernatant was added to 9 ml of 0.1 M phosphate buffer and 2 ml of

TABLE IV

Hydrolysis of Labeled α-Tocopheryl Esters in Bile-pancreatic Juice[a]

d,l-3,4-$^3H_2$-α-Tocopheryl	Radioactivity (%)[b]		
	As ester	α-Tocopherol	α-Tocopheryl quinone
Acetate	29	66	2
Propionate	25	60	7
Butyrate	29	63	2
Isobutyrate	89	7	1
Caprylate	22	68	3
Palmitate	27	49	10
Acid succinate	94	2	1
Benzoate	94	3	1
Nicotinate	86	3	2
o-Hydroxybenzoate	93	3	2
o-Acetatybenzoate	15 (83)[c]	1	1
Pivalate	95	1	1

[a]Each ester (0.13 μmole, 2 μCi) was incubated in the bile-pancreatic of rats for 60 min. Incubation conditions were described in the text.

[b]Mean of 2 experiments.

[c]Number in the parenthesis shows the percentage of o-hydroxybenzoate.

0.5% Tween 80 solution containing 0.27 μmole (4 μCi) of d,l-3,4-$^3H_2$-α-tocopheryl ester and incubated at 37 C for 60 min. After incubation, 10 ml of acetone:ethyl alcohol (1:1, v/v), was added to 1 ml of incubation mixture and heated for 2 min in an 80 C water bath. The mixture was centrifuged for 10 min at 3,500 rev/min. To the supernatant, 200 μg each of α-tocopherol, corresponding α-tocopheryl ester, α-tocopheryl quinone, and dimers and/or trimers of α-tocopherol (13-15) were added as carriers and the mixture evaporated in vacuo at 45-50 C. The residue was dissolved in 5-6 drops of ethyl acetate, spotted on a thin layer plate, and developed in the solvent system shown in Table III. The bands were visualized by ultraviolet lamp (2536 A) in the dark and divided into 7 zones. Each zone was scraped into a scintillation vial and the radioactivity determined as described previously.

In Vivo Experiments

Under ethyl ether anesthesia, the thoracic duct of each rat was cannulated following the procedure of Bollman, et al., (19). The rats used in the lymphatic fistula experiment were kept in restraining cages (20) overnight and given normal saline solution ad libitum (21). In the morning, the rats which drained 20 ml or more of lymph were orally administered 1.15 μmoles labeled α-tocopheryl ester in 1.5 ml/rat in the emulsion as described by Gallo-Torres, et al., (21). Lymph was collected for 12 hr at hourly intervals in tubes to which 2 drops of heparin had been added previously (4). During the experiment, the lymph fistula rats were given ad libitum normal saline solution (21).

After the volumes of lymph were recorded, 2 ml aliquots of lymph were placed in 50 ml centrifugation tubes with 40 ml ethyl alcohol:diisopropyl ether, (2:1, v/v), and centrifuged at 3,500 rev/min for 10 min to precipitate proteins. The supernatant was evaporated in vacuo at 45-50 C. The residue was dissolved in 1 ml ethyl acetate of which 0.05 ml was put in a vial for counting. Liquid scintillator was added and the radioactivity of the extract was determined in the liquid scintillation counter. Ethyl acetate solution (0.05 ml) of lipid extracts were analyzed on TLC using the solvent systems shown in Table III, as described above.

RESULTS

Incubation of d,l-3,4-$^3H_2$-α-Tocopheryl Esters in Bile-pancreatic Juice of Rats

The hydrolysis rates of the twelve α-tocopheryl esters were expressed as α-tocopheryl ester, α-tocopherol, and α-tocopheryl quinone remaining after being incubated for 60 min in rat bile-pancreatic fluid. No dimer or trimer of α-tocopherol was found in the in vivo or in vitro experiments (14,15,22,23). As shown in Table IV, most of the radioactivity administered as α-tocopheryl ester was accounted for in the product and substrates after TLC. Irrespective of carbon chain length, straight chain esters were hydrolyzed equally well, as typified by acetate and palmitate esters. Branched chain esters, such as isobutyrate and pivalate, were hydrolyzed at a much slower rate. Aromatic esters of α-tocopherol generally showed mini-

TABLE V

Hydrolysis of Labeled α-Tocopheryl Esters in Small Intestine Homogenate of Rats[a]

d,1-3,4-$^3H_2$-α-Tocopheryl	Radioactivity (%)[b]		
	As ester	α-Tocopherol	α-Tocopheryl quinone
Acetate	60	35	1
Propionate	63	25	2
Butyrate	74	22	2
Isobutyrate	80	15	1
Caprylate	97	2	1
Palmitate	97	2	1
Acid succinate	71	24	4
Benzoate	87	5	2
Nicotinate	88	9	2
o-Hydroxybenzoate	95	2	1
o-Acetoxybenzoate	63 (30)[c]	1	2
Pivalate	94	1	1

[a]Each ester (0.27 μmole, 4 μCi) was incubated in the 9,000 x g supernatant of rat small intestine homogenate for 60 min at 37 C.

[b]Mean of 2 experiments.

[c]Number in the parenthesis shows the percentage of o-hydroxybenzoate.

TABLE VI

Hydrolysis of Labeled α-Tocopheryl Esters in Liver Homogenate of Rats[a]

d,1-3,4-$^3H_2$-α-Tocopheryl	Radioactivity (%)[b]		
	As ester	α-Tocopherol	α-Tocopheryl quinone
Acetate	65	32	2
Propionate	66	26	1
Butyrate	75	23	1
Isobutyrate	77	17	1
Caprylate	94	1	1
Palmitate	94	1	1
Acid succinate	95	1	3
Benzoate	89	3	1
Nicotinate	86	8	1
o-Hydroxybenzoate	95	1	1
o-Acetoxybenzoate	91 (6)[c]	1	1
Pivalate	94	1	1

[a]Each ester (0.27 μmole, 4 μCi) was incubated in the 9,000 x g supernatant of rat liver homogenate for 60 min at 37 C.

[b]Mean of 2 experiments.

[c]Number in the parenthesis shows the percentage of o-hydroxybenzoate.

mal rates of hydrolysis in the bile-pancreatic juice. o-Acetoxybenzoate disappeared rapidly to yield o-hydroxybenzoate.

Incubation of d,1-3,4-$^3H_2$-α-Tocopheryl Esters in Small Intestine and Liver Supernatants

Rates of hydrolysis after incubation for 60 min of 12 α-tocopheryl esters in 9,000 x g supernatants of small intestine and liver of rats are shown in Tables V and VI, respectively. Hydrolysis of α-tocopheryl acetate took place at similar rates in the 2 supernatants during the 180 min incubation. Susceptibility to hydrolysis of 12 α-tocopheryl esters incubated in both supernatants was very similar. The rate of hydrolysis decreased in the following order: acetate > propionate, butyrate, acid succinate > isobutyrate > caprylate, palmitate, and aromatic esters. Generally long chain fatty acid esters and aromatic esters were hyrolyzed very slowly. An extensively branched ester such as pivalate resisted hydrolysis in all of the examined systems including bile-pancreatic juice.

Lymphatic Absorption of d,1-3,4-$^3H_2$-α-Tocopheryl Esters

After oral administration of d,1-3,4-$^3H_2$-α-tocopheryl esters in emulsion,

TABLE VII

Cumulative Lymph Absorption of Radioactivity for 12 Hr of d,l-3,4-$^3H_2$-α-Tocopheryl Esters to Thoracic Duct Fistula Rats after Administration in Emulsion

d,l-3,4-$^3H_2$-α-Tocopheryl	N[a]	Dose level/rat		Absorption percentage of radioactivity in lymph (% of dose)[b]
		μmole	μCi	
Acetate	5	1.15	50	52.3 ± 3.8
Palmitate	3	1.15	50	52.7 ± 5.5
Acid succinate	3	1.15	50	30.8 ± 2.9
Nicotinate	3	1.15	50	45.5 ± 3.2
o-Hydroxybenzoate	3	1.15	20	9.6 ± 1.2
Pivalate	3	1.15	24	13.5 ± 3.4

[a]N = number of experiments

[b]Mean ± S.E.

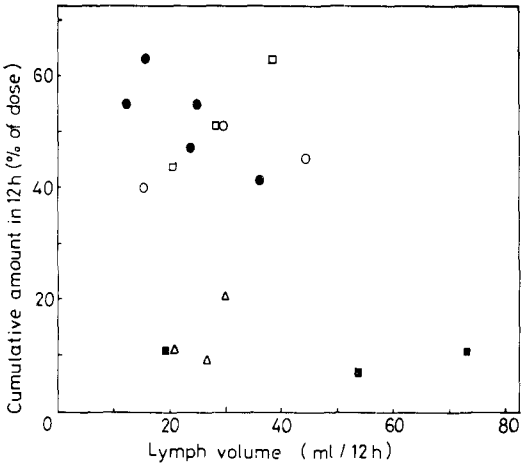

FIG. 1. Correlation between lymph volume and appearance of radioactivity in lymph. ● = d,l-3,4-$^3H_2$-α-tocopheryl acetate; ○ = d,l-3,4-$^3H_2$-α-tocopheryl nicotinate; □ = d,l-3,4-$^3H_2$-α-tocopheryl palmitate; ■ = d,l-3,4-$^3H_2$-α-tocopheryl o-hycroxybenzoate; △ = d,l-3,4-$^3H_2$-α-tocopheryl pivalate.

absorption of radioactivity was determined by obtaining lymph samples through a fistula in the thoracic duct. The results appear in Table VII. The transfer rate of radioactivity into lymph was found to be the highest in acetate and palmitate, followed by nicotinate, acid succinate, pivalate, and o-hydroxybenzoate. Correlation between lymph volume and appearance of radioactivity in the lymph is shown in Figure 1. The correlation between them was small.

The lymph samples collected during the first 12 hr after administration were analyzed by TLC and the results shown in Table VIII. Rats fed acetate, palmitate, and acid succinate esters absorbed the vitamin as α-tocopherol, whereas, the pivalate ester appeared predominately unchanged in the lymph. It should be noted that the tocopheryl pivalate had the slowest rate of hydrolysis in the in vitro incubation as well.

DISCUSSION

It has been established that vitamin E must be solubilized in order to be absorbed in the gut (24,25). Morgan, et al., (26) have shown that bile serves this function. Similarly, a hydrolase found in pancreatic juice was active on lipid esters (26), but α-tocopheryl nicotinate administered by a stomach tube was absorbed in the esterified form (2,27). Jayanthi Bai, et al., (6) demonstrated that vitamin E esterase in chicken tissues was active in the presence of bile salt.

TABLE VIII

Composition of Radioactivity Recovered in Lymph after Gastric Intubation of d,l-3,4-$^3H_2$-α-Tocopheryl Ester in Emulsion

d,l-3,4-$^3H_2$-α-Tocopheryl	Dose level/rat		Administered radioactivity in lymph for 12 hr (%)		
	μmole	μCi	As ester	α-Tocopheryl	α-Tocopheryl quinone
Acetate	1.15	50	2	82	8
Palmitate	1.15	50	2	57	16
Acid succinate	1.15	50	2	75	8
Nicotinate	1.15	50	9	55	15
o-Hydroxybenzoate	3.00	50	72	22	3
Pivalate	2.42	50	86	9	4

The markedly different features of hydrolysis in several α-tocopheryl esters in in vitro experimental conditions have been shown in the present paper. In the bile-pancreatic juice, straight chain fatty acid esters of α-tocopherol were hydrolyzed at similar rates. In the supernatant of intestine and liver homogenates, the increase of chain length of fatty acids reduced the rate of hydrolysis, while aromatic esters of α-tocopherol resisted hydrolysis. Pivalate in particular was hydrolyzed slowly in the bile-pancreatic juice and the supernatant of small intestine incubation experiments. The results suggested the esterase enzymes in pancreatic juice had no preference for straight chain fatty acid esters of α-tocopherol and did not significantly act on branched chain esters, as shown in the case of isobutyrate and pivalate. The resistance of o-hydroxybenzoate and pivalate to intestinal enzyme hydrolysis may have been due to the steric hindrance of ester moiety.

The in vivo experiments clearly showed the easily hydrolyzed esters of α-tocopherol were hydrolyzed before absorption, i.e., acetate, palmitate, acid succinate, while slowly hydrolyzed esters, pivalate and o-hydroxybenzoate, were absorbed unchanged into the lymph, though to a lesser degree, as shown in Table VII. As illustrated in Table VIII, the appearance of radioactivity and α-tocopherol in lymph after oral administration of ^{3}H-α-tocopheryl palmitate was found to be similar to that of ^{3}H-α-tocopheryl acetate. This fact supported the result obtained in the incubation experiments of acetate and palmitate esters. The absorption rate of labeled α-tocopheryl acetate was 10% of a 2 mg dose given to rats as reported by Gallo-Torres (2) and 52% of 0.55 mg in this present paper. This discrepancy may have been due to differences in the experimental conditions. The fact that unhydrolyzed ester and α-tocopherol, corresponding to 4% and 25% of the dose, respectively, appeared in lymph during the first 12 hr after oral administration of ^{3}H-α-tocopheryl nicotinate was comparable to the results of Gallo-Torres (5). On the other hand, the ^{3}H-α-tocopheryl pivalate was absorbed without being hydrolyzed previously.

While it has been thought that hydrolysis of esters is a prerequisite process in the intestinal absorption of the other fat soluble vitamin esters (28), intestinal hydrolysis of vitamin E esters appeared to be unnecessary for absorption.

The present paper demonstrates a close correlation between the intestinal hydrolysis of α-tocopheryl esters in vitro and the form absorbed into lymph in vivo. The metabolic fates of α-tocopheryl esters used in this study are not well known, except for α-tocopheryl acetate, acid succinate, and nicotinate. Further investigation on the biochemical behavior of scarcely hydrolyzable esters of α-tocopherol absorbed in lymph will be presented in a subsequent paper.

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Analysis of Methylsterol Fractions from Twenty Vegetable Oils

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ABSTRACT

The 4-monomethylsterol and 4,4-dimethylsterol fractions were separated from the unsaponifiables of 20 vegetable oils by preparative thin layer chromatography, and their compositions were determined by gas liquid chromatography. Tentative identification of the individual components of these fractions was carried out by gas liquid chromatography and combined gas liquid chromatography-mass spectrometry. Among 4-monomethylsterols, obtusifoliol, gramisterol, and citrostadienol occur abundantly in most of the oils. Cycloeucalenol also occurs in some of the oils as a major component of 4-monomethylsterols. Other 4-monomethylsterols tentatively identified are: lophenol, 31-norlanosterol, 31-norcycloartenol, and 31-norlanostenol and/or 4α-methylzymostenol. Among 4,4-dimethylsterols, cycloartenol and 24-methylenecycloartanol followed by β-amyrin and cycloartanol are common to most of the oils. Butyrospermol, α-amyrin, lupeol, and cyclobranol together with a 4,4-dimethylsterol, presumably lanostenol, occur in some of the oils. Cyclolaudenol is present in poppy seed oil. Besides these compounds, each of the oils contains some unidentified members of 4-monomethylsterols and 4,4-dimethylsterols. The methylsterol fraction of capsicum seed oil as compared with that of the other oils is characterized by its very high content of lophenol and cycloartanol together with three other members, presumably 31-norlanostenol, 4α-methylzymostenol, and lanostenol.

INTRODUCTION

A number of vegetable oils have been analyzed in this laboratory for their sterol constituents, i.e., 4-desmethylsterols, 4-monomethylsterols and 4,4-dimethylsterols, leading to many findings of scientific and practical interest (1-5). According to a study (2) on the methylsterol compositions of 19 commercially prepared vegetable oils, obtusifoliol, gramisterol (24-methylenelophenol), and citrostadienol occur in the 4-monomethylsterol fractions of all oils, and another 4-monomethylsterol, cycloeucalenol, in most of the oils. Cycloartenol and 24-methylenecycloartanol are present in all oils as their predominant 4,4-dimethylsterol components. α- And β-amyrins, cycloartanol, and cyclobranol also are of widespread occurrence in the oils examined. On the other hand, 3 *Theaceae(Camellia japonica* L., *Camellia Sasanqua* Thunb., and *Thea sinensis* L.) seed oils, alfalfa and garden balsam seed oils and shea butter show characteristic compositions of the 4,4-dimethylsterol fractions; the fractions contain scarcely any of cycloartenol and 24-methylenecycloartanol, and consist mainly of pentacyclic triterpene alcohols, such as α- and β-amyrins and lupeol, and butyrospermol (3).

In a previous study (5) on the desmethylsterol compositions of 20 vegetable oils hitherto less investigated, the unsaponfiables were separated into 4 fractions, i.e., fractions of 4-desmethylsterols, 4-monomethylsterols, 4,4-dimethylsterols and less polar compounds (hydrocarbons, etc.) by preparative thin layer chromatography (TLC). The present paper is concerned with compositions of 4-monomethylsterol and 4,4-dimethylsterol fractions obtained on that occasion.

The following trivial names are used in this paper:

cholesterol = cholest-5-en-3β-ol
zymostenol = 5α-cholest-8-en-3β-ol
zymosterol = 5α-cholesta-8,24-dien-3β-ol
β-sitosterol = (24*R*)-24-ethylcholest-5-en-3β-ol
lophenol = 4α-methyl-5α-cholest-7-en-3β-ol
31-norlanostenol = 4α,14α-dimethyl-5α-cholest-8-en-3β-ol
31-norlanosterol = 4α,14α-dimethyl-5α-cholesta-8,24-dien-3β-ol
4α-methylzymostenol = 4α-methyl-5α-cholest-8-en-3β-ol
4α-methylzymosterol = 4α-methyl-5α-cholesta-8,24-dien-3β-ol
obtusifoliol = 4α,14α-dimethyl-24-methylene-5α-cholest-8-en-3β-ol
31-norcycloartenol = 4α,14α-dimethyl-9β,1-cyclo-5α-cholest-24-en-3β-ol
cycloeucalenol = 4α,14α-dimethyl-9β,19-cyclo-24-methylene-5α-cholestan-3β-ol
gramisterol = 4α-methyl-24-methylene-5α-cholest-7-en-3β-ol

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TABLE I

Relative Retention Times of the Authentic Specimens of Sterol on OV-17 Column

Compounds	Position of double bond	Other structural characteristics	RRT[a]
4-Desmethylsterols			
(Cholestane series)			
Zymostenol	8	—	0.63
Zymosterol	8, 24	—	0.78
4-Monomethylsterols			
(4α-Methylcholestane series)			
Lophenol	7	—	0.83
31-Norlanosterol	8, 24	14α-CH_3	0.86
4α-Methylzymosterol	8, 24	—	0.87
Obtusifoliol	8, 24(28)	14α-CH_3, 24-CH_2	0.94
31-Norcycloartenol	24	14α-CH_3, 9,19-cyclo[b]	0.99
Cycloeucalenol	24(28)	14α-CH_3, 24-CH_2, 9,19-cyclo	1.10
Gramisterol	7, 24(28)	24-CH_2	1.12
Citrostadienol	7, 24(28)	24Z-C_2H_4	1.52
4,4-Dimethylsterols			
Lanostane series			
Lanostenol	8	—	0.89
Cycloartanol	—	9,19-cyclo	1.02
Cycloartenol	24	9,19-cyclo	1.24
Cyclolaudenol	25(26)	9,19-cyclo,24S-CH_3	1.35
24-Methylenecycloartanol	24(28)	9,19-cyclo, 24-CH_2	1.38
Cyclobranol	24	9,19-cyclo, 24-CH_3	1.68
Euphane series			
Butyrospermol	7,24	—	1.17
Pentacyclic triterpenes			
β-Amyrin			1.13
α-Amyrin			1.28
Lupeol			1.33

[a]RRT = Retention time for β-sitosterol (30 min) is taken as 1.00.

[b]9,19-cyclo = 9β,19-cyclopropane ring.

citrostadienol = 4α-methyl-(24Z)-24-ethylidene-5α-cholest-7-en-3β-ol
lanostenol = 5α-lanost-8-en-3β-ol
cycloartanol = 9β,19-cyclo-5α-lanostan-3β-ol
cycloartenol = 9β,19-cyclo-5α-lanost-24-en-3β-ol
cyclolaudenol = (24S)-24-methyl-9β,19-cyclo-5α-lanost-25-en-3β-ol
24-methylenecycloartanol = 24-methylene-9β,19-cyclo-5α-lanostan-3β-ol
cyclobranol = 24-methyl-9β,19-cyclo-5α-lanost-24-en-3β-ol
butyrospermol = 5α-eupha-7,24-dien-3β-ol
β-amyrin = 5α-olean-12-en-3β-ol
α-amyrin = 5α-urs-12-en-3β-ol
lupeol = 5α-lup-20(29)-en-3β-ol

EXPERIMENTAL PROCEDURES

Materials, Saponification and Preparative Thin Layer Chromatography

The origin of the 20 vegetable oils, saponification of the oils, and preparative thin layer chromatography (TLC) of the unsaponifiables were described in the previous paper (5). Twenty sterols: obtusifoliol, cycloartanol, cycloartenol, 24-methylenecycloartanol, cyclobranol, and α- and β-amyrins (2), butyrospermol and lupeol (3), cyclolaudenol (4), gramisterol and citrostadienol (6), cycloeucalenol (7), zymosterol, zymostenol, 4α-methyl zymosterol and lanostenol (8); lophenol (9); and a fraction containing 31-norlanosterol and 31-norcycloartenol separated from the unsaponifiables of rapeseed oil (T. Itoh, T. Tamura and T. Matsumoto, unpublished data, 1974), were used as the authentic specimens for gas liquid chromatography (GLC) and combined GLC-mass spectrometry (GLC-MS). Table I shows relative retention times (RRT) for these reference specimens.

GLC and Combined GLC-MS

GLC for the analyses of the sterol fractions was performed using an OV-17 column (1.5%) as mentioned previously (5). For determining the separation factors between the members of 4-monomethylsterols (Table III), QF-1 column also was used with a 2 m x 3 mm internal diameter (ID) glass column packed with a 1.5% QF-1 on Shimalite-W, 80-100 mesh, prepared by Nihon Chromato Works Ltd. (Tokyo, Japan). The column temperature was 203 C, and the detector, 230 C. RRT on both columns are ex-

TABLE II

Composition of 4-Monomethylsterol Fractions of Twenty Vegetable Oils Determined by GLC(OV-17)

Relative retention time[a] of individual 4-monomethylsterols	Compositions (%)[b]													
		I	II	III	IV	V	VI	VII					VIII	
	0.63	0.70	0.83	0.86	0.94	0.99	1.10	1.12	1.20	1.28	1.36	1.46	1.52	Others
Walnut *(Juglandaceae)* Japan				2	22			15		8	19		31	3
Pecan nut *(Juglandaceae)* US				2	5	2		13			4		74	
Cashew nut *(Anacardiaceae)* India		1	3		17	1	61			tr	tr	1	16	
Pistachio nut *(Anacardiaceae)* Iran	tr[c]			tr	36			21		11	8		24	
Japan wax *(Anacardiaceae)* Japan			1		21	5		53			6	1	13	
Pine nut *(Pinaceae)* China					5	2		35			2		55	1
Almond nut *(Rosaceae)* US				1	17	tr		30		1	4	3	44	
Sal fat *(Dipterocarpaceae)* India		1		2	10		32			2	2	2	10	39
Tohaku *(Lauraceae)* Korea		2	1	4	27	2		24			1	19	20	
Chaulmoogra *(Flacourtiaceae)* India				1	29			33		2	1	8	20	6
Perilla *(Labiatae)* Japan				15	37	6		25		tr	2		15	
Tung *(Euphorbiaceae)* China	5		1		19		16			5	12		39	3
Akamegashiwa *(Euphorbiaceae)* Japan					17	9		52			1		21	
Hemp seed *(Moraceae)*	1		2		6		25			1	11		52	2
Mustard *(Cruciferae)* Canada	2	2		2	31			47			1	1	9	5
Illipé butter *(Sapotaceae)*	1	1			13		54				tr	1	3	27
Poppy seed *(Papaveraceae)*	4			2	32		36		3		6			17
Pumpkin seed *(Cucurbitaceae)* China			2		24	3		40	3	3	16	8	1	
Capsicum seed-*Solanaceae)* Korea		26[d]	30		8	3		7			4		10	12
Fagara seed *(Rutaceae)* Korea		2		8	35	7		28				4	16	

[a]Retention time for β-sitosterol (30 min) is taken as 1.00.

[b]I = 31-norlanostenol and/or 4α-methylzymostenol; II = lophenol; III = 31-norlanosterol; IV = obtusifoliol; V = 31-norcycloartenol; VI = cycloeucalenol; VII = gramisterol; VIII = citrostadienol.

[c]tr = trace, less than 0.5%.

[d]Mixture of 31-norlanostenol and 4α-methylzymostenol.

TABLE III

Relative Retention Times[a] of Sterols Determined on OV-17 and QF-1 Columns

Compounds	Substituents[b]		OV-17	QF-1
	Skeleton	Side chain		
Gramisterol[c]	Δ^7, 4α-methyl	24-methylene	1.117	0.984
Lophenol[c]	Δ^7, 4α-methyl	---	0.832	0.741
Obtusifoliol[c]	Δ^8, 4α,14α-dimethyl	24-methylene	0.944	0.953
31-Norlanostenol (Sterol-Ia)	Δ^8, 4α,14α-dimethyl	---	0.703	0.716
Zymosterol[c]	Δ^8	Δ^{24}	0.782	0.701
4α-Methylzymosterol[c]	Δ^8, 4α-methyl	Δ^{24}	0.874	0.719
Zymostenol[c]	Δ^8	---	0.628	0.647
4α-Methylzymostenol (Sterol-Ib)	Δ^8, 4α-methyl	---	0.703	0.665

[a]Retention time for β-sitosterol (30 min) is taken as 1.000.

[b]Based on 5α-cholestan-3β-ol structure.

[c]Authentic specimens.

TABLE IV

24-Methylene and 4α-Methyl Substituents Separation Factors on OV-17 and QF-1 Columns

Compounds compared	Separation factors[a]	
	OV-17	QF-1
24-Methylene/24-Unsubstituted		
Gramisterol/Lophenol	1.343	1.328
Obtusifoliol/31-Norlanostenol	1.343	1.331
4α-Methyl/4-Desmethyl		
4α-Methylzymosterol/Zymosterol	1.118	1.026
4α-Methylzymostenol/Zymostenol	1.119	1.028

[a]Calculated from the data in Table III.

pressed by the ratio of the retention time for the substance under examination to the retention time (30 min) for β-sitosterol. Estimation of the approximate compositions of 4-monomethylsterol and 4,4-dimethylsterol fractions are based upon area percent values obtained from GLC peaks using the triangulation method. Small differences in GLC response, if any, for different sterols are disregarded. Hence, the data recorded in Tables II and IV may not be strictly accurate, but provide a measure sufficient enough to know relative proportions of component sterols.

Combined GLC-MS was performed in the same way as described previously (5). An OV-17 column (3%) was used for the GLC.

RESULTS

4-Monomethylsterols

The approximate compositions of the 4-monomethylsterol fractions from individual oils determined by GLC (OV-17) are shown in Table II. Sterol-II (RRT, 0.83) in the 4-monomethylsterol fraction of capsicum seed oil showed molecular ion (M^+) at m/e 400 ($C_{28}H_{48}O$) with other principal ions at m/e 385, 382, 367, 287, 269, 260, 245, 243, and 227 in the mass spectrum. The RRT and the mass spectrometric fragmentation pattern were identical with those of authentic specimen of lophenol (RRT, 0.83, mol wt, 400), and, hence, the sterol was recognized as lophenol. Sterols-IV, -VI, -VII, and -VIII were tentatively identified as obtusifoliol, cycloeucalenol, gramisterol, and citrostadienol, respecitvely, on the basis of the GLC and mass spectrometric evidence. The mass spectra of the 4-monomethylsterol fractions from most of the oils showed, however, that sterol-VI contained a minute quantity of gramisterol besides cycloeucalenol and sterol-VII contained a small proportion of cycloeucalenol besides gramisterol. Sterols-III and -V were regarded as 31-norlanosterol and 31-norcycloartenol, respectively, on the basis of their GLC behavior.

Mass spectrum of the GLC peak at RRT 0.70 in the 4-monomethylsterol fraction of capsicum seed oil exhibited 2 M^+ at m/e 414 and 400, indicating that this GLC peak comprises at least 2 components. One component, sterol-Ia (M^+ m/e 414, $C_{29}H_{50}O$) exhibited principal

TABLE V

Composition of 4,4-Dimethylsterol Fractions of Twenty Vegetable Oils Determined by Gas Liquid Chromatography (OV-17)

Relative retention time[a] of individual 4,4-dimethylsterols	Composition (%)[b]																
	0.74	0.81	0.86	i 0.89	ii 1.02	1.07	iii 1.12	iv 1.17	1.20	v 1.24	vi 1.28	vii 1.33	viii 1.38	1.52	ix 1.68	1.71	Others
Walnut	11	1		tr[c]	2		1		17[d]	42[d]			23	3[d]		tr	
Pecan nut	1	tr		tr	1		10			63			24		1		
Cashew nut	tr		tr	tr	10		6			39		16[d]	28[c]	1			
Pistachio nut				2	6		2			33			57			tr	
Japan wax	17				2		27	3[d]			20		21[d]	7[d]		2	1
Pine nut	1	tr	tr		2		tr		11[d]	10[d]			76				
Almond nut	3	tr		tr	5					9			83				
Sal fat	1			1	tr		26			59			8		5		
Tohaku	1	tr			2					16			77			4	
Chaulmoogra					2		12		6[d]	10[d]			69		1		
Perilla	tr	tr	tr		2		7			70			21				
Tung	tr	tr		1	4			39		9[d]			39	3[d]		3	2
Akamegashiwa	3				1[d]	3[d]	4[d]	6[d]		32			49	2	tr		
Hemp seed	2		3		2	4[d]	16[d]	14[d]		7		28[d]	17[d]		7		
Mustard	1	tr		tr	4	3	tr			77			14				1
Illipé				1	1[d]	5[d]	30				21		35	3[d]	4		
Poppy seed	1	tr	tr		1		18			19		13[d,e]	40[d]	1[d]	7		
Pumpkin seed		tr		1	3		10		44[f]	10[d]			30				2
Capsicum seed		1		16	22	5[d]				50		6	tr				2
Fagara seed	2	2		1	7		2		12[d]	13[d]			53	6[d]		2	

[a]Retention time for β-sitosterol (30 min) taken as 1.00.

[b]i = lanostenol; ii = cycloartanol; iii = β-amyrin; iv = butyrospermol; v = cycloartenol; vi = α-amyrin; vii = lupeol; viii = 24-methylenecycloartanol; ix = cyclobranol.

[c]tr = trace, less than 0.5%.

[d]Roughly calculated values.

[e]RRT = 1.35, cyclolaudenol.

[f]RRT = 1.19, unidentified.

ions at m/e 399(M-CH_3), 381(M-CH_3-H_2O), 301(M-C_8H_{17}[side chain]), 299(M-C_8H_{17}-2H), 273(M-C_8H_{17}-28), 245(M-C_8H_{17}-C_3H_6[part of ring D] -CH_2[14α-CH_3-H]), 227(M-C_8H_{17}-C_3H_6-CH_2-H_2O), and 201(m/e 227-26). Further, the weak ions, which may be taken as indicative of the presence of Δ^8-bond in the molecule/(10,11), were observed at m/e 412(M-2H), 397(M-CH_3-2H), and 379(M-CH_3-H_2O-2H) in the spectrum. The fragmentation pattern was essentially identical with that of 31-norlanostenol (mol wt, 414) reported by Atallah and Nicholas (11). The other component, sterol-Ib(M^+ 400, $C_{28}H_{48}O$) showed principal ions at m/e 385(M-CH_3), 367(M-CH_3-H_2O), 287(M-C_8H_{17} [side chain]), 285(M-C_8H_{17}-2H), 269(M-C_8H_{17}-H_2O) and 259(M-C_8H_{17}-28), and weak ions characteristic for the sterols with Δ^8-bond (10,11) at m/e 398(M-2H), 383(M-CH_3-2H) and 365(M-CH_3-H_2O-2H), and, hence, the sterol-Ib is concluded to be a sterol possessing Δ^8-bond and C_8-saturated side chain, i.e., 4α-methylzymostenol. The ΔRAc-value (RRT of acetate/RRT of the corresponding free sterol) (12) for the sterol-I is calculated from RRT-values (free sterol, 0.70; acetate, 0.91) as 1.30, a typical value for the members of 4α-monomethylsterol series.

The conclusion that the sterol-I in the 4-monomethylsterol fraction of capsicum seed oil comprises 31-norlanostenol and 4α-methylzymostenol is supported by an inspection of another GLC data mentioned below. Though the two components in the sterol-I fraction of capsicum seed oil showed the same RRT(0.703) on OV-17 column, they showed 2 separate peaks, Ia at RRT 0.716 (peak area 68%) and Ib at RRT 0.665 (peak area 32%), respectively, on QF-1 column. Table III indicates the RRT of several authentic specimens of sterols and of the above mentioned 2 components, sterol-Ia and sterol-Ib, precisely determined on both OV-17 and QF-1 columns, and Table IV shows the separation factors relating to the 24-methylene and 4α-methyl substituents of sterols, calculated from the data in Table III.

4,4-Dimethylsterols

Table V shows approximate compositions of the 4,4-cimethylsterol fractions of 20 vegetable oils determined by GLC. Tentative identification of the following 4,4-dimethylsterols, triterpene alcohols, was based on GLC and combined GLC-MS evidence: lanostenol, cycloartanol, β-amyrin, butyrospermol, cycloartenol, α-amyrin, lupeol, 24-methylenecycloartanol, cyclobranol, and cyclolaudenol. 4,4-Dimethylsterol with RRT 0.89 in the 4,4-dimethylsterol fraction from capsicum seed oil showed M^+ at m/e 428($C_{30}H_{52}O$, relative intensity 21%) with other principal ions at m/e 413(M-CH_3, 100%), 395(M-CH_3-H_2O, 71%), 313([M-C_8H_{17}[side chain]-2H, 3%), 297(M-C_8H_{17}-H_2, 3%), 273(M-C_8H_{17}-42[part of ring D], 18%), 271(M-C_8H_{17}-OH-27, 6%), 259(M-C_8H_{17}-42-CH_2[14α-CH_3-H], 15%), 255(M-C_8H_{17}-42-H_2O, 6%), and 241(M-C_8H_{17}-42-CH_2-H_2O, 15%), and the ions characteristic for the sterols with Δ^8-bond at m/e 426 (M-2H, 6%) and 411(M-CH_3-2H, 9%) (10,11) in the mass spectrum. The RRT and the fragmentation pattern, as well as the M^+, are in good agreement with those observed for the authentic specimen of lanostenol.

Mass spectrum of the peak at RRT 1.35 in the 4,4-dimethylsterol fraction of poppy seed oil indicated M^+ at m/e 440($C_{31}H_{52}O$, 6%), and the other principal fragmented ions at m/e 425(12%), 422(13%), 407(23%), 379(10%), 353(10%), 315(8%), 300(21%), 297(9%), 285(8%), and 175(39%). The RRT and M^+, as well as the fragmentation pattern, are quite identical with those of the authentic specimen of cyclolaudenol.

DISCUSSION

It is recognized from Table II that 3 4-monomethylsterols, i.e., obtusifoliol, gramisterol, and citrostadienol, occur abundantly in most of the oils. Cycloeucalenol also occurs in some of the oils as a major component of 4-monomethylsterols. An unidentified component with RRT 1.36 also is of common occurrence. Both 31-norlanosterol and 31-norcycloartenol whose ubiquitous distribution in higher plant kingdom has been supposed by Heintz and Benveniste (13), are present, mostly in a minor amount, in many of the oils. Table II shows that small amounts of lophenol, 31-norlanostenol and/or 4α-methylzymostenol, and further the compounds with RRT 0.63, 1.28, and 1.46 also are found occasionally in the 4-monomethylsterol fractions.

As for 4,4-dimethylsterols, it is found that cycloartenol and 24-methylenecycloartanol followed by β-amyrin and cycloartanol are common to most of the oils. Butyrospermol, α-amyrin, lupeol, and cyclobranol together with a 4,4-dimethylsterol, presumably lanostenol, occur in some of the oils. Cyclolaudenol, whose occurrence in opium has been reported previously (14), is present in poppy seed oil. Pumpkin seed oil, which contains exclusively Δ^7-sterols in the 4-desmethylsterol fraction (5), shows an unusual feature also in the 4,4-dimethylsterol fraction containing an unidenti-

fied compound with RRT 1.19 as the most predominant component.

It was noted in the previous paper (5) that the 4-desmethylsterol fraction of capsicum seed oil contained an exceptionally high proportion of cholesterol (10). As is seen from Tables II and V, this oil differs strikingly from other oils also in the composition of 4-monomethyl- and 4,4-dimethyl-sterol fraction. This oil contains large amounts of lophenol, 31-norlanostenol, and 4α-methylzymostenol in the 4-monomethylsterol fraction, and a relatively high proportion of lanostenol in the 4,4-dimethylsterol fraction. Neither of 4α-methylzymostenol and lanostenol has been reported before, to our knowledge, to occur in higher plants.

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SHORT COMMUNICATIONS

Dietary Alteration of Fatty Acid Composition of Lipid Classes in Mouse Mammary Adenocarcinoma

ABSTRACT

The composition of total fatty acids in serially transplanted mammary adenocarcinomas of C_3H mice which were fed a fat free diet or a stock diet containing 4% fat for 8 weeks were significantly different, although fatty acid amounts were similar. The difference in composition was manifested in the triglyceride, phosphatidyl choline, and phosphatidyl ethanolamine fractions. Tumors of mice fed fat free diet had appreciable amounts of eicosatrienoic acid, whereas neoplasms of stock diet fed animals had none. In addition, higher levels of oleic acid and lower contents of linoleic acid were found in tumors from mice fed fat free diet than in those from mice fed the stock diet. Thus, mechanisms which maintained the triglyceride fatty acid composition in some tumors, such as 7288CTC hepatoma, were not observed in mouse mammary adenocarcinomas, and, therefore, were not a general phenomena associated with carcinogenesis.

INTRODUCTION

Recently, we reported that dietary fat produced alterations in the composition of total fatty acids of mammary adenocarcinomas in C_3H mice (1). However, it was not certain whether the fatty acid composition of some lipid classes was unaffected, while that of others was changed by the diet. Wood, et al., (2) have observed that the triglyceride fatty acid composition of the 7288CTC hepatoma was unchanged regardless of whether the animals were fed a stock or fat free diet for 4-5 weeks. We report here that fatty acid composition of major lipid classes, triglycerides and phospholipids, of a mammary adenocarcinoma reflect changes due to diet fat.

MATERIALS AND METHODS

Mammary adenocarcinomas used in this investigation arose from a hyperplastic alveolar nodule outgrowth implanted into a cleared mammary fat pad of a 3 week old C_3H mouse (3). This tumor had been transplanted serially into 3 month old C_3H female mice which were raised and maintained in our laboratory on a commercial stock diet containing 4% fat (Wayne Lab Blox, Allied Mills, Inc., Chicago, IL). For this study, mice also were maintained on a fat free diet (4) subsequent to tumor transplantation.

TABLE I

Fatty Acid Composition of Lipid Classes of Mammary Adenocarcinoma of Mice Fed Different Diets

Fatty acid	Stock diet[a]					Fat free diet[a]			
	Diet fatty acid	Total lipid	PC[b]	PE[b]	TG[b]	Total lipid	PC[b]	PE[b]	TG[b]
14	2.3	0.8	0.8	0.6	1.0	0.6	1.3	0.3	1.8
15	0	2.1	0	4.5	0.5	0	0	0	0
16	17.1	18.3	27.1	8.7	21.1	18.7	31.6	8.8	22.3
16:1	3.1	4.1	6.4	3.9	6.6	4.9	4.3	2.0	4.0
17	0	1.3	0	2.1	0	0	0	0	0
18	4.5	15.5	8.6	16.2	9.5	15.2	4.1	9.9	4.8
18:1	24.0	26.5	29.7	23.8	37.1	36.2	40.4	51.9	51.8
18:2	44.5	11.4	9.8	7.1	18.1	1.6	1.1	1.0	1.0
18:3	4.5	0.6	0.8	0.7	0.8	1.1	1.2	1.5	1.9
20:3	0	0	0	0	0	10.1	6.4	9.0	5.8
20:4	0	19.5	16.9	32.4	5.4	11.3	9.5	15.6	6.9

[a]Figures given are the means of 2 closely agreeing values from duplicate determinations with different animals, and represent the % total fatty acids in each fraction.

[b]PC = Phosphatidyl choline; PE = phosphatidyl ethanolamine; and TG = triglyceride.

Eight weeks after tumor implantation, tumors were removed and a small piece (200 mg) was treated under reflux with 2 ml of 30% KOH in 50% methanol at 90 C for 16 hr. For quantitation of total fatty acid content, pentadecanoic acid (200 μg) was added to the saponification medium. Following acidification, total fatty acids were extracted with ether. Extracts were dried over anhydrous Na_2SO_4 and gently evaporated to dryness under a stream of N_2. Fatty acid methyl esters were prepared with diazomethane and were separated at 180 C by gas liquid chromatography (5). The instrument used was a Varian aerograph (Model 2740) which was provided with flame ionization detectors and stainless steel columns (6 ft x 1/8 in.) packed with 15% diethylene glycol succinate on H/P Chromosorb G. The percent composition of various fatty acids was calculated by triangulation, and when required the total fatty acid content was determined from the amount of pentadecanoic acid added as an internal standard.

Total lipids were extracted from tumors with chloroform:methanol (2:1, v/v) and separated into various lipid classes by thin layer chromatography (TLC) (6). TLC plates were sprayed with a 0.1% solution of 2',7',dichlorofluorescein in methanol, and after visualizing the lipid classes under UV light, they were individually extracted from the gel by vigorous shaking with chloroform:methanol (2:1, v/v) followed by sonication for 30 seconds. The dye was removed from the chloroform:methanol extract by washing twice with 0.5 N ammonium hydroxide in 50% methanol and once with "Folch Upperphase" (7). In preliminary experiments with labeled compounds, we found that this procedure of lipid extraction from gels and subsequent dye removal yielded >95% of the neutral- and phosphoglycerides, and did not result in any hydrolysis.

RESULTS AND DISCUSSION

Total fatty acid content of mammary tumors in mice maintained on a fat free diet (10.2 ± 2.1 mg/g wet wt, mean ± SD of 4 samples) was quite similar to that observed when they were fed a stock diet (10.8 ± 2.6 mg/g wet wt, mean ± SD of 4 samples). Whereas, tumors taken from mice fed fat free diet contained only 1.6% of their total fatty acids as linoleate, those neoplasms removed from animals fed stock diet had almost 8 times that much (Table I). This difference in composition of total fatty acids was not restricted to any single lipid class, but was true for the phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and triglyceride (TG) fractions. Determinations of fatty acid compositions of free fatty acid, cholesterol ester, sphingomyelin, and lysophospholipid fractions were not carried out, because these lipid classes were shown to be minor constituents of the total lipid, <10%. Appreciable amounts of penta- (2-5%) and heptadecanoic acid (1-2%) were found in the total lipid and PE fractions from tumors of mice fed the stock diet. Because these acids were not present in the diet, their origin is not known. They could have arisen by synthesis from propionate (8) or any other odd chain fatty acid present in the commercial stock diet.

In the total lipids of tumors of mice fed the fat free diet, we found appreciable amounts of eicosatrienoic acid and elevated levels of oleic acid. Such observations also were made with the PC, PE, and TG fractions. Although the origin of $C_{20:3}$ acid is not known with absolute certainty, we have, in other experiments (1), observed high levels of it in the livers of mice fed fat free diet for 8 weeks. We, therefore, suggest that the liver was the source. However, whether the tumor synthesized $C_{20:3}$ from $C_{18:1}$ is not known at this time.

Although the level of arachidonic acid in the TG fraction was not affected by feeding the fat free diet, values obtained with PC and PE fractions from tumors of mice fed fat free diet were one-half those for tumors from mice fed commercial stock diet. Regardless of dietary condition, PE contained relatively more arachidonic acid and less palmitic acid than did either PC or TG.

Recently, Wood, et al., (2) reported that in the 7288CTC hepatoma, fatty acid composition of neutral lipids was unaffected by fat in the diet and suggested that these results are in agreement with the lack of feedback control of fatty acid biosynthesis in hepatomas (9). However, the very significant effect that dietary fatty acids had on the composition of glycerides in the mammary adenocarcinoma shown here indicates that this is not a general characteristic of neoplasia. As we have demonstrated previously, not all tissues which show dietary adaptation of fatty acid composition respond metabolically with decreased lipogenic capacities. Indeed, mammary gland (4) and fetal liver (10) are prime examples of such tissues.

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[Received May 12, 1975]

Composition of Liver Lipids of the Rat During Pregnancy and Lactation

ABSTRACT

Triglyceride concentrations in rat liver rose during late pregnancy, declined at peak lactation, and then rose again during involution. The percentage of oleate in the triglycerides rose at peak lactation, but that of linoleate fell. Although phospholipid concentrations were unchanged, the percentage of palmitate in this fraction rose, and those of stearate and arachidonate fell during pregnancy and lactation. These changes may be related to the changes in lipogenesis and fat mobilization that occur during pregnancy and lactation.

INTRODUCTION

Although some of the effects of pregnancy and lactation on the composition of the rat liver have been known for many years (1), the changes in the lipid composition are not so well characterized. There is some evidence that the concentrations of cholesterol, triglyceride and lipid phosphorus do not change during pregnancy (2), but other work (3) suggests that there are changes in the fatty acid composition of rat liver lipids during late pregnancy. There is little information about changes during lactation, and in the present study we have investigated the effects of both pregnancy and lactation on the lipid composition of the rat liver.

MATERIALS AND METHODS

Animals

Hooded Norway rats from the Institute colony were used and were 3 months old at the time of mating. They were fed a commercial diet for laboratory animals ad libitum and were allowed access to water at all times. In addition to a group of nonpregnant animals, further groups of rats were studied on the 21st day of pregnancy, the 2nd and 14th days of lactation, and on the 3rd day after removing the young. Parturition occurred on the 22nd day after mating, and the young were removed on the 21st day after birth.

Analytical Methods

Rats were killed by breaking their necks. Samples of liver (ca. 1 g) were frozen in liquid nitrogen and powdered. Total lipids were extracted from this powder (4), and the mixed lipids obtained were resolved by column chromatography on silicic acid. Cholesteryl esters were eluted from the column with hexane containing 1% ether; free cholesterol and triglycerides (TG) were eluted with ether, and phospholipids (PL) were eluted with chloroform: methanol (1:1, v/v). These fractions were ana-

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TABLE I

Effects of Pregnancy and Lactation on Composition of Rat Liver Triglycerides[a]

Group	Triglyceride (mg/g liver)	Fatty acid (% by wt) 16:0	18:1	18:2
Unmated	2.6 ± 0.4	28.6 ± 1.0	29.8 ± 1.5	18.9 ± 1.2
21 days pregnant	4.2 ± 0.5[b]	26.1 ± 0.6	32.3 ± 1.4	23.5 ± 1.2[b]
2 days lactating	5.1 ± 0.9[b]	26.7 ± 0.7	29.4 ± 0.7	24.8 ± 1.4[b]
14 days lactating	1.6 ± 0.1	31.8 ± 1.4	36.1 ± 1.4[b]	10.2 ± 0.4[b]
3 days after weaning	7.2 ± 0.6[b]	28.3 ± 1.0	42.0 ± 1.3[b]	12.6 ± 1.0[b]

[a]Values are means ± SE for 6 rats in each group.

[b]Values significantly (<0.05) different from corresponding value for unmated rats (multiple range test).

TABLE II

Effects of Pregnancy and Lactation on Composition of Rat Liver Phospholipids[a]

Group	Lipid phosphorus (mg/g liver)	Fatty acid (% by wt) 16:0	18:0	20:4
Unmated	1.07 ± 0.04	12.8 ± 0.5	28.5 ± 0.5	22.0 ± 0.6
21 days pregnant	1.09 ± 0.05	19.1 ± 0.5[b]	23.8 ± 0.5[b]	15.6 ± 0.3[b]
2 days lactating	0.97 ± 0.03	19.2 ± 0.4[b]	22.7 ± 0.3[b]	15.9 ± 0.5[b]
14 days lactating	1.14 ± 0.02	16.2 ± 0.6[b]	22.8 ± 0.8[b]	13.5 ± 0.7[b]
3 days after weaning	0.95 ± 0.05	14.1 ± 0.6	29.5 ± 0.9	15.9 ± 0.6[b]

[a]Values are means ± SE for 6 rats in each group.

[b]Values significantly (P<0.05) different from corresponding value for unmated rats (multiple range test).

lyzed for cholesterol (5), glyceride-glycerol (6), and phospholipid phosphorus (7). Fatty acid composition of each fraction was determined by gas liquid chromatography (GLC) after preparation of the methyl esters of the component fatty acids (8).

GLC was carried out in a Pye series 104 chromatograph fitted with a flame ionization detector. A stainless steel column (200 cm x 6 mm internal diameter [ID]) was used and was packed with 10% (w/w) EGSS-X on Diatomite-C (100-120 mesh, acid washed, and treated with dimethyldiclorosilane). Nitrogen at a flow rate of 30 ml/min was used as the carrier gas, and the column was operated under isothermal conditions at 200 C. The instrument was linked to a Chromatographic Data Processor (Digital Equipment Co. Ltd., Reading, England). The output of this equipment gave the percentage contribution by wt of the individual fatty acid methyl esters in the mixture applied to the column.

RESULTS AND DISCUSSION

Preliminary studies established that there were no changes in the composition of liver lipids on the 7th and 16th days of pregnancy. The latter result agrees with an earlier report (2).

There was an increase in the concentration of TG during the period immediately before and after parturition, but on the 14th day of lactation, the value was only ca. 30% of that observed on the 2nd day (Table 1). There was a big increase in TG content after removal of the young. The changes in the concentration of lipid phosphorus were small and not statistically significant (Table II). Mean concentrations of free and esterified cholesterol were 1.7 and 0.18 mg/g liver, respectively, for unmated rats and were unchanged in pregnancy. At peak lactation, the concentrations were significantly lower (1.1 and 0.09 mg/g liver for free and esterified cholesterol, respectively), but after weaning they were similar to those for unmated rats.

McKay and Kaunitz (3) found that there were changes in fatty acid composition of rat liver total lipids during late pregnancy, but did not attempt to fractionate their total lipid extract. In the present study, we have found that there are changes in fatty acid composition of both TG and PL, and the most important of

these changes are shown in Tables I and II. The percentage of oleic acid in the TG fraction increased at peak lactation (14) days) and was at a maximum during involution (Table I). The percentage of linoleic acid increased during late pregnancy, but then declined markedly during peak lactation and involution. Although the concentration of lipid phosphorus did not change during pregnancy and lactation, there was an increase in the percentage of palmitic acid in the PL fraction and a decrease in the percentages of stearic and arachidonic acids (Table II).

In the period immediately before parturition, there were considerable changes in lipid metabolism in the rat. These included an abrupt fall in lipoprotein lipase activity in adipose tissue and an increase in plasma TG levels (9). Furthermore, food intake was greater and there was an increase in the synthesis of fatty acids in the liver (10). The accumulation of TG in the liver at this time may have been related to these changes. The concentration of TG in the liver remained high in early lactation, but plasma TG fell rapidly soon after parturition, probably due to a rapid increase in lipoprotein lipase activity in mammary tissue which allowed it to take up TG for milk production (9). Although food intake and the synthesis of fatty acids in the liver were even higher during lactation (10), the values for the concentration of TG in both plasma and liver were low at peak lactation (9, Table I), and probably reflected a rapid turnover of plasma TG and their uptake by the mammary gland. At weaning there was a rapid fall in mammary lipoprotein lipase activity (9) which gave rise to hypertriglyceridemia and the accumulation of TG in the liver.

The changes in fatty acid composition may be a further consequence of these changes in lipid metabolism. McKay and Kaunitz (3) attributed the changes in pregnant rats to a mobilization of fat depots and an increase in the extra mitochondrial synthesis of fatty acids relative to mitochondrial transformations to stearic and arachidonic acids. These factors may have been important in the lactating rat also. It seems relevant also to draw attention to the similarity between the changes in fatty acid composition that occurred during lactation and those observed after feeding fat deficient diets (11). In both cases, the changes coincided with an increase in hepatic lipogenesis. As the animals used in the present work received a nutritionally adequate diet ad libitum, the importance of this similarity remains to be established.

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Changes with Diet in the Composition of Phosphatidyl Choline of Sheep Bile

ABSTRACT

Bile phosphatidyl choline from sheep, in contrast to that from nonruminants, contains low levels of the normal range of polyunsaturated fatty acids. A comparison has been made of the composition of bile phosphatidyl cholines from sheep receiving either a control diet, a control diet supplemented with unprotected maize oil, or a control diet supplemented with soybean oil or tallow that had been protected against hydrolysis and hydrogenation in the rumen. The composition of bile phosphatidyl choline from sheep

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Bile phosphatidyl choline from sheep, in contrast to that from nonruminants, contains low levels of the normal range of polyunsaturated fatty acids. A comparison has been made of the composition of bile phosphatidyl cholines from sheep receiving either a control diet, a control diet supplemented with unprotected maize oil, or a control diet supplemented with soybean oil or tallow that had been protected against hydrolysis and hydrogenation in the rumen. The composition of bile phosphatidyl choline from sheep

receiving protected soybean oil supplement was virtually indistinguishable from that from nonruminants.

INTRODUCTION

Phosphatidyl choline constitutes the principal glyceroplipid in bile of all mammalian species examined to date. In ruminants (1), fatty acid composition of bile phosphatidyl choline is characterized by low levels of linoleic acid and its longer chain metabolites. It also is characterized by appreciable amounts of polyunsaturated fatty acids not normally found in phospholipids in quantity, such as linolenic acid and certain conjugated fatty acids that are intermediates in the ruminal biohydrogenation of linoleic and linolenic acids. Bile phosphatidyl choline from nonruminant species, e.g., the pig (1), rat, dog, and man (2), however, contain linoleic acid as the major unsaturated fatty acid component. In this study, the composition of bile phosphatidyl choline has been examined in sheep fed control diets or diets containing unprotected maize oil, or tallow or soybean oil protected by treatment with formaldehyde treated protein against biohydrogenation by rumen microorganisms (3).

MATERIALS AND METHODS

Four groups of 5 6-month-old Cheviot lambs were fed either a control diet of hay and concentrates or diets in which a proportion of the concentrates, i.e., high energy cereals such as rolled oats or flaked maize, was replaced either by protected soybean oil (3), protected tallow (3), or by unprotected maize oil (30 g/day of supplementary fat or 12% or total calories). The total fat intake of lambs on the control diet was 12 g/day, and on the fat supplemented diets, it was 40 g/day. The fats protected with formaldehyde-treated protein were donated by Alta Lipids UK Ltd. (London, England). Animals were maintained on the diets for 6 weeks when they were killed and the gall bladders removed. The gall bladder contents were acidified to pH 4 with concentrated HCl before being extracted with chloroform:methanol (2:1, v/v). Phosphatidyl choline was isolated by silicic acid column chromatography followed by preparative thin layer chromatography (TLC) as described earlier (1). The methyl ester derivatives, prepared by sodium methoxide catalyzed methanolysis, were separated by gas liquid chromatography (GLC) on packed columns of EGSS-X, and were identified by their retention times relative to authentic standards and by silver nitrate TLC (1). *trans*-Monoenoic components were estimated by GLC after separation along with saturated components by TLC on silica gel G layers impregnated with silver nitrate (10 % by wt); hexane:diethyl ether (9:1, v/v) was the developing solvent. Phosphatidyl choline was converted to diglycerides by phospholipase C hydrolysis and acetylated prior to separation into molecular species by silver nitrate TLC by methods described in detail elsewhere (1).

RESULTS AND DISCUSSION

The yield of phosphatidyl choline from bile of sheep on the 4 diets did not vary significantly, and averaged 0.342 g/100 ml bile, an amount only one-third of the level in more mature animals (1). Fatty acid compositions of bile phosphatidyl cholines are listed in Table I. In that of the control animals, 16:0, 18:0, and 18:1 (*cis*) were the major components, along with an appreciable amount of 18:2 (n-6) and considerably more 20:4 (n-6) than was detected in the same lipid from more mature sheep (1). Also, 18:3 (n-3) and fatty acids containing conjugated double bond systems, which were major components in the bile of the mature animals, were only present in trace amounts in the younger animals used in this study. The reason for this effect was probably because the mature sheep in the earlier work were pasture fed, whereas the younger ones in this work received hay concentrate diets; the latter have much higher 18:2 contents relative to 18:3 than fresh grass (4).

Not unexpectedly, the composition of bile phosphatidyl choline from sheep receiving the protected tallow supplement did not differ markedly from that of the control animals, and, indeed, the only significant difference was a drop in the concentration of 16:1. This fatty acid is often present in higher concentrations in the tissues of animals on low fat diets than in those receiving high fat diets (5). With sheep receiving the protected soybean oil supplement, on the other hand, there was a greater than 3-fold increase in the concentration of linoleic acid in the bile phosphatidyl choline entirely at the expense of the *cis*-monoenoic acids. Indeed, the fatty acid composition in this instance was very similar to that of bile phosphatidyl choline from nonruminant animals. Bile phosphatidyl choline from sheep fed unprotected maize oil supplement contained twice as much 18:2 (n-6) as that of controls, again at the expense of the *cis*-monoenoic fatty acids. Therefore, an appreciable portion of the linoleic acid in the maize oil supplement must have escaped biohydrogenation. With none of the fat supplements were the levels of the longer chain metabolites of

TABLE I

Variation in Fatty Acid Composition (% by wt) and Amount of Bile Phosphatidyl Choline with Dietary Treatment

Dietary fat supplement	Fatty acid composition (% by wt)									Amount of phosphatidyl choline[c] g/100 g bile)
	16:0	16:1	18:0	18:1 *cis*	18:1 *trans*	18:2 (n-6)	18:2 conj[a]	20:4	other[b]	
None (control	25.9 ±4.6	3.3 ±0.7	15.9 ±3.8	29.8 ±5.6	1.6 ±0.8	8.9 ±3.2	1.2 ±0.5	6.9 ±1.7	6.5	0.451 ±0.221
Protected tallow	26.1 ±3.7	1.5[e] ±0.2	17.1 ±1.4	24.6 ±3.3	1.1 ±0.5	10.3 ±0.9	0.9 ±0.5	10.2 ±4.0	8.2	0.185 ±0.073
Protected soybean oil	22.7 ±2.1	1.4[e] ±0.3	17.7 ±1.4	14.3[d] ±4.8	1.4 ±0.6	28.1[e] ±6.0	0.6 ±0.2	9.4 ±1.8	4.4	0.344 ±0.123
Unprotected maize oil	24.6 ±4.1	1.7[e] ±0.7	17.7 ±3.9	18.6 ±5.6	2.5 ±0.7	19.5 ±4.7	1.6 ±0.6	8.3 ±2.4	5.5	0.388 ±0.172

[a]9-*cis*, 11-*trans*-Octadecadienoic acid.

[b]Including 18:3 (n-6), 18:3 (n-3), *cis*-9, *trans*-11, *cis*-15-octadecatrienoic acid, 20:3 (n-6), 20:4 (n-3), 22:3 (n-6), 22:4 (n-6), 22:5 (n-3) and 22:6 (n-3).

[c]Mean ± SD of 5 animals.

[d]Significantly ($P<0.01$) different from control group.

[e]Significantly ($P<0.001$ different from control group.

TABLE II

Variation in Proportions (% of the Principal Molecular Species in Bile Phosphatidyl Cholines with Dietary Treatment

Dietary fat supplement	Molecular species (Mol %)					
	Saturated-saturated	Saturated-monoenoic	Monoenoic-monoenoic	Saturated-dienoic	Monoenoic-dienoic	Polyenoic[a]
Protected tallow	2.4	44.0	5.1	19.7	4.1	24.7
Protected soybean oil	1.6	23.6	3.3	43.2	8.1	20.2
None (control)	3.4	52.6	7.6	12.2	3.1	21.1
Unprotected maize oil	1.5	34.0	4.9	32.0	4.4	23.2

[a]Three or more double bonds.

linoleic acid, in bile, such as 20:4 (n-6), altered significantly.

Phosphatidyl cholines from the bile of sheep in each group were pooled and separated into molecular species, in the form of diglyceride acetate derivatives by means of silver nitrate chromatography. The results are listed in Table II. More than half of the control phosphatidyl choline consisted of the molecular species containing one saturated and one monoenoic fatty acid (SM). In bile phosphatidyl choline from sheep on fat supplemented diets, this species tended to decrease and was replaced largely by the molecular species containing one saturated and one dienoic fatty acid (SD). In that from the sheep receiving the protected soybean oil supplement, for example, the proportion of the SM fraction was only half that of the control, while the proportion of the SD fraction was more than 3 times that of the control. Although the amounts of the molecular species from the 4 groups varied markedly, fatty acid compositions of corresponding fractions were very similar.

These results provided confirmation of the suggestion (1) that the principal structural requirement of phosphatidyl choline for its detergent function in bile is that it be liquid, for which purpose it contains in one position, probably position 2, those polyunsaturated fatty acids most readily available or those not required for more essential purposes. In the normal pasture fed ruminant, linoleic acid and related metabolites are in short supply because of biohydrogenation. The animal then utilizes any available linolenic acid, the essential status of which has been questioned (6), and conjugated unsaturated fatty acids for the synthesis of bile phosphatidyl choline. In nonruminants on an adequate diet, tissue concentration of linoleic acid would not be limiting, and this is the polyunsaturated fatty acid most readily available for the purpose. However, it was clear that when the supply of linoleic acid to ruminant tissues was not limited, as with the sheep given the protected soybean oil supplement, the biliary phosphatidyl choline was almost indistinguishable in composition from that of nonruminants.

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Effect of Diet on the Composition of Cholesteryl Esters of Sheep Adrenals

ABSTRACT

Fatty acid components of cholesteryl esters from the adrenals of sheep, like those of nonruminants, were characterized by significant amounts of the longer chain metabolites of linoleic acid. Administration to sheep of diets rich in linoleic acid and protected against biohydrogenation did not alter the concentration of these components significantly. Although 18:2 levels were elevated, this was largely at the expense of *cis*-monoenoic fatty acids.

INTRODUCTION

The fatty acid composition of adrenal cholesteryl esters of a number of species of monogastric animals including the rat (1-4), guinea pig (2), rabbit (5,6), dog (7), pig (8), and man (4) have been determined. They are characterized by high concentrations of longer chain linoleic acid metabolites, including homo-γ-linolenic acid (20:3 [n-6]), arachidonic acid (20:4 [n-6]), and, in particular, the so-called adrenic acid (22:4 [n-6]). Administration of adrenocorticotropic hormone (ACTH) increases the concentration of these C_{20} and C_{22} fatty acids in adrenal cholesteryl esters of the rabbit (6), and it has been suggested that they may play a part in the biosynthesis of steroid hormones in the adrenals (9,10). Compositions of adrenal cholesteryl esters of ruminant species do not appear to have been recorded. In this study, the composition of adrenal cholesteryl esters of the sheep have been examined in animals fed either a control diet or diets supplemented with unprotected maize oil or with tallow or soybean oil which had been protected by formaldehyde treated protein (11) against hydrolysis and biohydrogenation by rumen microorganisms.

MATERIALS AND METHODS

Four groups of 5 6-month-old Cheviot lambs were fed either a control diet of hay and concentrates, i.e., a mixture of high energy cereals, such as rolled oats, or flaked maize, or diets in which a proportion of the concentrates was replaced either by protected soybean oil (11), protected tallow (11), or by unprotected maize oil (30 g/day of supplementary fat or 12% of total calories). The fats protected with formaldehyde treated protein were donated by Alta Lipids UK Ltd. (London, England). Further details of the compositions of the feeds will be published elsewhere. Animals were maintained on the diets for 6 weeks when they were killed and the adrenals removed. Lipids were extracted with chloroform:methanol (2:1, v/v) and stored in chloroform at -20 C. Cholesteryl esters were isolated by preparative thin layer chromatography (TLC) on layers of silica gel G (0.5 mm thick); hexane:diethyl ether (9:1, v/v) was the developing solvent. Methyl ester derivatives, prepared by sodium methoxide catalyzed methanolysis, were separated by gas liquid chromatography (GLC) on packed columns (2 m x 4 mm) of EGSS-X (15% on Gas-Chrom P) isothermally at 180 C, and were identified by their retention times relative to authentic standards, i.e., 16:0 to 18:2 (n-6) and 20:4 (n-6) (Applied Science Laboratories Inc., State College, PA), by means of equivalent chain length (ECL) values and by silver nitrate TLC (12). *trans*-Monoenoic components were estimated by GLC after separation along with the saturated components by TLC on silica gel G layers impregnated with silver nitrate (10% by wt); hexane:diethyl ether (9:1, v/v) was again the developing solvent.

RESULTS AND DISCUSSION

Much smaller amounts of cholesteryl esters (0.66 mg/g tissue) and free cholesterol (1.27 mg/g of tissue) were found in sheep adrenals than has been reported for nonruminant tissues, and these amounts did not vary significantly with dietary treatment.

Fatty acid compositions of cholesteryl esters from adrenals of sheep on the 4 diets are listed in Table I. In those of animals on control diet, 16:0, 18:0, 18:1 (*cis*), and 18:2 (n-6) were the major components, along with smaller quantities of branched chain, largely the *iso*- and *anteiso*-isomers, odd chain, saturated and unsaturated, and longer chain fatty acids derived from linoleic acid. These last components, comprising mainly 18:3 (n-6), 20:3 (n-6), 20:4 (n-6), and 22:4 (n-6), are characteristic constituents of the cholesteryl esters of the adrenal glands of numerous nonruminant species (1-8) in which they are normally present in somewhat higher concentrations than was found in this instance. While ruminant tissues tend to contain much less linoleic acid and its metabolites in total than those of nonruminants, because of biohydrogenation of dietary unsaturated fatty acids in the rumen, the portion that

TABLE I

Variation of Fatty Acid Composition of Adrenal Cholesteryl Esters with Dietary Treatment

Dietary fat supplement	Fatty acid composition (% by wt)[a]														
	16:0	16:1	17 br[b]	17:0	17:1	18:0	18:1 *cis*	18:1 *trans*	18:2 (n-6)	18:3 (n-6)	20:3 (n-6)	20:4 (n-6)	20:5 (n-3)	22:4 (n-6)	other[c]
Protected tallow	14.7 ±2.0	2.8 ±0.8	3.6[d] ±0.5	0.7 ±0.1	1.4 ±0.2	8.0 ±1.9	36.7 ±1.3	1.6 ±0.9	15.6 ±3.1	1.6 ±0.3	1.2 ±0.5	3.8 ±1.3	1.3 ±0.3	1.7 ±0.3	5.3
Protected soybean	8.5[e] ±1.5	1.7 ±1.2	2.4[d] ±1.0	0.5 ±0.1	0.6[f] ±0.2	7.8 ±2.6	20.8[f] ±4.7	1.5 ±0.6	35.4[f] ±9.1	1.6 ±0.5	4.7 ±3.3	5.0 ±1.1	1.2 ±0.7	2.9 ±1.2	5.4
None (control)	13.2 ±2.5	3.1 ±1.6	5.8 ±2.2	1.0 ±0.4	1.4 ±0.4	6.6 ±2.1	33.4 ±2.1	1.6 ±0.9	18.1 ±5.0	1.7 ±0.5	1.4 ±0.6	3.7 ±0.4	2.0 ±1.7	2.0 ±0.9	5.0
Maize oil	10.7 ±2.1	1.7 ±0.5	3.4[d] ±1.5	0.7 ±0.4	0.7[f] ±0.1	10.0 ±1.1	24.4[e] ±5.2	3.7[e] ±1.0	26.0[d] ±4.7	1.4 ±0.6	2.5 ±1.6	3.9 ±0.6	1.7 ±0.4	3.0 ±2.2	6.2

[a]Mean ± SD of 5 animals.

[b]A mixture of C_{17} *iso*- and *anteiso*-branched chain components.

[c]Components that always comprised <1% of the total, including 18 br, 18:3 (n-3), *cis*-9, *trans*-11-octadecadienoic acid, 20:2 (n-6), 22:3 (n-6), 22:4 (n-3), 22:5 (n-3), and 22:6 (n-3).

[d]Significantly ($P<0.05$) different from control group.

[e]Significantly ($P<0.01$) different from control group.

[f]Significantly ($P<0.001$) different from control group.

escapes biohydrogenation tends to be concentrated in the cholesteryl ester and phospholipid fractions (13).

Not unexpectedly, the composition of adrenal cholesteryl esters from animals receiving the diet supplemented with protected tallow did not differ markedly from that of the control animals. Indeed, the only significant difference was a decrease in the concentration of the branched chain components. There were considerable changes in the composition of adrenal cholesteryl esters of the sheep receiving the protected soybean oil supplement, however. The 18:2 concentration doubled with a concomitant decrease largely in the concentration of 18:1 (*cis*), but also in those of 17:1, 16:0, and of the branched chain components. With sheep receiving the diet containing unprotected maize oil supplement, there was again a marked increase (ca. 50%) in the proportion of 18:2 in the adrenal cholesteryl esters, indicating that some of the 18:2 must have escaped biohydrogenation, and this was again accompanied by a decrease in the concentrations of the C_{17} and C_{18} *cis*-monoenoic components and of the branched chain fatty acids. A 2-fold increase in the concentration of *trans*-octadecenoic acid, an intermediate in the biohydrogenation process, probably served as an indication that a significant amount of biohydrogenation was occurring nonetheless in the rumen, and that this was not going to completion.

The decrease in concentration of *cis*-monoenoic fatty acids in adrenal cholesteryl esters of sheep receiving large amounts of 18:2 may have been a result of inhibition of desaturation of saturated fatty acids in the tissue by linoleic acid (14). The branched chain fatty acids found in ruminant tissues are formed largely by microorganisms in the rumen, and the decrease in concentration of these compounds in animals on all the fat supplemented diets, relative to those on the control diet, possibly reflected some interference by these diets with the metabolism of the microorganisms.

With none of the dietary treatments did the concentrations of longer chain fatty acids of the linoleate family in adrenal cholesteryl esters differ significantly from those of the sheep on control diet. In contrast, an increase in linoleic acid content of the diet of rabbits resulted in marked increases in the concentrations of 20:3 (n-6), 20:4 (n-6), and 22:4 (n-6) in the adrenal cholesteryl esters (5). Thus, it would seem that low levels of C_{20} and C_{22} polyunsaturated fatty acids that are normally present in the adrenal cholesteryl esters of sheep are adequate for any essential metabolic role (9,10) that these fatty acids may have in the adrenal glands of this species.

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ERRATUM

An error occurred in the article by G.A. Dhopeshwarkar and C. Subramanian entitled "Metabolism of 1-^{14}C Linolenic Acid in Developing Grain: II. Incorporation of Radioactivity from 1-^{14}C Linolenate into Brain Lipids," (Lipids 10:242 [1975]). In the Introduction, the reference by J. Tinoco, M.A. Williams, I. Hicenbergs, and R.L. Lyman (J. Nutr. 101:937 [1971]) was misquoted. The sentence, "In the case of rats, Tinoco, et al., (5) found that linolenic acid was not essential for normal growth and whole tissue lipid analysis did not show any differences between controls and those fed an 18:3 deficient diet," should read, "In the case of rats, Tinoco, et al., (5) found linolenic acid was not essential for normal growth or reproduction in spite of very low levels of tissue ω3 fatty acids found in animals fed an 18:3 deficient diet."

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Lipogenesis by Intact Hepatocytes from Normal and Diabetic Rats

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ABSTRACT

Intact hepatocytes isolated from livers of diabetic rats demonstrate the characteristic decreased lipogenic capacities as compared to normal. Administration of insulin to diabetic rats restores these capacities to near normal levels. The results emphasize the potential that the hepatocyte system has for the study of hormonal regulation of lipogenesis.

INTRODUCTION

Although several techniques have been described for the isolation of metabolically active liver cells, generally, it is now accepted that the enzymatic digestion method of Berry and Friend (1) provides the best means for the isolation of morphologically intact hepatocytes in high yield. Cells isolated by this method have been used to investigate several aspects of protein synthesis (2), gluconeogenesis (3), and fatty acid (4-6) metabolism. In a recent report (5), we pointed out that the rates of fatty acid synthesis in intact parenchymal cells prepared by this method (1) are of the same order as those observed in vivo. The results of the present study further show that the types of lipids synthesized by hepatocytes are also comparable to those observed in experiments with liver tissue (7). Furthermore, cells prepared from livers of diabetic rats have lowered abilities to synthesize fatty acids, and administration of insulin to diabetic rats restores the synthetic capacities to near normal levels.

MATERIALS AND METHODS

Intact parenchymal cells were isolated from livers of male rats, 200-300 g body wt, of the Long-Evans strain. All rats were fed an adequate chow diet ad libitum. Experimental diabetes was induced by intraperitoneal (IP) injection of alloxan (200 mg/kg body wt) or intravenous (IV) injection of streptozotocin (60 mg/kg body wt). Blood sugar assays were performed by the glucostat method (Worthington Biochemical Corp., Freehold, NJ). The washed hepatocytes were incubated in Krebs-Henseleit bicarbonate-buffer (8) for 30, 60, 90, and 120 min at 37 C with a mixture of 95% O_2 and 5% CO_2 as gas phase. All other incubation conditions have been described elsewhere (5) and are outlined in Table I. Fatty acid synthesis from endogenous substrates was studied by measuring the incorporation of ^3H from ^3H_2O into total lipids in the absence of added acetate. The contribution of exogenous acetate to fatty acid synthesis was determined by incubating the cells with 1-^{14}C-acetate (10 mM). Total fatty acid synthesis was determined by incubation with ^3H_2O in the presence of 10 mM acetate. Cellular lipids were saponified with ethanolic KOH and the fatty acids separated from digitonin-precipitable sterols (9). In experiments where distribution of radioactivity among the various lipid classes was required, total lipids were extracted with chloroform:methanol (2:1) and washed according to the method of Folch, et al., (10). The lipids were separated by thin layer chromatography (11) on Silica Gel G, using hexane:diethyl ether:glacial acetic acid (70:20:1) as solvent system. All radioactive assays were performed by liquid scintillation spectrometry and corrected for quenching.

RESULTS AND DISCUSSION

Results presented in Table I confirm our earlier findings (5) that fatty acid synthesis in isolated hepatocytes proceeds at rates appreciably higher than those obtained with tissue slices. Under the conditions employed here, the lipogenic rate is a linear function of time for at least 2 hr. In addition, it can be seen that the conversion of acetate carbon to fatty acids is about 10 times higher than that to cholesterol (Table I). Studies on the distribution of the isotopes among the various lipid classes show (Table II) that the major portion of the activity was present in phospholipids and triglycerides with only 15% in the cholesterol fraction. These data are in accord with the results obtained in tissue slice experiments (7). On the other hand, Capuzzi, et al., (4) have shown in their studies on fatty acid synthesis by hepatocytes that over half of the incorporated acetate was found in the cholesterol fraction. Though it is difficult to explain this discrepancy, it is likely that some of the variation is attributable to the differences in the methodology used for the isolation of hepatocytes. As has been pointed out by Capuzzi, et al., (4), the open perfusion method employed in their studies

TABLE I

Effect of Diabetes on Lipogenesis in Isolated Hepatocytes[a]

Hepatocytes prepared from:	Blood sugar at sacrifice (mg/100 ml)	Radioactive substrate incubated							
		1-^{14}C-acetate			^3H_2O		^3H_2O + acetate		
		CO_2	Fatty acids	Cholesterol	Fatty acids	Cholesterol	Fatty acids	Cholesterol
Normal Rats	120 ± 15 (6)	362 ± 21 (6)	23.5 ± 0.8 (16)	3.4 ± 0.8 (7)	46.3 ± 0.5 (7)	6.3 ± 1.3 (3)	51.2 ± 1.6 (5)	6.5 ± 1.2 (3)
Diabetic Rats								
Alloxan	538 ± 100 (5)	387 (2)	2.4 ± 0.9 (5)	0.6 ± 0.3 (4)	8.1 ± 0.7 (4)	2.4 ± 0.5 (5)	11.7 ± 1.0 (5)	2.2 ± 0.7 (4)
Streptozotocin	444 ± 42 (3)	410 (2)	2.3 (2)	1.8 (2)	8.4 ± 1.6 (3)	3.8 ± 0.8 (3)	14.0 ± 2.4 (3)	4.7 ± 1.1 (3)
Alloxan diabetic injected with insulin[b]	95 (2)	293 (2)	21.1 (2)	2.6 (2)	31.6 (2)	4.5 (2)	39.7 (2)	5.2 (2)

[a]Values are expressed as μmoles of substrate incorporated/g wet wt/hr, and are given as the means ± S.E. The number of experiments is given in parentheses.
[b]Insulin was injected daily (5 units/100 g body wt; intraperitoneally) for 3 days. In addition, each rat received a fourth injection 2 hr before sacrifice.

TABLE II

Incorporation of 1-^{14}C-Acetate and of ^3H_2O into Various Lipid Classes by Hepatocytes Obtained from Normal Rats[a]

Lipid class	Percentage distribution of isotope from:	
	1-^{14}C-acetate (6)	^3H_2O + acetate (3)
Phospholipids	34.2 ± 0.8	39.6 ± 0.3
Monoglycerides	2.0 ± 0.1	3.0 ± 0.4
1,3 Diglycerides	1.8 ± 0.2	15.2 ± 0.6
Cholesterol	15.4 ± 0.6	15.2 ± 0.6
1,2 Diglycerides	8.6 ± 0.6	3.7 ± 0.1
Triglycerides	33.2 ± 1.3	30.3 ± 0.7
Fatty acids	2.1 ± 0.3	4.5 ± 0.1
Cholesterol esters	2.5 ± 0.1	3.7 ± 0.3

[a]Freshly-prepared liver cells were incubated with 1-^{14}C-acetate or with ^3H_2O and 10 mM acetate. The diglycerides and cholesterol, which migrate to the same location on thin layer plates with the hexane:diethyl ether:glacial acetic acid solvent mixture, were scraped from the plates, eluted from the gel and were separated further by thin layer chromatography with chloroform:benzene:ethyl acetate:glacial acetic acid (150:50:25:4). Results are the means ± S.E. of the experiments given in parentheses.

carries the risk of subjecting the liver to hypoxic conditions during the perfusion period. When oxygen is excluded from the medium during perfusion, hepatocytes lose glycogen (H.V. Werner, personal communication). We have shown (Abraham, unpublished data) that such glycogen depleted cells exhibit a severely diminished capacity for fatty acid synthesis; however, they do not show a proportionate decrease in cholesterol synthesis. Thus, it is possible that exposure of the liver to hypoxic conditions during perfusion results in the isolation of cells which, though morphologically intact, incorporate acetate carbon preferentially into sterols.

Results presented in Table I show that the well known block in lipogenesis in livers of diabetic rats (12-14) is also reflected in the metabolic properties of isolated hepatocytes. Hepatocytes prepared from streptozotocin, or alloxan, diabetic rats possess reduced capacities for lipogenesis. This diminished incorporation is observed when either ^{14}C-acetate or ^3H_2O is used as substrate. Administration of insulin to the diabetic rats results in a marked improvement in the capacities of the hepatocytes prepared for these rats to synthesize fatty acids (Table I).

It is worth noting that the decrease in the case of acetate is more dramatic than in the case of tritiated water. Although the incorporation of tritium from water and carbon from acetate into fatty acids gives us some informa-

tion about the relative contributions of the two pathways of fatty acid synthesis, elongation and de novo, the effect of diabetes on these processes cannot be assessed from the present data.

We may conclude that the enzymatic dispersion method of Berry and Friend (1) results in the isolation of hepatocytes whose capacities for lipogenesis are reflective of intact liver and in which metabolic consequences of in vivo manipulation of hormonal status are still manifested. The data thus establish the usefulness of these cells as a powerful tool for the elucidation of the mechanisms which control regulation of hepatic lipogenesis.

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Synthesis and Analysis of Phytyl and Phytenoyl Wax Esters

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ABSTRACT

An efficient procedure for preparing phytenic acid methyl ester, free of isomers, from phytol is reported. Phytyl phytenate and other isoprenoid wax esters were synthesized. Gas liquid chromatography of these wax esters and other compounds related to phytol and phytenic acid is described. The alkyl constituents of isoprenoid wax esters can be analyzed after alkaline methanolysis and the acyl constituents after acidic methanolysis. The applicability of these methods to natural mixtures was demonstrated with wax esters from mosses which contained both types of isoprenoids and with wax esters from healthy and frost damaged grass which contained phytol, but not phytenic acid.

INTRODUCTION

The occurrence of phytol as constituent of chlorophyll has been known for more than half a century, but its occurrence in wax esters from plant materials has been reported only recently. Phytol has been found in wax esters of leaves from *Acer platanoides* (1) and from *Fatsia japonica* (2). Phytol and phytenic acid have been found as major constituents of wax esters from bryophytes (3).

Analysis of wax esters containing phytol and phytenic acid requires alkaline methanolysis for determination of the alkyl moieties and acidic methanolysis for determination of the acyl moieties. The former method isomerizes phytenic acid to a great extent, and the latter method is destructive to phytol. These two methods must be combined to obtain satisfactory analyses of the isoprenoid moieties. Suitable methods described here now have been established using synthetic isoprenoid wax esters and then were tested by analyses of waxes from two mosses, *Hygrohypnum luridum* and *Paraleucobryum longifolium*, and from timothy grass, *Phleum pratense*.

MATERIALS AND METHODS

Preparation of Phytenic Acid

Phytol (Nutritional Biochemicals Corp., Cleveland, OH) was purified by column chromatography over Unisil (100-200 mesh, Clarkson Chemical Co., Williamsport, PA) with petroleum ether (bp 60-70 C):diethyl ether (95:5) as eluting solvent. The alcohol was oxidized to aldehyde with CrO_3-pyridine in methylene chloride (4) under rigorous exclusion of moisture. The oxidant was prepared in 30 ml CH_2Cl_2 by addition of 24 mmole pyridine, followed by 12 mmole CrO_3. After clearing of the initial turbidity, 2 mmole phytol in 4 ml CH_2Cl_2 was added. The mixture was stirred for 1 hr, and the solution then was decanted into a separatory funnel. Additional product was recovered from the precipitate by extraction with diethyl ether and added to the decanted portion. The combined solution was washed with aqueous 5% NaOH, 5% HCl, 5% $NaHCO_3$, and finally with saturated NaCl to neutrality. After purification by column chromatography, as described for phytol, phytenal was obtained in 75% yield. This was oxidized to the acid by Ag_2O (5). To the solution of 1.5 mmole phytenal in 10 ml ethanol was added 9 mmole $AgNO_3$ in 2.2 ml water, followed by 27 mmole NaOH in 15 ml water. The latter was added slowly from a dropping funnel under vigorous stirring, which was continued overnight at room temperature.

The reaction mixture was filtered through sintered glass, and the precipitate was rinsed twice with 20 ml of 30% ethanol in water. The filtrate was diluted with 300 ml water and extracted once with 200 ml petroleum ether (bp 30-40 C): diethyl ether (1:1). This extract from the alkaline solution was discarded. The aqueous phase was acidified to about pH 2 with 4 N HCl. It was extracted 3 times with the above solvent mixture, and the combined extracts then were washed to neutrality with water.

After purification by column chromatography as specified above for phytol, phytenic acid was obtained in a yield of 60% in reference to phytol. It contained ca. 15% contaminants as indicated by faster migrating material in thin layer chromatography (TLC) and in gas liquid chromatography (GLC) after esterification with CH_2N_2. Most likely the contaminants were positional and geometric olefinic isomers as described in the literature (6). Column chromatography over Unisil of the esters with petroleum ether:diethyl ether (99:1) yielded about half of the material as methyl phytenate (3,7,11,15-tetramethylhexadec-*trans*-2-enoate) in 99% purity and

TABLE I

Gas Liquid Chromatography of Methyl Phytenate and Related Compounds

Compound[a]	Equivalent chain length[b] on EGS[c]	Equivalent chain length[b] on DEGS[c]	Composition % Crude synthetic methyl ester	Composition % Treated with $NaOCH_3V$, reesterified
Isomer	14.1			3.6
Isomer	15.2	15.3	4.0	
Phytanate	16.8	17.0		
Isomer	17.0			29.4
Isomer	17.7	17.7	10.0	32.7
Oleate	18.7	18.4		
Phytenate[d]	18.9	18.8	86.0	26.1
Isomer	19.8			8.3

[a]For possible structures of isomers, see reference 6.

[b]ECL in reference to straight chain saturated fatty methyl esters.

[c]EGS = ethylene glycol succinate; DEGS = diethylene glycol succinate.

[d]3,7,11,15-Tetramethylhexadec-*trans*-2-enoate.

about one-third of it in 93% purity.

Synthesis of Wax Esters (7)

The chlorides of 0.20 mmoles palmitic, oleic, linolenic, or phytenic acids were prepared in 1 ml dry benzene by addition of 0.28 mmole oxalyl chloride under stirring at 60-65 C for 2 hr in a flask equipped with a condenser and $CaCl_2$ drying tube. Excess oxalyl chloride, HCl, and solvent then were carefully removed under high vacuum. Dry diethyl ether was added to the acid chloride, followed by 0.20 mmole phytol or hexadecanol in the same solvent and 0.25 ml dry pyridine. The mixture was heated to reflux and stirred for 3 hr. The product was recovered in diethyl ether: petroleum ether (bp 30-40 C) (1:1) and washed once with water, 3 times with 1N H_2SO_4, once with 5% $NaHCO_3$, and finally with water to neutrality.

Wax esters were purified by TLC using trichloroethylene for development. Pure phytyl palmitate (Phy-16:0), Phy-18:1, Phy-18:3, Palmityl phytenate (16:0-Phy), and Phy-Phy were prepared with yields between 50-80%. The abbreviated notation for wax esters is alcohol-acid, using Phy for phytol and for phytenic acid and the common short form for the straight chain constituents. Acidic catalysis by *p*-toluenesulfonic acid (8) for esterification was not applicable to the synthesis of wax esters from phytol or from phytenic acid.

Isolation of Wax Esters from Plants

The aquatic moss *Hygrohypnum luridum* was collected from rocks in a small stream (Exshaw, Alberta, Canada). Moss of dry habitat, *Paraleucobryum longifolium*, was collected from glacial boulders in deep shade (Somerset Co., Maine). Lipids were extracted from moss gametophores with $CHCl_3$:CH_3OH and fractionated by column chromatography on Unisil as previously described (3,9). Wax esters were obtained together with hydrocarbons and steryl esters and were separated from these components by column chromatography on MgO (10,11).

Timothy grass, *Phleum pratense*, was harvested in September, 5 days after a heavy frost from a location near Austin, MN, where brown grass leaves, damaged by cold, were next to green leaves which did not show any sign of deterioration. The samples of leaves were extracted with $CHCl_3$:CH_3OH (2:1), and chlorophyll was determined in the extracts (12). The lipids were recovered, and a fraction containing wax and steryl esters was obtained by TLC on Silica Gel H, 1 mm thick, with trichloroethylene as solvent. This mixture was not further fractionated.

Hydrogenation of Wax Esters

Wax esters containing the phytyl moiety had to be hydrogenated for GLC analysis, because they were destroyed at the high temperature (280 C) used. Hydrogenation of the double bond in phytol and its derivatives met with difficulties (13), but nearly quantitative yields of phytanyl wax esters were obtained when samples of 1-10 mg were hydrogenated with 20 mg of catalyst, 10% Pd on $CaCO_3$ (K and K Laboratories, Plainview, NY) in 3 ml petroleum ether (bp 60-70 C) with a slow stream of H_2 and magnetic stirring for 1 hr.

GLC of Wax Esters

An F&M 402 apparatus (Hewlett-Packard Instruments Co., Avondale, PA) was used with

TABLE II

Gas Liquid Chromatography of Compounds Related to Phytol

Compound	Equivalent chain length[a] on EGS	DEGS	Compound	Equivalent chain length[b] on EGS	DEGS
Phytanyl acetate	16.9	17.1	Phytane[c]	8.1	11.2
Phytyl acetate	18.4	18.3	Phytadienes[d]	11.2	12.5
				11.8[e]	13.1[e]
Oleyl acetate	18.8	18.4		12.3	13.5
				12.7	14.0
			Phytyl methyl ethers[d]	14.2[e]	15.0[e]
				15.2	15.8
			Phytenal	20.2	19.9

[a]ECL in reference to straight chain saturated alkyl acetates.

[b]ECL in reference to straight chain saturated fatty acid methyl esters.

[c]From phytol by hydrogenation with Pd or PtO_2.

[d]From phytyl esters by acidic hydrolysis in methanol.

[e]Major component of the mixture which had been isolated by thin layer chromatography.

helium as carrier gas and with a flame ionization detector. The aluminum column, 0.3 mm internal diameter and 1.2 m long, was packed with OV-1, 5% on Gas Chrom Q, 100-120 mesh (Applied Science Laboratories, State College, PA). The column was operated at 280 C, and under these conditions, the time required for elution of arachidyl arachidate was 1 hr. Equivalent chain length (ECL) in reference to 16:0-16:0, 32.0; 18:0-18:0, 36.0; and 20:0-20:0, 40.0 was for phytanyl-16:0 and 16:0-phytanate, 33.6; phytanyl phytanate, 35.1; and phytanyl-18:0, 35.6. All phytyl wax esters were destroyed by these GLC conditions, but 16:0-Phy emerged without detectable alteration, ECL 34.6.

Methanolysis of Phytenoyl Wax Esters

Presence of the phytenoyl moiety required acidic methanolysis for analysis of acyl constituents. Samples of 1-10 mg were heated for 1 hr at 90 C with 1-2 ml dry 2 N HCl in CH_3OH, in a teflon lined screw cap tube with magnetic stirrer. Methanol and HCl were removed first by a stream of N_2, then in high vacuum. TLC showed methanolysis to be virtually complete. The methyl esters were separated from alcohols by preparative TLC before analyzing them by GLC.

Complete methanolysis also can be achieved with BF_3, 14% in CH_3OH (Applied Science Laboratories, State College, PA) under the same conditions, but the reagent was slightly destructive to phytenic acid. Phytol was destroyed by both acidic reagents and yielded mainly phytadienes and phytyl methyl ethers. The identity of these products was verified, after isolation by TLC, by mass spectrometry which gave molecular ions m/e 278 and 310, respectively.

Methanolysis of Phytyl Wax Esters

Presence of the phytyl moiety required alkaline methanolysis for analysis of the alkyl constituents. Samples of 1-10 mg were heated under the above conditions with 1-2 ml 2 N $NaOCH_3$ in CH_3OH. The solutions then were poured into 20 ml water, slightly acidified with 4 N HCl, extracted 3 times with petroleum ether (bp 30-60 C):diethyl ether (1:1) and the extracts then were washed with water. The samples were exposed to CH_2N_2 (14) to methylate any fatty acids which had been formed under these conditions. All synthetic samples were hydrolyzed completely and phytol was not altered. The alcohols were separated from methyl esters by TLC and then converted to acetates by heating for 1 hr at 90 C with 0.1 ml acetic anhydride.

Phytenic acid was extensively isomerized by the alkaline reagent at 90 C. At room temperature, wax esters were not hydrolyzed completely by the reagent within 24 hr. Reflux with aqueous 2N KOH in methanol for 2 hr isomerized ca. 15% of the phytenic acid. These latter conditions completely hydrolyzed phy-16:0, but only 30% of Phy-Phy.

GLC of Isoprenoid Methyl Esters and Acetates

A Hewlett-Packard Model F&M 830 apparatus was used at 180 C with helium as carrier gas and hydrogen flame detector. Columns were 0.3 mm ID and 1.8 m long, packed with ethyl-

TABLE III

Analysis of Hydrogenated Wax Esters from Mosses[a]

Compound	*Hygrohypnum luridum*	*Paraleucobryum longifolium*
C_{32}	1.4	-
Phy-C_{16}	10.6	9.4
C_{34}	1.1	-
Phy-Phy	78.4	39.8
Phy-C_{18}	5.9	5.3
C_{36}	0.9	-
Phy-C_{20}	1.7	2.4
C_{38}	-	4.0
C_{40}	+	12.2
C_{42}	+	13.2
C_{44}	+	8.9
C_{46}	-	4.7

[a]Percent of peak areas in gas liquid chromatography on OV-1 at 280 C.

[b](-) = not detectable; (+) detectable in small quantities.

ene glycol succinate (EGS), 15% HI-EFF-2BP on 100-120 mesh Gas Chrom P (Applied Science Laboratories, State College, PA); and 0.3 mm ID and 2.4 m long packed with diethylene glycol succinate (DEGS) (Varian Aerograph, Walnut Creek, CA), 20% on siliconized Chromosorb W (Johns-Manville, Chicago, IL).

Table I gives GLC data on compounds related to phytenic acid as they occurred in the crude preparation and after treatment of esters with $NaOCH_3$. Table II gives such data on compounds that are related to phytol and may arise with hydrogenation of the alcohol or by acidic hydrolysis of its esters in methanol. Methyl phytenate migrated distinct from oleate on DEGS, but not on EGS; whereas, phytyl and oleyl acetates were resolved on EGS but not on DEGS. Phytyl methyl ethers are listed because they are not separated well from methyl esters by TLC, and may lead to misinterpretations when analyzing the latter by GLC.

RESULTS FROM NATURAL MIXTURES

GLC of wax esters from both mosses, *Hygropypnum luridum* and *Paraleucobryum longifolium*, showed some peaks of straight chain wax esters and an extended solvent front as had been observed in GLC of authentic phytyl esters. Presence of *n*-alkyl phytenates was not indicated, although the experiments with authentic compounds had shown that this type ester could be chromatographed. The presence of isoprenoid esters was revealed by GLC of the hydrogenated wax esters which showed a peak corresponding to phytanyl phytanate and representing the major portion of both samples (Table III). They also contained some phytanyl esters of straight chain acids together with common wax esters. One can conclude that all phytenic acid was bound to phytol. Phytol was in molar excess over phytenic acid, and the excess was bound to straight chain fatty acids.

From analyses of the constituents (Table IV), it was seen that phytenic acid was a major component of wax esters from both mosses at levels of 68.2 and 29.5% of acyl groups. However, GLC of the hydrogenated wax samples (Table III) had indicated the presence of 78.4 and 39.8% phytanate. Corresponding values for total phytol by analysis of alkyl groups were 85.1 and 52.7% (Table IV), but 96.6 and 56.9% by GLC of the hydrogenated wax esters (Table III). The discrepancies of 4-11% may have been due to the presence of minor amounts of saturated isoprenoid structures. An additional and perhaps the major cause was the great difference in GLC conditions which lead to some quantitative deviations. Minor decomposition of phytyl acetate was shown by a shift of the base line which consistently occurred shortly before this peak emerged. We did not attempt further refinement of the quantitative analyses.

Table IV shows that alkyl and acyl groups of moss wax esters could not be analyzed by a single procedure. Phytol was lost completely by the $HCl:CH_3OH$ method, and phytenic acid was isomerized largely by the $NaOCH_3$ method and yielded peaks obscuring the chromatogram.

When analyzing wax esters of the grass, *Phleum pratense*, it was of interest also to determine chlorophyll, because it is a likely source of phytol in wax esters of plants (1). From the green leaves, 148 mg lipid extract per gram dry wt was obtained, of which 15.8 mg was chlorophyll and 8.9 mg was wax plus steryl esters. The frost damaged sample gave 77 mg lipid extract per gram dry wt, of which 1.5 mg

TABLE IV

Constituents of Wax Esters from Mosses[a]

Structure	*Hygrohypnum luridum*		*Paraleucobryum longifolium*	
	Alcohol by $NaOCH_3$[b]	Acid by HCl/CH_3OH[c]	Alcohol by $NaOCH_3$[d]	Acid by HCl/CH_3OH[c]
16:0	0.8	12.0	+	18.8
16:1	1.1	5.1	+	7.2
18:0	1.2	1.4	3.1	6.6
18:1	+	+	-	+
Phy	85.1	68.2	52.7	29.5
18:2	-	1.7	-	3.7
18:3	-	6.3	-	10.4
20:0	0.9	+	2.8	5.6
20:4	-	1.4	-	4.3
20:5	-	3.4	-	-
22:0	11.0	+	21.5	8.3
24:0	+	-	16.9	5.8
26:0	+	-	2.9	-

[a]Percent of peak areas in gas liquid chromatography on ethylene glycol succinate and diethylene glycol succinate at 180 C.

[b]Analysis of methyl esters obtained from hydrolysis with $NaOCH_3$ showed 11.5% methyl phytenate and 52% isomers.

[c]Analysis of alcohol acetates obtained from hydrolysis with HCl/CH_3OH did not indicate the presence of phytyl acetate or of isomers.

[d]Analysis of methyl esters obtained from hydrolysis with $NaOCH_3$ showed 4.5% methyl phytenate and 22.5% isomers.

TABLE V

Analysis of Wax and Steryl Esters from *Phleum pratense*[a]

Structure of moieties	Acids from wax + steryl esters[b]		Alcohols from wax esters[c]	
	Viable	Frost damaged	Viable	Frost damaged
12:0	2.8	3.7	-	-
14:0	11.2	13.0	-	-
16:0	12.4	11.3	9.6	-
18:0	6.4	8.4	9.8	-
18:1	4.2	2.2	-	-
Phy	-	-	37.8	83.5
18:2	12.6	8.6	-	-
18:3	33.0	33.2	-	-
20:0	5.1	9.1	17.4	7.8
22:0	6.3	5.6	25.4	8.7
24:0	3.3	2.7	-	-

[a]Percent of peak areas in gas liquid chromatography on ethylene glycol succinate and diethylene glycol succinate at 180 C.

[b]HCl-CH_3OH method.

[c]$NaOCH_3$ method.

was chlorophyll and 8.8 mg was wax plus steryl esters. Apparently, due to freezing of the tissue, ca. 50% of the lipid and 90% of the chlorophyll was lost, but the absolute amount of wax plus steryl esters remained the same. However, in this mixed fraction from the undamaged grass, steryl esters were the main components, whereas, in the damaged grass, wax esters were the greater portion.

The $NaOCH_3$ method showed that in these waxes, phytol was prominent among alkyl groups and that its amount was increased greatly upon frost damage (Table V). The $HCl:CH_3OH$ method showed that neither sample contained phytenic acid. Linolenic was the major acid, but its relative concentration did not change under the adverse conditions.

Phytenic acid was a major acyl component, and phytol was the predominant alkyl component in wax esters of several mosses analyzed in

this laboratory (3). The habitats of these mosses ranged from aquatic to extremely dry, and from temperate to polar conditions. Phytenic acid occurrence spanned all genera analyzed, but levels were highest in the order *Hypnobryales*. Although phytol occurs in wax esters of higher plants, phytenic acid has not as yet been reported from them (1,2,15). The presence of phytenic acid in waxes may be a distinctive feature of mosses. This difference and the changes caused by frost as described here for grass leaves warrant further investigation of isoprenoid wax esters in plants.

