Fatty Acid Composition of Seed Oils from Six *Adansonia* Species with Particular Reference to Cyclopropane and Cyclopropene Acids

AUGUSTE RALAIMANARIVO[a], EMILE M. GAYDOU[b] and JEAN-PIERRE BIANCHINI[a,*],
[a]Ecole Supérieure de Chimie de Marseille, Université de Droit, d'Ecònomie et des Sciences, Centre Scientifique de Saint-Jérôme, rue Henri Poincaré, 13 397 Marseille Cedex 4, France; and [b]Département des Industries Agricoles et Alimentaires, Etablissement d' Enseignement Supérieur des Sciences Agronomiques, Université de Madagascar, B.P. 175, Antananarivo, République Démocratique de Madagascar

ABSTRACT

The oil content of six *Adansonia* species (Bombacaceae family) of Madagascar (*Adansonia grandidieri, A. za, A. digitata, A. fony, A. madagascariensis* and *A. suarenzensis*) and Africa (*A. digitata*) ranges from 8 to 46%. All the oils give a positive response to the Halphen test. Malvalic, sterculic and dihydrosterculic acids were detected using gas liquid chromatography-mass spectrometry (GLC-MS). Epoxy or hydroxy fatty acids were not found in these oils. Fatty acid composition was determined by GLC using glass capillary columns coated with BDS and Carbowax 20 M. Results obtained for cyclopropenic fatty acids (CPEFA) were compared to those given by glass capillary GLC after derivatization with silver nitrate in methanol, by hydrogen bromide titration and by proton magnetic resonance (PMR). Good agreement was observed for the results given by the various methods. Malvalic acid content ranges from 3 to 28%, sterculic acid from 1 to 8% and dihydrosterculic acid from 1.5 to 5.1%. Odd-numbered fatty acids (Pentadecanoic and heptadecanoic) were also observed in minute amounts (0.1-1.1%). Among the normal fatty acids, we observed mainly palmitic (21-46%), oleic (15-40%) and linoleic (12-32%). The relationship between fatty acid composition and *Adansonia* species is discussed.

Lipids 17:1-10, 1981.

INTRODUCTION

It was reported in some papers and reviews (1-6) that cyclopropanic fatty acids (CPEFA) such as malvalic (8,9-methylene-heptadec-8-enoic) and sterculic (9,10-methylene-octadec-9-enoic) acids are widespread in higher plant families, especially in, e.g., Malvaceae, Sterculiaceae, Tiliaceae, Sapindaceae and Bombacaceae. Recent works show that the oils containing CPEFA are frequently accompanied by smaller proportions of cyclopropanic fatty acids (CPAFA), such as dihydrosterculic and dihydromalvalic acids (7,8), which are the dihydro analogs of CPEFA. In some cases, the CPAFA are the major components of the oils (6,9,10). Because these CPEFA are responsible for some physiological disorders and should have some cocarcinogenic properties (8,11-14), assays of seed oils for CPEFA have been done during the past decade.

The genus *Adansonia* (Bombacaceae family) comprises some species in Africa, Madagascar and Australia. The African baobab, *A. digitata*, was not reported to contain CPEFA (15-17), although it was said in earlier investigations that baobab seed oils should contain CPEFA as these oils gave a positive Halphen test (18-20). In a recent work, we have found that a Malagasy baobab species, *A. grandidieri*, contains malvalic (6-7%), sterculic (4-8%) and dihydrosterculic (1.5-2%) acids (21).

In our interest of ascertaining whether any consistent relationship exists between the fatty acid composition and the taxonomical arrangement of plants, we have made a more extensive study of fatty acid composition from various species of *Adansonia*. In this work, the fatty acid composition, with special attention to CPEFA and CPAFA of 6 Malagasy baobab species, was studied. Among the 7 species of *Adansonia* found in Madagascar (22), the 6 species studied in this paper were: *A. grandidieri, A. za, A. digitata, A. fony, A. madagascariensis* and *A. suarezensis*. A sample of the African baobab seed oil, *A. digitata*, from Haute-Volta was also investigated. In some cases, seeds of different origin or seeds of different varieties were studied and 11 samples in all were investigated, as shown in Table 1, to determine the variation in fatty acid composition. Identifications of malvalic, sterculic and dihydrosterculic acids were made using gas liquid chromatography-mass spectrometry (GLC-MS). CPEFA were analyzed by GLC of the derivatives obtained according to the method of Schneider et al. (23). Results were compared to those obtained using direct GLC analysis of the total fatty acid methyl ester fractions (24), hydrobromic acid titration (25,26) and the proton magnetic resonance (PMR) method of Pawlowski et al. (27).

TABLE 1

Origin of Baobab Seeds

Number	Species	Origin[a]
1	*A. grandidieri*	Morondava area
2	*A. grandidieri*	Morondava Market
3	*A. grandidieri*	Refined oil (SICA, Morondava)
4	*A. za*	Betsipotika forest (Morondava)
5	*A. za* var. Boinensis	Ambovombe area
6	*A. za* var. Bozy	Ambanja area
7	*A. digitata*	Mahajanga
8	*A. digitata*	Bobo-Dioulasso (Haute-Volta)
9	*A. fony*	Andranomena (Belo/Tsiribihina)
10	*A. madacascariensis*	Ramana beach (Antsiranana)
11	*A. suarezensis*	Andrakaka area (Antsiranana)

[a]All the seeds were collected in Madagascar but sample 8 was collected in Haute-Volta.

EXPERIMENTAL PROCEDURES

Materials

The baobab fruits 1, 2, 4-7 and 9-11 were collected in Madagascar areas as shown in Table 1 during 1978-79. The African baobab fruit 8 was collected in Haute-Volta in 1978.

Ten oils, 1, 2 and 4-11, were prepared from the corresponding dried seeds by Soxhlet extraction using light petroleum (40-60 C). The oil content of these seeds is given in Table 2. A specimen of pure industrial and refined oil of *A. grandidieri* 3 was purchased from SICA Society (Morondava, Madagascar). The iodine, saponification, acidic and hydroxyl values, as well as refractive index and density, are indicated in Table 2 and were determined according to NF methods (28).

General Methods

Infrared (IR) spectra were determined as liquid films or as 5% solutions in carbon tetrachloride on a Perkin Elmer (Norwalk, CT) Model 457 instrument; ultraviolet (UV) spectra were measured using methanolic solution with a Beckman (Gagny, France) DB-G spectrophotometer.

Halphen Color Test

The method of Halphen (29) was used for characterization of CPEFA. Equal volumes (about 1-3 ml) of oil, amyl alcohol and carbon disulfide containing 1% of sulfur were placed in a test tube and warmed on a steam bath during 10-15 min. A characteristic red pink color appeared for the 11 oils.

Other Chemical Procedures

Durbetaki's method (25,26) was used at 2 temperatures (3 and 55 C) for direct quantitative hydrogen bromide titration of oxiranic and cyclopropenic functions of fatty acids. Results were expressed in Table 2 as vernolic acid for the oxirane oxygen and as sterculic acid for the cyclopropene ring. Methyl esters were prepared from oils (5 g) by methanolysis (30) with 0.2 molar sodium methoxide in methanol (40 ml). Argentation of methyl esters was performed as described by Schneider et al. (23).

Gas Liquid Chromatography

An Intersmat IG 12 DFL gas chromatograph (Chelles-les-Coudreaux, France) equipped with a flame ionization detector and a glass injector was used. Two glass capillary columns were employed: a 45 m long, 0.35 mm id column coated with Carbowax 20 M (0.15 μmphase thickness) and a 25 m long, 0.40 mm id column coated with BDS (0.20 μm thickness). Temperatures were 190 C for the Carbowax 20 M column and 150 C for the BDS column; 200 C for the detector and 220 C for the inlet ovens. Flow rate of hydrogen as carrier gas was 1 ml/min; split 5/100. The injections averaged about 0.5-1 μl with a 5% solution of methyl esters. A Perkin Elmer Model 56 recorder was used and peak areas were integrated by a Spectra-Physics Minigrator integrator (Santa Clara, CA).

Gas Liquid Chromatography-Mass Spectrometry

Analyses were performed on a Varian M 112 S gas chromatograph spectrometer (Varian Europe, Zug, Switzerland). The BDS glass capillary column was used for GLC. Operating conditions were: column, 150 C; helium, 1 ml/min; molecular separator, 200 C; ion source, 100 C; ionizing voltage, 70 eV. The data system was a Varian Spectro System 200.

TABLE 2

Analytical Data of *Adansonia* Species Seeds and Oils

	A. grandidieri			*A. za*		*A. digitata*		*A. fony*	*A. madagascariensis*	*A. suarerensis*
	1	2	3	4	5[a]	7	8	9	10	11
Oil content (%)[b]	30.7	36.4	–[c]	10.9	13.4	8.4	13.2	10.5	13.8	46.2
Iodine value (Wijs)	63	57	61	87	96	79	80	88	88	56
Saponification value	193	194	195	192	192	190	190	193	194	193
Acidic value	0.6	0.7	2.5	2.0	2.8	9.7	3.6	2.1	3.2	1.1
Hydroxyl value	0.5	0.6	1.0	0.7	1.4	–[d]	–[d]	1.2	1.1	0.6
HBr-equiv. 3 C[e]	0.1	0.1	0.1	0.1	0.2	–[d]	0.5	0.3	0.1	0.2
HBr equiv. 55 C[f]	13.1	18.5	14.6	8.8	6.2	–[d]	1.8	3.8	7.5	11.7
Refractive index, n_D^{40}	1.4598	1.4604	1.4612	1.4633	1.1635	1.4621	1.4613	1.4629	1.4629	1.4590
Density, d_D^{40}	0.895	0.900	0.903	0.902	0.900	0.899	0.906	0.902	0.900	0.894
Halphen test	+	+	+	+	+	+	+	+	+	+

[a] *A. za* var. Boinensis.
[b] Oil content for the whole seed.
[c] Industrial oil sample.
[d] Not determined.
[e] Expressed as % vernolic acid.
[f] Expressed as % sterculic acid.

Proton Magnetic Resonance

The oils were dissolved (diluted 1:20, v/v) in a carbon tetrachloride/chloroform (90:10, v/v) mixture. A Perkin Elmer Model R 32 NMR Spectrometer (Norwalk, CT) was used for the analysis. The percentage CPEFA was calculated as previously described (27). Areas were measured using a planimeter because the 2 peaks of interest were too close together to allow satisfactory use of the instrument's integrator. Twice-repeated scanning of samples were made using a 250-cps sweep width.

RESULTS

Seeds of the various *Adansonia* species when extracted with petroleum ether gave 8-46% of oil (Table 2). For sample 6, oil content was very low (1.5%) because an immature fruit was investigated. The seed oils obtained are yellow fluids which crystallize in the refrigerator. The analytical values of oils are summarized in Table 2. de Bruin et al. (31) and, later, Bohannon and Kleiman (32) reported that *Pachira aquatica* (bombacaceae) seed oil contains α-hydroxy sterculic acid. The low results obtained for hydroxyl values (see Table 2) show that hydroxy acids are in minute amounts or absent in baobab oils. Hydrogen bromide titration at 3 C (25,26) revealed that epoxy acids are in very low concentration or nonexistent, because results expressed as vernolic acid (9,10-epoxy-oleic acid) are about 0.1-0.5%. Epoxy acids were not detected using the picric acid thin layer chromatography (TLC) test (33). All the oils gave a positive red-pink Halphen color test, showing the presence of a cyclopropenic group. The oils gave a characteristic infrared (IR) band at 1009-1010 cm^{-1} for the cyclopropene moiety and hydrogen bromide titration at 55 C (25,26) revealed that total cyclopropenoid material ranges from 1.8 to 18.5% (results expressed as sterculic acid, Table 2). Proton magnetic resonance (PMR) spectra showed proton singlet at 9.2 τ. There was no ultraviolet (UV) maximum in the range 220-230 nm, indicating no conjugated unsaturation. On the basis of these results, methyl esters were examined by GLC and GLC-MS.

GLC Analysis Using CPEFA Derivatives

Fatty acids methyl esters of the 11 samples of baobab seed oils were prepared by transmethylation of the oils. CPEFA were transformed into more stable derivatives using the method of Schneider et al. (23). For each CPEFA, 2 methoxy ether compounds and 2 ketonic compounds are formed, as shown in

Figure 1, from both sterculic and malvalic acids.

The derivatized methyl esters of 4 baobab seed oils, 1, 4, 9 and 11, were analyzed using glass capillary columns coated with Carbowax 20 M and BDS. The equivalent chain length (ECL) obtained for normal fatty acids were in good agreement with those given for Carbowax 20 M (34,35) and for BDS (36,37). Malvalic and sterculic acids each gave 2 peaks for the ether derivatives (18:CM_1 and 18:CM_2; 19:CM_1 and 19:CM_2) and 2 peaks for the ketonic derivatives (18:CC_1 and 18:CC_2; 19:CC_1 and 19:CC_2) as has been shown for cottonseed and kapok oils (24). The mass spectrum of the fatty acid methyl ester with ECL 19.27 on BDS showed M^+ at m/e 310 (calculated for $C_{20}H_{38}O_2$, MW 310) with other ions at m/e 278, 236, 194, 139, 97, 84, 69, 55 and 41. The presence of the ions at m/e 278 (M-CH_4O), 236 (M-$CH_2C(OH)CH_3$), 194 (M-$C_6H_{12}O_2$) suggests that this fatty acid methyl ester is methyl dihydrosterculate as reported by several authors (38-40). Under the present GLC conditions, the peaks of methyl dihydrosterculate (19:CA) and methyl linolenate (18:3ω3) could not be resolved on Carbowax 20 M (Table 3). The fatty acid composition is given in Table 3 and both sets of results obtained with the 2 glass capillary columns are in the same range.

GLC Analysis without Derivatization

Direct GLC analysis of total methyl esters, without previous CPEFA transformation, on BDS and Carbowax 20 M glass capillary columns permits the appearance of 2 new peaks which were identified using GLC-MS. The mass spectrum of the fatty acid methyl ester with ECL 17.94 on BDS and 17.92 on Carbowax 20 M showed M^+ at m/e 294 (calculated for $C_{19}H_{34}O_2$, MW 294) with other abundant ions at m/e 263, 223, 209, 164, 149, 123, 109, 95, 81, 68, 55 and 41. The fragmentation pattern is basically similar to that of authentic methyl malvalate (41). The mass spectrum of the methyl ester with ECL 18.90 on BDS and 18.97 on Carbowax 20 M gave M^+ at m/e 308 (calculated for $C_{20}H_{36}O_2$, MW 308) with other ions at m/e 277, 237, 193, 163, 137, 123, 109, 95, 81, 68, 55 and 41 and similar to that of methyl sterculate reported by Pawlowski et al. (41). Quantitative analysis results obtained for the 11 samples investigated are presented in Table 4 with BDS and in Table 5 with Carbowax 20 M columns. Note that in most cases, total CPEFA content is higher with BDS than with Carbowax 20 M columns. It

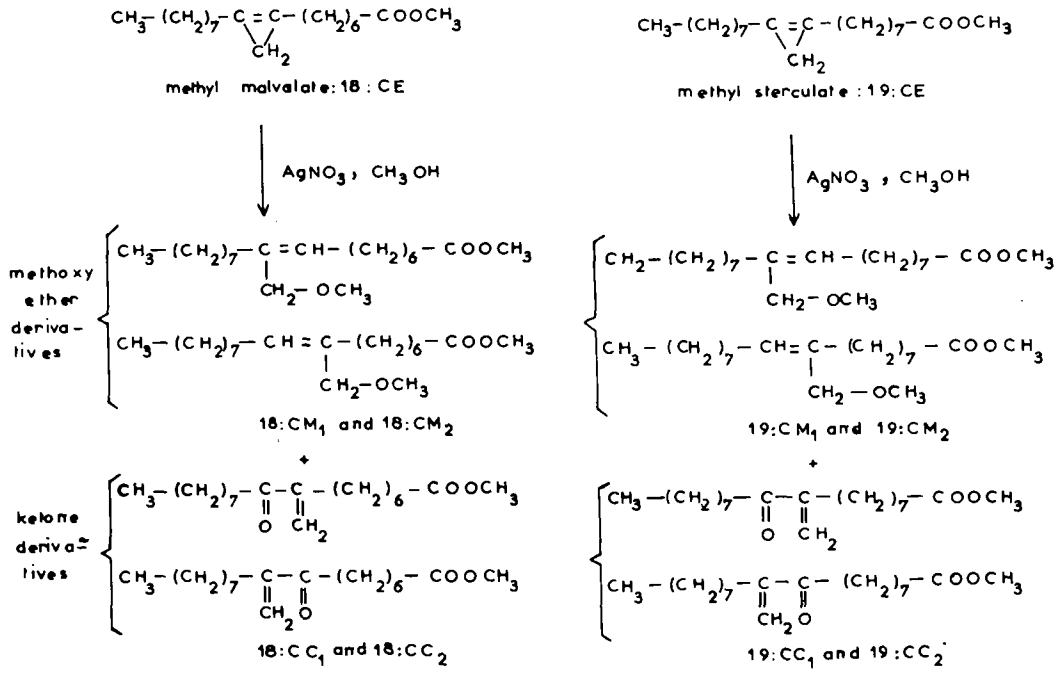


FIG. 1. Methoxy ether and ketone derivative formation of methyl malvalate and methyl sterculate by silver nitrate reaction in anhydrous methanol. Symbols used for the derivatives.

TABLE 3

Fatty Acid Composition[a] of Some Samples of *Adansonia* Seed Oils after Reaction with Silver Nitrate in Anhydrous Methanol

	ECL[c]		Oil samples							
			A. grandidieri 1		*A. za* 4		*A. fony* 9		*A. suarezensis* 11	
Fatty acid[b]	BDS[d]	Carbowax[e] 20 M	BDS	Carbowax 20 M	BDS	Carbowax 20 M	BDS	Carbowax 20 M	BDS	Carbowax 20 M
14:0	–	–	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.1
15:0	–	–	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
16:0	–	–	37.3	37.5	26.8	27.3	31.6	29.8	46.2	45.6
16:1	16.35	16.29	0.4	0.5	0.2	0.2	0.4	0.4	0.3	0.3
17:0	–	–	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3
17:1	17.25	17.21	0.4	0.4	0.6	0.6	0.5	0.5	0.8	0.7
17:2 ?	17.80	17.66	0.2	0.1	0.3	0.3	0.3	0.3	0.3	0.3
18:0	–	–	3.4	3.4	3.0	3.0	3.7	3.8	3.4	3.5
18:1ω9	18.27	18.21	23.2	21.7	29.8	29.3	30.6	32.5	21.2	21.2
18:1ω7	18.32	18.27	–	1.6		0.9	1.2		0.8	1.2
18:2ω6	18.82	18.66	15.5	15.8	22.6	22.3	20.0	20.4	12.2	12.5
19:CA[f]	19.27	19.26	4.8	5.1	5.5	6.6	3.5	4.4	1.8	2.2
18:3ω3	19.50		0.4		1.1		0.7		0.5	
20:0	–	–	1.0	0.7	0.7	0.7	1.0	1.0	0.6	0.5
20:1	20.16	20.13	0.1	0.1	0.2	0.2	0.2	0.2	0.1	tr
22:0	–	–	0.2	0.4	0.2	tr	0.1	0.1	tr	0.2
$18:CM_1$	20.70	20.37	2.7	2.4	2.6	2.4	2.0	1.9	3.3	3.1
$18:CM_2$	20.77	20.42	2.6	2.7	2.6	2.6	2.0	2.1	3.3	3.4
$19:CM_1$	21.65	21.34	3.6	3.0	1.3	1.2	0.7	0.8	1.9	1.8
$19:CM_2$	21.72	21.39	2.9	3.4	1.2	1.4	0.7	0.8	2.0	2.0
$18:CC_1$	22.33	21.70	0.5	0.3	0.5	0.3	0.2	0.3	0.6	tr
$18:CC_2$	22.67	21.82	tr	0.1	0.1	0.2	tr	0.3	tr	0.5
$19:CC_1$	23.50	22.70	0.1	0.2	0.1	tr	tr	tr	0.1	tr
$19:CC_2$	23.56	22.80	0.2	0.2	tr	0.1	tr	tr	0.1	0.5

[a]Percent by wt.

[b]The corresponding formula of CPEFA derivatives is given in Fig. 1.

[c]Equivalent chain length.

[d]BDS glass capillary column at 150 C.

[e]Carbowax 20 M glass capillary column at 190 C.

[f]19:CA:dihydrosterculic acid.

TABLE 4

Fatty Acid Analysis[a] of *Adansonia* Seed Oils by Direct GLC Analysis of Their Methyl Esters on BDS Glass Capillary Column at 150 C

		Oil samples										
		A. grandidieri			*A. za*			*A. digitata*		*A. fony*	*A. mad.*	*A. suar.*
Fatty acid[b]	ECL[c]	1	2	3	4	5[d]	6[e]	7	8	9	10	11
14:0	–	0.2	0.1	0.2	0.1	tr	0.3	0.1	0.3	0.2	0.1	0.1
15:0	–	0.1	0.1	0.1	tr	tr	tr	tr	0.1	0.1	tr	0.1
16:0	–	41.5	42.8	39.9	24.5	20.9	25.4	21.3	27.2	29.9	26.1	45.9
16:1	16.35	0.5	0.6	0.5	0.2	0.2	0.4	0.2	0.2	0.5	0.2	0.3
17:0	–	0.2	0.2	0.2	0.1	0.2	0.4	0.2	0.2	0.2	0.1	0.3
17:1	17.25	0.3	0.3	0.4	0.6	0.5	0.9	0.2	0.3	0.7	0.5	0.7
17:2 ?	17.80	0.1	0.1	0.1	0.4	0.4	0.5	0.2	0.1	0.4	0.3	0.3
18:CE	17.94	6.3	6.9	7.7	6.7	4.9	27.7	6.2	3.1	5.1	5.9	7.7
18:0	–	2.0	3.7	3.7	2.4	3.2	–	4.4	5.4	2.0	2.2	3.2
18:1ω9	18.27	19.8	21.0	21.1	28.8	27.6	15.0	39.9	36.1	29.7	27.0	21.3
18:1ω7	18.32	1.1	1.0	1.0	0.9	1.1	0.9	0.9	0.7	1.2	1.1	0.8
18:2ω6	18.82	14.1	12.2	14.5	25.6	32.4	20.9	18.7	21.7	23.3	28.8	12.1
19:CE	18.90	7.6	7.4	7.6	2.9	2.1	2.9	1.2	1.6	1.6	2.2	4.3
19:CA	19.27	4.4	1.8	1.8	4.6	4.5	2.2	5.1	1.5	2.6	4.0	1.7
18:3	19.50	0.2	0.1	0.2	1.4	1.3	1.7	0.3	0.1	1.0	0.7	0.5
20:0	–	0.6	0.7	0.9	0.4	0.4	0.4	0.7	1.0	0.7	0.4	0.5
20:1	20.16	0.1	0.1	0.1	0.1	0.1	0.3	0.2	0.1	0.1	0.1	0.1
22:0	–	0.1	0.1	0.1	0.1	0.1	tr	0.1	0.1	0.1	0.1	0.1
Total CPEFA[a]		13.9	14.3	15.3	9.6	7.0	30.6	7.4	4.7	6.7	8.1	12.0
Sterculic/malvalic	–	1.21	1.07	0.99	0.43	0.43	0.11	0.19	0.52	0.31	0.37	0.56
Sterculic/dihydrosterculic	–	1.73	4.11	4.75	0.63	0.47	1.32	0.23	1.07	0.61	0.55	2.53

[a]Percent by wt.

[b]18:CE, malvalic acid; 19:CE, sterculic acid; 19:CA, dihydrosterculic acid.

[c]Equivalent chain length.

[d]*A. za* var. Boinensis.

[e]*A. za* var. Bozy.

TABLE 5

Fatty Acid Analysis[a] of *Adansonia* Seed Oils by Direct GLC Analysis of Their Methyl Esters on Carbowax 20 M Glass Capillary Column at 190 C

Fatty acid[b]	ECL[c]	Oil samples										
		A. grandidieri			*A. za*			*A. digitata*		*A. fony*	*A. mad.*	*A. suar.*
		1	2	3	4	5[d]	6[e]	7	8	9	10	11
14:0	–	0.1	0.1	0.2	0.1	tr	tr	tr	0.2	0.1	0.1	0.1
15:0	–	0.2	0.1	0.1	0.1	tr	tr	tr	–	0.1	tr	0.1
16:0	–	37.5	41.8	40.6	26.3	19.7	23.6	21.3	27.2	27.5	26.2	46.7
16:1	16.29	0.5	0.7	0.5	0.2	0.1	–	0.3	0.2	0.5	0.3	0.4
17:0	–	0.2	0.1	0.1	0.2	0.2	0.3	0.1	0.2	0.2	0.1	0.3
17:1	17.21	0.3	0.4	0.4	0.6	0.4	0.7	0.2	0.2	0.6	0.5	0.7
17:2 ?	17.66	0.2	0.1	0.2	0.4	0.4	0.4	0.2	0.1	0.4	0.3	0.3
18:CE	17.92	6.0	6.1	6.5	5.7	4.9	31.0	5.0	3.2	4.3	5.1	6.4
18:0	–	3.4	4.7	4.5	2.8	3.6	2.0	5.4	5.8	3.8	2.8	3.9
18:1ω9	18.21	21.7	21.9	21.0	29.1	27.6	13.2	42.5	36.8	29.9	26.5	21.2
18:1ω7	18.27	1.4	1.5	1.4	1.1	1.4	1.2	–	–	2.0	1.7	1.3
18:2ω6	18.66	15.0	12.4	14.6	23.5	32.8	19.9	18.2	21.6	24.1	29.0	12.1
19:CE	18.87	6.7	6.9	6.9	2.6	2.4	3.0	1.0	1.9	1.5	2.1	3.9
19:CA + 18:3	19.26	5.0	2.1	2.0	6.5	5.9	3.3	5.6	1.6	3.9	4.7	2.2
20:0	–	0.8	0.8	0.8	0.6	0.4	0.3	0.2	0.7	0.8	0.4	0.4
20:1	20.13	0.1	0.1	0.1	0.2	0.1	0.3	tr	0.1	0.1	0.1	tr
22:0	–	0.4	0.2	0.1	tr	0.1	0.4	tr	0.2	0.2	0.1	tr
Total CPEFA[a]	–	12.7	13.0	13.4	8.3	7.3	34.0	6.0	5.1	5.8	7.2	10.3
Sterculic/malvalic	–	1.12	1.13	1.06	0.46	0.49	0.11	0.20	0.60	0.35	0.41	0.61

[a]Percent by wt.

[b]18:CE, malvalic acid; 19:CE, sterculic acid; 19:CA, dihydrosterculic acid.

[c]Equivalent chain length.

[d]*A. za* var. Boinensis.

[e]*A. za* var. Bozy.

was shown in a previous paper (24) that temperature effects the CPEFA stability and that a better temperature for Carbowax 20 M would be 170 C.

Comparative Results of CPEFA Determination

CPEFA quantitation has long been a problem because of instability of their methyl esters on the GLC columns (23). Coleman (42) reviewed 5 methods for determination of cyclopropenoid fatty acids. Among them, we chose Durbetaki's method (25) with hydrobromic acid at 55 C, the PMR method of Pawlowski et al. (27) and compared the results obtained with those given by GLC using direct analysis or after derivatization of CPEFA. Results obtained for the 11 samples of *Adansonia* species are presented in Table 6. Notable is that the results given by our method are in good agreement with those given by the others. These show that the direct GLC method appears as an accurate and rapid method to simultaneously assay CPEFA and CPAFA methyl esters.

DISCUSSION

Among the 18 fatty acids investigated in the 6 baobab species, one can observe the presence of odd-numbered fatty acids such as pentadecanoic, heptadecanoic, heptadecenoic and heptadecadienoic acids. It should be noted that presence of such odd-numbered fatty acids was recently reported in various species of, e.g., Bombacaceae, Malvaceae, Sterculiaceae, Sapotaceae, Ebenaceae and Anacardiaceae (6,32). In all the cases, these fatty acids are found in small amounts (0.1-0.6%). Although epoxy and hydroxy acids occur in the Bombacaceae family (32), these acids were absent in the *Andansonia* genus. The same fatty acids were found in all the species and the most important were palmitic (21-46%), oleic (15-40%), linoleic (12-32%), malvalic (2-28%) and sterculic (1-8%) acids. The finding in sample 6 of an abnormally large amount of malvalic acid (*A. za* var. Bozy: 28-31%, see Tables 4 and 5) may have resulted because it was an immature fruit, and it is known that fatty acid composition evolves during seed maturation (43). On the other hand, fatty acid composition shows very little variation in the same species (as *A. grandidieri* samples 1, 2, 3 and *A. digitata* samples 7 and 8).

Few results have been obtained in the search for a relationship between biochemical evidence and the taxonomical arrangement of the *Adansonia* genus. In a caryologic study of 8 *Adansonia* species, Miege and Burdet (44)

TABLE 6

Comparative Composition of CPEFA of *Adansonia* Seed Oil Using Various Methods

Method	Column	Temp. (C)	Oil samples[a]								
			A. grandidieri			*A. za*		*A. dig.*	*A. fony*	*A. mada.*	*A. suar.*
			1	2	3	4	5[b]	8	9	10	11
HBr equivalent	–	55	13.1	10.5	14.6	8.8	6.2	1.8	3.8	7.5	11.7
PMR	–	Ambient	13.7	14.8	14.7	8.9	8.2	3.3	4.1	5.8	12.1
GLC after	BDS	150	12.6	–	–	8.4	–	–	5.6	–	11.3
derivatization	Carbowax 20 M	190	12.3	–	–	8.2	–	–	6.2	–	11.3
GLC	BDS	150	13.9	14.3	15.3	9.6	7.0	4.7	6.7	8.1	12.0
(Direct analysis)	Carbowax 20 M	190	12.7	13.0	13.4	8.3	7.3	5.1	5.8	7.2	10.3

[a]Percent by wt of CPEFA.
[b]*A. za* var. Boinensis.

and Miege (45) showed that there was a polyploid series between the species, but in a later work, Miege ascertained that the increasing complexity of the seed proteins of 4 species (*A. digitata*, *A. fony*, *A. grandidieri* and *A. za*) was related to the increases in chromosome number (46). We can remark, taking into account the variation in fatty acid composition, that it is possible to make some distinction between the various species. A content of palmitic acid near or above 40% is characteristic of *A. grandidieri* and *A. suarezensis* species. *A. digitata* species of Madagascar (sample 7) and of Africa (sample 8) are characterized by a higher content of oleic acid (35-40%). *A. za*, *A. madagascariensis* and *A. fony* species have almost equal proportions of palmitic, oleic and linoleic acids, which is about 25-30% (Tables 4 and 5). The content of malvalic, sterculic and dihydrosterculic acids vary in a notable manner from one species to another. It was shown by Yano et al. (47) that the biosynthetic pathway of CPEFA and CPAFA involves the initial formation of dihydrosterculic acid from oleic acid, with subsequent desaturation to sterculic acid, and α-oxidation to malvalic acid. It is possible to make some distinction between the various species taking into account the sterculic/malvalic ratio (Table 4). *A. grandidieri* could be distinguished from *A. suarezensis* because the sterculic/malvalic ratio goes from 1.0-1.2 to 0.6, respectively. It seems more difficult to use this ratio to distinguish *A. za*, *A. madagascariensis* and *A. fony* because the variation is slight (0.11-0.52) and large variation may be observed in the same species (7:0.19; 8:0.52). The sterculic/dihydrosterculic ratio seems even more difficult to use (Table 4) if one considers the variation within the same species, such as *A. grandidieri* (1.73-4.75).

ACKNOWLEDGMENTS

The authors are grateful to P. Garnier for providing seeds of African *A. Digitata*; P. Rakotomanga and the "Service des Eaux et Forêts" of Morondava, Mahajanga and Antiserana (Madagascar) for help in the collection of the 6 species of *Adansonia* seeds. *A. za* var. Boinensis was kindly supplied by M. Ralaivao. GLC-MS analyses were performed with the help of Dr. Gerster, Department de Biologie, Service de Radioagronomie, Cadarache (France). The Fonds d'Aide et de Coopération (F.A.C.) from France afforded a grant to A. Ralaimanarivo who carried out this work at the "Ecole Supérieure de Chimie de Marseille."

REFERENCES

1. Carter, F.L., and Frampton, V.L. (1964) Chem. Rev. 64, 497-525.
2. Phelps, R.A., Shenstone, F.S., Kemmerer, A.R., and Evans, R.J. (1965) Poult. Sci. 44, 358-394.
3. Recourt, J.H., Jurriens, G., and Schmitz, M. (1967) J. Chromatogr. 30, 35-42.
4. Smith, C.R., Jr. (1970) in Progress in the Chemistry of Fats and Other Lipids (Holman, R.T., ed.) Vol. 11, Part I, pp. 139-177, Pergamon Press, New York, NY.
5. Christie, W.W. (1970) in Topics in Lipid Chemistry (Gunstone, F.D., ed.) Vol. 1, pp. 1-49, Logos Press ltd, London.
6. Vickery, J.R. (1980) J. Am. Oil Chem. Soc. 57, 87-91.
7. Smith, C.R., Jr., Wilson, T.L., and Mikolajczak, K.L. (1961) Chem. Ind. (London) 256-258.
8. Wilson, T.L., Smith, C.R., Jr., and Mikolajczak, K.L. (1961) J. Am. Oil Chem. Soc. 38, 696-699.
9. Lie Ken Jie, M.S.F., and Chan, M.F. (1977) J. Chem. Soc. Chem. Commun. 3, 78.
10. Kleiman, R., Earle, F.R., and Wolff, I.A. (1969) Lipids 4, 317-320.
11. Abou-Ashour, A.M., and Edwards, H.M. (1970) J. Nutr. 100, 1347-1352.
12. Roehm, J.N., Lee, D.J., Wales, J.H., Polityka, S.D., and Sinnhuber, R.O. (1970) Lipids 5, 80-84.
13. Lee, D.J., Wales, J.H., and Sinnhuber, R.O. (1971) Cancer Res. 31, 960-963.
14. Sinnhuber, R.O., Lee, D.J., Wales, J.H., and Ayres, J.L. (1968) J. Natl. Cancer Inst. 41, 1293-1301.
15. Thomas, V., and Boiry, F. (1913) Bull. Soc. Chim. Fr. 13, 827-832.
16. Cmelik, S.H.W. (1963) J. Sci. Food Agric. 14, 287-291.
17. Gaydou, E.M., Bianchini, J.P.,and Ralaimanarivo, A. (1979) Rev. Fr. Corps Gras 26, 447-448.
18. Milliau, E. (1904) C.R. Acad. Sci. 139, 807-808.
19. Milliau, E. (1910) Bull. Halles, through Matieres Grasses 2, 1545 (1910); Chem. Abstr. 4, 1655.
20. Pavolini, T., and Pavolini, L. (1941) Olii Miner. Grassi Saponi Colori Vernici 21, 233 (1942); Chem. Abstr., 36, 1203[1].
21. Gaydou, E.M., Bianchini, J.P., and Ralaimanarivo, A. (1981) Fette Seifen Anstrichm., in press.
22. Perrier de la Bathie, H., and Hochreutiner, B.P.G. (1955) in Flore de Madagascar et des Comores (Humbert, H., ed.) Vol. 130, pp. 1-17, Museum, Paris.
23. Schneider, E.L., Loke, S.P., and Hopkins, D.T. (1968) J. Am. Oil Chem. Soc. 45, 585-580.
24. Bianchini, J.P., Ralaimanarivo, A., and Gaydou, E.M. (1981) Anal. Chem., in press.
25. Durbetaki, A.J. (1956) Anal. Chem. 28, 2000-2001.
26. Harris, J.A., Magne, F.C., and Skau, E.L. (1963) J. Am. Oil Chem. Soc. 40, 718-720.
27. Pawlowski, N.E., Nixon, J.E., and Sinnhuber, R.O. (1972) J. Am. Oil Chem. Soc. 49, 387-392.
28. Wolff, J.P. (1968) Manuel d'Analyse des Corps Gras, Azoulay, Paris.
29. Halphen, G. (1897) J. Pharm. 6, 390-392.
30. Luddy, F.E., Barford, R.A., and Riemen-Schneider, R.W. (1960) J. Am. Oil Chem. Soc. 37, 447-451.
31. de Bruin, A., Heesterman, J.E., and Mills, M.R. (1963) J. Sci. Food Agric. 14, 757-760.
32. Bohannon, M.B., and Kleiman, R. (1978) Lipids 13, 270-273.
33. Fioriti, J.A., and Sims, R.J. (1968) J. Chromatogr. 32, 761-763.
34. Flanzy, J., Boudon, M., Leger, C., and Pihet, J. (1976) J. Chromatogr. Sci. 14, 17-24.

35. Mordret, F., Prevot, A., LeBarbanchon, N., and Barbati, C. (1977) Rev. Fr. Corps Gras 24, 467-475.
36. Ackman, R.G. (1972) in Progress in the Chemistry of Fats and Other Lipids (Holman, R.T., ed.) Vol. 12, pp. 165-284, Pergamon Press, Oxford.
37. Jamieson, G.R. (1970) in Topics in Lipid Chemistry (Gunstone, F.D., ed.) Vol. 1, pp. 107-159, Logos Press Ltd., London.
38. Hallgren, B., Ryhage, R., and Stenhagen, E. (1959) Acta Chem. Scand. 13, 845-847.
39. Wood, R., and Reiser, R. (1965) J. Am. Oil Chem. Soc. 42, 315-320.
40. Christie, W.W., and Holman, R.T. (1966) Lipids 1, 176-182.
41. Pawlowski, N.E., Eisele, T.A., Lee, D.J., Nixon, J.E., and Sinnhuber, R.O. (1974) Chem. Phys. Lipids 13, 164-172.
42. Coleman, E.C. (1970) J. Assoc. Off. Anal. Chem. 53, 1209-1213.
43. Yano, I., Nichols, B.W., Morris, L.J., and James, A.T. (1972) Lipids 7, 30-34.
44. Miege, J., and Burdet, H. (1968) Candollea 23, 59-66.
45. Miege, J. (1974) Candollea 29, 457-475.
46. Miege, J. (1975) Biossiera 24, 345-352.
47. Yano, I., Morris, L.J., Nichols, B.W., and James, A.T. (1972) Lipids 7, 35-45.

[Received May 11, 1981]

Photosensitized Oxidation of Methyl Linoleate: Secondary and Volatile Thermal Decomposition Products[1]

E.N. FRANKEL*, W.E. NEFF, E. SELKE and D. WEISLEDER, *Nothern Regional Research Center, Agricultural Research, U.S. Department of Agriculture, Peoria, IL 61604*

ABSTRACT

Studies of photosensitized oxidation of methyl linoleate show that the greater relative concentration of 9- and 13-hydroperoxides than 10- and 12-hydroperoxides is characteristic of singlet oxygenation and not due to either simultaneous autoxidation or type 1 photosensitized oxidation. Cyclization of the internal 10- and 12-hydroperoxides accounts for their lower relative concentrations. Secondary products separated by silicic acid and high pressure liquid chromatography were characterized spectrally (IR, UV, ^{1}H-NMR, ^{13}C-NMR, GC-MS). Major secondary products included diastereomeric pairs of 13-hydroperoxy-10,12-epidioxy-*trans*-8-octadecenoate (I and III) and 9-hydroperoxy-10,12-epidioxy-*trans*-13-octadecenoate (II and IV); minor secondary products included hydroperoxy oxygenated and epoxy esters. Thermal decomposition of the hydroperoxy cyclic peroxides produced hexanal and methyl 10-oxo-8-decenoate as major volatiles from I and III and methyl 9-oxo-nonanoate and 2-heptenal from II and IV. Hydroperoxy cyclic peroxides may be important sources of volatile decomposition products of photooxidized fats.
Lipids 17:11-18, 1981.

There is evidence that oxygen activated in the singlet state ($^1O_2$) is involved in many biological processes (1-4) and that its reaction products may play a part in initiating the normal free radical autoxidation of unsaturated fats (5-7). For these reasons, many studies have been published recently on the reactions of $^1O_2$ with unsaturated fatty acids (8-14), and several reviews have also appeared (4,15-18).

According to the "ene" addition mechanism recognized for the reaction of $^1O_2$ with unsaturated compounds (19-21), concerted addition would occur at each unsaturated carbon of the fatty acid chains. Some reports (10,13) indicate that the oxidation of methyl linoleate with $^1O_2$ forms conjugated (9- + 13-OOH) and unconjugated (10- + 12-OOH) hydroperoxides in approximately equal amounts; but other reports (6,11,12,14) show a product ratio favoring the conjugated hydroperoxides. With linolenate, we found an isomeric distribution favoring the external 9- and 16-hydroperoxides (23-25%) over the internal 10-, 12-, 13- and 15-hydroperoxides (12-14%) (12). These studies using methylene blue as photosensitizer were fully confirmed by those of Terao and Matsushita (14,16) using chlorophyll as photosensitizer.

In addition to hydroperoxidation, other reactions of $^1O_2$ with unsaturated fatty acids have been investigated. Geometric isomerization of *trans,cis* to *trans,trans* conjugated hydroperoxides has been reported in the chlorophyll-sensitized photooxygenation of *cis*-4,*cis*-7-undecadiene, studied as a model of methyl linoleate (6). Secondary oxidation products that have been recently reported from methyl linoleate treated with $^1O_2$ include di- and trioxygenated compounds (14), hydroperoxy-cyclic peroxides and an unsaturated epoxy alcohol (22). Our previous studies identified hydroperoxy cyclic peroxides as major, and dihydroperoxides as minor secondary products in autoxidized methyl linolenate (23). Hydroperoxy cyclic peroxides from oxidized linoleate and linolenate were also implicated as important precursors of volatile decomposition products (24). This paper presents studies of the secondary products of photosensitized oxidation of methyl linoleate showing that cyclization of the internal 10- and 12-hydroperoxides accounts for their lower relative concentration than the external 9- and 13-hydroperoxides. Decomposition of the resulting hydroperoxy cyclic peroxides contributes to the volatiles in photooxidized methyl linoleate.

EXPERIMENTAL

Pure methyl linoleate (100% by GLC) was prepared by counter double current distribution (25) of safflower methyl esters followed by silicic acid chromatography and vacuum distillation. Silicic acid chromatography with hexane elution was repeated on small samples (1-2 g) of methyl linoleate just before each oxidation to remove traces of peroxides, as detected by peroxide value determination and by TLC. The same reducing agent (Ph_3P bonded

*Address correspondence to: Dr. E.N. Frankel, Northern Regional Research Center, 1815 N. University St., Peoria, IL 61604.

[1]Presented at 72nd Annual Meeting of the American Oil Chemists Society, New Orleans, LA, May 1981.

on styrene-divinyl benzene copolymer) and silylating reagent were used as previously described (23). Procedures for reduction, catalytic hydrogenation and silylation were also the same.

Photosensitized oxidations were done on samples free of peroxides in CH_2Cl_2 solution by bubbling O_2 at 0 C using 10 mg methylene blue/g of methyl linoleate in an open tube exposed to a 1,000-W, air-cooled tungsten light source through a 1-in. layer of water to filter IR radiation. Alternate light and dark exposures were achieved by interrupting the irradiation and sealing the reactor with aluminum foil to avoid stray light exposure. Rates of oxidation were followed by peroxide value determinations (AOCS Method Cd 8-53).

Oxidized samples were first separated on a silicic acid chromatographic column with diethyl ether/hexane eluants (26). Selected fractions were further separated by high pressure liquid chromatography (HPLC) with a 50 x 0.94 cm, 10-μ silica column (Magnum-9, Partisil 10, Whatman, Inc., Clifton, NJ). The solvent pumped at 27 C and a rate of 1 ml/min consisted of a mixture of hexane/methylene chloride/ethyl acetate (6:4:1, v/v/v). A refractive index detector was used and sample size varied from 15 to 20 mg.

Purified oxidation products were characterized by infrared (IR), gas chromatography-mass spectrometry (GC-MS), ^{1}H-NMR and ^{13}C-NMR, by the same methodology described previously (23,27). TLC was done on Silica Gel 60 plates with fluorescent indicator (E. Merck, Darmstadt, Germany) with the same developing solvent system as used for HPLC. Isomeric hydroperoxide analyses were made by GC-MS (28) after hydrogenation and silylation. Hydroperoxycyclic peroxide fractions were decomposed in the injector port of a gas chromatograph at 210 C (24). The volatile decomposition products were identified by computerized GC-MS and GC-retention (29), but the GC conditions were different: 3% J X R stationary phase in a 6 ft x 1/8 in. glass column, 0 to 250 C at 2 C/min temperature program, 20 ml/min flow rate was used.

RESULTS AND DISCUSSION

Isomeric Hydroperoxide Analysis

Because both oxidation of methyl linoleate with $^1O_2$ and free radical autoxidation produce the same conjugated 9- and 13-hydroperoxides, the isomeric ratio of hydroperoxides can vary if these 2 processes occur concurrently and if there is a photosensitized free radical process. Two reports (11,14) show a change in isomeric ratio with level of oxidation, and Thomas and Pryor (13) suggested that this change is due to excited-state dye-hydroperoxide interactions. Some photosensitizers, such as riboflavin, also produce the same isomeric hydroperoxides in linolenate as the free radical autoxidation, by a type 1 mechanism involving diene radicals (9). In contrast to type 2 photooxidation, in which the sensitizer reacts with O_2, in type 1 photooxidation, the sensitizer reacts with the substrate usually with the formation of free radicals (19-21).

To determine whether the relatively high concentration of 9- and 13-hydroperoxides compared to the 10- and 12-hydroperoxides might be due to either concurrent autoxidation or type 1 photosensitization, oxidation of linoleate in the presence of methylene blue was measured during successive dark and light periods. The almost exclusive occurrence of sensitized photooxidation was demonstrated as no oxidation occurred in the dark periods (Fig. 1). The oxidation level either decreased slightly or remained essentially unchanged during each successive dark period. Samples of linoleate photooxidized in the presence of methylene blue were further analyzed for isomeric hydroperoxides by GC-MS at a wider range of oxidation levels than previously reported (12,14). Results in Table 1 show a consistently higher ratio of the 9- and 13-hydroperoxides than of the 10- and 12-hydroperoxides (1.4:2.3) at *all* peroxide levels studied between 149 and 1,567. It is concluded, therefore, that the greater concentration of the 9- and 13-hydroperoxides compared to the 10- and 12-hydroperoxides is not due to either simultaneous autoxidation or type 1 photosensitized oxidation. This isomeric distri-

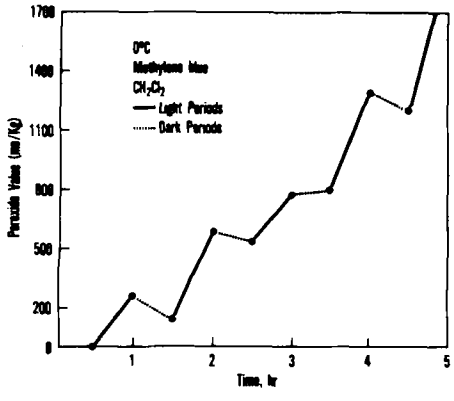


FIG. 1. Methylene blue-sensitized photooxidation of methyl linoleate. Illumination switched on and off as indicated.

TABLE 1

GC-MS Analysis of Isomeric Hydroxystearates[a] from Photosensitized-Oxidized Methyl Linoleate[b]

Time (hr)	Peroxide value (me/kg)	Relative %			
		9-OH	10-OH	12-OH	13-OH
0.5	149	32	15	16	37
1.5	544	28	19	21	32
2.5	743	28	19	21	32
3.5	1,129	25	17	22	36
4.5	1,567	25	19	23	33

[a]From hydroperoxides after hydrogenation and silylation (28).
[b]Conditions given in Experimental section.

bution is clearly characteristic of oxidation with $^1O_2$; i.e., both conjugated and nonconjugated hydroperoxides are unique $^1O_2$ products.

Hydroperoxy Cyclic Peroxides

An answer to the question of why the isomeric ratio of hydroperoxides did not conform to the statistical distribution expected from the concerted ene addition mechanism for $^1O_2$ (19-21) was sought by a study of the secondary photosensitized oxidation products of linoleate. Our previous studies on the autoxidation of methyl linolenate indicated that the lower relative concentration of the internal 12- and 13-hydroperoxides could be accounted for by their cyclization into hydroperoxy cyclic peroxides (23). The internal 10- and 12-hydroperoxides from linoleate oxidized with $^1O_2$ have the same homoallylic *cis* unsaturation required for cyclization as the corresponding internal 12- and 13-hydroperoxides of linolenate. Indeed, a mixture of hydroperoxy cyclic peroxides was recently identified by Mihelich (22) in sensitized photooxidized methyl linoleate, but the positional isomers of these hydroperoxy cyclic peroxides were not separated. In the present work, 4 isomeric hydroperoxy cyclic peroxides were completely separated and identified. By achieving this separation, we were able to trace the origin of the hydroperoxy cyclic peroxides and study their role as precursors of volatiles (see section on Volatile Decomposition Products).

A sample of methyl linoleate subjected to sensitized photooxidation was analyzed by GC after hydrogenation and silylation. Although no quantitative estimate can be made by GC of the hydrogenated silyl derivatives, the chromatogram in Figure 2 shows peaks due to epoxy-, hydroxy-, dihydroxy- and trihydroxystearates (28). The hydroxy- and dihydroxystearates come from mono- and dihydroperoxides (see section on Hydroperoxy-Oxygenated Derivatives). Trihydroxystearates come from the hydroperoxy cyclic peroxides (28). Partial resolution of the peak due to trihydroxysterate indicates the presence of 2 positional isomers that were identified below by GC-MS.

Samples of methyl linoleate photooxidized at different peroxide levels were fractionated by silicic acid column chromatography. Various oxidation products were separated and identified by TLC on the basis of functional groups by comparison with previously characterized compounds (27,28). Quantitative analyses show that the hydroperoxy cyclic peroxides are major, and hydroperoxy-oxygenated compounds are minor secondary oxidation products (Table 2). These secondary and other unidentified polar materials increased in concentration with the level of oxidation. At peroxide values above 2,000, the proportion of hydroperoxy cyclic peroxides was ca. one-third the concentration of monohydroperoxides.

Silicic acid chromatographic fractions (eluted

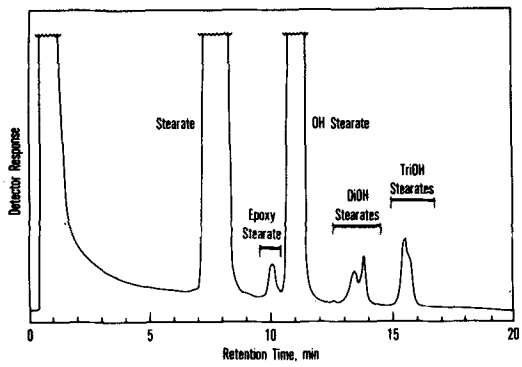


FIG. 2. Gas chromatogram of hydrogenated-sensitized-photooxidized methyl linoleate (TMS ether), peroxide value 1383. Conditions: see ref. 27.

TABLE 2

Weight-Percent Composition of Silicic Acid Fractions from Photosensitized-Oxidized Methyl Linoleate at 0 C

Compounds	Peroxide value (me/kg)			
	863	1947	2262	2543
	Weight (%)			
Linoleate	85	70.2	62.8	51.9
Epoxy esters	0.3	1.2	0.3	0.4
Monohydroperoxides[a]	12.6	24.3	30.3	30.7
Hydroperoxy cyclic peroxides	1.3	2.8	3.6	9.7
Hydroperoxy-oxygenated cpds	0.3	0.9	1.5	5.3
Polar material (?)	0.5	0.6	1.5	2.0

[a]9 + 10 + 12 + 13-OOH (12).

with 1:1 ether/hexane mixture) containing the hydroperoxy cyclic peroxides were further resolved by HPLC into 4 components (2 positional and 2 diastereoisomers) with the ternary hexane/methylene chloride/ethyl acetate, solvent system (Fig. 3). Cyclic peroxide fractions I-IV were characterized spectrally, and the structures established are given in Figure 4. The pairs of diastereoisomers I-III and II-IV differ by the configuration of the hydroperoxy group relative to that of the cyclic peroxide ring. The stereochemistry shown is based on the results of Mihelich (22), assuming that the more polar diastereoisomer produces, upon hydrogenation, the all R methyl 9,10,12-trihydroxystearate.

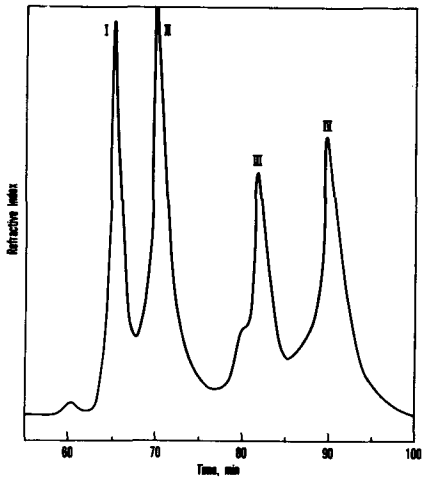


FIG. 3. Microporous silica HPLC chromatogram of hydroperoxy-cyclic peroxide mixture from linoleate photosensitized oxidation at 0 C, peroxide value 2543 (flow 1.0 ml/min, mobile solvent: 6:4:1, hexane/methylene chloride/ethyl acetate, refractive index detector).

TLC (6:4:1, hexane/methylene chloride/ethyl acetate) showed 4 UV inactive spots of respective R_f 0.45, 0.41, 0.32 and 0.29 for cyclic peroxide fractions I, II, III and IV. IR (CS_2) of all fractions: 3530 (free OH or OOH), 3700-3150 (bonded C-OH or C-OOH), 968 (isolated *trans* unsaturation) cm^{-1}.

Studies of ^{1}H-NMR (Table 3) and ^{13}C-NMR (Table 4) proved the identity of each individual hydroperoxy cyclic peroxide diastereoisomers (I-IV) and confirmed those reported by Mihelich (22) for mixtures of positional isomers. Although the positional isomers I-II and III-IV could not be distinguished by their ^{1}H-NMR, the diastereoisomers I-III and II-IV could be differentiated by the shifts of the protons on carbons bearing the hydroperoxy groups (3.93 and 4.20 ppm) on one hand, and on the methylene proton C-11α (2.13 and 2.41 ppm),

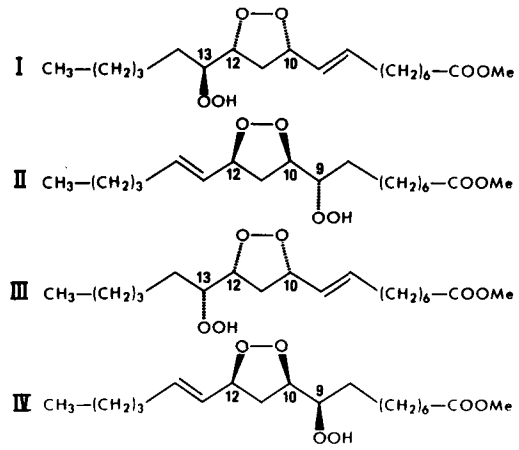


FIG. 4. Structures of hydroperoxy cyclic peroxide fractions isolated by HPLC (see Fig. 3). Each structure shown consists of pairs of enantiomers and only one isomer is shown.

TABLE 3

^{1}H-NMR of Methyl 13-Hydroperoxy-10,12-epidioxy-*trans*-8-octadecenoate (I,III) and 9-Hydroperoxy-10,12-epidioxy-*trans*-13-octadecenoate (II,IV),[a] δ ppm, J Hz

I	III	II	IV	Multiplicity[b]	Protons	I,III; II,IV	Assignment I,III	Assignment II,IV
10.21	9.71	9.58	9.53	br s	1	–	13-OOH	9-OOH
5.91	5.87	5.91	5.89	dt	1	J: 6.5; 15	H-8	H-14
5.45	5.40	5.45	5.40	dd	1	J: 7.5	H-9	H-13
4.70	4.66	4.69	4.66	q	1	J: 7.5	H-10	H-12
4.45	4.45	4.44	4.45	ddd	1	J: 8,6,7; 3,5,8	H-12	H-10
4.20	3.94	4.17	3.93	m	1	–	H-13	H-9
3.67	3.66	3.66	3.66	s	3	–	OCH_3	
2.78	2.83	2.77	2.82	ddd	1	J: 12.5	H-11β	
2.41	2.13	2.40	2.13	ddd	1	–	H-11α	

[a]See Fig. 4 for structures.

[b]Multiplicity: br = broad, s = singlet, d = doublet, q = quartet, m = multiplet.

on the other hand. The coupling constant for the proton on the carbon bearing the hydroperoxy and the adjacent proton on the ring carbon is also significantly different for the 2 diastereoisomers (Table 3). The resonance on methylene C-11 (δ 40.8-43.3 ppm) was the only signal showing a significant difference between the pairs of diastereoisomers I-III and II-IV (Table 4). Small differences are apparent between positional isomers in the resonances due to oxygenated C-10 and C-12 (0.5 and 1.1 ppm) and due to C-14 (I and III) and C-8 (II and IV) α to the hydroperoxide group (0.8 and 0.9 ppm).

Further identification of positional isomers I-II and III-IV was based on GC-MS of hydrogenated and reduced hydroperoxy cyclic peroxides. The hydrogenated derivatives of I and III after silylation produced the same MS fragments as 10,12,13-triOTMS-stearate, C-10: m/e (rel intensity) 273 (100) and C-13: 173 (47). The corresponding hydrogenated derivatives of II and IV after silylation produced 9,10,12-triOTMS-stearate, C-9: 259 (38) and C-12: 187 (100) (28). After reduction with polymer-bound Ph_3P, molecular related peaks were very weak: 414 (M^+, 0.05), 398 (M-16, s), 382 (M-32, 1). Cleavage at the carbon-bearing

TABLE 4

^{13}C-NMR of 13-Hydroperoxy-10,12-epidioxy-*trans*-8-octadecenoate (I,III) and 9-Hydroperoxy-10,12-epidioxy-*trans*-13-octadecenoate (II,IV)[a], δ ppm

I	III	C-Assignment	II	IV	C-Assignment
174.3	174.4	1	174.4	174.4	1
34.1	34.1	2	34.1	34.1	2
24.9	24.9	3	24.9	24.9	3
28.9	28.8	4,5,6	29.6	29.3	4,5,6
28.7	28.5		29.2	29.1	
28.5			29.0	28.7	
32.3	32.3	7	32.1	32.1	15
139.3	139.0	8,9	139.5	139.3	13,14
124.2	124.4		124.0	124.2	
83.3	82.9	10	83.8	84.0	12
40.8	43.3	11	40.9	43.3	11
84.0	84.1	12	83.3	82.9	10
86.1	85.9	13	86.1	85.9	9
31.7	31.8	14	30.9	30.9	8
25.5	25.3	15	25.7	25.6	7
31.7	31.8	16	32.1	32.1	16
22.5	22.6	17	22.5	22.2	17
14.0	14.0	18	13.8	13.9	18
51.4	51.5	OCH_3	51.8	51.5	OCH_3

[a]See Fig. 4 for structures.

OTMS produced base peaks at C-9: 259 (100) for II and IV and at C-13: 173 (100) for I and III.

The mechanism for cyclization of internal 10-hydroperoxide is depicted in Figure 5. This free radical cyclization is based on previous studies with autoxidized linolenate (30), autoxidized, enzymatically produced 13-linolenate hydroperoxide (31), and photosensitized-oxidized linoleate (22). The structural feature present in each precursor of cyclic peroxides is a *cis*-double bond homoallylic to the carbon bearing the hydroperoxides. Cyclization of the internal 10- and 12-hydroperoxides having this structural feature accounts for their significantly lower concentration than the conjugated external 9- and 13-hydroperoxides (see section on Isomeric Hydroperoxide Analysis). If molar concentrations for hydroperoxy cyclic peroxides (Table 2) are added to those of 10- and 12-hydroperoxides (Table 1), the calculated ratios for 9 + 13/10 + 12-isomers approaches 1. Our observation that this isomeric ratio favors the conjugated 9- and 13-hydroperoxides at all levels of oxidation studied (Table 1) indicates that cyclization is a rapid process. Further evidence for this rapid cyclization is provided by the relatively high proportion of the cyclic peroxides among the secondary oxidation products (Table 2), in agreement with our GC-analyses showing trihydroxystearates (derived from cyclic peroxides I-IV) as important secondary oxidation products (Fig. 2). Furthermore, our evidence in support of an independent, not photosensitized, free radical process (Fig. 5) is based on our observation that chromatographically purified monohydroperoxides from photosensitized-oxidized methyl linoleate form significant amounts of hydroperoxy cyclic peroxides upon standing in the dark at 0-5 C (as detected by TLC and HPLC).

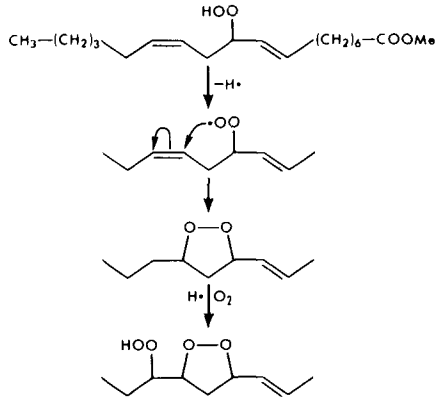


FIG. 5. Mechanism for cyclization of internal 10-hydroperoxide from photosensitized-oxidized methyl linoleate.

Hydroperoxy-Oxygenated Derivatives

A more polar fraction than the hydroperoxy cyclic peroxides separated by silicic acid column chromatography (3:2, ethyl ether/hexane) was identified as due to a mixture of isomeric dihydroperoxides or hydroperoxy-oxygenated compounds. TLC (1:1, ethyl ether/hexane) showed 2 slightly UV active spots of R_f 0.36 and 0.29 that gave a positive KI peroxide test (32). IR (CS_2) 3510 (OH, OOH), 3005 (olefin), 960 (isolated *trans* unsaturation) cm^{-1}. ^{1}H-NMR (100 MHz, $CDCl_3$) δ 5.61 ppm (m, 4H, CH=CH), 8.5 (broad s, 2H, OOH), 4.38 (m, 2H, C<u>H</u>-OOH). After hydrogenation, the resulting dihydroxystearates were silylated and analyzed by GC-MS, m/e (rel intensity): 459 (M-15,0.64), 443 (M-31,0.64). Cleavage at carbon-bearing OTMS indicated isomers with one hydroxy on C-9: 259 (41.8), C-10: 273 (18) and C-12: 301 (17.5) on the ester end, and one hydroxy on C-10: 215 (20), C-12: 187 (24.1) and C-13: 173 (49.3) on the hydrocarbon end of the molecule. Therefore, we apparently deal with a mixture of dihydroperoxides or hydroperoxy-oxygenated derivatives in which one hydroperoxide group is located between C-9, C-10 and C-12, and/or the other hydroperoxide between C-10, C-12 and C-13.

The 6 dioxygenated isomers that can be considered include: 9-10, 9-12, 9-13, 10-12, 10-13 and 12-13. Because 9,13-dioxygenated compounds were previously identified as prominent secondary products of 9- and 13-linoleate hydroperoxides (27,28), the 9-12, 10-12 and 10-13 isomers are apparently derived from the 10- and 12-monohydroperoxides of methyl linoleate. The 9-10 and 12-13 isomers may be derived from the corresponding epoxides that have been detected in small amounts by GC (Fig. 2) and identified by GC-MS: 312 (M^+, 0.2), 381 (M-31, 14.7), 9,10-epoxy: 199 (7.4), 155 (5.7), 12,13-epoxy: 241 (2.2), 113 (11.1) (27,28). Decomposition of the hydroperoxy cyclic peroxides I-IV may also provide a source of 10,12-dihydroperoxides (by loss of OOH and reaction with ·OH and ·H radicals). However, more direct evidence is needed by isolation of the individual isomeric dihydroperoxides.

Volatile Decomposition Products

Pure hydroperoxides from autoxidized and photosensitized-oxidized fatty esters were previously decomposed in the injector port of a

TABLE 5

GC-MS Analysis[a] of Volatiles from Thermally Decomposed Methyl 13-Hydroperoxy-10,12-epidioxy-*trans*-8-octadecenoate (I,III) and Methyl 9-Hydroperoxy-10,12-epidioxy-*trans*-13-octadecenoate (II,IV)[a]

Compound	Elution temp. (C)	Relative %	
		I + III	II + IV
Pentane	8	2.4	–
Pentanal	17	0.2	0.5
Acetic acid	22	0.2	0.4
Hexanal	38	44.6	2.5
2-Hexenal	46	0.1	0.3
2-Heptanone	54	1.7	–
2-Heptenal	75	1.1	27.3
2-Pentylfuran	76	0.2	1.9
3-Octen-2-one	85	–	4.9
Methyl heptanoate	83	0.4	–
Methyl octanoate	99	1.7	5.0
Hexanoic acid	102	0.5	–
2,4-Nonadienal	108	–	1.5
Methyl 8-oxooctanoate	122	1.3	0.3
Methyl 9-oxononanoate	150	3.8	39.3
Methyl 10-oxo-8-decenoate	160	28.6	2.6
Unidentified		13.2	13.5

[a]See Experimental section.

GC-MS system to clarify the sources of undesirable flavors (24). The same approach was used with the pure hydroperoxy-cyclic peroxides isolated in the present work (see section on Hydroperoxy Cyclic Peroxides) to compare the volatile decomposition products with those of the corresponding monohydroperoxides. Table 5 compares the GC-MS analysis of volatiles from methyl 13-hydroperoxy-10,12-epidioxy-*trans*-8-(I + III) and methyl 9-hydroperoxy-10,12-epidioxy-*trans*-13-octadecenoate (II + IV). Hexanal and methyl 10-oxo-8-decenoate were major volatile products from cyclic peroxides I + III, and methyl 9-oxo-nonanoate and 2-heptenal, from cyclic peroxides II and IV. On one hand, hexanal and methyl 9-oxo-nonanoate were previously found as major volatiles from both autoxidation- and photosensitized oxidation-produced hydroperoxides (24). On the other hand, methyl 10-oxo-8-decenoate, and especially 2-heptenal, were characteristic of the photooxidized-produced hydroperoxides (24). The other minor volatiles were generally the same as those from monohydroperoxides, with the exception of 2-heptanone (from I + III) and 3-octene-2-one (from II and IV), which appear to be unique products of the cyclic peroxides.

The same scheme recognized for decomposition of monohydroperoxides (24,29) involving carbon-carbon cleavage about alkoxy radical intermediates, explains the formation of pentane and hexanal from cyclic peroxides I and III, and of methyl octanoate and methyl 9-oxo-nonanoate from cyclic peroxides II and IV (Fig. 6). The cleavage between the peroxy ring and the alkoxy group is the most important. A less favorable cleavage between the double bond and the peroxy ring would produce olefin radicals that may react with hydroxyl radical to form vinyl alcohols, which then would tautomerize to saturated aldehydes (24). This process explains the formation of methyl 9-oxo-nonanoate from cyclic peroxides

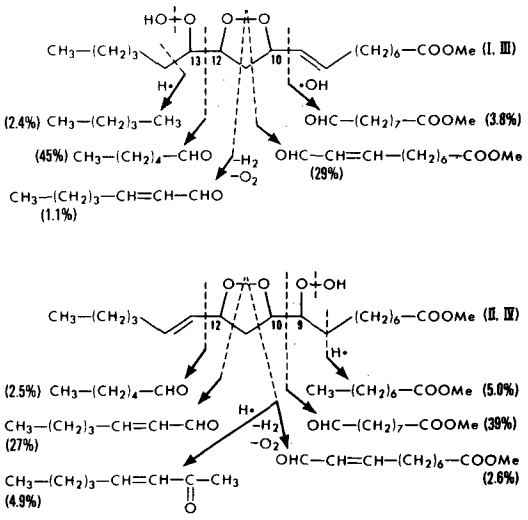


FIG. 6. Thermal decomposition cleavage of hydroperoxy cyclic peroxides I-IV.

I and III and hexanal from cyclic peroxides II and IV. Cleavage of the peroxy ring and carbon-carbon bonds β to the *trans* olefinic bond is another important process producing major amounts of methyl 10-oxo-8-decenoate from I and III and 2-heptenal from II and IV. A less favorable cleavage of the peroxy ring and carbon-carbon bonds β to the hydroperoxy group produced, after removal of the hydroperoxide (by elimination of H_2O_2), small amounts of 2-heptenal from I and III, methyl 10-oxo-8-decenoate and 3-octene-2-one from II and IV. The unsaturated methyl ketone ester expected also from I and III was not identified. Malonaldehyde was postulated as decomposition product by cleavage on either side of the cyclic peroxides (30). However, this dialdehyde was not identified under our GC-MS conditions.

The results of this work confirm our previous postulate (24) that hydroperoxy cyclic peroxides may be important sources of volatile decomposition products, and account for the common products observed from hydroperoxides of both autoxidized and photosensitized-oxidized methyl linoleate.

ACKNOWLEDGMENT

We thank C.R. Smith, Jr., and H.W. Gardner for useful discussions of the stereochemistry of structures in Figure 4.

REFERENCES

1. Kaplan, M.L. (1971) Chem. Tech. 621-626.
2. Foote, C.S. (1976) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. II, pp. 85-133, Academic Press, New York, NY.
3. Bland, J. (1976) J. Chem. Educ. 53:274-279.
4. Krinsky, N.I. (1977) Trends in Biochem. Sci. 2, 35-38.
5. Rawls, H.R., and van Santen, P.J. (1970) J. Am. Oil Chem. Soc. 47, 121-125.
6. Clements, A.H., Van den Engh, R.H., Frost, D.J., Hoogenhout, K., and Nooi, J.R. (1973) J. Am. Oil Chem. Soc. 50:325-330.
7. Carlsson, D.J., Suprunchuk, T., and Wiles, D.M. (1976) J. Am. Oil Chem. Soc. 53:656-660.
8. Cobern, D., Hobbs, J.S., Lucas, R.A., and MacKenzie, D.J. (1966) J. Chem. Soc. (C) 1897-1902.
9. Chan, H.W.-S. (1977) J. Am. Oil Chem. Soc. 54, 100-104.
10. Terao, J., and Matsushita, S. (1977) J. Am. Oil Chem. Soc. 54, 234-238.
11. Terao, J., and Matsushita, S. (1977) Agric. Biol. Chem. 41, 2467-2468.
12. Frankel, E.N., Neff, W.E., and Bessler, T.R. (1979) Lipids 14, 961-967.
13. Thomas, M.J., and Pryor, W.A. (1980) Lipids 15, 544-548.
14. Terao, J., and Matsushita, S. (1980) J. Food Process. Preserv. 3, 329-337.
15. Korycka-Dahl, M.B., and Richardson, T. (1978) CRC Crit. Rev. Food Sci. Nutr. 11, 209-291.
16. Matsushita, S., and Terao, J. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 27-44, Plenum Press, New York, NY.
17. Frankel, E.N. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 141-170, Plenum Press, New York, NY.
18. Frankel, E.N. (1980) Progr. Lipid Res. 19, 1-22.
19. Foote, C.S. (1968) Acc. Chem. Res. 1, 104-110.
20. Gollnick, K. (1978) in Singlet Oxygen Reactions with Organic Compounds and Polymers (Rånby, B., and Rabek, J.F., eds.) pp. 111-134, John Wiley, New York, NY.
21. Frimer, A.A. (1979) Chem. Rev. 79, 359-387.
22. Mihelich, E.D. (1980) J. Am. Chem. Soc. 102, 7141-7143.
23. Neff, W.E., Frankel, E.N., and Weisleder, D. (1981) Lipids 16, 439-448.
24. Frankel, E.N., Neff, W.E., and Selke, E. (1981) Lipids 16, 279-285.
25. Butterfield, R.O., Dutton, H.J., and Scholfield, C.R. (1966) Anal. Chem. 38, 86-88.
26. Gardner, H.W. (1970) J. Lipid Res. 11, 311-321.
27. Neff, W.E., Frankel, E.N., Scholfield, C.R., and Weisleder, D. (1978) Lipids 13, 415-421.
28. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977) Lipids 12, 901-907, 908-913, 1055-1061.
29. Selke, E., Rohwedder, W.K., and Dutton, H.J. (1977) J. Am. Oil Chem. Soc. 54, 62-67.
30. Pryor, W.A., Stanley, J.P., and Blair, E. (1976) Lipids 11, 370-379.
31. Chan, H.W.-S., Matthew, J.A., and Coxon, D.T. (1980) J. Chem. Soc. Chem. Commun. 235-236.
32. Vioque, E., and Holman, R.T. (1962) Arch. Biochem. Biophys. 99, 522-528.

[Received June 15, 1981]

The Effect of Environmental Temperature on Sebum Composition in Tropical and Temperate Breeds of Cattle

J.C. O'KELLY and H.P. REICH, *C.S.I.R.O., Division of Animal Production, Tropical Cattle Research Centre, PO Box 545, North Rockhampton, Qld. 4701, Australia*

ABSTRACT

This study compared the effect of environmental temperature on sebum composition in 2 breeds of cattle, British (SH) and Brahman (GB), which differ in their abilities to tolerate heat. By long-term exposure of both breeds to environmental temperatures of 24 C and 32 C and the more heat-tolerant GB breed to 38 C, it was possible to make breed comparisons at (a) different body temperatures, i.e., when all animals were exposed to the same environmental temperature, and (b), at the same body temperature, i.e., when the 2 breeds were exposed to different ambient temperatures. The composition of sebum excreted to saturation level on the skin surface was determined. At the same body temperatures, the amounts of fatty acids in each lipid class were higher in GB than in SH animals except during hyperthermia when the amounts of triglyceride fatty acids were similar in both breeds. The total amounts of individual fatty acids except 14:1, 16:1, 20:0 and 14:OH were higher in both breeds at 32 C than at 24 C. The GB cattle excreted more essential fatty acids (EFA) than the SH cattle at 24 C and at 32 C. There was a significant genotype by environment interaction in the amounts of EFA partitioned between triglycerides and wax esters; in GB cattle, the amount of EFA excreted in triglycerides decreased whereas the amount excreted in wax esters increased with rising body temperature.

Lipids 17:19-26, 1981.

INTRODUCTION

Each mammalian species has characteristically different compositions of skin surface lipids (1) and there is diversity in compositions of lipid classes among bovine species (2). Sebum output in cattle, as in many other species, is influenced by environmental temperature and humidity (3,4), but the physiological role of sebum is not well understood. Pan (5) has suggested that the level of skin surface lipids on cattle may be related to differences in heat tolerance among breeds and O'Kelly and Reich (4) demonstrated that total sebum output in thermoneutral and hot environments varies significantly with genotype and coat type. There are, however, no comparative studies of the effects of environmental temperature on the lipid class composition and fatty acid profiles of sebum in different genotypes of bovines.

This paper compares the sebum composition of British (*Bos taurus*) and Brahman (*Bos indicus*) steers at the same level of feed intake in thermoneutral and hot environments.

MATERIALS AND METHODS

Animals

Four Brahman and 4 British (½ Shorthorn × ½ Hereford) steers, all about 14 months old, were used and the experiment, conducted in summer, was divided into 3 periods of 10 days each. During the experimental periods, the animals were housed in a controlled temperature room. Feed (lucerne hay) was consumed in portions of 3 kg each in the morning and in the afternoon, and water was freely available throughout. The mean body weights (kg ± standard errors) at the beginning of the experiment were: Brahman 196 ± 2; British 173 ± 2.

All 8 animals were used in the first 2 periods whereas only the 4 Brahmans were used in the third period. During the first period, a thermoneutral temperature, 24 C, was selected. In the second period, the temperature of the room was adjusted to 32 C dry bulb (DB), 28 C wet bulb (WB), so that rectal temperatures of the British animals were about 1 C higher than at 24 C and the animals were considered to be suffering the metabolic derangements of hyperthermia (6). In the third period, the Brahman steers were housed in the room at a temperature 38 C DB, 26 WB, which maintained their mean rectal temperature about 1 C higher than at 24 C, and the animals were considered to be suffering consequences of hyperthermia (6).

Sebum Collection

The skin was prepared for sebum collection before the animals entered the climate room. The hair on both sides of the midline in the dorsal thoracico-lumbar region was cut with electric clippers; then the skin was shaved with a safety razor and washed thoroughly with water. After one day in the climate

room, the shaved area of skin was washed thoroughly with methanol to remove lipid material. Four areas of 63.5 cm^2 were delineated on both sides of the mid-line; the areas were paired, each area on the left side being matched to an area in a corresponding position on the right side, and the pairs were numbered 1-4 from the head toward the tail region. Sebum was collected from the first pair at 6 hr, from the second pair at 1 day, from the third pair at 2 days and from the fourth pair at 3 days after cleaning the skin with methanol. Immediately after sebum collection, the area was washed with methanol to provide time 0 for the next sampling. Subsequently, sebum was collected from the paired areas, in the same sequence as the first sampling, at 4, 5, 6 and 7 days after washing with methanol.

Smith and Ahmed (7), using the cup/methanol method (3) for collecting sebum, have confirmed that freshly collected bovine skin surface lipid is largely sebum, and the method used in this study follows that of Smith et al. (3). For sebum collection, an open glass cylinder of 9 cm id was placed over the marked area of skin. The 63.5 cm^2 area of skin inside the cylinder was then irrigated with 1 × 10 ml followed by 2 × 5 ml of methanol using a Manostat Pipette. The methanol washings were combined and filtered through paper (Whatman No. 3) to remove skin debris and the filtrate was taken to dryness in a rotary vacuum evaporator under nitrogen. The residue was taken up in *n*-hexane and transferred to a tared glass weighing bottle. The *n*-hexane was then evaporated under nitrogen at 55 C to constant weight.

Extraction and Analysis of Lipids

The total lipid extract was fractionated into individual lipid classes by thin layer chromatography (TLC) using glass plates coated with 0.5 mm of Kieselgel G (E. Merck, Darmstadt, Germany) and the 3-stage solvent system of Nicolaides et al. (8). All TLC plates were sprayed with a 0.1% w/v solution of 2,4-dichlorofluorescein in methanol and the lipid bands were visualized under UV light. The bands were scraped from the plates and the lipids were eluted with chloroform/methanol (2:1, v/v). Identification of lipid bands, except the wax esters, was by comparison with known standards and the material remaining on the origin of the plate was designated polar lipid.

The isolation of the fatty acids and the identification of the hydroxy fatty acids from the bands scraped from the plates were done as described previously (2). Methyl esters of the fatty acids were prepared with methanolic HCl and analyzed by gas liquid chromatography (GLC) in a Varian 3700 apparatus with a hydrogen flame ionization detector and a CDS III Chromatography Data System. A 1.8-m stainless steel column of 3 mm id was packed with Supelcoport (80-100 mesh, Supelco, Bellefonte, PA) coated with 10% DEGS-PS. The column temperature was 180 C and nitrogen was the carrier gas. Peak identifications were based on retention times relative to methyl stearate and cochromatography with known standards of methyl ester mixtures (Nu-Chek-Prep, Inc., Elysian, MN). The absolute amount of fatty acids in each lipid class was determined using *n*-heptadecanoic acid as an internal standard (9). Free cholesterol was determined by the method of MacIntyre and Ralston (10).

Statistical Analysis

The results were subjected to analyses of variance using the method of least squares.

RESULTS

The pattern of changes in sebum excretion over the 7-day period following cleaning of the skin with solvent was similar for each breed and environmental temperature. The weight of sebum collected increased progressively over the 1-, 2-, 3- and 4-day periods. Thereafter, the weight of sebum which was collected over the 5-, 6- and 7-day periods was relatively constant and similar to the amount of lipid which accumulated over 4 days. Consequently, the mean value of the sebum collected from each animal over the 4- to 7-day periods was used as the measure of the sebum saturation level (SSL), which represents the maximal amount of lipid that accumulates on the surface under given environmental conditions as described by O'Kelly and Reich (4). Only sebum collected at the saturation level was analyzed.

The 3 fractions which chromatographed between sterol ester and triglyceride bands were designated wax ester bands 1, 2 and 3 as described previously (2) and the sum of the fatty acids from all 3 fractions was subsequently called "wax ester fatty acid." There was generally a high inverse correlation between the amounts of fatty acids in the triglyceride and nonesterified fatty acid (NEFA) fractions, suggesting that the NEFA were derived from triglycerides. Consequently, the sum of the fatty acids from these 2 fractions was used in the statistical analyses.

The results of the lipid composition of sebum excreted to saturation level are given in

TABLE 1

Mean Rectal Temperature (C), *Weights of Fatty Acids* Contained in the Sterol Ester, Wax Ester, Triglyceride, NEFA and Polar Lipid Fractions and the Mean Weight of Free Sterol in the Sebum Excreted to Saturation Level in British (SH) and Brahman (GB) Cattle at Different Environmental Temperatures

Air temp.	24 C			32 C			38 C
	SH	GB	SE[a]	SH	GB	SE[a]	GB
Rectal temperature	38.6	38.4	0.1[b]	39.4	38.5	0.2[c]	39.3
Sterol ester	209	293	57	375	495	85	817
Wax ester	1091	1159	180	2262	3004	280[b]	3344
Triglyceride + NEFA	1606	1594	162	2113	2596	398	2236
Polar lipid	259	235	67	588	779	88	1013
Total fatty acids	3164	3281	349	5339	6874	301[c]	7411
Free sterol	1541	1531	1	1979	2011	363	4370

Values are expressed as $\mu g/100\ cm^2$ of skin surface.

[a]Standard error of the differences between breeds.

[b]Breed differences significant at $p < 0.05$.

[c]Breed differences significant at $p < 0.01$.

Tables 1-6. At each environmental temperature, there were no significant differences within animals in the weights of fatty acids contained in the individual lipid fractions nor in the amounts of free sterol in sebum sampled on days 4 and 7. Consequently, the mean values of the amount of lipids on these 2 days are reported in the tables.

Between breeds, there were no significant differences in the weights of fatty acids contained in the individual lipid classes nor in the amounts of free sterol on the skin surface at 24 C, but at 32 C, the amount of fatty acid in the wax ester fraction and the amount of total fatty acids in sebum were significantly higher in Brahman than in British animals (Table 1). Within breeds, the amount of free sterol and the weights of fatty acids contained in all lipid classes were higher ($p < 0.01$) in hot environments than at 24 C, but in the Brahman breed, only the amounts of free sterol and the weights of fatty acids contained in the sterol ester fraction were higher ($p < 0.01$) at 38 C than 32 C.

The total amounts of the individual fatty acids in sebum excreted to saturation level are shown in Table 2. The amounts of all fatty acids except 14:1, 16:1, 20:0 and 14:OH were higher ($p < 0.01$) in both breeds at 32 C than at 24 C. In the Brahman breed, the amounts of all the individual fatty acids were higher ($p < 0.01$) at 38 C than at 24 C, but only the amounts of 14:1, 16:1, 20:4 and 14:OH were higher ($p < 0.05$) at 38 C than at 32 C. There were breed differences in the amounts of 18:2 and 18:3 at both 24 C and 32 C, but of the saturated acids, only 16:0 and 18:0 showed significant breed differences at 32 C (Table 2).

Sterol Esters (Table 3)

In the sterol ester fraction, the fatty acids 16:0 and 18:0 were the only ones found in significantly greater ($p < 0.01$) amounts at 32 C than at 24 C and the levels of these saturated acids were higher ($p < 0.05$) in Brahman than in British animals at both 24 C and 32 C. However, in the Brahman breed, the amounts of all the fatty acids except 18:2 and 20:0 were higher ($p < 0.01$) at 38 C than at 24 C.

Wax Esters (Table 4)

There were only minor amounts of the EFA 18:2ω6 and 18:3ω3 in the wax ester fractions at 24 C, but the amounts of these acids increased significantly ($p < 0.001$) in both breeds in hot environments. At 32 C, the levels of the EFA were higher ($p < 0.05$) in Brahman than in British animals. On the other hand, the amounts of the α-hydroxy acids 14:OH and 16:OH were lower ($p < 0.05$) in Brahman animals than in British animals at 32 C. The amount of 16:OH in the wax esters was higher ($p < 0.05$) at 32 C than at 24 C in British cattle. In contrast, the level of 16:OH in the wax ester fraction in Brahman cattle at 32 C was similar to that at 24 C, although the level of this acid at 38 C was higher ($p < 0.05$) than at 24 C. The amounts of all the unsubstituted fatty acids except 20:0 in Brahman cattle were higher at 38 C ($p < 0.001$) and at 32 C ($p < 0.01$) than at 24 C whereas, in the British animals, only the amounts of the saturated acids 14:0 and 16:0 were higher ($p < 0.01$)

in the hot environment than at 24 C.

Triglycerides (Table 5)

In the fatty acids derived from triglycerides, there were significant breed differences in the amounts of 18:2ω6 and 18:3ω3 at 24 C and in 14:OH and 16:OH at 32 C. The weights of 18:2ω6 and 18:3ω3 in the triglyceride fraction from British cattle were not significantly different during exposure to 24 C or 32 C. However, the amounts of these acids in the triglyceride fraction of Brahman cattle were lower ($p < 0.01$) at 32 C than at 24 C and lower ($p < 0.05$) at 38 C than at 32 C. In both breeds, the amount of 16:OH was higher ($p < 0.01$) at 32 C than at 24 C and, in the Brahman breed, was higher ($p < 0.01$) at 38 C than at 32 C. The fatty acid 20:3 was the only unsubstituted acid in the triglyceride fraction from British cattle to differ significantly ($p < 0.01$) in amount at 32 C compared to 24 C. In contrast, the amounts of 14:0 and 18:0 as well as 20:3 were higher ($p < 0.01$) at 32 C than at 24 C in Brahman cattle. The amounts of 18:0 and 20:3 in Brahman cattle were also higher ($p < 0.01$) at 38 C than at 24 C but the

TABLE 2

Mean Total Weights of Individual Fatty Acids in the Sebum Excreted to Saturation Level in British (SH) and Brahman (GB) Cattle at Different Environmental Temperatures

Fatty acid	24 C			32 C			38 C
	SH	GB	SE[a]	SH	GB	SE[a]	GB
14:0	785	751	109	1196	1600	225	1339
14:1	44	39	7	45	50	9	95
16:0	862	813	71	1295	1663	112[b]	1691
16:1	39	46	7	55	60	7	79
18:0	270	321	30	387	591	48[c]	686
18:1	99	104	10	141	171	24	175
18:2ω6	221	278	17[b]	317	489	34[c]	446
18:3ω3	75	156	6[d]	95	209	8[d]	238
20:0	87	91	15	96	140	25	89
20:3	63	52	14	181	259	68	214
20:4	–	–	–	8	25	10	170
20:5	–	–	–	222	250	61	378
14:OH	58	46	12	92	55	21	119
16:OH	527	524	99	985	1075	108	1441

Values are expressed as μg/100 cm^2 of skin surface.

[a]Standard error of the differences between breeds.

[b]Breed differences significant at $p < 0.05$.

[c]Breed differences significant at $p < 0.01$.

[d]Breed differences significant at $p < 0.001$.

TABLE 3

Mean Weights of Individual Fatty Acids Contained in the Sterol Ester Fraction of Sebum Excreted to the Saturation Level in British (SH) and Brahman (GB) Cattle at Different Environmental Temperatures

Fatty acid	24 C			32 C			38 C
	SH	GB	SE[a]	SH	GB	SE[a]	
14:0	46	71	21	75	101	34	194
16:0	67	78	3[b]	102	126	4[b]	271
16:1	–	–	–	14	18	3	27
18:0	49	74	8[b]	75	124	18[b]	172
18:1	9	10	2	11	13	4	17
18:2ω6	4	8	2	7	9	3	8
20:0	15	24	4	15	25	7	21
20:3	–	–	–	13	17	11	13
20:5	–	–	–	49	33	30	21

Values are expressed as μg/100 cm^2 of skin surface.

[a]Standard error of the differences between breeds.

[b]Breed differences significant at $p < 0.05$.

amounts of 14:0 and 16:0 were lower ($p < 0.01$) at 38 C than at 32 C.

Polar Lipid (Table 6)

There were no significant breed differences in the amounts of fatty acids contained in the phospholipid fractions of animals at 24 C and at 32 C. In both breeds, the amounts of the fatty acids 16-0, 18:1, 18:2ω6, 20:3 and 16:OH increased ($p < 0.01$) with increasing environmental temperature.

Breed Comparisons at the Same Body Temperatures

The rectal temperatures were not significantly different when the British animals (T_{re} 38.5) exposed to 24 C were compared to the Brahman animals (T_{re} 38.6) exposed to 32 C, and similarly, when the British animals (T_{re} 39.5) at 32 C were compared to the Brahman animals (T_{re} 39.4) at 38 C. It was thus possible to make breed comparisons at the same normal (T_{re} 38.55) and hyperthermic (T_{re} 39.45)

TABLE 4

Mean Weights of Individual Fatty Acids Contained in the Wax Ester Fractions of Sebum Excreted to the Saturation Level in British (SH) and Brahman (GB) Cattle at Different Environmental Temperatures

	24 C			32 C			38 C
Fatty acid	SH	GB	SE[a]	SH	GB	SE[a]	GB
14:0	368	393	67	717	940	130	850
14:1	16	15	3	16	24	5	44
16:0	108	110	13	354	759	244	846
16:1	10	9	2	12	15	3	25
18:0	58	80	10	104	209	30[b]	260
18:1	19	15	3	28	52	13	62
18:2ω6	–	–	–	52	245	24[b]	316
18:3ω3	8	16	4	26	108	20[b]	152
20:0	12	19	4	15	34	6[b]	26
20:3	–	–	–	32	44	11	50
20:5	–	–	–	–	–	–	50
14:OH	56	45	11	84	31	24[b]	37
16:OH	426	423	38	737	445	90[b]	520

Values are expressed as μg/100 cm^2 of skin surface.

[a]Standard error of the differences between breeds.

[b]Breed differences significant at $p < 0.05$.

TABLE 5

Mean Weights of Individual Fatty Acids Contained in the Triglyceride + Nonesterified Fatty Acid Fractions of Sebum Excreted to the Saturation Level in British (SH) and Brahman (GB) Cattle at Different Environmental Temperatures

	24 C			32 C			38 C
Fatty acid	SH	GB	SE[a]	SH	GB	SE[a]	GB
14:0	318	248	49	349	472	106	224
14:1	24	16	6	23	17	7	29
16:0	620	566	68	721	649	90	466
16:1	23	26	4	23	21	5	19
18:0	135	144	15	174	222	25	228
18:1	57	67	6	73	76	14	56
18:2ω6	191	242	15[b]	210	170	60	69
18:3ω3	61	121	5[c]	55	65	13	21
20:0	38	33	6	48	69	17	42
20:3	37	33	8	92	137	41	111
20:5	–	–	–	62	95	22	150
14:OH	–	–	–	8	24	4[b]	3
16:OH	85	77	13	173	521	43[b]	709

Values are expressed as μg/100 cm^2 of skin surface.

[a]Standard error of the differences between breeds.

[b]Breed differences significant at $p < 0.05$.

[c]Breed differences significant at $p < 0.001$.

TABLE 6

Mean Amounts of Individual Fatty Acids Contained in the Polar Lipid Fraction of Sebum Excreted to the Saturation Level in British (SH) and Brahman (GB) Cattle at Different Environmental Temperatures

Fatty acid	24 C			32 C			38 C
	SH	GB	SE[a]	SH	GB	SE[a]	GB
14:0	55	39	21	55	87	33	71
16:0	66	59	15	118	130	40	108
18:0	27	24	6	33	36	7	28
18:1	14	12	3	29	30	3	40
18:2ω6	26	23	7	48	65	10	51
18:3ω3	4	15	5	13	36	12	66
20:0	23	15	6	18	12	2	
20:3	23	14	6	45	62	23	40
20:4	–	–	–	8	25	10	170
20:5	–	–	–	110	122	18	158
14:OH	–	–	–	–	–	–	19
16:OH	14	23	8	74	108	30	213

Values are expressed as μg/100 cm^2 of skin surface.

[a]Standard error of the difference between breeds.

body temperatures.

At both normal and hyperthermic body temperatures, the amounts of (a) total individual fatty acids (Table 2), (b) all fatty acids in sterol esters except the polyunsaturated fatty acids (Table 3), (c) all the unsubstituted fatty acids in wax esters (Table 4) and (d) all the major fatty acids in phospholipids (Table 6) were higher ($p < 0.01$) in Brahman than in British animals.

The amounts of the individual lipid fractions measured were higher ($p < 0.01$) in sebum from Brahman than from British steers at T_{re} 38.55 and similarly at T_{re} 39.45 except for the weights of fatty acids in the triglyceride fractions which did not differ significantly between breeds (Table 1). In the wax ester fractions, the amounts of the α-hydroxy fatty acids 14:OH and 16:OH were similar in both breeds at normal body temperature but were lower ($p < 0.01$) in the Brahman than in the British animals during hyperthermia (Table 4). At T_{re} 38.55, the amounts of the fatty acids 14:0, 18:0, 20:3, 20:5, 14:OH and 16:OH were higher in the triglyceride fractions from Brahman than from British cattle (Table 5). However, during hyperthermia, the amounts of 18:0, 20:3, 20:5 and 16:OH were higher ($p < 0.01$) and the amounts of 14:0, 16:0, 18:2ω6 and 18:3ω3 were lower ($p < 0.01$) in triglyceride fractions from Brahman than from British steers.

DISCUSSION

Brahman cattle are known to be more tolerant of heat than British cattle. So, when the 2 breeds were exposed to the same hot environment (32 C), the Brahman cattle, as expected, maintained a lower body temperature than the British cattle. Clearly, in explaining breed differences in sebaceous gland activity in the present study, the interactions between genotype and environmental temperature had to be considered. Therefore, the experimental findings were interpreted in relation to breed differences in sebum output and composition at (a) different body temperatures, i.e., when all animals were exposed to the same environmental temperature and (b) at the same body temperature, i.e., when the 2 breeds were exposed to different ambient temperatures.

In considering the functional attributes of sebum, it is accepted that the surface lipids are designed to withstand the adverse effects of environmental constraints and it has been pointed out that polyunsaturated compounds, which are highly susceptible to oxidation by atmospheric oxygen, are virtually absent in surface waxes whereas such compounds are very significant components of internal lipids (11). The production of sebum of such chemical stability in cattle would be highly advantageous from a nutritional aspect because biohydrogenation reactions within the rumen drastically reduce the metabolic supply of EFA from dietary sources. It is surprising, then, that cattle sebum is relatively rich in linoleic acid (2,12) and, in this study, the polyunsaturated fatty acids formed a significant proportion of the total fatty acids in sebum, being 14% in both breeds at 24 C ambient temperature and as high as 20% in the Brahmans at 38 C ambient temperature. As elevated body temperature in cattle has the effect of reducing food intake and because

prolonged exposure of British cattle to heat resulted in an increased output of linoleic acid in the sebum (3), the loss of EFA through the skin of cattle in the tropics might be expected to be lower in adapted than in unadapted breeds. Contrary to expectations, it was found that, at both 24 C and 32 C ambient temperatures, the Brahman cattle excreted significantly greater amounts of the EFA linoleic and linolenic (18:2ω6 and 18:3ω3) in sebum than the British cattle. There were also significant genotype by environment interactions in the amounts of EFA partitioned between the triglyceride and wax ester fractions. During hyperthermia in the British animals, the amount of EFA excreted in the triglyceride fraction was not significantly different from the amount excreted at normal body temperature. On the other hand, in Brahman animals, the amounts of EFA excreted in the triglyceride fraction decreased whereas the amount excreted in the wax esters increased with rising body temperature.

Thus, the question is posed whether the diversion of EFA into wax esters at the expense of triglycerides in Brahman cattle during heat exposure has an adaptational significance. Linoleic acid is known to be important in the regulation of cutaneous moisture; however, the major barrier against water loss is not in the lipid film on the skin surface, but in the stratum corneum. However, the derivatives of the EFA 20:3, 20:4 and 20:5, which were present in only trace amounts in the sebum of animals at 24 C, were found in significant quantities in the sebum of animals in hot environments. Blood flow to the skin would increase with rising body temperature in both breeds and would presumably accelerate the supply of metabolites to the glands. Thus, the increasing amounts of polyunsaturated fatty acids incorporated in sebum components with increasing body temperature may be a reflection of an elevated metabolism rather than a directed excretion onto the skin surface for a specific role in thermal balance. It might be noted that, although it appears most of the lipids are synthesized de novo by the gland (13), the EFA are not synthesized in the skin but originate from the food via the plasma lipoproteins and an increased blood flow would be a likely source of part of the increased amounts of these acids excreted in the sebum of animals exposed to hot environments.

The density of hair follicles and, hence, sebaceous glands is greater in *Bos indicus* than in *B. taurus* cattle (14). The higher sebum output in Brahman than in British cattle at the same body temperature might, then, be supposed to be explained by the greater density of sebaceous glands in the Brahman animals. It is possible, however, that the breed difference in sebum output at the same body temperature does not arise solely from differences in sebaceous gland density. For example, it has been proposed that the differences in heat tolerance between these 2 breeds might be explained largely by differences in the ability to lose heat by nonevaporative pathways (4). If this is the case, it could be expected that, compared to British cattle, the blood flow to the skin of Brahman cattle would be higher with an associated higher metabolic activity of the sebaceous glands.

In this study, the wax esters and triglycerides, lipid classes known to be synthesized in the sebaceous glands and exported in the sebum, contained a high proportion of hydroxy acids. As none of these acids were found in the sterol esters, it seems likely that the esterified sterols of cattle surface lipids are mainly of epidermal origin as suggested by O'Kelly et al. (2). While it was assumed that, in the skin surface lipids of humans, the free cholesterol originates from the epidermis (15), there has been no evidence that the free sterol present in the surface film of cattle is the result of any process other than keratinization of epidermal cells. It could be anticipated, then, that the amount of free sterol/unit surface area would be similar in the 2 breeds because it would not be influenced by breed differences in the density of sebaceous glands. Yet, at the same body temperature, the amount of free sterol was higher in Brahman than in British steers and, within breeds, the amount of free sterol which accumulated on the skin surface increased with increasing body temperature as did the amounts of wax esters and triglycerides from the sebaceous glands. These findings suggest that, in cattle, a significant portion of the free sterol in the surface lipid film originates in the sebaceous glands and is supported by the histochemical evidence for the presence of free cholesterol within the sebaceous glands of zebu and Ayrshire cattle (16).

REFERENCES

1. Nicolaides, N. (1974) Science 186, 19-26.
2. O'Kelly, J.C., Reich, H.P., and Mills, S.C. (1980) Comp. Biochem. Physiol. 67B, 217-220.
3. Smith, M.F.,Noble, R.C., and Jenkinson, D.M. (1975) Res. Vet. Sci. 19, 253-258.
4. O'Kelly, J.C., and Reich, H.P. (1981) J. Therm. Biol. 6, 97-101.
5. Pan, Y.S. (1970) J. Agric. Sci. Camb. 75, 41-46.
6. O'Kelly, J.C. (1973) Br. J. Nutr. 30, 211-220.
7. Smith, M.E., and Ahmed, S.U. (1976) Res. Vet. Sci. 21, 250-252.

8. Nicolaides, N., Fu, H.C., and Rice, G.R. (1968) J. Invest. Dermatol. 51, 83-89.
9. Christie, W.W., Noble, R.C., and Moore, J.H. (1970) Analyst London 95, 940-944.
10. MacIntyre, I., and Ralston, M. (1954) Biochem. J. 56, 43.
11. Kolattukudy, P.E. (1976) in Chemistry and Biochemistry of Natural Waxes (Kolattukudy, P.E., ed.) pp. 1-15, Elsevier, Amsterdam.
12. Noble, R.C., Crouchman, M.L., and Moore, J.H. (1974) Res. Vet. Sci. 17, 372-376.
13. Downing, D.T. (1976) in Chemistry and Biochemistry of Natural Waxes (Kolattukudy, P.E., ed.) pp. 17-48, Elsevier, Amsterdam.
14. Turner, H.G., Nay, T., and French, G.T. (1962) Aust. J. Agric. Res. 13, 960-973.
15. Nicolaides, N. (1963) in Advances in Biology of the Skin (Montagna, W., Ellis, R.A., and Silver, A.P., eds.) pp. 167-187, Pergamon Press, Oxford.
16. Yang, S.H. (1952) J. Agric. Sci. Camb. 42, 155-158.

[Received May 26, 1981]

Interrelationship between Dietary *trans* Fatty Acids and the 6- and 9-Desaturases in the Rat

REMI DE SCHRIJVER[1] and **ORVILLE S. PRIVETT**[2], *The Hormel Institute, University of Minnesota, Austin, MN 55912*

ABSTRACT

Studies are reported on the effects of dietary *trans* fatty acids on the 6- and 9-acyl desaturase activities in the liver microsomes of rats fed essential fatty acid (EFA)-deficient and non-EFA-deficient diets. In experiment I, weanling male rats were fed a semisynthetic diet with either 10% safflower oil (SAF) or 10% hydrogenated coconut oil (HCO). At the age of one year, half of the dietary fat was replaced by a supplement containing elaidate, linolelaidate and *cis,trans-trans,cis*-18:2 (TRANS) for 12 weeks. In experiment II, male rats which were kept from weaning on a 10% SAF diet for one year received one of the following fat supplements for a 12-week period: 10% HCO, 9% HCO + 1% TRANS, or 5% HCO + 5% TRANS. Feeding TRANS depressed the 6-desaturase activity in the liver microsomes, especially in the EFA-deficient rats (HCO + TRANS group of experiment I). Unlike the 6-deaturase activity, the 9-desaturase activity was not inhibited by the dietary *trans* fatty acids and was significantly stimulated in the non-EFA-deficient rats (SAF + TRANS group of experiment I and HCO + TRANS groups of experiment II). This was evidenced by incubation reactions and by comparisons of fatty acid consumptions and microsomal fatty acid levels, showing extra biosynthesis of 16:1 and 18:1 when TRANS was fed. The biosynthesis of essential (n-6) fatty acids was depressed by the TRANS supplement in EFA-deficient as well as in non-EFA-deficient animals.
Lipids 17:27-34, 1981.

INTRODUCTION

It has been well demonstrated that dietary *trans* fatty acids are incorporated in animal and human tissues (1-10) in more or less significant amounts, depending on the dietary concentration, the length of dietary treatment and the particular tissue and lipid fraction. The pathways for metabolism of *trans* fatty acids are somewhat different than for the *cis* acids. Lemarchal and Munsch (11) and Lemarchal (12) proved that elaidic acid can be desaturated in vivo to some extent to 5-*cis*,9-*trans*-octadecadienoic acid, only when other substrates for the 5-desaturase are lacking. On the other hand, elaidic acid is also converted to stearic acid by a direct hydrogenation process, as shown by Dhopeshwarkar and Mead (13) in guinea pigs. Privett et al. (3) and Karney and Dhopeshwarkar (14) showed that linolelaidic acid is not converted in vivo into an eicosatetraenoic acid. It has also been demonstrated that *cis,trans*-18:2, but not *trans,cis*-18:2, is converted to 20:4 with a *trans* double bond (3,15), indicating that the 6-desaturase requires a substrate with a 9-*cis* double bond. Although some of the *trans* isomers of linoleic acid may be converted into *trans*-arachidonic acid, it should be pointed out that they do not have the properties of essential fatty acids (EFA) (16).

Because of their structural characteristics, *trans* fatty acids are potentially able to affect the alternating enzymatic desaturation and chain elongation reactions which occur in animal and human tissues and by which dietary and endogenous fatty acids are converted into higher homologs. At the enzyme level, Brenner and Peluffo (17) demonstrated by in vitro experiments that linolelaidate inhibits the microsomal system that converts linoleic acid to γ-linolenic acid. Also using in vitro experiments, Mahfouz et al. (18) found that positional isomers of *trans*-18:1 acids inhibit 5-, 6- and 9-desaturase activities in liver microsomes of EFA-deficient rats. In nutrition experiments, Anderson et al. (15) found that increasing levels of dietary 9-*trans*,12-*trans*-18:2 resulted in decreased levels of arachidonic acid in the rat liver even when appropriate amounts of linoleic acid were fed. However, a dietary mixture of *cis,trans*-18:2 and *trans,cis*-18:2 did not inhibit the synthesis of arachidonic acid. In previous nutrition studies, Takatori et al. (1), Privett et al. (2) and Kurata and Privett (19) showed that elaidate and particularly linolelaidate impaired the conversion of linoleic acid to arachidonic acid and also the conversion of oleic acid to eicosatrienoic acid. Simultaneously, it was observed that the level of *cis*-18:1 in the liver microsomes increased. The accumulation of *cis*-18:1 may be due to several factors, such as increased consumption, decreased catabolism, inhibition of the 6-desaturase depressing the conversion of 18:1 to higher homologs, and increased de novo

[1]Present address: Laboratory of Animal Nutrition, University of Ghent, 19, Heidestraat, Merelbeke 9220, Belgium.

[2]Person to whom reprint requests should be addressed.

synthesis indicating that the 9-desaturase activity is not inhibited by *trans* fatty acids. The present nutrition experiments were designed to investigate the effect of dietary *trans* fatty acids, particularly on the 9-desaturase activity, as compared to the 6-desaturase activity, in EFA-deficient and non-EFA-deficient rats. Desaturase activities were measured in incubation reactions using the microsomal fraction of the liver. Also, the fatty acid pattern of the hepatic microsomes was analyzed to determine relationships between the synthesis of unsaturated fatty acids and the role of dietary *trans* fatty acids in these metabolic processes.

MATERIALS AND METHODS

Animals

Two experiments were performed. In the first experiment, weanling male Sprague-Dawley rats were fed a semisynthetic diet (Table 1) based on casein, sucrose, cellulose, and containing a 10% fat supplement, either safflower oil (SAF) or hydrogenated coconut oil (HCO). At the end of a one-year period, a mixture of *trans* fatty acids (TRANS) was included in each of these diets: 6 rats from the SAF group were shifted to a diet containing 5% SAF + 5% TRANS and 6 rats from the HCO group were fed a 5% HCO + 5% TRANS supplement. The TRANS diets were supplied for an additional 12 weeks. At the same time, 2 groups of 6 rats remained on the 10% SAF or 10% HCO diet. The TRANS concentrate contained 13.5% *trans*-18:1, 51% *trans,trans*-18:2 and 18% *cis,trans-trans,cis*-18:2. It was not determined if both *cis,trans*-18:2 and *trans,cis*-18:2 or only one of these fatty acids was present in the TRANS supplement.

In the second experiment, one-year-old Sprague-Dawley rats which had been kept on the 10% SAF diet (Table 1) were switched to EFA-deficient diets, either containing 10% HCO or 9% HCO + 1% TRANS or 5% HCO + 5% TRANS, also for an additional 12 weeks. Each group consisted of 6 animals. It is known that EFA-deficient rats show elevated enzyme activities, as also reported in this study. By switching non-EFA-deficient rats to an EFA-deficient diet for a relatively short period, it was hoped that higher enzyme activities would occur and that, consequently, the effect of dietary *trans* fatty acids on the desaturase activities would be more pronounced and measured more accurately, while the rats were still non-EFA-deficient at the end of the 12-week experimental period.

The animals were weighed at weekly intervals and food consumption was measured on a daily basis. The diets were stored at -20 C and were fed fresh daily. The rats were housed in individual cages with raised wire floors. The temperature was kept constant at 23 C and the relative humidity at 55%. At the end of the experiments, the rats were sacrificed by exsanguination after light ether anesthesia. The animals were not fasted before killing in order to be able to measure maximal activities of rat liver desaturases (20).

TABLE 1

Diet Composition (% by wt)

Casein (vitamin test)	22.50
L-Cystine[a]	0.20
Wesson salt mixture plus $ZnCl_2$ and $MnSO_4 \cdot H_2O$[b]	4.03
Choline mix[c]	1.00
Vitamin mix[d]	1.00
Cellulose (Alphacel)	16.50
Sucrose	44.77
Fat[e]	10.00

[a]L-Cystine was added to the diet to bolster the level of sulfur-containing amino acids.

[b]Wesson salt mixture did not contain zinc or manganese, hence these elements were added to the mix as follows: 0.60 g $ZnCl_2$ and 0.90 g $MnSO_4 \cdot H_2O$/200 g salt mixture.

[c]Choline mix consisted of 22% choline dihydrogen citrate in vitamin test casein.

[d]Vitamin mix (1 kg) contained 2.5 g thiamine HCl, 2.5 g riboflavin, 9.0 g nicotinic acid, 9.0 g calcium pantothenate, 2.0 g pyridoxine HCl, 4.0 g cyanocobalamin (B_{12}), 7.5 g *p*-aminobenzoic acid, 0.1 g folic acid, 0.02 g biotin, 20.0 g meso-inositol, 0.5 g menadione (vitamin K), and 943.0 g vitamin test casein. Fat and vitamins A, D and E were mixed into the diet daily and stored at 0 C overnight. Vitamin D_2, 5.0 mg; retinol acetate, 6.9 mg; α-tocopherol acetate, 300 mg/kg of diet.

[e]Propyl gallate as an antioxidant was added to safflower oil and TRANS-concentrate (66 mg/100 g of fat).

Enzyme Preparation

Immediately upon killing of the animals, their livers were excised, minced and rinsed with ice-cold saline solution and homogenization solution consisting of 0.15 M KCl, 5 mM $MgCl_2$, 0.25 M sucrose, 1.5 mM glutathione and 50 mM potassium phosphate buffer, pH 7.3. Livers were homogenized in a Potter-Elvehjem homogenizer in 2 vol of solution. Homogenates were centrifuged at 800 × g for 10 min to remove cell debris and the fat pad. The post-mitochondrial supernatant, obtained by spinning at 12,000 × g for 30 min, was further centrifuged at 100,000 × g for 1 hr. The 100,000 × g pellet was resuspended in the

homogenization solution (0.5 ml/g of original liver), put into tubes under N_2 and stored at -70 C. All operations were done at maximal 5 C. Protein concentration was measured according to Lowry et al. (21).

Incubations

GSH, ATP, CoASH, NADH and bovine serum albumin, containing less than 0.005% fatty acid, were purchased from Sigma Chemical Co., St. Louis, MO. For measuring the 6- and 9-desaturase activities, [1-^{14}C] linoleic acid and [1-^{14}C] stearic acid were used, respectively, purchased from New England Nuclear, Boston, MA.

The microsomes were diluted with the homogenization solution to a concentration of 20 mg protein/ml. Each incubation was done in 2 ml of 0.15 M KCl-0.25 M sucrose solution containing: ATP, 10 μM; CoA, 0.6 μM; NADH, 2.5 μM; $MgCl_2$, 10 μM; GSH, 3 μM; potassium phosphate buffer, pH 7.3, 100 μM; microsomal protein, 5 mg; and 200 nmol of radioactive substrate. The incubation reaction was started by addition of the substrate solution. Incubation vials were gently agitated at 37 C in air for 10 min. The reaction was stopped by the addition of 5 ml of a 10% (w/v) KOH solution in methanol and the mixture was allowed to saponify at 85 C for 2 hr under N_2. After acidification, the fatty acids were extracted with petroleum ether. The extract was dried under a stream of nitrogen and esterified with anhydrous 10% (w/v) HCl in methanol at 85 C for 2 hr.

Radioactivities of the methyl esters were determined on fractions isolated by gas liquid chromatography (GLC) using a Hewlett Packard instrument, Model 5840A, equipped with a 9:1 splitter and a 12 ft x 0.125 in. id column packed with 10% Silar 10C on 100-200 mesh Gas-Chrom Q at 170-250 C, programmed at 2.0 C/min with a flow rate of helium of 10 cc/min. The fractions corresponding to the radioactive substrates 18:0 and 18:2(n-6), and the radioactive products 18:1(n-9) and 18:3(n-6) were collected in glass tubes directly attached to the outlet of the splitter, coincident with their detection by the flame detector. The collecting tubes were washed with 15 ml of toluene-based scintillation fluid (5.5 g of Permablend I/ℓ of toluene). The radioactivity was measured in a Beckman LS 7500 liquid scintillation counter. The recovered radioactivity was 85% of the amount used. The percentage of desaturation was calculated as the ratio of the counts in the product to the sum of the counts in substrate plus product, corrected for background.

Fatty Acid Analysis

Fatty acid composition of dietary fats was determined by GLC of methyl esters prepared by interesterification with methanol using HCL as the catalyst (22). The fatty acid composition of the liver microsomal lipid was determined according to the saponification and methylation procedures previously described in this report. Instrumental conditions were also the same. The fatty acid standards were purchased from Nu-Chek-Prep, Elysian, MN.

RESULTS

Experiment I

The growth of the rats, kept for one year on either the 10% HCO diet or the 10% SAF diet, had plateaued. The rats of the 10% HCO group had developed dermal symptoms on feet and tail, typical of an EFA deficiency, and their growth was suppressed compared to the 10% SAF group. The average body weight of the 10% HCO rats was 316 ± 35 g (± SE) and the 10% SAF rats weighed 491 ± 42 g. The inclusion of *trans* fatty acids in the HCO and SAF diet for 12 weeks did not significantly ($p < 0.05$) affect the growth or the food consumption of these animals.

Generally, the fatty acid composition of the diet was well reflected in the fatty acid pattern of the liver microsomal lipid (Table 2). The 10% SAF group showed high levels of linoleic acid and arachidonic acid in the liver microsomes. The 10% HCO rats exhibited the typical fatty acid compositional pattern of an EFA deficiency: elevated levels of 16:1, 18:1 and 20:3(n-9) and low levels of 18:2(n-6) and 20:4(n-6). Also, minor amounts (<1%) of 18:2(n-9), 20:2(n-9) and 20:4(n-7) characterized the fatty acid pattern of the liver microsomes of the 10% HCO group. As observed by others (20,23-25), the incubation reactions showed elevated 6- and 9-desaturase activities in the liver microsomes of the EFA-deficient 10% HCO rats (Table 3).

Including the TRANS supplement in the SAF diet increased the 18:2(n-6) concentration in the liver microsomes, in spite of the much lower consumption of this fatty acid, which indicated that the metabolism of 18:2(n-6) was inhibited. In agreement with this observation were the results of the incubation reactions showing that the conversion of 18:2(n-6) to 18:3(n-6) was significantly suppressed by the dietary *trans* fatty acids (Table 3). Feeding the TRANS supplement to the SAF animals increased the level of 18:1, and particularly of 16:1, in the liver microsomes, notwithstanding the fact that the intake of 16:1 was

TABLE 2

Experiment I: Fatty Acid Composition of Liver Microsomal Lipid and Dietary Fat (% wt)

Group[a]	10% SAF (0-15 months)		5% SAF + 5% TRANS (12-15 months)		10% HCO (0-15 months)		5% HCO + 5% TRANS (12-15 months)	
	LM[b]	D[b]	LM	D	LM	D[c]	LM	D
16:0[d]	19.3 ± 0.7[e]	6.7	20.7 ± 0.9	8.4	19.3 ± 0.7	9.3	21.3 ± 0.9	9.7
16:1	1.2 ± 0.2	0.1	3.3 ± 0.2	0.1	6.8 ± 0.3	–	8.0 ± 0.3	–
18:0	20.2 ± 0.9	2.5	19.1 ± 0.6	3.1	16.9 ± 0.7	14.4	13.9 ± 1.0	9.0
trans-18:1	–	–	1.1 ± 0.3	6.7	–	1.6	1.2 ± 0.4	7.5
cis-18:1	6.1 ± 0.9	11.8	8.4 ± 0.6	7.4	21.9 ± 1.2	tr[f]	22.7 ± 1.3	1.5
trans,trans-18:2	–	–	2.3 ± 0.2	25.5	–	–	4.6 ± 0.3	25.5
cis,trans-trans,cis-18:2	–	–	tr	9.0	–	–	10.3 ± 0.6	9.0
18:2(n-6)	13.0 ± 0.8	77.1	18.3 ± 0.9	38.6	2.4 ± 0.1	0.1	1.8 ± 0.1	0.1
20:3(n-9)	0.1 ± 0.1	–	tr	–	15.1 ± 0.7	–	5.8 ± 0.7	–
20:4(n-6)	27.5 ± 1.2	–	20.6 ± 0.9	–	2.9 ± 0.2	–	1.6 ± 0.2	–
22:4(n-6)	2.5 ± 0.3	–	1.1 ± 0.2	–	1.6 ± 0.2	–	0.6 ± 0.3	–
22:5(n-6)	4.2 ± 1.2	–	1.9 ± 0.7	–	1.8 ± 0.5	–	0.5 ± 0.10	–

[a]SAF = safflower oil, HCO = hydrogenated coconut oil, TRANS = concentrate of *trans* fatty acids. For additional details, see Materials and Methods.

[b]LM = liver microsomes, D = diet.

[c]HCO also contained 3.4% 8:0, 5.1% 10:0, 47.7% 12:0 and 17.9% 14:0.

[d]Shorthand designation of fatty acids: number before colon = chain length; number after colon = number of double bonds. The digit after n states the number of carbon atoms from the methyl end of the acyl chain to the nearest double bond.

[e]M ± SD, n = 6.

[f]tr = trace, less than 0.5%.

TABLE 3

Experiment I: Liver Microsomal Enzyme Activities (nmol/min/mg protein)[a]

Group[b]	10% SAF (0-15 months)	5% SAF + 5% TRANS (12-15 months)	10 HCO (0-15 months)	5% HCO + 5% TRANS (12-15 months)
6-Desaturase	0.26 ± 0.03[c]	0.20 ± 0.03[c]	0.56 ± 0.05[c]	0.13 ± 0.03[c]
9-Desaturase	0.83 ± 0.05[d,e,f]	1.66 ± 0.06[d]	1.57 ± 0.07[e]	1.83 ± 0.06[f]

[a]M ± SD, n = 6 for groups 10% SAF and 10% HCO; n = 5 for groups 5% SAF + 5% TRANS and 5% HCO + 5% TRANS.

[b]SAF = safflower oil, HCO = hydrogenated coconut oil, TRANS = concentrate of *trans* fatty acids. For additional details, see Materials and Methods.

[c,d,e,f]Values with a common superscript are significantly different ($p < 0.05$).

not changed by shifting the rats to the 5% SAF + 5% TRANS diet and that the 18:1 consumption was decreased considerably. In accord with this, the 9-desaturase activities measured in vitro were very significantly ($p < 0.01$) increased in the 5% SAF + 5% TRANS animals.

Including the TRANS supplement in the HCO diet did not alter the levels of 16:1 and 18:1 and decreased substantially the levels of 18:2(n-6), 20:4(n-6) and 20:3(n-9) in the liver microsomes. The measurements of the enzyme activities revealed a strong inhibition of the 6-desaturase activity ($p < 0.01$) and a slight elevation of the 9-desaturase activity ($p < 0.1$) for the 5% HCO + 5% TRANS group.

Experiment II

Switching the rats from the 10% SAF diet either to the 10% HCO, 9% HCO + 1% TRANS or 5% HCO + 5% TRANS diet did not significantly affect the body weights of these rats, but their food consumption increased. However, the food intake among these groups was not significantly different ($p < 0.05$).

Shifting the rats from the 10% SAF diet to the 10% HCO diet decreased the 18:2(n-6) level in the liver microsomes from 11.7 to 7.8% and the 20:4(n-6) level remained unchanged, indicating that these rats did not become EFA-deficient, although they received the EFA-deficient diet for 12 weeks (Table 4). Another indication for this phenomenon was the absence of 18:2(n-9) and 20:4(n-7) and the minor amount (0.2%) of 20:3(n-9) in the liver microsomes of the 10% HCO group. The in vitro experiments showed that the 6-desaturase activity was not altered by the 12-week feeding period of the 10% HCO diet, but the 9-desaturase activity was significantly increased ($p < 0.01$). This was reflected in the fatty acid pattern of the liver microsomes of the 10% HCO group, which showed substantially elevated levels of 16:1 and 18:1, although shifting the rats from the 10% SAF diet to the 10% HCO diet did not change 16:1 consumption and 18:1 intake became nil (Table 5).

Shifting the rats from the 10% SAF to the 1% or 5% TRANS-containing HCO diet increased the amount of 16:1 in the hepatic microsomes to the same extent as was observed with the 10% HCO group. The 18:1 level in the microsomes increased even more, as compared to the 10% HCO group, probably partly due to some higher 18:1 consumption. Measurements of the enzyme activities revealed significantly ($p < 0.01$) enhanced 9-desaturase activities in both TRANS-fed groups. On the contrary, the 6-desaturase activity was inhibited, as also reflected in the fatty acid patterns of the liver microsomes. Including the TRANS supplement in the HCO diet resulted in accumulation of 18:2(n-6) and depression of the 20:4(n-6) level, proportional to the *trans* fatty acid concentration in the diet and in the microsomal lipid.

DISCUSSION

The experiments showed that the 6-desaturase activity in the liver microsomal fraction was impaired by feeding the TRANS supplement. This could be concluded from the results of the incubation reactions in which liver microsomes of TRANS-fed rats showed depressed rates of the 6-desaturase-catalyzed conversion of 18:2(n-6) to 18:3(n-6). Also, Brenner and Peluffo (17) demonstrated, with in vitro experiments using liver microsomes of rats fed a fat-free diet, that linolelaidate inhibits the conversion of linoleic acid to γ-linolenic acid. In our incubation reactions, inhibition of the 6-desaturase was shown in both EFA-deficient and non-EFA-deficient rats. Evidence for the *in vivo* 6-desaturase inhibition was provided by the accumulation of 18:2(n-6)

TABLE 4

Experiment II: Fatty Acid Composition of Liver Microsomal Lipid and Dietary Fat (% wt)

Group[a]	10% SAF (0-12 months)		10% HCO (12-15 months)		9% HCO + 1% TRANS (12-15 months)		5% HCO + 5% TRANS (12-15 months)	
	LM[b]	D[b]	LM	D[c]	LM	D	LM	D
16:0[d]	19.9 ± 0.7[e]	6.7	19.4 ± 1.1	9.3	20.2 ± 1.2	9.4	14.1 ± 0.9	9.7
16:1	1.2 ± 0.2	0.1	4.4 ± 0.9	–	4.7 ± 0.8	–	3.6 ± 1.1	–
18:0	20.6 ± 0.9	2.5	18.5 ± 1.3	14.4	16.4 ± 0.5	13.3	12.8 ± 0.9	9.0
trans-18:1	–	–	–	1.6	1.0 ± 0.1	2.8	2.7 ± 0.2	7.5
cis-18:1	5.8 ± 0.9	11.8	8.2 ± 1.3	tr[f]	10.6 ± 0.8	0.3	11.1 ± 0.5	1.5
trans,trans-18:2	–	–	–	0.1	3.1 ± 0.5	5.2	8.6 ± 0.8	25.5
cis,trans-trans,cis-18:2	–	–	–	–	tr	1.8	tr	9.0
18:2(n-6)	13.8 ± 0.8	77.1	8.1 ± 0.5	0.13	9.4 ± 1.6	0.12	15.7 ± 1.0	0.06
20:3(n-9)	0.1 ± 0.1	–	0.2 ± 0.1	–	0.3 ± 0.1	–	0.1 ± 0.1	–
20:4(n-6)	27.5 ± 1.2	–	27.4 ± 0.9	–	21.2 ± 1.9	–	18.6 ± 1.4	–
22:4(n-6)	2.8 ± 0.3	–	2.6 ± 0.2	–	1.4 ± 0.4	–	1.3 ± 0.3	–
22:5(n-6)	4.6 ± 1.2	–	3.9 ± 1.1	–	2.0 ± 0.9	–	2.2 ± 0.4	–

[a]SAF = safflower oil, HCO = hydrogenated coconut oil, TRANS = concentrate of *trans* fatty acids. For additional details, see Materials and Methods.

[b]LM = liver microsomes, D = diet.

[c]HCO also contained 3.4% 8:0, 5.1% 10:0, 47.7% 12:0 and 17.9% 14:0.

[d]Shorthand designation of fatty acids: number before colon = chain length; number after colon = number of double bonds. The digit after n states the number of carbon atoms from the methyl end of the acyl chain to the nearest double bond.

[e]M ± SD, n = 6.

[f]tr = trace, less than 0.5%.

TABLE 5

Experiment II: Liver Microsomal Enzyme Activities (nmol/min/mg protein)[a]

Group[b]	10% SAF (0-12 months)	10% HCO (12-15 months)	9% HCO + 1% TRANS (12-15 months)	5% HCO + 5% TRANS (12-15 months)
6-Desaturase	0.26 ± 0.03[c,e]	0.25 ± 0.04[d]	0.20 ± 0.03[c]	0.16 ± 0.05[d,e]
9-Desaturase	0.82 ± 0.05[f,g]	1.43 ± 0.05[f,g]	2.45 ± 0.10[g]	2.83 ± 0.09[f]

[a]M ± SD, n = 6 for 10% SAF group; n = 5 for other groups.

[b]SAF = safflower oil, HCO = hydrogenated coconut oil, TRANS = concentrate of *trans* fatty acids. For additional details, see Materials and Methods.

c,d,e,f,g6-Desaturase activities with a common superscript are significantly different for $p < 0.05$. 9-Desaturase activities with a common superscript are significantly different for $p < 0.01$.

in the liver microsomes of the TRANS-fed rats (unaffected by consumption of this fatty acid) and by consequent depression of the 20:4(n-6), 22:4(n-6) and 22:5(n-6) biosynthesis in these animals.

An interesting observation relative to the effect of dietary *trans* fatty acids on the 6-desaturase activity was that the inhibition of this enzyme system was more pronounced in EFA-deficient conditions. In experiment I, shifting the rats from the 10% SAF diet to the 5% SAF + 5% TRANS diet lowered the 6-desaturase activity from 0.26 to 0.20 nmol/min/mg protein while including the same amount of TRANS in the EFA-deficient HCO diet revealed a striking inhibition of the 6-desaturase: the activity dropped from 0.56 to 0.13 nmol/min/mg protein. This seemed to be related to the degree of accumulation of dietary *trans* fatty acids in the liver microsomes: the rats accumulated substantially more *trans,trans*-18:2 and *cis,trans-trans,cis*-18:2 when shifted to the TRANS-containing HCO diet than when fed the 5% SAF + 5% TRANS diet. In accord with this observation was that of Privett et al. (2), who concluded from the fatty acid compositions of rat liver and serum that primarily linolelaidate, compared to elaidate, impaired the interconversion of 18:2(n-6) to 20:4(n-6).

Unlike the 6-desaturase, the incubation experiments showed that dietary *trans* fatty acids do not inhibit the 9-desaturase. In vivo it was shown that elevated levels of 16:1 and 18:1 in the liver microsomes of the TRANS-fed rats generally were not affected by the consumption of these fatty acids; rather they appeared to be related to 2 factors: extra biosynthesis of 16:1 and 18:1 and inhibition of the 6-desaturase system. Assuming that the same 6-desaturase system is involved in the different fatty acid pathways, the 6-desaturase-catalyzed conversions of 16:1(n-7) to 16:2(n-7) and 18:1(n-9) to 18:2(n-9) will be inhibited in the TRANS-fed rats, similar to the inhibition of the 18:2(n-6) desaturation in the linoleate pathway. Consequently, some accumulation of 16:1 and 18:1 in the liver microsomes of the TRANS-fed rats may occur. Simultaneously, extra 16:1 and 18:1 may be synthesized as well, indicated by the elevated 9-desaturase activities in the TRANS-fed groups. This was well demonstrated by the 5% SAF + 5% TRANS group in experiment I: the consumption of 16:1 was negligible and, as a consequence, the elevated level of microsomal 16:1 was essentially produced by biosynthesis, which proved that the 9-desaturase activity in vivo was not inhibited, but even enhanced. With regard to the increased 9-desaturase activity in these experiments, Guo and Alexander's (26) work should be mentioned. They reported that dietary elaidic acid stimulated the capacity for hepatic de novo fatty acid synthesis of saturated and monounsaturated fatty acids from acetate.

The effect of the TRANS supplement on the increase of the 9-desaturase activity in the EFA-deficient rats (5% HCO + 5% TRANS group of experiment I) was only slight as compared to the pronounced effect in the case of the non-EFA-deficient rats. This can be explained by the fact that, unlike the 10% SAF rats, the liver microsomes of the 10% HCO rats contained high levels of 16:1 and 18:1 at the time these animals were switched to the 5% HCO + 5% TRANS diet. Consequently, some product inhibition could be expected when 16:1 and 18:1 were to be extra synthesized upon switching the animals to the TRANS-containing HCO diet.

The results from the present study indicate that, by feeding the TRANS supplement, the inhibition of the 6-desaturase system appears to be responsible for the decrease of the biosynthesis of essential long-chain (n-6) fatty acids. However, the animal requires the highly unsaturated metabolites and attempts

to respond, however unsuccessfully, by intensifying the production of the substrates it is able to synthesize itself, 16:1(n-9) and 18:1(n-9), as shown by the elevation of the 9-desaturase activity.

ACKNOWLEDGMENTS

This work was supported in part by U.S. Public Health Service Grant AG-00174 from the National Institute on Aging; AM-04942 from the Institute of Arthritis and Metabolic Diseases; Grant No. HL-08214 from the Program Projects Branch, Extramural Programs, National Heart, Lung and Blood Institute; and by The Hormel Foundation.

REFERENCES

1. Takatori, T., Phillips, F.C., Shimasaki H., and Privett, O.S. (1976) Lipids 11, 272-280.
2. Privett, O.S., Phillips, F.C., Shimasaki, H., Nozawa, T., and Nickell, E.C. (1977) Am. J. Clin. Nutr. 30, 1009-1017.
3. Privett, O.S., Stearns, E.M., and Nickell, E.C. (1967) J. Nutr. 92, 303-310.
4. Privett, O.S., and Blank, M.L. (1964) J. Am. Oil Chem. Soc. 41, 292-297.
5. Johnston, P.V., Kummerow, F.A., and Walton, C.H. (1958) Proc. Soc. Exp. Biol. Med. 99, 735-736.
6. Sgoutas, D., and Kumerow, F.A. (1970) Am. J. Clin. Nutr. 23, 1111-1119.
7. Egwim, P.O., and Kummerow, F.A. (1972) J. Nutr. 102, 783-792.
8. Schrock, C.G., and Connor, W.E. (1975) Am. J. Clin. Nutr. 28, 1020-1027.
9. Wood, R., and Wiegand, R.D. (1975) Lipids 10, 746-749.
10. Heckers, H., Körner, M., Tüschen, T.W.L., and Melcher, F.W. (1977) Atherosclerosis 28, 389-398.
11. Lemarchal, P., and Munsch, N. (1965) C.R. Soc. Biol. 260, 714-716.
12. Lemarchal, P. (1966) C.R. Soc. Biol. 262, 816-819.
13. Dhopeshwarker, G.A., and Mead, J.F. (1962) J. Lipid Res. 3, 238-242.
14. Karney, R.I., and Dhopeshwarkar, G.A. (1978) Biochim. Biophys. Acta 531, 9-15.
15. Anderson, R.L., Fullmer, C.S., and Hollenbach, E.J. (1975) J. Nutr. 105, 393-400.
16. Houtsmuller, U.M.T. (1978) Fette Seifen Anstrichm. 80, 162-169.
17. Brenner, R.R., and Peluffo, R.O. (1969) Biochim. Biophys. Acta 176, 471-479.
18. Mahfouz, M.M., Johnson, S., and Holman, R.T. (1980) Lipids 15, 100-107.
19. Kurata, N., and Privett, O.S. (1980) Lipids 15, 1029-1036.
20. Inkpen, C.A., Harris, R.A., and Quackenbusch, F.W. (1969) J. Lipid Res. 10, 277-282.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
22. Privett, O.S., and Nutter, L.J. (1967) Lipids 2, 149-154.
23. Marcel, Y.L., Christiansen, K., and Holman, R.T. (1968) Biochim. Biophys. Acta 164, 25-34.
24. Castuma, J.C., Catala, A., and Brenner, R.R. (1972) J. Lipid Res. 13, 783-789.
25. Sprecher, H. (1974) Biochim. Biophys. Acta 360, 113-123.
26. Guo, L.S.S., and Alexander, J.C. (1974) Biochim. Biophys. Acta 369, 411-420.

[Received August 28, 1981]

Volatile Hydrocarbons from Photosynthetic Membranes Containing Different Fatty Acids

GERHARD SANDMANN and **PETER BÖGER**, *Lehrstuhl für Physiologie und Biochemie der Pflanzen, Universität Konstanz, D-7750 Konstanz, Germany*

ABSTRACT

A cell-free plant system was developed generating short-chain volatile hydrocarbons as markers of light-induced copper-mediated peroxidation of fatty acids present either as endogenous constituents of photosynthetic membranes or added exogenously. Different polyunsaturated fatty acids are present in the blue green algae *Anacystis nidulans*, *Anabaena variabilis* and *Spirulina platensis*. The first species has no polyunsaturated acids. Thylakoids isolated from these algae produce different short-chain volatile hydrocarbons. The location of the double bond of dienoic or higher polyunsaturated fatty acids most distant from the carboxyl group determines the chain length of hydrocarbons evolved. Their number of C-atoms is the same as found beyond this double bond of the fatty-acid molecule (ω-1). This pattern of volatile hydrocarbons produced is in contrast to thermolytic cleavage. Malondialdehyde is formed only when at least 3 double bonds are present in the fatty acid. Peroxidation of endogenous thylakoidal and added fatty acids is completed within 24 hr; a maximum of 1% of the carbon skeleton can be recovered as volatile hydrocarbons.

Lipids 17:35-41, 1981.

INTRODUCTION

Short-chain volatile hydrocarbons are some of the possible degradation products formed during peroxidation of bound fatty acids (1). There are reports of significant formation of pentane and ethane by carbon-tetrachloride-treated rats (2), and evolution of ethane and ethylene when intact algae were illuminated in the presence of herbicides or toxic copper amounts (3,4). Evolution of propane was reported as the major gaseous hydrocarbon induced by herbicide treatment of the xanthophycean alga *Bumilleriopsis* (5,6).

From the reaction of fatty acids in model systems, it was found that the various hydrocarbon gases can be formed from different fatty acids (7-10). However, it is doubtful whether the origin of peroxidatively formed hydrocarbon gases in biological systems can be shown by thermal and chemical peroxidative degradation of fatty acids.

Up to now, the origin of short-chain hydrocarbons evolved by isolated thylakoids has not been investigated. In the present communication, this has been achieved using 3 species of blue-green algae which exhibit a qualitatively different fatty acid pattern, and from which active cell-free assay systems can be prepared.

Thylakoid membranes isolated from these organisms were found to actively decompose their endogenous fatty acids as well as those which were added to the reaction mixture during illumination in the presence of copper (50 μM $CuSO_4$). The different hydrocarbons measured in such experiments allowed for a correlation with the composition of polyunsaturated fatty acid(s) present in this algal system.

MATERIALS AND METHODS

Algal Growth and Fatty Acid Determination

Anabaena variabilis (strain 1403-4) and *Anacystis nidulans* (strain 1402-1) were obtained from the Algal Culture Collection, University of Göttingen, whereas *Spirulina platensis* was from our own stock. They were cultivated as described previously (11,12) with the following modifications: *Anacystis* was grown at 35 C; the medium for *Spirulina* contained an additional 16 g of $NaHCO_3$/ℓ of culture medium, which allows growth without gassing by air and CO_2.

Lipids were extracted from the wet paste of 1-ℓ cell suspension batches under nitrogen and in the dark by successive treatments with methanol once at 65 C and twice with chloroform/methanol (2:1, v/v) at 55 C (50 ml each). The combined fractions were pooled and mixed with an equal volume of saturated NaCl solution in a separatory funnel. The lower organic phase was dried over anhydrous Na_2SO_4 and chromatographed on activated Silica Gel G plates in chloroform/methanol/water (65:25:4, v/v/v) (13) to a front distance of about 5 cm to expel colored compounds. The layer between the origin and the lowest colored band was scraped off the extracted by mixing the extract with an equal volume of 0.5 M methanolic KOH and boiling for 2 min. The fatty acids were esterified by adding another aliquot of 14% borontrifluoride in methanol (14). After boiling for another 2 min, the fatty acid methyl

esters were extracted with hexane and separated by gas liquid chromatography (GLC) on a 15% Reoplex column at 200 C. Identification and quantitation of fatty acid esters was done with purchased standards, i.e., methyl esters from the fatty acids of Table 1.

Spheroplast and Thylakoid Preparation; Lipid Peroxidation

Spheroplasts are cells from which the wall has been removed by treatment with lysozyme. They are disrupted ("shocked") by introducing them into the incubation medium as described next; this suspension contains photosynthetic membranes (thylakoids) which are able to perform the Hill reaction without loss of activity for about 2 hr. Spheroplasts were prepared according to the method of Spiller (15) with a lysozyme concentration of 1 mg/ml and an incubation time of 2 hr at 35 C. Cell-free photosynthetic activity was routinely measured as oxygen evolution with the Clark electrode (16) using the $H_2O \rightarrow$ ferricyanide system (15). Average values of about 100 μmol O_2 produced/mg chlorophyll x hr were obtained.

The incubation medium for volatile hydrocarbon evolution consisted of phosphate buffer, pH 7.8, 10 mM; NH_4Cl, 5 mM; $MgCl_2$, 5 mM; $CuSO_4$, 50 μM; and thylakoids (shocked spheroplasts) equivalent to 200 μg of chlorophyll a in a final volume of 2 ml as described previously (12). In the experiments of Tables 2 and 3 and Figure 1, 3 mg of the fatty acid mentioned were pipetted by drops into the 2-ml incubation medium containing the isolated *Anacystis* thylakoid material. For that purpose, palmitic, stearic and arachidonic acids were dissolved in methanol (3 mg/10 μl); the other fatty acids were used directly. Incubation was done in sealed headspace vessels for 2 hr under shaking in a Warburg apparatus, in light, at 25 C (4). Immediately after the onset of shaking, the mixture became dispersed. The vessels fit into the automatic headspace sampler of Perkin-Elmer gas chromatograph Model F22, where they were warmed to 40 C and immediately withdrawn automatically. Hydrocarbons were separated on a 5-ft column of activated alumina with a temperature program from 100 to 170 C at a rate of 20 C/min, and from 170 to 185 C at a rate of 4 C/min and a nitrogen flow of 30 ml/min. Mixtures of standard hydrocarbon gases were prepared in similar concentrations as found in the samples. The retention times were for: C_2H_5, 0.8; C_2H_4, 1.1; C_3H_8, 1.6; C_3H_6, 2.2; *n*-C_4-H_{10}, 2.9; *n*-C_4H_8, 3.2; *i*-C_4H_8, 3.9; C_5H_{12}, 4.8; and C_5H_{10}, 6.1 min. The hydrocarbon gases were further identified on a Poropack-R column. The detection limits for the volatile hydrocarbons were 1-5 pmol. All experiments were done in duplicates. One representative set of data was documented from 3 to 5 separate experiments. The standard error of these experiments was about 10-15%. After hydrocarbon measurement, malondialdehyde was determined from the same samples according to the method of Takahama and Nishimura (17). The values were corrected for the amount of thiobarbiturate-reactive material detected in the dark-treated controls, which was about 20% of the corresponding samples.

To determine the degree of disappearance of added C-18 fatty acids (Table 3), samples were extracted 3 times with 2 ml of diethylether, and the ether was evaporated in a stream of nitrogen. The resulting residue was redissolved in 0.5 M methanolic KOH and the solution

TABLE 1

Concentration of Fatty Acids in Blue-Green Algae

Fatty acids present	*Anacystis nidulans* (μg/μg chlorophyll)	(%)	*Anabaena variabilis* (μg/μg chlorophyll)	(%)	*Spirulina platensis* (μg/μg chlorophyll)	(%)
14:0	0.1	0.5	1.7	1.8	0.8	1.5
16:0	8.8	50.9	19.5	20.2	24.2	43.6
16:1ω7	5.3	30.6	25.8	26.7	4.9	8.9
16:2ω6	0	0	0	0	0	0
16:3ω3	0	0	0.3	0.3	0	0
18:0	0.2	1.1	0.4	0.4	3.2	5.7
18:1ω9	2.9	16.5	12.7	13.1	3.2	5.7
18:2ω6	0	0	14.9	15.4	9.2	16.6
18:3ω3	0	0	19.9	20.6	0	0
18:3ω6	0	0	0	0	12.8	23.1
20:4ω6	0	0	0	0	0	0

Values < 0.5% have been omitted; zero denotes undetectable quantities (see Methods).

TABLE 2

Formation of Volatile Hydrocarbons (nmol) from Exogenously Added Fatty Acids Peroxidized by Illuminated Isolated *Anacystis* Thylakoids in the Presence of 50 μM $CuSO_4$

Fatty acids (3 mg/2 ml) added to thylakoids	Ethane	Ethylene	Propane	Pentane	Pentene
Control	0	0	0	0	0
+18:1	0.003	0	0	0	0
+18:2ω6	0.32	0.19	0.09	8.14	1.19
+18:3ω3	6.31	3.71	0	0	0
+18:3ω6	0.29	0.25	0.21	17.42	4.30
+20:4ω6	0.46	0.28	0.11	28.64	2.62
+β-carotene (15 mg/2 ml)	0	0	0	0	0

Control: no fatty acids were added to the reaction mixture. Three mg of the fatty acids used here are equivalent to about 10 μmol. These were introduced into the incubation medium as given in Methods and illuminated for 2 hr. Addition of 16:0, 16:1 and 18:0 fatty acids produced no hydrocarbons. Zero denotes undetectable quantities.

TABLE 3

Formation of Volatile Hydrocarbons and Thiobarbiturate-Reactive Material (Malondialdehyde, MDA) from C-18 Fatty Acids during Copper-Mediated Peroxidation with Illuminated *Anacystis* Thylakoids in Short- and Long-Term Experiments

	Measurement over 1 hr			Measurement over 24 hr	
Additions[a]	Sum of volatile[b] hydrocarbons (nmol)	MDA (nmol)	Molar ratio (MDA/hydrocarbon)	Residual fatty acid (μg)	Molar ratio (hydrocarbons/fatty acids degraded X 10^{-3})
18:0	0	<1	–	ND[c]	–
18:1	0	<1	–	ND[c]	–
18:2	7.7	3	0.4	36.3	2.8
18:3ω3	5.6	23	4.1	80.7	2.4
18:3ω6	11.8	47	4.0	21.8	8.6

[a]Three mg fatty acid/2 ml reaction volume equivalent to 10 μmol/2 ml.

[b]Ethane and ethylene, but pentane and pentene in the last line.

[c]Not determined.

treated as already described for fatty acid determination.

Chemicals

Fatty acids, their methyl esters (both ca. 99% pure), β-carotene, and boron-trifluoride/methanol mixture were purchased from Sigma, München; standard hydrocarbon gases were purchased from Linde, Höllriegelskreuth, Germany; liquid pentane and pentene, silica gel plates, and other analytical-grade chemicals were from Merck, Darmstadt, Germany.

RESULTS

Anacystis, Anabaena and *Spirulina* were investigated for their fatty acid contents (Table 1). Common to all 3 species were the large amounts of stearic (18:0), oleic (18:1) and palmitoleic acid (16:1). Myristic (14:0) and palmitic (16:0) acid were found as minor components in each of these species. Other fatty acids, especially unsaturated ones, were not detectable in *Anacystis*. In contrast, *Spirulina* and *Anabaena* contained both linoleic (18:2) and linolenic (18:3) acid. However, as shown in Table 1, the 18:3 is present as the α-isomer (18:3ω3) in *Anabaena* and as the γ-isomer (18:3ω6) in *Spirulina*.

In a similar fashion, qualitative differences could be observed in the volatile hydrocarbons evolving during light incubation of thylakoids from all 3 algae (Table 4). *Anacystis* did not form any measurable volatile hydrocarbons with a chain length of 2 or 5 C-atoms. The hydrocarbon gases produced by the other 2

species were ethane, ethylene, pentane and pentene for *Anabaena*, and pentane plus pentene only in the case of *Spirulina*. Substantial quantities of the 2 saturated volatile hydrocarbons were formed by control samples of *Anabaena* and *Spirulina* without addition of copper. Evolution of saturated gaseous hydrocarbons was increased 2- or 3-fold by 50 μM $CuSO_4$. The unsaturated hydrocarbons, ethylene and pentene, were produced in substantial amounts only during the copper-mediated peroxidation. Hydrocarbons with 3 or 4 C-atoms were not detected with *Anabaena* and *Spirulina*.

Anacystis thylakoids which did not exhibit any endogenous hydrocarbon-gas formation were used to peroxidize different fatty acids added to the isolated thylakoid membranes. In these experiments, copper-catalyzed, light-induced peroxidation of palmitic (16:0), palmitoleic (16:1), stearic (18:0) and oleic (18:1) acids did not result in the formation of any detectable hydrocarbon gases with a 2- to 5-carbon skeleton (Table 2). In contrast, all the 2-, 3- and 4-fold unsaturated fatty acids that were used yielded ethane, ethylene, propane, pentane and pentene, depending on the position of the double bonds. Application of α-linolenic acid (18:3ω3) to the *Anacystis* thylakoids yielded ethane and ethylene exclusively. Linoleic acid (18:2ω6), γ-linolenic acid (18:3ω6) and arachidonic acid (20:4ω6) predominantly yielded pentane and pentene with traces of propane, ethane and ethylene. A compound with a conjugated system of double bonds was added as a control. As expected, β-carotene did not produce volatile hydrocarbons (Table 2).

Volatile hydrocarbon evolution by *Anacystis* thylakoids after addition of α-linolenic acid was traced over 24 hr (Fig. 1). In the presence of 50 μM Cu^{2+}, light-induced ethane evolution started immediately and leveled off between 7 and 24 hr. In contrast, ethylene formation exhibited a lag phase of 1 to 2 hr. In the linear part of the curve between 2 and 6 hr, the ethane-to-ethylene ratio was more or less constant with 2 to 2.5 times more release of ethane than ethylene from α-linolenic acid.

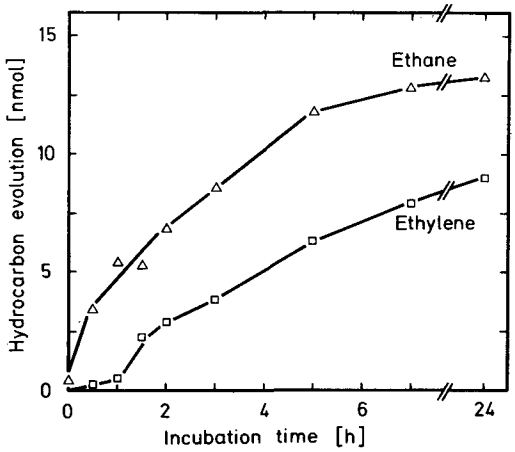


FIG. 1. Light-induced formation of ethane and ethylene from added α-linolenic acid (3 mg/2 ml) using isolated *Anacystis* thylakoids in the presence of 50 μM $CuSO_4$. Dark controls or light controls in which copper was omitted yielded about 1 nmol C-2 hydrocarbons after 4 hr of incubation. The α-linolenic acid used was taken from a fresh vial opened immediately before the experiment.

Table 3 demonstrates the quantitative relationship of the peroxidation of linoleic, α-linolenic and γ-linolenic acid, each present with 3 mg at the start. After 1 hr, the formation of volatile hydrocarbons and malondialdehyde from the C-18 fatty acids as indicated was determined. All fatty acids with 2 or more double bonds yielded short-chain hydrocarbon gases. Substantial amounts of malondialdehyde, another marker product of lipid peroxidation, were only found when trienoic fatty acids were

TABLE 4

Production of Volatile Hydrocarbons (pmol) with Illuminated, Copper-Treated Thylakoids from Three Species of Blue-Green Algae

Hydrocarbons evolved	*Anacystis*		*Anabaena*		*Spirulina*	
	Control	Cu^{2+}	Control	Cu^{2+}	Control	Cu^{2+}
Ethane	0	0	16.9	43.9	0	0
Ethylene	0	0	2.7	45.2	0	0
Pentane	0	0	20.1	52.4	11.7	37.0
Pentene	0	0	1.2	18.2	0	27.6

Fifty μM $CuSO_4$ and isolated thylakoids were present in the incubation medium (see Methods); illumination was for 2 hr. In the control experiments, copper was omitted. Zero denotes undetectable quantities.

degraded. In a parallel experiment over 24 hr, the maximal hydrocarbon evolution reached (Fig. 1) was determined together with the remaining fatty acid which had not been peroxidized. All polyunsaturated fatty acids were degraded to more than 99%. Stoichiometrically, the yield of volatile hydrocarbons produced was, however, less than 1%.

DISCUSSION

The composition of fatty acids in *Anacystis* has been reported previously (18). This was confirmed for our strain; polyunsaturated acids were absent. The detection of additional linoleic and α-linolenic acids in *A. variabilis* and linoleic and γ-linolenic acids in *S. platensis* again is in accordance with previous investigations (19-21). Furthermore, we determined the quantitative amount of these fatty acids in relation to chlorophyll contents (Table 1). This was necessary to estimate the amounts of volatile hydrocarbons evolved in relation to fatty acids of thylakoids.

In a recent paper (12), the proposed mechanism of peroxidative formation of hydrocarbon gases was outlined. Radicals arising from the action of copper ions (or certain herbicides such as substituted *p*-nitrodiphenylethers like oxyfluorfen [5]) initiate light-induced peroxidative degradations of fatty acids via the corresponding hydroperoxides. Copper ions are important for initiation of hydroperoxide formation and further conversion to hydrocarbons (12). Presumably, the ratio of ethane to ethylene is dependent on the relationship of Cu^{2+} to Cu^{+} ions in the medium, which is again influenced by the redox activity of the thylakoid material during the experimental time (Fig. 1). As shown in this paper, light-induced hydrocarbon-gas evolution from copper-treated thylakoids was clearly correlated with their endogenous fatty acids present in the photosynthetic membranes.

Absence of polyunsatured fatty acids in *Anacystis* was paralleled by lack of detection of gaseous hydrocarbon formation (Table 4). The main qualitative difference in the polyunsaturated fatty acids between *Spirulina* and *Anabaena*, i.e., the presence of two ω-6 fatty acids in the first species and both an ω-3 and an ω-6 fatty acid in the *Anabaena*, was demonstrated by the formation of volatile hydrocarbons of 5 C-atoms in *Spirulina*, and by volatile hydrocarbons of 2 and 5-C atoms in *Anabaena*. Our conclusion that 2 C-atom hydrocarbon gases derive from ω-3 and 5 C-atom hydrocarbons are formed from ω-6 polyunsaturated fatty acids was further corroborated by the addition of fatty acids to *Anacystis* under peroxidative conditions (Table 2).

With our cell-free thylakoid system from blue-green algae, we have been able to confirm and generalize that at least 2 double bonds are necessary to degrade fatty acids to volatile hydrocarbons, and that the ω-6 isomers of polyunsaturated fatty acids yield hydrocarbons with 5 C-atoms and the ω-3 species produce hydrocarbon gases with 2-C atoms.

The following difference was evident between peroxidation of either endogenous or exogenously added fatty acids. In the exogenously added fatty acids (Table 2), small amounts of hydrocarbons with 2 and 3 C-atoms could always be detected with pentane and pentene as the major gaseous hydrocarbons. Less than 2% of volatile hydrocarbons shorter than 5 C-atoms were found during chemical decomposition of γ-linolenic acid hydroperoxide to pentane (9). However, when thylakoids from *Spirulina* were used, which endogenously contain only an ω-5 species as polyunsaturated fatty acid, pentane and pentene were found, but no other short-chain hydrocarbon gases.

Comparison of the data from Tables 2 and 4 supports the assumption that very small amounts of isomers (different localization of double bonds) present as impurities in the purchased fatty acids are the source of ethane, ethylene and propane (for which the amount was below 0.5 nmol/vessel over a 2-hr incubation time; see Table 2). This conclusion does not agree with the interpretation of Dumelin and Tappel (8), who discussed formation of shorter chain hydrocarbons by β-scission of longer chain alkyl radicals. Another argument against, e.g., the formation of propane from the pentane radical by β-scission, is our finding of large amounts of propane in the xanthophycean alga *Bumilleriopsis filiformis* when it was treated with paraquat (6). We succeeded in isolating from this alga a fatty acid that is decomposed mainly to propane in the *Anacystis* assay system of Table 2. Investigation of the structure of this fatty acid is underway.

As demonstrated by our data with the biological system presented here, peroxidative formation of volatile hydrocarbons by chemical decomposition of fatty acids cannot be reliably generalized. In particular, the results of thermolytic scission of fatty acids reported by Arnaud and Wuhrmann (7) cannot be compared to oxidative degradation under physiological conditions. Their finding that all unsaturated fatty acids, including the monounsaturated ones, were split to hydrocarbons suggests a mechanism different from hydrocarbon formation in biological systems. In the previously reported

model system (8) using fatty acid methyl esters and corresponding peroxides as well as iron and copper ions, pentane from added ω-6, γ-linoleic and arachidonic acids, methyl ester was formed as well as ethane from the ω-3, α-linolenic acid methyl ester. These data are similar to ours. The chemical system described by Dumelin and Tappel (8) best resembles our enzymatically catalyzed reaction.

The usefulness of applying fatty acids to suspensions of intact algal cells appears doubtful as a means of gaining insight into biological peroxidation processes, as was reported recently (10). High fatty acid concentrations were necessary as compared to low yield of volatile hydrocarbons. The fatty acid uptake by the cells is the crucial point in using intact cells for quantitative measurements of biological fatty acid degradation and corresponding volatile hydrocarbon evolution. Still, the possibility remains that part of the gas production was caused by chemical decomposition outside the cells due to metal ions present. The assay presented in this paper comes closer to the physiological situation of the cells, because it takes advantage of the endogenous thylakoid-bound fatty acids.

Besides short-chain volatile hydrocarbons, thiobarbiturate-reactive material is a product of lipid peroxidation. During long-term incubation, malondialdehyde as a reactive molecule can undergo side reactions by cross-linking with various biological compounds (22). Therefore, its relationship to hydrocarbons was determined after a 1-hr incubation time (Table 3). Degradation of fatty acids and formation of hydrocarbon gases comes to an end after 24 hr. Consequently, the total amount of volatile hydrocarbons evolved could only be measured after a longer reaction time (24 hr, Fig. 1). As a source of both malondialdehyde and volatile hydrocarbons during peroxidation by our thylakoid system, 3 types of fatty acids can be distinguished: first, saturated and monounsaturated fatty acids yield neither gaseous hydrocarbons nor malondialdehyde; second, the dienoic acids form hydrocarbon, but very little malondialdehyde; and third, we have ω-3 and ω-6 trienoic acids that produce hydrocarbons and malondialdehyde simultaneously. Absence of malondialdehyde from a dienoic fatty acid was already demonstrated for the autoxidation of linoleic acid (23). This can be explained by the mechanism proposed by Pryor et al. (24), who assumed malondialdehyde to be formed from a prostaglandin-like endoperoxide. This mechanism is only possible with a trienoic or more highly unsaturated fatty acid.

The amount of malondialdehyde formed from α- or γ-linolenic acids exceeded about 4 times that of volatile hydrocarbons (Table 3). Ratios in the same order were reported by Riley and Cohen (25). In brain and liver homogenates from mice treated with carbon tetrachloride to induce peroxidation, they found 5 to 9 times more malondialdehyde formation compared to ethane production.

During a 24-hr incubation, about 99% of the added polyunsaturated acids were decomposed, and about 0.3-0.9% could be recovered as volatile hydrocarbons. These ratios were also reported for other peroxidative systems to which fatty acids had been added (8). As our data show, fatty acids present as part of membrane lipids seem to be less accessible, possibly due to a closer association with endogenous antioxidants (26). The thylakoids from *Anabaena* and *Spirulina* used in the peroxidation experiments (Table 4) contained roughly the same amount of endogenous polyunsaturated fatty acids as were exogenously added to *Anacystis* thylakoids (Table 2). Nevertheless, they produce only 1/1,000 of the volatile hydrocarbons produced by exogenously given fatty acids as indicated by the data in Table 3.

ACKNOWLEDGMENT

This research was supported by the Deutsche Forschungsgemeinschaft. We appreciate the helpful comments of Drs. A.L. Tappel and K.J. Kunert, both at the University of California, Davis, CA. Thanks are expressed to S. Kuhn for expert technical assistance.

REFERENCES

1. Tappel, A.L. (1980) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. 4, pp. 1-47, Academic Press, New York.
2. Tappel, A.L., and Dillard, C.J. (1981) Fed. Proc. 40, 174-178.
3. Elstner, E.F., and Pils, J. (1979) Z. Naturforsch. 34c, 1040-1043.
4. Sandmann, G., and Böger, P. (1980) Z. Pflanzenphysiol. 98, 53-59.
5. Lambert, R., and Böger, P. (1981) Z. Pflanzenkr. Pflanzenpathol. Pflanzenschutz, special issue no. 9, 147-152.
6. Boehler-Kohler, B.A., Läpple, G., Hellman, V., and Böger, P. (1981) Pestic. Sci., in press.
7. Arnaud, M., and Wuhrman (1974) Proc. IV Int. Congr. Food Sci. and Technol. Vol. 1, 186-193.
8. Dumelin, E.E., and Tappel, A.L. (1977) Lipids 12, 894-900.
9. Donovan, D.H., and Menzel, D.B. (1978) Experientia 34, 775-776.
10. Schobert, B., and Elstner, E.F. (1980) Plant Physiol. 66, 215-219.
11. Sandmann, G., and Böger, P. (1980) Plant Sci. Lett. 17, 417-424.
12. Sandmann, G., and Böger, P. (1980) Plant Physiol. 66, 797-800.
13. Allen, C.F., and Good, P. (1971) Methods

Enzymol. 23A, 523-547.
14. Metcalfe, L.C., Schmitz, A.A., and Pelka, J.R. (1966) Anal. Chem. 38, 514-515.
15. Spiller, H. (1980) Plant Physiol. 66, 446-450.
16. Böger, P., and Schlue, U. (1976) Weed Res. 16, 149-154.
17. Takahama, U., and Nishimura, M. (1975) Plant Cell Physiol. 16, 737-748.
18. Parker, P., van Baalen, C., and Maurer, L. (1967) Science Washington 155, 707-708.
19. Oro, J., Tornabene, T.G., Nooner, D.W., and Gelpi, E. (1967) J. Bacteriol. 93, 1811-1818.
20. Nichols, W.B., and Wood, B.J.B. (1968) Lipids 3, 46-50.
21. Schneider, H., Gelpi, E., Bennett, E.O., and Oro, J. (1970) Phytochemistry 9, 613-617.
22. Goldstein, B.D. (1979) in Oxygen Free Radicals and Tissue Damage, Ciba Foundation (New Series) Vol. 65, pp. 295-314, Excerpta Medica, Amsterdam.
23. Dahle, L.K., Hill, E.G., and Holman, R.T. (1962) Arch. Biochem. Biophys. 98, 253-261.
24. Pryor, W.A., Stanley, J.P., and Blair, E. (1976) Lipids 11, 370-379.
25. Riley, C.A., and Cohen, C. (1974) Science Washington 183, 208-210.
26. Witting, L.A. (1980) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. 4, pp. 295-319, Academic Press, New York, NY.

[Received July 6, 1981]

Lung Phosphatidylcholine and Fasting in Male and Female Rats

MASAO ABE*, MAKOTO ENDO and **HIROSHI NAGAI**, *Department of Biochemistry, Sapporo Medical College, Sapporo, 060, Japan*

ABSTRACT

Lung from male and female rats fasted for 4 days were used. Phospholipid, phosphatidylcholine and its molecular species were analyzed in lungs from these rats and effects of fasting upon the biosynthesis of phosphatidylcholine in lungs from both sexes were determined using radioactive choline. The molecular species of phosphatidylcholine in both male and female rats did not differ with fasting except the monoenoic species. Incorporation of choline into phosphatidylcholine in both male and female rats significantly increased after fasting, but distribution of radioactivity in phosphatidylcholine yielded similar values in each group. These results suggest that the decrease of saturated phosphatidylcholine content after fasting may be not due to specific change in saturated phosphatidylcholine.

Lipids 17:42-45, 1981.

INTRODUCTION

Pulmonary surfactant, which prevents the collapse of the alveoli during expiration and the filling of alveoli with transudate from the capillaries (1), appears to be lipoprotein (2). The lipid part of this lipoprotein thought to be primarily responsible for the surface-active properties is the phospholipid saturated (dipalmitoyl) phosphatidylcholine (3,4).

It has been reported that the synthesis and secretion of components of the surfactant can be decreased by interfering with lung oxidative metabolism, and that fasting (food deprivation) depresses lung oxidative metabolism. Gail et al. (5) have reported that saturated phosphatidylcholine (PC) of both tissue and lavage from lung tissue (male rat) significantly decreased in fasted rats. In spite of these decreases, the air pressure-volume curves of excised lungs of fasted animals were the same as those for control animals. These reports led us to question whether only saturated PC in lung decreased specifically after fasting.

The purpose of this study was to examine in greater detail the amount and the composition of molecular species of PC in lungs of fasted rats.

MATERIALS AND METHODS

Male and female Wistar rats weighing ca. 180 g were used in these experiments. Experimental rats were fasted for 4 days while control rats were maintained on an ad libitum diet of Oriental rat feed (composition: carbohydrate, 53.0%; protein, 24.1%; lipid, 4.6%; water, 7.0%; mineral, 7.1%; fiber, 4.2%) (Oriental Yeast Co., Tokyo). All animals were allowed free access to drinking water. Rats were anesthetized by the intraperitoneal injection of pentobarbital (50 mg/kg body wt). In rapid sequence, we opened the thorax and transected the inferior vena cava and aorta. The lung was then removed and weighed.

After the lungs were removed, they were sliced with a tissue slicer set at 1 mm. The slices were immediately weighed and placed into 50-ml Erlenmeyer flasks containing 4.0 ml of Krebs-Ringer bicarbonate buffer with bovine serum albumin (5 g/100 ml) (6); 1.6 μCi of [^{14}C]choline was added to the medium. The flasks were capped, gassed with 95% O_2 and 5% CO_2, and then shaken at a frequency of 120/min in an incubator at 37 C for 90 min. After incubation, the slices and medium were centrifuged at 10,000 x g for 10 min; the sediment was resuspended in cold 0.9% NaCl solution, recentrifuged and then homogenized for lipid extraction.