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Failure to Demonstrate Degradation of [4-^{14}C] Cholesterol to Volatile Hydrocarbons in Rats and in Human Fecal Homogenates

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ABSTRACT

The inability of previous workers to recover completely the radioactivity from ingested [4-^{14}C] cholesterol has led to the hypothesis that the colonic flora of some individuals degrade the sterol nucleus to volatile hydrocarbons, particularly CH_4. In the present investigation, the production of radioactive volatiles was measured following incubation of [4-^{14}C] cholesterol with 8 human fecal homogenates or after instillation of the labeled sterol into the cecum of 3 rats housed in a closed rebreathing system. Three of the 8 homogenates and each of the 3 rats produced copious CH_4. However, analysis by combustion demonstrated no radioactivity above background in the volatile headspace of the homogenates or the gas space of the closed system housing the rats, indicating that <0.001% of the number 4 carbon of [4-^{14}C] cholesterol could have been converted to volatile hydrocarbons. This study, therefore, provides no support for the concept that volatile products account for the incomplete recovery of ingested sterols observed in certain subjects. However, this hypothesis can not be excluded entirely until similar results are obtained with subjects who can be shown to degrade cholesterol.

INTRODUCTION

Grundy, Ahrens, and Salen (1) have proposed that the intestinal flora of some individuals are capable of degrading the ring structure of neutral sterols. This proposal is based on their finding in some individuals of an incomplete fecal recovery of ingested [4-^{14}C] cholesterol and β-sitosterol, despite complete recovery of simultaneously administered nonabsorbable markers. Loss of sizeable quantities of plant sterols during passage through the intestinal tract of certain individuals also has been reported by Connor, et al. (2) and Borgström (3). The apparent disappearance of [4-^{14}C] cholesterol during incubation with human fecal homogenates was reported by 3 groups (1,4,5), but was not observed by a fourth group (6).

The products of this proposed degradation process have never been identified, however. The possibility that the disappearing ^{14}C of [4-^{14}C] cholesterol might be excreted as more polar fecal compound or $^{14}CO_2$ (1,7,8) has been excluded. Ahrens (8), therefore, has speculated that sterols may be degraded to volatile hydrocarbons, such as methane (CH_4), which would be lost in the flatus, or absorbed and then exhaled by the lungs.

The hypothesis that CH_4 might be derived from cholesterol was appealing to us because our previous studies (9) had shown that: a) certain individuals excrete sufficient CH_4, .05-.005 moles per day, to account for the carbon in the 4 position of the cholesterol ring structure, which disappears during the proposed degradation; b) some individuals consistently excrete CH_4, whereas others excrete little or none, analogous to the finding that certain individuals apparently degrade large quantities of sterols, whereas there is little or no degradation in others; c) the percentage of the population (20-30%) that excrete sufficient CH_4 to account for degradation is roughly comparable to the fraction of the population which degrades cholesterol, if < 90% recovery of sitosterol is arbitrarily assumed to indicate appreciable degradation (3); and d) CH_4 is excreted despite prolonged fasting implicating an endogenous carbon source.

The present study was designed to determine if the ^{14}C of [4-^{14}C] cholesterol is converted to volatile hydrocarbons during incubation with human fecal homogenates or following instillation into the colon of the rat.

METHODS

Incubation of Human Fecal Samples with Cholesterol-4-^{14}C

Fecal samples were obtained from 8 healthy subjects. Analysis of expired air of these subjects for CH_4 (9) showed that 3 of the 8 subjects excreted appreciable CH_4, while the other 5 excreted little or no CH_4.

Because the colonic lumen is normally anerobic, an attempt was made to handle the fecal samples in an anaerobic fashion. One-

hundred ml of 0.1 molar Na phosphate (pH 7.0) in isotonic saline was added to the honogenizing vessel. A stream of N_2 was then bubbled through the buffer for ca. 20 min to remove dissolved O_2. Under a rapid flow of N_2, ca. 10 g of freshly collected fecal sample was added to the vessel which then was purged further with N_2. The vessel was sealed, placed in ice water, and the specimen was homogenized for 10 min. Under a stream of N_2 20 ml of homogenate was aspirated into each of 2 100-ml syringes, previously purged with N_2, which were fitted with stopcocks. Two ml of a deoxygenated solution containing 10 mmolar taurocholate, 20 mmolar lecithin, and a known amount (ca. 1 μc) of [4-^{14}C] cholesterol (specific activity 0.2 μc/mg) was added to each syringe. Nitrogen containing 10% CO_2 then was aspirated into the syring until the total gas content was 50 ml. The syringes were vigorously mixed for 5 min, and the control syringe was frozen by immersion in dry ice and acetone and stored at -30 C. The other syring was constantly mixed on a rotating wheel during incubation at 38 C.

After 72 hr, 10 ml of gas from the incubated syring was removed for analysis for CH_4 by gas chromatography (9). An additional 30 ml of gas was quantitatively transferred to another 100 ml syring and was analyzed for the presence of $^{14}CO_2$ and combustible ^{14}C as follows. Seventy ml of O_2, to promote combustion, and 2 ml cold carrier CH_4 were added to the syringe. This gas then was aspirated slowly through a combustion train consisting of, in series, a CO_2 absorber, a combustion chamber, and a second CO_2 absorber. The CO_2 absorbing fluid was 4 ml of 0.5% hyamine hydroxide: methanol (1:1). The combustion chamber consisted of a ceramic tube, 30 cm in length, maintained at 1050 C in an oven. An additional 500 ml air was aspirated through the system after the syringe had been emptied to flush the dead space.

Preliminary studies demonstrated that > 99% of cold methane was combusted as it passed through the oven, and virtually all of this CH_4 was recovered as CO_2.

The radioactivity of the CO_2 trapping fluid was determined by adding 15 ml of Flouralloy (Beckman Instruments, Fullerton, CA) in toluene and counting in a liquid scintillation counter to at least ± 5% accuracy.

The radioactivity of 5 ml aliquots of the test and thawed control homogenates was determined using the saponification and extraction technique of Grundy, Ahrens, and Miettinen (10). An internal standard was used to determine the recovery of radioactivity from the aqueous and petroleum ether phases.

Four additional fecal samples, 3 of which were obtained from the original 8 subjects, were studies as follows. After preparation of the homogenate as previously described, 4-ml aliquots were aspirated into each of 2 10-ml syringes. One μc of [4-^{14}C] cholesterol was added, the contents of the syringes were mixed for 5 min, and 1 sample was frozen and the other incubated as described previously. At the end of the 72 hr incubation period, the entire homogenates were instilled into dialysis bags. To determine the radioactivity, samples were dried at room temperature and combusted in a Packard Tri-Carb Oxidizer, (Packard Instruments, Inc., Downers Grove, IL).

Studies in Rats

Rats with chronically inplanted cecal cannulas (11) were employed. Three rats, which were known to excrete large amounts of CH_4 were studied.

One ml of a solution containing 10 mM taurocholate, 20 mM lecithin, and 2 uc of [^{14}C] cholesterol was instilled into the cecum. The animal then was placed for 24 hr in a closed, rebreathing system which has been previously described (12). At the end of this period, an aliquot of the gas in the closed system was analyzed for CH_4. To determine if any of the ^{14}C had been metabolized to a volatile form, the entire gas phase in the closed system was aspirated through the combustion train. Radioactivity of the CO_2 absorbing fluid was measured as described previously.

RESULTS

Table 1 summarizes the results of studies with the 8 fecal homogenates which were analyzed for radioactivity following saponification and extraction. The gas phase of the fecal samples from each of the 3 subjects whose expired air contained high concentrations of CH_4 (subjects 6,7, and 8) contained at least 20 times more CH_4 than did any of the other 5 samples. No significant radioactivity (< 8 CPM, or < 11 DPM, above background) was observed in the CO_2 or the combustible gases of any sample. Because ca. three-fifths of the head space of the homogenates was analyzed, it can be calculated that the maximum percent of the radioactivity of [4-^{14}C] cholesterol that might have been converted to a combustible gas was ca. 0.001%. Nevertheless, the incubated fecal sample consistently had less recoverable radioactivity than did its frozen control when extracted and then counted. This disparity had no relation to the quantity of CH_4 produced. The radioactivity recovered in the incubated sample averaged 84

TABLE I

^{14}C in Gas phase

Fecal sample	CH_4 produced (ml/72 hr)	CO_2 (CPM)	Combustible gases (CPM)	^{14}C Recovery (incubated/control x 100)
1	0.0017	0[a]	0	85
2	0.014	0	0	82
3	0.0011	0	0	80
4	0.099	0	0	88
5	0.066	0	0	91
6	2.5	0	0	69
7	4.4	0	0	87
8	11	0	0	88
				Mean 84 ± 2.4[b]

[a]0 indicates less than 8 CPM above background.

[b]Variability expressedaas ± 1 SEM

± 2.4% (1 SEM) of the frozen control ($p<0.01$). The radioactivity of both the incubated and control samples was present in the petroleum ether phase, while the aqueous phase contained negligible counts. The recovery of ^{14}C from the control homogenates averaged 95 ± 2.0% of that initially added.

In contrast to the results with the extraction procedure, recovery of ^{14}C was virtually complete in each of the 4 studies in which radioactivity was determined by combustion of the entire homogenate. By this method, the ratio of radioactivity of the incubated sample to the control averaged 102% (Range 95-106%).

The CH_4 production of the 3 rats averaged 2.4 ml per 24 hr. None of the rats excreted detectable ^{14}C in the form of a combustible gas over the 24 hr study period. Because the combustion products of the entire gas space of the closed system could be trapped in 1 scintillation vial, this finding indicates that < 0.0005% of the instilled [4-^{14}C] cholesterol could have been converted to CH_4.

DISCUSSION

In the present study, no detectable radioactivity was liberated in the form of $^{14}CO_2$ or $^{14}CH_4$ during incubation of human fecal homogenates with [4-^{14}C] cholesterol. Three of these homogenates produced relatively large quantities of CH_4, and, thus, it is apparent that the carbon in the 4 position of cholesterol did not serve as a carbon source for CH_4 in these homogenates. Similarly, no $^{14}CH_4$ was detected after instillation of labeled cholesterol into the cecum of rats which were producing CH_4. Because the combustion temperature (1050 C) used to convert CH_4 to CO_2 is sufficient to oxidize virtually all hydrocarbons present in the gas phase, degradation of the number 4 carbon of cholesterol to volatile hydrocarbons other than CH_4 is excluded also.

Because we did not determine if the subjects used in the present study degraded cholesterol, it might be argued that the fecal specimens may all have been obtained from nondegraders. The concept that the population can be divided into degraders and nondegraders has been derived from recovery studies. The results of these studies suggest, however, that there is a continuum of sterol recoveries from the population rather than a clear cut segregation into 2 groups. For example, in 7 subjects studied by Grundy, et al., (1) fecal recoveries of [4-^{14}C] cholesterol were 95%, 95%, 90%, 90%, 87%, 75%, and 69%. Of 24 subjects studied by Borgström (3), recovery of plant sterols from 23 individuals showed a continuum from 110 to 77%, with a recovery of 41% from the remaining subject. Because this technique is relatively crude, some value such as < 90% recovery, is arbitrarily considered to be evidence of degradation. Greater than 90% recovery seems to be considered evidence that no degratation occurred, although this value should be interpreted to indicate that degradation was of insufficient magnitude to be detected by the technique employed. Thus, there is little data to support the concept that some individuals do not degrade cholesterol, while others degrade readily detectable quantities.

In contrast to the insensitivity of recovery studies, if degradation of [4-^{14}C] cholesterol results in labeled volatile products, analysis of the gas space for ^{14}C provides an extremely sensitive means of detecting this degradation. Our failure to detect ^{14}C in quantities above background radiation in these gases indicate that < 0.001% of the ^{14}C of cholesterol was converted to a volatile hydrocarbon. While the possibility remains that fecal specimens from other individuals might have shown appreciable conversion of cholesterol to volatile products, this

seems unlikely in view of the essentially zero conversion observed in the 8 fecal specimens used in the present investigation.

It is also theoretically possible that bacteria might degrade sterols to volatile hydrocarbons in the gut, but lose this ability when studied in fecal homogenates. However, it is apparent that the bacteria in the homogenates continued to produce CH_4. It seems unlikely that, if bacteria were utilizing cholesterol as a substrate for CH_4 production in the colon, they would not utilize cholesterol in the fecal homogenates which had no exogenously added carbon source other than CO_2.

In an attempt to assess the in situ ability of the colonic flora to degrade [4-^{14}C] cholesterol to volatile radioactive products, labeled cholesterol was instilled into the cecum of intact rats. The interpretation of the findings of this experiment is somewhat weakened by the previous inability of several groups to demonstrate sterol degradation in rats (8,13). However, these groups did not study the CH_4 excretion of their experimental animals. Methane is excreted in extremely variable quantities by rats, and it seems possible that the rats utilized in previous studies were not CH_4 producers. In addition, as discussed previously, measurement of $^{14}CH_4$ accumulation is about 20,000 times more sensitive than are recovery studies for the detection of degradation in the rat. Our studies in rats demonstrate that there was virtually zero, 0.0005%, conversion of [4-^{14}C] cholesterol to radioactive volatiles, despite brisk CH_4 production.

In addition to the preceding experimental evidence, there is a theoretical argument against the possibility that CH_4 might be derived from the ^{14}C of [4-^{14}C] cholesterol. All CH_4 producing bacteria studied to date produce CH_4 via the reduction of CO_2 with H_2 (14). Because careful studies of degraders have demonstrated no appreciable excretion of $^{14}CO_2$ (7,8), one would have to postulate a yet unrecognized metabolic pathway for the production of CH_4 from cholesterol.

The present study does not indicate whether carbons in positions other than number 4 might be converted to volatile products. However, the concept of degradation of cholesterol to volatile metabolites is based on failure to recover the radioactive carbon in the 4 position. Thus, our failure to demonstrate in vitro conversion of [4-^{14}C] cholesterol to volatile metabolites provides atrong negative evidence concerning this proposed pathway of enteric cholesterol degradation.

Despite our failure to demonstrate the production of radioactive volatiles when fecal homogenates incubated with [4-^{14}C] cholesterol were extracted by the technique of Grundy, et al., (10), recovery of ^{14}C was significantly ($P<0.005$) less (84 ± 2.4%) than that from the nonincubated control. Rosenfield and Hellman (5), using the Soxhlet technique, similarly recovered a mean of only ca. 86% of labeled cholesterol or β-sitosterol following incubation of human fecal homogenates. Grundy, et al., (1) also noted losses of 15-40% [4-^{14}C] cholesterol during incubation with some fecal specimens. These workers found the remaining radioactivity in the form of cholesteryl sulfate, which remains in the aqueous phase and is not recovered using their extraction technique.

When the entire homogenate was combusted, we obtained complete recovery of ^{14}C. Thus, it appears that in vitro the fecal bacteria converted cholesterol into some form, such as cholesteryl sulfates, which was not extractable with the technique employed. While unlikely, we cannot rule out the possibility that the homogenate was not truly homogeneous, thus resulting in a sampling error. Against this latter hypothesis is the consistant finding of less than the expected recovery of radioactivity, whereas one might expect variable recovery, either greater or less than expected, if there was a nonhomogeneous distribution of the sterol.

Enteric sterol catabolism may not be simulated in incubated fecal homogenates. For example, Grundy, et al., (1) detected very little cholesteryl sulfate in the feces of degraders, whereas incubated fecal homogenates converted 15-40% of [4-^{14}C] cholesterol into sulfate esters. In addition, these investigators found that extraction or combustion of fecal samples from apparent degraders gave similar recoveries of ^{14}C activity in contrast to the consistent discrepancy observed in our in vitro studies. Until these disparities between in vivo and in vitro observations are reconciled, it is uncertain whether the degradation of sterols to a nonextractable form in homogenates pertains to the apparent phenomenon of degradation in vivo.

While we have not obtained evidence to support the hypothesis that [4-^{14}C] cholesterol is converted to volatile metabolites, to exclude this possiblity with absolute certainty, it will probably be necessary for investigators, who have clearly identified subjects as degraders, to carry out studies in which the radioactivity of the volatile excretory products of these subjects is measured after ingestion of [4-^{14}C] sterols. If such studies prove negative, the possibility still remains that sterols could be degraded to products which are absorbed and enter metabolic pools which turn over slowly. The excretory rate of ^{14}C from such pools might be

too slow to be detectable by the technique employed to date.

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Examination of Acetolysis Products of Phosphatidylcholine by Gas Chromatography-Mass Spectrometry

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ABSTRACT

A comparison of monoacetyldiglycerides obtained from authentic phosphatidylcholines by acetolysis with those obtained by phospholipase C-acetylation was made to examine the intermolecular acyl migration, the intramolecular acyl migration between C-1 and C-2, and the formation of 1,3-isomer in the acetolysis reaction. Egg yolk phosphatidylcholine also was used. It was revealed that in acetolysis, the intermolecular acyl migration and selective degradation of polyunsaturated fatty acids did not take place at all. The intramolecular acyl migration, including the formation of 1,3-isomer, occurred to a small extent. Appreciable difference was not found in comparison of molecular species compositions of monoacetyldiglycerides derived by both methods from egg yolk phosphatidylcholine, except small differences found in the contents of two kinds of molecular species.

INTRODUCTION

Fractionation of intact phospholipids into individual molecular species are difficult due to their strong polarity. The use of monoacetyldiglyceride (MADG) for subfractionation of glycerophospholipid was proposed by Renkonen (1). In this less polar form, the usual fractionation methods for triglyceride, including thin layer chromatography (TLC) on silver nitrate impregnated silica gel and gas liquid chromatography (GLC) on nonpolar liquid phase, are able to be used efficiently. The conversion of glycerophospholipids into MADG can be carried out by two methods: a) acetolysis in a mixture of acetic anhydride and acetic acid, and b) treatment with phospholipase C followed by acetylation (1). Though phospholipase C-acetylation method is milder than the former method, there are some limitations in regard to the kind of phospholipids applicable due to the substrate specificity of the enzyme. On the other hand, the acetolysis method is preferred over the latter method because of simplicity of the procedure and the lack of specificity. However, some intramolecular and intermolecular acyl migrations during the acetolysis reaction have been reported in some experiments, in which the positional distribution of fatty acid of phosphatidylcholine (PC) from egg yolk were compared by the both methods (2-4). The possibility of a selective degradation of polyunsaturated fatty acids in the reaction also has been described (5). Reviewing carefully the reports which were critical to the acetolysis, we realized that unnecessarily prolonged heating was employed in every case. In addition, the critical data were obtained from the natural mixture of PC, but not from authentic pure compounds. Because MADG is very useful for the identification of molecular species by gas chromatography-mass spectrometry (GC-MS) as reported previously (6), it would be valuable to reexamine the kind and extent of undesirable side reaction occurring in acetolysis.

The present paper deals with a comparison of MADG obtained from authentic PC by acetolysis with those obtained by phospholipase C-acetylation. TLC, GLC, GC-MS, and mass fragmentography were used to examine the intermolecular acyl migration, intramolecular acyl migration between C-1 and C-2, and the formation of 1,3-isomer in the reaction. Egg yolk PC also was used for the examination of selective degradation of unsaturated fatty acids. Intermolecular acyl migration and selective degradation of polyunsaturated fatty acids did not take place, and the intramolecular acyl migration including the formation of 1,3-isomer occurred to a small extent. Comparison of the molecular species of MADG obtained from egg yolk PC by the two methods revealed no differences.

EXPERIMENTAL PROCEDURE

Materials

Synthetic 1,2-dipalmitoyl-PC, 1,2-dioleoyl-PC, 1-palmitoyl-2-palmitoleoyl-PC, 1-stearoyl-2-oleoyl-PC, 1,2-dipalmitin, 1,3-dipalmitin, 1,2-distearin, and 1,3-distearin were purchased from Applied Science Laboratories (State College, PA). Egg yolk PC was prepared according to the Pangborn's method (7), and then purified on silicic acid (8,9). Phospholipase C of *Clostridium welchii* was purchased from Sigma Chemical Co. (St. Louis, MO). Specially prepared acetic anhydride and redistilled, dried

TABLE I

Fatty Acid Composition of Egg Yolk Phosphatidylcholine Before and After Acetolysis

Fatty acid	Before acetolysis (% by wt)	5 Hr after acetolysis (% by wt)
16:0	34.5	35.8
16:1	1.6	1.2
17:1	0.7	0.5
18:0	12.4	12.7
18:1	32.3	32.2
18:2	15.0	15.1
20:4	3.5	2.6
22:6	---[a]	---[a]

[a]Not identified by the present gas liquid chromatographic condition.

pyridine were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). Silver nitrate impregnated silica gel plate was prepared by immersing a precoated Silica Gel G plate (E. Merck, A.G., Darmstadt, Germany) in a 10% solution of silver nitrate in acetonitrile for 1 hr. All other solvents and chemicals were reagent grade.

Acetolysis of PC

The reaction was carried out in a Teflon-lined screw capped vial of 7 ml capacity (Pierce Chemicals Co., Rockford, IL). About 2-5 mg of PC were dissolved in 1 ml of an acetic acid: acetic anhydride mixture (3:2, v/v) in the vial. The tip of the tightly capped vial was dipped in an oil bath kept at 150 C for 5 hr. The progress of the reaction was checked by TLC on Silica Gel G developed with petroleum ether:ethyl ether (85:15, v/v). After completion of the reaction, MADG was extracted from the reaction mixture with chloroform-methanol-water (8:4:3, v/v/v) 3 times, and followed by washing with water and drying over anhydrous sodium sulfate for 1 hr. The dried solution was concentrated in vacuo for further analyses.

Phospholipase C and Acetylation

About 5 mg of PC was dissolved with 1 ml of ethyl ether in a similar screw capped vial as above, then 0.5 ml 0.4 M $CaCl_2$ and 0.1 ml Tris buffer (pH 7.4) containing 4 units of phospholipase C were added. The reaction was carried out at room temperature with vigorous shaking for 10 min. The completion of the reaction was checked on a Silica Gel G plate developed with n-hexane:ethyl ether (1:1, v/v). The diglyceride released was extracted with ethyl ether, followed by washing with water and drying over anhydrous sodium sulfate. Acetylation of diglycerides was carried out in a mixture of acetic anhydride and pyridine (10). The purity of MADG obtained was checked by TLC.

Analytical Methods

TLC for MADG was run on a silver nitrate impregnated Silica Gel G plate using benzene: chloroform:methanol (98:2:0.1, v/v/v) as developing solvent (11), except otherwise stated. In this TLC, molecular species of MADG different in the degree of unsaturation, as well as 1,2- and 1,3-isomers, were separated simultaneously.

MADG separated on the TLC plate was extracted according to the method of Bligh and Dyer (12) after the addition of a known amount of tricaprin as an internal standard. The extracted MADG was determined quantitatively by GLC using a Shimadzu gas chromatograph GC-5A, equipped with a 35 cm x 3 mm column packed with 1% silicone OV-1 on 60-80 mesh Chromosorb W (AW and HMDS). The column temperature was programmed from 200 C to 290 C with an increasing rate at 3 C/min.

GC-MS of MADG was carried out on an LKB-9000S gas chromatograph-mass spectrometer. The analytical conditions were the same as described in the previous paper (6), except as otherwise stated.

Mass fragmentography was carried out on an LKB-9000S gas chromatograph-mass spectrometer, equipped with a high speed multiple ion detector-peak matcher (MID–PM), and with a 35 cm x 3 mm column packed with 3% Dexsil 300GC on 70-80 mesh Anakrom ABS. The column temperature was 280 C or 290 C. The electron voltage was 20 eV and the speed of oscillograph paper 5 mm/min. The MID was adjusted to record the intensity of m/e 367 and 369 for stearoyl-oleoyl-acetyl glycerol, and m/e 339 and 341 for palmitoyl-palmitoleoyl-acetyl glycerol.

Fatty acid methyl esters (13) were analyzed by a Shimadzu gas chromatograph GC-5A, equipped with a 2.5 m x 3 mm column packed with 15% DEGS on 60-80 mesh Neopak AS. The column oven temperature was 187 C. The nitrogen flow rate was 60 ml/min. To calibrate the eluted peaks, NIH fatty acid standard mixture (Type KE) (Applied Science Lab.) was used, and the peak areas were determined by a Shimadzu digital integrater ITG-2A.

RESULTS AND DISCUSSION

To examine the selective degradation of unsaturated fatty acids in the acetolysis reaction, the composition of the fatty acid of MADG prepared by acetolysis from egg yolk PC was compared with that of the intact egg yolk PC. As will be seen in Table I, there was no appre-

ciable change in fatty acid compositions before and after acetolysis.

Intermolecular acyl migration was examined by using an equimolar mixture of 1,2-dipalmitoyl PC and 1,2-dioleoyl PC, which was subjected to the acetolysis for 5 hr and 16 hr. The MADG obtained by acetolysis for 5 hr was examined by TLC, GLC, and GC-MS. If the intermolecular acyl migration was actually caused by the acetolysis as reported by Privett and Nutter (4), a hybrid molecular species, palmitoyl-oleoyl-acetyl glycerol, must be detected by the methods mentioned above. Figure 1 shows the results of TLC. Although as much as 200 μg of the sample was spotted on the plate, no spot corresponding to the monoene type MADG consisting of palmitoyl-oleoyl-acetyl glycerol was detected on the plate, on which as small as 1 μg of MADG was detectable. Moreover, no peak corresponding to palmitoyl-oleoyl MADG was detected between the peaks of dipalmitoyl MADG and dioleoyl MADG on the gas chromatogram as shown in Figure 2. In the results of GC-MS, no fragment attributable to palmitoyl-oleoyl-acetyl glycerol was detected either. The same results also were obtained with MADG prepared by acetolysis for 16 hr.

The formation of 1,3-isomer was examined by TLC by using authentic PC. In the cases of 1-palmitoyl-2-palmitoleoyl PC, 1-stearoyl-2-oleoyl PC, and 1,2-dioleoyl PC, there was no formation of the 1,3-isomer after acetolysis for 5 hr. In the case of a mixture of 1,2-dipalmitoyl PC and 1,2-dioleoyl PC, a small amount of 1,3-dipalmitoyl-2-acetyl glycerol and a slight amount of 1,3-dioleoyl-2-acetyl glycerol were detected on the silver nitrate impregnated Silica Gel G plate. The percentage of 1,3-dipalmitoyl MADG in the total dipalmitoyl MADG was ca. 9.9%, whereas, 1,3-dioleoyl MADG in the total dioleoyl MADG was only 2%. The proportion of 1,3-isomer seemed to vary with the kind of acyl residue in the molecular species. The 1,3-isomer can be removed from 1,2-MADG by TLC on a plate of Silica Gel G developed with petroleum ether:ethyl ether (85:15), prior to further analysis.

Intramolecular acyl migration in the acetolysis reaction was examined by means of GC-MS and mass fragmentography. Two preparations of MADG were prepared from 1-stearoyl-2-oleoyl PC, one of them by acetolysis for 5 hr, and the other by phospholipase C-acetylation, and the mass spectra were compared to each other. As shown in Figure 3, no appreciable difference could be detected between both procedures, indicating that intramolecular acyl migration did not occur during the acetolysis.

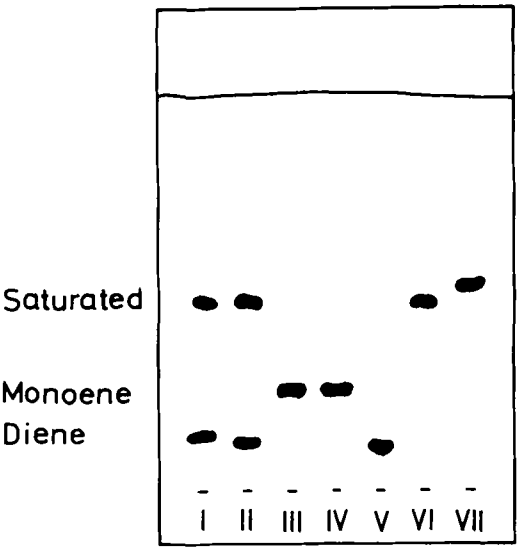

FIG. 1. Thin layer chromatogram of monoacetyldiglycerides (MADG) obtained from authentic phosphatidylcholines (PC) and diglycerides. Solvent: benzene:chloroform:methanol (98:2:0.1). (I) = MADG from a mixture of 1,2-dipalmitoyl PC and 1,2-dioleoyl PC by acetolysis for 5 hr. (II) = MADG from the same mixture as I by acetolysis for 16 hr. (III) = MADG from 1-palmitoyl-2-palmitoleoyl PC by acetolysis for 5 hr. (IV) = MADG from 1-stearoyl-2-oleoyl PC by acetolysis for 5 hr. (V) = MADG from 1,2-dioleoyl PC by acetolysis for 5 hr. (VI) = MADG from 1,2-distearin by acetylation. (VII) = MADG from 1,3-distearin by acetylation.

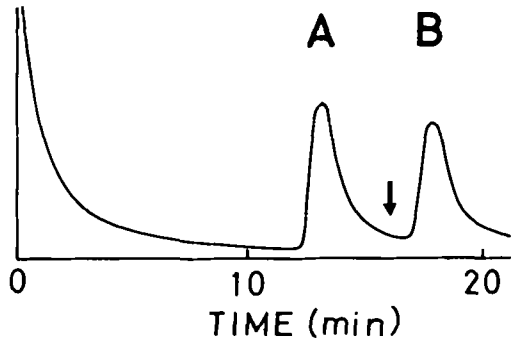

FIG. 2. Gas chromatogram of monoacetyldiglyceride (MADG) obtained from a mixture of 1,2-dipalmitoyl phosphatidylcholine and 1,2-dioleoyl phosphatidylcholine by acetolysis. (A) = 1,2-dipalmitoyl MADG. (B) = 1,2-dioleoyl MADG. The arrow shows the elution position of palmitoyl-oleoyl MADG.

However, because the $[M - RCOOCH_2]^+$ ions, which were important for determination of the position of acyl residue in glycerol, were small in the usual mass spectra, it was difficult to determine quantitatively the extent of conversion to 1-oleoyl-2-stearoyl-3-acetyl glycerol only

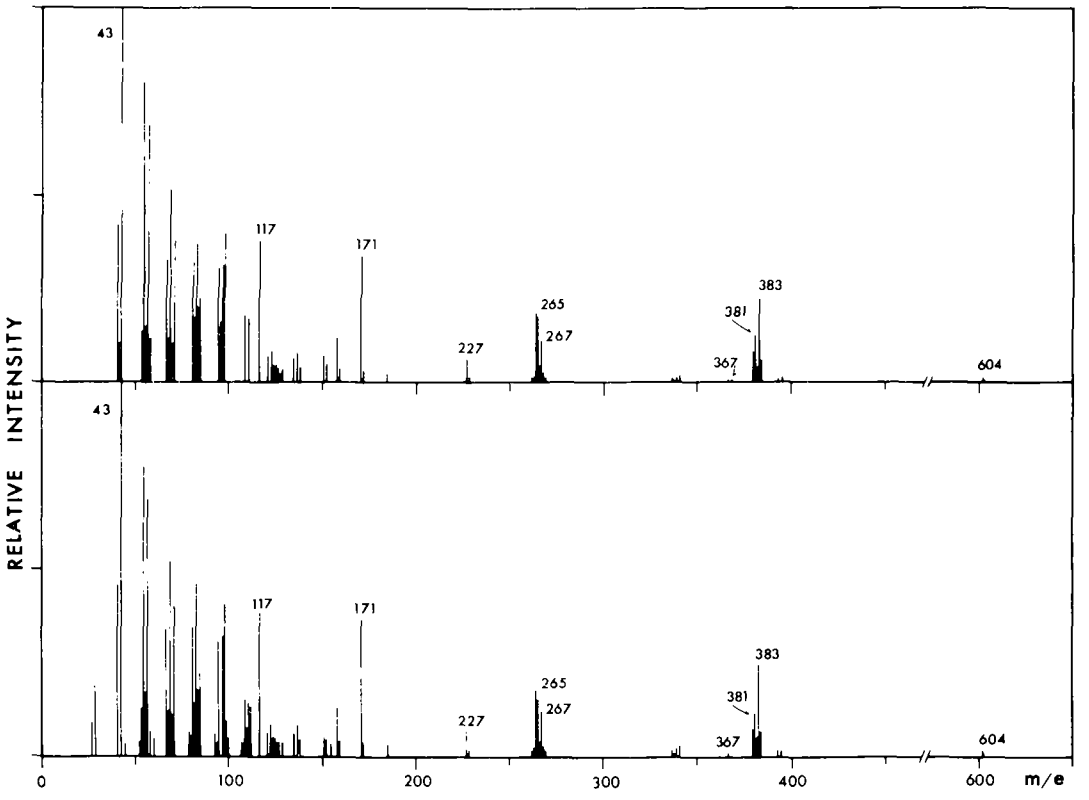

FIG. 3. Mass spectra of monoacetyldiglycerides prepared from 1-stearoyl-2-oleoyl phosphatidylcholine by phospholipase C-acetylation (upper) and by acetolysis (lower).

by using the $[M - RCOOCH_2]^+$ ions on the usual mass spectra.

Therefore, the materials were analyzed by mass fragmentography. Mass number of MID were focused at m/e 367 for the elimination of stearoyloxymethylene and at m/e 369 for the elimination of oleoyloxymethylene from 1-stearoyl-2-oleoyl-acetyl glycerol. The results are shown in Figure 4. The percentage of the ht at the former mass number to the sum of the hts at the both mass numbers, $P_{(367)}$, was calculated according to the following equation:

$$P_{(367)} = [H_{367}/(H_{367} + H_{369})] \times 100, (\%), \quad (I)$$

where H_{367} and H_{369} are the peak hts at the respective mass numbers. The percentage for the MADG prepared by phospholipase C-acetylation, $P_{c(367)}$, was 82.0%, whereas, the percentage for the MADG prepared by acetolysis, $P_{a(367)}$, was 77.5%. To obtain the percentage of acyl migration, A, from 1-stearoyl-2-oleoyl-acetyl glycerol to 1-oleoyl-2-stearoyl-acetyl glycerol, a calculation was made according to the following equation:

$$A = [(P_{c(367)} - P_{a(367)})/(P_{c(367)} - P_{c(369)})] \times 100, (\%), \quad (II)$$

where $P_{c(369)}$ is the percentage of ht at m/e 369, corresponding to the elimination of oleoyloxymethylene from an assumed reverse molecular species, 1-oleoyl-2-stearoyl-acetyl glycerol, to the sum of those at m/e 367 and at m/e 369, and assumed to be equal to 100 - $P_{c(367)}$. That is,

$$A = [(82.0 - 77.5)/\{82.0 - (100 - 82.0)\}] \times 100 = 7.0, (\%). \quad (III)$$

This value represents the percentage of 1-oleoyl-2-stearoyl-3-acetyl glycerol formed by the intramolecular acyl migration in the acetolysis of 1-stearoyl-2-oleoyl PC.

1-Palmitoyl-2-palmitoleoyl PC was examined in a similar manner. The mass number of MID was focused at m/e 339 for the elimination of palmitoyloxymethylene and at m/e 341 for the elimination of palmitoleoyloxymethylene from 1-palmitoyl-2-palmitoleoyl-acetyl glycerol. In

this case, the acyl migration was calculated to be 16.0%.

The values found for intramolecular acyl migration were rather small compared to those previously reported (2-4), and seemed not to be a serious hindrance to the determination of molecular species by GC-MS.

A further comparison of both methods was carried out by the use of egg yolk PC as material. The MADG prepared by acetolysis or by phospholipase C-acetylation from egg yolk PC was fractionated on silver nitrate impregnated Silica Gel G plates. Six spots, corresponding to saturated, monoene, diene, triene, tetraene, and hexaene MADG, were obtained, and there was no difference in the relative amounts of the 6 MADG between the both methods. No difference was observed in GLC of each subfraction of MADG, either. Mass spectra of corresponding components of MADG from the two methods were identical. The compositions of the molecular species of egg yolk PC by both methods were very similar (Table II). The recovery of molecular species containing higher unsaturated fatty acids such as $C_{22:6}$ was somewhat lower for the acetolysis method than for the phospholipase C-acetylation.

The present examination of the acetolysis products of PC demonstrated that the acetolysis procedure described above did not cause intermolecular acyl migration and selective degradation of unsaturated fatty acid, that the extents of intramolecular acyl migration including the formation of the 1,3-isomer were not so large as to affect the mass spectra of the original MADG, and that no appreciable difference was observed between the molecular species of MADG derived by the both methods from egg yolk PC. These results show that acetolysis and GC-MS (6) is a useful convenient method for the determination of molecular species of phospholipids. The phospholipase C-acetylation method, however, is preferable to the acetolysis method for preparation MADG

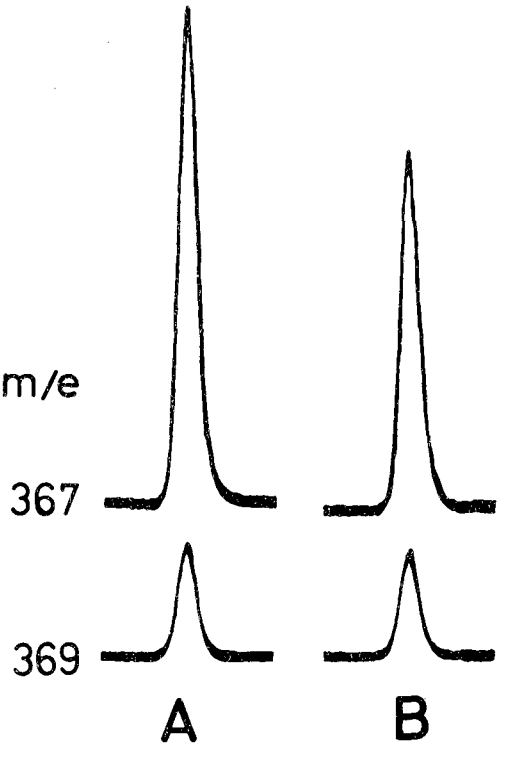

FIG. 4. Mass fragmentograms of $[M - RCOOCH_2]^+$ ions of monoacetyldiglycerides prepared from 1-stearoyl-2-oleoyl phosphatidylcholine by phospholipase C-acetylation (A) and acetolysis (B).

TABLE II

Molecular Species Compositions of Monoacetyldiglyceride Derived by Two Different Methods from Egg Yolk Phosphatidylcholine

Double bonds	Sum of FA[a]	Fatty acid		Content (%)	
		1 Position	2 Position	Acetolysis	Phospholipase C-acetylation
0	32	16:0	16:0	0.6	0.6
0	34	16:0	18:0	---	0.5
1	34	16:0	18:1	49.4	47.5
1	36	18:0	18:1	14.9	8.0
2	34	16:0	18:2	16.2	23.2
2	36	18:0	18:2	12.7	11.3
		18:1	18:1		
3	36	18:1	18:2	1.8	1.2
4	36	16:0	20:4	1.8	1.6
4	38	18:0	20:4	2.3	2.0
6	38	16:0	22:6	0.5	3.0
6	40	18:0	22:6	---	1.0

[a]Sum of carbon numbers of fatty acyl (FA) residues.

from the molecular species containing highly unsaturated fatty acids.

The acetolysis method is available for phosphatidylethanolamine and phosphatidylserine (1,14). Applicability of the method to other glycerophospholipids, such as phosphatidylglycerol, phosphotidylinositol, cardiolipin, phosphatidic acid should be investigated. Especially in the case of phosphatidic acid, the method could be a most useful one, because the compound cannot be attacked by phospholipase C, but by phosphatidic acid phosphatase.

The characteristics of the mass spectra of MADG in comparison with those of trimethylsilyldiglyceride will be published elsewhere.

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Inhibition of Hepatic Lipogenesis by Adenine Nucleotides

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ABSTRACT

Incubation of liver slices and isolated liver cells with adenosine cyclic-3′,5′-monophosphate at concentrations which inhibit lipogenesis was found to expand the pool size of the noncyclic adenine nucleotides in the intact cells of the preparations. This observation led to studies which demonstrated that adenosine and adenosine-5′-monophosphate also inhibited lipogenesis and expanded the adenine nucleotide pool size. It is proposed but not proven that the increase in intracellular nucleotides produced by adenosine-5′-monophosphate, adenosine cyclic-3′,5′-monophosphate, and adenosine may have an adverse effect upon the synthesis of fatty acids. Because of the expansion of the adenine nucleotide pool size, high concentrations of adenosine cyclic-3′,5′-monophosphate should not be used to investigate the mechanism responsible for hormonal regulation of lipogenesis. As an added complication, exogenous adenosine-5′-monophosphate was found to produce a small but significant increase in the intracellular concentration of adenosine cyclic-3′,5′-monophosphate of isolated liver cells. This effect also may be a factor in the inhibition of lipogenesis by adenosine-5′-monophosphate. Low concentrations of N^6,$O^{2'}$-dibutyryl adenosine cyclic-3′,5′-monophosphate were found to inhibit lipogenesis without increasing the intracellular adenine nucleotide content of either liver slices or isolated liver cells. It is concluded that studies on the mechanism of glucagon regulation of lipogenesis should be carried out with glucagon or low concentrations of N^6,$O^{2'}$-dibutyryl adenosine cyclic-3′,5′-monophosphate.

INTRODUCTION

Adenosine cyclic-3′,5′-monophosphate (cyclic AMP) has been reported in a number of studies (1-4) to inhibit lipogenesis in liver tissue preparations. An inhibitory action of the cyclic nucleotide on acetyl CoA carboxylase (E.C. 6.4.1.2) has been suggested to account for the inhibition of lipogenesis (5,6). In addition, an inhibition of aerobic glycolytic activity has been shown to be responsible in part for N^6,$O^{2'}$-dibutyryl adenosine-3′,5′-monophosphate (dibutyryl cyclic AMP) and glucagon inhibition of lipogenesis (7). On the other hand, Raskin, et al., (8) have seriously questioned the physiological significance of this response to glucagon and cyclic AMP. We demonstrate here that high concentrations of cyclic AMP expand the intracellular nucleotide pool size, and, therefore, should not be used in studies on the mechanism responsible for glucagon inhibition of lipogenesis.

METHODS AND MATERIALS

Male Wistar rats weighing ca. 200 g were starved for 48 hr and refed for 48 hr on a high sucrose diet ("Fat-Free" test diet, Nutritional Biochemicals Corp., Cleveland, OH) (9). Liver slices were prepared with a Harvard Tissue slicer. Isolated liver cells were prepared by the method of Berry and Friend (10) with modifications described previously (7). Incubations were carried out in Krebs-Henseleit saline equilibrated with 95% oxygen:5% carbon dioxide in a Dubnoff metabolic shaking incubator at 37.5 C. In experiments conducted with isolated cells, the medium was supplemented with 2.5% albumin which had been charcoal treated to remove the fatty acids (11).

Incubations conducted for determining the rate of fatty acid synthesis were carried out in 4 ml of medium containing 100-200 mg of slices or isolated cells wet wt and 1 mCi of ^{3}HOH. Incubations were terminated by the addition of 0.4 ml of 50% (w/v) perchloric acid. The precipitate was extracted twice for total lipids with methanol:chloroform (2:1) as described by Kates (12). The total lipids were saponified at 73 C with a solution which was 0.3 M in NaOH and contained 90% (v/v) methanol (12). The nonsaponifiable fraction was extracted and discarded. Fatty acids were extracted and counted for radioactivity with a scintillation counter. Benzene was added to the total lipid extract and to the final fatty acid extract before complete evaporation of the solvent under nitrogen. Addition of benzene reduced zero time controls to background levels of radioactivity as traces of water were removed more effectively. Calculations of the rate of fatty acid synthesis were based on the assumption (13) that 1.0 μmole of ^{3}HOH incorporated

corresponded to 1.15 μmole of acetyl groups incorporated.

In studies involving the determination of tissue adenine nucleotide and metabolite concentrations of tissue slices, incubations were conducted in triplicate and terminated after 30 min by rapid filtration of the slices from the incubation medium with a tea strainer. The slices were tapped cleanly onto a liquid nitrogen cooled freeze clamp and frozen with a second clamp. In this manner, the slices were isolated from the medium and frozen solid in less than 3 sec. The slices were homogenized in 6% perchloric acid with a Polytron PT-10 homogenizer operated at full speed for 20 sec. Residues obtained after centrifugation were re-extracted with 6% perchloric acid. The combined extracts were adjusted rapidly to pH 5-6 with 20% KOH and centrifuged to remove potassium perchlorate. In studies involving the determination of the adenine nucleotide content of isolated liver cells, incubations were terminated by rapid separation of the cells from the medium by centrifugation into 6% perchloric acid with the hepatocyte separation tubes described in detail previously (14). Perchloric acid extracts of the cells were treated as described above for slices. Inorganic phosphate was determined by the method of Martin and Doty as described by Lindberg and Ernster (15). Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and α-ketoglutarate were determined by enzymatic analysis using methods described by Williamson and Corkey (16); lactate, malate, and pyruvate were determined as described by Hohorst, et al., (17); and citrate was determined as described by Möllering and Gruber (18). Cytoplasmic-free nicotinamide adenine dinucleotide (NAD^+) to NADH ratios were calculated from the concentrations of lactate and pyruvate and the equilibrium constant of lactate dyhydrogenase (19).

For the determination of cyclic AMP, isolated liver cells were incubated and then centrifuged into 5% trichloroacetic acid via the hepatocyte separation tubes. Tritiated, general labeled, cyclic AMP in tracer quantities was added to the extract to determine percent recoveries. Purification of the extracts was carried out by column chromatography on AG 1-X8 formate as described by Kneer, et al., (20). Cyclic AMP content was determined by the protein kinase binding assay of Gilman (21,22). The quantity of other adenine nucleotides in the purified extracts was established by enzymatic analysis to be too low to interfere with the determination of cyclic AMP.

The oxidation of succinate to malate was used to estimate the viability of liver slice preparations (14). The slices, 100 mg wet wt in a volume of 8 ml of Krebs-Henseleit saline, were preincubated for 5 min at 37 C. Succinate was added to the flasks at an initial concentration of 20 mM and the incubation terminated after 2 and 5 min with perchloric acid. The rates of malate accumulation by slices were compared with the rates by homogenates prepared with a Potter-Elvehjem homogenizer in which all cells were established to be broken by failure to exclude trypan blue.

Radioactive 5′-AMP was prepared enzymatically from [$^{14}C_8$] adenosine triphosphate by the combined action of purified mitochondrial ATPase (F_1) and adenylate kinase (E.C. 2.7.4.3). Purification was carried out by ascending paper chromatography with the solvent system of n-butanol:acetone:acetic acid: aq. 5% NH_4OH:H_2O: 0.1 M ethylene diamine tetracetic acid (EDTA), (45:15:10:10:19:1, v/v) (23). [$^{14}C_8$] Adenosine cyclic 3′,5′-monophosphate was purified prior to use by ascending paper chromatography with the solvent system of isopropanol:NH_4OH:H_2O (7:1:2, v/v). Radioactive adenine nucleotides of perchloric acid extracts of tissue slices were separated by column chromatography on AG 1-X8 formate by the method described by Groot and Van den Bergh (24). All radioactive isotopes were obtained from New England Nuclear (Boston, MA). All enzymes, except the purified ATPase, and most chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Studies with Liver Slices

In an attempt to gain insight into the mechanism responsible for cyclic AMP inhibition of lipogenesis, freeze stop studies were conducted with liver slices incubated with and without cyclic AMP. As shown in Table I, cyclic AMP was found to produce a substantial increase in the ATP, ADP, AMP, and inorganic phosphate content of freeze clamped liver slices. In contrast, significant decreases were observed in the concentrations of lactate, pyruvate, malate, citrate, and α-ketoglutarate. The cytoplasmic-free NAD^+ to NADH ratio also was decreased significantly by the cyclic nucleotide.

Adenosine-5′-monophosphate (5′-AMP) also was found to inhibit lipogenesis (Table II) and to increase the adenine nucleotide content of liver slices (Table I). As shown in Table II, both cyclic AMP and 5′-AMP brought about an inhibition of lipogenesis at similar concentrations with a trend noted for cyclic AMP to be more effective. The changes in metabolite levels

TABLE I

Effect of 3′,5′-cyclic AMP and 5′-AMP on the Adenine Nucleotide and Metabolite Levels of Liver Slices[a]

Metabolite or metabolite ratio[b]	Experiment I		Experiment II	
	No addition (μmol/g)	3′,5′-cyclic AMP (μmol/g)	No addition (μmol/g)	5′-AMP (μmol/g)
ATP	0.67 ± 0.07	0.88 ± 0.08[c]	0.61 ± 0.02	0.81 ± 0.03[c]
ADP	0.24 ± 0.01	0.39 ± 0.02[c]	0.23 ± 0.02	0.28 ± 0.01[c]
AMP	0.09 ± 0.01	0.14 ± 0.02[c]	0.07 ± 0.01	0.05 ± 0.01
Σ ATP + ADP + AMP	1.00 ± 0.10	1.51 ± 0.10[c]	0.91 ± 0.02	1.17 ± 0.04[c]
Inorganic phosphate	3.2 ± 0.3	3.9 ± 0.3[c]	3.6 ± 0.3	5.6 ± 0.3[c]
Lactate	2.6 ± 0.4	1.4 ± 0.2[c]	2.3 ± 0.2	2.0 ± 0.2
Pyruvate	0.16 ± 0.01	0.04 ± 0.01[c]	0.14 ± 0.01	0.07 ± 0.01[c]
Malate	0.61 ± 0.06	0.30 ± 0.05[c]	0.58 ± 0.04	0.45 ± 0.09
Citrate	0.19 ± 0.03	0.12 ± 0.02[c]	0.14 ± 0.03	0.08 ± 0.01[c]
α-ketoglutarate	0.12 ± 0.01	0.05 ± 0.01[c]	0.09 ± 0.02	0.05 ± 0.01[c]
Free [NAD]/[NADH]cyto	603 ± 63	278 ± 64[c]	540 ± 72	341 ± 33[c]

[a]The initial concentrations were 5 mM for 3′,5′-cyclic adenosine monophosphate (AMP) in Experiment I and 5 mM for 5′-AMP in Experiment II. Concentrations of metabolites are expressed as μmol/g wet wt of slices, as means ± S.E.M. for liver slices prepared from 6 animals in Experiment I and 5 animals in Experiment II. Values which are significantly ($P < 0.05$) different from the no addition controls by the Student's t test for paired data.

[b]ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; NAD = nicotinamide adenine dinucleotide.

[c]Values which are significantly ($P<0.05$) different from the no additions controls by the student's t test for paired data.

TABLE II

Effect of 5′-AMP, 3′, 5′-cyclic AMP, and Dibutyryl Cyclic AMP on Fatty Acid Synthesis by Liver Slices[a]

Addition (mM)	Rate of fatty acid synthesis (nmoles/min/g wet wt)
None	52 ± 12
5′-AMP (0.5)	32 ± 13
5′-AMP (2.5)	7 ± 3
5′-AMP (5.0)	8 ± 2
3′, 5′-cAMP (0.5)	18 ± 5
3′, 5′-cAMP (2.5)	4 ± 1
3′, 5′-cAMP (5.0)	2 ± 1
Dibutyryl cyclic AMP (0.05)	3 ± 1

[a]AMP = adenosine monophosphate; rates of lipogenesis are expressed as nmoles of "C_2" units incorporated into fatty acids/min/g wet wt of slices, as means ± S.E.M. for liver slices prepared from 3 animals. Rates were determined for the last 30 min of 60 min incubations with the additions listed. All values are significantly ($P < 0.05$) different from the no addition control by the Student's t test for paired data.

caused by 5′-AMP (Table I) mimicked in part those produced by cyclic AMP. Significant increases were found in ATP, ADP, and inorganic phosphate levels, whereas, significant decreases were found in pyruvate, citrate, α-ketoglutarate, and the cytoplasmic-free NAD^+ to NADH ratio. However, significant changes were not induced by 5′-AMP in the liver slice content of lactate, malate, and 5′-AMP. The lack of effect on the 5′-AMP content was surprising in view of the fact that the slices were not washed free of the external medium. However, much of the exogenous 5′-AMP had been lost from the medium during the incubation.

Experiments conducted with radioactive cyclic AMP and 5′-AMP demonstrated that uptake and phosphorylation of these nucleotides accounted for the observed increase in the intracellular content of ATP (Table III). Based on the specific activity of the exogenous nucleotides and the total radioactivity of the isolated ATP, ca. 75% of the total ATP of the slices was derived from the exogenous nucleotides. No attempt was made to determine whether the nucleotides were dephosphorylated prior to penetration into the cells (see Discus-

TABLE III

Incorporation of [$^{14}C_8$]-AMP and [$^{14}C_8$]-cyclic AMP into the ATP of Liver Slices[a]

Addition	Total ATP (μmol/g wet wt)	ATP derived from exogenous nucleotide (μmol/g wet wt)	ATP derived from exogenous nucleotide (%)
None	0.54	-------	-------
[^{14}C]AMP (5 mM)	0.88	0.76	77
[^{14}C]cyclic AMP (5 mM)	0.93	0.68	73

[a]AMP = adenosine monophosphate; ATP = adenosine triphosphate; incubations were conducted for 60 min in duplicate with 500 mg wet wt liver slices per flask. Incubations were terminated by freeze clamping. Perchloric acid extracts were analyzed for the quantity and radioactivity of ATP.

TABLE IV

Effect of Dibutyryl Cyclic AMP on the Adenine Nucleotide and Metabolite Levels of Liver Slices[a]

Metabolite or metabolite ratio[b]	No addition (μmol/g)	Dibutyryl cyclic AMP (μmol/g)
ATP	0.41 ± 0.06	0.37 ± 0.02
ADP	0.22 ± 0.02	0.22 ± 0.04
AMP	0.09 ± 0.02	0.12 ± 0.02
Σ ATP + ADP + AMP	0.72 ± 0.07	0.71 ± 0.06
Inorganic phosphate	2.16 ± 0.54	2.64 ± 0.44
Lactate	1.4 ± 0.3	0.66 ± 0.20[c]
Pyruvate	0.12 ± 0.01	0.05 ± 0.06[c]
Malate	0.34 ± 0.07	0.25 ± 0.05[c]
Citrate	0.10 ± 0.02	0.08 ± 0.01
α-ketoglutarate	0.06 ± 0.01	0.04 ± 0.01
free [NAD]/[NADH]cyto	878 ± 120	969 ± 303

[a]The initial concentration of dibutyryl cyclic adenosine monophosphate (AMP) was 0.05 mM. Concentrations of metabolites are expressed as μmol/g wet wt of slices, as means ± S.E.M. for liver slices prepared from 4 animals incubated for 30 min.

[b]ATP = adenosine triphosphate; ADP = adenosine diphosphate; NAD = nicotinamide adenine diphosphate.

[c]Values which are significantly ($P < 0.05$) different from controls by the Student's t test for paired data.

TABLE V

Effect of 5′-AMP, Cyclic AMP, ADP, ATP, Adenosine and Dibutyryl Cyclic AMP on Fatty Acid Synthesis by Isolated Cells[a]

Addition (mM)	Rate of fatty acid synthesis nmoles/min/gm wet wt
None	260 ± 40
5′-AMP (0.1)	170 ± 30
5′-AMP (0.5)	20 ± 10
Cyclic AMP (0.1)	100 ± 10
Cyclic AMP (0.5)	60 ± 10
ADP (0.1)	140 ± 10
ADP (0.5)	20 ± 10
ATP (0.1)	100 ± 50
ATP (0.5)	10 ± 5
Adenosine (0.1)	100 ± 20
Adenosine (0.5)	20 ± 10
Dibutyryl cyclic AMP (0.05)	50 ± 10

[a]AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate. Rates of lipogenesis are expressed as nmoles of "C_2" units incorporated into fatty acids/min/g wet wt of cells, as means ± S.E.M. for isolated cells prepared from 3 animals. Rates were determined for the last 30 min of 60 min incubations with the additions listed. All values are statistically ($P < 0.05$) different from the no addition control by the Student's t test for paired data.

TABLE VI

Effect of Cyclic AMP, 5′-AMP, Adenosine, and Dibutyryl Cyclic AMP on the Adenine Nucleotide Content of Isolated Liver Cells[a]

Addition (mM)	ATP[b] (μmol/g)	ADP[b] (μmol/g)	AMP (μmol/g)	ΣATP + ADP + AMP (μmol/g)
None	2.46 ± 0.11	1.09 ± 0.07	0.38 ± 0.18	3.93 ± 0.21
Cyclic AMP (0.5)	3.76 ± 0.37[c]	1.57 ± 0.08[c]	0.50 ± 0.04	5.83 ± 0.42[c]
5′-AMP (0.5)	4.37 ± 0.49[c]	1.54 ± 0.19	0.48 ± 0.03	6.39 ± 0.69[c]
Adenosine (0.5)	3.96 ± 0.39[c]	1.67 ± 0.05[c]	0.50 ± 0.03	6.13 ± 0.41[c]
Dibutyryl cyclic AMP (0.05)	2.33 ± 0.03	1.17 ± 0.04	0.45 ± 0.04	3.95 ± 0.07

[a]AMP = adenosine monophosphate; values are expressed as μmol/g wet wt of liver cells, as means ± S.E.M. for liver cells prepared from 3 animals and incubated for 45 min.

[b]ATP = adenosine triphosphate; ADP = adenosine diphosphate.

[c]Values which are significantly ($P < 0.05$) different from no addition controls by the Student's t-test for paired data.

sion). Nevertheless, it can be concluded that the cells of liver slices take up at least the adenine moiety of 5′-AMP and cyclic AMP and that this uptake leads to an increase in the pool size of adenine nucleotides within liver slices.

As reported by other investigators (3) and previously (7), dibutyryl cyclic AMP inhibited lipogenesis at much lower concentrations than cyclic AMP (Table II). At such low concentrations, dibutyryl cyclic AMP was not found to affect the adenine nucleotide content of liver slices (Table IV). However, significant decreases in the concentrations of lactate, pyruvate, and malate were produced by dibutyryl cyclic AMP.

Studies with Isolated Liver Cells

Concern as to whether the above studies represented an artifact of the liver slice preparation prompted an investigation of the effect of adenine nucleotides on isolated hepatocytes. As shown in Table V, fatty acid synthesis was effectively inhibited with this preparation by comparable concentrations of 5′-AMP, 3′,5′-cyclic AMP, ADP, ATP, and adenosine. The isolated cells, however, were considerably more sensitive to these inhibitors than the liver slice preparation. For example, 0.5 mM 5′-AMP produced only 38% inhibition with liver slices, but produced 92% inhibition with isolated liver cells. The observation that ADP, ATP, and adenosine have the same action as 5′-AMP makes it unlikely that the inhibitory effect is caused by some contaminant of the 5′-AMP used in these studies. As reported previously (7), dibutyryl cyclic AMP was found to be a very effective inhibitor of fatty acid synthesis by isolated liver cells (Table V) and was considerably more effective than the other nucleotides.

As reported above for slices, 3′,5′-cyclic AMP and 5′-AMP increased dramatically the adenine nucleotide content of the isolated liver cells (Table VI). Because adenosine also was found to inhibit lipogenesis (Table V), the study was extended to include this nucleoside which was found to greatly expand the adenine nucleotide pool size (Table VI). Dibutyryl cyclic AMP, however, did not have this effect (Table VI) at a concentration which produced a striking inhibition of lipogenesis (Table V). Hence, the results obtained with liver cells are qualitatively similar to those reported with liver slices. The rates of lipogenesis were greater with liver cells as was the sensitivity of this process to adenine nucleotides. In addition, the expansion produced in the intracellular nucleotide pool size by the various adenine derivatives was more pronounced with liver cells than with liver slices.

The possibility that exogenous 5′-AMP affected an increase in the 3′,5′-cyclic AMP content of the cells also was investigated. As shown in Figure 1, glucagon at 10^{-6} M produced a dramatic but transient increase in 3′,5′-cyclic AMP. A much smaller but nevertheless significant increase in 3′,5′-cyclic AMP was observed with exogenous 5′-AMP. The increase was significant ($P < 0.05$) at 15 min, but not at 30 and 45 min of incubation.

DISCUSSION

This study developed during an investigation of the mechanism responsible for cyclic AMP inhibition of lipogenesis. Incubation of liver slices with this nucleotide was found to increase the noncyclic adenine nucleotide content of the slices. This observation suggested that the adenine nucleotide derived by the hydrolysis of cyclic AMP might be involved in the inhibition of lipogenesis. Therefore, studies were conducted with 5′-AMP which also was found to inhibit lipogenesis and to increase the adenine

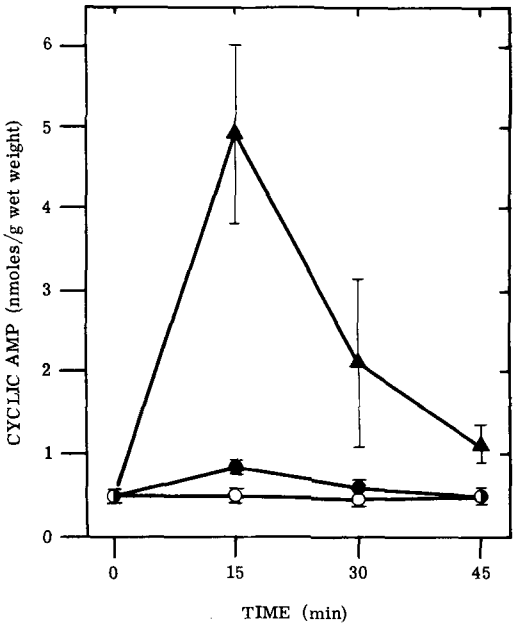

FIG. 1. Effect of 5'-AMP (adenosine monophosphate) and glucagon on the cyclic AMP content of isolated liver cells. ○ = no additions; ● = 5'-AMP at 0.5 mM; and ▲ = glucagon at 10^{-6} M. The results are given as means ± S.E.M. for 5-7 liver cell preparations. The increase caused by glucagon was statistically ($P < 0.05$) significant at all time points; 5'-AMP only at 15 min.

nucleotides of liver slices. These observations would suggest that one mechanism by which cyclic AMP inhibits lipogenesis is by the generation of 5'-AMP or adenosine, which in turn increases the adenine nucleotide content of the cell to bring about an inhibition of lipogenesis. The mere slicing of liver is well established to cause a dramatic decrease in the adenine nucleotide content of this tissue (25). Hence, at first inspection, it might seem paradoxical to suggest that an expansion of the pool size of the adenine nucleotides of the cells of liver slices would lead to an inhibition of a metabolic process such as lipogenesis. Indeed, exogenous 5'-AMP and cyclic AMP failed to increase the apparent intracellular content of adenine nucleotides back to the level of the intact liver. Hence, it might be argued that exogenous adenine nucleotides should tend to restore normal metabolic activity. However, this requires the assumption that all cells of liver slices are deficient in adenine nucleotides. From the results reported above with isolated liver cells and below with succinate oxidation to malate by liver slices, it can be suggested that this assumption is not valid. A more likely explanation is that part of the cells are damaged and completely void of adenine nucleotides, whereas, other cells are intact and contain a complete complement of adenine nucleotides. Thus, the only cells which would be competent to respond to exogenous adenine nucleotides would be the intact cells which already contain a full complement of adenine nucleotides. By this analysis, the increase in adenine nucleotides of liver slices caused by exogenous 5'-AMP or cyclic AMP would represent a considerable expansion of the pool size of the intact cells of the tissue. This interpretation is supported by the results obtained with isolated liver cells. Most of the cells of this preparation are intact, i.e., >95% exclude trypan blue, and contain a full complement of adenine nucleotides (26) (Table VI). Exogenous cyclic AMP, 5'-AMP, and adenosine were observed to expand greatly the adenine nucleotide pool size of these cells and to inhibit concurrently fatty acid synthesis. Although the percentage is difficult to estimate, it is recognized that liver slices contain a great number of damaged cells. With the proposal stated above that only intact cells of liver slices contain significant quantities of adenine nucleotides, it can be calculated from the adenine nucleotide content of intact liver, 4 μmoles/g wet wt (26), that only ca. 25% of the cells of liver slices used in this study and that of Krebs (25) would meet this criterion of intactness. The notion discussed by Krebs (25), that the adenine nucleotides of liver slices might be confined to the mitochondrial compartment, is untenable. Liver cells which lacked cytoplasmic adenine nucleotides would be unable to maintain transmembrane ion gradients. The resulting influx of Ca^{++} would lead to swelling and disruption of the mitochondria with a resulting loss of intramitochondrial adenine nucleotides. The viability of isolated liver cells can be estimated by either trypan blue exclusion or succinate oxidation to malate (14). Although the former method cannot be applied to liver slices, the latter method can be used to give a minimum estimate of the number of cells damaged. Succinate is oxidized to malate only by damaged cells of a liver preparation because intact cells are nearly impermeable to succinate and malate (14). It was determined that the liver slices used in this study produced malate from succinate at a rate of 7.1 ± 0.3 μmoles/min/g wet wt (mean ± S.E.M. for liver slices prepared from 4 animals and incubated under the conditions described above). Homogenates of these livers produced malate from succinate at a rate of 19.5 ± 0.5 μmoles/min/g wet wt. Hence, by this analysis, at least 36% of the cells of the slices were not intact. In contrast to the estimate based on

ATP content, malate production probably gave a minimal estimate of the percentage of damaged cells. This is because cells which are damaged, yet not accessible to exogenous succinate and/or oxygen, would not convert succinate to malate. A considerably number of such cells would be expected to exist within the interior of liver slices. Hence, the suggestion from this analysis is that liver slices consist of a mixed population of extensively damaged cells and relatively intact cells, and that only the latter cells could be expected to respond like isolated cells to exogenous adenine nucleotides. Although it is clear that isolated liver cells should be used for metabolic studies in preference to liver slices, there is an important point to be made here. Slices represent the only system for studying human liver in which there is maintenance of structural integrity of the cells. The isolation of intact cells from human liver appears impossible. Therefore, as long as it is appreciated that the slice is composed of a mixed population of damaged and intact cells, meaningful studies can be carried out with slices of human liver. Such studies are currently in progress in this laboratory.

Cyclic AMP may inhibit lipogenesis by limiting the activity of acetyl CoA carboxylase (5,6), either by phosphorylation of the enzyme into an inactive form (27) or by induced changes in established effectors of the enzyme (28-30). Another attractive possibility is that cyclic AMP limits substrate supply for lipogenesis by suppressing glycolytic activity (7) and/or pyruvate dehydrogenase activity. However, no attempt is made here to define the mechanism responsible for cyclic AMP inhibition of lipogenesis nor to explain why Raskin, et al., (8) failed to observe inhibition of lipogenesis by cyclic AMP in the perfused liver. Instead, this report makes it clear that studies attempting to deal with the mechanism of action of cyclic AMP should be conducted under experimental conditions that preclude increases in intracellular adenine nucleotides. This could be accomplished most readily by the generation of intracellular cyclic AMP by hormonal activation of adenylate cyclase or by the judicious use of the dibutyryl derivative.

This study should not be construed to support the work of Raskin, et al., (8) in suggesting that cyclic AMP inhibition of lipogenesis is an artifact of the liver slice preparation. Glucagon and dibutyryl cyclic AMP now have been shown to inhibit lipogenesis by liver slices and isolated liver cells without affecting the adenine nucleotide content of the cells. Indeed, studies in this laboratory now have demonstrated that dibutyryl cyclic AMP and glucagon effectively inhibit lipogenesis in the perfused liver as well (R.A. Harris, J.P. Mapes, and C.S. Stewart, unpublished data).

The inhibition of lipogenesis by adenine nucleotides reported in this study suggests that some step in fatty acid synthesis per se or in substrate supply may be affected by changes in the balance of adenine nucleotides and/or inorganic phosphate within the liver slices. Indeed, adenine nucleotides are recognized as important regulators of metabolic processes (31). On the other hand, exogenous 5′-AMP was found to lead to a significant increase in the cyclic AMP content of isolated liver cells. Although this increase was small compared to the increased caused by glucagon, it nevertheless could be a factor in the inhibition of lipogenesis caused by 5′-AMP. Exogenous adenine nucleotides have been shown in other studies to increase the cyclic AMP content of slices of guinea pig cerebral cortex (32) and cultured astrocytoma cells (33). Whether this response with liver cells was due to a surface receptor which activates adenylate cyclase or a secondary effect caused by the increase in intracellular adenine nucleotides is not known.

In retrospect, it is not surprising that exogenous 5′-AMP and cyclic AMP increase the adenine nucleotide content of liver cells. Adenosine has been shown to increase the adenine nucleotide content of liver in vivo (34) and liver cells in vitro (P. Lund, N. Cornell, and H.A. Krebs, personal communication). In addition, 5′-AMP at concentrations similar to those used in this study have been shown to increase the adenine nucleotide and inorganic phosphate content of perfused and sliced kidney (35) and to exert strong metabolic effects in both kidney (35) and liver (36). Evidence has been presented in other studies that exogenous nucleotides such as 5′-AMP are degraded rapidly by enzymes located at the outer surface of the plasma membranes of liver cells (37) and granulocytes (38). Presumably cyclic AMP, 5′-AMP, ADP, and ATP are all degraded to the neutral molecule adenosine prior to penetration into liver cells in quantities sufficient to expand the adenine nucleotide pool size. The lack of an effect of dibutyryl cyclic AMP on the adenine nucleotide content of liver slices can be explained on the basis that this compound must be a poor substrate for the external enzymes, as well as the phosphodiesterases that hydrolyze cyclic AMP (39). Also, very low concentrations of dibutyryl cyclic AMP were used in these studies because of its greater effectiveness as an inhibitor of lipogenesis. The mechanism responsible for the action of adenosine, adenine nucleotides, dibutyryl cyclic AMP, and gluca-

gon on lipogenesis is being investigated further with the isolated cell and perfused liver preparations.

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Lipoxygenase Isozymes of Peanut

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ABSTRACT

Lipoxygenase was isolated and partially purified from peanut seed by ammonium sulfate precipitation, gel filtration, and ion exchange column chromatography. Three isozymes of lipoxygenase were identified. Two had pH optima of 6.2, and the other an optimum of 8.3. Molecular weight of each isozyme was 7.3 x 10^4, as determined by gel filtration. The alkaline optimum isozyme was not inhibited by NaCN and was inhibited by $CaCl_2$ except at very low concentrations. The acid optimum isozymes were inhibited by NaCN and were stimulated by $CaCl_2$ concentrations up to ca. 0.7 mM.

INTRODUCTION

Lipoxygenase (E.C. 1.13.1.13) catalyzes the oxidation of *cis, cis*-1,4-pentadiene systems in the presence of molecular oxygen and has been implicated in flavor production in several commodities. It is present in a variety of plants, with soybean lipoxygenase receiving the most attention. Investigations have shown that crude and purified lipoxygenase from various sources differ in pH optima, substrate specificity, number of isozymes, and other characteristics (1-4).

Siddiqi and Tappel (1), using crude extracts and a pH 7.0 assay, reported that peanuts contained only 1% of the lipoxygenase activity found in soybeans. This perhaps has contributed to the lack of investigation of peanut lipoxygenase. Dillard, et al., (2), using crude peanut lipoxygenase, found two pH optima with linoleic acid, trilinolein, and cottonseed oil substrates. This indicated that more than one isozyme of the enzyme might be present. A pH optimum of ca. pH 6 was reported by St. Angelo and Ory (5) who used ammonium sulfate to fractionate the enzyme. Pattee, et al., (6) investigated pentane production by peanut lipoxygenase and attempted to isolate a separate pentane producing enzyme, purified peanut lipoxygenase wieth ammonium sulfate and column chromatography. Possibly due to a single pH assay of fractions and use of a relatively steep NaCl gradient on DEAE-Sephadex, they did not observe the isozyme separation reported here. This paper reports the first identification, purification, and characterization of peanut lipoxygenase isozymes.

EXPERIMENTAL PROCEDURES

Sources of Materials

Peanuts (*Arachis hypogaea* L. var. NC-5) were obtained from the North Carolina Peanut

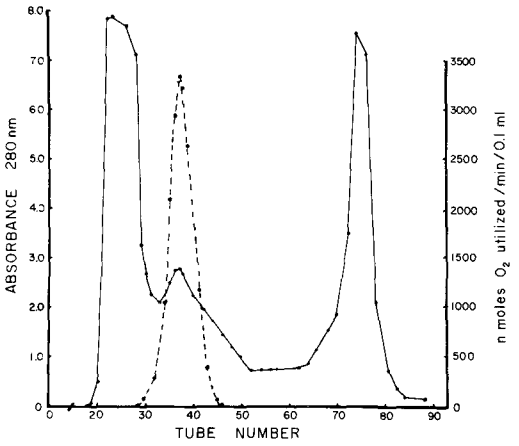

FIG. 1. Elution of peanut lipoxygenase from Sephadex G-150; 878 mg applied to 2.5 X 75 cm column. (------) - protein absorbance at 280nm; (- - -) = lipoxygenase activity.

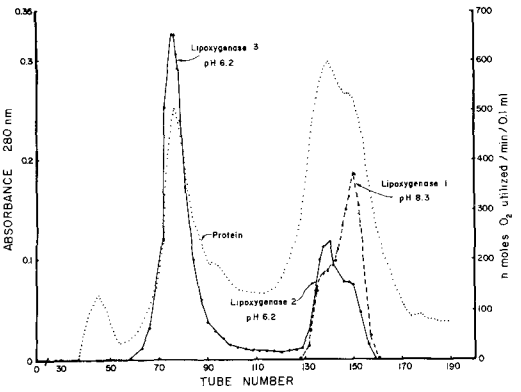

FIG. 2. Purification of peanut lipoxygenase on DEAE-Sephadex A-50; 118 mg applied to 2.5 X 35 cm column. (. . .) = protein absorbance at 280nm; (------) = lipoxygenase activity pH 6.2; (- - -- = lipoxygenase activity pH 8.3.

TABLE I

Purification and Isolation of Lipoxygenase Isozymes from Peanuts

Fraction	Purification[a]					Purification[b]				
	Total activity	Specific activity	From crude	Per step	Recovery (%)	Total activity	Specific activity	From crude	Per step	Recovery (%)
Crude	2184000	472				169260	37			
$(NH_4)_2SO_4$ Supernatant (40%)	1848600	557	1.2	1.2	84.6	478500	145	4	4	282.7
$(NH_4)_2SO_4$ Precipitate (65%)	2968875	3383	7.2	6.1	135.9	258825	295	8	2.0	152.9
Sephadex G-150	1105650	9342	19.8	2.8	50.6	185850	1570	43	5.3	109.8
DEAE - Sephadex A-50 (peak fractions)										
Isozyme 3	30702	23755	50.3	2.5	1.4					
Isozyme 2	11272	7181	15.2	0.8	0.5					
Isozyme 1						17413	12776	349	8.1	10.2

[a]Activity measured at pH 6.2.
[b]Activity measured at pH 8.3.

Belt Research Station (Lewiston, NC), high purity grade linoleic acid from the Hormel Institute (Austin, MN), Sephadex G-150, DEAE-Sephadex, and standard proteins for mol wt determination from Pharmacia Fine Chemicals (Piscataway, NJ), and special enzyme grade ammonium sulfate from Schwarz/Mann (Orangeburg, NY).

Enzyme Assay

Lipoxygenase activity was measured polarographically in a reaction vessel fitted with a Clark oxygen electrode. The vessel contained 3.3 μmoles of linoleic acid and 0.08% Tween 20 in 0.4 ml of 0.1 M borate pH 7.0, enzyme, and enough 0.05 M phosphate buffer pH 6.2 or 0.05 M Tris-HCl buffer pH 8.3 for a total volume of 1.5 ml. Activity was calculated from the slope of the polarographic trace on assumption that the initial O_2 concentration was 260 nmoles/ml (7). Generally all experiments were repeated at least 3 times.

Enzyme Purification

Lipoxygenase was extracted from acetone powders prepared from peanut seed according to the methods of Pattee and Swaisgood (8) by stirring for 1 hr at room temperature in 20 volumes of 0.5 M Tris-HCl buffer, pH 7.0. All other procedures were performed at 4 C; centrifugations were performed at 11,700 x g for 10 min, and only 0.05 M Tris-HCl buffer pH 7.0 was used. The slurry obtained was centrifuged, and the supernatant filtered through Whatman No. 4 filter paper. Protein precipitating between 40-65% ammonium sulfate saturation was pelleted by centrifugation, and the supernant was discarded. The precipitate was redissolved in a small volume of buffer and placed on a Sephadex G-150 column (2.5 cm X 75 cm), which then was eluted with buffer using a flow rate of 16 ml/hr. Active fractions were combined and placed on a DEAE-Sephadex A-50 column (2.5 cm X 35 cm) that had been equilibrated with buffer. The column was developed at 32 ml/hr with an 800 ml linear gradient of 0.04-0.25 M NaCl in buffer. Aliquots of 4.2 ml were collected, and protein elution was monitored continuously at 280 nm. Generally, the peak activity fraction (tube) was used for characterization studies of each of the 3 isozymes. Single observations were made using the fraction 2-3 tubes removed to the outside of the peak activity fraction to verify results where cross contamination of isozymes might occur in the peak activity fraction.

Mol wt was determined by reverse flow gel filtration on a Sephadex G-150 column (2.5 cm X 35 cm). Protein content was determined

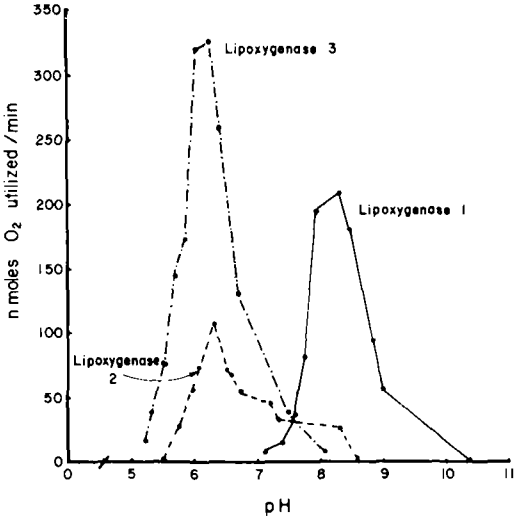

FIG. 3. Effect of pH on activity of peanut lipoxygenase isozymes. Isozyme 1, = 26.9 ug/assay; Isozyme 2, 38.5 ug/assay; Isozyme 3 = 8 ug/assay.

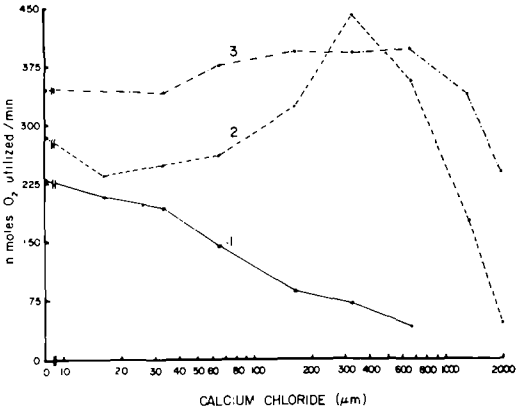

FIG. 4. Effect of $CaCl_2$ on activity of peanut lipoxygenase isozymes. Isozyme 1 = 39.1 μg/assay, Isozyme 2=53.7 μg/assay, Isozyme 3=8 μg/assay. Data plotted on 3 cycle semilogarithmic paper.

spectrophotometrically as described by Layne (9).

RESULTS AND DISCUSSION

Purification

Lipoxygenase isozymes have been purified to various degrees with up to 4 isozymes in peas identified by disc-gel electrophoresis (4). Peanut, like soybean (3), appears to have three isozymes. Purification by Sephadex G-150 column chromatography resulted in 3 major protein peaks; and lipoxygenase activity was concentrated in the small middle peak (Fig. 1). A 19.8-fold purification of pH 6.2 activity and a 43-fold purification of pH 8.3 activity was achieved through this step (Table I). Purification on DEAE-Sephadex A-50 provided further purification and revealed the presence of 3 isozymes (Fig. 2). The isozymes are numbered as the soybean lipoxygenase isozymes, that is, in the reverse order of their elution (3). Table I indicates the effectiveness of the various steps in purification of the isozymes.

pH Optima

Optimum pH's of the isozymes of peanut lipoxygenase resemble those of soybean lipoxygenase isozymes, 2 acid and one alkaline (3,10) (Fig. 3). The pH optima reported here of 6.2 and 8.3 are similar to those reported for crude peanut lipoxygenase by Dillard, et al., (2) using linoleic acid substrate. Some reports (3,11) indicate that isozyme 1 (pH 9.0) is the most active of the 3 soybean isozymes; whereas, our data indicate that isozyme 3 (pH 6.2) is the most active of the peanut isozymes.

Stability

Activity of the enzyme in acetone powders stored at -20 C appeared to be very stable during the 10-month duration of the study. After DEAE-Sephadex purification, stability of the isozymes varied. Held at 5 C, isozymes 1 (pH 8.3) and 2 (pH 6.2) lost activity in 2-3 days, while isozyme 3 (pH 6.2) remained relatively active for 5-6 days. Sephadex G-150 purified enzyme taken to 70% ammonium sulfate saturation and stored at 5 C lost $<$ 30% of its pH 6.2 activity in 60 days.

Effect of $CaCl_2$

The effects of calcium concentration on the lipoxygenase isozymes of peanuts (Fig. 4) and of soybeans were not the same. Soybean isozyme 1 (pH 9.0) was activated by calcium (12), while peanut isozyme 1 (pH 8.3) was progressively inhibited by increased calcium concentrations. Isozymes 2 and 3 of soybeans, pH 6.8-7.0, respectively were activated and inhibited by calcium (3,12,13), while peanut isozymes 2 and 3, both pH 6.2, were activated by 40-400 μM calcium and inhibited by higher concentrations (Fig. 4). The mechanism by which calcium activates lipoxygenase has received considerable attention, and recently Zimmerman and Snyder (13) presented convincing evidence that calcium interacts with the linoleate substrate rather than directly with soybean lipoxygenase 2. Because actual substrate availability was concerned, such a mechanism may hold true for inhibitory effects.

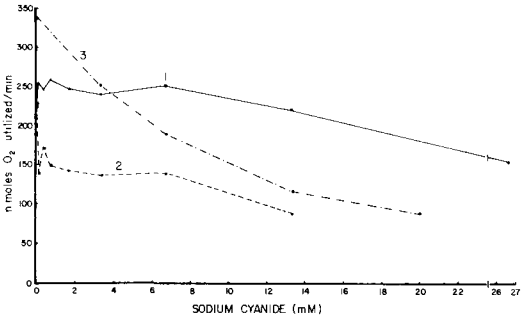

FIG. 5. Effect of NaCN on activity of peanut lipoxygenase isozymes. Isozyme 1 = 39.1 μg/assay; Isozyme 2 = 53.7 μg/assay; Isozyme 3=8 μg/assay.

Effect of NaCN

When added to the reaction mixture before enzyme addition, NaCN inhibited peanut lipoxygenase isozymes 2 and 3, but concentrations below 14 mM had little effect on the activity of isozyme 1 (Fig. 5). A general inhibition of isozyme 3 was noted, while isozyme 2 activity decreased quickly and remained relatively constant at NaCN concentrations of up to 7 mM. Haydar and Hadziyev (4) reported up to 40% inhibition of pea lipoxygenase by cyanide concentrations up to 50 mM, but attributed a portion of the decrease to inhibition of other lipid oxidizing enzymes in the extract. St. Angelo and Ory (5) incubated peanut lipoxygenase in 1 mM KCN for 1 hr at 4 C before testing at about pH 6.0, and found no influence on lipoxygenase activity. Siddiqi and Tappel (1) reported that incubation of crude peanut lipoxygenase in 1 mM cyanide produced no inhibition at a reaction pH of 7.0. Cyanide generally is not considered an inhibitor of lipoxygenase activity (1); however, Johns (14) indicated that 3.3 mM NaCN inhibited the pH 7 lipoxygenase isozyme of soybean by ca. 87%. This lends support to our data, because both pH 6.2 isozymes of peanut were inhibited. Isozyme purity may affect the inhibitory effects of cyanide. Most reports on cyanide inhibition are from relatively crude lipoxygenase preparations, while the work of Johns (14) and that reported here are on relatively pure preparations. Therefore, it is quite possible that earlier reports overlooked specific isozyme effects.

Molecular Weight

Mol wts of the peanut isozymes purified by DEAE-Sephadex were determined by Sephadex gel filtration. All the isozymes had the same elution volume, which relative to the elution volumes of the protein standards, indicated a

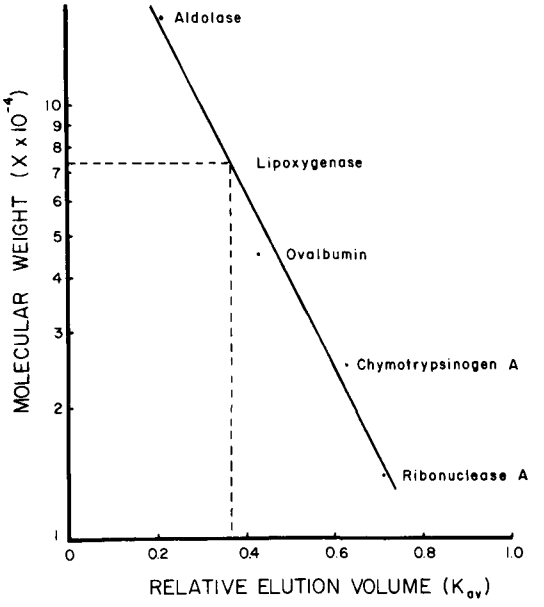

FIG. 6. Calibration curve for determination of peanut lipoxygenase mol wt. Column: 2.5 X 35 cm reverse flow Sephadex G-150 eluted at 16 ml/hr with 0.05 M Tris - HCl buffer pH 7.0; Standard proteins: 8-10 mg each; lipoxygenase; 5-6 mg each.

mol wt of 73,000 (Fig. 6). Mol wts of just over 100,000 have been reported for soybean lipoxygenase (3,14-16). The value reported here agrees well with the mol wt of 74,000 for pea lipoxygenase reported by Haydar and Hadziyev (4).

Further work on isolation, characterization, and stabilization of peanut lipoxygenase isozymes is in progress pursuant to studies involving product identification and possible physiological relationships of the isozymes.

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Comparison of Phospholipid Composition of *Aedes aegypti* and *Aedes albopictus* Cells Obtained from Logarithmic and Stationary Phases of Growth

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ABSTRACT

Aedes aegypti and *Aedes albopictus* cells were grown in tissue culture and harvested at logarithmic and stationary phases of development. The phospholipids were extracted, separated into lipid classes, and fatty acid composition of each fraction determined. The phosphatidylethanolamine fraction was the major lipid (42-54%). With aging, the *A. aegypti* cells showed an increase in polyenes in the phosphatidylcholine and phosphatidylethanolamine fractions and in monoenes and polyenes in the phosphatidylinositol fraction. The lysophosphatidylcholine fraction had an increase in chain length of the fatty acids with aging of the *A. aegypti* cells. The *A. albopictus* cells, with aging, showed increases in chain length and in the relative percentage of polyenes in the lysophosphatidylcholine, phosphatidylcholine, and phosphatidylserine fractions. In the phosphatidylinositol fraction, chain elongation of fatty acids occurred as the cells aged. In the ceramide phosphorylcholine fraction, there were increases in saturation and chain elongation of the fatty acids from the logarithmic to the stationary phase of the *A. albopictus* cells. An increase in polyenes was observed with aging of the cells in the phosphatidylethanolamine fraction.

INTRODUCTION

It has been shown in studies of changes of fatty acid composition of lipids of insects through various stages of development that greater amounts of unsaturated acids developed with aging (1). Municio, et al., (2,3), using labeled homogenates of larvae and adults of the insect *Ceratitis capitata*, reported differences in chain elongation and desaturation of fatty acids at two stages of development of the insect.

Sedee (4) demonstrated that saturated and unsaturated fatty acid synthesis in insects takes place by two separate pathways without interconversion by dehydrogenation. Wakil (5) presented evidence of two distinct systems for synthesis of fatty acids in mammals; the mitochondrial system for elongation of existing fatty acids and the non-mitochondrial or malonyl CoA pathway. Fast (6) reviewed publications that indicated both systems were present in insects.

Mosquitos as well as some other insects of the Dipteran order have been found to have phosphatidylethanolamine as the main component of the phospholipids (7), whereas, the major fraction of the phospholipids of mammals and other insects was phosphatidylcholine (6). Other unique features of the Diptera were associated with the sphingophospholipids (8,9) and the larger amounts of 16:0 and 16:1 acids (7).

Two species of mosquito cells, *A. aegypti* and *A. albopictus*, were selected for in vitro studies. The phospholipid fractions were analyzed at two phases of growth for changes in the fatty acid composition that could occur with aging.

MATERIALS AND METHODS

Preparation of Cells

A. albopictus larval cells, Singh's strain (10), were obtained from F. Paul (Naval Medical Research Institute, Bethesda, MD). The *A. aegypti* larval cells, Singh's strain (10), were supplied by S.H. Hsu (Naval Medical Research Unit No. 2, Taipei, Taiwan). The cells were grown in suspension culture and harvested as reported previously by McMeans, et al., (11).

Lipid Extraction and Fractionation

The lipids from mosquito cells were extracted as described by Makino, et al., (12), and fractionated into neutral lipids and phospholipids by column chromatography (13). The total phospholipid was chromatographed on plates coated with Silica Gel H using the solvent system, chloroform:methanol:acetic acid:water (25:15:4:2) to separate the lipids (14). Known quantities of standard compounds and the sample were applied to lanes on each plate. Positions of the classes of phospholipid were determined by spraying the reference lanes on the developed plate with molybdenum blue reagent (15). Separated lipids were scraped

from the plates. For a better separation of the phosphatidylserine and phosphatidylinositol fractions, compounds were extracted from the silica gel and rechromatographed on a Silica Gel G plate using the same developing solvent system and spray as in the original separation.

Methylation

Silica gel bands containing separated phospholipid classes were scraped from the plate and transesterified with 5% HCl in methanol (16).

Gas Liquid Chromatography

The gas liquid chromatographic (GLC) analyses were made on a Fisher-Victoreen gas chromatograph model 4000. The methyl esters were analyzed on columns 8 ft by 1/8 in. (internal diameter [ID], 244 cm x 0.32 cm), packed with 15% EGSS-X on Gas Chrom P, 100-120 mesh (Applied Science Laboratories, State College, PA).

Standards

Authentic phospholipid standards were obtained from the Lipid Preparation Laboratory, The Hormel Institute (Austin, MN), Applied Science Laboratories (State College, PA), or Supelco, Inc. (Bellefonte, PA), or prepared in our laboratory. The methyl ester standards for GLC references were purchased from the Lipid Preparation Laboratory, The Hormel Institute.

RESULTS

Results were obtained from two experiments conducted at logarithmic and stationary phases of growth of the insect cells. The fatty acid compositions of the classes of phospholipids in the species of mosquito cells and in the mammalian source in the original growth medium were compared. Also reported were the differences occurring between species and phases of growth of each type of mosquito cell.

The relative percentages of the classes of phospholipids are shown in Table I. Ca. 50% phosphatidylethanolamine and 30% phosphatidylcholine were present in the total phospholipid fraction of both species at the two phases of development. There were increases in the amount of the ceramide phosphorylcholine fraction in both species and of the phosphatidylinositol fraction in *A. aegypti* cells, and a decrease in the amount of the phosphatidylethanolamine fraction in *A. albopictus* cells with aging.

A. aegypti Cells: Fatty Acid Profiles from Logarithmic and Stationary Phases of Growth

Changes occurred in the fatty acid profiles of the lysophosphatidylcholine fraction (Table II) as the *A. aegypti* cells aged. There were smaller amounts of 16:1 and 18:1 and larger amounts of 20:0 fatty acids in the stationary phase of growth. Chain elongation with aging was demonstrated by the increase of C-20 acids from 15 to 40%. The amount of saturated acids increased, and the monoenes decreased in the lysophosphatidylcholine fraction of the mosquito cells with aging.

The fatty acid profile and relative percentage of saturated acids were approximately equal in the ceramide phosphorylcholine fraction at the two phases of growth of the mosquito cells (Table III). There was an increase in the amount of 18:2 and a decrease in 18:1 in the fatty acids of the phosphatidylcholine fraction with aging of the mosquito cells (Table IV). An increase in polyenes of 13 to 23% was observed in the cells.

In the phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine fractions

TABLE I

Distribution of Phospholipids of *Aedes aegypti* and *Aedes albopictus* Cells in Late Logarithmic and Stationary Phases of Growth

Phospholipid classes	Leafhopper medium	*Aedes aegypti*		*Aedes albopictus*	
		Log[a]	Stat[b]	Log[a]	Stat[b]
Lysophosphatidylcholine	8.2 ± 1.2[c]	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
Ceramide phosphorylcholine	16.0 ± 1.2	1.7 ± 0.4	3.4 ± 0.1	3.2 ± 0.4	5.7 ± 0.5
Phosphatidylcholine	71.8 ± 0.8	37.4 ± 0.6	27.9 ± 5.1	30.7 ± 1.2	34.6 ± 2.3
Phosphatidylserine	0.3 ± 0.1	3.7 ± 1.2	4.1 ± 0.3	3.0 ± 0.6	5.6 ± 1.0
Phosphatidylinositol[d]	2.0 ± 0.3	8.3 ± 1.0	15.9 ± 0.3	9.9 ± 0.6	12.0 ± 2.2
Phosphatidylethanolamine	1.7 ± 0.3	48.8 ± 0.2	48.6 ± 4.3	53.6 ± 2.0	42.0 ± 1.7

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Relative percentage of lipid classes; mean ± differences from the mean.

[d]Includes ceramide phosphatidylethanolamine.

TABLE II

Constituent Fatty Acids of the Lysophosphatidylcholine Fraction

Fatty acid	Leafhopper medium	*Aedes aegypti*		*Aedes albopictus*	
		Log[a]	Stat[b]	Log[a]	Stat[b]
14:0[c]	0.6 ± 0.1[d]	1.7 ± 0.6	2.2 ± 1.2	1.5 ± 1.2	2.5 ± 0.7
15:0	0.5 ± 0.1			Tr[e]	0.7 ± 0.3
16:0	28.6 ± 2.3	23.6 ± 3.1	24.7 ± 1.2	35.7 ± 0.6	25.8 ± 1.7
16:1	2.3 ± 1.3	11.4 ± 2.9	3.8 ± 0.4	10.8 ± 0.4	6.4 ± 2.8
17:0	1.6 ± 0.2		Tr		0.8 ± 0.4
18:0	39.5 ± 0.5	13.7 ± 0.3	11.8 ± 1.5	21.2 ± 0.6	15.5 ± 2.9
18:1	19.5 ± 1.8	28.7 ± 6.6	8.2 ± 2.1	24.4 ± 2.2	22.0 ± 2.8
18:2	2.4 ± 0.5	2.8 ± 0.1	2.5 ± 0.5	1.5 ± 0.1	6.2 ± 1.2
18:3	2.9 ± 1.1	1.0 ± 0.9	0.7 ± 0.7		Tr
20:0		13.0 ± 2.5	39.4 ± 0.6	3.8 ± 1.8	9.3 ± 0.6
20:3	0.5 ± 0.1				
20:4	1.0 ± 0.1	1.0 ± 0.9	Tr	0.7 ± 0.0	3.3 ± 1.2
20:5		0.7 ± 0.6	0.6 ± 0.6		1.7 ± 1.6
22:0[f]	Tr	2.6 ± 0.5	5.6 ± 1.0	Tr	5.8 ± 4.4
Saturates	70.8	54.6	83.7	62.2	60.4
Monoenes	21.8	40.1	12.0	35.2	28.4
Polyenes	6.8	5.5	3.8	2.2	11.2

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

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

[d]Relative percentages of fatty acid; mean ± differences from the mean.

[e]Tr = trace, <0.5%.

[f]22 carbon fatty acid series.

TABLE III

Constituent Fatty Acids of the Ceramide Phosphorylcholine Fraction

Fatty acid	Leafhopper medium	*Aedes aegypti*		*Aedes albopictus*	
		Log[a]	Stat[b]	Log[a]	Stat[b]
12:0[c]				Tr[d]	Tr
14:0	0.6 ± 0.1[e]	Tr	0.9 ± 0.3	1.4 ± 0.7	1.7 ± 0.5
14:1	Tr			Tr	Tr
15:0					Tr
16:0	36.1 ± 1.5	10.7 ± 2.3	7.6 ± 1.6	40.0 ± 1.0	14.0 ± 2.7
16:1		1.7 ± 0.4	1.0 ± 0.3	5.8 ± 1.1	1.7 ± 1.2
17:0	0.9 ± 0.1	Tr	Tr		Tr
18:0	11.4 ± 1.7	5.8 ± 1.3	5.0 ± 0.3	8.8 ± 0.3	5.3 ± 0.5
18:1	4.0 ± 3.0	4.2 ± 0.4	1.8 ± 0.1	4.8 ± 1.9	3.1 ± 1.4
18:2	0.5 ± 0.2	0.6 ± 0.6	0.9 ± 0.3	Tr	1.5 ± 1.0
20:0	1.2 ± 0.2	61.3 ± 2.3	61.3 ± 5.3	12.9 ± 1.2	51.0 ± 4.4
22:0	8.7 ± 1.1	10.0 ± 3.8	11.7 ± 4.3	9.1 ± 0.5	11.6 ± 4.0
22:1	0.7 ± 0.1				
23:0	5.0 ± 0.2	1.2 ± 0.3	2.0 ± 0.7	2.4 ± 0.2	1.9 ± 1.1
23:1	0.7 ± 0.2		1.8 ± 1.8		0.5 ± 0.5
24:0	10.1 ± 1.5	1.1 ± 0.4	4.5 ± 2.2	5.6 ± 0.4	3.6 ± 0.9
24:1	20.3 ± 3.1	2.5 ± 1.4	2.0 ± 1.5	9.2 ± 2.8	3.2 ± 0.0
Saturates	74.0	90.1	93.0	80.2	89.1
Monoenes	25.7	8.4	6.6	19.8	8.5
Polyenes	0.5	0.6	0.9	0.0	1.5

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

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

[d]Tr = trace, < 0.5%.

[e]Relative percentages of fatty acid; mean ± differences from the mean.

(Tables V-VII) there was an increase in the relative percentage of 18:2 fatty acid from the logarithmic to stationary phase of growth. The phosphatidylinositol fraction also showed a decrease in the 20:0 acid, and the phosphatidylethanolamine showed a slight decrease in the

TABLE IV

Constituent Fatty Acids of the Phosphatidylcholine Fraction

Fatty acid	Leafhopper medium	*Aedes aegypti*		*Aedes albopictus*	
		Log[a]	Stat[b]	Log[a]	Stat[b]
12:0[c]	Tr[d]	Tr	0.8 ± 0.1[e]	Tr	0.6 ± 0.1
14:0	Tr	3.1 ± 0.2	4.1 ± 0.4	2.8 ± 0.2	3.9 ± 0.1
14:1		1.1 ± 0.2	1.0 ± 0.1		0.7 ± 0.0
15:0	0.9 ± 0.3	0.5 ± 0.1	1.0 ± 0.1	0.9 ± 0.5	0.5 ± 0.1
16:0	15.4 ± 2.2	22.7 ± 0.6	21.9 ± 0.5	21.7 ± 1.3	18.7 ± 0.4
16:1	1.9 ± 0.7	12.9 ± 0.4	12.7 ± 0.3	14.3 ± 0.6	11.5 ± 0.4
17:0	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.8 ± 0.9	0.9 ± 0.1
17:1	0.7 ± 0.3				
18:0	21.4 ± 0.3	7.9 ± 0.5	5.8 ± 0.2	10.8 ± 0.3	6.2 ± 0.0
18:1	31.2 ± 3.7	36.9 ± 0.1	28.5 ± 0.7	37.3 ± 0.3	29.8 ± 0.3
18:2	10.5 ± 0.9	6.5 ± 0.3	14.2 ± 1.4	4.8 ± 0.8	15.2 ± 0.0
18:3	0.9 ± 0.4	0.7 ± 0.1	1.0 ± 0.3	0.5 ± 0.0	1.2 ± 0.0
20:0		0.9 ± 0.3	0.8 ± 0.2	Tr	0.9 ± 0.1
20:1		Tr			0.8 ± 0.0
20:2	0.9 ± 0.5	0.6 ± 0.2	0.9 ± 0.3		0.6 ± 0.6
20:3	4.1 ± 0.4	0.9 ± 0.2	0.5 ± 0.5	0.5 ± 0.0	0.5 ± 0.0
20:4	6.8 ± 0.5	3.8 ± 0.8	5.6 ± 1.6	2.7 ± 0.1	6.3 ± 0.1
20:5	0.6 ± 0.1	0.6 ± 0.5	0.7 ± 0.7	0.8 ± 0.1	1.9 ±0.2
22:5	1.5 ± 1.0				
22:6	1.7 ± 0.1				
Saturates	38.6	36.0	35.3	38.0	31.7
Monoenes	33.8	50.9	42.2	51.6	42.8
Polyenes	27.0	13.1	22.9	9.3	25.7

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

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

[d]Tr = trace, < 0.5%.

[e]Relative percentages of fatty acid; mean ± differences from the mean.

TABLE V

Constituent Fatty Acids of the Phosphatidylserine Fraction

Fatty acid	Leafhopper medium	*Aedes aegypti*		*Aedes albopictus*	
		Log[a]	Stat[b]	Log[a]	Stat[b]
14:0[c]	1.0 ± 0.3[d]		0.9 ± 0.2	0.6 ± 0.1	1.0 ± 0.2
15:0	0.7 ± 0.3	Tr[e]		Tr	
16:0	15.7 ± 2.4	5.7 ± 0.5	7.4 ± 0.4	9.1 ± 0.2	7.0 ± 0.7
16:1	1.1 ± 0.0	8.3 ± 3.0	9.1 ± 1.0	10.9 ± 0.4	6.6 ± 0.3
17:0	1.2 ± 0.0	Tr	0.8 ± 0.1		0.6 ± 0.0
17:1	1.2 ± 1.2				
18:0	35.8 ± 2.5	16.9 ± 0.7	15.2 ± 1.0	21.0 ± 1.6	10.5 ± 0.5
18:1	20.9 ± 1.5	59.7 ± 0.4	55.8 ± 3.0	53.2 ± 1.1	51.4 ± 2.4
18:2	3.0 ± 0.2	3.5 ± 0.7	6.6 ± 0.3	2.3 ± 0.4	10.2 ± 0.7
18:3	0.6 ± 0.1	Tr	1.3 ± 0.6		1.0 ± 0.2
20:0	2.6 0.8	1.8 ± 0.6	1.3 ± 0.1	1.1 ± 0.2	2.4 ± 0.1
20:1		Tr	Tr		0.9 ± 0.8
20:2		Tr			1.4 ± 0.0
20:3	4.7 3.2	0.8 ± 0.2	0.5 ± 0.3	0.6 ± 0.1	1.4 ± 0.5
20:4	7.1 0.7	0.7 ± 0.7	1.1 ± 1.0	0.8 ± 0.0	4.2 ± 0.6
20:5		0.6 ± 0.6			1.6 ± 0.2
22:4	5.0 4.9				
Saturates	57.0	24.4	25.6	31.8	21.5
Monoenes	23.2	68.0	64.9	64.1	58.9
Polyenes	20.4	5.6	9.5	3.7	19.8

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

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

[d]Relative percentages of fatty acid; mean ± differences from the mean.

[e]Tr = trace, < 0.5%.

TABLE VI

Constituent Fatty Acids of the Phosphatidylinositol Fraction[a]

Fatty acid	Leafhopper medium	*Aedes aegypti*		*Aedes albopictus*	
		Log[b]	Stat[c]	Log[b]	Stat[c]
14:0[d]	Tr[e]	Tr	0.8 ± 0.5[f]	Tr	0.5 ±
16:0	10.4 ± 0.0	6.1 ± 0.8	8.0 ± 1.7	10.0 ± 0.3	5.7 ± 0.1
16:0	1.5 ± 0.3	5.2 ± 1.4	6.7 ± 1.4	7.7 ± 0.9	3.4 ± 0.2
17:0	1.4 ± 0.0				
18:0	33.5 ± 0.8	7.1 ± 0.4	7.1 ± 1.1	10.9 ± 1.2	5.8 ± 0.1
18:1	19.8 ± 2.1	17.9 ± 0.7	19.5 ± 5.3	37.5 ± 5.8	16.6 ± 0.9
18:2	3.0 ± 0.5	5.7 ± 0.0	9.1 ± 0.9	5.9 ± 0.1	8.6 ± 0.2
18:3	2.3 ± 1.8	Tr	1.1 ± 1.1	1.7 ± 0.8	1.2 ± 0.1
20:0	Tr	42.3 ± 0.2	31.5 ± 1.9	13.0 ± 4.8	36.2 ± 0.3
20:2	Tr	Tr	1.6 ± 1.6		
20:3	6.3 ± 0.3	8.2 ± 0.4	5.6 ± 1.3	4.8 ± 1.4	8.4 ± 0.4
20:4	16.9 ± 0.3	4.9 ± 0.0	7.0 ± 1.0	6.8 ± 0.7	9.6 ± 0.2
20:5	Tr	1.4 ± 0.1	2.2 ± 0.1	1.7 ± 0.2	3.9 ± 0.2
22:4	0.7 ± 0.3				
22:5	1.8 ± 1.0				
22:6	0.8 ± 0.1				
Saturates	45.3	55.5	47.4	33.9	48.2
Monoenes	21.3	23.1	26.2	45.2	20.0
Polyenes	31.8	20.2	26.6	20.9	31.7

[a]Includes ceramide phosphatidylethanolamine fraction.

[b]*Logarithmic phase of growth.*

[c]Stationary phase of growth.

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

[e]Tr = trace, < 0.5%.

[f]Relative percentages of fatty acid; mean ± differences from the mean.

TABLE VII

Constituent Fatty Acids of the Phosphatidylethanolamine Fraction

Fatty acid	Leafhopper medium	*Aedes aegypti*		*Aedes albopictus*	
		Log[a]	Stat[b]	Log[a]	Stat[b]
12:0[c]		1.4 ± 0.1	Tr[e]		Tr
14:0	0.7 ± 0.1[d]	Tr	3.0 ± 0.1	1.1 ± 0.0	3.1 ± 0.2
14:1		Tr	Tr		Tr
15:0	2.9 ± 1.2		Tr	1.2 ± 0.4	Tr
16:0	7.7 ± 0.0	18.6 ± 0.6	19.6 ± 2.2	20.1 ± 0.9	17.4 ± 0.4
16:1	1.4 ± 0.0	13.0 ± 1.6	11.6 ± 0.0	11.1 ± 0.7	9.9 ± 0.2
17:0	1.1 ± 0.6	1.8 ± 0.9	0.8 ± 0.1	1.4 ± 0.7	0.8 ± 0.0
17:1	1.0 ± 0.1				
18:0	19.8 ± 0.2	7.0 ± 0.1	5.2 ± 0.1	7.3 ± 0.1	5.8 ± 0.0
18:1	26.8 ± 1.1	44.6 ± 0.6	39.9 ± 1.4	44.6 ± 1.0	40.4 ± 0.2
18:2	9.7 ± 1.2	6.9 ± 1.3	11.9 ± 0.3	5.1 ± 0.2	13.2 ± 0.0
18:3	0.7 ± 0.5	Tr	0.6 ± 0.6	0.7 ± 0.2	1.2 ± 0.0
20:0	0.8 ± 0.0	1.7 ± 0.5	1.3 ± 0.2	4.4 ± 0.8	1.1 ± 0.4
20:1			Tr		0.7 ± 0.0
20:2	1.8 ± 1.4	0.5 ± 0.5	Tr		
20:3	2.9 ± 0.9	0.5 ± 0.2	Tr	1.2 ± 0.2	0.5 ± 0.2
20:4	15.7 ± 1.1	2.2 ± 0.5	3.1 ± 0.2	1.6 ± 0.2	3.7 ± 0.2
20:5	0.9 ± 0.9	1.0 ±0.2	1.2 ± 0.2	0.7 ± 0.1	1.6 ± 0.2
22:4	1.4 ± 0.3				
22:5	4.1 ± 1.7				
22:6	1.2 ± 1.2				
Saturates	33.0	30.5	29.9	35.5	28.2
Monoenes	29.2	57.6	51.5	55.7	51.0
Polyenes	38.4	11.1	16.8	9.3	20.2

[a]Logarithmic phase of growth

[b]Stationary phase of growth

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

[d]Relative percentages of fatty acid; mean ± differences from the mean.

[e]Tr = trace, < 0.5%.

amount of 18:1 acid with aging.

Chain elongation of the fatty acids occurred in the lysophosphatidylcholine fraction as the cells aged. The average chain length of the acids in the other phospholipid fractions examined was approximately the same in the logarithmic and stationary phases of growth of the *A. aegypti* cells.

The lysophosphatidylcholine fraction showed an increase in saturation from the logarithmic to stationary phase of growth. The phosphatidylcholine and phosphatidylethanolamine fractions had an increase in the percentage of polyenes and the phosphatidylinositol fraction showed an increase in monoenes and polyenes as the cells aged. In the other phospholipid fractions analyzed, there were small or no changes in the amount of unsaturation of the fatty acids with aging of the cells.

A. albopictus Cells: Fatty Acid Profiles From Logarithmic and Stationary Phases of Growth

In the fatty acid profiles of the lysophosphatidylcholine fraction (Table II). the *A. albopictus* cells have smaller percentages of 16:0 and 18:0 acids and greater percentages of 18:2 and 20:0 fatty acids in the stationary phase in comparison to the logarithmic phase of growth. An increase in unsaturated fatty acids was observed by a 2 to 11% increase in total polyenes with aging of the cells.

Changes occurred in fatty acid profiles of the ceramide phosphorylcholine fraction (Table III) with aging of the mosquito cells. *A. albopictus* cells had smaller amounts of 16:0, 18:0, and 24:1 fatty acids and larger amounts of 20:0 acid in the stationary phase than were found in the logarithmic phase. As the cells aged, there was a 20 to 9% decrease in monoenes and an increase in chain elongation.

The amounts of 18:2 and 20:4 acids became greater, and the amounts of 16:1, 18:0, and 18:1 acids decreased in the fatty acid profile of the phosphatidylcholine fraction (Table IV) as *A. albopictus* cells aged. Results from the comparison of the logarithmic and stationary phases of growth of the cells showed a 9 to 26% increase in polyenes, and the average chain length of acids increased with aging.

There were decreases in the percentages of 16:1 and 18:0 acids and increases in the percentages of 18:2 and 20-carbon unsaturated acids in the fatty acid pattern of the phosphatidylserine fraction (Table V) as the *A. albopictus* cells aged. The cells showed a 32 to 22% decrease in total saturated acids and an increase in polyenes of 4 to 20% with aging. There was an increase in the average chain length of fatty acids in the phosphatidylserine fraction from the logarithmic to stationary phase of growth of the cells.

In the fatty acid profile of the phosphatidylinositol fraction (Table VI), cells in the stationary phase had greater relative amounts of 18:2, 20:0, and 20-carbon unsaturated acids and smaller amounts of 16:0, 16:1, 18:0, and 18:1 than were observed in the logarithmic phase. There were increases from 34 to 48% in the amounts of saturated acids and polyenes (21 to 32%), and a decrease of 45 to 20% in the amount of monoenes with aging of the cells.

A. albopictus cells in the stationary phase had less 18:1 acid and more 18:2 acid in the fatty acid profile of the phosphatidylethanolamine fraction (Table VII). The total amount of polyenes, 9-20%, was greater in the stationary phase. The average chain length of fatty acids at both stages of growth of the cells was approximately the same.

Comparison of Profiles of Fatty Acids of *A. aegypti* and *A. Albopictus* in Logarithmic and Stationary Phases of Growth

There were species differences in both phases of growth in the lysophosphatidylcholine fraction (Table II). In the logarithmic phase, *A. aegypti* cells contained less 16:0 and 18:0 and more 20:0 fatty acids than *A. albopictus* cells. In the stationary phase, *A. aegypti* cells had less 18:1 and 18:2 acids and more 20:0 fatty acid than *A. albopictus* cells. *A. aegypti* cells had a larger amount of saturated acids and a smaller amount of monoenes in the stationary phase than *A. albopictus* cells.

In the ceramide phosphorylcholine (Table III), species differences occurred in the logarithmic phase of growth with *A. aegypti* cells having smaller percentages of 16:0, 24:0, and 24:1 fatty acids and a larger percentage of 20:0 acid. Also, in the logarithmic phase, *A. aegypti* cells had a smaller amount of monoenes and a larger amount of saturated acids than *A. albopictus* cells. In the stationary phase of growth, *A. aegypti* cells had a smaller percentage of 16:0 acid than *A. albopictus* cells.

In the phosphatidylcholine fraction (Table IV) there was more 18:0 acid found in the logarithmic phase of *A. albopictus* cells than in *A. aegypti* cells at the same growth phase. At the stationary phase, fatty acid profiles of the phosphatidylcholine fraction of the two species were similar. However, there was some increase in average chain length of *A. albopictus* cells with aging.

Fatty acid profiles of the phosphatidylserine fraction (Table V) showed *A. aegypti* cells having smaller percentages of 16:0 and 18:0 fatty acids and a greater percentage of 18:1 acid than *A. albopictus* cells in the logarithmic phase, and

smaller amounts of 18:2 and 20-carbon unsaturated acids and more 16:1 and 18:0 acids than *A. albopictus* cells in the stationary phase. *A. aegypti* cells contained a smaller percentage of saturated acids than *A. albopictus* cells in the logarithmic phase of growth.

Species differences occurred in the logarithmic phase of growth of cells in the phosphatidylinositol fraction (Table VI). *A. aegypti* cells had less 16:0, 18:0, and 18:1 and more 20:0 acids in the fatty acid profile than *A. albopictus* cells. *A. aegypti* cells also contained more saturated acids and less monoenes at the logarithmic phase than *A. albopictus* cells at the same growth phase.

In the phosphatidylethanolamine fraction (Table VII), fatty acid profiles of the two *Aedes* cells at either phase of growth were approximately the same.

Whenever changes in the amounts of 16:1, 18:0, 18:1, and 24:1 fatty acid occurred in one or several fractions of phospholipids, there was a decrease in the amount of these fatty acids with aging in both types of mosquito cells. When the amounts of 18:2, 20-carbon unsaturated, and 22:0 fatty acids changed in any fraction of phospholipid with aging of the mosquito cells, the acids were found in larger amounts in the stationary phase.

The lysophosphatidylcholine fraction of the *A. aegypti* cells showed an increase in amount of saturation of acids in the fatty acid profile as cells aged, whereas, the lysophosphatidylcholine fraction of *A. albopictus* cells showed an increase in the amount of polyenes with aging of cells. The ceramide phosphorylcholine fraction from *A. aegypti* cells at both growth phases and from *A. albopictus* cells, stationary phase, contained approximately the same percentages of saturated, monoene, and polyene acids. *A. albopictus* cells in the logarithmic phase had less saturates and more monoenes than the *A. aegypti* cells. The amount of polyenes is $< 2\%$ in either cell at either growth phase in the ceramide phosphorylcholine fractions. In the phosphatidylserine fraction, the amounts of saturated acids monoenes, and polyenes were approximately the same at both growth phases in *A. aegypti* cells, but there was an increase in polyenes with aging of *A. albopictus* cells. There was an increase in monoenes and polyenes in the phosphatidylinositol fraction of *A. aegypti* cells with aging. Variations in the amounts of saturated acid, monoenes, and polyenes occurred in the fatty acid profile of the phosphatidylinositol fraction with small changes in total unsaturation of the acids as *A. albopictus* cells aged. Phosphatidylcholine and phosphatidylethanolamine fractions showed increases in polyenes in both *Aedes* cells from the logarithmic to sationary phase of growth.

As *A. aegypti* cells aged, there was an increase in the average chain length of the fatty acid profile of the lysophosphatidylcholine fraction, but the chain length of acids remained about the same in the other phospholipid fractions analyzed. The average chain length of the fatty acids increased with aging of *A. albopictus* cells in all phospholipid fractions, except phosphatidylethanolamine, which was approximately equal at both stages of growth.

Fatty Acid Profiles of Mosquito Cells and Growth Medium

Lysophosphatidylcholine fraction. Fatty acid profiles of the lysophosphatidylcholine fractions are listed in Table II. In comparison with the growth medium, *A. aegypti* cells had a larger percentage of 16:1 fatty acid in the logarithmic phase, a smaller percentage of 18:1 in the stationary phase, and less 18:0 and more 20:0 acids in both phases. *A. albopictus* cells showed more 16:0 and 16:1, and less 18:0 fatty acid in the logarithmic phase, and less 18:0, more 18:2 and 20:0 acids in the stationary phase than were found in the medium.

Ceramide phosphorylcholine fraction. Table III illustrates the fatty acid profile of the ceramide phosphorylcholine fraction. *A. aegypti* cells, in both phases of growth, had smaller amounts of 16:0, 18:0, 23:0, 24:0, and 24:1 fatty acids and larger amounts of 20:0 acid than were found in the leafhopper medium. *A. albopictus* cells, in both phases of growth, had smaller amounts of 23:0, 24:0, and 24:1 fatty acids and a larger amount of 20:0 acid than in the medium. In addition, *A. albopictus* cells after 2 days of growth had more 16:1 acid, and after 10 days of growth had smaller amounts of 16:0 and 18:0 than were found in the medium.

Phosphatidylcholine fraction. Fatty acid profiles of both species of mosquito cells at logarithmic and stationary phases had larger percentages of 16:1 acid and smaller percentages of 18:0 acid than the growth medium (Table IV). *Aedes* cells had smaller percentages of 18:2 in the logarithmic phase and larger percentages of 18:2 in the stationary phase than the medium. *A. aegypti* cells, at both growth phases, and *A. albopictus* cells, at the logarithmic phase, had larger percentages of 16:0 and smaller percentages of 20-carbon unsaturated acids than the medium.

Phosphatidylserine fraction. In comparing fatty acid profiles of the two mosquito cells with the growth medium (Table V), larger amounts of 16:1 and 18:1 acids were found in the logarithmic and stationary phases, and also larger amounts of

18:2 acid were found in the stationary phase of the species. There were smaller amounts of 16:0 and 18:0 acids in both phases of growth of the 2 cells than occurred in the medium. In comparison to the medium, there were smaller amounts of 20-carbon unsaturated acids in both phases of growth of *A. aegypti* cells and in the logarithmic phase of *A. albopictus* cells.

Phosphatidylinositol fraction. Fatty acid profiles of both *Aedes* cells, logarithmic and stationary phases (Table VI), differed from the leafhopper medium by having larger percentages of 18:2 and 20:0 acids, larger or a slight increase in 16:1 acid, and smaller percentages of 18:0 and 20:4 acids. In addition, *A. aegypti* cells, in the logarithmic phase, contained a smaller percentage of 16:0 acid than the medium. Also, *A. albopictus* cells in the logarithmic phase contained more 18:1 acids and in the stationary phase less 16:0 acid than the medium.

Phosphatidylethanolamine fraction. Table VII shows fatty acid profiles of the phosphatidylethanolamine fractions. Both species of mosquito cells had larger percentages of 16:0, 16:1, and 18:1 fatty acids and smaller percentages of 18:0 and 20:4 acids than found in the medium. *A. albopictus* cells at the logarithmic phase contained less 18:2 acid and at the stationary phase contained more 18:2 acid than the medium.

DISCUSSION

Sphingolipids of Diptera have been found to be different from those found in mammals (8) Luukkonen, et al., (9) examined cultured *A. albopictus* cells and found the sphingophospholipid fraction composed of ceramide phosphorylthanolamine and ceramide phosphorylcholine. Yang and co-workers (17) investigated the sphingophospholipids of species of *Aedes* and *Culex* mosquito cells grown in cell culture. Two components of the sphingophospholipids, ceramide phosphorylcholine, and ceramide phosphorylethanolamine, were isolated and identified. The unique fatty acid composition of sphingophospholipids and increases in the amount of the ceramide phosphorylcholine fraction with aging of cells may be associated with differences in metabolic functions of sphingophospholipids specific for each genus. It has been reported that sphingomyelin (ceramide phosphorylcholine) was not present in species of Diptera (8). The presence of ceramide phosphorylcholine could be characteristic of a certain genus of mosquitos and not of Diptera in general.

Fatty acid composition of the various phospholipid classes of the mosquito cells differed from those found in insects and mammals when phosphatidylcholine was the major phospholipid (7). De Gier and van Deenen (18) reported the changing of fatty acid composition of membrane phospholipid could alter the permeability of the membrane. Diptera, with a different phosphatidylcholine-phosphatidylethanolamine ratio than other insects, could be used for further study of membrane permeability, other phospholipid function, and the role of fatty acid in phospholipid fractions.

There was some tendency for *A. albopictus* cells to show greater changes in fatty acid composition than *A. aegypti* cells from logarithmic to stationary phases of growth. Whether the changes were due to species differences of factors in selecting the exact phase of development of cells had not been shown.

There is no doubt that the mosquito cells can synthesize their own phospholipids, because the medium and the mosquito cell fatty acid profiles of the phospholipids differed greatly.

Increases in the chain length of long chain fatty acids, when observed, were found in the older cells. An investigation of the activity of chain elongating enzymes of fatty acids should be examined to correlate such activity with aging of cells. This may be an attempt of the cell to stabilize its cell membrane. Mammalian cells we have used do not show the same magnitude of results. The reason for cell death in arbovirus infection in mammalian cells versus the increase in time it takes to kill mosquito cells may be associated with capacity of the mosquito cell to repair its lipid membranes more rapidly than mammalian cells with aging.

It is apparent also that studies of fatty acid analyses of mosquito cell profiles must be performed at different stages of cell development to be meaningful, because a variety of profiles can be observed which is dependent on the age of the cell. Further, age of these cells may be associated with differences in cell susceptibility and yield of arboviruses.

Although there were some differences found in the patterns of the fatty acids of the phospholipid fractions between the two species of mosquito cells in this study, it is doubtful if these could be used as a means of identification of species.

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Paul, Naval Medical Research Institute, Bethesda, MD, provided *Aedes albopictus* larval cells, Singh's strain, and S.H. Hsu, Naval Medical Research Unit No. 2, Taipei, Taiwan, provided *Aedes aegypti* larval cells, Singh's strain.

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Furanoid Fatty Acids from Fish Lipids[1]

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ABSTRACT

Fatty acids, recently reported as constituents of certain fish lipids, were identified to be derivatives of furan (furanoid fish fatty acids). 12,15-Epoxy-13,14-dimethyleicosa-12,14-dienoic acid is predominant among the furan acids and is associated with *bis*-homologs in regard to chain length. Monomethyl acids, such as 12,15-epoxy-13-methyleicosa-12,14-dienoic, are present in appreciable amounts. The structures were concluded from oxidative degradations, from mass spectrometry of methyl esters of the novel acids and fatty acids derived from them by opening the ring, and from nuclear magnetic resonance, infrared, and Raman spectra. The results from chemical procedures and from spectrometric methods were in agreement with those obtained with authentic methyl 9,12-epoxyoctadeca-9,11-dienoate. The number of substituents at the furan ring greatly influences hydrogenation, hydrogenolysis, and hydrolysis reactions of the ring.

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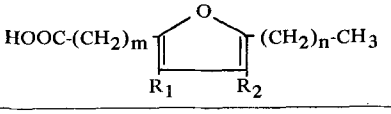
INTRODUCTION

A new series of fatty acids recently has been discovered by Glass and coworkers (1) as constituents of lipids from northern pike (*Esox lucius*) and other fish species. They are found primarily in the liver and testes lipids, and wide seasonal fluctuations of their amounts have been observed. For example, at spawning time of northern pike in early spring, the new acids are <5% of all acyl moieties in liver lipids, but they may reach levels of 65% in testes lipids. However, in late fall, they have been found in amounts up to 25% of the acids in the total liver lipids, where they may represent >90% of the acids bound in cholesteryl esters.

Altogether, eight memebers of the new series of acids have been detected so far. Their similarities in thin layer chromatography (TLC), gas liquid chromatography (GLC), and mass spectrometry (MS) indicated close structural relationships (1). It now has been established that they have a furan ring in common and it is expedient to use the term furanoid fatty acids for them, abbreviated F_1 to F_8. Abbreviations previously used (1), P or PMFA for pike or pike male fatty acids, respectively, are too narrow, as these acids have since been detected in many other fish and, at low levels, in some female specimens (Glass, et al., unpublished data). A

TABLE I

Structures of Furanoid Acids from Fish



Compound[a]	Empirical formula	m	n	R_1	R_2
F_1	$C_{18}H_{30}O_3$	8	2	CH_3	CH_3
F_2	$C_{19}H_{32}O_3$	8	4	CH_3	H
F_3	$C_{20}H_{34}O_3$	8	4	CH_3	CH_3
F_4	$C_{20}H_{34}O_3$	10	2	CH_3	CH_3
F_5	$C_{21}H_{36}O_3$	10	4	CH_3	H
F_6	$C_{22}H_{38}O_3$	10	4	CH_3	CH_3
F_7[b]	$C_{23}H_{40}O_3$	12	4	CH_3	H
F_8	$C_{24}H_{42}O_3$	12	4	CH_3	CH_3
Reference[c]	$C_{18}H_{30}O_3$	7	5	H	H

[a]The esters are numbered in the sequence of gas liquid chromatography retention times.

[b]$R_1 = CH_3$ is suggested for F_7 by inference from F_2 and F_5.

[c]9,12-Epoxyoctadeca-9,11-dienoic acid, semisynthetic from ricinoleic acid (3).

furanoid fatty acid also has been described from *Exocarpus cupressiformis* seed oil (2).

The furanoid acids, F_1 to F_8, represent a homologous series, with F_3 and F_4 being isomers. F_6 was the predominant furanoid acid in all materials and was used in most of the structure investigations. The formula of this acid, $C_{22}H_{38}O_3$, had been verified by high resolution MS, and elemental analysis was in agreement (1). According to the results, the new fatty acids from fish lipids contain a furan ring with 3 or 4 substituents. Specific structures as listed in Table I are proposed for F_1 to F_8. For example, furanoid fish acid F_6 is 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic acid.

EXPERIMENTAL PROCEDURES

Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out on layers of Silica Gel H (E.C. Merck, Darmstadt, Germany) with hexane:diethyl ether:acetic acid (85:15:1) as developing solvent. In argentation chromatography, the adsorbent contained 5% $AgNO_3$. The plates were activated at 110 C for 16 hr.

The mixture of F esters, as well as pure F_6 and pure F_5 esters, gave, with 50% H_2SO_4 + $K_2C_2O_7$, a red color on the chromatograms which appeared sooner than the red color of cholesterol or its esters. The semisynthetic reference compound, 9,12-epoxyoctadeca-9,11-dienoate (3), gave a yellow-orange stain prior to charring.

Gas Liquid Chromatography

Gas liquid chromatography conditions were (a) for analysis of fatty acid methyl esters and fatty alcohol acetates or trimethylsilyl ethers: 10% Silar 10C on Gas Chrom Q, 100/120 mesh (Applied Science Labs, Inc., State College, PA), in an aluminum column, 3.2 mm internal diameter (ID) and 240 cm long, at 190 C; (b) for preparative separation of F methyl esters: 6% SE-30 (Fisher Scientific Co., Chicago, IL) on Chromosorb W 60/80 mesh (Johns Manville, Celite Division, New York, NY), in a column, 7.9 mm ID and 180 cm long, at 212 C; (c) for analysis of short chain monoesters: as for fatty acid methyl esters, but at 105 C; (d) for analysis of diesters: 15% HI-EFF-2BP (ethylene glycol succinate) on Gas Chrom P, 100/120 mesh (Applied Science Labs) in a column, 3.2 mm ID and 180 cm long, at 180 C.

Furanoid Fish Acid Methyl Esters

According to previously described procedures (1), lipids were extracted from tissues in a Waring Blendor by chloroform:methanol (2:1) and recovered as usual. Alkaline methanolysis yielded the methyl esters which were subjected to hydrogenation in chloroform for 20 min by H_2 at atmospheric pressure with PtO_2 catalyst. Under these conditions, only the common fatty acid methyl esters were hydrogenated, and they were removed by crystallization as urea complexes (1). GLC and MS showed that F methyl esters were not changed by these procedures. Unsaturated straight chain fatty esters also can be removed by argentation-TLC, where the F esters migrate closely behind saturated fatty esters which are in the mixtures.

Individual F esters were obtained by GLC after the above purification or enrichment. Minor amounts of saturated straight chain esters did not interfere with GLC of the F esters. However, F_3 and F_4 methyl esters have similar retention times so that their preparative separation was not efficient.

A reference compound, methyl 9,12-epoxyoctadeca-9,11-dienoate, was prepared from ricinoleic acid according to published procedures (3).

Hydrogenations

Hydrogenation of F_6 methyl ester with 10% Pd on charcoal (ICN-K&K Laboratories, Inc., Plainview, NY) in chloroform yielded a product which had, in TLC, an approximate R_f value of 0.6. However, GLC indicated that this was a mixture of at least two compounds, I and II, in about equal amounts. They emerged as well separated peaks with equivalent chain length (ECL) values (4) 24.2 (I) and 25.7 (II) on Silar 10C, while the F_6 ester had ECL 24.8. MS indicated the uptake of 4 H for each fraction, and their fragmentation patterns were not distinguishable. Therefore, tetrahydro F_6-I and II must be stereoisomers, but each of them may still represent a mixture of stereoisomers. When hydrogenating F_6 methyl ester with PtO_2 (Engelhardt Industries, Inc., Chemical Division, Newark, NJ) in acetic acid, the ratio of I and II was greatly changed in favor of the latter, so that tetrahydro F_6-I represented $<5\%$ of the hydrogenated product.

Hydrogenation of F_5 ester and of the lower homologs with Pd on charcoal in chloroform yielded a mixture that was analogous in TLC, GLC, and MS to the mixture of isomers obtained from F_6 ester. However, hydrogenation of F_5 ester with PtO_2 in acetic acid yielded a more complex mixture which could be fractionated by TLC. A rapidly migrating fraction, R_f 0.6, consisted according to GLC and MS of stereoisomers as had been encountered in hydrogenation of F_6. A slow migrating fraction, R_f 0.3, represented about half of the

product from hydrogenation of F_5. This R_f was very close to that of 12-hydroxystearate.

Hydrogenation of the reference compound, 9,12-epoxyoctadeca-9,11-dienoate, with the Pd catalyst under nonacidic conditions, led to the product of R_f 0.6, which, according to GLC, consisted of isomers, ECL 22.1 and 22.5. Hydrogenation of the compounds with PtO_2 in glacial acetic acid yielded mainly the slow migrating TLC fraction, R_f 0.3, but some tetrahydrofuranoid ester, R_f 0.6, also was formed.

The saturated compound, 9,12-epoxyoctadecanoic acid, also was prepared directly from 9,12-dihydroxystearic acid by cyclization (3) and its methyl ester had the expected R_f 0.6. GLC indicated that the product was a mixture of esters similar to that obtained from 9,12-epoxyoctadeca-9,11-dienoate by hydrogenation with Pd. Mass spectra of the tetrahydro compounds obtained by the different routes were identical.

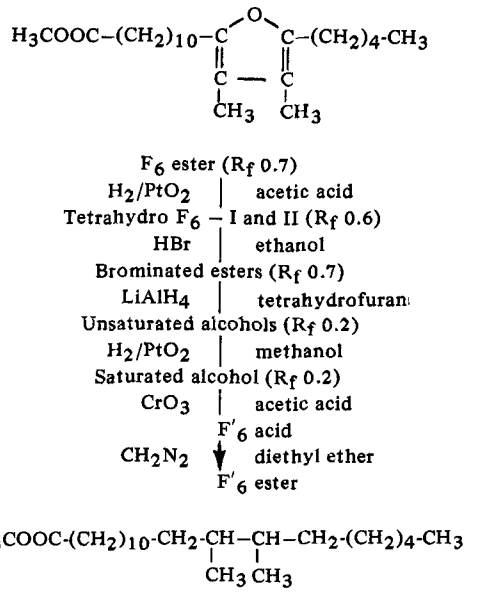
Oxidative Degradations

Methyl esters of F_4, F_5, F_6, and the reference compound were oxidized by O_3-H_2O_2 as described for olefinic straight chain fatty esters (5), but they were exposed to ozone twice as long as necessary for reaction with common olefinic fatty esters. The products were recovered by cautious removal of H_2O_2 and solvents, and then were esterified with diazomethane or diazobutane (6) before analysis by GLC and MS.

The same esters, as well as tetrahydro F_6-I plus II and 9,12-epoxyoctadecanoate were oxidized by CrO_3 in glacial acetic acid (7) for 16 hr at room temperature. Excess oxidant was destroyed by $NaHSO_3$, and the resulting acids were recovered and esterified for analysis as in the foregoing.

Conversion of Tetrahydro F to Alkanoic (F′) Acids

Ca. 50 mg of tetrahydro F_6 methyl ester consisting mainly of isomer II or 50 mg of the corresponding mixed tetrahydro F_{1-6} esters, R_f 0.6, was dissolved in 3 ml absolute ethanol and treated with a stream of purified HBr at 0 C by bubbling the gas through the solution (8). After at least 6 hr under saturation conditions at that temperature, the material was poured on crushed ice, and the lipid was extracted. TLC revealed two major spots, R_f 0.6 consisting of original esters, and R_f 0.7 which is similar to that of straight chain fatty esters. Unreacted esters (ca. 35%) were recovered and treated again with HBr to increase the amount of product. Fractions having R_f 0.7 were isolated, and elemental analysis of samples originating from both tetrahydro F_6 and from mixed tetrahydro F esters showed a content of ca. 1.7 Br/molecule. GLC of the residual tetrahydro F_6 esters indicated that the relative amount of isomer II had decreased considerably.



SCHEME I. Reactions leading from methyl 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoate (F_6) to 13,14-dimethyleicosanoate (F'_6).

Treatment of the brominated fractions with excess $LiAlH_4$ in refluxing tetrahydrofuran for 4 hr (9) yielded mono- and dialcohols, according to R_f's in TLC. The monoalcohol fractions were purified by TLC and did not contain any bromine. MS of aliquots as acetates and trimethylsilyl (TMS) ethers showed that they were unsaturated. Hydrogenation with PtO_2 in methanol yielded the saturated fatty alcohols.

Oxidation of 10-15 mg alcohols with 16 mg CrO_3 in 2 ml acetic acid for 7.5 min at room temperature yielded fatty acids, F'_6 and F'_{1-6}, which were recovered and esterified with CH_2N_2 (10) for purification by TLC. GLC showed for F'_6 methyl ester ECL 20.8. The range of ECLs of the mixture F'_{1-6} was 17.1 to 20.8.

The sequence of reactions and intermediates which led for F to F′ esters is summarized in Scheme I with F_6 as example.

Spectrometry

MS was carried out using an LKB-9000 spectrometer, when necessary in conjunction with GLC (1).

NMR spectra were recorded with a Varian CFT-20 instrument (Varian, Inc., Palo Alto, CA) equipped for proton observance at 80

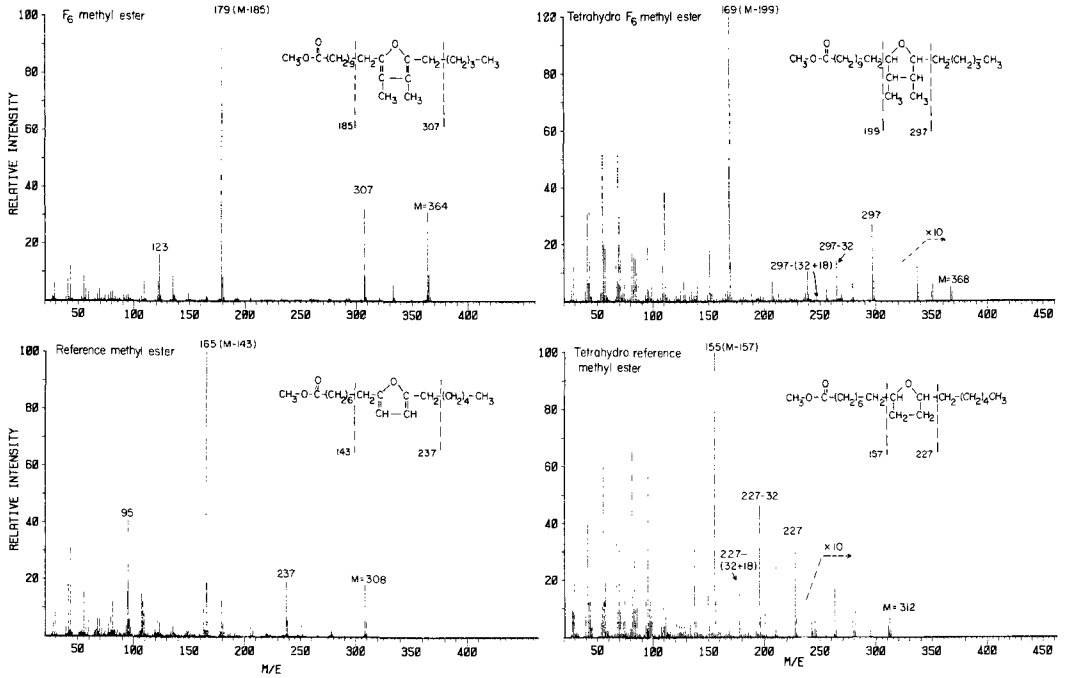


FIG. 1. Mass spectra of methyl 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoate (F_6); its product of hydrogenation, 12,15-epoxy-13,14-dimethyleicosanoate (tetrahydro F_6 isomer II); and authentic reference esters, 9,12-epoxyoctadeca-9,11-dienoate and 9,12-epoxyoctadecanoate.

MHz. Spectra were obtained on 5-10 mg samples dissolved in $CDCl_3$ at ambient temperature. Signals are reported in reference to TMS. Varian CFT-20 and XLFT-100 instruments were used for recording natural abundance ^{13}C NMR spectra at 20 and 25.2 MHz, respectively, using $CDCl_3$ solutions.

IR spectra were measured with a Perkin Elmer instrument Model 21 (Perkin Elmer Corp., Norwalk, CT) equipped with a NaCl prism. Samples were in the form of liquid film between NaCl plates.

UV spectra were taken with a Beckman DK-2 ratio recording spectrophotometer (Beckman Instrument Co., Fullerton, CA) using purified hexane as solvent.

Raman spectra were obtained with a Laser Raman Spectrophotometer (Japan Electron Optics Laboratory, Tokyo, Japan, JRS-SL) using the 4880 Å exciting line from an argon ion laser. Samples were placed neat in a sealed capillary tube.

Optical rotation was not detectable with a mixture of F or with pure F_6 ester.

RESULTS

Degradation of F_6 and of F_5 methyl esters by O_3-H_2O_2 and subsequent procedures yielded dodecanedioate and hexanoate. Accordingly, these compounds have a C_{11} chain with a terminal carboxyl group, a C_5 chain with a terminal methyl group, and double bonds between C-12 and C-13 and between C-ω6 and C-ω7.

Degradation of F_6, F_5, and F_4 methyl esters with CrO_3 and subsequent procedures yielded from each, dodecanedioate plus undecanedioate in ca. equal amounts. F_6 and F_5 also yielded hexanoate plus pentanoate. The anticipated smaller fragments from F_4 were not identified. Apparently, oxidation proceeds not only at C-12, but also at C-11, and the same applies to C-ω6 and C-ω5.

A mixture of tetrahydro F_6-I plus II methyl esters yielded with CrO_3 the same homologous pairs of fragments in relative amounts similar to those obtained from nonhydrogenated F_6. The identical oxidation products from F_6 and tetrahydro F_6 indicate that, besides a double bond, an additional function is located on C-12 and on C-ω6 which leads to oxidation and partial loss of these C atoms.

The reference compound was oxidized by the same methods and yielded with O_3-H_2O_2, nonanedioate and heptanoate, whereas, with CrO_3, nonanedioate plus octanedioate and heptanoate plus hexanoate were obtained. The

TABLE II

Characteristic Ions in Mass Spectra of F[a] and Tetrahydro F Methyl Esters[b]

Compound	Molecular ion	M-(alkyl)	M(alkyl ester)[c]	Furan fragment
F_1	308(15.5)	279(15.8)	151	123(10.7)
F_2	322(17.9)	265 (7.4)	165	109(14.6)
F_3	336(23.0)	279(34.5)	179	123(18.3)
F_4	336(19.2)	307(22.7)	151	123(12.4)
F_5	350(21.8)	293 (8.2)	165	109(14.1)
F_6	364(25.0)	307(31.8)	179	123(16.2)
F_7	378(23.8)	321 (8.1)	165	109(18.0)
F_8	392(28.2)	335(27.7)	179	123(20.7)
Reference[d]	308(23.0)	237(20.3)	165	95(46.0)
Tetrahydro F_5-II	354 (0.3)	283(17.4)	155	
Tetrahydro F_6-I	368 (0.3)	297(26.8)	169	
Tetrahydro F_6-II	368 (0.4)	297(23.3)	169	
Tetrahydro-reference I + II[e]	312 (0.1)	227(40.5)	155	

[a]For structures, see Table I.

[b]Values in parentheses are intensities as percentages of base peaks.

[c]Base peak.

[d]9,12-Epoxyoctadeca-9,11-dienoate.

[e]9,12-Epoxyoctadecanoate.

results are in agreement with the foregoing interpretations.

The oxidations of F_6 and F_5 accounted for 16 C atoms as members of two aliphatic straight chains, including a carboxyl group. Specific reagents for carbonyl groups failed to identify the third O atom, and spectrometric methods did not indicate the presence of a free hydroxyl group. However, similarities in the mass spectra of F, tetrahydro F, and the respective reference esters made likely the presence of a furan ring, which would account for this oxygen. Pertinent spectra are shown in Figure 1.

The oxygen in the unsaturated ring exerts a stabilizing effect which leads to a pronounced molecular ion peak and virtually eliminates peaks characteristic of straight chain methyl esters. Allylic cleavage of the alkylcarboxyl chain leads to the base peaks, m/e 165 (M-143) of 9,12-epoxyoctadeca-9,11-dienoate and 179 (M-185) of F_6 ester. Allylic cleavage of the alkyl chain gives m/e 237 (M-71) from the reference and 307 (M-57) from F_6 ester. A metastable peak, 258.9 of F_6, confirms the interpretation.

The reference compound shows a peak at m/e 95 which also has been reported for the identical methyl ester from *Exocarpus cupressiformis* (2). The fragment originates from the furan ring by cleavage of both allylic positions with H rearrangement and equivalent peaks for F_5 and F_6 esters are found at m/e 109 and 123. The increments of 14 units suggest for F_5 one methyl and for F_6 two methyl or one ethyl substituent of the ring.

Upon hydrogenation, cleavage next to the ring becomes prominent. The reference ester gives the base peak m/e 155 (M-157) by elimination of the alkylcarboxyl chain and the peak 227 (M-85) by elimination of the alkyl chain. The respective peaks from tetrahydro F_6 ester are m/e 169 (M-199) and 297 (M-71).

These and corresponding MS data with intensities are listed in Table II for all F compounds. The results are in accord with those from chemical degradations.

The alkanoate F'_6 was obtained from tetrahydro F_6 by treatment with HBr and subsequent reactions to confirm the foregoing deductions and to characterize the substituents. The molecular ion m/e 354 of F'_6 methyl ester shows that all C atoms have been retained. The fragmentation is typical for methyl branched fatty esters. Peaks at 213, 214, 241, 242, and 269, and barely detectable peaks at 227 and 255, indicate a chain with methyl branches at C-13 and C-14. This structure is confirmed also by the characteristic ions at 209 (241-32), 191 (241-[32+18]), 237 (269-32), and 219 (269-[32+18]) (11). The spectrum of F'_6 pyrrolidide (12) shows large peaks at 252, 280, and 308 with very small peaks at 266 and 294, which again indicates methyl branches at C-13 and C-14.

To obtain such information for other F esters, a mixture of F_{1-6} was converted into F'_{1-6} esters, which then were subjected to GLC-MS. As expected, the peak for F'_6 of this mixture gave a mass spectrum identical to that

of F'_6 derived from pure F_6 ester. The fragmentation patterns of F'_1, F'_3, and F'_4 are very similar to that of F'_6. It can be concluded that F'_1 and F'_3 have two methyl groups, one located on C-11 and one on C-12; F'_4 has methyl groups on C-13 and 14. In the spectrum of F'_5, only the peak m/e 227 is missing, while large peaks appear at 241, 209 (241-32), and 191 (241-[32+18]). This indicates one methyl group at C-13. The spectrum of F'_2 conforms with the pattern exhibited by F'_5, but the methyl group is at C-11. The methyl substituents are placed accordingly at the furan ring of the structures listed in Table I. Further confirmation of the substituted furan structures is found in other spectral data.

The proton NMR spectrum of methyl 9,12-epoxyoctadeca-9,11-dienoate shows a single peak at 5.8 ppm which can be assigned to the olefinic protons in ring positions 3 and 4. This signal appears also in F_5 ester at 5.7 ppm, but is not found in F_6 ester, where the ring is tetrasubstituted. The protons of the methyl groups on the unsaturated ring in F_5 and F_6 cause a singlet at 1.8 ppm. After hydrogenation with Pd, the methyl signals overlap with those of the terminal methyl group in the 0.8 ppm region. Protons next to an ether bond are revealed in 9,12-epoxyoctadecanoate and tetrahydro F_6-I plus II esters by signals in the region of 3.8 ppm which are close to and partially obscured by the methoxy signal at 3.7 ppm. Reference and unsaturated F_6 esters do not show absorption in the 3.8 ppm region. The methylene protons next to the furan ring cause a triplet at 2.6 ppm in the spectrum of the reference ester and at 2.5 for F_5 and F_6. All compounds discussed in the foregoing also show the triplet for the methylene next to the ester group at 2.3 ppm.

Chemical shifts in the proton-decoupled ^{13}C NMR spectra also agree with the structures proposed. The pertinent features are a signal at δ(ppm) 154.4 for ring C-2 and C-5, and at 104.6 for ring C-3 and C-4 for the reference methyl ester; corresponding to them are δ148.7 and 114.8 in F_6 methyl ester. F_6 ester also shows a signal at δ8.5 for the methyl substituents at ring C-3 and C-4. Other shifts are as expected for fatty acid methyl esters and are not listed here.

The IR spectrum of 9,12-epoxyoctadeca-9,11-dienoate shows strong sharp peaks at 770 cm^{-1} (out-of-plane deformation vibrations of the ring [13]) and 1010 cm^{-1} (ring breathing [13]). They are found with 2,5-dimethylfuran (14) and several other 2,5-dialkylfurans (15). The trisubstituted ring of F_5 shows weaker peaks at 785 cm^{-1} and 1025 cm^{-1}, but such peaks are absent from the spectrum of the tetrasubstituted ring of F_6.

Symmetrical and asymmetrical C=C stretching vibrations appear at 1565 and 1610 cm^{-1} for the model compound. These frequencies are raised to 1570 and 1630 for F_5 and to 1590 and 1645 cm^{-1} for F_6, suggesting increased double bond strength with additional substitution.

The frequency of the C-O stretching vibration of ethers normally is near 1100 cm^{-1}, and it appears as a strong peak in the hydrogenated reference and F esters. Adjacent double bonds shift this peak near 1250 cm^{-1}, where it is in the range of the methoxy absorption. The peak is recognized without interference when the ester group of F_6 is reduced to an alcohol by $LiAlH_4$, and to a methyl group by further reduction with $LiAlH_4$ of the derived methylsulfonate (16,17) (not described in the Experimental Procedures section). Other parts of the molecule are not changed by these reactions.

The UV spectrum of F_6 has a maximum at 227 nm, molar absorptivity 7400. The reference compound shows the absorption maximum at 222 nm, molar absorptivity 8990. Several substituted furans have been reported to absorb between 222 and 227 nm with molar absorptivities between 6000 and 8000 (18,19).

The Raman spectra of the reference, F_5 and F_6 esters, show absorption at 1752, 1575, and 1598 for double bonds. Absorption at 3120 nm indicates olefinic protons in the reference and F_5 esters, but is missing in F_6 ester. These bands are not observed with hydrogenated materials.

DISCUSSION

The furan ring is the characteristic feature of the newly identified fatty acids (Table I). The double bonds of the ring are distinct from olefinic bonds by their aromatic character. They are more resistant to hydrogenation, and selective saturation of olefinic bonds is possible in alkenylfurans (20-24). This difference can be used in separating the furanoid from the accompanying straight chain olefinic fatty esters by hydrogenating the latter for subsequent separation in the form of urea complexes (1). Similarly, the aromatic character greatly diminishes the interaction of the double bonds with silver ions so that furanoid fatty esters can be separated by argentation chromatography from the common unsaturated fatty esters. They then are to be fractionated only from the saturated esters originally present.

The reactivity of the furan ring is modified by the number of alkyl substituents. Differ-

ences become apparent when comparing reactions of methyl 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoate (F_6), of 12,15-epoxy-13-methyleicosa-12,14-dienoate (F_5), and of 9,12-epoxyoctadeca-9,11-dienoate which served as reference compound.

All furanoid fatty acid methyl esters can be hydrogenated in chloroform with Pd on charcoal to tetrahydrofuran compounds. When hydrogenating with PtO_2 in acetic acid, F_6 yields the tetrahydro F_6 esters, but F_5 yields tetrahydro F_5 together with ca. equal amounts of 12(15)-hydroxy-13-methyleicosanoate formed by hydrogenolysis of the ring. The reference ester yields predominantly the product of hydrogenolysis, 9(12)-hydroxystearate. Hydrogenolysis of the furan ring is well known as a reaction which may be concurrent with, even predominant over, hydrogenation (20-22,25). In our examples, hydrogenolysis becomes the major reaction as the number of alkyl substituents at the ring decreases from 4 to 3 and to 2. Similar observations were made in attempts to open the ring by hydrolysis.

Opening the ring was important in the sequence of reactions which led to identification and location of the substituents (Scheme I). 9,12-Epoxyoctadeca-9,11-dienoate as well as 9,12-epoxyoctadecanoate were hydrolyzed readily by concentrated HCl in dioxan and further reactions led from both starting materials to methyl stearate with overall yields of 25-60% (not described in the Experimental Procedures section). However, the same conditions failed to hydrolyze F_6 and $F_{1\text{-}6}$ esters to any appreciable extent.

Hydrogen bromide is a more effective reagent for opening the tetrahydrofuran ring (26) and gave products from tetrahydro F esters in yields which made further reactions feasible. The reaction rate of HBr with tetrahydro F_6 esters seems to be greater with isomer II than I. Mass spectra of these compounds do not reveal any structural differences. Therefore, the different reactivities cannot be explained by isomer I being a tetrahydropyran derivative, which would be more difficult to hydrolyze (27), but must be due to steric factors inherent to the substituted tetrahydrofuran ring. The product from tetrahydro F_6 and HBr seems to be a mixture of mono- and dibromo compounds. However, all bromine is eliminated in the alcohols obtained by reduction with $LiAlH_4$.

The alcohols derived from F_6 contain, according to molecular ions in MS, one and two double bonds. Unsaturation may have been introduced by HBr when converting the tetrahydrofuran ring into a monobromo compound (28), but examples for dehydrohalogenation by metal hydrides, including $LiAlH_4$, also are known (29,30). Hydrogenation of the alcohols is achieved easily and provides the saturated alcohol which is prerequisite for oxidation to the fatty acid F'_6 and subsequent mass spectrometry as methyl ester or pyrrolidide.

The series of fish furanoid acids which we are describing here probably will be further supplemented. For example, higher homologs, F_9 and F_{10}, also a F_6 methyl ester with one additional double bond, are detectable in some materials by GLC-MS. When encountered, the amounts of such components are minimal.

9,12-Epoxyoctadeca-9,11-dienoic acid from *E. cupressiformis* seed oil is the only natural furanoid fatty acid so far reported (2). It does not have methyl substituents and has the double bond closest to the terminal methyl group in $\omega 7$ position, while the furanoid fish acids have methyl substituents and $\omega 6$ or $\omega 4$ unsaturated structures. Disregarding their cyclic structure, the furanoid acids have a divinyl ether group. Two acids of such noncyclic structure have been described as products from linoleic and linolenic acids by action of *Solanum tuberosum* (potato tuber) lipoxygenase and sequential reactions (31,32). Methylated divinyl ether acids have not been reported from natural materials.

Substitution of a 5-membered ring by an alkyl and an alkylcarboxyl chain is reminiscent of the structure typical for prostaglandins. A physiological role of the furanoid fish acids may be indicated by their preferential esterification to cholesterol and by the fluctuations in amounts which reach a maximum at the time of reproduction and are at a minimum after that period. It is of interest in this connection that physiological activity resembling that of some prostaglandins has been claimed for several synthetic 2-furan- and 2-tetrahydrofuranoctanoates having a variety of aliphatic substituents in position 5 of the ring (33,34).

Similarly, the biosynthesis and catabolism of the furanoid fish acids presently are a matter of speculation. In regard to the biosynthesis, it seems significant that their unsaturation has $\omega 6$ or $\omega 4$ structure. This may be due to precursors of linoleic-$\omega 6$ or 9,12-16:2$\omega 4$ type. However, cyclization of 1,4-dioxo compounds to furans is very common (35,36) and the ω double bond of a F acid is not necessarily the same as in an unsaturated precursor fatty acid. The occurrence of F acids substituted with one and with two methyl groups, but otherwise of equal or very similar structure, suggests separate methylation steps in the course of their biosynthesis. However, a nonmethylated F acid, which one

might expect as an intermediate, has not been found yet in fish lipids. Specific search for such and radioactive syntheses prerequisite for study of the metabolism and physiological role of F acids are under way.

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Unsaturated C_{18} α-Hydroxy Acids in *Salvia nilotica*[1]

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ABSTRACT

The oil of *Salvia nilotica* Jacq. (Labiatae) seed contains 0.6% α-hydroxyoleic, 4.2% α-hydroxylinoleic, and 5.4% α-hydroxylinolenic acids. The first two have not been found previously in seed oils. In addition to the common fatty acids, also identified were small amounts of three unsaturated C_{17} acids and one branched chain C_{17} acid. Methyl esters of the component fatty acids were fractionated by both column and thin-layer chromatography. These esters were identified by combination of gas chromatography, GC-mass spectrometry, ozonolysis-GC, infrared, and nuclear magnetic resonance.

INTRODUCTION

Many oxygenated fatty acids have been reported as constituents of seed oil from a variety of plants. However, only a few acids with a hydroxyl group alpha to the carboxyl function have been reported as naturally occurring constituents in seed oils (1,2).

When the methyl esters derived from *Salvia nilotica* Jacq. (Labiatae) were investigated, components that had structures similar to the α-hydroxylinolenic in *Thymus vulgaris* were found (1). In this paper we report the isolation and proof of structure for two new α-hydroxy acids, α-hydroxyoleic, and α-hydroxylinoleic, in addition to α-hydroxylinolenic acid.

EXPERIMENTAL PROCEDURES

Methyl esters were prepared with 10% boron trifluoride in methanol by the procedure described by Metcalfe, et al., (3). The esters were analyzed by gas liquid chromatography (GLC) and thin layer chromatography (TLC) (4). The nonoxygenated esters were separated from the oxygenated esters on a 25-mm internal diameter (ID) column packed with a slurry of 75 g Hi-Flosil support 60/200 mesh in 250 ml of 20% ether in benzene. Before sample introduction, 200 ml of 2% ether in benzene was passed through the column. Nonoxygenated esters were eluted from the column with benzene; the oxygenated esters, with diethyl ether (5).

The esters were fractionated according to degree of unsaturation by preparative TLC on 10 X 36 cm plates coated with Silica Gel G containing 20% silver nitrate (6). Benzene was used as the developing solvent for the nonoxygenated esters and a 50:50 mixture of benzene and ether for the oxygenated esters. After development, all plates were sprayed with an alcoholic solution of dichlorofluorescein so that the bands could be observed under ultraviolet (UV) light. Each band, fractions I-IV [Table I] of the nonoxygenated esters and fractions A, B, and C of the oxygenated esters, was scraped from the plates and the esters were recovered with ether. The esters in fraction IV were fractionated according to chain length by preparative GLC on a 6 ft X 1/4 in. 5% LAC-2-R446 column.

Infrared (IR) absorption was measured from carbon disulfide solutions in 1-mm NaCl cells on a Perkin-Elmer Model 137 spectrophotometer. Optical rotatory dispersion (ORD) curves were recorded from a solution of the ester in either methanol or chloroform, or both, with a Cary Model 60 spectropolarimeter. Nuclear magnetic resonance (NMR) spectra were obtained from deuteriochloroform solutions of the samples containing 1% tetramethylsilane, as internal standard, with a Varian HA-100 spectrometer. Ozonolysis-GC (7) was used to locate the sites of unsaturation in the nonoxygenated esters. The olefinic bonds in the oxygenated esters were determined by GC-mass spectrometry of methoxy derivatives (8). On these derivatives the original hydroxyl group, which did not enter into the methoxylation reaction, was silylated with *bis*(trimethylsilyl)-trifluoroacetamide in acetonitrile and introduced into a Dupont Model 21-492-1 mass spectrometer by a GC inlet system from a Packard 7401 gas chromatograph equipped with a 6-ft glass column packed with Dexsil 300 GC 9(0. The temperature of the column and of the source of the mass spectrometer was held at 200 C. The equivalent chain lengths (ECLs) of methyl esters and ozonolysis products were determined by GLC (6,10) on a Packard Model 7401. Tentative identification of these components in the fractions were based on their ECLs from two columns having dissimilar liquid phases (10).

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TABLE I

Composition of Mixed Esters and Isolated Fractions from *Salvia nilotica*

Component	Mixed esters	Fraction I	II	III	IV	A	B	C
		(% by gas liquid chromatography)						
12:0	Tr[f]	0.1	0.1	---	---	---	0.2	---
13:0	Tr	0.2	Tr	---	---	---	0.2	---
14:0	Tr	0.2	Tr	---	---	---	0.1	---
15:0	Tr	0.2	---	---	---	---	---	---
15:1	---	---	Tr	---	---	---	---	---
16:0	8.2	61.3	2.0	---	---	1.0	0.2	---
16:1	0.1	---	0.5	---	---	---	---	---
17:0	0.1	0.8	---	---	---	1.2	---	---
17:0[a]	0.1	1.1	---	---	---	1.0	---	---
17:1	0.1	0.3	0.4	---	---	0.5	---	---
17:2	---	---	---	0.8	---	0.3	---	---
17:3	0.4	---	---	---	2.3	---	---	---
18:0	4.5	33.9	1.0	0.1	---	0.4	0.2	---
18:1	10.1	0.7	85.6	---	---	0.3	0.1	---
18:2	35.8	---	2.1	98.8	---	0.2	0.1	---
18:3	28.7	---	---	---	95.7	1.3	0.3	---
19:1	---	---	0.7	---	---	---	---	---
20:0	1.0	1.6	---	---	---	0.2	0.8	---
20:1	---	0.2	1.6	---	---	0.2	0.5	---
20:2	---	---	---	0.2	---	0.4	---	---
22:0	---	0.3	---	---	---	---	---	---
Unknown equivalent chain lengths								
R-446	APL							
12.4	---	0.3	---	---	---	---	0.2	0.1
22.3	18.7[b]	0.6	0.2	1.6	---	---	74.7	1.0	---
23.8	18.7[c]	4.3	0.3	2.4	Tr	Tr	14.2	96.0	2.6
24.3	18.7[d]	5.6	0.5	2.5	Tr	Tr	0.4	Tr	95.2
---	20.2	---	---	---	---	---	1.0	---	1.3
---	20.5	---	---	---	---	---	2.3	---	0.6

[a]A branch chain saturate.
[b]α-Hydroxyoleic acid.
[c]α-Hydroxylinoleic acid.
[d]α-Hydroxylinolenic acid.
[e]APL = Apiezon L.
[f]Tr = trace.

RESULTS AND DISCUSSION

GLC analysis of the mixed methyl esters derived from the seed oil of *Salvia nilotica* revealed the presence of at least 4 unusual components. Two of these had ECL values compatible with α-hydroxylinolenic and heptadecatrienoic acids reported by Smith, et al., (1). TLC analysis on Silica Gel G plates containing boric acid showed a migration pattern consistent with the pattern of nonhydroxy and hydroxy esters. These esters were separated accordingly by column chromatography (5).

IR spectra of the esters exhibited no maximum between 900-1000 cm^{-1}. Therefore, no olefinic bonds were *trans*.

Identification of Nonoxygenated Fatty Esters

On the basis of GLC data, fraction I is composed of 96% normal saturated esters. In addition, 1% of the fraction is a component with ECLs of 16.7 (Apiezon L. column) and 16.6 (LAC-2-R466 column). These ECLs suggest that the component is a C_{17} fatty ester with an iso structure. The mass spectrum from this component shows a parent ion (M) at m/e 284, indicative of a saturated C_{17} methyl ester, and other features similar to the spectrum of methyl heptadecanoate, which suggest that the branch chain component probably has a terminal isopropyl group (11).

Fraction II contains 85% C_{18} monoenes in addition to a small amount of a component that has ECLs consistent with those of a C_{17} monoene. Ozonolysis-GLC (7) of fraction II revealed fragments having the retention characteristics of a C_8 aldehyde-ester (8AE), 9AE, 11AE, and C_9 aldehyde (9A) produced from a mixture of 8-17:1, 9-18:1, and 11-20:1, respectively.

ECLs of the major component in fraction III are identical to those of methyl linoleate; the remainder of that fraction was shown to be methyl heptadecadienoate. The major ozonolysis fragments observed from fraction III were 8AE, 9AE, and 6A. These fragments established the parent esters as 8,11-17:2 and 9,12-18:2.

The trienoic esters (fraction IV) exhibited ECLs consistent with those of methyl linolenate and norlinolenate (1). The trienes were separated according to chain length by preparative GC before ozonolysis. Upon ozonolysis, the 17:3 ester yielded 8AE and the 18:3, 9AE. These fragments represent 8,11,14-17:3 and 9,12,15-18:3. The short chain aldehydes and dialdehydes expected as ozonolysis products of the trienes are not observed under the ozonolysis-GLC conditions (7). The sharp triplet at δ 0.96 in the NMR spectra from both the C_{17} and C_{18} triene indicates they contain omega-3 double bonds (12).

Identification of the Oxygenated Fatty Esters

ECLs of the esters in fractions A, B, and C (Table I) indicate that the major component in each is a hydroxy ester, differing only in degree of unsaturation. IR spectrum from each fraction exhibits prominent bands at 3550, 1265, 1210, and 1110 cm^{-1}. These bands are consistent with those of known α-hydroxy esters (1). NMR spectra revealed a signal at δ 4.1 (singlet) attributed to the methine proton and similar to that reported for α-hydroxylinolenic acid (1). In addition, these structures were confirmed by the area of the olefinic signal at δ 5.3-5.4 (multiplet). This area represented two protons or one double bond in fraction A, four protons or two double bonds in fraction B, and six protons or three double bonds in fraction C. Signals at δ 2.3-2.4 representing a methylene group alpha to a carboxyl function were not observed.

Mass spectra of the silylated α-hydroxy esters exhibit ions at M-15 (methyl), M-31 (methoxyl), M-43, and M-59, all of which are expected ions from silylated unsaturated monohydroxy esters (9). Also found is the ion at m/e 161, which results from cleavage between C-2 and C-3 (9). The molecular ions from the individual spectra were observed at m/e 384 for a fraction A, m/e 382 for fraction B, and m/e 380 for fraction C; these ions corroborate NMR results that show the respective esters contain one, two, and three double bonds.

The sites of unsaturation were defined by GC-MS of the silylated methoxy derivatives of these esters (8,9). The data shown in Figure 1 indicate that each of the α-hydroxy acids is in the Δ9 series. The ORD analyses had the following characteristics: α-hydroxylinolenate $[\alpha]^{25}_{589}$ -3.25°, $[\alpha]_{550}$ -3.75°, $[\alpha]_{500}$ -6.07°, $[\alpha]_{450}$ -7.75°, $[\alpha]_{400}$ -12.9° (c. 0.0016 g/ml in methanol), α-hydroxy-linolenate $[\alpha]^{25}_{589}$ -5.89°, $[\alpha]_{550}$ -9.23°, $[\alpha]_{500}$ -12.9°, $[\alpha]_{450}$ -17.0°, $[\alpha]_{400}$ -24.3° (c. 0.0016 g/ml in chloroform, α-hydroxylinoleate $[\alpha]^{25}_{589}$ -6.9°, $[\alpha]_{550}$ -8.4°, $[\alpha]_{500}$ -11.85°, $[\alpha]_{450}$ -15.6°, $[\alpha]_{400}$ -23.0° (c. 0.0016 g/ml in chloroform). The negative rotations observed for these acids are comparable to those reported by Smith, et al., (1) for α-hydroxylinolenic acid. No reliable ORD analysis of α-hydroxyoleic acid was made because the quantity of that component was insufficient.

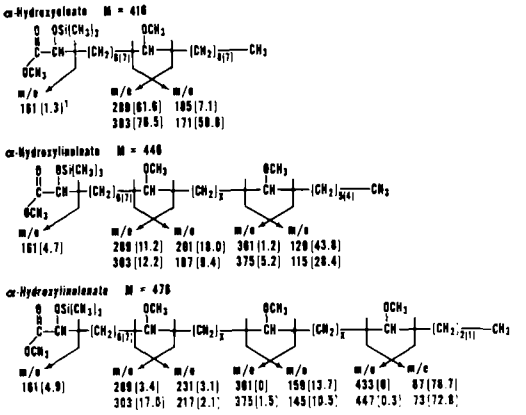

FIG. 1. Fragments from gas chromatography-mass spectrometry of silylated methoxy derivatives of the unsaturated α-hydroxy esters from *Salvia nilotica* esters.

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Distribution of Cholesteryl Esters and Other Lipids in Subcellular Fractions of the Adrenal Gland of the Pig

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ABSTRACT

Total lipids from whole pig adrenal glands as well as from their mitochondria, microsomes, liposomes, and cell sap were extracted and fractionated first into neutral lipids and phospholipids. The highest percentage of neutral lipids was found in the cell sap, and the lowest in the microsomal fraction. Neutral lipids were subfractionated into cholesteryl esters, free cholesterol, triglycerides, and free fatty acids. Cholesteryl esters were distributed throughout the liposomes. Free fatty acids represented a substantial part of cell sap lipids, but were present also in the mitochondria, microsomes, and liposomes. Fatty acids of all fractions were analyzed by gas liquid chromatography. Free fatty acids and cholesteryl ester fatty acids from all cellular fractions were similar in composition and were characterized by considerable quantities of linoleic and arachidonic acid. Triglycerides were characterized by an increased percentage of palmitic and a low content of arachidonic acid. Phosphatidyl choline, phosphatidyl ethanolamine, diphosphatidyl glycerol, and sphingomyelin plus phosphatidyl inositol were isolated from the lipids by preparative thin layer chromatography, and their fatty acids analyzed by gas liquid chromatography. Phosphatidyl choline and phosphatidyl ethanolamine from mitochondria, microsomes, and cell sap were very similar in respect of their fatty acid composition. Sphingomyelin plus phosphatidyl inositol was characterized by a high content of $C_{22:2}\omega 6$. Diphosphatidyl glycerol was present in mitochondria and in the cell sap.

INTRODUCTION

Work published in recent years (1-3) has shown large variations in total lipid and cholesteryl ester content in adrenal glands from various animal species. More recent work (4-6) has shed some light on the distribution of cholesteryl esters in the adrenal cells. It was established that cholesteryl esters were not only present in liposomes (7) but also in the mitochondria, microsomes, and the cell sap. These findings considerably complicated attempts to explain the metabolism of cholesteryl esters in the adrenal gland. Because of the great variability from species to species with respect to cholesteryl esters, it seemed that a comprehensive picture about their role would have to be built from as much information as possible. To satisfy this need, the present work on the pig adrenal gland was carried out.

MATERIALS AND METHODS

Materials and Conditions

These were described in previous papers (4-6).

Animal Material

Adrenal glands were obtained from the local abattoir. Animals were of the Landrace breed, 7-month old baconers of mixed sexes with an average wt of 150 lb. Material was collected on ice and processed within 2 hr from the time the animals were slaughtered. After removal of the superficial fat, the average wt of the glands was 2.2 g. Pooled samples of 10 glands were used for the extraction of the lipids.

Preparation of Subcellular Fractions

Mitochondria and crude microsomes were prepared as described previously (4,5). Liposomes were prepared from the supernatant after separation of the microsomal fraction. The cloudy part of the supernatant was siphoned off and spun in the rotor 40 of a Beckman 12 ultracentrifuge at 100,000 x g for 2 hr. The fatty pellicle was removed mechanically and dissolved in chloroform:methanol (2:1).

Extraction and Fractionation of Lipids

The procedure for extraction and fractionation of lipids was essentially the same as described previously (4-6). Preparative separation of phospholipids was carried out on one-dimensional Adsorbosil 5 thin layer chromatographic (TLC) plates (Applied Science Laboratories, State College, Pa.) using chloroform: methanol:water (65:35:4).

TABLE I

Percent Composition in Various Cell Fractions from the Adrenal Gland of the Pig

Fraction	Cell homogenate %	Mitochondria (%)	Microsomal fraction (%)	Cell sap (%)
Cholesteryl esters	3.9	6.2	3.9	17.9
Cholesterol	8.0	5.2	10.0	8.7
Triglycerides	20.3	3.0	4.7	12.2
Free fatty acids	3.3	5.1	7.6	26.5
Phospholipids	64.5	80.5	73.8	34.7

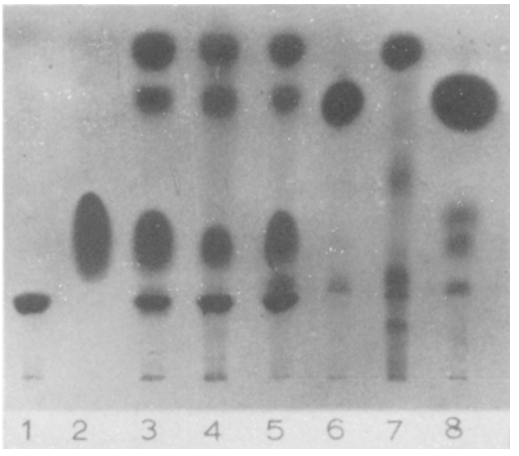

FIG. 1. Neutral lipids from various subcellular fractions of the adrenal gland of the pig on Silica Gel G developed in petroleum ether:ether:acetic acid (70:30:1). Detection of spots: 50% sulfuric acid and charring. 1, Cholesterol; 2, oleic acid; 3, neutral lipids from cell sap; 4, neutral lipids from the mitochondria; 5, neutral lipids from the microsomal fraction; 6, glycerol trioleate; 7, cholesteryl palmitate; 8, methyl oleate.

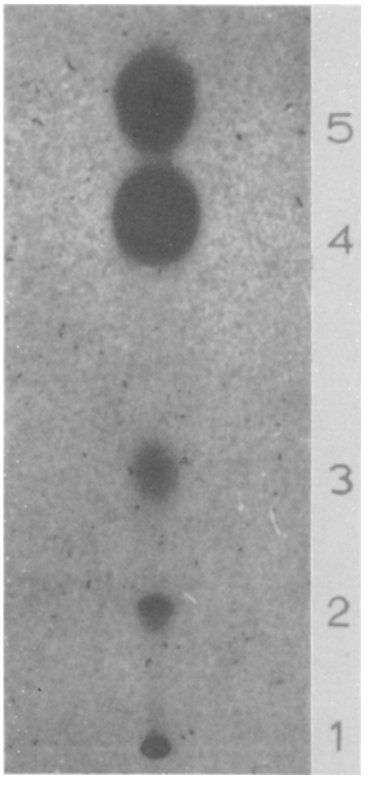

FIG. 2. Total lipids from the liposomes of the pig adrenal gland. Development and detection of spots as in Fig. 1. 1, Phospholipids; 2, cholesterol; 3, free fatty acids; 4, triglycerides; 5, cholesteryl esters.

Gas Chromatography of Lipids

Fatty acids were analyzed as described previously (8). In addition, a 2 m column packed with 2.5% Silar 5 CP (Applied Science Laboratories, State College, Pa.) was used. The column was operated at 180 C and a flow rate of 40 ml N_2/min. Fatty acids were identified by the combined use of retention times of standards relative to methyl stearate and the carbon number values as desceibed by Woodford and van Gent (9).

RESULTS AND DISCUSSION

Gas Chromatography of Fatty Acids

The analysis of fatty acids (FA) from mitochondrial and microsomal cholesteryl esters revealed several fast emerging peaks indicating the presence of FA with less than 12 carbon atoms, but the peaks were too small to be integrated. Accurate identification of eicosadienoic acid from various fractions proved to be rather difficult because of the small difference in the relative retention times between the $\omega 6$ and $\omega 9$ isomers. Docosadienoic acid, which appeared in all fractions, and represented a particularly large percentage in some of the phospholipids, was tentatively identified as $C_{22:2}\omega 6$.

Distribution and Composition of Neutral Lipids

Whole adrenal glands contained 54 mg lipids per gram of fresh tissue, of which 3.3% (1.8

TABLE II

Percent Fatty Acid Composition in Neutral Lipids from Subcellular Fractions of the Adrenal Gland of the Pig

Carbon number	Cholesteryl esters (%)			Free fatty acids (%)			Triglycerides (%)		
	Mitochondria	Microsomes	Cell sap	Mitochondria	Microsomes	Cell sap	Mitochondria	Microsomes	Cell sap
12:0	0.8	0.3	--	--	--	--	0.7	--	--
14:0	10.2	1.6	2.1	4.0	1.7	1.7	2.6	1.7	3.4
14:1	1.3	1.0	--	--	0.5	--	0.7	0.5	0.5
14:2	--	--	1.0	--	--	--	--	--	--
16:0	13.2	11.8	9.3	22.2	16.0	21.4	20.8	16.0	26.3
16:1	1.0	1.4	3.0	1.5	1.7	1.0	1.8	1.7	2.7
16:2	2.2	1.0	0.8	0.8	1.2	0.5	0.7	1.2	--
17:0	--	--	1.0	--	--	--	TR[a]	--	0.5
18:0	8.8	9.6	8.5	22.2	16.0	20.0	17.3	16.0	20.7
18:1	17.6	26.7	33.0	20.0	26.5	26.0	30.5	26.5	31.5
18:2	15.3	15.4	15.3	12.7	14.7	8.5	11.5	14.7	8.7
18:3ω6	2.2	4.4	3.0	2.3	3.0	1.4	2.6	3.0	1.0
20:1ω9	1.6	1.0	1.6	0.8	1.2	0.5	1.8	1.2	1.0
20:2ω9 or 20:2ω6	1.6	3.0	1.6	--	0.5	--	1.8	0.5	--
20:3ω6	2.6	7.0	5.5	3.0	3.6	12.0	2.2	3.6	1.0
20:4ω6	9.0	10.6	9.7	10.5	12.0	15.0	2.8	12.0	2.7
20:5ω3	2.6	1.0	1.3	--	--	0.5	--	--	--
22:0	1.6	--	--	--	--	0.5	--	--	--
22:2ω6	7.4	1.0	1.0	--	--	--	0.7	--	--
22:4ω6	--	2.2	--	--	TR[a]	--	--	TR[a]	--
22:5ω6	--	--	1.3	--	1.2	0.5	--	1.2	--
22:5ω3	--	1.0	--	--	--	--	--	--	--
22:6ω3	1.0	--	1.0	--	--	0.5	--	--	--
Unidentified	--	--	--	--	--	--	1.8	--	--

[a]TR = Traces.

TABLE III

Percent Fatty Acid Composition of the Liposomal Lipids from the Adrenal Gland of the Pig

Carbon number	(%)
$C_{14:0}$	5.3
$C_{14:2}$	0.5
$C_{16:0}$	16.4
$C_{16:1}$	2.7
$C_{16:2}$	0.8
$C_{17:0}$	0.5
$C_{18:0}$	10.6
$C_{18:1}$	20.0
$C_{18:2}$	12.8
$C_{18:3\omega6}$	4.4
$C_{20:1\omega9}$	1.3
$C_{20:2\omega6}$	1.6
$C_{20:3\omega6}$	5.7
$C_{20:4\omega6}$	12.4
$C_{20:5\omega3}$	0.5
$C_{22:2\omega9}$	3.2
$C_{22:4\omega6}$	1.3

mg/g fresh tissue) consisted of cholesteryl esters. This figure was considerably lower than figures given by Eberhagen and Jossiphov (1) for the cortex and medulla. Such variations could be explained by changes in physiological conditions and nutritional status (10).

The distribution of cholesteryl esters and other lipids in the total lipids from various subcellular fractions is given in Table I (Fig. 1). It shows a high concentration of cholesteryl esters in the cell sap and smaller quantities in the mitochondria and microsomal fraction. In terms of percentage of the total glandular lipids, these 3 cellular fractions comprised 94% of the total cholesteryl esters. Most of the unaccounted cholesteryl esters (0.1 mg/g fresh tissue) were found in the liposomal fraction, while a small quantity probably was present in the nuclei (11). The relative percentage figures were misleading if one thinks in terms of absolute quantities of various lipids in different parts of the cell. The highest yield actually was obtained from the microsomal fraction and the lowest one from the cell sap.

The origin and metabolic role of the cholesteryl esters in the adrenal gland is still not completely understood. It has been established earlier, on the basis of in vivo experiments, and in recent years with studies involving cell suspensions, that the metabolism of cholesteryl esters is under control of adrenocorticotropic hormone (ACTH) (10,12), and that they are precursors of the corticosteroids (13-15). The question of why certain animal species, such as the elephant (5), produce enormous quantities of cholesteryl esters in the adrenal gland, while others like the bovine animals (7,16) produce very little or none at all, still remains open. Fruhling, et al., (7) have been trying to correlate the quantity of cholesteryl esters to the intensity of cholesterol biosynthesis. This is apparently dependent upon the volume of the endoplasmic reticulum in the cell and also upon the internal structure of the mitochondria. According to these authors, animal species such as the guinea pig and the rat, whose adrenals can synthesize only part of the cholesterol necessary for the biosynthesis of the corticosteroids, draw the balance of the cholesterol from the plasma (17) and store it in form of esters in the liposomes. Indeed, the described species contained a considerable quantity of cholesteryl esters in their adrenal liposomes.

As it could be seen from our own experiments, cholesteryl esters were not always accumulated in the form of liposomes. The presence of large quantities of cholesteryl esters in the microsomal fraction (40% of the total in the cell) should not be considered as unusual because it has been established that cholesteryl ester synthetase appeared to be localized largely in this fraction (18). This also confirmed the assumption by Brecher, et al., (19) that cholesteryl esters must be stored in different pools of the adrenal cells.

The composition of the liposomes also seemed to be subject to considerable variation, not only from organ to organ (20,21), but in the same gland from species to species. In the elephant (6), they consisted exclusively of cholesteryl esters, while in other species (7), and in the pig as well (Fig. 2), they were a mixture of cholesteryl esters, free cholesterol, triglycerides, free fatty acids, (FFA), and phospholipids. The triglycerides in the liposomes apparently provided the source of fatty acids for the biosynthesis of cholesteryl esters (7). This seemed rather unlikely, because the FFA in the cell sap (6) would represent a far more reactive form. Besides, the composition of FFA showed a much greater similarity in comparison with those from cholesteryl esters than the FA from the triglyceride fraction. In recent experiments with adrenal cell suspensions (19), it was established that free oleic acid was being incorporated into cholesteryl esters and other lipids.

Cholesteryl esters from various cellular elements in the pig adrenal gland were very similar in their FA composition (Table II), except for one unsaturated acid which was only present in substantial quantities in the mitochondria and which was tentatively identified as $C_{22:2\omega6}$. Unfortunately, very few data were available for comparative purposes, because most of the studies on adrenal cholesteryl esters have dealt with whole glands (3,22,23). Although the presence of γ-linolenic, homo-γ-

TABLE IV

Percent Fatty Acid Composition in Phospholipids from Subcellular Fractions of the Adrenal Gland of the Pig

Carbon number	Phosphatidyl choline			Phosphatidyl ethanolamine			Sphingomyelin + phosphatidyl inositol			Diphosphatidyl glycerol	
	Mitochondria	Microsomes	Cell sap	Mitochondria	Microsomes	Cell sap	Mitochondria	Microsomes	Cell sap	Mitochondria	Cell sap
14:0	1.0	1.0	2.2	1.8	3.5	2.4	6.0	3.0	3.3	3.2	7.6
14:1	--	--	--	--	--	--	--	--	--	TR[a]	--
14:2	0.5	0.5	0.4	--	0.5	--	1.4	0.6	0.6	--	TR[a]
16:0	14.8	17.5	18.2	5.6	9.0	9.3	21.0	31.8	28.3	10.8	17.0
16:1	0.5	TR[a]	--	--	--	3.0	--	--	--	0.5	0.7
16:2	1.0	1.0	0.4	0.5	0.5	--	1.4	1.5	0.6	0.5	0.7
17:0	--	--	--	--	--	0.5	--	--	--	TR[a]	TR[a]
18:0	29.7	26.5	25.2	30.8	24.0	25.0	12.6	21.5	22.3	7.6	14.6
18:1	11.0	10.6	14.0	4.6	6.0	8.3	7.0	11.5	10.0	9.8	15.5
18:2	8.9	5.7	12.1	3.7	4.5	4.4	5.4	6.0	5.3	39.6	13.6
18:3ω6	1.5	1.5	3.0	0.5	1.5	0.5	1.4	--	3.3	4.3	--
20:1ω9	1.0	1.0	--	0.5	1.0	0.5	0.7	--	1.2	--	--
20:2ω6 or 20:2ω9	1.0	--	0.4	--	0.5	--	--	--	--	--	0.7
20:3ω6	2.4	2.4	2.2	1.8	2.0	2.0	3.6	3.0	3.3	3.2	3.4
20:4ω6	22.7	29.0	15.6	44.8	41.0	34.3	3.6	2.2	4.6	4.3	4.2
20:5ω3	--	1.0	0.8	1.5	1.5	2.0	--	--	2.6	--	--
22:0	--	--	--	--	--	--	--	--	--	2.2	--
22:2ω6	2.0	1.8	2.6	1.5	2.0	3.0	31.0	7.2	12.6	9.8	14.6
22:3ω6	--	--	--	--	--	--	3.5	--	--	2.7	--
22:4ω6	--	0.5	1.7	1.4	1.5	2.4	1.4	3.0	--	1.0	1.6
22:5ω3	1.0	--	--	0.5	1.0	--	--	--	--	--	--
22:5ω6	--	--	--	--	--	2.4	--	--	2.0	--	--
22:6ω3	--	--	0.8	0.5	--	--	--	--	--	--	--
24:0	1.0	--	--	--	--	--	--	3.0	--	0.5	4.2

[a]TR = Traces.

linolenic, and adrenic ($C_{22:4}\omega6$) acids seemed to be a common feature of the cholesteryl esters in adrenal glands from all investigated species (3,12,24-28), the ratios, particularly the one between homo-γ-linolenic and arachidonic acid, varies within the cell. The low ratio between these 2 acids in the mitochondria indicated a much faster rate of conversion than in the microsomal fraction where the ratio was considerably higher. The same seemed to be the case with the FAA fractions in the mitochondria and microsomes, but not with the triglycerides. In particular, the very low content of arachidonic acid in the triglycerides should be noted.

The docosatetraenoic acid, which represented a large part of the cholesteryl ester and phospholipid fractions of the rat adrenal gland (12) and also was found in the adrenals of the dog (25,26), the guinea pig (28), and the rabbit (3), was represented only in small quantities in the adrenal gland of the pig. It was localized in the cholesteryl esters of the mitochondria and the microsomes and was absent from all other lipid fractions.

The FA composition of the liposomes (Table III) was very similar to the FA composition of the cholesteryl esters from other parts of the cell. Because cholesteryl esters and triglycerides were represented in the liposomes in more or less equal proportions, one might assume that triglycerides would have had the same or similar homo-γ-linolenic arachidonic ratio as cholesteryl esters. This would mean that triglycerides from the liposomes could have a different composition from those in other parts of the cell.

When commenting on lipids of the cell sap, the high percentage of neutral lipids should be pointed out. This seemed to be a regular feature of cell sap lipids (6,19). In spite of this high perentage, the cell sap still held only 30% of the total neutral lipids in the cell, which was caused by the low absolute lipid content in the cell sap. The bulk of neutral lipids was located in the microsomal fraction which contained 50% of all the neutral lipids in the cell. It was rather difficult to speculate on the metabolic significance of the cell sap lipids because they were probably a mixture of substances synthesized in the cytoplasm, like the FA, and lipids in transit, like the cholesteryl ester and phospholipids.

Distribution and Composition of Phospholipids

Phosphatidyl choline and phosphatidyl ethanolamine were the main phospholipids in all cellular fractions. Phosphatidyl choline had a higher percentage of palmitic and linoleic acids than phsophatidyl ethanolamine, while the latter had a higher content of stearic and arachidonic acid (Table IV). These features agreed very well with the FA composition of these 2 compounds described in various organs of other species (30-32).

Some differences were found in the sphingomyelis plus phosphatidyl inositol fractions, and it concerned mainly the content of stearic and docosadienoic acids. Phosphatidyl inositol was normally characterized by a very high content of stearic acid (4,32), and, although this fraction was a mixture, the difference could not have been balanced out only by a low content of sphingomyelin in the mitochondria (21). The differences in the content of docosadienoic acid were particularly striking, and it seemed that this acid was replacing the adrenic acid.

Even more striking were the differences in FA composition of diphosphatidyl glycerol. The compound isolated from the mitochondria and the typical features of diphosphatidyl glycerol isolated from other sources (31,33), were manifested in a very high content of linoleic acid and a low content of arachidonic acid. Diphosphatidyl glycerol from cell sap had a rather unorthodox composition characterized by a very much reduced content of linoleic acid and increased quantity of stearic, oleic, and eicosadienoic acid. No diphosphatidyl glycerol was found in the microsomal fraction, although it had been isolated from the microsomes of the liver (34).

A docosadienoic acid has been found earlier in the neutral and phospholipid fractions fof pig muscles, but only in smaller quantities (35). Whether it was an acid characteristic for the lipid metabolism of the pig is not absolutely certain. It should be borne in mind that certain dietary changes chould affect the fatty acid pattern in all organs of the pig, even over a relatively short period (36).

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SHORT COMMUNICATIONS

Subnanogram Detection of *t*-Butyldimethylsilyl Fatty Acid Esters by Mass Fragmentography

ABSTRACT

The mass spectra of *t*-butyldimethylsilyl fatty acid esters all display a pronounced $(M\text{-}C_4H_9)^+$ ion. The proportion of the total ionization carried by this fragment, particularly for saturated and mono-, di-, and tri-unsaturated acid derivatives, facilitates their qualitative analysis at the subanogram level by mass fragmentography.

INTRODUCTION

Gas chromatography-mass spectrometry (GC-MS) is a technique widely used for the identification of fatty acids in biological mixtures, usually as their methyl esters (1,2). These derivatives, however, are not considered satisfactory for unequivocal identification or sensitive detection purposes, as the mass spectra obtained are typically dominated by intense ions at low mass, characteristic of the ester moiety rather than the structure of the acid itself.

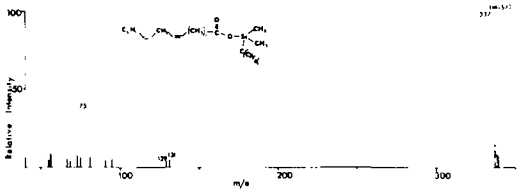


FIG. 1. Mass spectrum of linoleic acid *t*-BDMS ester.

We report here a study of the mass spectral properties of *t*-butyldimethylsilyl (*t*-BDMS) esters, whose characteristics facilitate specific analysis of fatty acids at very low levels.

EXPERIMENTAL PROCEDURES

Fatty acids were all purchased from Applied Science Laboratories, Inc. (State College, PA), except Phytanic acid which was donated by A. Poulos (Adelaide Childrens Hospital, South Australia). *t*-Butyldimethylsilyl esters of reference acids were prepared by adding 100 µl of a N,N-dimethylformamide solution (10 ml) containing 2 mmole imidazole and 1 mmole *t*-butyldimethylsilyl chloride to 500 µg acid. This procedure was reported originally by Corey and Venkateswarlu (3) for the formation of *t*-BDMS ethers as hydroxyl protecting groups. After heating the reaction mixture for 15 min in 60 C bath, brine was added, and the derivative was extracted into ether prior to GC-MS analysis.

GC-MS was carried out with an AEI MS-30 mass spectrometer, equipped with a multipeak monitor, and interfaced to a Pye 104 gas chromatograph using a single stage dimethyl silicone membrane separator. All spectra were determined at 25 eV. The gas chromatographic column was a glass coil (1 m x 2 mm internal diameter (ID) packed with 1% OV-225:Silar 10C (9/1) on 100-120 mesh support. The helium flow was 30 ml/min and the column temperature was 180 C.

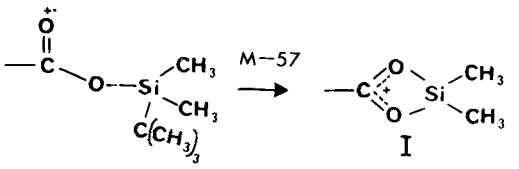
TABLE I

Retention Indices (I) and Major Mass Spectral Fragments of *t* - BDMS Esters

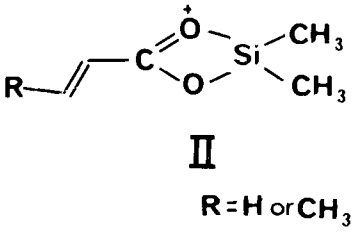
t-BDMS ester	I	Mass Fragments[a]
Palmitic	2430	313[b] (100),131(7),129(6),117(14),75(40).
Phytanic	2535	369[b] (100),201(11),145(10),143(6),117(16).
Oleic	2620	339[b] (100,131(11),129(3),75(26),73(6).
Elaidic	2625	339[b] (100),131(8),129(2),75(18).
Linoleic	2655	
Linolenic	2700	335[b] (100),131(20),95(28),79(21),75(75).
cis-5-Eicosenoic	2805	367[b] (100,171(35),157(16),117(40),75(40).
Arachidonic	2855	361[b] (23),131(23),129(35),93(40),91(40),81(35) 79(60),75(100),73(65),67(45).

[a]Expressed as *m/e* value with relative intensity in parenthesis.

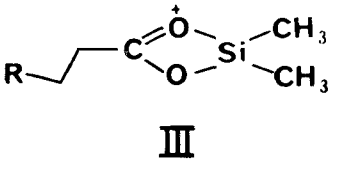
[b]$(M\text{-}C_4H_9)^+$ ion.



SCHEME I.



STRUCTURE II.



STRUCTURE III.

RESULTS AND DISCUSSION

All the acids investigated formed the respective *t*-BDMS esters in quantitative yield as estimated by thin layer chromatography (TLC), gas liquid chromatography (GLC), and, in one case, isolation. The *t*-BDMS esters show good gas chromatographic properties, but expectedly have appreciably longer retention times than the corresponding methyl esters (Table I).

The base peak in the electron impact spectrum of nearly all the *t*-BDMS esters studied (Table I and Fig. 1), which is postulated to have the resonance stabilized cyclic Structure I (Scheme I), is formed by fission of a *t*-butyl radical from the molecular ion. Ions at m/e 129 and 131 are characteristic of all the acids recorded, except phytanic, in which the 2 ions are shifted to m/e 143 and 145. The probable structures of these ions are thus Structures II and III.

It is apparent from the data in Table I that as the degree of unsaturation increases, the *t*-BDMS ester moiety is less able to direct the fragmentational mode of the molecule, and accordingly, the proportion of low mol wt hydrocarbon ions increases. A comparison of the *t*-BDMS and methyl ester (1) spectra of arachidonic acid shows many common ions, e.g., m/e 67, 79, 91, 93, 105, and 150. The

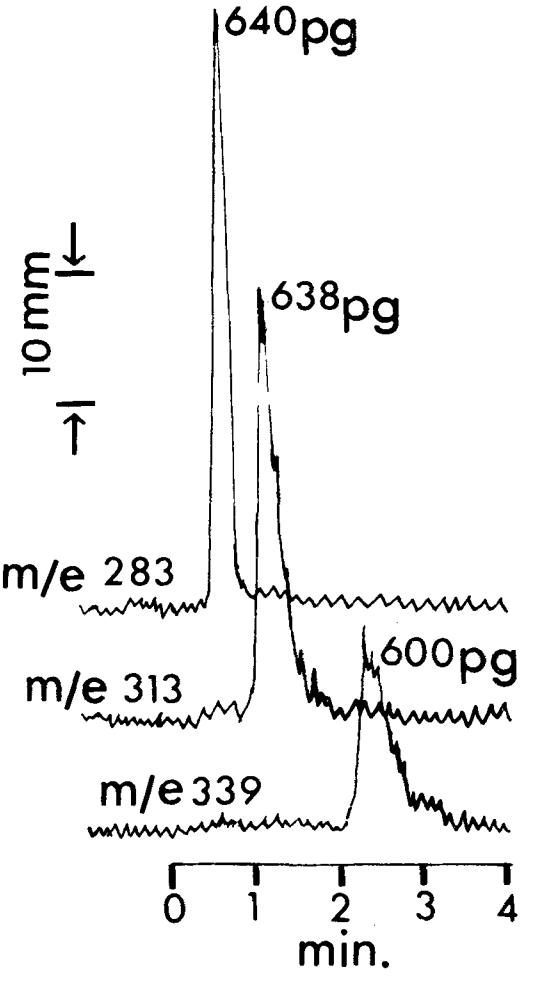


FIG. 2. Mass fragmentogram of myristoleic (m/e 283), palmitic (m/e 313) and elaidic (m/e 339) acids as their *t*-BDMS esters.

structural information available from these hydrocarbon ion fragments has been discussed previously (1).

Figure 2 shows the results of analysis of 3 fatty acid *t*-BDMS esters by the technique of mass fragmentography (4,5). The very small amounts detected and the specificity for each individual acid are noteworthy points. Furthermore, by monitoring the $(M\text{-}C_4H_9)^+$ ions, acids which differ in mol wt need not be resolved by GLC to be detected.

Because the (M-57) peak is usually the most intense, the use of these derivatives facilitates the measurement of the mol wt of trace amounts of unknown acids with much greater confidence than is possible when using methyl ester derivatives.

Work is now in progress to assess the scope

of these derivatives for analysis of biological mixtures.

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Mass Spectrometric Localization of Methyl Branching in Fatty Acids Using Acylpyrrolidines

ABSTRACT

Localization of a methyl branch in a fatty acid molecule by mass spectrometry is facilitated by using the pyrrolidide rather than the methyl ester. Branched fatty acid methyl esters are converted to pyrrolidides and are then analyzed by gas chromatography and mass spectrometry. The diagnostic fragments indicate position of the methyl branch.

Mass spectra of methyl esters of normal, methyl branched *iso* and *anteiso* fatty acids are rather similar, and the structure must be deduced from very small differences in intensity of diagnostic ions (1). Moreover, the isomers must be separated by gas liquid chromatography (GLC), the bleed from which is a disturbing factor in interpreting mass spectra. As part of our investigation of mass spectra of amides of fatty acids (2-4), the spectra of pyrrolidides of methyl branched fatty acids were studied. They were found to reveal readily the location of a methyl branch, and to distinguish between *normal, iso,* and *anteiso* structures. In this study, the pyrrolidides were prepared and the mass spectra measured under conditions identical to those previously published for pyrrolidides of unsaturated fatty acids 2,4).

Because the fatty acid pyrrolidides contain a nitrogen, it is very easy to deduce their fragmentation patterns, for the nitrogen containing fragments yield even numbered ions, and the odd numbered ions contain only C, H, and O. The mass spectra of fatty acid methyl esters contain mostly odd numbered masses to which

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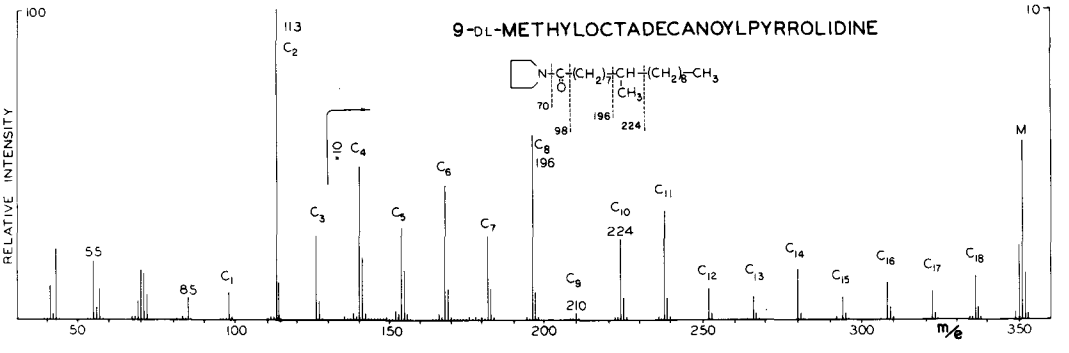

FIG. 1. Mass spectra of 9-DL-methyloctadecanoyl-pyrrolidine.

of these derivatives for analysis of biological mixtures.

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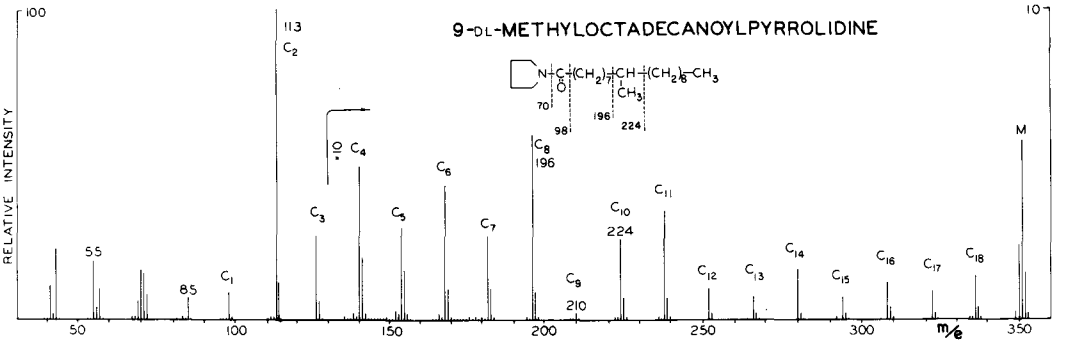

FIG. 1. Mass spectra of 9-DL-methyloctadecanoyl-pyrrolidine.

several ion structures may contribute.