The lipids were extracted from the lung tissue (ca. 500 mg) or slices (ca. 200 mg) with 30 ml of chloroform/methanol (2:1, by vol) according to the method of Folch et al. (7). The PC fraction was isolated by thin layer chromatography (TLC) using a solvent system of chloroform/methanol/water (70:30:5, by vol) as described previously (6). The PC fraction was further chromatographed to remove contaminated phosphatidylglycerol on thin layer plates (Silica Gel G plates prepared with 0.4 M boric acid) using a solvent system of chloroform/methanol/water/conc ammonia (70:30:3:2, by vol) according to the method of Poorthuis et al. (8).

PC was converted to diacylglycerol (DG) using phospholipase C according to the method of Renkonen (9). The DG was further converted to DG-acetate in the presence of pyridine and acetic anhydride. The resulting DG-acetate was separated into saturated, monoenoic, dienoic, trienoic, tetraenoic and hexaenoic species by TLC on $AgNO_3$-impregnated

TABLE 1

Phospholipid Content in Control and Experimental Groups

	Male (n=8)			Female (n=8)		
	Control	Experimental	P	Control	Experimental	p
Phospholipid						
μmol/g wet lung	31.6 ± 2.6	30.3 ± 1.9	NS[a]	31.7 ± 2.5	29.4 ± 1.0	NS
μmol/whole lung	34.4 ± 3.2	27.3 ± 2.7	<.001	33.8 ± 2.8	26.3 ± 1.9	<.001
Phosphatidylcholine						
μmol/g wet lung	16.1 ± 1.6	15.3 ± 1.3	NS	17.4 ± 1.4	17.9 ± 1.0[b]	NS
μmol/whole lung	17.5 ± 1.6	13.8 ± 1.6	<.001	18.5 ± 1.6	16.0 ± 1.7[c]	NS
% to phospholipid	50.9 ± 2.3	50.4 ± 2.3	NS	54.9 ± 3.6	60.8 ± 3.1[d]	<.05

Values are means ± SD; duplicate determinations were done in each group.

[a]NS = not significant.

[b]$p<.01$ vs male.

[c]$p<.05$ vs male.

[d]$p<.001$ vs male.

plates using a solvent system of toluene/methanol (97:3, by vol).

In tissue slice experiments, saturated PC was obtained by using the procedure of Shimojo et al. (10) which oxidizes unsaturated acyl groups and reduces the chromatographic mobility of the unsaturated PC.

Among the chemicals used in this study were [1,2-^{14}C] choline (sp act 10 mCi/mmol, from New England Nuclear), bovine serum albumin (Fraction V powder, from Seikagaku kogyo Co., Ltd., Tokyo), and phospholipase C from *Clostridium welchii* (from Sigma, St. Louis, MO).

Phosphorus was determined by the method of Bartlett (11). Glycerol was determined by the Van Handel and Zilversmit method (12). Protein content was determined in samples which had been homogenized in distilled water (13). Radioactivity was measured using a liquid scintillation counter and an external standard (6).

RESULTS

Table 1 shows some changes in phospholipid and PC contents of male and female rat lungs after fasting. Phospholipid contents/g wet lung in both sexes showed no difference between the control and experimental groups. The phospholipid content per whole lung in both male and female, however, significantly decreased after fasting. On the other hand, PC content/g wet lung in both male and female showed no difference between the control and experimental groups. The PC content per whole lung decreased significantly in male rats but only slightly in female rats after fasting. The percentage of PC in phospholipid after fasting showed no change in male rats but increased significantly in female rats. In addition, PC content (both per g wet and per whole lungs) and its percentage to phospholipid in the experimental group were significantly greater in females than in males.

Table 2 shows molecular species pattern of PC in control and experimental rat lungs (both male and female). Monoenoic species in both males and females significantly increased after fasting. Saturated PC, which is a major component of lung and surfactant PC, was similar in each group. The other species in both males and females showed no differences between control and experimental groups, indicating that fasting did not alter any molecular species of lung PC except the small changes in monoenoic species.

Table 3 shows incorporation of [^{14}C] choline into PC in the control and experimental rat lungs (both male and female). Sp act of PC in both males and females significantly increased after fasting, suggesting that utilization of choline increased in fasted rat lungs because PC content/g wet lung in both males and females showed no difference between the control and experimental groups (Table 1). Distribution of radioactivity in saturated and unsaturated PC showed no difference between the control and experimental lungs in both male and female rats.

DISCUSSION

Faridy (14) has reported that food deprivation causes a decrease in contents of phospholipid and PC/mg DNA of lung. However, his data also indicated that phospholipid and PC/g wet lung after fasting yielded similar values to

TABLE 2

Molecular Species of Phosphatidylcholine in Two Groups (Mol %)

	Male (n=8)			Female (n=8)		
	Control	Experimental	p	Control	Experimental	p
Saturated	34.0 ± 1.2	33.4 ± 0.9	NS[a]	33.8 ± 1.2	33.6 ± 1.7	NS
Monoene	25.8 ± 1.2	28.9 ± 0.9	<.01	24.9 ± 1.9	28.0 ± 1.4	<.01
Diene	17.5 ± 1.0	16.7 ± 1.0	NS	16.7 ± 0.7	15.7 ± 1.1	NS
Triene	3.0 ± 0.3	3.0 ± 0.4	NS	2.9 ± 0.3	3.1 ± 0.5	NS
Tetraene	15.3 ± 1.3	14.1 ± 0.6	NS	16.0 ± 0.9	14.6 ± 0.6	NS
Hexaene	4.4 ± 0.9	3.9 ± 1.0	NS	5.7 ± 0.9	4.8 ± 1.1	NS

Values are means ± SD; duplicate determinations were done in each group.
[a]NS = not significant.

TABLE 3

Incorporation of [^{14}C]Choline into Phosphatidylcholine in Two Groups

	Male (n=3)			Female (n=3)		
	Control	Experimental	p	Control	Experimental	p
Phosphatidylcholine (PC) (dpm × 10^3/μmol)	118.8 ± 8.3	135.6 ± 4.8	<.01	121.0 ± 1.0	136.5 ± 2.1	<.01
Distribution % of radioactivity						
Saturated-PC	33.6 ± 3.5	32.0 ± 0.1	NS[a]	32.1 ± 2.1	33.2 ± 1.0	NS
Unsaturated-PC	66.4 ± 3.5	68.0 ± 0.1	NS	67.9 ± 2.1	66.8 ± 1.0	NS

Values are means ± SD; duplicate determinations were done in each group.
[a]NS = not significant.

those of controls. Recently, Gail et al. (5) have extended Faridy's findings by measuring saturated PC, which is more specific than total PC and a major component of pulmonary surfactant, and found that starvation lowered both alveolar and lung tissue saturated PC. On the other hand, the present results indicated that the contents of phospholipid and PC changed after fasting when calculated per whole lung but not when calculated per g wet lung. Therefore, this observation suggests that the decrease in phospholipid and PC, including saturated PC, after fasting may be related to a decrease of lung weight caused by fasting.

It may be reasoned that food deprivation may alter lung surface forces because the rates of synthesis and secretion of surface-active material can be decreased by interfering with lung oxidative metabolism which is depressed by fasting. However, Rhoades (15), Weiss and Jurrus (16), and Gail et al. (5) have reported that pressure-volume curves of lungs excised from fasted rats were the same as those of control rats, and Faridy (14) has reported the slightly increased surface force. These observations are consistent with our results; the PC and saturated PC content/g wet lung after fasting showed no change in both male and female rats. However, our results appear to differ from the report by Gail et al. (5), who reported slight decreases in volume density of lamellar bodies in the type II cell cytoplasm of fasted rats.

The influence of fasting on the utilization of glucose and palmitate in lung reported by Rhoades (15) suggests some changes in PC metabolism to maintain essential lung lipids during an altered physiologic state. In addition, Gross et al. (17) have reported that food deprivation decreased the activity of several enzymes required for phospholipid synthesis. On the other hand, rats fasted for 4 days may have a relative choline deficiency because choline deficiency has been reported to develop by feeding diets without choline for 4-7 days (18). We have noted that the fasted rat livers have compositional changes of PC and phosphatidylethanolamine similar to those of choline-deficient rats (19), although the altered physiologic state in the fasted rat markedly differed from that in the choline-deficient rat (20). Therefore, the increased incorporation of radiolabel from choline into PC in both male and female rats after fasting (Table 3) suggests

contributions of increased de novo synthesis of PC, but also decreased pool size of free choline in the lung. Moreover, these results on choline incorporation into saturated and unsaturated PC indicate that fasting may not affect the specificity of CDP-choline:diacylglycerol cholinephosphotransferase for each molecular species of diacylglycerol in the lung because the distribution of radioactivity in saturated and unsaturated PC showed no difference between control and experimental groups.

ACKNOWLEDGMENT

The authors thank Prof. K. Ohno and Assoc. Prof. T. Shimojo, Department of Biochemistry, Sapporo Medical College, for their encouragement. This work was supported in part by a Scientific Research Grant from the Ministry of Education of Japan.

REFERENCES

1. Farrel, P.M., and Avery, M.E. (1975) Am. Rev. Resp. Dis. 111, 657-688.
2. King, R.J. (1974) Fed. Proc. 33, 2238-2247.
3. Van Golde, L.M.G. (1976) Am. Rev. Resp. Dis. 114, 977-1000.
4. Ohno, K., Akino, T., and Fujiwara, T. (1978) Rev. Perinat. Med. 2, 227-318.
5. Gail, D.B., Massaro, G.D., and Massaro, D. (1977) J. Appl. Physiol. 42, 88-92.
6. Abe, M., and Tierney, D.F. (1977) J. Appl. Physiol. 42, 202-205.
7. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
8. Poorthuis, B.J.H., Yazaki, P.J., and Hostetler, K.Y. (1976) J. Lipid Res. 17, 433-437.
9. Renkonen, O. (1966) Biochim. Biophys. Acta 125, 288-309.
10. Shimojo, T., Abe, M., and Ohta, M. (1974) J. Lipid Res. 15, 525-527.
11. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
12. Van Handel, E., and Zilversmit, D.B. (1957) J. Lab. Clin. Med. 50, 152-157.
13. Lowry, D.H., Rosebrough, N.J., Farr, A.L., and Randall, R.T. (1951) J. Biol. Chem. 193, 265-275.
14. Faridy, E.E. (1970) J. Appl. Physiol. 29, 493-498.
15. Rhoades, R.A. (1975) J. Appl. Physiol. 38, 513-516.
16. Weiss, H.S., and Jurrus, E. (1971) Respiration Physiol. 12, 123-129.
17. Gross, I., Rooney, S.A., and Warshaw, J.P. (1975) Biochem. Biophys. Res. Commun. 64, 59-63.
18. Leelavathi, D.E., Katyal, S.L., and Lombardi, B. (1974) Life Sci. 14, 1203-1210.
19. Abe, M., Endo, M., Nagai, H., and Shimojo, T. (1981) IRCS Med. Sci. 9, 34.
20. Abe, M., Endo, M., Nagai, H., and Shimojo, T. (1981) IRCS Med. Sci. 9, 410.

[Received May 26, 1981]

METHODS

Reactivity of Key Metabolic Sterols in Standard Colorimetric Assays for Cholesterol

CHITTA P. SARKAR and **RICHARD J. CENEDELLA**, *Department of Biochemistry, Kirksville College of Osteopathic Medicine, Kirksville, MO 63501*

ABSTRACT

The reaction of lanosterol, desmosterol and 7-dehydrocholesterol, key intermediates in cholesterol biosynthesis, were compared with cholesterol in 3 standard colorimetric assays for cholesterol based on formation of chomogens with acetic anhydride, ferric chloride and ferrous sulfate. Marked differences in the reaction of the sterols in the different assays were due both to formation of chomogens with qualitatively similar spectral patterns but with greatly different extinctions and to formation of chromogens with clearly different absorption maxima. For example, in all assays, cholesterol and desmosterol formed chromogens with very similar absorption spectra but with varying extinctions, whereas the lanosterol chromogen in all assays was different from cholesterol's in both absorption maxima and in extinctions. The findings show that attempts to measure tissue sterol levels by colorimetric methods can result in great errors when cholesterol is not the sole sterol. Also, the unique spectral properties of the lanosterol chromogen formed in the Liebermann-Burchard reaction (a sharp absorption peak at 450 nm) suggests the possible use of this method as a qualitative test for lanosterol. *Lipids* 17:46-49, 1981.

Differences in the reactivity of 7-dehydrocholesterol and cholesterol in the Liebermann-Burchard reaction has been used as a basis for estimating hepatic cholesterolgenesis (1). Knowledge about the reactivity of various sterols in colorimetric assays used to measure cholesterol have been important in pharmacological studies involving inhibition of cholesterolgenesis at late metabolic steps (2,3). For example, Peter et al. (2), in attempting to measure changes in tissue total sterols following treatment of rats with 20,25-diazacholesterol, a drug which causes accumulation of desmosterol, observed that desmosterol gave only 75% of the color produced by cholesterol when the assay of Zlatkis et al. (4) was used. The authors corrected their measurements of tissue sterols using this information. Dvornik and Hill (3) similarly used an observation that 7-dehydrocholesterol produced only 40% as much color as cholesterol in the Zlatkis assay to permit measurement of tissue sterol levels following treatment with AY-9944. Although the reactivity of some sterols in the Liebermann-Burchard reaction was compared many years ago (5,6), little information is available on possible differences in the reaction of important metabolic sterols in the various standard colorimetric assays for cholesterol and on the reason for such differences. This study compares the reaction of lanosterol, desmosterol and 7-dehydrocholesterol, key intermediates in cholesterol synthesis, with cholesterol in the methods of Sperry and Webb (modified Liebermann-Burchard) (7), Zlatkis et al. (4) and Searcy and Bergquist (8) for colorimetric estimation of cholesterol. These assays are based on the formation by cholesterol of chromogens with acetic anhydride, ferric chloride and ferrous sulfate, respectively.

MATERIALS AND METHODS

Chemicals

Ayerst Research Laboratories (Montreal, Que.) was the source of the AY-9944. Cholesterol, 7-dehydrocholesterol, desmosterol and lanosterol for gas liquid chromatography (GLC) and for generation of the standard reaction curves in the different colorimetric assays were obtained from Sigma Chemical Co. (St. Louis, MO). Mass spectrometry of the commercially available lanosterol established it to be composed of about 60% lanosterol and 40% dehydrolanosterol (unpublished observations). Cholesterol, 7-dehydrocholesterol and desmosterol each produced one peak in GLC analysis. All other chemicals and solvents were reagent-grade quality.

Animals and Treatments

Pregnant Sprague-Dawley rats were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA).

Beginning at 6 days of age, pups were injected (s.c.) every fourth day with 50 mg/kg of AY-9944 (*trans*-1,4-bis[2-chlorobenzylaminomethyl]cyclohexane dihydrochloride) suspended in olive oil. This dosage was continued for 4 weeks. Untreated pups served as controls.

Extraction and Saponification of Sterols

Treated and age-matched control rats were sacrificed by decapitation at 35 days of age. Total lipids were extracted from whole brain by homogenization in 20 vol of chloroform/methanol (2:1, v/v) as we described before (9). Aliquots of the total lipid extract were saponified and the nonsaponifiable lipids recovered (10). The sterol content of this fraction was then quantitated by GLC and by 3 separate colorimetric assays (4,7,8).

Estimation of Sterols by Gas Liquid Chromatography

Sterols were analyzed by GLC using a 6-ft coiled glass column (0.22 mm id) packed with 3% OV-17 on 100/120 Gas Chrom Q and operated isothermally at 265 C in a Becker 417 gas chromatograph with flame ionization detection (FID). Cholesterol and 7-dehydrocholesterol appeared as 2 well separated peaks in this system. Sterol concentrations were determined by comparing the area of peaks (determined by triangulation) with that produced by a known amount of 5-α cholestane added as internal standard. The ratio of the response of a given mass of cholesterol and 7-dehydrocholesterol to 5-α cholestane in the FID was also determined.

Colorimetric Assays of Sterols

The reactivity of cholesterol, lanosterol, 7-dehydrocholesterol and desmosterol in 3 standard colorimetric assays for cholesterol were compared. The reaction curves generated by known amounts of these sterols were determined for the acetic-anydride method of Sperry and Webb (7), the $FeCl_3$ method of Zlatkis et al. (4) and the $FeSO_4$ method of Searcy and Bergquist (8) as modified by Pollet et al. (11).

Absorption Spectra of Sterol Chromogens

The absorption spectrum of the chromogens developed by a given mass of the various sterols in each of the color reactions was determined for the 350 nm and 720 range using a Perkin-Elmer Lambda 3 recording spectrophotometer.

RESULTS AND DISCUSSION

Marked differences were seen in the reaction curves generated by the various sterols in the different color assays (Fig. 1). As noted earlier (5,6), 7-dehydrocholesterol yielded higher optical densities than cholesterol with the Liebermann-Burchard reagent (Fig. 1A) whereas lanosterol and desmosterol produced about only 10 and 40%, respectively, of the color generated by cholesterol. The poor reaction of lanosterol and desmosterol can be explained by lack of formation of a chromogen by either sterol which absorbed in the 660 nm range (Fig. 2A), the wavelength optimum of the assay. Although the absorption spectrum of 7-dehydrocholesterol and cholesterol were qualitatively similar, both having absorption peaks in about 660 nm and 400 nm, 7-dehydrocholesterol produced a greater extinction than

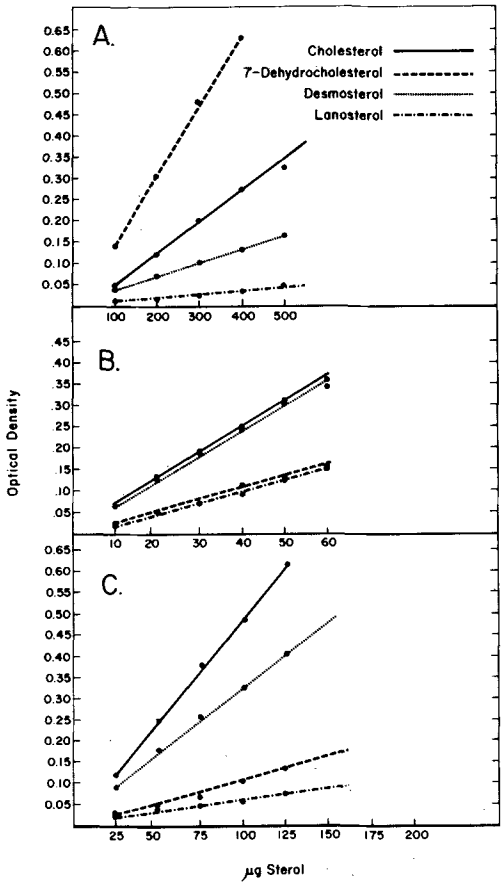


FIG. 1. Reaction curves of cholesterol, desmosterol, 7-dehydrocholesterol and lanosterol in 3 colorimetric assays for cholesterol. A. Method of Sperry and Webb (7) using the Liebermann-Burchard reagent (acetic anhydride): od read at 660 nm. B. Method of Searcy and Bergquist (8) as modified by Pollet et al. (11) using $FeSO_4$: od read at 460 nm. C. Method of Zlatkis et al. (4) using $FeCl_3$: od read at 560 nm.

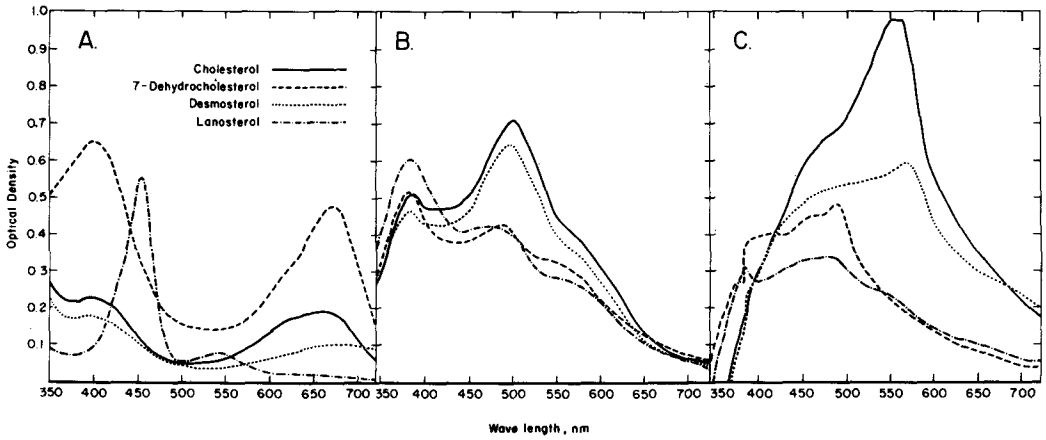


FIG. 2. Absorption spectra of chromogens produced by cholesterol, desmosterol, 7-dehydrocholesterol and lanosterol in 3 different colorimetric assays for cholesterol. Scans were made between 720 nm and 350 nm at a rate of 120 nm/min using a Perkin-Elmer Lambda 3 recording spectrophotometer. A. Acetic anhydride method of Sperry and Webb (7); each sterol present at 300 μg. B. $FeSO_4$ method of Pollet et al. (11); each sterol present at 100 μg. C. $FeCl_3$ method of Zlatkis et al. (4); each sterol present at 200 μg.

cholesterol at both of these wavelengths. The sharp absorption peak produced only by lanosterol at about 450 nm was interesting in that it suggests that the Liebermann-Burchard reagent might be used as a rather specific colorimetric test for lanosterol.

As with the Liebermann-Burchard reagent, the intensity of the color produced by the individual sterols in the $FeSO_4$ method of Searcy and Bergquist (8) as modified by Pollet et al. (11) correlated well with the absorption spectrum of the chromogens formed. Cholesterol and desmosterol each produced similar amounts of a chromogen with an absorption maximum at about 495 nm (Fig. 2B) and the standard curves generated by the 2 sterols were virtually superimposable (Fig. 1B). Although standard curves were prepared by measuring the absorbance at 460 nm according to Pollet et al. (11), the actual absorption maximum of the chromogen produced by cholesterol is around 490 nm. Little 490 nm absorbing chromogen was formed by 7-dehydrocholesterol and lanosterol (Fig. 2B) and each yielded standard curves for this reaction for which slopes were only about 40% of those generated by cholesterol and desmosterol (Fig. 1B). It is notable that the chromogens produced by all 4 sterols had an absorption peak at about 380 nm. This was the maximum absorbance for both 7-dehydrocholesterol and lanosterol chromogens (Fig. 2B).

The method of Zlatkis et al. (4) for quantitation of cholesterol is based on formation of a chromogen with $FeCl_3$ that possesses an absorption maximum at 560 nm. Both cholesterol and desmosterol formed chromogens which absorbed significantly at this wavelength (Fig. 2C); however, the extinction of the cholesterol chromogen was clearly greater than desmosterol's and the standard curve generated by cholesterol had a much steeper slope than that formed by desmosterol (Fig. 1C). 7-Dehydrocholesterol and lanosterol produced little color relative to cholesterol in this assay (Fig. 1C), since neither sterol formed a chromogen with $FeCl_3$ that absorbed appreciably in the 560 nm range (Fig. 2C). Both produced chromogen with an absorption maximum at about 490 nm.

Differences in the reaction of these sterols in the standard colorimetric assays for cholesterol were due both to formation of chromogens with qualitatively similar spectral patterns but with greatly different extinctions at the wavelength optimum for the method, and to formation of chromogens with very clearly different absorption maxima. For example, the chromogens formed by cholesterol and desmosterol in all 3 assays had very similar patterns of absorption with varying extinctions at the optimal wavelengths. On the other hand, the absorption spectrum produced by lanosterol in all assays were clearly different from those of cholesterol in both absorption maximum and in extinction.

These findings show that attempts to measure tissue total sterol levels by colorimetric methods when cholesterol is not the sole sterol present can result in great errors. Treatment of

TABLE 1

Brain Sterol Levels in Control and AY-9944-Treated Rats Measured by Various Methods

Method[b]	mg Total sterol/g brain (wet wt)[a]	
	Control	AY-9944[c]
Gas chromatography	12.1 ± 0.6	12.9 ± 0.4
Acetic anhydride	13.3 ± 0.9	33.2 ± 0.1
Ferrous sulfate	13.3 ± 0.5	6.5 ± 0.3
Ferric chloride	13.3 ± 0.3	6.8 ± 0.1

[a]Values are the mean ± SEM of 3 individual brains.

[b]Total sterols of whole brains of 35-day-old control and AY-9944-treated rats (50 mg/kg, s.c., every 4th day starting at 6 days of age) were quantified by gas chromatography and by 3 different colorimetric assays. Cholesterol was used to generate the standard curve for each assay.

[c]About 80% of the total sterol was determined by GLC to be 7-dehydrocholesterol.

the rat between 1 and 5 weeks of age with AY-9944 resulted in replacement of about 80% of the brain cholesterol with 7-dehydrocholesterol without significantly changing the total sterol concentration of this tissue (Table 1). Use of the 3 colorimetric assays to measure brain total sterols resulted in either a gross overestimation or underestimation of sterol levels when cholesterol was used to generate the standard curve. Based on the data presented in Fig. 1, a mixture of cholesterol and desmosterol is the only combination in which total sterol levels could be correctly estimated by a colorimetric method, but only with the $FeSO_4$ method of Zlatkis et al. (4). Any other combination of sterols must be measured by GLC to quantitate the total sterol content.

ACKNOWLEDGMENTS

We thank Dennis Schroeder and Susan Gearhart for providing technical assistance. Thanks are also due to Dixie Baxley for preparation of the manuscript and to David Welch for drawing the figures. The work was supported by NIH Grants NS 14446 and EY 02568.

REFERENCES

1. Goh, E.H. (1979) Biochem. Pharmacol. 28, 2969-2972.
2. Peter, J.B., Andiman, R.M., Bowman, R.I., and Nagatoma, T. (1973) Exp. Neurol. 41, 738-744.
3. Dvornik, D., and Hill, P. (1968) J. Lipid Res. 9, 587-595.
4. Zlatkis, A., Zak, B., and Boyle, A.J. (1953) J. Lab. Clin. Med. 41, 486-492.
5. Moore, P.R., and Baumann, C.A. (1952) J. Biol. Chem. 195, 615-621.
6. Idler, D.R., and Baumann, C.A. (1953) J. Biol. Chem. 203, 389-396.
7. Sperry, W.M., and Webb, M. (1950) J. Biol. Chem. 187, 97-106.
8. Searcy, R.L., and Bergquist, J.L. (1960) Clin. Chim. Acta 5, 192-199.
9. Bierkamper, G.G., and Cenedella, R.J. (1978) Brain Res. 150, 343-351.
10. Cenedella, R.J., and Belis, J.A. (1981) J. Lipid Res. 22, 122-130.
11. Pollet, S., Ermidou, S., Le Saux, F., Monge, M., and Baumann, M. (1978) J. Lipid Res. 19, 916-921.

[Received July 31, 1981]

High Performance Liquid Chromatography and Glass Capillary Gas Chromatography of Geometric and Positional Isomers of Long Chain Monounsaturated Fatty Acids[1]

LENNART SVENSSON*, LESLIE SISFONTES, GÖRAN NYBORG and ROLF BLOMSTRAND, *Department of Clinical Chemistry, Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden*

ABSTRACT

Positional and geometrical isomers of monounsaturated long chain fatty acids were analyzed by the combination of high performance liquid chromatography (HPLC) and glass capillary gas chromatography (GC). A preparative group separation of *cis* and *trans* isomers of the monounsaturated fatty acid methyl esters was achieved according to chain length by reversed-phase HPLC, and using a highly sensitive interference refractive index detector. After collection of the different fractions containing *cis* and *trans* forms of the monounsaturated fatty acid methyl esters, the fractions were analyzed for their content of positional isomers using glass capillary GC with Silar-5 CP as stationary phase. The preparative step in the HPLC was also used analytically for the determination of the ratio between the *cis* and *trans* monounsaturated fatty acids. A comparison was made between the results obtained with the HPLC technique and the results of a GLC technique with a packed OV-275 column. There was a good correlation between the 2 techniques with a tendency to higher *trans* values with the HPLC technique (4%). It was shown with reference substances that 18:1ω6-*cis* to ω11-*cis* and 18:1ω5-*trans* to ω12-*trans*, the most common monounsaturated fatty acid isomers in partially hydrogenated vegetable oils, could be almost quantitatively recovered in the HPLC step. Most of the individual positional isomers of monounsaturated fatty acids of varying chain length could be separated and determined in the glass capillary GC step with the exception of those isomers containing the double bond in a relatively high ω-position. The relative standard deviation of the technique as determined with reference substances was better than 4%. The described technique was applied to the analysis of the isomeric monounsaturated fatty acid content in partially hydrogenated vegetable and marine oils, and about 5 samples a day could be executed.
Lipids 17:50-59, 1981.

INTRODUCTION

Most of the vegetable and marine oils consumed today are partially hydrogenated to obtain a more desirable physical form and improved stability when stored. During this process, the polyunsaturated fatty acids in the natural oils are mainly reduced. Depending on the hydrogenation conditions used, the resultant monounsaturated fatty acids usually contain a large number of positional isomers with the double bond of both *cis* and *trans* configuration. When feeding experiments are made with partially hydrogenated vegetable and marine oils, it is very important to characterize these dietary oils with respect to the isomeric fatty acid content. An important biological problem is to follow the incorporation of isomeric fatty acids into organ lipids, and especially into the membrane phospholipids (1).

Traditionally, the determination of *trans* fatty acids has been done by infrared (IR) spectrophotometry, a technique which requires relatively large amounts of sample (2).

Gas liquid chromatography (GLC) on packed columns has been used for quantitative determination of the *cis* and *trans* percentages of monounsaturated fatty acids, either as epoxy derivatives of the methyl esters using conventional stationary phases (3), or as methyl esters using the very polar stationary phases Silar-10 C, SP-2340 or OV-275 (4-10). When these types of stationary phases were coated on capillary columns, positional and geometrical isomers of monounsaturated fatty acid methyl esters could be resolved to some extent, but some of the *cis* isomers generally overlapped with the *trans* isomers (11-14). Our experience is that it is difficult to prepare high-efficiency capillaries with these very polar stationary phases (15).

Because esters of monounsaturated fatty acids do not normally produce mass spectra useful for determination of the double bond position or geometrical configuration, suitable derivatives must be made. Attempts have been

*To whom correspondence should be addressed.

[1]Part of this work has been presented at the ISF/AOCS World Congress, New York (1980) *JAOCS* 58, (4), 1981, abstr. no. 184.

made with epoxy derivatives (16,17), trimethylsilyloxy derivatives (18), pyrrolidide derivatives (19) and methoxy derivatives (20), or to use chemical ionization mass spectrometry (21), but the techniques have not been applied to the analysis of complex fatty acid mixtures such as partially hydrogenated vegetable and marine oils.

In most common techniques used for the analysis of positional and geometrical isomers of monounsaturated long chain fatty acids in biological materials, the monounsaturated fatty acids are first isolated as methyl esters according to chain length by preparative GLC, then separated into their *cis* and *trans* forms by argentation thin layer chromatography (TLC), followed by GLC of the ozonide cleavage products of the monounsaturated fatty acids. The technique has been used successfully to determine the content of isomeric monounsaturated fatty acids in partially hydrogenated vegetable and marine oils as well as the degree of incorporation of different isomeric fatty acids into organ lipids after feeding partially hydrogenated oils (22-32). The technique is very precise and gives almost complete information on all isomeric monounsaturated fatty acids, but consists of many different steps and is therefore time-consuming and laborious. Two-step countercurrent distribution or rubber and silver-saturated resin liquid chromatography together with ozonolysis techniques have been used successively by Dutton (33) in the analysis of isomeric fatty acids of partially hydrogenated vegetable oils, but the analysis was limited to only one chain length.

In this work, we have used a simpler technique based on the combination of high performance liquid chromatography (HPLC) and glass capillary gas chromatography (GC). After a group separation on the HPLC according to geometry and chain length, the *cis* and *trans* monounsaturated fatty acids were collected and analyzed for their content of positional isomers using high-efficiency capillary columns (15).

MATERIALS AND METHODS

Reference Substances and Other Chemicals

Geometrical and positional isomers of octadecenoic acid (18:1ω1, 18:1ω2-*cis* to 18:1-ω15-*cis* and 18:1ω3-*trans* to 18:1ω13-*trans*) were a generous gift from Prof. F.D. Gunstone (University of St. Andrews, Scotland). Positional *cis* isomers of eicosenoic acid (20:1ω3 and 20:1ω9) and docosenoic acid (22:1ω3, 22:1ω7, 22:1ω9 and 22:1ω11) were obtained from Unilever Research (Vlaardingen, The Netherlands). All other fatty acids used were obtained from Nu-Chek-Prep. (Elysian, MN). Partially hydrogenated peanut oil was a gift from Dr. B. Andersen (Aarhus Oliefabrik A/S, Aarhus, Denmark), partially hydrogenated Norwegian capelin oil, a gift from Dr. R. Ohlson (AB Karlshamns Oljefabriker, Karlshamn, Sweden), and partially hydrogenated herring oil, a gift from Dr. J. Beare-Rogers (Dept. of Health and Welfare, Ottawa, Canada). Methanolic sodium methoxide (0.5 M) was obtained from Applied Science Europe B.V. (Oud-Beijerland, The Netherlands). Methanolic hydrogen chloride (1 M) was prepared from hydrogen chloride, purity 99% (AGA Special Gas, Lidingö, Sweden). All other reagents and chemicals used were purchased from Merck (Darmstadt, G.F.R.).

Preparation of Samples

TLC of phospholipids was done as previously described (34). Analysis of the positional distribution of fatty acids in phosphatidylcholine and phosphatidylethanolamine was performed by enzymatic hydrolysis with phospholipase A_2 (Ophiophagus Hannah, Sigma Chemical Co., St. Louis, MO) as described by Christie (35). Fatty acid methyl esters were prepared by transesterification of the dietary oils or phospholipids followed by acidic esterification with 1 M hydrogen chloride in methanol essentially described by Carreau and Dubacq (36). When methyl esters of free fatty acids were prepared, the transesterification step was omitted.

High Performance Liquid Chromatography (HPLC)

Instrumentation. An OPTILAB 931 HSRI high performance liquid chromatograph with a highly sensitive Interference Refractive Index (IRI) detector MULTIREF 902 (OPTILAB, Vällingby, Sweden) was used. The measuring cell vol was 12 μl and the light path length was 10 mm. The column used was a Lichroma 200 x 4.6 mm id stainless-steel column, slurry-packed with reversed-phase material, 5-μ Nucleosil C_{18} (Machery-Nagel, Düren, G.F.R.). This chromatographic system allowed measurement of changes in RI of 5×10^{-9} Δ RI, which was equal to a detection limit of about 10 ng for fatty acid methyl esters. The outlet of the refractive index detector was modified in order to collect fractions.

Column packing. The column was prepared by the upward slurry packing technique described by Bristow et al. (37). The packing apparatus consisted of an A 9512 LC air operated pump (Stansted Fluid Power, LTD, Essex, England) connected via a valve to the bottom of a sample cylinder with a vol of 75 ml. The

HPLC column was connected at the top of the sample cylinder. The reversed-phase packing material was slurried in chloroform, ultrasonicated for 5 min, transferred to the sample cylinder, and forced with acetone into the column at a pressure of 40 MPa. After passage of 150 ml of acetone, the column was turned 180° and a further 50 ml of acetone was pumped through the column. By this packing procedure, high-efficiency columns were obtained (about 9,000 plates/200 mm, calculated from palmitic acid methyl ester eluted at $k'=8$ under the chromatographic conditions used).

HPLC procedure. Twenty μl of a sample or standard mixture of fatty acid methyl esters dissolved in methanol containing BHT (20 ng/μl) was injected into the liquid chromatograph using a loop injector (Valco CV-6-UHPa-N60, Valco Instruments Co., Houston, TX). Distilled and filtered methanol/water (89:11, v/v) was used as the mobile phase, and the flow rate was 1.2 ml/min. Under these conditions, the back pressure obtained was 12-14 MPa. The detector sensitivity was set to 10^{-5}-10^{-6} ΔRI fullscale deflection (FSD) depending on the amount of sample to be analyzed. The ratio between *cis* and *trans* forms of monounsaturated fatty acid methyl esters was calculated by area integration using an Autolab System IV (Spectra-Physics, Mountain View, CA). Fractions corresponding to the *cis* and *trans* forms of the fatty acids were collected. The solvent was evaporated under nitrogen, and the residue was dissolved in hexane and analyzed for the content of positional isomers using glass capillary GC.

Glass Capillary Gas Chromatography

Instrumentation. Either the glass capillary GC system with a solventless injector (falling needle) as previously described (15) or a Fractovap 4160 gas chromatograph equipped with an "on-column injector" (Carlo Erba Strumentazione, Milano, Italy) was used. The column used (58 m × 0.3 mm id) was coated with Silar-5 CP (Applied Science Europe B.V., Oud Beijerland, The Netherlands).

Column preparation. Silar-5 CP was coated according to the static method on borosilicate glass capillary columns with barium carbonate deposition as previously described (15). By this procedure, high-efficiency capillary columns were obtained (more than 130,000 real plates/58 m and a separation number of about 24).

Glass capillary gas chromatographic procedure. Standard mixtures of isomeric fatty acids or *cis* and *trans* fractions from the preparative HPLC were injected into the column. The oven temperature was 160 C and the carrier gas was helium (0.6 ml/min). Retention times and peak areas were calculated using a CDS-111 chromatographic data system (Varian, Palo Alto, CA).

Packed Column Gas Liquid Chromatography

The ratio between *cis* and *trans* monounsaturated fatty acids was also determined with packed column GLC using 15% OV-275 on 100-120 mesh Chromosorb P, AW-DMCS (Supelco Inc., Bellefonte, PA). The analysis was performed in a HP 5710A gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a 8.5 m × 2 mm id glass column. The oven temperature was 220 C, and helium at a flow rate of 10 ml/min was used as carrier gas. Area calculation was made with an Autolab System IV.

RESULTS

In order to study the influence of the geometry and position of the double bond on the chromatographic behavior of monounsaturated fatty acid methyl esters in the HPLC system used, a series of positional isomers of *cis* and *trans* octadecenoic acid methyl esters (18:1) was used as model substances. The double bond in the *cis* isomers was distributed from ω2 to ω15 and in the *trans* isomers from ω3 to ω13; 18:1ω1 was included. In Figure 1, the relative retention time (the retention time for palmitic acid methyl esters was set to 1.0) of the different isomers of octadecenoic acid methyl esters

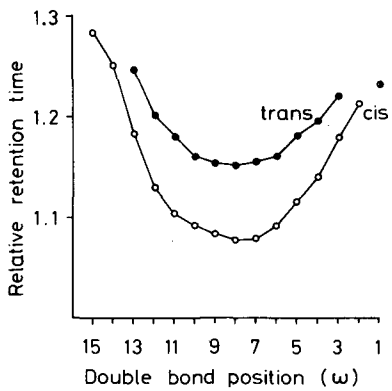


FIG. 1. Relative retention time in the reversed-phase HPLC of different isomers of octadecenoic acid methyl esters as a function of the position and geometry of the double bond. The retention times are related to the retention time of palmitic acid methyl ester. Double bond position (ω) is stated from the hydrocarbon end of the fatty acid molecule. Column: 5-μ Nucleosil C_{18}, 200 × 4.6 mm id; mobile phase: methanol/water (89:11, v/v); flow: 1.2 ml/min; pressure: 13 MPa.

is plotted vs the position and geometry of the double bond. The *cis* isomers eluted before the corresponding *trans* isomers. The *cis* isomers with the double bond in a position near the ends of the molecule cochromatographed with *trans* isomers with the double bond distributed around the middle of the molecule. Thus, it is obvious that, under the conditions used, a complete group separation into *cis* and *trans* cannot be achieved if a large number of positional isomers is present. In most partially hydrogenated oils, the *cis* isomers of the monounsaturated fatty acids have the double bond in a relatively narrow range around the middle of the molecule compared to the *trans* isomers (10). Therefore, when fractionating in the preparative HPLC procedure, most of the *cis* isomers should be collected as is predicted by Figure 1.

To investigate this assumption, a mixture of *cis* isomers of octadecenoic acid methyl esters with the double bond in the odd ω-positions 7-11 and a corresponding mixture of *trans* isomers with the double bond in the odd ω-positions 5-11 were first analyzed separately by glass capillary GC. Then, the 2 mixtures were mixed. After separation on HPLC, the *cis* and *trans* fractions were collected and each fraction was reanalyzed by glass capillary gas chromatography. The *cis* and *trans* isomers with the double bond in the even ω-positions (ω6-10 and ω6-12 for *cis* and *trans*, respectively) went through the same procedure. The reason for dividing the different isomers into 2 groups containing odd and even double bond position was to minimize the risk of different isomers overlapping in the glass capillary GC, giving more reliable results. The results of these investigations are presented in Table 1. The recovery of *cis* isomers ω6-11 and *trans* isomers ω5-12 are nearly complete. The relative standard deviation calculated from 6 analyses was better than 4%. If further *cis* isomers were included into the mixture, e.g., *cis* ω5 and *cis* ω12, they were partially separated from the *trans* material. This was not a problem with the *trans* isomers, because they eluted after the *cis* isomers and could all be quantitatively collected. However, they could be contaminated with small amounts of *cis* isomers with the double bond in a low or high ω-position if present in the sample.

Besides the preparative approach of the HPLC technique, the possibility of using it analytically at the same time in order to determine the ratio of *cis* and *trans* isomers of the monounsaturated fatty acids on the same runs was investigated. There were not any common fatty acids which interfered with octadecenoic, eicosenoic or docosenoic acids. However, arachidonic acid and docosahexaenoic acid cochromatographed with hexadecenoic acids. Thus, the ratio of *cis* and *trans* hexadecenoic acids could not be determined in phospholipids, which are rich in these polyunsaturated fatty acids. However, if a methoxy-bromomercuri-adduct separation (38) is included before the HPLC separation, the overlap or coincidence due to different chain length and number of double bonds could be avoided, as only the

TABLE 1

Effect of the Double Bond Position and Geometry on the Quantitative Collection of *cis* and *trans* Fractions of Octadecenoic Acid after HPLC Separation

	Distribution of 18:1 *cis* isomers (%)			Distribution of 18:1 *trans* isomers (%)			
	ω7	ω9	ω11	ω5	ω7	ω9	ω11
Before HPLC	34.1 ± 1.3	54.3 ± 1.4	11.6 ± 0.4	22.4 ± 0.1	24.7 ± 0.6	26.1 ± 0.2	26.8 ± 0.5
After HPLC	36.5 ± 0.3	52.7 ± 0.3	10.8 ± 0.2	23.2 ± 0.3	26.0 ± 0.4	25.6 ± 0.5	25.2 ± 0.7
	Distribution of 18:1 *cis* isomers (%)			Distribution of 18:1 *trans* isomers (%)			
	ω6	ω8	ω10	ω6	ω8	ω10 + 12	
Before HPLC	30.9 ± 0.2	27.0 ± 0.3	42.1 ± 0.4	24.8 ± 0.3	30.4 ± 0.1	44.8 ± 0.3	
After HPLC	31.1 ± 0.6	27.2 ± 0.8	41.7 ± 0.3	25.6 ± 0.5	30.6 ± 0.6	43.8 ± 0.4	

Mixtures of *cis* and *trans* octadecenoic acid methyl esters (18:1) with the double bond in odd and even ω-position were first analyzed separately by glass capillary gas chromatography (before HPLC). The different isomers were mixed into 2 mixtures containing *cis* and *trans* isomers of odd and even ω-position, respectively. After HPLC separation, the *cis* and *trans* fractions were collected and each fraction reanalyzed by glass capillary gas chromatography (after HPLC). Chromatographic conditions are given in Fig. 1. Double bond position (ω) is stated from the hydrocarbon end of the fatty acid molecule. The figures in the table are mean ± standard deviation of 6 analyses.

TABLE 2

Relative Detector Response of Different Geometrical and Positional Isomers of Octadecenoic Acid as Determined by HPLC using RI Detection and Gas Chromatography (GLC) on OV-275 Packed Column

ωn	$\left(\frac{18:1\omega n\ trans}{18:1\omega 9\ cis}\right)_{HPLC}$ / $\left(\frac{18:1\omega n\ trans}{18:1\omega 9\ cis}\right)_{GLC}$	$\left(\frac{18:1\omega n\ cis}{18:1\omega 9\ trans}\right)_{HPLC}$ / $\left(\frac{18:1\omega n\ cis}{18:1\omega 9\ trans}\right)_{GLC}$
7	1.03	0.96
8	1.07	0.93
9	1.07	0.95
10	1.07	0.95
11	1.06	0.96

monoene fraction needs to be analyzed by HPLC.

Before using peak area percentage in the calculation of the ratio between *cis* and *trans* monounsaturated fatty acids in quantitative HPLC analysis with refractive index detector, the relative response between *cis* and *trans* isomers and between different positional isomers has to be investigated. Unfortunately, the amount of the different isomeric octadecenoic acids available was not large enough to prepare accurate standard mixtures by weighing. To overcome this problem, mixtures with an approximate concentration of isomers were prepared, and the response in the HPLC procedure was compared to those obtained after gas chromatographic separation on OV-275-packed column using a flame ionization detector, for which the response is proportional to the mass concentration. The results of this investigation are presented in Table 2. The area ratios between different *trans* isomers of octadecenoic acid methyl esters (ω7-11) and oleic acid methyl ester (18:1ω9*c*) obtained in the HPLC analysis were divided with the corresponding area ratios obtained after GLC analysis on OV-275. The corresponding ratios were calculated for the different *cis* isomers using elaidic acid methyl ester (18:1ω9*t*) as reference. Ideally, the ratio should be 1.00. There was a very small difference in response between different positional isomers, but about 6% higher values were obtained for the *trans* isomers in the HPLC procedure compared to the GLC. Thus, a small difference in refractive index for *cis* and *trans* isomers cannot be excluded. Another explanation could be the opposite elution order obtained in the HPLC procedure compared to GLC. Tailing *cis* peaks in the HPLC procedure could interfere with the *trans* peaks and vice-versa in the GLC procedure.

The *trans* octadecenoic acids as percentage

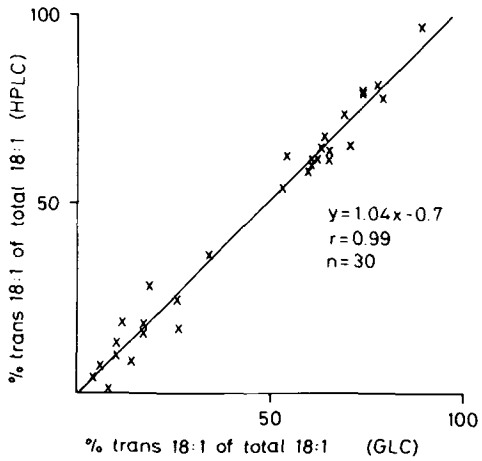


FIG. 2. Comparison of the HPLC method and the GLC method on OV-275 packed column for the determination of *trans* octadecenoic acid as % of total octadecenoic acids. The analyses were made on 1- and 2-position of mitochondrial phospholipids after feeding partially hydrogenated oils to rats. HPLC conditions as in Fig. 1. For experimental details of the GLC method, see Materials and Methods.

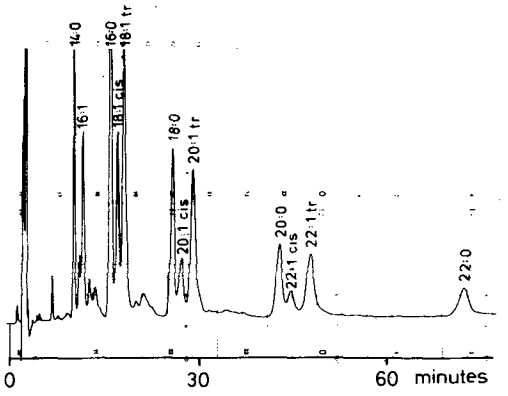


FIG. 3. Reversed-phase HPLC of fatty acid methyl esters obtained after transesterification of partially hydrogenated Norwegian capelin oil. Sample size: 25 μg. Detector sensitivity: 10^{-6} ΔRI FSD. For further information of the chromatographic conditions, see legend to Fig. 1. Shorthand notation is used for the fatty acids indicating chain length:number of double bonds. Note that the fatty acid methyl esters elute according to their relative solubility in the mobile and stationary phases. The more methanol-soluble monounsaturated fatty acids elute much faster than the corresponding saturated fatty acid and nearly coelute with the nearest lower saturated homolog.

TABLE 3

Comparison of the HPLC Method and the GLC Method on OV-275 Packed Column for the Determination of *trans* Monounsaturated Fatty Acids as Percentage of Total Fatty Acids in Some Partially Hydrogenated Oils

Oil	HPLC (%)	GLC (%)
Partially hydrogenated peanut oil	50.6	46.5
Partially hydrogenated Norwegian capelin oil	35.2	35.8
Partially hydrogenated herring oil	27.1	25.9

of total octadecenoic acids in rat heart mitochondrial phospholipids after feeding partially hydrogenated oils were determined by HPLC and GLC on OV-275. In Figure 2, the *trans* octadecenoic acid percentage obtained with the HPLC technique is plotted vs the corresponding values obtained with the GLC technique. Somewhat high values (4%) were obtained with the HPLC technique. and the correlation coefficient between the 2 methods was 0.99.

The reversed-phase HPLC separation of fatty acid methyl esters obtained after transesterification of partially hydrogenated Norwegian capelin oil is shown in Figure 3. As can be seen from the chromatogram, there was a separation according to chain length and number of double bonds. The monounsaturated fatty acids eluted before the corresponding saturated fatty acids. The *cis* forms of the monounsaturated fatty acids eluted before the *trans* forms.

In Table 3, the concentration of *trans* monounsaturated fatty acids of some partially hydrogenated oils obtained with the HPLC procedure is compared to the corresponding results obtained after GLC analysis on OV-275. The concentration was calculated from the total fatty acid composition determined by glass capillary GC and from the ratio between *trans* and *cis* monounsaturated fatty acids obtained with the 2 different techniques. There was a tendency to higher values for the *trans* fatty acid concentration when analyzed by HPLC.

The elution pattern of different geometrical and positional isomers of octadecenoic acid methyl esters in the glass capillary GC using Silar-5 CP as stationary phase has previously been described (15). As can be seen from Figure 4, some positional isomers of octadecenoic acid could not be resolved. From this chromatogram, the logarithm of the adjusted retention times for positional isomers with the double bond in the same ω-position could be plotted vs the carbon atom number (39), and could be used for a tentative identification of unknown isomeric monounsaturated fatty acids.

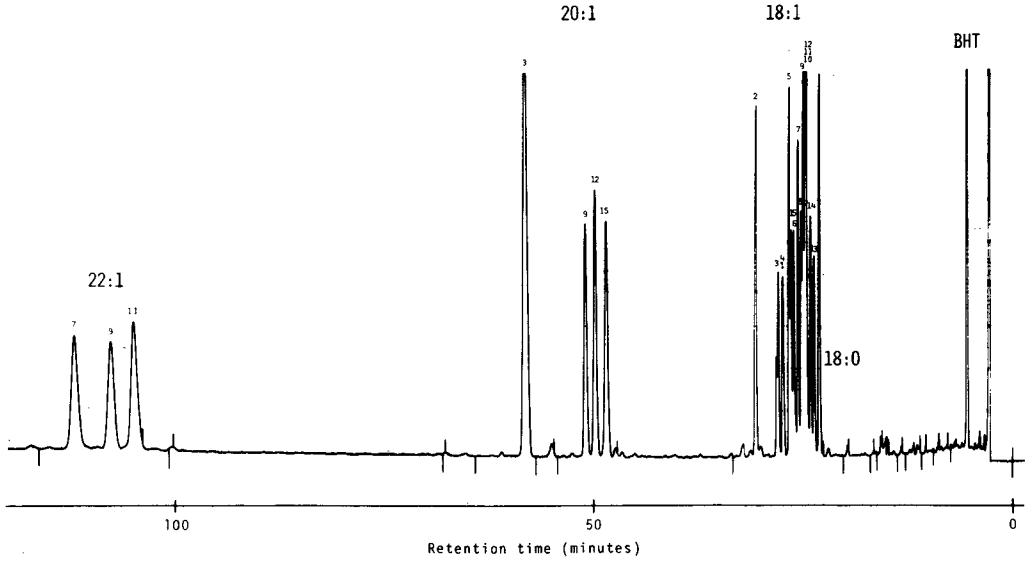


FIG. 4. Glass capillary gas chromatography of a standard mixture of different positional *cis* isomers of long chain monounsaturated fatty acids. Shorthand notation is used for the fatty acids indicating chain length:number of double bonds. The peak numbers refer to the double bond position (ω), which is stated from the hydrocarbon end of the fatty acid molecule. BHT = 2,6-di-*tert*-butyl-*p*-cresol. Silar-5 CP on borosilicate glass capillary column, 58 m × 0.3 mm id. Column temperature: 160 C. Carrier gas: helium, 0.6 ml/min.

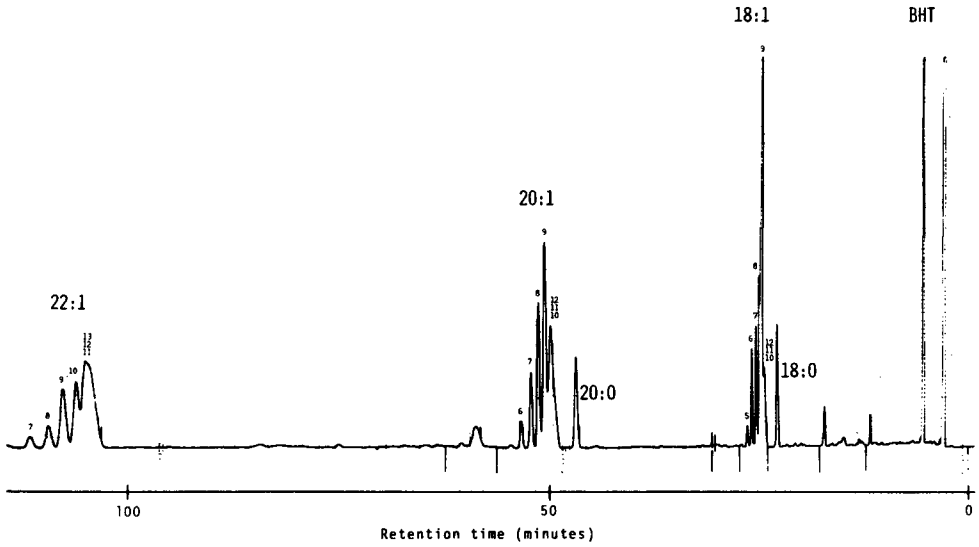


FIG. 5. Glass capillary gas chromatography of the *cis* fractions of monounsaturated fatty acid methyl esters isolated after HPLC separation of the methyl esters obtained after transesterification of partially hydrogenated Norwegian capelin oil. Chromatographic conditions and further informations are given in legend to Fig. 4.

The *cis* isomers of the monounsaturated fatty acids from partially hydrogenated Norwegian capelin oil obtained after HPLC separation and fractionation were analyzed for the content of positional isomers by glass capillary GC (cf. Fig. 5). Similar results were obtained with the *trans* fractions, but the different positional isomers were more difficult to separate (cf. Fig. 6).

From the results obtained in the analysis by HPLC and glass capillary GC, the partially hydrogenated Norwegian capelin oil could be characterized with respect to the distribution of geometrical and positional isomers of monounsaturated fatty acids of chain length 18-22 (cf. Fig. 7). As can be seen from the figure, most of the positional isomers of the monounsaturated fatty acids could be separated by glass capillary GC, but some isomers, especially those with the double bond in a higher ω-position, were not resolved. Because no positive identification such as ozonolysis was done on the different *cis* and *trans* fractions, one cannot exclude the possibility that a small amount of high ω number 22:1 isomers (e.g., 22:1ω16) from reduction of 22:6ω3 can coincide with lower ω

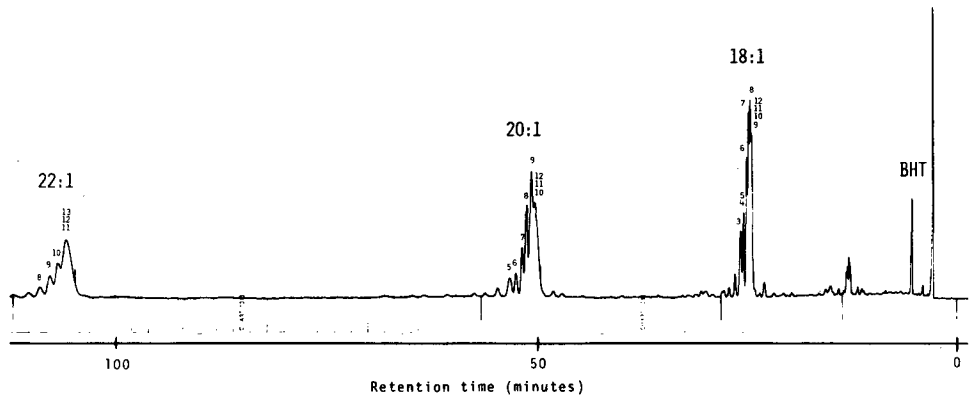


FIG. 6. Glass capillary gas chromatography of the *trans* fractions of monounsaturated fatty acid methyl esters isolated after HPLC separation of the methyl esters obtained after transesterification of partially hydrogenated Norwegian capelin oil. Chromatographic conditions and further informations are given in legend to Fig. 4.

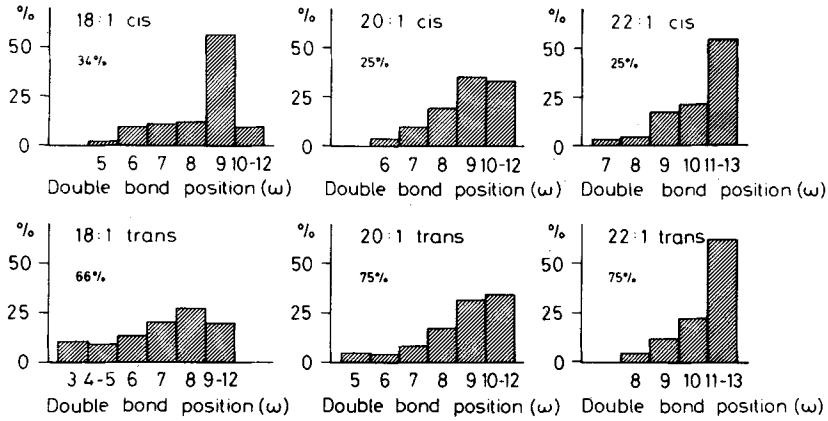


FIG. 7. The distribution of positional and geometrical isomers of monounsaturated fatty acids in partially hydrogenated Norwegian capelin oil as determined by the combination of HPLC and glass capillary gas chromatography. The distribution of *cis* and *trans* isomers is given as % of total monounsaturated fatty acids of each chain length. The double bond position (ω) is stated from the hydrocarbon end of the fatty acid molecule.

number isomers in the glass capillary GC. In the octadecenoic and eicosenoic acid *cis* fraction, the fatty acids with the double bond in ω9-position dominate. In the *trans* fractions, there was a somewhat wider distribution of positional isomers.

DISCUSSION

The technique described here for the analysis of geometrical and positional isomers of monounsaturated fatty acids as methyl ester derivatives was based on the combination of reversed-phase HPLC using RI detection and glass capillary GC. Borch (40) and Pei et al. (41) have used phenacyl and *p*-bromophenacyl esters, respectively, as fatty acid derivatives in the reversed-phase HPLC. These strongly UV-absorbing derivatives not only increase the sensitivity in the HPLC analysis but also the molecular weight. Thus, a subsequent analysis of positional isomers by glass capillary GC has to be performed at very high temperatures. By using methyl ester derivatives which have excellent gas chromatographic properties, these analyses could be done at relatively low temperatures.

Different silver-loaded stationary phases have been shown to give good separations of geometrical isomers of monounsaturated fatty acids. Emken et al. (42) and Scholfield (43) used silver-saturated cation exchange resins and Lam and Grushka (44) used silver-loaded aluminosilicate. Battaglia and Frölich (45) showed that even certain positional isomers could be resolved when using silver-nitrate-treated silica. However, a separation according to chain length could not be obtained with these techniques. Consequently, these techniques are unsuitable to differentiate between isomers of different chain lengths. On the contrary, the separation according to chain length was not a problem in the reversed-phase chromatography, but the separation power of geometrical isomers was not as high as in argentation chromatography. However, Schomburg and Zegarski (46) have shown that the *cis* and *trans* separation in reversed-phase chromatography could be improved by the addition of silver ions to the mobile phase.

The relative response in the HPLC analysis using RI detection of some geometrical and positional isomers of octadecenoic acid was investigated and compared with those obtained in the GLC analysis using an OV-275-packed column. For the isomers investigated, the response was practically independent of the double bond position. It should be pointed out that, in the quantitative analysis of the *trans* monounsaturated fatty acid content of partially hydrogenated oils and mitochondrial phospholipids (cf. Table 3 and Fig. 2), we cannot exclude the possibility that positional isomers other than those investigated could differ in response. A possible explanation to the fact that the *trans* values obtained by the HPLC method were somewhat higher (ca. 4%) than those obtained by the GLC method is either a small difference in response of the RI detector between *cis* and *trans* fatty acids or that the heterogeneity of the *trans* fatty acids is greater than that of the *cis* fatty acids (10). Thus, in

the GLC analysis, *trans* fatty acids with the double bond in a low ω-position probably will coelute with the *cis* isomers and give rise to too-low *trans* fatty acid values. These discriminating effects of *trans* fatty acids in the GLC procedure have also been confirmed in comparisons with IR spectrophotometry (10).

The method presented here has been used in connection with rat feeding experiments with partially hydrogenated vegetable and marine oils (1). The partially hydrogenated oils were characterized and the distribution of positional and geometrical isomers of monounsaturated acids in different phospholipids was estimated with this method. The sensitivity of the technique permitted analysis of less than 100 μg of lipid material. About 5 samples a day could be executed. The HPLC step used is a very mild procedure compared to preparative GLC, which has been used to isolate fatty acids according to chain length for the ozonolytic procedure. Unfortunately, not all of the positional isomers of monounsaturated fatty acids present in the biological samples could be resolved by the glass capillary GC step. In order to determine the complete distribution of all existing positional isomers, the *cis* and *trans* fractions isolated after HPLC separation should be subjected to ozonolytic cleavage with subsequent analysis with glass capillary GC. With these modifications, the often inefficient and troublesome fractionation with preparative GLC and argentation TLC commonly used in connection with ozonolytic cleavage could be replaced by a single and less laborious step.

ACKNOWLEDGMENTS

We thank Prof. F.D. Gunstone, University of St. Andrews, Scotland, for providing geometrical and positional isomers of octadecenoic acid. The skillful technical assistance of engineers B. Boström, O. Caspersson and G. Persson is gratefully acknowledged. This work was supported by grants to Prof. R. Blomstrand from The Swedish National Food Administration and The Swedish Margarine Industry's Association for Nutritional Physiological Research.

REFERENCES

1. Blomstrand, R., and Svensson, L., in preparation.
2. Scholfield, C.R. (1979) in Geometrical and Positional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 17-52, American Oil Chemists' Society, Champaign, IL.
3. Emken, E.A. (1972) Lipids 7, 459-466.
4. Ottenstein, D.M., Bartley, D.A., and Supina, W.R. (1976) J. Chromatogr. 119, 401-407.
5. Ottenstein, D.M., Witting, L.A., Walker, G., Mahadevan, V., and Pelick, N. (1977) J. Am. Oil Chem. Soc. 54, 207-209.
6. Perkins, E.G., McCarthy, T.P., O'Brien, M.A., and Kummerow, F.A. (1977) J. Am. Oil Chem. Soc. 54, 279-281.
7. Heckers, H., Dittmar, K., Melcher, F.W., and Kalinowski, H.O. (1977) J. Chromatogr. 135, 93-107.
8. Conacher, H.B.S., Iyengar, J.R., and Beare-Rogers, J.L. (1977) J. Assoc. Off. Anal. Chem. 60, 899-903.
9. Conacher, H.B.S., and Iyengar, J.R. (1978) J. Assoc. Off. Anal. Chem. 61, 702-708.
10. Heckers, H., Melcher, F.W., and Dittmar, K. (1979) Fette Seifen Anstrichm. 81, 217-226.
11. Heckers, H., Melcher, F.W., and Schloeder, U. (1977) J. Chromatogr. 136, 311-317.
12. Van Vleet, E.S., and Quinn, J.G. (1978) J. Chromatogr. 151, 396-400.
13. Ojanperä, S.H. (1978) J. Am. Oil Chem. Soc. 55, 290-292.
14. Kozuharov, S. (1980) J. Chromatogr. 198, 153-155.
15. Sisfontes, L., Nyborg, G., Svensson, L., and Blomstrand, R. (1981) J. Chromatogr. 216, 115-125.
16. Aplin, R.T., and Coles, L. (1967) Chem. Commun. 858-859.
17. Gunstone, F.D., and Jacobsberg, F.R. (1972) Chem. Phys. Lipids 9, 26-34.
18. Capella, P., and Zorzut, C.M. (1968) Anal. Chem. 40, 1458-1463.
19. Andersson, B.Å., and Holman, R.T. (1974) Lipids 9, 185-190.
20. Minnikin, D.E., Abley, P., McQuillin, F.J., Kusamran, K., Maskens, K., and Polgar, N. (1974) Lipids 9, 135-140.
21. Ariga, T., Araki, E., and Murata, T. (1977) Chem. Phys. Lipids 19, 14-19.
22. Conacher, H.B.S., and Page, B.D. (1972) J. Am. Oil Chem. Soc. 49, 283-286.
23. Conacher, H.B.S., Page, B.D., and Chadha, R.K. (1972) J. Am. Oil Chem. Soc. 49, 520-523.
24. Conacher, H.B.S., Page, B.D., and Beare-Rogers, J.L. (1973) Lipids 8, 256-258.
25. Wood, R., Chumbler, F., and Wiegand, R. (1977) J. Biol. Chem. 252, 1965-1970.
26. Wood, R., and Chumbler, F. (1978) Lipids 13, 75-84.
27. Wood, R. (1979) Lipids 14, 975-982.
28. Reichwald-Hacker, I., Ilsemann, K., and Mukherjee, K.D. (1979) J. Nutr. 109, 1051-1056.
29. Reichwald-Hacker, I., Grosse-Oetringhaus, S., Kiewitt, I., and Mukherjee, K.D. (1979) Biochim. Biophys. Acta 575, 327-334.
30. Reichwald-Hacker, I., Grosse-Oetringhaus, S., Kiewitt, I., and Mukherjee, K.D. (1980) J. Nutr. 110, 1122-1129.
31. Høy, C.E., and Hølmer, G. (1979) Lipids 14, 727-733.
32. Høy, C.E., and Hølmer, G. (1981) Lipids 16, 102-108.
33. Dutton, H.J. (1975) in Analysis of Lipids and Lipoproteins (Perkins, E.G., ed.) pp. 138-152, American Oil Chemists' Society, Champaign, IL.
34. Blomstrand, R., and Svensson, L. (1974) Lipids 9, 771-780.
35. Christie, W.W. (1973) in Lipid Analysis, pp. 273-275, Pergamon Press, Oxford.
36. Carreau, J.P., and Dubacq, J.P. (1978) J. Chromatogr. 151, 384-390.
37. Bristow, P.A., Brittain, P.N., Riley, C.M., and Williamson, B.F. (1977) J. Chromatogr. 131, 57-64.
38. Sebedio, J-L., and Ackman, R.G. (1981) Lipids 16, 461-467.
39. Ackman, R.G. (1972) in Prog. Chem. Fats Other

Lipids (Holman, R.T., ed.) Vol. 12, pp. 165-284, Pergamon Press, Oxford.
40. Borch, R.F. (1975) Anal. Chem. 47, 2437-2439.
41. Pei, P.T.S., Kossa, W.C., Ramachandran, S., and Henly, R.S. (1976) Lipids 11, 814-816.
42. Emken, E.A., Scholfield, C.R., and Dutton, H.J. (1964) J. Am. Oil Chem. Soc. 41, 388-390.
43. Scholfield, C.R. (1980) J. Am. Oil Chem. Soc. 57, 331-334.
44. Lam, S., and Grushka, E. (1977) J. Chromatogr. Sci. 15, 234-238.
45. Battaglia, R., and Fröhlich, D. (1980) Chromatographia 13, 428-431.
46. Schomburg, G., and Zegarski, K. (1975) J. Chromatogr. 114, 174-178.

[Received June 17, 1981]

Simultaneous Determination of *Sonchus arvensis* L. Triterpenes by Gas Chromatography-Mass Spectrometry

S.N. HOOPER[a], R.F. CHANDLER[a,*], E. LEWIS[b] and W.D. JAMIESON[b], *[a]College of Pharmacy, Dalhousie University, Halifax, Nova Scotia, Canada; and [b]Atlantic Research Laboratory, National Research Council, Halifax, Nova Scotia, Canada*

ABSTRACT

Sonchus arvensis L. or sow thistle has proved an excellent source of pentacylic triterpenes; triterpenes account for about 6% of the crude lipid extract or 0.2% of the dried plant. Composition of the triterpenoid fraction, as indicated by gas chromatography of the corresponding acetates, was α-amyrin (9%), β-amyrin (21%), lupeol (13%), taraxasterol (24%) and pseudo-taraxasterol (12%). A single, long-chain alcohol (16%) was also present. The nature of the triterpenoids was defined using gas chromatography and gas chromatography-mass spectrometry (GC/MS) and confirmed by direct comparison with authentic samples. The use of combined GC/MS proved extremely valuable in the simultaneous analysis of a mixture of triterpene acetates and greatly reduced the time needed to phytochemically evaluate these compounds.

Lipids 17:60-63, 1981.

During our studies of herbal remedies of the Micmac Indians (1), it became necessary to obtain enough pseudo-taraxasterol for its characterization by proton and carbon-13 nuclear magnetic resonance spectroscopy (NMR). *Sonchus asper* had previously been reported as an excellent source of several common triterpenoids including pseudo-taraxasterol (2). Although we did not have *S. asper*, we did have a small amount of dried *Sonchus arvensis* L. (sow thistle) from a previous screening program (3). Like other members of the Compositae (2,4), *S. arvensis* contains larger amounts of taraxasterol than pseudo-taraxasterol, a difficult pair to separate (4,5). Before separating and purifying pseudo-taraxasterol in *S. arvensis*, we decided to verify its identity and the amount present in the plant. Gas liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC/MS) were chosen as the analytical techniques.

Using appropriate standards and GC/MS, the identification of each triterpene in the mixture was possible following a single injection of the acetylated triterpenoid fraction of a plant extract. To the best of our knowledge, this technique has been applied to the analysis of a triterpene mixture only once before (6).

MATERIALS AND METHODS

Collection and Extraction

The aerial parts of *Sonchus arvensis* L. were collected during the flowering stage from an open field near Halifax, Nova Scotia, in Sept. 1980. The material was determined by Dr. M.J. Harvey, Department of Biology, Dalhousie University, and herbarium samples are now on file. The plant material was dried in a forced-air oven at 60 C and ground in a Wiley mill to a coarse powder (0.5 cm). This material (300 g) was placed in a beaker and macerated for at least 24 hr in each solvent—twice with petroleum ether and twice with chloroform. The solvents were removed in vacuo, producing a yellowish-green semisolid mass. These extracts were combined (10.3 g) and saponified via AOCS method Ca. 6b-53 (7). The nonsaponifiable portion was extracted with diethyl ether and the solvent was again removed in vacuo, yielding a yellow material (8.8 g).

Known weights of nonsaponifiable material and cholestane were mixed and GLC analysis

TABLE 1

Gas Chromatographic Relative Retention Times for *Sonchus arvensis* Triterpenes[a]

Compound	Column OV-1	OV-17	OV-225
Alcohols			
β-Amyrin	2.35	4.42	6.08
α-Amyrin	2.52	5.24	7.04
Lupeol	2.52	5.24	7.32
ψ-Taraxasterol	2.98	6.72	9.13
Taraxasterol	2.98	6.74	9.69
Acetates			
β-Amyrin	2.88	5.36	6.60
α-Amyrin	3.10	6.04	7.57
Lupeol	3.10	6.32	7.97
ψ-Taraxasterol	3.66	8.19	9.90
Taraxasterol	3.66	8.25	10.50

[a]All figures are recorded as relative retention times (RRT) using 5α-cholestane as the internal standard. Absolute retention times for 5α-cholestane were 3:00, 3:51 and 4:01 min, respectively, for the OV-1, OV-17 and OV-225 columns. All identifications were confirmed by spiking with authentic standards.

was conducted to determine the percentage of the triterpenes present.

Thin Layer Chromatography (TLC)

The material obtained above was fractionated by preparative TLC using plates coated with Silica Gel H. Following visualization procedures, the sterols appeared as a red band ($R_f \approx 0.25$) and the triterpenes as a red-brown band ($R_f \approx 0.40$). The material from both bands was recovered by extracting the silica gel with ether and chloroform (8).

Gas Liquid Chromatography

GLC was conducted on the crude extract and on fractions from TLC as free alcohols or as acetates (8). A Hewlett-Packard Model 5750 gas chromatograph, equipped with a dual flame ionization detector and a Model 3380A integrator, and containing 6 ft x 1/4 in. glass columns packed with OV-17, OV-1 (9) or OV-225 (3% on 100-120 mesh Chromosorb W, H.P.), was used for all GLC analyses. Column temperatures were maintained at 250, 290 and 250 C, respectively, and gas flows for each column were 60 ml/min for nitrogen (the carrier gas) and hydrogen, and 240 ml/min for the air.

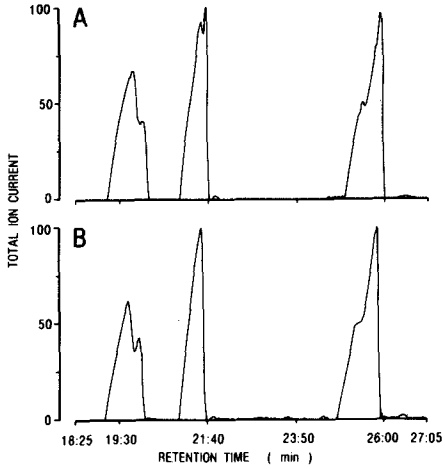


FIG. 1. Total ion current chromatogram of *S. arvensis* triterpene acetates: A. obtained by EI-MS; B. obtained by methane PCI-MS.

Gas Chromatography-Mass Spectrometry

GC/MS studies were performed on a Finnigan-MAT 4000 GC/MS quadrapole mass spectrometer coupled to an INCOS data system.

TABLE 2

Relative Intensities in the Electron Impact Mass Spectra[a] of *Sonchus arvensis* Triterpene Acetates

m/e	α-Amyrin	β-Amyrin	Taraxasterol	Pseudo-Taraxasterol	Lupeol
468[b]	0.4	0.4	1.7	0.2	2.5
453	–	–	–	–	0.6
408	–	–	1.6	0.3	0.7
393	–	–	0.8	0.5	1.0
249	0.7	0.6	6.7	4.3	7.5
229	–	–	3.6	–	9.4
218	100	100	5.0	2.2	26
204	–	–	14	–	23
203	27	51	15	9.3	32
191	7.5	3.4	31	16	33
189	44	19	100	100	99.8
175	12	11	23	19	31
161	18	8.6	23	14	34
149	15	7.2	18	12	29
147	23	15	31	18	40
137	10	–	11	8.1	20
135	42	18	55	44	72
133	32	15	28	32	40
123	30	9.2	32	36	54
121	38	19	74	77	90
119	–	–	–	–	55
109	44	20	81	64	89
107	45	23	72	65	100

[a]Retention times of the mass spectral scans, as obtained from the GC/MS (Fig. 1), used in the analysis of the triterpene acetates were: α-amyrin, 21:10; β-amyrin, 19:50; taraxasterol, 26:10; pseudo-taraxasterol, 25:00; and lupeol, 21:50 min. The shoulder at 20:15 is an unidentified triterpene acetate.

[b]Molecular ion (M+).

The column was a high-capacity OV-1 flexible quartz capillary (25 m x 0.3 mm id) which was directly interfaced to the source of the mass spectrometer. Source temperatures (indicated) were 260 C for electron impact (EI) MS studies and 230 C for the positive ion chemical ionization (PCI) MS studies. The EI spectra were obtained at 70 eV. Methane was used as the ionizing gas for the PCI studies at a source pressure of 0.02 *t* (indicated), and helium was used as the carrier gas (2 ml/min).

Authentic Samples

Authentic samples were purchased as follows: cholestane, β-sitosterol and stigmasterol–Sigma Chemical Co., St. Louis, MO; cholesterol–Fisher Scientific Co., Montreal, Quebec; campesterol–Applied Science, State College, PA; α- and β-amyrin–Pfaltz and Bauer, Inc., Stamford, CT. Taraxasterol was a gift from Dr. T.R. Watson, Pharmacy Department, University of Sydney, Australia, and pseudo-taraxasterol acetate was a gift from Dr. R.V. Madrigal, Northern Regional Research Laboratory, USDA, Peoria, IL.

RESULTS AND DISCUSSION

Although GC/MS could potentially be used to rapidly identify the components of a triterpene mixture, it apparently has been successfully employed for this purpose only once before our study (6). It has, however, been used to identify pure triterpenes (10).

Analyses by GLC (Table 1) demonstrated that the triterpenes of *S. arvensis*, which occurred as a mixture of alcohols and their acetates, represented about 6% of the crude lipid extract or about 0.2% of the dried plant material. Under the conditions described, retention times of the triterpene acetates differed sufficiently on OV-17 and OV-225 columns to permit identification of each acetate. Separation was incomplete on OV-17 at higher temperatures (275 C) and on OV-1 columns, however. Five triterpenes were identified by GLC analyses of their acetates, when compared

TABLE 3

Relative Intensities in the Methane Positive Chemical Ionization Mass Spectra[a] of *Sonchus arvensis* Triterpene Acetates

m/e	α-Amyrin	β-Amyrin	Taraxasterol	Pseudo-Taraxasterol	Lupeol
468[b]	0.2	0.1	0.2	0.1	–
453	0.1	0.2	0.1	0.1	–
409	63	38	58	55	24
408	49	31	28	32	14
394	–	–	–	6.3	1.5
393	24	16	25	25	9.6
327	–	–	–	0.4	–
326	–	–	–	0.2	–
249	2.3	2.3	3.1	2.3	2.1
229	–	–	–	–	18
219	38	34	39	38	32
218	58	43	9.0	4.0	12
205	60	64	73	64	76
203	38	49	49	42	57
192	–	–	–	9.6	9.8
191	62	69	99	75	7.3
189	37	42	47	39	45
175	20	25	20	16	21
161	20	25	16	16	19
149	47	47	47	44	56
147	21	22	14	13	16
137	46	51	35	34	41
135	41	39	30	33	38
133	23	24	15	19	17
123	72	66	55	48	67
121	43	41	41	41	34
109	100	100	100	100	100
107	32	35	21	20	23

[a]Retention times of the mass spectral scans as obtained from the GC/MS (Fig. 1) used in the analysis of the triterpene acetates were: α-amyrin, 21:10; β-amyrin, 19:30; taraxasterol, 25:50; pseudo-taraxasterol, 24:55; and lupeol, 21:35 min. The shoulder at 20:00 is an unidentified triterpene acetate.

[b]Molecular ion (M+).

to authentic samples. Taraxasterol was the major triterpene found (24% of the triterpene TLC band). β-Amyrin represented 21%, lupeol 13%, pseudo-taraxasterol 12%, and α-amyrin 9% of the band.

A single component, with a shorter GLC retention time (RT = 8:05 min; cf. Fig. 1) than the triterpenes and which produced a negative Lieberman-Burchard test, represented another 16% of the total. This compound was readily isolated by preparative argentation TLC (8) and was identified as a long-chain alcohol by its infrared and NMR spectra.

The total ion current chromatogram for the triterpene acetates of *S. arvensis*, obtained by using EI-MS, is shown in Figure 1A; Figure 1B shows the chromatogram produced by using methane PCI-MS. Although resolution of the triterpene acetates was incomplete on the OV-1 capillary column, we were able to separate these compounds by mass chromatography using the data system. In this way, mass spectra (Tables 2 and 3) were obtained for the 5 triterpenes that GLC indicated were present. In all cases, GLC retention times and the mass spectra agreed with those of the authentic samples. Also observed in the total ion current chromatograms was a compound appearing as a shoulder on the β-amyrin band. This compound appears to be the acetate of a monohydroxy-triterpene as its molecular ion occurs at 468; however, its identity has not been resolved.

Pentacylclic triterpenes are minor components of most plants and are seldom commercially available. Because this class of compounds has recently been shown to exhibit antitumor (11-16), antihypercholesteremic (17,18), anti-inflammatory (19-21), anticonvulsant (19), antibacterial, analgesic, antitussive and many other activities (20-22), it is important to find a source that can provide these compounds in quantities large enough to permit more extensive biological testing. Our investigation and other reports (2,10) indicate that thistles may be such a source.

This study has demonstrated how useful GC/MS is for rapid identification of triterpene acetates present in a complex mixture. This technique eliminates the need for tedious and time-consuming isolation, purification and characterization procedures usually applied to this group of products.

ACKNOWLEDGMENTS

The authors thank Dr. M.J. Harvey, Associate Professor, Department of Biology, Dalhousie University, for his help in determining the plant material; Dr. T.R. Watson, Professor, Pharmacy Department, University of Sydney, Sydney, Australia, for the gift of taraxasterol; Dr. R.V. Madrigal, Northern Regional Research Laboratory, USDA, Peoria, IL, for the gift of ψ-taraxasterol acetate; and Editorial Service, Faculty of Medicine, Dalhousie University, for help in preparing this manuscript.

This work was supported by the Medical Research Council of Canada (MA-6448).

REFERENCES

1. Chandler, R.F., Freeman, L., and Hooper, S.N. (1979) J. Ethnopharm. 1, 49-68.
2. Behari, M., Gupta, R., Itoh, T., and Matsumoto, T. (1980) Yukagaku 29, 82-85; Chem. Abstr. 92, 211784w.
3. Chandler, R.F., and Hooper, S.N. (1982) Lipids, in press.
4. Burrows, S., and Simpson, J.C.E. (1938) J. Chem. Soc. 2042-2047.
5. Chandler, R.F., Hooper, S.N., Hooper, D.L., Jamieson, W.D., Flinn, C.G. and Safe, L.M., J. Pharm. Sci., in press.
6. Bombardelli, E., Gabetta, B., Martinelli, E.M., and Mustichi, G. (1979) Fitoterapia 50, 11-24.
7. Method Ca. 6b-53 (1972) The Official and Tentative Methods of the American Oil Chemists' Society, 3rd edn., American Oil Chemists' Society, Champaign, IL.
8. Safe, L.M., Wong, C.J., and Chandler, R.F. (1974) J. Pharm. Sci. 63, 464-466.
9. Hooper, S.N., and Chandler, R.F. (1978) J. Pharm. Sci. 67, 1157-1159.
10. Madrigal, R.V., Plattner, R.D., and Smith, C.R., Jr. (1975) Lipids 10, 208-213.
11. Hartwell, J.L., and Abbott, B.J. (1969) Adv. Pharm. Chemother. 7, 117-209.
12. Hartwell, J.L. (1976) Cancer Treatment Rep. 60, 1031-1067.
13. Miles, D.H., Kokpol, U., Zalkow, L.H., Steindel, S.J., and Nabors, J.B. (1974) J. Pharm. Sci. 63, 613-615.
14. Pettit, G.R. (1977) Biosynthetic Products for Cancer Chemotherapy, Vol. 1, pp. 61-96, 177-186, Plenum Press, New York, NY.
15. Pettit, G.R., and Cragg, G.M. (1978) Biosynthetic Products for Cancer Chemotherapy, Vol. 2, pp. 11-60, 121-126, Plenum Press, New York, NY.
16. Pettit, G.R., and Ode, R.H. (1979) Biosynthetic Products for Cancer Chemotherapy, Vol. 3, pp. 9-18, 65-124, Plenum Press, New York, NY.
17. Chandler, R.F., Hooper, S.N., and Ismail, H.A. (1979) Can. J. Pharm. Sci. 14, 61-62.
18. Subbiah, M.T.R. (1973) Am. J. Clin. Nutr. 26, 219-225.
19. Chaturvedi, A.K., Parmar, S.S., Nigam, S.K., Bhatnagar, S.C., Misra, G., and Sastry, B.V.R. (1976) Pharmacol. Res. Commun. 8, 199-210.
20. Farnsworth, N.R., and Cordell, G.A. (1976) Lloydia 39, 420-455.
21. Benoit, P.S., Fong, H.H.S., Svoboda, G.H., and Farnsworth, N.R. (1976) Lloydia 39, 160-171.
22. Shibata, S. (1977) in New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutic Activity (Wagner, H., and Wolff, P., eds.) pp. 177-196, Springer-Verlag, New York, NY.

[Received August 14, 1981]

Essential and Nonessential Fatty Acid Oxidation in Mice Bearing Ehrlich Ascites Carcinoma

MURAD OOKHTENS* and **NOME BAKER**, *Tumor-Lipid Laboratory, Research Service, Veterans Administration Wadsworth Medical Center, Los Angeles, CA 90073, and the Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90024*

ABSTRACT

We tested the hypothesis that mobilized (essential) free fatty acids (FFA) are spared from oxidation in cancer-bearing animals. We injected tracers [1-^{14}C] linoleate, [1-^{14}C] palmitate and $NaH^{14}CO_3$ intravenously as single rapid doses in separate groups of mice bearing Ehrlich ascites tumor (EAT) and controls, and measured breath $^{14}CO_2$. The data from $NaH^{14}CO_3$ injections were used to develop kinetic, compartmental models of the HCO_3^--CO_2 systems. These models were integrated with our earlier model of plasma FFA turnover for control and EAT-bearing mice. The integrated multicompartmental models were then fitted to breath $^{14}CO_2$ data from mice injected with tracer FFA to compute the rates of FFA oxidation. FFA were not spared from an oxidative fate in our cancer-bearing vs normal animals; moreover, essential FFA were not preferentially spared from oxidation compared to nonessential FFA in the cancer-bearing mice.
Lipids 17:65-71, 1982.

INTRODUCTION

Very little is known about the fate of FA mobilized from the fat depots of tumor-bearing animals. In describing a lipid mobilizing factor in lymphoma-bearing mice, Kitada et al. (1) also noted that linoleate mobilized from labeled triglycerides of transplanted adipose tissue appeared to be spared an oxidative fate in their tumor-bearing mice. The linoleate mobilized from the fat depots seemed to be preferentially sequestered by the cancer cells, which require FA supplied by the host for tumor growth (2). Although this finding was not the main point of the study (1), it is of special interest in the light of earlier findings from our laboratory conducted on mice bearing EAT (3-5). Using tracer palmitate and linoleate, complexed to mouse serum albumin and injected intravenously (iv), we have shown that: (a) both essential and nonessential fatty acids are mobilized in EAT-bearing mice at rates that are indistinguishable from those of controls under either fed or fasted states (3,4); (b) essential and nonessential FFA are taken up from the tumor's extracellular fluid rapidly, but with no evidence of sequestration of essential FA (6); and (c) no more than 1% of the fat mobilized by the host is transported to the tumor (3,5). We have assumed that, eventually, most of the essential and nonessential FFA mobilized from the fat depots of our tumor-bearing mice are probably oxidized to CO_2. Thus, our working hypothesis is that both essential and nonessential FA are mobilized from the fat depots of cancer-bearing animals at about the same rates as in controls, and the FFA that enter the circulation are oxidized to CO_2 or stored again in the host's tissues with only a minor fraction being taken up by cancer cells.

In this study, as an initial test of this hypothesis, we have studied our cancer-host model, i.e., EAT grown in mice. Instead of using labeled adipose tissue grafts (1), we simplified the experimental design to a more direct one, i.e., injection of ^{14}C-labeled tracer FFA-serum albumin complex iv into EAT-bearing and control mice and measurement of breath $^{14}CO_2$. We also studied the kinetics of the HCO_3^--CO_2 system in EAT-bearing and control mice using tracer $NaH^{14}CO_3$ injections. We have used these data to estimate by multicompartmental analysis the effect of a large tumor on the rate of essential and nonessential FFA oxidation to CO_2 in mice.

Abbreviations: FA, fatty acids; FFA, free fatty acids; VLDL-TGFA, very low density lipoprotein-triglyceride fatty acids; 18:2, linoleate; 16:0, palmitate; EAT, Ehrlich ascites tumor.

MATERIALS AND METHODS

Animals and Tumors

Male, Swiss Webster mice (Hilltop Lab Animals, Inc., Chatsworth, CA), 5-10 weeks old, maintained on Purina Laboratory mouse chow and water ad libitum, were used. Tumor inoculations with 15×10^6 Ehrlich-Lettré hyperdiploid ascites tumor cells (chromosome number 44) were made using the transplanted cells from the subline maintained in Swiss Webster mice by Dr. Ralph McKee, Biological Chemistry Department, UCLA School of Medicine. The details of the transplantation procedure have been described previously (5). Mice were used 9-10 days following the inoculation. At that time, their body weights had

increased from 28.5 ± 0.6 g to 44.3 ± 0.9 g (mean ± SE, n = 28) and their whole tumor volumes (cells plus extracellular fluid) were 8-10 ml. The age-matched control animals had body weights equal to 32.6 ± 0.4 g (mean ± SE, n = 16) on the experimental days. The experiments started at 8-9 a.m. at which time the breath CO_2 collections from the first pair of mice (one control and one tumorous) were started. Commonly, 4 such pairs of mice were used in any single experiment on a given day. Breath CO_2 collection from the last pair started around 3-4 p.m. at the latest. From our previous experience, mice with access to food ad libitum under the circumstances of our experiments are likely to be post-absorptive or briefly fasted (3,4).

Sodium Bicarbonate

$NaH^{14}CO_3$, 4.2 mCi/mmol (Dhom Products, Ltd., Los Angeles, CA, Lot No. C03702), was used. Each mouse was injected iv with 1.5 μCi of this tracer, diluted to 50 μl in slightly alkaline saline.

Fatty Acid-Serum Albumin Complexes

[1-^{14}C]Palmitic acid, 58 mCi/mmol, and [1-^{14}C]linoleic acid, 52.5 mCi/mmol (Dhom Products, Ltd., Los Angeles, CA, Lot Nos. C16103B, C16103C, and C22904), 99% pure by thin layer chromatography on silica gel and silver nitrate silica gel systems, were used. Fatty acid-serum albumin complexes of each were prepared according to the procedure described previously (7). In the injected dose, the final molar ratio of the total FA (added and already present in serum) to serum albumin was ca. 2. In our experiments, 50 μl containing either 1 μCi of palmitate complex or 0.4 μCi of the linoleate complex was injected iv (tail vein).

Breath CO_2 Measurements

The apparatus and the techniques for collecting the expired air, trapping the CO_2 present in the breath of mice, and measuring $^{14}CO_2$ have been described in our earlier study (8).

Liquid Scintillation Counting

Aliquots (0.5 ml) of the trapping agent taken at different times during each experiment were dispersed in 10 ml of Insta-Gel/toluene (1:1, v/v) scintillation cocktail in glass counting vials (8). Appropriate blanks and dose assays were prepared similarly. All samples were assayed for ^{14}C-radioactivity in a Beckman LS 3133P liquid scintillation spectrometer. An external standard was used to determine the degree of quenching.

Multicompartmental Analyses

Multicompartmental models were formulated to fit the data and to compute the FFA transport and oxidation rates using the SAAM program (9), and an IBM 360/91 digital computer. Many of the general assumptions involved in the use of simplified models to study rates of oxidation of metabolic fuels have been described and discussed in an earlier article (10).

RESULTS

Bicarbonate-CO_2 Systems

Transport of $^{14}CO_2$ to the lungs and subsequent expiration in breath involves mixing and dilution of $^{14}CO_2$ in the intermediate pools of HCO_3^--CO_2 in both tumor-bearing and control mice. To identify the kinetics of these pools and to incorporate them in our analysis for estimation of the rates of FA oxidation, we have done the following 2 sets of experiments.

Breath $^{14}CO_2$ in Control and EAT-Bearing Mice after iv Injection of Tracer $NaH^{14}CO_3$: Kinetic Behavior of the Bicarbonate Pools

A group of normal and EAT-bearing mice were injected with tracer $NaH^{14}CO_3$ iv and the cumulative appearance of $^{14}CO_2$ in their breath was measured (8). Figure 1 shows the data from these experiments. As can be seen, in both the

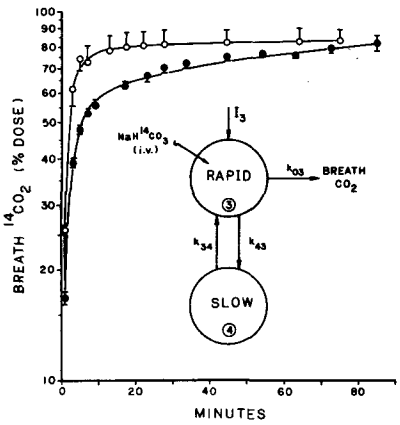


FIG. 1. Breath $^{14}CO_2$ following iv injections of tracer $NaH^{14}CO_3$ into control (open circles; n = 3) and EAT-bearing (dark circles; n = 4) mice. Bars, ± SE. Curves represent the least squares fits obtained with SAAM using the 2-compartment model shown and allowing all 3 fractional transport rates to adjust during iterations. The computed values for the rates are shown in Table 1. I_3 represents the influx of unlabeled CO_2-bicarbonate to maintain the steady-state.

TABLE 1

Fractional Transport Rates (min $^{-1}$) of the Bicarbonate-CO_2 System in Control and EAT-Bearing Mice

	k_{03}[a]	k_{43}	k_{34}
Control	0.34 ± 0.014[b]	0.085 ± 0.023	0.0034 ± 0.001
EAT-Bearing	0.20 ± 0.007	0.13 ± 0.013	0.018 ± 0.003

[a]Fractional rates correspond to those shown in Fig. 1.
[b]Mean ± SD (n = 4).

controls (open circles) and EAT-bearing (dark circles) mice, about 85% of the injected label was expired in breath $^{14}CO_2$; however, in control mice, almost all of the expired $^{14}CO_2$, was recovered in breath in the initial 15-20 min, whereas in the EAT-bearing group, the appearance of $^{14}CO_2$ was relatively delayed and reached 85% of the dose toward the very end of the experiment. This delayed appearance of $^{14}CO_2$ implies that EAT-bearing mice have larger or more bicarbonate pools than controls. Since our only aim in these studies was to define the kinetics of the HCO_3^--CO_2 system so that we could estimate the oxidation rates of FA to CO_2 (see below), we did not attempt any more detailed investigation of this difference. We used the 2-compartment model shown in Figure 1 to fit the data separately for each group of mice. Compartment 3 in the model includes all the HCO_3^--CO_2 pools that are in rapid equilibrium with the vascular pool where the injection (iv) of the tracer takes place and from which CO_2 is expired. Compartment 4 represents a lumping of those body HCO_3^--CO_2 pools which turn over slowly, but fast enough to recycle labeled bicarbonate into compartment 3 during the experiment. The best least squares fits obtained by allowing the adjustment of all 3 fractional transport rates (shown in Fig. 1) for control and EAT-bearing mice are shown by the curves drawn in Figure 1. The values of the fractional transport rates are shown in Table 1. In the control mice, only about 20% of the total flux of the rapid compartment mixes with the slow compartment $[k_{43}/(k_{43} + k_{03})]$; this mixing component reaches about 40% of the total flux in EAT-bearing mice. This increase is partly due to exchange of plasma bicarbonate with the large tumor extracellular fluid bicarbonate pool (M. Ookhtens and N. Baker, unpublished observations). The 2-compartment models for the HCO_3^--CO_2 systems of control and EAT-bearing mice were later integrated with our model for FFA turnover (4) to compute the rates of FFA oxidation to CO_2 (see below).

Breath $^{14}CO_2$ Measurements after iv Injection of Tracer [1-^{14}C] Palmitate or [1-^{14}C] Linoleate

Either of these labeled tracer FA complexed to mouse serum albumin was injected iv into separate groups of control and EAT-bearing mice and their breath $^{14}CO_2$ output was measured. As can be seen (Fig. 2), 30-40% of the injected doses were recovered as breath $^{14}CO_2$ in 1 hr. The apparent features of these data were the virtual identity of the breath $^{14}CO_2$ between the control and EAT-bearing groups for each tracer FA. There was no evidence that the essential FA 18:2 was being spared from oxidation any more than the nonessential FA 16:0. In fact, about 30% more $^{14}CO_2$ was expired after injection of [^{14}C] 18:2 compared to that expired after injection of [^{14}C] 16:0 (Fig. 2; about 40% vs 30% of injected dose). This difference is diminished when the relative plasma pool sizes of the 2 FA are considered in control and EAT-bearing mice (15-30% higher plasma 16:0 vs 18:2 concentrations [3]). Therefore, instead of attempting to highlight the minor differences in the molar rates of oxidation of each FA, we have used tracer data from each to estimate a range for the rates of total FA oxidation, considering each tracer to be representative of the kinetics of total FA oxidation (3).

Estimation of FFA Oxidation Rates by Multicompartmental Analysis

To estimate the rates of FFA oxidation from the data presented in Figure 2, we proceeded as follows. We had already established in an earlier study (3) that plasma palmitate and linoleate (as FFA-albumin complexes) turn over at the same rate in both EAT-bearing and control mice. Second, we used the 2-compartment model proposed by Baker et al. (4) which was based on a detailed study of the turnover of labeled palmitate complexed to serum albumin in fasted tumor-bearing and control mice. We believe that this simplification is warranted since our earlier study (4) had defined the kinetics of plasma FFA turnover within narrow

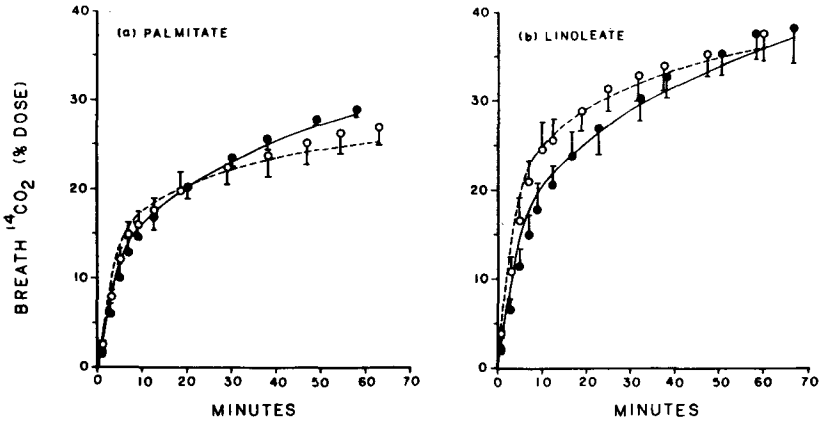


FIG. 2. Breath $^{14}CO_2$ following iv injections of (a) [1-^{14}C] palmitate and (b) [1-^{14}C]-linoleate complexed to mouse serum albumin into control (open circles) and EAT-bearing (dark circles) mice, respectively. Data points and vertical bars represent mean ± SE for 4 mice in each group. Broken and continuous curves represent the least squares fits to the data from control and EAT-bearing mice using the integrated model (Fig. 3) of the FFA and HCO_3^--CO_2 subsystems.

limits and all of the parameters were identical in the EAT-bearing and control mice. We then integrated this 2-compartment model that defined the plasma- and extraplasma-FFA kinetics with the bicarbonate model (Fig. 1) for each group (parameters listed in Table 1). The integrated model is shown in Figure 3. For each case, i.e., control and EAT-bearing mice, the fractional transport rates for the HCO_3^--CO_2 system were set to the values identified before (Table 1) and were kept constant during successive iterations using the SAAM program (9). Most of the fractional transport rates of the 2-compartment model representing plasma FFA kinetics were also kept constant. Thus, k_{12} and k_{21}, representing the exchange between compartments 1 and 2, and the total fractional irreversible disposal ($k_{01} + k_{31}$ in Fig. 3) were fixed at the values determined previously (4). In each case, k_{31} (i.e., the fractional rate of FFA oxidation) was the only parameter allowed to adjust independently while fitting the integrated models to the data presented in Figure 2. The value for k_{01} was a dependent variable, defined as $k_{01} = 1.2 - k_{31}$ (see Fig. 3). The computer-derived fits are shown by broken (controls) and solid (EAT-bearing) curves in Figure 2. Table 2 presents the values estimated by the SAAM program for the fractional rates (min^{-1}) of FA oxidation (k_{31}), previously measured FFA pool sizes (4), and our computed rates (μeq/min/mouse) of FFA oxidation in control and EAT-bearing mice using tracers [1-^{14}C] palmitate and [1-^{14}C] linoleate.

As shown in Table 2, the mean fractional rates of FFA oxidation using labeled palmitate as a tracer were lower than the corresponding values using labeled linoleate in both control and EAT-bearing mice (0.39 vs 0.55 min^{-1} and 0.48 vs 0.61 min^{-1}, respectively). Since the fractional SD of each rate obtained with the SAAM analysis was only about 2-3%, each of these differences was highly significant ($p < 0.01$). However, this statistical analysis is inconclusive for 2 reasons. First, the SD of k_{31} are unrealistically low due to the method of analysis used (fixed parameters for the FFA and bicarbonate subsystems). Second, as already noted, the pool sizes of plasma palmitate tend to be 15-30% greater than those of linoleate in both groups (3). Therefore, the oxidation rates, when expressed as μeq 16:0 or 18:2/min/mouse, are clearly not significantly different. Whether or not linoleate is oxidized significantly faster than palmitate, one point is clear: essential FFA oxidation is not reduced compared to nonessential FFA oxidation in these tumor-bearing mice.

The values of k_{31} shown in Table 2 also indicate clearly that, regardless of the tracer used, the fractional rates of FFA oxidation in tumor-bearing mice are not depressed compared to those of the controls. Thus, using labeled palmitate, the fractional rates were 23% faster in the tumor-bearing mice (0.48 vs 0.39 min^{-1}); using labeled linoleate, there was a 10% increase in the rate (0.61 vs 0.55 min^{-1}). In each case, the difference appeared to be statistically significant; however, as already noted, the fractional

SD obtained in the SAAM analysis were unrealistically low (2-3%). The mean of the fractional rate constants using 16:0 and 18:2 may give the best estimate of k_{31} for the total plasma FFA pool (Table 2, line 3). The grand mean was 17% faster in the tumor-bearing than in the control mice (0.55 ± 0.065 vs 0.47 ± 0.080 min^{-1}). The difference was not statistically significant. Therefore, based on the values for k_{31} shown in Table 2, the FFA being mobilized from the fat depots in the tumor-bearing mice are not spared from an oxidative fate when compared to the controls. This is also seen clearly when the mean fractional FFA oxidation rates are multiplied by the corresponding plasma FFA pool sizes of each group. As shown in the bottom of Table 2, the FFA oxidation rate to CO_2 tended to be faster in the tumor-bearing than in the control mice (1.00 ± 0.23 vs 0.79 ± 0.25 μeq FFA/min/mouse); the difference was not statistically significant. Based on these observations and our earlier study (3), we must conclude that both essential and nonessential FFA are mobilized from the fat depots at approximately equal rates in EAT-bearing and control mice and the mobilized FFA are oxidized to CO_2 at least as rapidly in the tumor-bearing mice as in the controls.

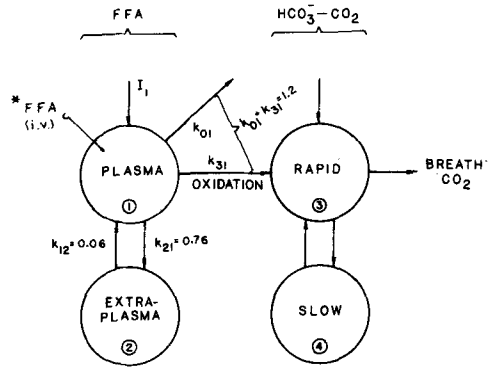


FIG. 3. Integrated model of FFA turnover and oxidation to CO_2. The FFA subsystem was defined in an earlier study (4), and the $HCO_3^- - CO_2$ subsystem was developed in this paper. This integrated model was used to estimate the fractional rate of FFA oxidation, k_{31}, which was submitted as the only independent adjustable parameter during the SAAM runs. The fractional rates between compartments 1 and 2, as well as the total rate of loss out of compartment 1, i.e. (k_{01} + k_{31}), were kept constant and equal to values determined before (4). The fractional transport rates of the $HCO_3^- - CO_2$ system were submitted as constants and equal to the ones presented in Table 1 for control and EAT-bearing mice. The arrow marked with *FFA represents the iv injection of tracer 16:0 and 18:2. I_1 represents the influx of cold FFA to maintain the steady-state.

DISCUSSION

Although many earlier studies (e.g., review by Begg [11]) have reported the mobilization and depletion of fat stores along with development of hyperlipidemia in cancer-bearing animals, we have been unable to find any published quantitative studies of plasma FFA oxidation rates in cancer-bearing mice. The only directly relevant study seems to be that of Waterhouse and Kemperman with human subjects (12). They found no difference in the rates of plasma FFA oxidation in cancer patients compared to controls. The early study of Medes et al. (13) clearly established that exogenously fed fatty acids are rapidly oxidized by tumor-bearing mice; however, rates of FA oxidation were not calculated (about 80-90% of ingested [1-^{14}C]palmitic acid was expired as $^{14}CO_2$ within 24 hr), and no control mice were studied.

Aside from the findings of Kitada et al. (1), we know of only one other study, that of Costa et al. (14), in which FA oxidation in the tumor-bearing host was reported to be impaired. Costa et al. described a marked depression of breath $^{14}CO_2$ formation from injected ^{14}C-labeled triglyceride in mice bearing Krebs-2 carcinoma vs controls. We have criticized (8) their experimental design in terms of both the form and route of tracer administration ([^{14}C]tripalmitin dissolved in carrier peanut oil, injected intraperitoneally), as well as their failure to measure any intermediate pool sizes involved in tracer dilution (e.g., the plasma TG pool). Our experiments (8) that were designed to verify the generality of the findings of Costa et al. (14) failed to show any depression of plasma VLDL-TGFA-^{14}C oxidation to $^{14}CO_2$ in mice bearing subcutaneous, *solid* Ehrlich tumors. Mice bearing Ehrlich *ascites* tumors show a significant (2-to-3-fold) depression in the breath $^{14}CO_2$ compared to controls when both are injected iv with VLDL-TGFA-^{14}C; however, this depression is almost entirely accountable (M. Ookhtens and N. Baker, unpublished observations) by tracer dilution due to the hypertriglyceridemia that develops in this cancer-host model (15,16).

Thus, the available literature would lead one to predict that FFA are readily oxidized by the tumor-bearing host. Yet, in a recently published study by Kitada et al. (1), the interesting incidental observation was made that, in mice bearing virus-accelerated lymphoma, the labeled essential FA (18:2) mobilized from the host adipose tissues are largely sequestered

TABLE 2

Fractional and Total Rates of Plasma FFA Oxidation in Control and EAT-Bearing Mice

Parameters (rates and pool sizes)	Control	EAT-Bearing
Fractional rates (k_{31}) of FFA oxidation (min^{-1}) for:		
[1-^{14}C]palmitate	0.39[a]	0.48
[1-^{14}C]linoleate	0.55	0.61
Mean (16:0 + 18:2)	0.47 ± 0.080[b]	0.55 ± 0.065[c]
Plasma FFA pool sizes (μeq/mouse)[d]	1.69 ± 0.53	1.82 ± 0.42
Rates of total FFA oxidation (μeq/min/mouse)[e]	0.79 ± 0.25	1.00 ± 0.23

[a]Fractional SD were of the order of magnitude of 2-3% but are not shown because of constraints used in the SAAM analysis (see text).

[b]± SD.

[c]NS (control vs tumor-bearing mice).

[d]Data from our previous study (4).

[e]Computed by multiplying the mean fractional rates (line 3) by the plasma FFA pool sizes (line 4) of control and tumorous mice, respectively, taking into account the variances.

by tumorous tissues and spared from an oxidative fate. This observation raises the possibilities that essential FA were sequestered by the tumor to a greater extent than nonessential FA and that essential FA were preferentially spared from oxidation to CO_2 by the host. (Since the oxidation of fatty acids was not the central point of their study, Kitada et al. did not compare essential with nonessential FA oxidation.) We did not detect any increased mobilization and preferential sequestration of the essential FA linoleate vs the nonessential FA palmitate by EAT growing in Swiss-Webster mice (3,6). In the present study, we have measured the oxidation rates of mobilized FA to CO_2, and we have tried to test directly the possibility that essential FFA are spared an oxidative fate in EAT-bearing mice. However, to avoid technical complications and possible artifacts introduced by grafting of prelabeled adipose tissue pieces (1), we selected a more straightforward experimental design, i.e., the iv injection of tracer FFA complexed to mouse serum albumin followed by measurement of breath $^{14}CO_2$ for periods of about 1 hr.

Using tracer techniques to identify the kinetics of the HCO_3^--CO_2 systems in both EAT-bearing and control mice, and integrating our previously described (4) compartmental model for plasma FFA kinetics with HCO_3^--CO_2 models developed in the present study, we estimated the rates of FFA oxidation in EAT-bearing and control mice. Our analyses failed to show any evidence for the sparing of FFA from an oxidative fate in EAT-bearing mice compared to controls, or any tendency toward the preferential sparing of the essential FA compared to the nonessential FA.

The interpretation of our results is dependdent on the assumptions inherent in tracer kinetic methods involving single-dose injections and multicompartmental analyses (10). It is unlikely that those assumptions could be instrumental in our failure to detect any significant differences between the FA oxidation rates in EAT-bearing mice vs controls. If, indeed, there are any differences in these rates, they must be so subtle that they cannot be detected by our measurement and/or analytical techniques. At any rate, these differences cannot be as dramatic (practically an all-or-none response with respect to FA oxidation) as those reported by Kitada et al. (1).

In order to reconcile our results with those of Kitada et al., several important variables need to be considered: the use of labeled, grafted fat pads vs that of labeled FFA-albumin complexes as tracer techniques for studying fat depot mobilization and FA oxidation; differences in our approaches to data analyses; interpretation of short-term vs long-term tracer studies; the use of a solid lymphoma rather than an ascites tumor and the attendant perfusion-transport factors; and, perhaps, nutritional states of the mice. Studies designed to explore these variables in both lymphoma-bearing and EAT-bearing mice and their controls are in progress.

ACKNOWLEDGMENTS

This investigation was supported by VA Medical Research Funds, NIH-USPHS Grant CA 15813 and

Post-Doctoral National Research Service Award USPHS CA 05651 to M. Ookhtens by the National Cancer Institute, DHEW. Computing assistance was obtained from the Health Sciences Computing Facility, UCLA, supported by NIH Grant RR-3. We thank A.G. Lowe and D.B. Learn for their excellent technical assistance and H. Rostami for biostatistical consultation.

REFERENCES

1. Kitada, S., Hays, E.F., and Mead, J.F. (1980) Lipids 15, 168-174.
2. Spector, A.A. (1975) Prog. Biochem. Pharmacol. 10, 42-75.
3. Baker, N., Sandborg, C., Morris, D., and Ookhtens, M. (1977) Cancer Res. 37, 2218-2225.
4. Baker, N., Hill, V.A., and Ookhtens, M. (1978) Cancer Res. 38, 2372-2377.
5. Mermier, P., and Baker, N. (1974) J. Lipid Res. 15, 339-351.
6. Kannan, R., Ookhtens, M., and Baker, N. (1980) Cancer Res. 40, 2447-2454.
7. Ookhtens, M., and Baker, N. (1979) Cancer Res. 39, 973-980.
8. Ookhtens, M., and Baker, N. (1979) Cancer Res. 39, 5118-5123.
9. Berman, M., and Weiss, M.F. (1977) SAAM Manual, Washington, DC, U.S. Government Printing Office, DHEW Publication No. (NIH) 76-730, 175 pp.
10. Baker, N. (1969) J. Lipid Res. 10, 1-24.
11. Begg, R.W. (1958) Adv. Cancer Res. 5, 1-54.
12. Waterhouse, C., and Kemperman, J.H. (1971) Cancer Res. 31, 1273-1278.
13. Medes, G., Paden, G., and Weinhouse, S. (1957) Cancer Res. 17, 127-133.
14. Costa, G., Lyles, K., and Ullrich, L. (1976) Cancer (Phila) 38, 1259-1265.
15. Brenneman, D.E., Mathur, S.N., and Spector, A.A. (1975) Eur. J. Cancer 11, 225-230.
16. Kannan, R., and Baker, N. (1977) Lipids 12, 153-158.

[Received August 20, 1981]

Ether Lipid Content and Fatty Acid Distribution in Rabbit Polymorphonuclear Neutrophil Phospholipids

HOWARD W. MUELLER[a], JOSEPH T. O'FLAHERTY[b] and ROBERT L. WYKLE[*,a],
Departments of Biochemistry[a] and Medicine[b], Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103

ABSTRACT

This study was undertaken to determine if rabbit neutrophils contain sufficient ether-linked precursor for the synthesis of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor) by a deacylation-reacylation pathway. The phospholipids from rabbit peritoneal polymorphonuclear neutrophils were purified and quantitated, and the choline-containing and ethanolamine-containing phosphoglycerides were analyzed for ether lipid content. Choline-containing phosphoglycerides (37%), ethanolamine-containing phosphoglycerides (30%), and sphingomyelin (28%) were the predominant phospholipid classes, with smaller amounts of phosphatidylserine (5%) and phosphatidylinositol (<1%). The choline-linked fraction contained high amounts of 1-*O*-alkyl-2-acyl- (46%) and 1,2-diacyl-*sn*-glycero-3-phosphocholine (54%), with a trace of the 1-*O*-alk-1'-enyl-2-acyl species. The ethanolamine-linked fraction contained high amounts of 1-*O*-alk-1'-enyl-2-acyl- (63%) and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (34%), and a low quantity of the 1-*O*-alkyl-2-acyl species (3%). The predominant 1-*O*-alkyl ether chains found in the *sn*-1 position of the choline-linked fraction were 16:0 (35%), 18:0 (14%), 18:1 (26%), 20:0 (16%), and 22:0 (9%). The major 1-*O*-alk-1'-enyl ether chains found in the *sn*-1 position of the ethanolamine-linked fraction were 14:0 (13%), 16:0 (44%), 18:0 (27%), 18:1 (12%) and 18:2 (3%). The major acyl groups in the *sn*-1 position of 1,2-diacyl-*sn*-glycero-3-phosphocholine and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine were 16:0, 18:0 and 18:1. The most abundant acyl group in the *sn*-2 position of all classes of choline- and ethanolamine-linked phosphoglycerides was 18:2. Although this work does not define the biosynthetic pathway for platelet activating factor, it does show that there is ample precursor present to support its synthesis by a deacylation-reacylation pathway.
Lipids 17:72-77, 1982.

INTRODUCTION

PAF is a very potent inducer of rabbit platelet aggregation and the release of serotonin, prostaglandins and granular constituents from platelets (1). It has recently been shown to have the structure 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-*O*-alkyl-2-acetyl-GPC) (2). Originally, PAF was found to be secreted from antigen-stimulated, IgE-sensitized basophils, and has since been shown to also be secreted by other cells including macrophages (3), monocytes and polymorphonuclear neutrophils (PMN) (4).

Two pathways for the synthesis of 1-*O*-alkyl-2-acetyl-GPC have been demonstrated in microsomal preparations of rat spleen and several other tissues. The first pathway involves an acetyltransferase enzyme which transfers acetate from acetyl-CoA to 1-*O*-alkyl-2-lyso-GPC (5). In the second pathway, 1-*O*-alkyl-2-acetyl-GPC is synthesized from 1-*O*-alkyl-2-acetylglycerol by a CDP-choline:1-*O*-alkyl-2-acetylglycerol cholinephosphotransferase reaction (6). For the first pathway to operate as part of a deacylation-reacylation cycle in whole cells, a pool of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine containing a long-chain residue at the 2-position would be expected. In this case, a phospholipase A_2 reaction (7) would make available the necessary substrate for the acyltransferase of the deacylation-reacylation pathway. Chap et al. (8) showed that ^{14}C-labeled acetate was incorporated into 1-*O*-alkyl-2-acetyl-GPC in stimulated platelets; however, this work did not define the pathway of synthesis.

In this study, we have characterized the lipids of rabbit neutrophils to determine if the cells contain enough 1-*O*-alkyl-2-acyl-GPC to serve as a possible precursor of 1-*O*-alkyl-2-acetyl-GPC. Our study was limited to the phospholipids because we were focusing on possible PAF precursors and because work has previously been done on PMN neutral lipid (9).

Abbreviations: PAF, platelet activating factor; GPC, *sn*-glycero-3-phosphocholine; PMN, polymorphonuclear neutrophils; TLC, thin layer chromatography; GPE, *sn*-glycero-3-phosphoethanolamine; GLC, gas liquid chromatography; PC, choline-containing phosphoglycerides; PE, ethanolamine-containing phosphoglycerides; PS, phosphatidylserine; PI, phosphatidylinositol.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade or better and solvents were distilled before use. Type II shellfish glycogen, phospholipase C (*Bacillus*

cereus) and phospholipase A_2 (*Ophiophagus hannah* venom) were obtained from Sigma Chemical Co. *Rhizopus delemar* lipase was obtained from Miles Biochemicals. 1-*O*-Alkylglycerol standards were purchased from Serdary Research Laboratories. All other lipid standards were from Supelco and Nu-Chek-Prep. Vitride ($NaAlH_2[OCH_2CH_2OCH_3]_2$) was purchased from Alfa Products.

Preparation of Cells

Rabbit peritoneal PMN were obtained from New Zealand Albino White rabbits 3-5 hr after the administration of Type II shellfish glycogen (10). The leukocyte population contained better than 95% PMN and there were less than 20 platelets/100 PMN. Cell yield from 3 rabbits was 1-2 x 10^9 PMN.

Extraction and Fractionation of Lipids

Cellular lipids were extracted by the method of Bligh and Dyer (11) immediately after cell collection. The choline-linked and ethanolamine-linked glycerolipid fractions were purified by preparative TLC on Silica Gel H. The plates were developed in chloroform/methanol/glacial acetic acid/water (50:25:8:4, v/v) and the lipids were recovered from the gel by Bligh and Dyer extraction (11).

Quantitation of Phospholipid

An aliquot of the total lipid extract (200-300 μg) was analyzed by 2-dimensional TLC using chloroform/acetone/glacial acetic acid/methanol/water (60:24:18:12:6, v/v) in the first dimension and *n*-butanol/glacial acetic acid/H_2O (80:26:26, v/v) in the second dimension (C.C. Cunningham, personal communication). The various phospholipids were visualized and assayed for lipid phosphorus using the method of Rouser et al. (12). Recovery of phospholipid phosphorus was better than 90%.

Separation and Quantitation of 1,2-Diacyl-, 1-O-Alkyl-2-acyl- and 1-O-Alk-1′-enyl-2-acyl-GPC and -GPE

Purified choline- and ethanolamine-containing fractions (1-3 mg) were coated inside a 100-ml round-bottomed flask, exposed to HCl gas for 5 min to hydrolyze the 1-*O*-alk-1′-enyl groups, and then flushed thoroughly with N_2. The cleaved aldehyde, the lysophospholipid, and the unreacted phospholipid were separated by preparative TLC as described in the previous section. The amount of lysophospholipid as determined by lipid phosphorus analysis of the separated products was taken as the 1-*O*-alk-1′-enyl content. Cleavage of the diacyl and alkyl-acyl phospholipids was negligible under these conditions.

The unhydrolyzed phospholipid was treated with phospholipase C and the products acetylated according to Waku et al. (13) to yield 1,2-diacyl-3-acetylglycerol and 1-*O*-alkyl-2-acyl-3-acetylglycerol. These 2 species were separated by preparative TLC on Silica Gel H using hexane/diethyl ether/glacial acetic acid (70:30:1, v/v). In order to measure the different classes, the purified acetylglycerides and the lysophospholipid from acid treatment were dissolved in 2.7 ml of methanol containing 7% H_2SO_4. The samples were sealed under N_2 and heated for 2.5 hr at 80 C. After cooling in ice 2 ml of H_2O were added and the methyl esters were extracted twice into hexane. These samples were analyzed by GLC as described next using 15:0 methyl ester as an internal standard. Each mol of methyl ester was taken to represent 1 mol of 1-*O*-alkyl-2-acyl-phospholipid, 1 mol of 1-*O*-alk-1′-enyl-2-acyl-phospholipid, or 0.5 mol of 1,2-diacyl phospholipid.

Determination of 1-O-Alk-1′-enyl Chain Distribution

Aldehydes formed from acid treatment of plasmalogen were reduced with Vitride ($NaAlH_2[OCH_2CH_2OCH_3]_2$) to the corresponding alcohol by the method of Snyder et al. (14). The fatty alcohols were then acetylated in the same manner as the diglycerides (13) and analyzed by GLC as described later using 15:0 alcohol acetate as an internal standard.

Determination of 1-O-Alkyl Chain Distribution

1-*O*-Alkyl-2-acyl-3-acetylglycerol (1-2 mg) was reduced with Vitride for 1.5 hr as already described to yield 1-*O*-alkylglycerol, which was purified by preparative TLC. The isopropylidene derivative was made by a modified method of Wood (15). Briefly, 1 ml of acetone containing 0.1 μl of 70% perchloric acid was added to the sample. The mixture was allowed to stand at room temperature for 20 min, 1-2 drops of concentrated NH_4OH were added, and the product was extracted 3 times with 1:1 hexane/diethyl ether. The isopropylidene derivatives were analyzed by GLC as described later. The 1-*O*-alkyl chain distribution was based on area percentages. Peak identities for these samples were also confirmed by GLC-mass spectroscopy (GLC-MS) analysis as described later.

Fatty Acid Distribution of 1,2-Diacyl-GPC and -GPE

Fatty acyl residues at positions 1 and 2 of diacyl-GPC and -GPE were analyzed by the method of Sugiura et al. (16).

GLC Analyses

All GLC analyses were performed on a Varian 3700 gas chromatograph with a CDS-111 data processor. Methyl esters were analyzed on a 62-m glass capillary column coated with OV-351. Column temperature ranged from 155 to 245 C with a programmed rate of 8 C/min up to 200 C, then 5 C/min up to 245 C. The helium flow rate was 1 ml/min. The injector and detector temperatures were 200 and 300 C, respectively.

Fatty alcohol acetates were separated on a 6-ft glass column packed with 10% Silar-10C on 100/120 mesh Gas-Chrom Q. Column temperature ranged from 160 to 235 C at a rate of 3 C/min. Isopropylidene alkylglycerols were analyzed on the same column; however, the temperature ranged from 180 to 235 C at 3 C/min. The nitrogen flow rate was 30 ml/min. Injector and detector temperatures were 200 C and 300 C, respectively.

GLC-MS analysis was performed under the auspices of Dr. D.L. Millington (University of North Carolina, Chapel Hill). The instrument was a VGMM-7070 double focusing mass spectrometer coupled to a HP-5710A gas chromatograph. The column was a 25-m HP "fused silica" SP2100 coated capillary, programmed from 60 to 200 C at 20 C/min, then from 200 to 270 C at 4 C/min. The carrier flow rate was 1 ml/min.

RESULTS AND DISCUSSION

The phospholipids of rabbit PMN, which constituted ca. 43% by mass of the total lipid fraction, were quantitated by TLC and lipid phosphorus analysis as described in Methods. As shown in Table 1, the phospholipids were found to be high in PC (37%), PE (30%) and sphingomyelin (28%). There was also a significant amount of PS (5%) and a trace of PI (<1%). No lysophospholipids were detected. These results are similar to the findings of Mason et al. (17), which showed guinea pig peritoneal PMN to be high in PC (34%), PE (32%) and sphingomyelin (19%) with smaller amounts of PS (8%) and PI (5%). Circulating human PMN, however, have been reported to contain higher amounts of PC (44%) and reduced levels of sphingomyelin (5%) in addition to PE (33%), PS (8%) and PI (7%) (9). In this study, platelet lipid contamination was calculated to be no more than 1% based on the phospholipid content of human platelets (18).

As shown in Table 2, the PC fraction of rabbit PMN contained 54% 1,2-diacyl-GPC and 46% 1-*O*-alkyl-2-acyl-GPC. To our knowledge,

TABLE 1

Phospholipid Composition of Polymorphonuclear Leukocytes

	mol % phosphorus (N = 9)[a]
Sphingomyelin	27.8 ± 1.9
Choline-containing phosphoglycerides	37.0 ± 1.9
Phosphatidylinositol	<1
Phosphatidylserine	4.8 ± 1.9
Ethanolamine-containing phosphoglycerides	29.8 ± 1.9

[a]Each determination represents a separate population of 1-2 × 10^9 cells. Standard deviations are given. There were 5350 ± 965 nmol of lipid phosphorus/10^9 PMN (N = 3) and 937 ± 65 nmol of lipid phosphorus/mg of total lipid (N = 3).

TABLE 2

Ether Class Composition of Choline- and Ethanolamine-Containing Phosphoglycerides

	Choline-containing phosphoglycerides (N = 4)[a]	Ethanolamine-containing phosphoglycerides (N = 3)[a]
1,2-Diacyl	54.3 ± 2.2[b]	33.7 ± 0.8
1-*O*-Alkyl-2-acyl	45.5 ± 2.5	3.0 ± 0.8
1-*O*-Alk-1′-enyl-2-acyl	trace	63.4 ± 2.4

[a]Each determination represents a separate population of cells.

[b]Data are presented as mol % ± standard deviation. Classes were quantitated as described in Methods.

TABLE 3

Fatty Chain Distribution of Choline-Containing Phosphoglycerides

	1,2-Diacyl (mol %)		1-*O*-Alkyl-2-acyl (mol %)	
	Position 1 (N = 2)[a]	Position 2 (N = 4)[a]	Position 1 (N = 3)[a,b]	Position 2 (N = 4)[a]
16:0	42.7 ± 1.4	16.2 ± 0.5	35.1 ± 2.4	13.1 ± 1.9
16:1	4.6 ± 2.3	3.5 ± 0.3	--	2.0 ± 0.4
18:0	15.3 ± 2.2	4.0 ± 1.6	14.1 ± 1.1	2.2 ± 0.7
18:1	25.7 ± 3.1	16.6 ± 2.0	26.1 ± 1.5	13.7 ± 2.7
18:2	8.9 ± 1.5	52.7 ± 4.5	–	65.0 ± 2.0
18:3	0.5 ± 0.5	1.2 ± 0.7	–	0.4 ± 0.4
20:0			16.0 ± 1.3	
20:3	0.8 ± 0.8	1.7 ± 1.0	--	
20:4	0.2 ± 0.2	2.7 ± 0.3	–	1.3 ± 0.7
20:5	–	0.2 ± 0.3	--	
22:0	--	–	8.7 ± 1.9	–
Other[c]	--	1.1 ± 0.6	–	0.1 ± 0.2

[a]Each determination represents a separate population of cells. Standard deviations are given.

[b]Based on area % rather than mol %.

[c]Minor chains present were 22:1, 22:4, 22:5 and 22:6.

this is the first report on the 1-*O*-alkyl content of neutrophil lipids; this level is unusually high when compared with the amounts in most other tissues (19). To confirm that these levels were present, a complete Vitride reduction (14) of the choline-containing fraction was performed. The alkylglycerol was quantitated by the method of Blank et al. (20) and found to agree with the GLC data. There was also a trace of choline-linked plasmalogen (1-*O*-alk-1′-enyl-2-acyl-GPC) as shown by lipid phosphorus analysis following acid hydrolysis, but further characterization was not pursued due to the minute quantities present. The PE fraction, on the other hand, contained high amounts of 1-*O*-alk-1′-enyl-2-acyl-GPE (63%), smaller amounts of 1,2-diacyl-GPE (34%), and a relatively low quantity of 1-*O*-alkyl-2-acyl-GPE (3%). A high plasmalogen content in ethanolamine phosphoglycerides (66%) has also been found in circulating human PMN (21).

The fatty acyl distribution of PC and PE is shown in Tables 3 and 4, respectively. The major acyl groups found in the PC and PE fractions were 16:0, 18:0, 18:1 and 18:2, which concurs with Elsbach's analysis of rabbit PMN total lipid (22). In the 1,2-diacyl-GPC, the major acyl group in the *sn*-1 position was 16:0 (43%), but there were also significant amounts of 18:1 (26%) and 18:0 (15%). However, the *sn*-1 position of the 1,2-diacyl-GPE contained mainly 18:0 (48%), with smaller amounts of 18:1 (26%) and 16:0 (11%).

The most abundant acyl group in the *sn*-2 position of both 1,2-diacyl-GPC (53%) and -GPE (48%) was 18:2, with lesser amounts of 16:0, 18:1 and other unsaturates. Arachidonate comprised only 3 and 6% of the choline- and ethanolamine-linked fractions, respectively, in the *sn*-2 position.

To determine the ether chain distribution, 1-*O*-alkyl-2-acyl phosphoglycerides were analyzed by GLC as the isopropylidene derivative of 1-*O*-alkylglycerol as already described. Analysis by GLC-MS yielded characteristic ions of M-15 and m/z = 101, both indicative of a glyceryl ether structure (2). In the 1-*O*-alkyl-2-acyl-GPC, the 5 major ether-linked chains were 16:0 (35%), 18:0 (14%), 18:1 (26%), 20:0 (16%) and 22:0 (9%). GLC-MS analysis also showed minute traces (<<1%) of 14:0, 17:0, 19:0 and 18:2. The mass spectra of the 20:0 and 22:0 isopropylidene derivatives are shown in Figure 1; their presence is surprising because the alkyl chains of most tissues analyzed have been restricted to 18 carbons and shorter. However, rat intestinal mucosa has been reported to contain 22% 20:0 in the 1-*O*-alkyl-2-acyl-GPC fraction (19). In the 1-*O*-alkyl-2-acyl-GPE fraction, these 5 major peaks were also found, but in slightly differing proportions. Due to the small amounts of 1-*O*-alkyl-2-acyl-GPE available, only one quantitative analysis of the chain distribution was done.

As was the case with the 1,2-diacyl phosphoglycerides, 18:2 was the predominant fatty acid residue in the *sn*-2 position of 1-*O*-alkyl-2-acyl-GPC and -GPE, accounting for 65 and 51%,

respectively, of the residues at the 2-position. Smaller amounts of 18:1, 16:0 and 18:0 were also found, but there was again very little arachidonate found in the *sn*-2 position, accounting for 1% in 1-*O*-alkyl-2-acyl-GPC and 7% in the 1-*O*-alkyl-2-acyl-GPE.

The vinyl ether chain distribution of 1-*O*-alk-1′-enyl-2-acyl-GPE was determined by GLC as already described. Analysis of the PE plasmalogen showed 14:0 (13%), 16:0 (44%), 18:0

TABLE 4

Fatty Chain Distribution of Ethanolamine-Containing Phosphoglycerides

	1-2-Diacyl (mol %)		1-*O*-Alkyl-2-acyl (mol %)		1-*O*-Alk-1′-enyl-2-acyl (mol %)	
	Position 1 (N = 2)[a]	Position 2 (N = 4)[a]	Position 1 (N = 1)[a,b]	Position 2 (N = 2)[a]	Position 1 (N = 3)[a]	Position 2 (N = 7)[a]
14:0	–	–	–	–	12.8 ± 2.8	–
16:0	10.8 ± 1.0	8.0 ± 3.3	17.0	10.0 ± 1.7	44.3 ± 3.9	1.9 ± 0.8
16:1	1.5 ± 0.8	2.0 ± 1.0	–	–	–	1.0 ± 0.4
18:0	47.8 ± 6.8	6.4 ± 2.6	26.0	7.7 ± 0.0	27.0 ± 3.2	1.1 ± 0.8
18:1	26.1 ± 6.3	21.7 ± 2.6	22.5	19.7 ± 1.5	11.8 ± 3.1	8.5 ± 0.5
18:2	6.0 ± 1.1	47.8 ± 12.2	–	50.9 ± 4.2	2.7 ± 1.2	80.2 ± 2.1
18:3	2.8 ± 1.4	2.2 ± 1.7	–	3.3 ± 3.3	–	1.0 ± 0.4
20:0	–	–	25.0	–	–	–
20:3	2.1 ± 2.1	1.7 ± 0.7	–	–	–	0.5 ± 0.2
20:4	0.3 ± 0.3	6.3 ± 0.8	–	6.7 ± 0.4	–	4.5 ± 1.6
20:5	–	1.2 ± 1.2	–	–	–	0.1 ± 0.1
22:0	–	–	9.5	–	–	–
Other[c]	3.2 ± 2.3	1.3 ± 1.5	–	1.9 ± 1.9	–	1.2 ± 0.9

[a]Each determination represents a separate population of cells. Standard deviations are given.

[b]Based on area % rather than mol %.

[c]Minor chains present were 22:1, 22:4, 22:5, 22:6 and 24:1.

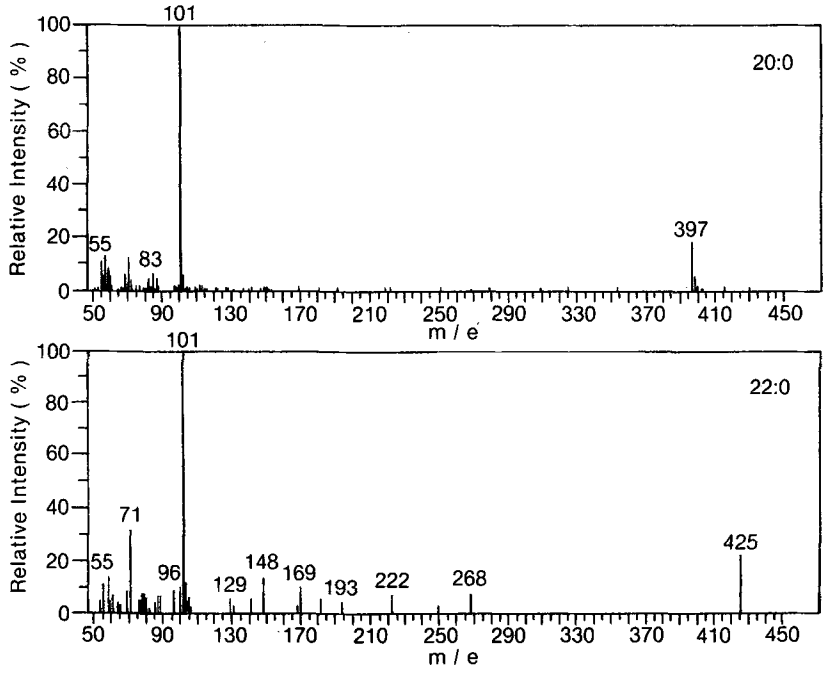


FIG. 1. Mass spectra of the 20:0 and 22:0 1-*O*-alkyl isopropylidene derivatives. Characteristic ions are shown at m/z = 101 and M - 15.

(27%), 18:1 (12%) and 18:2 (3%) as the 5 major vinyl ether chains in the *sn*-1 position. The *sn*-2 position was comprised mainly of 18:2 (80%), 18:1 (9%), and other unsaturates including arachidonate (5%).

This study provides no direct information on the pathway by which PMN synthesize PAF. However, it does show that rabbit neutrophils contain ample precursor to support the synthesis of PAF by a deacylation-reacylation pathway. It will be important to take into account the large amounts of 1-*O*-alkyl-2-acyl-GPC in studies of the synthesis of PAF in neutrophils.

ACKNOWLEDGMENTS

This work was supported by Grants HL-26818, HL-26257, and HL-27799 from the National Heart, Lung and Blood Institute. The authors also thank M. Samuel for technical assistance and G. Charles for help in preparation of this manuscript.

REFERENCES

1. Henson, P.M., and Pinckard, R.N. (1977) Monogr. Allergy 12, 13-26.
2. Hanahan, D.J., Demopoulos, C.A., Liehr, J., and Pinckard, R.N. (1980) J. Biol. Chem. 255, 5514-5516.
3. Mencia-Huerta, J.M., and Benveniste, J. (1979) Eur. J. Immunol. 9, 409-415.
4. Clark, P.O., Hanahan, D.J., and Pinckard, R.N. (1980) Biochim. Biophys. Acta 628, 69-75.
5. Wykle, R.L., Malone, B., and Snyder, F. (1980) J. Biol. Chem. 255, 10256-10260.
6. Renooij, W., and Snyder, F. (1981) Biochim. Biophys. Acta 663, 545-555.
7. Elsbach, P., and Rizack, M.A. (1963) Am. J. Physiol. 205, 1154-1158.
8. Chap, H., Mauco, G., Simon, M., Benveniste, J., and Douste-Blazy, L. (1981) Nature 289, 312-314.
9. Gottfried, E.L. (1972) Sem. Hematol. 9, 241-250.
10. O'Flaherty, J.T., Cousart, S., Lineberger, A.S., Bond, E., Bass, D.A., DeChatelet, L.R., Leake, E.S., and McCall, C.E. (1980) Am. J. Pathol. 101, 79-90.
11. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-918.
12. Rouser, G., Siakotos, A.N., and Fleischer, S. (1966) Lipids 1, 85-86.
13. Waku, K., Ito, H., Bito, T., and Nakazawa, Y. (1974) J. Biochem. 75, 1307-1312.
14. Snyder, F., Blank, M., and Wykle, R.L. (1971) J. Biol. Chem. 246, 3639-3645.
15. Wood, R. (1967) Lipids 2, 199-203.
16. Sugiura, T., Masuzawa, Y., and Waku, K. (1980) Lipids 15, 475-478.
17. Mason, R.J., Stossel, T.P., and Vaughan, M. (1972) J. Clin. Invest. 51, 2399-2407.
18. Broekman, M.J., Ward, J.W., and Marcus, A.J. (1980) J. Clin. Invest. 66, 275-283.
19. Horrocks, L.A. (1972) in Ether Lipids—Chemistry and Biology (Snyder, F., ed.) pp. 177-272, Academic Press, New York, NY.
20. Blank, M.L., Cress, E.A., Piantadosi, C., and Snyder, F. (1974) Biochim. Biophys. Acta 380, 208-218.
21. Gottfried, E.L. (1967) J. Lipid Res. 8, 321-327.
22. Elsbach, P. (1959) J. Exp. Med. 110, 969-980.

[Received August 17, 1981]

In vivo and in vitro Antilipolytic Effects of Some Various Substituted Homocysteine-Thiolactone-Nicotinamides: Structure-Activity Study

F. MACCARI*, O. GHIRARDI, P. DE WITT and **M.T. RAMACCI**, *Research Laboratories, Sigma-Tau S.p.A., Pomezia, Italy*

ABSTRACT

The antilipolytic activity of homocysteine-thiolactone-nicotinamide (ST22) and its 2-chloro (ST71), 6-chloro (ST82) and 6-hydroxy (ST90) derivatives was investigated by evaluation of serum free fatty acids (FFA) and triglycerides (TG) (in vivo) and FFA release from adipose tissue (in vitro). Increased FFA levels in 17-hr fasted rats at 60 min following treatment with $7 \cdot 10^{-4}$ mol kg^{-1} p.o. were reduced by 70% (ST22), 60% (ST82) and 18% (ST71), whereas ST90 provoked no change; TG levels showed similar changes. Basal FFA release from epididymal rat adipose tissue at 60 min following treatment with $7 \cdot 10^{-4}$ mol kg^{-1} p.o. of ST22 and ST82 was reduced by 79 and 45%, respectively. Lipid mobilization induced by noradrenaline (NA) was diversely affected by the compounds according to the tests employed: with in vivo experiments, serum FFA levels were reduced by 60, 70, 10 and 5% at 60 min following treatment with ST22, ST82, ST71 and ST90, respectively ($7 \cdot 10^{-4}$ mol kg^{-1} p.o.; NA bitartrate, 2 mg kg^{-1} s.c.); in vitro, ST22 produced no change, whereas the other compounds induced a significant mobilization of FFA. The results suggest that: (a) antilipolytic activity can be greatly modified when various substituents capable of influencing either the inductive (-I) or the resonance (+M) effect are introduced into the different positions of the pyridine ring; and (b) the lipolysis experiments did not evince any direct relationship between the effects obtained by the in vivo tests and those obtained by the in vitro tests.

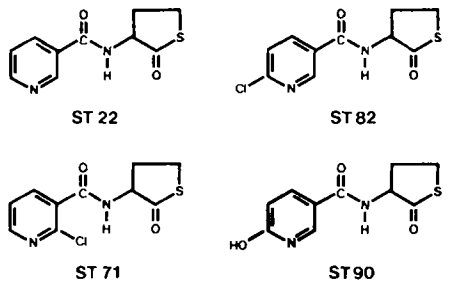
Lipids 17:78-83, 1982.

INTRODUCTION

A preliminary pharmacological investigation on a series of molecules corresponding to the formula homocysteine-thiolactone-nicotinamide that was done to detect new antilipemic agents showed that some of them were endowed with in vivo antilipolytic activity (1). A reduction in the serum free fatty acid (FFA) levels -increased either by fasting or noradrenaline (NA) stimulation in the rat--was found, but no antagonism of FFA mobilization from rat epididymal tissue in vitro occurred (1).

The absence of a relationship between the results obtained with the 2 experimental models was also observed in the case of other compounds, such as 3,5-dimethyl-pyrazole and 5-methyl-pyrazole 3-carboxylic acid, both of which are capable of depressing serum FFA levels in vivo, but only the latter of inhibiting lipid mobilization in vitro (2-4). This was shown to be due to the structure of the molecule and the capability of 3,5-dimethyl-pyrazole to convert itself in vivo to the active form: 5-methyl-pyrazole tricarboxylic acid (5).

The results obtained from the preliminary pharmacological screening of the molecules ST22, ST82, ST71 and ST90, represented by Scheme I,



SCHEME I

suggest that the electronic balance existing between the 2 functional groups of the molecules, aza and carbonyl, respectively, responsible for the pharmacological activity of the compounds, varies in relation to the changes brought about in the nicotinic acid ring.

This prompted us to investigate more closely how the chemical structure affects the response to the commonly used tests for antilipolytic activity.

MATERIALS AND METHODS

Animal Diet and Drugs

Albino Wistar rats from Sigma-Tau breeding stock of both sexes weighing 180-250 g were

TABLE 1

Effect of Some Homocysteine-Thiolactone-Nicotinamides on Lipolysis Induced by 17-hr Fasting in Rats

Compound	Dose (mol/kg/p.o.)	S-FFA (μEq/1)	(%)	(p)	S-TG (mg/100 ml)	(%)	(p)
Control	–	1119 ± 69	–	–	93.4 ± 9.2	–	–
ST22	$7 \cdot 10^{-4}$	335 ± 37	30	*	35.5 ± 3.8	38	*
ST82	$7 \cdot 10^{-4}$	447 ± 28	40	*	58.6 ± 4.1	62	**
ST71	$7 \cdot 10^{-4}$	917 ± 122	82	NS	99.9 ± 7.5	107	NS
ST90	$7 \cdot 10^{-4}$	1286 ± 125	115	NS	90.5 ± 10.7	97	NS

Serum free fatty acid and triglyceride average values ± SEM at 60 min after administration.

Student's t-test: *$p<0.001$ and **$p<0.01$, respectively, significant difference vs control; 10 rats/group.

used. The rats were fed ad libitum with MT Altromin-Rieper diet (Vandoies, Bolzano, Italy) with free access to water and kept in makrolon cages (42.5 x 26 x 15 cm) in groups of 5, at 23-25 C with 60% relative humidity, in a light-controlled room (12-hr light, 6 a.m.-6 p.m., and 12-hr dark, 6 p.m.-6 a.m.).

The compounds under investigation, synthetized at Sigma-Tau chemical laboratories, were dissolved in aqeuous solution and given by gavage in the in vivio experiments, whereas for the in vitro experiments, they were added directly to the medium.

The aqueous solvent was used as the placebo for both experimental models. Noradrenaline bitartrate (Merck) dissolved in water was subcutaneously injected into the animals (2 mg/kg) in the in vivo experiments, and added (0.15 mcg/ml) to the medium for the in vitro experiments.

Experiments

In lipolysis induced by fasting, the animals of both sexes were deprived of food for 17 hr with free access to water and then treated either with a single oral dose ($7 \cdot 10^{-4}$ mol/kg) of each compound or with placebo. FFA and serum triglyceride (TG) levels were determined

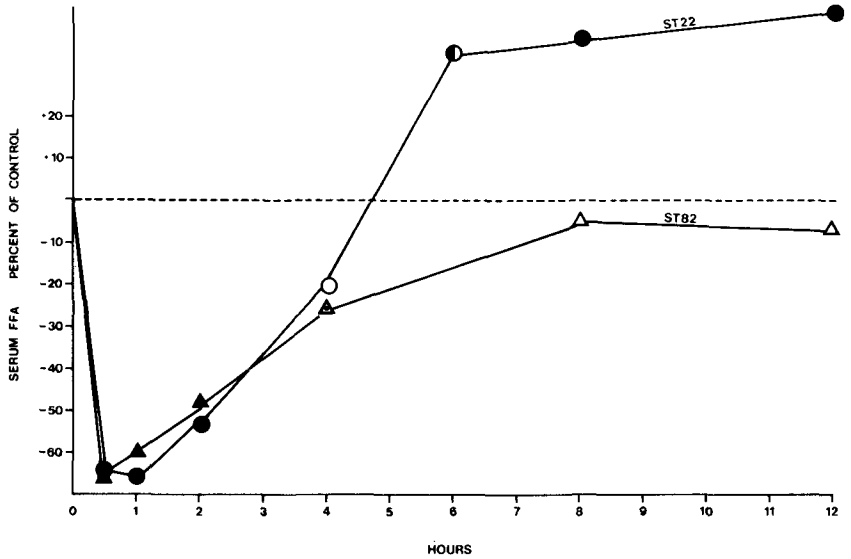


FIG. 1. Effect of ST22 (o—o $7 \cdot 10^{-4}$ mol/kg) and ST82 (Δ—Δ $7 \cdot 10^{-4}$ mol/kg) p.o. on lipid mobilization in 17-hr fasted rats. Mean value of serum FFA in percentage of controls (1119±69 μEq/1) at different intervals after administration. Student's t-test vs control: empty, dotted, half-filled and filled symbols indicate nonsignificant difference, $p<0.05$, <0.01 and <0.001, respectively; 10 rats/group.

at various intervals following administration.

In lipolysis induced by noradrenaline, freely fed animals received the single oral dose ($7 \cdot 10^{-4}$ mol/kg) of each compound or placebo and, 30 min later, they received noradrenaline bitartrate subcutaneously (2 mg/kg). FFA serum levels were determined at 30 min following the noradrenaline injection.

In order to evaluate the effect of the compounds on the basal release of FFA from adipose tissue, freely fed male animals were given a single oral dose ($7 \cdot 10^{-4}$ mol/kg) of each compound and, at different time intervals, they were killed and the epididymal adipose tissue rapidly removed, minced, pooled, distributed (200 mg ± 10) and incubated in flasks containing 5 ml of Krebs phosphate buffer with 3% bovine albumin (Fraction V Pentex) according to the methods described by Bizzi et al. (6,7).

Regarding the study of the effects produced by the compounds of the NA-induced FFA release from adipose tissue, epididymal adipose tissue removed from normally fed rats was homogenized and incubated for 30 min in the same medium as already described with the addition of NA (0.15 mcg/ml) and the compounds under investigation ($8 \cdot 10^{-4}$ M).

FFA levels were determined after an additional 60 min of incubation. Van Handel and Zilversmith's method (8) was employed for determining triglyceride and Dole's method (9) as modified by Trout et al. (10) for FFA.

RESULTS

Increased FFA levels in the serum of 17-hr fasted animals were significantly reduced 60 min after oral administration of ST22 and ST82, whereas no changes were caused by ST71 and ST90 (Table 1).

A similar effect was obtained in regard to serum triglycerides (Table 1). The capability of both ST22 and ST81 to reduce FFA levels in fasted rats lasted up to the 4th hr following oral administration with maximal activity at the 30th min.

However, the 2 compounds showed different behavior in affecting FFA levels: a rebound effect was significantly present at the 6th and 12th hr after ST22 treatment; the maximal increase caused by ST82 at 8-12 hr following administration did not exceed basal values (Fig. 1). The effect elicited by both compounds on serum TG levels was different in intensity and duration as one oral administration of ST22 determined a more marked and significant effect up to the 4th hr, whereas when ST82 was given, a less marked effect which was nevertheless highly significant beyond the 8th hr, was observed (Fig. 2).

The lipid mobilization induced by NA in vivo was antagonized by orally administering ST22 and ST81 which reduced serum FFA levels by 60 and 70%, respectively. Also in this case, ST71 and ST90 had no activity (Fig. 3).

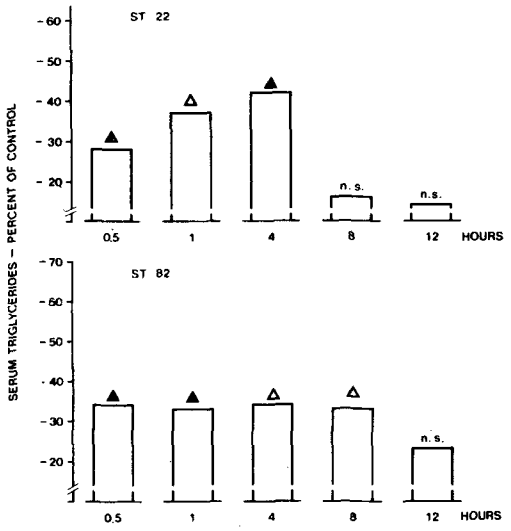


FIG. 2. Effect of ST22 ($7 \cdot 10^{-4}$ mol/kg p.o.) and ST82 ($7 \cdot 10^{-4}$ mol/kg p.o.) on 17-hr fasting-induced lipolysis in rats. Mean values of serum triglycerides in percentage of control (78.2±3.6 mg/100 ml) at different time intervals after administration. Student's t-test *vs control*; NS, dotted, half-filled and filled symbols indicate nonsignificant difference, $p<0.05$, <0.01 and <0.001, respectively; 10 rats/group.

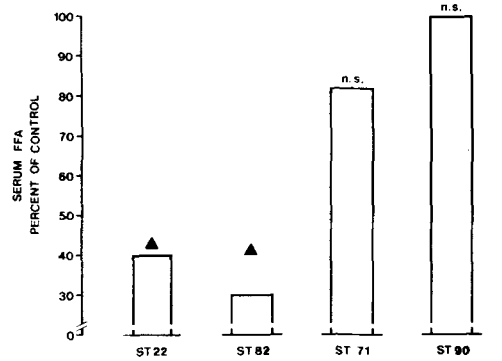


FIG. 3. Effect of some homocysteine-thiolactone-nicotinamides ($7 \cdot 10^{-4}$ mol/kg p.o.) on FFA mobilization stimulated by NA bitartrate (2 mg kg^{-1} s.c.) in rat. Mean of values serum FFA in percentage of control (normal = 428.74±27, 45 μEq/1; control = 973.72 ±34.26 μEq/1) at 30 min after NA and at 60 min after compound administration. Student's t-test vs control; NS and filled symbols indicate nonsignificant difference and $p<0.001$, respectively; 10 rats/group.

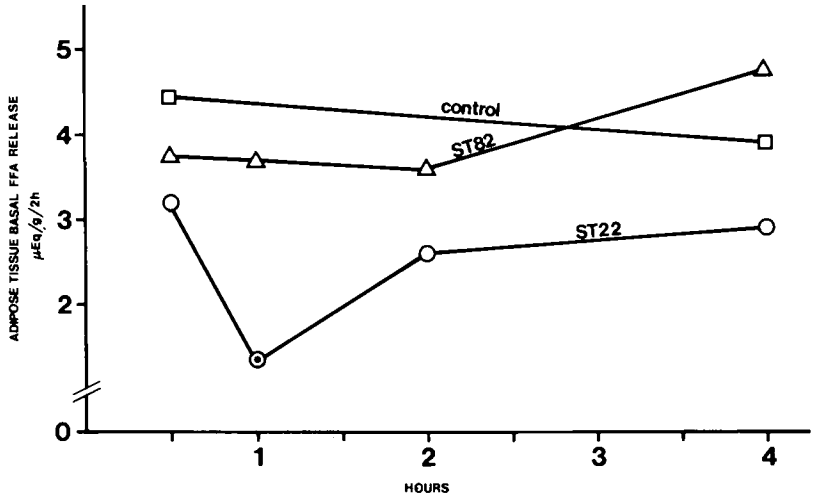


FIG. 4. Basal FFA release from epididymal adipose tissue in normally fed rats untreated (□—□) or treated with ST22 (○—○ $7 \cdot 10^{-4}$ mol/kg p.o.) and ST82 (△—△ $7 \cdot 10^{-4}$ mol/kg p.o.). Mean value of FFA, μEq/g/2 hr, released at different time after administration. Student's t-test vs control: empty and dotted symbols indicate nonsignificant difference and $p \leq 0.05$, respectively; 8 rats/group.

The in vitro FFA release from epididymal adipose tissue removed from pretreated animals was diversely affected by the compounds. ST22 at 1 hr caused a sharp reduction in FFA release values which showed a progressive increase from the 2nd through the 4th hr; ST82 did not produce any significant change, although a slight initial reduction followed by an augmentation of the same magnitude at the 4th hr was apparent (Fig. 4). Also in this case, ST71 and ST90 had no effect.

NA-induced FFA release from adipose tissue removed from untreated animals was unaffected by the presence of ST22 in the medium, but was significantly increased by ST82, ST71 and ST90 (Fig. 5). ST82 proved to be the most active and the intensity of effect was dose-related (Fig. 6).

DISCUSSION

The investigation of antilipolytic drugs is still of considerable interest due to the close correlation between the presence of impaired lipid metabolism and some high-risk diseases, e.g., myocardial infarction, atherosclerosis and diabetes. Because of this, new drugs with the capability of affecting lipid metabolism without causing hazardous side effects are of particular interest.

The results of our research summarized in Table 2 suggest several considerations. One of these concerns the methods used for evaluating antilipolytic activity which showed that there is not always a direct relationship between the results obtained by in vitro and in vivo experiments. Both ST22 and ST82 inhibited lipolysis in vivo, but in vitro, the ST22 showed no effect whereas the ST82 augmented the effects of the lipolytic process inducer. This property was also shared by ST71 and ST90 which had no

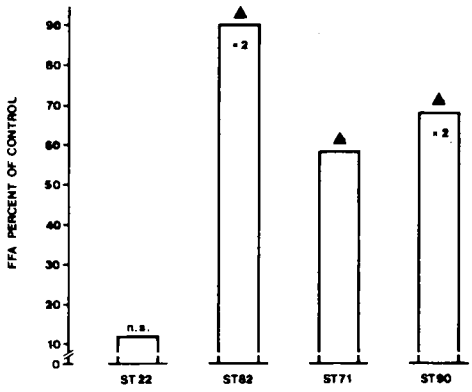


FIG. 5. Effect of some homocysteine-thiolactone-nicotinamides ($8 \cdot 10^{-4}$ M) on FFA release (μEq/g/hr) from rat epididymal adipose tissue stimulated by NA (0.15 mcg/ml of incubation medium). Mean value of FFA release in percentage of control. Student's t-test vs control: NS and filled symbols indicate nonsignificant difference and $p<0.001$, respectively; 6 rats/group. X2 = the corresponding value on the y axis is to be multiplied by 2.

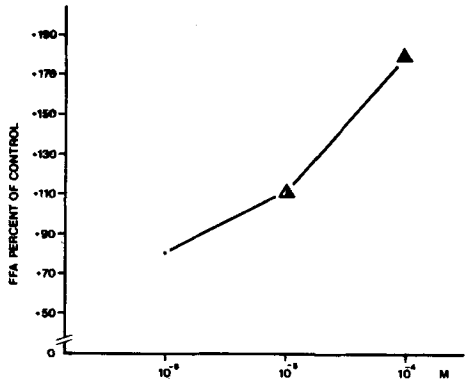
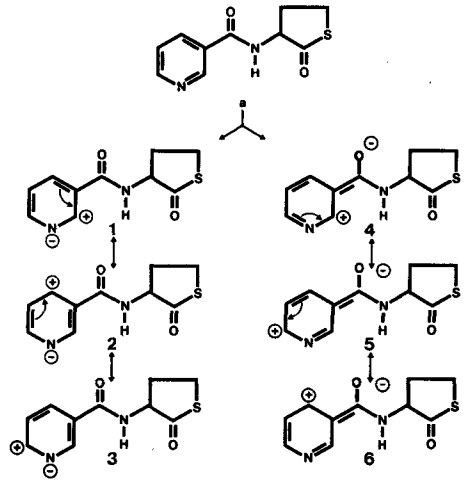


FIG. 6. Effect of ST82 at 3 dose levels (10^{-6}, 10^{-5}, 10^{-4} M) on NA (0.15 mcg/ml incubation medium) stimulating FFA release (μEq/g/hr) from rat epididymal adipose tissue. Mean value of FFA in percentage of control: half-filled and filled symbols indicate $p<0.01$ and <0.001, respectively; 6 rats/group.

in vivo activity.

The cause of these differences could be ascribed to a difference in experimental conditions, and the difficulty in an accurate evaluation of the multiple mechanisms which are responsible for the in vivo lipolytic process can only provide a partial, and not always easily interpreted, indication in regard to metabolic activities exhibited by these compounds. The in vitro study of the 4 molecules under investigation did not elicit antilipolytic activity which was present in vivo with elevated intensity exclusively for ST22 and ST82.

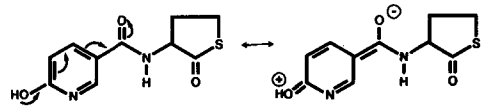
Considering the structural properties of these compounds, in ST22, the relative positions of the aza group and of the -C=O group could allow variation in the resonance structure identified in Scheme II. These electronic defects appear to occur in positions 2, 4 and 6.



SCHEME II

The introduction of a substituent in the foregoing positions could affect the charge distribution only when the electronic nature of the substituent significantly interacts with the basic molecule nucleus. In particular, in 2-chloro derivative (ST71), the remarkable -I effect of the chloride atom deeply influences the charge distribution of that part of the molecule between the aza group and the carbonyl group, whereas in the 6-chloro derivative (ST82), the remarkable -I effect and the mild +M effect of the alogen atom are insufficient to perturb the above charge distribution. A slight alteration in the resonance structures 3 and 5 occurs in the ST82.

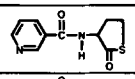
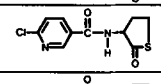
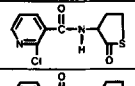
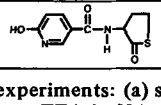
Conversely, the 6-hydroxy derivative (ST90), owing to the remarkable +M effect of the hydroxyl group, shows an electronic distribution of that part of the molecule. This is hypothesized to be structurally related to antilipolytic activity, and a loss of activity was observed (Scheme III).



SCHEME III

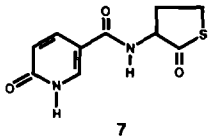
TABLE 2

Summary of the Effect of Some Variously Substituted Homocysteine-Thiolactone-Nicotinamides on Lipid Metabolism in the Rat

compound	structure	lipolysis			
		fasting[a]	in vivo NA[b]	basal[c]	in vitro NA[d]
ST22		↓↓	↓↓	↓	—
ST82		↓↓	↓↓	—	↑↑
ST 71		—	—	○	↑
ST 90		—	—	○	↑↑

In vivo experiments: (a) serum FFA in 17-hr fasted rats; (b) serum FFA in NA-treated (2 mg/kg s.c.) rats; (c) FFA release from epididymal adipose tissue of rats treated with the compounds; (d) in vitro experiments: release from the adipose tissue of untreated animals incubated with NA 0.15 mcg/ml and the compounds.
↑ = Enhancement.
↓ = Inhibition.
— = No effect.
○ = Untested.

Moreover, one should not overlook the possibility that a 7-ketone tautomeric form may exist. This could cause an electronic distribution other than the one for ST22 in the pyridine ring (Scheme IV).



7

SCHEME IV

ACKNOWLEDGMENTS

Technical assistance was provided by M.R. Pascale and R. Taurelli; English translation of the text was by I. Bartlett.

REFERENCES

1. Ramacci, M.T., Maccari, F., and Ghirardi, O., G.I.R.F.-S.I.S.F. Symposium, Rome, Oct. 2-4, 1975.
2. Gerritsen, G.C., and Dulin, W.E. (1965) Diabetes 14, 507-515.
3. Gerritsen, G.C., and Dulin, W.E. (1965) J. Pharmacol. Exp. Ther. 150, 491-498.
4. Gerritsen, G.C., Dulin, W.E., and Kupiecki, F.P. (1969) in Drugs Affecting Lipid Metabolism Holmes, W.L., Carlson, L.A., and Paoletti, R., eds.) pp. 93-103, Plenum Press, New York, NY.
5. Smith, D.L., Forist, A.A., and Dulin, W.E. (1965) J. Med. Chem. 8, 350-353.
6. Bizzi, A., Codegoni, A.M., and Garattini, S. (1967) Il Farmaco, Ed. Sci. 22, 709-716.
7. Bizzi, A., Codegoni, A.M., and Garrattini, S. (1969) Biochem. Pharmacol. 18, 2053-2060.
8. Van Handel, E., and Zilversmith, D.B. (1957) J. Lab. Clin. Med. 50, 152-157.
9. Dole, V.P. (1956) J. Clin. Invest. 35, 150-154.
10. Trout, D.L., Estes, E.H., and Friedberg, S.J. (1960) J. Lipid Res. 1, 199-202.

[Received April 8, 1981]

Hydroperoxides Formed by Ferrous Ion-Catalyzed Oxidation of Methyl Linolenate

I. TOYODA, J. TERAO and S. MATSUSHITA*, *Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan*

ABSTRACT

An emulsion of methyl linolenate was allowed to oxidize with a catalyst of ferrous sulfate and ascorbic acid. Three oxidation products were isolated, and their hydrogenated derivatives were characterized as the isomeric mixture of methyl monohydroxyoctadecanoate (monoOH), methyl 9,16-dihydroxyoctadecanoate (diOH), and the isomeric mixture of methyl trihydroxyoctadecanoate (triOH). The monoOH isomers and diOH apparently were derived from methyl monohydroperoxyoctadecatrienoate (monoHPO) and methyl dihydroperoxyoctadecatrienoate (diHPO), respectively. Two triOH isomers (the 9,10,12- and 13,15,16-isomers) were thought to be derived from the products containing cyclic peroxide-hydroperoxide structure. 9,16-diHPO was produced by the incubation of monoHPO with ferrous sulfate and ascorbic acid. Moreover, the experiment using $^{18}O_2$ demonstrated that monoHPO yielded 9,16-diHPO by reacting with oxygen molecule. 9,10,13- and/or 9,12,13- and 12,13,16- and/or 12,15,16-triOH isomers were also detected in the hydrogenated derivatives of oxidation products from monoHPO.