In the mass spectrum of a methyl branched fatty acid pyrrolidide, the same very simple fragmentation pattern occurs as with the straight chain ones (5). Beginning with the high mass region, the molecular ion (M) is accompanied by the ion formed via elimination of the terminal methyl group, and a series of ions with successively one less CH_2 group regularly spaced 14 a.m.u. apart, down to the McLafferty rearrangement ion m/e 113 (2-6). This latter ion is base peak in the spectra of all pyrrolidides investigated. Each ion contains the pyrrolidide group and is formed by a direct cleavage of the molecular ion as is shown by appropriate metastable ions, as is also the case with pyrrolidides of unsaturated fatty acids (2). The differences between pyrrolidides of straight chain and methyl branched fatty acids are variations in the intensities of the peaks corresponding to cleavages at either side of the branch. In Figure 1, the methyl branch is located on carbon 9 and the fragment (m/e 210) that corresponds to cleavage at this carbon is of very low intensity compared with the fragments containing one more or one less CH_2 group (m/e 196 and m/e 224).

In the mass spectrum of 10-methyloctadecanoyl pyrrolidine peaks of higher intensity, m/e 210 and m/e 238, 14 a.m.u. below and above a low intensity peak at m/e 224, indicate a methyl branch at carbon 10.

The same phenomenon was found in spectra of pyrrolidides of *iso* and *anteiso* 15:0 and 17:0 fatty acids. The high mass regions of the spectra of pyrrolidides of the 15:0 acids are presented in Figure 2. For *n*-15:0, a small peak is caused by the elimination of the terminal methyl group (m/e 280). The *iso*-15:0 has a peak of low intensity corresponding to elimination of C_2H_5. *Anteiso*-15:0 has a peak of similar low intensity by elimination of the terminal C_3H_7. The mass spectra of pyrrolidides of methyl branched fatty acids of other chain lengths and numbers of methyl groups confirmed that a minimum between two maxima in the fragment series $[M - CH_3(CH_2)_n]^+$ indicates the position of the methyl group.

Because it is necessary to separate the *normal, iso,* and *anteiso* fatty acid pyrrolidides by GLC before mass spectral analysis, equivalent chain length (ECL) values are given. They were measured on SILAR 10C (Applied Science Labs, State College, PA) at 240 C and on DEGS-PS (Supelco, Bellefonte, PA) at 220 C, respectively: *iso*-15:0, 14.28 and 14.43; *anteiso*-15:0, 14.62 and 14.75; *iso*-17:0, 16.40 and 16.43; *anteiso*-17:0, 16.77 and 16.72. Most of the GLC stationary phases used for

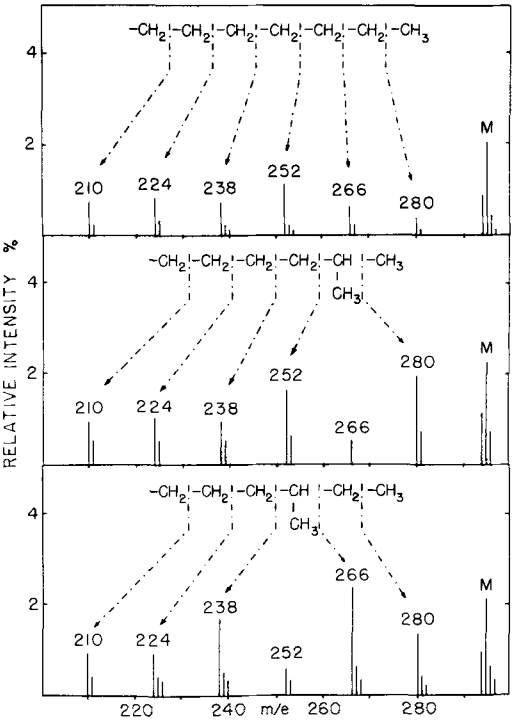

FIG. 2. The high mass region of the spectra of *normal, iso,* and *anteiso*-15:0 fatty acid pyrrolidides.

separation of fatty acid derivatives give background mass spectra containing fragments at odd masses. Because electron impact of pyrrolidides produces many main fragments of even mass numbers, the background from the GLC bleed does not interfere with the interpretation of a mass spectrum of a branched fatty acid pyrrolidide. In the spectra of methyl esters, it is hard to distinguish between *normal* and *iso* structures because the latter is characterized by an extremely low intensity peak (< 1%) caused by loss of 65 a.m.u. from the molecular ion (7) and which may be obscured by ions formed from bleeding of the stationary phase (8).

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ERRATUM

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Uptake and Metabolism of α-Monopalmitin by Rat Lung In Vitro[1]

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ABSTRACT

CO_2 production from and uptake of α-glyceryl mono (palmitate-1-^{14}C) were studied in an in vitro system using minced rat lung. Monoglyceride radioactivity was readily incorporated into lung tissue lipids. In a time course of 5-120 min, ca. 2.9-21.9% of the initial medium ^{14}C-radioactivity was recovered in tissue lipids, including free fatty acid and monoglyceride, per one g of tissue. From 93 to 72% of the initial radioactivity remained in the medium during the same incubation periods. The ratio of tissue neutral lipid to phospholipid radioactivity decreased from 2:1 at 5 min to ca. 1:2.1 at 120 min. Most of the phospholipid-^{14}C was in phosphatidyl choline, and this accounted for 80% of phospholipid-^{14}C. Analysis of the tissue lipid radioactivity pattern revealed that during early periods of incubation (5-15 min) there was a rapid accumulation of ^{14}C in monoglycerides and free fatty acids, which decreased with increasing incubation time concomitant with increase in radioactivity of tissue phospholipids and triglycerides. During the same time course, 6.5-85.3% of medium-^{14}C was in free fatty acid, indicating the presence of an active α-monopalmitin-hydrolyzing system. After 2 hr of incubation, only 1.8% of the initial medium-^{14}C had been oxidized to CO_2. Under the same experimental conditions, ^{14}C-α-monopalmitin and palmitate-1-^{14}C were almost equally utilized and the patterns of lipid incorporated from both substrates were similar. It is suggested that rat lungs can utilize α-monopalmitin in a similar manner as palmitate after the former is hydrolyzed.

INTRODUCTION

Previous reports from our laboratory (1-3) demonstrated that perfusion or incubation of rat lung tissue with various substrates, acetate pyruvate, glucose, xylitol, or fatty acids resulted in the formation of complex lipids, especially phospholipids. It has been observed that the T 1/2 disappearance of ^{14}C-α-monopalmitin from the blood circulation after its intravenous administration is similar to that of free fatty acid (Meng and England, unpublished data). This finding suggests a rapid uptake by tissues. Because monoglyceride (MG) is one of the products of hydrolysis of dietary fat and of chylomicron triglycerides, and because utilization of MG for the synthesis of higher glycerides, diglycerides, and triglycerides, occurs in intestine (4-6) and to a less extent in adipose tissue (7,8), it was thought interesting to study the uptake and metabolism of MG by the lung. In the present work, α-glyceryl mono (palmitin-1-^{14}C) was added to the medium for the incubation of rat lung. CO_2 production and ^{14}C incorporation into tissue complex lipids were used as criteria for α-monopalmitin metabolism. The utilization of ^{14}C-α-monopalmitin and palmitate-1-^{14}C by rat lung in the same test system was also compared.

MATERIAL AND METHODS

Animals

Normal male rats of the Sprague-Dawley strain weighing 250-300 g were used. The rats were maintained of Purina Laboratory Chow and tap water ad libitum.

Preparation of Tissue and Incubation System

The preparation of minced rat lung, incubation system, and experimental procedures have been reported (1). An emulsion of α-monopalmitin and albumin complex of ^{14}C-1-palmitate were used as substrates, and a substrate concentration of 1.3 μmoles/ml was selected in order to compare with results obtained in previous studies in which other substrates were used (1,2). The emulsion of α-monopalmitin was prepared by mixing gum acacia with α-monopalmitin in a ratio of 4:1 (w:w). The mixture was sonicated with a Branson Sonifier (Branson Instrument Inc., Stranford, CN) tuned

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TABLE I

Time Course of Oxidation and Uptake of ^{14}C-α-Monopalmitin by Minced Rat Lung[a]

Incubation time (min)	^{14}C-Tissue lipids (μmoles)	^{14}C-Tissue lipids (%)[b]	$^{14}CO_2$ (μmoles)	$^{14}CO_2$ (%)[b]
5	0.12	0.91 ± 0.09 (6)[c]	trace	trace (5)[c]
15	0.22	1.17 ± 0.10 (6)	0.01	0.09 ± 0.03 (4)
30	0.35	2.66 ± 0.18 (6)	0.04	0.27 ± 0.09 (6)
60	0.66	5.02 ± 0.25 (8)	0.10	0.76 ± 0.03 (8)
120	0.88	6.71 ± 0.28 (6)	0.24	1.85 ± 0.15 (6)

[a]Incubation system was a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.3 g tissue, 25 mg/ml bovine serum albumin and 4 μmoles ^{14}C-1-α-monopalmitin with a specific radioactivity of 0.25 μCi/μmole. The final volume was 3 ml. Incubation was at 37 C under 95% O_2, 5% CO_2 for various time intervals as indicated.

[b]Percent of initial radioactivity in medium.

[c]Figures in parentheses represent the number of animals and the results are expressed as means ± S.E.

TABLE II

Time Course of Lipid Radioactivity in Incubation Medium[a]

Time (min)	Monoglyceride (MG) (%)[b]	Free fatty acid (FFA) (%)[b]	Others (%)[b]
0 (4)[c]	99.5 ± 0.2	0.5 ± 0.1	<0.1
5 (4)	93.3 ± 0.8	6.5 ± 0.6	<1
15 (4)	81.7 ± 2.2	17.3 ± 2.3	<1
30 (4)	60.9 ± 1.5	37.8 ± 1.8	<1
60 (4)	36.5 ± 2.1	62.7 ± 2.2	<1
120 (4)	13.9 ± 2.9	85.2 ± 3.2	<1

[a]Incubation medium was extracted for lipids by Folch's method followed by thin layer chromatography with a solvent system of petroleum ether:ethyl ether:acetic acid (60:40:1).

[b]% Radioactivity = (MG, FFA, or other lipids/MG + FFA + other lipids) x 100.

[c]Figures in parentheses represent the number of animals.

to maximal output for 1 min at 5-10 C. The emulsion thus prepared was stable for at least 1 week, but was usually prepared 1 day prior to use in each experiment.

Analysis of Lipids

Lipid components, CO_2 production, and radioactivity were measured by procedures reported previously (1). Free fatty acid (FFA) production during incubation was determined either by the method of Dole (9) or by liquid scintillation counting of ^{14}C-FFA. For the latter method, ^{14}C-hydrolysates were extracted from the medium by the method of Folch, et al., (10) followed by separation by thin layer chromatography (TLC). The separated components were transferred from the TLC plates directly into the counting vials and the radioactivity was measured as reported previously (1). 1,2-Diglycerides and 1,3-diglycerides were identified using TLC by procedures suggested by Brown and Johnston (11) and Malins and Mangold (12).

Synthesis of α-glyceryl mono (palmitate-1-^{14}C) and α-monopalmitin was done in our laboratory according to the method of Hartman (13). The purity of both products was >99.5%. ^{14}C-1-palmitic acid was purchased from New England Nuclear Corp. (Boston, MA). Gum acacia and glycerol were obtained from Fisher Scientific Co. (Fairlawn, NJ).

RESULTS

CO_2 Production and Lipid Synthesis

A time course of $^{14}CO_2$ production and ^{14}C incorporation into tissue lipid is shown in Table I. Ca. 0.10-5.9% of the initial radioactivity in the medium was oxidized to $^{14}CO_2$ and 2.9-21.9% of the initial ^{14}C-radioactivity was recovered in lipids of one g of fresh minced tissue during a time course of 5-120 min; from 93 to 72.2% of the initial radioactivity remained in medium lipids during the same incubation periods. At the end of 2 hr of incubation, 0.24 μmoles and 0.88 μmoles of initial α-monopalmitin radioactivity were recovered as

$^{14}CO_2$ and ^{14}C-lipids in the tissue, respectively, per one gm of fresh minced lung; of the 0.88 μmoles of lipids in the tissue, 0.25 μmoles or 6.6% of the initial radioactivity was calculated to be in complex lipids other than MG and FFA.

Distribution of Radioactivity in Lipids of the Incubation Medium

Table II shows the distribution of radioactivity in medium lipids during the 2-hr incubation period. The relative percentage of α-monopalmitin in the incubation medium decreased from 100 to 14%, whereas that of FFA increased from 0.5 to 85%. There was only a negligible amount of radioactivity in other lipids present during the entire incubation period of 2 hr.

Fractionation of Tissue Lipids

Tissue lipids were separated into neutral lipids and phospholipids by silicic acid column chromatography. It can be seen in Figure 1 that after 5 min of incubation most of ^{14}C-radioactivity in the tissue was in the neutral lipid fraction. As incubation was continued, radioactivity in this fraction was increased gradually, while the radioactivity in phospholipids was increased more sharply. The radioactivity in this fraction exceeded that in the nonphospholipid fraction after 30 min of incubation. The synthesis of phospholipids was linear with time during the first hr.

Tissue lipids were further separated by TLC using various solvent systems (Table III). During the incubation periods of from 5 min to 2 hr, the radioactivity in phosphatidyl choline (PC) increased from 25 to 58% and that in triglyceride (TG) increased from 2 to 10%; diglyceride (DG) was also slightly increased with time. However, a decrease in MG from 25 to 5% and in FFA from 39 to 7% was observed. Fractionation of phospholipids by TLC revealed that the percentage of ^{14}C in PC (76-80%) relative to that in other phospholipids (PL) remained fairly constant during the entire incubation period.

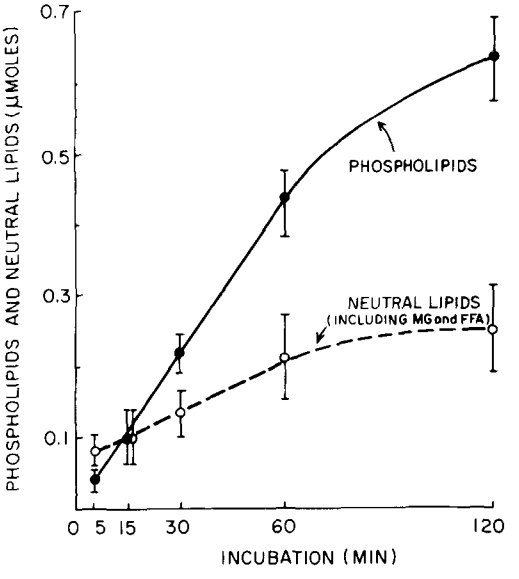

FIG. 1. Rate of cumulative incorporation of α-^{14}C-1-monopalmitate into phospholipids and neutral lipids. Neutral lipids included triglycerides, diglycerides, monoglycerides (MG), and free fatty acid (FFA).

TABLE III

Percent Distribution of Radioactivity in Tissue Lipid Fractions[a]

Incubation time (min)	MG[b] (%)[d]	DG[b] (%)[d]	FFA[b] (%)[d]	TG[b] (%)[d]	PC[b,c] (%)[d]
5 (6)[d]	25.4 ± 2.9	2.9 ± 0.4	38.8 ± 1.5	2.1 ± 0.4	24.8 ± 1.7
15 (6)	16.1 ± 1.5	3.9 ± 0.4	26.8 ± 2.1	3.4 ± 0.6	38.4 ± 1.7
30 (6)	9.1 ± 0.6	5.0 ± 0.3	15.2 ± 0.4	7.7 ± 0.6	50.5 ± 0.8
60 (6)	5.2 ± 0.5	5.6 ± 0.6	13.6 ± 0.6	9.1 ± 1.4	53.6 ± 0.8
120 (6)	4.7 ± 0.5	5.9 ± 1.1	7.1 ± 0.7	10.1 ± 1.1	58.0 ± 1.1

[a]Separated by thin layer chromatography. Solvent systems were: for neutral lipids, petroleum ether:ethyl ether:acetic acid (85:15:1); for phospholipids, chloroform:methanol:water:acetic acid (75:25:5:1).

[b]MG = monoglyceride; DG = diglyceride; FFA = free fatty acids; TG = triglyceride; and PC = phosphatidyl choline.

[c]Phospholipid fraction consisted of other phospholipids in addition to phosphatidyl choline.

[d]Percent of total lipid radioactivity, calculated from individual lipid fraction/total lipids) x 100.

[e]Figures in parentheses represent the number of animals.

TABLE IV

Oxidation and Uptake of ^{14}C-1-Palmitate and α-Glyceryl-Mono (palmitate-1-^{14}C) by Minced Rat Lung Tissue[a]

Substrate	CO_2 (μmoles/g/hr)[b]	CO_2 (%)[c]	Tissue lipids (μmoles/g/hr)[b]	Tissue lipids (%)[c]
^{14}C-MG (4)[d]	0.10 ± 0.01	0.73 ± 0.04	0.75 ± 0.02	5.61 ± 0.20
^{14}C-PA (4)	0.12 ± 0.01	0.89 ± 0.08	0.81 ± 0.02	5.94 ± 0.13

[a]Incubation system was a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.3 g tissue, 25 mg/ml bovine serum albumin and 4 μmoles of substrate with a specific radioactivity of 0.25 μCi/μmole. The final incubation volume was 3 ml. Incubation was carried out at 37 C under 95% O_2 5% CO_2 for one hr.

[b]μMoles of substrate/g wet tissue/hr.

[c]Percent of the initial radioactivity in medium.

[d]^{14}C-MG = α-glyceryl-mono (palmitate-1-^{14}C); ^{14}C-PA = ^{14}C-1-palmitate-Na. Figures in parentheses represent number of animals.

TABLE V

Distribution of Radioactivity in Tissue Lipid Fractions[a]

Lipid fraction[d]	^{14}C-MP[d] (%)[b]	^{14}C-PA[d] (%)
MG	3.9 ± 1.2[c]	1.8 ± 0.8
1,2-DG	4.6 ± 0.9	3.4 ± 0.8
1,3-DG	1.3 ± 0.4	1.1 ± 0.2
FFA	18.1 ± 2.6	19.4 ± 1.9
TG	11.7 ± 0.6	10.2 ± 1.8
PC	47.9 ± 4.7	47.1 ± 1.9
Other PL	11.8 ± 2.3	12.1 ± 3.7

[a]Lipid fractions were separated by thin layer chromatography.

[b]Percent of total tissue lipid radioactivity, calculated from (individual lipid fraction/total lipid) x 100.

[c]Results are average of four experiments ± standard error.

[d]MP = α-monopalmitin; PA = palmitate; MG = monoglyceride; DG = diglyceride; FFA = free fatty acids; TG = triglyceride; PC = phosphatidyl choline; PL = phospholipids.

Comparison of ^{14}C-1-Palmitate and α-Monopalmitin Utilization

A comparative study of the utilization and metabolism of ^{14}C-palmitate (PA) and ^{14}C-α-monopalmitin (MP) in rat lung tissue was made under similar experimental conditions, including substrate concentration. $^{14}CO_2$ production and tissue lipid-^{14}C were determined after 1 hr of incubation. Table IV shows that 0.7% (0.1 μmole) of the initial radioactivity of MP and 0.9% (0.12 μmoles) of PA were oxidized to CO_2, whereas 5.6% (0.75 μmoles) of MP and 5.9% (0.81 μmoles/g/hr) of palmitate (PA), respectively, were recovered in tissue lipids.

The distribution of radioactivity in neutral lipids (NL) and PL was compared after their separation by silicic acid column chromatography. It was observed that 40 and 42% of the total lipid radioactivity from MP and PA, respectively, were in NL, whereas the remaining 60 and 58% of the total lipid radioactivity, from MP and PA, respectively, were in PL fraction. The recovery of radioactivity of lipid standards from the column in our laboratory has been between 90-95%.

Further fractionation of tissue lipids in experiments using MP and PA as substrates showed no significant difference between the patterns of lipid incorporation (Table V) except that the radioactivity in MG was 3.9 and 1.8% of the total lipid radioactivity when MP and PA, respectively, were used as substrates. Of the total radioactivity in DG, ca. 70-80% was recovered in 1,2-DG, whereas the remaining 20-30% was found in 1,3-DG fraction. Ca. 80% of PL radioactivity from both substrates was recovered as PC.

DISCUSSION

This work was designed to study if monoglyceride, as a product of triglyceride lypolysis, is taken up and metabolized by the lung. The results show that this is the case. The results of a time course study of incubated lung tissue (Tables I and III) suggest the presence of an active system for oxidation of MP to CO_2 and incorporation into tissue lipids. Most of the newly formed complex lipid radioactivity was in PL, especially in PC. The high percentage of ^{14}C in PC relative to other lipids and its steady increase during the time course study provide further confirmation of our previous finding (1,2) that there is an active PC synthesizing system in lung tissue. Tombropoulas (14) has reported the incorporation of palmitate into lipids by lung subcellular fractions. Incorpora-

tion of other substrates into lung lipids and lipid metabolism in the lung have also been reported by other workers as reviewed by Naimark (15).

The progressive decrease in radioactivity of tissue MG and FFA and corresponding increase in PC, TG, and DG suggest that (a) MG is being hydrolyzed, (b) FFA, a hydrolytic product of MP, shows some accumulation, (c) some FFA is reesterified to DG, TG, and PC, which is apparently rate limiting, and (d) excessive FFA is released into the medium.

The question concerning the removal and metabolism of circulating glycerides by tissues is whether the intact glycerides or their hydrolytic products are removed by the lung. There are two pathways by which MG are incorporated into complex lipids: (a) the glycerol phosphate pathway described by Kornberg and Pricer (16) and Weiss and Kennedy (17) and (b) the monoglyceride pathway reported by Clark and Hubscher (4,18), Senior and coworkers (6,20,21), and Johnston and associates (5,7,8,19). In the first pathway, formation of complex lipids from MG is preceded by hydrolysis of MG, while the latter, complex lipids are formed via direct acylation of MG.

The rapid increase in labeled FFA in the medium suggests the existence of an active MP hydrolyzing system (22-28) in the lung. A preliminary report concerning the existence and characteristics of a MP lipase in rat lung has been reported (29).

The presence of an extremely active MG lipase system demonstrated in this study indicates that the glycerol phosphate pathway is the major pathway for complex lipid synthesis in the rat lung under normal physiologic conditions. The contribution of the monoglyceride pathway, if any, must be a minor one. Further evidence for the formation of complex lipids via glycerol phosphate pathway was supported by the findings that in the analysis of DG isomers, 70-80% of DG radioactivity was found in 1,2-DG, while the 1,3-DG accounted for only 20-30% of DG radioactivity (Table V).

There were no significant differences between α-monopalmitin and palmitic acid with regard to the rate of utilization and patterns of tissue lipid incorporation. The similarity in the rate of incorporation and patterns of tissue lipids between these two substrates indicates that the pathway for complex lipid synthesis (TG, PC) is identical. Apparently, α-monopalmitin was hydrolyzed by lung MG lipase and the resulting FFA (palmitic acid) was then reesterified to form higher glycerides or PL.

ACKNOWLEDGMENTS

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Uptake and Metabolism of Exogenous Eicosa-8,11,14-trienoic Acid in Minimal Deviation Hepatoma 7288 C Cells

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ABSTRACT

Minimal deviation hepatoma 7288 C cells were cultured in Swim's medium containing 10% serum for 48 hr. The growth medium was replaced with serum free media containing different concentrations of [1-^{14}C]eicosa-8,11,14-trienoic acid and the cells were incubated for 24 hr. Incorporation into cell lipids, oxidation to CO_2, and desaturation to arachidonic acid were studied. The oxidation of the acid was very low. It was preferentially incorporated into the polar lipids of the cell. The incorporation depended on the number of cells and fatty acid concentration. Saturation of the cells with the acid was reached when 144.7 nmoles per mg of cellular protein were incorporated. The acid was desaturated readily to arachidonic acid. The nmoles of eicosatrienoic acid converted to arachidonic acid per mg of cellular protein were hyperbolic function of the acid incorporated. Maximal desaturation, 23 nmoles per mg of cellular protein, was reached when the cells were saturated with the acid. The calculations of the desaturation capacity and of the endogenous pool of eicosatrienoic acid available for desaturation in the cell are discussed.

INTRODUCTION

Cultured cells provide an excellent experimental system for the study of metabolic reactions. This is due to their rather homogeneous character, which allows the examination of different events separated from the complex physiological interactions encountered in vivo. Moreover, their lipid composition can be easily controlled by modification of their nutritional environment (1,2).

Because established cell lines are often tumorigenic when transplanted in vivo (3), it is generally considered that cell transformations in vitro are analogous to the malignant process in vivo. For that reason, one can compare directly the metabolism in cultured tumor cells with the metabolism in normal cells.

It has been established that lipids containing particularly polyunsaturated fatty acids of the linoleic and α-linolenic series are involved intimately with metabolism and cellular structure and function, especially at the membrane level. Striking differences in lipid metabolism between normal and neoplasic tissues have been shown to occur (4). Information regarding lipids and their metabolism can be pertinent in any attempt to develop and evaluate methods for the prevention and treatment of neoplasia.

Cultured cells derived from Morris minimal deviation hepatoma (HTC) cells have little arachidonic acid (1). This is in accordance with their diminshed capacity to synthesize this acid from linoleic acid shown in the first paper of this series (1). For that reason, it has been suggested that some of the reactions involved in its synthesis could be altered. Eicosa-8,11,14-trienoic acid is the immediate precursor of arachidonic acid. Therefore, the objective of the present investigation was to do the ground work necessary for any other research on this line and to assess the ability of HTC cells to incorporate and metabolize exogenous eicosa-8,11,14-trienoic acid, studying particularly the kinetics of eicosa-8,11,14-trienoic acid incorporation and desaturation to arachidonic acid.

MATERIALS AND METHODS

Culture Conditions

HTC 7288 C cells were maintained and grown at 37 C in confluent layer attached to glass on Swim's 77 medium, supplemented with 10% calf serum (5), using conventional sterile conditions as described previously (1).

Radiochemicals

[1-^{14}C]Eicosa-8,11,14-trienoic acid (58 mCi/mmole) 98% pure was provided by New England Nuclear (Boston, MA). Eicosa-8,11,14-trienoic acid was purchased from Nu-Check-Prep, Inc. (Elysian, MN).

Assay Procedure

The cells were dislodged from the glass surface using a 0.25% trypsin solution and were counted in a hemocytometer. When not specified, 0.5 x 10^6 cells per bottle were cultured in

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70 cm^2 flasks with 20 ml Swim's 77 medium supplemented with 10% calf serum as described elsewhere (1). After 48 hr, when the cells were in the logarithmic phase of growth, the cultured medium was replaced by 10 ml of Swim's 77 medium without serum, to which different amounts of labeled eicosa-8,11,14-trienoic acid were added. The acid was added as sodium salt bound to defatted albumin (6) according to Spector, et al., (7) in a ratio of 2 moles fatty acid to 1 mole albumin.

After different periods of incubation, the attached cells were washed twice with 0.85% NaCl, removed from the container with a rubber policeman, collected in tubes, and leveled to 5 ml with 0.85% NaCl. An aliquot of the suspension was used to determine the amount of cellular proteins by the method of Lowry, et al., (8), and the rest was centrifuged. The saline solution was decanted and the cells were saponified with 2 ml of 10% KOH in ethanol for 45 min at 85 C. The fatty acids were extracted from the acidified solution with light petroleum (bp 30 C-40 C) and esterified with 3 M HCl in methanol. The radioactivity of the recovered methyl esters was determined in a Packard Tri-Carb scintillation counter with 86% efficiency for ^{14}C, using a scintillation solution prepared with 4 g of 2,5-diphenyl-oxazol (PPO) and 100 mg of 1,4-bis-2 (5-phenyloxazolyl)-benzene (POPOP) per liter in toluene. The distribution of radioactivity between eicosatrienoic and arachidonic acids was measured by gas liquid radiochromatography in a Packard apparatus with a proportional counter. The column was prepared with 15% diethyleneglycol succinate on Chromosorb W (80-100 mesh) and the analysis was performed at 176 C.

The labeled arachidonic acid formed from [1-^{14}C]eicosa-8,11,14-trienoic acid was identified by comparison with an authentic standard. It was also separated by gas liquid chromatography, collected, and the structure recognized by reductive ozonlysis (9). The products of ozonolysis, chromatographed in the same apparatus and column at 150 C, gave a labeled peak that corresponded to an aldehyde-ester of 5 carbons.

CO_2 Determination

In some experiments, the production of labeled CO_2 was also measured. For this purpose the CO_2 produced was collected in filter paper impregnated with Hyamine. After the incubation, the medium was acidified with sulphuric acid and the paper was then introduced into a vial containing 20 ml of Bray's scintillation solution (10). The radioactivity was measured as described previously. The radioactivity remaining in the medium and the saline solution used to wash the attached cells were also measured in Bray's solution (10). The attached cells were dislodged from the glass surface with a rubber policeman, suspended in 0.85% NaCl solution, and the radioactivity measured in Bray's solution (10).

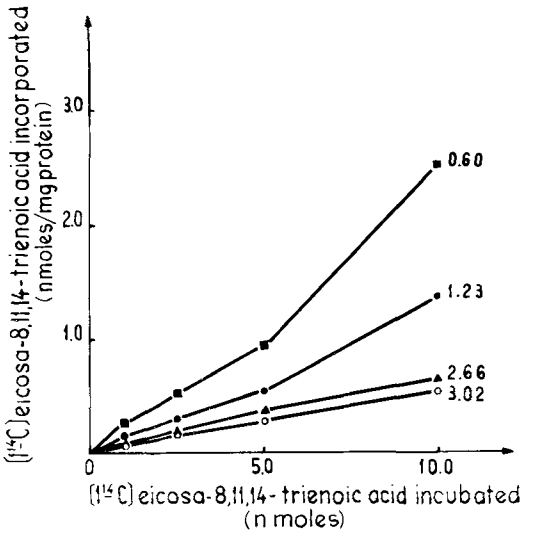

FIG. 1. Incorporation of [1-^{14}C]eicosa-8,11,14-trienoic acid in hepatoma 7288 C cells (HTC) as a function of cell concentration and substrate added. HTC cells were incubated at 37 C for 24 hr with the labeled acid in Swim's medium. On each curve are indicated the mg of cellular protein incubated per flask. Results are the mean of two incubation flasks.

Separation of Lipids

The incorporation of the eicosa-8,11,14-trienoic acid into cellular lipids was studied by incubation of 2.5 nmoles of [1-^{14}C]eicosa-8,11,14-trienoic acid with HTC cells (5 mg of cellular protein) for 24 hr at 37 C in the usual way.

The cellular lipids were extracted with chloroform:methanol (2:1, v/v) by the procedure of Folch, et al., (11). Individual lipid classes were separated by thin layer chromatography (TLC) on 500 μ plates of activated Silica Gel G. The solvent mixture used was chloroform:methanol:water (65:25:4, v/v/v). The spots of the lipids were scraped off and suspended in 10 ml of Bray's solution (10). Radioactivity was measured as usual in the Packard Tri-Carb Scintillation counter.

RESULTS AND DISCUSSION

Incorporation of Eicosatrienoic Acid in the Cells

To determine to what extent the amount of

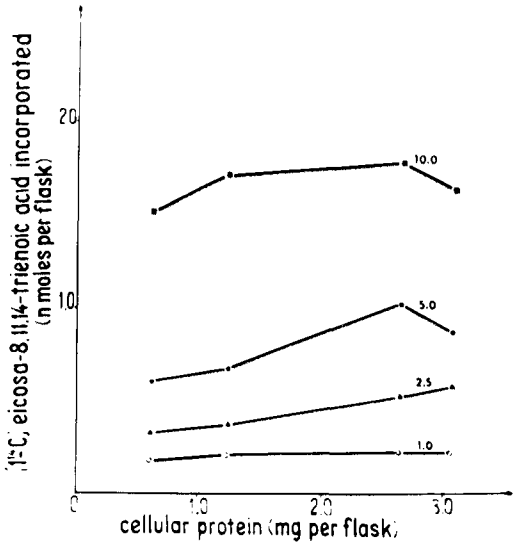

FIG. 2. Effect of cell concentration on total incorporation of [1-14C]eicosa-8,11,14-trienoic acid. Experimental conditions are the same as for Figure 1. nMoles of labeled acid incubated are on the top of each curve.

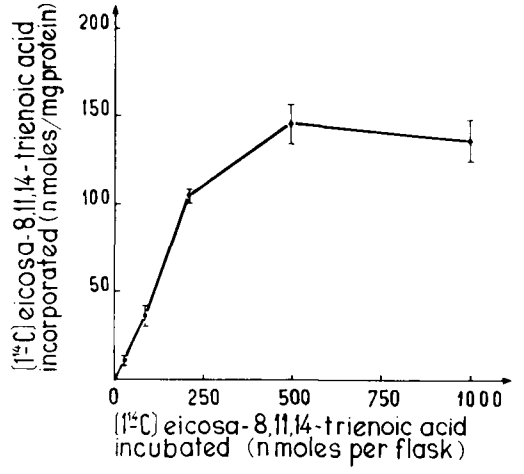

FIG. 3. Saturation of [1-14C]eicosa-8,11,14-trienoic acid incorporation in hepatoma 7288 C (HTC) cells. HTC cells were incubated at 37 C for 24 hr with increasing concentrations of the labeled acid in Swim's medium. Values are the means ± SE of 3 incubation flasks.

incubated acid and cell concentration affected eicosatrienoic acid uptake, a series of assay incubations with [1-14C]eicosa-8,11,14-trienoic acid was carried out. The amount of incubated acid was varied from 1 to 10 nmoles, and the cell concentrations from 0.6 to 3.02 mg of cellular protein per flask.

As is shown in Figure 1, [1-14C]eicosa-8,11,14-trienoic acid was taken up by the cells and the amount incorporated into the cells increased with the concentration of the incubated acid. Moreover, when the lowest cell concentrations (0.6 and 1.23 mg of cellular protein per flask) were incubated, a sigmoidal curve was observed. It is important to remark that a sigmoidal incorporation curve was also obtained when we analyzed the data published by Fulco (12) on the incorporation of palmitic acid in *Bacillus megaterium*. This type of curve can be explained in different ways. It may suggest the existence of a cooperative effect on the fatty acid incorporation in cells at low concentrations of the acid.

TABLE I

Distribution of Labeling Among Lipid Fractions After Incubation of Hepatoma 7288 C Cells with [1-14C]Eicosa-8,11,14-trienoic acid

	Radioactivity (%)[a]
Lysolecithin zone	11.8
Phosphatidylcholine	49.0
Phosphatidylethanolamine	21.5
Free fatty acids	7.3
Neutral lipids	6.8

[a]Minor fractions complete 100%. Results are the mean of two incubation flasks.

The amount of acid incorporated per mg of cellular protein increased when the amount of incubated cells was decreased (Fig. 1). When the lowest concentrations of labeled acid were incubated, the amount incorporated in the cells per bottle was almost independent of cell concentration (Fig. 2). However, with high concentrations of labeled acid, the incorporation increased slightly with cell concentration (Fig. 2).

Saturation of the cells with eicosatrienoic acid could be achieved by increasing the concentration of the acid in the medium (Fig. 3). When 500 nmoles or higher amounts of acid were incubated with 0.6 mg of cellular protein per flask, the cells were saturated and 144.7 nmoles per mg of cellular protein were taken up. The saturation curve followed a normal hyperbolic shape.

The distribution of labeling among the different lipid fractions after incubation of 2.5 nmoles of [1-14C]eicosatrienoic acid with 5 mg of cellular protein for 24 hr is shown in Table I. It is evident that the radioactivity is preferentially incorporated into the polar lipids.

Oxidation of Eicosa-8,11,14-trienoic Acid

The fate of exogenous eicosa-8,11,14-trienoic acid in HTC cells was studied by incubation of the cells with the labeled acid and esti-

TABLE II

Distribution of Radioactivity After Incubation of Different Amounts of [1-^{14}C] Eicosa-8,11,14-trienoic Acid with Hepatoma 7288 C Cells

Incubated acid (nmoles)	Distribution of radioactivity		
	CO_2 (%)[b]	Cells (%)	Medium (%)
0.4	0.9 (0.6-1.1)	28.3 (27.3-29.4)	70.8 (69.5-72.2)
0.9	0.2 (0.1-0.2)	36.8 (35.9-37.7)	63.0 (62.1-63.9)
2.0	0.6 (0.2-1.0)	48.3 (46.6-50.0)	51.1 (49.0-53.2)
4.0	0.4 (0.3-0.4)	43.6 (43.4-43.7)	56.0 (56.0-56.1)
10.0	0.5 (0.4-0.6)	40.9 (39.4-42.3)	58.6 (57.3-60.0)

[a]Cellular protein (0.6 mg) of HTC cells were incubated with [1-^{14}C]eicosa-8,11,14-trienoic acid for 24 hr using the techniques described in methods.

[b]Results are the mean of two determinations. Extreme values are between brackets.

mation of labeling distribution among CO_2, incubated cells, and culture medium. The results are shown in Table II. It is evident that the absolute amounts of labeled CO_2 by the cells increased with the amount of incubated acid. However, the production of CO_2 from [1-^{14}C]eicosatrienoic acid during the incubation was very low and most of the labeled acid was incorporated into the cells rather than oxidized to CO_2. Similar results were also shown by Wiegand and Wood (2) for palmitic acid. Very probably, as these authors proposed, the energy requirements of the HTC cells are satisfied by catabolizing glucose from the medium in preference to oxidizing the exogenous or endogenous fatty acids.

Conversion of Eicosa-8,11,14-trienoic Acid to Arachidonic Acid

HTC cells not only store the fatty acid in the lipids, but also desaturate eicosa-8,11,14-trienoic acid actively to arachidonic acid, as is shown in Figure 4. Figure 4 shows the time curve of [1-^{14}C]eicosatrienoic acid incorporation and simultaneous desaturation to arachidonic acid by the HTC cells. It is evident that the fatty acid incorporation follows a hyperbolic curve and that the rate of incorporation declines after 12 hr. However, the simultaneous desaturation of the [1-^{14}C]eicosatrienoic acid has a rather constant rate during the entire 24 hr period.

The effect of substrate concentration and amount of cells incubated on the desaturation of [1-^{14}C]eicosatrienoic acid is shown in Figure 5. At the low fatty acid concentration incubated per mg of cellular protein, the

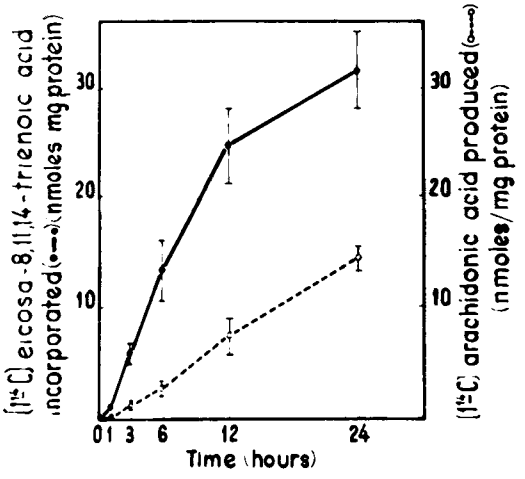

FIG. 4. Effect of incubation time on eicosa-8,11,14-trienoic acid incorporation and desaturation to arachidonic acid. 0.6 mg of cellular protein of hepatoma 7288 C (HTC) cells were incubated with 80 nmoles of [1-^{14}C]eicosa-8,11,14-trienoic acid in the conditions described in methods. Values are the means ± SE of 3 incubation flasks.

arachidonic acid produced increased linearly with the amount of acid incorporated per mg of cellular protein independently of cell concentration. It is evident that in these conditions the Δ5 desaturation capacity of the cells is not yet saturated by the substrate. To reach this saturation, it was necessary to increase the concentration of [1-^{14}C]eicosatrienoic acid incubated (Fig. 6). By comparison of Figure 6 with Figure 3, it is shown that the saturation of the desaturation reaction converting [1-^{14}C]eicosatrienoic acid to arachidonic acid was achieved

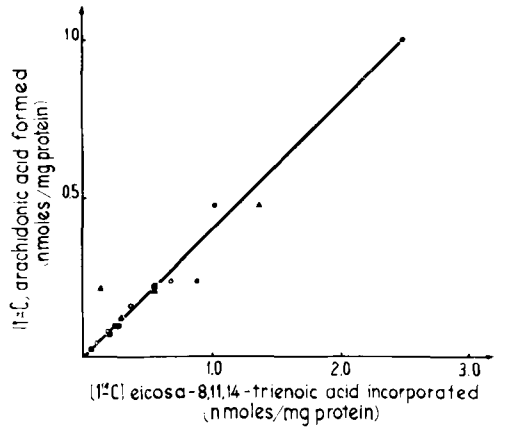

FIG. 5. Incorporation and desaturation of [1-14C]eicosa-8,11,14-trienoic acid to arachidonic acid. Different concentrations of hepatoma 7288 C (HTC) cells were incubated with the labeled acid during 24 hr at 37 C in the conditions described in the text. Cellular protein per flask; (●) = 0.6 mg; (▲) = 1.23 mg; (○) = 2.66 mg; and (■) = 3.02 mg.

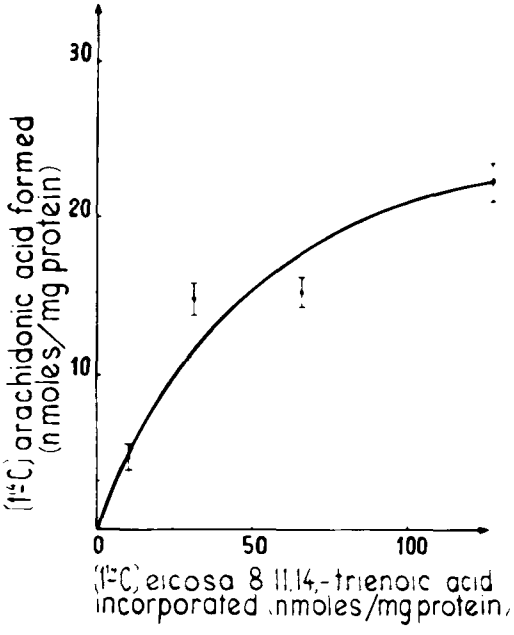

FIG. 6. Desaturation of [1-14C]eicosa-8,11,14-trienoic acid as a function of substrate concentration. Hepatoma 7288 C (HTC) cells (0.6 mg cellular protein) incubated as in Figure 3 with increasing concentrations of the labeled acid also were used to measure the conversion to arachidonic acid. Values are means ± SE of 3 incubation flasks.

apparently when the acid incorporated into the cells reached the plateau. This plateau corresponded to ca. 145 nmoles of labeled acid

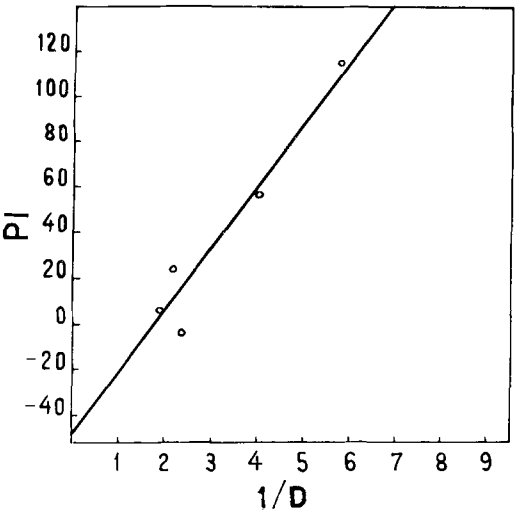

FIG. 7. Relationship between [1-14C]eicosa-8,11,14-trienoic acid incorporated per mg of cellular protein (PI) and fraction of PI desaturated (D) in hepatoma 7288 C (HTC) cells. Data of Figure 6 plotted according to equation 2 in the text, so that the interception gives the value for the endogenous pool (PE) size in nmoles of eicosa-8,11,14-trienoic acid desaturated per mg of cellular protein and the slope gives the nmoles of eicosa-8,11,14-trienoic acid desaturated per mg of cellular protein (K).

incorporated per mg of cellular protein. The total amount desaturated was ca. 23 nmoles of labeled eicosatrienoic acid per mg of cellular protein.

However, it is obvious that the cell is not only desaturating the exogenous labeled acid, but is also converting the endogenous pool. Therefore, it is important to measure, if possible, the whole desaturating capacity of the cell that may be related to precise metabolic situations. At the same time, it is also very important to measure the endogenous pool of the acid available for desaturation. Fulco (12) has shown in his work on palmitate incorporation and desaturation by the *B.megaterium* that it is possible to estimate the total desaturation of this acid, as well as the endogenous pool in the bacteria, by application of a simple equation. The equation used, adapted to our units, is:

$$K = (D)(PI + PE) \qquad (I)$$

where K = nmoles of acid desaturated per mg of cellular protein, D = the fraction of radioactive acid desaturated, PI = nmoles of [1-14C]acid incorporated per mg of cellular protein, and PE = nmoles of acid per mg of cellular protein in the endogenous pool available for desaturation.

Rearranging this equation in the same way as Fulco (12), it gives:

$$PI = K(1/D) - PE \quad (II)$$

If K and PE are constants, the equation is linear. When we took the data from Figure 6 and plotted PI versus 1/D, a straight line was obtained (Fig. 7). By application of statistical analysis, K and PE were calculated from the slope and intercept of the curve.

An endogenous pool size (PE) of 48.8 nmoles and a constant desaturation level of 27.2 nmoles of eicosa-8,11,14-trienoic acid per mg of cellular protein were obtained. This last figure is not very different from the 23 nmoles converted by the cells when they were saturated with the acid (Fig. 6). Obviously, these results may be considered as approximations and we cannot assure yet that they are real. However, this methodology will be tested with other fatty acids and it may be extremely important to study the effects of different regulatory factors on fatty acid incorporation and desaturation in HTC cells.

Eicosa-8,11,14-trienoic acid is generally found only in traces in HTC cells (1). Therefore, the existence of an endogenous pool, available for desaturation, of ca. 48.8 nmoles per mg of cellular protein is at first sight relatively large. It could possibly mean that this pool is very specific for desaturation and the lipids that incorporate this acid have a rapid turnover.

In previous work (1) it was shown that HTC cells incubated with 1-^{14}C linoleic acid incorporated the acid, but very little radioactivity was found in arachidonic acid. In the present work, it is clearly shown that the difficulty of HTC cells to convert exogenous 1-^{14}C linoleic acid to arachidonic acid is not due to the absence or low activity of the Δ5-desaturase. Because the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid is the last step in the biosynthesis of arachidonic acid from linoleic acid, the problem of the cell must reside in a step of the biosynthetic chain prior to this reaction. Dunbar and Bailey (13), confirmed these results and showed that a series of heteroploid cells did not Δ6-desaturate linoleic acid, but did Δ5-desaturate eicosa-8,11,14-trienoic acid. They concluded that the cells suffered a loss or modification of the Δ6-desaturase. However, Alaniz, et al., (1) and this work show that HTC cells apparently have an active Δ6-desaturation of α-linolenic acid and the corresponding elongating enzymes, as they readily convert α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid and eicosa-5,8,11,14,17-pentaenoic acid (1).

All the information gathered until now establishes that the same Δ6-desaturase desaturates oleic, linoleic, and α-linolenic acids (14). For this reason, it is difficult to think that in HTC cells there are different Δ6-desaturases for the different fatty acids, and the same may be admitted for the elongating enzymes (15). The Δ6-desaturase is a very important regulatory step in fatty acid biosynthesis (16). Therefore, although the inhibition of the synthesis of arachidonic acid must very probably occur at the level of the Δ6-desaturase, it is so specific that it only decreases linoleic acid desaturation and not α-linolenic conversion to octadeca-6,9,12,15-tetraenoic acid. It is still difficult to establish the mechanism of the inhibition, but it may play an important role in living cells and tissues.

ACKNOWLEDGMENTS

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Stereospecific Analysis of Some Cruciferae Species

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ABSTRACT

Results of stereospecific analysis of nine Cruciferae seed oils are presented. The major fatty acids in the oils investigated are nonrandomly distributed, not only between inner and outer positions, but also between sn-1 and sn-3 positions. When the positional data were plotted versus erucic acid content, a regular and characteristic distribution pattern for each fatty acid was obtained. Category I acids (saturated acids and acids with more than 18 carbon atoms) prefer either of the outer positions. Erucic acid, however, has a clear preference for the sn-3 position. When in the outer positions, Category II acids (unsaturated acids with 18 carbon atoms) as a rule prefer the sn-1 to the sn-3 position. This tendency increases with decreasing unsaturation.

INTRODUCTION

Procedures for stereospecific analysis of triacylglycerols have been available since 1965 (1,2). Before that date, a restricted positional analysis had been used which could only distinguish between the sn-2 and the combined sn-1 and sn-3 positions (α- and β-positions) (3,4).

Several results from analyses of triacylglycerols of Cruciferae species with respect to sn-2 and the combined sn-1,3 positions have been published (5,6). The fatty acid distribution between the sn-1 and sn-3 positions, however, has been investigated only by Brockerhoff and Yurkowski (7) and by Podlaha and Töregård (8). Brockerhoff and Yurkowski analyzed a single sample of *Brassica napus;* Podlaha and Töregård studied four different samples of *Brassica napus* and *B. campestris.* The results showed a clearly non-random distribution between the sn-1 and sn-3 positions for most major acids.

This paper presents further results of stereospecific analysis of the Cruciferae species.

MATERIALS AND METHODS

Seeds were obtained from the Swedish Seed Association (Svalöv, Sweden). The oil was cold-pressed from the grains and the triacylglycerol

TABLE I

Fatty Acid Composition in Cruciferae Seed Oils

Species and variety	Composition (mole %)							
	16:0	18:0	18:1	18:2	18:3	20:1	22:1	Others
Brassica napus, v. 'Oro'	4.8	1.2	62.6	18.9	9.5	1.6	0.6	0.3
Brassica napus, v. 'Sinus'	5.0	1.0	45.2	18.1	11.1	8.0	10.7	0.9
Brassica napus, v. 'SV71-6'	4.2	1.3	29.0	17.2	10.9	14.0	21.5	1.6
Brassica campestris, v. 'Bele'	2.6	0.8	27.8	16.7	9.9	11.2	27.8	2.7
Brassica napus, v. 'Gulle'	4.4	0.9	19.6	15.2	10.8	13.8	33.6	2.1
Brassica campestris, v. 'Duro'	2.2	0.8	15.6	14.5	10.6	10.1	43.4	3.1
Brassica napus, v. 'Panter'	3.3	0.7	11.7	13.7	10.6	7.1	50.2	2.4
Sinapis alba	3.4	0.9	26.8	9.1	11.9	9.7	35.2	3.6
Crambe abyssinica	2.1	0.6	17.4	9.2	7.7	2.5	56.6	3.8

TABLE II

Stereospecific Analysis of 1,2-Dipalmitoyl-3-myristoyl-glycerol

	C14:0			C16:0		
	Mole %	Theoretical	SD[a]	Mole %	Theoretical	SD[a]
sn-1 Position	3.7	0	0.5	96.3	100	0.5
sn-2 Position	1.9	0	0.2	98.1	100	0.2
sn-3 Position	99.5	100	0.5	0.5	0	2.0

[a]SD = Standard deviation.

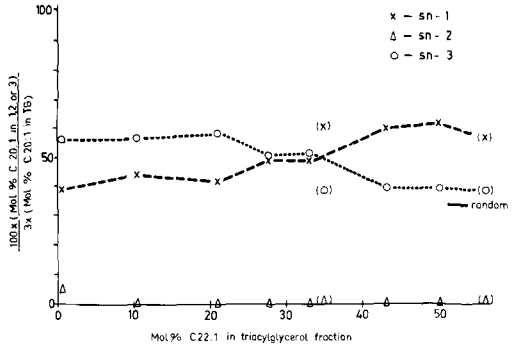

FIG. 1. Distribution of gadoleic acid between the three positions. TG = triacylglycerols.

FIG. 3. Distribution of oleic acid between the three positions.

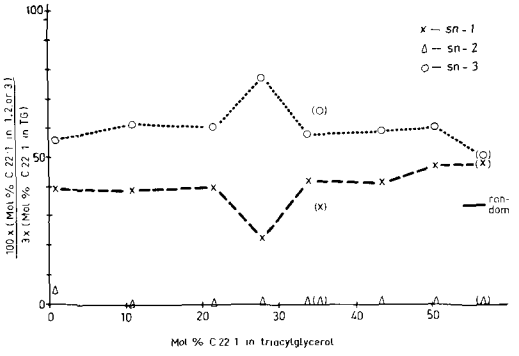

FIG. 2. Distribution of erucic acid between the three positions.

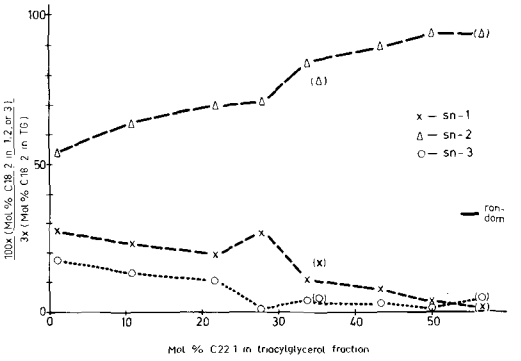

FIG. 4. Distribution of linoleic acid between the three positions.

fraction isolated by chromatography on silicic acid column. Seed materials investigated and the fatty acid composition of their triacylglycerols are presented in Table I.

Cleavage with ethylmagnesiumbromide was performed principally according to Brockerhoff's procedure (1,2) adapted for mg quantities by Christie and Moore (9) and Åkesson (10). The same day thin layer chromatography (TLC) separation of diacylglycerols and synthesis of phosphatidylphenols was carried out.

The next day, the phosphatidylphenols were isolated according to the procedure of Åkesson (10) and hydrolysis with phospholipase A (9) was started. Ether was evaporated with a stream of nitrogen the following morning. Thereafter, 1 ml of water and 2.5 ml chloroform:methanol mixture (2:1) were added, and extraction of lipids and isolation of the reaction products by TLC then were performed according to Åkesson (10).

The zones of interest on the TLC-plate were removed and the lipids transesterified with BF_3 immediately on silica gel adsorbent without previous extraction.

Pancreatic lipase hydrolysis was carried out according to Luddy's procedure for 50 mg samples (3).

Gas chromatography analyses were performed on a Varian A 2100 gas chromatograph equipped with a 6 ft x 2 mm internal diameter (ID) glass column packed with 6% butanediol succinate (BDS) on Anakrom ABS 110/120. Peak areas were integrated with a Hewlett-Packard, 3370 A Integrator.

RESULTS AND DISCUSSION

Table II shows results obtained from the analysis of the synthetized triacylglycerol, sn-1,2-dipalmito-3-myristin. If we accept that the triacylglycerol has that theoretical composition, the figures indicate that the method is slightly inaccurate, particularly for the sn-1 position. The precision is fair and this complicated and laborious method can, on the whole, be regarded as satisfactory.

Every actual stereospecific analysis was checked by calculating the fatty acid (FA) composition in position sn-3 in two independ-

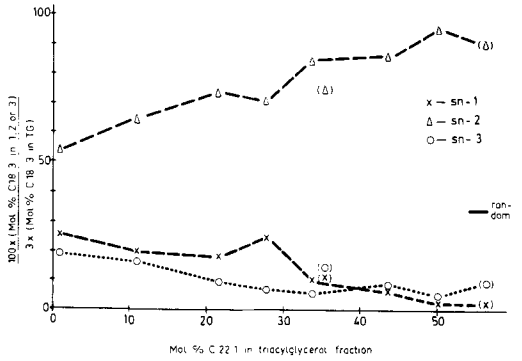

FIG. 5. Distribution of linolenic acid between the three positions.

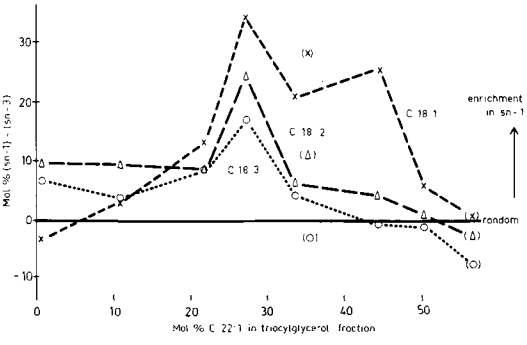

FIG. 6. Distribution differences between sn-1 and sn-3 positions for unsaturated C18-acids.

ent ways (from 1-acyl-3-lysophosphatidylphenols and from 2,3-diacyl-1-lysophosphatidylphenols). Results which showed differences > 2 mole % were rejected.

Figures 1-5 show the distribution pattern for the major fatty acids among the sn-1, sn-2 and sn-3 positions in the investigated seed oils as a function of their erucic acid content. The y-axis represents the percent distribution in moles of the fatty acids between the three triacylglycerol positions. Thus, a random distribution of an acid is indicated by 33.3% in each of the three positions. More than 33.3% is an indication of an enrichment in that particular position and anything less than 33.3%, the opposite. The points within brackets represent the non-*Brassica* species.

Because of the relatively large method error for such small components as palmitic and stearic acids, no significant difference between the sn-1 and sn-3 positions can be stated. However, they are strongly accumulated in the outer positions (not shown in figure).

Gadoleic acid has the same strong preference for the outer positions. However, between these positions it appears to be a systematic distribution pattern in that the preference for sn-3 is changed to a preference for sn-1 when the erucic acid content in triacylglycerols rises above ca. 30 mole %.

Erucic acid is almost completely concentrated at the outer positions, except for the very low erucic acid species. The concentration at the sn-3 position is clearly evident for the *Brassica* and *Sinapis* species, while for *Crambe* the distribution between sn-1 and sn-3 is symmetrical. The saturated C16, C18, and the C20:1, C22:1 fatty acids (Category I acids) are, according to the Evans distribution hypothesis (11), first esterified at the outer positions. According to the same theory, the unsaturated C18-acids (Category II acids) then are distributed on the remaining positions.

Oleic acid is concentrated on the inner position. The distribution between the sn-1 and the sn-3 positions is non-random and correlated to the erucic acid content. Oleic acid begins to be unequally distributed between sn-1 and sn-3 with erucic acid content in triacylglycerols above 10 mole %, and again fairly equally distributed when the erucic acid reaches the highest levels. The sn-1 position is preferred, in some instances very strongly.

Linoleic acid, mainly in the inner position, also shows random distribution between the outer positions in species high in erucic acid, but affinity for the sn-1 position in the others. Linolenic acid has the same overall distribution pattern as linoleic acid, but the asymmetry is not so pronounced.

A survey of the positional distribution of the Category II acids over sn-1 and sn-3 is presented in Figure 6. The curves represent the deviations from random distribution, using mole % at sn-1 minus mole % at sn-3. It is clearly evident that the non-random distribution decreases with increasing unsaturation.

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Hepatoma, Host Liver, and Normal Rat Liver Phospholipids as Affected by Diet

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ABSTRACT

Individual phospholipid classes derived from hepatoma, host liver, and normal liver of rats maintained on chow and fat free diets were examined in detail and the sphingomyelin and phosphoglyceride structures compared. The concentration of hepatoma sphingomyelin was higher while phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and diphosphatidylglycerol were only one-fourth to one-half normal liver concentrations, irrespective of diet. Hepatoma phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol contained higher percentages of 18:1 and, except phosphatidylinositol, much lower percentages of most polyunsaturated fatty acids than liver. The 1-position of host liver phosphatidylcholine and phosphatidylethanolamine, normal liver phosphatidylcholine and phosphatidylethanolamine, and hepatoma phosphatidylcholine from animals on both diets had the same approximate fatty acid composition, but the percentage of 16:0 in hepatoma phosphatidylethanolamine was reduced dramatically. The low percentage of 16:0 at the 1-position of both phosphatidylethanolamine and triglycerides suggests that the 1-position fatty acids of these two classes may have a similar origin. The fat free diet reduced the percentage of 18:2 in liver diphosphatidylglycerol 3-fold and the decrease was offset by increased percentages of 16:1 and 18:1; whereas the very low percentage of 18:2 in hepatoma diphosphatidylglycerol was offset by increased percentages of 18:0 and 16:0. Liver phosphatidylinositol and phosphatidylcholine from the animals fed the fat free diet contained the highest percentage of 20:3, which replaced 20:4. Hepatoma sphingomyelin contained a much higher concentration of 24:0 and 24:1 than liver. The hepatoma sphingomyelin also contained a C-24 dienoic acid, which was not detected in host and normal liver. Host liver contained a higher percentage of 22:6 than normal liver. The diglycerides derived from host liver PC contained a significantly higher percentage of carbon number 38 than normal liver. Diglycerides derived from hepatoma phosphatidylcholine and phosphatidylethanolamine exhibited a 1-random-2-random distribution of fatty acids, whereas diglycerides from liver phosphatidylcholine and phosphatidylethanolamine showed pairing of specific fatty acids.

INTRODUCTION

The occurrence of hyperlipemia and loss of body lipid in tumor bearing animals (1) indicate that lipid metabolism in the host is affected by the tumor. On the other hand, the correlation between dietary fat and the incidence of some tumors (2-4) suggest that host lipids may affect the tumor. The few scattered reports that describe the change or lack of change that occurs in the lipids of tumor bearing animals have been reviewed in the first paper of this series (5). This is the third in a series of papers that describes the results of experiments designed to investigate the relationship between host and tumor lipid metabolism. The data reported here compare quantitatively the phospholipids derived from hepatoma 7288CTC, normal rat liver, and host liver of animals maintained on normal and fat free diets. Preliminary reports of these studies have appeared (6,7).

METHODS AND MATERIALS

Hepatoma 7288CTC, livers from normal rats, and livers of rats bearing hepatoma 7288CTC (denoted throughout this report as host liver) were obtained from groups of three or more animals (175-225 g) maintained on chow and fat free diets for four to five weeks as described previously (5). Lipids were extracted by the Bligh and Dyer procedure (two extractions) (8), fractionated into neutral lipid and phospholipid fractions by silicic acid chromatography (9), and weighed. Phospholipids were resolved into individual classes by thin layer chromatography (TLC) (10) and quantified by the phosphorus method of Rouser, et al., (11). Individual phospholipid classes on preparative chromatoplates were visualized under UV light after spraying with 2′,7′-dichlorofluorescein or Rhodamine

TABLE I

Comparison of Phospholipid Class Percentages Derived from Normal Rat Liver, Host Liver, and Hepatoma of Rats Maintained on Normal and Fat Free Diets

Hepatic tissue	Phospholipid concentrations (mg/g wet wt)	Phospholipid class percentages[a]								
		Origin	Lyso-PC	SPH	PC	PI	PS	X[b]	PE	DPG + SF
Rat liver, normal, chow diet	36.6 ± 2.2	1.2 ± 0.4	3.2 ± 0.8	3.1 ± 0.2	42.4 ± 2.9	9.9 ± 0.7	4.0 ± 0.6	4.3 ± 0.2	24.4 ± 2.4	7.5 ± 2.0
Rat liver, normal, fat free diet	31.4 ± 2.8	1.2 ± 0.7	3.0 ± 0.2	3.2 ± 0.4	43.0 ± 2.6	11.6 ± 0.8	4.9 ± 0.6	3.1 ± 0.8	21.2 ± 1.1	8.8 ± 1.3
Rat liver, hepatoma host, chow diet	36.2 ± 1.9	1.1 ± 0.4	2.4 ± 0.4	4.6 ± 0.2	49.2 ± 3.4	9.3 ± 1.2	4.8 ± 1.6	4.4 ± 0.9	22.3 ± 2.0	5.3 ± 0.3
Rat liver, hepatoma host, fat free diet	17.0	0.9	2.3	4.0	42.5	7.7	5.0	-	32.2	4.5
7288CTC hepatoma chow fed host	14.2 ± 1.3	1.4 ± 0.9	2.6 ± 0.2	14.5 ± 0.2	37.3 ± 1.3	9.1 ± 0.2	5.9 ± 0.4	-	22.9 ± 1.1	4.7 ± 0.3
7288CTC hepatoma host fed fat free diet	10.9	1.3	3.2	17.7	38.6	9.2	5.3	-	19.0	5.7

[a]Lyso-PC = Lysophosphatidylcholine; PC = phosphatidylcholine; SPH = sphingomyelin; PI = phosphatidylinositol; PS = phosphatidylserine; X = tentatively identified as N, N-dimethyl phosphatidylethanolamine; PE = phosphatidylethanolamine; DPG = diphosphatidylglycerol; SF = solvent front. Values without standard deviations represent the mean of duplicate analysis of a single pooled sample from 3 animals, whereas values accompanied by standard deviations represent the mean of duplicate determinations on each of the 3 animals of the group. Percentages are based on phosphorous analysis and have not been corrected for small differences in mol wts of the various classes.

[b]Tentatively identified as N, N-dimethyl phosphatidylethanolamine. This compound was present in the phospholipid of the fourth row but was not observed in the hepatoma phospholipids.

6G. The localized bands were scraped from the chromatoplate, eluted from the absorbent, and checked for purity. Samples that were not >95% pure were repurified by chromatography. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were subjected to phospholipase A hydrolysis as described previously (12) to obtain the fatty acid compositions of the 1- and 2-positions.

The fatty acids released by hydrolysis of PC and PE, the lysophosphatides, and other phospholipid classes were converted to methyl esters and analyzed by gas liquid chromatography (GLC) on polar columns as described earlier (13). PC and PE were hydrolyzed to diglycerides with *Clostridium welchii* phospholipase C (14), diglyceride acetates were prepared by the p-toluenesulfonic acid catalyzed acetylation procedure (15), hydrogenated, and analyzed intact by high temperature GLC (13).

Diglyceride and fatty acid percentages represent the mean of two or more determinations on the pooled sample for each tissue and dietary condition. Agreement between determined percentages was usually ±5% for major components and ±10% for minor components. Identities of fatty acids were based upon cochromatography with standards before and after hydrogenation. Positions of double bond in C-16, C-18, and C-20 monoenes were determined and are reported in a companion paper.

Crotalus atrox venom (phospholipase A) was purchased from Ross Allen's Reptile Institute (Silver Springs, FL). Phospholipase C (*Cl. welchii*) was obtained from Calbiochem (LaJolla, CA). Diglyceride and phospholipid standards were supplied by Applied Science Lab (State College, PA) and Supelco, Inc. (Bellefonte, PA). All solvents were glass distilled and obtained from Burdick and Jackson Labs (Muskegon, MI).

RESULTS

Class Composition

The percentages of major phospholipid classes from hepatoma, host liver, and normal liver of animals maintained on chow and fat free diets are given in Table I. The class compositions of normal and host livers were essentially the same, and diet had little or no effect. Hepatoma contained less PC and much higher percentages of sphingomyelin than liver, and, as in the case of liver, diet had no effect on the hepatoma phospholipid class composition.

Concentrations (mg/g wet wt) of the various phospholipid classes can be calculated from the total phospholipid values given in the first column of Table I. Most phospholipid classes from normal livers of animals on both diets and from host livers of chow fed rats exhibited the same approximate concentrations, but host livers from rats fed the fat free diet contained ca. 50% or less PC, phosphatidylinositol (PI), phosphatidylserine (PS), and diphosphatidylglycerol (DPG). Hepatoma sphingomyelin concentrations were higher than those of liver, whereas all other phospholipid class levels were lower. Hepatoma PC and PE concentrations were only 25 to 50% of liver levels.

Positional Distribution of Fatty Acids

The fatty acid composition of the 1- and 2-positions of PC and PE derived from hepatoma, host liver, and normal liver of rats maintained on chow and fat free diets is given in Table II. The 1-position of all hepatic tissue PC exhibited the same composition: 16:0, 41 ± 2.8% and 18:0, 56.6 ± 4%. Host and normal liver PE were similar at the 1-position and contained primarily palmitate and stearate. In sharp contrast, the 1-position of hepatoma PE, affected little by diet, contained only 25 to 35% as much palmitate as liver PE. The decrease in palmitate was offset by an increase in stearate percentages.

Polyunsaturated fatty acids dominated the 2-position of PC and PE of livers but not the hepatoma. Generally, the 2-position of PC in host and normal liver from animals on the same diet was similar. This was also true in the case of PE. The exception to this generalization was the usually higher percentages of 18:2 and 22:6 observed in host livers. The fat free diet reduced 18:2 and 20:4 and increased 20:3, 18:1, and 16:1 percentages at the 2-position of liver and hepatoma PC. Hepatoma PC exhibited elevated percentages of 16:0 and 18:1 at the 2-position compared to liver PC. The 2-position of all PE samples contained twice as much 18:0 and, with the exception of hepatoma, less than one-half the percentage of 18:1 as the corresponding position of PC. The fat free diet reduced 18:2 percentages at the 2-position of liver and hepatoma PE, but 20:4 and 18:1 percentages showed little change. Hepatoma PE contained elevated 18:1, and reduced 20:4 and 22:6 percentages relative to the 2-position of liver PE.

Fatty Acid Composition of PS, PI and DPG

The fatty acid compositions of PS, PI, and DPG isolated from hepatoma, host liver, and normal liver of rats maintained on chow and fat free diets are shown in Table III. DPG used for these compositional analyses was purified by rechromatography in a less polar solvent system (12). All hepatic tissue PS was characterized by

TABLE II

Distribution of Fatty Acids at the 1- and 2-Positions of Phosphatidylcholine and Phosphatidylethanolamine Derived from Normal Rat Liver, Host Liver, and Hepatoma of Animals Maintained on Normal and Fat Free Diets

	Fatty acid percentages[a]											
	2-Position									1-Position		
Hepatic tissue	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:5	22:6	16:0	18:0	18:1
						Phosphatidylcholine						
Liver, normal, chow diet	7.8	0.7	8.6	14.8	31.8	0.9	29.1	T[b]	4.2	43.4	52.6	1.0
Liver, normal, fat free diet	8.0	4.9	9.0	32.3	8.6	11.5	21.8	1.8	2.0	38.8	60.2	0.8
Liver, host, chow diet	10.1	0.5	7.9	17.6	33.0	0.5	23.3	T	6.0	43.0	55.5	T
Liver, host fat free diet	8.9	1.8	7.7	25.9	15.7	6.6	22.7	T	6.8	37.4	62.3	T
Hepatoma, chow diet	17.2	1.0	5.0	36.3	26.5	T	11.1	T	1.0	43.8	52.2	1.2
Hepatoma, fat free diet	16.4	3.2	5.5	48.8	12.4	2.5	8.5	T	0.9	39.0	57.0	1.3
						Phosphatidylethanolamine						
Liver, normal chow diet	6.0		11.8	8.8	15.7		42.4	2.1	12.3	40.0	56.2	1.1
Liver, normal fat free diet	10.0	0.8	17.2	10.7	1.6	5.0	42.7	4.9	6.5	34.1	65.0	0.8
Liver, host chow diet	18.6		19.8	6.1	13.2		28.5	0.7	12.4	37.8	61.4	T
Liver, host fat free diet	8.1	T	11.6	5.6	4.0	4.7	42.5	2.4	20.8	28.4	70.9	0.6
Hepatoma, chow diet	8.4	0.8	14.5	34.2	18.1	1.0	14.4	T	2.4	9.6	85.0	3.7
Hepatoma, fat free diet	7.4	0.7	13.8	47.0	9.5	1.3	14.2		2.5	9.0	78.2	11.0

[a]Percentages represent the mean of two or more analyses. The differences between the sum of the percentages in any row at either position and 100% represents the sum of other acids present in small amounts but not given in the table.

[b]T = Detectable levels of < 0.5%.

TABLE III

Fatty Acid Composition of Phosphoglycerides Derived from Rat Liver, Host Liver, and Hepatoma Tissue of Animals Maintained on Normal and Fat Free Diets

Hepatic tissue	Fatty acid percentages[a]							
	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:6
Phosphatidylserine								
Liver, normal, chow diet	6.3		66.9	2.8	1.1		18.3	3.6
Liver, normal, fat free diet	6.4		61.9	6.2	T	1.2	20.7	1.8
Liver, hepatoma host, chow diet	5.4		45.8	3.2	3.9		24.2	15.8
Liver, hepatoma host, fat free diet	4.3		65.3	5.5	0.7	1.7	14.6	3.7
Hepatoma 7288CTC, chow diet	3.5		47.6	29.7	6.6		4.4	1.9
Hepatoma 7288CTC, fat free diet	4.5	T[b]	53.4	24.4	1.7	1.4	2.4	T
Phosphatidylinositol								
Liver, normal, chow diet	7.9		56.7	2.7	1.7		29.9	
Liver, normal, fat free diet	4.7		63.3	1.6		11.0	19.4	
Liver, hepatoma host, chow diet	5.7		45.8	2.2	3.6	1.1	37.0	3.7
Liver, hepatoma host, fat free diet	4.7		65.3	3.2	0.6	11.5	12.6	1.7
Hepatoma 7288CTC, chow diet	3.2		47.6	15.7	7.9		21.3	1.3
Hepatoma 7288CTC, fat free diet	4.3		53.4	15.4	3.4	4.1	16.2	1.0
Diphosphatidylglycerol								
Liver, normal, chow diet	3.9	2.1	1.8	16.2	75.9			
Liver, normal, fat free diet	5.6	15.5	1.8	45.4	25.4	4.5	1.7	
Liver, hepatoma host, chow diet	6.1	1.0	3.3	14.3	70.6		1.0	0.9
Liver, hepatoma host, fat free diet	7.2	10.8	7.8	31.1	33.5	2.6	3.7	1.5
Hepatoma 7288CTC, chow diet	16.0	1.2	25.1	26.0	16.8		7.3	4.3
Hepatoma 7288CTC, fat free diet	13.6	0.9	35.8	29.1	7.1		6.1	T

[a]The difference between the sum of the percentages in any row and 100% represents the sum of other fatty acids present in small amounts and not given in the table. The phospholipid classes from the hepatomas of animals on both diets contained 1.0-4.0% of each of the following acids: 20:1, 20:2, and 22:4. Normal liver and host liver did not contain more than trace levels of these acids.

[b]T = Detectable levels of $< 0.5\%$.

a high level of stearate. Hepatoma PS exhibited a severalfold increase in the percentage of 18:1 and a much decrease percentage of 20:4 relative to liver. The effects of the fat free diet were marginal in both hepatoma and liver PS. Host liver PS contained an increased percentage of 22:6.

Both hepatoma and liver PI, like PS, contained a high percentage of 18:0. Hepatoma PI 18:1 was severalfold higher than liver; but, unlike hepatoma PS, the percentage of 20:4 was not too different from liver. The fat free diet reduced 18:2 and 20:4 percentages in liver and hepatoma PI and elevated the percentage of 20:3. Again, as in PS, the percentage of some polyunsaturated fatty acids increased in the host liver PI.

High levels of 18:2 characterized the DPG of host and normal liver, but not the hepatoma. The percentage of 18:2 was reduced 55 to 65% in both host and normal liver DPG of animals fed the fat free diet relative to chow fed animals. The 18:2 was replaced with 16:1 and 18:1. Only 18:2 and 18:0 of hepatoma DPG responded to the fat free diet. Hepatoma DPG contained between 40 and 50% saturated fatty acids, whereas normal liver DPG contained 6-8% and host liver 10-15%.

The fatty acid composition of sphingomyelin from hepatoma and liver as affected by diet as shown in Table IV. Generally, the effects of the fat free diet on hepatic tissue sphingomyelin fatty acid composition were minimal. Palmitic acid percentages increased in host liver sphingomyelin relative to normal liver. Lignoceric acid percentages were lower in host liver sphingomyelins than in normal liver. The percentage of nervonic acid in hepatoma sphingomyelin was approximately double that of liver. Hepatoma sphingomyelin contained a C-24 dienoic acid which was not detectable in liver sphingomyelin.

Carbon Number Distributions of PC and PE Diglycerides

The carbon number distributions of diglycerides derived from PC and PE of hepatoma, host liver, and normal liver from animals fed chow and fat free diets are given in Table V. 1-random-2-random distribution data calculated with fatty acid percentages from Table II are also given in Table V for comparison. Diglycerides from normal liver PC of animals fed the fat

free diet contained a higher percentage of lower mol wt species than liver PC of chow fed animals. Host liver PC of both chow and fat free diet fed rats contained a higher percentage of higher mol wt species than either normal liver PC or hepatoma PC. The carbon number distribution of hepatoma PC, unaffected by diet, agreed more closely with normal liver PC percentages than with host liver PC percentages. Determined and calculated carbon number percentages of liver PC did not agree; however, a rather close agreement was observed for hepatoma PC.

The carbon number distribution of diglycerides derived from PE was dramatically different from PC diglycerides of both liver and hepatoma. In contrast to liver PC, the fat free diet caused an elevation in the higher mol wt species of both normal and host liver PE relative to chow fed noraml and host liver PE. Hepatoma PE, unaffected by diet, exhibited a carbon number distribution very different from liver PE. As observed for liver PC, the determined and calculated carbon number distributions for liver PE were not comparable. In contrast, hepatoma PE showed agreement between determined and calculated carbon number percentages.

DISCUSSION

Class Composition

Phospholipid class percentages were affected little by feeding the fat free diet or by the presence of the hepatoma. This is in general agreement with previously reported data (12, 16-18). The combination of hepatoma and fat free diet reduced host liver phospholipid mass dramatically (5), and not all classes were affected equally. Both percentage and mass data showed an elevation of hepatoma sphingomyelin. Bergelson, et al., (17) have shown that hepatoma 27, hepatoma 22, zajdela ascites hepatoma, and cellular fractions from these neoplasms contained higher percentages of sphingomyelin than liver. Transformed diploid cells grown in culture (19), cultured hepatoma cells (10), and a number of transplantable rat and mouse tumors (20) also have been shown to contain higher percentages of sphingomyelin than nontransformed cells and normal liver. Decreases in the other hepatoma phospholipid classes relative to normal liver may be more significant than the elevated concentrations of sphingomyelin. A number of different neoplasms examined show lower levels of phospholipids than do normal tissues, whether expressed on the basis of wet wt, protein, or total nitrogen (18,19,21). This decreased concentra-

TABLE IV

Fatty Acid Composition of Sphingomyelin from Normal and Neoplastic Tissue as Affected by Diet

Hepatic tissue	Sphingomyelin concentrations (mg/g wet wt)	Fatty acid percentages[a]								
		16:0	18:0	20:0	22:0	23:0	23:1	24:0	24:1	24:2[b]
Liver, normal, chow diet	1.1	15.4		1.5	11.1	11.9	5.2	32.3	12.2	
Liver, normal, fat free diet	1.0	20.2	16.4	1.8	10.6	6.5	4.5	27.9	11.8	
Liver, hepatoma host, chow diet	1.7	31.3	9.8	1.1	8.6	8.3	1.4	23.0	15.8	
Liver, hepatoma host, fat free diet	0.7	30.6	16.1	2.2	10.7	5.3	1.0	17.3	15.6	
Hepatoma 7288CTC, chow diet	2.1	33.5	5.6	T[c]	4.2	1.0		25.9	27.5	2.5
Hepatoma 7288CTC, fat free diet	1.9	22.7	5.1	0.7	6.9	0.7		30.3	28.1	2.2

[a]The difference between the sum of the percentages of any row and 100% represents minor amounts of other fatty acids not given in the table.

[b]Because of the small quantity of this acid, its absolute identity was not established in the hepatoma.

[c]T = Detectable quantities but < 0.5% of the total.

TABLE V

Comparison of Determined and Calculated Carbon Number Distributions of Diglyceride Acetates Derived from Normal Liver, Host Liver, and Hepatoma of Animals Maintained on Normal and Fat Free Diets

Hepatic tissue	Origin of data[b]	Carbon number percentages[a]						
		30	32	34	36	38	40	42
				Phosphatidylcholine				
Normal liver, chow diet	Determined		2.4	43.7	40.7	11.6	1.7	
	Calculated		3.8	29.3	44.0	18.6	2.7	T
Normal liver, fat free diet	Determined		12.6	48.9	32.7	5.8	T	
	Calculated		5.3	27.5	43.4	20.9	1.9	1.1
Host liver, chow diet	Determined		3.1	37.7	36.5	20.5	2.2	
	Calculated		4.5	31.4	43.5	16.6	3.7	
Host liver, fat free diet	Determined		3.1	27.7	33.8	30.0	5.3	
	Calculated		4.0	25.3	42.4	21.9	4.7	T
Hepatoma, chow diet	Determined	0.7	8.3	37.5	41.3	9.5	1.8	1.0
	Calculated		8.0	39.8	42.2	8.1	1.0	T
Hepatoma, fat free diet	Determined	0.4	6.6	36.4	42.7	11.0	1.9	1.0
	Calculated		6.6	37.5	46.2	8.2	0.8	T
				Phosphatidylethanolamine				
Normal liver, chow diet	Determined		1.6	19.2	30.8	46.9	1.6	
	Calculated		2.5	18.4	39.0	31.4	8.7	
Normal liver, fat free diet	Determined		2.3	8.2	25.5	61.5	2.6	
	Calculated		3.7	17.2	35.7	35.4	7.6	
Host liver, chow diet	Determined		1.4	16.0	27.9	49.7	5.0	
	Calculated		7.1	26.4	35.2	22.9	8.2	
Host liver, fat free diet	Determined		1.2	6.7	22.6	63.1	6.3	
	Calculated		2.4	12.0	28.6	40.4	16.5	
Hepatoma, chow diet	Determined		0.6	15.5	57.3	22.3	3.7	0.6
	Calculated		0.9	14.8	61.6	17.0	5.0	T
Hepatoma, fat free diet	Determined		0.8	17.0	61.6	17.9	2.1	0.6
	Calculated		1.2	17.2	62.8	15.1	4.0	

[a]The carbon atoms of acetate are not included in the carbon numbers.

[b]Calculated values were calculated from the determined abundance of each even carbon numbered fatty acid in the 1- and 2-positions (Table II) and the possible number of fatty acid combinations of each carbon number. A sample 1-random-2-random distribution calculation of carbon number 32 is given: 32=(16x16)+(14x18)+(18x14)x 100. Each multiplicand and multiplier represents a combination or permutation of the fatty acid chain lengths present that could give rise to carbon number 32. The multiplicand represents the 1-position and the multiplier the 2-position. Determined fatty acid percentages are substituted and the resulting sum of the products represents the 1-random-2-random distribution percentage of carbon number 32.

tion of phospholipids may be a characteristic of most neoplasms that has not been recognized because most data have been reported as relative percentages.

Phosphatidylcholine

Positional analysis of liver PC, the most studied phospholipid of hepatic tissue, confirms earlier reports (22-24) that indicated the 1-position, composed primarily of palmitate and stearate, was affected only slightly by diet. Likewise, the 1-position was not affected in hepatoma. The similarities in the composition of 1-position of PC in hepatoma and liver (Table II) have been reported in hepatoma 5123C (25), whereas other hepatomas (25,26) have elevated 18:1 percentages. The 2-position fatty acid percentages of normal and host liver PC from animals on both diets generally agreed well with reported percentages obtained from animals fed regular diets (22,25-28) and essential fatty acid deficient diets (22). The higher percentage of 16:0 and lower percentage of C-20 and C-22 polyunsaturated fatty acids at the 2-position of hepatoma PC relative to liver are in agreement with published data for other hepatomas (25,26) and Ehrlich ascites cells (14).

The similarities in the fatty acid composition at the 1- and 2-positions of normal and host liver PC of chow fed animals (Table II) might lead one to conclude that the hepatoma had no effect on host liver PC species, but comparison of determined diglyceride carbon number percentages (Table V) indicated that pairing of fatty acids yielding higher mol wt species had occurred. Higher mol wt species of PC in the host liver of the fat free fed animals was also observed. The lack of agreement between determined and calculated carbon number percentages for normal and host liver PC diglycerides illustrates, as shown previously (28), the preferential pairing of fatty acids. In contrast, the agreement between determined and calculated percentages of hepatoma PC diglycerides indicates lack of specificity in fatty acid pairing. The diglycerides derived from PC of Ehrlich ascites cells examined previously (14) also exhibited a 1-random-2-random distribution.

Phosphatidylethanolamine

The 1-position of liver PE was affected to a small degree by both diet and the tumor. 1-position percentages of liver PE from rats bearing Yoshida and Morris 5123 hepatomas (29) agreed well with the chow fed host liver PE percentages. Percentages previously reported for the 1-position of normal liver PE (28) were similar to the fat free fed–host liver PE percentages. Although the 1-position percentages for normal liver PE reported previously and the present percentages differ in the ratio of palmitate to stearate, it is of interest that in each case the 1-position compositions for PE and PC are similar. This similarity suggests that the fatty acids at the 1-positions of PC and PE arise from the same source. In contrast to the 1-position percentages, which were similar for liver and hepatoma PC, hepatoma PE contained only one-third to one-fourth the palmitate of liver PE. The low levels of palmitate at the 1-position of hepatoma PE (Table II) and hepatoma triglyceride (30) suggest that the 1-position fatty acids of PE and TG arise from the same source, whereas the 1-position fatty acids of normal rat liver PC and PE appear to arise from the same source. Ehrlich ascites cells (14), Yoshida hepatoma (29), and Morris 5123 hepatoma (29) all contained lower percentages of palmitate at the 1-position of PE than liver PE.

The level of C-20 and C-22 polyunsaturated fatty acids at the 2-position of hepatoma PE was much reduced in comparison to liver. This decreased level was offset by a rise in the percentage of 18:1, following the trend of two hepatomas (29) and Ehrlich ascites cells (14).

The molecular species of liver PE were affected differently than liver PC; normal liver PE from the animals fed the fat free diet contained an elevated percentage of higher mol wt species, and the hepatoma did not affect the host liver PE carbon number distribution. It is of interest that both hepatoma PC and PE diglycerides showed only very small peaks on the chromatograms that could have resulted from odd carbon numbered diglycerides or ether linked diglycerides. These data demonstrate that the high level of ether linked phospholipids found in some neoplasms (31) is not characteristic of all neoplasms.

Phosphatidylserine, Phosphatidylinositol, and Diphosphatidylglycerol

Liver and hepatoma PS and PI were characterized by a high percentage of stearate, as reported previously for rat liver (12) and Ehrlich ascites cells (32). The fat free diet had only marginal effects on the percentage of 20:4 in liver PS, whereas the percentage of 20:4 in liver PI was reduced. The reduced percentage of 20:4 in PI appeared to be replaced with 20:3. If the 20:3 acid was esterified at the 2-position of PI as was the case for PC, then PI contained the highest quantity of 20:3 per mole, followed by PC and DPG. The percentage of 20:4 in hepatoma PS was reduced dramatically, whereas the percentage of this acid in hepatoma PI was

within the range of liver values. Both PS and PI of Ehrlich ascites cells exhibited similar types of fatty acid profiles (30). The high percentage of 18:2, characteristic of liver DPG (12), was partially replaced with 16:1 and 18:1 fatty acids in liver of animals fed fat free diet. The percentage of 18:2 in hepatoma DPG, lower than liver DPG from animals fed the fat free diet, was not replaced with monoenoic acids but with stearate and palmitate.

Sphingomyelin

The fatty acid profile of liver sphingomyelin, characterized by a high percentage of C-22, C-23, and C-24 saturated and monoenoic fatty acids (32), was not greatly affected by the fat free diet. The lower percentages of 18:0, 20:0, 22:0, 23:0, and 23:1 in hepatoma sphingomyelin were replaced with a higher percentage 24:1 (Table IV). Actually, the percentages do not reflect the real pictures because of various tissue levels of sphingomyelin. Concentrations (mg/g wet wt) calculated from the data in Table IV show that 24:0 and 24:1 hepatoma sphingomyelin species greatly exceed liver concentrations. Hepatoma sphingomyelin also contained a C-24 dienoic acid shown previously to be present in Ehrlich ascites cells (32), cultured hepatoma cells (10), and a large number of transplantable rat and mouse tumors (20), but absent or present in only trace amounts of sphingomyelin of normal tissue (12,20,32).

Effect of Hepatoma on Host Lipids

Some phospholipid classes of host liver showed an increase in the percentage of 18:2 and 20:4 over normal liver percentages, whereas all host liver phospholipid classes, except sphingomyelin, showed an increase in the percentage of 22:6. The increased percentage of 22:6 would only be apparent if the phospholipid species containing polyunsaturated fatty acids were being conserved at the expense of other class species. However, in a number of instances the increased percentages could have only occurred due to a net increase in the concentration of host liver 22:6. Neifakh and Lankin (33) have reported that the concentrations of 18:1, 18:2, and 20:4 were higher in total lipids derived from rats bearing Walker carcinoma and hepatoma 22 at 14 and 11 days, respectively, than normal liver. They (33) apparently did not observe or measure the concentration of host liver 22:6, which showed the most pronounced increases in the present study.

The increased percentage of carbon number 38 of diglycerides derived from host liver PC (Table V) indicates an apparent effect of the tumor on the distribution of molecular species. It would appear that the tumor can affect phospholipid biosynthesis or can be instrumental in affecting the turnover of some molecular species in the host liver.

Phosphoglyceride Molecular Species

If one accepts the concept that specific molecular species of phosphoglycerides are vital cellular components and serve specific functions, then it is not surprising that liver and hepatoma behave differently. Not only does the hepatoma cell have to function with only one-fourth to one-half the amount of PC, PE, PS, and DPG of normal liver cells, but it has to manage with different molecular species of phosphoglycerides than found in liver. The C-20 and C-22 polyunsaturated fatty acids of the aforementioned classes are dramatically reduced, which means the quantity of molecular species containing these fatty acids is further reduced. In addition, the hepatoma has to contend with a 1-random-2-random distribution of fatty acids in PC and PE that further reduces the quantity of some molecular species. All of these abnormalities in the lipids of the hepatoma make it clear that cellular components and functions which require specific molecular species containing polyunsaturated fatty acids will be severely affected. Because of the abnormalities in the lipid constituents of neoplastic cells, it is not surprising that they function differently than normal cells.

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Hepatoma, Host Liver, and Normal Rat Liver Lipids: Distribution of Isomeric Monoene Fatty Acids in Individual Lipid Classes

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ABSTRACT

Monoenoic acid fractions were isolated from phosphatidycholine, phosphatidylethanolamine, triglycerides, and cholesteryl esters of hepatoma 7288CTC, host liver, and normal liver from animals maintained on chow and fat free diets. Hexadecenoate (16:1), octadecenoate (18:1), and eisosenoate (20:1) fractions were analyzed quantitatively for their isomeric composition. The fat free diet had little or no effect relative to the chow diet on the isomeric composition of 16:1, 18:1, and 20:1 from any lipid class in either hepatoma, host liver, or normal liver. Host livers were reduced in palmitoleic acid, and oleic and eicos-11-enoic acids were increased relative to normal liver. The 16:1 fraction from triglyceride of normal liver, host liver, and hepatoma contained 90, 80, and 75% palmitoleic acid, respectively. The 20:1 fraction from triglycerides of normal liver, host liver, and hepatoma contained ca. 55, 70, and 60% eicos-11-enoic acid, respectively, with the remainder consisting of eicos-13-enoic acid. The proportion of vaccenic acid in the 18:1 fraction was 60, 50, 20, and 25% for phosphatidylethanolamine, phosphatidylcholine, triglycerides, and cholesteryl esters, respectively, with oleic acid making up the balance. In contrast, all hepatoma lipid classes exhibited the same proportion of oleic (70%) and vaccenic (30%) acids. These data appear to be the first to demonstrate lipid class specificity for isomeric octadecenoic acids in normal liver and the loss of this specificity in a neoplasm.

INTRODUCTION

Evidence obtained from mass data (1) and metabolism data (2) has shown that cultured minimal deviation hepatoma cells contained and synthesized large amounts of vaccenic acid. The present study was designed to determine the distribution of isomeric monoenoic acids in individual lipid classes from a host grown hepatoma, host liver, and normal rat liver of animals maintained on chow and fat free diets. A preliminary report of the results has appeared (3).

EXPERIMENTAL PROCEDURES

Normal rat livers, livers of rats bearing hepatoma 7288CTC (denoted hereafter as host liver), and 7288CTC hepatomas were obtained from groups of animals maintained on chow and fat free diets for 4-5 weeks (4). The lipids were extracted, individual lipid classes isolated, and methyl esters prepared as described previously (4-6). Unsaturated esters were resolved by preparative gas liquid chromatography (GLC) on polar columns (7). In addition, 16:1 and

TABLE I

Distribution of Isomeric Hexadecenoic Acids of Triglycerides Derived from Normal Liver, Host Liver, and Hepatoma 7288CTC of Rats Maintained on Normal and Fat Free Diets

Hepatic tissue	Isomeric percentages[a]		
	Δ6	Δ7	Δ9
Rat liver, normal chow diet	4	4	92
Rat liver, normal, fat free diet	7	2	91
Rat liver, hepatoma host, chow diet	10	12	78
Rat liver, hepatoma host, fat free diet	16	5	79
Hepatoma 7288CTC, host fed chow diet	7	19	74
Hepatoma 7288CTC, host fed fat free diet	7	18	75

[a]Percentages represent the mean of duplicate analyses of a composite sample from 3 to 6 animals per group, except for the normal chow and fat free fed animal values which represent the mean of analyses from two groups of animals. The percentages are based on comparison of the quantities of the aldesters, except for the Δ6 isomer which is based on the aldehyde portion of the molecule because of the low yield of C-6 and shorter aldesters.

TABLE II

Percentage Distribution of Isomeric Octadecenoic Acids in Individual Neutral Lipids and Phospholipid Classes Derived from Normal Liver, Host Liver, and Hepatoma 7288CTC of Rats Maintained on Normal and Fat Free Diets

	Isomeric octadecenoic acid percentages[a]							
	PE[b]		PC[b]		TG[b]		CE[b]	
Hepatic tissue	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
Rat liver, normal, chow diet	40	60	49	51	83	17	75	25
Rat liver, normal, fat free diet	42	58	54	46	82	18	73	27
Rat liver, hepatoma host, chow diet	58	42	68	32	85	15	86	14
Rat liver, hepatoma host, fat free diet	55	45	74	26	88	12	88	12
Hepatoma 7288CTC, host fed chow diet	73	27	70	30	73	27	73	27
Hepatoma 7288CTC, host fed fat free diet	64	36	66	34	71	29	70	30

[a]Percentages represent the mean of duplicate analyses of a composite sample from 3 to 6 animals per group, except for the normal chow and fat free fed animal values which represent the mean of analyses from two groups of animals.

[b]PE = Phosphatidylethanolamine; PC = phosphatidylcholine; TG = triglyceride; CE = cholesterol ester.

20:1 fractions were rechromatographed on a nonpolar column (3% SE-30) to remove contaminating unsaturated acids of other chain lengths. Ozonides, prepared by a modification of the Beroza and Bierl procedure (8), were subjected to GLC and the resolved cleavage products quantitated as described previously (1). The source and purity of reagents and solvents were the same as given earlier (1).

RESULTS

The distribution of isomeric 16:1 acids of triglycerides (the only class that contained a sufficient quantity of 16:1 to permit analysis) obtained from hepatoma, normal liver, and host liver of rats maintained on normal and fat free diets is given in Table I. The identity of the Δ6 isomer, which was based on the aldehyde portion of the molecule, instead of the aldester, must be considered tentative because the small quantities did not permit further characterization. The percentage of the Δ6 isomer in host liver was about double that of normal liver and hepatoma percentages. Diet had little or no effect on the triglyceride 16:1 isomeric composition. Normal liver contained the highest percentage of palmitoleic acid; the Δ9 isomer, host liver intermediate, and hepatoma only about 75%. The balance of hepatoma 16:1 was composed primarily of the Δ7 isomer. This was in contrast to cultured hepatoma cells which contained palmitoleic acid and the Δ11 isomer (1).

The percentage compositions of the isomeric octadecenoic acids derived from phosphatidylcholine (PC), phosphatidylethanolamine (PE), triglycerides (TG), and cholesteryl esters (CE) of the various hepatic tissues from animals fed the two diets are shown in Table II. Oleic and vaccenic accounted for more than 98% of all isomers. The effect of the fat free diet was minimal on all lipid classes of all hepatic tissue. Each lipid class of normal liver exhibited a characteristic isomeric distribution; the percentage of oleic was TG > CE > PC > PE. The lipid classes of host liver contained higher percentages of oleic acid than normal liver. Host liver TG and CE isomeric octadecenoic acid compositions were the same, but PC and PE remained different. Hepatoma, in contrast to liver, did not exhibit a specificity for isomeric octadecenoic acids in its lipid classes; all classes contained ca. 30% vaccenic and 70% oleic acid.

The percentage compositions of the eicosenoic acid fraction isolated from normal and host liver TG and several hepatoma lipid classes are given in Table III. The Δ11 and Δ13 isomers accounted for 97% or more of the fraction. Again, the effect of diet on the isomeric composition was minimal. Normal liver and hepatoma TG contained ca. the same composition, whereas host liver contained a higher percentage of the Δ11 isomer. The isomeric composition of the hepatoma eicosenoic acids was ca. the same for all lipid classes.

Two dienoic acid fractions of some samples were also analyzed for isomeric compositions. Fractions of 18:2 and 20:2 isolated from hepatoma TG and CE contained more than 94% Δ9,12 octadecadienoic and Δ11,14 eicosadienoic acids. The 18:2 fractions isolated from TG of host and normal livers from animals on both diets likewise contained 95% or more of the Δ9,12 isomer.

Agreement between duplicate determinations was usually within ± 7% for major components (25% and higher) and ± 15% for minor components. Agreement between data from the two groups of normal animals fed chow and fat

TABLE III

Distribution of Isomeric Eicosenoic Acids Derived from Lipid Classes of Normal Liver, Host Liver, and Hepatoma 7288CTC of Rats Maintained on Normal and Fat Free Diets

Hepatic tissue	Isomeric percentages[a] Δ11	Δ13
Triglycerides		
Rat liver, normal, chow diet	53	45
Rat liver, normal, fat free diet	58	40
Rat liver, hepatoma host, chow diet	70	27
Rat liver, hepatoma host, fat free diet	69	29
Hepatoma 7288CTC, host fed chow diet	63	34
Hepatoma 7288CTC, host fed fat free diet	54	45
Sterol esters		
Hepatoma 7288CTC, host fed chow diet	63	35
Hepatoma 7288CTC, host fed fat free diet	54	44
Phosphatidylcholine		
Hepatoma 7288CTC, host fed chow diet	54	45
Phosphatidylethanolamine		
Hepatoma 7288CTC, host fed fat free diet	58	40

[a]Percentages represent the mean of duplicate analyses of a composite sample from 3 to 6 animals per group, except for the normal chow and fat free fed animals' values which represent the mean of analyses from two groups of animals.