Lipids 17:84-90, 1982.

ABBREVIATIONS

MonoHPO (methyl monohydroperoxyoctadecatrienoate); diHPO (methyl dihydroperoxyoctadecatrienoate); HPLC (high performance liquid chromatography); GLC (gas liquid chromatography); GC-MS (gas chromatography-mass spectrometry); TLC (thin layer chromatography); TMS (trimethylsilyl); TBDMS (*tert*-butyldimethylsilyl); monoOH (methyl monohydroxyoctadecanoate); diOH (methyl dihydroxyoctadecanoate); triOH (methyl trihydroxyoctadecanoate).

INTRODUCTION

It has been generally accepted that metal ions and metallo-proteins catalyze lipid peroxidation, resulting in the deterioration of edible oils and oil-containing foods (1). Hemoglobin and other iron porphyrins are the major catalysts of lipid oxidation in meat and meat products (2-4). Non-heme iron (5,6) and inorganic ferrous ions (7) have also been shown to catalyze lipid oxidation in foods.

Chan and Newby (8) reported on the isomeric composition of monohydroperoxides formed by the oxidation of linoleic acid and its ester catalyzed by hemoproteins and transition metal ions using HPLC. Products formed by hemoglobin-catalyzed decomposition of linoleic acid monohydroperoxides were also studied by Hamberg (9). Furthermore, Gardner et al. (10,11) identified various nonvolatile products formed by the decomposition of linoleic acid monohydroperoxides with Fe(III)-cysteine and suggested that alkoxy radicals have a part in the homolytic decomposition. However, the mechanisms of oxidation and decomposition of linolenic acid oxidation products have not been completely clarified because of the complexity of the reactions.

In this paper, products formed by Fe(II)-catalyzed oxidation of methyl linolenate were isolated and characterized by GC-MS. GC-MS analysis was further applied to investigate secondary oxidation products formed from monoHPO. The oxidation pathway of methyl linolenate during ferrous ion catalysis is also discussed.

EXPERIMENTAL PROCEDURES

Materials

Methyl linolenate (99% grade) was purchased from Nakarai Chem. Co., Kyoto, Japan. Before use, methyl linolenate was purified by column chromatography with Florisil (100/200 mesh) to exclude any peroxides (12). $H_2{}^{18}O$ (99.75%) and $^{18}O_2$ (99%) were purchased from Commissariat a L'Energie Atomique, France.

Preparation of monoHPO

Methyl linolenate was autoxidized at room temperature with bubbling air for 2 days. MonoHPO were separated from autoxidized methyl linolenate by silica gel chromatography (13) and then purified by preparative TLC (14). The 9-isomer and 13-isomer of monoHPO were prepared from monoHPO mixture by HPLC with repeated injection (15).

Oxidation Procedure

Emulsion of methyl linolenate or monoHPO

were prepared as follows: 2.0 ml of 0.1 M phosphate buffer, pH 6.2, containing 0.1% Tween 20, EDTA (10^{-5} M), and methyl linolenate or monoHPO (7.8×10^{-3} M) were thoroughly emulsified with an ultrasonic vibrator (28 kHz) for 1 min or 10 sec, respectively. The reaction was initiated by addition of the catalysts (50 μl aqueous solution of $FeSO_4$ and 100 μl aqueous solution of ascorbic acid [final conc, $FeSO_4$ = 10^{-5} M, ascorbic acid = 2×10^{-3} M]). Incubation was done at 25 C with continuous shaking.

Isolation of Oxidation Products

The reaction mixture was extracted with chloroform/methanol (1:1, v/v). The extracts were concentrated in vacuo, and then applied to Silica Gel G (Merck, Silica Gel PF-254)-coated TLC plate (0.5 mm thick) in a solvent for development, *n*-hexane/diethyl ether/acetic acid (60:90:1, v/v/v). The bands of the products were detected under UV light and were scraped off, and then extracted with chloroform. For clarifying the TLC pattern of oxidation products, a Shimadzu double wavelength TLC scanner CS-910 was used for monitoring the plate.

Incubation of monoHPO with $H_2{}^{18}O$

Tween 20 (final conc, 0.1%), 0.5 μl of EDTA aqueous solution (final conc, 10^{-5} M)

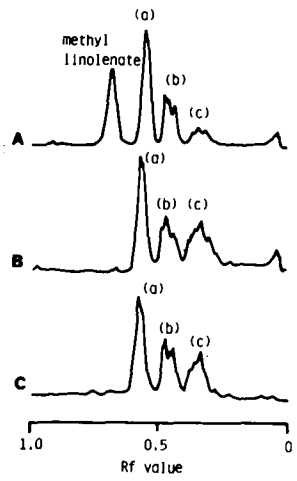


FIG. 1. TLC patterns of oxidized methyl linolenate. Methyl linolenate after incubation with Fe(II)-ascorbic acid for 4 hr was spotted on a TLC plate. Development was performed with *n*-hexane/diethyl ether/acetic acid (60:90:1, v/v/v). After it was sprayed with 50% H_2SO_4 in saturated K_2CrO_7 solution (A) or 5% KI in ethylcellosolve solution (B), the plate was heated for 10 min prior to monitoring at 350 nm. Chromatogram of (C) was obtained by monitoring at 235 nm without spraying any solution.

and 1 mg of monoHPO were added to 300 μl of $H_2{}^{18}O$ and emulsified in a sonicating bath (Bransonic 42, 98-V) for 3 min. The emulsion was incubated with addition of $FeSO_4$ (10^{-5} M) and ascorbic acid (2×10^{-3} M) for 7 hr at 25 C.

Incubation of monoHPO with $^{18}O_2$

An emulsion of monoHPO (2.0 ml, 7.8×10^{-3} M) containing 0.1% of Tween 20 and EDTA (10^{-5} M) was placed in a reaction vessel with an adjunct cell containing a solution of 150 μl aqueous solution of $FeSO_4$ (10^{-5} M) and ascorbic acid (2×10^{-3} M), which was then sealed. After the solutions were evacuated and headspace was replaced by argon gas, the vessel was evacuated, followed by saturation with $^{18}O_2$. Incubation was started by mixing the emulsion with a solution of $FeSO_4$ and ascorbic acid. The reaction mixture was extracted after a 3-hr incubation.

Derivatization

Reduction by $NaBH_4$ and hydrogenation with palladium on carbon were done in the same manner as described previously (16). The procedures of trimethylsilylation and *tert*-butyldimethylsilylation were the same as those described in the previous paper (17).

Gas Liquid Chromatography

After the derivatization, the reaction products were analyzed by GLC with a Shimadzu GC-7A apparatus equipped with a glass column packed with 2% OV-1 on Neopack 2A, 60/80 mesh. The quantities were calculated by using methyl heptadecanoate as internal standard.

Gas Chromatography-Mass Spectrometry

A PAC 300 system consisting of an LKB-9000S gas chromatograph-mass spectrometer with OKITAC minicomputer were used with a glass column packed with 2% OV-1 on Neopack 2A. The column temperature was set at 200-260 C (3 C/min). The conditions for operating the mass spectrometer were described previously (17). The selected ion retrieval was performed from scans obtained at 5-sec intervals. The isomeric composition of monoHPO was calculated by a computer summation of the peak areas of fragment ions which were due to the α-cleavage of the trimethylsilyloxy group of each positional isomer of monoOH formed by the reduction and hydrogenation of monoHPO (18).

RESULTS

Characterization of Oxidation Products

Figure 1 shows the densitometric pattern of

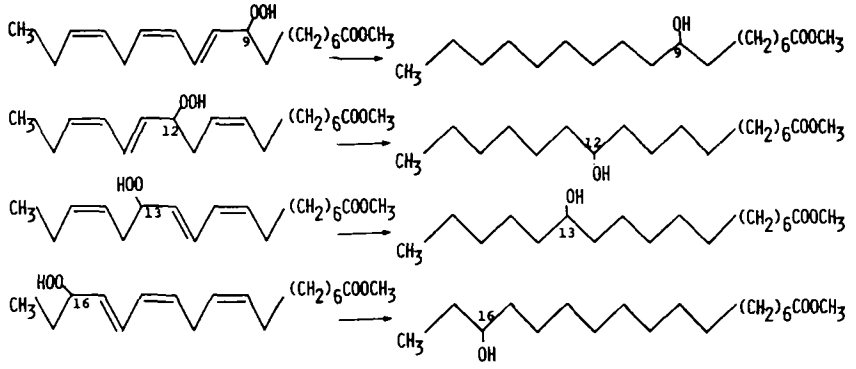
oxidized methyl linolenate. Three spots, a, b and c, were positive to the peroxide-detecting reagent, i.e., a solution of potassium iodide.

The UV spectra of fractions a and b gave the λ_{max}^{EtOH} at 234.5 and 235.5 nm, respectively, due to conjugated diene. The spectrum of fraction c showed the absorption peaks due to conjugated triene (λ_{max}^{EtOH} = 259.5, 269 and 280 nm). The 3 fractions had almost the same infrared (IR) spectra in which strong absorption appeared at the vicinity of 3,400 cm^{-1} due to a hydroperoxy group.

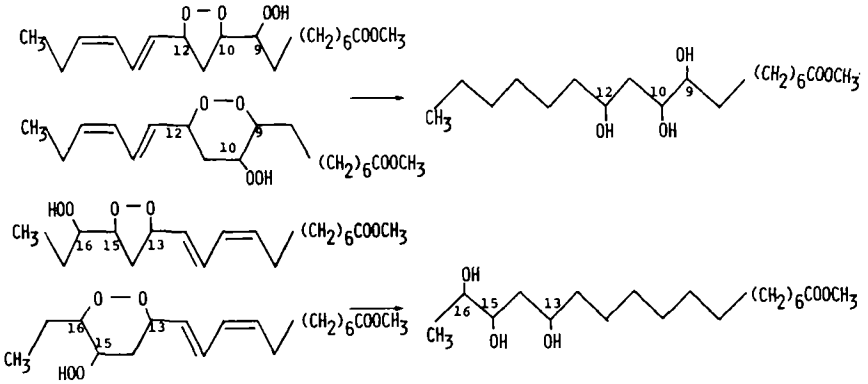
The TMS derivative of hydrogenated fraction a was separated into 2 peaks on a chromatogram, and their mass spectra corresponded to those of the isomeric mixture of monoOH and the relative proportions of the monoOH isomers (9-isomer, 36.5%; 12-isomer, 10.0%; 13-isomer, 13.1%; 16-isomer, 40.4%) almost agreed with those obtained from the autoxidation mixture of methyl linolenate reported by Frankel et al. (19,20). Therefore, fraction a was identified as an isomeric mixture of monoHPO (Scheme I).

The TMS derivative of hydrogenated fraction b was also separated into 2 peaks on the gas chromatogram and the fragmentation patterns in their mass spectra corresponded to those of methyl 9,10,12-trihydroxyoctadecanoate (9,10,12-triOH) and methyl 13,15,16-trihydroxyoctadecanoate (13,15,16-triOH) (20). Begemann et al. (21) previously reported that the 9,10,12 and 13,15,16-triOH can be produced by reduction and hydrogenation of 6- or 5-membered cyclic peroxide-hydroperoxides. Thus, it is apparent that fraction b (Scheme II) contains the isomeric mixture of cyclic peroxide-hydroperoxide structures (20).

Fraction c gave only 1 peak on the chromatogram. Figure 2 shows the mass spectra of TMS and *tert*-butyldimethylsilyl (TBDMS) derivatives of the hydrogenated fraction c. Although the molecular ion [M^+] did not appear, characteristic ions for the TMS derivative of methyl dihydroxyoctadecanoate (diOH) were present at m/z 459 [M-15, loss of CH_3] (Fig. 2-A). For the TBDMS derivative (Fig. 2-B), fragment ions were present at m/z 543 [M-15], 501 [M-57, loss of $C(CH_3)_3$], 369 [501-132, loss of *tert*-



SCHEME I. Structure and reactions of fraction a.



SCHEME II. Structure and reactions of fraction b.

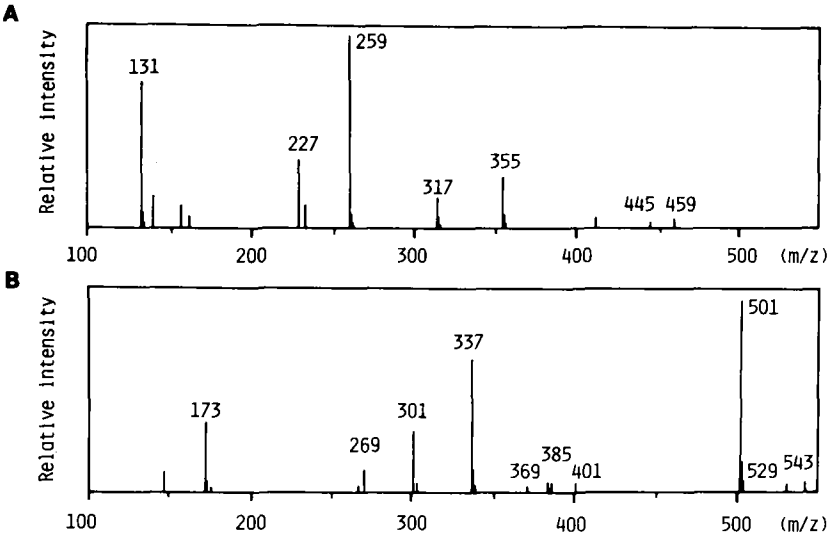
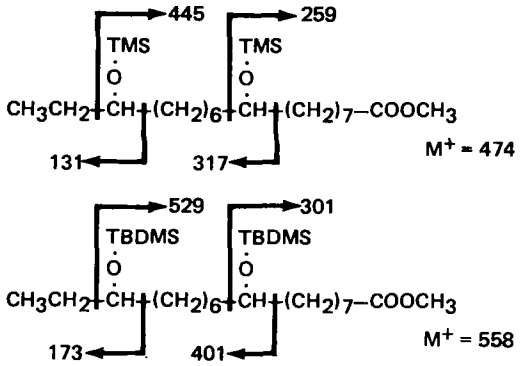


FIG. 2. Mass spectra of hydrogenated fraction c. (A) TMS derivative; (B) TBDMS derivative.

butyldimethylsilanol].

Fragment ions due to α-cleavage of trimethylsilyloxy and *tert*-butyldimethylsilyloxy group were represented in the formula:

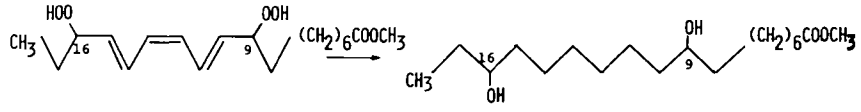


Fragment ions, m/z 355, 227, were formed by the loss of trimethylsilanol from m/z 445 and 317, respectively. Other α-cleavage ions such as m/z 187 or 315 did not appear in the spectra of TMS derivative. Thus, 2 hydroxy groups were apparently located at C-9 and C-16 positions. From the spectral data just described, fraction c (Scheme III) was tentatively identified as methyl 9,16-dihydroperoxyoctadecatrienoate (diHPO).

Nonvolatile Products Formed by the Reaction of monoHPO with Fe(II)-Ascorbic Acid

The reduction, hydrogenation and trimethylsilylation of the reaction mixture after incubation of methyl linolenate or monoHPO with Fe(II) and ascorbic acid produced a gas chromatogram, a typical example of which is shown in Figure 3. Five major peaks observed in the chromatogram for methyl linolenate (Fig. 3-A) were attributed to the derivatives of oxidation products, monoOH, diOH and triOH. On the other hand, the reaction products of monoHPO gave one prominent peak of 9,16-diOH, minor peak of 13,15,16-triOH and no peak of 9,10,12-triOH. The other peaks (peak 6 and 7) were identified as 9,10,13- and/or 9,12,13-triOH, and 12,13,16- and/or 12,15,16-triOH by their mass spectra described next.

Fragmentations characteristic for the TMS derivative of triOH were present at m/z (rel. intensity), 457 [M-(90+15)] (5), and 441 [M-(90+31), loss of trimethylsilanol and OCH_3] (6). Fragment ions due to α-cleavage of trimethylsilyloxy group appeared at 389 (5), 299 [389-



SCHEME III. Structure and reactions of fraction c.

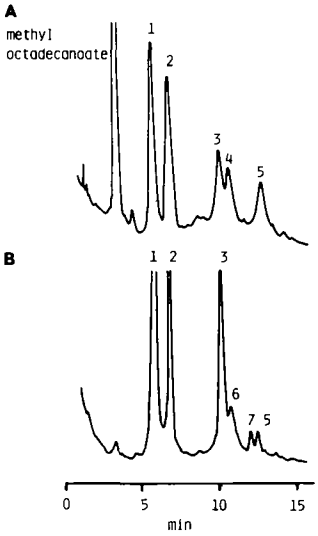
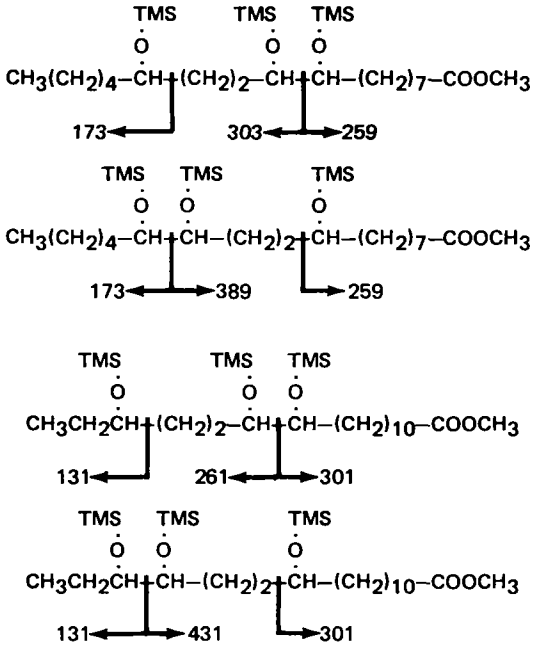


FIG. 3. Gas chromatograms of the reaction mixture of methyl linolenate (A) and monoHPO (B). Incubation of methyl linolenate or monoHPO with Fe(II)-ascorbic acid was done at 25 C for 4 hr (A) or 2 hr (B). Peaks were identified as: 1, a mixture of 9-, 12-, and 13-monoOH; 2, 16-monoOH; 3, 9,16-diOH; 4, 9,10,12-triOH; 5, 13,15,16-triOH; 6, 9,10,13- and/or 9,12,13-triOH; 7, 12,13,16- and/or 12,15,16-triOH.

90] (100), 259 (49), 213 [303-90] (70), and 173 (55) for peak 6, and 431 (3), 341 [431-90] (58), 301 (50), 261 (26), 171 [261-90] (100), 131 (41) for peak 7.



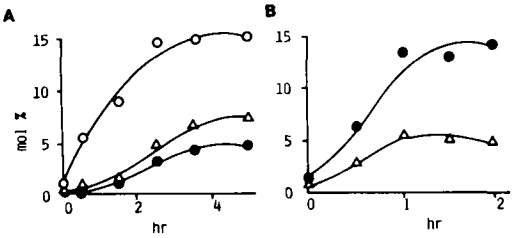


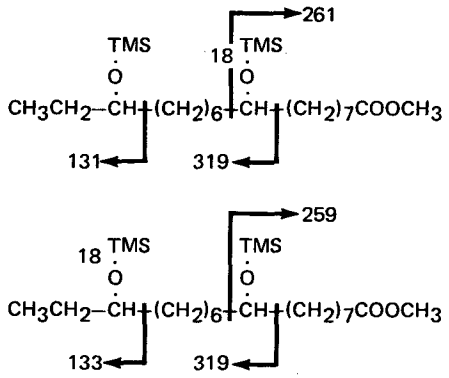
FIG. 4. Changes in the amounts of the reaction products of methyl linolenate (A) or monoHPO (B). The amounts of each product was determined by the peak area in the chromatogram as shown in Fig. 3. ○: Isomeric mixture of monoOH; ●: 9,16-diOH; △: isomeric mixture of triOH.

Figure 4 shows the changes in the amounts of 9,16-diOH and the isomeric mixture of triOH during the incubation of methyl linolenate (A) or monoHPO (B) with Fe(II) and ascorbic acid. In the reaction of methyl linolenate, about 15 mol % of monoOH was detected by GC after a 3-hr incubation. The amounts of 9,16-diOH and triOH isomers also increased with time in the reaction of both methyl linolenate and monoHPO. Although the total amounts of triOH isomers accumulated during the incubation of monoHPO was significantly smaller than that of 9,16-diOH, the reverse was true throughout the incubation of methyl linolenate. Isomeric mixtures of monoHPO after 4 hr of incubation produced 9,16-diOH (17.8%), 9,10,12-triOH (0.5%), 13,15,16-triOH (1.0%), 9,10,13- and/or 9,12,13-triOH (5.3%), 12,13,16- and/or 12,15,16-triOH (0.8%). On the other hand, 9-monoHPO isomer produced 9,16-diOH (15.7%) and 9,10,13- and/or 9,12,13-triOH (4.3%), and 13-monoHPO isomer produced 9,16-isomer (1.3%), 13,15,16-triOH (4.0%) and 9,10,13- and/or 9,12,13-triOH (5.4%).

Origin of Oxygen in the Reaction Products of monoHPO

Two experiments were done to determine the origin of oxygen in the reaction products of monoHPO: (I) incubation of monoHPO with Fe(II)-ascorbic acid in $H_2{}^{18}O$ and air; (II) the same incubation in normal H_2O and $^{18}O_2$. Mass chromatograms of 9,16-diOH and 9,10,13- and/or 9,12,13-triOH produced from the 2 experimental conditions were obtained by monitoring the principal α-cleavage ions of their TMS derivatives (Fig. 5). When the emulsion of monoHPO was incubated in $^{18}O_2$, unique fragment ions appeared at m/z 133, 229 [319-90], and 261 for 9,16-diOH (A) accompanied by the principal α-cleavage ions specific to the TMS

derivatives of 9,16-diOH, m/z 131, 227 and 259.



In the case of 9,10,13- and/or 9,12,13-triOH (B), unique fragment ions also appeared at m/z 175, 215 and 301 [391-90]. These fragmentations indicate that the TMS derivatives of diOH and triOH contain $(CH_3)_3Si^{18}O$ groups (22). In contrast, these unique fragment ions were hardly detected at all when monoHPO was incubated in the emulsion of $H_2{}^{18}O$ (Fig. 5-I). It is therefore concluded that the diOH and triOH were derived by incorporation of oxygen into monoHPO.

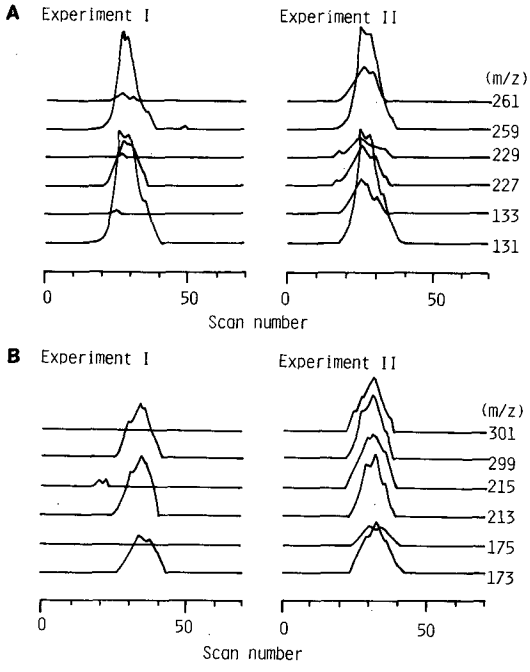


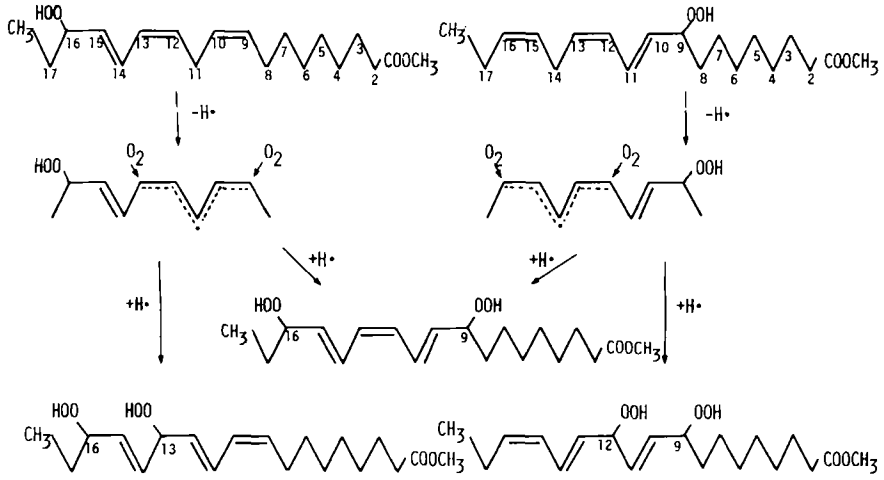
FIG. 5. Mass chromatograms of 9,16-diOH (A) and 9,10,13- and/or 9,12,13-triOH (B) obtained from the reaction mixture of monoHPO. I: Incubation with $H_2{}^{18}O$; II: incubation with $^{18}O_2$.

DISCUSSION

Free radical oxidation of methyl linolenate produces an isomeric mixture of monoHPO (9-, 12-,13- and 16-isomers) (19,20). Moreover, 9,10,12- and 13,15,16-triOH were previously detected in the more polar products in oxidized methyl linolenate and were assumed to be derived from cyclic peroxide-hydroperoxides (21). Frankel et al. (20) suggested that cyclic peroxide-hydroperoxides are produced by cyclization of 12- and 13-monoHPO isomers. Pryor et al. (23) also indicated that cyclization of 12- and 13-peroxy radicals would produce hydroperoxides containing 5-membered cyclic peroxides or prostaglandin-like endoperoxides. However, the results obtained from incubation of the isomeric mixture of monoHPO with inorganic iron (Figs. 3 and 4) suggest that cyclic peroxide-hydroperoxides hardly accumulate during decomposition of monoHPO, compared to the oxidation of methyl linolenate. Relatively low proportions of 12- and 13-isomers of monoHPO may affect the yields of 9,10,12- and 13,15,16-triOH in the products of monoHPO.

On the other hand, 9,10,13- and/or 9,12,13-, and 12,13,16- and/or 12,15,16-triOH were present in the hydrogenated derivatives of oxidation products from monoHPO. Formation of the latter triOH isomers cannot be explained by the cyclization of 12- and 13-monoHPO. It seems reasonable that an alternative pathway other than cyclization of 12- and 13-monoHPO participates in the formation of cyclic peroxide-hydroperoxides from monoHPO, e.g., cyclization of 9-monoHPO or 16-monoHPO followed by hydroperoxidation.

Formation of diHPO is a characteristic for secondary oxidation of monoHPO. The results obtained from incubation of monoHPO with $^{18}O_2$ demonstrated that hydroperoxidation of monoHPO leads to the formation of diHPO. The hydroperoxidation pathway of 9- and 16-isomers is postulated as shown in Scheme IV. Hydroperoxidation may occur in the pentadiene structure of the 9- and 16-isomers, similar to the free radical oxidation of methyl linoleate (24). The pentadiene radical which is formed by hydrogen abstraction at C-14 position of 9-monoHPO would be subjected to an attack of oxygen, resulting in 9,12- and 9,16-diHPO isomers. On the other hand, the pentadiene radical formed by hydrogen abstraction of the C-11 position of 16-monoHPO isomer would yield 9,16- and 13,16-diHPO isomers. It was not clarified why no 9,12- and 13,16-isomers were detected in the secondary oxidation products. This result may be explained by selective attack of oxygen at C-9 and C-16 positions or



SCHEME IV. Formation of diHPO from monoHPO.

by the difference of the stability among 3 diOH isomers.

Hydroperoxides, including diHPO and cyclic peroxide-hydroperoxides, probably act as precursors of secondary decomposition products such as volatile carbonyl compounds and polymeric products. It is therefore considered that the formation of these volatile oxygenated compounds play a large part in the deterioration of edible oils and oil-containing foods.

When we were preparing this paper, a report by Frankel et al. (25) was published. He and his colleagues identified isomeric mixture of cyclic peroxide-hydroperoxides and diHPO produced from autoxidized methyl linolenate and suggested that cyclic peroxide-hydroperoxides are formed by cyclization of 12- and 13-monoHPO, and diHPO, by hydroperoxidation of 9- and 16-monoHPO. Our studies on secondary oxidation of monoHPO demonstrated that diHPO is formed by hydroperoxidation of monoHPO. However, the formation pathway of cyclic peroxide-hydroperoxides could not be entirely clarified. We indicated that an alternative pathway which produces 9,10,13- and/or 9,12,13- and 12,13,16- and/or 12,15,16-triOH as hydrogenated derivatives also participates in the formation of cyclic peroxide-hydroperoxides from monoHPO.

REFERENCES

1. Waters, W.A. (1971) J. Am. Oil Chem. Soc. 48, 427-433.
2. Tappel, A.L. (1953) Food Res. 18, 104-108.
3. Tappel, A.L. (1955) J. Biol. Chem. 217, 721-733.
4. Younathan, M.T., and Watts, B.M. (1959) Food Res. 24, 728-734.
5. Love, J.D., and Pearson, A.M. (1974) J. Agric. Food Chem. 22, 1032-1034.
6. Kwoh, T.L. (1971) J. Am. Oil Chem. Soc. 48, 550-555.
7. Wills, E.D. (1965) Biochim. Biophys. Acta 98, 238-251.
8. Chan, H.W.-S., and Newby, V.K. (1980) Biochim. Biophys. Acta 617, 353-362.
9. Hamberg, M. (1975) Lipids 10, 87-92.
10. Gardner, H.W., Kleiman, R., and Weisleder, D. (1974) Lipids 9, 696-706.
11. Gardner, H.W., Weisleder, D., and Kleiman, R. (1978) Lipids 13, 246-252.
12. Carrol, K.K. (1961) J. Lipid Res. 2, 135-141.
13. Terao, J., and Matsushita, S. (1975) Agric. Biol. Chem. 39, 397-402.
14. Terao, J., and Matsushita, S. (1975) Agric. Biol. Chem. 39, 2027-2033.
15. Terao, J., Hirota, Y., and Matsushita, S. (1981) J. Jpn. Oil Chem. Soc. 30, 10-14.
16. Terao, J., and Matsushita, S. (1977) J. Am. Oil Chem. Soc. 54, 234-238.
17. Terao, J., and Matsushita, S. (1981) Agric. Biol. Chem. 45, 587-593.
18. Frankel, E.N., Neff, W.E., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977) Lipids 12, 901-907.
19. Frankel, E.N., Evans, C.D., McConnel, D.G., Selke, E., and Dutton, H.J. (1961) J. Org. Chem. 26, 4663-4669.
20. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977) Lipids 12, 1055-1061.
21. Begemann, P.H., Woestenberg, W.J., and Leers, S. (1968) J. Agric. Food Chem. 16, 679-684.
22. Gerristsen, M., Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1976) FEBS Lett. 67, 149-152.
23. Pryor, W.A., Stanley, J.P., and Brair, E. (1976) Lipids 11, 370-379.
24. Frankel, E.N. (1962) in Lipids and Their Oxidation (Schultz, H.W., ed.) pp. 51-78, AVI Publishing Co., Westport, CT.
25. Neff, W.E., Frankel, E.N., and Weisleder, D. (1981) Lipids 16, 439-448.

[Received April 20, 1981]

On the Specificity of a Phospholipase A_2 Purified from the 106,000 × g Pellet of Bovine Brain

N.C.C. GRAY and **K.P. STRICKLAND***, *Department of Biochemistry, University of Western Ontario, London, N6A 5C1, Ontario, Canada*

ABSTRACT

Assessment has been made of the specificity of a purified phospholipase A_2 from the 106,000 × g pellet (microsomal fraction) of bovine grey matter which shows strong activity toward phosphatidylinositol (PI). In the first series of experiments involving the utilization as substrates of PI with different ^{14}C- or ^{3}H-labeled fatty acids in the 2-position, the purified phospholipase A_2 most readily removed 16:0 palmitic acid, followed by 18:0 stearic acid, 18:1 oleic acid and 20:4 arachidonic acid. In the second series of experiments, the purified phospholipase A_2 showed preferential action toward PI (100%) compared to phosphatidylcholine (PC, 62.5%), phosphatidic acid (PA, 32.6%), phosphatidylethanolamine (PE, 25.1%) and phosphatidylserine (PS, 21.5%), where each phosphoglyceride was labeled in the 2-position with [1-^{14}C] oleic acid. In the third series of experiments, fatty acids were shown to cause inhibition of action of the purified phospholipase A_2 on 1-acyl, 2-[1-^{14}C] oleoyl PI in the order 20:4 > 18:1 > 18:0 > 16:0 which is the reverse order to that just noted. In the final series of experiments, the addition of the phosphoglycerides PC, PE, PS and PA in amounts of 5 or 10 μM caused either no inhibition (PE, 2%), slight inhibition (PC, 15%) or reasonably significant inhibition (PA, 20% and PS, 40%) of action of the purified phospholipase A_2 on 1-acyl, 2-[1-^{14}C]-oleoyl PI. The pattern of specificity observed for the purified phospholipase A_2 combined with its microsomal location are the expected properties of a phospholipase A_2 that might function in a deacylation-reacylation cycle for modifying the fatty acid distribution in PI.

Lipids 17:91-96, 1982.

INTRODUCTION

Recent studies from this laboratory (1,2) have demonstrated in a 106,000 × g pellet (microsomal fraction) of bovine or rat brain the presence of a phospholipase A_2 which has a pH optimum of 7.5 and shows good activity toward phosphatidylinositol (PI). This enzyme has been purified to homogeneity. It requires Ca^{2+} for activation and is stimulated by low concentrations of Triton X-100. The enzyme is stabilized by glycerol, asolecithin and β-mercaptoethanol, which are necessary additions for assay.

Our interest in this enzyme and its purification and characterization arose from earlier studies in which attempts were made to determine how the predominant 1-stearoyl, 2-arachidonoyl molecular species of PI in brain and other tissues arose (see ref. in 1). It was concluded that a major reshaping of the PI, synthesized de novo, must occur by a deacylation-reacylation cycle such as that existing for other phosphoglycerides (3-5). Although good evidence existed for a selectivity toward arachidonoyl CoA in the reacylation of 1-acyl lyso PI, there was no good definitive evidence for a suitable deacylation mechanism until our studies (1,2) were completed.

The findings reported on the purified phospholipase A_2 from bovine brain (2) do not include any consideration of the selectivity shown by this enzyme in respect to the nature of the fatty acid in the 2-position. This paper reports the results obtained for the action of the purified phospholipase A_2 from bovine brain of PI containing different labeled fatty acids in the 2-position and on different phosphoglycerides containing [1-^{14}C] oleic acid in the 2-position. The effects of the addition of different fatty acids and of different phosphoglycerides on the deacylation of 2-[1-^{14}C]-oleoyl-PI have also been examined and the results obtained are reported.

MATERIALS AND METHODS

Materials

The following acids, [1-^{14}C] stearic acid (40-60 mCi/mmol), [1-^{14}C] oleic acid (56 mCi/mmol) and [5,6,8,9,11,12,14,15-^{3}H (N)] arachidonic acid (60-100 Ci/mmol) were all purchased from New England Nuclear Corp., Boston, MA. [1-^{14}C] Palmitic acid (59 mCi/mmol) came from Amersham Searle Co., Oakville, Ontario. Phosphatidylinositol (pig liver), phosphatidylcholine (beef brain), phosphatidylethanolamine (egg), phosphatidylserine (beef brain) and phosphatidic acid (egg) were all obtained from Serdary Research Laboratory, London, Ontario. Phospholipase A_2 (400-500 U/mg) purified from *Crotalus adamanteus* venom was purchased from Serdary Research Laboratory, London, Ontario. Triacylglycerol lipase (EC 3.1.1.3), which is predominantly a phospholipase A_1 toward PI (6) from *Rhizopus arrhizus*, was obtained from Sigma Chemical Co., St. Louis, MO.

Preparation and Purification of 2-^{14}C or ^{3}H-Labeled Acyl PI

The 2-^{14}C- or ^{3}H-labeled acyl PI were essentially prepared as described earlier (1) for the preparation and purification of 1-acyl, 2-[1-^{14}C]oleoyl GPI (GPI = *sn*-glycerol-3-phosphoinositol). 1-acyl *lyso*-PI was formed by action of phospholipase A_2 partially purified from *C. adamanteus* venom on pig liver PI. The reacylation of the *lyso*-PI was done using a modified system of Webster and Alpern (7), as described by Shum et al. (1). The reacylation was done using the fatty acids [1-^{14}C]oleic, [1-^{14}C]palmitic, [1-^{14}C]stearic and [5,6,8,9,-11,12,14,15-^{3}H (N)]arachidonic and a rat liver microsomal fraction as the enzymatic source. Each of the different 2-^{14}C- or ^{3}H-labeled acyl PI was extracted according to Folch et al. (8) and purified essentially as described by Hanahan et al. (9). Each of the labeled PI was examined for location of the ^{14}C- or ^{3}H-labeled acyl groups by gas liquid chromatographic (GLC) analysis (1,10) using a Beckman GC-65 (Beckman Instruments, Inc., Fullerton, CA) fitted with a Beckman DEGS metal column (6′ x 1/8″) and assessment of the radioactivity removed by phospholipase A_2 action. The results obtained indicated that the labeled PI substrates contained approximately the following amount of label in the 2-acyl position: 20:4, 98%; 18:1, 94%; 18:0, 90% and 16:0, 80%. Rather rapid transacylation was evident with the 16:0-labeled PI. These substrates were used as quickly as possible because of the problem of transacylation.

Preparation and Purification of 2-[1-^{14}C] Oleoyl-Labeled Phosphoglycerides

Of the five 2-[1-^{14}C]oleoyl-labeled phosphoglycerides used in this study, only the 1-acyl, 2-[1-^{14}C]oleoyl phosphatidylcholine (PC) was purchased (Applied Science Div., Milton Roy Co. Lab. Group, State College, PA). This phosphoglyceride was further purified by 2 consecutive runs on silicic acid/celite (2:1, w/w) columns eluted with a chloroform/methanol gradient (9) prior to being used in the assay.

The preparation and purification of 1-acyl-2-[1-^{14}C]oleoyl phosphatidic acid (PA), 1-acyl-2-[1-^{14}C]oleoyl phosphatidylethanolamine (PE) and 1-acyl-2-[1-^{14}C]oleoyl phosphatidylserine (PS) were done in a similar manner to that already described for the preparation and purification of 1-acyl-2-[1-^{14}C]oleoyl-PI. Instead of using PI, unlabeled preparations of each of the other phosphoglycerides were substituted into the procedures for deacylation by phospholipase A_2 and subsequent reacylation of the *lyso*-intermediates with [1-^{14}C]-oleic acid to yield the appropriately labeled phosphoglyceride. The method of Hanahan et al. (9) was used to separate the newly formed 2-[1-^{14}C]oleoyl-labeled phosphoglycerides. The location of the [1-^{14}C]oleic acid within each phosphoglyceride was determined by GLC analysis (1,10) and shown to be comparable to that reported earlier for PI (1).

Preparation and Purification of 1-Acyl-Labeled PI

A series of PI labeled in the 1-position with [1-^{14}C]palmitic acid, [1-^{14}C]stearic acid, [1-^{14}C]oleic acid and [5,6,8,9,11,12,14,15-^{3}H (N)]arachidonic acid was prepared and purified as described by Holub and Piekarski (6). All resulting 1-acyl-labeled PI were checked for the location of the incorporated fatty acid by GLC analysis, as described previously (1) and as applied to the 2-acyl-labeled PI.

Purified Phospholipase A_2 Preparation

The preparation and purification of the phospholipase A_2 extracted from the 106,000 x g pellet of bovine grey matter has recently been described (2). The purification involved extraction from the 106,000 x g pellet (i.e., microsomal fraction) by Triton X-100, followed by ammonium sulfate fractionation, consecutive column chromatography runs on Sephadex G-200 and DEAE-Sephacel and preparative polyacrylamide gel electrophoresis. The procedure yielded a Ca^{2+}-activated phospholipase A_2 preparation that showed a single band on SDS-polyacrylamide gel electrophoresis with a molecular weight of 18,300 daltons (2). Kinetic and other properties of the enzyme are reported elsewhere (2). This purified phospholipase A_2 was used as the enzyme preparation for the studies reported here.

Phospholipase A_2 Assay

The basic assay system (2) contained in 0.5 ml was: Tris-maleate-acetate buffer, 50 mM, pH 7.5; $CaCl_2$, 5 mM; 2-[1-^{14}C]oleoyl-labeled phosphoglyceride (3.0-5.5 x 10^4 dpm), 0.3 mM; and 25.0-40.0 μg purified phospholipase A_2. The radioactive phosphoglycerides, dissolved in chloroform, were dried down under nitrogen and dispersed in the buffer by sonication in an ultrasonicator bath (Branson Instruments Ltd., Danbury, CT). The assay tubes were incubated for 10 min at 37 C in an Aquatherm water bath shaker (New Brunswick Scientific, New Brunswick, NJ) at 200 rpm.

In the experiments where various fatty acids

or phosphoglycerides were tested for their competitive effect on the deacylation of 1-acyl-2-[1-^{14}C]oleoyl-PI, each was added to the assay system at the level of the Tris-maleate-acetate buffer (50 mM; pH 7.5). Each was sonicated in an ultrasonicator bath prior to addition to the assay tube.

Extraction and Separation of Reaction Products

The extraction and separation of the reaction products (fatty acids and *lyso*-phosphoglycerides) formed from the different [2-^{14}C]- or [^{3}H]acyl PI or 1-acyl, 2-[1-^{14}C]oleoyl-labeled phosphoglycerides were done as described by Shum et al. (1).

Measurements of Radioactivity and Enzyme Activity

Appropriate areas from thin layer chromatographic plates were scraped into scintillation vials and the radioactivity was determined by liquid scintillation counting as described earlier (1,2) using aqueous counting scintillant (ACS) supplied by Amersham Corp. (Oakville, Ontario). Phospholipase A_2 activity was expressed as μmol fatty acid released/min/mg protein. The protein was measured by the method of Bradford (11) using the kit supplied by Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ontario).

RESULTS

Those PI, prepared with different labeled fatty acids in the 2-position as described in Materials and Methods, were first used as substrate in assessing the specificity of the phospholipase A_2 purified from the 106,000 x g pellet of bovine brain in this laboratory (2). Table 1 shows the results obtained from this study. An interesting pattern emerged. The PI with 16:0 palmitic acid in the 2-position was the most active in terms of released fatty acid followed by 18:0 stearic acid, 18:1 oleic acid and 20:4 arachidonic acid. The 2 saturated fatty acids were released at 2-5 times the rate of the monoenoic (oleic) fatty acid and 4-10 times the rate of the tetraenoic (arachidonic) fatty acid studied in this series. It should be noted that the purified phospholipase A_2 showed little or no activity toward any PI synthesized with labeled fatty acids in the 1-position. The small activity that was observed was accountable on the basis of a small amount of transmigration of labeled fatty acid to the 2-position.

The second series of substrates to be purchased or prepared for assessment of the above phospholipase A_2 included the phosphoglycerides, PC, PE, PS, phosphatidic acid (PA) and PI, all with [1-^{14}C]oleic acid in the 2-position. As noted in Table 2, the purified phospholipase A_2 showed greatest activity toward PI; with this activity set at 100%, the following descending order of activity resulted: PC (62.5%), PA (32.6%), PE (25.1%) and PS (21.5%). The 60% higher activity shown toward PI compared to PC, the next highest activity, indicates a reasonable selectivity on the part of the purified phospholipase A_2 toward PI.

The third series of experiments undertaken was to examine the effect of the addition of unlabeled fatty acids (corresponding to those used to label PI in Table 1) on the release of [1-^{14}C]oleic acid from the 2-position of PI. The results obtained (Table 3) show that arachidonic acid causes the greatest reduction in release of labeled oleic acid followed by oleic

TABLE 1

Specificity of the Purified Phospholipase A_2 from the 106,000 X g pellet Microsomes) of Bovine Brain Acting on PI Labeled with Different Fatty Acids in the 2-Position

Substrate[a]	Number	Fatty acid released[b] (μmol/min/mg protein ± SEM)	Fatty acid released relative to 2-[1-^{14}C]oleoyl-PI (%)
2-[1-^{14}C]Palmitoyl-PI	4	2.67 ± 0.305	496
2-[1-^{14}C]Stearoyl-PI	3	1.03 ± 0.144	194
2-[1-^{14}C]Oleoyl-PI	17	0.53 ± 0.029	100
2-[5,6,8,9,11,12,14,15-^{3}H] Arachidonoyl-PI	4	0.23 ± 0.019	43

[a]The labeled substrates noted were prepared as described in Materials and Methods based on the procedures described earlier (1).

[b]The phospholipase A_2 was purified as outlined in Materials and Methods and described elsewhere (2). The activity of the enzyme was measured as described in Materials and Methods based on the method reported earlier by Shum et al. (1).

TABLE 2

Specificity of the Purified Phospholipase A_2 from the 106,000 X g Pellet (Microsomes) of Bovine Brain Acting on Different Phosphoglycerides Labeled with [1-^{14}C]-Oleic Acid in the 2-Position

Substrate[a]	Number	Fatty acid released[b] (μmol/min/mg protein ± SEM)	Activity relative to 2-[1-^{14}C]oleoyl-PI (%)
1-Acyl, 2-[1-^{14}C]oleoyl-PI	17	0.534 ± 0.0294	100.0
1-Acyl, 2-[1-^{14}C]oleoyl-PC	4	0.334 ± 0.0265	62.5
1-Acyl, 2-[1-^{14}C]oleoyl-PA	4	0.174 ± 0.0139	32.6
1-Acyl, 2-[1-^{14}C]oleoyl-PE	4	0.134 ± 0.0265	25.1
1 Acyl, 2-[1-^{14}C]oleoyl-PS	2	0.115[c] (0.105, 0.124)	21.5

[a]Substrates were either purchased (PC) or prepared (PI, PE, PS and PA) as described in Materials and Methods based on procedures described earlier (1,2).

[b]The phospholipase A_2 was purified and its activity measured as noted for Table 1.

[c]Average of the 2 values shown in parentheses.

TABLE 3

Effect of Different Fatty Acids on the Action of Purified Phospholipase A_2 from the 106,000 X g Pellet (Microsomes) of Bovine Brain toward PI Labeled with [1-^{14}C] Oleic Acid in the 2-position

Fatty acid added[a]	Concentration (μM)	nmol [1-^{14}C]oleic acid released/min/mg protein[b]		% of control		
		Expt. 1	Expt. 2	Expt. 1	Expt. 2	Ave.
Control	–	493.3	617.6	100.0	100.0	100.0
Palmitic acid	5	437.6	561.0	88.7	90.8	89.8
(16:0)	10	441.3	538.0	89.5	87.1	88.3
Stearic acid	5	412.1	519.8	83.5	84.2	83.9
(18:0)	10	402.3	537.2	81.6	87.0	84.3
Oleic acid	5	372.9	496.3	75.6	80.4	78.0
(18:1)	10	321.4	463.1	65.2	75.0	70.1
Arachidonic acid	5	249.5	221.4	50.6	35.9	43.3
(20:4)	10	186.8	198.6	37.9	32.2	35.1

[a]Fatty acids were added to buffer to give the final concentrations shown and then sonicated as detailed in Materials and Methods.

[b]The phospholipase A_2 was purified and its activity measured as noted for Table 1.

acid, then stearic acid and finally palmitic acid. This order of what might be termed product inhibition is the exact reverse to that observed for the release of the corresponding labeled fatty acids from the 2-position.

The final study to be reported is concerned with the examination of the addition of different unlabeled phosphoglycerides on the release of [1-^{14}C]oleic acid from the 2-position of PI (Table 4). Both PS and PA show significant inhibitions (40 and 20%, respectively) of release of labeled oleic acid from PI whereas the reductions observed with PC (15%) and PE (2%) are both less and of either borderline or no significance. The types of inhibition observed are not inconsistent with what might be expected with a phospholipase A_2 showing selectivity toward PI.

DISCUSSION

The discovery (1) and subsequent purification (2) of a phospholipase A_2 present in the microsomal fraction of brain which showed good activity toward PI was of considerable interest to us in regard to studies that were in progress on how the predominant 1-stearoyl, 2-arachidonoyl species of PI may be formed. As cited elsewhere (1,2), only a limited number of studies are reported which focus primarily on the deacylation of PI. Yet it is essential for this process to go on if there is to be significant reshaping of the PI molecule in terms of its fatty acid composition by a deacylation-reacylation cycle (3-5). Furthermore, considerable in vivo and whole-cell studies with labeled precursors yield data which strongly suggest a

TABLE 4

Effect of Adding Different Phosphoglycerides on the Action of Purified Phospholipase A_2 from the 106,000 X g Pellet (Microsomes) of Bovine Brain toward PI Labeled with [1-^{14}C]Oleic Acid in the 2-Position

Phosphoglyceride added	Concentration (μM)	nmol [1-^{14}C]oleic acid released/min/mg protein[a]	% of control
Control	–	483.2	100.0
Phosphatidylcholine	5	421.4	87.2
	10	408.3	84.5
Phosphatidylethanolamine	5	473.2	97.9
	10	474.1	98.1
Phosphatidylserine	5	321.6	66.6
	10	282.1	58.4
Phosphatidic acid	5	396.8	82.1
	10	384.6	79.6

[a]The phospholipase A_2 was purified and its activity measured as noted in Table 1.

major role for this cycle in regard to PI metabolism. The experiments done in this study were designed to assess the specificity of this phospholipase A_2, particularly in respect to its action on PI with a view to determining whether this enzyme might have the right characteristics and, hence, the potential to function in a deacylation-reacylation cycle.

The results obtained showed a number of patterns which are consistent with the functional role just described for the purified phospholipase A_2. The finding that the saturated fatty acids (16:0 first and then 18:0) are preferentially released compared to unsaturated fatty acids and that 18:1 oleic acid is preferentially released compared to 20:4 arachidonic acid is the type of pattern to be expected for a phospholipase A_2 if the arachidonoyl group in the 2-position of PI is to be conserved. Such a selectivity would be useful for the removal of fatty acids other than arachidonic from the 2-position to form *lyso*-PI which could then be acylated by acyltransferases showing selectivity for arachidonoyl CoA (6,12,13). Also, disaturated-GPI, if present, would be preferentially attacked. However, in contrast to the situation for PC (14), the possibility of this happening significantly for PI seems unlikely in view of the finding of Holub et al. (15) that only trace amounts of disaturated-GPI exist in the PI of bovine brain although on a purely random basis up to 21% could exist in this form. The pattern of fatty acid inhibition observed is also consistent with that interpretation. Thus, the greater inhibition noted with arachidonic acid is to be expected if arachidonoyl groups are to be prevented from being released. Finally, the somewhat greater preference shown by the purified phospholipase A_2 toward PI than other phosphoglycerides points to the possibility that this enzyme has a prime role to play in respect to PI metabolism. This raises the interesting possibility that several membrane-bound A_2 phospholipase may exist in a tissue such as brain which show different substrate specificities.

Until truly in vitro or reconstitution experiments with purified enzymes (phospholipase A_2 and acyltransferases) are done, it is not possible to demonstrate a direct involvement of the purified phospholipase A_2 under study in a deacylation-reacylation cycle for PI. However, the pattern of action of this phospholipase A_2 and the fact that it is located in the microsomal fraction where the acyltransferases are found (6,12,13) provide strong support for the role described here for this enzyme. Once the appropriate *lyso*-PI is formed, there is good evidence for the presence of acyltransferases (6,12,13) that show selectivity in terms of the fatty acid added.

An alternate physiological role for the purified phospholipase A_2 which is somewhat negated by the results reported here is that of producing arachidonic acid for prostaglandin synthesis (16,17). Actually, much controversy exists in respect to whether arachidonate derived from PI for prostaglandin synthesis is provided via the consecutive actions of PI-phosphodiesterase and diacylglycerol lipase (18) or by a PI-hydrolyzing phospholipase A_2 (19). The fact that the purified phospholipase A_2 shows less activity toward PI with arachidonate in the 2-position would argue against a major role for this enzyme in respect to the release of arachidonate, unless, as yet undiscovered, regulatory mechanisms operating in the membrane locus of the enzyme alters its specificity.

ACKNOWLEDGMENT

This work was supported by Grant MT-617 from the Medical Research Council of Canada.

REFERENCES

1. Shum, T.Y.P., Gray, N.C.C., and Strickland, K.P. (1979) Can. J. Biochem. 57, 1359-1367.
2. Gray, N.C.C., and Strickland, K.P., Can. J. Biochem., in press.
3. Hill, E.E., and Lands, W.E.M. (1970) in Lipid Metabolism (Wakil, S.J., ed.) pp. 185-277, Academic Press, New York, NY.
4. Van Den Bosch, H. (1974) Ann. Rev. Biochem. 43, 243-277.
5. Van Den Bosch, H. (1980) Biochim. Biophys. Acta 604, 191-246.
6. Holub, B.J., and Piekarski, J. (1979) Lipids 14, 529-532.
7. Webster, G.R., and Alpern, R.J. (1964) Biochem. J. 90, 35-42.
8. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-409.
9. Hanahan, D.J., Dittmer, J.C., and Warashina, E. (1957) J. Biol. Chem. 228, 685-700.
10. Bishop, H.H., and Strickland, K.P. (1970) Can. J. Biochem. 48, 267-277.
11. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
12. Holub, B.J. (1976) Lipids 11, 1-5.
13. Baker, R.R., and Thompson, W. (1973) J. Biol. Chem. 248, 7060-7065.
14. Montfoort, A., Van Gelde, L.M.G., and Van Deenen, L.L.M. (1971) Biochim. Biophys. Acta 231, 335-342.
15. Holub, G.J., Kuksis, A., and Thompson, W. (1970) J. Lipid Res. 11, 558-564.
16. Haye, B., Champion, S., and Jacquemen, C. (1973) FEBS Lett. 30, 253-259.
17. Haye, B., and Jacquemin, C. (1977) Biochim. Biophys. Acta, 487, 231-242.
18. Irvine, R.F., Letcher, A.J., and Dawson, R.M.C. (1980) FEBS Lett. 119, 287-289.
19. Hong, S.L., and Deykin, D. (1981) J. Biol. Chem. 256, 5215-5219.

[Received August 13, 1981]

Gas Chromatographic Assay of the Diastereomeric Composition of *all-rac*-α-Tocopheryl Acetate[1]

CLIFFORD G. SCOTT*, NOAL COHEN, PATRICK P. RIGGIO and **GIUSEPPE WEBER**, *Chemical Research Department, Hoffmann-La Roche Inc., Nutley, NJ 07110*

ABSTRACT

It has been established by an evaluation of 6 production samples of *all-rac*-α-tocopheryl acetate that all 4 racemates (RRS/SSR, RRR/SSS, RSR/SRS and RSS/SRR) are consistently present in equimolar amounts (SD < 0.3, RSD < 1.2%). An analysis of variance indicated variance due to signal noise to be consistent within a sample run but to vary from day to day. Variance due to area measurement was greater for the first and last eluted racemates than for the second and third. Peak width and asymmetry were found to be extremely sensitive to sample loading and, even within acceptable limits for good quantitation, the distortion was sufficient to give the elution profile the appearance of a sample composed of 4 components in unequal proportions increasing according to the order of elution.

Lipids 17:97-101, 1982.

INTRODUCTION

A recent publication by Slover and Thompson (1) describes an elegant gas chromatographic procedure for the analytical separation and quantitation of the 4 racemates present in *all-rac*-α-tocopherol. The paper included data indicating the composition of 2 commercially available synthetic *all-rac*-α-tocopherols to have a skew distribution of the 4 racemates; thus normalized peak areas for the racemates are shown as 22.8, 25.0, 25.6 and 26.7% in order of their elution (data averaged over 8 determinations between the 2 samples). Similar skew distributions were reported for an *all-rac*-α-tocopherol USP reference sample and for material extracted from a vitamin E capsule.

These results contrast with our own published data (2) on ROCHE production *all-rac*-α-tocopherols which were obtained using a slight modification of the Slover-Thompson procedure in which the methyl ether rather than the trimethylsilyl ether derivative was gas chromatographed. Our data indicated the racemates to be present in essentially equal amounts (24.9, 25.1, 24.8 and 25.0%, in order of elution).

Because the biological activity of *all-rac*-α-tocopherol can be a function of the racemate distribution, it is of the utmost importance that these differences be resolved, not only with respect to the distribution itself, but also to the constancy of the distribution from sample to sample. The work reported in this paper addresses these questions as they relate to ROCHE production *all-rac*-α-tocopheryl acetate.

[1]Dedicated to the memory of Dr. Willy Leimgruber, who died July 8, 1981.

EXPERIMENTAL

Samples

Six samples were obtained, 1 from each of 6 batches of production NF/USP grade *all-rac*-α-tocopheryl acetate chosen at random from batches made during March 1980-March 1981. The acetates were reduced to the alcohol with lithium aluminum hydride and then methylated with dimethyl sulfate as previously described (2). Solutions of the methyl ether in methylene chloride at 0.01% and 0.02% levels were used for gas chromatographic analysis.

Gas Chromatography

Gas liquid chromatography (GLC) was done on a 100 m × 0.25 mm id glass capillary column coated with SP2340 liquid phase (Quadrex Corp., New Haven, CT). The gas chromatograph was a Hewlett-Packard Model 5700 equipped with flame ionization detector and standard splitter. Hydrogen was used as carrier gas at an average linear velocity of ca. 14 cm sec^{-1}, and the column temperature was held at 190 C. The injector and detector temperatures were 250 and 300 C, respectively.

Analysis Scheme

The analysis scheme was to inject each of the 6 samples 5 times (30 chromatograms) and replicate the area measurements 5 times on each chromatogram (150 sets of data). Because of the lengthy retention time of ca. 180 min, the injections for each sample were piggybacked at 20-min intervals. Also, for expediency, 6 rather than 5 injections were piggybacked on each sample to cover the eventuality that a noise spike or obvious baseline pertubation occurred during the elution of any one

of the first 5 injections. Only for 1 out of the 6 samples did this situation arise. For the other 5 samples, data were obtained for the first 5 injections. In order to cover what was believed to be an acceptable range of mass injected, injections ranged from ca. 1 μl of the 0.01% solution to 2 μl of the 0.02% solution of methyl ether derivative in methylene chloride.

Quantitation

The detector output was fed to an auto-ranging analog-to-digital converter (Digimetry *unit ex Computer Inquiry Systems*, Waldwick, NJ) coupled to a Hewlett-Packard Model 21MX computer (128K) and 7900A cartridge disc system (10M bytes). The detector output was sampled 15 times/sec and data, averaged every sec, were analyzed by area internal normalization using a combination of custom supplied software for area integration (Computer Inquiry Systems) and in-house user-written software for direct interaction with the CRT of the data system. Briefly, the quantitation was achieved by plotting the 4 eluted racemates of a sample full-scale on a Tektronix 4010-1 screen and using the cross-hairs to indicate to the computer where the baseline should commence and terminate and where the perpendiculars should be dropped at the valleys. The areas enclosed within the indicated boundaries were then automatically integrated and normalized by the computer. For the 5 sets of area measurement made on each chromatogram, the operator attempted to eliminate the subjectivity of the assessment, and at the same time obtain the maximal variances, by randomly including all possible variations of, e.g., baseline and perpendicular drop positions, when, due to random noise, several options were available. Calculated data for each sample were held in the computer and not printed out until after the fifth and last analysis had been completed.

Chromatographic Data

Plate numbers at 0.607 of the peak height were calculated by the computer for all 4 components for every injection of all 6 samples. Asymmetry ratio defined as the ratios of peak widths, rear/front, on either side of the perpendicular dropped from the peak maximum and measured at 0.1354 of the peak height were also determined.

RESULTS AND DISCUSSION

Quantitative Results

The overall chromatogram obtained for the run of 6 piggy-backed injections on sample lot

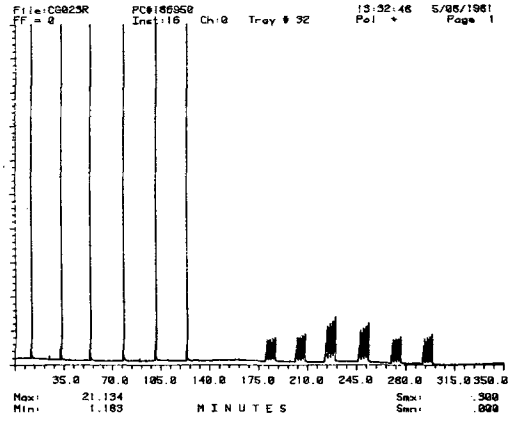


FIG. 1. Chromatographic run of 6 piggy-backed injections for the *all-rac-α*-tocopheryl methyl ether of lot #473050 on 100 m × 0.25 mm id glass capillary column coated with SP 2340 liquid phase.

#473050 is shown in Figure 1. The computer-enlarged chromatogram for the elution of the first injection is shown in Figure 2 together with 2 options for base-line position. The continuous baseline and perpendiculars from the valleys shown in Figure 2 were the boundaries which gave 25.45, 24.95, 24.91 and 24.69% for the racemates in order of elution. Another option for baseline choice (dashed line) gave results of 25.20, 24.94, 24.93 and 24.93% for the racemates.

The detailed results obtained for the first 5 injections of the sample shown in Figure 1 are given in Table 1. A summary of the overall mean values obtained for the 6 samples are given in Table 2. The mean values, corrected to the fourth significant figure are RRS/SSR

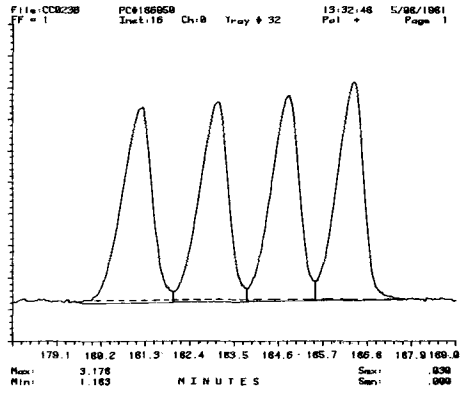


FIG. 2. Elution profile for the first injection of lot #473050 (elution period between 178 and 180 min on Fig. 1) showing 2 options for baseline position.

TABLE 1

Normalized Area Percentages for Lot #473050

First eluted racemate RRS/SSR

Injection #	1	2	3	4	5
Area 1	25.01	24.63	24.90	24.82	24.86
Det. # 2	25.20	24.76	24.74	25.01	24.92
3	25.04	24.99	24.92	25.05	24.89
4	25.45	24.78	24.75	24.83	24.95
5	25.28	25.26	24.79	24.81	24.55
Mean	25.196	24.884	24.820	24.904	24.834
Overall mean			24.928		

Second eluted racemate RRR/SSS

Injection #	1	2	3	4	5
Area 1	24.88	24.60	25.08	24.92	24.81
Det. # 2	24.94	24.71	24.98	24.90	24.77
3	24.85	24.72	25.11	24.95	24.74
4	24.95	24.64	25.07	24.93	24.75
5	24.97	24.74	25.09	24.92	24.71
Mean	24.918	24.682	25.066	24.924	24.756
Overall mean			24.869		

Third eluted racemate RSR/SRS

Injection #	1	2	3	4	5
Area 1	25.05	25.27	25.13	25.19	25.19
Det. # 2	24.93	25.23	25.09	25.17	25.21
3	25.09	25.18	25.07	25.11	25.22
4	24.91	25.33	25.19	25.15	25.18
5	24.98	25.05	25.22	25.24	25.38
Mean	24.992	25.212	25.140	25.172	25.236
Overall mean			25.150		

Fourth eluted component RSS/SRR

Injection #	1	2	3	4	5
Area 1	25.06	25.50	24.88	25.08	25.14
Det. # 2	24.93	25.30	25.20	24.92	25.10
3	25.02	25.10	24.91	24.88	25.15
4	24.69	25.24	24.99	25.10	25.12
5	24.77	24.95	24.90	25.03	25.37
Mean	24.894	25.218	24.976	25.002	25.176
Overall mean			25.053		

TABLE 2

Summary of Normalized Area Percentages for 6 Samples

Lot #	Racemate			
	RRS/SSR	RRR/SSS	RSR/SRS	RSS/SRR
256011	24.901	24.999	25.098	25.009
473050	24.928	24.869	25.150	25.053
208110	25.020	24.965	25.050	24.966
164090	24.996	25.173	25.054	24.780
408030	24.979	24.980	25.130	24.910
333031	25.058	24.804	25.092	25.046
Mean	24.980	24.965	25.096	24.961
SD[a]	0.293	0.245	0.158	0.283
Upper limit[b]	25.736	25.597	25.504	25.691
Lower limit[b]	24.224	24.333	24.688	24.231

[a]Standard deviations are based on the 150 data points obtained for each racemate.

[b]Upper and lower limits at 99% confidence levels.

racemate = 24.98, RRR/SSS = 24.97, RSR/SRS = 25.10 and RSS/SRR = 24.96. Standard deviations calculated for each racemate on the total of 150 data points obtained for each (based on the assumption that all 6 samples can be regarded as belonging to the same population) and without any rejection of statistical outliers are 0.293, 0.245, 0.158 and 0.283, respectively, in order of elution. The data confirm our previous findings using considerably less data, that the 4 racemates are present in equal amounts and the ratios do not vary between samples. While this contrasts with the results reported by Slover and Thompson, it should be noted that, in their paper (1), they draw attention to the difficulty of the chromatography and the smallness of their area measurements, and indicate confirmatory work is required in other laboratories before conclusions should be drawn regarding tocopherols in general.

Evaluation of the Quantitative Procedure

The quantitative procedure used was one of area normalization of 4 closely eluted peaks having identical physical properties and, therefore, the variances in the analytical measurement can be narrowed down to 2 sources. The first source is the fluctuation in response of the detector output and signal transmission over the time of an elution period and the second source is in the assessment of the areas on the chromatogram. The design of the analytical scheme allowed these variances to be estimated by an analysis of variance. The results are given as standard deviations in Table 3.

The estimates of the standard deviations of the detector output range from a low of 0.08 to a high of 0.43. The only pattern that emerges is that the variation between samples is greater than within a sample. This is compatible with the situation of working at high sensitivity with noise levels showing a greater day-to-day variation (each sample run on a different day) than over the 2-hr elution period for 6 piggy-backed injections of a single sample.

The estimates of the standard deviations of the area measurements (given in parentheses in Table 3) have values between 0.14 and 0.24 for the first and last eluted peaks and between 0.05 and 0.09 for the 2 middle peaks. These results tend to suggest that the variability in the area measurement is attributable to the positioning of the start and end of the baseline, rather than the positioning of the perpendiculars dropped from the valleys. This tendency is reflected in the overall standard deviations reported in Table 2. In this respect, it is of interest to note that in 5 of the 7 analyses reported by Slover and Thompson (their Table 2, ref. 1) this trend is clearly observed, with the standard deviations for the data on the 2 center peaks being lower than for the 2

TABLE 3

Standard Deviations Attributable to Detector Output and (Area Normalization)

	Racemate			
Lot #	RRS/SSR	RRR/SSS	RSR/SRS	RSS/SRR
256011	0.12 (0.20)	0.16 (0.06)	0.16 (0.09)	0.15 (0.19)
473050	0.14 (0.17)	0.15 (0.05)	0.09 (0.08)	0.12 (0.15)
208110	0.43 (0.21)	0.22 (0.08)	0.16 (0.08)	0.19 (0.24)
164090	0.35 (0.14)	0.39 (0.06)	0.22 (0.05)	0.39 (0.15)
408030	0.11 (0.20)	0.09 (0.07)	0.14 (0.06)	0.08 (0.21)
333031	0.19 (0.16)	0.24 (0.05)	0.11 (0.06)	0.22 (0.16)

TABLE 4

Plate Numbers and (Asymmetry Values) over 6-fold Range of Sample Size Injected

Area mv. sec	RRS/SSR	RRR/SSS	RSR/SRS	RSS/SRR
22	354[a] (0.93)	357 (0.86)	379 (0.85)	420 (0.82)
61	295 (0.67)	316 (0.61)	340 (0.62)	403 (0.56)
74	276 (0.58)	308 (0.57)	320 (0.58)	393 (0.46)
110	248 (0.50)	287 (0.42)	302 (0.50)	394 (0.36)
129	210 (0.44)	254 (0.43)	278 (0.46)	360 (0.32)

[a]Values in table to be multiplied by 1,000 to obtain plate number.

outer peaks. It is thus conceivable that their main source of variance occurred in the positioning of the start and end of the baseline and instrumental error biasing one or other (or both) of these positions could readily introduce the skew distribution observed by them.

Evaluation of Chromatographic Data

As reported by Slover and Thompson, separation efficiency and peak shape of the racemates are unusually sensitive to sample size. The sample sizes used in this study were varied over a narrow range representing a compromise between obtaining adequate separation and accurate area measurement. Plate numbers and asymmetry values are given in Table 4 for injections covering the range from minimum to maximum used in this study. For the minimal injection, the peaks are essentially symmetrical even though there is a distinct trend for introduction of asymmetry progressing from the first to the fourth eluted racemate. Plate numbers are also essentially the same, although it might be that the slight increase in efficiency for the fourth eluted racemate is just significant. As the sample size is increased, the asymmetry increases and the peaks broaden (as indicated by plate numbers which, due to the asymmetry, are somewhat artificial). The broadening is consistently greater for the first eluted racemate than it is for the last. The increase in broadening for the second and third eluted racemates is intermediate between the first and last eluted. The net effect of these changes is to distort the appearance of the chromatogram, as shown in Figure 3, which compares the elution profile for the smallest injection with the elution profile for the largest injection. The elution profile for the larger injection could certainly lead to the belief that the racemates were present in a skewed distribution.

The data reported herein show quite conclusively that the 4 racemates present in synthetic, i.e., *all-rac*-α-tocopheryl acetate, are present in equimolar amounts (RSD < 1.2%) in 6 samples chosen at random from production lots spanning a period of 12 months.

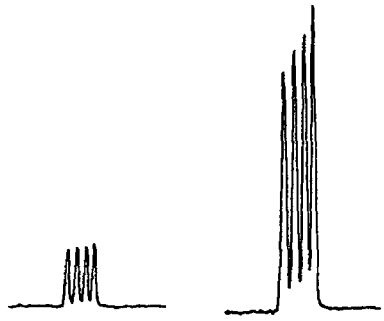


FIG. 3. Elution profiles for the minimal and maximal sample injections used in the study.

The variances of determination are greater for the first- and last-eluted racemates than they are for the second and third and this appears attributable to the poorer definition in choosing baseline start and end rather than choosing the position for dropping perpendiculars at the valleys.

Elution peak shape is particularly sensitive to sample load with, in addition to peak fronting, the earlier eluting peaks broadening more quickly than the later eluting peaks. The net result is that, even without overload affecting quantitative precision, the visual effect is to suggest a skew distribution of the racemates.

ACKNOWLEDGMENTS

The authors are grateful to H.T. Slover of the USDA Nutrient Composition Laboratory, Beltsville, MD, for providing prepublication details of that laboratory's gas chromatographic procedure and for subsequent discussions. We are also appreciative of the support given this work by G. Saucy, Associate Director of Chemical Research, Hoffmann-La Roche Inc., Nutley, NJ.

REFERENCES

1. Slover, H.T., and Thompson, R.H. (1981) Lipids 16, 268-275.
2. Cohen, N., Scott, C.G., Neukom, C., Lopresti, R.J., Weber, G., and Saucy, G., (1981) Helv. Chim. Acta 64, 1158-1173.

[Received October 5, 1981]

Herbal Remedies of the Maritime Indians: Sterols and Triterpenes of *Tanacetum vulgare* L. (Tansy)

R.F. CHANDLER*,[a], S.N. HOOPER[a], D.L. HOOPER[b], W.D. JAMIESON[c] and E. LEWIS[c],
[a]*College of Pharmacy*, [b]*Department of Chemistry, Dalhousie University, Halifax, Nova Scotia, Canada; and* [c]*Atlantic Research Laboratory, National Research Council, Halifax, Nova Scotia, Canada*

ABSTRACT

Plant sterols and triterpenes exhibit a wide range of pharmacological activities. As part of our ongoing studies of the medicinal aspects of Maritime flora, particularly the herbal remedies of the Micmac and Malecite Indians, we determined the nature of the sterols and triterpenes of *Tanacetum vulgare* L. (Compositae)–a widely used herbal remedy usually referred to as tansy. By using thin layer and gas chromatographics, nuclear magnetic resonance (NMR) spectroscopy and combined gas chromatography-mass spectrometry, we were able to identify β-sitosterol as the major sterol and α-amyrin as the major triterpene of tansy. We also identified the sterols stigmasterol, campesterol and cholesterol, and the triterpenes β-amyrin and taraxasterol. A fourth triterpene was tentatively identified as pseudo-taraxasterol. The successful therapeutic application of this herb may be due partly to the presence of one or more of these compounds. The sterols and triterpenes of tansy have not been previously reported; neither, to our knowledge, have the NMR spectra of the amyrins and the NMR and mass spectra of taraxasterol.
Lipids 17:102-106, 1982.

Our literature searches of the medicinal aspects of Maritime flora, particularly the herbal remedies of the Micmac and Malecite Indians (1,2), revealed only 3 references (3-5) to the sterols and triterpenes of *Tanacetum vulgare* L. (Compositae), commonly known as tansy, despite the extensive use of this plant as a traditional medicine (6-11) and the numerous phytochemical studies reported (12-22).

Plant sterols and triterpenes (pentacyclic triterpenes) exhibit a wide range of pharmacological activities. These include antitumor and cytotoxic (23-28), antihypercholesteremic (29-30), anti-inflammatory (31-33), anticonvulsant (31), antibacterial, analgesic, antitussive and expectorant (32-34) activities.

Some of the traditional uses of tansy could conceivably be related to the presence of the sterols and triterpenes which we had shown were present in the plant in an earlier study (2). We, therefore, decided to investigate the nature of these compounds in tansy.

Even though its oil is toxic (8,35-39), tansy has a rich heritage as a traditional herbal remedy. It was officially listed in the *United States Pharmacopeia* as a stimulant, anthelmintic and emmenagogue from 1820 to 1905 (6,40). Tansy was widely used for these purposes and for its tonic, diaphoretic, emetic, nervine, analgesic, anti-inflammatory and other activities for problems such as rheumatism, bruises, swellings, dermatologic afflictions, fevers, headaches, diarrhea, sore throat, prevention of pregnancy and some gynecological problems (6-11,35,36,41). Tansy has also been employed as an insect repellent (36,42) and an insecticide (8,35,36). The Micmac Indians used the plant to prevent pregnancy (largely as an abortifacient) and as a diuretic (43).

MATERIALS AND METHODS

Collection and Extraction

The aerial parts of *T. vulgare* L. were collected during the flowering stage from the roadside and railway slopes near Port Williams, Nova Scotia, during Sept. 1979. The material was determined by Dr. M.J. Harvey, Department of Biology, Dalhousie University, and herbarium samples are now on file. The plant material was dried in a forced-air oven at 60 C and ground in a Wiley mill to a coarse powder (0.5 cm). This material (11.7 kg) was placed in a stainless steel tank and macerated in chloroform/methanol (1:1, v/v) for at least 24 hr. The extract was then drained from the tank. This procedure was repeated 3 times; the extracts were combined; and the solvent was removed in vacuo to produce a dark green semisolid mass (456 g). This mass was saponified following AOCS method Ca 6b-53 (44). The nonsaponifiable materials were extracted with ether and the solvent was again removed in vacuo, yielding a yellowish solid (81.4 g).

Thin Layer Chromatography

The nonsaponifiable material obtained was fractionated by preparative thin layer chromatography (TLC) using plates coated with silica gel H. Following visualization procedures, the sterols appeared as a red band ($R_f \approx 0.25$) and the triterpenes as a red-brown

TABLE 1

Gas Chromatographic Relative Retention Times for Tansy Sterols and Triterpenes[a]

Compound	Column			
	SE-30	OV-1	OV-17	OV-225
Sterols				
Cholesterol	1.53	1.57	2.15	–
Campesterol	1.86	1.91	2.65	–
Stigmasterol	2.10	2.02	2.90	–
β-Sitosterol	2.14	2.25	2.96	–
Unknown A	ND[b]	ND	3.08	–
Unknown B	ND	ND	3.14	–
Triterpenes				
β-Amyrin	2.48	–	3.26	6.38
α-Amyrin	2.66	–	3.70	7.39
Ψ-Taraxasterol[c]	2.92	–	4.53	9.60
Taraxasterol	2.92	–	4.55	10.20
Unknown A	3.72	–	5.01	11.07
Unknown B	3.94	–	6.53	12.01
Unknown C	ND	–	7.11	13.60

[a]All figures are for the free alcohols and are recorded as relative retention times (RRT) using 5α-cholestane as the internal standard.

[b]Not detected under the stated operating conditions.

[c]Tentative structural identification.

band ($R_f \cong 0.40$). The material from both bands was recovered by extracting the silica gel with ether and chloroform (45).

The triterpene band was acetylated (45) and further fractionated using silica gel H impregnated with silver nitrate (46-48). The 6 bands present, shown with 2′,7′-dichlorofluorescein (46,48), were numbered according to increasing R_f. Following elution from the plates with ether and chloroform, bands 1, 2 and 6 produced negative Liebermann-Burchard tests and were not studied further. Bands 3, 4 and 5 produced positive tests (49) for triterpenes. To obtain a few mg of 3 triterpene acetates that were at least 80% pure, we repeated the silver nitrate process for bands 3 and 5.

Gas Liquid Chromatography

The sterol fraction was analyzed by gas liquid chromatography (GLC) using SE-30, OV-1 and OV-17 columns, and authentic reference standards (50). The triterpenes were similarly analyzed using SE-30, OV-17 and OV-225 (3% on 100-120 mesh Chromosorb W, H.P.) columns and respective oven temperatures of 290, 275 and 250 C. Relative retention times (RRT) were calculated using 5α-cholestane as the internal reference. Results are recorded in Table 1.

Nuclear Magnetic Resonance Spectroscopy

The NMR spectra were recorded on a Varian HR-220 spectrometer located at the Canadian 220 MHz NMR Centre, Department of Medical Genetics, University of Toronto. Preliminary NMR spectra were recorded on the Varian A-60, T-60, and CFT-20 spectrometers of Dalhousie University, Halifax, Nova Scotia. All spectra were recorded on dilute solutions in deuterated solvents using tetramethylsilane as the internal reference. Results are presented in Table 2.

Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC/MS) studies were performed on a Finnigan-MAT 4000 quadrapole mass spectrometer coupled to an INCOS data system. The column was a high capacity 3% OV-1 flexible quartz capillary (25 m × 0.3 mm id) which was directly interfaced to the source of the mass spectrometer. Source temperatures (indicated) were 260 C for electron impact (EI) MS studies and 230 C for the positive ion chemical ionization (PCI) MS studies. Methane was used as the ionizing gas for the PCI studies at a source pressure of 0.02 torr (indicated), and helium was used as the carrier gas (2 ml/min). The EI studies were determined at 70 eV. Results are presented in Table 3.

Authentic Samples

Authentic samples were purchased as follows: cholestane, β-sitosterol and stigmasterol–Sigma Chemical Co., St. Louis, MO; cholesterol

–Fisher Scientific Co., Montreal, Quebec; campesterol–Applied Science, State College, PA; and α- and β-amyrin–Pfaltz and Bauer, Inc., Stamford, CT. Taraxasterol was a gift from Dr. T.R. Watson, Pharmacy Department, University of Sydney, Australia.

RESULTS AND DISCUSSION

Quantitative TLC indicated that the sterols and triterpenes, respectively, represented about 33 and 32% of the nonsaponifiable fraction.

GLC of the sterol band recovered from the thin layer chromatograms showed 6 sterols present (Table 1). β-Sitosterol, representing 63% of the sterols, was the major one found. Other sterols that could be identified were stigmasterol (22%), campesterol (8%) and cholesterol (5%). Co-injection of the sterol fraction sequentially with each of the authentic sterols produced single, symmetrical peaks, verifying the identity of these 4 sterols in tansy. Traces of 2 additional, unidentified sterols accounted for the remaining 2% of the sterol fraction.

Similar treatment of the triterpene fraction indicated 6 triterpenes present when the SE-30 column was used. However, when we used the OV-17 column, one of the triterpene peaks appeared as a poorly resolved pair, RRT of 4.53 and 4.55. Complete separation was obtained on the OV-225 column, establishing the presence of 7 triterpenes in tansy. The GLC data (Table 1) demonstrated the presence of α-amyrin (59%), β-amyrin (25%), taraxasterol (9%), and a fourth triterpene (4%) tentatively identified as pseudo-taraxasterol. This latter triterpene also appears in *Achillea millefolium* L. (yarrow) as the others do. The remaining triterpenes (3%) were not identified.

The triterpene acetates that were obtained by repeated silver nitrate chromatography, α-amyrin, β-amyrin and taraxasterol, were analyzed by both NMR (Table 2) and GC/MS (Table 3).

The NMR spectra of these 3 triterpene acetates were in keeping with those of similar compounds (51,52). The appearance of a triplet at 5.14 ppm and 5.18 ppm in α- and β-amyrin, respectively, was consistent with an olefinic hydrogen at C-12. The chemical shifts of the acetate group (2.05-2.06 ppm) and of the corresponding methine hydrogen (4.50-4.53 ppm) suggest an equatorial acetate. In C_6D_6 solution, the associated coupling constants (5 and 12 Hz) of the methine hydrogen suggest a slight conformational change, but both were in keeping with 2 vicinal coupling interactions: one axial-axial (J = 12 Hz) and the other axial-equatorial (J = 5 Hz). The fact that no other coupling constants appeared to be present supports the presence of a vicinal gemdimethyl group at C-4.

The spectrum of taraxasterol acetate showed a doublet (1.02, J = 6 Hz) corresponding to the methyl group at the tertiary carbon (C-19)

TABLE 2

Nuclear Magnetic Resonance Assignments for Tansy Triterpene Acetates

Group assignment	α-Amyrin		β-Amyrin		Taraxasterol	
	Identity[a]	Shift[b]	Identity[a]	Shift[b]	Identity[a]	Shift[b]
CH_3–C	3H,s	0.81	6H,s	0.84	9H,s	0.85
CH_3–C	9H,s	0.89	12H,s	0.89	3H,s	0.87
CH_3–C	3H,s	0.93	6H,s	0.98	3H,s	0.93
CH_3–C	3H,s	1.00	–	–	–	–
CH_3–C	3H,s	1.02	–	–	3H,s	1.02
CH_3–C	3H,s	1.08	–	–	–	–
CH_3–CH	–	–	–	–	3H,d[c]	1.02
CH_2–CH=C	2H,m	1.90	2H,m	1.90	–	–
CH_3–CO	3H,s	2.06	3H,s	2.06	3H,s	2.05
CH_3–CH	–	–	–	–	1H,m	2.10
CH_2=C–CH	–	–	–	–	1H,m	2.40
CH–OAC	1H,t[d]	4.53	1H,t[d]	4.53	1H,t[d]	4.50
CH_2=C	–	–	–	–	2H,m	4.60
CH=C	1H,t[e]	5.14	1H,t[f]	5.18	–	–

[a]In $CDCl_3$, number of hydrogens, multiplicity (d=doublet, m=multiplet, s=singlet, t=triplet).

[b](δ), ppm.

[c]J (coupling constant) = 6 Hz.

[d]J = 8 Hz.

[e]J = 3-4 Hz.

[f]J = 4 Hz.

TABLE 3

Relative Intensities in the Mass Spectra of Tansy Triterpene Acetates

Mass-to-charge rates	Electron impact spectra			Chemical ionization spectra		
	α-Amyrin	β-Amyrin	Taraxasterol	α-Amyrin	β-Amyrin	Taraxasterol
468[a]	0.4	0.4	1.9	0.2	0.1	0.2
453	–	–	–	0.1	0.2	0.1
409	–	–	–	63	38	58
408	–	–	1.6	49	31	28
393	–	–	0.8	24	16	25
249	0.7	0.6	6.7	2.3	2.3	3.1
229	–	–	3.6	–	–	–
219	–	–	–	38	34	39
218	100	100	5.0	58	43	9.0
205	–	–	–	60	64	73
204	–	–	14	–	–	–
203	27	51	15	38	49	49
191	7.5	3.4	31	62	69	99
189	44	19	100	37	42	47
175	12	11	23	20	25	20
161	18	8.6	23	20	25	16
149	15	7.2	18	47	47	47
147	23	15	31	21	22	14
137	10	–	11	46	51	35
135	42	18	55	41	39	30
133	32	15	28	23	24	15
123	30	9.2	32	72	66	55
121	38	19	74	43	41	41
109	44	20	81	100	100	100
107	45	23	72	52	35	21

[a]M^+ (molecular ion).

and a broad, 2-proton multiplet centered at 4.60 ppm consistent with an exocyclic vinylic group (C-20(30)).

The EI mass spectra of α- and β-amyrin acetates agreed with published data (46), and the spectrum of taraxasterol acetate was in keeping with predicted fragmentation patterns (53,54).

The PCI mass spectra for the triterpenes were very similar, differing only in the relative abundance of the fragment ions; however, all showed a base peak at m/e 109.

In summary, direct comparison with authentic material by GLC, NMR and GC/MS confirmed the identity of α- and β-amyrins (band 5) and taraxasterol (band 3). Band 4 was a 1:1 mixture of taraxasterol and the substance we are tentatively calling pseudo-taraxasterol. This compound is being analyzed as part of our studies on yarrow, in which it is more prevalent. For this reason, we have not investigated its identity further in this study beyond confirming that it is identical with the compound that occurs in yarrow.

This is apparently the first time that sterols (β-sitosterol, stigmasterol, campesterol and cholesterol) and triterpenes (α-amyrin, β-amyrin, and taraxasterol) have been identified in *T. vulgare*, although the presence of sterols (2,4) and phytosterols or triterpenic alcohols (2-5) have been recorded earlier. No triterpenic diols (4) were identified, but the possibility remains that they could be the compounds observed with the greater RRT. Although the mass spectra of α- and β-amyrins and pseudo-taraxasterol have previously been documented (46,53,54), this appears to be the first recording of the actual NMR of the amyrin acetates and taraxasterol acetate, and of the mass spectrum of taraxasterol acetate.

ACKNOWLEDGMENTS

The authors thank Dr. M.J. Harvey, Associate Professor, Department of Biology, Dalhousie University, and Mr. A. Wilson, Curator of Botany, Nova Scotia Museum, for their help in the collection and determination of the plant material; Dr. T.R. Watson, Professor, Pharmacy Department, University of Sydney, Sydney, Australia, for the sample of taraxasterol; Dr. A.A. Grey, Director, Canadian 220 MHz NMR Centre, Department of Medical Genetics, University of Toronto, for the NMR spectra and his valuable comments; and Editorial Service, Faculty of Medicine, Dalhousie University, for help in preparing this manuscript.

This work was supported by the Medical Research Council of Canada (MA-6448).

REFERENCES

1. Chandler, R.F., Freeman, L., and Hooper, S.N. (1979) J. Ethnopharm. 1, 49-68.

2. Chandler, R.F., and Hooper, S.N., Can. J. Pharm. Sci., in press.
3. Favreau, G., and Mockle, J.A. (1962) Can. Pharm. J. 95, 32-34.
4. Kasprzyk, Z., and Kozierowska, T. (1966) Bull. Acad. Pol. Sci., Ser. Sci. Biol. 14, 645-649.
5. Matthes, H., and Serger, H. (1909) Arch. Pharm. 247, 418-431.
6. Vogel, V.J. (1970) in American Indian Medicine, p. 380, University of Oklahoma Press, Norman, OK.
7. Moerman, D.E. (1977) American Medical Ethnobotany, pp. 188-189, Garland Publishing, New York, NY.
8. Hutchens, A.R., Tretchikoff, N.G., and Tretchikoff, N.K. (1973) Indian Herbalogy of North America, pp. 279-280, Merco, Windsor, Canada.
9. Millspaugh, C.F. (1974) American Medicinal Plants, pp. 338-343, Dover Publications, New York, NY.
10. Grieve, M. (1974) A Modern Herbal, pp. 789-790, Hafner Press, New York, NY.
11. Levy, J. de B. (1966) Herbal Handbook for Everyone, p. 125, Faber and Faber, London.
12. Izvestiya Akademii Nauk Armyanskoi SSR, Biologischeskie Nauki. Armenia, Russia 7, 27 (1954); as cited in Willaman, J.J., and Li, H.L. (1970) Lloydia 33(3A), 81.
13. Rudloff, E. Van, and Underhill, E.W. (1965) Phytochemistry 4, 11-17.
14. Banthorpe, D.V., and Mann, J. (1972). Phytochemistry 11, 2589-2591.
15. Nano, G.M., Bicchi, C., Frattini, C., and Gallino, M. (1979) Planta Med. 35, 270-274.
16. Tetenyi, P., Kaposi, P., and Hethelyi, E. (1975) Phytochemistry 14, 1539-1544.
17. Samek, Z., Holub, M., Grabarczyk, H., Drozdz, B., and Herout, V. (1973) Collec. Czech. Chem. Commun. 38, 1971-1976.
18. Yunusov, A.I., Sidyakin, G.P., and Nigmatullaev, A.M. (1979) Chem. Nat. Compd. USSR 15, 88-89.
19. Wagner, H., Flores, G., and Geissman, T.A. (1972) Phytochemistry 11, 451.
20. Ban'kovskii, A.I., Glyzin, V.I., and Smirnova, L.P. (1978) Farmatsiya 27, 24-28 (Chem. Abstr. 89, 72955a).
21. Banthorpe, D.V., and Wirz-Justice A. (1972) J. Chem. Soc., Perkin Trans. 1, 1769-1772.
22. Schulte, K.E., Ruecker, G., and Dressen, F.P. (1966) Planta Med. 14, 162-170.
23. Hartwell, J.L., and Abbott, B.J. (1969) Adv. Pharmacol. Chemother. 7, 117-209.
24. Hartwell, J.L. (1976) Cancer Treatment Rep. 60, 1031-1067.
25. Miles, D.H., Kokpol, U., Zalkow, L.H., Steindel, S.J., and Nabors, J.B. (1974) J. Pharm. Sci. 63, 613-615.
26. Pettit, G.R. (1977) Biosyn. Prod. Cancer Chemother. 1, 61-96, 177-186.
27. Pettit, G.R., and Cragg, G.M. (1978) Biosyn. Prod. Cancer Chemother. 2, 11-60, 121-126.
28. Pettit, G.R., and Ode, R.H. (1979) Biosyn. Prod. Cancer Chemother. 3, 9-18, 65-124.
29. Chandler, R.F., Hooper, S.N., and Ismail, H.A. (1979) Can. J. Pharm. Sci. 14, 61-62.
30. Subbiah, M.T.R. (1973) Am. J. Clin. Nutr. 26, 219-225.
31. Chaturvedi, A.K., Parmar, S.S., Nigam, S.K., Bhatnagar, S.C., Misra, G., and Sastry, B.V.R. (1976) Pharmacol. Res. Commun. 8, 199-210.
32. Farnsworth, N.R., and Cordell, G.A. (1976) Lloydia 39, 420-424.
33. Benoit, P.S., Fong, H.H.S., Svoboda, G.H., and Farnsworth, N.R. (1976) Lloydia 39, 160-171.
34. Shibata, S. (1977) in New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutic Activity (Wagner, H., and Wolff, P., eds.) pp. 177-196, Springer-Verlag, New York, NY.
35. Krochmal, A., and Krochmal, C. (1973) A Guide to the Medicinal Plants of the United States, pp. 212-213, Quadrangle, New York, NY.
36. Lewis, W.H., and Elvin-Lewis, M.P.F. (1977) Medical Botany. Plants Affecting Man's Health, pp. 58, 84, 370, Wiley, New York, NY.
37. The Pharmacopeia of the United States of America, 6th revision, (1882) pp. 316-317, William Wood and Co., New York, NY.
38. Kingsbury, J.M. (1964) Poisonous Plants of the United States and Canada, p. 437, Prentice-Hall, Englewood Cliffs, NJ.
39. Hardin, J.W., and Arena, J.M. (1974) Human Poisoning from Native and Cultivated Plants, 2nd edn., pp. 151-152, Duke University Press, Durham, NC.
40. Claus, E.P. (1956) Pharmacognosy, 3rd edn., p. 316, Lea and Febiger, Philadelphia, PA.
41. Albert-Puleo, M. (1978) Econ. Bot. 32, 65-74.
42. Hussey, J.S. (1974) Econ. Bot. 28, 311-337.
43. Mechling, W.H. (1959) Anthropologica 8, 239-263.
44. Method Ca 6b-53 (1972) The Official and Tentative Methods of the American Oil Chemists' Society, 3rd edn., American Oil Chemists' Society Champaign, IL.
45. Safe, L.M., Wong, C.J., and Chandler, R.F. (1974) J. Pharm. Sci. 63, 464-466.
46. Madrigal, R.V., Plattner, R.D., and Smith, C.R., Jr. (1975) Lipids 10, 208-213.
47. Vroman, H.E., and Cohen, C.F. (1967) J. Lipid Res. 8, 150-152.
48. Morris, L.J. (1966) J. Lipid Res. 7, 717-732.
49. Fong, H.H.S. (1973) in Experiments in the Pharmaceutical Biological Sciences (Malone, M.H., and McLaughlin, J.L., eds.) pp. 43-51, American Association of Colleges of Pharmacy, Stockton, CA.
50. Hooper, S.N., and Chandler, R.F. (1978) J. Pharm. Sci. 67, 1157-1159.
51. Shamma, M., Glick, R.E., and Mumma, R.O. (1962) J. Org. Chem. 27, 4512-4517.
52. Bhacca, N.S., and Williams, D.H. (1964) Applications of NMR Spectroscopy in Organic Chemistry, pp. 13-41, 77-89, Holden-Day, San Francisco, CA.
53. Budzikiewicz, H., Wilson, J.M., and Djerassi, C. (1963) J. Am. Chem. Soc. 85, 3688-3699.
54. Budzikiewicz, H., Wilson, J.M., and Djerassi, C. (1964) Structure Elucidation of Natural Products by Mass Spectrometry, Vol. II. Steroids, Terpenoids, Sugars and Miscellaneous Classes, pp. 121-140, Holden-Day, San Francisco, CA.

[Received March 23, 1981]

METHODS

A Convenient Method for the Preparation of Asialo-G_{M1}[1]

NORIYUKI KASAI, LAUREL O. SILLERUD and ROBERT K. YU*, *Departments of Neurology and Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06510*

ABSTRACT

A convenient and efficient procedure has been devised for the large-scale preparation of asialo-G_{M1} from bovine brain gangliosides. The procedure relies on the complete desialylation of brain gangliosides, consisting primarily of G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, by mild formic acid hydrolysis (0.1 N, 100 C; 2 hr). Following the hydrolysis step, asialo-G_{M1} can be isolated and purified by Folch partitioning and Iatrobeads column chromatography, with an overall yield of more than 50%.
Lipids 17:107-110, 1982.

INTRODUCTION

Asialo-G_{M1} (gangliotetraosyl ceramide: galactosyl, β 1-3, N-acetylgalactosaminyl, β 1-4, galactosyl, β 1-4, glucosyl, β 1-1, ceramide) is a minor, but important, neutral glycosphingolipid in animal tissues. It has recently been reported that this glycolipid can serve as a cell-surface marker of mouse natural killer cells (2,3) and may be associated with natural cell-mediated cytotoxicity (4). It has also been suggested to be a differentiation antigen of mouse (5) and rat (6,7) thymocytes. Furthermore, this glycolipid appears to be a cell-surface marker of leukemic cells from patients with acute lymphoblastic leukemia (8). The detection of asialo-G_{M1} antibody in sera from patients with Graves' disease and Hashimoto's thyroiditis (9) and systemic lupus erythematosus (10) suggests that this glycolipid may serve as an autoantigen that is involved in the pathogenesis of these diseases. In addition to this, asialo-G_{M1} has been shown to be a precursor of G_{M1b} (11-13), a naturally occurring ganglioside found in rat ascites hepatoma cells (14) and human erythrocytes (15).

Because the concentrations of asialo-G_{M1} in normal tissues are generally extremely low, most investigators prepare this glycolipid from G_{M1} ganglioside by removing the sialic acid residue under mild acid conditions (16,17). The amount of pure G_{M1} is usually limited; therefore, it is impractical to use this approach for the large-scale preparation of asialo-G_{M1}. The procedure devised by Yip and Dain (18), employing bovine brain ganglioside mixtures as the starting material, offers a reasonable alternative. However, the procedure is not efficient for obtaining large amounts of asialo-G_{M1} because considerable breakdown of the neutral gangliotetraosylceramide backbone can occur under the hydrolytic conditions (0.1 N HCl, 100 C; 1 hr) and the use of preparative thin layer chromatography (TLC) for sample isolation is time-consuming. In this paper, we describe a modified procedure for the large-scale preparation of highly purified asialo-G_{M1} from bovine brain gangliosides.

EXPERIMENTAL PROCEDURES

Purification of Bovine Brain Gangliosides

A commercial preparation of crude bovine brain gangliosides (Supelco, Inc., Bellefonte, PA, lot. no. LA 01280) was purified by a scaled-up version of the method of Ledeen et al. (19). Briefly, the ganglioside sample, about 1.5 g, was dissolved in 1,000 ml of chloroform/methanol/water (30:60:8, v/v/v). The solution was applied to a column of DEAE-Sephadex A-25 (bed vol 34 x 3 cm, acetate form). After slow elution (1 ml/min) of the sample solution, the column was eluted with an additional 1,000 ml of the same solvent. Analysis of the above neutral lipid fraction by TLC in the solvent system of chloroform/methanol/0.02% aq. $CaCl_2$ (55:45:10, v/v/v) did not reveal the presence of any gangliosides. This fraction was heavily contaminated with cholesterol, cerebrosides and phosphatides. The acidic lipids, which included gangliosides, were eluted with 1,000 ml of chloroform/methanol/0.8 M sodium acetate (30:60:8, v/v/v). This fraction was reduced to a vol of about 200 ml by evapora-

[1]The ganglioside nomenclature used is that of Svennerholm (1).

tion and treated with 90 ml of 0.1 N NaOH in methanol at 37 C for 1 hr in order to destroy the contaminating acidic phosphatides. The solution was then dialyzed against cold distilled water for 3 days and lyophilized. Final purification of the gangliosides was achieved by silicic acid column chromatography. The lyophilized sample, dissolved in 10 ml of chloroform/methanol (85:15, v/v), was applied to a Unisil column (200-325 mesh, Clarkson, Williamsport, PA, bed vol 27 x 2.3 cm) packed in the same solvent. The contaminating fatty acids (from phosphatides) and sulfatides were eluted from the column with 500 ml of chloroform/methanol (85:15, v/v). Gangliosides were recovered by elution with 1,000 ml of chloroform/methanol (1:2, v/v). After evaporating the solvent, we obtained 380 mg of pure gangliosides. The phosphorus content of this preparation was <0.1% as estimated by the method of Bartlett (20).

Preparation of Asialo-G_{M1}

A portion of the purified ganglioside sample, 178 mg, was dissolved in 18 ml of 0.1 N aq. formic acid in a 50-ml, screw-capped tube. The solution was heated at 100 C for 2 hr and lyophilized. The hydrolyzate was then partitioned in a biphasic system of chloroform/methanol/water (8:4:3, v/v/v) (21). After standing, the upper phase was removed and the lower phase washed with a "theoretical upper phase" consisting of chloroform/methanol/water (3:48:47, v/v/v). The upper phase and the washings were discarded and the lower phase evaporated to dryness to give a crude mixture of neutral glycolipids.

In order to isolate asialo-G_{M1}, the glycolipid sample, dissolved in 3 ml of chloroform/methanol/water (83:16:0.5, v/v/v), was applied to an Iatrobeads (6 RS 8060, Iatron Laboratories, Inc., Tokyo, Japan) column (52 x 1.2 cm) packed in the same solvent mixture (22). The column was eluted with a linear gradient solvent system prepared from a total of 500 ml of chloroform/methanol/water (83:16:0.5 and 55:42:3, v/v/v) followed by 250 ml of the latter solvent. Fractions of 10 ml were collected and the glycolipid elution profile was monitored by silica gel TLC using the solvent system of chloroform/methanol/0.02% aq. $CaCl_2$ (55:45:10, v/v/v). Fractions 35-44 contained only asialo-G_{M1}, and were pooled and evaporated to yield 61 mg of white material, corresponding to a yield of 51%.

Analytical Procedures

The purity of the isolated asialo-G_{M1} was examined by high performance TLC (Silica Gel 60, E. Merck, Darmstadt, West Germany). The plates were developed with the following solvent systems: chloroform/methanol/0.02% aq. $CaCl_2$ (60:40:9, v/v/v), chloroform/methanol/3.5 M ammonium hydroxide (60:35:8, v/v/v) and 1-propanol/conc. ammonium hydroxide/water (70:15:15, v/v/v). Gangliosides were visualized by spraying with resorcinol/HCl (23) followed by heating the covered plate at 95 C on an aluminum block heater (24). For visualization of neutral glycolipids, the plates were sprayed with anthrone/H_2SO_4 reagent followed by heating at 105 C for 10 min.

The molar ratios of the carbohydrate and long-chain base constituents were determined by gas liquid chromatography as their N,O-trifluoroacetyl derivatives (25). The asialo-G_{M1} was also analyzed by ^{13}C-nuclear magnetic resonance (^{13}C NMR) spectrometry. The lipid was run as a 75 mM solution in $[^{12}C]DCl_3$/$[^{12}C]D_3OD/D_2O$ (deuterated phosphate buffer, pH 7.5) (1:2:2, v/v/v) on a Bruker Fourier transform WH 360 spectrometer at 90.55 MHz. The proton decoupled spectrum was obtained at 20 C and the chemical shifts were referenced to the ω-1 carbon of the ceramide set at 23.45 ppm (referenced to tetramethylsilane) to coincide with our previous work with G_{M1} (26).

RESULTS AND DISCUSSION

Bovine brains contain 4 major ganglioside species, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}; together they account for nearly 95% of the total gangliosides (27). Structurally, these gangliosides share the common gangliotetraosylceramide backbone. Hence, they serve as excellent sources for the preparation of asialo-G_{M1}. The commercial preparation of bovine brain gangliosides proves to be a convenient starting material. However, the commercial preparations are generally heavily contaminated by various nonganglioside materials (28). These contaminants can be effectively removed by a scaled-up version of the method of Leedeen et al. (19), as described in this paper. Our results indicated that the particular preparation we used contained only about 25-30% of pure gangliosides. Caution, therefore, must be exercised when using commercial preparations of gangliosides for biochemical and physical studies.

Desialylation of gangliosides can be effected by the mild acid conditions (e.g., 0.1 N HCl, 100 C, 1 hr) as described by Yip and Dain (18). However, considerable partial degradation of the neutral carbohydrate backbone can occur, resulting in a rather low yield (about

20%) of asialo-G_{M1} (Yu, R.K., and Ledeen, R.W., unpublished observations). The use of formic acid instead of HCl offers an alternative because it has been reported that hydrolysis of certain gangliosides in 1 M formic acid at 100 C for 1 hr removes the sialic acid with very little degradation of the oligosaccharide chain (29). Prolonged heating results in the degradation of the oligosaccharide chain (29). In the present study, we chose the even milder hydrolytic conditions of 0.1 N formic acid and 100 C. Initially, on small-scale trial experiments, the heating time varied from 10 min to 3 hr. The products were examined by TLC. The maximal yield of asialo-G_{M1} was achieved after heating for 2 hr. Therefore, the final hydrolytic conditions were chosen as 0.1 N formic acid, 2 hr and 100 C.

Following a Folch partitioning step to remove water-soluble degradation products and the small amounts of unhydrolyzed gangliosides, the neutral glycolipids could be easily fractionated by Iatrobeads column chromatography (22). TLC revealed that fractions 5-7 contained glucocerebroside (wt 1.6 mg), fractions 8-13 contained lactosyl ceramide (wt 5.3 mg), fractions 18-26 contained asialo-G_{M2} (wt 3.2 mg), fractions 35-44 contained asialo-G_{M1} (wt 61 mg), and fractions 45-55 contained a mixture of asialo-G_{M1} and a slow-migrating neutral glycolipid which probably corresponded to a pentahexosyl ceramide (wt 20 mg). A typical thin layer chromatogram of the various fractions is shown in Figure 1. No resorcinol-positive material was present in any of these fractions.

The asialo-G_{M1} is homogeneous as examined by TLC in 3 different solvent systems. Analysis of the carbohydrate and long-chain base constituents revealed the presence of glucose, galactose, N-acetylgalactosamine and long-chain base in the molar ratios of 1.0:2.2:1.2:0.7. No sialic acid peak could be detected. The ^{13}C NMR spectrum shows the presence of only 4 peaks with the following chemical shifts in the anomeric carbon region: 103.34 ppm (*β-D*-glucosyl), 103.77 ppm (inner *β-D*-galactosyl), 104.44 ppm (*β-D*-acetylgalactosaminyl) and 105.90 ppm (terminal *β-D*-galactosyl). No resonance peaks corresponding to sialic acid could be detected. The complete spectrum, which will be published elsewhere (30), is consistent with an asialo-G_{M1} structure. The lipophilic constituents of asialo-G_{M1} were not determined. However, it is reasonable to assume that they consist of stearic acid as the major fatty acid and (4E)-sphingenine and (4E)-eicosasphingenine as the major long-chain bases (31).

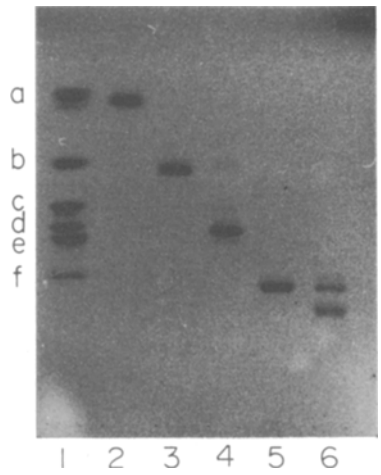


FIG. 1. Thin layer chromatogram of the neutral glycolipids fractionated by Iatrobeads column. The plate was developed with chloroform/methanol/aq. 0.02% $CaCl_2$ (60:40:9, v/v/v) and visualized by anthrone/H_2SO_4 reagent. Lane 1, standard glycolipids (a: glucocerebroside; b: lactosyl ceramide; c: globotriosyl ceramide; d: gangliotriosyl ceramide; e: globoside I; f: asialo-G_{M1}); lane 2, fraction 5-7; lane 3, fraction 8-13; lane 4, fraction 18-26; lane 5, fraction 35-44; lane 6, fraction 45-55.

In summary, we have devised a simple and convenient procedure for the large-scale preparation of highly purified asialo-G_{M1} from commercially available bovine brain gangliosides. The availability of large quantities of this neutral glycolipid should facilitate further studies on the physical, biological and biochemical properties of this molecule.

ACKNOWLEDGMENTS

This study was supported by USPHS grant NS-11853 and grant RG 1289-A-2 from the National Multiple Sclerosis Society.

REFERENCES

1. Svennerholm, L. (1973) J. Neurochem.10, 613-623.
2. Kasai, M., Iwamori, M., Nagai, Y., Okumura, K., and Tada, T. (1980) Eur. J. Immunol. 10, 175-180.
3. Young, W.W., Jr., Hakomori, S., Durdik, J.M. and Henney, C.S. (1980) J. Immunol. 124, 199-203.
4. Schwarting, G.A., and Summers, A. (1980) J. Immunol. 124, 1691-1694.
5. Habu, S., Kasai, M., Nagai, Y., Tamaoki, N., Tada, T., Herzenberg, L.A., and Okumura, K. (1980) J. Immunol. 125, 2284-2288.
6. Momoi, T., Wiegandt, H., Arndt, R., and Thiele, H.-G. (1980) J. Immunol. 125, 2496-2500.
7. Arndt, R., Thiele, H.-G., Hamann, A., Graning,

G., Raedler, A., Momoi, T., and Wiegandt, H. (1981) Eur. J. Immunol. 11, 21-26.
8. Nakahara, K., Ohashi, T., Oda, T., Hirano, T., Kasai, M., Okumura, K., and Tada, T. (1980) New Eng. J. Med. 302, 674-677.
9. Sawada, K., Sakurami, T., Imura, H., Iwamori, M., and Nagai, Y. (1980) Lancet 11, 198.
10. Hirano, T., Hashimoto, H., Shiokawa, Y., Iwamori, M., Nagai, Y., Kasai, M., Ochiai, Y., and Okumura, K. (1980) J. Clin. Invest. 66, 1437-1440.
11. Stoffyn, A., Stoffyn, P., and Yip, M.C.M. (1975) Biochim. Biophys. Acta 409, 97-103.
12. Stoffyn, P., and Stoffyn, A. (1980) Carbohydr. Res. 78, 327-340.
13. Taki, T., Hirabayashi, Y., Ishiwata, Y., Matsumoto, M., and Kojima, K. (1979) Biochim. Biophys. Acta 572, 113-120.
14. Hirabayashi, Y., Taki, Y., and Matsumoto, M. (1979) FEBS Lett. 100, 253-257.
15. Watanabe, K., Powell, M., and Hakomori, S.-I. (1979) J. Biol. Chem. 254, 8223-8229.
16. Naiki, M., Marcus, D.M., and Ledeen, R. (1974) J. Immunol. 113, 84-93.
17. Momoi, T., and Wiegandt, H. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1201-1210.
18. Yip, M.C.M., and Dain, J.A. (1969) Lipids 4, 270-277.
19. Ledeen, R.W., Yu, R.K., and Eng, L.F. (1973) J. Neurochem. 21, 829-839.
20. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
21. Folch, J., Lees, M.B., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
22. Ando, S., Isobe, M., and Nagai, Y. (1976) Biochim. Biophys. Acta 424, 98-105.
23. Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604-611.
24. Ando, S., Chaug, N.-C., and Yu, R.K. (1978) Anal. Biochem. 89, 437-450.
25. Ando, S., and Yamakawa, T. (1971) J. Biochem. (Tokyo) 70, 335-340.
26. Sillerud, L.O., Prestegard, J.H., Yu, R.K., Schafer, D.E., and Konigsberg, W.H. (1978) Biochemistry 17, 2619-2628.
27. Suzuki, K. (1964) Life Sci. 3, 1227-1233.
28. Carter, T.P., Sampugna, J., and Campagnoni, A.T. (1972) Lipids 7, 271-273.
29. Svennerholm, L., Mansson, J.-E., and Li, Y.-T. (1973) J. Biol. Chem. 248, 740-742.
30. Sillerud, L.O., and Yu, R.K. (submitted).
31. Yohe, H.C., Roark, D.E., and Rosenberg, A. (1976) J. Biol. Chem. 251, 7083-7087.

[Received August 17, 1981]

COMMUNICATIONS

Oral Contraceptive and Platelet Lipid Biosynthesis in Female Rats: Dose-Response Relationship

M. CIAVATTI[a], E. DAVENAS[a], G. MICHEL[b] and S. RENAUD[a,*], [a]*INSERM, Unit 63, 22 avenue du Doyen Lépine, 69500 Bron, France;* and [b]*Laboratoire de Biochimie Microbienne, Université Claude Bernard, 69622 Villeurbanne Cedex, France*

ABSTRACT

Female rats were treated with different doses of an oral contraceptive (ethinyl estradiol + lynestrenol) and lipid biosynthesis was studied in blood platelets by acetate incorporation into different fractions separated by thin layer chromatography. A marked increase in lipid biosynthesis was observed, especially in the sterol fractions (cholesterol and lanosterol-dihydrolanosterol). It was dose-dependent, observed after a lag-phase, maximal in 3 days and normalized in 8 days. Thus, the oral contraceptive studied here appears to modify platelet lipid biosynthesis for the entire life of the platelets. *Lipids* 17:111-114, 1982.

INTRODUCTION

It has been reported that treatment by estrogen influences serum lipids (1,2). Recently, we have found that the biosynthesis of platelet lipids in rats was considerably modified by the administration of oral contraceptive, mostly of the estrogen type (3). Although there was an increase in all fractions, the major difference appeared to be at the level of the sterols (cholesterol and lanosterol) (4). The main interest in this finding is that lanosterol markedly potentiates platelet aggregation and blood coagulation (5). Because oral contraceptives have been reported to be associated with increased risks of thromboembolic phenomena (6,7), these risks might be related to the platelet hyperactivity resulting from the modifications induced in platelet biosynthesis of lanosterol.

Under normal conditions, it seems that there is no synthesis of cholesterol in human platelets (8). By contrast, in female rats treated for 3 days by an oral contraceptive, we observed a 5-fold increase in the platelet synthesis of cholesterol and lanosterol + dihydrolanosterol from radioactive acetate (4).

The purpose of this study was to determine the influence of the dose of oral contraceptives on platelet biosynthesis of total lipids and of each fraction. The sequence of the response was also observed to further characterize this somewhat unique effect of oral contraceptives.

MATERIALS AND METHODS

Twelve female Sprague-Dawley rats (180-200 g) were used for each study and each study was repeated twice. The control group (3 rats for each set) received by intubation 0.5 ml olive oil/100 g body wt, whereas the experimental groups (3 rats/group) received the same amount of olive oil containing the different doses of the mixture ethinyl estradiol-lynestrenol. One dose contained 10 μg ethinyl estradiol + 250 μg lynestrenol. Ethinyl estradiol (19-nor-17α-pregna-1,3,5[10]-trien-20-yn-3,17 diol) was obtained from ICN Pharmaceuticals Inc. (Cleveland, OH); lynestrenol (19-nor-17α-pregn-4-en-20-yn-17-ol) was from Organon Co. (Oss, The Netherlands).

Platelets were isolated from blood as previously described (3). From a pool of 3 rats, 4×10^9 platelets were resuspended in 4 ml of incomplete Tyrode solution (8.7 g NaCl, 0.2 g KCl, 0.8 g $NaHCO_3$, 1 g glucose, 0.07 g KH_2PO_4, 1.2 g $MgCl_2$, 2.5 g gelatin and distilled water to 1,000 ml, adjusted to pH 6.8). Platelets were incubated with 100 μl of 0.12 mM sodium [^{14}C]acetate solution (20 μCi) at 37 C for 90 min under normal atmosphere. [U-^{14}C]Acetic acid, sodium salt (sp act 96.8 Ci/mol) was obtained from the Commissariat à l'Energie Atomique (Saclay, France).

After incubation, platelets were washed 3 times with a tyrode solution (pH = 6.8) at 4 C, and platelet lipids were extracted by the method of Folch et al. (9). The extract was evaporated under nitrogen and lipids separated by thin layer chromatography (TLC) on Silica Gel G (Merck) with the solvent of Kunz (10) (22 ml diethyl ether, 0.65 ml methanol, 0.7 ml ethanol, 0.3 ml propionic acid, 0.7 ml acetic

acid, 0.9 ml formic acid, 0.05 ml distilled water [well mixed], 2.0 ml ethyl acetate, 0.8 ml diisopropanol, 20 ml benzene, 14 ml heptane and 19 ml light petroleum [bp below 40° C] as eluent). The radioactive spots were localized by autoradiography and scraped into scintillation vials for the determination of the radioactivity as previously described (3).

Standard error was calculated by the usual method (11). Student's t-test was used to determine the significance of the data.

RESULTS

Effect of Dose of the Contraceptive on Acetate Incorporation into Platelet Lipids

Two series of experiments were performed. In series 1, shown in Figure 1, one dose of contraceptive was administered to groups of animals for 1, 2 or 3 days and the platelets were examined on the fourth day of the experiment.

In series 2, each group of animals received 1, 2 or 3 doses of contraceptive on the first day of the experiment and all the animals were studied on the fourth day.

As shown in Figure 1, the incorporation of [^{14}C]acetate increased with the dose of the contraceptive. The increase in lipid biosynthesis was ca. 100% when 3 doses of contraceptive were given, either together on the first day or spread over a period of 3 days. With one dose only, a small increase could be observed provided the platelets were studied 3 days after the administration. In contrast, there was no increase when platelets were studied 1 day after the administration of a single dose (group B, series 1). Thus, it appears that the response of platelets to an oral contraceptive is dose-dependent and occurs after a lag-phase.

Effect of Time after Administration of Contraceptive

In all experiments, 3 doses of the contraceptive (30 μg ethinyl estradiol + 750 μg lynestrenol) in 0.5 ml olive oil/100 g body wt were given on the first day and platelets were studied after 1, 3, 5 and 8 days. Results obtained are reported in Figure 2. The incorporation was maximal at 3 days, declined at 5 days and was normal at 8 days.

Distribution of [^{14}C] Acetate between the Different Lipid Fractions

In each series of studies, the different labeled fractions (phospholipids, cholesterol, lanosterol + dihydrolanosterol and other lipids) were separated by TLC and counted for radioactivity. The results obtained are shown in Table 1. In all series, the only difference as compared to the controls was in the sterol fractions, i.e., cholesterol, but mostly lanosterol + dihydrolanosterol. In series 3 and 4, it was only in this last fraction that a significant increase could be noted in the contraceptive-treated animals.

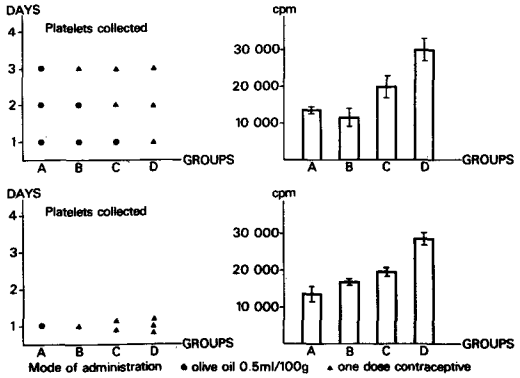


FIG. 1. Influence of the dose and sequence of administration of the contraceptive on acetate incorporation into platelet lipids of female rats. In both series, platelets were collected on the 4th day and results expressed in counts/million (cpm)/10^9 platelets (mean ± SE of 3 studies). Series 1: as indicated in the figure, group A (control) received daily, for 3 days, 0.5 ml olive oil/100 g body wt; group B, olive oil for 2 days and one dose (10 μg ethinyl estradiol + 250 μg lynestrenol) of contraceptive on the 3rd day; group C, olive oil on the 1st day, and one dose of contraceptive on the 2nd and 3rd day; group D, one dose of contraceptive for the first 3 days. Series 2: olive oil (0.5 ml/100 g) alone (group A) or olive oil containing one dose (group B), 2 doses (group C) or 3 doses (group D) of the contraceptive were given on the first day only. In both series, group D was significantly ($p<0.01$) different from group A.

The increase in the biosynthesis of cholesterol, or of the cholesterol precursors, was closely related to the dose of the contraceptive (series 1 and 2). However, the time at which the synthesis was studied after the drug administration was also of crucial importance (series 3-5). After 1 day (series 3), the synthesis of lanosterol was almost doubled. After 3 days (series 2, group D), cholesterol synthesis was 5 times higher and lanosterol synthesis 6 times higher. After 5 days (series 4), the synthesis of lanosterol was still doubled compared to the controls, whereas that of cholesterol was back to normal.

DISCUSSION

We have shown in previous work (4) that the administration of an oral contraceptive markedly modifies the biosynthesis of platelet lipids in female rats. In the platelets of untreated rats, as in the human (8), cholesterol is not synthe-

sized because the step from acetate to mevalonate seems to be inoperative (8).

When female rats are treated with oral contraceptives, mevalonate can apparently be synthesized. This results in the formation of sizable amounts of cholesterol and lanosterol (4). Under these conditions, the incorporation of [^{14}C] acetate into the platelet lipids is markedly increased.

In the contraceptive, the estrogen moiety (ethinyl estradiol) appears to be a factor responsible for the changes in platelet lipid metabolism (4). However, we have observed (4) that ethinyl estradiol added to incubation medium containing [^{14}C] acetate and platelets does not modify platelet lipid metabolism. This is also consistent with the results of the present study which show that several days are needed after the contraceptive administration for obtaining the maximal increase in the lipid biosynthesis. Thus, it seems that estrogen does not act directly on the platelets—at least does not induce immediately the change in lipid metabolism. This is particularly striking with the 3 doses of the contraceptive administered on the first day with a maximal response observed 3 days later (Fig. 2). On the other hand, there was still a sizable effect of the contraceptive 5 days after its administration. Consequently, it seems that platelets were irreversibly modified either by ethinyl estradiol or by one of its metabolites, because the survival time of rat platelets appears to be 4-5 days (12).

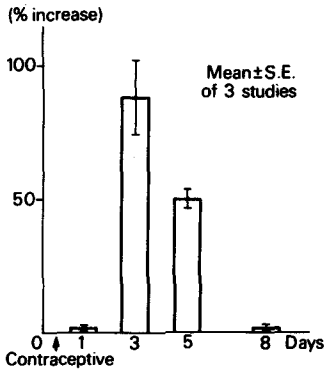


FIG. 2. Time-response of acetate incorporation into platelet lipids with the contraceptive (3 doses) being administered solely on the first day. Platelets were collected 1, 3, 5 and 8 days after the oral contraceptive administration. Results are expressed as % increase in incorporation compared to the controls. The response at days 3 and 5 was significantly different ($p<0.001$) from this at days 1 and 8.

In this study, the effect of the contraceptive disappeared completely after 8 days. This corresponds to the total elimination of the platelets which were initially present at the time of the contraceptive administration. However, the response of lipid biosynthesis did not run parallel to the disappearance of platelets: the effect of the contraceptive started after 1 day and was

TABLE 1

Incorporation of [^{14}C] Acetate into Platelet Lipid Fractions of Female Rats Treated with an Oral Contraceptive[a]

	Groups	Phospholipids	Cholesterol	Lanosterol + dihydrolanosterol	Other lipids
Series 1	A	76.5 ± 5.2	0.6 ± 0.2	6.9 ± 2.4	15.6 ± 4.4
	B	75.5 ± 4.5	0.8 ± 0.1	7.0 ± 2.2	15.8 ± 3.9
	C	60.2 ± 2.6	1.0 ± 0.4	20.2 ± 4.1	16.8 ± 4
	D	43.6 ± 2.1	3.2 ± 1.8	37.8 ± 4.0	14.2 ± 3.7
Series 2	A	73.0 ± 2.4	0.5 ± 0.2	4.1 ± 1.9	21.9 ± 2.0
	B	66.3 ± 2.0	1.1 ± 0.4	10.0 ± 0.6	22.3 ± 1.8
	C	57.0 ± 3.3	2.0 ± 0.4	21.1 ± 3.3	19.7 ± 2.0
	D	53.1 ± 3.9	2.5 ± 0.3	24.8 ± 2.4	19.3 ± 2.2
Series 3	A	65.1 ± 1.5	2.3 ± 1.1	7.5 ± 1.3	24.7 ± 0.8
	D	59.0 ± 2.3	2.2 ± 0.6	12.8 ± 1.3	25.7 ± 3.0
Series 4	A	73.1 ± 5.2	1.4 ± 0.6	5.5 ± 1.7	19.7 ± 4.2
	D	66.8 ± 2.8	1.7 ± 0.6	12.2 ± 2.1	18.9 ± 4.2
Series 5	A	65.1 ± 0.9	1.0 ± 0.3	6.6 ± 1.9	27.0 ± 1.4
	D	70.0 ± 2.1	0.8 ± 0.2	4.3 ± 0.7	24.7 ± 2.0

[a]The results are expressed as % of total lipids (mean ± SE of 3 studies). Series 1 and 2 are similar to those of Fig. 1. In series 3, 4 and 5, the oil alone (group A) or the contraceptive (triple dose, group D) were given on the first day. Platelets were collected 1 (series 3), 5 (series 4) and 8 (series 5) days after the contraceptive administration.

maximal after 3 days.

These results might be explained by one of the following hypotheses: (a) the estrogen acted on the platelets which were present at the time of the administration, with a lag-time, still unexplained, but needed for the estrogen to modify the response of the enzymes involved in the cholesterol biosynthesis; or (b) the estrogen is able to act solely on platelets not yet in the blood, i.e., on the megakaryocytes.

Whatever the mechanism might be, the resulting increase in lanosterol, not in cholesterol, greatly increases the platelet activities involved in both coagulation and aggregation (5). This marked influence of an oral contraceptive on the biosynthesis of sterols in platelets might be related to the predisposition to thromboembolic phenomena associated with this treatment. Nevertheless, the dose-related effect observed in this study would suggest less adverse manifestations with the much smaller dosage of estrogen used at the present time in the oral contraceptives.

ACKNOWLEDGMENTS

The authors are greatly indebted to Organon (Oss, The Netherlands) for supplying the Lynestrenol used in these studies and to Astra-Calve (France), through the courtesy of M.T. Juillet, for financial support.

REFERENCES

1. Stokes, T., and Wynn, V. (1975) Lancet 2, 677-681.
2. Kannel, W.B. (1979) Circulation 60, 490-491.
3. Ciavatti, M., and Renaud, S. (1979) Hormon. Metab. Res. 11, 441-444.
4. Ciavatti, M., Michel, G., and Renaud, S. (1980) Biochim. Biophys. Acta 620, 297-307.
5. Ciavatti, M., Dumont, E., Benoit, C., and Renaud, S. (1980) Science 210, 642-644.
6. Inman, W.H., Vessey, M.P., Westerholm, B., and Engelund, A. (1970) Br. Med. J. 2, 203-209.
7. Markush, R.E., and Seigel, D.G. (1969) Am. J. Publ. Health 59, 418-434.
8. Derksen, A., and Cohen, P. (1973) J. Biol. Chem. 248, 7396-7403.
9. Folch, J.M., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
10. Kunz, F. (1973) Biochim. Biophys. Acta 296, 331-334.
11. Burn, J.H., Finney, D.J., and Godwin, L.L. (1950) in Biological Standardization, pp. 42-43, 2nd Edn., Oxford University Press, Oxford, U.K.
12. Ginsburg, A.D., and Aster, R.H. (1974) Haemostasis 2, 287-293.

[Received October 16, 1981]

Tissue Fatty Acid Changes and Tumor Incidence in C3H Mice Ingesting Cottonseed Oil

IAN J. TINSLEY*, GLENN WILSON and ROBERT R. LOWRY,
Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331

ABSTRACT

The incidence of spontaneous mammary tumors in C3H mice at 35 wk was higher in mice fed rations containing cottonseed oil than in mice fed fats of comparable fatty acid composition. The time to 50% (T_{50}) incidence was also shorter in the first group. The fatty acid composition of tissue lipids from mice fed the cottonseed oil showed changes indicating the presence of cyclopropene fatty acids–higher levels of saturates and lower oleate/stearate and palmitoleate/palmitate ratios. A possible association between the development of a spontaneous mammary tumor in the C3H mouse and the presence of cyclopropene fatty acids in the cottonseed oil is indicated.
Lipids 17:115-117, 1982.

INTRODUCTION

Sterculic and malvalic acids, cyclopropenoid fatty acids present in cottonseed, have been shown to have a synergistic effect on the induction of liver tumors in rainbow trout by aflatoxin B_1 (1). Subsequently, it has been demonstrated that both are primary hepatocarcinogens in this organism (2). Further experiments have shown that hepatocellular carcinomas can be induced in rainbow trout by feeding diets containing either glandless cottonseed kernels or a lightly processed cottonseed oil (3).

At this stage, these cyclopropenoid fatty acids have not been shown to have either a synergistic or direct carcinogenic effect in mammals. A recent study in our laboratory provides evidence suggesting that these components could be affecting the development of a spontaneous mammary tumor in mice. Mice fed rations containing cottonseed oil developed tumors at a higher rate than animals fed rations containing other oils of comparable fatty acid composition. In addition, the fatty acid composition of tissues from mice fed the cottonseed oil suggested the presence of cyclopropenoid fatty acids.

MATERIALS AND METHODS

The development of spontaneous mammary tumors was observed in female C3H mice fed nutritionally adequate (4) semisynthetic rations containing 10% fat. A total of 20 different fats were used including 11 natural fats and oils and mixtures of those fats and oils. These fats and mixtures were selected such that the levels of different fatty acids were not significantly correlated and statistical procedures were used to define the effect of individual fatty acids on the development of the tumor system. The composition of the 20 fats and other experimental details have been described (5). A food-grade cottonseed oil was used in one experimental diet and was combined with butterfat (60:40) in another.

Four animals from each dietary group were sacrificed after 4 months on experiment and samples of subcutaneous fat from the mammary region and the liver were excised. The fatty acid compositions of the triglyceride fraction of the subcutaneous fat and of the liver lipid were determined (6,7). These analyses were intended to monitor feeding efficiency, confirming fatty acid patterns expected from the different fats. Similar samples were taken from animals (6-7 from each dietary group) with tumors upon termination of the experiment.

RESULTS AND DISCUSSION

The major conclusions of the overall study was that the substitution of linoleate for the other fatty acids enhanced tumorigenesis whereas a comparable substitution of stearate tended to reduce tumor development (5). For the purposes of this analysis, short-term tumor incidence data and calculated time to 50% tumor incidence ($\hat{T}_{50}$) are repeated for those fats showing the 6 lowest values for the second parameter (Table 1). By contrast, mice ingesting diets containing 10% tallow and 10% lard showed the longest $\hat{T}_{50}$, i.e., 68.5 and 70.1 wk, respectively. Data are also included for animals fed olive oil or butter–the butter for comparison with those fed the cottonseed/butter mixture. The fatty acid composition of the dietary fat is also given (Table 2).

It is significant that the lowest $\hat{T}_{50}$ values were observed in mice fed cottonseed oil even when it comprised only 5% of the diet (Table 1). The differential associated with feeding cottonseed oil is even more pronounced if one considers tumor incidence at 35 wk. Tumor incidence in animals ingesting the other 13

TABLE 1

Dietary Fat and Tumor Incidence

Dietary fat	$\hat{T}_{50}$ (wk)	Mice with palpable tumor (%)	
		35 wk	45 wk
Cottonseed	56.3	19	30
Butter (0.6), cottonseed (0.4)	56.3	18	18
Safflower	56.6	10	21
Safflower (0.5), olive (0.5)	59.0	5	15
Span	59.5	8	18
Corn	59.9	9	21
Olive	61.3	0	12
Butter	62.0	8	21
Approx. standard error	± 3-5	± 4	± 6

TABLE 2

Fatty Acid Composition[a] (% by wt)

	14:0	16:0	18:0	18:1	18:2	18:3
Cottonseed	0.59	14.9	3.08	22.5	56.5	2.78
Butter (0.6), cottonseed (0.4)	6.52	27.3	7.98	24.6	30.3	1.61
Safflower	–	6.2	2.48	10.4	80.8	
Safflower (0.5), olive (0.5)	–	9.0	2.51	24.3	46.0	
Span (rapeseed–low erucic)	–	3.9	1.92	60.6	22.7	9.48
Corn	–	10.6	2.11	25.8	60.6	1.0
Olive	–	12.9	2.70	75.7	8.12	
Butter	12.7	39.7	13.8	28.3	2.5	

[a]Each value represents the average of results of analyses of 8 diet samples. Standard deviations are less than 10% and are omitted for clarity.

rations was 8% or less at 35 wk with one exception (a ration containing coconut/safflower, 50:50) where a 15% incidence was observed. The data suggest that lauric acid may be a factor in this case. The tumorigenic effect of the cottonseed oil thus appears to be more pronounced than that of corn oil which has a comparable fatty acid composition, and equivalent to safflower oil which contains considerably higher levels of linoleate. The latter contrast may be tenuous, in that there is some evidence that the tumorigenic response to dietary linoleate is not necessarily linear over an extended range (8).

The feeding of cottonseed oil to C3H mice increases the level of both palmitate and stearate in the triglycerides of subcutaneous fat (Table 3). A significant reduction in the oleate/stearate and palmitoleate/palmitic ratios is also observed. Comparison of the means of the 2 subsets, cottonseed-no cottonseed, using the Students' t-test, indicated a highly significant ($p<.001$) effect of the cottonseed oil in all cases. In animals fed the cottonseed/butter mixture, the effect of the cottonseed is over and above that produced by feeding butter alone. These responses were observed in animals fed for 4 months or until tumor development and sacrifice. Similar changes were observed in liver and tumor lipids. Data from animals fed the other 13 rations were consistent with these observations, i.e., lower saturate content and higher monoene/saturate ratios.

These changes are clearly indicative of the presence of cyclopropene fatty acids in the cottonseed oil and reflect the inhibition of the Δ9-desaturase system (9). Such changes in fatty acid composition may be observed before functional changes are produced (10). Analysis of the cottonseed oil by nuclear magnetic resonance (NMR) spectroscopy did not show the characteristic resonance peaks of the cyclopropene protons (11), suggesting that, if the cyclopropene acids were present, the level was considerably less than 1%.

Allen et al. (10) have produced comparable changes in fatty acid composition in chickens, feeding 2.8 mg/kg/day of methyl sterculate for 12 months. If a comparison between species is valid, a 35-g mouse consuming 4 g of diet a day would require ca. 25 ppm of cyclopropene acid in the diet or 250 ppm (.025%) in the oil.

TABLE 3

Saturate and Monoene Fatty Acids from Triglycerides of Mammary Tissue—4-Month Feeding

	Fatty acid level (% by wt)				Monoene/saturate ratios	
	16:0	16:1	18:0	18:1	16:1/16:0	18:1/18:0
Cottonseed	15.6 ± 4.5[a]	3.33 ± .23	3.60 ± .87	34.8 ± 2.5	.23 ± .07	9.9 ± 1.9
Butter (0.6), cottonseed (0.4)	20.3 ± 1.6	6.15 ± 1.10	3.25 ± .77	45.0 ± 1.2	.31 ± .06	14.5 ± 3.8
Safflower	10.3 ± 0.4	3.83 ± .46	1.33 ± .17	21.9 ± 0.5	.37 ± .05	16.7 ± 1.8
Safflower (0.5), olive (0.5)	10.2 ± 0.7	3.63 ± .36	1.32 ± .17	49.7 ± 2.2	.36 ± .03	37.8 ± 3.5
Span	8.73 ± 1.0	3.17 ± .69	1.18 ± .15	65.1 ± 1.5	.37 ± .07	56.1 ± 7.1
Corn	12.4 ± 0.9	4.13 ± .38	1.40 ± .20	36.7 ± 1.0	.34 ± .03	26.7 ± 5.1
Olive	10.2 ± 0.2	4.43 ± .38	1.07 ± .03	76.9 ± 1.3	.43 ± .04	76.1 ± 21
Butter	17.5 ± 1.4	12.3 ± 0.3	1.70 ± .10	60.1 ± 1.7	.67 ± .02	35.4 ± 2.6

[a]Mean and standard deviation.

This level would not be detected by NMR but is substantially lower than that reported for some cottonseed oils (12,13).

It is not valid to conclude from these data that cyclopropene fatty acids enhance the development of mammary tumors in the C3H mouse. The response of the mice to the cottonseed oil is different from those fed comparable oils and tissue fatty acid data are suggestive of the presence of low levels of cyclopropene acids in the cottonseed oil. This possible association certainly warrants further investigation, given the tumorigenic effects observed in trout (3). Observations of Carroll and Khor would also tend to reinforce these conclusions (14). In rats exposed to 7,12-dimethylbenz(α)-anthracene and fed either corn, cottonseed or soybean oils (oils with comparable fatty acid compositions), invariably the trend, though not necessarily significant, is to observe the more tumorigenic effect in animals fed the cottonseed oil.

This analysis also illustrates the complex problem of defining the effects of different fats and oils on tumorigenesis. Not only is it necessary to establish the contribution of the major fatty acids, but it is important to consider some potentially active minor constituents. These could include the cyclopropene acids or food additives (15) and sterols (16).

ACKNOWLEDGMENTS

The research was supported in part by USPHS Grant no. CA20998 from the National Cancer Institute. Oregon State University Agricultural Experiment Station Technical Paper no. 6000.

REFERENCES

1. Sinnhuber, R.O., Lee, D.J., Wales, J.H., Landers, M.K., and Keyl, A.C. (1974) J. Natl. Cancer Inst. 53, 1285-1288.
2. Sinnhuber, R.O., Hendricks, J.D., Putnam, G.B., Wales, J.H., Pawlowski, N.E., Nixon, J.E., and Lee, D.J. (1976) Fed. Proc. 35, 505.
3. Hendricks, J.D., Sinnhuber, R.O., Loveland, P.M., Pawlowski, N.E., and Nixon, J.E. (1980) Science 208, 309-311.
4. National Research Council Nutritional Requirements of Laboratory Animals, p. 47, Washington, DC, National Academy of Sciences.
5. Tinsley, I.J., Schmitz, J.A., and Pierce, D.A. (1981) Cancer Res. 41, 1460-1465.
6. Saddler, J.B., Lowry, R.R., Krueger, H.M., and Tinsley, I.J. (1966) J. Am. Oil Chem. Soc. 43, 321-324.
7. Lowry, R.R., and Tinsley, I.J. (1975) J. Am. Oil Chem. Soc. 52, 298-299.
8. Carroll, K.K., and Hopkins, G.J. (1978) Lipids 14, 155-158.
9. Raju, P.K., and Reiser, R. (1967) J. Biol. Chem. 242, 379-384.
10. Allen, E., Johnson, A.R., Fogerty, A.C., Pearson, J.A., and Shenstone, F.S. (1967) Lipids 2, 419-423.
11. Pawlowski, N.E., Nixon, J.E., and Sinnhuber, R.O. (1972) J. Am. Oil Chem. Soc. 49, 387-392.
12. Nixon, J.E., Sinnhuber, R.O., Lee, D.J., Landers, M.K., and Harr, J.R. (1974) J. Natl. Cancer Inst. 53, 453-458.
13. Bailey, A.V., Harris, J.A., and Skau, E.L. (1966) J. Am. Oil Chem. Soc. 43, 107-110.
14. Carroll, K.K., and Khor, H.T. (1971) Lipids 6, 415-420.
15. McCay, P.B., and King, M.M. (1979) Fed. Proc. 38, 1073.
16. Littman, M.L., Taguchi, T., and Mosbach, E.H. (1966) Cancer Chemother. Rep. 50, 25-45.

[Received October 19, 1981]

ERRATA

The following erratum was received for the article "Effect of Chronic Ingestion of DDT on Physiological and Biochemical Aspects of Fatty Acid Deficiency," by D.A. Sampson, R.E. Pitas and R.G. Jensen (*Lipids* 15:815-822, 1980).

Tinsley and Lowry (3) reported that "... DDT depressed growth in the deficient but not the EFA-supplemented females.... No such interaction was observed with male rats." Their data in Table II, however, show that, in their study, DDT depressed 12-week body weights in EFA-deficient and -supplemented male and female rats. Apparently, data were transposed in their Table II, so that treatment means and standard deviations for the +EFA+ DDT groups and the -EFA-DDT groups were switched for both male and female rats. If one looks at the corrected data, the authors' text is still incorrect, in that it appears that DDT depressed 12-week body weights in both male and female EFA-deficient, but not EFA-supplemented, rats (95% confidence intervals[a] for treatment mean differences for -EFA-DDT rats: males 11-47 g; females 5-23 g). The present study did not confirm a growth-diminishing effect of DDT on EFA-deficient rats; here, DDT did not significantly affect final body weights in either EFA-deficient or EFA-supplemented rats.

---

[a]Calculated as $(\bar{X}_1 - \bar{X}_2) \pm t_{.05,\ 6df} \times \sqrt{2(.5[s_1^2 + s_2^2])/4}$, where $(.5[s_1^2 + s_2^2])$ is taken as the pooled variance.

In "Incorporation into Lipid Classes of Products from Microsomal Desaturation of Isomeric *trans*-Octadecenoic Acids," by T. Riisom and R.T. Holman (*Lipids* 16:647-654, 1981), please note the following corrections to Table 1. The caption should read "Desaturation of *t*-Octadecenoic Acids to *c,c*- and *c,t*-Octadecadienoates by Rat Liver Microsomes[a]" instead of "Desaturation of *t*-Octadecenoic Acids to *c,t*- and *c,t*-Octadecadienoates by Rat Liver Microsomes[a]." Under the heading "% Conversion," the middle subheading should read "*c,t*-18:2," not *c,c*-18:2."

ERRATA

The following erratum was received for the article "Effect of Chronic Ingestion of DDT on Physiological and Biochemical Aspects of Fatty Acid Deficiency," by D.A. Sampson, R.E. Pitas and R.G. Jensen (*Lipids* 15:815-822, 1980).

Tinsley and Lowry (3) reported that "... DDT depressed growth in the deficient but not the EFA-supplemented females.... No such interaction was observed with male rats." Their data in Table II, however, show that, in their study, DDT depressed 12-week body weights in EFA-deficient and -supplemented male and female rats. Apparently, data were transposed in their Table II, so that treatment means and standard deviations for the +EFA+ DDT groups and the -EFA-DDT groups were switched for both male and female rats. If one looks at the corrected data, the authors' text is still incorrect, in that it appears that DDT depressed 12-week body weights in both male and female EFA-deficient, but not EFA-supplemented, rats (95% confidence intervals[a] for treatment mean differences for -EFA-DDT rats: males 11-47 g; females 5-23 g). The present study did not confirm a growth-diminishing effect of DDT on EFA-deficient rats; here, DDT did not significantly affect final body weights in either EFA-deficient or EFA-supplemented rats.

---

[a]Calculated as $(\bar{X}_1 - \bar{X}_2) \pm t_{.05,\ 6df} \times \sqrt{2(.5[s_1^2 + s_2^2])/4}$, where $(.5[s_1^2 + s_2^2])$ is taken as the pooled variance.

In "Incorporation into Lipid Classes of Products from Microsomal Desaturation of Isomeric *trans*-Octadecenoic Acids," by T. Riisom and R.T. Holman (*Lipids* 16:647-654, 1981), please note the following corrections to Table 1. The caption should read "Desaturation of *t*-Octadecenoic Acids to *c,c*- and *c,t*-Octadecadienoates by Rat Liver Microsomes[a]" instead of "Desaturation of *t*-Octadecenoic Acids to *c,t*- and *c,t*-Octadecadienoates by Rat Liver Microsomes[a]." Under the heading "% Conversion," the middle subheading should read "*c,t*-18:2," not *c,c*-18:2."

Different Pools of Esterified Arachidonic Acid in Rabbit Kidney Medulla: Relationship to Ca^{2+}-Stimulated Prostaglandin Biosynthesis

A. ERMAN, R. AZURI and A. RAZ*, *Department of Biochemistry, George S. Wise Center of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel*

ABSTRACT

We investigated the effect of Ca^{2+} ions on renal medulla metabolism of endogenous esterified arachidonic acid in contrast to that of radioactive arachidonate incorporated into medullary lipids. Some striking differences between the release of unlabeled prostaglandin E_2 and of ^{14}C-labeled prostaglandin E_2 and arachidonic acid were seen in incubations in absence or presence of Ca^{2+} ions. These differences indicated that exogenous [^{14}C] arachidonate incubated with medulla slices is incorporated into both Ca^{2+}-sensitive and Ca^{2+}-insensitive lipid pools of esterified arachidonate and furthermore, the Ca^{2+}-sensitive pool is itself heterogeneous and consists of at least 2 functionally different lipid pools of esterified arachidonate. The first Ca^{2+}-sensitive pool is characterized by a higher arachidonate turnover rate and incorporates more rapidly added radioactive arachidonate. The acylhydrolase activity which releases arachidonate from this pool is not efficiently coupled to prostaglandin endoperoxide synthase. In contrast, the second Ca^{2+}-sensitive lipid pool has a slower arachidonate turnover rate and, consequently, a slower incorporation of added ^{14}C-acid. The acylhydrolase activity associated with this pool is more tightly coupled to prostaglandin endoperoxide synthase, so that a higher portion of released arachidonate is converted to prostaglandin E_2. Studies on arachidonic acid metabolic transformations using exogenously radioactive free acid added to tissues should therefore be interpreted with caution because the results obtained may not reflect accurately the metabolic fate of endogenous, lipid-esterified arachidonate which is released and metabolized under physiological conditions in vivo.

Lipids 17:119-123, 1982.

INTRODUCTION

Metabolic studies on the profile of arachidonic acid oxygenated products generated by specific tissues and cells are often done by adding the free radioactive acid to the system investigated. Yet, studies in several laboratories have shown significant differences in the amounts and types of prostaglandin products formed in response to physiological stimuli vs addition of free arachidonate. Recently, studies from our laboratory and others (1,2) have shown that: (a) the peptide hormones bradykinin and angiotensin II elicit in the perfused kidney the release of arachidonate from a unique lipid pool, (b) this lipolytic process is tightly coupled to prostaglandin synthesis so that added free arachidonate is not readily available as a substrate for this pool, and (c) the pattern of prostaglandin products generated after peptide hormone administration is quite different from that obtained from free arachidonate administration.

Ca^{2+} ions have been shown to activate phospholipase A_2 activity and prostaglandin E_2 (PGE_2) generation in renal medullary slices and in medulla subcellular fractions (3,4). As Ca^{2+} ions have been suggested to participate in the intracellular mediation and/or modulation of a variety of physiological stimuli, we investigated the effect of Ca^{2+} ions on renal release of endogenous arachidonate and its conversion to prostaglandin E_2 in comparison to that of exogenous added [^{14}C] arachidonate.

MATERIALS AND METHODS

Incubation of Medulla Slices

Rabbits (male, New Zealand-white-derived, local strain, 2.5-3.0 kg) were sacrificed by air injection into the heart, both kidneys were removed and medulla slices prepared as described elsewhere (3). The slices (0.7-1.0-g) were rinsed twice with 0.1 M Tris-HCl buffer (pH 8.0), and incubated in 2.0 ml of the same buffer in the absence or presence of different concentrations of Ca^{2+} ions or arachidonic acid. Incubations were done at 37 C for 120 min in open air with shaking. The incubation media were changed every 30 min and analyzed for prostaglandin E_2 and arachidonic acid as described previously (3). Briefly, the media were extracted with 2 × 3 vol of ethyl acetate (pH 3.5) and separated by thin layer chromatography (TLC) using the chromatography system chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v/v/v). The prostaglandin E_2 zones were extracted with chloroform/methanol (2:1, v/v), evaporated, dissolved in Tris-HCl buffer and determined by bioassay on rat stomach strip (3).

Incorporation of Radioactive Arachidonic Acid into Renal Medulla Lipids

Medulla slices (5-6 g) were incubated with 7.0 μCi of [1-^{14}C]arachidonic acid for 30 min at 37 C with shaking; 40-45% of the added [^{14}C]arachidonate was incorporated into cellular lipids (phospholipids, 30-35%; neutral lipids, 9-11%). Following the incubation the medium was discarded and the slices rinsed twice with 0.1 M Tris-HCl buffer (pH 8.0) containing bovine serum albumin (2 mg/ml).

MATERIALS

Prostaglandins E_2, D_2, $F_{2\alpha}$ and A_2 were kindly supplied by Drs. U. Axen and J.E. Pike of Upjohn Co. (Kalamazoo, MI). Fatty acid standards were obtained from Supelco (Bellefonte, PA). [1-^{14}C]Arachidonic acid (S.A. 55 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, England). (5,6,8,11,-12,14,15[n]-^{3}H)prostaglandin E_2 (S.A. 86 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Fatty-acid-poor bovine serum albumin was obtained from Calbiochem (San Diego, CA). Other reagents were analytical grade.

RESULTS

Renal medullary slices incubated in 0.1 M tris buffer (pH 8.0) release both arachidonic acid and prostaglandin E_2 (PGE_2). This basal efflux is higher during the initial period of incubation, and apparently reflects metabolic changes following certain injury to the tissue during preparation of the slices and initial equilibration with the incubating medium. After 60 min of incubation and thereafter, the basal rate of PGE_2 generation is already fairly constant at ca. 1.7 μg/g tissue/30 min; this rate is ca. 1/2 of the initial rate (Fig. 1). Calcium ions added to the incubation medium stimulate PGE_2 formation in a dose-dependent manner. In contrast to the basal efflux, the rate of Ca^{2+}-stimulated PGE_2 release is independent of the time following preparation of the slices (Fig. 1, inset). Consequently, at 2 mM concentration, net Ca^{2+}-stimulated PGE_2 release comprised ca. 35% of the total PGE_2 released during the initial incubation period and 65% of the total released at the 91-120-min incubation period (Fig. 1).

Some striking differences were seen when we contrasted basal and Ca^{2+}-stimulated release of unlabeled PGE_2 with the release of [^{14}C]-PGE_2 and [^{14}C]arachidonic acid from slices prelabeled with [1-^{14}C]arachidonate. In comparison to unlabeled PGE_2, a sharper

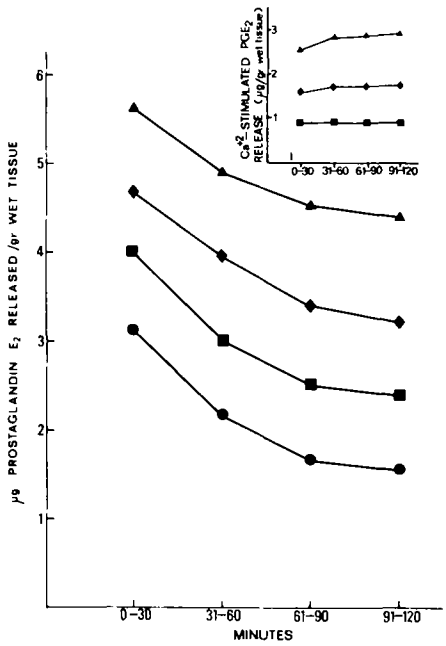


FIG. 1. Effect of Ca^{2+} ions on medullary prostaglandin E_2 release. Medulla slices were incubated in 0.1 M Tris-HCl (pH 8.0) in the absence or presence of different concentrations of $CaCl_2$. The incubation media were replaced with fresh media every 30 min and the media PGE_2 content determined. For details, see Methods section. Values are means of 6 experiments in duplicate. SEM were less than 10%. Ca^{2+} concentrations used were: 0 (●—●), 1 mM (■—■), 2 mM (◆—◆) and 5 mM (▲—▲). Insert: Values for prostaglandin E_2 release at zero Ca^{2+} were subtracted for the corresponding values at the different Ca^{2+} concentrations and the obtained differences, which represent net Ca^{2+}-stimulated prostaglandin E_2 release, were plotted.

decline in the release of [^{14}C]PGE_2 is seen during the time course of incubation (Fig. 2). An initial release of 12,300 cpm/g tissue/30 min is reduced by 70% to 3,900 cpm/g tissue/30 min at the 91-120-min period. Furthermore, compared to unlabeled PGE_2, net Ca^{2+}-stimulated release of [^{14}C]PGE_2 comprises significantly less of the total [^{14}C]PGE_2 release. At 2 mM concentration, net Ca^{2+}-stimulated release of [^{14}C]PGE_2 comprises only 14% of the total released during the initial incubation period and 22% of the total released during the 91-120-min period (Fig. 2). These differences between basal generation and Ca^{2+}-stimulated generation of unlabeled and labeled PGE_2 strongly suggest that: (a) release of ^{14}C-esterified arachidonic from prelabeled slices is originating from both a Ca^{2+}-sensitive and Ca^{2+}-insensitive pool of esterified acid, and

(b) unlabeled and ^{14}C-labeled PGE_2 fractions originate from different pools of precursor arachidonic acid.

As the basal efflux of PGE_2 becomes fairly constant after 90 min of incubation, we used the results from the 91-120-min incubation period to obtain a more precise comparison of the kinetic parameters for the release of labeled and nonlabeled arachidonate and PGE_2. These results (Table 1) show that an increase in Ca^{2+} concentration from 1 mM to 2 mM produces ca. 100% increase in the release of unlabeled arachidonate and PGE_2 but only 39 and 32% increases in the release of [^{14}C]arachidonate and [^{14}C]PGE_2, respectively. We define the term "coupling ratio" as a ratio of generated PGE_2 to released arachidonate. This ratio provides a quantitative measure for the degree of coupling between the arachidonate-releasing lipolysis and the subsequent conversion of arachidonic acid to PGE_2. For Ca^{2+}-stimulated release of unlabeled arachidonate and PGE_2, the coupling ratio is ca. 0.62 whereas for radioactive species, it is significantly lower at 0.26 (Table 1). These results provide additional evidence that Ca^{2+} ions elicit the release of radioactive arachidonate and prostaglandin E_2 and of nonradioactive arachidonate and prostaglandin E_2 from functionally different pools of arachidonic acid.

This conclusion is supported by results of additional experiments with slices prelabeled with [1-^{14}C]arachidonate in which we compared the effect of added unlabeled arachidonic acid on the release of [^{14}C]arachidonate and the generation of radioactive and nonradioactive prostaglandin E_2 (Table 2). Added exogenous arachidonate produced a ca. 3-fold increase in the release of [^{14}C]arachidonate and a 4.5-fold increase in the production of unlabeled prostaglandin E_2. In sharp contrast, release of [^{14}C]prostaglandin E_2 was *not* increased and, in fact, was slightly reduced. Concurrent addition of arachidonate and Ca^{2+} ions yielded additive increase in the efflux of [^{14}C]arachidonate. Production of unlabeled prostaglandin E_2 was also similarly increased whereas release of [^{14}C]prostaglandin E_2 was decreased to a level below that observed with Ca^{2+} ions added alone.

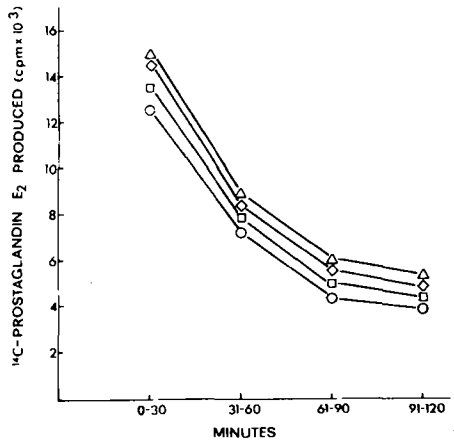


FIG. 2. Effect of Ca^{2+} ions on radioactive prostaglandin E_2 release. Medulla slices (5-6 g) were preincubated with 7.0 μCi of [1-^{14}C]arachidonic acid for 30 min. Following the preincubation, the slices were rinsed twice, incubated as described in Fig. 1 and the media analyzed for [^{14}C]PGE_2. For details, see Methods section. Ca^{2+} concentrations used were: 0 (○—○), 1 mM (□—□), 2 mM (◇—◇) and 5 mM (△—△). For each time point, values obtained at 0 and 1 mM were significantly different at $p > 0.8$. All other values were significantly different at $p > 0.9$ (Student's t-test).

TABLE 1

Ca^{2+}-Stimulated Release of Radioactive and Nonradioactive Arachidonic Acid and Prostaglandin E_2 from Medulla Slices[a]

$CaCl_2$ concentration in incubation medium (mM)	Net release[b]			
	AA (μg)	PGE_2 (μg)	[^{14}C]AA (cpm)	[^{14}C]PGE_2 (cpm)
1	1.40 ± 0.09	0.95 ± 0.08	2980 ± 140	980 ± 60
2	2.80 ± 0.15	1.82 ± 0.13	4130 ± 180	1280 ± 80
Coupling ratio[c]	0.62		0.26	

[a]Medulla slices were prelabeled with [1-^{14}C]arachidonic acid as described in Methods. The slices were then incubated in the absence or presence of $CaCl_2$ (1 or 2 mM) for 120 min, changing the incubation medium every 30 min. Release of radioactive and nonradioactive PGE_2 and arachidonic acid (AA) was determined during the 91-120 min period. Values are mean ± SEM of 3 experiments.

[b]After subtraction of the corresponding values obtained in incubations in the absence of $CaCl_2$.

[c]Ratio of increase in PGE_2 synthesis to increase in arachidonic acid release upon increase in $CaCl_2$ concentration from 1 to 2 mM.

TABLE 2

Release of Radioactive Arachidonate, PGE_2 and Nonradioactive PGE_2 from Medulla Slices[a]

Addition to incubation medium	[^{14}C]Arachidonate (cpm)	[^{14}C]PGE_2 (cpm)	PGE_2 (μg)
	5600	3470	1.47
Arachidonic acid	15000	3160	6.56
$CaCl_2$	11700	4860	4.90
Arachidonic acid + $CaCl_2$	23800	4150	8.32

[a]Slices prelabeled with [1-^{14}C]arachidonic acid were incubated in the presence of either 5 mM $CaCl_2$ or arachidonic acid (50 μg) or with both and the release of PGE_2 and arachidonic acid during the 91-120 min period determined. For details, see Methods. Results are given for a single experiment. Similar results were obtained in a total of 5 such experiments.

DISCUSSION

Numerous studies on the metabolic conversions of arachidonic acid into prostaglandins and other oxygenated products have employed exogenously added radioactive or nonradioactive arachidonic acid as a substrate. These studies have provided valuable data on the various metabolic pathways for arachidonate oxygenation. Recent studies have, however, indicated certain limitations in these investigations, showing that added arachidonate may undergo metabolic transformations which are different from those of endogenous, lipid-esterified arachidonate. Thus, Isakson et al. showed that the perfused rabbit kidney prelabeled with [^{14}C]arachidonic acid released, after bradykinin or angiotensin II stimulation, only radioactive PGE_2 (5). This is in sharp contrast to the demonstrated conversion of added free radioactive arachidonate to PGE_2, $PGF_{2\alpha}$, PGD_2 and HHT during incubations with renal medullary slices or medulla microsomes (6-8). Hseuh and Needleman (9) have shown that the molecular form in which unesterified, radioactive arachidonate is administered (free or albumin-bound) to the perfused kidney or heart has a profound effect on the pattern of its incorporation into individual lipids in specific regions of the organ.

More recently, studies from our laboratory have indicated that, functionally, the molecular form of arachidonate serving as substrate for hormone-induced PGE_2 generation is not the free acid, but rather the esterified acid present in a unique hormone-sensitive lipid pool (2). We have also demonstrated (4) the presence of distinct phospholipase A_2-prostaglandin synthase systems in mitochondria and plasma membrane fractions from rabbit kidney medulla. These subcellular fractions generate, in response to Ca^{2+} ions addition, mainly PGE_2 from endogenous tissue arachidonate whereas added free arachidonate is converted to PGE_2, $PGF_{2\alpha}$, PGD_2 and HHT (4).

These present studies demonstrate that Ca^{2+} ions stimulate release of arachidonic acid from 2 functionally different lipid pools of esterified acid. The first pool is characterized by a higher turnover rate of arachidonate; added free radioactive acid is incorporated mainly into this pool. The second pool has a slower arachidonate turnover rate; added radioactive acid is only slowly incorporated into this pool. A major difference between the 2 lipid pools resides in the extent of metabolic coupling between the amount of released arachidonate and the amount of PGE_2 produced. Arachidonate release from the fast-equilibrating pool is less coupled to subsequent conversion to PGE_2, with a coupling ratio of 0.26 μg PGE_2 formed/μg arachidonate released. In contrast, release of arachidonic acid from the slow-equilibrating pool is more tightly coupled to PGE_2 synthesis with a coupling ratio of 0.62 μg PGE_2 formed/μg arachidonate released (Table 1). The 2 lipid pools of esterified arachidonate are also differentially affected by addition of free arachidonate. In medulla slices prelabeled with [^{14}C]arachidonate, added acid increased the release of [^{14}C]acid from the fast-equilibrating pool whereas production of [^{14}C]PGE_2 was significantly reduced because its generation is not tightly coupled and added free arachidonate effectively competes with released [^{14}C]arachidonate for the same prostaglandin synthease enzyme. At the same time, added free acid does not significantly effect endogenous PGE_2 production from the slow-equilibrating arachidonate pool, indicating that endogenously released acid is preferred over added exogenous acid as a substrate for the prostaglandin synthase coupled to this pool.

These results and conclusions demonstrate significant differences in the metabolism of endogenously esterified arachidonate vs exogenously added free arachidonate in the rate

and amount of conversion to oxygenated products. Therefore, assessment of arachidonate metabolic studies using exogenously administered free acid should be interpreted with care, because the results obtained may differ considerably from those obtained from endogenous, lipid-esterified acid which is released by physiological stimuli in vivo.

REFERENCES

1. Needleman, P., Wyche, A., Bronson, S.D., Holmberg, S., and Morrison, A.R. (1979) J. Biol. Chem. 254, 9772-9777.
2. Schwartzman, M., Liberman, E., and Raz, A. (1981) J. Biol. Chem. 256, 2329-2333.
3. Erman, A., and Raz, A. (1979) Biochem. J. 182, 821-825.
4. Erman, A., and Raz, A. (1981) Biochem. J. 194, 957-961.
5. Isakson, P.C., Raz, A., Denny, S.E., Wyche, A., and Needleman, P. (1977) Prostaglandins 14, 853-871.
6. Crowshaw, K. (1973) Prostaglandins 3, 607-619.
7. Whorton, A.R., Smigel, M., Oates, J.A., and Frölich (1978) Biochim. Biophys. Acta 529, 176-180.
8. Blackwell, G.J., Flower, R.J., and Vane, J.R. (1975) Biochim. Biophys. Acta 398, 178-190.
9. Hseuh, W., and Needleman, P. (1979) Prostaglandins 16, 661-681.