TABLE IV

Quantity of Monenoic Fatty Acids in Various Lipid Classes Isolated from Rat Liver, Host Liver, and Hepatoma of Animals Maintained on Normal and Fat Free Diets

		Dietary regimen					
		Chow			Fat free		
		Monoenoic acids[a] (mg/g wet wt)			Monoenoic acids[a] (mg/g wet wt)		
Hepatic tissue	Lipid class	16:1	18:1	20:1	16:1	18:1	20:1
Rat liver, normal	TG	0.55	3.65	<0.05[b]	5.50	17.0	<0.2
	PC	0.05	1.18	<0.07	0.34	2.21	<0.07
	PE	<0.04	0.42	<0.04	0.03	0.37	<0.03
Rat liver, host	TG	0.14	2.0	<0.03	1.52	9.93	<0.1
	PC	<0.09	1.62	<0.09	0.07	1.0	<0.04
	PE	<0.04	0.28	<0.04	0.03	0.18	<0.03
Hepatoma	TG	0.05	1.16	0.14	0.12	1.47	0.18
	PC	0.03	1.01	0.05	0.02	0.63	0.03
	PE	0.07	1.02	0.04	0.01	0.57	0.02

[a]Monoene concentrations were calculated from triglyceride (TG) (4) and phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (6) data published in other papers of this series.

[b]Values preceded by < were calculated based on the assumption that all fatty acid percentages given as trace (T) were 0.5%, but in most cases, especially 20:1 of normal and host liver, the percentage was much less.

free diets was similar to the agreement between duplicate determinations. The use of classical names of fatty acids does not imply that double bond configurations were determined; the *cis* configuration was assumed.

DISCUSSION

This study is apparently the first to investigate the isomeric monoene content of individual lipid classes of rat liver. The occurrence of different proportions of oleic and vaccenic acids in each lipid class examined demonstrates the need to analyze individual lipid classes instead of total neutral lipid or total phospholipid fractions. Spence (9) has shown that the proportions of monoene isomers at the 1- and 2-positions of rat brain PC and PE differed, while differences between lipid classes were minimal.

One of the most surprising aspects of the present study was the fact that the fat free diet

had little or no effect on 16:1, 18:1, or 20:1 isomeric monoene composition in any lipid class from either normal liver, host liver, or hepatoma. Monoene concentrations given in Table IV show that TG 18:1 and 16:1 concentrations increased 5 and 10 fold, respectively, in normal and host livers of animals fed the fat free diet relative to chow fed animals. The effect of the fat free diet on the concentration of 18:1 in liver PC and PE and all three hepatoma lipid classes was less pronounced. Despite the range in monoene concentration from no change to a change of several fold brought about by the fat free diet, the proportion of isomers remained relatively unchanged. These data indicate that isomer monoene ratios in hepatic lipid classes remain constant regardless of rate of monoenoic fatty acid biosynthesis. Spence (10) has shown that the 18:1 isomeric composition of total lipids from rat kidney, lung, brain, and lumbar fat was relatively unchanged by feeding a fat free diet for 17-20 weeks, whereas heart 18:1 and 16:1 of heart, kidney, lung, and brain showed higher percentages of the $\Delta 9$ isomers than chow fed animals.

The percengage of polyunsaturated fatty acids and the distribution of fatty acids in PC of host liver have been shown to be affected by the hepatoma in a companion paper (6). The present data indicate that the hepatoma also has an effect on the isomeric composition of host liver monoene fatty acids. The increased percentage of eicos-11-enoic acid in host liver TG is consistent with the increased percentage of oleic acid. Likewise, the decreased percentage of vaccenic acid is consistent with the decreased percentage of palmitoleic acid observed in host liver TG.

The isomeric monoene composition of the hepatoma was distinctly different from liver. The uniqueness of the hepatoma composition may be less important than the fact that the hepatoma did not show the preferential incorporation of oleic and vaccenic into lipid classes as was exhibited by liver. Hepatoma eicosenoic isomers also exhibited a lack of lipid class preference. Our experiments with cultured hepatoma cells grown on medium containing varying levels of serum and lipids likewise showed only marginal changes in the proportions of isomeric monoenes isolated from PC, PE, and TG (1). The significance of these observations may not become clear until the function of vaccenic acid is established. The present data, which show that the two major phospholipid classes of liver contain vaccenic acid percentages equal to or greater than oleic acid, suggest that vaccenic acid does serve a useful function. Vaccenic acid has been shown to be a normal constituent of rat liver cells and organelles (11) and human liver (12). Holloway and Wakil (11) showed more than 10 years ago that biosynthesis of vaccenic acid in liver proceeded via elongation of palmitoleic acid. We have demonstrated recently (2) that this is also the route of vaccenic acid biosynthesis in cultured hepatoma cells. The present data show that 18:2, which was unaffected in hepatoma TG by the fat free diet (4,5), consisted of more than 95% linoleic acid. The 20:2 acid fraction that was elevated in hepatoma TG and CE (4) was derived from linoleic by elongation.

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Lipid and Lipoprotein Measurements in a Normal Adult American Population[1]

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ABSTRACT

From a parent population of 774, a subpopulation of 160 normal adults ages 27-66 was randomly selected, 20 from each decade and sex. A detailed comparison was made by analytic ultracentrifugation and complete agarose gel electrophoresis on serum and the 1.006 g/ml top and bottom preparative ultracentrifuge lipoprotein fractions. The latter was internally standardized by total lipid and plasma total cholesterol and triglyceride determinations giving normal reference lipoprotein values. The reading procedure allowed the identification and quantification of floating β and sinking pre-β. In the subpopulation, there were two of the former and 13 of the latter. For large scale clinical application of such quantitative lipoprotein electrophoresis full automation of the microdensitometry and calculations will be required.

INTRODUCTION

With current interest in the presence of elevated blood lipids and their relationship to excessive and premature heart disease (1,2), there exists a need for a simple lipoprotein quantitative screening test, as well as normal population data, for comparative and reference purposes. The measurement of plasma total triglyceride (TG) and total cholesterol (TC), although useful clinically, does not provide as much information as that given by the combination of lipid and quantitative lipoprotein measurements. This follows from the fact that nearly all lipids exist as lipoproteins and different combinations of lipoprotein patterns can result from similar TG and TC lipid values. Although typing (3,4) is not a completely satisfactory method of diagnosis of lipoprotein abnormalities, it nonetheless is a framework on which most hyperlipoproteinemias can be classified. The most important need for such classification is to provide a basis for prescribing either dietary (5) and/or drug therapy. After therapy is begun, lipid and/or quantitative lipoprotein measurements are periodically needed to evaluate the effectiveness of therapy.

Ideally, an appropriate quantitative lipoprotein analysis should be: a) inexpensive, b) applicable to small quantities of serum, c) in a form allowing a permanent record for qualitative and quantitative evaluation and re-evaluation at a later date, d) capable of being automated, and e) should allow standardization in many laboratories if widespread clinical use is contemplated. Micro electrophoresis on a suitable medium potentially can fulfill the above requirements. Previously we have favorably

[1]Presented in part at the AOCS meeting, Philadelphia, September, 1974.

TABLE I

Comparison of Mean Total Cholesterol and Triglyceride Values of Subpopulation with Values of Parent Modesto Population

	Males			Females		
Age group	n[a]	TC[a]	TG[a]	n	TC[a]	TG[a]
27-36	115	185	113	100	180	93
	20	186 (7)[b]	129 (12)	20	178 (6)	91 (6)
37-46	122	202	148	111	196	100
	20	197 (6)	157 (16)	20	196 (6)	109 (12)
47-56	129	211	153	118	212	112
	20	209 (5)	156 (15)	20	209 (8)	115 (8)
57-66	89	222	135	114	228	128
	20	224 (7)	147 (23)	20	222 (6)	103 (8)

[a]n = Number; TC = total cholesterol; TG = triglycerides.

[b]Values in parenthesis are standard error of mean of subpopulation values. All results are from Technicon AA_1 procedure (8).

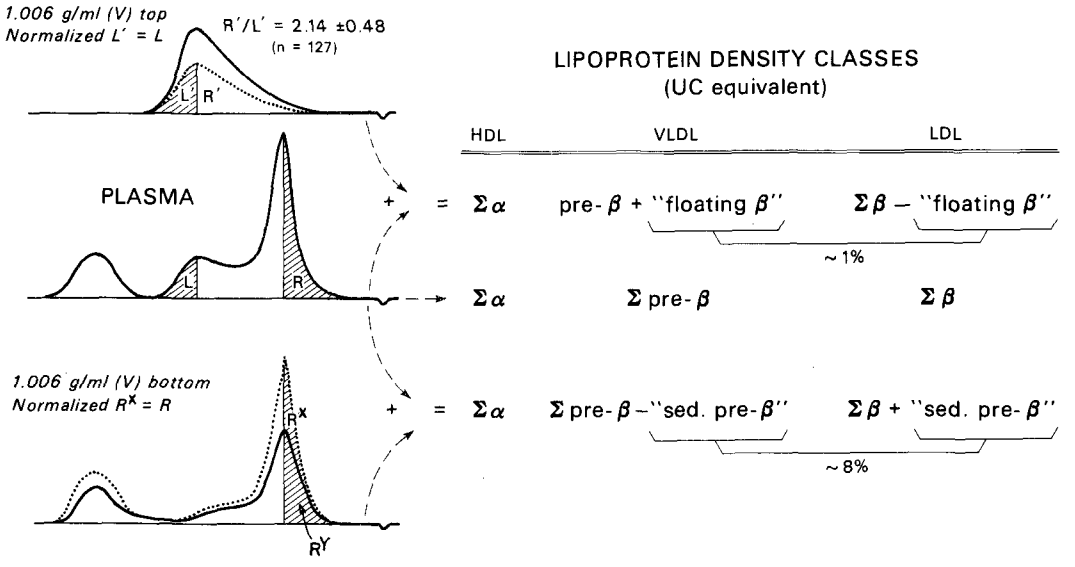

FIG. 1. Schematic method of combing the 1.006 g/ml very low density lipoprotein (VLDL) top and bottom preparative lipoprotein fraction scans with the plasma densitometric scan. The dotted top and bottom scans have been normalized and these are individually combined with the plasma scan to yield two sets of data. HDL = high density lipoprotein; LDL = low density lipoproteins.

evaluated and calibrated one off-the-shelf agarose gel electrophoresis kit and have provided a basis for internal standardization using plasma TG and TC values (6). We will consider here normal population values evaluated by this agarose gel electrophoretic procedure and by analytic ultracentrifugation.

EXPERIMENTAL PROCEDURES

Because the purpose of this study was to establish normal lipoprotein values by agarose gel electrophoresis and calibrate such a procedure, there were certain restrictions, namely the limited number of samples over a short period of time that could be analyzed with the analytic ultracentrifuge. One of us (PDW) was involved in re-studying a rather large population of normals ($n > 1000$) which provided a unique opportunity to select a random sample subpopulation that could be studied conveniently both by complete agarose gel electrophoresis and by analytic ultracentrifugation. From a previous screening study of 1118 volunteers in the Modesto, CA. area (7), which involved a complete physical examination and serum TG and TC measurement, a normal population of 933 was asked by letter and phone follow-up to return 2 years later. The criteria for normalcy were: a) no overt diabetes, b) no obvious ECG abnormality, c) diastolic blood pressure $<$ 100 mm Hg, and d) no participants were eliminated if lipoproteins were found to be abnormally high. Of the 933 contacted, 774 volunteered to return and were scheduled for blood specimens and physical examinations over a 2-week period. From this schedule, a random subpopulation of 20 subjects was selected in each decade of each sex. The selection was randomized with respect to day of examination and time of blood sampling. Disodium ethylene diamine tetraacetate (EDTA) plasma (100 mg/100 ml) was prepared from blood samples obtained after an overnight fast and an essentially fat free breakfast. Because data on the TG and TC obtained 2 years earlier were available on all returning subjects, a satisfactory comparison was made of the original population and the selected subpopulation.

Table I lists by sex the mean TG and TC of the total population in each decade as compared with the corresponding subpopulation data, which incorporated the standard error of the mean. (All these measurements were made 2 years prior to this study.) In each case, TG and TC were determined according to the methods of the Lipid Research Clinics (8). All but one borderline group (females, 57-66 years) were clearly representative samplings of the larger normal population.

The following analyses were performed on all 160 samples: a) plasma TG and TC (AAI) and total gravimetric lipid (TGL), b) agarose gel electrophoresis on plasma, and on the

TABLE II

Plasma Lipoproteins (Analytic Ultracentrifuge) and Lipid Concentrations[a] of Modesto Normals

Males Age	Lipid concentrations (mg/100 ml) TGL[b]	TC[b]	TG[b]	Plasma lipoproteins $F_{1.20}(0\text{-}9)$ (HDL)[c]	Sf 20-400 (VLDL)[c]	Sf 0-20 (LDL)[c]
27-36	603 ± 91	188 ± 28	110 ± 25	235 ± 55	89 ± 40	352 ± 92
37-46	673 ± 110	202 ± 35	118 ± 59	275 ± 84	102 ± 85	401 ± 82
47-56	771 ± 117	212 ± 32	154 ± 81	283 ± 76	136 ± 104	453 ± 86
57-66	748 ± 142	245 ± 31	128 ± 81	287 ± 81	115 ± 78	452 ± 85
Females Age						
27-36	606 ± 93	184 ± 25	86 ± 29	342 ± 42	47 ± 31	309 ± 64
37-46	628 ± 121	190 ± 35	85 ± 35	382 ± 116	51 ± 37	305 ± 97
47-56	723 ± 141	212 ± 32	123 ± 73	402 ± 102	87 ± 83	373 ± 105
57-66	839 ± 149	245 ± 31	158 ± 98	413 ± 90	140 ± 136	422 ± 94

[a]n = 20 in each age group.

[b]TGL = total gravimetric lipid; TC = total cholesterol; TG = triglyceride.

[c]HDL = high density lipoproteins; VLDL = very low density lipoproteins; LDL = low density lipoproteins.

1.006 g/ml very low density lipoprotein (VLDL) top and bottom preparative ultracentrifuge fractions, and c) analytic ultracentrifugation on the total low and high density lipoprotein fractions, as described elsewhere (9).

The agarose gel electrophoresis was performed essentially as described by Hatch, et al., (6). Scanning was performed with a model RFT densitometer (Transidyne Corp., Ann Arbor, MI). Normal conditions utilized a full scale of 0.30 optical density (OD) units with a slit setting of 0.4 x 10.0 mm and a wavelength setting of 520 nm. The actual slit dimensions, however, were 0.4 x 2.6 mm when focused on a sheet of white bond in the plane of the gel. Such slit focusing is recommended before the scanning of slides. The microdensitometry was done in 2 ways. First, to compile comparative data, the plasma scan was divided in the total α, pre-β, and β components, and internally standardized by a gravimetric total lipid determination. This procedure, exclusive of calculations, required ca. 2 min. In the second more complex analysis, potentially capable of automation, the total plasma scan was combined with one or both of the 1.006 g/ml VLDL top and bottom ultracentrifuge fractions. This was done to identify properly all VLDL and low density lipoproteins (LDL), taking into consideration the potential presence of floating β in VLDL and sedimenting, or sinking pre-β in the LDL lipoprotein fraction. In this second process, the asymmetry of the pre-β peak was evaluated from the scan of the 1.006 g/ml top fraction, and a ratio of L^1 to R^1 was used to resolve or extrapolate the pre-β component present in the plasma scan (Fig. 1). This total normalized pre-β component then was subtracted from the partially resolved pre-β and β components in the plasma scan, resulting in pre-β and β values. The scan of the 1.006 g/ml botton fraction, however, was normalized using a ratio of R^x to R^y (Fig. 1). This ratio was applied to the β peak and sinking pre-β component, if present, of the 1.006 g/ml bottom fraction. This normalized β value, and sinking pre-β, if present, then was subtracted from the total pre-β and β peaks in the plasma scan, producing a VLDL pre-β value. This procedure using all 3 scans normally yielded 2 sets of data. Mean values for all 3 components then were calculated. However, when sinking pre-β was present in the 1.006 g/ml bottom fraction, the bottom fraction and the plasma scan were utilized. Similarly, when floating β was present in the 1.006 g/ml VLDL top fraction, the top fraction and the plasma scan were used. In these 2 circumstances, only one set of data was obtained from each process. These manipulations are schematically shown in Fig. 1 and are intended to provide unambiguous VLDL and LDL data, equivalent to the analytic ultracentrifuge results, through the complete agarose electrophoresis procedure utilizing the preparative lipoprotein fractions. This procedure, exclusive of calculations, required ca. 5 min for all 3 scans. It was anticipated that, in routine screening, only a small fraction of the samples would require this complete agarose procedure, the plasma scan alone being sufficient in most cases for lipoprotein quantification.

RESULTS

Table II presents the plasma lipid and the

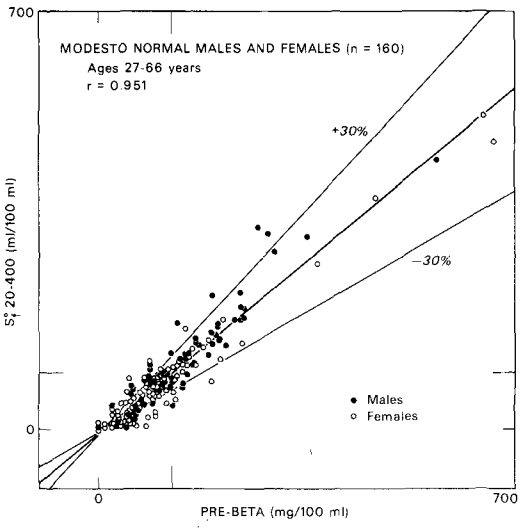

FIG. 2. Comparison of agarose gel electrophoresis (AGE) data with analytic ultracentrifuge (An U.C.) lipoprotein data. S_f^0 20-400 vs very low density lipoprotein (VLDL) (pre-β).

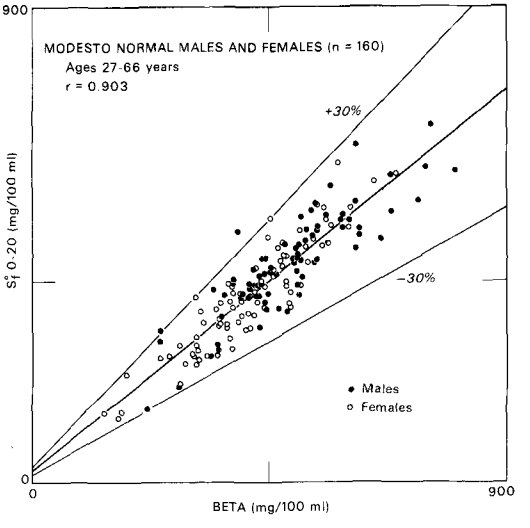

FIG. 3. Comparison of agarose gel electrophoresis (AGE) data with analytic ultracentrifuge (An U.C.) lipoprotein data. S_f^0 0-20 vs low density lipoprotein (LDL) (β).

analytic ultracentrifuge lipoprotein data for all age groups studied. As expected, all lipids increase progressively with age in both sexes with the exception of triglycerides in the 57-66 year old males. For all age groups, HDL was significantly higher in the females as compared with the males.

The results of the complete agarose gel electrophoresis procedure and comparison with the analytic ultracentrifuge data are given in Figs. 2, 3, and 4. All data are included in each scatter plot and the results demonstrate a high degree of correlation between the analytic ultracentrifuge and the agarose gel electrophoresis results. Table III gives a summary of the results, including regression formulae $Y = B + AX$ for converting agarose data into equivalent analytic ultracentrifuge data. Also given is the standard error of measurement $S_{y.x}$ which is the error of estimating the assumed true or analytic ultracentrifuge concentrations from the agarose electrophoresis data utilizing the appropriate regression formula for each lipoprotein class. This error is ca. 12-25% of the mean lipoprotein values, and, thus, the agarose procedure approaches that of an analytic measurement. Of particular interest is the improved measurement of α or HDL.

Of the 160 normals studied, two subjects exhibited floating β in their 1.006 g/ml VLDL fractions (type III) and 13 exhibited resolvable sinking pre-β in their 1.006 g/ml bottom fractions. The latter low incidence reflected the sensitivity of detection of this lipoprotein

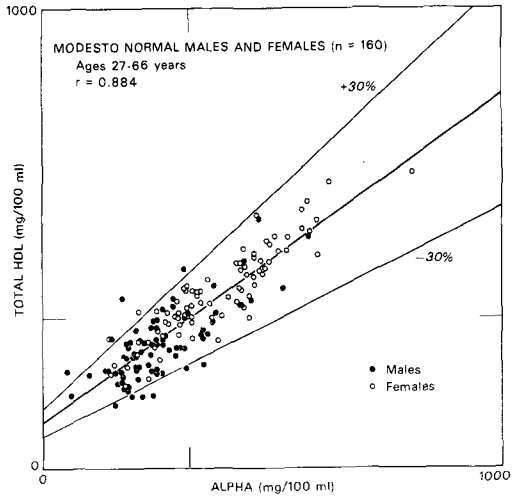

FIG. 4. Comparison of agarose gel electrophoresis (AGE) data with analytic ultracentrifuge (An U.C.) lipoprotein data. $F_{1.20}^0$ 0-9 vs total high density lipoprotein (HDL) (α).

component (LP)(a) which may exist at low concentrations in all individuals (10).

Of additional value are groupings of the 27-46 and 47-66 age groups for the purpose of comparison and ranking of data. Table IV gives these values which provide an appropriate reference for any subject's results. For example, results expressed merely in mg/100 ml values alone have little meaning to a patient or his physician. However, when they are converted

TABLE III

Conversion of Agarose Gel Electrophoresis Data to Equivalent Analytic Ultracentrifuge Data, Modesto Normal Males and Females[a]

Lipoprotein[b] class	Analytic Ultracentrifuge (Y)	Agarose Gel Electrophoresis (X)	Sy.x	B + AX[c]
HDL	327 ± 104	320 ± 129	49	100 + 0.714X
VLDL	96 ± 88	125 ± 101	28	-7 + 0.828X
LDL	383 ± 104	488 ± 118	45	25 + 0.800X

[a]Mean data combining 1.006 g/ml very low density lipoprotein fractions with plasma and 1.006 g/ml bottom fraction with plasma scans; n = 160.

[b]HDL = High density lipoprotein; VLDL = very low density lipoprotein; LDL = low density lipoprotein.

[c]Regression formula Y = B + AX is from total data as presented in the scatter plots of Figures 2, 3, and 4.

TABLE IV

Modesto Normal Agarose Gel Electrophoresis Data Summarized for Comparative Purposes with Typical Example of Appropriate Ranking[a]

	Age	n	α (HDL)[b]	pre-β (VLDL)[b]	β (LDL)[b]
Males	27-46	39	251 ± 91	124 ± 74	430 ± 89
	47-66	40	259 ± 103	149 ± 93	539 ± 112
Females	27-46	40	367 ± 126	70 ± 40	375 ± 93
	47-66	40	400 ± 121	155 ± 144	449 ± 112
	Male age				
Case 3030	34		168	220	886
Ranking[c]	27-46		(18%, -0.91σ)	(90%, 1.31σ)	(100%, 5.15σ)
	all males		(19%, -0.89σ)	(84%, 0.99σ)	(100%, 3.48σ)

[a]Data in mg/100 ml are from complete agarose gel electrophoresis procedure standardized by plasma total cholesterol and triglyceride determinations (Technicon AA_1 procedure).

[b]HDL = high density lipoprotein; VLDL = very low density lipoprotein; LDL = low density lipoprotein.

[c]Note the usefulness of the σ value for comparative ranking when the percentile rank is 100%. This is a type IIb patient with moderate VLDL elevation.

to percentile rank and standard score values, expressed in standard deviation units above or below the mean, they allow a convenient, understandable means of reference with respect to normalcy, as well as providing a useful criterion of degree of abnormality. The value of expressing ranking in σ units is particularly useful when lipoprotein values are either very high or very low. For example, above 3σ, the % rank has no quantitative discrimination. Thus, the computer processed results shown in Table IV provide such convenient and comparative rankings for all 3 lipoprotein components. As suggested, these values may be useful in adding a quantification to the typing system, in this case a type IIb. Thus, the II'ness, referring to LDL elevation, and the B'ness, referring to associated VLDL elevation, might appropriately be expressed quantitatively as I1(5.15 σ) and B(1.31 σ). Here the lipoprotein concentrations are quantitatively expressed as σ values with respect to normal population lipoprotein concentrations.

DISCUSSION

Although there have been numerous electrophoresis studies of plasma lipoproteins, it was not easy to find appropriate studies for comparison with our data. Perhaps the most comparable quantitative agarose gel electrophoresis study is that of Dyerberg and Hjorne (11). They utilized a laboratory prepared agarose system patterned after Noble (12) standardized by total gravimetric lipid with assumptions that dye uptake of the sudan black stain was proportional to lipoprotein lipid content. Our data obtained from plasma scans alone standardized by total gravimetric lipid are compared with their data in Table V and VI for the

TABLE V

Comparison of Dyerberg & Hjorne (D & H) and Agarose Gel Electrophoresis Standardized by Total Plasma Gravimetric Lipid, Normal Males[a]

n	Age	Source	α	pre-β	β
20	27-36	Modesto	267 ± 69	113 ± 45	480 ± 101
25	31-40	D & H	290 ± 69	117 ± 59	455 ± 85
20	37-46	Modesto	298 ± 125	122 ± 100	537 ± 91
27	41-50	D & H	284 ± 108	181 ± 87	519 ± 152
20	47-56	Modesto	291 ± 136	164 ± 132	619 ± 112
36	51-60	D & H	278 ± 89	158 ± 77	532 ± 108
19[b]	57-66	Modesto	294 ± 90	119 ± 64	645 ± 121
21	61-70	D & H	275 ± 80	145 ± 102	522 ± 120

[a]See Reference 11.

[b]One type III excluded.

TABLE VI

Comparison of Dyerberg & Hjorne (D & H) and Agarose Gel Electrophoresis by Total Plasma Gravimetric Lipid, Normal Females[a]

n	Age	Source	α	pre-β	β
20	27-36	Modesto	389 ± 90	72 ± 42	450 ± 92
20	31-40	D & H	371 ± 92	99 ± 54	429 ± 123
20	37-46	Modesto	455 ± 181	68 ± 45	439 ± 139
21	41-50	D & H	357 ± 110	111 ± 49	486 ± 122
20	47-56	Modesto	450 ± 152	102 ± 93	526 ± 135
20	51-60	D & H	435 ± 104	98 ± 55	602 ± 145
19[b]	57-66	Modesto	473 ± 121	152 ± 156	580 ± 118
22	61-70	D & H	378 ± 97	123 ± 68	626 ± 152

[a]See Reference 11.

[b]One type III excluded.

males and females, respectively. High density lipoproteins (HDL) concentrations were comparable by the two techniques, but the concentrations of VLDL were lower and LDL generally higher in our studies. This is, in part, the result of differences in the shapes of the pre-β component. Our pre-β asymmetry $R^1/L^1 = 2.14 \pm 0.48$ would lead to underestimating the total pre-β component and overestimating the total β component by manual two component resolution in the total plasma scans. Because many low level VLDL fractions were not resolvable, a uniform procedure of correcting for this bias was not possible. Nonetheless, it is encouraging and of considerable interest that two similar independent studies of normal lipoprotein values have yielded comparable data. Inherent differences in the populations, environmental factors, and different dietary habits undoubtedly contribute to some of the differences observed in the VLDL and LDL levels.

There are differences in lipoprotein values obtained by our three possible reading procedures. In general, the serum scan alone will provide the lowest VLDL and highest LDL values. Higher VLDL and lower LDL is obtained from plasma + 1.006 g/ml VLDL top scans and highest VLDL and lowest LDL is obtained from the plasma + 1.006 g/ml bottom scans. Our full agarose procedure takes the mean of the latter two combinations when available, and such data (Table IV) are more comparable to the results of Dyerberg and Hjorne (11).

The main theme of this study is the comparison of lipoprotein values obtained by an internally standardized agarose gel electrophoretic technique with results using the analytic ultracentrifuge. However, some discussion of the differences in lipoprotein concentrations obtained by each of the two methods, as well as comparison with older ultracentrifugal data, appear to be in order. A comparison is given between the analytic ultracentrifuge values and the serum lipid values (Table II) as well as with the agarose gel electrophoresis results (Table III). Considering the content of protein in each lipoprotein class, and assuming the total gravimetric lipid represents recovery of total lipo-

protein lipid, the analytic ultracentrifuge results appear low by as much as 15%. Similarly, the agarose gel lipoprotein results standardized by serum cholesterol and triglyceride determinations are ca. 15% higher than the ultracentrifuge results (Table III). These discrepancies may be explained, in part, as the result of some 5% ultracentrifugal loss in the prep tube and prep cap during pipetting. Also, there is some unrecovered HDL and very high density lipoprotein (VHDL) (σ's $>$ 1.21 g/ml) that may account for 5-8% of the total lipid.

An additional consideration is the absolute error of the specific refractive increments used for the lipoproteins (9) which may be in error by as much as 5-8%. However, it should be understood that, although the ultracentrifuge may underestimate certain classes of lipoproteins, ultracentrifuge measurements are consistent, reproducible, and, thus, appropriate for calibration purposes.

Comparison of the present data with earlier ultracentrifugal data (13) measured by the same technique (9) reveal somewhat higher values for LDL and HDL in the 35-49 yr age group studied, particularly in females. Values for HDL were 300 and 457 mg/100 ml for the males and females, respectively. A possible explanation for this unusually high female value was that it was from a small nonrandom sample of middle class working women. In a further study of these women, those with hysterectomies (n = 5) had substantially higher HDL levels than the others (503 ± 142 mg/100 ml and 420 ± 86 mg/100 ml, respectively). The incidence of hysterectomies in our present normal study was unknown, but could have explained in part the lower HDL values. Also, another change since 1965 among those women practicing birth control has been the almost universal usage of birth control pills. These might be expected to have effects on blood lipids and lipoproteins, although definitive effects on HDL levels have not been reported. Despite the conflict with earlier data, the present study represents a random selection from a population of some 800 normals with differing backgrounds, and, hence, these data should be more authentically normal.

Because the manipulations and manual calculations are very tedious, but systematic, for our complete agarose procedure, they, nonetheless, would be vastly simplified by automation. A preliminary test using a 1000 channel analyzer, a paper punch tape, and a computer program has proven that such a system will work as well or better than the tedious manual procedure (14). Thus, the potential of an automated microdensitometry system has been demonstrated, which would be essential if widespread clinical application of quantitative lipoprotein electrophoresis is considered. Of particular interest was the potential improvement in the measurement of HDL, undoubtedly the result of baseline corrections. Accurate HDL measurements could be very important, because, independent of the level of other lipoproteins, it may be advantageous to have a high HDL level and potentially deleterious to have an abnormally low level (15).

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Regulatory Function of Pyruvate Dehydrogenase and the Mitochondrion in Lipogenesis

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ABSTRACT

The activity of pyruvate dehydrogenase from freshly isolated mitochondria was shown to be dependent upon the nutritional and metabolic state of the animal prior to sacrifice, such that mitochondria from the livers of 48 hr starved, diabetic, or high fat fed rats had lower enzyme activity than normal, chow fed rats. The activity of pyruvate dehydrogenase and the rate of lipogenesis were shown to correlate to a certain extent when a reconstituted, cell free system consisting of 105,000 x g supernatant of rat liver and isolated mitochondria was used. This sytem was employed so that the role of the mitochondrion and pyruvate dehydrogenase in lipogenesis could be investigated. Dichloroacetate increased the activity of pyruvate dehydrogenase and increased the rate of lipogenesis, suggesting that the activity of pyruvate dehydrogenase is an important factor in determining the rate of lipogenesis in the reconstituted system. It was observed, however, that dichloroacetate was more effective in stimulating the activity of pyruvate dehydrogenase than the rate of lipogenesis when mitochondria from starved animals were used to reconstitute lipogenesis. Furthermore, the cytoplasmic adenosine triphosphate/adenosine diphosphate ratios and phosphorylation potentials (ATP/ADP x Pi) maintained in the reconstituted system by mitochondria isolated from starved animals were found to be significantly lower than those maintained by mitochondria isolated from chow fed animals. It is proposed that the lower "energy pressure" maintained in the reconstituted system by mitochondria isolated from starved animals severely limits lipogenesis at the ATP requiring steps of the process.

INTRODUCTION

The enzymes of both the cytosolic and mitochondrial compartments are required for lipogenesis from glucose. These points have been reviewed previously (1). The glycolytic enzymes furnish the carbon from glucose for fatty acid synthesis in the form of pyruvate, which is oxidized by the mitochondrial enzyme, pyruvate dehydrogenase (E.C. 1.2.4.1), to form acetyl-CoA. Acetyl-CoA combines with oxaloacetate to form citrate, which can exit through the inner mitochondrial membrane to be cleaved in the cytosol. Thus, for lipogenesis from carbohydrate substrates, the mitochondrion is directly involved in 3 necessary reactions: (a) oxidation of pyruvate; (b) synthesis of citrate; and (c) exit of citrate from the mitochondria. A less complex system than the complete cell to study the involvement of pyruvate dehydrogenase and the mitochondrion in lipogenesis would be a cell free system consisting of particle free cytoplasmic fraction plus mitochondria prepared from rat liver. Such a system has been described (2,3) and modified by Watson and Lowenstein (4) for the incorporation of labeled alanine into fatty acids. As shown previously (4,5), reconstitution of the cell free system is dependent upon the presence of the mitochondria. With this system it has been shown that the capacity of isolated mitochondria to support lipogenesis is dependent upon the nutritional and metabolic condition of the animal prior to sacrifice and isolation of the mitochondria (5), such that liver mitochondria from 48-hr starved animals are not able to support lipogenesis to as great an extent as liver mitochondria prepared from chow fed rats (5). This study was carried out to investigate the explanation for the difference in lipogenesis supported by mitochondria prepared from the livers of chow fed and 48-hr starved rats.

METHODS AND MATERIALS

Preparation of Mitochondria and 105,000 x g Supernatant

Mitochondria were prepared from rat liver by the method of Johnson and Lardy (6). The 105,000 x g supernatant (4,5) was prepared from the livers of rats which had been starved 48 hr and then refed ad libitum 48-72 hr on a high sucrose diet ("Fat-Free" test diet, Nutritional Biochemicals Corp., Cleveland, OH) (7).

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The 105,000 x g supernatant was activated as described by Watson and Lowenstein (4). This preparation of soluble enzymes was found stable to freezing in a liquid nitrogen storage tank for at least 6 weeks. Protein was determined by the biuret method (8) and, on occasion, by the Lowry method (9).

Reconstituted Cell-Free System Capable of Lipogenesis

The incubation conditions used in the reconstituted system have been described previously (5). The incubations were carried out in 25-ml Erlenmeyer flasks stoppered with serum caps at 37 C with 95% O_2 and 5% CO_2 as the gas phase and constant shaking at 120 cycle/min. The incubation mixture was 20 mM in $KHCO_3$, 9 mM in dithiothreitol, 12 mM in $MgCl_2$, 4 mM in L-malate, 2 mM in Adenosine triphosphate (ATP), 4 mM in glucose-6-phosphate, 5 mM in 2-oxoglutarate, 4 mM in phosphate buffer, pH 7.4, 12.5 mM in glycylglycine-NaOH buffer, pH 7.4, ca. 50 mM in sucrose, 0.84 mM in nicotinamide adenine dinucleotide phosphate ($NADP^+$), 0.1 mM in NAD^+, and 0.16 mM in Coenzyme A. The incubation mixture (1.5 ml) also contained ca. 4.5 mg of activated 105,000 x g supernatant protein, ca. 7.5 mg of mitochondrial protein, and ca. 1 mCi of ^3HOH.

Fatty acids were extracted from the incubation medium after saponification as described by Watson and Lowenstein (4). The extracts were evaporated to dryness in scintillation vials. Benzene was added to the extracts to remove the last traces of ^3HOH as an azeotrope. Zero time controls were reduced to background levels by this step. The fatty acids were counted in a toluene base scintillation fluid (0.01% *p-bis* [2-(5-phenyloxazolyl)]-benzene and 0.4% 2,5-diphenyloxazole) with a Searle Isocap/300 liquid scintillation spectrometer. Radioactive CO_2 was collected in Hyamine hydroxide after acidification of the incubation medium and counted in the scintillation fluid described above.

Assay of the Active Form of Pyruvate Dehydrogenase

The mitochondria were homogenized on dry ice in a solution 20 mM in potassium phosphate, pH 7.0, and 60% in glycerol (v/v) at temperatures below -5 C by a Polytron Type PT-10 homogenizer operated at full speed for 15 sec. When pyruvate dehydrogenase was measured on samples that had been incubated in the medium for measuring fatty acid synthesis, the samples were poured into 4 ml ice cold buffer and centrifuged for 5 min at 12,000 x g in a Sorvall Type SM-24 rotor at 2 C. The supernatant was discarded and the tubes were dried with tissue paper. The samples were homogenized in 2 ml of phosphate/glycerol as above.

The active form of pyruvate dehydrogenase was assayed in 1 dram shell vials stoppered with serum caps. The incubation medium, pH 8.0, was 20 mM in potassium phosphate, 0.5 mM in $MgCl_2$, 1.2 mM in NAD^+, 13 mM in mercaptoethanol, 1.0 mM in CoA, 0.5 mM in dithiothreitol, 5 mM in 2-oxoglutarate, and 5 mM in [1-^{14}C]alanine (5 x 10^4 CPM). The incubation mixture (0.2 ml) also contained 640 mU of lactate dehydrogenase (E.C. 1.1.1.27), 192 mU of glutamate-pyruvate transaminase (E.C. 2.6.1.2), and 0.18 mg of mitochondrial protein. The assay was conducted for 30 min at 30 C in a shaking water bath at 120 cycle/min. The reaction was stopped with an injection through the serum cap of 0.5 ml of 6 N HCl and the $^{14}CO_2$ released was collected in hanging cups containing Hyamine hydroxide. After acidification, the vials were shaken for an additional hour. The CO_2 was counted in the toluene scintillation fluid described above. The production of $^{14}CO_2$ was linear with protein up to 0.7 mg per assay and linear with time up to 60 min.

Metabolite and Nucleotide Assays

Incubations were stopped with perchloric acid at final concentration 6%, the protein was sedimented by centrifugation of 10,000 x g for 10 min, and the supernatant was neutralized with KOH. Inorganic phosphate was measured by the method described by Lindberg and Ernster (10). Enzymatic assays were used to measure ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) by the methods of Williamson and Corkey (11); lactate, pyruvate, and malate were determined by the methods of Hohorst et al. (12); and citrate was determined by the method of Mollering and Gruber (13). Results are expressed as the means ± SEM and were analyzed for statistical significance by the 2-tailed Student's t test.

Assay of Respiratory Control and State 3 Respiration

State 3 respiration and the respiratory control ratio always were assayed as a check on the integrity of the mitochondrial preparations (14). The incubation mixture used (5 ml) was 225 mM in sucrose, 10 mM in potassium phosphate, pH 7.4, 5 mM in $MgCl_2$, 20 mM in KCl, 20 mM in triethanolamine, pH 7.4, 5 mM in glutamate, 5 mM in L-malate, and contained 10-12 mg of mitochondrial protein. Respiration

was released with ADP at an initial concentration of 0.2 mM.

ATPase activity was measured in the same buffer used for respiratory control determination, except that oxidizable substrates and inorganic phosphate were omitted and 10 mM ATP and 2 μg/ml rotenone were added. The amount of inorganic phosphate released in 10 min at 30 C was measured by method of Lindberg and Ernster (10).

Diet and Rats

Male rats of the Wistar strain (185-250 g) were used in all experiments. The animals were maintained on stock colony diet (chow fed), unless otherwise indicated. The high fat, carbohydrate free diet was 58% in corn oil margarine, 25% in casein, 11% in alphacel cellulose, and supplemented with salts and vitamins.

Materials

All enzymes and most chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Radioactive compounds and Hyamine hydroxide were obtained from New England Nuclear, (Boston, MA). Hanging cups for the collection of $^{14}CO_2$ in Hyamine hydroxide were obtained from Kontes Glass Company, (Evanston, IL).

RESULTS

Pyruvate Dehydrogenase and Lipogenesis

The activity of pyruvate dehydrogenase from freshly isolated liver mitochondria was found to be dependent upon the nutritional and metabolic condition of the rat prior to sacrifice (Table I), such that mitochondria from starved, diabetic, and fat fed animals had a lower activity of pyruvate dehydrogenase than mitochondria from chow fed rats. Harris et al. (5), using a reconstituted cell free system, showed that liver mitochondria from starved, diabetic, and fat fed animals support a lower rate of lipogenesis than mitochondria from chow fed controls. Therefore, it appears that the activity of pyruvate dehydrogenase and lipogenesis correlate posibively with each other. This question was investigated further using only mitochondria from chow fed and starved animals.

Dichloroacetate has been shown to bring about an activation of pyruvate dehydrogenase in muscle and adipose tissue (15-17). It was reasoned that the rates of fatty acid synthesis would be increased by dichloroacetate if the

TABLE I

Activity of Pyruvate Dehydrogenase in Isolated Rat Liver Mitochondria Prepared from the Livers of Rats in Various Metabolic Conditions[a]

Liver mitochondrial source	Pyruvate dehydrogenase activity
	(nmoles/min/mg protein)
Chow fed (6)	6.7 ± 0.2
Starved 48 hr (6)	2.6 ± 0.2[d]
Diabetic[b] (6)	3.8 ± 0.5[d]
High fat diet[c] (4)	1.3 ± 0.2[d]

[a]Freshly isolated liver mitochondria were homogenized in glycerol-phosphate buffer and the activity of pyruvate dehydrogenase was measured.

[b]Diabetic rats were produced by the intravenous injection of alloxan (40 mg/kg). Rats were maintained for 2 weeks on adequate insulin to maintain normal blood glucose. Insulin was withdrawn 2 days prior to sacrifice. Only animals with blood glucose greater than 300 mg % were used in this study.

[c]Rats of this groups were maintained on the high fat diet for 6 days prior to sacrifice.

[d]$P<0.05$, Student's t test. All comparisons are made to the liver mitochondria isolated from chow fed rats. Results are expressed as means ± SEM with the number of mitochondrial preparations used indicated in parentheses.

TABLE II

Pyruvate Dehydrogenase and Fatty Acid Synthesis in Reconstituted Cell Free System[a]

Mitochondrial source	Pyruvate dehydrogenase activity	Fatty acid synthesis
	(nmoles "C2" units/min/mg protein)	
Chow fed	13.0 ± 0.9	4.2 ± 0.3
Chow fed + DCA[b]	15.0 ± 1.4	5.1 ± 0.2[c]
Starved 48 hr	9.2 ± 0.8[c]	2.8 ± 0.2[c]
Starved 48 hr + DCA[b]	14.0 ± 1.1[d]	3.7 ± 0.3[d,e]

[a]The incubations were terminated at 20 min.

[b]DCA = dichloracetate; 2 mM was included in the incubation.

[c]Significantly ($P<0.05$) different from chow fed group.

[d]Significantly ($P<0.05$) different from 48-hr starved group.

[e]Significantly ($P<0.05$) different from chow fed plus DCA group.

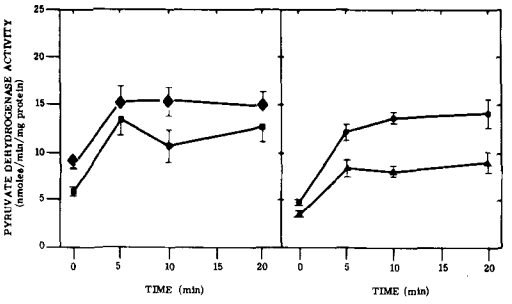

FIG. 1. The effect of dichloroacetate (♦,●) on the activity of pyruvate dehydrogenase in the reconstituted system with liver mitochondria prepared from chow-fed (■,♦) and 48-hr starved (▲,●) rats.

activity of pyruvate dehydrogenase activity was limiting for lipogenesis. As shown in Table II, the rates of fatty acid synthesis were found to be increased by dichloroacetate. It should be noted, however, that the activity of pyruvate dehydrogenase was significantly increased by dichloroacetate only when mitochondria were isolated from starved rats. A comparison of the activities of pyruvate dehydrogenase of mitochondria freshly isolated (Table I) and of mitochondria incubated for 20 min (Table II) suggested that incubation resulted in an activation of the enzyme. This probably can be explained on the basis that the incubation medium contains Mg^{++} and a fairly high final concentration of pyruvate, both established activators of pyruvate dehydrogenase (18,19). Figure 1 shows that the activity of pyruvate dehydrogenase was increased by incubation of mitochondria in this reconstituted system. This figure also shows that dichloroacetate caused a more rapid activation and a greater total amount of activity. This was particularly true when the mitochondria were isolated from starved animals, the increase being statistically ($P<0.05$) significant throughout the time study. The increase in pyruvate dehydrogenase as a result of dichloroacetate at time zero probably can be explained by the experimental procedure. The complete incubation medium minus the mitochondria was brought to the incubation temperature, the mitochondria were added, and the mixture was poured immediately into centrifuge tubes containing ice cold buffer. This explanation required that dichloroacetate effect a very rapid activation of liver pyruvate dehydrogenase. An alternative but less likely explanation would be that residual dichloroacetate not removed from the mitochondria during centrifugation might cause a slow activation of the enzyme during the 30 min assay period. Regardless, the results suggest that the activity of pyruvate dehydrogenase can be rate limiting to the process of lipogenesis. Indeed, the lag in fatty acid synthesis by the reconstituted system noted previously (4,5) can probaly be explained in part on this basis. However, it appears that other factors besides the activity of pyruvate dehydrogenase must become involved in limiting the rate of lipogenesis. This is apparent in Table II, which shows that the activity of pyruvate dehydrogenase of mitochondria from starved rats was increased by dichloroacetate to the value of chow-fed rats, whereas the rate of lipogenesis was still substantially less.

Cytoplasmic Phosphorylation Potential and $NADP^+/NADPH$ Ratio

The $NADP^+/NADPH$ ratio and phosphorylation potential (ATP/ADP x Pi) could be important in determining the rate of lipogenesis in this system. If the $NADP^+/NADPH$ ratio is too oxidized or the phosphorylation potential too low, then lipogenesis would be inhibited because NADPH is needed for reduction and ATP is needed for the cleavage of citrate and the formation of malonyl CoA. Table III shows that no apparent difference was found in the free $NADP^+/NADPH$ ratio maintained by mitochondria from starved of chow fed rats, as measured from the concentrations of malate, pyruvate, the calculated concentration of CO_2 (1.14 mM), and the equilibrium constant for malic enzyme (E.C. 1.1.1.37). However, the cytoplasmic phosphorylation potential and the ATP/ADP ratio were found to be lower with mitochondria isolated from starved animals. Table III also shows that the decrease in the phosphorylation potential and ATP/ADP ratio is due mainly to a large change in ADP concentration. Figures 2 and 3 demonstrate that the phosphorylation potential and ATP/ADP ratio fall very rapidly for the first 15 min of incubation and then begin to approach a steady state. These results show that the fall in the phosphorylation potential and ATP/ADP ratio is more rapid and a lower steady state is approached when the system contains liver mitochondria from starved rats. It appears that these decreases in phosphorylation potential and ATP/ADP ratio could account, to a large extent, for the difference in the capabilities for lipogenesis of mitochondria isolated from chow fed compared to starved rats.

The lower phosphorylation potential and ATP/ADP ratio maintained by mitochondria from starved animals may be due to the lower respiratory control ratio of the mitochondria (Table IV). The lower respiratory control ratio is due to a significantly slower rate of state 3 respiration (Table IV). Although the intrinsic ATPase activity of mitochondria from starved

rats was found to be higher (Table IV), this could not account for the lower respiratory control ratio because state 4 respiration was not affected.

DISCUSSION

With this system for studying the involvement of the mitochondrion in lipogenesis, it was reported previously that the capacity of the system to carry out lipogenesis was dependent upon the metabolic condition of the animal prior to sacrifice and isolation of the liver mitochondria (5). In the current study, all incubations were conducted under similar conditions. The only difference was the type of animals, starved 48 hr or chow fed, from which the mitochondria were isolated. Therefore, the changes observed in the incubations are due presumably to the type of mitochondria added. The mitochondrion is involved in 3 major steps for lipogenesis to occur from carbohydrate substrate. Also, the mitochondrion is believed to be important in maintaining an adequate extra mitochondrial phosphorylation potential or ATP/ADP ratio.

The oxidation of pyruvate to acetyl-CoA by pyruvate dehydrogenase is one of the major functions of the mitochondrion in lipogenesis. Pyruvate dehydrogenase is an interconvertible enzyme (18,19) and is less active when fatty acid synthesis is slow (5,18) (Table I), and more active when the rate of fatty acid synthesis is increased (Table II and Fig. 1). It would seem important from a teleological standpoint for metabolism to be regulated at the level of pyruvate dehydrogenase. When pyruvate dehydrogenase is phosphorylated, the enzyme is completely inactive. Thus, the cell can regulate the flow of carbon by the percent of dephosphorylated enzyme. Pyruvate, one of the substrates of this enzyme, is an intermediate in a number of pathways including glycolysis, citrate formation, lipogenesis, ketogenesis, and gluconeogenesis. Pyruvate is also an activator of pyruvate dehydrogenase (18,19) so that when its concentration increases, so will the percent of dephosphorylated enzyme and the rate of flux of pyruvate through the enzyme. Thus, pyruvate exerts an influence on the activity of pyruvate dehydrogenase and also its steady state concentration is dependent in part on the activity of the enzyme. Therefore, under conditions in which the concentration of pyruvate is elevated in the liver, e.g., well fed state, the activity of pyruvate dehydrogenase is elevated (18) (Table I and II) and the rate of lipogenesis is increased (5) (Table II). In an opposite situation of low concentrations of liver pyruvate, e.g.,

TABLE III

Extramitochondrial Phosphorylation Potential, NAD^+/NADH Ratio and $NADP^+$/NADPH Ratio of the Reconstituted Cell Free System for Fatty Acid Synthesis[a]

Mitochondrial source	$\frac{NAD^+}{NADH}$	$\frac{NADP^+}{NADPH}$	$\frac{ATP}{ADP \cdot HPO_4}$[d]	$\frac{ATP}{ADP}$	AXP[e]	ATP	ADP	AMP	Inorganic Phosphate
					(μmole/ml)				
Chow-fed	500 ± 54	.011 ± .002	2800 ± 170	10.4 ± 0.9	2.7 ± .02	2.4 ± .03	.23 ± .01	.059 ± .009	4.8 ± 0.3
Chow-fed + DCA[b]	440 ± 91	-	2700 ± 330	11.6 ± 1.2	2.5 ± .13	1.3 ± .14	.20 ± .02	.063 ± .005	4.5 ± 0.4
Starved 48 hr	440 ± 72	.016 ± .003	1000 ± 130[c]	4.3 ± 0.4[c]	2.8 ± .23	2.2 ± .20	.51 ± .10	.089 ± .025	5.4 ± 0.3
Starved 48 hr + DCA[b]	370 ± 54	-	1500 ± 330[c]	4.8 ± 0.3[c]	2.5 ± .05	2.0 ± .05	.42 ± .11	.071 ± .019	5.1 ± 0.4

[a]Incubations were terminated at 20 min; ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; NAD = nicotinamide adenine diphosphate.

[b]DCA = dichloroacetate.

[c]Student's t test was used. Results are expressed as means ± SEM with 4 mitochondrial preparations for each group. The phosphorylation potential and the ATP/ADP ratio maintained by the mitochondria from chow-fed was significantly higher ($P<0.05$) than the mitochondria from starved.

[d]The HPO_4^{-2} concentration was taken as 79.6% of the total inorganic phosphate at pH 7.4.

[e]AXP denotes the total adenine nucleotides.

TABLE IV

Respiratory Control Ratio and Intrinsic ATPase Activity of Mitochondria Isolated from Chow Fed and 48-hr Starved Rats

	Respiration rate		Respiratory control ratio	ATPase
	State 3	State 4		
	(natoms oxygen/min/mg protein)			(nmoles/min/mg protein)
Chow fed	501 ± 8	17 ± 1	6.1 ± 0.1	28 ± 2
Starved 48 hr	72 ± 3[a]	18 ± 1	4.1 ± 0.1[a]	42 ± 1[a]

[a]$P<0.01$, Student's t test was used. Results are expressed as means ± SEM with 6 mitochondrial preparations in each group.

starvation, the activity of pyruvate dehydrogenase is less (18) (Table I) and the rate of lipogenesis is decreased (5) (Table II). Therefore, under conditions of high pyruvate, pyruvate dehydrogenase should direct the flow of carbon to acetyl-CoA which then can be used for citrate formation. Citrate is transported into the cytoplasm to be cleaved for use in fatty acid synthesis. There should be an abundance of oxaloacetate for synthesis of citrate under conditions of this system because pyruvate carboxylase (E.C. 6.4.1.1) is activated by acetyl-CoA (20). From this discussion it can be seen that the activity of pyruvate dehydrogenase would be expected to correlate with the rate of lipogenesis and the data in this report and the data previously reported (5) support this conclusion. Further support for this suggestion was obtained with studies conducted with dichloroacetate.

Dichloroacetate apparently increases the activity of pyruvate dehydrogenase by inhibiting the kinase that phosphorylates pyruvate dehydrogenase (15,16). In this reconstituted system, the increase in pyruvate dehydrogenase activity caused by dichloroacetate is correlated with an increase in the rate of lipogenesis (Fig. 1 and Table II). When mitochondria from starved rats are treated with dichloroacetate, the rate of lipogenesis is not increased to the level of mitochondria of the chow-fed rats treated with dichloroacetate, even though the activity of pyruvate dehydrogenase is increased to the level of the chow fed mitochondria treated with dichloroacetate. If the activity of pyruvate dehydrogenase were the only controlling factor in this reconstituted system, then the rate of fatty acid synthesis should be increased to the level of mitochondria from chow fed rats.

Thus, it is concluded that the pyruvate dehydrogenase activity of freshly isolated liver mitochondria of starved animals is lower than that of chow fed animals. It also is concluded that this is a factor in differentiating the capacity of these two types of mitochondria to support lipogenesis in the reconstituted system. From the action of dichloroacetate, it is apparent that there is no significant difference in the total pyruvate dehydrogenase activity of the two types of mitochondria; rather the difference exists in the percent of the total enzyme in the active form. These results are consistent with the difference reported by Wieland et al. (21) in the activity of pyruvate dehydrogenase of liver of fed and starved animals. It also is apparent, however, from the results reported above that the activity of pyruvate dehydrogenase is not the only factor involved in the difference between mitochondria prepared from fed and starved animals. Therefore, other factors were investigated.

The NADP/NADPH ratio might be expected to regulate lipogenesis, because NADPH is necessary for the reductive process of fatty acid synthesis. However, as shown in Table III, the $NADP^+$/NADPH ratio is not dependent on the type of mitochondria used in the system.

The levels at which the mitochondria in this system maintain the concentration of ATP and ADP, cytoplasmic phosphorylation potential, or energy charge may be important factors in limiting the rate of lipogenesis. It would appear that the increasing concentration of ADP has the largest influence in this system, because it is twice as high with mitochondria from starved animals, whereas, the concentration of ATP only drops a small amount. The cytoplasmic phosphorylation potential and ATP/ADP ratio would be important because citrate lyase (E.C. 4.1.3.6) and acetyl-CoA carboxylase (E.C. 6.4.1.2) are ATP utilizing enzymes and are essential for lipogenesis to occur from carbohydrate sources. Citrate lyase activity has been shown to be dependent upon the ratio of ATP/ADP such that the smaller the ratio the less effective the enzyme (22). It has been shown that when livers are freeze clamped, the livers from well fed animals have a higher cytoplasmic phosphorylation potential than the livers from animals that have been starved (23). Therefore,

under these conditions, a high phosphorylation potential is correlated with a high rate of lipogenesis and a lower phosphorylation potential with a lower rate of lipogenesis. Also, it has been shown that fructose in high concentrations inhibits fatty acid synthesis, although at low concentrations it is a good lipogenic substrate (24,25). The difference in the response to high and low concentrations of fructose appears to be that at high concentrations fructose drastically lowers the concentration of ATP and the phosphorylation potential to bring about an inhibition of lipogenesis. Bhaduri and Srere (2) and Iliffe and Myant (3) have shown that there must be a high ATP concentration for the rate of lipogenesis to be rapid in a similar reconstituted system. Thus, it might be expected that the cytoplasmic phosphorylation potential, the concentration of ADP, the concentration of ATP, and the ratio of ATP/ADP are important factors in determining the rate of lipogenesis in this reconstituted system.

The phosphorylation potential and ATP/ADP ratio in this system are maintained primarily by the mitochondria. The data in Table III and Figures 2 and 3 show that the mitochondria isolated from starved animals cannot maintain as high a phosphorylation potential or ATP/ADP ratio as mitochondria isolated from chow fed animals. Also, this table shows that dichloroacetate does not alter the phosphorlyation potential or ATP/ADP ratio. Therefore, it is suggested that the phosphorylation potential and ATP/ADP ratio may become limiting factors in this system for lipogenesis when the activity of pyruvate dehydrogenase is increased by dichloroacetate and the mitochondria are isolated from starved animals. The explanation for why mitochondria from starved animals do not maintain as high a phosphorylation potential is not known. However, the lower rates of state 3 respiration, the lower respiratory control ratio, and the higher intrinsic ATPase activity of these mitochondria may be involved (Table IV). Long chain acyl CoA esters have been shown to inhibit the adenine nucleotide antiport at low concentrations (26-31) and lead to a lower extra-mitochondrial phosphorylation potential and ratio of ATP/ADP in a reconstituted system (32). Therefore, in the starved condition in which there are more long chain acyl CoA esters in the liver, the mitochondria from starved animals may be isolated with more bound CoA esters (33) which could inhibit the adenine nucleotide antiport and lead to a lower ATP/ADP ratio. Another factor that might be involved is that mitochondria from starved animals have been reported to have less components of the electron transfer chain (34,35).

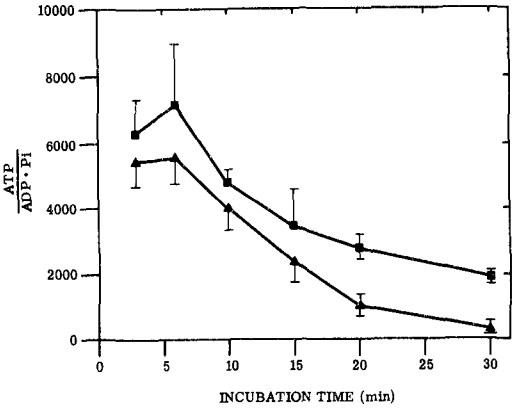

FIG. 2. The extramitochondrial phosphorylation potential in the reconstituted system with liver mitochondria prepared from chow fed (■) and 48-hr starved (▲) rats. ATP = adenosine triphosphate; ADP = adenosine diphosphate.

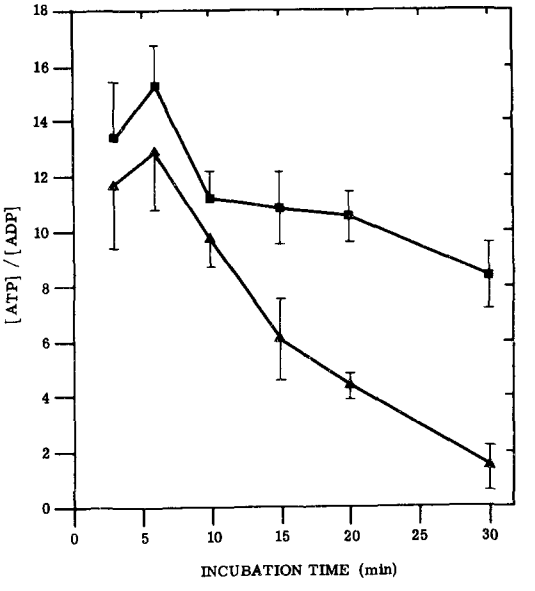

FIG. 3. The extramitochondrial ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) in the reconstituted system with liver mitochondria prepared from chow fed (■) and 48-hr starved (▲) rats.

The high speed supernatant fraction also may contain enough ATPase-like activity to keep the mitochondria near state 3 respiration. If this is the case, then the mitochondria would be synthesizing ATP at nearly maximal rates and the mitochondria from starved animals would not be able to maintain as high a rate of ATP synthesis. This would result in a lower extramitochondrial phosphorylation potential and ATP/ADP ratio and lower rates of lipogenesis.

In summary, the rate of lipogenesis in the cell free system used in this study is less when mitochondria are used that have been isolated from starved animals when compared to mitochondria isolated from chow fed animals. This appears to be due in part to a lower activity of pyruvate dehydrogenase and in part to a lower phosphorylation potential or ATP/ADP ratio maintained in this system by mitochondria isolated from starved animals.

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Effect of Ethanol on Utilization of Plasma Free Fatty Acids for Liver Triacylglycerol Synthesis and Its Relation to Hepatic Triacylglycerol Accumulation in Rats

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ABSTRACT

The effect of 3 different single doses of ethanol on the liver triacylglycerol concentration and on the metabolism of intravenously injected ^{14}C-oleic acid in fasted rats was studied. All 3 doses (2, 3.75, and 6 g ethanol/kg body wt) caused a rapid increase in the liver triacylglycerol concentration during the first 5-6 hr after the ethanol was given. Until the plasma ethanol concentration had fallen to low values, the high liver triacylglycerol levels were raised and were independent of the ethanol dose given. The incorporation of radioactivity from intravenously injected ^{14}C-oleic acid into liver triacylglycerols was increased over control values to the same extent in all rats given ethanol as long as the plasma ethanol concentration was above a low level. High rates of ethanol oxidation and increased utilization of plasma free fatty acids for liver triacylglycerol synthesis were closely correlated with the development and maintenance of the ethanol induced liver triacylglycerol accumulation.

INTRODUCTION

A single large dose of ethanol given to a fasted rat leads to a reversible increase in the liver lipid concentration (1). The major part of the fatty acids in the accumulated triacylglycerols is derived from the adipose tissue (2). The underlying mechanism was first believed to be an increased mobilization of free fatty acids from the adipose tissue (3). However, another possible explanation is, that during oxidation of ethanol in the liver, a larger fraction of the fatty acids entering the liver from the plasma is used for triacylglycerol synthesis and a lesser fraction is oxidized (4-6). Much evidence has been gathered in favor of the latter hypothesis (6-8), and recently it was shown using a technique with serial determination of the plasma free fatty acid concentration, that ethanol may cause a liver triacylglycerol accumulation in fasted rats without an increase in the plasma free fatty acid concentration during the period of lipid accumulation (9). This strongly suggests that increased lipolysis in the adipose tissue is not a prerequisite for the development of the acute ethanol-induced fatty liver in the rat.

In the present investigation we have studied the utilization of plasma free fatty acids for liver triacylglycerol synthesis and the liver triacylglycerol concentration in fasted rats during a 16-hr period after different single doses of ethanol were given. This was done to elucidate if there might be a causal relationship between the effects of ethanol on these parameters and also to investigate the possible relationship between the dose of ethanol given and the accumulation of liver triacylglycerols.

MATERIALS AND METHODS

Preparation of Labeled Injection Solution

1-^{14}C-oleic acid (Radiochemical Centre, Amersham, England) was used as supplied without further purification. It was complexed to 5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in saline (10).

Experimental Design

Female Sprague-Dawley rats (Anticimex, Stockholm, Sweden) weighing 140-160 g were used. Up to the experiment, they were fed a standard laboratory diet (AB EWOS, Södertälje, Sweden) and water ad libitum. After a 24-hr fast, the unanaesthetized rats were tube fed ethanol as a 38% solution (v/v) or saline (0.9% w/v NaCl). Ethanol was given in 3 different doses, 2.00, 3.75 or 6.00 g/kg body wt. Rats killed after 0 hr received no treatment. After tube feeding, the rats continued to fast, but had free access to tap water. In experiment I, at 0, 6, or 16 hr after treatment, rats from different groups were anaesthetized with ether and 0.5 ml of the fatty acid-albumin complex (containing 10 x 10^6 cpm/ml) was injected into the left jugular vein. Five min later, the rats were killed by exsanguination through the aortic bifurcation. The blood was transferred to a chilled heparinized centrifuge tube, and plasma was collected after centrifugation at 4 C.

In experiment II, rats were treated identically, except that no labeled fatty acid was injected, and rats were killed after 0 and 5 hr. The liver was removed rapidly, rinsed in water,

TABLE I

Distribution of Radioactivity in Liver and Carcass Lipids After ^{14}C-Oleic Acid Injection[a]

Ethanol dose (g/kg body wt)		Time after treatment (h)	Plasma ethanol concentration (mM))	Radioactivity (% of injected dose)	
				Liver triacylglycerol (whole liver)	Carcass fatty acids
0	(12)[b]	0		15.4 ± 0.9	33.3 ± 0.5
0	(6)	6		15.8 ± 1.0	33.9 ± 0.8
2.00	(6)	6	4.0 ± 1.7	20.8 ± 1.1[c]	36.5 ± 1.5
3.75	(6)	6	35.7 ± 3.2	29.0 ± 1.0[d]	35.9 ± 1.1
6.00	(5)	6	61.0 ± 4.4	31.2 ± 1.1[d]	36.0 ± 1.6
2.00	(6)	11	1.0 ± 0.3	16.3 ± 1.1	36.4 ± 1.4
3.75	(5)	11	1.5 ± 0.5	19.8 ± 1.9	35.7 ± 1.2
6.00	(6)	11	53.7 ± 5.4	26.8 ± 0.4	37.6 ± 0.7
0	(5)	16		12.1 ± 0.9	34.9 ± 1.6
2.00	(6)	16	0.6 ± 0.2	13.0 ± 0.6[c]	35.8 ± 1.5
3.75	(6)	16	1.7 ± 1.0	13.7 ± 0.7[c]	33.7 ± 1.0
6.00	(5)	16	3.3 ± 1.6	19.9 ± 1.8[c]	35.0 ± 2.3

[a]Values (mean ± SE) collected 5 min after intravenous injection of ^{14}C-Oleic Acid into fasted female rats fed different doses of ethanol or saline.

[b]( ) = Number of rats.

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

[d]Significantly ($P<0.01$) different from corresponding control group.

blotted dry, weighed, and homogenized in chloroform:methanol (2:1). The rat carcass was transferred to a 1-liter flask containing 300 ml 30% ethanolic KOH.

Analyses

Plasma ethanol concentration was determined by gas chromatography as described previously (6), but with 2-butanol instead of methanol as internal standard. A chloroform extract of the liver lipids was prepared according to Folch, et al., (11). Lipid classes were separated by thin layer chromatography (TLC) as described previously (12). Aliquots of the triacylglycerol extracts were taken for determination of esterlinkages (13).

Carcass extracts were heated at 65 C overnight, the volume made up to 1000 ml with 95% ethanol, and fatty acids extracted from aliquots of the digest (14). The radioactivities of aliquots of carcass fatty acids and liver triacylglycerols then were determined in a Packard, model 3320, Tri-Carb scintillation spectrometer. Quenching was corrected for by use of external standard.

Statistics

Differences between groups of rats were tested using Wilcoxon's rank sum test (15). Differences were considered significant for $P < 0.05$.

RESULTS

Rats given 3.75 or 6 g of ethanol/kg body wt were markedly ataxic between 0 and 6 hr. In contrast, rats given 2 g of ethanol/kg body wt could not be distinguished in behaviour from control rats.

Six hr after the ethanol was given, all 3 groups of rats in experiment I had increased liver triacylglycerol concentrations compared with the controls ($P < 0.01$ in all groups), (Fig. 1). The groups given 3.75 and 6 g of ethanol/kg body wt had similar high triacylglycerol concentrations. These rats also had high plasma ethanol concentrations (Table I). The triacylglycerol concentration was somewhat lower in the group of rats given 2 g of ethanol/kg body wt. In this group, the mean plasma ethanol concentration was 4 mM (Table I), and in 3 of the 6 rats, the concentrations were 2 mM or less. At these low plasma concentrations, the rate of liver ethanol metabolism is decreased (16), i.e., the liver alcohol dehydrogenase probably is not saturated (16,17). In a second experiment (experiment II), identically treated rats were killed after 5 hr. In this experiment the mean plasma ethanol concentrations in all 3 groups of rats were high enough, in the 2 g group 9.8 mM, to maintain oxidation of ethanol at about the same high rate in all groups of rats. There was no statistically significant difference in liver triacylglycerol concentration between the groups ($P > 0.05$, Fig. 2).

After 6 hr, the liver triacylglycerol concentration decreased in the 2 g group and reached almost control levels at 16 hr (Fig. 1). Between 6 and 11 hr, the mean values of the liver triacylglycerol concentrations increased in both the 3.75 and 6 g groups (Fig. 1). However, the differences between the values at 6 and 11 hr within each of these groups were not statisti-

cally significant. Also at 11 hr the liver triacylglycerol concentrations were similar in the 3.75 and the 6 g groups (Fig. 1). At 11 hr the rats given 3.75 g ethanol/kg body wt had little or no ethanol left in the blood, whereas, the rats given 6 g ethanol/kg body wt still had a high plasma ethanol concentration (Table I). Between 11 and 16 hr, the liver triacylglycerol concentration did not decrease in the 6 g group, while it decreased in the 3.75 g group ($P < 0.05$, Fig. 1). Thus, the liver triacylglycerol concentration increased or remained high as long as the plasma ethanol concentration was above a very low level. Furthermore, when the plasma ethanol concentration had fallen to lower levels, the liver triacylglycerol concentration decreased (Table I, Fig. 1).

To study the utilization of plasma free fatty acids for synthesis of liver triacylglycerols, the incorporation of radioactivity into this liver lipid from intravenously (IV) injected oleic acid was measured. Long chain fatty acids entering liver cells are ultimately oxidized to carbon dioxide and ketone bodies or esterified to form triacylglycerols, phospholipids, and other fatty acid esters. IV injected labeled fatty acids are rapidly incorporated into liver lipids, and 3 min after injection only negligible amounts of radioactivity are present in free fatty acids in the liver triacylglycerols reaches a maximum ca. 3 min after injection, and remains essentially unchanged up to 20 min after injection (14,18). In this and other studies (5,6,9) the incorporation of radioactivity from labeled oleic acid into liver triacylglycerols 5 min after injection has been used to estimate the utilization of plasma free fatty acids for synthesis of liver triacylglycerols.

Rats that still had a high level of ethanol in their plasma incorporated more radioactivity from the injected oleic acid into their liver triacylglycerols than did the control rats or the rats that already had cleared the ethanol from the plasma (Table I). The rats that were probably just removing the last ethanol from the plasma, i.e., the 2 g group at 6 hr, the 3.75 g group at 11 hr, and the 6 g group at 16 hr, showed intermediate values. The incorporation of radioactivity into carcass lipids was not much altered after ethanol treatment (Table I), suggesting that the partition of the flux of plasma free fatty acids between the liver and the rest of the body was not affected by the ethanol treatment.

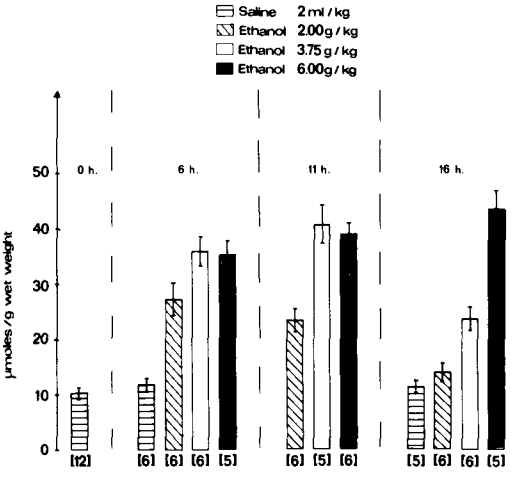

FIG. 1. Liver triacylglycerol concentrations in fasted female rats tube fed different doses of ethanol and in control rats tube fed saline. Control rats in the 0 hr group were not tube fed. Rats were killed at different times after tube feeding as indicated in the figure. Values are Mean ± SE; number of rats is in parentheses.

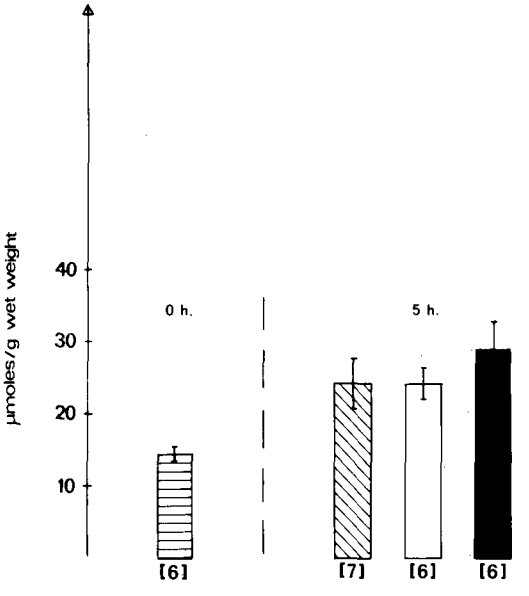

FIG. 2. Liver triacylglycerol concentration in fasted female rats tube fed different doses of ethanol and in control rats tube fed saline. For indications see Figure 1. Control rats in the 0 hr group were not tube fed. Values are Mean ± SE; number of rats is in parentheses.

DISCUSSION

In previously fasted rats fed a large dose of ethanol, i.e., the conditions of the present experiments, the rate of fatty acid synthesis is low (19). Therefore, the main source of fatty acids for the liver is the plasma free fatty acids. Their metabolism in the liver ultimately leads to oxi-

dation or esterification. It has been shown previously that as a consequence of the ethanol oxidation in the liver a larger fraction of the plasma free fatty acids is incorporated into liver triacylglycerols in ethanol fed rats than in control rats (5,9). This was suggested to be a major factor for the observed increase in the liver triacylglycerol concentration. Our present data are in agreement with this hypothesis, because at all times when the liver triacylglycerol concentration increased or was maintained at a high level, there was an increased incorporation of IV injected labeled free fatty acids into the liver triacylglyerols (Table I). Both in the present and in previous experiments, ethanol probably did not change the distribution of the plasma free fatty acid flux between the liver and the rest of the body, because the fatty acid radioactivity in the carcass after IV injection of labeled fatty acid was not altered by ethanol treatment. Thus, this effect of ethanol is most likely brought about by a shift in the metabolism in the liver cells of free fatty acids derived from the plasma from oxidation to esterification. This is in agreement with the observation that ethanol cause such a change in the free fatty acid metabolism in isolated liver cells (20). The lipid load on the liver by this mechanism is dependent on the uptake of plasma free fatty acids into the liver which is proportional to the plasma free fatty acid concentration (21). In experiments with single large doses of ethanol, the plasma free fatty acid concentration has been reported to be either unchanged (4-6,22) or increased (3). In either case, the influx of plasma free fatty acids into the liver cells is large, and in conjunction with the increased utilization for triacylglycerol synthesis, probably is the major cause of the acute ethanol induced fatty liver in rats.

Although conflicting results exists (23), the acute ethanol induced lipid accumulation in fasted rats probably is dependent on a rapid oxidation of ethanol in the liver (6-8,24). The ethanol oxidation is a zero-order process at all but very low blood ethanol concentrations probably owing to the dominating role of liver alcohol dehydrogenase (EC 1.1.1.1) in this process (17). Makar, et al., (16) found a constant rate of ethanol metabolism in the rat at blood ethanol concentrations above 0.02% (4.3 mM). Thus, a major prerequisite for the liver lipid accumulation should be a blood ethanol concentration at, or above this level.

All 3 doses of ethanol used in the present study led to an increase in the liver triacylglycerol concentration (Fig. 1). Moreover, as long as the plasma ethanol concentration was above a low level, the high liver triacylglycerol concentrations were maintained (Table I, Fig. 1). Due to the rapid ethanol oxidation in the rat, an exact determination of this critical plasma ethanol level is not possible with the present experimental design. No difference between the ethanol treated groups was seen in the magnitude of the liver triacylglycerol accumulation as long as the blood ethanol concentration were above a low level. Thus, a larger dose led to a higher triacylglycerol accumulation only because it provided a longer period of triacylglycerol accumulation. This does not necessarily imply that all lipid parameters influencing the liver triacylglycerol concentration are independent of the ethanol dose. However, the rate of liver lipid accumulation is strikingly independent of the amount of ethanol given. These findings differ somewhat from those of Maling, et al., (25), who found an increase in the liver triacylglycerols in only a few rats given 2 g ethanol/kg body wt at 6 hr after ethanol treatment. However, in the corresponding group in the present investigation, the liver triacylglycerol concentration was above that seen in control rats, but was probably decreasing at this time. Thus, small differences in the time course of the liver triacylglycerol concentration in experiments performed in different laboratories may well explain the discrepancy.

Recently, we have shown in rats with constant blood ethanol levels maintained by continuous IV infusion of ethanol, that in contrast to a moderately high blood ethanol concentration (45 mM) a low blood ethanol concentration (10 mM), although high enough to maintain a high rate of ethanol oxidation, did not lead to an increase in the liver triacylglycerol concentration (9). This was probably due to a transient decrease in the plasma free fatty acid concentration. Thus, the blood ethanol level above which a rapid liver lipid accumulation occurs is not identical with the level required to maintain a constant rate of ethanol oxidation.

When the ethanol was cleared, the liver triacylglycerol concentration decreased in the 2 g and 3.75 g groups (Fig. 1). In other experiments, we have found that this is true also for rats give 6 g ethanol/kg body wt (19). Thus, increased utilization of liver triacylglycerols occurs at this time. Probably, this compensatory increased utilization of the liver triacylglycerols starts around 6 hr, because after this time no further rapid rise in liver triacylglycerol concentration was observed in the 2 groups of rats that still showed high blood ethanol concentrations. This is in good agreement with our previous results (12), showing that ethanol after a lag period of several hours increased the

plasma triacylglycerol concentrations in previously fasted female rats, probably due mainly to an increase in the liver triacylglycerol secretion.

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Tumor Extracellular Triglycerides in Mice During Growth of Ehrlich Ascites Carcinoma[1]

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ABSTRACT

Our earlier work with Swiss-Webster mice has shown that most of the lipid in Ehrlich ascites tumor extracellular fluid is in the form of free fatty acids. This finding is in direct contradiction to earlier and subsequent reports from another laboratory that has found free fatty acids to be a very minor component and triglycerides to be the major lipid of Ehrlich ascites tumor extracellular fluid. In light of these contradictory reports, we have carried out a study patterned after that of other workers, but using our Swiss-Webster mice. As predicted from our earlier study, we have found very little triglyceride in Ehrlich ascites tumor extracellular fluid. Although we could demonstrate a significant, transient hypertriglyceridemia during tumor growth, maximum plasma triglyceride concentrations were an order of magnitude lower than those reported by other workers. In addition, and again in contrast to other reports, we found that plasma triglyceride and tumor extracellular fluid triglyceride levels in tumorous mice fell significantly with fasting. Thus, interesting differences in triglyceride metabolism between mouse and/or tumor strains seem to exist. Our present findings suggest, but do not prove, that triglycerides in the tumor extracellular fluid probably are not a major source of the rapidly turning over, tumor extracellular fluid free fatty acid in our mice.

INTRODUCTION

Free fatty acids (FFA) in the extracellular fluid of Ehrlich ascites carcinoma are replaced rapidly (1,2); however, very little of the FFA passing through the tumor's extracellular fluid FFA pool is derived from plasma FFA (2). Recently Brenneman, et al.,(3) suggested that one possible important source of FFA for lipid synthesis during tumor growth may be very low density lipoprotein (VLDL) triglyceride fatty acids (3). In support of this possibility was the observation that their tumor-bearing mice were severly hyperlipidemic due to elevated VLDL-triglycerides and cholesterol levels (3). Moreover, extremely high concentrations of VLDL-triglycerides were found in the tumor extracellular fluid.

This finding was at variance with observations from our laboratory using a different strain of mouse in which very little lipid fatty acid (FA) other than FFA was found in Ehrlich ascites carcinoma extracellular fluid (2). However, in view of the marked discrepancy between the data of Brenneman, et al., (3) and those obtained by Mermier and Baker (2), we have carried out a detailed study of triglyceride levels in plasma and tumor extracellular fluid during Ehrlich ascites tumor growth in Swiss-Webster mice. Despite a moderate and transient hypertriglyceridemia that developed during tumor growth, tumor extracellular fluid remained relatively free of triglycerides, especially in the fasted state. Although these data cannot rule out the possibility that FFA in tumor extracellular fluid is derived to a large extent from plasma VLDL-triglycerides, they do not lend strong support to this hypothesis.

EXPERIMENTAL PROCEDURES

Tumor

Male Swiss-Webster mice (Hilltop Lab. Animals, Inc., Chatsworth, CA), 6-10 weeks old, were used. Mice bearing Ehrlich-Lettré hyperdiploid carcinoma were originally obtained from R. McKee, (Biological Chemistry Department, UCLA School of Medicine, Los Angeles, CA), and transplanted as described previously (2). The common chromosome pattern of our Ehrlich ascites tumor was close to diploidy with a number of 44, occasionally 45. Polyploidisation occurred in about 10-15% of the cells examined. The inoculum of washed tumor cells was 1.5 x 10^7 cells/mouse, injected intraperitoneally. Although the number of tumor cells injected was the same as that used in previous studies from this laboratory (2), the inoculum was 5 times larger than that used by Brenneman, et al., (3) in their earlier study of hypertriglyceridemia in tumorous CBA mice. The tumor size was at its maximum on day 14.

[1]Veterans Administration Project RI 3-60, MRIS No. 0790.

TABLE I

Triglyceride Concentrations of Blood Plasma and Tumor Extracellular Fluid in Mice During Growth of Ehrlich Ascites Tumor

Day of tumor	Triglyceride (mg/dl) Normal, blood plasma[a]	Tumor, blood plasma[a]	Tumor extracellular fluid[a]
2	48 ± 9	52 ± 14	---
4	148 ± 24	178 ± 20	---
6	138 ± 25	228 ± 32[b]	---
8	129 ± 10	281 ± 51[c]	17.5 ± 6.5
10	63 ± 14	224 ± 45[d]	5.4 ± 3.9
12	100 ± 8	87 ± 29 (5)	1.4 ± 1.4 (5)
14	91 ± 11	6 ± 3.8 (4)	< 0.2 (4)

[a]Mean ± S.E. of 6 animals from each group unless otherwise marked in parenthesis.
[b]Significantly ($P<0.05$) different when compared to normal, blood plasma group.
[c]Significantly ($P<0.02$) different when compared to normal, blood plasma group.
[d]Significantly ($P<0.01$) different when compared to normal, blood plasma group.

TABLE II

Effect of Fasting on Triglyceride Levels of Blood Plasma and Tumor Extracellular Fluid in Tumorous Mice

Nutritional state	Triglyceride (mg/dl)[a] Normal, blood plasma	Tumor, blood plasma	Tumor extracellular fluid
Fed[b]	55 ± 8.7	310 ± 36	19 ± 8.5
6-hr fast[b]	27 ± 5.8	108 ± 26	4.5 ± 2.8
24-hr fast[c]	42 ± 8.7	44 ± 20	1.0 ± 1.0

[a]Triglyceride levels were determined on the 8th day after tumor transplantation.
[b]Mean ± S.E. of 6 animals from each group.
[c]Mean ± S.E. of 4 animals from each group.

The mean survival time of tumor-bearing mice was 15 days. The animals were fed Purina chow diet and water ad libitum. Initial body wts were 30-35 g. Body wts were recorded every other day to monitor tumor growth.

Sampling

Blood from tumorous and control animals was obtained from the opthalmic venous sinus in heparinized capillaries around 10 AM on days 2, 4, 6, 8, 10, 12, and 14. The blood was immediately centrifuged at 1000 rpm for 1 min at 4 C and the plasma separated from the red cells. Tumor samples were obtained by puncturing the abdomens of tumor-bearing animals with a needle. The needle was removed from the peritoneal cavity, and ca. 0.1-0.2 ml tumor was allowed to drip into heparinized capillaries by gently squeezing the abdomen. Extracellular fluid was separated from the tumor cells by centrifugation as described above. The tumor could not be obtained readily on days 2, 4, and 6 employing this method.

To study the effect of fasting, another group of 12 animals, 6 normal mice and 6 mice bearing 8-day old tumors, was used. The sampling was done at ca. 12 noon in the fed state, 3 PM, 6-hr fasted, and 10 AM the next day, 24-hr fasted. It was found, however, that the blood from 24-hr fasted tumorous animals had very low hematocrit values. To exclude the possibility that repeated sampling had caused hemodilution, blood from another set of 8, 24-hr fasted mice (4 normal and 4 bearing 8-day old tumors) was used for the 24-hr values.

Analyses of Triglycerides

Triglycerides of blood plasma and extracellular fluid were estimated using the method of Galletti (4) with slight modifications. Twenty μl plasma and 20 μl saline were taken instead of 40 μl plasma, and 1 ml of isopropyl ether was used instead of 0.5 ml. Plasma and extracellular fluid triglyceride values were read from a simultaneously run tripalmitin standard curve.

RESULTS AND DISCUSSION

Blood plasma triglyceride levels of Ehrlich

ascites carcinomatous mice increased significantly ($P<0.05$) relative to normal controls within 6 days after tumor inoculation, as shown in Table I. Maximum hypertriglyceridemia was observed on the 8th day of tumor growth. Plasma triglyceride levels fell after that and were at their lowest values by the time the tumor grew to its maximum size (ca. 18 ml in 14 days [5]). Very little triglyceride was found in the tumor extracellular fluid at the time that plasma triglyceride was maximal in the tumorous mice. Triglyceride levels in the tumor extracellular fluid fell after the 8th day to levels that were undetectable by the 14th day (Table I).

Plasma triglyceride values of the normal ad libitum fed mice were in the range of those reported by others (6); however, samples taken at noon instead of 10 AM (Table I, days 2 and 10; Table II, fed controls) tended to be lower than the others. This may have resulted from a fasting effect, because food deprivation for 6 hr was shown to lower plasma triglyceride concentration significantly (Table II). The fasting effect on plasma triglyceride levels was pronounced even more in tumorous mice, falling from an average of 310 mg/dl (Fed) to 44 mg/dl after a 24-hr fast (Table II).

The effect of fasting on plasma and extracellular fluid triglyceride levels may be related to the marked fall in both of these values seen during the late stages of tumor growth (Table I). That is, as the tumor grows, the animals either may lose their appetites or become unable to reach their food. Although food intake was not measured in our study, the results in Table II show that it is imperative to control food intake carefully to study triglyceride metabolism in tumorous mice.

In some respects, our present findings confirm the results of Brenneman, et al., (3); namely, a rise and fall of blood plasma triglyceride concentration during tumor growth. However, the magnitude of the tumor-induced hypertriglyceridemia was very much greater in their study than in ours. Brenneman, et al., (3) found plasma triglyceride values as high as 1600 mg/dl on the 11th day of tumor growth. This is about an order of magnitude higher than we have observed. Even more interesting are the differences in triglyceride levels in the tumor extracellular fluid. Whereas, Brenneman, et al. (3) found average values of 336 mg/dl in 12-day old tumors, we observed a barely detectable value of 1.4 mg/dl. This then confirms the earlier work of Mermier and Baker who found that only a minor fraction of the tumor extracellular total lipid fatty acid was esterified (2).

Another interesting difference between the behavior of triglycerides in our studies and those of Brenneman, et al., (3) is the response of tumor extracellular fluid triglyceride levels to fasting. They reported that the very high levels of extracellular fluid triglycerides found in 12-day old tumors were not changed during a 16-hr fast. They cited this observation as evidence that this triglyceride was of endogenous origin, independent of dietary lipid. However, there was a dramatic, total disappearance of triglycerides from the extracellular fluid during a 24-hr fast in the mice in our study.

The present results strongly suggest that the tumor strains and/or the mouse strains used by Spector's group and by us have vastly different control systems governing the mobilization and metabolism of FFA and triglycerides during tumor growth. Moreover, the fact that the tumors of the mice in our study have been found to have the same FFA pool size, replacement rate, and metabolic fate as those of Spector (2,7), even though they have dramatically different concentrations of extracellular triglycerides, leads us to conclude that VLDL-triglyceride is probably not a major source of tumor extracellular FFA in either mouse strain. Comparative kinetic studies using labeled triglycerides will be required to establish the quantitative importance of VLDL-triglycerides with respect to net tumor lipid accumulation during tumor growth and with respect to the tumor's energy needs in these 2 very different populations of tumor-bearing mice.

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Effects of Dietary Triglyceride on the Properties and Lipid Composition of Plasma Lipoproteins: Acute Experiments in Rats Fed Safflower Oil

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ABSTRACT

Male rats were administered 1.5 ml safflower oil by gastric intubation 0, 4, and 8 hr after a 16 hr fast. Plasma, liver, and adipose tissue were collected 16 hr after the last fatty meal. Rats fasted for 16 hr served as controls. Following fat feeding, the fatty acid composition of the very low density lipoprotein, triglyceride, and hepatic triglyceride were similar, as were the percentages of 18:2 in the very low density lipoprotein and hepatic cholesteryl esters. The phospholipids of liver and plasma lipoproteins were similar in the control groups, except that more 16:0 was present in the plasma lipoproteins. After fat feeding, the plasma lipoprotein phospholipids were enriched with 18:2 more than were the hepatic phospholipids. Furthermore, the percentage of 18:2 in phospholipid was much less than in triglyceride or cholesteryl esters. Clearly, esterified lipids of liver and plasma lipoproteins (very low density lipoprotein, low density lipoprotein, and high density lipoprotein), and to a lesser extent, adipose tissue, were enriched with 18:2 derived from dietary triglyceride fatty acid even 16 hr after the terminal meal. A major proportion of the very low density lipoprotein isolated by ultracentrifugation in zonal rotors from plasma of fat fed animals had a faster rate-zonal mobility than did the very low density lipoprotein isolated from plasma of control animals. The very low density lipoprotein isolated from plasma of fat fed rats contained fewer moles of phospholipids, cholesterol, and cholesteryl esters, relative to triglyceride than did the very low density lipoprotein from plasma of animals not receiving safflower oil. The molar ratio triglyceride:phospholipid:cholesterol:cholesterol esters in the very low density lipoprotein was 100:42.0:22.1:44.5 in the control group and 100:35.4:17.8:19.5 in the fat fed animals. It is postulated that an important biochemical mechanism by which dietary triglyceride fatty acids consumed by the animal over a long period of time alter plasma concentrations of triglyceride, phospholipids, and cholesterol esters is the directive influence of plasma free fatty acid, derived from dietary triglyceride, on the secretion of very low density lipoprotein lipids by the liver.

INTRODUCTION

The very low density lipoprotein (VLDL) secreted by the isolated perfused rat liver in response to infusion of oleate contains fewer moles of cholesterol and phospholipid (PL) relative to triglyceride (TG) and has a faster flotation rate in the zonal ultracentrifuge than does the VLDL secreted when palmitate is infused (1). Similar observations were made when other common long chain saturated (14:0, 18:0) fatty acids were compared with unsaturated (16:1, 18:2) fatty acids (2). These differences in lipid composition and rate-zonal mobility suggested to the authors that the VLDL synthesized and secreted by the liver in vitro from unsaturated free fatty acids (FFA) are larger and less dense particles than those synthesized from saturated FFA. Apparently, more of the relatively polar cholesterol and PL are incorporated into the structure of the VLDL particle when the TG was synthesized predominantly from saturated long chain fatty acids than when synthesized from unsaturated fatty acids. The size of the VLDL particle secreted by the liver may depend also on the rate of snythesis of VLDL TG, such that the size of the particle appears to increase with increasing output of TG (2).

It is clear that FFA stimulate the formation and secretion of TG and other lipid components of the VLDL by the isolated rat liver, and that the properties of the VLDL, in turn, are dependent on the properties of the FFA perfusing the liver (3). In the intact rat (4) and in man (5), the composition of the plasma FFA varies with the fatty acid composition of the

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TABLE I

Concentration of Plasma Lipids[a]

Group	Lipid class				
	Triglycerides	Phospholipid	Free cholesterol	Cholesterol ester	Free fatty acid
Control (5)[b]	40.8 ± 1.7 [36.6 - 46.2][c]	127.7 ± 4.2	46.7 ± 4.8	110.6 ± 5.4	59.7 ± 5.1
Fat fed (7)	86.2 ± 19.9[d] [51.3 - 203.9][c]	151.6 ± 4.8[d]	44.4 ± 1.6	111.0 ± 5.9	53.1 ± 5.2

[a]μMoles ± SEM/100 ml plasma.
[b]Figures in parentheses indicate number of observations.
[c]Range of values.
[d]Significantly different from controls, $p < 0.05$.

dietary TG. It is reasonable to assume, therefore, that in vivo, as in vitro, the ultracentrifugal properties and fatty acid composition of the plasma VLDL will change in response to the dietary TG. We wish to report that such changes in the properties of the plasma VLDL of intact rats were observed after feeding safflower oil, an oil high in content of linoleic acid. The VLDL isolated from the plasma of rats deprived of food after feeding safflower oil had a more rapid rate-zonal mobility in the ultracentrifuge, and contained less PL and cholesterol per mole TG than did the VLDL isolated from plasma of fasted control animals.

A preliminary report of this work has appeared (6).

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague-Dawley (Holtzman Inc., Madison, WI) rats were maintained on Standard Purina Laboratory Chow from the time of their arrival in the laboratory until the day prior to the experiment (2-3 weeks). Body wts of the rats ranged from 175-300 g at the time of the experiments. Animals were fasted for 16 hr. One group was then sacrificed to serve as control while another group was fed 1.5 ml safflower oil at 0 time (after the 16 hr overnight fast), 4 hr, and 8 hr later by gastric intubation under light ether anesthesia. The animals in the control group and in the experimental group (16 hr after the third meal) were anesthetized with ethyl ether and blood was collected from the abdominal aorta. Samples of liver and adipose tissue (epididymal fat pad) were also obtained.

Analytical Procedures

The blood was collected in tubes containing ethylenediamine tetraacetic acid sodium salt (EDTA) (1 mg/ml blood), centrifuged to remove formed elements, and plasma was collected. The plasma was pooled (5-7 rats), and aliquots were taken and extracted immediately for lipid analysis. Total lipids, extracted from plasma and from plasma lipoproteins with $CHCl_3$:CH_3OH (2:1, v/v), were separated into lipid classes by chromatography on thin layer plates (250 μ thick, Silica Gel G) (7). The liver was perfused briefly with a solution of 0.9% NaCl to wash out residual blood; it was removed, cleansed of adherent diaphragm and other tissue, blotted, weighed, and homogenized with 10 vol of 95% ethanol. The hepatic residue was extracted further with ethyl ether

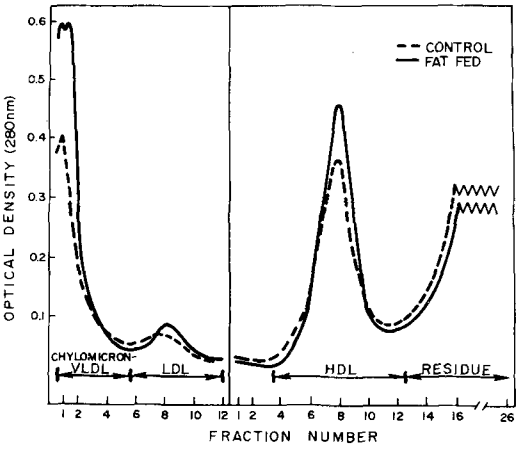

FIG. 1. Zonal ultracentrifugation of rat plasma lipoproteins. The pattern is typical for each group. Twenty-five ml of rat plasma was adjusted to a density of 1.4 with solid NaBr and was introduced into the Ti-14 (Spinco) zonal rotor behind a 600 ml linear density gradient (d1.0-1.4, NaBr). Sufficient NaBr solution, d=1.4, was added to fill the rotor. Centrifugation was carried out for 2 hr at 43,000 rpm, after which 300 ml were displaced from the core of the rotor at 3000 rpm in 25 ml fractions to isolate the very low density lipoprotein and low density lipoprotein. 250 ml distilled water was then introduced at the core of the rotor. Centrifugation was continued for 20 hr at 43,000 rpm after which the entire contents of the rotor were displaced and the high density lipoprotein and residue were obtained. All solutions contained 1 mM ethylenediamine tetraacetic acid sodium salt.

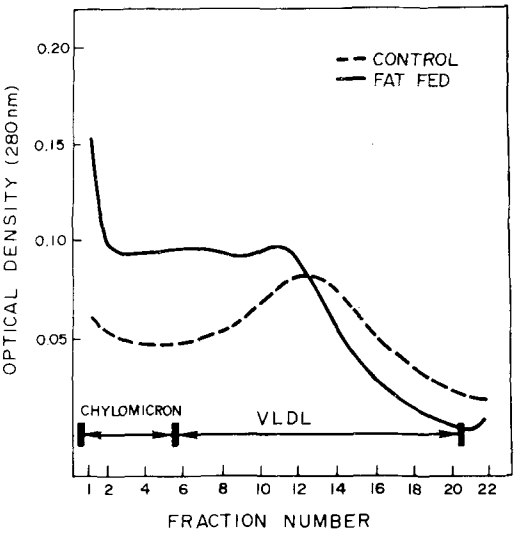

FIG. 2. Zonal ultracentrifugation of very low density lipoprotein (VLDL) and chylomicrons. Patterns are typical for both groups. The chylomicron-VLDL fraction obtained in the initial isolation (Fig. 1) was concentrated and adjusted to d=1.4 with NaBr. The initial gradient consisted of a 200 ml overlay of water, 380 ml of a linear density gradient (d1.0-1.4), and a sample volume of 70 ml. Centrifugation was carried out for 20 min at 30,000 rpm. Fractions of 25 ml each were displaced from the rotor.

in a Soxhlet extractor (8). The crude extract was dried in vacuo and solubilized in petroleum ether. An aliquot of this extract was reduced in volumn in vacuo, and applied to the thin layer plates. A sample of epidiymal fat pad was homogenized in 20 ml $CHCl_3$:CH_3OH (2:1) and then treated, as were extracts of plasma. Duplicate separations by thin layer chromatography (TLC) were carried out in all cases. The thin layer plates after development were sprayed lightly with 0.1% aqueous Rhodamine 6G, and the bands of lipid were visualized under ultraviolet light. The bands were scraped from the plates and eluted with 10 ml $CHCL_3$. Aliquots of the extracts were analyzed for cholesterol or cholesteryl esters (CE) (9) and for triglyceride (10). Total lipid soluble phosphorus (calculated as PL) was determined by the method of King (11) after digesting the band scraped from the origin (2). Samples of TG, FFA, PL, and CE isolated by TLC were methylated with BF_3 (12) and the percentage composition of the fatty acid methyl esters in each lipid class was measured by gas liquid chromatogrphy (5). An aliquot of the pooled plasma (20-25 ml) was used for the isolation of the plasma lipoproteins by density gradient ultracentrifugation in zonal rotors. Isolation was started on the same day that the plasma was collected from the animals. The general methodology for this procedure has been described previously in detail (13). Protein was determined by the method of Lowry, et al., (14) using bovine serum albumin as the standard.

Materials

All solvents were of reagent grade and were redistilled from glass prior to use. Safflower oil was generously provided by F. Mattson and Proctor-Gamble Co. (Cincinnati, OH) The composition of the oil was: 6.2%, 16:0; 1.8%, 18:0; 12.4%, 18:1; and 79.6%, 18:2. Precoated (Silica Gel G, schedule A) thin layer plates for chromatography were obtained from Analtech, Inc. (Newark, DE). Statistical analyses were carried out by Student's t test or the Mann-Whitney U test.