[Received March 30, 1981]

Effects of Ethanol Diets on Cholesterol Content and Phospholipid Acyl Composition of Rat Hepatocytes

THOMAS L. SMITH*, ALISON E. VICKERS, KLAUS BRENDEL and MARY J. GERHART,
Veterans Administration Medical Center, Research Service (151), Tucson, AZ 85723;
and Department of Pharmacology, Arizona Health Sciences Center,
University of Arizona, Tucson, AZ 85724

ABSTRACT

Chronic treatment of adult male rats with ethanol liquid diets resulted in alterations in phospholipid and cholesterol contents as well as the acyl composition of phosphatidylethanolamine (PE), phosphatidylinositol (PI)-phosphatidylserine (PS) mixture, and phosphatidylcholine (PC) of isolated hepatocytes. The influence of ethanol on these lipids was largely dependent on the proportion of dietary fat. Phospholipid and total cholesterol contents were elevated 23 and 27%, respectively, by ethanol when offered in a low-fat diet (5% corn oil). Only the percentage of arachidonic acid from PI-PS was significantly reduced in the low-fat ethanol group. Exposure to a high-fat (34% corn oil) diet in the presence of ethanol for 4-5 weeks resulted in a significant decrease in arachidonate/linoleate ratios of hepatic PE, PS-PI and PC, while total phospholipid content remained constant. In the high-fat, ethanol-treated group, hepatic cholesterol content was increased 2-fold. These results suggest that the level of dietary fat plays an important role in determining the effects of chronic ethanol consumption on hepatic cholesterol content and phospholipid acyl composition.
Lipids 17:124-128, 1982.

INTRODUCTION

Cellular tolerance to the continued presence of ethanol is thought to be due to a change in the physical properties of the lipid bilayer (1,2), which, in turn, is presumably due to alterations in membrane lipid composition (3-5). The effects of chronic ethanol intake on hepatic lipids have been documented in several studies. In general, long-term exposure to ethanol results in an increase in fatty acid saturation with arachidonic/linoleic acid ratios being decreased in both mitochondrial and whole liver preparations (6,7). In addition, increases in liver cholesterol and total phospholipids, i.e., phosphatidylcholine (PC), have been reported (8-10). Except for a few studies, most investigators have administered ethanol in diets containing relatively high fat (>18%). Such high levels per se can influence the net effect of ethanol on various hepatic enzymes (11). Thus, in order to discern the influence of dietary fat content on hepatic lipid changes resulting from chronic ethanol consumption, a direct comparison between high- and low-fat ethanol diets on hepatic cholesterol and phospholipid composition seemed warranted.

MATERIALS AND METHODS

Animal Diets and Tissue Preparation

Male Sprague-Dawley rats (Simonsen Labs, Gilroy, CA) weighing 150-200 g were housed individually and pair-fed nutritionally balanced liquid diets (6), which contained either low-fat (5%) or high-fat (34%) corn oil. Ethanol was substituted isocalorically for sucrose and amounted to 36% of the total caloric intake. Rats consumed these diets for a total of 4-5 weeks. The average daily ethanol intake as measured by calibrated glass feeding tubes (Bio-Serv, Inc., Frenchtown, NJ) was 12 g/kg body wt for both high and low fat dietary groups.

Animals were killed by decapitation, the livers were perfused with collagenase (Type II, Worthington, Freehold, NJ) and parenchymal cells were isolated as described before (12). The freshly isolated hepatocytes were washed several times in 100 ml Krebs-Henseleit buffer, pH 7.4. After centrifugation at 40 x g for 2 min, the cells were resuspended to a final concentration of 15 mg wet wt tissue/ml with 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) (Sigma) which contained: NaCl, 144 mM; KCl, 5 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.5 mM and glucose, 5 mM. Representative aliquots of these suspensions were used for all lipid analyses.

Because the ratios of protein content to mg wet wt tissue were the same for all experimental groups, the results for cholesterol and phospholipid contents are expressed as μg/mg wet wt tissue rather than on a protein basis.

Lipid Analyses

Hepatocyte lipids were extracted in 20 vol $CHCl_3/CH_3OH$ (2:1, v/v) containing 0.02% butylated hydroxytoluene (BHT). The lipid extracts were first partitioned with 0.2 vol 0.01 N HCl followed by 2 additional washes of the lower phase with $CHCl_3/CH_3OH/H_2O$

(3:48:47, v/v) and neutralization. Phosphatidylethanolamine (PE), phosphatidylinositol-phosphatidylserine (PI-PS) and PC were isolated by one-dimensional thin layer chromatography (TLC) on Silica Gel H plates (Applied Science, Gardena, CA) with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25:15:4:2, v/v) (13) containing 0.02% BHT. Lipid spots were visualized by iodine vapors. Aliquots of the lipid extracts representing 1-4 mg of original tissue wt were taken for total phospholipid (14) and cholesterol (15) determinations. In some experiments, fatty acid analyses were performed on individual lipids isolated by TLC. Each lipid spot was scraped directly into screw-capped vials and was methylesterified with boron trifluoride/14% methanol reagent (Supelco, Bellefonte, PA). Fatty acid methyl esters were separated using a GC-Mini 2 gas chromatograph (Shimadzu, Columbia, MD) equipped with a dual flame ionization detector and 6′ × 1/8″ glass column packed with Silar-10C (Applied Science Lab, State College, PA). A C-RIA data processor (Shimadzu) was used to calculate percentages of various fatty acid methyl esters. Retention times of each methyl esters were compared to those of known standards (Nu-Chek-Prep, Elysian, MN). The double bond index was calculated from the sum of (percentage of each unsaturated fatty acid) × (number of double bonds).

RESULTS

Effects of Diets on Fatty Acid Composition of Major Hepatic Phospholipids

The major fatty acids constituting PE in rats fed a low-fat (corn oil) diet were palmitic, stearic, oleic and arachidonic. The inclusion of ethanol into this diet did not significantly alter the fatty acid composition of hepatic PE when compared to low-fat control animals (Table 1). When the fat content was increased to 34%, significant reductions in the proportions of palmitic and oleic acids were observed whereas linoleic acid increased, presumably reflecting the dietary fat source. The addition of ethanol to this high-fat diet resulted in a small increase in the proportion of linoleic acid ($p<0.07$) and a significant decrease in arachidonate. Thus, the arachidonic/linoleic acid ratios were 2.94 for high-fat controls and 1.90 for ethanol-treated rats, respectively. The double bond index (DBI) for PE was significantly reduced in the ethanol-high-fat-treated animals (Table 1).

The main fatty acids of the combined PS-PI phospholipids were stearic and arachidonate (Table 2). In rats given a 34% corn oil diet, only arachidonic acid was increased compared to control low-fat diet. Ethanol significantly reduced the proportion of arachidonate in both high- and low-fat diets. In the high-fat diet, ethanol reduced the arachidonic/linoleic ratio from 9.0 to 5.8. Consumption of both high- and low-fat ethanol diets caused a significant reduction in the DBI of PS-PI.

Table 3 illustrates the effects of low- and high-fat diets on the fatty acid composition of PC. The principal fatty acids of this phospholipid were palmitic, stearic, oleic, linoleic and arachidonic. Ethanol given in a low-fat diet did not significantly alter any of the fatty acids just listed. In control animals that were offered a high-fat diet, the proportions of palmitoleic and oleic acids decreased whereas those of linoleic and arachidonic acids increased. PC arachidonic acid was significantly reduced when ethanol was added to the high-fat diet. Palmitic acid was also reduced in the high-fat ethanol diet group compared to control or ethanol low-fat diets. The high-fat ethanol diet decreased PC arachidonic/linoleic ratio from 1.9 to 1.5. Other PC fatty acids were unaffected by ethanol. Ethanol given either in high- or low-fat diets did not reduce the DBI for PC.

Total Phospholipid and Cholesterol Content

The effects of high- and low-fat ethanol diets on total phospholipid levels, as assessed by total phosphorus analyses, were measured in the isolated hepatocyte preparation. In the low-fat dietary group, total phospholipids tended to increase ($p < 0.08$) when ethanol was added: control values were, 1.67 ± 0.11 μg P/mg wet wt; ethanol, 2.06 ± 0.17 μg P/mg wet wt. Interestingly, the high-fat diet by itself or in conjunction with ethanol had no effect on total phospholipid content. Consumption of both low- and high-fat ethanol diets caused an increase in total cholesterol levels which was much more dramatic in the second diet (Table 4).

DISCUSSION

This study has examined the influence of ethanol given in liquid diets with differing fat content on hepatic cholesterol and phospholipid content as well as phospholipid acyl composition. The fatty acid compositions of the PC, PI-PS, and PE of isolated hepatocytes were essentially unaffected by ethanol given in a low-fat (5%) liquid diet. When the dietary fat content was increased to 34%, ethanol significantly decreased the proportion of arachidonate in all of the phospholipids examined (Tables 1-3). Thus, a decrease in phospholipid

arachidonate/linoleate ratios after ethanol treatment is more apparent when a high-fat diet is employed. Using the same diets, others have reported similar findings in total mitochondrial fatty acids (6). In addition, decreases in arachidonate/linoleate ratios have been demonstrated in guinea pig adrenal (4) and mouse liver (7) mitochondria. Unfortunately, the dietary fat levels were not given in these (4,7) investigations. However, in a recent study of rat liver mitochondrial phospholipids, arachidonate/linoleate ratios were unaltered after chronic treatment with ethanol in a high-fat diet (16). The reasons for the discrepancy between our results and those of the Waring et al. study are not apparent, because both diets used corn oil as the chief fat source.

There are numerous conflicting reports concerning the effects of chronic ethanol intake on total phospholipid levels in liver. Thus, in rats fed a low-fat ethanol diet, total hepatic phospholipid levels have been reported to increase (10), decrease (6) or remain the same (9,17). In addition, phospholipid content has been reported to increase after exposure to high-fat (>20%) ethanol diets (6,18,19). Under our experimental conditions, a small increase in phospholipid levels was achieved

TABLE 1

Effect of Various Liquid Diets on Fatty Acid Composition of PE from Rat Hepatocytes

Fatty acids	Low-fat diet		High-fat diet	
	Control	Ethanol	Control	Ethanol
16:0	19.7 ± 4.8	18.6 ± 4.3	14.9 ± 0.8*	13.0 ± 1.1*
16:1	1.4 ± 0.6	1.5 ± 0.6	0.6 ± 0.3	0.5 ± 0.1
18:0	26.8 ± 2.7	28.9 ± 2.5	30.0 ± 2.0	33.1 ± 2.9
18:1	10.1 ± 0.6	10.5 ± 0.6	6.6 ± 0.3*	7.4 ± 0.4*
18:2	5.1 ± 1.4	5.1 ± 1.0	9.1 ± 1.3*	12.5 ± 1.2*
20:3	1.3 ± 0.7	0.7 ± 0.2	0.3 ± 0.2	0.5 ± 0.1
20:4	27.5 ± 1.3	25.6 ± 2.7	27.0 ± 0.2	23.8 ± 0.4**
22:6	3.9 ± 0.7	3.9 ± 0.8	4.4 ± 0.2	4.1 ± 0.8
Double bond index	159.2 ± 6.7	153.1 ± 8.1	162.0 ± 2.3	154.1 ± 3.4**

Male rats were offered either a low (5%) or high (34%) fat (corn oil) liquid diet with or without ethanol (36%) for 4-5 weeks (see Methods for details).

Values are the mean ± SEM percentages of total fatty acids separated by GLC. PE was isolated by one-dimensional TLC. Results are derived from duplicate samples of 3 animals from each dietary group.

*$p < 0.05$ when compared to low-fat control diet values.

**$p < 0.05$ when compared to high-fat control diet values.

TABLE 2

Effect of Various Liquid Diets on Fatty Acid Composition of PS-PI from Rat Hepatocytes

Fatty acids	Low-fat diet		High-fat diet	
	Control	Ethanol	Control	Ethanol
16:0	8.6 ± 4.0	7.4 ± 2.4	7.4 ± 1.0	6.4 ± 1.0
16:1	0.7 ± 0.3	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.2
18:0	49.0 ± 3.1	53.0 ± 2.9	48.4 ± 2.7	48.7 ± 3.7
18:1	4.5 ± 1.0	4.3 ± 0.6	4.4 ± 0.3	4.4 ± 0.7
18:2	3.0 ± 1.0	2.3 ± 0.6	3.6 ± 0.6	5.0 ± 1.0
20:3	1.6 ± 0.5	23.0 ± 0.3	0.6 ± 0.4	1.8 ± 0.2**
20:4	25.3 ± 0.7	1.7 ± 1.0*	32.5 ± 0.6*	29.0 ± 0.7**
22:6	1.8 ± 0.5	1.7 ± 0.3	1.6 ± 0.2	1.7 ± 0.3
Double bond index	127.9 ± 3.1	116.5 ± 2.2*	145.9 ± 4.5	133.8 ± 2.7**

Results are derived from duplicate samples of 3 animals from each dietary group.

*$p < 0.05$ compared to low-fat control diet values.

**$p < 0.05$ compared to high-fat control diet values.

See Table 1 for experimental conditions.

TABLE 3

Effects of Various Liquid Diets on Fatty Acid Composition of PC from Rat Hepatocytes

Fatty acids	Low-fat diet		High-fat diet	
	Control	Ethanol	Control	Ethanol
16:0	24.1 ± 3.4	24.6 ± 1.9	22.7 ± 0.7	17.9 ± 0.8*
16:1	3.6 ± 1.4	3.1 ± 1.3	0.8 ± 0.2*	0.5 ± 0.2*
18:0	19.1 ± 3.3	20.5 ± 2.8	23.5 ± 1.2	25.9 ± 0.6*
18:1	15.1 ± 1.3	14.2 ± 1.2	5.9 ± 0.2*	6.0 ± 0.4*
18:2	10.4 ± 1.2	10.9 ± 0.7	14.2 ± 1.2*	16.2 ± 0.7*
20:3	1.9 ± 0.4	2.1 ± 0.4	0.8 ± 0.1	1.2 ± 0.5
20:4	19.9 ± 2.0	19.7 ± 0.5	26.5 ± 0.7*	24.9 ± 0.4**
22:6	1.7 ± 0.2	1.6 ± 0.2	1.8 ± 0.2	1.9 ± 1.1
Double bond index	134.9 ± 6.2	133.4 ± 1.8	154.1 ± 11.8	155.9 ± 3.1

Results are derived from duplicate samples of 3 animals from each dietary group.
*$p < 0.05$ compared to low-fat control diet values.
**$p < 0.05$ compared to high-fat control diet values.
See Table 1 for experimental conditions.

TABLE 4

Effect of Chronic Ethanol Consumption on Total Cholesterol Content of Hepatocytes

	μg/mg wet wt ± SEM	
	Low-fat diet	High-fat diet
Controls	4.0 ± 0.2	6.0 ± 0.4
Ethanol	5.1 ± 0.3*	13.7 ± 0.6**

n = 4 separate animals for each dietary group.
*$p<0.05$; **$p < 0.001$.

only in the low-fat ethanol diet. No change in phospholipid levels was observed after treatment with the high-fat ethanol diet. These discrepancies among different laboratories might be attributed, in part, to different methodologies and units of measure, i.e., mg lipid, μg phosphorus, or mg fatty acid methyl esters/tissue wt. For example, if an experimental treatment increased the proportion of longer chain fatty acid acyl groups, the phospholipid weight would be increased proportionally with no increase in the actual number of moles of phospholipid. Quantification of phospholipid levels by measuring inorganic phosphorus, on the other hand, more closely reflects the actual number of moles of phospholipid in a given sample.

Altering the fat content of ethanol liquid diets was observed to have a striking effect on cholesterol accumulation in isolated hepatocytes (Table 4). The most interesting aspect of our data was that total cholesterol more than doubled when ethanol was present in the high-fat diet, whereas in the low-fat diet, the effects of ethanol were only modest. Others have also observed similar increases in rat liver cholesterol after chronic ethanol feeding. Furthermore, both acetyl-CoA and cholesterol synthesis have been shown to increase after chronic high-fat ethanol diets (8,17). The mechanism for these increases is still unclear. However, our results suggest that increased hepatic cholesterol content after chronic ethanol consumption is clearly a function of the amount of corn oil intake.

ACKNOWLEDGMENTS

This research was supported by a Veterans Administration High Priority Alcohol Research Grant and by NIH Grant AM 14977.

REFERENCES

1. Hill, M.W., and Bangham, A.D. (1975) Adv. Exp. Med. Biol. 59, 1-9.
2. Chin, J.H., and Goldstein, D.B. (1977) Science 196, 684-685.
3. Chin, J.H., Parsons, L.M., and Goldstein, D.B. (1978) Biochim. Biophys. Acta 513, 358-363.
4. Sun, G.Y., and Sun, A.Y. (1978) Res. Commun. Chem. Pathol. Pharmacol. 21, 355-358.
5. Starich, G.H., and Reitz, R.C. (1979) Toxicol. Lett. 4, 1-5.
6. Thompson, J.A., and Reitz, R.C. (1978) Lipids 13, 540-550.
7. Miceli, J.N. (1973) Lipids 8, 722-727.
8. Lefevre, A.F., DeCarli, L.M., and Lieber, C.S. (1972) J. Lipid Res. 13, 48-55.
9. Mendenhall, C.L., Bradford, R.H., and Furman, R.H. (1969) Biochim. Biophys. Acta 187, 501-509.
10. Fallon, H.J., Gertman, P.M., and Kemp, E.J. (1969) Biochim. Biophys. Acta. 187, 94-104.
11. Joly, J.G., and Hetu, C. (1977) Can. J. Physiol. Pharmacol. 55, 34-41.
12. Hayes, J.S., and Brendel, K. (1976) Biochem.

Pharmacol. 25, 1495-1500.
13. Smith, T.L., Eichberg, J., and Hauser, G. (1979) Life Sci. 24, 2179-2184.
14. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
15. Rudel, L.L., and Morris, M.D. (1973) J. Lipid Res. 14, 364-366.
16. Waring, A.J., Rottenberg, H., Ohnishi, T., and Rubin, E. (1981) Proc. Natl. Acad. Sci. USA 78, 2582-2586.
17. Forsander, C.A., and Lindros, K.D. (1967) Acta Chem. Scand. 21, 2568-2575.
18. Lieber, C.S., Jones, D.P., and DeCarli, L.M. (1965) J. Clin. Invest. 44, 1009-1021.
19. Goheen, S.C., Larkin, E.C., Manix, M., and Rao, G.A. (1980) Lipids 15, 328-336.

[Received August 20, 1981]

Effects of Zinc Deficiency and Castration on Fatty Acid Composition and Desaturation in Rats

SANDA CLEJAN[a,*], MARIANO CASTRO-MAGANA[b], PLATON J. COLLIPP[b], ERNESTO JONAS[a], and VADDANAHALLY T. MADDAIAH[b], [a]*Nassau County Medical Center, Department of Medicine, Division of Cardiology; and* [b]*Department of Pediatrics and SUNY at Stony Brook, East Meadow, NY 11554*

ABSTRACT

The effects of zinc deficiency and testosterone on fatty acid composition of plasma lipids and microsomes of liver, intestine and testes were studied. The activities of fatty acid desaturase (Δ6 and Δ5) in rat liver and testes were also measured. A significant decrease in the level of arachidonic acid was observed in plasma of normal rats fed the zinc-deficient diet. Castration significantly decreased arachidonic acid but increased 20:3 fatty acid, which is negligible in normal rats. Testosterone and zinc administration restored arachidonic acid to normal values. Zinc deficiency does not significantly change the fatty acid profile in liver, but castration decreased both arachidonic and 22:6 fatty acid. Intestinal mucosal microsomes showed that the predominant fatty acid in this tissue, palmitic acid, is independent of zinc status, whereas polyunsaturated fatty acids 18:2 and 20:4 were decreased by zinc-deficient diet or castration. Zinc deficiency sharply decreased 22:5 fatty acid and to some extent, other polyunsaturated fatty acids in testis microsomes. These changes in fatty acids are in agreement with increased Δ9 desaturation and decreased Δ5 desaturase activity. In testes, both Δ6 and Δ5 desaturase activities are decreased in zinc deficiency. It appears that zinc influences the conversion of linoleic to arachidonic acid, whereas testosterone influences Δ6 desaturase activity. The data suggest that zinc deficiency may be one of the important factors in the causation of polyunsaturated fatty acid deficiency, which, in turn, may induce serum hypertriglyceridemia.
Lipids 17:129-135, 1982.

INTRODUCTION

Recent evidence suggests that zinc (Zn) deficiency causes an increase in the proportion of saturated and monounsaturated fatty acids and a marked reduction of polyunsaturated fatty acids profile of hepatic diglyceride (1). Stearoyl CoA desaturase, which was shown to be a control enzyme for hepatic lipogenesis (2), increased in zinc-deficient and in castrated rats (3).

An interaction between dietary Zn and essential fatty acids (EFA) exists in rats (4,5) as well as in chicks (6), but in chicks, polyunsaturated fatty acids increase the signs of Zn deficiency, whereas in rats, the effect is opposite. Because Zn deficiency with or without testosterone deficiency produces a number of abnormalities in lipid metabolism (1,3), we studied the fatty acid compositions of plasma lipids, liver, intestine and testis microsomes. Because of the changes in saturated, and especially polyunsaturated, fatty acids in plasma and different microsomal membranes, it is important to understand how Zn deficiency affects fatty acids. Therefore, the purpose of this study was to determine if the changes in fatty acid composition in experimental Zn deficiency and castration with and without testosterone correspond to changes in fatty acid desaturation in liver and testis microsomes. Testis was chosen for analysis because of the known relationship between Zn and testicular function (7).

MATERIALS AND METHODS

Experimental Design

Thirty-eight male Sprague-Dawley rats, weighing 90 ± 20 g, were randomly assigned to 6 groups. Castration was performed by mid-line abdominal incision in 22 rats under anesthesia with 3.5% chloral hydrate injected intraperitoneally (ip).

Group 1 consisted of 8 rats which were fed a purified Zn-supplemented diet containing 100 mg/kg Zn as Zn chloride in addition to 15% sucrose, 19.9% egg white, 3% soy flour, RP vitamin mix (providing in mg/kg: thiamin HCl, 10; riboflavin, 10; pyridoxine HCl, 10; Ca pantothenate, 30; niacin, 50; inositol, 50; biotin, 0.4; folacin, 2; cyanocobalamin, 0.03; retinylacetate, 6.9; cholecalciferol, 10.1; and DL-α-tocopherol, 23), 5.0% RP mineral mix without Zn (the mixture supplied the following concentration in mg/kg of salts: $CaHPO_4$, 25; K citrate·H_2O, 74; K_2SO_4, 26; NaCl, 37; MgO, 12; Fe citrate, 3; $MnCO_3$, 1.7; $CuCO_3$, 0.15; CrK $(SO_4)_2 \cdot H_2O$, 0.19; $Na_2Se_2O_3 \cdot 5H_2O$, 0.003), 0.15% DL-methionne, 0.2% choline chloride, 5.0% corn oil, 5.0% lard, 44.6% dextrin and 0.0005% biotin. Group 2 consisted of 8 rats fed a Zn-deficient diet (1.2 ppm Zn) and deionized water ad libitum;

Zn content was the only difference between the 2 diets. Group 3 consisted of 6 castrated rats on Zn-supplemented diet and group 4 contained 6 castrated rats on Zn-deficient diet. Groups 5 and 6 consisted of 5 castrated rats each, which were also fed Zn-supplemented and Zn-deficient diets, respectively, and after 4 weeks, the animals received an injection of testosterone enanthate (20 mg 1M). All the animals were maintained on the diets for 2 more weeks; therefore, the total experimental period was 6 weeks.

Rats on Zn-deficient diets became hypophagic after 2-3 days on the diet. To ensure equicaloric consumption of food, pair-feeding procedures were instituted. Food allowed to the control animals (group 1) and groups 3 and 5 on Zn-supplemented diet was limited to be equal to the amount consumed by the Zn-deficient groups (groups 2, 4 and 6). Animals were housed in stainless steel cages and all materials with which the rats came in contact were stainless steel, glass or plastic. In this period, the rats gained 160 ± 32 g, and the Zn-deficient groups showed clear signs of Zn deficiency (heavily scaled and ridged tail, and loss of hair).

Rats were killed after anesthetization with 3.5% aq. solution of chloral hydrate 1 cc/100 g body wt injected ip. Blood was collected by cardiac puncture for the determination of Zn and fatty acids. Liver and a section of jejunum from all groups and testes from rats in groups 1 and 2 were immediately removed and placed in iced 0.25 M sucrose solution. Zn-deficient diet produced a significant reduction in the Zn content of hair (43%), liver (35%), jejunum (44%) and testicles (30%) compared to the Zn-sufficient groups. Serum Zn was also reduced by 25-32% in the Zn-deficient groups.

Preparation of Microsomes from Liver, Testes and Intestine

Livers were homogenized in 0.33 M sucrose, 0.01 M Tris (pH 7.4) in a Potter-Elvenhjen homogenizer (1 g liver/10 ml). The microsomal 105,000 x g supernatant fractions were isolated from the liver homogenates as described previously (3).

Microsomal membranes from small intestine were harvested by the procedure described by Ray (8). The mucosa was desquamated, scraped (9), and homogenized gently in a medium containing 250 mM sucrose, 0.2 mM EDTA and 2 mM piperazine-N, N^1-bis[2-ethanesulfonic acid) buffer (pH 7.0) using a loose pestle. The homogenate was centrifuged at 8,000 x g for 5 min. The supernatant was then centrifuged at 100,000 x g for 2 hr and the pellet was resuspended in the homogenizing medium.

Testes were excised, decapsulated and pooled (3,4). They were homogenized with 6 vol of 0.15 M KCl, 5 mM $MgCl_2$, 1.5 μM glutathione, 62 mM phosphate buffer (pH 7) and 0.25 M sucrose in 0.1 mM EDTA. The homogenate was centrifuged at 10,000 x g for 20 min, and the supernatant was recentrifuged at 10,000 x g for 1 hr. The pellet was suspended in the homogenizing solution as described by us (3).

Protein contents of the various subcellular fractions were estimated by the method of Lowry et al. (10). Homogenization and centrifugation procedures were done at 0 C.

Determination of Lipid Fatty Acid Composition

Fatty acids in plasma were determined by a method which involved saponification, extraction into hexane and on-column methylation with trimethyl-6α-trifluoro-*m*-tolyl) ammonium hydroxide (11). The fatty acids in microsomal preparations were analyzed by gas liquid chromatography after conversion to methyl esters by refluxing for 2 hr with 6 ml of a solution of 1 ml H_2SO_4 conc/61.5 ml methanol/123 ml benzene. Identification of methyl esters was done with a Hewlett-Packard 5710A gas chromatograph equipped with dual column and dual flame ionization detectors. The stainless-steel column (6 ft x 1/8 in. id) was packed with 80-100 mesh Chromosorb (acid-washed) coated with 20% (w/w) ethylene glycol succinate/2% phosphoric acid. Routine analyses were performed isothermally at 185 C with a carrier gas-flow rate 30 ml/min. Peak identifications were based on relative retention times relative to methyl stearate and by comparison with a known mixture of standard methyl ester. Relative peak areas were measured by a Hewlett-Packard 3380A electronic integrator, as described previously (12).

Fatty Acid Desaturase Assays

The desaturation reactions were done as described previously (3) for steroyl CoA desaturase (Δ9). For Δ6 and Δ5 desaturases, 90 nmol of linoleic acid and 100 nmol of eicosa-8,11,14-trienoic acid were used. [1-^{14}C]-Linoleic acid (50 mCi/mmol) and [1-^{14}C]-eicosa-8,11-14-trienoic acid (61 mCi/mmol) were provided by New England Nuclear, Boston, MA. The solution contained 5 mM $MgCl_2$, 2 mM ATP, 50 mM CoA, 1 mM NADH, and 50 mM potassium phosphate buffer (pH 7.4) in a total vol of 1 ml. This solution was incubated at 37 C for 3 min and the reaction was initiated with the addition of 5 mg of

microsomal protein in 0.2 ml of 0.25 M sucrose. The reactions were terminated by the addition of 2 ml of alcoholic KOH (25% 10 M KOH and 75% ethanol). The reaction mixtures were hydrolyzed at 70 C for 1 hr and titrated to a pH of 3 using bromphenol blue as indicator. The fatty acids were extracted with petroleum ether and methylated as described previously (12). The fatty acid methyl esters were separated by argentation thin layer chromatography (TLC) using a hexane/benzene (50:50) solvent system for Δ9 assay and a toluene/acetone (95:5) system for Δ6 assay. ^{14}C Radioactivity in the substrate and the product was counted in a Packard Model 330 liquid scintillation spectrometer. Recovery of radioactivity by this technique was 80%. For the assay conditions described, the amount of 18:2, or 18:3 or 20:4 fatty acid, respectively, formed was proportional to reaction time for 20 min and the resulting rates were proportional to protein concentration.

RESULTS

Plasma and Microsomal Fatty Acid Composition

The effects of Zn status on the composition of fatty acids was examined by analyzing the lipids in circulation (plasma), in liver, in cells that rapidly turn over (intestine mucosal microsomes) and in cells of the reproductive system (testis) (Tables 1-4). In order to assess interaction of Zn and testosterone, the data were tested for significance by the analysis of variance. Table 1 shows that a significant decrease in the relative levels of 20:4 and fatty acids higher than 20:4 was observed in plasma of rats fed the Zn-deficient diet ($F = 36.71$, $p < 0.001$). Castration, whether associated with Zn restriction or not (groups 3 and 4), showed a 5 times reduction in arachidonic acid. Another important observation is that 20:3 acid, nonexistent in normal rats, appeared in increasing proportion in castrated rats, as well as the proportion of 18:1. The 18:2 was increased significantly in Zn-deficient rats, but sharply decreased in castrated rats without relation to the Zn-deficient diet. Testosterone administration partially returned 20:4 fatty acid to a more normal value, but only administration of testosterone and zinc together had a complete effect on castrated rats. Table 2 shows the results of the fatty acid analysis of the liver microsomal lipid extracts. No differences were found between groups 1 and 2, and therefore, Zn deficiency alone does not change the fatty acid profile in liver, but castration significantly decreased both 20:4 and 22:6 fatty acids. Testosterone administration restored the levels of 20:4 to normal values, and 22:6 over the normal.

The fatty acid compositions of intestinal mucosal microsomes are shown in Table 3. The predominant fatty acids are the saturated palmitic, 16:0, which remains constant independent of the Zn status whereas stearic acid (18:0) and the monoenoic 18:1 both increased in Zn-deficient rats ($F = 382.6$, $p < 0.001$). Among the polyunsaturated fatty acids, 18:2 and 20:4 were significantly decreased by the

TABLE 1

Effect of Zinc Status on the Fatty Acid Composition of Rat Plasma Lipids[a]

Fatty acid (%)	Groups 1 N + Zn (8)[b]	2 N - Zn (8)	3 C + Zn (6)	4 C - Zn (6)	5 C + T + Zn (5)	6 C + T - Zn (5)
16:0	19.5 ± 1.2[c]	20.2 ± 1.4	20.6 ± 1.2	18.8 ± 1.0	19.8 ± 1.0	20.3 ± 1.5
16:1	4.3 ± 0.4	4.8 ± 0.8	9.6 ± 0.4	9.0 ± 0.5	4.5 ± 0.3	4.5 ± 0.2
18:0	8.8 ± 0.9	5.0 ± 0.5	9.5 ± 0.3	6.2 ± 0.2	8.0 ± 0.5	8.7 ± 0.5
18:1	16.2 ± 1.0	18.2 ± 1.0	33.5 ± 1.1[d]	34.4 ± 2.0	15.8 ± 1.4	20.4 ± 1.6
18:2	22.4 ± 1.0	26.5 ± 0.9[d]	9.9 ± 1.4[d]	10.1 ± 0.6	22.0 ± 1.3	23.5 ± 1.4
20:3	0.0	0.0	12.5 ± 0.4[d]	12.3 ± 0.5	0.0	4.2 ± 0.1[d],[e]
20:4	27.5 ± 2.4	22.8 ± 2.1[d]	5.4 ± 1.0[d]	9.2 ± 1.1[d]	28.2 ± 2.1	17.8 ± 0.9[d],[e]
> 20:4	1.3 ± 0.1	0.5 ± 0.1	0.0[d]	0.0	1.0 ± 0.1	0.6 ± 0.0

[a]Fatty acid composition was determined by gas chromatography as described in Materials and Methods. Fatty acids 14:0, 22:4, 22:5 and 22:6 were less than 0.2%.

[b]The number of rats in each group is given in parentheses.

[c]Mean ± SD.

[d]Statistical comparison was by analysis of variance and computation of F ratio. $p < 0.001$ compared to group 1.

[e]$p < 0.001$ compared to group 5.

TABLE 2

Effect of Zinc Status on the Fatty Acid Composition of Rat Liver Microsomes

Fatty acid[a] (%)	Groups 1 N + Zn (8)	2 N - Zn (8)	3 C + Zn (6)	4 C - Zn (6)	5 C + T + Zn (5)	6 C + T - Zn (5)
16:0	19.5 ± 2.1	19.8 ± 2.4	20.4 ± 2.2	20.2 ± 1.9	19.3 ± 1.3	19.8 ± 2.0
16:1	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.2
18:0	16.8 ± 1.0	18.4 ± 0.8	17.7 ± 0.8	17.2 ± 0.6	16.4 ± 0.4	19.9 ± 0.4
18:1	9.2 ± 0.3	7.9 ± 0.2	9.9 ± 0.4	9.8 ± 0.4	9.0 ± 0.1	7.9 ± 0.1
18:2	24.0 ± 1.4	25.6 ± 2.0	26.0 ± 1.6	27.0 ± 1.5	23.5 ± 0.9	24.2 ± 1.0
20:3	0.0	0.0	4.3 ± 0.2	4.5 ± 0.1	0.0	0.0
20:4	20.9 ± 2.1	18.8 ± 0.6	16.8 ± 0.4[b]	16.9 ± 0.6	20.6 ± 0.5	20.8 ± 0.8
22:4	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.0
22:5	1.3 ± 0.2	1.2 ± 0.2	1.1 ± 0.4	1.0 ± 0.3	1.4 ± 0.2	1.0 ± 0.2
22:6	7.1 ± 2.0	7.0 ± 1.4	2.9 ± 0.6[b]	2.5 ± 0.4	8.3 ± 0.9	8.2 ± 0.7

[a]The fatty acid 14:0 was less than 0.1%.

[b]$p < 0.001$ compared to group 1. Statistical comparison was by analysis of variance and computation of F ratio.

TABLE 3

Effect of Zinc Status on the Fatty Acid Composition of Rat Intestine Mucosal Microsomes

Fatty acid (%)	Groups 1 N + Zn (8)	2 N - Zn (8)	3 C + Zn (6)	4 C - Zn (6)	5 C + T + Zn (5)	6 C + T - Zn (5)
14:0	4.6 ± 0.1	3.9 ± 0.3	3.8 ± 0.4	3.7 ± 0.1	3.9 ± 0.2	4.2 ± 0.1
16:0	29.8 ± 2.4	24.2 ± 1.5	30.3 ± 1.8	27.2 ± 2.0	29.2 ± 1.6	29.0 ± 1.8
16:1	3.2 ± 0.4	6.0 ± 0.2[a]	5.4 ± 0.6	6.2 ± 0.2	3.6 ± 0.1	4.4 ± 0.6
18:0	20.2 ± 2.0	28.1 ± 2.1[a]	22.8 ± 0.9	23.4 ± 0.8	20.6 ± 1.4	21.8 ± 1.1
18:1	24.2 ± 1.4	29.6 ± 1.3[a]	30.4 ± 1.6[a]	32.0 ± 1.5[a]	25.0 ± 1.4	24.8 ± 1.2
18:2	13.2 ± 1.0	6.8 ± 0.3[a]	6.3 ± 0.6[a]	6.9 ± 0.5[a]	13.0 ± 0.2	12.1 ± 0.4[b]
20:4	4.0 ± 0.3	1.4 ± 0.1[a]	0.8 ± 0.1[a]	0.6 ± 0.0[a]	4.2 ± 0.1	3.7 ± 0.3[b]
> 20:4	0.8 ± 0.1	0.0	0.0	0.0	0.5 ± 0.0	0.0

[a]$p < 0.001$ compared to group 1, by computation of F ratio.

[b]$p < 0.005$ compared to C + T + Zn group.

Zn-deficient diet. The same changes are seen in castrated rats. Testosterone administration together with Zn corrected the deficiency in polyunsaturated fatty acids. The capacity of testicular microsomes to produce the polyunsaturated 22:5 fatty acid is well known (13). As seen in Table 4, this fatty acid is sharply reduced in Zn-deficient rats ($p < 0.001$). Some decrease in other polyunsaturated fatty acids (20:4 and 22:4) is also evident in Zn-deficient rats. This decrease is compensated by an increase in 18:0, 18:1 and 18:2.

Influence of Zn Deficiency and Castration on Fatty Acid Desaturation

In liver microsomes, Δ6 desaturase activity is similar in noncastrated rats independent of the diet, whereas Δ5 desaturase activity is low in Zn-depleted rats (51.3%) compared to controls. In contrast, castration decreased both Δ6 and Δ5 desaturase activities (Table 5). Testosterone administration corrected the defect in Δ6 but not Δ5 desaturase activities. In vitro oxidative desaturation of stearic acid and linoleic acid in testes is seen in Table 6. Zn deficiency increased Δ9 desaturase activity by 40% but slightly decreased Δ6 desaturase by 25%.

DISCUSSION

Our studies demonstrated that Zn status in the rat causes changes in the fatty acid composition of plasma and tissue microsomal membranes. Polyunsaturated fatty acids decreased and monoenoic acid increased, which

TABLE 4

Effect of Zinc Status on the Fatty Acid Composition of Rat Testes Microsomes

Fatty acid (%)	Groups	
	N + Zn	N - Zn
16:0[a]	40.6 ± 2.2	40.0 ± 2.4
16:1	0.8 ± 0.1	1.9 ± 0.3
18:0	8.2 ± 1.0	12.0 ± 0.6[b]
18:1	10.8 ± 1.2	18.0 ± 1.4[b]
18:2	5.0 ± 0.1	8.8 ± 0.4[b]
20:4	15.0 ± 0.3	12.0 ± 1.0[c]
22:4	2.9 ± 0.4	1.2 ± 0.6[c]
22:5	16.5 ± 0.9	5.9 ± 0.8[b]

[a]The fatty acid 14:0 was less than 0.2%.

[b]$p < 0.001$ compared to N + Zn group; statistical analysis was by Student's t-test.

[c]$p < 0.005$ compared to N + Zn group.

is consistent with the increased Δ9 desaturase activity (3), and the decreased Δ5 desaturase activity found in this study. Castration resulted in the same trend but increased proportion of 18:1 and 20:3 and decreased proportion of 18:2 and 20:4 are consistent with the defect in both Δ6 and Δ5 desaturase activity and increased Δ9 desaturase activity. Testosterone therapy, especially together with Zn, restored the desaturase activities to levels higher than controls. The changes in plasma and microsomal fatty acid compositions were likewise reversed. It is known that decreased Δ5 desaturase activity results in increased 20:3 (8,11,14) but decreased 20:4 fatty acid proportion (15). Our observations, therefore, indicate that the influence of the microsomal fatty acid composition on Δ5 desaturase is a major factor in the control of fatty acid biosynthesis. But, in addition to alterations induced by changes in Δ6, Δ9 and Δ5 desaturase activities, diets, fatty acid elongation, membrane lipid degradation and fatty acid oxidation may be important. This becomes clear from the changes in fatty acids which do not correspond to changes in desaturation. This emphasizes the complexity of the factors that regulate membrane fatty acid composition by hormonal factors.

As seen from our results, testosterone does not correct Δ5 desaturase. Hence, it appears that Zn influences the enzyme system in the conversion of linoleic to arachidonic acid (Δ5 desaturase), whereas testosterone is active on Δ6 desaturase activity. This change in fatty acid desaturation results in alteration in membrane lipid composition in the same way it might be expected by an effect on a common property of the membranes such as fluidity. We have already shown (3) that feeding a Zn-deficient diet to castrated rats decreased phospholipid content and consequently increased the cholesterol-to-phospholipid ratio in liver microsomes. An increase in cholesterol-to-phospholipid ratio also occurred in small intestine and testis microsomes from rats fed the Zn-deficient diet. On the basis of these results, it could be postulated that Zn deficiency alters the lipid composition and fluidity of microsomal membranes and may have a profound effect on every membrane-associated process. Furthermore, Zn is involved in the metabolism of arachidonic acid which is a precursor for prostaglandins. Experiments in progress in our laboratory point to the direction of Zn influence on prostaglandin.

The importance of polyunsaturated acids in the male reproductive system has been well documented (14). Elongation of arachidonic

TABLE 5

In vitro Oxidative Desaturation of Linoleic Acid to γ-Linolenic Acid (Δ6) and 5,8,11-Eicosatrienoic Acid to Arachidonic Acid (Δ5) by Liver Microsomes of Normal and Castrated Rats on Different Zinc Diets[a]

	Δ6 Desaturase activity (nmol/mg protein/15 min)	% of control	Δ5 Desaturase activity (nmol/mg protein/15 min)	% of control
N + Zn (8)	1.48 ± 0.38	100	0.302 ± 0.007	100
N - Zn (8)	0.42 ± 0.07	96.1 ± 4.7	0.155 ± 0.023[b]	51.3
C + Zn (6)	0.57 ± 0.29[b]	38.5 ± 19.9	0.194 ± 0.025[b]	64.2
C - Zn (6)	0.27 ± 0.18[b]	18.2 ± 12.4	0.110 ± 0.030[b,c]	36.4
C + T + Zn (5)	1.74 ± 0.06	117.6 ± 3.8	0.240 ± 0.012	79.5
C + T - Zn (5)	1.47 ± 0.09[d]	99.3 ± 6.2	0.200 ± 0.015	66.2

[a]Microsomal fatty acid desaturase activity was done as described in Materials and Methods.

[b]Statistical comparison was by analysis of variance and computation of F ratio. $p < 0.001$ compared to N + Zn group.

[c]$p < 0.001$ compared to C + Zn group.

[d]$p < 0.005$ compared to C + T + Zn group.

TABLE 6

In vitro Oxidative Desaturation of Stearic Acid to Oleic Acid and of Linoleic to γ-Linolenic Acid by Testes Microsomes of Rats on Zinc-Deficient and Zinc-Supplemented Diet

	N + Zn (8)	N - Zn (8)
Δ9 Desaturase activity (nmol/mg protein/15 min)	4.24 ± 0.10	5.96 ± 0.56[a] (140.6%)
Δ6 Desaturase activity (nmol/mg protein/15 min)	2.72 ± 0.30	2.05 ± 0.30[a] (75.4%)

[a]$p < 0.01$ by Student's t-test.

acid to 22:4 and the Δ4 desaturation to 22:5 occur in liver in a very limited extent, but are important in testis (13), as also demonstrated by our results. The high concentration of Zn in the male genital tract (16) the relationship between hypogonadism as a prominent feature of Zn deficiency (17), and the ability of arachidonic acid to cure testicular atrophy (18) show the importance of studying the synthesis of polyunsaturated fatty acid by testes. Table 4 shows that Zn deficiency provoked a decrease in the relative proportion of 20:4, and especially 22:5, of the testes. On the other hand, atrophy of the testes of Zn-deficient rats has similarities with the atrophy produced by EFA deficiency (18). Hence, a decreased biosynthesis of 20:4 and 22:5 in testes of Zn-deficient animals may be the very factor, or at least one of the principal factors, evoking the typical testicular problems resulting from the Zn-deficient state. Marked changes in testis lipids characterized by decrease in total phospholipids and 22:5 fatty acid were seen also by Bieri and Prival (19). They also reported a moderate increase in arachidonic (20:4) acid similar, but less marked, than those resulting from vitamin E deficiency.

The second objective of this investigation was to show the relationship between fatty acids and the hypertriglyceridemia observed in serum of Zn-deficient rats (1). We found that Zn supplementation returned serum triglycerides to normal levels.

Liver lipids were characterized in Zn deficiency by increased diglycerides, triglycerides and phosphatidylcholines, but by decreased phosphatidylethanolamines (1). Good correlation was found between serum and liver lipids. Huang and Williams (20) showed that the rate of hepatic triglyceride secretion was 2-3 times greater in EFA-deficient rats than in nondeficient controls. The increase in triglyceride secretion as well as a higher level of liver TG as was seen by us in Zn deficiency (1) can be then related to the EFA deficiency induced by Zn as reported in this paper. This will trigger increased lipogenesis and increased mobilization of fatty acid from adipose tissue, explaining the high triglyceride levels in serum. Bettger's experiments (4,6) strongly suggest a physiological interaction between Zn and EFA. Thus, Zn deficiency is responsible for the defect in desaturation which drastically decreases polyunsaturated fatty acid, creating EFA deficiency. EFA deficiency triggers increase of hepatic triglyceride secretion and, by this, hypertriglyceridemia in serum.

REFERENCES

1. Clejan, S., Castro-Magana, M., Chen, S.Y., Maddaiah, V.T., and Collipp, P.J. (1982) Nutr. Res., in press.
2. Jeffcoat, R., Roberts, P.A., Ornesher, J., and James, A.T. (1979) Eur. J. Biochem. 101, 439-445.
3. Clejan, S., Maddaiah, V.T., Castro-Magana, M., and Collipp, P.J. (1981) Lipids 16, 454-460.
4. Bettger, W.J., Reeves, P.G., Moscatelli, E., Reynolds, E.A., and O'Dell, B.L. (1979) J. Nutr. 109, 480-488.
5. O'Dell, B.L., Reynolds, G., and Reeves, P.G. (1977) J. Nutr. 107, 1222-1228.
6. Bettger, W.J., Reeves, P.G., Moscatelli, E.A., Savage, J.E., and O'Dell, B.L. (1980) J. Nutr. 110, 50-58.
7. Lei, K.Y., Abbasi, A., and Prasad, A.S. (1976) Am. J. Physiol. 230, 1730-1732.
8. Ray, T.K. (1978) FEBS Lett. 92, 49-52.
9. Forte, J.G., Ray, T.K., and Poulter, J.L. (1972) J. Appl. Physiol. 32, 714-717.
10. Lowry, O.M., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
11. Gerhart, K.O., and Gehrke, C.W. (1977) J. Chromatogr. 143, 335-346.
12. Clejan, S., Collipp, P.J., and Maddaiah, V.T. (1980) Arch. Biochem. Biophys. 203, 744-752.
13. Davis, J.T., and Coniglio, J.G. (1966) J. Biol. Chem. 241, 610-612.
14. Conigliou, G. (1977) The Testes (Johnson, A.D., and Gomes, W.R., eds.) Vol. 4, pp. 432-445, Academic Press, New York, NY.
15. Brenner, R.R.(1974) Mol. Cell. Biochem. 3, 41-52.
16. Hidiroglou, M., Williams, O.J., Ho, S.K., and Tryphonas, L. (1979) Am. J. Vet. Res. 40,

103-107.
17. Underwood, E.J. (1977) Trace Elements in Humans and Animal Nutrition, pp. 214-216, Academic Press, New York, NY.
18. Schoffing, K., Federlin, K., Schmitt, W., and Pfeiffer, T. (1967) Acta Endocrinol. 54, 335-346.
19. Bieri, J.G., and Prival, E. (1965) Comp. Biochem. Physiol. 15, 275-282.
20. Huang, M.T., and Williams, A.A. (1980) Am. J. Physiol. 238, E499-E505.

[Received August 6, 1981]

Rates of Depletion of Linoleic Acid from Fat Depots of Selected Lines of Mice Differing in Growth Rate and Adiposity

E.J. EISEN*, A.L. CARTWRIGHT, K.M. WELLER[1] and K.J. SMITH[2],
Department of Animal Science, North Carolina State University, Raleigh, NC 27650

ABSTRACT

Rates of depletion and half-lives of linoleic acid from epididymal, subcutaneous and retroperitoneal fat pads and the residual body were compared among 5 genetically diverse strains of mice: M16, a polygenic obese line developed by selection for high postweaning gain; ICR, a control line from which M16 was derived; H_6, a nonobese strain selected for large 6-week body weight; L_6, selected for small 6-week body weight; and C_2, a control line from which H_6 and L_6 were initiated. Rates of depletion and half-lives of linoleic acid were obtained on a fat-free diet following an enrichment period of feeding a diet high in linoleic acid. The M16 mice have an increased capability of synthesizing fat from carbohydrates as shown by a continued increase in fat depot weights when fed the fat-free diet. The 4 other lines showed no subsequent increase in fat depot weights on the fat-free diet. Rates of depletion of linoleic acid were significantly different among lines in each of the 4 depots. Ranking of lines for depletion rates was similar among the 3 discrete depots, but a more rapid rate of depletion was observed in subcutaneous and retroperitoneal fat depots than in the epididymal fat depot. Rates of depletion in line M16 were slower than in the ICR control line. Line H_6 had a slower rate of depletion than line L_6. Line L_6 deviated more from the C_2 control than did line H_6, indicating an asymmetric correlated selection response. The decreased depletion rate of linoleic acid in fat tissue of M16 and H_6 mice suggests the possibility that the turnover rates of fatty acids have been reduced in these lines as a result of a reduction in lipolytic activity. The increased depletion rate of linoleic acid in L_6 mice suggests that selection for small body size has substantially increased the rate of fat turnover. The experiment demonstrates that genetic differences among lines in fat turnover have accrued as correlated responses to selection for growth rate.
Lipids 17:136-148, 1982.

Selection for fast and slow growth rate in laboratory mice often leads to positively correlated responses in adiposity (1,2). Fast growing lines typically become moderately obese (3,4), but exceptions do occur (5,6). Correlated changes in fat deposition have been repeated sufficiently to indicate that they are a consequence of pleiotropy and not transient effects of linkage disequilibrium or fortuitous effects of genetic drift. The pleiotropic effects of many groups of genes are likely to be involved, each group mediating different aspects of the development of obesity.

Obesity in rapidly growing lines is characterized by increases in adipocyte size and number (7-9). Selection for rapid growth under ad libitum feeding results in an increased appetite, thus supplying metabolizable energy above maintenance requirements that is deposited primarily as fat in older animals (10). Apart from the increase in appetite, the higher fat deposition is due to increased fat synthesis on a limited feed intake. Mice which had been selected for rapid growth (M16) were fed a maintenance diet during the period of expected rapid juvenile gain from 4 to 6 weeks of age (8). The selected mice which were fed the restricted diet had a 77% higher body fat percentage than ad-libitum-fed unselected mice of similar body weight (≅ 30 g) and age (6 weeks). These results suggest that selection for rapid gain has increased the capacity for lipogenesis. Limited evidence that lipogenesis has been increased in the M16 line is indicated by elevated levels of serum insulin and cholesterol and increased activity of fatty acid synthetase in the liver and lipoprotein lipase in the epididymal fat pad (11). Another mechanism which may contribute to the increased fat deposition is a decrease in rate of fat turnover.

This study focuses on fatty acid turnover in 5 genetically diverse strains of mice, including 2 rapidly growing lines and 1 slow growing line. Several approaches have been proposed to measure rate of depletion or half-life of a fatty acid (12-19). The procedure chosen was the in vivo rate of linoleic acid depletion obtained on a fat-free diet following an enrichment period of feeding a diet high in linoleic acid (12). As linoleic acid is not synthesized by the mouse, depletion curves of linoleic acid in fat depots may provide a relative estimate of overall fat turnover. Specific objectives were to measure rates of depletion of linoleic acid from the epididymal, subcutaneous and retroperitoneal fat pads and the residual body in 5 lines of mice differing in growth rate and adiposity as a consequence of selection. The proposed

[1]Present address: School of Science, Purdue University, West Lafayette, IN 47907.

[2]Present address: College of Veterinary Medicine, Cornell University, Ithaca, NY 14853.

hypothesis is that a line selected for rapid growth and showing a positively correlated response in percentage body fat has a slower rate of linoleic acid depletion than an unselected control or a line selected for slow growth.

MATERIALS AND METHODS

Genetic Stocks

Male mice were sampled from the following 5 lines: M16, developed by long-term selection for high 3- to 6-week postweaning gain (20); ICR, the control line from which mice were originally sampled to initiate selection in M16; H_6 and L_6, developed by long-term selection for high and low 6-week body weight (21); C_2, a replicate of the foundation population from which H_6 and L_6 were originally started. Of the 2 large lines, M16 is moderately obese whereas H_6 is not obese (22). M16 mice exhibit a hypertrophic-hyperplastic form of obesity (7,8,23). Line H_6 has an increased number of adipocytes in the epididymal fat pad compared to the C_2 control (23), suggesting that H_6 mice may have a propensity toward adiposity. Line L_6 does not show a consistently correlated response in percentage body fat (6), and its adipose cellularity has not been investigated.

Experimental Design

Litters were standardized at one day of age to 6 pups in L_6 and 8 pups in the other lines to minimize postnatal maternal differences in competition for nutrients. Mice were weaned at 3 weeks of age, caged singly and fed coarsely ground Purina Laboratory Chow until they were placed on the enrichment diet. Only male mice were used in the experiment. Mice were housed in a laboratory with artificial light from 0700 to 1900 hours, with temperature at 22 ± 1 C and relative humidity of 50-60%.

The enrichment phase consisted of feeding 3- to 4-week-old mice for 27 days a diet which was high in linoleic acid. The linoleic acid diet was formulated by adding 15% safflower oil (Hollywood Health Foods, Los Angeles, CA) by wt to a fat-free diet (Table 1). Linoleic acid content of safflower oil varies from 76 to 79% (24). At the end of the enrichment phase (day 27), mice were bathed in baby shampoo to remove any particles of the linoleic acid diet adhering to their bodies. During the depletion phase, mice were fed a fat-free diet (Table 1) for 33 days. Feed was offered ad libitum in glass jars with a wire mesh screen placed over the feed to reduce spillage. Feed jars were changed every 2-4 days, at which time feed consumption was recorded. Mice were weighed every 6-8 days.

TABLE 1

Fat-Free Diet

Ingredients	g/kg
Casein[a]	207
Fiber[b]	161
Starch[c]	196
Sucrose	196
Glucose	180
Salt mix[d]	39
Vitamin-mix[e]	21

[a]Vitamin-free casein, U.S. Biochemical Corp., Cleveland, OH.

[b]Celufil, nonnutritive bulk, U.S. Biochemical Corp., Cleveland, OH.

[c]Corn starch, washed, dried and ground to prevent dust.

[d]USP XIV mixture, U.S. Biochemical Corp., Cleveland, OH.

[e]Vitamin supplement, catalog #23430, U.S. Biochemical Corp., Cleveland, OH.

Approximately 5 mice (range 4-7) within each line were killed with ether every 2-6 days during both the enrichment and depletion phases. Epididymal, retroperitoneal and subcutaneous fat pads were excised immediately, placed in a tared glass scintillation vial, weighed and frozen at -18 C until they were analyzed for fatty acids. The gastrointestinal tract was excised, flushed with water to remove ingesta, blotted and replaced in the body cavity. Empty body weight was determined and the carcass was frozen until analysis.

The carcass was lyophilized for 7 days and weight of the dry body determined. The carcass was then ground following the procedure described by Eisen and Leatherwood (25). Gross energy content of the empty body, consisting of the sum of carcass energy and estimates of energy content of the excised fat pads, was determined by bomb calorimetry on a Parr Adiabatic Calorimeter Model 1241.

Analysis of Fatty Acids

Total lipids were extracted from samples of the fat pads to be representative of the triglycerides from these tissues. Fat pads (0.5 g or less) were minced and extracted overnight on an orbital shaker with 10 ml of chloroform/methanol (1:1, v/v). Fat pads were extracted 2 additional times with 5 ml and 7.5 ml of the chloroform methanol mixture. All of the filtered extracts were pooled in a separatory funnel. The extract was washed with 6 ml 0.1 M KCl, and the triglyceride phase was collected and dried under a stream of nitrogen and

stored in a vacuum with a desiccant. Triglycerides were weighed and their density determined with calibrated pipettes.

Analysis of fatty acids in the carcass was based on the composition of neutral fats rather than total lipids because of the higher phospholipid content in the whole body. Triglycerides were extracted from 0.5-g samples of dried, ground carcass using the procedure of Dole (26). One ml of an internal standard of pentadecanoic fatty acid (15:0) in heptane (10 mg/ml) was added at the beginning of the extraction procedure.

Quantitative measurement of fatty acids was based on the ratio of an added internal standard fatty acid to that of the sample fatty acids. Gas liquid chromatography (GLC) was used to separate the methyl esters of the individual fatty acids.

Methyl esters of the fatty acids were prepared in the presence of the internal standard with a transesterification procedure. Accurately weighed quantities of triglycerides (≅ 50 mg) in 1.0 ml of methyl chloroform/toluene (2:8, v/v) were mixed with 2.0 ml of anhydrous methanolic HCl (5%). A water scavenger of 0.5 ml of 2,2-dimethoxypropane was added and the mixture was sealed in screw-capped vials. The reaction was complete after overnight incubation at room temperature. Five ml of water and 5 ml of petroleum ether were added to the sample. The petroleum ether layer was removed and the extraction was repeated twice. The pooled fractions of petroleum ether extracts were dried with anhydrous ammonium sulfate and anhydrous sodium bicarbonate (4:1). The filtered extract was evaporated with a stream of nitrogen, and the methyl esters of fatty acids were dissolved in 1.0 ml heptane and stored in septum-sealed and crimped vials.

Methyl esters of the fatty acids were separated by GLC on a 15% DEGS column (Supelco) at 180 C and were quantitated by integration of peak areas and comparison to the 15:0 fatty acid internal standard. The following fatty acids were assayed: myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1) and linoleic (18:2). The number to the left of the colon indicates the number of carbon atoms in the fatty acids and the number to the right indicates the number of double bonds.

Statistical Analysis

Data from the enrichment and depletion feeding periods were analyzed separately. Response variables were analyzed in factorial analyses of variance that included the effects of line, time (days), line by time and an error term (27). Age on the day that the animals were weighed or killed was included as a covariate in the model when it reached statistical significance at $p<.05$.

Time trends in the response variables were analyzed by linear regression procedures (27). Fatty acid weights were transformed to natural logarithms (ln) to remove nonlinear trends. The logarithmic weight of each fatty acid within each fat depot was analyzed by multiple linear regression using time in days and ln weight of the fat pad as independent variables. Ln weight of the dry, residual body was used as an independent variable for the fatty acids extracted from that source. Ln depot weight was included in the analysis to adjust for changes in depot size with time.

Linoleic acid percentage in the 3 fat depots increased on the high-fat diet. The nonlinear increase was described reasonably well by the equation $C_t = A(1 - be^{-kt})$, where C_t = linoleic acid percentage at time t in days, A = asymptotic linoleic acid percentage, k = rate of linoleic acid increase and b = an integration constant. Nonlinear regression procedures were used to find estimates of the parameters (28).

Linoleic acid as a percentage of total triglycerides in the fat depot generally followed an exponential decay curve with time on the fat-free diet. The curve has the form $C_t = C_o e^{kt}$, where C_t = linoleic acid percentage at time t in days, C_o = linoleic acid percentage at initiation of the fat-free diet and k = rate of decline in linoleic acid percentage. The half-life of linoleic acid percentage was estimated as $t_{1/2}$

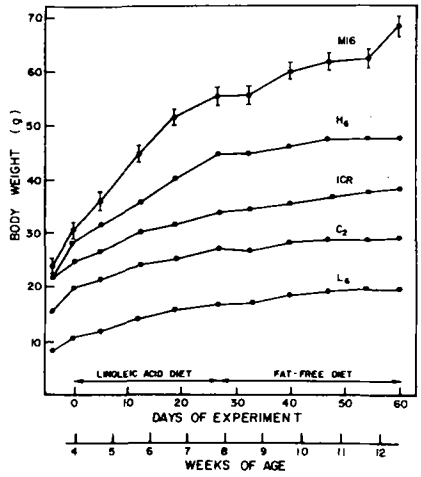


FIG. 1. Body weight means of the 5 lines. Vertical lines represent standard errors. Arrows indicate number of days and approximate ages of the mice when fed the linoleic acid and fat-free diets.

= (ln.5)/k. Estimates of C_0 and k for the depots of each line were obtained by nonlinear regression procedures (28).

Correlations were calculated between linoleic acid percentage and the percentage of each of the other fatty acids in the same depot (29). Correlations pooled within line and time provide estimates of the degree of spatial association between linoleic acid percentage and the percentages of the other fatty acids. Correlations calculated over time pooled within lines estimate corresponding temporal associations. Correlations within line by time classes were calculated between the same fatty acid (percentage or weight) located in different depots. These correlations provide an indication of the degree of association between the amounts of a specific fatty acid located in 2 distinct depots.

Significance of differences between means and between regression coefficients were determined by t-tests to provide insight into the changes brought about by selection (30): M16-ICR = correlated response due to selection for rapid gain as a deviation from control; H_6-L_6 = divergent correlated response due to selection for high and low body weight; $H_6+L_6-2C_2$ = asymmetric response, i.e., the deviation from a response of equal absolute value in the high and low direction of selection; M16-H_6 = comparison of differences between selected lines; and ICR-C_2 = comparison of differences between unselected lines.

RESULTS

Growth curves of the 5 lines show that differences in body weight due to selection for either 6-week body weight or 3- to 6-week weight gain were already established at weaning (Fig. 1). Mean body weights of each line were significantly ($p<.01$) different from one another throughout the experimental period. Lines M16 and H_6 had positively correlated responses (exceeded the respective ICR and C_2 controls) in weight gain, feed consumption and gross feed efficiency whereas L_6 had a negatively correlated response (less than the C_2 control) in feed consumption (Table 2).

Epididymal, subcutaneous and retroper-

TABLE 2

Line Means for Gain, Feed Intake and Feed Efficiency

Line	Days 0-12[f]	12-27	27-40	40-60
		Gain (g/day)		
ICR	.40[a] (49)[g]	.31[a] (39)	.12[a,c] (20)	.08[a] (5)
M16	1.20[b] (52)	.74[b] (42)	.33[b] (20)	.23[b] (4)
L_6	.31[c] (58)	.19[c] (48)	.16[a] (26)	.10[a] (7)
C_2	.34[a,c] (57)	.22[c] (47)	.07[c] (24)	.03[a] (7)
H_6	.65[d] (49)	.46[d] (40)	.08[c] (20)	.02[a] (5)
SE[h]	.024	.018	.032	.048
		Feed intake (g/day)[i]		
ICR	6.0[a]	5.6[a]	6.3[a]	6.2[a]
M16	7.2[b]	7.4[b]	8.4[b]	8.8[b]
L_6	3.5[c]	3.6[c]	3.8[c]	3.6[c]
C_2	4.3[d]	4.2[d]	4.7[d]	4.5[d]
H_6	6.1[a]	6.1[e]	6.6[e]	7.1[e]
SE[h]	.07	.08	.10	.27
		Feed efficiency (g gain/g feed)		
ICR	.068[a]	.055[a]	.019[a]	.014[a]
M16	.168[b]	.100[b]	.038[b]	.026[a]
L_6	.088[c]	.055[a]	.041[b]	.027[a]
C_2	.079[a,c]	.052[a]	.016[a]	.007[a]
H_6	.107[d]	.075[c]	.012[a]	.003[a]
SE[h]	.005	.003	.004	.009

[a-e]Column means not sharing a common superscript are significantly different at $p<.05$.

[f]Linoleic acid diet fed from 0 to 27 days; fat-free diet fed from 27 to 60 days.

[g]Number of mice in parentheses.

[h]Approximate standard error of the mean.

[i]To convert to kcal/g, multiply columns 1 and 2 by 4.567 kcal and columns 3 and 4 by 3.914 kcal.

itoneal fat pad weights, residual body fat weight and body energy/empty body weight increased with days on the linoleic acid diet in all lines (Fig. 2, Tables 3 and 4). Only the M16 line continued to show a significant increase in fat depot weight when the mice were transferred to the fat-free diet.

Depot fat means ($\bar{X}$) and rates of increase (b) were greater in M16 than in ICR mice during both the linoleic acid and fat-free feeding periods (Tables 3 and 4). H_6 mice had higher means and rates of fat deposition than L_6 mice on the linoleic acid diet, whereas on the fat-free diet the mean differences were maintained with no differences in rate. Generally, M16 mice exceeded H_6 mice in rate of fat deposited whereas ICR mice did not differ significantly from C_2 mice.

Cumulative fatty acid percentages for 14:0, 16:0, 16:1, 18:0, 18:1 and 18:2 in the 5 lines are presented in Figures 3 and 4 for the epididymal and subcutaneous fat pads. The effect of feeding a diet high in safflower oil on linoleic acid percentage in each fat depot was dramatic. The initial low linoleic acid percentage was increased rapidly in the fat pads of the 5 lines. Response of linoleic acid percentage in the residual dry body was more erratic; only the L_6 line exhibited an increase in linoleic acid percentage, but then declined toward the end of the high-fat period.

Nonlinear regression equations that describe the increase in linoleic acid percentage in each depot were homogeneous among the 5 lines, and were pooled to provide an empirical description of the response of each depot to feeding a diet high in linoleic acid (Fig. 5). Parameter estimates of the equations and F-tests (12 and 159 d.f.) for heterogeneity among lines were:

Fat depot	A	k	b	F	Pheterog.
Epididymal	50.3 ± .8%	.48 ± .05	.77 ± .03	1.64	>.05
Subcutaneous	51.3 ± .7%	.70 ± .12	.67 ± .03	1.67	>.05
Retroperitoneal	45.5 ± .9%	.06 ± .03	.80 ± .03	1.29	>.05

Linoleic acid percentage increased rapidly in the subcutaneous and epididymal fat depots, reaching a plateau at ca. 5 and 10 days, respectively. Linoleic acid percentage increased more slowly in the retroperitoneal fat pads, and by day 27, had attained about 85% of the estimated asymptote. Changes in linoleic acid percentage in the residual dry body could not be ascertained in the manner just described because of irregular fluctuations across time.

Transferring mice to the fat-free diet resulted in an immediate reduction in linoleic acid percentage in each fat depot as represented

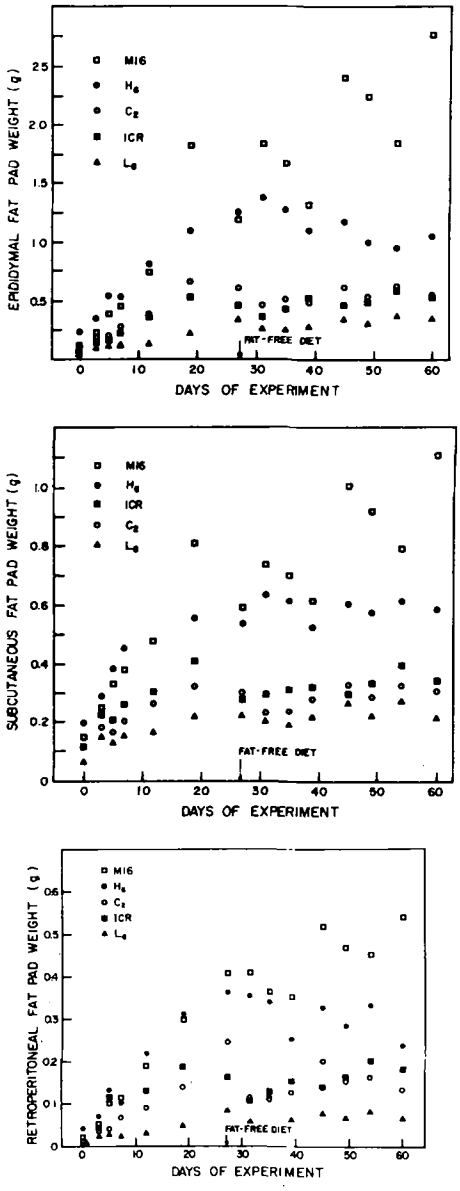


FIG. 2. Epididymal (E), subcutaneous (S) and retroperitoneal (R) fat pad weights plotted against days on experiment. Each mean represents 4-7 mice.

by the epididymal (Fig. 3) and subcutaneous (Fig. 4) fat pads, but not for the residual dry body of lines M16 and H_6. The depletion curves of linoleic acid percentage fitted to the data (Fig. 6) were heterogeneous ($p<.01$) among lines. The decay curves agreed reasonably well with the observed means and the coefficients of determination varied from .90 to .98, indicating a good fit statistically. Rates

TABLE 3

Mean ($\bar{X}$) Fat Pad Weight, Residual Body Fat and Energy Values Averaged over Days on the Linoleic Acid Diet, Regression Coefficients (b) of Variables on Days after Initiating the Linoleic Acid Diet, and Linear Contrasts

Line	Epid. fat pad wt (g)		Subcut. fat pad wt (g)			Retroper. fat pad wt (g)		Residual body fat wt (g)[a]			Energy/empty body wt (kcal/g)		
	$\bar{X}$	b^c	$\bar{X}$	b^c_L	$b^{c,d}_Q$	$\bar{X}$	b^c	$\bar{X}$	b^c_L	$b^{c,d}_Q$	$\bar{X}$	b^c_L	b^c_Q
ICR	.27	.16**	.25	.36**	-.008**	.10	.05**	.90	.19*	–	1.68	.54**	-.015**
M16	.70	.55**	.42	.64**	-.012**	.17	.13**	1.82	2.71**	-.048**	1.90	.88**	-.023**
L_6	.15	.10**	.15	.05**	–	.03	.02	.48	.07	–	1.76	.08	–
C_2	.33	.21**	.21	.22**	-.004*	.10	.10**	.85	.17*	–	1.86	.63**	-.016**
H_6	.68	.39**	.39	.38**	-.007**	.18	.12**	1.61	.56**	–	2.00	.59**	-.010*
SE[b]	.03	.04	.012	.02	.002	.015	.017	.06	.09	–	.03	.14	.005
Contrast													
M16-ICR	.43**	.29**	.17**	.28**	-.004	.07**	.08**	.92**	–	–	.22**	.34	-.008
H_6-L_6	.53**	.29**	.24**	–	–	.15**	.10**	1.13**	.43**	–	.24**	–	–
$H_6+L_6-2C_2$	.17**	.07	.12**	–	–	.01	-.06	.30*	.29	–	.05	–	–
M16-H_6	.02	.16**	.03	.26**	-.005	-.01	-.01	.21*	–	–	-.10*	.29	-.013
ICR-C_2	-.06	.05	.04*	.14	-.004	.00	-.05*	.05	.02	–	-.17**	-.09	-.001

*$p<.05$, **$p<.01$.

[a]Excludes excised fat pads.

[b]Standard error.

[c]X 10.

[d]Quadratic trend.

TABLE 4

Mean ($\bar{X}$) Fat Pad Weight, Residual Body Fat and Energy Values Averaged over Days on the Fat-free Diet, Regression Coefficients (b) of Variables on Days after Initiating the Fat-free Diet, and Linear Contrasts

Line	Epid. fat pad wt (g)		Subcut. fat pad wt (g)		Retroper. fat pad wt (g)		Residual body fat wt (g)[a]		Energy/empty body wt (kcal/g)	
	$\bar{X}$	b[c]	$\bar{X}$	b[c]	$\bar{X}$	b[c]	$\bar{X}$	b	$\bar{X}$	b[c]
ICR	.48	.04	.32	.02	.15	.02	1.43	.21	1.84	.07
M16	1.91	.36**	.81	.13**	.42	.07**	4.24	1.14**	2.44	.19**
L_6	.31	.02	.22	.01	.08	.00	.77	.13	1.90	.04
C_2	.55	.02	.28	.02	.16	.02	1.32	.16	2.08	.06
H_6	1.18	-.08	.58	.00	.31	.02	2.74	.19	2.37	.00
SE[b]	.06	.06	.02	.02	.02	.02	.13	.12	.04	.04
Contrast										
M16-ICR	1.43**	.32**	.49**	.11**	.27**	.05	2.81**	.93**	.60**	.12*
H_6-L_6	.87**	-.10	.36**	-.01	.23**	.02	1.97**	.06	.46**	-.04
$H_6+L_6-2C_2$	.39**	-.10	.22**	-.01	.07	.00	.87**	.00	.10	-.08
M16-H_6	.73**	.44**	.23**	.13**	.11**	.05	1.50**	.95**	.07	.19**
ICR-C_2	-.07	.02	.04	.00	-.01	.00	.09	.05	-.25**	.01

*$p<.05$, **$p<.01$.

[a]Excludes excised fat pads.

[b]Standard error.

[c]X 10.

of depletion (k) or, alternatively, half-lives ($t_{1/2}$) were significantly different among lines (Table 5). A more rapid rate of depletion was observed in subcutaneous and retroperitoneal fat depots than in the epididymal fat depot. The $t_{1/2}$ of linoleic acid varied among lines from 7 to 16 days in subcutaneous, 6 to 20 days in retroperitoneal and 11 to 43 days in epididymal fat depots.

The ranking of lines for depletion rates of linoleic acid were similar among the 3 discrete depots. Rates of depletion in line M16 were slower than in line ICR in all 4 fat depots, but did not reach statistical significance in the epididymal fat pad. Line H_6 had slower ($p < .01$) rates of depletion than line L_6 in all 4 depots. There was a tendency for the correlated response in k to be asymmetric because L_6

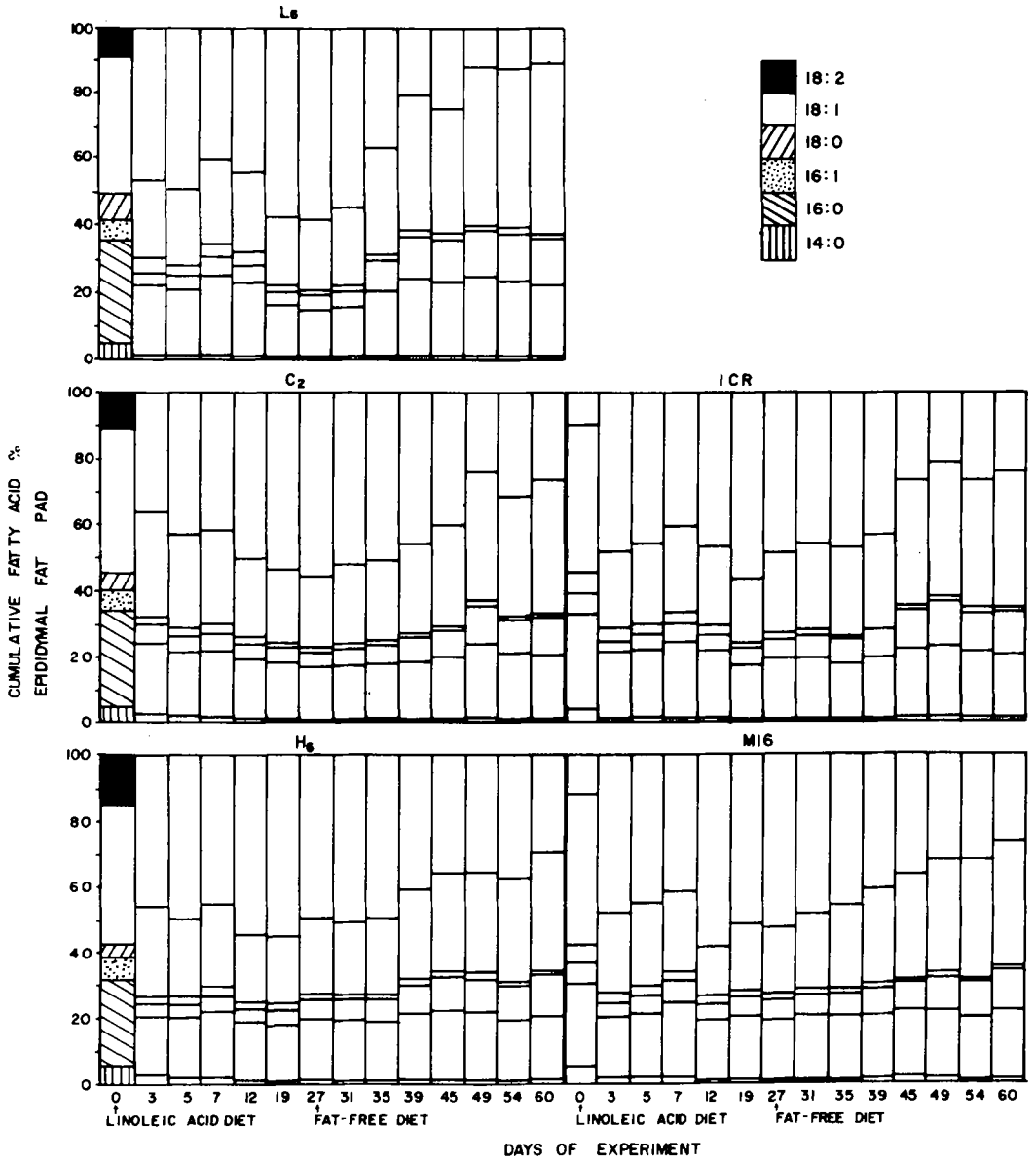


FIG. 3. Cumulative fatty acid percentages in the epididymal fat pads of the 5 lines plotted against days on experiment. Approximate standard errors for 14:0, 16:0, 16:1, 18:0, 18:1 and 18:2 are .3, 1.3, .5, .5, 1.3 and 2.6 for the linoleic acid feeding period and .1, .8, .6, .1, 1.5 and 2.5 for the fat-free feeding period.

deviated more from the C_2 control line than did H_6. Lines M16 and H_6 were not different, whereas ICR had a significantly faster rate of depletion than C_2.

The partial regression coefficients of ln fatty acid weight (14:0, 16:0, 16:1, 18:0 and 18:1) on days after initiating the fat-free diet, adjusted for ln fat pad weight were positive with the one exception of ln 18:0 (Table 6). This fatty acid accounted for only a small percentage of the total fatty acids, however (Figs. 3 and 4). Significant line differences among the partial regressions were sporadic and showed no consistent trend. Linoleic acid content was analyzed in a similar manner, but the results are not presented because they agree with the nonlinear regression analysis.

Correlations over time between linoleic acid

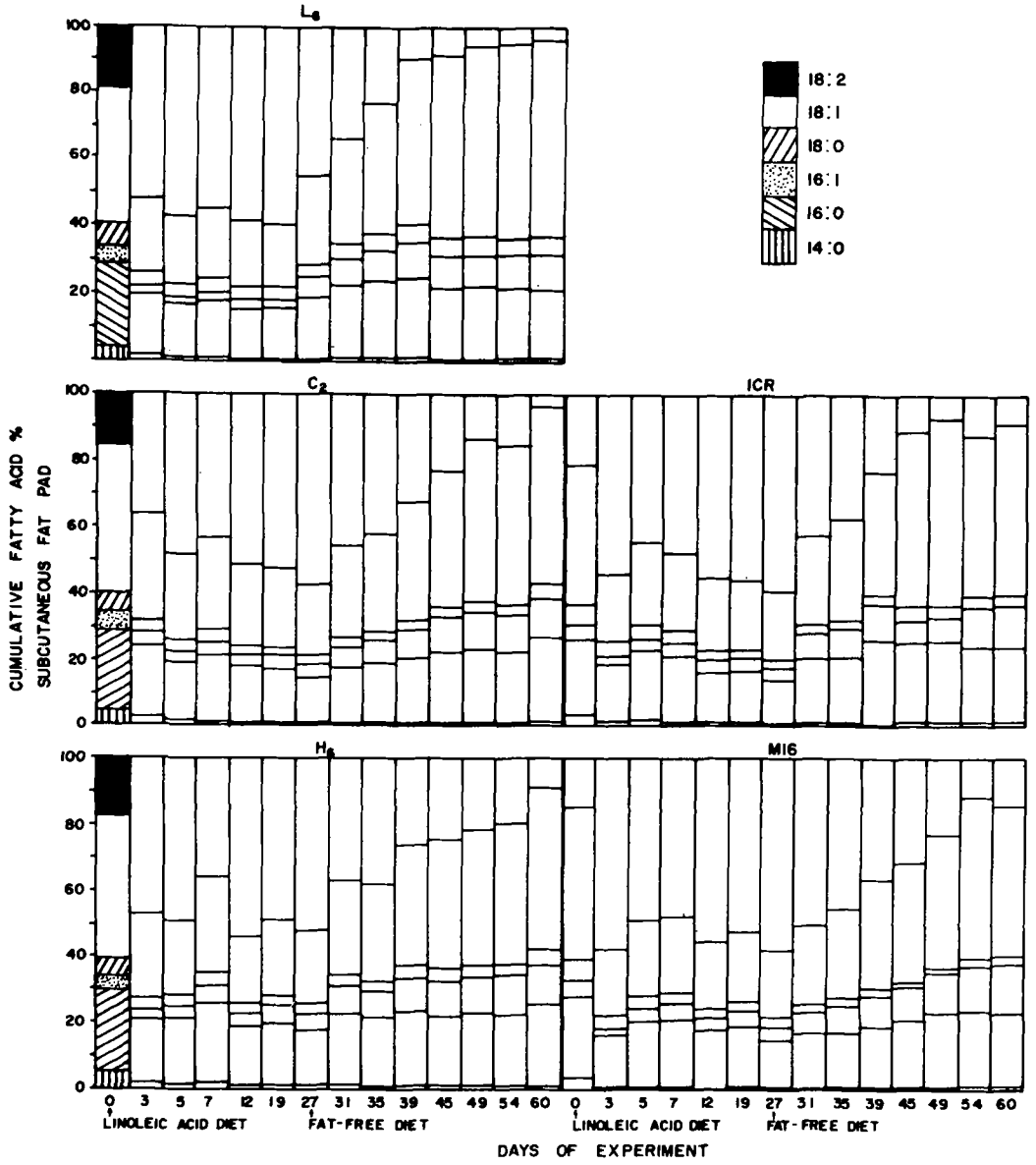


FIG. 4. Cumulative fatty acid percentages in the subcutaneous fat pads of the 5 lines plotted against days on experiment. Approximate standard errors for 14:0, 16:0, 16:1, 18:0, 18:1 and 18:2 are .3, .8, .5, .4, 1.2 and 1.8 for the linoleic acid feeding period and .1, .8, .5, .4, 1.6 and 2.5 for the fat-free feeding period.

percentage and the percentage of each of the other fatty acids (r_2) were highly negative in most cases. Thus, the linoleic acid percentage of each depot increased with the number of days on the safflower oil diet, the percentages of the other fatty acids decreased, and the reverse was true on the fat-free diet (Figs. 3 and 4). The corresponding spatial correlations (r_1) were also negative, but smaller on the average than r_2. Obviously, these are correlations between percentages of the total fatty acid weight so that the negative sign reflects the fact that, as the percentage of linoleic acid changes, the other fatty acid percentages must change in the opposite direction. The correlations provide a quantitative measure of the reciprocal relationship.