RESULTS

The concentrations of various lipids in the plasma are shown in Table I. The concentration of plasma TG and PL was significantly ($P<0.05$) elevated in the fat fed group. No changes in the

TABLE II

Composition of Plasma Lipoproteins

Group	Lipids[a]				Protein[b]
	Triglycerides	Phospholipid	Free cholesterol	Cholesterol ester	
VLDL					
Control (5)[c]	55.1 ± 1.5	20.3 ± 0.6	5.8 ± 0.3	18.9 ± 1.5	10.0 ± 1.7
Fat fed (6)	67.0 ± 2.2[d]	18.9 ± 1.4	5.0 ± 0.4	9.1 ± 1.2	9.6 ± 2.0
LDL					
Control (5)	11.3 ± 1.0	32.8 ± 1.2	12.7 ± 0.6	43.2 ± 1.6	12.6 ± 1.6
Fat fed (6)	14.8 ± 1.2	32.0 ± 1.1	11.4 ± 0.3	41.7 ± 1.2	12.3 ± 1.0
HDL					
Control (5)	1.0 ± 0.2	48.5 ± 2.3	7.5 ± 0.8	43.0 ± 2.6	39.5 ± 1.0
Fat fed (6)	1.0 ± 0.1	53.3 ± 1.8	7.0 ± 0.2	38.8 ± 1.9	39.7 ± 1.2

[a]Data are means ± SEM, expressed as % by wt of the lipid portion of the lipoprotein. Mol wts used in the calculations were cholesterol (C), 387; cholesteryl ester (CE), 651; phospholipid (PL), 751; and triglyceride (TG), 850.

[b]Data are means ± SEM, expressed as % by wt of the total lipoprotein (lipid plus protein).

[c]Numbers in parentheses indicate number of observations.

[d]Significantly ($P < 0.05$) different from control values.

concentrations of plasma FFA, cholesterol or CE due to fat feeding were observed.

The pattern of the various lipoproteins in the density gradient following zonal ultracentrifugation of plasma from control and fat fed animals can be seen in Figure 1. Each of the lipoproteins was identified by rate of migration in the density gradient, by electrophoresis on paper, and by formation of a precipitin arc when reacted with antiserum to rat lipoproteins by the technique of double immunodiffusion (15). The fractions containing chylomicrons plus VLDL (tubes 1-5) were pooled and recentifuged to obtain the pattern seen in Figure 2. The contents of tubes 1-5 (chylomicra) and 6-20 (VLDL), respectively, were combined, and analyzed. The chylomicron fraction (tubes 1-5) did not react with antiserum to rat VLDL, whereas fraction 6-20 did react. The peak of the VLDL from plasma of control animals always appeared in a more dense portion of the gradient than did the peak of the VLDL isolated after feeding of safflower oil.

The percentage composition of the various lipids and of protein in the plasma lipoproteins is presented in Table II. Of particular interest is the increase of TG and the concommitant decrease of CE in the VLDL after fat feeding. No other differences in the percentage lipid or protein composition of the lipoproteins were observed.

A calculation of the moles of PL, cholesterol, and CE relative to TG in the plasma VLDL is presented in Table III. The proportion of PL, cholesterol, and CE relative to TG in the VLDL decreased after feeding of safflower oil; furthermore, considerably less sterol (cholesterol plus CE) was observed to be present per mole TG after safflower oil feeding than in the control. Similar ratios were obtained in vitro using the isolated perfused rat liver (2). Infusion of unsaturated FFA produced a VLDL containing less PL and cholesterol per mole TG. An important difference, however, between the studies with the intact animal and with the isolated liver, is that there is much more CE present in the VLDL isolated from plasma of rats than in the VLDL secreted by the isolated perfused liver.

The fatty acid composition of the FFAs and esterified lipids of plasma are shown in Table IV. After safflower oil feeding, there was a marked increase in 18:2 content of all these lipids, with corresponding decreases in 16:0 and 18:1; 20:4 decreased in all classes except FFA after fat feeding. The fatty acid composition of various lipoprotein lipids is presented in Table V, VI, and VII. Esterified lipids in all

TABLE III

Molar Composition of VLDL[a] Lipids

Group	Ratio[b]				Molar ratio	
	TG[a]	PL[a]	mmmC[a]	CE[a]	TG+CE:PL+C	PL:C
Control (5)[c]	100	42.0 ± 2.3	22.1 ± 0.6	44.5 ± 4.8	2.26 ± 0.08	1.91 ± 0.13
Fat fed (6)	100	35.4 ± 1.8[d]	17.8 ± 1.3[c]	19.5 ± 2.5[d]	2.25 ± 0.08	2.02 ± 0.16

[a]VLDL = Very low density lipoprotein; TG = triglyceride; PL = phospholipid; C = free cholesterol; CE = cholesteryl esters.
[b]Values are mean molar ratios ± SEM, relative to TG (=100).
[c]Numbers in parentheses indicate number of observations.
[d]Significantly ($P < 0.05$) different from control values.

TABLE IV

Fatty Acid Composition of Plasma Lipids[a]

Group[c]	Fatty acid analyzed				
	16:0	18:0	18:1	18:2	20:4
Control (5)[b]					
FFA	35.8 ± 1.3	4.9 ± 0.7 (3)	31.2 ± 2.2 (3)	22.0 ± 0.8	3.8 ± 0.4
TG	26.0 ± 0.7	2.5 ± 0.2 (3)	22.8 ± 0.7 (3)	31.4 ± 0.7	5.7 ± 0.4
CE	11.1 ± 0.1	<1.0	16.2 ± 0.5	20.7 ± 0.4	49.0 ± 0.7
PL	24.4 ± 0.9	23.8 (2)	5.7 (2)	14.8 ± 0.5	23.5 ± 0.7
Fat fed (7)					
FFA	28.1 ± 1.1[d]	6.7 ± 0.6 (5)	24.9 ± 1.4 (5)[d]	35.5 ± 2.7[d]	3.4 ± 0.7
TG	15.6 ± 0.3[d]	3.2 ± 0.3 (6)[d]	13.2 ± 0.4 (6)[d]	58.8 ± 2.5[d]	3.4 ± 0.4[d]
CE	7.4 ± 0.6[d]	<1.0	6.5 ± 0.6[d]	42.0 ± 1.2[d]	43.1 ± 1.5[d]
PL	19.0 ± 0.7[d]	27.0 (2)	3.8 (2)	28.3 ± 1.2[d]	16.8 ± 1.2[d]

[a]% by wt of total ± SEM. Fatty acids with retention times > 20:4 are not included.
[b]Numbers in parentheses indicate the number of observations.
[c]FFA = Free fatty acids; TG = triglyceride; CE = Cholesteryl esters; PL = phospholipid.
[d]Significantly ($P < 0.05$) different from controls.

TABLE V

Fatty Acid Composition of Lipoprotein Triglyceride[a]

Group[c]	Fatty acid analyzed					
	16:0	18:0	18:1	18:0 + 18:1	18:2	20:4
Control						
Chylomicron (3)[b]	36.3 ± 2.7	7.8 ± 0.2	20.2 ± 2.3	28.0 ± 2.4	21.1 ± 1.6	8.2 ± 1.5
VLDL (5)	24.6 ± 0.5	2.5 (2)	21.4 (2)	24.2 ± 0.2	31.3 ± 0.6	5.4 ± 0.6
LDL (5)	19.7 ± 1.1	2.0 ± 0.4 (3)	26.4 ± 4.4 (3)	26.6 ± 2.8	25.8 ± 1.6	9.3 ± 0.8
HDL (3)	36.5 ± 3.1	5.6 ± 1.1	25.4 ± 1.8	31.0 ± 2.9	17.8 ± 3.7	6.9 ± 0.9
Fat fed						
Chylomicron (5)	14.9 ± 1.7[d]	6.1 ± 0.5 (4)	14.8 ± 1.4 (4)	20.1 ± 1.2[d]	59.4 ± 2.7[d]	2.7 ± 0.5[d]
VLDL (6)	16.5 ± 1.3[d]	3.1 ± 0.4 (4)	13.7 ± 0.8 (4)	16.8 ± 0.8[d]	57.0 ± 2.9[d]	2.8 ± 0.5[d]
LDL (7)	12.8 ± 0.9[d]	2.5 ± 0.2 (5)	11.6 ± 1.5 (5)[d]	13.0 ± 1.1[d]	53.7 ± 1.9[d]	9.8 ± 1.5
HDL (7)	22.9 ± 0.6[d]	6.7 ± 0.9 (6)	19.1 ± 1.1 (6)[d]	24.0 ± 2.4[d]	44.7 ± 2.4[d]	6.7 ± 2.5

[a]% by wt of total ± SEM. Fatty acids with retention times > 20:4 are not included.
[b]Figures in parentheses indicate number of observations.
[c]VLDL = Very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein.
[d]Significantly different from control, $P < 0.02$.

lipoprotein classes were enriched with 18:2 after feeding safflower oil. The percentage of 18:2 in all lipid classes of the lipoproteins approximately doubled, but the magnitude of the percentage increase of 18:2 in PL was considerably less than in TG and CE. The differences in composition of fatty acids in lipoprotein PL after safflower oil feeding resulted apparently from substitution of 18:2 for 16:0 and 20:4. Of particular interest is the fatty acid composition of the CE of the various lipoprotein fractions. In both control and fat fed groups, the percentage of 20:4 in CE of VLDL was small in contrast to intermediate values for low density lipoprotein (LDL), and high values for high density lipoprotein (HDL) and residual fraction. After safflower oil feedings, 18:2 replaced primarily 16:0 and 18:1 in VLDL and LDL; 18:2 also replaced some 20:4 in HDL and residue, although 20:4 remained the most prominent fatty acid of total plasma CE (Table IV).

The fatty acid composition of the lipids of liver and adipose tissue is presented in Table VIII. The percentage of 18:2 in hepatic lipids increased in all classes, although the increase was least in PL, as might be expected with the large pool and relatively slow turnover of PL. The percentage of 18:2 in CE of liver increased to the same extent as it did in hepatic TG after fat feedings. The percentage of 18:2 in hepatic TG was identical to that of total plasma and VLDL TG, indicative of equilibration of these pools at this time. The percentage of 18:2 in adipose tissue TG increased from 25.2 to 30.2 after safflower oil feedings.

DISCUSSION

TGs are synthesized by the liver from fatty acids derived from various sources and are secreted in the VLDL and stored in hepatic depots. It has been reported previously from this laboratory that the structure and quantity of the FFA perfusing the liver in vitro will affect the quantity, characteristics, and composition of the VLDL secreted by the liver (2). Clearly then, the plasma FFA must determine the properties of the VLDL secreted by the liver and circulating in the plasma in vivo. In both animals and man in the fed state, a major fraction of the plasma FFA is derived from the dietary neutral fat (4,5). The TG pool of adipose tissue is large and relatively stable and the fatty acid composition is relatively resistant to change (16). Even though the adipose tissue TG was enriched significantly ($P<0.05$) with 18:2 under the conditions of this experiment (Table VIII), the magnitude of the change was

TABLE VI

Fatty Acid Composition of Lipoprotein Phospholipid[a]

Group[c]	Fatty acid analyzed			
	16:0	18:0 + 18:1	18:2	20:4
Control				
Chylomicron (3)[b]	30.7 ± 3.1	43.7 ± 4.6	10.9 ± 5.5	12.5 ± 1.6
VLDL (5)	21.3 ± 0.1	30.0 ± 0.8	15.5 ± 0.5	23.4 ± 0.9
LDL (5)	24.8 ± 1.0	29.5 ± 0.7	14.2 ± 0.2	20.9 ± 0.8
HDL (5)	24.2 ± 2.6	31.1 ± 1.9	13.5 ± 1.6	21.4 ± 1.8
Residue (4)	33.2 ± 0.8	32.0 ± 1.1	14.4 ± 0.6	16.4 ± 0.5
Fat fed				
Chylomicron (5)	22.2 ± 3.2	32.1 ± 0.5[d]	28.5 ± 4.5[d]	13.5 ± 1.9
VLDL (6)	18.3 ± 0.8[d]	31.0 ± 0.8	27.2 ± 2.8[d]	16.1 ± 2.0[d]
LDL (7)	19.1 ± 0.8[d]	30.8 ± 1.2	27.3 ± 1.2[d]	15.6 ± 1.4[d]
HDL (7)	18.0 ± 0.5[d]	29.8 ± 0.5	29.2 ± 1.3[d]	16.2 ± 1.2[d]
Residue (5)	28.9 ± 1.2[d]	31.0 ± 0.6	25.8 ± 1.4[d]	12.4 ± 1.1[d]

[a]% by wt of total fatty acids ± SEM. Fatty acids with retention times ≥ 20:4 are not included.

[b]Figures in parentheses indicate number of observations.

[c]VLDL = Very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein.

[d]Significantly different from control, $P < 0.05$.

TABLE VII

Fatty Acid Composition of Cholesteryl Esters in Plasma Lipoproteins[a]

Group[e]	Fatty acid analyzed			
	16:0	18:1[b]	18:2	20:4
Control				
Chylomicrons (3)[c]	IQ[d]	IQ	IQ	IQ
VLDL (5)	17.2 ± 1.0	44.8 ± 1.2	27.6 ± 0.1	7.3 ± 0.5
LDL (5)	14.4 ± 0.4	33.4 ± 1.1	26.2 ± 0.3	23.5 ± 0.9
HDL (5)	8.4 ± 0.2	4.8 ± 0.2	18.2 ± 0.5	66.7 ± 1.2
Residue (4)	9.8 ± 1.2	3.5 ± 1.2	14.9 ± 0.3	66.2 ± 3.4
Fat fed				
Chylomicrons (3)	8.2 ± 4.1	30.6 ± 5.3	61.2 ± 3.2	none detected
VLDL (6)	7.6 ± 0.6[f]	24.9 ± 2.0[f]	58.2 ± 2.4[f]	8.0 ± 0.4
LDL (7)	7.6 ± 0.5[f]	13.4 ± 0.8[f]	56.3 ± 1.7[f]	21.9 ± 1.2
HDL (7)	6.7 ± 0.4[f]	2.0 ± 0.2[f]	36.2 ± 1.8[f]	54.7 ± 2.2[f]
Residue (5)	6.4 ± 2.8	11.9 ± 0.8[f]	33.6 ± 1.4[f]	55.0 ± 4.4

[a]% by wt of total fatty acid ± SEM. Fatty acids with retention times > 20:4 are not included.

[b]18:0 was negligible (<1.0%).

[c]Numbers in parentheses indicate number of observations.

[d]IQ = insufficient quantity to analyze accurately.

[e]VLDL = Very low density lipoprotein; LDL = low density lipoprotein; HDL high density lipoprotein.

[f]Significantly ($P<0.01$) different from control values.

small in comparison to other TG pools. The fatty acid composition of adipose tissue TG will begin to reflect the dietary fat only when the diet has been maintained for a long period of time. The dominant influence of plasma FFA in the Fed animal, therefore, probably is dietary fat while the contribution from lipolysis of adipose tissue TGs may be of lesser importance. In addition, the hepatic TG storage pool also contributes to the TG secreted in the VLDL (4).

The VLDL secreted by the isolated perfused rat liver during infusion of unsaturated fatty acids was shown to have a faster rate-zonal mobility in the ultracentrifuge and to contain less PL and cholesterol per mole TG than did the VLDL produced from saturated fatty acids or when FFA was not infused (2). The data reported here suggest that similar regulatory mechanisms exist in the intact animal. The VLDL isolated from the plasma after feeding safflower oil had a faster rate-zonal mobility

TABLE VIII

Fatty Acid Composition of Lipids of Liver and Adipose Tissue[a]

Group[c]	Fatty acid analyzed				
	16:0	(18:0 + 18:1)[b]	18:2	20:4	22:6
Liver					
TG					
Control (4)[d]	26.7 ± 0.8	26.9 ± 0.5	30.6 ± 0.6	4.3 ± 0.3	6.9 ± 0.8
Fat fed (9)	16.9 ± 0.5[e]	14.8 ± 0.5[e]	59.8 ± 1.4[e]	2.7 ± 0.2[e]	3.5 ± 0.5[e]
PL					
Control (4)	16.6 ± 0.2	30.5 ± 0.8	14.6 ± 0.6	24.8 ± 0.9	9.2 ± 0.6
Fat fed (9)	14.4 ± 0.2[e]	31.1 ± 0.5	21.3 ± 0.6[e]	23.2 ± 0.6	7.2 ± 0.5[e]
CE					
Control (4)	27.2 ± 2.4	42.8 ± 0.7	23.3 ± 1.3	4.8 ± 0.6	ND[f]
Fat fed (9)	13.1 ± 1.2[e]	22.9 ± 0.9[e]	56.5 ± 1.6[e]	6.3 ± 0.9	ND
Adipose tissue					
TG					
Control (4)	30.8 ± 0.7	39.5 ± 0.5	25.2 ± 0.6	ND	ND
Fat fed (4)	28.8 ± 0.8	36.3 ± 1.6	30.2 ± 1.2[e]	ND	ND

[a]% by wt of total fatty acid ± SEM. Table does not include small amounts of 14:0, 18:3, 20:5, 22:4, and 22:5 (1-2%).

[b]18:0 was 2-4% of total analyzed fatty acid.

[c]TG = triglyceride, PL = phospholipid, CE = cholesteryl esters.

[d]Numbers in parentheses indicate number of observations.

[f]ND = not detected.

and had a lower proportion of PL and cholesterol relative to TG than did the VLDL from plasma of control rats. Presumably these differences between the plasma VLDL from the two groups is a consequence of the higher proportion of unsaturated fatty acids available to the liver in the safflower oil fed rats. The rate-zonal mobility and the lipid composition of VLDL from the safflower oil fed rats are both suggestive of a larger average particle size than that of the control group.

Of particular interest is the large amount of CE relative to the other lipid components in the VLDL isolated in vivo compared to the VLDL secreted by the isolated perfused liver (1,2). Presumably, the VLDL secreted by the liver in vitro is composed primarily of newly synthesized VLDL, whereas the VLDL isolated in vivo includes, in addition, partially degraded VLDL particles and some VLDL originating from the intestine (17-19). If the VLDL TG were metabolized by peripheral tissues more rapidly than was the sterol ester, one might expect the ratio CE:TG to increase as the VLDL is metabolized. In recent experiments, degradation of plasma VLDL lipids was reduced in vivo by treatment of the animal with Triton WR 1339. Under these conditions, the ratio of CE:TG in the VLDL was considerably less than in the plasma VLDL of non-Tritonized animals (20) and approached the ratio found in VLDL secreted by the perfused rat liver (2). These data suggest that the proportion of CE relative to TG increases as the result of catabolism of the VLDL. Conclusions regarding effects of Triton WR-1339 on the catabolism of VLDL are improper unless comparisons are made with animals consuming identical diets. The CE of the plasma VLDL probably is derived directly from the liver, because the fatty acid composition of CE in these two pools is quite similar (Tables VII and VIII) and is markedly different from that of the CE of LDL and HDL. A large portion of the CE in LDL and HDL is produced in the plasma by action of lecithin-cholesterol-acyl transferase (LCAT) (21). The present data would suggest, in agreement with studies of others, that only a small proportion of the CE in VLDL arises as a result of this reaction (22).

Even though feedings of safflower oil reduced the moles of PL, cholesterol, and CE in the VLDL relative to TG, the molar ratio of core lipids (TG + CE) to surface lipids (PL + C) remained unchanged in the plasma VLDL from control and fat fed animals (Table III). The ratio PL:C in lipids of the VLDL was also unchanged with fat feedings, analogous to observations with livers perfused in vitro with either saturated or unsaturated fatty acids (2). These data suggest that, although the proportion of the nonpolar core lipids may vary, there is a specific molecular requirement for the composition of surface lipids and ratio of core to surface lipids which is essential for the stability of the VLDL particle. These observations are consistent with the proposal of Schumaker and Adams (23) that, during peripheral metabolism of VLDL, as TG is hydrolyzed and removed from the lipoprotein under the influence of lipoprotein lipase, LCAT removes lecithin and cholesterol in a ratio of 1:1 to maintain the assumed spherical lipoprotein structure.

An important concept derived from the data reported in this manuscript is the proposed mechanism, now demonstrated in vivo as well as in vitro (1,2), by which the FFA affects the lipid composition and physical properties of the VLDL. By these means, the fatty acids, whether of dietary or endogenous origin, can affect simultaneously the concentrations of all classes of plasma lipids. Of particular importance is the specific demonstration that unsaturated fats decrease the concentration of cholesterol (and CE, in vivo) and PL in the VLDL relative to TG. The regulatory effects of the FFA on the hepatic secretion and properties of the VLDL in these model systems may explain partially the biochemical mechanism for the simultaneous decrease in plasma concentrations of TG, PL, cholesterol, and CE in man and animals maintained for long periods of time on diets containing a high proportion of polyunsaturated fats relative to saturated fats.

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Studies In Vitro of Lipogenesis in Rat Testicular Tissue

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ABSTRACT

Testicular tissue was shown to contain the full complement of enzymes required for de novo synthesis of fatty acids. The enzymes capable of snythesizing palmitic acid from citrate, acetate, or acetyl CoA were found to be present in the soluble (cytoplasmic) fraction. These included fatty acid synthetase, acetyl CoA carboxylase, citrate-cleavage enzyme, malic enzyme, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. Optimal conditions for assaying activities of fatty acid synthetase and acetyl CoA carboxylase in the soluble fraction from rat testes were established, and the activities of these two enzymes were determined to be 0.54 ± 0.1 and 0.030 ± 0.002 (nmoles of substrate incorporated into fatty acid per min per mg of soluble fraction protein), respectively. The activities of citrate-cleavage enzyme, malic enzyme, and the glucose-6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase pair were also measured. The activities were 6.0 ± 0.7, 34.9 ± 4.2, and 29.9 ± 9.3 nmoles/min/mg, respectively.

INTRODUCTION

De novo synthesis of fatty acids has been extensively investigated, particularly in liver and adipose tissue (1,2). However, little is known about the corresponding system in testicular tissue. The ability of testicular tissue to synthesize palmitic and stearic acids in vitro from 1-^{14}C-acetate was shown by Hall, et al., (3) using slices of rabbit testis. Davis, et al., (4) demonstrated that palmitic acid synthesized in testes of rats injected intratesticularly with 1-^{14}C-acetate was synthesized entirely de novo. These results indicate that acetyl CoA carboxylase and fatty acid synthetase are present in testicular tissue.

The aim of this study was to examine these enzymes in rat testicular tissue with regard to existence, optimal conditions for assay, and normal levels of activity. The existence and activities of several enzymes thought to supply substrate and reduced nicotinamide adenine dinucleotide phosphate (NADP) for de novo synthesis were also studied. These included citrate-cleavage enzyme, malic enzyme, and the glucose-6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase pair (5).

[1]This study was submitted in partial fulfillment of the requirements for the PhD degree, Department of Biochemistry, Vanderbilt University.

EXPERIMENTAL PROCEDURES

Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) were maintained on a standard purified diet containing 10% corn oil. The animals were killed at 3-4 months of age, and the testes were removed, weighed, and homogenized in ice cold 0.1M potassium phosphate buffer (pH 7.4) containing 0.25M sucrose and 0.001M cysteine (Buffer I). The soluble fraction (cytosol) was isolated after centifugation at 105,000 x g for 1 hr and used in assaying acetyl CoA carboxylase and fatty acid synthetase. Testicular mitochondria and microsomes were isolated by the method of DeDuve, et al., (6). The soluble fraction isolated from testes homogenized in 0.05M Hepes buffer (pH 7.4) containing 0.25M sucrose and 0.001 M cysteine (Buffer II) was used in assaying activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, because glucose-6-phosphate dehydrogenase is inhibited by phosphate (7).

Fatty acid synthetase activity was measured in the soluble fraction isolated from rat testis by determining the incorporation of 1,3-^{14}C-malonyl CoA (New England Nuclear Corporation, Boston, MA., 35.4 mCi/mmole) into fatty acids, using a modification of the procedure of Foster and McWhorter (8). The assay medium contained 120 μmoles of potassium phosphate buffer (pH 7.4), 300 μmoles of sucrose, 1.2 μmoles of cysteine, 32 μmoles of acetyl CoA, 15 μmoles of $MgCl_2$, 10 μmoles of adenosine triphosphate (ATP), and a reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system consisting of 2.8 units of glucose-6-phosphate dehydrogenase, 4 μmoles of $NADP^+$, and 20 μmoles of glucose-6-phosphate. (All enzymes and cofactors were obtained from Sigma Chemical Co., St. Louis, MO). Aliquots of the soluble fraction plus Buffer I were added to flasks containing the above incubation medium to give a final volume of 1.5 ml. The flasks were preincubated at 37 C for 2 min, after which the reaction was initiated by addition of 1,3-^{14}C-malonyl CoA.

TABLE I

Marker Enzymes in Fractions
Isolated from Homogenates of Rat Testes

Subcellular fraction	Enzyme activity (% of total activity recovered)[a]		
	Succinate-cytochrome c reductase	Nucleoside diphosphatase	Lactate dehydrogenase
Nuclear	11.3	6.6	8.3
Mitochondrial	85.4	11.2	9.7
Microsomal	3.2	79.8	4.2
Soluble[b]	0	2.4	77.8

[a]> 90% of total activity in homogenate was recovered.
[b]Cytosol.

TABLE II

Subcellular Localization of Enzymes
Involved in Synthesis of Fatty Acids in Rat Testes

Subcellular fraction	Incorporation[a] (dpm/mg protein)	Total mg protein/fraction	Total activity (dpm)
Soluble fraction	5750	99.0	569,250
Microsomal fraction	132	45.0	5,940
Micochondrial fraction	2551	21.3	54,336

[a]Incubation with 1-^{14}C-acetyl CoA (0.0093 μmoles, 0.5 μCi) at 37 C for 30 min in an air atmosphere.

Labeled malonyl CoA was diluted with unlabeled malonyl CoA (Sigma Chemical Co., St. Louis, MO) and a total of 0.15 μmoles (0.233 μCi) was added to each incubation flask. Incubations were done in an air atmosphere and, after a specified incubation time, the reactions were stopped by addition of 2 ml of 40% potassium hydroxide followed by 6 ml of 95% ethanol and 0.2 ml of 0.5% hydroquinone (w/v in 95% ethanol). Hydrolysis of any esterified fatty acids was complete if left at room temperature overnight. ^{14}C Fatty acids synthesized by fatty acid synthetase were extracted after acidification of the hydrolysate as previously described (4,9), and the total radioactivity determined by liquid scintillation spectrometry. Specific activity was calculated as nmoles of ^{14}C-malonyl CoA incorporated into extracted fatty acids per min per mg soluble fraction protein.

Acetyl CoA carboxylase activity was determined in the soluble fraction from testes by the combined reaction of acetyl CoA carboxylase and fatty acid synthetase similar to the procedure of Foster and McWhorter (8). (Acetyl CoA carboxylase is the rate limiting enzyme (10) in the overall conversion of acetyl CoA to fatty acids.) Incubations were done in medium containing 105 μmoles of potassium phosphate (pH 7.4), 263 μmoles of sucrose, 1.05 μmoles of cysteine, 7.5 μmoles of $MgCl_2$, 15 μmoles of malonate, 15 μmoles of $KHCO_3$, 10 μmoles of ATP, and the NADPH generating system described above. Aliquots of the soluble fraction were added to the assay medium and the mixture preincubated at 37 C for 2 min. 1-^{14}C-Acetyl CoA (New England Nuclear Corp., Boston, MA, 55 mCi/mmole) was diluted with unlabeled acetyl CoA, and 0.1 μmole (0.5 μCi) was added to initiate the reaction. The incubations were done in air and the specific activities determined as described for fatty acid synthetase.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed according to Glock and McLean (11), malic enzyme according to Ochoa (12), and citrate-cleavage enzyme by the procedure of Cottam and Srere (13). The rate of reaction of each of these enzymes was determined spectrophotometrically by monitoring absorbance changes at 340 nm. Activities are expressed as nmoles of pyridine nucleotide reduced (or oxidized) per min per mg of soluble fraction protein.

Marker enzyme assays were done on fractions isolated from homogenates of rat testes. Succinate-cytochrome c reductase activity, a marker for mitochondria, was assayed according to Fleischer and Fleischer (14), and lactate

TABLE III

Substrates for Fatty Acid Synthesis by Soluble Fraction from Rat Testes

Substrate	Incorporation into total fatty acids[a] (dpm/mg soluble fraction)	Soluble fraction protein in incubation (mg)
Citrate[b]	1844	15.2
Acetate[c]	3304	11.9
Acetyl CoA[d]	6622	4.98

[a]Incubations were done at 37 C for 30 min. It was not determined if saturation type kinetics with respect to substrate concentration were being observed in experiments with citrate and acetate.

[b]Incubation with 1,5-^{14}C-citrate (0.96 μmoles or 2.58 μCi) contained 0.6 μmole CoA.

[c]Incubation with 1-^{14}C-acetate (1.0 μmole or 2.2 μCi) contained 0.6 μmole CoA.

[d]Incubation with 1-^{14}C-acetyl CoA (0.1 μmole or 0.5 μCi).

TABLE IV

Cofactor Requirements for Fatty Acid Synthetase in Soluble Fraction Isolated from Homogenates of Rat Testes

Cofactor	Amount (μmoles)	Incorporation of ^{14}C-malonyl CoA[a] (cpm/min mg)
NADH[b]	10	255
NADPH[b]	10	1224
Generating system[c]	10	1275
ATP[b]	10	1224
	0	732
Acetyl CoA	0.032	1224
	0.064	1198

[a]Incubation with 1,3-^{14}C-malonyl CoA (0.15 μmoles) for 15 min at 37 C in an atmosphere of air.

[b]NADH = reduced nicotinamide adenine dinucleotide; NADPH = reduced nicotinamide adenine dinucleotide phosphate; ATP = adenosine triphosphate.

[c]Generating system—2.8 units of glucose-6-phosphate dehydrogenase, 4 μmoles of NADP, and 20 μmoles of glucose-6-PO_4.

dehydrogenase, a marker for cytosol (soluble fraction), was assayed according to Kornberg (15). Because no detectable glucose-6-phosphatase activity could be measured in testicular microsomes, nucleoside diphosphatase was used as a microsomal marker and determined by the method of Plaut (16).

Analysis of the products of lipogenesis was done by analytical gas liquid radiochromatography of the fatty acid methyl esters using columns packed with 15% EGSSX on Gas Chrom P (100/120 mesh) as previously described (4,17). ^{14}C Palmitic acid isolated from incubations with testicular soluble fraction by preparative gas liquid chromatography (GLC) (17) was degraded by the procedure of Dauben, et al., (18).

RESULTS

Analyses of marker enzymes for mitochondrial, microsomal, and soluble fractions isolated from testes indicated that these preparations were relatively pure (Table I) and could be used for locating the enzymes for fatty acid synthesis. The incorporation of 1-^{14}C-acetyl CoA into fatty acids in each subcellular fraction was determined after incubating each fraction as described for acetyl CoA carboxylase. The data presented in Table II demonstrate the presence of the enzyme system for synthesis of fatty acids in the soluble fraction of the testis. The incorporation of ^{14}C into fatty acids in the mitochondrial fraction probably represents elongation of endogenous fatty acids by an elongation system similar to that known to be present in rat liver (19).

The soluble fraction was also able to synthesize fatty acids when 1-^{14}C-acetate or 1,5-^{14}C-citrate was used as substrate (Table III).

The study of individual enzymes involved in

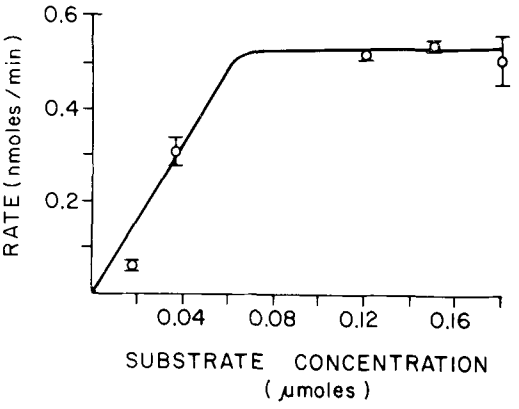

FIG. 1. Rate of fatty acid synthesis by fatty acid synthetase from rat testes as a function of 1,3-^{14}C-malonyl CoA concentration. The assay medium contained 120 μmoles of potassium phosphate buffer (pH 7.4), 300 μmoles of sucrose, 1.2 μmoles of cysteine, 32 μmoles of acetyl CoA, 15 μmoles of $MgCl_2$, 10 μmoles of adenosine triphosphate, testicular soluble fraction (1-4 mg), and the nicotinamide adenine dinucleotide phosphate generating system described in the text. Incubations were for 20 min in air at 37 C. All rates were normalized to 1 mg of protein. Each point represents an average ± range of duplicate assays.

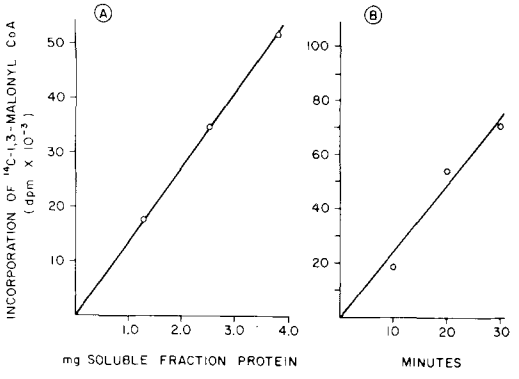

FIG. 2. Rate of fatty acid synthesis by fatty acid synthetase as a function of (A) protein concentration or (B) time. Incubations, as in Figure 1, used 1,3-^{14}C-malonyl CoA (0.15 μmoles) as substrate. Each point represents an average of duplicate incubations.

de novo synthesis was begun with a study of fatty acid synthetase. The requirement for reducing cofactors was studied to determine whether optimal synthesis could be obtained with reduced nicotinamide adenine dinucleotide (NADH), NADPH, or an NADPH generating system. As seen in Table IV, NADPH was ca. 4 times more effective in supplying reducing equivalents than was NADH. The NADPH generating system was as effective as NADPH. This generating system was used in all other experiments. It was also found that ATP was required by this reaction for maximal levels of synthesis. There was no effect on the amount of ^{14}C incorporated into fatty acids between the two concentrations of acetyl CoA used (ratios of acetyl CoA: malonyl CoA of 1:5 and 1:2.5).

The saturating concentration of ^{14}C-malonyl CoA was found to be above 0.12 μmoles (Fig. 1). Using saturating levels of ^{14}C-malonyl CoA (0.15 μmoles), the rate of reaction was found to be linear with respect to protein concentration (Fig. 2A) and time (Fig. 2B).

The specific activity of fatty acid synthetase measured in the soluble fractions from rat testes according to the above conditions was found to be 0.54 nmoles of malonyl CoA incorporated into fatty acid per min per mg protein (Table V). Fatty acids produced by the synthetase were shown by gas radiochromatography to be palmitic acid (89% of total ^{14}C fatty acids), stearic acid (9%), and myristic acid (2%). This is similar to the pattern reported by Brady, et al., (20) for liver.

Optimal conditions for measuring the activity of acetyl CoA carboxylase were also determined. Because the assay of acetyl CoA carboxylase depended on the activity of fatty acid synthetase, the incubation medium contained the cofactors shown to be optimal for fatty acid synthetase in addition to the cofactors required for the carboxylase.

The need for a carboxylic acid activator of the carboxylase was investigated. As shown in Table VI, malonate and citrate were both effective, but α-ketoglutarate was not. Because citrate is also a substrate for fatty acid synthesis in testicular tissue (Table III), malonate was used in all subsequent experiments. The substitution of a nitrogen atmosphere for an air atmosphere had no effect on activity. The use of Hepes buffer (pH 7.4) did not give optimal activity even though the pH of the incubation was maintained at 7.4. The optimal level of malonate concentration was found to be ca. 12mM (Fig. 3).

The assay conditions for the carboxylase were also optimized with respect to ATP and $MgCl_2$ concentrations. Figure 4 shows that, while Mg^{2+} ions were necessary for optimal activity, the reaction was not dependent on the concentration of this ion between concentrations of 3 and 12 mM. Optimal levels for ATP were in the range of 8-12mM, while concentrations above that were inhibitory. The rate of reaction was linear with respect to time (up to 40 min) and protein concentration (up to 8 mg soluble fraction protein).

Using the above conditions, the activity of

TABLE V

Specific Activity of Enzymes
Involved in De Novo Synthesis of Fatty Acids
in the Soluble Fraction from Rat Tissues

Enzyme	Specific activity[a] Testis	Liver
Fatty acid synthetase	0.54 ± 0.10 (9)[b]	2.71 ± 0.62 (2)
Acetyl CoA carboxylase	0.030 ± 0.002 (6)	
G-6-PDH and 6-PGDH[c]	29.9 ± 5.3 (7)	154 ± 36 (2)
Malic enzyme	34.9 ± 4.2 (4)	56 ± 11 (1)
Citrate-cleavage enzyme	6.0 ± 0.7 (4)	21.7 (1)

[a]Mean ±S.D. nmoles/min mg soluble fraction protein.

[b]( ) = Number of animals.

[c]Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

TABLE VI

Factors Affecting Acetyl CoA Carboxylase
Activity in the Soluble Fraction from Rat Testes

Assay conditions	Activity (dpm/mg)
Normal[a]	7103
20 mM malonate	6191
20 mM citrate	6332
12 mM α-Ketoglutarate	131
N_2 atmosphere	6895
38.3 mM Hepes buffer, pH 7.4	3180

[a]Incubations with 1-^{14}C-acetyl CoA (0.1 μmole) at 37 C for 20 min in an atmosphere of air. Incubations were 12 mM with respect to malonate and 76.6 mM with respect to phosphate concentrations.

acetyl CoA carboxylase was measured in incubations containing varying concentrations of 1-^{14}C-acetyl CoA. Using a double reciprocal plot, the K_m for acetyl CoA was calculated to be 6.0 X 10^{-5} M. The specific activity of acetyl CoA carboxylase measured in the soluble fraction from rat testis under optimal conditions was found to be 0.030 nmoles of ^{14}C-acetyl CoA incorporated into fatty acid per min per mg protein (Table V).

A sample of pooled labeled fatty acids from incubations with 1-^{14}C-acetyl CoA was analyzed by gas liquid radiochromatography. The principal fatty acid synthesized was palmitic (87% of the total ^{14}C recovered in fatty acids). The other fatty acids synthesized were stearic (8.5%) and myristic (4.5%) acids.

An amount of the synthesized ^{14}C-palmitic acid was isolated (> 98% radiochemically pure) by gas chromatography (GC) and used for chemical degradation (18). This procedure allows the determination of the ratio, specific radioactivity of the carboxyl carbon:specific radioactivity of the average carbon in the fatty acid. A theoretical ratio of 2.0 is expected for fatty acids synthesized de novo. A value of 2.2 was obtained in this experiment, indicating that palmitic acid isolated from these incubations was synthesized de novo.

Fatty acid synthesis in rat testis was further investigated by studying several enzymes associated with de novo synthesis. These enzymes included citrate-cleavage enzyme, malic enzyme, and the first two enzymes in the hexose monophosphate shunt, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The spectrophotometric procedures used in assaying these enzymes gave linear initial reaction rates; the rate of reaction of each of these enzymes was linear with respect to time. Each initial reaction rate was also proportional to protein concentration. The specific activities for these enzymes in rat testes are given in Table V, along with values for the corresponding enzymes measured in hepatic cytosol.

DISCUSSION

These studies establish testicular tissue as one which is capable of lipogenesis. Enzymatic activities of fatty acid synthetase, acetyl CoA carboxylase, citrate-cleavage enzyme, malic enzyme, and the glucose-6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase pair were demonstrated in the soluble fraction of

testes of normal rats. These enzymes allow the complete synthesis of fatty acids de novo by supplying the required cofactors and substrates for acetyl CoA carboxylase and fatty acid synthetase shown to be active in the testes in this study. Glucose-6-phosphate dehydrogenase activity (21) and malic enzyme (22) activity have been shown previously to be present in rat testes. The rather high activity of these two enzymes relative to fatty acid synthetase, acetyl CoA carboxylase, and citrate-cleavage enzyme indicates that there is an abundant supply of NADPH for both lipogenesis and steroidogenesis. As expected, the enzymes required for de novo synthesis were localized in the soluble fraction of the testes. The system was shown to use either citrate, acetate or acetyl CoA as efficient substrates for de novo synthesis, thereby establishing the existence of citrate-cleavage enzyme and acetyl CoA synthetase in addition to the other lipogenic enzymes in the testicular soluble fraction.

The products of the testicular synthetase system were found to be 87% palmitic, 4.5% myristic, and 8.5% stearic acid. These data agree well with the finding of Brady, et al. (20) that 81-87% of the products produced by the liver fatty acid synthetase was palmitic acid.

Acetyl CoA carboxylase as assayed in the soluble fraction of the testes seems to be similar to the liver enzyme. It was activated by either citrate or by malonate. Levels of malonate used in these experiments (12 mM) activate acetyl CoA carboxylase as effectively as 20 mM citrate. The K_m of acetyl CoA carboxylase for acetyl CoA was found to be 6.0×10^{-5} M. This value agrees well with values for the K_m of rat liver acetyl CoA carboxylase of 6.5×10^{-5} M for acetyl CoA (23).

Testicular tissue has been shown to synthesize polyunsaturated and saturated fatty acids (4,9). A continuous need for lipid synthesis may exist for the continuous production and development of spermatocytes to spermatids in the production of spermatozoa in the sexually mature animal. De novo synthesis may also be important in the development of testicular structure in the immature animal. Evans, et al., (9) found that young rats (30-day old) incorporated more ^{14}C-acetate into fatty acids (primarily 16:0) than did adult rats, suggesting a more active de novo synthesis system in young rats. This, perhaps, is important for synthesizing the complex lipids required for establishment of testicular structure and function. The importance of lipids in this process has been suggested by others (24). Thus, testicular de novo synthesis may be important in providing a readily available, independent source of palmitic acid (hence, also stearic and oleic acids) for synthesis of these lipids.

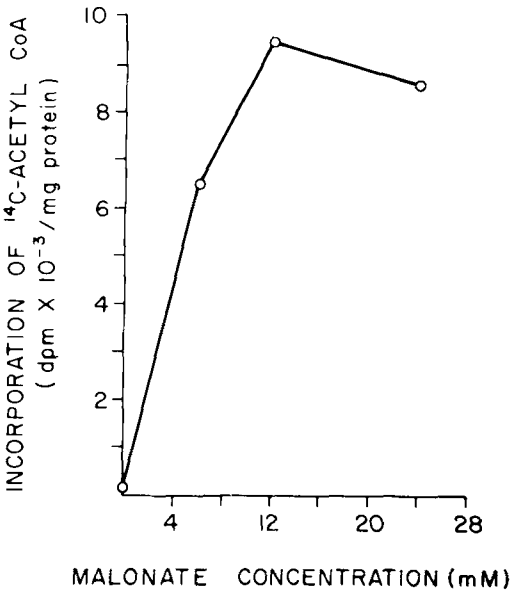

FIG. 3. Activity of acetyl CoA carboxylase as a function of malonate concentration. Incubations were as in Figure 1. Each flask contained 3 mg of soluble fraction protein. The data represent averages of duplicate incubations.

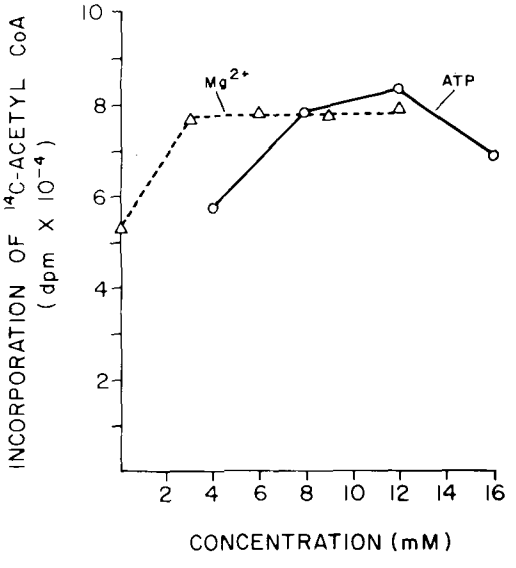

FIG. 4. Effect of Mg^{2+} and adenosine triphosphate concentration on acetyl CoA carboxylase activity in the soluble fraction isolated from rat testes. Incubations were as in Figure 1. Each point represents an average of duplicate incubations.

ACKNOWLEDGMENTS

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Synthesis of Cholesterol Esters in the Plasma and Liver of Sheep

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ABSTRACT

A study was made with sheep on the formation in vitro of long chain fatty acid esters of cholesterol by the lecithin-cholesterol-acyltransferase system present in the plasma and the acyl CoA-cholesterol-acyltransferase system present in the liver. The rate of cholesterol esterification in the plasma was 0.024 μmoles/ml/hr. The relative pattern of fatty acids esterified during incubation of the plasma remained constant over the 8 hr period of incubation and was similar to the fatty acids in the plasma cholesteryl esters before incubation began and to the fatty acids in the 2-position of the plasma lecithin. The predominant cholesteryl esters synthesized contained monoenoic and dienoic fatty acids. Unlike the bovine, there was no apparent discrimination in favor of the 18:2 containing species of plasma lecithin as donors of fatty acids. This difference could be accounted for by the similarity in the 18:2 content of the phospholipids present in the high density (density $>$ 1.062 and $<$ 1.21) and the low density (density $>$ 1.006 and $<$ 1.063) lipoprotein fractions of the sheep plasma. The possibility of some discrimination against 20:4 during cholesterol ester synthesis in the plasma of the sheep cannot be excluded. In the liver, the predominant cholesteryl esters synthesized contained saturated and monoenoic fatty acids; cholesteryl linoleate was synthesized to a very much less extent. There was considerable similarity between the composition of the unesterified fatty acid fraction of the liver before incubation began and the fatty acid composition of the cholesteryl esters synthesized during incubation. Addition of sonicated suspensions of free fatty acids altered markedly the fatty acid pattern of the cholesteryl esters synthesized by the liver slices. From the evidence presented it is concluded that the cholesteryl esters in sheep plasma are syntheized mainly by the plasma lecithin-cholesterol-acyltransferase system. The results are discussed in relation to cholesterol esterification systems demonstrated in the plasma and liver of monogastric animals.

INTRODUCTION

Both the plasma and the liver of animals are known to contain enzyme systems that catalyze the synthesis of cholesteryl esters of long chain fatty acids (1,2,3). Together these systems constitute the major source of cholesteryl esters circulating in the blood (3), although during absorption of dietary lipids a significant, if transitory, contribution to the plasma cholesteryl esters by the intestine also occurs. In the plasma, an enzyme, lecithin-cholesteol-acyltransferase (LCAT), catalzes the transfer of an acyl group from the 2-position of lecithin to the 3-hydroxyl group of cholesterol. In the liver, cholesteryl ester synthesis occurs principally via an acyl-CoA-cholesterol acyltransferase associated with the particulate fraction of the cell. Although the cholesteryl ester hydrolase present in the lysosomal fraction of the liver is also known to possess some cholesterol esterifying properties (4,5), its main function is that of ester hydrolysis. The relative importance of the liver and plasma cholesterol esterification processes in the synthesis of the plasma cholesteryl esters has been shown to vary considerably between animal species. Thus, in man the plasma LCAT system is credited with the formation of most of the plasma cholesteryl esters (6), whereas, in the rat a proportion of the plasma cholesteryl esters is of hepatic origin via the acyl-CoA-cholesterol acyltransferase system (1). In the ruminant animal, the cholesteryl ester fraction constitutes a much higher proportion of the total plasma lipids than it does in the plasma of nonruminant animals, and it may account for as much as 47% and 57%, respectively, of the total lipids present in the plasma of the sheep and ox (7,8). Furthermore, the choelsteryl esters in the plasma of ruminant animals are characterized by a higher concentration of polyunsaturated fatty acids than in most other species. In spite of the predominance of cholesteryl esters in the plasma lipids of ruminant animals, little is known about the metabolic function and synthetic origin of these plasma lipids, although an LCAT anzyme has been shown to be active in bovine plasma (9). An investigation into the formation of long chain fatty acid esters of

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cholesterol by both the plasma and the liver of sheep is now reported.

EXPERIMENTAL PROCEDURES

Animals and Diet

The animals used were 4 castrated male sheep of the Cheviot breed; they were 3 years old and their wt ranged between 20 and 25 kg. The animals were housed in individual pens and were fed a diet of hay and concentrates at 07.00 hr and 17.00 hr; water was available ad libitum.

Determination of Cholesterol Esterification in the Plasma

Blood samples were obtained from the jugular vein of each animal at 10.00 hr by means of heparinized Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) that had been chilled to 4 C. After centrifiguing the blood at 4 C, incubations were carried out with 2 ml aliquots of plasma in sterilized glass-stoppered tubes. Incubations were done in a shaking water bath maintained at 38 C. Two different methods were used for the determination of esterifying activity in the plasma.

Method 1. In this method, esterifying activity was determined by measuring the change that occurred in the concentration of unesterified cholesterol in the plasma during incubation. After incubation periods of 1, 2, 3, 4, 6, and 8 hr, the plasma esterification reaction was stopped by the addition of 8 ml methanol containing "carrier free" (4-^{14}C)-cholesterol (1.2 x 10^5 dpm). Methanol containing labeled cholesterol was also added directly to unincubated plasma to provide control samples. The total lipids were extracted by the addition of 8 ml chloroform. After heating for 20 min at 66 C, a further 8 ml of chloroform was added and the extract was filtered. The filtrate was washed with 6 ml 0.88% (w/v) KCl and the chloroform layer separated off. After removal of the solvent on a rotary film evaporator, the residue was applied to thin layer chromatoplates coated with Kieselgel G (E. Merck, Darmstadt, Germany) and developed in a solvent system of hexane:diethyl ether:formic acid (80:20:1 v/v). The band corresponding to free cholesterol was scraped off the plate and eluted with diethyl ether. The solvent was removed and the free cholesterol dissolved in 1 ml of diethyl ether; to this solution of cholesterol in diethyl ether was added 100 μl of a solution of 5-αcholestane in diethyl ether (21 mg/100 ml) as an internal standard. A portion of this solution was then introduced into a scintillation vial together with 10 ml of Scintimix II (Koch Light Laboratories Ltd., Slough, England) and the radioactivity determined in a Packard 2425 Liquid Scintillation Spectrometer (Packard Instrument Co., Downers Grove, IL). The remainder of the solution was reduced to a small volume under nitrogen and the ratio of cholestane:cholesterol determined by gas liquid chromatography on a 6 ft column packed with 3% OV17 adsorbed onto Gas Chrom Q (100-120 mesh; Applied Science Laboratories Inc., State College, PA). The column was housed in a Pye 104 chromatograph (Pye Unicam Ltd., Cambridge, England) equipped with flame ionization detectors and an electronic integrator (Hewlett Packard Ltd., Wokingham, Berks, England). The concentration of unesterified cholesterol was calculated from the peak area relative to that of the added cholestane; correction for recovery error was determined from the relative amount of radioactivity obtained in the final solution compared with that at the beginning of the extraction procedure.

Method 2. Cholesterol esterification in the plasma was also determined by a radioassay technique. A solution of "carrier free" (4-^{14}C)-cholesterol (1 x 10^5 dpm) in 100 μl of ethanol containing 0.5% Tween 20 (polyoxyethylene sorbitan, Koch-Light Laboratories Ltd., Slough, England) was added to each incubation tube. The ethanol was evaporated under nitrogen prior to the addition of the plasma. After incubation for 1, 2, 3, 4, 6, and 8 hr, the plasma esterification reaction was stopped by the addition of 8 ml methanol to each tube. Methanol was added directly to unincubated plasma to provide control samples. The lipids were extracted from each incubation mixture as in Method 1, and the purified lipid dissolved in 1 ml of chloroform. Ca. 100 μl of this solution was applied to a thin layer chromatoplate of Kieselgel G, and developed in hexane:diethyl ether:formic acid (80:20:1 v/v). The cholesteryl ester and free cholesterol bands were scraped from the plate directly into scintillation vials and counted as suspensions in a mixture of 10 ml Univolve 1 (Koch-Light Laboratories Ltd., Slough, England) and 4 ml of water. From the remainder of the lipid extract, the cholesteryl ester fraction was separated by thin layer chromatography (TLC) on Kieselgel G. The mixture of cholesteryl esters was then resolved into saturated, monoenoic, dienoic, trienoic, and tetraenoic fractions by argentation TLC (10) with a solvent system of benzene:hexane (1:1 v/v). These fractions were eluted from the chromatoplate with diethyl ether directly into scintillation vials and the radioactivity in each fraction was determined by scintillation counting.

Determination of Cholesterol Esterification in Liver

The animals were killed at 10.00 hr with a captive bolt humane killer. Each liver was perfused in situ via the hepatic portal vein with ice cold isotonic saline to remove all traces of blood. Slices ca. 0.3 mm thick were cut by hand. Homogenates were prepared at 4 C from 10 g of liver and 25 ml of 0.1M phosphate buffer (pH 7.4) in a Potter-Evehjem homogenizer with a loose fitting pestle. The homogenate was centrifuged at 200 x g for 10 min to remove cellular debris. All incubations were done in 25 ml Ehrlenmeyer flasks in a shaking water bath maintained at 38 C; air was the gas phase. Each flask contained 2 ml homogenate or 100 mg slices to which were added the following cofactors or substrates all in 0.1M phosphate buffer; 1.5 μmoles CoA, 15 μmoles adenosine triphosphate (ATP), 2 2 μmoles $MgCl_2$, and 50 μl of a solution of (4-^{14}C)-cholesterol complexed with albumin. The (4-^{14}C)-cholesterol-albumin solution was prepared as follows; 1 μCi of ^{14}C-cholesterol in 100 μl acetone was added slowly with continuous stirring to a solution of 100 mg ovine albumin (Fraction V, Sigma Chemical Co. Ltd., Surrey, England) in 1 ml of 0.1M phosphate buffer. This solution was then placed under nitrogen until the acetone was removed, 50 μl of this solution contained "carrier free" (4-^{14}C)-cholesterol with a radioactivity of 1 x 10^6 dpm, and 3 mg albumin. When appropriate, exogenous fatty acid substrates were included in the system by the addition of a sonicated suspension of 20 μmoles each of 18:0, 18:1, and 18:2 in 0.1M phosphate buffer. The final volume in each flask was adjusted to 3 ml with phosphate buffer. To serve as controls, slices or homogenates were incubated similarly, but in the absence of CoA and ATP. Incubations were terminated by the addition of 8 ml methanol and the lipids extracted according to the procedure of Folch, et al., (11). The final chloroform extract was reduced to ca. 1 ml and the extent of esterification determined from the distribution of radioactivity between the free cholesterol and cholesteryl ester fractions. The cholesteryl ester fraction was also fractionated by argentation TLC and the distribution of radioactivity between the saturated, monoenoic, dienoic, trienoic, and tetraenoic fractions determined as described above.

Other Methods

Serum lipoproteins were separated by ultracentrifugation according to the method of Lindgren, Jensen and Hatch (12). Fractions collected were very low density lipoproteins (VLDL), density <1.006; low density lipoproteins (LDL), density >1.006 and <1.063; high density lipoproteins (HDL), density >1.062 and <1.21; remainder, all material with density >1.21. VLDL fractions were washed by resuspension in physiological saline followed by recentrifugation under the conditions of the original separation. All other lipoprotein fractions were obtained without washing. Electrophoretic separations of the lipoprotein fractions on cellulose acetate (13) indicated little contamination of lipoprotein classes between fractions.

Lecithin was prepared from extracts of the total plasma phospholipids by separation on thin layer chromatoplates of Kieselgel G without binder (Camag, Muttenz, Switzerland) with a solvent system of chloroform:methanol:acetic acid:water (25:15:4:2 v/v) as described by Skipski, et al., (14). The lecithin was subjected to hydrolysis with the phospholipase A of snake venom (Ophiophagus Hannah) and the positional distribution of the fatty acids determined by the method of Robertson and Lands (15).

General lipid analyses and the determination of the fatty acid compositions of the various lipid fractions were by methods described previously (16-18). All chromatoplates were visualised under UV light after spraying with a solution of dichlorofluorescein (0.1% w/v). Identification of lipid fractions was by comparison with known standards. Solvents were distilled before use.

RESULTS

The values for the esterification of cholesterol during the incubation of sheep plasma at 38 C as determined by the gas liquid chromatographic procedure (GLC) (Method 1) and the radioassay technique (Method 2) are given in Table I. Although it is generally accepted that the most accurate method of measuring the rate of cholesterol esterification is to determine the decrease in the concentration of plasma free cholesterol during incubation, the precision of this method may be limited by the accuracy with which small changes in the concentration of free cholesterol may be measured. For example, from the results given in Table I it may be seen that a positive error of 1% in the determination of the free cholesterol concentration in the plasma before incubation began (14.28 instead of 14.14 mg/100 ml) would result in a positive error of 11.5% in the amount of cholesterol esterified during the first 2 hr of incubation (1.36 instead of 1.22 Δ mg/100 ml). (^{14}C)-Cholesterol has been used widely to measure the rate of esterification of plasma free

TABLE I

Esterification of Cholesterol During the Incubation of Sheep Plasma at 38 C[a]

Time of incubation (hr)	Method 1 Concentration of free cholesterol (mg/100 ml plasma)	Method 2 Plasma free cholesterol esterified during incubation (%)
0	14.14 ± 0.25	0.0
1	13.13 ± 0.32	5.52 ± 0.29
2	12.92 ± 0.21	9.77 ± 0.33
3	12.09 ± 0.27	13.43 ± 0.48
4	11.90 ± 0.27	16.38 ± 0.64
6	11.22 ± 0.29	20.69 ± 0.92
8	10.68 ± 0.21	25.21 ± 0.51

[a]Plasma samples from each of the 4 sheep were incubated in triplicate. Mean values are given with their standard errors.

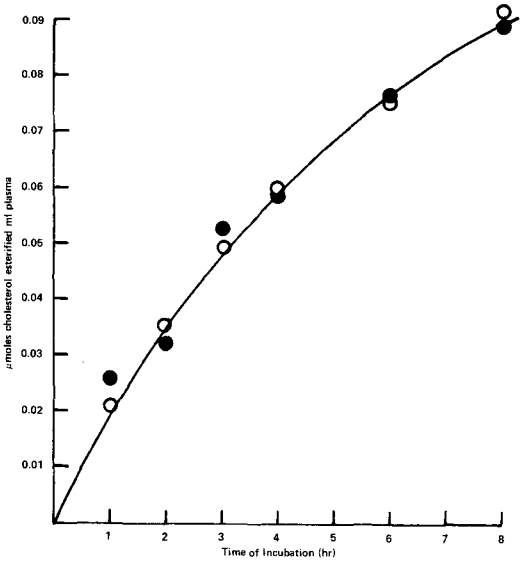

FIG. 1. Amount of free cholesterol (μmoles/ml plasma) esterified during the incubation of sheep plasma at 38 C. ● = Method 1, gas liquid chromatographic procedure; ○ = method 2, radioassay procedure.

cholesterol, but difficulties might be encountered from an inability to attain isotopic equilibrium between the various plasma lipoproteins (1). Due to these problems, both methods of measuring cholesterol esterification were used in the investigation now reported. Because there is good agreement between the results obtained by the two methods and from the curve obtained by plotting the amount of cholesterol esterified against the time of incubation (Fig. 1), it may be concluded that the rate of cholesterol esterification in sheep plasma amounted to 0.024 μmoles/ml/hr.

From the distribution of (^{14}C)-cholesterol in the cholesteryl ester fractions separated by argentation TLC (Method 2), it was possible to study the fatty acid specificity of the cholesterol esterification process in the plasma throughout the complete incubation period. The fatty acid compositions of the newly synthesized cholesteryl esters after 1 and 8 hr incubation are given in Table II together with the composition of the fatty acids esterified to cholesterol before incubation began and the composition of the fatty acids esterified in the 2-position of the plasma lecithin. It is clear that the pattern of fatty acids that became esterified to cholesterol remained constant over the complete 8 hr period of incubation. Moreover, the pattern of fatty acids esterified to cholesterol during incubation was very similar to that of the fatty acids in the plasma cholesteryl esters before incubation began and to that of the fatty acids in the 2-position of the plasma lecithin. However, in the 2-position of the plasma lecithin, the proportion of saturated fatty acids was somewhat less, and the proportion of tetraenoic acids was somewhat greater than in the plasma cholesterol esters.

The fatty acid compositions of the cholesteryl esters and phospholipids in the various lipoproteins of sheep plasma are given in Table III; for comparison, values for bovine lipoproteins obtained by Evans, et al., (19) and McCarthy, et al., (20) are also included in Table III. The mean percentages of the total cholesteryl esters circulating as VLDL, LDL, and HDL in sheep plasma were 2.2, 26.6, and 70.3 respectively; the mean percentages of the total plasma phospholipids circulating as VLDL, LDL, and HDL were 2.2, 19.2, and 77.6 respectively. The remainder of the plasma lipoproteins ($d > 1.21$) contained only ca. 1% of the total plasma cholesteryl esters and phospholipids. The cholesteryl esters in the LDL and HDL of sheep plasma contained higher concentrations of 18:1 and lower concentrations of the saturated fatty acids than did the choles-

TABLE II

Composition of Fatty Acids in Cholesteryl Esters and 2-Position of Lecithin in Sheep Plasma

	Fatty acids[a]				
	Saturated	Monoenoic	Dienoic	Trienoic	Tetraenoic
Plasma cholesteryl esters before incubation	16.5 ± 0.75	43.4 ± 0.67	36.1 ± 0.97	2.0 ± 0.09	1.5 ± 0.18
2-Position of plasma lecithin before incubation	11.1 ± 0.43	38.4 ± 0.84	34.2 ± 1.74	8.8 ± 0.25	7.5 ± 0.66
Cholesteryl esters synthesized after incubation of plasma for 1 hr	17.2 ± 0.29	37.2 ± 1.82	35.5 ± 1.18	9.7 ± 0.90	2.6 ± 0.25
Cholesteryl esters synthesized after incubation of plasma for 8 hr	16.5 ± 0.18	38.3 ± 0.37	34.2 ± 0.31	8.9 ± 0.79	2.2 ± 0.14

[a]Mean (g/100 g total fatty acids) ± standard error.

teryl esters in the other two lipoprotein fractions; the highest concentrations of 18:2 in the plasma cholesteryl esters were observed in the HDL fraction. Compared with the plasma cholesteryl esters, there appeared to be less variation in the fatty acid compositions of the phospholipids in the various lipoproteins of sheep plasma.

Appreciable esterification occurred with (^{14}C)-cholesterol was incubated with liver slices or homogenates at pH 7.4 for 2 hr (Table IV); there was little or no increase in the extent of cholesterol esterification when the time of incubation was extended to 4 hr. No experiments were conducted with incubation times of < 2 hr, and, therefore, no details were obtained on the linearity of the esterification process during incubation. In experiments with either slices or homogenates, $< 0.5\%$ of the (^{14}C)-cholesterol was esterified when ATP and CoA were omitted from the incubation medium. The addition of a sonicated suspension of fatty acids to the incubation medium resulted in a small but statistically ($P<0.05$) significant decrease in the extent of cholesterol esterification with both slices and homogeantes (Table IV). During the incubation lf slices or homogenates, the predominant cholesteryl esters synthesized were those containing saturated and monoenoic fatty acids; cholesteryl linoleate was synthesized to some extent, but there was very little esterification of cholesterol with trienoic or tetraenoic fatty acids. The fatty acid pattern of the synthesized cholesteryl esters did not change when the period of incubation was extended to 4 hr. There was considerable similarity between the composition of the unesterified fatty acid fraction of the liver before incubation began and the fatty acid composition of the cholesteryl esters synthesized during incubation (Table IV). However, it should be noted that the ratio of saturated:monoenoic fatty acids in the liver unesterified fatty acid fraction was 1.31, whereas, the corresponding ratios in the cholesteryl esters synthesized by the slices and homogenates were 1.08 and 1.03, respectively. The fatty acid pattern of the cholesteryl esters synthesized by the liver slices was altered markedly when the 1:1:1 molar mixture of 18:0, 18:1, and 18:2 was included in the incubation medium; the proportion of saturated cholesteryl esters synthesized was considerably reduced, whereas, the proportions of monoenoic and dienoic esters synthesized were increased. From the calculated amounts of the various unesterified fatty acids in each incubation flask (Table IV), it is clear that the effect of added substrate fatty acids on the composition of the cholesteryl esters synthesized by the slices can-

TABLE III

Comparison of the Fatty Acid Compositions of Cholesteryl Esters in Lipoproteins of Sheep and Bovine Plasma[a]

Cholesteryl esters	Sheep				Bovine[b]	
	VLDL[c] (d < 1.006)	LDL[c] (d > 1.006, < 1.063)	HDL[c] (d > 1.063, < 1.21)	Remainder (d > 1.21)	LDL[c] (d < 1.063)	HDL[c] (d > 1.063)
16:0	23.2 (21.1 - 24.8)	13.5 (11.0 - 14.8)	13.7 (11.1 - 18.9)	20.0 (17.1 - 24.7)	29.8 (23.4 - 30.0)	10.1 (5.2 - 19.2)
18:0	26.4 (26.2 - 26.5)	7.2 (4.4 - 11.7)	1.7 (1.0 - 2.8)	14.3 (4.2 - 22.5)	26.4 (10.0 - 52.0)	3.1 (0.8 - 7.2)
18:1	24.0 (22.4 - 26.1)	43.6 (39.2 - 50.1)	40.0 (37.7 - 42.9)	31.6 (27.2 - 39.6)	13.8 (3.8 - 23.6)	6.7 (3.4 - 11.5)
18:2	14.8 (8.1 - 21.5)	23.2 (16.0 - 29.7)	37.6 (33.7 - 41.2)	23.9 (23.1 - 24.7)	15.0 (1.2 - 38.4)	68.2 (50.1 - 78.6)
Phospholipids						
16:0	20.3 (16.4 - 28.2)	17.6 (16.9 - 18.0)	13.2 (12.7 - 14.3)	19.6 (18.8 - 21.4)	36.8 (26.0 - 42.6)	27.7 (18.0 - 37.2)
18:0	19.8 (14.0 - 31.5)	22.5 (20.1 - 23.7)	25.6 (23.6 - 29.7)	25.7 (22.5 - 32.0)	28.3 (17.6 - 44.0)	25.1 (21.7 - 31.0)
18:1	34.7 (29.4 - 37.4)	31.3 (29.6 - 34.9)	29.4 (26.0 - 31.2)	26.2 (23.6 - 27.5)	19.6 (11.0 - 21.3)	20.1 ((18.2 - 22.2)
18:2	17.0 (10.9 - 20.0)	22.5 (20.7 - 26.2)	22.1 (19.3 - 23.5)	20.6 (15.7 - 23.1)	5.7 (0.1 - 13.0)	19.6 (12.5 - 27.0)

[a]Mean values (g/100 g total fatty acids) and ranges for 3 animals are given in each instance.

[b]From Evans, et al., (19) and McCarthy, et al., (20).

[c]VLDL = Very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein.

TABLE IV

Esterification of (4-^{14}C) Cholesterol During the Incubation of Sheep Liver Slices or Homogenates[a]

		Fatty acids				
	(^{14}C) Cholesterol esterified (%)	Saturated	Monoenoic	Dienoic	Trienoic	Tetraenoic
Fatty acid composition of liver lipids before incubation (g/100 g total fatty acids)						
Cholesteryl esters		35.1 ± 2.31[b]	53.7 ± 2.76	11.1 ± 0.48	< 1.0	< 1.0
Unesterified fatty acids		48.4 ± 2.29	36.7 ± 2.83	10.6 ± 2.57	< 1.0	4.2 ± 0.62
Incubation of slices with no added fatty acids	7.0 ± 0.42					
Endogenous unesterified fatty acids/flask (μg)		6.74	5.06	1.46	0.14	0.57
Fatty acid composition of cholesteryl esters synthesized (g/100 g total fatty acids)		44.8 ± 0.91	41.4 ± 0.59	10.9 ± 0.79	1.6 ± 0.43	1.3 ± 0.25
Incubation of slices with added fatty acids	6.2 ± 0.16					
Endogenous plus exogenous fatty acids/flask (μg)		12.4	10.7	7.06	0.14	0.57
Fatty acid composition of cholesteryl esters synthesized (g/100 g total fatty acids)		20.9 ± 0.43	62.1 ± 0.92	15.2 ± 0.85	0.9 ± 0.22	0.8 ± 0.10
Incubation of homogenates with no added fatty acids	12.8 ± 0.49					
Endogenous unesterified fatty acids/flask (μg)		50.7	38.2	10.9	1.11	4.32
Fatty acid composition of cholesteryl esters synthesized (g/100 g total fatty acids		41.1 ± 0.39	39.8 ± 0.79	13.2 ± 0.93	2.8 ± 0.18	3.1 ± 0.49
Incubation of homogenates with added fatty acids	11.6 ± 0.42					
Endogenous plus exogenous unesterified fatty acids/flask (μg)		56.4	43.8	16.5	1.11	4.32
Fatty acid composition of cholesteryl esters synthesized (g/100 g total fatty acids)		39.8 ± 0.71	41.0 ± 0.81	13.3 ± 0.64	2.4 ± 0.26	3.4 ± 0.31

[a]Liver slices or homogenates from each of the 4 sheep were incubated in triplicate for 2 hr at 38 C.

[b]Mean ± standard error.

TABLE V

Fatty Acid Composition of Cholesteryl Esters and 2-Position of Lecithin in Bovine Plasma[a]

	Fatty acids				
	Saturated	Monoenoic	Dienoic	Trienoic	Tetraenoic
Cholesteryl esters in plasma before incubation	6.0	6.4	73.1	5.2	5.4
2-position of total lecithin in plasma before incubation	3.9	21.9	44.2	14.6	14.5
Cholesteryl esters synthesized during incubation[b]	10.3	3.9	67.9	7.6	10.2
2-position of plasma lecithin utilized in LCAT reaction during incubation[b]	10.1	4.4	68.5	7.9	9.0

[a]Mean values (g/100 g total fatty acids) calculated from results obtained by Noble, O'Kelly, and Moore (9).
[b]Incubation was for 12 hr at 38 C.

not be explained simply by the change in the composition of the unesterified fatty acids available for esterification, but must involve some form of fatty acid specificity during the esterification process. In contrast, the inclusion of substrate fatty acids in the incubation medium had little effect on the fatty acid pattern of the cholesteryl esters synthesized by the liver homogenates (Table IV). This observation may be related to the fact that the amount of added substrate fatty acids was relatively small compared with the endogenous unesterified fatty acids contained in the liver homogenate; each incubation flask contained 105 μg unesterified fatty acids derived from the liver homogenate and only 16.9 μg derived from the incubation medium. The results given in Table IV also show that the fatty acid composition of the cholesteryl esters synthesized by the liver preparations during incubation was different from that of the cholesteryl esters present in the liver before incubation began.

DISCUSSION

There is considerable variation in plasma LCAT activity between various animal species; the highest activities reported by Stokke (21) were for man (0.079 μmoles/ml/hr) and monkeys (0.105 μmoles/ml/hr), whereas, the activities for ruminant animals appear to be among the lowest reported. In addition to the activity of 0.024 μmoles/ml/hr found for sheep plasma in the present investigation, activities of 0.009 and 0.023 μmoles/ml/hr have been found for calf (21) and goat (Noble and Moore, unpublished data) plasma, respectively.

The results given in Table II show that, apart from the 20:4 content, the fatty acid composition of the cholesteryl esters synthesized during the incubation of sheep plasma in vitro was very similar to the composition of the fatty acids in the 2-position of the lecithin in the plasma before incubation began. This observation may be interpreted to indicate that, with the possible exception of 20:4, the LCAT system in sheep plasma does not discriminate between the various molecular species of plasma lecithin as donors of fatty acids. Thus, there is a marked contrast in the fatty acid specificities of the LCAT systems in sheep and bovine plasma. For direct comparison with the results obtained with sheep plasma (Table II), values obtained by Noble, O'Kelly, and Moore (9) from a study of the LCAT system in bovine plasma have been recalculated and summarized in Table V. The fatty acid composition of the cholesteryl esters synthesized during the incubation of bovine plasma for 12 hr at 38 C was quite different from the composition of the fatty acids in the 2-position of the plasma lecithin before incubation began. In particular, the concentration of dienoic acids in the newly synthesized cholesteryl esters was much greater than that in the 2-position of the total plasma lecithin (Table V). However, the concentration and structure of the bovine plasma lecithin were determined at various stages during the incubation so it was possible to calculate the composition of the fatty acids in the 2-position of that fraction of the plasma lecithin that had taken part in the LCAT reaction. Very good agreement was obtained between these calculated values and the values for the fatty acid composition of the cholesteryl esters that had been synthesized during incubation (Table V). It is clear then that the LCAT system in bovine plasma discriminates between the various molecular species of plasma lecithin as fatty acid donors, and preferentially utilizes those molecular species with 18:2 esterified in the 2-position. This preferential utilization may be due to the fact that the specificity of the LCAT enzyme in bovine plasma is such that the enzyme-substrate complex is formed more read-

ily with those molecules of lecithin that contain linoleic acid esterified in position 2, whereas the LCAT enzyme in sheep plasma might have a much broader substrate specificity (1). Alternatively, because the plasma LCAT enzyme reacts primarily with substrates circulating as HDL (6), it is possible that the distribution between the various lipoproteins of lecithin with 18:2 esterified in the 2-position differs in sheep and bovine plasma. Evidence to this effect is presented in Table III from which it may be seen that there was little variation in the 18:2 content of the phospholipids in the various plasma lipoproteins of sheep plasma. On the other hand, the results for bovine plasma, taken from Evans, Patton and McCarthy (19) and McCarthy, et al., (20) show that the phospholipids in the HDL contained much higher concentrations of 18:2 than did the phospholipids in the LDL (Table III). This difference in distribution could partly explain why in sheep plasma, but not in bovine plasma, the fatty acid composition of the cholesteryl esters synthesized by the LCAT system in vitro was very similar to the composition of the fatty acids in the 2-position of the total plasma lecithin (Table II). However, to explain why bovine plasma synthesizes so little monoenoic cholesteryl ester requires the involvement of some direct fatty acid discrimination by the LCAT system itself. As with bovine plasma, the LCAT system in human plasma has been shown to exert some selectivity in the utilization of different molecular species of plasma lecithin as fatty acid donors (1, 22, 23).

The requirement for ATP and CoA for the synthesis of cholesteryl esters by sheep liver slices or homogenates at pH 7.4 indicated that this synthesis was catalyzed by acyl CoA:cholesterol acyltransferase, the presence of which has been demonstrated in rat, pig, dog, monkey, rabbit, guinea pig, and calf liver, but not in human liver (21). Because the addition of free fatty acids to the medium had little effect on the rate of cholesterol ester synthesis by sheep liver slices or homogenates, and because the composition of the unesterified fatty acids in the liver was similar to the fatty acid composition of the cholesteryl esters synthesized in the absence of exogenous fatty acid substrate, it may be concluded that the magnitude and composition of the endogenous pool of unesterified fatty acids in sheep liver were sufficient to maintain optimum rates of cholesteryl ester synthesis. Similar conclusions were reached by Goodman, et al., (2) from the results of experiments in which rat liver preparations were incubated with exogenous palmitic or linoleic acids; however, the inclusion of oleic acid in the incubation medium increased the rate of cholesterol esterification by rat liver. The inclusion of the equimolar mixture of 18:0, 18:1, and 18:2 resulted in 2-fold increases in the amounts of saturated and monoenoic acids in each flask (Table IV), yet in the cholesteryl esters synthesized during incubation, the concentration of saturated fatty acids was reduced from 45 to 21% but the concentration of monoenoic acids was increased from 41 to 62%; the 5-fold increase in the amount of dienoic acid in each flask only resulted in an increase from 11 to 15% in the concentration of dienoic acid in the synthesized cholesteryl esters. Analogous results were obtained by Goodman, et al., (2) who incubated (^{3}H)-cholesterol with rat liver microsomes in the absence or presence of an equimolar mixture of 16:0, 18:1, and 18:2. The cholesteryl esters synthesized in the presence of exogenous fatty acids contained reduced concentrations of saturated fatty acids and increased concentrations of monoenoic and dienoic fatty acids. The saturated, monoenoic, and dienoic fatty acid contents of the cholesteryl esters synthesized by sheep liver homogenates were not altered by the inclusion of the equimolar mixture of 18:0, 18:1, and 18:2 in the incubation medium. These findings for the saturated and monoenoic acids are consistent with the fact that the inclusion of the exogenous fatty acids in the incubation medium only increased the amounts of saturated and monoenoic acids in each flask by ca. 12%. On the other hand, the inclusion of the exogenous fatty acid mixture in the incubation medium increased the amount of dienoic acid in each flask by ca. 60%, yet there was not change in the dienoic acid content of the cholesteryl ester synthesized by the homogenates. Thus the results in Table IV may be considered to be consistent with the view that the fatty acid specificity of cholesteryl ester synthesis in sheep liver was monoenoic > saturated > dienoic. A similar fatty acid specificity has been reported for cholesteryl ester synthesis in rat liver (2).

From the results of the present investigation, it may be concluded that the cholesteryl esters in sheep plasma are synthesized mainly by the plasma LCAT system. This conclusion is supported by the following observations. (a) The fatty acid composition of the total cholesteryl esters in sheep plasma is similar to that of the cholesteryl esters synthesized during incubation of the plasma in vitro, and to the composition of the fatty acids esterified in the 2-position of the plasma lecithin (Table II). (b) According to Glomset (1,6), the LCAT system acts primarily on plasma lipoproteins of densities greater than

1.006 g/ml, but especially on the HDL with densities greater than 1.063 g/ml. The fatty acid composition of the cholesteryl esters synthesized by the LCAT reaction in vitro (Table II) was similar to the composition of the cholesteryl esters in the lipoproteins with densities between 1.06 and 1.21 g/ml (Table III); this similarity was particularly noticeable with the HDL cholesteryl esters. (c) The cholesteryl esters in the LDL and HDL of sheep plasma accounted for ca. 97% of the total plasma cholesteryl esters. (d) There were pronounced differences between the fatty acid compositions of the cholesteryl esters present in the plasma (Tables II and III) and those of the cholesteryl esters present in, or synthesized by, the liver (Table IV). As regards the origin of the plasma cholesteryl esters, the sheep would thus appear to be similar to man in which the LCAT reaction is thought to be responsible for the formation of virtually all of the plasma cholesteryl esters, with the possible exception of a very small proportion derived from the intestinal tract (21). In the rat, on the other hand, the VLDL cholesteryl esters, which under certain dietary conditions form a high proporation of the total plasma cholesteryl esters, are though to be synthesized in the liver by the microsomal acyl CoA:cholesteryl acyl transferase (2, 24-26). The fatty acid composition of the VLDL cholesteryl esters in rat plasma is very similar to that of the cholesteryl esters in rat liver (24). Since the cholesteryl esters present in or synthesized by sheep liver contained much higher concentrations of monoenoic acids (Table IV) than did the VLDL cholesteryl esters in the plasma (Table III), it is unlikely that this small fraction (about 2%) of the total plasma cholesteryl esters was of hepatic origin; it is conceivable that the VLDL cholesteryl esters were derived from the intestine (2).

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Quantitative, Multicomponent Analysis of Fatty Acids from Cholesteryl Esters by Chemical Ionization Reconstructed Mass Chromatography

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ABSTRACT

Reconstructed mass chromatography using methane as a carrier and reagent gas for chemical ionization gas chromatography-mass spectrometry of the derived methyl esters allows rapid, quantitative microdeterminations of complete cholesteryl ester fatty acid profiles. The sensitivity of this method is consistent with completely specific, multicomponent assay at the picomole level. Introduction of two homologues as internal standards, one into the intact biological specimen and the other after derivatization, provides a measure of the net efficiency of the processes of extraction and derivatization. This procedure may be extended readily to the determination of fatty acid profiles in most biological fluids.

INTRODUCTION

Many techniques have been developed for quantitative estimation of fatty acids (1,2); however, many of these are nonspecific, insensitive, or tedious. Although gas chromatography (GC) of derivatized fatty acids, usually as their methyl esters (FAMEs), has been used for quantitative analysis for a number of years (3-5), sensitivity and accuracy of GC procedures may be compromised by interference from column bleed and coelution of peaks, whereas, specificity of detection depends upon completeness of separation by the column and must be verified at regular intervals by the use of authentic mixtures. Because of the exquisite selectivity of detection by mass spectrometry (MS), the technique of chemical ionization (CI) reconstructed mass chromatography (RMC) suffers from none of these problems, even for peaks having extremely low GC signal/noise ratio. In addition, the mass spectrum can be consulted if unambiguous peak identification is desired. By this technique, quantitation of individual fatty acids and total fatty acids in a complete lipid fraction is accomplished in a single step. Cholesteryl esters (CE) are chosen as illustration in this report.

MATERIALS AND METHODS

Sample Preparation for GC-MS Analysis

A mixture of 1 ml of pooled, normal human plasma and 600 μg (901 nmoles) of cholesteryl nonadecanoate was extracted with two 4-ml

TABLE I

Parameters[a] for Converting[b] Measured RMC Peak Areas into Molar Concentrations of Individual Fatty Acid Methyl Esters

FAME	m	b	r	range
14:0	0.871	0.262	.99999	0.01-10
16:0	0.826	0.426	.99986	0.01-50
16:1	0.835	1.308	.99983	0.01-10
18:0	0.853	0.497	.99982	0.01-10
18:1	0.832	0.403	.99995	0.01-50
18:2	0.863	1.747	.99996	0.1 -10
18:3	0.836	0.946	.99998	0.01-10
19:0	0.918	0.649	.99996	0.1 -100
20:4	0.868	1.259	.99994	0.1 -10

[a]FAME = Fatty acid methyl ester; m = slope; b = intercept; r = correlation coefficient of linear regression.

[b]According to the expression: $[FAME_i] = [17:0] \exp\{m \ln(Area_i/Area_{17:0}) + b\}$.

[c][17:0] added = 370 nmole/ml in this study.

TABLE II

Amounts of Fatty Acids and Cholesterol from Cholesteryl Ester Fraction of Normal Human Plasma

Fatty Acid[a]	Coefficient of variation (%)	Concentration (μmole/ml)
14:0	7.0	0.027
16:0	5.5	0.540
16:1	5.2	0.300
18:0	11.1	0.108
18:1	4.0	0.865
18:2	3.8	2.38
18:3	10.1	0.032
20:4	4.6	0.430
Total concentration		4.68
Total plasma cholesterol[b]		7.69±1.86
Free plasma cholesterol[b]		2.84±0.12
Cholesterol in CE[c] (difference)		4.85

[a]Determined as fatty acid methyl ester by chemical ionization reconstructed mass chromatography.

[b]Determined by gas chromatography.

[c]CE = *cholesteryl esters.*

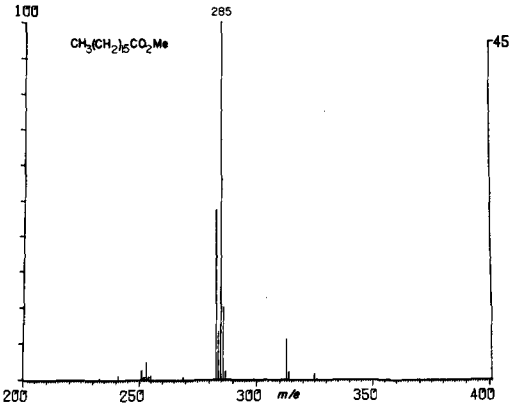

FIG. 1. Chemical ionization mass spectrum of methyl heptadecanoate (17:0 fatty acid methyl ester) using methane as reagent gas.

portions of methanol:chloroform (2:1); insoluble matter was dispersed partially by intermittent, vibrational agitation, and sedimented by centrifugation prior to decantation. The combined extracts were concentrated to dryness in a stream of nitrogen, affording a residue that was redissolved in the minimum amount, ca. 100 μl, of spectroquality heptane and quantitatively transferred to a 5 x 80 cm thin layer chromatographic (TLC) plate (Adsorbosil-5, Applied Science Labs, State College, PA). The TLC plate was developed in hexane:ether:acetic acid (90:20:1), and components were visualized by exposure to iodine vapors. The region containing the cholesteryl esters (CE) was scraped into a test tube, to which was added a 5-ml portion of chloroform:methanol:ether (1:1:1). The mixture was agitated vigorously and centrifuged, and the solvent was decanted. The residue was similarly extracted with a second 5-ml portion of solvent. The combined solutions were concentrated in a stream of dry nitrogen, dissolved in a mixture of 1 ml benzene and 2 ml 0.5 *N* sodium methoxide in methanol and heated to 80 C for 20 min. The transesterified mixture then was cooled and diluted with 3 ml distilled water and 3 ml ether. The organic layer was separated, washed with a 3-ml portion of distilled water, and dried by the addition of anhydrous sodium sulfate. After decantation from the desiccant, the solution was concentrated to dryness in a nitrogen stream and re-dissolved in 100 μl of ethyl acetate containing 100 μg (352 nmole) of methyl heptadecanoate (17:0 FAME).

Analysis of FAMEs by GC-MS

FAMEs were measured using a Finnigan 1015D MS interfaced to a Finnigan 9500 GC and a Finnigan 6000 Interactive Data System (DS) (Finnigan Corp., Sunnvale, CA). The GC was fitted with a 0.5 m x 2 mm internal diameter (ID) glass column packed with 3% EGSS-X coated on Gas Chrom Q. The solution to be analyzed (1 μl) was injected at a column temperature of 130 C; the effluent from the GC was diverted into a vacuum bypass for 30 sec, allowing the solvent to elute, whereupon the column temperature was increased at a rate of 6 C/min to a final temperature of 210 C. The flow rate of methane, 17 ml/min, through the GC was selected to afford a pressure of 0.95 torr, which was optimal for CI in the MS source; electron energy was 150 eV, multiplier setting was 1.7 kV, and pre-amplifier sensitivity was 10^{-7} a/V.

The DS scanned the range 230-330 amu at a uniform rate of 20 msec/amu during the GC run. After the termination of data acquisition, the DS was directed to recall individual records of the intensity of proton capture (M + 1) ions corresponding to each of the FAMEs present. The DS was used to calculate the area under the peak, reproducible within 5% for duplicate injections, in each such reconstructed mass chromatogram (RMC), and to normalize this area relative to that of 17:0 FAME. Values thus obtained were inserted into the appropriate expression from Table I to give the concentration of CE corresponding to that FAME in the original sample. Isolation efficiency was calculated as the amount of 19:0 FAME measured divided by the amount of 19:0 CE added. Calibration curves were calculated from quadruplicate determinations using gravimetrically prepared standard mixtures of FAMEs. The data in Table II are averages of triplicate

measurements on 3 identical preparations of normal human plasma.

Determination of Cholesterol by GC

Total cholesterol and free cholesterol were measured using a Barber-Coleman Model 5000 GC fitted with flame ionization detection and a 46 cm x 4 mm ID glass column packed with 3% OV-1 on Gas Chrom Q. The system was operated isothermally at 240 C, using argon as the carrier gas. Peak areas were estimated by triangulation, and samples were determined in triplicate. The preparation was essentially that of Blomhoff (6) except that (a) sitosterol was introduced into the plasma as an internal standard prior to the extraction procedures, and (b) measured values of cholesterol were adjusted to correct for losses in isolation and handling.

RESULTS AND DISCUSSION

The characteristic, proton capture (M + 1) ion appears as the most abundant species in the CI mass spectrum (Fig. 1) of each of the FAMEs in this study, accounting for ca. 50% of the total ionization in all examples. Conversely, the electron impact mass spectrum of a FAME exhibits only low intensity ions at the characteristic, high mass region, the most prominent fragments, m/e 74 and 87, being common to the entire series of FAMEs (7). Thus, CI MS offers both enhanced sensitivity and extreme selectivity as a means of quantitatively detecting FAMEs in a GC effluent; this selectivity carries the added advantages that such potential interferences as biological background and column bleed are generally transparent at the mass number being detected, so that baselines normally are close to zero in the RMCs. Figure 2a illustrates the output from the GC detected as the sum of the ion currents from 230 to 330 amu. Therein, methyl myristate (14:0 FAME) presents a weak signal, that of methyl arachidonate (20:4 FAME) is distorted in shape by interfering column bleed, and those of methyl linoleate (18:2 FAME) and 19:0 FAME coelute. In the RMCs (Figure 2b) of m/e 243, 285, 295, 313, and 319 (M + 1 for 14:0, 17:0, 18:2, 19:0 and 20:4 FAME, respectively), each peak is found on a separate trace, where its area may be measured accurately, in the absence of the interferences present in Figure 2a.

We found that the calibration curves empirically gave a linear approximation to a log-log expression (Table I and Fig. 3) rather than to a simple cartesian plot. Published calibration curves for the closely related technique of selective ion monitoring are commonly limited to

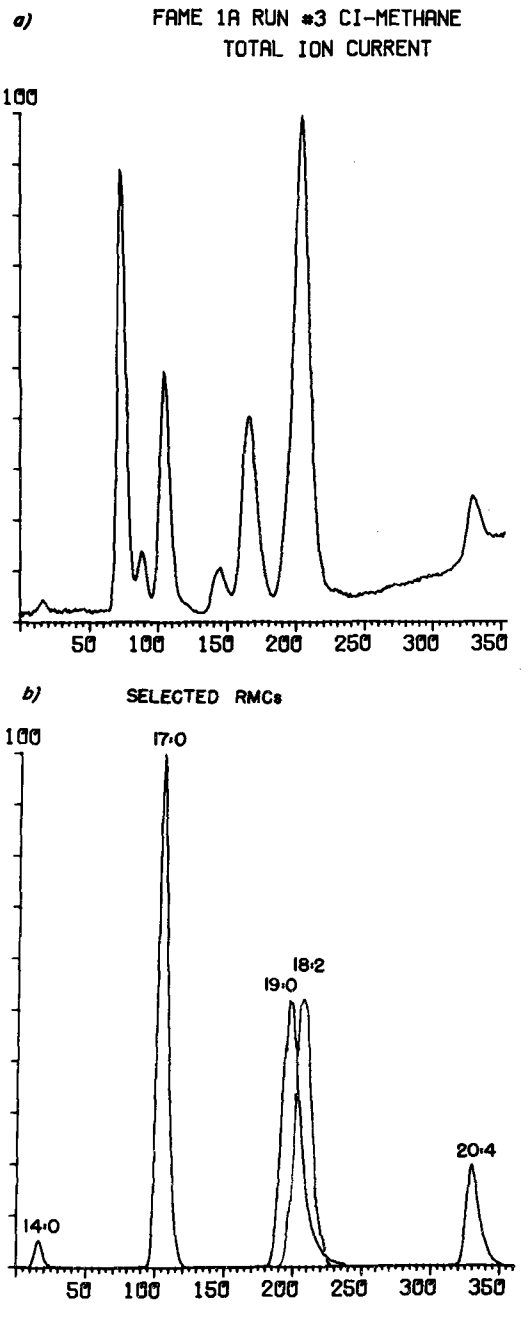

FIG. 2a. Gas chromatogram of fatty acid methyl ester (FAME) mixture detected by summing all of the ion currents measured by the mass spectrometer; 2b. Composite of reconstructed mass chromatograms (RMCs) for M + 1 ions of 14:0, 17:0, 18:2, 19:0 and 20:4 FAMEs.

a tenfold dilution range (8). However, Clark and Foltz (9) reported separate calibration curves having different slopes for 3,6-bis(tri-

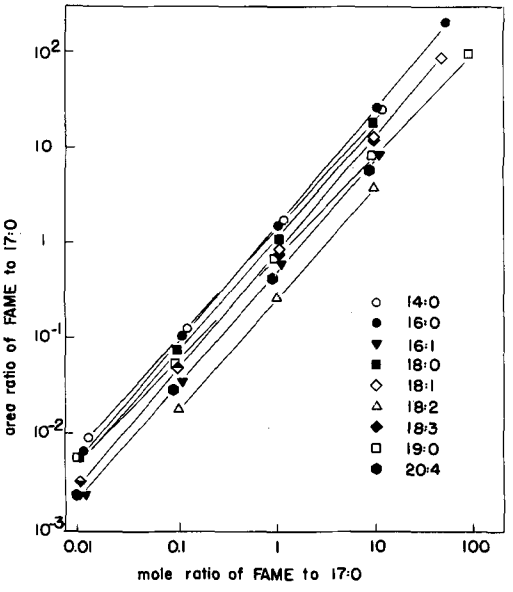

FIG. 3. Calibration plot for area response of fatty acid methyl esters (FAME) relative to 17:0 FAME versus mole ratio of FAMEs to 17:0 FAME; best-fit regression expressions and correlation coefficients are recorded in Table II.

methylsilyl)-morphine over two regions of a wider dilution range. The significance of this observation is unclear at present.

Table II contains the amounts of each fatty acid present in the original CE fraction as determined by CI-RMC, together with a value for the amount of cholesterol in the CE fraction, determined by subtracting free from total cholesterol content. Agreement between the two values is clearly within the reliability limits of either method of determination.

Thus, the present communication demonstrates that CI-RMC offers numerous advantages as a technique for simultaneous, multicomponent assay of fatty acids, i.e., simplicity of operation, speed, and broad dynamic range for determination. Furthermore, (a) CI-RMC requires only inexpensive internal standards, (b) the selectivity of detection minimizes the GC resolution needed for reliable quantitation, and (c) the mass spectrum is available to verify the identity of the component being measured. Multiple selective ion monitoring "mass fragmentography" would be slightly more sensitive for this analysis, but (a) commercial instruments presently have facilities to monitor no more than 4 ions, and (b) the mass spectrum would not be available for verification.

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Lecithin:Cholesterol Acyltransferase Activity in Hypercholesterolemic Subjects and in Hypercholesterolemic Subjects Treated with Clofibrate

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ABSTRACT

The lecithin:cholesterol acyl transfer reaction in the plasma of hypercholesterolemic subjects and of hypercholesterolemic subjects treated with clofibrate was studied. An increased enzyme activity was found in the first group of patients, while lecithin:cholesterol acyl transfer activity tended to normalize in the second group. This increased enzyme activity might be a defense mechanism against the accumulation of cholesterol in the arterial wall.

INTRODUCTION

The presence in human blood plasma of an enzyme that esterifies cholesterol has been known for some time (1). Glomset (2) has shown that an acyltransferase transfers fatty acids from the C-2 position of high density lipoprotein (HDL) lecithins to the hydroxyl group of cholesterol. Lecithin:cholesterol acyltransferase (LCAT) is involved in the metabolism of circulating lipoproteins because the few subjects affected by familial deficiency of this enzyme show serious alterations in lipoproteins, in as much as these, when they enter the circulatory stream, are not affected by the action of the enzyme (3). Schumaker and Adams (4) have suggested that lipoprotein lipase (LPase) and LCAT are enzymes that are involved in the metabolism of circulating lipoproteins. According to these authors, the LPase removes triglycerides from the interior of the lipoprotein micelles, thereby producing a reduction of their volume, while LCAT, by removing a molecule of phosphatidyl choline and of cholesterol from the surface of the micelle, allows it to remain intact and spherical.

Because of the possible connection between LCAT and the metabolism of lipoproteins, it was decided to study this enzyme activity in subjects affected by hypercholesterolemia and in hypercholesterolemic subjects treated with clofibrate. The predominance of esterified cholesterol in the plasma suggests that the synthesis of these compounds may be an important step in the metabolism of cholesterol.

Cholesterol can be synthesized by cells, but these cannot catabolize it; therefore, some mechanism must exist for the transport of cholesterol to the liver. Glomset (5) has hypothesized that this enzyme enters a metabolic cycle which he calls "membrane homeostasis." LCAT supposedly removes the cholesterol from the cellular membranes and transfers it to the lipoproteins, and particularly to the HDL, for esterification and subsequent elimination from the organism. Consequently, the enzyme might act as a defense mechanism by removing cholesterol accumulated in the arterial wall, in the same manner that the increased synthesis of phospholipids in atheromatous lesions (6) supposedly does.

MATERIALS AND METHODS

5,5′-Dithiobis 2-nitrobenzoic acid (Ellman reagent) and 2-mercaptoethanol were purchased from Eastman Organic Chemicals (Rochester, NY). (7α-^{3}H)Cholesterol with specific activity of 9.4 Ci/mmole was obtained from the Radiochemical Center (Amersham, England); it was found to be 97% radiochemically pure when tested by thin layer chromatography (TLC) and was used without further purification. Crystalline human albumin was 100% electrophoretically pure and was obtained from Behringwerke AG (Marburg-Lahn, West Germany). 2,5-Diphenyloxazole (PPO) and 2,2′-paraphenylenebis(4-methyl-5-phenyloxazole) (Dimethyl-POPOP) were obtained from E. Merck (Darmstadt, West Germany). All chemicals were reagent grade and were used without further purification.

Plasmas for the determination of LCAT activity were taken from 61 subjects of both sexes, 20-90 years old. Subjects with coronary artery diseases were excluded, as were those with endocrine, liver, and renal diseases. Only a few subjects affected by hypercholesterolemia were also affected by diabetes mellitus. Seventeen subjects had a plasma cholesterol concentration $<$ 250 mg/100 ml of plasma. Thirty-two subjects had a blood cholesterol concentration from 280 to 460 mg/100 ml of plasma. Twelve subjects in the past had had a high cholesterol concentration in the plasma, but this had been reduced to ca. 270 mg/100 ml of plasma with

TABLE I

Lecithin:Cholesterol Acyltransferase in Plasma from Young and Old Subjects

	Number	Age	Total cholesterol (mg/dl)	Free cholesterol (mg/dl)	Cholesterol esterification	
					Percentage	(μmoles/liter/hr)
Young	11	20-40	204 ± 28.5[a]	59 ± 15.7	5.7 ± 1.0	86 ± 20.6
Old	6	50-90	181 ± 31.5	59 ± 18.0	5.7 ± 0.3	88 ± 27.7

[a]Mean ± standard deviation.

TABLE II

Lecithin:Cholesterol Acyltransferase Activity in Plasma from Normal, Hypercholesterolemic and Hypercholesterolemic Subjects Treated with Clofibrate

	Number	Total cholesterol (mg/dl)	Free cholesterol (mg/dl)	Cholesterol esterification	
				Percentage	(μmoles/liter/hr)
Normal	17	196 ± 30.7[a]	59 ± 16.3	5.7 ± 0.76	86 ± 22.5
Hypercholesterolemic	32	321 ± 41.0	107 ± 22.7	3.9 ± 0.61	109 ± 26.7
Treated	12	257 ± 13.4	79 ± 12.5	4.2 ± 0.83	86 ± 22.9

[a]Mean ± standard deviation.

clofibrate in doses varying from 1 to 1.5 g per day. All the subjects had a plasma triglyceride concentration < 200 mg/100 ml of plasma. To study the possible influence of age on enzyme activity, the group having blood cholesterol concentrations up to 250 mg/100 ml of plasma was divided into two subgroups according to age: the first group included subjects between the ages of 20-40 and the second, subjects between the ages of 50-90. Blood was collected after an overnight fast and mixed with K_2EDTA (2 mg/ml blood), and blood samples were placed in ice immediately. Plasma was separated by centrifugation at 4 C and stored at 0 C until tested the same day.

LCAT activity was determined according to the method of Stokke and Norum (7). Plasma (100 μl) was preincubated with 30 μl of 5% albumin solution containing ca. 0.12 μCi of ^{3}H-cholesterol and with 20 μl of 10 mM Ellman reagent for 4 hr at 37 C in a shaking water bath. The preincubation period, in which LCAT activity is inhibited by the disulphide, was used to obtain a satisfactory isotopic equilibration between labeled cholesterol and the endogenous free cholesterol of the lipoproteins. This inhibition was reversed by adding 20 μl of 0.1 M mercaptoethanol, and the addition of 3 ml of chloroform:methanol (2:1, v/v) stopped the reaction after an hr incubation at 37 C. The mixture was filtered and the precipitate washed 3 times with chloroform:methanol. The extract was combined with the wash and evaporated under a stream of nitrogen. The dry residue was dissolved in a small amount of chloroform and the organic solvent was evaporated again under a nitrogen stream. The lipid residue was dissolved in a very small amount of n-heptane and transferred to precoated thin layer plates of silica gel 60 (E. Merck, Darmstadt, West Germany). TLC was carried out in light petroleum (bp 40-60 C):diethyl ether:acetic acid (85:15:1, by vol). The air dried plates were stained by exposure to iodine vapor; and after the evaporation of the excess iodine, the areas containing cholesterol and cholesterol esters were scraped off into liquid scintillation counting vials. The scintillation fluid was made up as described by Stokke and Norum (7). LCAT activity was expressed as μmoles of cholesteryl esters formed per liter of plasma per hr or as a percentage of labeled cholesterol acylated per hr. The cholesterol both in its free form and in its esterified form was determined by the method of Sperry and Webb (8).

RESULTS

Our data do not show notable variations in LCAT activity according to age. In fact, the esterification percentage was 5.7 ± 1.0% in the group of subjects aged 20-40 years and 5.7 ± 0.3% in the group of subjects aged 50-90. Such a percentage of esterification corresponds to 86 ± 20.6 μmoles/liter/hr of esterified cholesterol in the first group and to 88 ± 27.7 μmoles/liter/hr of esterified cholesterol in the second group. Such differences were not significant (Table I), and, therefore, subjects with plasma cholesterol concentration <

250 mg/100 ml were considered a homogeneous control group. LCAT activity in the hypercholesterolemic subjects was 3.95 ± 0.6% if expressed as a percentage of esterification corresponding to 109 ± 26.7 μmoles/liter/hr of esterified cholesterol. Such values were significantly ($P<0.01$) different from the values of the control group. In hypercholesterolemic subjects treated with clofibrate, the percentage of esterification was 4.19 ± 0.8% corresponding to 86.7 ± 22.5 μmoles/liter/hr. Only the percentage of esterification differs significantly ($P<0.01$) from the values of the control subjects (Table II).

To exclude the fact that the increased enzyme activity in the hypercholesterolemic subjects and the decreased percentage of esterification in subjects treated with clofibrate were dependent on the higher plasma cholesterol concentration in these two groups, we calculated the regression lines of enzyme activity on cholesterol concentration (Fig. 1). The comparison of the regression lines between hypercholesterolemic subjects and control subjects differs significantly ($P<0.05$) in residual variances while the comparison of the regression line of the subjects treated with clofibrate differs significantly ($P<0.01$) from that of the control subjects only in elevation.

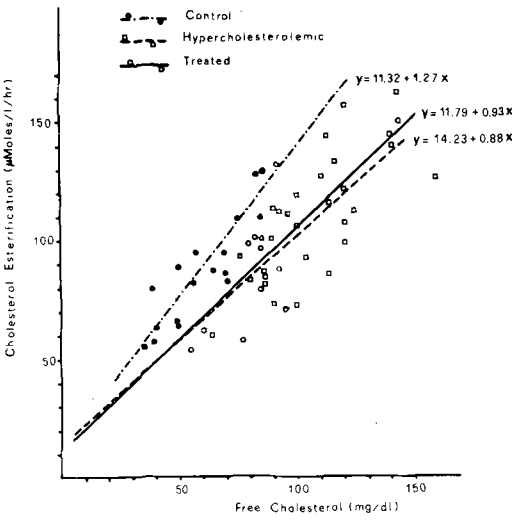

FIG. 1. Regression lines of lecithin:cholesterol acyl transferase activity on cholesterol concentration in normal, hypercholesterolemic, and hypercholesterolemic subjects treated with clofibrate.

DISCUSSION

Our results do not show a marked variation in enzyme activity according to the age of the subjects. This fact contrasts with the results of some authors (9,10) and may be explained by the different way the LCAT reaction was determined. In fact, Wagner and Poindexter (9) and Gherondace (10) measured the net esterification and not the initial rate of the reaction. On the contrary, our data agree with results obtained recently by Akanuma, et al., (11) who found no correlation between age and LCAT activity, but did notice a positive correlation between enzyme activity and body wt. This, too, suggests a connection between the enzyme and the metabolism of cholesterol because it is known that the adipose tissue is an important storage tissue for cholesterol in the organism (12). As far as hypercholesterolemic subjects are concerned, enzyme activity results decreased if expressed as a percentage of esterification but increased if expressed in μmoles/liter/hr. This finding is only seemingly contradictory because it is known that, while enzyme activity is dependent on the concentration of plasma free cholesterol (13), the rate of esterification does not increase linearly in the hypercholesterolemic subjects; thus, the relative value of the enzyme activity results decreased. This fact may also depend on the presence of inactive HDL as substrate in the plasma of hypercholesterolemic subjects, as Fielding, et al., (14) showed. In hypercholesterolemic subjects treated with clofibrate, no difference resulted in enzyme activity expressed in absolute values, while the percent of esterification decreased significantly ($P<0.01$) compared with that of the control subjects. This finding is related to the fact that the concentration of free cholesterol in the plasma of hypercholesterolemic subjects treated with clofibrate is significantly ($P<0.01$) higher in comparison to the control subjects.

If we consider the regression lines of enzyme activity on plasma free cholesterol concentration, significant variations are apparent both in hypercholesterolemic subjects and in treated ones. Clofibrate treatment, apart from normalizing the blood cholesterol concentration, tends to normalize the regression line as well, which only shows a a significant ($P<0.01$) difference in the elevation.

Shapiro, et al., (15) found a net decrease in LCAT activity in experimental hypercholesterolemia; however, thse authors did not measure the initial rate of the reaction, but considered the net esterification. Langer, et al., (16) noticed a reduced catabolism of low density lipoprotein (LDL) in the hyperlipoproteinemia of type II and reduced LCAT activity was found in this hyperlipoproteinemia (17). On the other hand, Marcel, et al., (18) found enhanced LCAT activity in human primary hyperlipidemia, but they measured the net esterification

and not the initial rate of the reaction. Modifications of LCAT activity was noticed in some congenital deficiencies of the lipoproteins, such as abetalipoproteinemia (19).

It should also be noted that enzyme activity is decreased in patients with coronary heart disease, one of the most important clinical manifestation of atherosclerosis (20). We feel that LCAT activity depends on the type of fatty acid present in the lecithins (21); the presence in C-2 position of polyunsaturated fatty acids increases enzyme activity (22). Gjone, et al., (23) found a reduction of LCAT activity in subjects given a diet rich in unsaturated fatty acids as well as a significant fall in the plasma concentration of the cholesterol and lecithins. Because it is known that in hypercholesterolemic subjects there is a tendency towards a decrease in polyunsaturated fatty acids, this factor might be important in enzyme activity (24). Glomset's hypothesis (5) that LCAT might be important in the cholesterol exchange between membranes and plasma could be confirmed by the data of Rutenberg and Soloff (25); that is, that the exchange of cholesterol between plasma and arterial wall is related to the presence of LCAT activity in the plasma. Furthermore, an inverse correlation between the tendency of various animal species towards spontaneous atherosclerosis and LCAT activity (26) has been noticed recently.

Our data and those of the literature in question confirm that LCAT is important in the metabolism of lipoproteins and cholesterol. The increase in its activity which we found in hypercholesterolemic subjects and its tendency to become normal again during treatment with clofibrate may depend on the change of the substrate (free cholesterol) concentration produced by the drug. But this hypothesis does not seem to be confirmed by the comparison of the regression lines of normal and hypercholesterolemic subjects which, being statistically ($P<0.05$) significant, exclude a close dependence of enzyme activity on cholesterol concentration. We think it possible that increased enzyme activity in hypercholesterolemic subjects may be a defense mechanism to remove the excess cholesterol in the arterial wall or to inhibit the deposition of cholesterol in the arterial wall by esterification of free cholesterol. Such a defense mechanism has already been suggested with a view to the increased synthesis of phospholipids in human atheromatous lesions.

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24-Methylenedammarenol: A New Triterpene Alcohol from Shea Butter

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ABSTRACT

A new triterpene alcohol was isolated from shea butter and its structure was shown to be 24-methylenedammarenol (24-methylene-5α-dammar-20[21]-en-3β-ol). Dammaradienol (5α-dammara-20[21],24-dien-3β-ol) also was isolated from shea butter.

INTRODUCTION

Previous work has indicated the presence of α-amyrin (1), β-amyrin (1,2), butyrospermol (1,3,4), parkeol (1,5), and 24-methylenedihydroparkeol (6) in the triterpene alcohol fraction of shea butter. Shea butter, obtained from the kernels of *Butyrospermum parkii,* a sapotaceous tree of tropical Africa, is a substance used as food by the natives and for the manufacture of soap and other products. This paper describes the isolation and identification of a new dammarane series triterpene alcohol, 24-methylenedammarenol (24-methylene-5α-dammar-20[21]-en-3β-ol) (Scheme I,II), and dammaradienol (5α-dammara-20[21],24-dien-3β-ol) (Scheme I,I) from shea butter. Dammaradienol (Scheme I,I) has only been known as a component of dammarane series triterpenes in dammar resin originating from trees of the Dipterocarpaceae family (7-11).

EXPERIMENTAL PROCEDURES

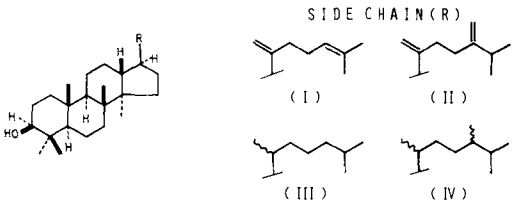
Melting points were determined with a Micro mp apparatus (Yanagimoto Seisakusho Ltd., Kyoto, Japan) and uncorrected. All recrystallizations were performed in acetone:methanol. Infrared (IR) spectra (KBr) were obtained with a Type IRA-2, IR spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan). Optical rotations were measured in $CHCl_3$ using a Carl Zeiss Polarimeter 0.01° (Carl Zeiss, Oberkochen, Germany). Concentrations used were indicated in parentheses as g/100 ml. Nuclear magnetic resonance (NMR) spectra in $CDCl_3$ were recorded on a JNM-C-60-HL instrument (60 MHz, Japan Electron Optics Laboratory Co., Tokyo, Japan) and calibrated against internal tetramethylsilane as 0 ppm. Mass spectra were taken with a Hitachi RMU-7M mass spectrometer (Hitachi Ltd., Tokyo, Japan), electron energy 70 eV, target current 57 μA, ion source temp 160-180 C, sample temp 100-120 C, and accelerating high voltage 4.6 kv. The samples were introduced directly into the ion source through a vacuum lock.

Preparative argentation thin layer chromatography (TLC) for the fractionation of triterpene acetates was carried out on the 20 x 20 cm plates coated with 0.5 mm of silica gel (Wakogel B-10, Wako Pure Chemical Industries Ltd., Osaka, Japan) impregnated with 10% or 20% silver nitrate, using a Toyo continuous flow development preparative TLC (Toyo Roshi Kaisha Ltd., Tokyo, Japan). The plate was developed for 1 hr using hexane:benzene (7:3) as solvent. Separated zones were observed under ultraviolet light (3600 A) after spraying of a rhodamine-6G solution in ethanol on the developed plate, and were cut off and quantitatively extracted with ether. The argentation silicic acid column was prepared as described previously (1) in a similar manner as proposed by Vroman and Cohen (12). Gas liquid chromatography (GLC) was performed under the same operating conditions as described previously (1), using a Shimadzu GC-5A gas chromatograph (Shimadzu Seisakusho Ltd., Kyoto, Japan) equipped with a flame ionization detector and a 2 m x 3 mm inside diameter glass column packed with 3% OV-17 on Gas Chrom-Z, 80-100 mesh. Relative retention time (RRT) was expressed by the ratio of the retention time for the substance under examination to the retention time for sitosterol. Other procedures such as saponification of fat, and hydrogenation (platinum oxide catalyst, ether solution), acetylation, and hydrolysis of triterpenes were carried out in a similar manner as described previously (1).

RESULTS AND DISCUSSION

Isolation of Dammaradienol and 24-Methylenedammarenol from Shea Butter

Unsaponifiable material (156 g) separated from shea butter (3 kg) was acetylated in the same manner as described previously (1). The acetate (150 g) was then chromatographed on argentation silicic acid (430 g) using hexane as eluent at first. The eluate was monitored periodically by GLC to remove the eluate fractions (130.2 g in total, eluted with 5 liters hexane), consisting almost exclusively of the acetates of



SCHEME I. Diagram of the skeleton and side chains of dammarane series of triterpene alcohols.

α-amyrin, lupeol, and butyrospermol. The column was then eluted with benzene (13 liters in total) to give an eluate fraction (17.5 g) which contained considerable amounts of several other triterpene acetates besides the above mentioned three triterpene acetates. This fraction was further fractionated on argentation silicic acid column (packing 178 g) to give a fraction (4.3 g) eluted with benzene: ether (4:1, 1 liter), preceded by a fraction (6.5 g) eluted with benzene (4 liter). The fraction (4.3 g) was then fractionated by preparative argentation TLC to give a fraction (800 mg) which indicated a R_f-value almost identical with that of the authentic specimen of dammaradienyl acetate isolated from dammar resin and was found by GLC to contain the acetates of I and II (Scheme I) in nearly equal proportions. Because the repetition of preparative argentation TLC of this fraction gave a single broad zone instead of two separate zones, the upper and lower portions of the zone were cut off separately and the material from each portion was repeatedly worked up by preparative TLC in a similar manner to give eventually two fractions; the fraction from the upper portion of the zone gave the I acetate (Scheme I) (68 mg, GLC purity 95%) and the other fraction from the lower portion of the zone afforded the II acetate (Scheme I) (80 mg, GLC purity 93%).

Both the hydrolyzed triterpene alcohols I and II (Scheme 1) showed a mobility identical with that of the authentic dammaradienol in TLC using silica gel plate (eluent, hexane:ether 4:1).

Dammaradienol from Shea Butter and Its Tetrahydro Derivative

The I acetate (Scheme I) (RRT 1.35) thus isolated from shea butter crystallized as plates, mp 151-153 C, $[\alpha]_D^{21}+59^\circ$ (c 0.81) (lit [7] dammaradienyl acetate $[\alpha]_D$ $+60^\circ$). Hydrolyzed I (Scheme I) (RRT 1.11) showed mp 136-138 C (fine needles). Mass spectrum of the alcohol (Scheme I) showed M^+ at m/e 426.3836 ($C_{30}H_{50}O$, requires 426.3859, relative intensity 16%) with the other principal ions at m/e 393.3537 (M - [$CH_3 + H_2O$], 393.3519, 2%), 315.2659 (M - [side chain + 2H], 315.2686, 5%), and 297.2570 (M - [side chain + 2H + H_2O], 297.2581, 3%), and base peak at m/e 109.0997. Because the sterols with unsaturated side chain are known to give a characteristic ion resulting from the loss of the entire C-17 substituent together with the rearrangement of two hydrogen atoms (13), this alcohol, giving the ion at m/e 315.2659, is expected to have a C_8-side chain with two double bonds. These double bonds were indicated by the IR spectrum of the I acetate (Scheme I) to exist as trisubstituted double bond (831 and 820 cm^{-1}) and terminal methylene group (3080, 1642 and 885 cm^{-1}). The I acetate (Scheme I) showed methyl signals at 0.88 (strong), 0.99, 1.64, 1.71, and 2.05 (3β-$OCOCH_3$) ppm in the NMR spectrum. The other signals also were observed at 4.51 (3α-H, multiplet [m]), 4.76 (ca. 2H, broad singlet [b], terminal methylene), and 5.14 (1H, triplet [t], J 4.2 Hz) ppm. Because a pair of the signals at 1.64 and 1.71 ppm is characteristic to the 26,27-dimethyl protons of the Δ^{24}-sterols (14), the trisubstituted double bond shown by IR spectrum is attributable to Δ^{24}-bond. The triplet at 5.14 ppm is, hence, assignable to 24-H. In the spectrum of the free alcohol I (Scheme I), the signals at 0.88 and 0.98 ppm, the part of which might be attributable to the angular methyls, and, further, the signals at 1.64 and 1.72 (26,27-dimethyl), 4.74 (b, terminal methylene), and 5.16 (t, J 4.2 Hz, 24-H) ppm, were still observed with scarcely any changes in their chemical shifts from those observed for the I acetate. On the other hand, the signals appeared also at 0.79 (ca. 3H), 2.05 (b, ca. 2H), and 3.24 (3α-H) ppm in the spectrum of the free alcohol I. The triterpene I was found identical with the authentic specimen of dammaradienol isolated from dammar resin described later in its IR, NMR, and mass spectra, RRT in GLC and mp, as well as TLC behavior. The alcohol (Scheme I,I) isolated from shea butter is, therefore, identified as dammaradienol (5α-dammara-20[21],24-dien-3β-ol).

Hydrogenation of the I acetate for 3 hr at room temperature gave the tetrahydro compound, dammaranyl (Scheme I,III) acetate (a mixture of the C-20 epimers), which on recrystallization showed mp 139-140 (plates) (lit [8], a mixture of the C-20 epimers of dammaranyl acetate, mp 139-141 C). The IR spectrum showed no absorptions correlated to any of the terminal methylene and the trisubstituted double bond. Mass spectrum of the alcohol (Scheme I,III) showed M^+ at m/e 430 ($C_{30}H_{54}O$, 30%) with the other ions at m/e

415 (M - CH_3, 10%), 412 (M - H_2O, 9%), 397 (M - [CH_3 + H_2O], 14%), 317 (M - side chain, 73%), and 299 (M - [side chain + H_2O], 98%), and base peak at m/e 95. The presence of the strong ion (M - side chain), which is characteristic for the sterols with a saturated side chain (13), indicates that the side chain of the alcohol (Scheme I,III) is fully saturated. The free alcohol III (Scheme I) showed a predominant peak, related to one of the C-20 epimers, at RRT 0.91 with a shoulder in the front side in GLC. In the NMR spectrum of the III acetate, the signals attributable to the skeletal methyls appeared, with almost identical chemical shifts with those observed for the I acetate, at 0.87 (strong), 0.97, and 2.06 (3β-$OCOCH_3$) ppm. A triplet assignable to 3α-H was observed at 4.50 (*J* 7.2 Hz) ppm. On the other hand, the 26,27-geminal dimethyl protons gave a doublet at 0.89 (*J* 5.4 Hz) ppm, indicating that the triterpene III (Scheme I) has a C-24 saturated side chain (14). The disappearance of the signals related to the C-20 terminal methylene and 24-H shows further that the side chain of the III is fully saturated. The free alcohol III (Scheme I) gave the methyl signals at 0.80, 0.87, 0.89 (d, *J* 6.0 Hz), 0.97, and 1.00 ppm, and a triplet at 3.22 (*J* 7.2 Hz, 3α-H) ppm.

24-Methylenedammarenol from Shea Butter and Its Tetrahydro Derivative

The new triterpene II acetate (Scheme I) (RRT 1.47) isolated from shea butter showed mp 145-147 C (plates), $[\alpha]_D^{21}$+62° (c 0.81). Hydrolysis of the II acetate gave free alcohol II (Scheme I) (RRT 1.21), mp 131-133 C (fine needles). In the mass spectrum, the free alcohol II showed M^+ at m/e 440.4017 ($C_{31}H_{52}O$, 440.4015, 20%), with the other principal ions at m/e 315.2652 (M - [side chain + 2H], 20%) and 297.2577 (M - [side chain + 2H + H_2O], 70%), and base peak at m/e 109.1028. The presence of a relatively strong ion M - (side chain + 2H) at m/e 315.2652, as is the case with the alcohol I, reveals that this alcohol (Scheme I,II) has a saturated ring system and a C_9-side chain with two double bonds. The two double bonds in the side chain were recognized to exist as the terminal methylene groups because the absorptions at 3080, 1640, and 886 cm^{-1} were observed without any of other olefinic bands in the IR spectrum of the alcohol. The chemical shifts of the skeletal methyl signals in the NMR spectra of the triterpene alcohol II (Scheme I) and its acetate were found almost identical with those of the respective derivatives of the triterpene I (Scheme I) (dammaradienol)——the acetate II (Scheme I) indicated those at 0.88 (strong), 0.99, and 2.06 (3β-$OCOCH_3$) ppm; and the free alcohol II gave those at 0.80, 0.88, and 0.99 ppm. This fact shows unequivocally that the ring system structure of the new triterpene II (Scheme I) is identical with that of the triterpene I, i.e., 5α-dammarane type ring system. On the other hand, the triterpene II (Scheme I) gave a signal of the 26,27-geminal dimethyl protons as a doublet at 1.06 (*J* 6.6 Hz) ppm. This is characteristic for the sterols possessing a C-24 terminal methylene group such as 24-methylenecycloartanol (24-methylene-9β,19-cyclo-5α-lanostan-3β-ol) (15). Hence, the new triterpene II is considered to possess a C-24 terminal methylene group. In the NMR spectra of the triterpene alcohol II (Scheme I) and its acetate, a broad singlet related to terminal methylene group was observed also at 4.76 ppm with an intensity (ca. 4H) stronger than that observed for the triterpene I (Scheme I) (dammaradienol) possessing one terminal methylene group at C-20. This indicates that the triterpene II (Scheme I) carries two terminal methylene groups in the side chain consistent with the observations on the IR and mass spectra mentioned above. As indicated above, because one of the terminal methylene groups was shown to be located at C-24, the other must be located at C-20 just as in the case of dammaradienol (Scheme I,I). Accordingly, the structure of the new triterpene alcohol (Scheme I,II) may be determined as 24-methylenedammarenol (24-methylene-5α-dammar-20[21]-en-3β-ol).

Moreover, two broad singlets were observed in the NMR spectra of the triterpene alcohol II and its acetate, and these signals may be attributable to the methylenic protons on C-22 and C-23, both being located at α-positions relative to the olefinic carbons (16).

Hydrogenation of the II acetate in the same way as described above afforded the tetrahydro compound (Scheme I,IV), 24-methyldammaranol (a mixture of the C-20 epimers), which on recrystallization showed mp 151-154 C (plates). The IR spectrum showed no peaks correlating with the terminal methylene group observed in the spectrum of the II. Recrystallization followed by hydrolysis of the acetate gave free alcohol as needles, mp 121-122 C. Mass spectrum of the alcohol (Scheme I,IV) showed M^+ at m/e 444 ($C_{31}H_{56}O$, 21%) with the other principal ions at m/e 429 (M - CH_3, 6%), 426 (M - H_2O, 8%), 411 (M - [CH_3 + H_2O], 5%), 317 (M - side chain), and 299 (M - [side chain + H_2O], 60%), and base peak at m/e 95. The presence of the relatively strong ion at m/e 317 (M - side chain) accompanied with the stronger ion involving loss of water, as is the case with the alcohol III, reveals that the side chain of the

alcohol (Scheme I,IV) is fully saturated. The IV acetate showed the signals related to the skeletal methyls at 0.87 (strong), 0.97, and 2.06 (3β-$OCOCH_3$) ppm, and the free alcohol IV indicated those at 0.79, 0.87, 0.97, and 0.99 ppm, respectively. The chemical shifts of these signals are almost identical with those observed for the triterpene II. On the other hand, a doublet related to the 26,27-geminal dimethyl protons was observed at 0.88 ppm, while the broad singlets appeared at 2.16, 2.18, and 4.76 ppm in the spectrum of the triterpene II were disappeared in the spectra of the IV for both the acetate and free alcohol. These facts suppose that during hydrogenation the side chain of the II was fully saturated and produced the IV (Scheme I).

Gas chromatographic evidence affords further support to the structure IV for the hydrogenated product of the new triterpene II. The free alcohol IV indicated a major peak, one of the C-20 epimers, at RRT 1.19 with a shoulder peak in the front side in GLC as was observed also in the case of dammaranol (Scheme I,III) (major peak RRT 0.91) mentioned above. From these retention data, the separation factor IV/III was calculated as 1.31——the value was found identical with the corresponding separation factor of 24-methyl/24-unsubstituted determined on 5α-lanostane series compounds under the same GLC condition (17). Thus, the triterpene (Scheme I,IV) is regarded as a 24-methyl homologue of dammaranol (Scheme I,III), i.e., 24-methyldammaranol.

Dammaradienol from Dammar Resin

An authentic specimen of dammaradienol used for the identification of the triterpene I was isolated from dammar resin as follows. Dewaxed dammar (70 g) was prepared from coarsely ground resin in a similar manner as described by Mills and Werner (7). It was suspended in hexane:benzene (4:1, 500 ml), poured onto the column of alumina (650 g), and eluted with the same solvent system. The first fraction (22.8 g) eluted with 7 liters of the eluent was left aside. The second fraction (8.4 g) eluted with 6 liters of the eluent was found to consist of nearly equal proportions of dammaradienol (RRT 1.11) and an unidentified keto alcohol (RRT 2.11). A portion (2.2 g) of the second fraction was subjected to preparative TLC (0.5 mm thick silica gel plates; eluent, hexane:ether 4:1). The zone closer to the solvent front gave dammaradienol (0.5 g), mp 136-138 C (fine needles), after recrystallization (lit [7], mp 136-138 c). This on acetylation afforded the acetate (>99% pure by GLC), mp 150-152 C (plates) (lit [7], mp 151-153 C). In the literature (7), Mills and Werner reported that free dammaradienol showed IR absorptions at 3620, 3070, 1640, and 895 cm^{-1} in CS_2. The IR spectrum of dammaradienyl acetate isolated in this study showed the presence of terminal methylene group (3070, 1641, and 898 cm^{-1}) and trisubstituted double bond (828 and 818 cm^{-1}). Mass spectrum of free dammaradienol gave M^+ at m/e 426 ($C_{30}H_{50}O$, 30%), with the other principal ions at m/e 411 (M - CH_3, 3%), 408 (M - H_2O, 2%), 393 (M - [CH_3 + H_2O], 4%), 315 (M - [side chain + 2H], 17%), and 297 (M - [side chain + 2H + H_2O], 11%), and base peak at m/e 109.

In the NMR spectrum, dammaradienyl acetate indicated methyl signals at 0.87 (strong), 0.98, 1.63, 1.70, and 2.05 (3β-$OCOCH_3$) ppm. Taking advantage of the observations by Lehn (18) on dammarane series triterpenes, the strong overlapping singlets at 0.87 ppm are attributed to 10β-, 14α, 4α-, and 4β-methyl protons, and the singlet at 0.98 ppm to 8β-methyl protons. A pair of the two signals at 1.63 and 1.70 ppm is related to the 26,27-dimethyl protons deshielded by Δ^{24}-bond (14), and the broad singlet observed at 4.76 (ca. 2H) ppm is assignable to C-20 terminal methylene protons. Further, the signals appearing at 4.51 (1H, m) and 5.14 (1H, t, J 4.2 Hz) are attributable to 3α-H and 24-H, respectively. Free dammaradienol gave the signals of the angular methyls, 26,27-dimethyl, C-20 terminal methylene, and 24-H, with scarcely any changes in their chemical shifts from those observed for the acetate mentioned above: 8β-methyl (0.99 ppm), 10β-, and 14α-methyls (0.88 ppm), 26,27-dimethyl (1.63 and 1.70 ppm), C-20 terminal methylene (4.74 ppm), and 24-H (5.16 ppm). The 4α- and 4β-methyl signals were, in this case, observed as two separate singlets, one of which was at 0.78 ppm and the other was overlapped with the signal at 0.99 ppm. Taking into consideration the observation of Δ^8-lanostane series compounds by Barton, et al. (19), the signals at 0.78 and 0.99 ppm were attributed to 4β-methyl and 4α-methyl protons, respectively. Moreover, a broad singlet observed at 2.05 (ca. 2H) ppm in the spectrum is presumably related to the C-22 methylenic protons which are located at α-position relative to the olefinic carbon (16). In the spectrum of the acetate mentioned above, the presence of this signal remained indistinct because of the overlapping 3β-acetoxyl signal just at 2.05 ppm.

Consequently, the triterpene I isolated from shea butter is reasonably identified as dammaradienol, and the new triterpene II isolated along with the triterpene I from shea butter

TABLE I

Chemical Shifts of NMR Signals of Dammarane Series Triterpenes

Compound	3-C substituent	Chemical shifts, (ppm)[a]								
		Angular methyls								
		8β-CH_3	10β-CH_3	14α-CH_3	4α-CH_3	4β-CH_3	26,27-CH_3	3β-$OCOCH_3$	3α-H	Others
I	3β-OAc	0.99s[b]	0.88s	0.88s	0.88s	0.88s	1.64s,1.71s	2.05s	4.51m	2.05(22-H_2 ?) 4.76b(21-H_2) 5.14t(24-H) *J* 4.2 Hz
	3β-OH	0.98s	0.88s	0.88s	0.98s	0.79s	1.64s,1.72s		3.24m	2.05b(22-H_2 ?) 4.74b(21-H_2) 5.16t(24-H) *J* 4.2 Hz
III	3β-OAc	0.97s	0.87s	0.87s	0.87s	0.87s	0.89d *J* 5.4 Hz	2.06s	4.50t *J* 7.2 Hz	
	3β-OH	0.97s	0.87s	0.87s	1.00s	0.80s	0.89d *J* 6.0 Hz		3.22t *J* 7.2 Hz	
II	3β-OAc	0.99s	0.88s	0.88s	0.88s	0.88s	1.06d *J* 6.6 Hz	2.06s	4.52m	2.16b,2.18b(22,23-H_2 ?) 4.76b(21-H_2,24-CH_2)
	3β-OH	0.99s	0.88s	0.88s	0.99s	0.80s	1.06d *J* 6.6 Hz		3.23m	2.16b,2.18b(22,23-H_2 ?) 4.76b(21-H_2, 24-CH_2)
IV	3β-OAc	0.97s	0.87s	0.87s	0.87s	0.87s	0.88d *J* 5.4 Hz	2.06s	4.51m	
	3β-OH	0.97s	0.87s	0.87s	0.99s	0.79s	0.88d *J* 6.0 Hz		3.21m	

[a]60 MHz, $CDC1_3$ solution, internal tetramethylsilane = 0 ppm.

[b]s = Singlet, b = broad singlet, d = doublet, t = triplet, and m = multiplet.

must be a 24-methylene homologue of the I, i.e., 24-methylenedammarenol. Table I summarizes the NMR signals of the dammarane series triterpenes measured in this study.

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Fatty Acid Synthesis from 2-^{14}C-Acetate in Rat Testis Mitochondrial and Cytosol Fractions In Vitro

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ABSTRACT

An in vitro system for acetate incorporation into fatty acids by the mitochondrial and the cytosol fractions of rat testis is described. The rate of incorporation of acetate into fatty acids was twice as fast with the mitochondrial as with the cytosol fraction; both systems were stimulated in the presence of adenosine triphosphate, reduced nicotinamide adenine dinucleotide phosphate, coenzyme A, and $MgCl_2$. The optimum pH was between 7.0-7.5 for the mitochondrial fraction and between 6.5-8.0 for the cytosol fraction. Radio gas chromatography showed that palmitic acid was the most highly labeled acid, followed by stearic acid, in the mitochondrial fraction in accord with the pathway of de novo fatty acid synthesis. Some of the labeled acetate was also incorporated into the 16:1 and 18:1 fatty acids of this fraction. Distribution of radioactivity among the mitochondrial lipid classes was highest in the phospholipids and monoglycerides, followed by diglycerides and cholesterol; little radioactivity was present in the triglyceride fraction. These observations are in accord with studies of the incorporation of labeled metabolites into testicular lipids following intratesticular injection and indicate the validity of the in vitro system for studies of specific reactions occurring in vivo.

INTRODUCTION

The enzymatic pathways for the de novo synthesis of fatty acids have been elaborated in extensive studies with subcellular liver preparations (1-3). These and other investigations (4-8) indicated the existence of 2 regions that are responsible for the de novo synthesis of fatty acids within the cell, a mitochondrial one and an extramitochondrial one. During the past several years, in vivo studies have been performed on the metabolism of polyunsaturated fatty acids (9,10) and on the incorporation of linoleic acid into lipid classes of rat testes (11). The importance of these and other results with respect to maturation of the spermatids has been discussed (12,13). Evans, et al. (14) reported the incorporation of radioactive acetate into testicular lipids in vitro using whole tissue. However, this system gave a distribution of radioactivity among the lipid classes that differed considerably from that obtained in vivo upon the intratesticular injection of acetate. The present study was undertaken to develop an in vitro system for acetate incorporation into fatty acids using the mitochondrial and the cytosol fractions of rat testes.

MATERIALS AND METHODS

Adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), Coenzyme A (CoA) glycose-6-phosphate, 6 phosphogluconic acid, cytochrome c, and β-glycerophosphate were purchased from Sigma Chemicals Co. (St. Louis, MO).

Adult male rats of the Sprague-Dawley strain weighing 300-400 g were killed and the testes immediately removed and freed of the tunica albuginea. The soft testis tissue was gently homogenized with a total of 10 volumes of 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.4, using a Potter-Elvehjem homogenizer. After separation of the red cells and debris by centrifugation at 600 x g, the mitochondrial and particle free supernatant fractions were prepared by differential centrifugation (15) with minor modifications as previously described (16).

The presparations were checked for contamination with other subcellular material using a number of marker enzymes (17,18); succinate cytochrome c reductase for mitochondria, acid phosphatase for lysosomes, glucose-6-phosphatase for microsomes, and 6-phosphogluconate dehydrogenase for the cytosol, and also by electron microscopy. Protein was determined according to Lowry, et al., (19).

The incubation mixture for acetate incorporation into fatty acids contained 1 mM sodium 2-^{14}C-acetate (specific acitivity 1 μC/1 μmole), 10 mM $MgCl_2$ 5 mM $MnCl_2$, 20 mM $KHCO_3$, 1-4 mM ATP, 0.2-0.8 mM NADPH 0.5-2.0 mM CoA, 30 mM glutathione, 40 mM phosphate buffer, pH 7.4, and from 0.5 to 2.0 mg of mito-

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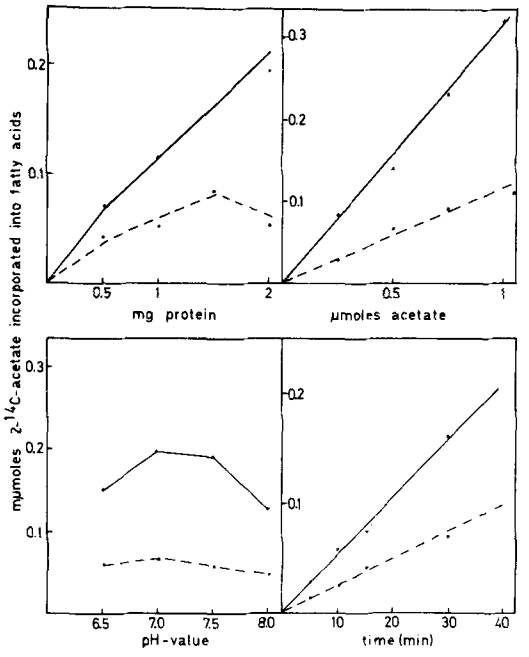

FIG. 1. In vitro incorporation of 2-^{14}C-acetate (0.5 μC/0.5 μmoles) into fatty acids at 30 C as a function of protein and acetate concentration, pH and incubation time; (—) mitochondrial fraction, (- - -) particle free supernatant fraction. Each pair of curves was selected from individual animals as typical of a series of experiments.

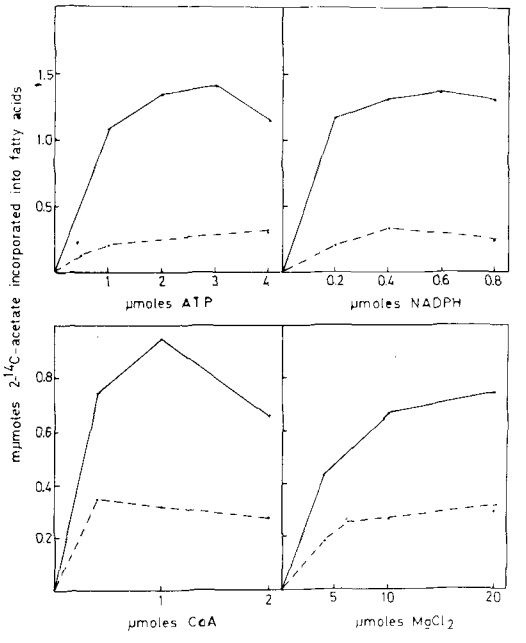

FIG. 2. In vitro incorporation of 2-^{14}C-acetate (1 μC/4 μmoles) into fatty acids using various concentrations of adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), CoA, and $MgCl_2$, (—) mitochondrial fraction, (- - -) particle free supernatant fraction, 2 mg protein each. The samples were incubated for 45 min at 30 c in the presence of the various cofactors. Each pair of curves was selected from individual animals as typical of a series of experiments.

chondrial or supernatant (cytosol) protein in a total volume of 1.0 ml. The incubation was carried out in a Dubnoff metabolic shaker for 45 min at 30 C.

Total lipid was obtained by extraction of the samples with methylene chloride as described by Ziboh and Hsia (20). The fatty acids were obtained by saponifying the samples for 3 hr at 100 C with 2.0 ml of 5 N NaOH. Upon acidification with 1.25 ml of 10 N H_2SO_4, the fatty acids were extracted 3 times with 5.0 ml of petroleum ether. The extracts were washed twice with water, dried over Na_2SO_4 and recovered by evaporation of the solvent. Radioactivity measurements were made with a Packard Tri-carb Model 3002 spectrometer on solutions of the samples dissolved in a toluene scintillator (PPO and POPOP, Packard Instrument Co., Downers Grove, IL).

Thin layer chromatography (TLC) analysis of the neutral and polar lipids was carried out on Silica Gel H plates using the solvent systems petroleum ether:ether:acetic acid (85:15:2) and chloroform:methanol:ammonia (28%):water (70:36:5:4). The lipids were visualized by iodine vapor and by charring with 50% H_2SO_4.

Isolation of the lipid classes was performed using silicic acid column chromatography (21). Fatty acid methyl esters were analyzed on a Varian Aerograph gas chromatograph equipped with a hydrogen flame detector and an 8 ft x 1/8 in. column packed with 10% EGSS-X (Applied Science Laboratories, State College, PA) on Chromosorb W at 200 C. Nitrogen was used as carrier gas. Radio gas chromatography of the fatty acid methyl esters was conducted on an F & M Model 500 apparatus (F & M Scientific Corp., Hillside, IL) equipped with a thermal conductivity detector (150 mA), and a 6 ft x 1/4 in. column packed with Gas Chrom P containing 16% EGSS-X at 200 C. The temperature of the block heater was 250 C, and helium was used as carrier gas (70 ml/min). The radioactivity of collected samples was counted with a Packard Tricarb Model 3002 spectrometer using the toluene scintillation solution of POP and POPO.

RESULTS AND DISCUSSION

The mitochondrial enzyme system of testis was more than twice as active as that of the

TABLE I

Distribution of ^{14}C Radioactivity Between the Lipid Fractions Obtained from in Vitro Experiments

Compounds	Counts/min[b]
Triglycerides	28.3 ± 15.4
Cholesterol	85.0 ± 38.1
Diglycerides	79.7 ± 48.5
Monoglycerides	193.3 ± 129
Phospholipids	506.3 ± 100

[a]Using rat testis mitochondria (6.25 mg) and 16 μmoles ^{14}C-acetate (1 μC/4 μmoles), including the various cofactors after a 45 min incubation time at 30 C.

[b]Mean ± standard deviations obtained from 2 column chromatographic separations of 2 individual experiments.

TABLE II

Distribution of ^{14}C Radioactivity Among Fatty Acids Obtained from in Vitro Experiments Using Rat Testis Mitochondria

Fatty acid	Counts/10 min[a]
16:0	373 ± 137
16:1	130 ± 68
18:0	162 ± 87
18:1	155 ± 69

[a]Mean ± standard deviations obtained from 2 radio gas chromatograms of 2 individual experiments.

cytosol for the incorporation of acetate into fatty acids. Acetate incorporation was not stimulated in mitochondrial free supernatant fractions by the addition of up to 1 mM of citrate to the incubation mixture. Figure 1 shows typical results of the effect of cofactors, protein and substrate concentration, incubation time, and pH on the rate of incorporation of acetate into testicular fatty acid. These results show that the reaction was linear for at least 40 min at a concentration of protein of 2 mg and 1 μmole of acetate in the incubation mixture. The optimum pH was 7.0-7.5 for the mitochondrial fraction, and the cytosol system was less sensitive to pH changes between 6.5-8.0. Both the mitochondrial and, to a lesser extent, the cytosol system were stimulated by the addition of ATP, NADPH, CoA and $MgCl_2$, and illustrated in Figure 2.

Studies with liver preparations (2,22) show that acetyl-CoA, the building blocks for the de novo biosynthesis of fatty acids, is carboxylated to malonyl-CoA which, in the presence of NADPH, gives rise to palmitic and stearic acids. The chief source of acetyl-CoA is within the mitochondria, whereas the chief site of fatty acid synthesis is the particle free cytoplasm with liver (23). The synthesis of fatty acids in the mitochondrial fraction of testes might be explained by high activity of acid phosphatase in the cytosol which could interfere in reactions involving activation by ATP. Because acetyl-CoA itself cannot readily penetrate the mitochondrial membrane (24), acetyl-carriers such as carnitine or the citrate synthetase system may not be present in testis mitochondria in sufficient amounts to exert an effective carrier function for transporting acetate into the cytoplasm. It is also possible that the cytosol contains only a small amount of acetyl-CoA synthetase in view of the relatively low stimulation of this system in the presence of ATP and CoA. The substitution of ^{14}C-acetyl CoA for ^{14}C-acetate could be used to check this possibility, but was not studied in the present work. The bimodal intracellular localization of this enzyme, both in mitochondria and in the cytosol fractions, has been established by Aas and Bremer (25).

The distribution of radioactivity between the various lipid classes for the mitochondrial lipids is presented in Table I. Comparable data could not be obtained for the cytosol fraction because the total radioactivity was too low to be fractionated. Very little of the triglyceride was labeled; cholesterol and diglycerides were labeled to a greater extent with high activity in the monoglyceride fraction. The formation of monoglycerides by the mitochondrial enzyme system of rat liver has been described by several authors (26-28). A large portion of radioactivity was also observed in the phospholipid fraction, i.e., choline-, ethanolamine-, and inositol phosphatides. These results differ from those of Evans, et al., (14) who found that the radioactivity was distributed fairly evenly among the triglycerides, phospholipids, and free fatty acids, which contrasts sharply from their in vivo experiments upon the intratesticular injection of acetate. In previous work from this laboratory (11), it was shown that 1-^{14}C linoleic acid injected intratesticularly was rapidly and preferentially incorporated into phosphatidylcholine; over 44% of the radioactivity was present in this lipid, compared to 5.7% in the triglycerides, 1 hr after administration of the acid. Moreover, upon the intratesticular injection of radioactive trilinolein (glyceryl 1-^{14}C trilinoleate) (29), the incorporation of radioactivity increased to a greater extent in the phosphatidylcholine than in any other lipid class as it decreased from the triglyceride fraction. Hence, it appears that the present in vitro system approximates that which occurs in vivo starting with the de novo syntheses of fatty acids.

Table II shows the distribution of radioactivity among fatty acids of the mitochondrial fractions obtained after lipid hydrolysis and radio gas chromatography of the fatty acid methyl esters. Palmitic acid was labeled to the greatest extent followed by stearic acid as expected by de novo synthesis. Myristic acid also should be labeled by this pathway, but no radioactivity was detected in this fraction. However, it could have been missed because of the low concentration of this fatty acid in testicular lipid and the generally low radioactivity of the other fatty acids.

The radioactivity in the 16:1 and 18:1 fatty acids might arise from chain elongation inasmuch as these systems have been reported in liver (5), brain (30), and heart sarcosomes (31). However, in view of the small amount of endogenous precursors, especially 14:1 in testicular tissue, it is probable that they arise from a small amount of desaturase activity inasmuch as it is very difficult to prepare mitochondria completely free of microsomal material. Whether or not a chain elongation pathway is involved in the synthesis of these fatty acids in testis may be determined from their positional isomer composition, which will have to await upon further studies. Regardless, it appears that the in vitro system described herein should be useful for the investigation of specific reactions in pathways of lipid synthesis in vivo.

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Fat Metabolism in Higher Plants:[1] Metabolism of Medium Chain Fatty Acids

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ABSTRACT

Cell free preparations of avocado mesocarp and spinach leaf tissue rapidly convert lauryl CoA to DL-3-hydroxyl lauric acid as well as 2-, and 3-dodecanoic acids. The conversion does not occur under anaerobic conditions unless a suitable redox carrier such as ferredoxin is present. $H_2{}^{18}O$ is incorporated into the 3-hydroxyl function, but $O_2{}^{18}$ is not. The characteristics of this system are presented and a possible function of this system is proposed.

INTRODUCTION

It was previously reported that a high speed (105,000 x g 60 min) supernatant from avocado mesocarp catalyzed the conversion of medium chain length saturated fatty acids to their corresponding beta hydroxy derivatives (1). Hawke and Stumpf (2), using barley tissue slices, observed that radioactive β-OH derivatives of these acids were incorporated into long chain saturated and unsaturated fatty acids. They concluded, however, that these derivatives were probably first converted to saturated acids and then desaturated aerobically, because O_2 was a requirement for the formation of unsaturated fatty acids with either the β-OH derivative or the corresponding saturated fatty acid. Although there is evidence that α-linolenic acid is a product of sequential desaturation of stearic acid (3,4), it was recently observed that in disrupted chloroplasts, 18:3 was synthesized by elongation of endogenous 16:3 when ^{14}C-acetate was added to the incubation (5). In addition, synthesis of 18:3 was not affected by addition of CN^-, whereas formation of 18:1 and 18:2 was markedly inhibited in the presence of CN^-. No fatty acid synthesis took place in presence of avidin when ^{14}C-acetate served as the substrate, except for 18:3 formation. Kannangara, et al., (6) showed that medium chain length saturated fatty acids served as precursors to α-linolenic acid in in vivo experiments. It was postulated that the 3 double bonds of 18:3 were introduced at the C-12 hydrocarbon chain level and the product, a dodecatrienoic fatty acid, was then elongated to 18 carbons. Hence, an in vitro demonstration of desaturation at the C-12 level is of prime importance. The present paper describes an enzyme system that can account for the introduction of a single double bond at the C-12 level. Whether this is the first of the 3 double bonds introduced at the C-12 level remains for further evaluation.

[1]Number LXIX of a series.

EXPERIMENTAL PROCEDURES

Plant Material

Avocado (*Persea americana*) var. Fuerte) and spinach were purchased from local markets. Avocado mesocarp high speed supernatants were prepared according to Harwood, et al., (1). Spinach chloroplasts were isolated according to Jacobson, et al., (5). Whole homogenates were obtained by grinding young spinach leaves in a Waring Blendor. The cytosol fraction was obtained by centrifuging out the chloroplasts at 3,600 g for 45 sec.

Chemicals

Adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome c, and G-6-P dehydrogenase were from Sigma Chemical Co. (St. Louis, MO). Methyl β-hydroxy and methyl α-hydroxy fatty acids were from Analabs, Inc. (North Haven, CT). [1-^{14}C]Acetate (58 mCi/mmole), [1-^{14}C]octanoic acid (17 mCi/mmole), [1-^{14}C]decanoic (14.3 mCi/mmole), [1-^{14}C]hexadecanoic (55 μCi/mmole), and $NaBT_4$ (130 mCi/mmole) were obtained from Amersham-Searle (Des Plaines, IL). [1-^{14}C]Tetradecanoic (31.2 mCi/mmole) was from Nuclear-Chicago (Des Plaines, IL), [1-^{14}C]dodecanoic (31.2 mCi/mmole) from Amersham-Searle, and [1-^{14}C]dodecanoic (1.62 mCi/mmole) from Mallinckrodt (St. Louis, MO) were pooled and purified on gas liquid chromatography (GLC) yielding a final specific activity of ca. 6 μCi/μmole. [U-^{14}]Hexadecanoic acid (0.05 mCi/0.018 mg) and ^3H_2O (100 mCi/gm) were purchased from New England Nuclear (Boston, MA). $^{18}O_2$ (0-17, 0.609 atom %; 0-18, 93.97 atom %) was from Miles Laboratories (Elkhart, IN) and was a generous gift

from E.E. Conn, University of California, Davis. $H_2{}^{18}O$ (0-17, 0.17 atom %; 0-18, 20.6 atom %) was obtained from Bio-Rad Laboratories (Richmond, CA). Acyl carrier protein (ACP) (*Escherichia coli*) was kindly provided by J.G. Jaworski of this laboratory (7). Purified spinach ferredoxin was a generous gift from R. Buchanan of the University of California, Berkeley. All other chemicals were reagent grade and obtained from commercial sources.

15% HI-EFF 2BP on Gas Chrom Q and 10% EGSS-X on Gas Chrom P were from Applied Sciences Laboratory, (State College, PA). 1% OV-17 on Supelco AWD MCS and n-butylboronic acid were purchased from Supelco (Bellefonte, PA).

Assays and Incubations

The basic assay system for the avocado mesocarp system was as described previously (1). Where [1-^{14}C]acyl CoA substrates were used, all other cofactors were omitted. An assay system employing chloroplast preparations was described by Jacobson, et al., (5). With whole homogenates or cytosol fractions, the NADH and NADPH generating systems were omitted unless otherwise specified. Incorporation of [1-^{14}C]-acetate and of other [^{14}C]-fatty acids into spinach leaf slices was according to Applequist, et al., (8) and Hawke and Stumpf (2), respectively. Anaerobic studies were performed in the Gilson oxygen electrode cell with a continuous flushing of the incubation mixture with N_2. Protein concentrations were determined according to Lowry, et al., (9) and chlorophyll according to Jacobson, et al., (5).

Extraction and Identification of Products

Fatty acid extraction was done according to Bligh and Dyer (10). Methyl esters of fatty acids were prepared by either the BF_3 methanol (11) or by the diazomethane procedure (12). Methyl esters were analyzed on GLC using either 15% HI-EFF-2BP or 10% EGSS-X stainless steel columns, 6 or 10 ft in length with a helium/butane gas flow rate of 60 ml/min. Thin layer chromatography (TLC) was according to Harwood, et al., (1). Separation of fatty acids according to their degree of unsaturation was done according to the argentation TLC procedure of Morris (13). Authentic unlabeled fatty acid methyl ester standards were used along with the radioactive products, both on GLC and TLC.

Unsaturated fatty acids were converted to their corresponding saturated fatty acids by H_2/PtO reduction at 30 lb pressure and room temperature for 1-1.5 hr. Reductive ozonolysis was done according to Stein and Nicolaidis (14). Mass spectrophotometric analysis of ^{18}O samples was kindly performed by C.C. Sweeley of the Department of Biochemistry, Michigan State University, East Lansing, MI. Acetylmandeloxy derivatives of β-OH acids were done according to Hitchcock and Rose (15); their D-phenyl propionate derivatives were kindly performed and analyzed by S. Hammerström of the Department of Chemistry, Karolinska Institutet, Stockholm 60, Sweden.

Determination of the double bond position was as follows: to the ozonized and reductively cleaved radioactive sample was added 0.5-1 ml of 0.1 M $KMnO_4$ in 0.2 M H_2SO_4; the test tube was tightly capped and shaken for 30 min at 0 C. Excess permanganate was destroyed by addition of small amounts of $NaHSO_3$, and the product was extracted 6 times with 1-2 ml of diethyl ether. Methylation was with diazomethane and the product analyzed by GLC on 10 ft HI-EFF-2BP column at 140 C along with authentic dimethyl esters of oxalic, malonic, and succinic acids.

Synthesis

Decanoyl and lauryl CoA were synthesized by a modified procedure of Goldman and Vagelos (16). After the final acidification of the predominantly aqueous solution, the preparation was placed on a semi-solidly packed glass wool column (4 x 0.5 cm). The acyl CoA was eluted using a modified procedure of Galliard and Stumpf (17). The column was washed with several volumes of cold diethyl ether (-10 C); the acyl CoA product was then eluted at room temperature with 10-15 ml of 2-propanol:pyridine:water (1:1:1) solvent. The solvent was removed on a rotary evaporator and the residue resuspended in an appropriate volume of 0.1 M potassium phosphate buffer, pH 59. Estimation of the thioester formed was done spectrophotometrically at 260 mμ, chromatographically according to the method of Huang (18), and chemically by the Barron and Mooney procedure (19). Palmityl CoA was synthesized by the same procedure, except that the aqueous solution was directly lyophilized and the residue was suspended in phosphate buffer as above. Decenoyl CoA was synthesized according to Seubert, et al., (20) and Wieland, et al., (21). Crotonyl CoA was made according to Stern (22).

Radioactivity of these acyl CoA esters was determined by counting aliquots in Bray's solution in the Beckman Scintillation counter.

RESULTS

Nature of Product and Characterization of the System

The radioactive polar product obtained when [1-^{14}C]octanoic, [1-^{14}C]decanoic or

TABLE I

Effect of Pyridine Nucleotides on β-Hydroxylation[a]

Additions		Conversion of control (%)
Control		100
NAD^+	5×10^{-4} M	0
	5×10^{-2} M	0
NADH	5×10^{-4} M	100
	5×10^{-2} M	55.3
NADP	5×10^{-4} M	100
	5×10^{-2} M	42
NADPH	5×10^{-4} M	96.8
	5×10^{-2} M	61.8
NADPH generating system		27.9
FAD[b]	5×10^{-4} M	99.8
	5×10^{-2} M	99.2

[a]Assay conditions given in Methods with additions indicated above. The nicotinamide adenine dinucleotide phosphate (NADPH) generating system consisted of the following: 0.15 units of Torula yeast dehydrogenase (1 unit = amount of enzyme oxidizing 1.0 μmole of G-6-P to 6-P-gluconate per min at pH 7.4, at 25 C in presence of NADP, 4 μmoles G-6-P and 0.5 μmoles of NADP. Substrate used was either 10 μmoles of 1-^{14}C-decanoic or 1-^{14}C-lauric acid. In the control, 4.8 μmoles were formed per mg protein in 60 min.

[b]FAD = flavin adenine dinucleotide.

TABLE II

Effect of Reducing Agents[a]

Reducing agents[b]	Concentration used	Control (decanoyl CoA as substrate) (%)
-GSH	8 mM	100
+GSH	8 mM	100
+Ascorbic acid	8 mM	100
+DTT	8 mM	100
+Cysteine	18 mM	100
+Mercaptoethanol	8 mM	0
+Mercaptoethanol	1 mM	0

[a]Assay conditions as given in Methods with additions indicated above. Incubation without any reductant was taken as control. The rate of conversion in it was ca. 0.045 μmoles/min/mg protein.

[b]GSH = Glutathione; DTT = dithioltreitol.

[1-^{14}C]dodecanoic acid was incubated with 105,000 x g supernatant from the avocado mesocarp was identified as the corresponding β-OH derivative of the particular fatty acid by both chromatographic and chemical methods described previously (1). Conversion of this product along with the appropriate nonradioactive standards to the acetylmandeloxy and to D-phenyl propionate derivatives showed that D and L β-OH isomers were produced in ca. 1:1 molar ratio. The system was active only with the aforementioned fatty acids in the presence of ATP, CoA, and Mg^{++}. The corresponding CoA thioesters were equally active. Addition of ACP to the complete reaction mixture did not result in additional stimulation.

External Reductants and Inhibitors

It was of interest to learn what electron donor/acceptor might be involved in this enzyme system. Tables I and II show that none of the pyridine nucleotides or external reductants seemed to be involved in an electron accepting/donating capacity. In fact, NAD^+ and mercaptoethanol were potent inhibitors. These results would seem to exclude the enzyme as a member of the monooxygenases since most of these require either NADH or NADPH in addition to molecular oxygen. As shown in Table III a number of inhibitors were tested to further characterize the enzyme system. Of the metal-protein inhibitors, only NaN_3 caused inhibition (42%). Iodoacetate exerted 17% inhibition, while pCMB was totally ineffective. On the basis of these results, it is unlikely that either transition metals or SH groups are important for the catalytic function of the enzyme(s) involved. These observations therefore

TABLE III

Effect of Inhibitors on Hydroxylation[a]

Inhibitor	Concentration used	Inhibition (%)
None (control)	--	0
Aminopterin	3×10^{-4} M	0
2,2'-bipyridyl	1×10^{-6} M	5.1
pCMBb	1×10^{-3} M	0
Iodoacetate	1×10^{-3} M	17.6
Menadione	5×10^{-2} M	0
NaCN	1×10^{-1} M	0
NaN_3	15×10^{-3} M	41.6

[a][1-^{14}C] Fatty acyl CoAs were used as substrates. In the control with no inhibitors added ca. 0.028 μmoles of product were formed in 30 min from the original 0.039 μmoles of [1-^{14}C] decanoyl CoA.

[b]pCMB = p-chloro-mercuribenzolate.

seem to exclude this enzyme system from the general class of oxygenases which are responsible for hydroxylation of both aromatic and aliphatic compounds and most often require a reduced pyridine nucleotide, ascorbate, or pteridine derivatives, or metal ion in addition to molecular oxygen.

That the observed β-OH fatty acid products may be related to crotonase activity was ruled out by taking advantage of the observation that crotonase is stable to heat treatment of 55 C for 20-25 min, whereas the present enzyme was not. Whereas heat treated enzyme preparation retained full crotonase activity, it could not synthesize the β-OH fatty acids. In addition, pCMB completely inhibited crotonase, but did not effect the present hydroxylation activity.

Role of Oxygen

To establish whether molecular oxygen is the donor of oxygen atom to the β-OH group, an experiment was conducted in $^{18}O_2$. If molecular oxygen was the donor, one would expect from a mass spectral analysis of the resulting fragments of the product, a high intensity signal at 103, the mol wt of the fragment $\overset{^{18}OH}{\overset{|}{CH}}\text{-}CH_2\text{-}COCH_3$. None would be expected were the experiment conducted in $H_2{}^{18}O$. When using $^{18}O_2$, which contained 93.97 atom % of 0-18 and 0.609 atom % of 0-17, the analysis of the product showed <3% of $^{18}O_2$ was associated with the OH containing fragment. Therefore, a second experiment was run in $H_2{}^{18}O$ (20.6 atom % of 0-18; 0.17 atom % of 0-16), the results of which showed 100% incorporation of ^{18}O into the OH fragment. Hence, the oxygen of the β-OH acyl function is derived from $H_2{}^{18}O$ and not from $^{18}O_2$. Thus, molecular oxygen was not involved as a monooxygenase in the formation of β-hydroxy group. Consequently, the reaction was run under N_2 to determine whether the insertion of the hydroxl function would proceed anaerobically. As seen from Table IV, there was an absolute dependence of the reaction on molecular oxygen. When the reaction was carried out under N_2 but in presence of electron acceptors such as ferredoxin, flavin adenine dinucleotide (FAD), or cytochrome c, partial or almost complete restoration of activity was observed as shown in Table V.

From these studies it appears that molecular oxygen was not directly involved in the hydroxylation reaction. The anaerobic studies would seem to implicate oxygen as terminal electron acceptor with another electron chain

TABLE IV

Formation of β-Hydroxy Fatty Acid as a Function of Oxygen Tension[a]

Gas composition		Conversion of Control (%) Experiment		
% O_2	% N_2	1	2	3
20.5 (air control)	0	100	100	100
0	100	0	0	0.9
100	0	93.6	90	89.2
80	20	73.2	81.8	98.3
55	45	83	100	95
25	75	100	98.1	100
12	88	93.3	100	99
10	90	100	99.4	100
7	93	100	--	--
3-5	92-95	66	97.7	--
2-3	97-98	67.9	80.1	83.3

[a]Experimental conditions as previously described. In the air control, using 1-^{14}C-decanoyl CoA as the substrate, the approximate rate of conversion was 1.20 μmoles/mg protein/30 min at 30 C.

TABLE V

Replacement of Molecular Oxygen by Electron Acceptors in the Formation of β-Hydroxy Fatty Acids[a]

Gas phase	Electron acceptor	Conversion to β-OH (%)
20.5% O_2 (air control)	--	26
100% N	--	0
100% N	15 μg Ferredoxin	12.6
100% N	60 μg Ferredoxin	13.9
100% N	120 μg Ferredoxin	8.8
100% N	2 μg FAD[b]	19.6
100% N	4 μg FAD	18.2
100% N	8 μg FAD	12.1
100% N	20 μg FAD	9.1
100% N	40 μg FAD	0
100% N	4 μg cytochrome c	13
100% N	20 μg cytochrome c	25
100% N	40 μg cytochrome c	15.4
100% N	80 μg cytochrome c	19.1
100% N	120 μg cytochrome c	26.2

[a]When similar reactions with these electron acceptors were performed aerobically, there was no detectable effect on the reaction. In the above experiments, approximate rate of conversion in the air control was 0.78 μmoles/mg protein in 30 min at 30 C.

[b]FAD = flavin adenine dinucleotide.

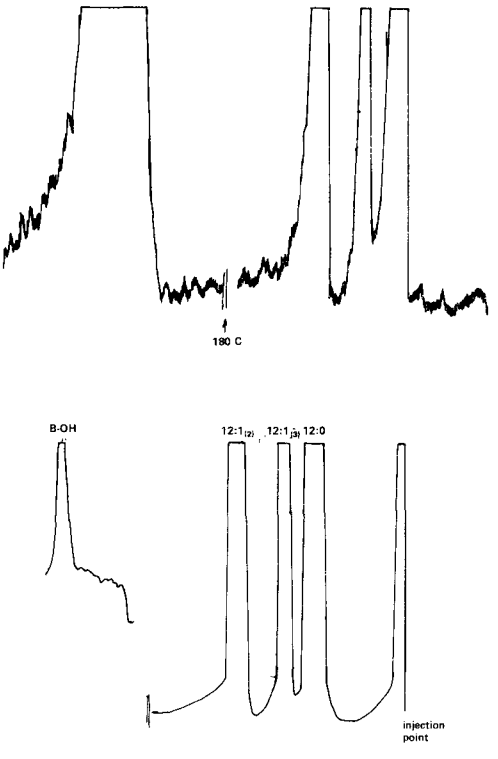

FIG. 1. Typical pattern obtained when the reaction products were analyzed on gas liquid chromatography using 10 ft HI-EFF-2BP columns at 120 C. To obtain the β-OH peak, the temperature had to be increased to 180 C. The β-OH peak is much broader than usual, because of its longer retention on the column due to lower injection temperatures.

system involved between the substrate and molecular oxygen.

Detection of Additional Radioactive Products

With the use of longer GLC columns and lower column temperatures, 2 additional radioactive peaks were observed as shown in Figure 1. The 2 peaks cochromatographed with the monoenoic standards. Reductive ozonolysis and subsequent $KMnO_4$ oxidation and methylation of the fragments resulted in radioactive dimethyl oxalate from 12:1(2) and dimethyl malonate from 12:1(3), as shown in Figure 2. Authentic unlabeled dimethyl oxalate and malonate were used as mass standards.

The synthesis of the 2 monoenoic acids was affected similarly to the synthesis of the β-OH fatty acids by the presence of pyridine nucleotides and inhibitors. An interrelationship between the monoenoic and the hydroxy fatty acids appeared probable. Time course studies were performed in order to observe possible precursor-product relationship. However, this relationship could not be established as rapid equilibrium appeared to be achieved between the monoenoate and the hydroxy acid at all times. A similar observation was made in *E. coli* system where β-OH decanoyl ACP was dehydrated to *cis*-3 and to *trans*-2-decanoyl ACP, and the 3 compounds were always found in equilibrium amounts (23,24). In addition, [3-^{3}H]β-OH lauryl CoA was synthesized and used as the substrate. Since tritium was on the β-carbon, the label could not be lost if β-OH acid was dehydrated to the 2- and 3-monoenoates. When the reaction was performed,

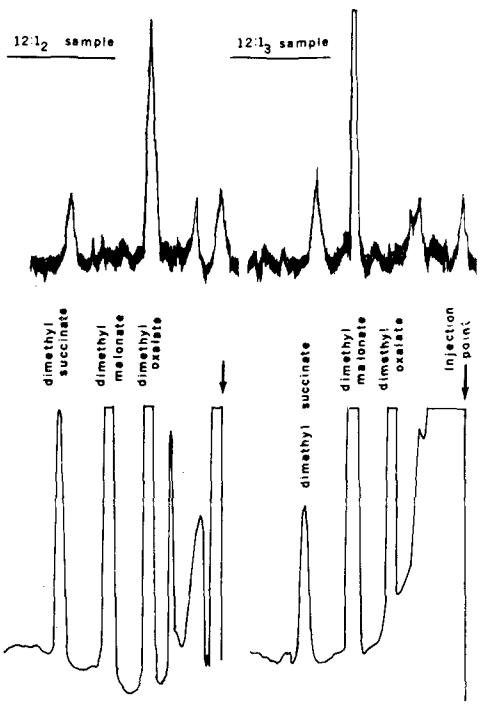

FIG. 2. Gas liquid chromatographic (GLC) analysis of the permanganate oxidized ozonolysis fragments of 12:1 samples. Ozonolysis, permanganate oxidation, and methylation are given in Methods. GLC conditions were as follows: 10 ft EGSS-X column, 120 C, flow rate of 60 ml/min, attenuation of 1 and radioactive scale of 1K. Nonradioactive peaks are authentic dimethyl ester standards of oxalate, malonate, and succinate.

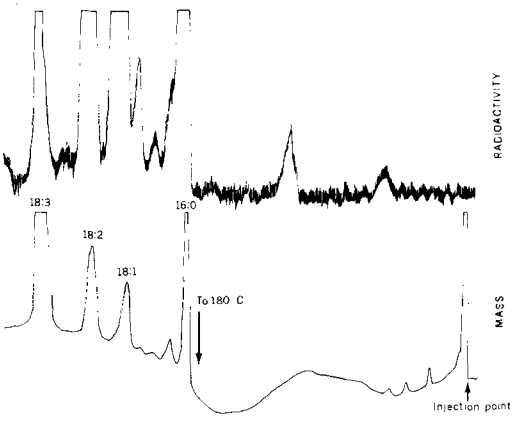

FIG. 3. 1-^{14}C-Acetate incorporation into young spinach leaf slices was assayed according to the method of Applequist, et al., (10). The extracted and methylated fatty acids were analyzed on gas liquid chromatography using a 10 ft EGSS-X column with attenuation 1 and radioactive scale of 1K. The column temperature at the time of injection was 130 C; after 25-30 min, it was raised to 180 C to obtain the longer chain fatty acids.

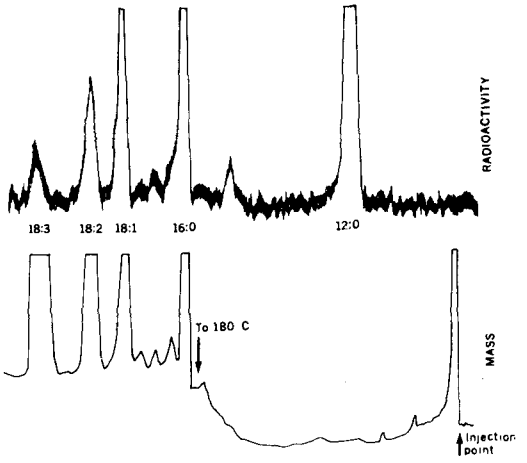

FIG. 4. 1-^{14}C-Laurate incorporation into young spinach leaf slices was determined by the method of Hawke and Stumpf (2). The extracted and methylated fatty acids were analyzed on gas liquid chromatography under conditions identical to those in Figure 3.

both monoenoic acids contained tritium and were produced in amounts equivalent to those obtained when corresponding saturated fatty acid was the substrate.

In summary, the enzyme system described appears rather unusual in that both β-OH and monoenoic acids were produced, yet neither a monooxygenase nor crotonase appear to be involved. Because a rapid equilibrium between the 3 products was achieved, it was not possible to establish whether the hydroxy acid was formed first and was subsequently dehydrated to 2- and 3-monoenoic acids, or whether the desaturation occurred first, followed by hydration.

Spinach System

It was not possible to show in vitro further metabolism of either the hydroxy or monoenoic fatty acids in the avocado system. To gain some insight as to the function of this enzyme system, we turned to spinach leaf tissue which contains large amounts of α-linolenic acid. A desaturase which would convert 18:2 to 18:3 has not been isolated. Work done in our laboratory (5,6) seemed to indicate that 18:3 was synthesized by a pathway different than the one involved in synthesis of other unsaturated fatty acids. In addition, medium chain length saturated fatty acids appeared to be precursors of 18:3 (6). Hence, we proceeded to examine whether the hydroxylase-desaturase system was present in spinach.

Localization of the System for the Formation of Medium Chain Monoenes in Spinach Leaf

Figures 3 and 4 show a typical pattern ob-

TABLE VI

Incorporation of 1-^{14}C-Acetate and 1-^{14}C-Laurate into Slices of Young Spinach Leaves[a]

^{14}C substrate	Incubation temperature (C)	Radioactivity (%)									
		12:0	12:1(2)	12:1(3)	14:0	16:0	16:1	18:0	18:1	18:2	18:3
1-^{14}C-acetate	15	1.1	---	---	6.1	17.8	2.3	4.7	25.7	21.1	11.4
1-^{14}C-laurate	15	36.1	---	---	4.6	21.5	Tr	Tr	17.9	12.2	7.7
1-^{14}C-laurate	30	45.1	---	---	31.2	18.5	---	3.3	2.1	---	---

[a]Experimental conditions same as given under Figures 3 and 4. The approximate amount of 1-^{14}C-acetate was 0.2 μmoles (5 μC) and that of 1-^{14}C-laurate was 0.5 μmoles (1.5-2 μC). Aerobic incubations were performed at designated temperatures for 4 hr with shaking and with 1000 ft candles light intensity.

TABLE VII

Incubation of 1-^{14}C-Laurate with Various Fractions of Young Spinach Leaf Preparations[a]

Preparation	^{14}C substrate	Incubation temperature (C)	Radioactivity (%)										
			8:0	10:0	12:0	12:1(2)	12:1(3)	14:0	16:0	18:0	18:1	18:2	18:3
Whole homogenate	1-^{14}C-laurate	15	--	--	47.2	10.5	6	5.4	3.5	4.1	24	1.1	0.91
		30	--	--	50.2	11	8	Tr	Tr	4.4	34.6	---	--
	1-^{14}C-acetate	15	1.4	3	2.4	--	--	4.1	18.8	27.3	40.1	8.9	3.2
		30	0.9	1.9	4	--	--	6.1	23.8	14.8	42.8	3.7	--
Cytosol fraction	1-^{14}C-laurate	15	--	--	62.8	9.3	5.5	--	3.5	2.4	16.5	--	--
		30	--	--	67.9	13.4	5.9	--	5.9	Tr	6.9	--	--
	1-^{14}C-acetate	15	3.2	5.9	6.7	--	--	4.2	11.2	46.6	22.3	--	--
		30	--	10.4	6.4	--	--	7.9	20.1	36.2	18.9	--	--
Pressates from chloroplasts	1-^{14}C-laurate	15	--	--	99	--	--	--	--	--	--	--	--
		30	--	--	88.6	--	--	--	--	--	--	--	--
	1-^{14}C-acetate	15	--	--	Tr	--	--	Tr	14.1	44.4	41.6	--	--
		30	--	--	--	--	--	--	20	61.8	18.2	--	--

[a]Incubation mixtures contained in μmoles: 2 adenosine triphosphate, 1 dithioltreitol, 0.2 CoA, 2 Mn^{++}, 0.5 1-^{14}C-laurate (2 μC) or 0.2 1-^{14}C-acetate (5 μC). In addition, they contained 4 mg protein and 5-10 μg acyl carrier protein. For whole homogenate the rate of conversion at 15 C of laurate to 12:1(2) and 12:1(3) is ca. 12.5 nmoles and 7 nmoles/mg protein/60 min, respectively.

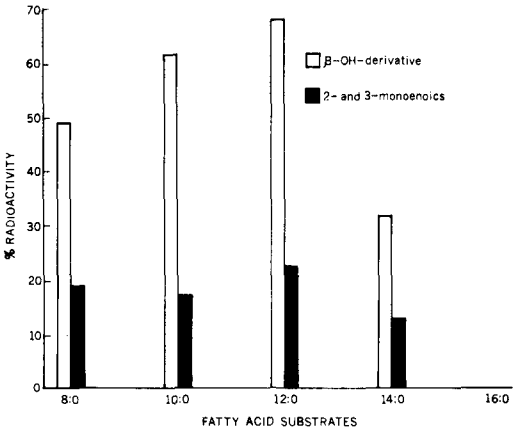

FIG. 5. Substrate specificity with respect to β-OH and 2-, 3-monoenoate formation. The amount of each substrate used as in the range of 0.5-0.8 μmoles and ca. 1.5-2.0 μC.

tained when [1-^{14}C]acetate and [1-^{14}C]laurate, respectively, were used as substrates for young spinach leaf slices. Table VI shows that no β-OH laurate, 12:1(2), or 12:1(3) was formed when either acetate or laurate was the substrate, while 18:3 was produced from both substrates when the incubation was done at 15 C. At 30 C no unsaturation took place.

Because no β-OH or monoenoic acids were formed when tissue slices were used, a disrupted spinach system was tested next. Whole homogenates, cytosolic fractions, and French pressured chloroplasts were prepared and challenged with [1-^{14}C]-laurate. Table VII summarizes the results obtained. 2- and 3-Dodecenoic acids were formed only in whole homogenate and cytosol. In addition, measurable amounts of α-linolenic acid were synthesized only in whole homogenates. The 18:3 synthesized here and in the leaf slices was not a product of a resynthesis of C-2 units resulting from possible degradation of the original 1-^{14}C-12:0 substrate, because on ozonolysis all of the radioactivity was found in the C_{1-9} fragment. Sucrose density gradient centrifugation of the cytosol fraction showed that the activity responsible for 12:1(2) and 12:1(3) formation was found at the top of the gradient. Hence, the system responsible for the synthesis of these monoenes is soluble and not particle associated.

Substrate Specificity

The system displayed highest activity with 12:0 as shown in Figure 5. No activity was observed with fatty acids longer than C-14 or shorter than C-8. It should be pointed out that while no β-OH fatty acid was observed in the initial experiments, it was a consistently ob-

TABLE VIII

Cofactor Requirement of the Cytosol Fraction from Young Spinach Leaves[a]

Substrate	Omission[b]	Total counts	Radioactivity (%)									
			10:0	12:0	12:1(2)	12:1(3)	β-OH-12:0	14:0	16:0	18:0	18:1	18:2	18:3
1-^{14}C-acetate	None	84,700	1.9	18.1	--	--	--	1.2	11	38.5	14.9	8	2.3
1-^{14}C-laurate	None (control)	502,200	--	19	14.5	16.3	46.6	0.9	1.2	1.4	--	--	--
1-^{14}C-laurate	DTT	608,900	--	8.6	15.3	8.1	62.8	1.3	2	1.9	--	--	--
1-^{14}C-laurate	ACP	660,100	--	24.7	15.7	7	50.3	2.3	--	--	--	--	--
1-^{14}C-laurate	CoA	430,400	--	57	4.1	Tr	31.2	--	5.2	2.4	--	--	--
1-^{14}C-laurate	ATP	883,100	--	92.4	--	--	Tr	--	--	--	--	--	--
1-^{14}C-laurate	All	600,000	--	97.8	--	--	--	--	--	--	--	--	--

[a]Supernatants of whole homogenates (cytosol) were used in the experiment. In the control incubation approximate rates of conversion of 12:0 to 12:1(2), 12:1(3), and to β-OH-12:0 are 18:1, 20:3, and 58.2 nmoles/mg protein/60 min, respectively.

[b]DTT = dithioltreitol; ACP = acyl carrier protein; ATP = adenosine triphosphate.

TABLE IX

Effect of Nicotinamide Adenine Dinucleotide (NAD^+), N_2 and CN^- on Whole Homogenates of Young Spinach Leaves[a]

Substrate	Treatment	Total counts	Radioactivity (%) 8:0	10:0	12:0	12:1(2)	12:1(3)	β-OH-12:0	16:0	16:1	18:0	18:1	18:2	18:3
1-^{14}C-acetate	None	24,200	1.9	5.2	4.5	--	--	--	10.6	7	49.7	12.5	9.2	0.9
1-^{14}C-laurate	None (control)	293,900	--	--	29.6	11.4	11.6	35	0.7	Tr	1.5	Tr	10.2	0.3
1-^{14}C-laurate	NAD^+,10^{-4}M	536,900	--	--	68.1	2.5	4.6	21.4	1.7	--	--	--	--	--
	NAD^+,10^{-2}M	584,400	--	--	83.1[b]	--	--	--	Tr	--	Tr	--	--	--
1-^{14}C-laurate	100% N_2	238,100	--	--	82.2[b]	--	--	Tr	3.9	--	0.9	--	--	--
	95-97% N_2, 3-5% O_2	993,400	--	--	59.3	6.1	5.6	20.1	2.1	--	1.1	Tr	1.5	Tr
1-^{14}C-laurate	CN^-,10^{-3}M	615,900	--	--	42.7	10.1	9.6	37.7	Tr	--	Tr	--	--	1.9

[a]In the control reaction, ca. 0.05, 0.05, and 0.175 μmoles of 12:1(2), 12:1(3), and β-OH-12:0 are formed, respectively, from 0.5 μmoles (1.5-2.0 μC) of 1-^{14}C-laurate substrate.

[b]The remaining radioactivity is in other unidentified peaks.

TABLE X

Effect of Pyridine Nucleotides on the Whole Homogenates of Young Spinach Leaves[a]

Substrate	Treatment[b]	Total counts	Radioactivity (%) 10:0	12:0	12:1(2)	12:1(3)	β-OH-12:0	16:0	16:1	18:0	18:1	18:2	18:3
1-^{14}C-acetate	None	532,100	2.9	1.7	--	--	--	18.4	Tr	11.7	36.4	15.3	13.6
1-^{14}C-laurate	None (control)	455,900	--	33	16.7	10.7	31.3	3	--	2.8	2.5	--	2.2
	NADPH generating system	850,300	--	72.4	7.4	14.1	5.8	Tr	--	3.4	--	--	2.7
	NADPH	768,600	--	41.6	11.3	12	38.7	1.8	--	3.2	1.5	--	2.3
	NADH	795,600	--	44.1	5.4	9.1	20	4.3	Tr	4.8	2.6	Tr	9.7
	ascorbate, ferredoxin + DCPIP	704,900	--	26.7	12.8	10.9	43.8	1.8	--	2.1	1.8	--	1.5

[a]In the control 0.08, 0.05, and 0.15 μmoles of 12:1(2), 12:1(3), and β-OH-12:0 are formed, respectively, from 0.5 μmoles (2 μC) of the 1-^{14}C-laurate.

[b]NADPH = Reduced nicotinamide adenine dinucleotide phosphate; NADH = reduced nicotinamide adenine dinucleotide; DCPIP = dichlorophenolindophenol.

served product in most of the subsequent studies. The condition of the particular batch of spinach used may very well have been responsible for this variation. A definite difference in metabolic activity, as measured by the amount of C^{14} acetate incorporation into fatty acids, was observed with different spinach preparations. This activity also varied with the seasons of the year.

All the monoenoic acids here had the single double bond at the 2 or 3 positions. Assuming that the 3,4-double bond system formed first and actually represents the first double bond of the final product, linolenate, then only the 3-dodecanoate would have the double bond in the correct position, i.e., on elongation by 3 C_2 units, the double bond would be in 9,10 position of the C_{18} fatty acid. When [3-^{3}H]β-OH lauryl CoA was used as the substrate, tritium label in the β-carbon was retained in both 12:1(2) and 12:1(3) fatty acids which again were produced in amounts equivalent to those obtained when lauric acid was used as a substrate. It may be a possibility that the first double bond is produced by a unique aerobic sequence, but the succeeding 2 double bonds may be formed by a more conventional aerobic mechanism.

Cofactor and Inhibitor Studies

The spinach system, like the avocado, was CoA specific as seen from Table VIII. Table IX shows the effect of NAD^+, N_2 and CN^- on the whole homogenates. NAD^+ and N_2 completely inhibited the synthesis of both β-OH and dodecanoic acids. The inhibition by N_2 was appreciably alleviated when 3-5% of oxygen was returned to the incubation mixture. Of interest was the observation that CN^- inhibits the synthesis of all unsaturated fatty acids except 18:3. The CN^- effect was previously reported by Kannangara, et al., (6) who used ^{14}C-acetate as the substrate. As seen from Table X, there appeared to be no appreciable effect on either the 12:1 or 18:3 synthesis by any of the pyridine nucleotides used except for NADH. In the case of the latter, ca. 4-fold increase in 18:3 synthesis occurred as compared to the control. There was also an overall decrease in the 12:1(2) and 12:1(3) synthesized. NADH has been shown to be a requirement in other desaturases. Possibly this indicates that NADH may be involved in further desaturations of 12:1 to 12:2 and 12:3 which are then elongated to 18:3. A number of yet unsuccessful attempts were made to accumulate 12:2 and 12:3 in addition to 12:1.

DISCUSSION

These studies indicated the presence of a system in spinach similar to that described in the avocado, in that β-OH, 2- and 3-monoenoates were synthesized from the corresponding medium chain length saturated fatty acids. The experiments using ^{3}H-β-OH-lauryl-S-CoA suggested that the 3 derivatives of lauric acid were formed by a pathway presumably different from beta-oxidation. The preferred substrates were the CoA esters of C-8, C-10, and C-12 fatty acids. Lauric acid was the most highly active substrate, not only with respect to formation of its hydroxy derivative and the 2- and 3-dodecenoates, but also with respect to 18:3 formation. From the in vivo studies of Hawke and Stumpf (2) and of Kannangara, et al., (6) it was observed that β-OH derivatives of even carbon chain fatty acids of C-8 to C-14 could serve as substrates for longer chain polyenoics, and that traces of 12:3 and 14:3 were found in spinach tissue slices. Detailed characterization of the latter 2 polyenoics was, however, difficult because they were not observed consistently and, when observed, were found in very small amounts.

In attempts to increase the level of C-12 polyenoic fatty acids, avidin, fluoride, and arsenite were used in the incubation, with little or no effect. The detection of these intermediates was, of course, greatly desired to confirm the postulated scheme for α-linolenic acid synthesis, which is:

$$12{:}0 \xrightarrow{3\ \text{desaturations}} 12{:}3(3,6,9) \xrightarrow{+C_2} 14{:}3(5,8,11) \xrightarrow{+C_2} 16{:}3(7,10,13) \xrightarrow{+C_2} 18{:}3(9,12,15) \qquad \text{(I)}$$

Major evidence so far favoring this pathway consists of the following observations: (a) in vivo incorporation of medium chain length fatty acids into longer chain polyenoic fatty acids (2); (b) insensitivity of 18:3 synthesis to CN^-, while other desaturations were inhibited (6); (c) avidin did not inhibit 18:3 synthesis, thus excluding its formation via de novo pathway (5); (d) 14:0 could not serve as the precursor for 18:3, probably eliminating the possible alternate pathway of 12:0 → 12:1 → 14:2 → 16:3 → 18:3 (6); and (e) ca. 0.4% of 14:3 and 12% of 16:3 was found in spinach chloroplast lipids (6). The present experiments with tissue slices confirm that 12:0 serves as precursor to 18:3 synthesis. In a cell free system, points a-d above were observed. It was also shown that the present system for the formation of the 2-, 3-dodecenoates, and β-OH fatty acids was not CN^- sensitive. This enzyme system was localized in the cytosol. Whereas whole homogenates were able to convert lauric acid to 18:3, the cytosol fraction alone could not. In

addition, chloroplasts alone were not capable of metabolizing lauric acid. It appeared, therefore, that both the cytosol and the chloroplast fractions were required for the synthesis of 18:3 from the 12:0 precursor. Hence, it seems feasible to postulate that 12:0 fatty acid is desaturated to 12:3 in the cytosol and is then transported into the chloroplast where elongation to 18:3 takes place. The transport may occur through membrane continuities which have been observed between various cytosol organelles and the chloroplast (25).

The system described in the present work would account for the formation of the first double bond. The 3-dodecanoate contains the double bond at the correct position, i.e., this double bond would be equivalent to the 7,8 double bond of 16:3(7,10,13) and 9,10 of 18:3(9,12,15). The results imply a separate C-12 pool for the synthesis of 18:3, and the narrow substrate specificity of the enzyme systems seems pertinent to this implication.

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Viral Stimulation of Choline Phosphotransferase in Spleen Microsomes

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ABSTRACT

Choline phosphotransferase and phosphatidyl ethanolamine methyltransferase enzymatic activities (nmoles phosphatidyl choline/min/mg protein) have been determined in spleen microsomes of Rauscher virus infected BALB/c male mice at 5, 10, 14, and 21 days following inoculation of the virus. There is a significant stimulation of the choline phosphotransferase activity in the virus infected spleens with the peak of activity at about 10 days of viral infection. The specific activity of choline phosphotransferase is 10 times that of the phosphatidyl ethanolamine methyltransferase at 10 days of viral infection. There is a 51-fold increase over controls for the total microsomal choline phosphotransferase at 14 days of viral infection and only an 18-fold increase over controls for the phosphatidyl ethanolamine methyltransferase activity. There is a significant ($P<0.001$) increase over controls in the concentration of total phospholipid-P, phosphatidyl choline-P, and phosphatidyl choline-P fractions as separated by argentation chromatography of microsomes from spleens of mice infected with Friend virus or Rauscher virus for 14 days. The choline phosphotransferase and phosphatidyl ethanolamine methyltransferase specific activities in liver microsomes of 14 day Friend and/or Rauscher virus are unaltered during viral infection.

INTRODUCTION

Phosphatidyl choline biosynthesis in microsomes is known to occur by 2 different major pathways. The Kennedy (1) pathway involves choline phosphotransferase which catalyzes the following reaction: cytidine diphosphocholine + α,β-diglyceride to form phosphatidyl choline + CMP. The Greenberg (2) pathway involves phosphatidyl ethanolamine methyltransferase which catalyzes the following reaction: phosphatidyl ethanolamine + S-adenosyl methionine to form phosphatidyl choline. Phosphatidyl choline is the major phospholipid of membranes of the cell. Phosphatidyl choline represents 48.5% of total lipid-P of microsomes (3), 45% of total lipid-P of mitochondria (3), and 37.4% of the total lipid-P of plasma membrane (4). Increased phospholipid synthesis has been observed with a number of viral infections (5-8). In this report, the enzymatic activity of choline phosphotransferase and phosphatidyl ethanolamine methyltransferase has been determined in spleen microsomes of mice during the development of tumors by Rauscher virus. The concentration or "pool size" of total phospholipid-P, phosphatidyl choline-P, and phosphatidyl choline-P fractions, as separated by argentation chromatography of microsomes from spleens of mice infected with Friend virus and Rauscher virus for 14 days, has been determined. Rauscher virus was discovered in 1958 as a murine leukemia infecting spleen tissue (9) and is a RNA virus (10).

MATERIALS AND METHODS

Sprague-Dawley BALB/c male mice were divided into two groups and fed Purina laboratory chow ad libitum. Group I served as controls. The animals of Group II were infected with Rauscher virus by intraperitoneal injection of 0.2 ml of virus as a 33% cell-free extract of spleen obtained from 14-day old infected animals.

The animals were killed by cervical dislocation at 5, 10, 14, and 21 days following viral inoculation. The spleen was removed, rinsed with cold water, blotted, weighed, pooled, and homogenized with ice cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with Teflon pestle. The microsomal fraction was isolated by differential centrifugation (11). The nuclear and mitochondrial fractions were separated from the homogenate by centrifuging for 10 min at 14,500 g. The supernatant solution was centrifuged at 78,450 g for 45 min to sediment the microsomal pellet. Protein was determined by a modified Biuret method (12).

Choline Phosphotransferase Assay

The reaction catalyzed by the enzyme CDP-choline:1,2-diglyceride choline phosphotransferase (EC 2.7.8.2) was assayed according to the method of Kennedy (13). The materials used were cytidine diphosphate-1,2-^{14}C-choline (ICN Tracerlab Chemical and Isotope Division, Irvine, CA) and Tween-20 (Sigma Chemical Co.,

TABLE I

Total Enzymatic Activity of Choline Phosphotransferase and Phosphatidyl Ethanolamine Methyltransferase of Whole Spleen Microsomes of Mice Infected with Rauscher Virus

Time after injection (days)	Choline phosphotransferase		Phosphatidyl ethanolamine methyltransferase	
	(nmoles phosphatidyl choline/min) x 10	Increase over control (%)	(nmoles phosphatidyl choline/min) x 10	Increase over control (%)
0	0.78 ± 0.18[a]	0	0.34 ± 0.19	0
5	5.23 ± 0.39	571	1.33 ± 0.62	291
10	20.23 ± 4.04	2494	1.85 ± 0.36	444
14	40.40 ± 8.88	5079	6.49 ± 1.00	1808
21	27.87 ± 4.68	3473	13.20 ± 2.08	3782

[a]Numbers preceded by ± are standard deviations.

St. Louis, MO). Diglycerides were prepared from egg lecithin by the method of Gurr, et al., (14) and purified by the chromatography method of Barron and Hannahan (15). Each reaction mixture contained 50 μmoles Tris-HCl (pH 8.0), 2 μmoles 1,2-diglycerides emulsified in 0.1 ml of 1% Tween-20, 10 μmoles $MgCl_2$, 0.5 μmoles CDP-1,2-^{14}C-choline (specific activity, 4 x 10^5 cpm/μmole), and 10 mg microsomal protein. The final volume of the reaction mixture was 1.3 ml. Reaction time was 6 min.

Phosphatidyl Ethanolamine Methyltransferase Assay

The assay of the enzyme phosphatidyl ethanolamine S-adenosyl methionine methyltransferase (EC 2.1.1.c) was done by the method of Rehbinder and Greenberg (16) and used L-distearoyl-α-glycerol phosphoryl-N,N-dimethylethanolamine as substrate. The materials used were ^{14}C-methyl-S-adenosyl methionine (New England Nuclear Corp., Boston, MA), unlabeled S-adenosyl methionine (Calbiochem., Los Angeles, CA), and L-distearoyl-α-glycerylphosphoryl-N,N-dimethylethanolamine (Schwarz-Mann, Orangeburg, NY). Each reaction mixture contained 1 μmoles L-distearoyl-α-glycerylphosphoryl-N,N-dimethylethanolamine emulsified in 1 ml of 0.2 M Tris-HCl (pH 8.6) containing 0.4% deoxycholate, 0.2 μmoles S-adenosyl-L-methionine methyl-^{14}C (specific activity, 2.3 x 10^5 cpm/μmole), and 6 mg of microsomal protein. The final volume of the reaction mixture was 1.7 ml. The reaction time was 10 min.

Total Phospholipid-P, Phosphatidyl Choline-P, and Phosphatidyl Choline-P Fractions

Additional mice were inoculated intraperitoneally with either Rauscher or Friend virus and killed after 14 days. The spleens were removed and microsomes prepared by differential centrifugation (11) and lipids extracted by the method of Folch, et al., (17) and lipid phosphorus determined (18). Phosphatidyl cholines were isolated from the lipid extract by thin layer chromatography (TLC) by the method of Parker and Peterson (19) and lipid phosphorus determined (20). Fractionation of the phosphatidyl choline fractions was carried out by TLC on Silica Gel H impregnated with silver nitrate by the method of Arvidson (21). The phosphatidyl choline fractions were extracted from the gel and the lipid phosphorus determined (20). The details of the methods were reported previously (18).

Assay of the Methylation of Phosphatidyl Ethanolamine to Phosphatidyl Choline

Additional mice were inoculated intraperi-

TABLE II

Spleen Wt in BALB/c Male Mice Infected with Friend Virus or Rauscher Virus

Time of infection (days)	Number of mice	Friend virus (mg)	Number of mice	Rauscher virus (mg)
0	388	82	200	85
3	30	108		
5	97	523	44	243
10	41	1855	43	648
14	52	1679	72	1572
21	30	1777	29	1917

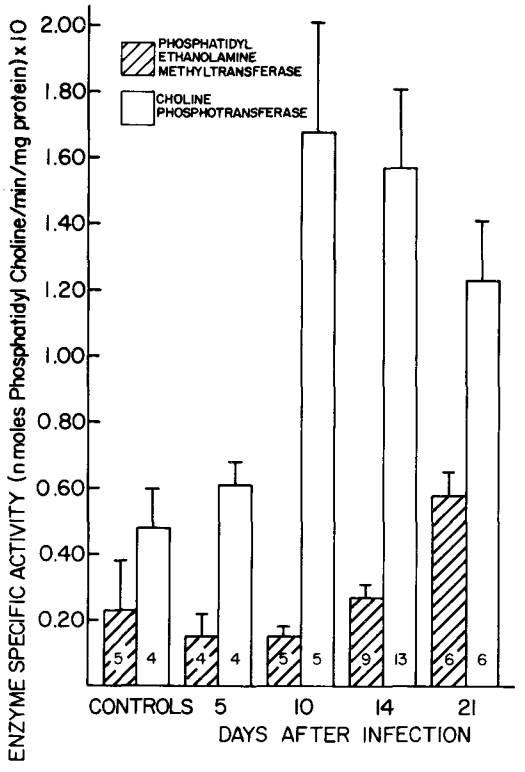

FIG. 1. Specific activities of choline phosphotransferase and of phosphatidyl ethanolamine methyltransferase (nmoles/min/mg protein) x 10 of spleen microsomes from control and Rauscher virus infected BALB/c male mice at various time intervals following inoculation of the virus. The vertical lines intersecting the tops of the bars indicate values for standard deviation of means. The number of animals is indicated in each bar.

toneally with either Rauscher or Friend virus and killed after 14 days. The spleens were removed and microsomes prepared by differential centrifugation. The assay of the methylation of phosphatidyl ethanolamine to phosphatidyl choline was accomplished by a modification of the method of Bremer and Greenberg (22). The reaction mixture contained the following components in a total volume of 1.1 ml: Tris-HCl, pH 8.6, 400 μmoles, S-adenosyl-L-methionine-methyl-^{14}C (specific activity, 2.3 x 10^5 cpm/μmole), 0.3 μmole, 1.2 μmole sodium deoxycholate, and 0.5 ml microsomal protein (5-10 mg). The assay was found to be linear with time from 5 to 60 min and with enzyme concentrations from 2 to 20 mg microsomal protein. The incubation of the complete reaction mixture was stopped after 10 min with 0.2 ml HCl, and lipids were extracted with n-butanol (22).

RESULTS AND DISCUSSION

The enzymatic activity of choline phosphotransferase and phosphatidyl ethanolamine methyltransferase was shown to be linear with time and concentration of enzyme (23). An oncogenic virus, Friend virus stimulates choline phosphotransferase during the production of the tumor (24). Vance and Burke (25) have reported that Semliki Forest virus, a noncancerigenic virus, inhibits choline phosphotransferase during infection. Figure 1 gives the enzymatic activity of choline phosphotransferase and phosphatidyl ethanolamine methyltransferase of microsomes of spleen from control and infected mice with Rauscher virus at 5, 10, 14, and 21 days.

The increase in the specific activity of the choline phosphotransferase over the specific activity of the phosphatidyl ethanolamine methyltransferase at 5, 10, 14, and 21 days of Rauscher viral infection is 4.3, 10.5, 5.8, and 2.1 times greater, respectively. The % increase over the controls of the specific activity of the choline phosphotransferase at 5, 10, 14, and 21 days of viral infection is 20%, 211%, 191%, and 130%, respectively. However, the % increase over control of the specific activities of the phosphatidyl ethanolamine methyltransferase at 5, 10, 14, and 21 days of viral infection is -38%, -33%, +13%, and +138%. It is apparent from the data that there is a greater stimulation of the choline phosphotransferase specific activity than the phosphatidyl ethanolamine

TABLE III

Concentration of Total Phospholipid-P, Phosphatidyl Choline-P, and Phosphatidyl Choline-P Fractions of Microsomes from Spleen of Mice Infected with Friend Virus or Rauscher Virus for 14 Days

Lipid Fraction	Number of determinations: 10 Controls μg/whole tissue	15 Friend virus μg/whole tissue	15 Friend virus Increase over control (%)	21 Rauscher virus μg/whole tissue	21 Rauscher virus Increase over control (%)
Total phospholipid-P	19.1 ± 3.4[a]	258.8 ± 50.8[b]	1255	234.1 ± 58.1[b]	1126
Phosphatidyl choline-P	7.12 ± 1.89	124.8 ± 27.8[b]	1653	94.7 ± 37.1[b]	1230
Phosphatidyl choline-P (% of total P)	46.5 ± 3.8	49.6 ± 4.0	6.7	52.7 ± 7.2	13.3
Phosphatidyl choline-P, fraction 1	0.43 ± 0.19	4.23 ± 1.79[b]	884	3.42 ± 1.68[b]	695
Phosphatidyl choline-P, fraction 2	0.40 ± 0.12	3.94 ± 1.70[b]	885	4.08 ± 1.78[b]	902
Phosphatidyl choline-P, fraction 3	1.36 ± 0.42	14.9 ± 7.6[b]	996	14.9 ± 3.3[b]	910
Phosphatidyl choline-P, fraction 4	3.60 ± 1.3	43.7 ± 11.1[b]	1114	51.8 ± 15.3[b]	1339

[a]Numbers preceded by ± are standard deviations.

[b]The test of significance was applied to difference between mean values for the viral infected and the controls. The probability for chance occurrence of this difference was $P < 0.001$.

methyltransferase, with peak of activity at about 10 days of viral infection. Table I gives the total whole spleen microsmal enzymatic activity of the 2 enzymes involved in phosphatidyl choline biosynthesis. It is apparent from the data that the viral infection greatly stimulates the choline phosphotransferase. There is a 24-fold, 50-fold, and 34-fold increase over control for the choline phosphotransferase at 10, 14, and 21 days of viral infection, respectively. However, there is only a 4-fold, 18-fold, and 37-fold increase over control for the phosphatidyl ethanolamine methyltransferase at the same time intervals of viral infection. Table II shows the progressive increase in size of spleen, from 5 to 14 days after Rauscher virus and/or Friend virus infection. The spleen hypertrophy or increase in wt after viral infection indicates tumor growth. The Friend virus tumor reaches a maximum wt after 10 days of viral infection and represents a 21-fold increase over controls. At 21 days of viral infection, the animals began to die from rupture of the spleen and metastasis of the tumor (24). The average growth of Rauscher virus tumor is somewhat slower, and at 21 days of viral infection a 19-fold increase over controls in wt occurred. The greatest increase over control for the total spleen choline phosphotransferase enzymatic activity is a 51-fold increase after 14 days of Rauscher virus infection (Table I), and for the spleen hypertrophy, a 16-fold increase over controls. At this time of viral infection, the total spleen phosphatidyl ethanolamine methyltransferase enzymatic activity has a 18-fold increase over control. The specific activity of the choline phosphotransferase is 5.8 times the specific activity of the phosphatidyl ethanolamine methyltransferase after 14 days of viral infection (Fig. 1). There is, during tumor growth, a significant viral stimulation of choline phosphotransferase. Table III gives the concentration or "pool size" of total phospholipid-P, phosphatidyl choline-P, and phosphatidyl choline-P fractions as separated by argentation chromatography of microsomes from spleen of mice infected with Rauscher virus or Friend virus for 14 days. Rauscher and Friend virus stimulated significantly ($P<0.001$) an increase over control in the total phospholipid-P, phosphatidyl choline-P, and phosphatidyl choline-P fractions. This viral stimulation in choline phosphotransferase activity is reflected in the increase in "pool size" of phosphatidyl choline-P. Table IV gives the liver microsomal activity of the 2 enzymes involved in phosphatidyl choline biosynthesis following viral infection. It is apparent from the data that the viruses do not stimulate phosphatidyl choline biosynthesis in

TABLE IV

Choline Phosphotransferase and Phosphatidyl Ethanolamine Methyltransferase Specific Activities in Liver Microsomes of 14-Day Rauscher or Friend Virus Infected BALB/c Male Mice

	Number of tissues	Choline phosphotransferase (nmoles/min/mg protein) x 10	Phosphatidyl ethanolamine methyltransferase (nmoles/min/mg protein) x 10
Normal	6	5.43 ± 2.01[a]	9.69 ± 0.53
Rauscher infected	4	5.40 ± 1.92	11.44 ± 1.57
Normal	3	5.06 ± 1.59	9.64 ± 0.45
Friend infected	3	3.10 ± 0.33	11.85 ± 2.31

[a]Numbers preceded by ± are standard deviations.

TABLE V

Assay of the Methylation of Phosphatidyl Ethanolamine to Phosphatidyl Choline (PC) in Spleen Microsomes of Mice Infected with Friend Virus or Rauscher Virus for 14 Days

	Specific activity (nmoles PC/min/mg protein) x 10	Total activity (nmoles PC/min) x 10
Control	0.174 ± .057[a]	20.59 ± 7.00
Friend infected	0.201 ± .038	41.40 ± 10.20
Rauscher infected	0.086 ± .023[b]	14.73 ± 5.13

[a]Numbers preceded by ± are standard deviations.

[b]The test of significance was applied to difference between the mean values for the viral infected and the controls. The probability for chance occurrence of this difference was $P < 0.001$.

the liver. The virus effect of stimulating phosphatidyl choline biosynthesis is apparently specific to that tissue where tumor is being produced.

To further substantiate the low viral stimulation of the phosphatidyl ethanolamine methyltransferase, the assay of the methylation of phosphatidyl ethanolamine to phosphatidyl choline was made. Table V shows that the specific activity and total activity in the spleen microsomes of mice infected with Friend virus or Rauscher virus for 14 days are not stimulated. This stimulation of phospholipid synthesis may only be observed in those viruses that cause tumors.

The existence of 2 pathways of phosphatidyl choline biosynthesis provides a source of different lecithin molecules for the normal function and integrity of the membranes of the cell. Friend and Rauscher viruses which are cancerigenic stimulate this pathway; non-cancerigenic viruses such as Semliki Forest virus inhibits choline phosphotransferase (25). These phospholipid changes in the membrane of the cell that are due to the viral infection may help to alter the characteristics of the cell membrane and thus be a part of the malignant process that is seen in the cells in the production of viral tumors.

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Stearoyl-CoA Desaturase Activity in Mammary Adenocarcinomas Carried by C_3H Mice

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ABSTRACT

Transplantable mammary adenocarcinomas and livers of C_3H mice fed a stock diet or a linoleate rich diet (15% corn oil) contain similar amounts of oleate (ca 3 mg/gm tissue). On feeding either a high carbohydrate, fat free or a high carbohydrate, saturated fat-containing (15% hydrogenated coconut or cottonseed oil) diet for 6 weeks, oleate levels increased 2-fold in tumor and 5-fold in liver. The specific activity of stearoyl-CoA desaturase in liver microsomes was similar to that in the corresponding fractions of mammary glands of lactating mice. In liver, this activity was enhanced 2- to 3-fold by feeding a high carbohydrate, fat free or a high carbohydrate, saturated fat diet. The desaturase activity in mammary tumor microsomes, while only 10% of that in hepatic microsomes, remained unaltered regardless of the type of diet fed. These observations suggest that (a) a major portion of the oleate in the mammary tumor is not produced within the tissue, (b) dietary adaptation is not a general characteristic of stearoyl-CoA desaturase in neoplastic tissues, and (c) enhanced desaturase activity in liver is directly related to the absence of linoleate or oleate, or to a large decrease in oleate in the diet.

INTRODUCTION

The total fatty acid composition of a transplantable mouse mammary adenocarcinoma derived from a naturally occurring preneoplastic hyperplastic alveolar nodule (1) resembles that of liver (2). Furthermore, the proportion of oleate in the total fatty acids of these tissues is greater when taken from mice fed a fat free high carbohydrate diet or a saturated fat-containing high carbohydrate diet than when taken from mice fed a stock diet (2). Many investigations (3-9) have shown that the activity of the enzyme responsible for the production of oleate (stearoyl-CoA desaturase) increases in the livers of animals fed a fat free diet. In the present study, we sought to determine (a) whether the mammary tumor enzyme responds to dietary manipulations and (b) whether the response is related to the type of dietary fat or merely to its absence. It was not our intention to compare the levels of desaturase activity in liver with those in mammary tumors, for all such comparisons between normal and neoplastic tissues are only valid if made between tissue counterparts. Liver and adenocarcinoma were selected as examples of tissues whose responses to long term dietary treatments are different whereas their fatty acid compositions are similar. A preliminary report has already appeared (10).

MATERIALS AND METHODS

The mammary adenocarcinomas used arose from a hyperplastic alveolar nodule outgrowth implanted into a cleared mammary fat pad of a 3-week old C_3H mouse (1). The tumor, once developed, was serially transplanted into several 3-month old isologous females maintained on a stock diet (Wayne Lab Blox, Allied Mills, Chicago, IL).

In the present study, after tumor transplantation, mice were fed either a stock diet, a fat free high carbohydrate diet (11), or diets containing 15% of various types of fats (corn oil, hydrogenated coconut oil, or hydrogenated cottonseed oil). After 6 weeks on the respective diets, the mice were sacrificed by cervical fracture and their livers and tumors carefully excised and trimmed clean. Pieces of each tissue (100 mg liver and 200 mg tumor) were immediately taken for analysis of total fatty acid content and determination of individual fatty acid composition as given below. The remaining tissues were separately homogenized in 0.25 M sucrose at 0-2 C and the microsomal fractions were isolated by the centrifugal procedures described previously (12).

Stearoyl-CoA desaturase activities were measured with either (Assay I) the potassium salt of (1-^{14}C) stearic acid or (Assay II) (1-^{14}C) stearoyl-CoA, as substrate by established methods (8,13). Protein was determined by the method of Lowry, et al., (14) with bovine serum albumin as standard.

To determine the fatty acid content and composition, tissues were heated at 90 C overnight under reflux with 30% KOH in 50%

TABLE I

Oleate Content of Livers and Mammary Tumors from Mice Fed Different Diets[a]

Diet			(% of total fatty acid)		(mg/g wet wt tissue)	
Designation	Fat content (%)	Tissue	Oleate	Eicosatrienoate	Total fatty acid	Total oleate
Stock[b]	4	Liver	16.3 ± 0.5	0	21.7 ± 3.8	3.5 ± 0.1
		Tumor	20.9 ± 0.8	0	12.5 ± 1.3	2.6 ± 0.1
Corn oil[c]	15	Liver	15.9 ± 0.5	0	19.2 ± 2.4	3.1 ± 0.1
		Tumor	20.7± 1.7	0	12.2 ± 1.7	2.5± 0.2
Fat free	0	Liver	47.1± 3.0	4.7 ± 1.9	37.6 ± 4.5	19.5 ± 1.8
		Tumor	36.0 ± 2.9	4.1 ± 1.1	11.2 ± 2.0	4.5 ± 0.4
Hydrogenated[d] coconut oil	15	Liver	41.8 ± 0.7	3.0 ± 0.2	32.5 ± 4.1	14.6 ± 0.3
		Tumor	37.2 ± 1.6	4.7 ± 0.5	11.1 ± 3.4	4.7 ± 0.2
Hydrogenated[e] cottonseed oil	15	Liver	44.1 ± 3.2	4.8 ± 1.6	27.4 ± 1.4	13.4 ± 1.3
		Tumor	36.8 ± 1.6	4.6 ± 0.9	10.4 ± 0.5	4.3 ± 0.3

[a]Mean ± standard deviation of values obtained from separate determinations with 3-5 mice on each diet.

[b]Contained C14:0, 2.3%; C16:0, 17.1%; C16:1, 3.1%; C18:0, 4.5%; C18:1, 24%; C18:2, 44.5%; and C18:3, 4.5%.

[c]Contained C16:0, 7.9%; C18:0, 1.4%; C18:1, 28.6%; C18:2, 59.9%; and C18:3, 2.2%.

[d]Contained C10:0, 5%; C12:0, 47.9%; C14:0, 22.9%; C16:0, 11.1%, C18:0, 11%; and C18:1, 2.1%.

[e]Contained C16:0, 26%; and C18:0, 74%.

TABLE II

Stearoyl-CoA Desaturase Activity of Microsomal Fractions from Livers and Mammary Tumors[a]

Microsomes from:		Oleate produced in:	
Liver	Tumor	Assay I[b]	Assay II[c]
(mg)		(nmoles)	
0.45	0	4.35	1.72
0.90	0	9.42	3.08
1.35	0	12.71	4.63
0	0.5	0.49	0.22
0	1.0	0.91	0.46
0	1.5	1.30	0.58

[a]Reactions were carried out at 37 C in a Dubnoff metabolic shaker for 5 min with air as gas phase. They were stopped by the addition of 2 ml 1 N KOH in methanol. Fatty acids were extracted after saponification and acidification and converted to their methyl esters (8). The methyl oleate was separated using $AgNO_3$ impregnated silica gel thin layer chromatography and quantitated (13).

[b]Incubation medium of assay I contained potassium salt of (1-^{14}C) stearic acid (0.2 mM), reduced Coenzyme A (0.2 mM), adenosine triphosphate (5 mM), reduced glutathione (10 mM), $MgCl_2$ (10 mM), reduced nicotinamide adenine dinucleotide (2 mM), DL-glycerol 3-phosphate (10 mM), potassium phosphate buffer (pH 7.4, 0.1 M), and microsomal protein in indicated amounts, all in a total volume of 1 ml.

[c]The medium of Assay II contained (1-^{14}C) stearoyl-CoA (50 μM), NADH (1 mM); potassium phosphate buffer (pH 7.4, 0.2 M) and microsomal protein in indicated amounts in a total volume of 0.5 ml.

methanol. Potassium pentadecanoate (200 μg) was added as an internal standard. After acidification, the fatty acids were extracted into ether and converted to their methyl esters (15). Separation of methyl esters was carried out isothermally at 180 C by gas liquid chromatography (GLC) in a Varian aerograph model 2740 which had dual flame ionization detectors and dual stainless steel columns (6 ft x 1/8 in.), each packed with 15% diethylene glycol succinate on H/P Chromosorb G. The peak areas corresponding to the various fatty acid methyl esters were calculated by triangulation and the percent distribution of individual fatty acids was determined. Total fatty acid content (mg/g wet wt) was calculated from tissue wts by using the areas corresponding to the total fatty acids and that of the known amount of added pentadecanoic acid.

Stearic and pentadecanoic acids, pure as judged by GLC, were purchased from Applied Science Labs (State College, PA). (1-^{14}C) Stearic acid and (1-^{14}C) stearoyl-CoA were obtained from New England Nuclear Corp. (Boston, MA). Stearoyl-CoA, reduced nicotinamide adenine dinucleotide (NADH), adenosine 5'-triphosphate (ATP), reduced Coenzyme A (CoASH), reduced glutathione (GSH), DL-glycerol 3-phosphate (GP), and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). Fully hydrogenated coconut and cotton seed oils were generous gifts from Pacific Vegetable Oil Corp. (Richmond, CA).

RESULTS AND DISCUSSION

The feeding of fat free or saturated fat diets, rather than stock or corn oil diets, for 6 weeks to mice resulted in an increase in the proportion of oleate in the total fatty acids of livers and mammary adenocarcinomas (Table I). Under these conditions, both tissues contained eicosatrienoic acid which originates from oleate (16,17). Therefore, in all subsequent calculations, we have added the values for oleate and eicosatrienoate to arrive at the total oleate amount in the tissues. Oleate levels in liver increased 4- to 6-fold and in tumor, 2-fold by feeding the mice the fat free or the saturated fat diets (Table I).

To determine whether the increased levels of oleate were accompanied by and presumably due to parallel increases in the stearoyl-CoA desaturase activities in these tissues, the initial experiments were carried out to establish optimal conditions for enzyme assay. Production of oleate from (1-^{14}C) stearate or (1-^{14}C) stearoyl-CoA was shown to be directly proportional to microsomal protein from both liver and mammary tumor (Table II). However, the desaturase activity in the microsomal fraction of liver was about 10-fold higher than that of the corresponding fraction from the tumor. The low enzymatic activity in the tumor microsomes was not due to the presence of endogenous inhibitors because, in experiments with microsomes from liver in the presence of those from tumor, an additive effect on enzyme activity was observed. During a 10 min incubation period, oleate production from each substrate increased linearly with time, using microsomal fractions from either tissue.

Several recent investigations (18-21) have shown the superiority of using acyl-CoA generating systems over exogenous acyl-CoA when studying acylation reactions. Such is also the case with the stearoyl-CoA desaturase system. When a stearoyl-CoA generating system was used rather than stearoyl-CoA, the desaturase was more active. The lower activity with stearoyl-CoA may be due, in part, to the detergent nature of the substrate.

Stearoyl-CoA desaturase specific activities of the microsomal fractions of livers and mammary adenocarcinomas of mice maintained

TABLE III

Specific Activity of Stearoyl-CoA Desaturase in the Microsomal Fractions of Livers and Mammary Tumors of Mice Fed Different Diets[a]

Diet		Oleate produced with microsomes from: (nmoles/min/mg protein)	
Designation[b]	Fat content (%)	Liver	Tumor
Stock	4	1.81 ± 0.43	0.18 ±0.07
Corn oil	15	1.75 ± 0.46	0.17 ±0.04
Fat free	0	4.58 ± 1.10	0.16 ±0.05
Hydrogenated coconut oil	15	4.29 ± 0.77	0.17 ± 0.04
Hydrogenated cottonseed oil	15	4.27 ± 0.80	0.17 ± 0.03

[a]Mice were innoculated with the tumor and were then fed the indicated diets for 6 weeks prior to sacrifice. Stearoyl-CoA desaturase activity was determined using Assay I as described in Table II with two different protein concentrations (0.5 and 1 mg). The values from microsomal preparations obtained from the respective tissues from 4 different mice in each dietary regimen are given as the mean ± standard deviation.

[b]See Table I for fatty acid composition of each diet.

under various dietary conditions were determined with (1-^{14}C) stearic acid as substrate, under conditions where oleate production was linear (Table III). Hepatic desaturase activities of mice which were fed the stock or the corn oil diet were similar and of the same order of magnitude as those observed previously from the mammary glands of lactating mice (12). However, the enzyme activities in the mammary tumors were small, ca. 10% of the values for the hepatic desaturase. Because a 2- to 3-fold increase in enzyme activities was observed with preparations from livers of mice maintained on a fat free or a saturated fat diet, it would appear that this increase is associated with the absence of linoleate or oleate, or with a large decrease in oleate in the diet. Regardless of the dietary conditions employed, the desaturase activities in mammary tumors remained unchanged.

Recent investigations with the Fisher sarcoma (R-3259) (7) and Morris hepatomas (5123c and 7777) (9) have shown that the stearoyl-CoA desaturase activity in the microsomal fractions of these neoplasms increased 2- to 3-fold when host animals were maintained on a fat free diet for 3-4 months. On the other hand, feeding fat free diets for only 1 week did not alter desaturase activities in Morris hepatomas 5123c and 7800 (8). We have shown here that, even though the fat free and the saturated fat diets were fed to mice for 6 weeks, the desaturase activity in a mammary adenocarcinoma was not altered. Thus, the capacity of stearoyl-CoA desaturase to adapt to diet as seen in liver is not a general characteristic of the enzyme and is manifest only in some neoplasms, not in all.

In general, those hepatomas which have low desaturase activities (8,9) also have low fatty acid synthetase activities. However, Morris hepatoma 7800 (8) which has a desaturase activity 2-fold greater than that in liver, shows a fatty acid synthetase level similar to that in liver. It has been suggested (8) that low desaturase activity may be related to an overall reduction of the lipogenic capacity of the tissue. This conclusion can also be extended to mouse mammary adenocarcinomas. This neoplasm, with its low desaturase activity, has only a limited lipogenic capacity (22,23) and contains very low levels of fatty acid synthetase (24) when compared with mammary glands of lactating mice.

While the oleate content of the liver and the mammary tumor are somewhat similar, the specific activity of the desaturase in liver is 10-fold greater than it is in the neoplasm. In both tissues, the oleate content increases at the expense of linoleate; i.e., when the polyunsaturated fatty acid is removed from the diet. However, the hepatic desaturase activity is the only one which responds. We may conclude that the low desaturase activity and the lack of adaptation to diet observed here suggest that a major portion of the oleate in the mammary tumor may not be synthesized within the tissue, but is derived from the diet or from other tissues through the circulation.

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Intestinal Lipid Absorption: Evidence for an Intrinsic Defect of Chylomicron Secretion by Normal Rat Distal Intestine

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ABSTRACT

Intracellular triglyceride accumulation and delayed chylomicron secretion were demonstrated in distal but not in proximal rat intestine following prolonged steady state fat absorption. After 1 and 4 hr of intraduodenal triolein infusion, the mucosal triglyceride content in distal intestinal segments was greatly increased compared with proximal segments. Electron microscopy at each time interval disclosed greater quantities of lipid droplets within the distal cells and these were much larger than the intracellular droplets in the proximal cells, some attaining an enormous size (9000 mμ). When proximal and distal cells were compared at intervals after cessation of a 4 hr triolein infusion, the differences in intracellular lipid accumulation were also evident because, even after 6 hr the distal cells were still filled with large droplets, whereas the proximal cells were almost devoid of fat. These results indicate a basic cellular difference between proximal and distal intestine in the processing of fat and, in contrast to current concepts, suggest that defective chylomicron secretion is not necessarily associated with limited B-apoprotein availability.

INTRODUCTION

The intestinal absorption of dietary fats begins with the intraluminal lipolysis of long chain triglycerides (TG) to fatty acids and 2-monoglycerides by pancreatic lipase. These lipolytic products, dispersed in bile salt mixed micelles, are then taken up by the intestinal mucosal epithelial cells, reconverted to TG, and complexed with free and esterified cholesterol, phospholipids, and specific apoproteins within the endoplasmic reticulum to form lipoproteins (chylomicrons) which are secreted into the lymph.

Despite considerable previous research, the precise factors which are essential to the final assembly and secretion of chylomicrons remain unknown. Evidence derived from human abetalipoproteinemia and animal experiments with protein synthesis inhibitors has inferred an obligatory role for the apoproteins in this process (1,2). In abetalipoproteinemia, the hereditary defect in B-apoprotein biosynthesis results in a marked impairment of chylomicron secretion and an accumulation of TG within the mucosal epithelial cells (3,4). Impaired chylomicron secretion accompanied by intracellular TG accumulation has been produced experimentally in rats treated with protein synthesis inhibitors (5-7). Recently Glickman, et al., have shown that, in the rat, the biosynthesis of one of the major chylomicron apoproteins is inhibited by acetoxycycloheximide, resulting in impaired lipid absorption and the secretion of abnormal chylomicrons (8,9).

Gastric emptying normally controls the pattern and rate of intestinal fat absorption. Because absorption is rapid and efficient, only the proximal intestine usually participates; however, when increasing amounts of emulsified TG are infused directly into rat duodenum, the entire length of the small intestine may be utilized in the absorptive process and a maximal rate of absorption is readily demonstarted (10). In a recent study, a triolein infusion of 1000-1030 μEq fatty acid (FA) per hr resulted in a maximum absorption rate of about 500 μEq FA/hr, which continued for at least 4 hr (11). In these experiments, during prolonged steady maximal triolein absorption, the distal intestine (segments 6-8 of 10 equally divided segments between the pylorus and ileocecal valve) accumulated TG progressively, whereas the concentration of TG in proximal intestine (segments 3-5) remained constant. In the present investigation, ultrastructural and biochemical studies were performed on intestinal tissues after 1 and 4 hr of maximal rate emulsified triolein-1-^{14}C infusion. The results of these experiments indicate that TG accumulation and defective chylomicron release occurs in distal intestine during conditions of prolonged steady state fat absorption.

MATERIALS AND METHODS

A detailed analysis of the utilization of tri-

olein-1-^{14}C-labeled lipid emulsions to investigate intestinal lipid transport under steady state conditions following intraduodenal infusion has recently been reported by Clark, et al., (11).

Preparation of Triolein Emulsions

Triolein oil (K and K Laboratories, Plainfield, IL) washed with ethanol until the free fatty acid (FFA) content was reduced to <2% and the residual ethanol removed under reduced pressure to constant wt, yielding a saponifiable fat content of 3016 μC/100 g oil).

The oil was labeled with triolein-1-^{14}C (Amersham-Searle Corporation, Arlington Heights, IL) by magnetic stirring at room temperature for 2 hr (200 μC/100 g oil). When isotopically homogenous, replicate 10 μl aliquots were weighed and counted and the specific activity of the oil was determined (ca. 1400 dpm per μEq FA).

A solution containing dextrose, 4.15%; pluronic F-68, 0.30% (Wyandotte Chemicals Corp., Wyandotte, MI); and vegetable lecithin, 1.20%, in water was prepared by sonication with a Branson sonifer at 45 C for 2 min. Next, 692 ml of this solution was mixed with 108 ml of the ^{14}C-labeled triolein oil and the mixture sonicated intermittently at 40-45 C until the emulsion which resulted was isotopically homogenous. The triolein-1-^{14}C emulsion was stored at 4 C for several days with occasional brief sonication to ensure homogeneity. When infused at 2.33 ml/hr (1012-1030 μEq FA/hr), maximal absorption rates of about 50% were expected (11).

Animal Methods

Male Wistar strain rats, 240-280 g. were maintained on Purina rat chow until the morning of surgery. Duodenal and gastric cannulas were implanted under anesthesia and the animals allowed to recover for 48 hr in restraint cages. For the first 24 hr, the animals were allowed 2% dextrose in water ad libitum. The dextrose was replaced by water for an additional 24 hr and, on the evening of the second post operative day, each rat was given 0.3 ml of olive oil plus 1.0 ml of 4.15% dextrose solution via the gastric cannula. Earlier experiments using a radioactive triolein tracer established that this amount of oil was 95-100% absorbed in 16 hr after gastric administration. The following morning, emulsified triolein was infused intraduodenally at a constant rate of ca. 1 mEq FA per hr for a total of 1 or 4 hr. At the end of the infusion period, the animals were sacrificed immediately by cervical dislocation. The entire small intestine from duodenum to terminal ileum was washed once in situ with 30 ml ice cooled sodium-taurocholate (2 mM) in isotonic saline, and washed again immediately after removal. The combined washes were collected and stored at -20 C. The small intestine was then divided into 10 equal segments and at least 8 blocks of tissue obtained from each segment for ultrastructural study.

Tissue Methods

Samples of intestinal tissue < 1 mm^3 were fixed for 2 hr in 2% phosphate buffered glutaraldehyde, rinsed in buffer and post fixed in 2% osmium tetroxide for 2 hr. After repeated rinsing in several changes of buffer, the blocks were dehydrated in a graded series of alcohols and embedded in epoxy resins. Ultrathin sections cut with diamond knives on a Porter-Blum MT-2A microtome, mounted on 100-mesh grids stained in 2% uranyl acetate and in lead citrate, were examined in a Zeiss EM-10 electron microscope at 60 kv.

After sampling the intestine for electron microscopy, the segments were transferred immediately to homogenizing tubes containing 2 ml of acid saline (pH <1). All tissues were in acid saline within 8 min of stopping the infusion. Separate measurements established that mucosa wt was a constant fraction of the total intestinal wt in all segments. Therefore, all segments contained equal amounts of mucosal tissue. Next, the tissues were homogenized, extracted with toluene:ethanol (2:1) aliquots of the toluene phases removed for determination of total radioactive lipid, and other aliquots separated into lipid classes by thin layer chromatography (TLC). Intestinal washes were mixed thoroughly and duplicate 2 ml aliquots immediately transferred to centrifuge tubes containing 6 ml acidified toluene:ethanol (final pH <1) for lipid extraction. The contents of stomach, cecum, and colon, if considerable fecal material was present, were washed separately and the washes thoroughly mixed as above.

Thin Layer Chromatography

Lipids extracted into toluene or chloroform were separated on Silica Gel G into origin (phospholipids), monoglycerides, FAs, 1:2 diglycerides, 1:3 diglycerides, and TG using a 2-stage separation procedure (12). The chromatographic fractions were counted without further extraction of labeled lipid from the silica gel.

All samples were counted in Bray's solution in a Beckman LS-250 liquid scintillation system. The data was normalized to unit wet wt of whole intestinal tissue, which is approximately equivalent to surface area, because the

% by wt of mucosa did not vary with distance along the intestine and because it has been shown that, in most regions of rat intestine, surface area is proportional to mucosal wt (13).

In other experiments, the intestinal tissues were obtained and similarly analyzed 3,6,9,12, and 24 hr after cessation of a 4 hr triolein infusion.

RESULTS

After 1 hr of triolein infusion, proximal intestinal segments contained less lipid (44 μEq FA per g wet wt) than distal segments (68 μEq FA per g wet wt), and by electron microscopy it was evident that the distal intestinal villous epithelium contained more, and generally larger, droplets than those found in proximal cells (Figs. 1 and 2). In both regions of intestine, the intracellular lipid droplets were bounded by smooth endoplasmic reticulum membranes, and the appearance of chylomicrons in dilated intercellular spaces suggested active secretion. In proximal segments, the intracellular droplets measured 110-875 mμ (average 350 mμ) compared to a larger size in distal segments, 105-1860 mμ (average 800 mμ).

These differences in intracellular lipid accumulation and in droplet size were greatly accentuated following a 4 hr triolein infusion. Distal segments now contained 204 μEq FA per g wet wt (segment 7) compared with 55 μEq FA per g wet wt in the proximal intestine (segment 3). By electron microscopy, the proximal cells appeared similar at 1 and 4 hr (compare Figures 1 and 3). Distal epithelial cells at 4 hr were engorged with lipid droplets, many of enormous size (compare Figures 3 and 4). In many distal cells (Fig. 4), the droplets measured 180-3570 mμ (average 1665 mμ); in some cells, droplets measuring almost 9000 mμ with an average diameter of 2700 mμ (Fig. 5) were found. In many of these engorged cells, the Golgi could not be readily identified because they were distended with large lipid droplets which altered their characteristic ultrastructural appearance. Although the ultrastructural observations indicated an impairment in lipid transport, the distal epithelial cells were still capable of active chylomicron secretion because the adjacent intracellular spaces and lymphatics were filled with chylomicrons. It is of considerable interest that proximal and distal lymphatic chylomicrons (Figs. 6 and 7) were relatively similar in size (50-550 mμ), in marked contrast to the striking differences in the size of the respective intracellular lipid droplets.

Proximal and distal segments were also studied at intervals between 3 and 24 hr after cessation of a 4 hr triolein infusion. A progressive decrease in the quantity of lipid in proximal and distal segments was observed which reflected the gradual secretion of intracellular TG into lymphatic channels. Three hr following cessation of the infusion, distal cells (Fig. 9) still contained many more and much larger lipid droplets than cells in the proximal segments (Fig. 8). By 6 hr, the proximal segments were almost devoid of intracellular deoplets (Fig. 10), whereas many distal cells still contained large droplets, some measuring 8000 mμ (Fig.

FIG. 1. Electron micrograph of rat proximal intestine 1 hr after intraduodenal triolein infusion. The epithelial cells are filled with 107-875 mμ membrane-bound lipid droplets and the Golgi (arrow) are distended with lipid. (x 6572)

FIG. 2. Distal intestinal segment 1 hr after intraduodenal triolein infusion. In comparison with proximal segments (Fig. 1), the intracellular lipid droplets are larger in size measuring 106-1860 mμ. (x 3360)

FIG. 3. Proximal intestine 4 hr after intraduodenal triolein infusion. The cells still contain many small membrane-bound lipid droplets. The appearance is similar to that of proximal segments (Fig. 1) at 1 hr (x 2800)

FIG. 4. Distal intestine 4 hr after triolein infusion. The distal cells are engorged with lipid droplets many of which are very large, measuring as much as 3570 mμ compared with a maximum of 1640 mμ in the proximal intestine (Fig. 3) at 4 hr. (x 2800)

FIG. 5. Distal intestine 4 hr after triolein infusion. This electron micrograph illustrates the extremely large size (9000 mμ) which some droplets attain, in the distal intestinal segments. (x 2660)

FIG. 6. Proximal intestinal lymphatic 4 hr after intraduodenal triolein infusion. The chylomicrons measure 50-550 mμ. (x 12,000)

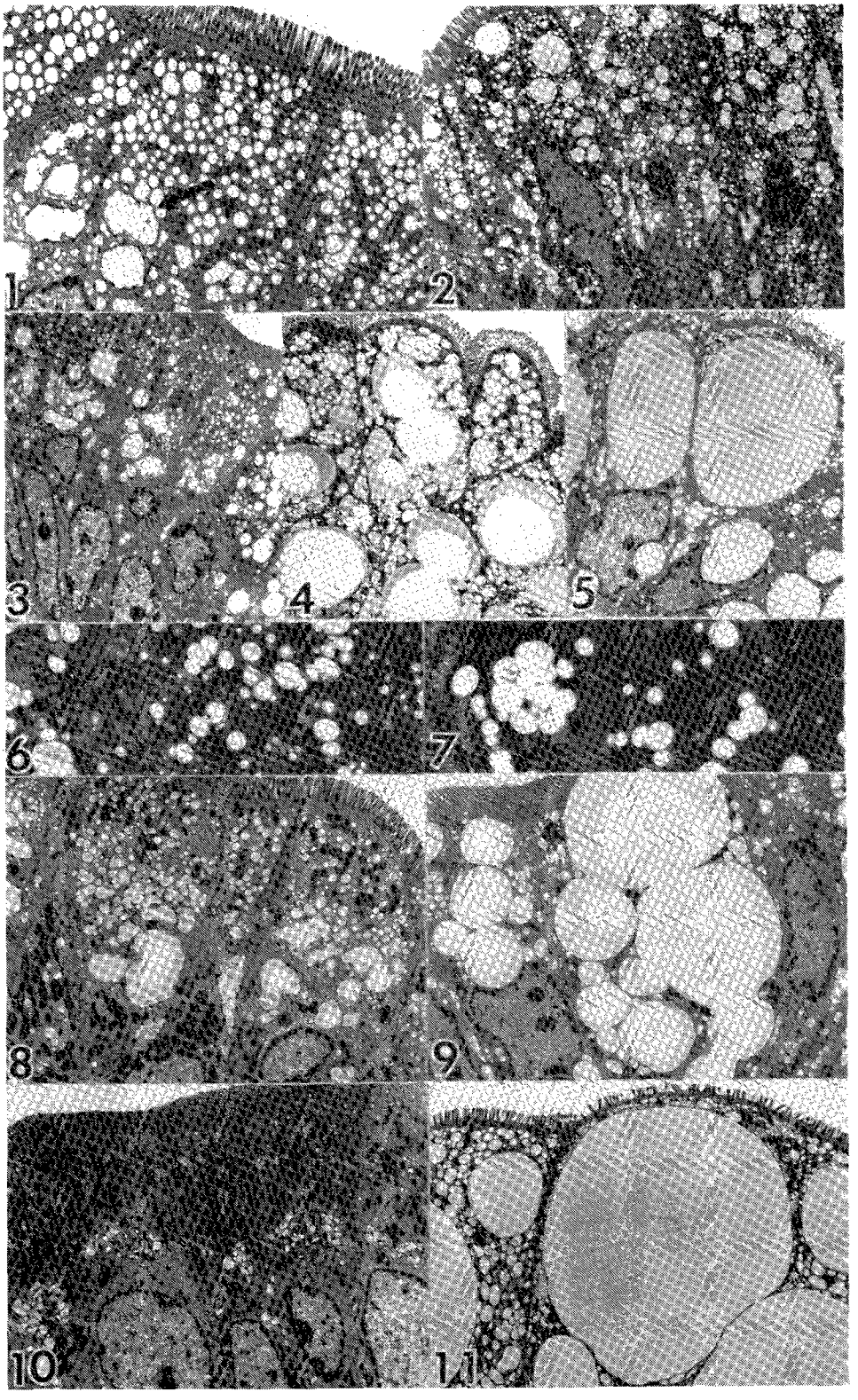
FIG. 7. Distal intestinal lymphatic 4 hr after fat infusion. In contrast to the enormous size of the intracellular lipid droplets (Figs. 4,5), the chylomicrons within the lymphatic are of normal size measuring 50-500 mμ. (x 12,000)

FIG. 8. Proximal intestine 3 hr after cessation of a 4 hr triolein infusion. The cells still contain many lipid droplets measuring 107-880 mμ. (x 3920)

FIG. 9. Distal intestine 3 hr after cessation of a 4 hr triolein infusion. In contrast to proximal intestine (Fig. 8), the cells are still filled with large quantities of retained lipid. Some of the droplets measure 8500 mμ. (x 3920)

FIG. 10. Proximal intestine 6 hr after cessation of a 4 hr triolein infusion. The proximal cells have now secreted almost all of the lipid which had been previously absorbed. The few droplets remaining measure 71-500 mμ. (x 3696)

FIG. 11. Distal intestine 6 hr after cessation of a 4 hr triolein infusion. Even at this time many cells are filled with lipid droplets which have not yet been secreted. (x 3584)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11). Nine hr after cessation of the triolein infusion, mobilization of retained lipid was still apparent by electron microscopy of distal cells; however, by 12 hr the distal cells were almost completely devoid of lipid droplets, although chylomicrons were still observed in distal lymphatics.

To exclude the possibility that prolonged uptake of luminal fat by distal mucosa might have contributed to the delayed clearance and also to measure lymph chylomicron output directly in proximal and distal intestine, proximal and distal intestinal segments were perfused directly in different animals prepared with abdominal thoracic duct fistulas (14). In these studies, differences in intraluminal fat digestion were avoided by diversion of bile and pancreatic juice in all animals with infusion of donor bile and pancreatic juice directly into the test segments. Emulsified triolein-1-^{14}C, 140 μEq FA per hr submaximal rate, was perfused through cannulas placed either in the proximal duodenum or into the midportion of the small intestine. The output of lipid-^{14}C in lymph from proximal intestine reached a steady state within 2 hr and, after cessation of infusion, the t-1/2 of disappearance of lipid-^{14}C from lymph was calculated to be 0.84 hr. A defect in the exit of absorbed triolein from distal intestine was directly demonstrated because a steady state was not achieved until 5 hr and the t-1/2 of lipid-^{14}C disappearance in lymph in this area was prolonged to 1.15 hr (14). The mucosal uptake was complete in both segments of intestine at the end of the perfusion; therefore, the difference in decay half time was due only to differences in output from proximal and distal mucosal cells.

The ultrastructural appearance of segments 3 and 6 of these animals showed changes similar to those found in rats perfused at maximal rates. Lipid particles of diameters reaching 8500 mμ were present in distal segments, whereas the proximal droplets were similar in size to those found after maximal intraduodenal perfusion. Distal intestinal segments also contained more TG at the end of the 6 hr perfusion (100 vs 31 μEq FA per g wet wt). There was no evidence of defective mucosal function in these experiments because proximal and distal segments had identical lymphatic chylomicron appearance rates when measured on the second day of the study following repeated triolein infusion. Additional evidence against functional damage was the observation that mesenteric lymph flow, a sensitive indicator of mucosal damage, was not reduced during distal perfusion.

Chylomicrons harvested from thoracic duct lymph after proximal and distal intestinal perfusion also differed in composition. During steady lymphatic lipid-^{14}C output, the TG to phospholipid molar ratio was 11.4 ± 0.4 (SEM) in chylomicron from animals receiving proximal perfusion, compared with 14.0 ± 0.8 (SEM) in animals with distal perfusion ($p<0.025$). Furthermore, chylomicron size, determined by electron microscopy, showed a significant shift towards larger particles in lymph from distal intestine compared with those from proximal intestine.

This observation was unexpected because chylomicrons in proximal and distal lymphatics were similar in size following intraduodenal triolein perfusion. There is as yet no adequate explanation for the larger chylomicrons in the distal lymphatics; however, ultrastructural studies currently in progress may serve to clarify the reasons for the size differences.

DISCUSSION

This study thus demonstrates that chylomicron production in distal intestine is limited, resulting in massive intracellular TG accumulation during both maximal and submaximal fat abosorption. The prevailing view of the mechanism for defective intestinal chylomicron secretion accompanied by mucosal TG accumulation attributes the defect to changes in B-apoprotein availability. Our current experiments, however, demonstrate that similar changes occur in normal animals without inhibited intestinal protein synthesis. Indeed, preliminary studies performed in collaboration with P. Roheim, Albert Einstein College of Medicine (New York, NY), indicate that B-apoprotein concentrations in chylomicrons synthesized by distal intestinal mucosa were at least as great as chylomicrons formed by proximal intestine following direct triolein perfusion. One interpretation of these results is that B-apoproteins are available in proximal and distal segments for chylomicron formation and that the regional difference in chylomicron secretion is related to other factors. It is possible, of course, that the turnover of the B-peptides is slower in the distal segments. This possibility could be tested by specific isotopic studies to determine B-apoprotein synthesis and secretion rates in mucosal preparations from different segments. However, further support for the conclusion that B-apoprotein synthesis may not be the sole factor determining chylomicron secretion rates comes from studies in essential FA deficiency rats (15) and in bile fistula rats fed a lecithin free micellar solution of monolein and fatty acid (16). In recent studies, Clark, et

al., (15) demonstrated that rats made deficient in essential FA, when fed a fat free diet supplemented with 4% triolein, had a decreased capacity for chylomicron synthesis or release, although uptake of FA from the intestinal lumen was normal. Because there was no defect in intraluminal fat digestion and the mucosal lipids were present as TG, it appeared plausible to attribute the chylomicron secretory defect to alterations in the membrane phospholipid composition or on phospholipid availability induced by the essential FA deficiency. This could result in compositional alterations in the chylomicrons or in defective Golgi function, in either case leading to imparied chylomicron secretion in the absence of direct effects on B-apoprotein biosynthesis. In another recent study, O'Doherty and coworkers (16) provided additional evidence that phospholipid availability is important for chylomicron synthesis and release. They demonstrated an impairment of fat release from the intestinal mucosa in rats deprived of dietary or biliary lecithin or choline. Because the phospholipid deficit also resulted in an overall depression of mucosal protein synthesis, an effect on B-apoprotein synthesis was not excluded. However, lecithin comprises 70-80% of rat chylomicron phospholipids, most of which is derived from dietary or biliary sources. Thus, decreased lecithin availability might produce compositional alterations in chylomicrons and in intracellular membranes, thereby, influencing chylomicron release by mechanisms still to be determined. Striking mucosal accumulation of absorbed fat have been demonstrated in the jejunum of cottontop marmosets fed a high fat, high cholesterol diet (17) and in inositol deficient gerbils fed diets rich in TG containing large amounts of saturated FA (18).

In each of these studies, intracellular TG accumulation similar to that observed in the distal intestine in the present studies was observed. In all of the models discussed, effects on intestinal B-apoprotein synthesis were not specifically excluded; however, the importance of phospholipids in membrane function and in chylomicron composition suggest that secretory defects could be just as easily explained by phospholipid deficiency. In this regard, the work of Friedman and Cardell is noteworthy (19). They confirmed the previous observations of Sabesin and Isselbacher (5) regarding the inhibition of lipid absorption by puromycin; however, they related the secretory defect to a puromycin induced deficiency of endoplasmic reticulum and Golgi membranes. In Friedman's and Cardell's study (19) a lack of endoplasmic reticulum and Golgi membranes was correlated with impaired chylomicron release and thus intracellular lipid accumulation. This might be explained by a puromycin induced inhibition of phospholipid synthesis (20).

The observations in the present study imply a basic cellular difference between proximal and distal rat intestine in the processing of fat. Differences in mucosal esterification are not responsible because accumulated fat in mucosa is always TG. Furthermore, no restriction in the transfer of chylomicrons into the intercellular spaces on lymphatics could be detected by electron microscopy. Regional differences in Golgi function between upper and lower intestine could be responsible for the observed differences in chylomicron secretion and the resulting distal intracellular TG accumulation. This may involve regional differences in Golgi membrane biosynthesis or altered incorporation of phospholipid, cholesterol, or glycoprotein into chylomicrons. Our results strongly suggest that B-apoprotein availability is not responsible for the regional differences, but additional studies will have to be performed to substantiate this concept. It is more intriguing, perhaps, to speculate on regional differences in membrane availability or Golgi function to account for the observations, as recent studies have emphasized the importance of the Golgi in the final processing of chylomicrons, with particular emphasis on the role of the Golgi in glycoprotein synthesis (21).

In normal animals, including man, the distal intestine does not participate in fat absorption. However, after proximal intestinal resection or bypass and in biliary obstruction, ileal fat absorption does occur in man, and chylomicrons of unusual composition and size may result. Because chylomicrons of differing size and composition have altered systemic metabolism, their metabolic fate may also be altered in these clinical situations.

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Conversion of Linoleic Acid Hydroperoxide by Soybean Lipoxygenase in the Presence of Guaiacol: Identification of the Reaction Products[1]

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ABSTRACT

Linoleic acid hydroperoxide formed by soybean lipoxygenase was metabolized by the same enzyme in the presence of guaiacol. The products of this reaction included trihydroxyoctadecenoic acids, hydroperoxydihydroxyoctadecenoic acids, hydroxyepoxyoctadecenoic acids, dihydroxyoctadecenoic acids, hydroxyoctadecadienoic acids, and oxooctadecadienoic acids.

INTRODUCTION

Lipoxygenase (E.C. 1.13.11.12.) is an enzyme which has been found in many plant sources. It is present in high concentrations, especially in cereals and legumes (1,2). The enzyme is known to catalyze the oxidation of essential unsaturated fatty acids with oxygen from air forming hydroperoxides with *trans-cis* conjugated double bonds. In 1966, Zimmerman (3) found an isomerase in linseed which converted linoleic acid hydroperoxide (LOOH) to an unsaturated α-ketol. Similar enzymes were later detected in barley, wheat, soybeans, corn, and mung beans by Zimmerman and Vick (4). Gardner (5) investigated this reaction in corn, and Graveland (6-8) reported the formation of an abundance of compounds arising after enzymatic LOOH formation during the incubation of linoleic acid with flour suspensions from wheat, rye, barley, oats, and corn. The enzymes participating in the courses of these reactions are unknown, but in wheat, Graveland (9) found that when lipoxygenase was adsorbed on glutenin, the formation of trihydroxy derivatives was favored. Heimann, et al., (10-12) described a lipoperoxydase in oats, which reduced LOOH to the corresponding hydroxyoctadecadienoic acids.

As early as 1943, Balls, et al., (13) reported that crude preparations of soybean lipoxygenase destroyed the previously formed LOOH slowly. It was shown later (14,15) that purified enzyme preparations destroyed LOOH only in the presence of a cofactor, guaiacol, and that it was impossible to separate lipoxygenase activity from the LOOH destroying factor.

The present report describes the formation and structures of compounds formed from LOOH in the presence of soybean lipoxygenase and guaiacol.

MATERIALS AND METHODS

Enzyme and Substrate

Soybean lipoxygenase was prepared from soybeans as reported earlier (15). We used enzyme L-1 according to the nomenclature of Christopher, et al., (16). The substrate was either [1-^{14}C] linoleic acid (Amersham Buchler, Braunschweig, West Germany) diluted with unlabeled material (Roth, Karlsruhe, West Germany) to 30 μCi/mol or [1-^{14}C] linoleic acid hydroperoxide. These substrates did not show any detectable chemical or radiochemical impurities in our chromatographic systems.

LOOH was prepared by incubation of 0.4 g linoleic acid with soybean lipoxygenase (2 portions of 2 mg, the second portion being added 15 min later) at 3 C in sodium borate buffer (0.05 M, pH 8.5). Oxygen was bubbled through the solution during the time of incubation. After 30-40 min, the mixture was acidified with 2N HCl to pH 2-3 and extracted twice with diethyl ether. The ether layer was washed with water until neutral and dried over anhydrous sodium sulfate. The solvent was evaporated in vacuo, and the residue subjected to preparative thin layer chromatography (TLC) using diethyl ether:heptane:acetic acid (50:50:1, v/v/v) as developing solvent mixture. After detection under ultraviolet (UV) light, the band containing the LOOH was scraped off, eluted with diethyl ether, and finally dissolved in ethanol. The concentration of the LOOH was determined photometrically at 234 nm using 25,000 $mol^{-1} cm^{-1}$ as the extinction coefficient.

Conversion of LOOH and Isolation of Products

For preparative isolation of the degradation products, 4 x 10^{-5} moles LOOH or linoleic acid were incubated with 2 mg lipoxygenase in 2

[1]These results were presented in part at the 12th International Society for Fat Research Congress, Milan, September, 1974.

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liters sodium borate buffer (0.05 M, pH 8.5) containing guaiacol (20 mM). To follow the reaction, aliquots of 3 ml were removed and the change in absorbance at 234 nm was recorded.

When no further change in absorbance occurred, another portion of LOOH was added. When the reaction was complete, an additional 1-2 mg lipoxygenase was added and the process was repeated 3-4 times. The total process took ca. 4-5 hr. The reaction mixture was subsequently acidified to pH 2-3 with 2N HCl and extracted twice with diethyl ether. The ether phases were washed with water until neutral, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was treated with diazomethane and subjected to TLC using double development with acetone:heptane (1:2, v/v).

Chromatographic Methods

TLC was carried out on glass plates (40 x 20 cm or 20 x 20 cm) coated with 0.5 or 0.25 mm Silica Gel HF_{254} (Merck, Darmstadt, West Germany). Methyl esters of the reaction products were separated by double development with acetone:heptane (1:2, v/v) as solvent as shown in Figure 1a. For further purification of single fractions of methyl esters, solvent mixtures of diethyl ether and heptane were used.

Spots and bands were located by spraying with 50% H_2SO_4 and heating to 120 C for 10 min. Carbonyl compounds were seen as yellow spots after spraying with 0.05% 2,4-dinitrophenylhydrazine (DNPH) in 30% $HClO_4$. Peroxides were detected as violet spots by spraying a KI solution followed by 1% starch solution. The KI solution had been freshly prepared from 40 mg KI in 10 ml water mixed with 5 ml acetic acid and a small amount of Zn powder. This solution was filtered immediately before use.

Radioactivity assay of TLC plates was performed with a thin layer scanner LB 2720 (Berthold, Wildbad, West Germany).

GLC was performed with a Hewlett Packard gas chromatograph 7620 A and stainless steel columns (diameter 1/8 in., length 6 ft) packed with 3% JXR on Gaschrom Q 100/120 mesh at 210 C isothermally. Carrier gas was helium at a flow rate of 35 ml/min. All compounds were chromatographed as methyl esters and as trimethylsilyl ethers if hydroxyl groups were present in the molecule.

Diazomethane in diethyl ether containing 10% methanol was used for methylation of carboxylic acids (17). Silylation prior to GLC analyses was carried out on 10-50 μg of the methyl esters with 40 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 20 μl pyridine at 70 C for 10 min. The resulting mixture of silylated compounds was injected directly.

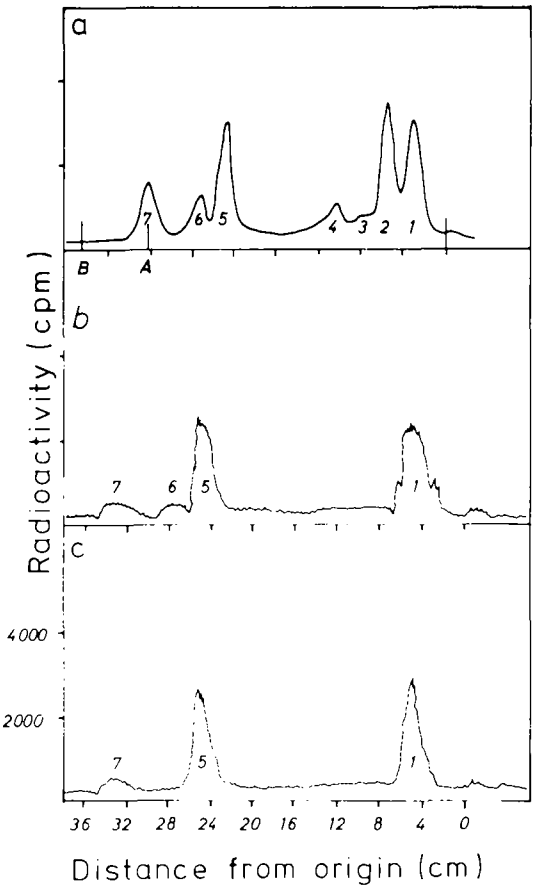


FIG. 1. Thin layer chromatograms of the methyl esters of the reaction mixture on Silica Gel HF_{254}, double development with acetone:heptane (1:2, v/v). A = Front of first development; B = front of second development. a = Untreated mixture, b = mixture after treatment with $SnCl_2$; c = mixture after treatment with $NaBH_4$. The numbers correspond to the compounds shown in Figure 4.

Infrared, Ultraviolet, and Mass Spectral Analyses

Infrared (IR) spectra were obtained with a Perkin Elmer Infracord Spectrophotometer 157E. Spectra were taken mostly from liquid films. UV measurements were obtained with a Zeiss PMQ-2 spectrophotometer. Mass spectra were recorded with the double focusing mass spectrometers Varian MAT CH5-D and Varian MAT 311 coupled with a gas chromatograph Varian 2700. For the coupling, a dual stage Helium separator (Watson-Biemann type) was used. The temperature was 210 C in the gas liquid chromatography (GLC) column as well as

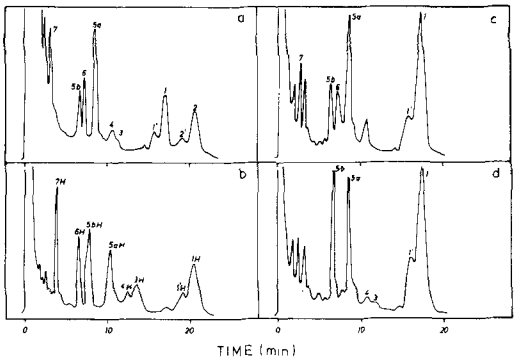


FIG. 2. Gas liquid chromatograms of the silylated methyl esters of the reaction mixture on 3% JXR. a = Untreated mixture; b = mixture after hydrogenation; c = mixture after treatment with $SnCl_2$; d = mixture after treatment with $NaBH_4$. The numbers correspond to the compounds shown in Figure 4.

in the separator and the ion source of the mass spectrometer. Ionization was performed by electron impact with an electron energy of 70 eV. The mass spectra were scanned magnetically with a scan rate of 100 m/e per second. A Packard 3375 liquid scintillation counter was used for counting the radioactivity of liquid samples.

Microchemical Methods

Hydrogenation of small samples of unsaturated hydroxy fatty acid methyl esters was performed in 10 ml ethanol with 10 mg PtO_2 as catalyst. Reduction of hydroperoxides and ketones was carried out with an excess of $NaBH_4$ in methanol at room temperature for 30 min.

Reduction of hydroperoxide with an excess of $SnCl_2$ was carried out in methanol.

Periodic acid oxidation was performed on a microscale with isolated fractions from TLC separations. The substance (0.1-0.5 mg) was dissolved in 1 ml acetic acid and mixed with 0.2 ml of 0.02 M KIO_4 solution. The mixture was stirred 60 min at room temperature. After 30 min of the reaction time, one drop of ethylene glycol was added. The oxidation products were converted to 2,4-dinitrophenylhydrazones (DNPs) by adding 2 ml 0.4% DNPH solution in 4N CHl (18). The DNPs were separated by TLC on Silica Gel G with cyclohexane:diethylether: methanol (100:10:2, v/v/v) as solvent. Ozonolysis of linoleic acid was performed with an apparatus constructed as described by Bonner (19). Linoleic acid was dissolved in methyl acetate and cooled to -78 C for ozonolysis. After 5 min, the reaction was stopped and triphenylphosphine was added. The aldehydes formed were converted to 2,4-dinitrophenyldrazones.

Quantitative determination of peroxides was performed in 5 ml 60% methanol. The solution was acidified with 10 µl concentrated HCl, and after 1 min 10 µl 3.6% $FeSO_4$ solution in 3.6% HCl was added. Exactly 30 sec later 0.5 ml of 20% KSCN solution was added; 150 sec after the addition of the KSCN solution, the absorption was measured at 505 nm against a blank. Peroxide values were calculated from a calibration curve obtained with purified LOOH of the same specific radioactivity as the formed reaction products.

RESULTS

Formation and Separation of Reaction Products

Because of strong irreversible substrate inhibition with LOOH concentrations higher than 6×10^{-5} M (15), the reaction had to be carried out in relatively large volumes to obtain sufficient quantities of products. The substrate ([1-^{14}C]LOOH or [1-^{14}C] linoleic acid) was added in 8-10 portions, each subsequent portion being added when the substrate of the previous addition had been fully converted. In this way, the products of the reaction were pooled without causing substrate inhibition. Radio-TLC showed that at least 6 different compounds were formed (Fig. 1a). TLC fraction 7 consisted of linoleic acid and was present only when LOOH was produced from linoleic acid in the same reaction mixture without preceding purification. The main TLC fractions (1,2, and 5) contained ca. equal amounts of radioactivity with little variation in different incubations. A difficult problem in the purification of the radioactively labeled products was the large number of mainly yellow compounds, which were formed from guaiacol during the course of the reaction. These compounds were distributed from TLC fraction 1 to fraction 5, making it difficult to use IR, nuclear magnetic resonance (NMR), or spray reagents and other chemical methods for the structure elucidation of the radioactively labeled compounds. GLC of the whole reaction mixture (Fig. 2a) was carried out after esterification with diazomethane and silylation. To compare the TLC and GLC peaks, the fractions from TLC were gas chromatographed separately. Each of the TLC fractions 1, 2, and 5 were split into two GLC peaks.

Influence of Different Reaction Conditions

Lipoxygenase isoenzyme L-1 according to Christopher, et al., (16) was used in most of the experiments described here. This isoenzyme, which has a pH optimum of 8.5 for the formation and degradation of LOOH corresponds to

that described by Theorell (20). It was purified and shown to be homogeneous by disk electrophoresis. The reaction of the pure enzyme was compared with that of commercially available soybean lipoxygenase (Fluka, Neu-Ulm, West Germany). Both enzyme preparations showed identical pH optima and product formation. The same reaction product pattern could be obtained by using purified LOOH as well as linoleic acid as substrate. When LOOH was prepared in situ, the only difference found was that some unchanged linoleic acid was left in the reaction mixture (Fig. 1a).

The reaction took place even with very low oxygen concentrations. In preliminary experiments, we found that when the reaction was carried out under nitrogen the usual products were formed. Experiments with $^{18}O_2$, however, showed clearly that atmospheric oxygen was taken up during the reaction (Streckert and Stans, unpublished data).

Lipoxygenase isoenzyme L-2 (16) with a pH optimum for the formation of LOOH at 6.5 also catalyzed the degradation of LOOH in the presence of guaiacol. The pH optimum for the degradation was found to be 8.5 as was the case for isoenzyme L-1.

In earlier reports (15), it was shown that lipoxygenase was irreversibly destroyed during the course of the reaction. The enzyme is destroyed only when both LOOH and guaiacol are present, because preincubation with either LOOH or guaiacol separately did not lead to significant changes in enzyme activity.

Although the physiological role of this reaction is unknown, it is of interest that when the reaction is carried out in soybean meal suspensions the enzyme is active for several hr (Streckert and Stan, unpublished data). Under these conditions, most of the products were identical to those produced in the reactions described above.

Identification of Reaction Products

Determination of functional groups. Fluorescence quenching on developed TLC plates showed 3-6 distinct bands indicating compounds with conjugated double bonds. Because of interference with products from guaiacol, this indication was unambiguous only for TLC fraction 6. Spraying with I_2:starch gave a strong reaction with TLC fraction 2 indicating the presence of peroxy groups, and weaker reaction with fraction 5, possibly due to some unconverted LOOH. Spraying with 2,4-dinitrophenylhydrazine showed a broad band due to products from guaiacol and distinct coloring of TLC fraction 6. Upon reduction of the product mixture with $NaBH_4$, TLC fractions 2 and 6

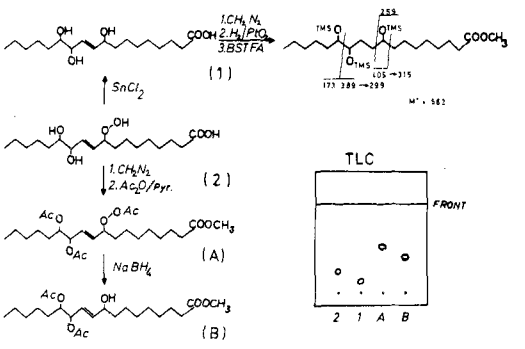


FIG. 3. Reactions carried out on thin layer chromatography fraction 2 on Silica Gel HF_{254} developed with acetone:heptane (2:3, v/v).

disappeared (Fig. 1c) and were not replaced by any new fractions. With the isolated fractions 2 and 6, it could be shown that fraction 2 was converted into fraction 1 and fraction 6 into fraction 5. With $SnCl_2$ TLC fraction 2 again was converted to fraction 1 (Fig. 1b), but fraction 6 remained unchanged.

When the reaction mixture was not silylated, GLC analysis indicated that peaks 6 and 7 remained unchanged, but peaks 1 and 5 were absent. When acetylation was used instead of silylation peaks 1, 1′, 2, 2′, 5a and 5b shifted to slightly higher Rf values (peaks corresponding to TLC fractions 3 and 4 were not clearly detectable because of the low concentrations), whereas, peaks 6 and 7 again remained unchanged. Retention times of hydrogenated products were only slightly different from those of unsaturated compounds except that peaks 2 and 2′ did not appear and peak 6 changed place with peak 5b (Fig. 2b). When TLC fraction 2 was isolated, hydrogenated, and subjected to GLC, it was identical with hydrogenated fraction 1 (Fig. 2b, peaks 1H and 1′H). Reduction with $NaBH_4$ prior to GLC showed that peak 6 was converted to peak 5b, whereas, with $SnCl_2$ peak 6 remained unchanged. Peaks 2 and 2′ disappeared after reduction with either $NaBH_4$ or $SnCl_2$ and were replaced by peaks 1 and 1′ (Fig. 2c and 2d).

The results of these peak shift experiments can be summarized as follows. Peak 7 remained unchanged in all reactions except hydrogenation; it was identified as linoleic acid (Cochromatography and GC-MS). Peak 6 seemed to contain a keto group. All peaks from 1 to 5b contained hydroxyl groups; they all remained unchanged after reduction except for TLC fraction 2. The latter seemed to contain a functional group which could easily be reduced to a hydroxyl group and, therefore, was probably a hydroperoxy or a peroxy group. When TLC

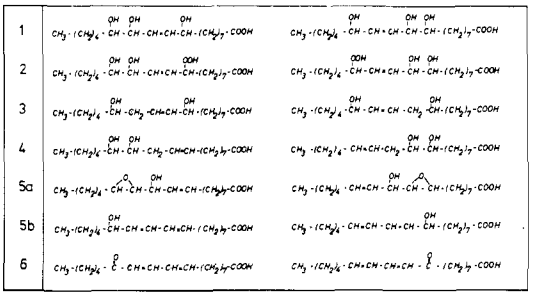


FIG. 4. Structures of reaction products formed from linoleic acid hydroperoxide on incubation with lipoxygenase and guaiacol. **1.** 9,12,13-Trihydroxy-10-*trans*-octadecenoic acid and 9,10,13-trihydroxy-11-*trans*-octadecenoic acid. **2.** 9-Hydroperoxy-12,13-dihydroxy-10-octadecenoic acid and 9,10-dihydroxy-13-hydroperoxy-11-octadecenoic acid. **3.** 9,13-Dihydroxy-10-octadecenoic acid and 9,13-dihydroxy-11-octadecenoic acid. **4.** 12,13-Dihydroxy-9-octadecenoic acid and 9,10-dihydroxy-12-octadecenoic acid. **5a.** 11-Hydroxy-12,13 epoxy-9-*cis*-octadecenoic acid and 11-hydroxy-9,10-epoxy-12-*cis*-octadecenoic acid. **5b.** 13-Hydroxy-9-*cis*-11-*trans*-octadecadienoic acid and 9-hydroxy-10-*trans*-12-*cis*-octadecadienoic acid. **6.** 13-Oxo-9,11-octadecadienoic acid and 9-oxo-10,12-octadecadienoic acid.

fraction 2 was isolated and subjected to GLC, four peaks were found (1, 1′, 2, 2′), showing the high thermal lability of this fraction.

Structures of products. Figure 4 contains all the structures determined for the reaction products. The basis for the assignments of these structures will be discussed below.

TLC fraction 1 consisted of a mixture of 9,12,13-trihydroxy-10-*trans*-octadecenoic acid and 9,10,13-trihydroxy-11-*trans*-octadecenoic acid. Mass spectra of the silylated methyl esters in the GLC peaks 1 and 1′ were identical and showed ions at m/e 545 ($M-CH_3$), 460 (M-hexanal), 387, 301, 297, 259, 211, 173, 155 and ions at m/e 147, 129, 103, 75, and 73, which are typical for trimethylsilyl ether derivatives. The mass spectra are in accordance with those obtained by Graveland (6), Heimann and Dresen (12), Arens and Grosch (21), and Tsuchida, et al., (22) for the same compounds. The hydrogenated compounds showed significant ions at m/e 389, 315, 299, 259, 213, and 173 together with the usual ions for silylated compounds, which is in accordance with spectra reported by Graveland (6). Comparison of the mass spectra of compound 1 before and after hydrogenation showed a double bond between C-9 and C-13. The IR spectrum of TLC fraction 1 showed a strong absorption band at 970 cm^{-1} (*trans* double bond). Several repeated chromatographies of fraction 1 with different mixtures of acetone:heptane gave 0.5 mg of a substance which had a sharp mp at 94 C after three recrystallizations from heptane:acetone. The crystalline substance gave only one peak on GLC (peak 1). The mass spectrum of this substance measured with a direct inlet system was identical with that of peak 1 or 1′ obtained using GC-MS. Periodic acid oxidation carried out on TLC fraction 1 followed by formation of DNP of the cleavage products gave four compounds on TLC. Two of the compounds were ^{14}C-labeled as could be expected from the following cleavage reactions:

$$CH_3\text{-}(CH_2)_4\text{-}CHOH\text{-}CH{=}CH\text{-}CHOH\text{-}CHOH\text{-}(CH_2)_7\text{-}{}^{14}CO_2CH_3 \longrightarrow \quad (I)$$
$$CH_3\text{-}(CH_2)_4\text{-}CHOH\text{-}CH{=}CH\text{-}CHO + \text{(A)}$$
$$OHC\text{-}(CH_2)_7\text{-}{}^{14}CO_2CH_3 \text{ (B)}$$

$$CH_3\text{-}(CH_2)_4\text{-}CHOH\text{-}CHOH\text{-}CH{=}CH\text{-}CHOH\text{-}(CH_2)_7\text{-}{}^{14}CO_2CH_3 \longrightarrow \quad (II)$$
$$CH_3\text{-}(CH_2)_4\text{-}CHO + \text{(C)}$$
$$OHC\text{-}CH{=}CH\text{-}CHOH\text{-}(CH_2)_7\text{-}{}^{14}CO_2CH_3 \text{ (D)}$$

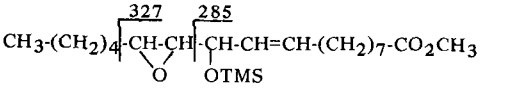
The DNPs of compounds B and C were synthesized by reductive ozonolysis of linoleic acid and used as references.

The DNPs of compounds B (Rf = 0.33) and C (Rf = 0.56) had absorption maxima at 358 nm (DNPs of saturated aldehydes), whereas those of compounds A (Rf = 0.26) and D (Rf = 0.15) had absorption maxima at 371 nm (DNPs of α,β-unsaturated aldehydes). With the crystalline compound (peak 1 on GLC), all four cleavage products were found again, which showed that the separation of TLC fraction 1 on GLC into two peaks (1 and 1′) was not due to a separation of positional isomers, but might be explained by a separation of stereoisomers.

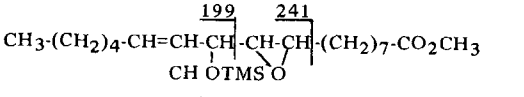
TLC fraction 2 was completely identical (TLC, GLC, GC-MS) with fraction 1 after reduction with $NaBH_4$, $SnCl_2$, or after hydrogenation of both fractions (Fig. 3). Reduction with NaB^2H_4 showed no uptake of deuterium. Quantitative determination of the peroxide content of fraction 2 gave a ratio of 1:1.1 compared with the same amount of LOOH, showing that one hydroperoxy group was present in compound 2. The position of the hydroperoxy group has not yet been definitely determined, but it is most probably attached to carbon atom 9 or 13 as shown in Figure 4. Acetylation followed by reduction with $NaBH_4$ (Fig. 3) and subsequent analysis by GC-MS of the silylated compound gave a significant ion at m/e 259, which is typical for a trimethylsilyl ether group at position 9. Labeling experiments with $^{18}O_2$ also suggest this position (Streckert and Stan, unpublished data).

TLC fraction 5 could be separated into two TLC fractions when rechromatographed using acetone:heptane (3:7, v/v). It also was separated into two peaks (5a and 5b) by GLC.

Fraction 5a consisted of an isomeric mixture of 11-hydroxy-12,13-epoxy-9-*cis*-octadecenoic acid and 11-hydroxy-9,10-epoxy-12-*cis*-octadecenoic acid. The IR spectrum showed bands at 3300 cm^{-1} (hydroxyl), 1750 cm^{-1} (ester), and 890 cm^{-1} (epoxy), but no band at 970 cm^{-1} (*trans* double bond). Mass spectra taken on the ascending GLC peak showed significant ions at m/e 398 (M^+), 383 (M-CH_3), 367 (M-OCH_3), 327 and 285 (base peak), whereas, on the descending peak in addition to the above peaks, ions at m/e 199 (base peak), 241, 211, and 225 were found. The main ions can be explained by the following fragmentations:

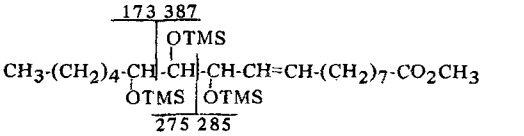


(5a-1) (III)

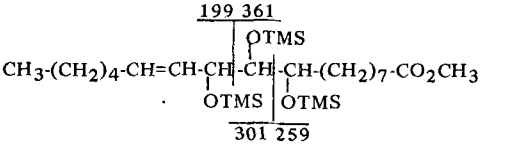


(5a-2) (IV)

Compound 5a-1 was recently described by Hamberg and Gotthammar (23). The mass spectrum reported by these authors is very similar to that obtained on the ascending peak of fraction 5a. When TLC fraction 5a was hydrogenated, significant ions were obtained at m/e 385 (M-CH_3), 369 (M-OCH_3), 287 (base peak), 271, 215, 201, and 183 together with the ions for silylated compounds. The strong ions at m/e 287 and 201 are due to cleavage between the epoxy and the trimethylsilyl ether groups. Comparison of the mass fragmentation pattern of both original and hydrogenated compounds 5a indicated that one double bond is present, which is most probably located between C-12 and C-13 or C-9 and C-10. After hydrolysis with acetic acid (23), TLC fraction 5a gave a more polar compound. On TLC this compound was a little less polar than those in fraction 1, whereas, on GLC it behaved in a similar manner to the compounds in fraction 1. Mass spectra showed significant ions at m/e 460 (M-100, loss of hexanal), 387, 361, 297 (387-90), 285, 275, 271 (361-90), 259, 185 (275-90), 173, and 155. The main ions can be explained by the following fragmentations:



(V)

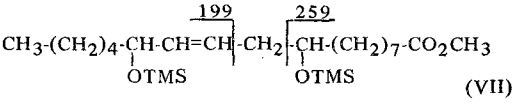


(VI)

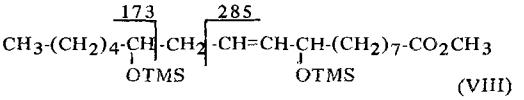
The ions at m/e 199, 301, and 211 (301-90) deriving from the minor compound are weak compared to those of the major compound. For reference we prepared a sample of compound D previously described by Hamberg and Gotthammar (23). This compound was found to be identical (IR, TLC, GLC, and GC-MS) with fraction 5a, but in disagreement with the findings of Hamberg and Gotthammar, the mass spectra showed additional ions at m/e 199 and 241. This might be due to a higher content of 9-LOOH in our preparation. The exact ratio of positional isomers was not determined in this preparation.

Fraction 5b consisted of an isomeric mixture of 13-hydroxy-9-*cis*-11-*trans*-octadecadienoic acid and 9-hydroxy-10-*trans*-12-*cis*-octadecadienoic acid. A sample prepared by reduction of LOOH with $NaBH_4$ was identical to fraction 5b according to UV, TLC, GLC, and GC-MS data. Mass spectra showed significant ions at m/e 382 (M^+), 367 (M-CH_3), 351 (M-OCH_3), 311, 225, 155. Mass spectra recorded on the hydrogenated fraction 5b showed significant ions at m/e 371 (M-CH_3), 355 (M-OCH_3), 339, 315, 259, 229, and 173. The appearance of ions at m/e 173 and 315, as well as 259 and 229, showed that fraction 5b was a mixture of isomers.

TLC fraction 3 behaved on TLC and GLC as expected for a dihydroxy acid. The small amount of material present in fraction 3 was not sufficient for detailed studies. Mass spectra showed significant ions at m/e 472 (M^+), 457 (M-CH_3), 441 (M-OCH_3), 429, 285, 259, 199, 173, and 155. The ions are in accordance with the following scheme of fragmentations:



(VII)



(VIII)

Mass spectra recorded on fraction 3 after hydrogenation showed ions at m/e 459 (M-CH_3), 443 (M-OCH_3), 427, 403, 374 (M-hexanal), 317, 313 (403-90), 259, 227 (317-90), 173, and 155. This clearly established the position of the hydroxyl groups at carbons 9 and 13 and the position of the double bonds.

TLC fraction 4 behaved on TLC and GLC as expected for a dihydroxy acid. The small amount of material again was not sufficient for detailed studies. Mass spectra showed significant ions at m/e 457 (M-CH_3), 441 (M-OCH_3), 382 (M-90), 401, 362 (M-hexanal), 361, 311 (401-90), 299, 275, 271 (361-90), 259, 213, 185 (275-90), and 173. This is in accordance with mass spectra obtained by Graveland (9) for the compounds 4 in Figure 4. The positions of the hydroxyl groups were confirmed by mass spectra of fraction 4 after hydrogenation. They showed ions at m/e 459 (M-CH_3), 443 (M-OCH_3), 374 (M-hexanal), 301, 259, 215, 173, and 155.

TLC fraction 6 was less polar on TLC than fraction 5b, but was eluted later on GLC than 5b. Silylation did not change the retention time on GLC. Reduction with $NaBH_4$ gave a compound, which was identical with fraction 5b according to TLC, GLC, and GC-MS. Treatment with $SnCl_2$ did not have any effect on fraction 6. Analysis of an aliquot by UV spectrophotometry showed an absorption band at 278 nm typical for unsaturated ketones (24). Bands in the IR spectrum (inter alia: 1730 cm^{-1} [methyl ester], 1680 cm^{-1} [unsaturated ketone], 3050, 990, and 960 cm^{-1} [*cis-trans*-conjugated double bond]) and the mass spectrum of fraction 6 was in accordance with that reported by Arens and Grosch (21) for the oxooctadecadienoic acids shown in Figure 4.

The mass spectrum of the fraction after hydrogenation showed ions at m/e 312 (M^+), 281 (M-OCH_3), 256, 241, 200, 185, 170, 150, 114, and 99. This indicated clearly the presence of an isomeric mixture of 9-oxo- and 13-oxo-octadecanoic acid methyl esters (25).

DISCUSSION

Our investigations show that purified soybean lipoxygenase catalyzes a reaction between LOOH and guaiacol, which leads to a number of products, none of which is in predominance. Some of the compounds have been described earlier and are formed enzymatically in plant material. The unsaturated trihydroxy compounds (Fig. 4:1) for example arise from linoleic acid in flours of different cereals. In wheat flour they are the main products, whereas, in flours of rye, oats, and barley they are formed to a lesser extent (8).

The hydroperoxydihydroxyoctadecenoic acid (Fig. 4:2) is an interesting compound which has not been described previously. The proposed structure is based on the ease of reduction to trihydroxyoctadecenoic acid and the occurrence of one hydroperoxy group in the molecule. Because the position of the hydroperoxy group has not yet been determined unequivocally, it is difficult to discuss the mechanism of its formation. It is clear, however, that it cannot arise by isomerization or decomposition, but rather by oxidation of LOOH. Experiments with $^{18}O_2$ confirm that this compound is formed by the incorporation of molecular oxygen into LOOH (Streckert and Stan, unpublished data). Possibly hydroperoxydihydroxyoctadecenoic acid is the precursor of the trihydroxyoctadecenoic acids which can be formed easily by reduction of the hydroperoxy group. In soybean meal suspensions, fraction 2 could not be detected, and the trihydroxyoctadecenoic acids were the main products (Streckert and Stan, unpublished data). This suggests that the hydroperoxy group is rapidly reduced in natural media. Further investigations on the mechanism of formation of the trihydroxy compounds are in progress.

The third group of products, which are present in larger amounts, consisted of hydroxyepoxyoctadecenoic acids (Fig. 4:5a). These compounds have not been detected previously in plant material. Hamberg and Gotthammar (23) have recently described their formation by heating LOOH in aqueous ethanol solution. Obviously, these compounds are quite stable and are hydrolyzed to vicinal trihydroxyoctadecenoic acids only by acetic acid at elevated temperatures. This explains the fact that the vicinal trihydroxy acids were not present in our incubation mixtures.

Monohydroxyoctadecadienoic acids are the main products in oats, where they are formed by a specific lipoperoxidase, which requires reducing agents (10-12). The same compounds were reported to be formed in suspensions of flour from wheat and other cereals by Graveland (8). He suggested, however, that the reduction of the hydroperoxy group was due mainly to nonenzymatic reductions by sulfhydryl groups present in the proteins of cereals. In our system only small amounts of monohydroxyoctadecadienoic acids were formed (Fig. 4:5a). Oxooctadecadienoic acids are known to be by-products in the lipoxygenase reaction (24). Arens and Grosch (21) have found these compounds in peas. Garssen, et al., (26) have reported that 13-oxo-9,11-octadecadienoic acid is formed during the anaerobic

reaction of 13-LOOH in the presence of lipoxygenase and linoleic acid. In the reaction reported here, LOOH was converted to a mixture of 9- and 13-oxooctadecadienoic acids in the absence of linoleic acid as well as in the presence of oxygen. Therefore, the mechanism suggested by Garssen, et al., (26) can be excluded.

Dihydroxyoctadecenoic acids (Fig. 4:3 and 4) were formed only in low concentrations in the reaction reported here, and therefore, could only be studied by GS-MS analysis. The formation of a mixture of vicinal dihydroxy acids in cereals was reported earlier by Graveland, et al., (7) and Arens and Grosch (21), whereas, the 9,13-dihydroxyoctadecenoic acids have not been reported previously.

Remarkably, some of the compounds formed in the reaction with guaiacol and lipoxygenase were found recently in several model reactions with LOOH, i.e., heat treatment in ethanol (23), Fe-ions in 80% ethanol (27), hemoglobin in phosphate buffer (28), and lipoxygenase L-2 from peas at pH 6.5 (21). This suggests that these compounds could be formed in plant material not only by enzymatic reactions, but also by a more general mechanism initiated by the formation of peroxy or oxy radicals from LOOH.

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SHORT COMMUNICATIONS

Effect of Vitamin B_{12} Status on the Occurrence of Branched-chain and Odd-numbered Fatty Acids in the Liver Lipids of the Baboon

ABSTRACT

Branched chain fatty acids of the anteiso series and others with methyl substitution nearer to the carboxyl group were found, together with odd numbered, straight chain fatty acids, in very small proportions in baboon liver lipids. The proportions were increased in vitamin B_{12}-depleted animals, especially after administration of a B_{12} analogue.

INTRODUCTION

In a recent study with baboons (1), it was observed that animals fed on a vitamin B_{12}-deficient diet excreted methylmalonate, a metabolite of propionate and of valine, in their urine in excess of the amount excreted by controls. Further, it was observed that vitamin B_{12}-depleted baboons excreted additional methylmalonate following the oral administration of propionate or valine, and that the amounts excreted were also enhanced after the intramuscular injection of an inactive analogue of vitamin B_{12} (cyanocobalamin monocarboxylic acid). It was therefore of interest to examine the lipids of some of these baboons, and others of differing vitamin B_{12} status, for the presence of odd numbered and branched chain fatty acids since it was inferred (2-4) that such acids can be formed when impaired metabolism of propionate leads to the accumulation of propionyl CoA and of its carboxylation product, methylmalonyl CoA.

EXPERIMENTAL PROCEDURES

Liver biopsy samples (ca. 250 mg) were obtained from 4-year old, male baboons (*Papio cyanocephalus*) which had been fed as previously described (1) on a synthetic diet with or without a supplement of vitamin B_{12}. Samples also were obtained from two vitamin B_{12}-depleted baboons immediately after they had been given a B_{12} analogue (cyanocobalamin monocarboxylic acid) by intramuscular injection in daily doses of 500 μg per kg body wt for 1 month and daily doses of 250 μg per kg body wt for a further 2 months. In addition, liver biopsy samples were obtained from 4-year old, male baboons which had received a stock diet of fresh fruit, vegetables and high-protein biscuits. Biopsy samples of omental adipose tissue (ca. 500 mg) were also obtained (at the same time as the liver samples) from at least one animal in each treatment group.

TABLE I

Odd Numbered, Straight Chain Fatty Acids and Branched Chain Fatty Acids in Liver Lipids of Baboons of Different Vitamin B_{12} Status[a]

Diet	Odd numbered acids[b]	Branched chain acids		Liver vitamin B_{12} ($\mu g/g^{-1}$ wet wt)
		Anteiso[c]	Others[d]	
Stock (4)[e]	0.7	0.2	0.2	0.47
Synthetic with vitamin B_{12} (4)	0.7	0.2	0.6	0.76
Synthetic without vitamin B_{12} (3)	1.2	0.2	1.4	0.22
Synthetic without vitamin B_{12}: given analogue (2)	2.0	1.1	4.0	0.18

[a]Fatty acid proportions (% by wt of total fatty acids) are given for pooled liver lipids from the animals in each dietary treatment group and vitamin B_{12} results are means of individual values within each treatment group.

[b]Mostly 15:0 and 17:0, with some 13:0.

[c]Mostly 12-methyltetradecanoic acid and 14-methylhexadecanoic acid.

[d]Tentatively identified as branched chain components and confirmed subsequently (see Table II).

[e]Number of animals from which the pooled liver lipids were derived is given in parentheses.

TABLE II

Equivalent Chain Length (ECL) Values of Branched Chain Fatty Acid Methyl Esters from Liver Lipids of Baboons[a]

Identity of methyl ester	ECL value			
	A	B[b]	C[c]	D[d]
4-Me-Dodecanoate	12.40	-	12.40	12.40
4,8-diMe-dodecanoate	12.64	12.68	12.68	-
4-Me-tridecanoate	13.39	-	13.38	13.38
4,8-diMe-tridecanoate	13.58	13.53	13.55	13.55
6-Me-tetradecanoate	14.30	14.30	14.28	14.30
8-Me-tetradecanoate	14.34	-	14.33	14.33
4-Me-tetradecanoate	14.37	14.38	14.38	14.38
12-Me-tetradecanoate	14.70	-	14.68	14.72
4-Me-pentadecanoate	15.36	15.33	15.38	15.35
8-Me-hexadecanoate	16.32	16.30	16.31	16.30
4-Me-hexadecanoate	16.36	16.38	16.35	16.35
12-Me-hexadecanoate	16.42	16.40	16.40	16.40
14-Me-hexadecanoate	16.70	16.70	16.70	16.68
6-Me-heptadecanoate	17.28	-	17.25	17.28

[a]Corresponding to those previously characterized by gas liquid chromatography and mass spectrometry (column A).

[b] Animal fed on stock diet.

[c]Animal fed on synthetic diet with vitamin B_{12}.

[d]Animal fed on synthetic diet without vitamin B_{12} and which had been given cyanocobalamin monocarboxylic acid 15 months previously.

TABLE III

Proportions of Branched Chain Acids in Liver Lipids of Three Baboons Receiving Different Diets

Diet	Branched chain acids[a] (% by wt)	
	Anteiso	Others
Stock	0.2	0.1
Synthetic with vitamin B_{12}	0.1	0.5
Synthetic without vitamin B_{12}: given analogue 15 months earlier	1.0	1.2

[a]For components which were identified see Table II.

A portion of each liver sample was used for determination of vitamin B_{12} (5) and total lipids were extracted from the remainder with chloroform:methanol (2:1,v/v). To obtain sufficient fatty acids for analysis, lipids from similarly treated baboons were pooled and saponified with excess 0.5M ethanolic KOH. The recovered fatty acids were converted to methyl esters which were analyzed (2) by conventional gas liquid chromatography (GLC). To facilitate the determination of the content of branched chain components in the methyl esters, a weighed portion was treated as described before (3) with mercuric acetate to remove unsaturated esters and then with urea to remove most of the straight chain esters. The resulting concentrate of branched chain methyl esters was weighed and subjected to conventional GLC.

These analyses (Table I) indicated that more detailed analyses were required to identify the branched chain fatty acids. Accordingly, large pieces of liver (ca. 50 g) were subsequently obtained from three of the baboons, given different dietary treatments, which were killed fifteen months after the biopsy samples were taken. During the intervening time, one animal continued to receive the stock diet, one the B_{12}-supplemented diet, and the other, which had earlier been given the B_{12} analogue, continued to receive the B_{12}-deficient diet. Fatty acid methyl esters were prepared as outlined above, and the concentrates of branched chain components were subjected to open tubular column GLC, using a 100 m column (internal diameter 0.25 mm) coated with polymerized butanediol succinate (3). The equivalent chain length (ECL) values were compared with those obtained previously from barley-fed lambs

under the same GLC conditions (2) for branched chain fatty acids which were identified by mass spectrometry. Those components to which identities could thus be assigned are listed in Table II.

RESULTS AND DISCUSSION

The liver fatty acids of baboons given the stock diet or the vitamin B_{12}-supplemented diet contained < 1% by wt of branched chain or odd numbered components (Table I). Vitamin B_{12} depletion resulted in the production of more of each of these groups of fatty acids, and, when the depletion was exacerbated by the administration of an analogue of B_{12}, the proportions of these fatty acids were increased further (Tables I and III). These findings substantiate the suggestion (1) that cyanocobalamin monocarboxylic acid exerts an antagonistic effect on the function of vitamin B_{12}, and indicate that in vitamin B_{12} deficiency propionyl CoA and methylmalonyl CoA can accumulate and become incorporated into fatty acids to give amounts of odd numbered and branched chain components in excess of those produced when vitamin B_{12} is not limiting. As Table II shows, the branched chain acids comprised members of the anteiso series, together with other acids in which one or two methyl groups were present in positions nearer to the carboxyl group of the molecule. Conclusive evidence that methylmalonyl CoA can be utilized for the production of such acids has recently been obtained from studies in vitro with fatty acid synthetase preparations from animal tissues (6).

Though there was an association between vitamin B_{12} status and occurrence in liver lipids of fatty acids derived from propionate and methylmalonate, correspondingly different proportions of such acids were not found in adipose tissue triacylglycerols. In no sample from any treatment group did the odd-numbered and branched chain fatty acids exceed 0.5 and 0.3%, respectively, of the total fatty acids.

The presence of very small amounts of branched chain acids in the liver and adipose tissue of the baboons which had apparently received an adequate intake of vitamin B_{12} suggests that, even under normal metabolic conditions, not all the methylmalonyl CoA produced from branched chain amino acids in the liver is converted to succinate, and some of it can be incorporated into newly synthesized fatty acids.

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Effect of Diet on Fatty Acids in the Lipoprotein Cholesteryl Esters of Type IIa and Normal Individuals[1]

ABSTRACT

Four normal and two individuals with type IIa hyperlipoproteinemia were placed on the National Heart and Lung Institute Type IIa Diet (<300 mg of cholesterol per day, high polyunsaturated, low saturated fat diet) for 1 week and on a normal diet the following week. Plasma samples were obtained and the cholesterol contents of plasma and of very low density, low density and high density lipoproteins determined. The cholesteryl esters in one type IIa and two normal individuals were identified.

The cholesteryl esters in type IIa very low density lipoproteins from blood drawn 45 min after the last meal in each dietary period, contained less 18:2 than from the normal. After the first dietary period, the very low density lipoprotein cholesteryl ester 18:2 content for the type IIa was 37.2M% and for the normals, 54.7M%. After the second dietary period, the corresponding values were 49.7M% and 56.7M%. Fasting samples had lower 18:2 contents in the low density lipoproteins from the Type IIa subject following both dietary periods and in the high density lipoproteins following the Type II diet.

INTRODUCTION

In Type II hyperlipoproteinemia, the individual has an elevated cholesterol level due to a decreased turnover of the low density lipoprotein (LDL) (1). This decrease in the catabolism of the LDL has been linked to the formation of atheromatous plaques (1,2). Because cholesterol and cholesteryl esters (CE) are components of the plaques and are carried in the blood by lipoproteins (LP), differences in fatty acid (FA) composition of CE between normal and type II individuals are of interest. The FA composition of CE in various LP fractions from a type IIa has not been reported. However, one publication has appeared giving these data for seriously ill patients following a 100 g corn or coconut oil meal (3). These data are available for whole plasma (4,5).

In this paper we present the FA composition of CE from two normal and one type IIa individual as affected by one week on a type IIa diet (6) and one week on a normal American diet.

MATERIALS AND METHODS

Materials and methods are described in detail in a previous publication (7). Given below is a brief synopsis of the experimental protocol.

Potential subjects were screened for hyperlipoproteinemia using the criteria of Frederickson, et al., (8) including plasma cholesterol and triglyceride (TG) levels, qualitative LP pattern by agarose gel electrophoresis, and quantitative determination of cholesterol in the major LP separated by sequential preparative ultracentrifugation. Two control and one Type IIa subject were selected for the study.

The subjects were placed on the type IIa diet (6) for one week, followed by a second week on a normal American high calorie, high cholesterol, high saturated fat diet. Blood was collected at the end of each dietary period using 0.1% Na_2 ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. One collection was made 45 min after the last meal on the diet for analysis of exogenous CE and another the following morning, after a 14 hr fast, for identification of endogenous CE.

Plasma and the major LP fractions isolated by ultracentrifugation were analyzed for cholesterol as described previously (7). CE were isolated by thin layer chromatography (TLC) following Folch extraction of the LP fraction and separation of polar and nonpolar lipids by column chromatography. TLC of the neutral lipid fraction was accomplished on 20 x 20 cm, 0.5 mm thick plates of Silica Gel G using a solvent system of petroleum ether:ethyl ether:acetic acid (90:30:2) and fractions were made visible by brief exposure to iodine vapors. The samples were eluted from the scraped CE band using chloroform:methanol (2:1) and stored under nitrogen at -20 C until analyzed.

Samples were esterified by first evaporating to dryness and adding 5 ml of 2.5N sodium methoxide. After 15 min at room temperature, the esters were extracted, concentrated (9), and analyzed by gas liquid chromatography (GLC). A Barber Colman series 5000 GLC with hydrogen flame ionization detectors was used for separations at 190 C on a 3.12 m stainless steel column (0.45 mm internal diameter) packed

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TABLE I

Fatty Acids in Lipoprotein Cholesteryl Esters of Type IIa and Normal Individuals on a Type II Diet

Subject[a] and lipoprotein	Dietary period I[b]	Fatty acid (M%)							
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	20:4
RH VLDL[c]	1/14[d]	-	-	18.3 ± 2.1[e]	2.0 ± 0.3	-	24.8 ± 1.5	53.8 ± 1.6	1.1 ± 0.2
KW VLDL	1/14	-	-	22.8 ± 1.6	-	-	21.6 ± 2.3	55.7 ± 1.7	-
FG VLDL	1/14	3.8 ± 0.7	4.2 ± 1.3	27.7 ± 1.9	1.4 ± 0.4	2.2 ± 1.1	20.0 ± 0.9	37.2 ± 2.4	3.4 ± 0.8
RH VLDL	1/15	-	-	19.3 ± 2.3	0.9 ± 0.2	-	28.9 ± 2.8	45.4 ± 2.3	5.5 ± 0.7
KW VLDL	1/15	0.3 ± 0.1	-	14.7 ± 2.5	1.0 ± 0.3	-	20.3 ± 3.2	55.8 ± 1.9	7.8 ± 0.5
FG VLDL	1/15	1.3 ± 0.3	1.6 ± 0.5	17.7 ± 1.9	0.6 ± 0.2	1.0 ± 0.4	23.6 ± 1.7	48.8 ± 2.5	5.4 ± 0.7
RH LDL	1/15	0.3 ± 0.1	-	13.2 ± 0.9	0.3 ± 0.2	0.4 ± 0.1	23.0 ± 2.4	58.9 ± 1.8	4.0 ± 0.2
KW LDL	1/15	0.4 ± 0.1	-	15.7 ± 1.4	0.7 ± 0.1	0.5 ± 0.2	20.1 ± 2.3	57.3 ± 1.7	5.2 ± 0.8
FG LDL	1/15	0.2 ± 0.1	-	13.1 ± 1.8	1.3 ± 0.3	0.5 ± 0.1	22.7 ± 1.8	55.3 ± 2.0	6.9 ± 0.9
RH HDL	1/15	0.7 ± 0.2	-	14.2 ± 1.6	0.2 ± 0.1	0.8 ± 0.1	28.5 ± 1.7	54.9 ± 2.0	0.6 ± 0.8
KW HDL	1/15	0.6 ± 0.1	-	14.1 ± 2.0	0.9 ± 0.1	0.5 ± 0.2	19.7 ± 1.6	57.2 ± 2.1	7.1 ± 1.1
FG HDL	1/15	0.3 ± 0.1	-	16.8 ± 1.9	1.5 ± 0.2	0.2 ± 0.1	25.5 ± 2.1	50.7 ± 1.8	4.9 ± 0.6

[a]RH and KW = normal individuals, FG = Type IIa.

[b]2000 Kcal, 270 mg cholesterol, high polyunsaturated/saturated fat diet.

[c]Very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL).

[d]1/14 obtained 45 min after last meal on diet I (exogenous), 1/15 after overnight fast.

[e]Mean ± standard error of mean. Based upon 3 gas liquid chromatography runs of the sample.

TABLE II

Fatty Acids in Lipoprotein Cholesteryl Esters of Type IIa and Normal Individuals on a Normal American Diet

Subject[a] and lipoprotein	Dietary period II[c]	Fatty acid (M%)							
		14:0	16:0	16:1	18:0	18:1	18:2	20:4	Minor constituents[b]
RH VLDL[d]	1/21[e]	-	11.1 ± 1.3[f]	2.0 ± 0.4	-	26.1 ± 2.9	58.5 ± 3.0	2.4 ± 0.9	-
KW VLDL	1/21	0.3 ± 0.1	14.0 ± 1.9	0.3 ± 0.1	2.1 ± 0.8	23.8 ± 3.0	54.7 ± 1.9	4.1 ± 1.3	0.7 ± 0.2
FG VLDL	1/21	0.5 ± 0.2	15.3 ± 2.4	0.7 ± 0.1	0.1 ± 0.1	27.1 ± 2.7	49.7 ± 2.3	6.6 ± 1.4	-
RH VLDL	1/22	-	11.0 ± 2.2	13.9 ± 2.3	-	23.9 ± 2.4	48.5 ± 1.9	2.4 ± 1.1	0.4 ± 0.1
KW VLDL	1/22	-	14.6 ± 2.1	1.1 ± 0.1	0.2 ± 0.1	22.7 ± 1.8	56.4 ± 2.9	5.1 ± 1.3	-
FG VLDL	1/22	0.3 ± 0.1	13.7 ± 1.8	1.6 ± 0.6	0.1 ± 0.1	26.4 ± 1.9	53.9 ± 2.5	4.0 ± 0.8	-
RH LDL	1/22	0.7 ± 0.2	13.7 ± 2.5	2.2 ± 0.7	-	23.7 ± 2.0	54.4 ± 1.8	4.6 ± 0.8	0.7 ± 0.2
KW LDL	1/22	0.3 ± 0.1	12.8 ± 1.8	0.8 ± 0.2	0.6 ± 0.2	18.5 ± 2.0	60.1 ± 2.3	6.5 ± 1.2	0.5 ± 0.1
FG LDL	1/22	0.3 ± 0.1 1	19.3± 1.7	1.4 ± 0.2	0.4 ± 0.2	26.9 ± 1.9	48.1 ± 2.2	3.7 ± 1.2	-
RH HDL	1/22	0.9 ± 0.1	15.2 ± 3.0	1.8 ± 0.5	0.1 ± 0.1	23.8 ± 2.5	51.2 ± 2.4	6.5 ± 1.9	0.3 ± 0.1
KW HDL	1/22	1.0 ± 0.2	11.4 ± 2.4	0.6 ± 0.2	0.2 ± 0.1	20.1 ± 2.3	60.6 ± 2.2	6.2 ± 1.8	-
FG HDL	1/22	0.7 ± 0.1	15.7 ± 1.9	0.5 ± 0.2	0.3 ± 0.1	23.0 ± 1.6	56.3 ± 2.6	3.5 ± 1.1	-

[a]RH and KW = normal individuals; FG = type IIa.

[b]12:0, 15:0, 20:0, and 22:0.

[c]2,800 Kcal, 570 mg cholesterol, low polyunsaturated/saturated fat ratio diet.

[d]Very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL).

[e]1/21 obtained 45 min after last meal in Diet II (exogenous), 1/22 after overnight fast.

[f]Mean ± standard error of mean. Based upon 3 gas liquid chromatography runs of the sample.

with 18% DEGS on Anakrom ABS 70/80 mesh. Component esters were quantitated with a Disc Chart Integrator Model 205 and calculated as mole %. Identification was by comparison of carbon numbers with those of standard methyl esters. Each sample was analyzed 3 times, and a standard error of the mean was calculated for each fatty acid.

RESULTS

The effect of diet, on the cholesterol levels of plasma very low density lipoproteins (VLDL), LDL, and high density lipoproteins (HDL) has been published (7). Following the first dietary period (Type IIa diet), the plasma cholesterol level in the Type IIa individuals was reduced ca. 15%, whereas the control group had an 8% reduction over the same 7 day period. During the second week (normal diet), the plasma cholesterol levels were not altered further.

Blood samples were taken 45 min after completion of a meal to obtain exogenous FA in VLDL-CE. Endogenous FA of CE for the VLDL, LDL, and HDL was obtained from blood samples drawn after a 14 hr overnight fast. The effects of the diets on CE FA are reported in Table I (low cholesterol) and Table II (high cholesterol).

Following the first dietary period (Table I), the Type IIa individual had a markedly lower 18:2 level in the exogenous VLDL-CE than did the normal; 37.2M% versus an average of 54.7M% for the normal subjects. Other differences noted in the exogenous fraction following the IIa diet were an increase of 14:0, 15:0, 16:0, and 18:0 relative to controls. Endogenous VLDL-CE in the Type IIa subject following dietary period 1 showed a higher level of 14:0 and 15:0, while 18:2 in the HDL fraction was depressed when compared to controls.

After dietary period 2 (Table II), a small reduction in 18:2 relative to the controls was still apparent in the exogenous VLDL-CE for the Type IIa individual and in the endogenous LDL fraction.

The differences above are in excess of variation noted in GLC analysis of the samples.

DISCUSSION

Through the first dietary period, total plasma cholesterol levels decreased, and reduced LDL-cholesterol concentrations were observed. During the second dietary period, in which there was an increased caloric intake (118%), increased dietary cholesterol, and increased saturated fat over period I, there was no change in plasma cholesterol, and a continued decrease in LDL-cholesterol. A probable explanation is that, due to the short duration of the therapeutic diet, a carry over effect influenced the continued decrease in the LDL-cholesterol.

Recent studies by Allard, et al., (10), in which the fatty acid profiles of the plasma lipid classes from coronary heart disease (CHD), primary hyperlipoproteinemic, and healthy subjects were obtained, found no differences between the fatty acid patterns of LP-CE from healthy and CHD patients. However, in contrast, the fatty acid patterns of plasma lipids from the hyperlipoproteinemic patients showed that the CE and other lipid classes were significantly richer in polyunsaturated fatty acids, (PUFA, mostly 18:2) in the primary Type II than in other types of hyperlipoproteinemia. Therefore, Allard, et al., (10) did not support the view proposed by Sinclair (11) and more recently by Kingsbury, et al., (5) that atherosclerosis is accompanied by a decrease in the proportion of plasma PUFA. We observed a slightly reduced 18:2 content in CE from the Type IIa individual's endogenous LDL, and HDL following dietary period 1 (Table I). Following dietary period II (Table II), 18:2 in the Type II subject was reduced only in the endogenous LDL. Following both dietary periods (Tables I and II), a reduction in 18:2, for the Type II subject, was observed in the exogenous VLDL. This may indicate a reduced transport of 18:2 from the intestine of our one subject with Type II hyperlipoproteinemia.

Kayden, et al., (3) noted some differences in the composition of CE from the VLDL of seriously ill patients 8 hr after single meals (100 g) of corn or coconut oil. As compared to the fasting samples, both 18:1 and 18:2 contents were reduced after feeding coconut oil, while only 18:1 was reduced after corn oil. The differences were not as large as those we observed for 18:2, and, because the status of the subjects and the experimental protocols were markedly different, comparisons are probably not meaningful.

In evaluating these data, one should remember that the two dietary periods were short and that we used few subjects, two normals and one Type IIa individual. However, to our knowledge, these are the first available data on the composition of the cholesteryl esters from an individual with Type IIa hyperlipoproteinemia and should be considered along with the information in our recently published paper (7).

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