Correlations between triglyceride weights in different fat depots were high, ranging from .64 to .92, but the correlations between triglycerides as a percentage of depot weight were much lower (.16-.68). In general, the correlations between weights of the same fatty acids in different depots were moderately high, indicating that weights of the same fatty acid tend to change similarly in the different depots. An exception was the low correlations between linoleic acid weight in the residual dry body and that in each of the fat pads. Correlations involving percentages of the same fatty acids were lower than those involving weights. Again, the correlations involving 18:2 in the residual dry body and each of the discrete depots were low and significant in one case only. The lower correlations, particularly involving linoleic acid percentage, suggest that percentage of a fatty acid in one depot accounts for little of the variation in percentage of the same fatty acid in a second depot.

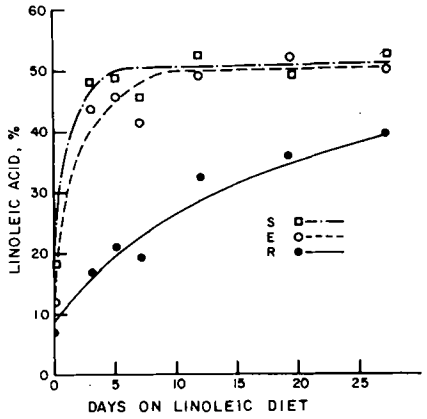


FIG. 5. Fitted response curves and observed means of linoleic acid percentage in the epididymal (E), subcutaneous (S) and retroperitoneal (R) fat depots averaged over lines when mice were fed a linoleic acid diet. The nonlinear regression equations were pooled over lines.

DISCUSSION

Previous studies have demonstrated that the rate of fatty acid turnover in mice or rats is affected by age (12), diet (13,31,32), sex (12), exercise (33) and tissue (31,34). Differences among 2 inbred lines and their F_1 crosses have been reported for lauric, myristic, palmitoleic, stearic, oleic and linoleic acid percentages (35).

In this study, genetic differences among lines of mice have been demonstrated in the depletion rate of linoleic acid percentage in 3 fat depots and the residual dry body. Furthermore, the direction of these differences follows the pattern of correlated responses predicted by the hypothesis set forth. M16 mice which are moderately obese as a result of selection for high growth rate exhibited a decrease in the depletion rate (increase in half-life) of linoleic acid compared with ICR nonobese mice. Line H_6, selected for large 6-week body weight, had a low depletion rate compared to line L_6, which was selected for small 6-week body weight. However, the response was asymmetric when compared to the C_2 control line. The rates of depletion in the 3 fat depots of H_6 were less than that of the C_2 control, whereas L_6 was greater than that of C_2. The asymmetry was such that the downward response in L_6 was 2.6-4.8 times as large as the upward response in H_6.

Although H_6 mice do not have an increased body fat percentage when fed a diet containing 5% fat (23), H_6 had an increased fat percentage when fed the linoleic acid diet in the present experiment. Therefore, the decreased depletion rate of linoleic acid in H_6 mice is consistent with the hypothesis that more obese animals have a lower depletion rate. In contrast, selection for small body weight in L_6 mice has led to an increase in fat turnover.

The increased half-life of linoleic acid in fat pads of M16 and H_6 mice suggests the possibility that the turnover rate of fatty acids has been reduced in these lines as a result of a reduction in lipolytic activity which reduces mobilization of fatty acids from adipose tissue. Mears and Mendel (36) reported that a moderately obese line with a history of selection similar to M16 has a decreased capacity for fatty acid mobilization from isolated adipocytes treated with epinephrine.

The M16 mice have greater capability for

synthesizing fat from carbohydrates as shown by the increased fat depot weights when the mice were fed a fat-free diet. This was not the case for the other rapidly growing line, H_6, or for the controls (ICR, C_2) and slow growing line (L_6). Line M16 also has demonstrated a higher rate of fat deposition when dietary intake is restricted during either preweaning or postweaning growth (7,8).

Cycles of triglyceride synthesis and mobilization are proposed as possible biochemical mechanisms by which chemical energy might be wasted or excess energy released for purposes of weight control (37,38). These mechanisms are likely to be under hormonal and nervous regulation. Hormonal differences between the selected lines in this study exist (11,39). To the authors' knowledge, this is the

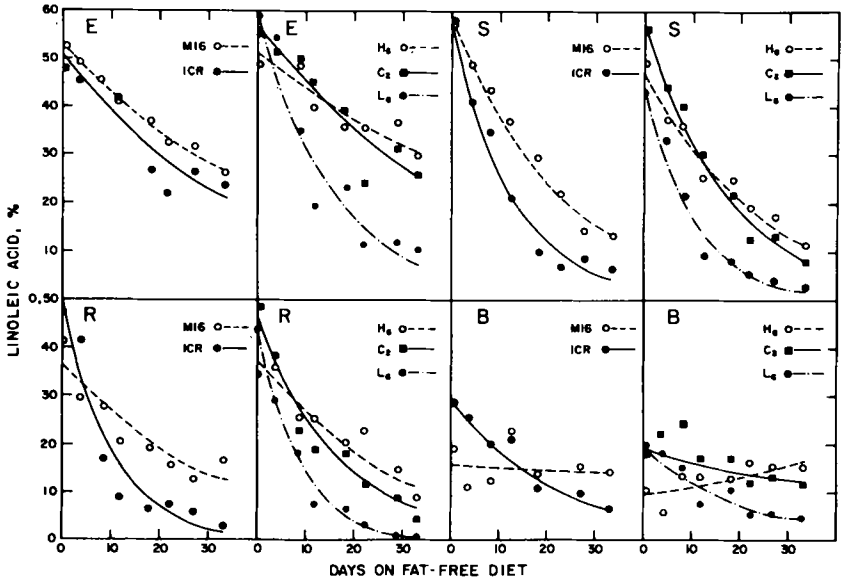


FIG. 6. Fitted depletion curves and observed means of linoleic acid percentage in the epididymal (E), subcutaneous (S) and retroperitoneal (R) fat depots and residual dry body (B) of the 5 lines when mice were fed the fat-free diet.

TABLE 5

Rates of Depletion ± Standard Error and Half-Lives of Linoleic Acid Percentage When Mice Were Fed a Fat-free Diet[a]

	Epid. fat pad		Subcut. fat pad		Retroper. fat pad		Residual dry body	
	k ± SE	$t_{½}$	k ± SE	$t_{½}$	k ± SE	$t_{½}$	k ± SE	$t_{½}$
Line								
ICR	-.27 ± .03	26	-.79 ± .05	9	-1.01 ± .12	7	-.46 ± .06	15
M16	-.21 ± .02	33	-.44 ± .04	16	-.34 ± .08	20	-.03 ± .08	–
L_6	-.63 ± .05	11	-.97 ± .06	7	-1.25 ± .16	6	-.46 ± .09	15
C_2	-.24 ± .02	29	-.58 ± .04	12	-.61 ± .10	12	-.14 ± .07	50
H_6	-.16 ± .03	43	-.43 ± .05	16	-.37 ± .09	19	.16 ± .10	–
Contrast								
M16-ICR	.06 ± .04	–	.34 ± .06**	–	.67 ± .14**	–	.43 ± .10**	–
H_6-L_6	.47 ± .05**	–	.54 ± .08**	–	.88 ± .18**	–	.62 ± .13**	–
H_6+L_6-$2C_2$	-.30 ± .07**	–	-.25 ± .11*	–	-.41 ± .28	–	-.02 ± .19	–
M16-H_6	-.05 ± .03	–	-.01 ± .06	–	.03 ± .12	–	-.19 ± .13	–
ICR-C_2	-.02 ± .04	–	-.21 ± .07**	–	-.40 ± .16*	–	-.33 ± .09**	–

*p<.05, **p<.01.

[a]$C_t = C_0 e^{kt}$ = 18:2% on day t, k = rate of decline in 18:2% with time (values have been multiplied by 10), C_0 = predicted 18:2% at initiation of fat-free diet.

TABLE 6

Regression Coefficients of ln Fatty Acid Weights (mg) on Days after Initiating the Fat-free Diet and Linear Contrasts[a,b]

	Fatty acid					Fatty acid				
	14:0	16:0	16:1	18:0	18:1	14:0	16:0	16:1	18:0	18:1
	Epididymal fat pad					Subcutaneous fat pad				
Line										
ICR	.21**	.12**	.36**	-.07*	.28**	.27**	.15**	.34**	.17**	.32**
M16	.13**	.07**	.30**	-.12**	.26**	.16**	.10**	.33**	-.06	.24**
L_6	.30**	.20**	.44**	.01	.36**	.21**	.11**	.23**	.17**	.35**
C_2	.16**	.11**	.36**	-.12**	.24**	.22**	.13**	.31**	.13**	.25**
H_6	.25**	.06**	.28**	-.05	.18**	.11**	.09**	.26**	.15**	.25**
SE[d]	.03	.02	.03	.03	.02	.04	.03	.04	.04	.03
Contrast										
M16-ICR	-.08	-.05	-.06	.05	-.02	-.11*	.05	.01	-.23**	-.08
H_6-L_6	-.05	-.14**	-.16**	-.06	-.18**	-.10	-.02	.03	-.02	-.10*
$H_6+L_6-2C_2$	.23**	.04	.00	.08	.06	-.12	-.06	-.13	.06	.10
M16-H_6	-.12**	.01	.02	-.07	.08**	.05	.01	.07	-.21**	-.01
ICR-C_2	.05	.01	.00	-.02	.04	.05	.06	.03	.04	.07
	Retroperitoneal fat pad					Residual dry body[c]				
Line										
ICR	.33**	.17**	.36**	.08	.32**	.25**	.14**	.45**	-.01	.38**
M16	.21**	.11**	.32**	.00	.34**	.19**	.09**	.39**	-.06	.36**
L_6	.32**	.18**	.34**	.27**	.42**	.12**	.03	.30**	-.16**	.33**
C_2	.36**	.25**	.40**	.17**	.42**	.20**	.12**	.37**	.02	.38**
H_6	.12*	.09*	.25**	.10*	.27**	.10*	.07*	.34**	.02	.27**
SE[d]	.06	.04	.05	.05	.05	.04	.03	.05	.04	.04
Contrast										
M16-ICR	-.12	-.06	-.04	-.08	.02	-.06	-.05	-.06	-.05	-.02
H_6-L_6	-.20*	-.09	-.09	-.17*	-.15*	-.02	.04	.04	.18**	-.06
$H_6+L_6-2C_2$	-.28	-.23*	-.21	.03	-.15	-.18	-.14	-.10	-.18	-.16
M16-H_6	.09	.02	.07	-.10	.07	.09	.02	.05	-.08	.09
ICR-C_2	-.03	-.08	.11	.09	-.10	.05	.07	.08	.03	.00

*$p<.05$, **$p<.01$.

[a]All slopes are adjusted by covariance analysis for ln fat pad weight (mg).

[b]All slopes have been multiplied by 10.

[c]Excludes excised fat pads.

[d]Standard error of the regression coefficient.

first report specifically indicating a difference in triglyceride turnover in animals in which selection resulted in differences in body size, body composition and efficiency of energy deposition.

ACKNOWLEDGMENTS

This is paper no. 8038 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh. This work was supported, in part, by NIH Grant no. AM21950. The many helpful suggestions of Dr. S.B. Tove are appreciated.

REFERENCES

1. Eisen, E.J. (1974) First World Congress on Genetics Applied to Livestock Production, Vol. 1, pp. 467-492, Madrid.
2. Roberts, R.C. (1979) Livest. Prod. Sci. 6, 93-104.
3. Bakker, H. (1974) Mededelingen Landbouwhogeschool, Wageningen, The Netherlands.
4. Hayes, J.F., and McCarthy, J.C. (1976) Genet. Res. 27, 389-403.
5. Fowler, R.E. (1958) J. Agric. Res. 51, 137-148.
6. Lang, B.J., and Legates, J.E. (1969) Theor. Appl. Genet. 39, 306-314.
7. Eisen, E.J., and Leatherwood, J.M. (1978a) J. Nutr. 108, 1652-1662.
8. Eisen, E.J., and Leatherwood, J.M. (1978b) J. Nutr. 108, 1663-1672.
9. Martin, R., White, J., Herbein, J., and Ezekwe, M.O. (1979) Growth 43, 167-173.
10. McCarthy, J.C. (1979) in Animal Models of Obesity (Festing, M.F.W., ed.) pp. 1-14, MacMillan Press Ltd., London.
11. Robeson, B.L., Eisen, E.J., and Leatherwood, J.M. (1981) Growth 45:198-215.
12. Tove, S.B., and Smith, F.H. (1959) Arch. Biochem. Biophys. 5, 352-265.
13. Tove, S.B., and Smith, F.H. (1960) J. Nutr. 71, 264-272.
14. Stein, Y., and Stein, O. (1962) Biochim. Biophys. Acta 60, 58-71.
15. Stein, Y., and Stein, O. (1965) in Handbook of Physiology. Section 5: Adipose Tissue (Renold, A.E., and Cahill, G.F., Jr., eds.) pp. 431-435,

Am. Physiol. Soc., Washington, DC.
16. Campbell, R.G., and Hashim, S.A. (1969) Am. J. Physiol. 217, 1614-1619.
17. Campbell, R.G., and Hashim, S.A. (1972) Proc. Soc. Exp. Biol. Med. 141, 652-655.
18. Christie, W.W., Hunter, M.L., and Vernon, R.G. (1979) Int. J. Biochem. 10, 517-521.
19. Klein, R.A., and Pittet, P.G. (1980) Lipids 15, 572-579.
20. Eisen, E.J. (1975) Genetics 79, 305-323.
21. Legates, J.E. (1969) Genetics Lectures, Vol. 1 (Bogart, R., ed.) pp. 149-165, Oregon State University Press, Corvallis.
22. Eisen, E.J., Bakker, H., and Nagai, J. (1977) Theor. Appl. Genet. 49, 21-34.
23. Eisen, E.J., Hayes, J.F., Allen, C.E., Bakker, H., and Nagai, J. (1978) Growth 42, 7-25.
24. Hilditch, T.P., and Williams, P.N. (1964) The Chemical Constitution of Natural Fats, 4th edn., John Wiley & Sons, New York, NY.
25. Eisen, E.J., and Leatherwood, J.M. (1976) J. Anim. Sci. 42, 52-62.
26. Dole, V.P. (1956) J. Clin. Invest. 35, 150-154.
27. Snedecor, G.W., and Cochran, W.G. (1967) Statistical Methods, 6th edn., Iowa State University Press, Ames.
28. Draper, N.R., and Smith, H. (1966) Applied Regression Analysis, John Wiley & Sons, Inc., New York, NY.
29. Kempthorne, O. (1957) An Introduction to Genetic Statistics, John Wiley & Sons, Inc., New York, NY.
30. Falconer, D.S. (1960) Introduction to Quantitative Genetics, Ronald Press Co., New York, NY.
31. Ostwald, R., Okey, R., Shannon, A., and Tinoco, J. (1962) J. Nutr. 76, 341-352.
32. Lang, K., and Reimold, W.V. (1970) Z. Ernährungswiss 10, 137-144.
33. Askew, E.W., Hecker, A.L., and Wise, W.W., Jr. (1977) J. Nutr. 107, 132-142.
34. Nørby, J.G. (1965) Br. J. Nutr. 19, 209-224.
35. Boylan, W.J., and Roberts, W.K. (1968) J. Agric. Sci. 70, 73-76.
36. Mears, G.J., and Mendel, V.E. (1974) J. Physiol. 240, 609-624.
37. Ball, E.G., and Jungas, R.L. (1961) Proc. Natl. Acad. Sci. U.S.A. 47, 932-941.
38. Newsholme, E.A. (1980) New Eng. J. Med. 302, 400-405.
39. Cartwright, A.L., Leatherwood, J.M., and Eisen, E.J. (1980) J. Nutr. 110, 1262-1273.

[Received October 29, 1981]

Hepatic Bile Acid Elution by Albumin and Bile Acid Content in Isolated Rat Hepatocytes

SHIGERU HASHIMOTO*, KIYOHISA UCHIDA and **MASAHARU HIRATA**, *Shionogi Research Laboratories, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan*

ABSTRACT

Bile acid contents were determined for isolated rat hepatocytes. During the course of isolating the hepatocytes, perfusion of rat liver with buffer containing 2% albumin eluted a significant amount of bile acids. The elution was proportional to the volume of the buffer and attributable to albumin in the buffer. The isolated hepatocytes prepared by perfusion with 0.1% albumin buffer, which eluted a negligible amount of bile acids, contained 95 ± 12 $\mu g/10^8$ cells of bile acids. The major bile acids were cholic acid (22%), β-muricholic acid (34%) and hyodeoxycholic acid (10%). Levels of the other bile acids were less than 3%. Peak 8, unidentified but presumed to be a trihydroxycholanoic acid, accounted for 19%.

Lipids 17:149-154, 1982.

ABBREVIATIONS

EGTA, ethyleneglycol, tetraacetate; TLC, thin layer chromatography; GLC, gas liquid chromatography.

The elevation of bile acid synthesis in bile-duct-ligated rats (1) and biliary fistular rats (2) is attributed to the deficiency of bile acids returning to the liver due to interruption of the enterohepatic circulation. The bile acids are transported from portal blood into bile canaliculi through hepatocytes. When a large amount of bile acids returns to the liver, the bile acid concentration in hepatocytes increases, and the bile acid formation is depressed by a negative feedback mechanism.

However, little information is available on bile acid contents in rat hepatocytes, though those in the whole liver have often been documented (3,4). In order to determine the bile acid contents in hepatocytes, we attempted to isolate rat hepatocytes. During the course of the experiment, we found a significant amount of bile acids was eluted from the liver by perfusion with a buffer containing EGTA and bovine serum albumin.

In this report, we describe the effect of albumin on bile acid elution and bile acid contents of isolated rat hepatocytes determined under the conditions that minimize the bile acid efflux.

MATERIALS AND METHODS

Animals

Wistar strain male rats (10-wk-old, weighing 260-320 g) were kept in an air-conditioned room (25 ± 1 C, 50-60% humidity) lighted 12 hr/day (8:00-20:00). They were maintained on a commercial balanced stock diet (Japan CLEA CA-1, Tokyo, Japan).

Materials

Bovine serum albumin, Fraction V, was purchased from Sigma Chemical Co., St. Louis, MO. EGTA was obtained from Yoneyama Chemical Co. Ltd., Osaka, Japan. Collagenase was purchased from Boehringer-Manheim, West Germany, and glass plates for TLC precoated with Silica Gel GF (Uniplate, No. 2011, 0.25-mm-thick layer) were bought from Analteck, Newark, DE. Amberlite XAD-2, from Rohm & Haas, Philadelphia, PA, was washed by the method of Makino et al. (5) prior to use. All other chemicals were of reagent grade.

Perfusion of Rat Liver

The rat was laparotomized under anesthesia with sodium pentobarbital (Somnopentyl®, Pitman-Moore, Washington, NJ; 65 mg/kg, ip), and the bile duct was ligated to prevent contamination of bile. Heparin (0.1 ml, 1,000 unit/ml) was injected via the caval vein. A stainless-steel cannula (2.1 mm id) connected to an oxygenator cylinder and a buffer reservoir was inserted into the portal vein and fixed with ligature. Perfusion of the liver was immediately started in situ with an albumin buffer (a modified Hanks buffer, pH 7.4, containing 0.5 mM EGTA and 2% bovine serum albumin) at 37 C. The liver was quickly removed and placed on a rack over a beaker to collect the flowing perfusate. The perfusion was continued at a flow rate of about 80 ml/min and 4 perfusates of 100 ml each were collected. The intact liver was perfused with saline to remove blood and was used as a control. Bile acids were not eluted by the saline perfusion.

In another experiment, the liver was perfused with albumin-free buffer and albumin buffer alternately to examine the effect of albumin, first with albumin-free buffer, second with 2% albumin buffer, third with albumin-

free buffer and finally with 2% albumin buffer. The volume of each perfusion buffer was 100 ml.

In both experiments, the perfusate was collected as soon as the cannula was inserted in order to minimize volume loss of the perfusate. Therefore, these perfusates contained blood.

Preparation of Rat Hepatocytes

The hepatocytes were isolated by the method of Moldéus et al. (6) using either 2% or 0.1% albumin buffer for perfusion and cell sedimentation. The liver was perfused by recirculation of 200 ml of the buffer for about 4 min, but the first part of the perfusate that contained blood was discarded. The buffer was then replaced by 100 ml of the modified Hanks buffer (pH 7.4) containing 0.12% collagenase and 4 mM calcium chloride, and was circulated for about 6 min. The softened liver was immersed in Krebs-Henseleit buffer (pH 7.4) containing 2% or 0.1% albumin. The capsule was cut open, and the dispersed cells were filtered through cheesecloth to remove the connective tissues and small clumps of unisolated cells. The cells were sedimented 3 times at 50 x g for about 3 min. The average yield of hepatocytes was 7-8 x 10^8 cells/liver in 3 types of experiments; in the first experiment, 2% albumin buffer was used for perfusion and washing, in the second, 0.1% albumin buffer for perfusion and 2% albumin buffer for washing, and in the third, 0.1% albumin buffer for perfusion and washing. The viability judged by the LDH latency test (7), was 98-99% for all preparations.

Extraction of Bile Acids

The perfusate was percolated through Amberlite XAD-2 column (2 cm in diameter, 10 cm high) and the bile acids were eluted with methanol after washing with water (5). The supernatant of cell sedimentation and washings were combined and centrifuged at about 1,900 x g for 15 min to remove a minute amount of the cells, and subjected to Amberlite XAD-2 column chromatography as already described. The sedimented cells were disrupted by freezing and thawing 3 times and diluted to 20 ml with water. The suspension was further homogenized with an ULTRA-TURRAX TP 18-10 (IKE-WERK, Janke & Kunkel KG, West Germany) and lyophilized. The dried residue was refluxed for 1 hr with 100 ml of 95% ethanol containing 0.1% ammonium hydroxide, and the extract was filtered after cooling to room temperature. The extraction procedure was repeated 3 times. The ethanolic extracts were combined and evaporated under reduced pressure. The residue was dissolved in 10 ml of 70% methanol and extracted twice with 10 ml of *n*-hexane to remove neutral fats. The methanolic layer was evaporated to dryness. The whole liver perfused with either saline or albumin buffer was homogenized in 20 ml of water, and the bile acids were extracted similarly.

Determination of Bile Acids

The extracts just described were hydrolyzed in 1.25 N sodium hydroxide solution at 120 C for 6 hr. After removal of the neutral substances by extraction with 40 ml of diethyl ether twice, the reaction mixture was acidified with 2 N hydrochloric acid solution, and bile acids were extracted with 40 ml of diethyl ether twice. The bile acid residue was methylated with freshly prepared ethereal diazomethane, acetylated with trifluoroacetic anhydride by being left at room temperature for 1 hr, and subjected to GLC analysis. The procedure for determination of bile acids by GLC on a QF-1 column has been described in a previous paper (8).

Because cholic and β-murichollic acids gave similar retention times on the QF-1 column, part of the methylated bile acid extract was applied to TLC and continuously developed for 2 hr with benzene/acetone (7:3, v/v) according to the method of Truter (9). The corresponding bands for methyl cholate and β-murichollate, made visible by exposure to iodine vapor, were scraped off, eluted with methanol and evaporated to dryness. The residue was trifluoroacetylated and analyzed by GLC on the QF-1 column.

RESULTS

Elution of Bile Acids from Liver

When the liver was perfused with the buffer containing 2% albumin, a considerable amount of bile acids was eluted into the perfusate in linear relation to the volume of the buffer, and the mean elution rate was about 80 μg/100 ml perfusion buffer (Fig. 1). However, bile acids were not eluted when albumin was excluded from the buffer. Figure 2 shows the changes in the elution of bile acids caused by alternately using the albumin-free buffer and the albumin buffer. In the first perfusate, though albumin was not added, about 40 μg of bile acids was eluted, but when the buffer was replaced with that containing 2% albumin, a larger amount of bile acids was eluted. In the third perfusate, no bile acids were found, but in the fourth perfusate, bile acids were eluted in an amount comparable to that in the second perfusate. The bile acids in the first perfusate

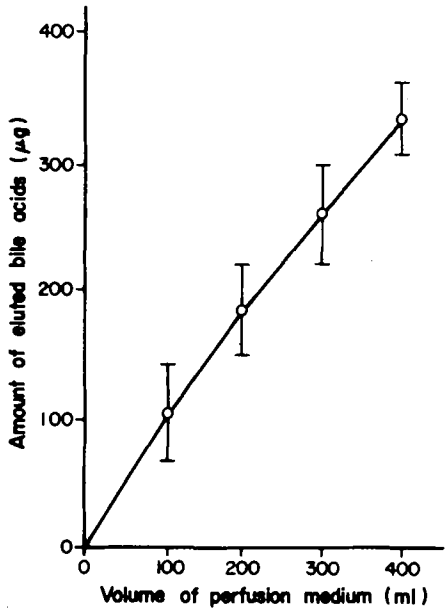


FIG. 1. Elution of bile acids from rat liver by perfusion with a modified Hanks buffer (pH 7.4) containing 2% albumin and 0.5 mM EGTA. Each point and vertical bar indicate the mean value and SE of 4 rats.

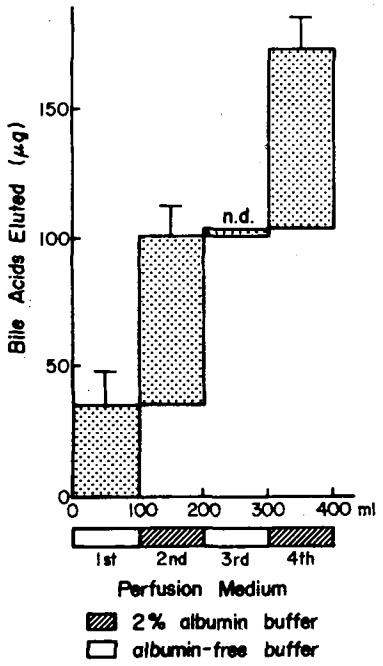


FIG. 2. Effect of albumin in perfusion buffer on elution of bile acids from rat liver. The first perfusion was done with a modified Hanks buffer (pH 7.4) containing 0.5 mM EGTA, the second, with the buffer containing 0.5 mM EGTA and 2% albumin, the third, with the buffer for the first perfusion, and the fourth, with the buffer for the second perfusion. Each column and vertical bar indicate the mean value and SE of 4 rats.

were considered to originate from the portal blood present in the liver, because part of the blood was combined with the first perfusate in this experiment.

Bile Acid Contents in Perfused and Intact Livers

Table 1 shows the amounts and proportions of cholic acid and β-muricholic acid in the liver perfused with albumin buffer and the perfusate and in the blood-free liver (perfused with saline). When the liver was perfused with 400 ml of 2% albumin buffer, the amounts of bile acids found in the perfusate and the tissue were 334 and 583 μg, respectively, but the total amount was similar to that in the blood-free liver.

Of the eluted bile acids, cholic acid and β-muricholic acid were the major components, accounting for 44 and 18%, respectively. On the other hand, cholic acid remaining in the perfused tissue accounted for 24% and β-muricholic acid for 37%. When the bile acids in the perfusate and tissue were combined, cholic acid was 29% of the total bile acids and β-muricholic

TABLE 1

Bile Acid Contents in Perfusate, Perfused Liver and Blood-Free Liver

	Perfused liver[a]			Blood-free liver[b]
	Perfusate	Tissue	Total	
Total bile acids (μg/liver)	334 ± 26[c]	583 ± 132[c]	917 ± 134[c]	1184 ± 125[c]
Cholic acid (%)	44 ± 3	24 ± 3	29 ± 2	32 ± 1
β-Muricholic acid (%)	18 ± 1	37 ± 4	28 ± 1	31 ± 3

[a]Rat liver was perfused with 400 ml of 2% albumin buffer.

[b]Rat liver was perfused with saline to remove blood from the tissue.

[c]Values presented in this column are mean ± SE of 4 rats.

acid 28%, which was similar to the proportion in the blood-free liver. This evidence suggests that cholic acid is transported faster than β-muricholic acid.

Amounts of Bile Acids in the Perfusate, Washings, Remnants and Isolated Hepatocytes

The hepatocytes were prepared with buffers containing different amounts of albumin, and the amounts of bile acids in the perfusate, collagenase perfusate, washings (the supernatant after cell sedimentation), remnants (mainly consisting of the remaining connective tissues and a small amount of cells), and isolated hepatocytes are shown in Table 2.

The amount of eluted bile acids was 153 μg when the liver was perfused with the 2% albumin buffer, whereas it was negligible when perfused with 0.1% albumin buffer. Bile acids found in the washings with the 0.1% albumin buffer amounted to 26 μg, far less than those in the washings with 2% albumin (200 μg). On the other hand, the bile acid contents in the hepatocytes were largest when prepared with only 0.1% albumin buffer. The bile acid contents in the collagenase perfusate and the remnants were not influenced by the albumin concentration.

Bile Acid Concentration in Rat Hepatocytes

The bile acid concentration in the hepatocytes isolated using 0.1% albumin buffer was 95 $\mu g/10^8$ cells. As shown in Table 3, cholic acid and β-muricholic acid were the major constituents, comprising about 22 and 34% of the total bile acids, respectively. Peak 8, which was not yet identified but presumed to be a trihydroxycholanoic acid, accounted for 19% and hyodeoxycholic acid for about 10%. The other bile acids usually found in rat bile such as chenodeoxycholic acid, deoxycholic acid, α-muricholic acid, and lithocholic acid accounted for no more than 3%.

DISCUSSION

A current method used for isolating rat hepatocytes is principally based on perfusion of collagenase (10) and requires prior perfusion with a buffer containing a chelating agent. A number of preperfusion media have been devised to improve the yield of viable cells (11).

This study indicated that bile acids in the hepatocytes were removed when the perfusion medium contained 2% albumin. The amount of the eluted bile acids was almost proportional to the volume of the perfusion medium. Cell

TABLE 2

Bile Acid Contents (μg/liver) in Perfusates, Washings, Hepatocytes and Remnants Obtained during the Isolation of Rat Hepatocytes

Experiment no.	1	2	3
Albumin conc.			
perfusate[a]	2.0%	0.1%	0.1%
washing[b]	2.0%	2.0%	0.1%
Recirculated preperfusate	153[d] (163, 143)	3 ± 1[e]	3 ± 1[e]
Collagenase perfusate	80 (71, 89)	85 ± 9	116 ± 31
Washings	224 (198, 250)	203 ± 25	26 ± 6
Hepatocytes	294 (250, 337)	604 ± 89	715 ± 87
($\mu g/10^8$ cells)	43	71.4 ± 8.1	94.9 ± 11.7
Remnants[c]	258 (258, lost)	323 ± 26	199 ± 31
Total	1009	1217 ± 80	1059 ± 142

[a]Liver was perfused with a modified Hanks buffer (pH 7.4) containing 0.5 mM EGTA and bovine serum albumin at the concentration indicated.

[b]Cells were suspended and sedimented 3 times in Krebs-Henseleit buffer (pH 7.4) containing bovine serum albumin at the concentration indicated.

[c]This fraction contained connective tissues, vascular tubes and small clumps of cells.

[d]Values presented in this column are mean of 2 experiments; 5 rats were used in each experiment, and the perfusates, and the other fractions, were combined and analyzed.

[e]Values presented in this column are mean ± SE of 4 rats.

TABLE 3

Bile Acid Content and Composition in Isolated Hepatocytes

Total bile acids ($\mu g/10^8$ cells)	94.9 ± 11.7[a]	
Composition	$\mu g/10^8$ cells	% of total
Cholic acid	20.9 ± 2.4[a]	21.5 ± 1.9[a]
β-Muricholic acid	31.3 ± 2.3	34.1 ± 3.5
Peak 8[b]	18.3 ± 3.8	18.8 ± 1.8
Hyodeoxycholic acid	9.1 ± 1.5	9.5 ± 1.1
α-Muricholic acid	2.4 ± 0.2	2.6 ± 0.4
Deoxycholic acid	1.1 ± 0.3	1.1 ± 0.2
Chenodeoxycholic acid	1.2 ± 0.3	1.1 ± 0.3
Ursodeoxycholic acid	0.3 ± 0.2	0.4 ± 0.2
Lithocholic acid	0.4 ± 0.4	0.4 ± 0.4
Others[c]	8.0 ± 2.3	10.3 ± 1.4

[a]Values presented in this column are mean ± SE of 4 rats.

[b]This is an unidentified peak.

[c]This includes ω-muricholic acid and some keto bile acids.

damage, however, was unlikely, because no elution occurred when albumin was excluded from the perfusion medium. The major bile acid in the perfusate was cholic acid, whereas that in the perfused tissue was β-muricholic acid. As both bile acids were initially present almost at the same proportion (Table 1), the change suggested that cholic acid had a higher affinity for albumin than β-muricholic acid.

The bile acid elution depended on the albumin concentration in the medium used for perfusion and washing. A higher concentration produced more elution of bile acids and resulted in a decrease of bile acids in the isolated hepatocytes. The cells sedimenting in 0.1% albumin buffer contained more bile acids than those in 2% albumin buffer. However, the combined amounts of bile acids in the 3 experiments with different albumin concentrations were similar to each other (Table 2) and very close to the value in the blood-free liver, suggesting that bile acids were not appreciably synthesized during the perfusion. Therefore, we concluded that the eluted bile acids and a part of those found in the washings (Table 2) had probably existed in the hepatocytes.

Anwer et al. (12) reported that their isolated rat hepatocytes contained no detectable amount of bile acids. On the other hand, Yousef et al. (13) found 190 ± 23 nmol of bile acids/g wet liver cells (equivalent to 75 ± 9 μg/g wet liver cells) in isolated hepatocytes. In this study, 95 ± 12 $\mu g/10^8$ cells, or 122 ± 13 μg/g wet liver cells, was found. The latter value was calculated on the basis of 1 g wet liver cells containing 128 × 10^6 cells (11) and was slightly higher than that of Yousef et al. (13). The major components of bile acids in the hepatocytes were cholic acid, β-muricholic acid and an unidentified peak 8. Among them, cholic acid has been shown to inhibit bile acid synthesis (14), but the physiological characteristics of β-muricholic acid and peak 8 are still unclear.

Low-molecular-weight cell constituents, such as methionine (15), glutathione (7), or bile acids in this study, are lost during the liver perfusion and preparation of the hepatocytes. Högberg and Kristoferson (7) reported that a simple way to minimize loss of cell constituents was to shorten the time required for cell isolation. However, the composition of perfusion medium was another important factor as shown in this study. Seglen (11) suggested that the beneficial effect of albumin in the perfusion medium was related to uniform penetration of collagenase into the liver tissue. The yield and viability of the cells were independent of the albumin concentration in the perfusion medium.

REFERENCES

1. Greim, H., Trülzsch, D., Roboz, J., Dressler, K., Czygan, P., Hutterer, F., Schaffner, F., and Popper, H. (1972) Gastroenterology 63, 837-845.
2. Danielsson, H. (1973) in The Bile Acids; Chemistry, Physiology, and Metabolism (Nair, P.P., and Kritchevsky, D., eds.) Vol. 2, p. 22, Plenum Press, New York-London.
3. Fisher, M.M., Kakis, G., and Yousef, I.M. (1976) Lipids 11, 93-96.
4. Oh, S.Y., and Dupont, J. (1975) Lipids 10, 340-347.
5. Makino, I., Shinozaki, K., Nakagawa, S., and Mashimo, K. (1974) J. Lipid Res. 15, 132-138.
6. Moldéus, P., Högberg, J., and Orrenius, S. (1978) in Methods in Enzymology (Fleisher, S., and Packer, L., eds.) Vol. LII (c), pp. 61-64, Academic Press, New York, NY.
7. Högberg, J., and Kristoferson, A. (1977) Eur. J. Biochem. 74, 77-82.
8. Uchida, K., Nomura, Y., Kadowaki, M., Miyata,

K., and Miyake, T. (1970) Endocrin. Jpn. 17, 101-121.
9. Truter, E.V. (1964) J. Chromatogr. 14, 57-61.
10. Berry, M.N., and Friend, D.S. (1969) J. Cell Biol. 43, 506-520.
11. Seglen, P.O. (1977) in Methods in Cell Biology (Prescott, D.M., ed.) Vol. 13, pp. 29-83, Academic Press, New York, NY.
12. Anwer, M.S., Kroker, R., and Hegner, D. (1975) Biochem. Biophys. Res. Commun. 64, 603-609.
13. Yousef, I.M., Ho, J., and Jeejeebhoy, K.N. (1978) Can. J. Biochem. 56, 780-783.
14. Shefer, S., Hauser, S., Bekersky, I., and Mosbach, E.H. (1969) J. Lipid Res. 10, 646-655.
15. Krebs, H.A., Hems, R., and Tyler, B. (1976) Biochem. J. 158, 341-353.

[Received September 10, 1981]

Changes of Fatty Acid Composition of Phospholipids in Liver Mitochondria and Microsomes of the Rat during Growth

ANTTI KERÄNEN[a], PAAVO KANKARE[a], and MIKKO HALLMAN[b,1,*],
[a]Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki 17, Finland; and [b]Children's Hospital, Helsinki University Central Hospital, Stenbäckinkatu 11, SF-00290 Helsinki 29, Finland

ABSTRACT

The fatty acid patterns of rat liver mitochondrial and microsomal phospholipids were analyzed from term fetuses, 1 and 4 days old, and adult rats. The main fatty acids of phosphatidylethanolamine and -choline were stearic and palmitic acids, although the patterns differed slightly. The fatty acid composition of corresponding phospholipids in mitochondria and microsomes was similar. The fatty acid pattern of cardiolipin was dominated by linoleic acid. The most consistent feature of the developmental changes in the fatty acid patterns of all phospholipids studied was a decrease in the relative amount of monounsaturated fatty acids. The percentages of saturated fatty acids in phosphatidylethanolamine and -choline increased during neonatal development. It is suggested that the high levels of fetal monounsaturated fatty acids were due to low availability of polyunsaturated fatty acids. *Lipids* 17:155-159, 1982.

INTRODUCTION

The roles of phospholipids in membrane structure and function are still quite obscure, as are the roles of their various fatty acid patterns.

Mitochondria have a unique pattern of phospholipids (1,2) and have the capacity to synthesize cardiolipin (3,4). Other mitochondrial phospholipids are probably derived from microsomes (5-10). Differences in the fatty acid patterns of phospholipids corresponding to the separate sites of their synthesis and to dietary factors have been described (11-15). Furthermore, the enzymes involved in the biosynthesis of phospholipids may select certain fatty acid structures. It has been shown that the quality of the pathway for phosphatidylcholine (PC) synthesis in the liver determines the fatty acid structure of the product (16,17).

During the perinatal period, the quantities of the major mitochondrial and microsomal phospholipids of the liver change only a little in relation to each other (18,19). We have further measured the fatty acids of the 3 mitochondrial and 2 microsomal phospholipids. Consistent developmental change was found that may be due to variation in the quality of fatty acids available at biosynthetic surfaces.

EXPERIMENTAL PROCEDURES

Animals

The rats were of the Sprague-Dawley strain. Adults received an ordinary laboratory diet ad libitum (Hankkija Oy, Helsinki, Finland). The percentage fatty acid composition of the diet was as follows: 14:0 (0.8%); 16:0 (23.9%); 16:1 (1.3%); 18:0 (1.9%); 18:1 (32.4%); 18:2 (39.0%); 18:3 (0.7%). The young rats were kept with their mothers. The age of fetal rats was calculated on the basis of the gestation period (± 12 hr). There was random sex distribution among 4-day-old and younger rats. The adults were 3-6-month-old females. At the gestational age of 21-22 days, a hysterectomy was performed, and the term fetuses (age = 0 days) were quickly shelled out of their amniotic sacs.

Isolation of Cell Organelles

The animals were sacrificed by exsanguination, and the liver was quickly removed and washed several times in a 270 mM sucrose, 1 mM EDTA, 5 mM tris-HCl solution, pH 7.4, at 0 C. The subsequent steps were done in a 270 mM sucrose, 1 mM EDTA solution, pH 7.4 at 4 C. The livers were homogenized 3-5 times with a motor-driven Teflon pestle homogenizer (Arthur Thomas Co.). The nuclear fraction was spun down at 750 × g for 10 min and the mitochondria were sedimented at 7,000 × g for 10 min, and then washed twice by sedimenting the organelles at 6,000 × g for 10 min from the washing solution. The resulting preparation contained well preserved mitochondria and only a few nonmitochondrial membranes as viewed by electron microscopy and by measuring the glucose-6-phosphatase activity (20).

Microsomes were obtained as follows: postmitochondrial supernatant was spun at

[1]Address for correspondence: Mikko Hallman, M.D., University of California, San Diego, Department of Pediatrics, C-019, La Jolla, CA 92093.

11,000 x g for 10 min and the resulting supernatant was centrifuged for 1 hr at 105,000 x g. The microsomal pellet obtained was washed once with the isolation medium.

Isolation and Purification of Phospholipids

The phospholipids were extracted according to Folch et al. (21) and purified with column chromatography on a silicic acid/celite (2:1, w/w) column eluting phospholipids with methanol (12). The phospholipids were separated from each other by thin layer chromatography (TLC) on a Silica Gel HR plate, chloroform/methanol/glacial acetic acid/water (65:43:1:3, v/v/v/v) as the developing solvent (12). After development, the plates were sprayed with 0.2% 2,7-dichlorofluorescein in ethanol (w/v) and spots were outlined under UV-light and scraped off. The phospholipids were eluted from the gel, using 1,2-dichloromethane/methanol/water (2:8:1, v/v/v). No phosphorus could be detected in silica gel after the extraction. Inorganic phosphorus was assayed according to Bartlett's method (22) after the lipids were oxidized with 72% $HClO_4$ (w/v).

To obtain the fatty acid methyl esters, phospholipids were methanolyzed (0.5 M anhydrous methanolic HCl, 24 hr, 70 C) and the resulting methyl esters were extracted with hexane. Samples of each group were tested on TLC to detect the hydroxy fatty acid methyl esters (23). Organic solvents (E. Merck, Darmstadt, Germany, except *n*-hexane, J.T. Baker Chemicals N.V., Deventer, Holland) were distilled prior to use. To prevent autoxidation in preparative work, 2,6-di-tert-butyl-*p*-cresol (BHT, Sigma Chemical Co., St. Louis, MO) was added to the solvent (24).

Gas Liquid Chromatography and Mass Spectrometry

The fatty acid methyl esters were analyzed on a 3% EGSS-X column isothermally at 200 C and on a 15% diethyleneglycoladipate column isothermally at 195 C. The fatty acid methyl esters were identified by using the modified equivalent chain-length correlation and by calculating separation factors for polyunsaturated fatty acid methyl esters (25). The molecular weight was confirmed by gas liquid chromatography-mass spectrometry (GC-MS) on a 3% EGSS-X column. The weight percentages of various fatty acids were determined by calculating the peak areas by triangulation. The fatty acid standards were obtained from Supelco Inc. (Bellefonte, PA).

The GC was performed with a Perkin-Elmer Model 900 gas chromatograph (Perkin-Elmer Corporation, Norwalk, CT) equipped with flame ionization detectors. The columns were 3% EGSS-X on Supelcort 80-100 mesh (Supel-

TABLE 1

Percentage Fatty Acid Composition of Phosphatidylethanolamine in Rat Liver Mitochondria and in Microsomes[a]

Fatty acid	Mitochondria				Microsomes	
	At birth	1-Day old	4-Day old	Adult	1-Day old	Adult
16:0	22.4[b]	16.1	29.5	30.8	21.0	23.6
16:1ω7	1.5	0.2	3.6	0.3	3.0	0.3
18:0	36.6	42.5	41.7	43.9	27.4	34.7
18:1ω9	12.4[c]	7.2	6.3	5.6	8.2[d]	4.6
18:2ω6	4.1	2.4	0.2	3.2	2.6	3.3
18:3ω3	0.2	0.2	0.2	0.2	0.4	0.7
20:3ω6	0.2	0.3	0.2	0.2	1.8	0.3
20:4ω6	10.8	16.5	10.6	7.3	16.7	14.1
22:4ω6	2.2	3.0	3.5	2.6	4.1	0.7
22:5ω3	0.2	1.0	0.2	0.2	2.2	1.8
22:6ω3	9.4[b]	10.6[b]	4.0	5.7	12.6	16.0
Saturated	59.0[b]	58.6[b]	71.2	74.7	48.4[d]	58.3
ω7 + ω9	13.9[c]	7.4	9.9	5.9	11.2[d]	4.9
ω6	17.3	22.2[d]	14.5	13.3	25.2[d]	18.4
ω3	9.8[b]	11.8[b]	4.4	6.1	15.2	18.5

[a]Figures are weight percentages of fatty acid methyl esters and means of from 3 to 5 determinations that were run in duplicate. Standard deviation was 15% or less of the means. In 4-day-old or younger rats, the whole litter was used for the single analysis.

[b]$p<0.05$ as compared to 4-day-old animals and adults (t-test).

[c]$p<0.05$ as compared to 1-day-old, 4-day-old, and adult animals.

[d]$p<0.05$ as compared to adults.

co Inc., Bellefonte, PA) and 15% diethyleneglycoladipate on Gas Chrom Q 80-100 mesh (Applied Science Laboratories Inc., State College, PA). Column sizes were 2 m × 3 mm and the carrier gas was argon.

GC-MS was performed with a Varian Aerograph 1700 gas chromatograph combined with a Varian MAT CH-7 mass spectrometer equipped with a Spectro System 100 MS data handling system. Column size of the gas chromatograph was 2 m × 2 mm and the carrier gas was helium. Electron energy was 70 eV. The total mass range was from m/e 50 to m/e 500.

RESULTS

Phosphatidylethanolamine (PE)

The fatty acid pattern of rat liver mitochondrial and microsomal PE is presented in Table 1.

The major fatty acids at all ages were palmitic (21-31% of the total) and stearic acid (27-44% of the total). In the microsomes, the amount of fatty acids belonging to the linoleic acid series was at least twice as high as in mitochondria. No hydroxy fatty acids were found.

The most prominent feature of the maturation process was the saturation of the fatty acids, which was mainly due to the increase in the amount of palmitic acid. The relative amount of mono- and polyunsaturated fatty acids decreased.

Phosphatidylcholine (PC)

The fatty acid pattern of rat liver mitochondrial and microsomal PC was also dominated by saturated fatty acids (42-60% of the total as presented in Table 2). In the fetal liver PC, the amount of monounsaturated fatty acids was exceptionally high (about 31% of the total). The amount of fatty acids belonging to the linoleic acid series (ω6) was about 3 times higher than the amount of fatty acids belonging to the linolenic acid series (ω3) (9-6% of the total).

The maturation was characterized by a decrease in monounsaturated fatty acids. Monounsaturated fatty acids were replaced by stearic acid and polyunsaturated fatty acids.

Cardiolipin

The fatty acid pattern of both fetal and adult rat liver mitochondrial cardiolipin consisted nearly exclusively of linoleic, oleic and palmitoleic acids, amounting to more than 90% of the weight of the total (Table 3).

The maturation was characterized by an increase in the amount of linoleic acid and by a corresponding decrease in the amount of oleic and palmitoleic acids.

TABLE 2

Percentage Fatty Acid Composition of Phosphatidylethanolamine in Rat Liver Mitochondria and in Microsomes[a]

Fatty acid	Mitochondria				Microsomes	
	At birth	1-Day-old	4-Day-old	Adult	1-Day-old	Adult
16:0	24.6	28.2	30.3	28.6	26.1	29.2
16:1ω7	4.2	2.0	0.2	1.2	2.9	1.2
18:0	20.4[b]	22.1	28.7	30.9	16.2[d]	28.5
18:1ω9	26.7[c]	15.9	8.5	11.2	21.9[d]	7.8
18:2ω6	10.2	7.1	7.8	10.6	10.9	10.8
18:3ω3	0.5	0.2	0.2	0.2	0.4	0.2
20:3ω6	1.2	0.8	0.2	0.2	0.9	1.8
20:4ω6	5.2[c]	14.7	15.2	8.8	11.5	11.2
22:4ω6	0.9	0.3	2.5	2.8	2.7	0.2
22:5ω3	0.2	0.4	0.2	0.2	0.6	1.0
22:6ω3	5.9	8.2	6.2	5.3	6.0	8.3
Saturated	45.0[b]	50.3	59.0	59.5	42.3[d]	57.7
ω7 + ω9	30.9[c]	17.9	8.7	12.4	24.8[d]	9.0
ω6	17.5[c]	22.9	25.7	22.4	26.0	24.0
ω3	6.6	8.8	6.6	5.7	7.0	9.5

[a]Figures are weight percentages of fatty acid methyl esters and means of from 3 to 5 determinations that were run in duplicate. Standard deviation was 15% or less of the means. In 4-day-old or younger rats, the whole litter was used for the single analysis.

[b]$p<0.05$ as compared to 4-day-old animals and adults (t-test).

[c]$p<0.05$ as compared to 1-day-old, 4-day-old, and adult animals.

[d]$p<0.05$ as compared to adults.

DISCUSSION

The fatty acid patterns of various phospholipids differed from each other. On the other hand, the fatty acid patterns of PE and PC in mitochondria were similar to those in microsomes. This finding was expected because mitochondrial PE and PC seem to be synthetized in microsomes (5-10).

The changes in the fatty acid patterns of corresponding phospholipids in mitochondria and microsomes resembled each other. The maturation process of these phospholipids is characterized by a relative increase in the amount of saturated fatty acids and by a replacement of monounsaturated fatty acids with polyunsaturated fatty acids.

Dobiášová et al. (26) have analyzed the changes in the fatty acid pattern of total phospholipids in the developing rat liver. Their material consisted of phospholipids of the whole liver and is therefore not directly comparable with ours. Nonetheless, we found that the fatty acid pattern of mitochondrial PE and PC, as well as the developmental changes, were similar to those analyzed by Dobiášová et al. It seems that mitochondrial needs for PE and PC fatty acids do not differ markedly from those of other cell organelles.

In rat liver microsomes, there is an increase in the activity of the synthesis of long-chain fatty acids, probably by chain-elongation, just after delivery with a maximum at the age of 11 days (27). At the same time, ethanolamine incorporation into phospholipids, which is low in fetal liver, increases rapidly reaching a level of 60-80% of the adult level at the age of one day (28). The possible correlation between these activities and the marked saturation of the fatty acids in microsomal and mitochondrial PE and PC remains to be studied. The minor differences which exist between microsomal and mitochondrial PE and PC are probably reflections of local needs. The mitochondrial chain-elongation system is known to be able to influence the phospholipids in such a way as to make their fatty acids more suitable for mitochondrial membranes (29).

It has been suggested that the different pathways for PC synthesis preferentially produce molecules with a certain fatty acid structure. According to the present evidence, 1-palmitoyl-2-oleoyl-glyceryl-phosphoryl choline and hexanoic lecithins (see for review ref. 30). According to studies on liver slices, the incorporation of methionine into PC rapidly increased during the neonatal period, whereas the development of choline incorporation was more gradual (31). These changes in the biosynthetic pathways barely explain the development in the fatty acid structure of PC and explain even less those of other phospholipids.

TABLE 3

Fatty Acid Composition of Cardiolipin in Rat Liver Mitochondria[a]

Fatty acid	Age	
	1-Day-old	Adult
16:0	0.9	0.6
16:1ω7	5.9[b]	3.8
18:0	2.6	1.6
18:1ω9	26.6[b]	17.3
18:2ω6	58.6[b]	70.1
18:3ω3	0.7	1.5
20:3ω6	1.7	1.6
20:4ω6	0.9	1.2
22:4ω6	0.2	0.2
22:5ω3	0.2	0.2
22:6ω3	1.7	1.9
Saturated	3.5	2.2
ω7 + ω9	32.5[b]	21.1
ω6	61.4[b]	73.1
ω3	2.6	3.6

[a]Figures are weight percentages of fatty acid methyl esters and means of 3 to 5 determinations. Standard deviation was 15% or less of the means. In 1-day-old rats, the whole litter was used for the analysis.

[b]$p<0.05$ as compared to adults (t-test).

The lipids are mostly synthesized de novo in the fetus (32). The high contents of palmitic, stearic and oleic acids of PE and PC in mitochondria and microsomes from fetal liver seem to support this conclusion. As a result of the lack of polyunsaturated fatty acids, the amount of monounsaturated fatty acids increases (33). This could be due to slow transport of fatty acids through the placenta in the fetus (34). On a balanced diet, such as milk, the synthesis of ω9-fatty acids is depressed and the polyunsaturated fatty acids from the diet are incorporated into phospholipids. In agreement with these findings, the relative amounts of polyunsaturated fatty acids increase and monounsaturated fatty acids decrease following delivery.

According to Peluffo et al., in essential fatty acid deficiency, the fatty acid pattern of microsomal phospholipids in weanling rats is similar to that in our normal newborn rats (35). Moreover, newborn animals and humans rapidly develop signs of essential fatty acid deficiency in the absence of suitable food (36,37).

Cardiolipin occurs in mammalian tissues exclusively in mitochondria (38) partly tightly bound to cytochrome oxidase (14,39). Its fatty acid pattern is totally different from that

of other phospholipids, characterized by a high amount of linoleic acid as found in the present and earlier studies (12,14). Of the fatty acids in cardiolipin, oleic acid is replaced by linoleic acid during neonatal growth. Therefore, it seems that the decrease in the relative amount of monounsaturated fatty acids and the corresponding increase in the amount of polyunsaturated fatty acids is a general phenomenon during the perinatal maturation of the phospholipid structure in the liver. It is not bound to the site of phospholipid synthesis, which indicates a cause somewhere outside this system. The explanation can be a relative lack of polyunsaturated fatty acids in the fetus, and an increase in their availability during the newborn period.

REFERENCES

1. Getz, G.S., Bartley, W., Stripe, F., Notton, B., and Renshaw, A. (1962) Biochem. J. 83, 181-191.
2. Fleischer, S., Rouser, G., Fleischer, B., Casu, A., and Kritchevsky, D. (1967) J. Lipid Res. 8, 170-180.
3. Davidson, J.B., and Stanacev, N.Z. (1971) Biochem. Biophys. Res. Commun. 42, 1191-1199.
4. Hostetler, K.Y., Van den Bosch, H., and Van Deenen, L.L.M. (1971) Biochim. Biophys. Acta 239, 113-119.
5. McMurray, W.C., and Dawson, R.M.C. (1969) Biochem. J. 112, 91-108.
6. Jungalwala, F.B., and Dawson, R.M.C. (1970) Eur. J. Biochem. 12, 399-402.
7. Block, M.C., Wirtz, K.W.A., and Scherphof, G.L. (1971) Biochim. Biophys. Acta 233, 61-75.
8. Sauner, M.T., and Lévy, M. (1971) J. Lipid Res. 12, 71-75.
9. Wirtz, K.W.A., Kamp, H.H., and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 274, 606-617.
10. Landriscina, C., and Marra, E. (1973) Life Sci. 13, 1373-1381.
11. Yamamoto, A., Isozaki, M., Hirayama, K., and Sagai, Y. (1965) J. Lipid Res. 6, 295-300.
12. Parkes, J.G., and Thompson, W. (1970) Biochim. Biophys. Acta 196, 162-169.
13. Colbeau, A., Nachbaur, J., and Vignais, P.M. (1971) Biochim. Biophys. Acta 249, 462-492.
14. Awasthi, Y.C., Chuang, T.F., Keenen, T.W., and Grane, G.L. (1971) Biochim. Biophys. Acta 226, 42-52.
15. Sgoutas, O.S., Jones, R., and Leight, M. (1973) Int. J. Biochem. 4, 437-445.
16. Trevehella, M.A., and Collins, F.D. (1973) Biochim. Biophys. Acta 296, 51-61.
17. Van Golde, L.M.G., Scherphof, G.L., and Van Deenen, L.L.M. (1969) Biochim. Biophys. Acta 176, 635-637.
18. Miller, J.E., and Cornatzer, W.E. (1966) Biochim. Biophys. Acta 125, 534-541.
19. Jakovcic, S., Haddock, J., Getz, G.S., Rabinowitz, M., and Swift, H. (1971) Biochem. J. 121, 341-347.
20. Hallman, M. (1971) Biochim. Biophys. Acta 253, 360-372.
21. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
22. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
23. Nikkari, T. (1965) Scand. J. Clin. Lab. Invest. 17, suppl. 85, 58-59.
24. Wren, J.J., and Szczepanowska, A.D. (1964) J. Chromatogr. 14, 405-410.
25. Supina, W.R. (1964) In Biomedical Applications of Gas Chromatography Szymanski, H.A., ed.) pp. 33-51, Plenum Press, New York, NY.
26. Dobiášová, M., Hahn, P., and Koldovský, O. (1964) Biochim. Biophys. Acta 84, 538-549.
27. Warshaw, J.B., and Kimura, R.E. (1973) Biol. Neonate 22, 133-140.
28. Weinhold, P.A., and Sanders, R.D. (1971) Biochemistry 10, 1090-1095.
29. Landriscina, C., Gnoni, G.V., and Quagliariello, E. (1972) Eur. J. Biochem. 29, 188-196.
30. Van den Bosch, H. (1974) Ann. Rev. Biochem. 43, 248-250.
31. Weinhold, P.A. (1969) Biochim. Biophys. Acta 187, 85-93.
32. Popják, G., and Beeckmann, M.L. (1950) Biochem. J. 46, 547-561.
33. Sprecher, H.W. (1972) Fed. Proc. 31, 1451-1457.
34. Van Duyne, C.M., Havel, R.J., and Felts, J.M. (1962) Am. J. Obstet. Gynecol. 84, 1069-1074.
35. Peluffo, R.O., Nervi, A.M., and Brenner, R.R. (1976) Biochim. Biophys. Acta 441, 25-31.
36. Holman, R.T., Caster, W.O., and Wiese, H.F. (1964) Am. J. Clin. Nutr. 14, 70-75.
37. Friedman, Z. (1980) Am. J. Dis. Child. 134, 397-408.
38. G. Rouser, Nelson, G.J., Fleischer, S., and Simon, G. (1968) in Biological Membranes, pp. 5-69 (Chapman, D., ed.) Academic Press, London, England.
39. Chuang, T.F., and Crane, F.L. (1973) J. Bioenerget. 4, 563-578.

[Revision received December 14, 1981]

Regional Distribution of Glycosylceramide-Sulfates in Human Kidney

BO E. SAMUELSSON, *Department of Medical Biochemistry, University of Göteborg, Box 33031, S-400 33 Göteborg, Sweden*

ABSTRACT

Glycosylceramide-sulfates were prepared separately from human kidney cortex and medulla. Glycosylceramide-sulfates were characterized with respect to long-chain bases, fatty acids, carbohydrates and sulfuric ester group position. Monogalactosylceramide I^3-sulfates were 3 times more concentrated in medulla compared to cortex, whereas lactosylceramide II^3-sulfates were 3 times more concentrated in cortex compared to medulla. The results were discussed in relation to the possible role of glycosylceramide-sulfates in sodium-potassium ion transport.
Lipids 17:160-165, 1982.

The finding of high concentrations of monogalactosylceramide I^3-sulfates in the outer part of bovine kidney medulla (3,4), the main site for corticosteroid-dependent sodium ion transport (5), was the incitement to the thought that glycosylceramide-sulfates are in some way related to sodium ion transport. This gave rise to a series of papers describing the lipid pattern and the activity of Na^+-K^+-ATPase in a number of vertebrate tissues (e.g., refs. 6-9). The results obtained from those studies showed that the concentration of monogalactosylceramide I^3-sulfate is stoichiometrically related to the activity of Na^+-K^+-ATPase in tissues such as brain gray matter, bovine kidney, salt glands of cartilaginous fish and marine birds, electric organ and erythrocytes. A correlation between monoglycosylceramide-sulfate metabolism and Na^+-K^+-ATPase activity in total mouse kidneys has also been shown (10). A model for a possible function of this lipid in ion transport was recently described (7,8,11).

All glycosylceramide-sulfates so far studied (6), including the glycolipid sulfate from the extreme halophile *Halobacterium salinarium* (11), show an identical polar section, galactose-3-sulfate, indicating a strict structural demand on this part of the molecule, possibly related to ion (K^+) transport (6-9,11). Lactosylceramide II^3-sulfates not found in the organs and tissues studied in this respect before, including bovine kidney cortex and medulla (6-9,11), have earlier been identified and characterized in lipid extracts from whole human kidneys (12, 13). Furthermore, human kidney has been shown to contain a higher Na^+-K^+-ATPase activity in medulla than in cortex (14,15) although, as in bovine kidney (4), there is not as pronounced a difference as in most vertebrate kidneys (16). It was considered of interest to study the distribution of both types of glycosylceramide-sulfates in different regions of human kidney. Such a study has not been done before and may add to the possible involvement of these lipids in ion transport. Preliminary results from the present work was reported earlier (3).

The lipophilic composition of individual sphingolipids in different regions of bovine kidney has been shown to differ considerably (7,17, and unpublished) and the total long-chain base pattern of human kidney has also shown major regional differences (18). These differences have been interpreted as a result of different physicochemical demands on the plasma membrane in the different regions (7, 17). It was thus considered of further interest to study the lipophilic composition of both types of glycosylceramide-sulfates isolated from different regions of human kidney.

MATERIALS AND METHODS

Kidneys

Adult human kidneys from 3 individuals were collected from autopsies at Sahlgren's hospital within 48 hr after death. The kidneys showed no macroscopic abnormalities and were stored at -20 C during the collection period (1 wk).

Reference Compounds

Monogalactosylceramide I^3-sulfates and lactosylceramide II^3-sulfates were kindly provided

The IUPAC-IUB recommendations (1) for nomenclature of glycosphingolipids carrying a sulfuric ester (sulfate) group, formally called sulfatides, are used here. The IUPAC-IUB recommendations of 1970 (2) for assignment of double bond position are used here for methyl branch positions. Thus, n-3 means position 3 from the methyl end. In the short-hand designations for long-chain bases, d means dihydroxy, t means trihydroxy and br is a methyl branched chain. The number before the colon means chain length and the number after the colon is degree of unsaturation. Na^+-K^+-ATPase = sodium-potassium adenosine triphosphatase (EC 3.6.1.3.).

by Dr. E. Mårtensson of this University (see refs. 12,13,19). Reference aldehydes were obtained by oxidation of long-chain bases of bovine kidney sphingomyelins (17).

Dissection

At the end of the collection period, the kidneys were thawed and freed from capsules and pelvices. Rough dissection into cortex, medulla and a small intermediate zone was performed as described for bovine kidney (3). The intermediate zone was discarded and the remaining 2 parts of the tissue were worked-up separately. The tissue was homogenized in a Turmix blender, lyophilized and dry wt was determined.

Preparation of Glycosylceramide-Sulfates

Total lipid extract was prepared from human kidney cortex or medulla and worked-up as described in detail elsewhere (4). The extract was subjected to mild alkaline hydrolysis and subsequent partition to eliminate glycerol ester lipids. The alkali-stable lipids were separated into nonacidic and acidic lipids by means of DEAE-cellulose column chromatography (4). The acidic fraction was dialyzed and lyophilized. From this material, monogalactosylceramide- and lactosylceramide-sulfates were separated by Florisil column chromatography using methanol in chloroform as eluant.

Characterization of Intact Glycosylceramide-Sulfates

The purity of the intact glycosylceramide-sulfate fractions was tested by thin layer chromatography (for conditions, see refs. 20, 21). The amount of pure glycosylceramide-sulfates was estimated gravimetrically and by colorimetric determination of hexose (20). For the determination of sulfate group position, acetylated and trimethylsilylated glycosylceramide-sulfates were analyzed by direct inlet mass spectrometry (22).

Characterization of Glycosylceramide-Sulfates after Degradation

Acid hydrolysis and separation of produced fatty acids and long-chain bases were performed as described elsewhere (17,23). Fatty acids were converted to methyl esters (24) and separated into normal and hydroxy fatty acid esters as described (6). After trimethylsilylation, the fatty acids were analyzed by gas chromatography (24). Total long-chain bases were converted to their corresponding dinitrophenyl derivatives, purified from acid-induced by-products and subjected to oxidation with lead tetraacetate (23). The aldehydes produced were analyzed by gas chromatography (17).

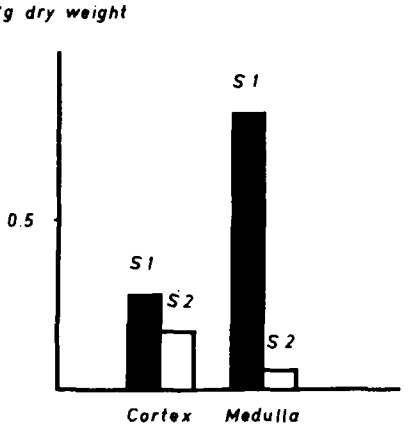


FIG. 1. Diagram showing the quantitative distribution of monogalactosylceramide I^3-sulfates (S1) and lactosylceramide II^3-sulfates (S2) between cortex and medulla of human kidney.

Qualitative carbohydrate analysis was performed principally as described elsewhere (25).

RESULTS AND COMMENTS

The quantitative figures obtained gravimetrically and by photometric determination of hexose agreed well and the quantitative distribution of monogalactosyl- and lactosylceramide-sulfates between cortex and medulla is shown in Figure 1.

Characterization of Intact Glycosylceramide-Sulfates

Intact glycosylceramide-sulfates were characterized on thin layer plates with known references.

A 3-position for the sulfate group was confirmed by the use of mass spectrometry of acetylated and trimethylsilylated derivatives (22). The partial spectrum of the monogalactosylceramide-sulfate was almost identical to that of a similar sample obtained from human brain (22). The spectrum of the lactosylceramide-sulfate is reproduced in Figure 2; m/e 361 originates from the substituted terminal hexose and indicates a monotrimethylsilylated acetylated terminal hexose. The presence of an intense peak at m/e 169 and the very low abundance of ions at m/e 199 strongly suggest the trimethylsilyloxy group and thus the original sulfate group to be in position 3 of the terminal hexose (22).

Characterization of Glycosylceramide-Sulfates after Degradation

Carbohydrate analysis of monoglycosylceramide-sulfate revealed only galactose, whereas

diglycosylceramide-sulfate gave glucose and galactose in the ratio of 1:1.

Results of the fatty acid analysis are shown in Table 1. Both normal and hydroxy fatty acids were present (12). Regarding monogalactosylceramide-sulfates, there were slightly more hydroxy fatty acids in medulla than in cortex, and lactosylceramide-sulfates from cortex contained relatively more normal fatty acids. Concerning the chain length distribution and unsaturation, there were no major differences between cortex and medulla. Results from the long-chain base analyses were collected in Table 2. As can be seen, mono- and diglycosylceramide-sulfates have roughly the same long-chain base pattern with the presence of both di- and trihydroxy bases as well as straight chain, branched chain, saturated and unsaturated dihydroxy bases (19). With monogalactosylceramide-sulfates, there is a 4-fold increase of trihydroxy bases in medulla compared to cortex. There is also a slight increase of species with longer chain length both for di- and trihydroxy bases (the appearance of t20:0 and d20:0 in the medulla).

DISCUSSION

The structures of glycosylceramide-sulfates from human kidneys are well documented (12, 13,19). The confirmatory micro-identification of sulfate group position by mass spectrometry of derivatized intact molecules offers an alternative to permethylation studies. This type of analysis has earlier been done on monogalactosylceramide-sulfates (20,22) and on the glycolipid sulfate of *H. salinarium* (11). As expected, the spectrum (Fig. 2) gives strong evidence for a 3-position of the sulfate group.

The high amount of trihydroxy bases in medulla compared to cortex for glycosylceramide-sulfates is in accordance with earlier findings made during studies of the total long-chain base pattern in human kidney cortex and medulla (18). So, also, was the case for bovine kidney (18), which was subjected to a detailed analysis to ascertain the regional distribution of sphingolipids (4), as well as a detailed characterization of individual sphingolipids from different regions (4,17, and unpublished). These interesting variations in ceramide structure might be a result of an adaptation to the different physicochemical demands on the membranes in different regions of the kidney (4,7,17). Model studies on synthetic sphingolipids containing phytosphingosine and related bases in combination with nonhydroxy and hydroxy fatty acids indicate significant effects on molecular packaging and conformation (26-28).

As can be seen from Figure 1, the concentration of monogalactosylceramide-sulfates is

TABLE 1

Fatty Acid Compositions of Glycosylceramide-Sulfates from Human Kidney Cortex and Medulla

Fatty acid chain length and unsaturation	Normal fatty acids			Hydroxy fatty acids		
	Monogalactosylceramide I^3-sulfates		Lactosylceramide II^3-sulfates	Monogalactosylceramide I^3-sulfates		Lactosylceramide II^3-sulfates
	Cortex (%)	Medulla (%)	Cortex (%)	Cortex (%)	Medulla (%)	Cortex (%)
16:1	tr[a]	tr	1	–	–	–
16:0	7	7	8	4	2	4
18:1	tr	tr	1	–	–	–
18:0	2	1	3	tr	tr	2
20:0	4	3	6	2	2	5
21:0	tr	–	–	tr	tr	–
22:1	tr	2	2	1	2	2
22:0	21	24	22	17	13	19
23:1	tr	tr	tr	tr	tr	tr
23:0	8	13	3	16	14	12
24:1	19	19	23	22	37	19
24:0	35	25	29	31	24	31
25:1	tr	1	1	2	3	tr
25:0	tr	tr	tr	1	1	tr
26:1	tr	tr	tr	2	1	4
26:0	tr	tr	tr	tr	–	–
% of total fatty acids	60	46	82	40	54	18

[a]Trace.

TABLE 2

Long-Chain Base Composition of Monogalactosylceramide I^3-sulfates and Lactosylceramide II^3-sulfates from Human Kidney Cortex and Medulla

Aldehyde	Relative amounts (%)			Probable parent base
	Cortex		Medulla	
	Monogalactosylceramide I^3-sulfates	Lactosylceramide II^3-sulfates	Monogalactosylceramide I^3-sulfates	
14:0	tr[a]	tr	1	d16:0 and t17:0
	tr	tr	tr	
15:0	8	8	34	t18:0 (and d17:0)
14:1	1	3	2	d16:1
16:0	4	3	2	d18:0
15:1	2	3	2	d17:1
17:0	tr	tr	2	t20:0 (and d19:0)
br(n-2) 16:1	1	2	tr	br(n-2) d18:1
16:1	71	72	46	d18:1
18:0	3	2	1	d20:0
br(n-2) 17:1	7	4	4	br(n-2) d19:1
br(n-3) 17:1	2	2	2	br(n-3) d19:1
17:1	tr	tr	1	d19:1
18:1	tr	tr	2	d20:1
Straight-chain bases	88	91	93	
Branched-chain bases	10	8	6	

[a]Trace.

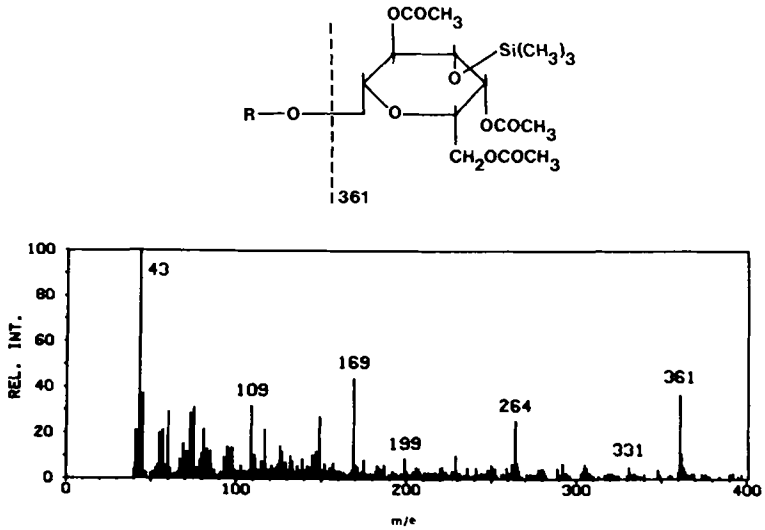


FIG. 2. Partial mass spectrum of monotrimethylsilylated acetylated derivative (22) of the lactosylceramide II^3-sulfate. A simplified formula is shown at top for the interpretation. Peaks below m/e 40 and above m/e 400 were not reproduced. The direct inlet system of an LKB-9000 instrument was used with the following conditions: acceleration voltage 3.5 kV, trap current 60 μA, electron energy 70 eV, source temperature 270 C and probe temperature at evaporation 200 C.

almost 3 times higher in medulla compared to cortex. This situation is similar to that found in bovine kidney although not as pronounced (9-fold difference in concentration for bovine kidney, see refs. 3 and 4). Lactosylceramide-sulfates have a lower concentration than monogalactosylceramide-sulfates both in cortex and in medulla, but show the reverse regional distribution with 3 times higher concentration in cortex compared to medulla. Added together, there is almost twice as much glycosylceramide-sulfates in medulla compared to cortex. This difference is roughly the same as that found for the Na^+-K^+-ATPase activity in human kidney cortex and medulla (14,15).

The very suggestive correlation between glycosylceramide-sulfate and Na^+-K^+-ATPase activity has earlier been shown only for monogalactosylceramide-sulfates and primarily in functionally more clear-cut tissues, such as the salt glands or marine animals (6), rectal gland of elasmobranchs (6) and the electric organ (9). The kidney is functionally more complex and the presence of several Na^+-K^+-ATPase has been indicated (6,29). A correlation between glycosylceramide-sulfate concentration and enzyme activity may therefore be less informative in this respect. This has been discussed in detail before (6). The new and unexpected finding of an uneven and different distribution of the 2 different glycosylceramide-sulfates between cortex and medulla of human kidney may, however, indicate additional functional aspects. If the terminal galactose I^3-sulfate of these molecules is, as postulated (6-9,11), an essential part in ion (K^+) transport, the present findings may be interpreted that different parts of the same glycosphingolipid molecules have different functions in the membrane (6,7). The ceramide as a part of the membrane matrix may contribute to the physicochemical characteristics, the carbohydrate chain may serve as a spacer between the membrane surface and the terminal saccharide, and the terminal carbohydrate may have a more specific function (e.g., in ion transport). The spacer function with different carbohydrate chain length may be a result of different membrane anatomy (4). Glycosylceramide-sulfates with even longer carbohydrate chains and a terminal galactose-3-sulfate have been shown to exist in hog gastric mucosa (30). It is also of interest that extremely halophilic bacteria, *H. salinarium*, living in almost saturated sodium chloride solution, have a glycolipid-sulfate with a terminal galactose-3-sulfate bound to mannosylglucosyldiphytanylglycerol (11 and references cited therein). Halophilic bacteria also contain an Na^+-K^+-ATPase.

Earlier work has shown that monogalactosyl I^3-sulfates were unique among lipids in tissue distribution following the extent of Na^+-K^+-ATPase activity and a possible function as a

receptor for K^+ ions has been proposed (7-9, 11). The present work shows that lactosylceramide II^3-sulfates in human kidney does not have the same distribution between cortex and medulla as monogalactosylceramide I^3-sulfates. This new finding does not exclude a possible role for the terminal galactose-3-sulfate in ion transport, but may indicate additional functional adaptation.

ACKNOWLEDGMENTS

Dr. K.-A. Karlsson is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (No. 3967), Medical Faculty of Göteborg and Svenska Sällskapet för Medicinsk Forskning.

REFERENCES

1. IUPAC-IUB Commission on Biochemical Nomenclature (1977) Eur. J. Biochem. 79, 11-21.
2. IUPAC-IUB Commission on Nomenclature of Organic Chemistry (1970) Eur. J. Biochem. 12, 1-2.
3. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1968) Acta Chem. Scand. 22, 2723-2724.
4. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1973) Biochim. Biophys. Acta 316, 317-335.
5. Murayama, Y., Morel, F., and LeGrimellec, C. (1972) Pflügers Arch. 333, 1-16.
6. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1974) Eur. J. Biochem. 46, 243-258.
7. Karlsson, K.-A., (1977) in Structure of Biological Membranes (Abrahamsson, S., and Pascher, I., eds.) pp. 245-274, Plenum Publishing Corporation, New York, NY.
8. Hansson, G.C., Karlsson, K.-A., and Samuelsson, B.E. (1978) J. Biochem. (Tokyo) 83, 813-819.
9. Hansson, G.C., Heilbronn, E., Karlsson, K.-A., and Samuelsson, B.E. (1979) J. Lipid Res. 20, 509-518.
10. Umeda, T., Egawa, K., and Nagai, Y. (1976) Jpn. J. Exp. Med. 46, 87-94.
11. Falk, K.-E., Karlsson, K.-A., and Samuelsson, B.E. (1980) Chem. Phys. Lipids 27, 9-21.
12. Mårtensson, E. (1966) Biochim. Biophys. Acta 116, 521-531.
13. Stoffyn, A., Stoffyn, P., and Mårtensson, E. (1968) Biochim. Biophys. Acta 152, 353-357.
14. Tullock, B.R., Gibson, K., and Harris, P. (1972) Clin. Sci. 42, 265-275.
15. Pettersson, S., and Scherstén, T. (1973) Eur. Surg. Res. 5, 282-291.
16. Hendler, E.D., Toretti, J., and Epstein, F.H. (1971) J. Clin. Invest. 50, 1329-1337.
17. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1973) Biochim. Biophys. Acta 316, 336-362.
18. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1968) Acta Chem. Scand. 22, 1361-1363.
19. Karlsson, K.-A., and Mårtensson, E. (1968) Biochim. Biophys. Acta 152, 230-233.
20. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1971) J. Membr. Biol. 5, 169-184.
21. Karlsson, K.-A., and Pascher, I. (1971) J. Lipid Res. 12, 466-472.
22. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1969) Biochem. Biophys. Res. Commun. 37, 22-27.
23. Karlsson, K.-A. (1970) Chem. Phys. Lipids 5, 6-43.
24. Karlsson, K.-A., Nilsson, K., Samuelsson, B.E., and Steen, G.O. (1969) Biochim. Biophys. Acta 176, 660-663.
25. Sweeley, C.C., and Vance, D.E. (1967) in Lipid Chromatographic Analysis (Marinetti, G.V., ed.) Vol. I, pp. 465-495, Marcel Dekker, New York, NY.
26. Abrahamsson, S., Dahlén, B., Löfgren, H., Pascher, I., and Sundell, S. (1977) in Structure of Biological Membranes (Abrahamsson, S., and Pascher, I., eds.) pp. 1-23, Plenum Publishing Corporation, New York, NY.
27. Löfgren, H., and Pascher, I. (1977) Chem. Phys. Lipids 20, 273-284.
28. Boggs, J.M. (1980) Can. J. Biochem. 58, 755-770.
29. Whittembury, G., and Proverbio, F. (1976) in Intestinal Ion Transport (Robinson, J.W.L., ed.) pp. 1-11, William Cloves and Sons Ltd., London.
30. Slomiany, B.L., Slomiany, A., and Horowitz, M.I. (1974) Biochim. Biophys. Acta 348, 388-396.

[Received July 21, 1981]

COMMUNICATIONS

Occurrence of 3-Oxo Triterpenes in the Unsaponifiable Matter of Some Vegetable Fats

J.N. KOLHE, ANIL BHASKAR and **N.V. BRINGI***, *Hindustan Lever Research Centre, Chakala, Andheri East, Bombay 400 099, India*

ABSTRACT

Fourteen vegetable oils were examined and 3-oxo triterpenes are present in the unsaponifiable matter of shea (*Butyrospermum parkii*), illipe (*Shorea stenoptera*), mango kernel (*Mangifera indica*), kokum (*Garcinia indica*) and phulwara (*Madhuca butyracea*) fats but not in cocoa butter, dhupa fat (*Vateria indica*), soybean, peanut, mustard, corn, palm, coconut or mowrah (*Madhuca latifolia*) fats. *Lipids* 17:166-168, 1982.

INTRODUCTION

The occurrence of 3-oxo triterpenes in the unsaponifiable matter of sal (*Shorea robusta*) was reported earlier (1). It is both of academic and practical interest to determine if the occurrence of oxo triterpenes is a general phenomenon in vegetable oils or if it is restricted to certain species. Accordingly, the unsaponifiable matter of 14 vegetable fats was analyzed and the results demonstrating the presence of 3-oxo triterpene in some fats which are generally characterized by the presence of substantial proportions of symmetrical triglycerides are reported in this communication.

EXPERIMENTAL PROCEDURE

Isolation of Unsaponifiable Matter

A typical procedure consists of saponifying the fat (100 g) in ethanol/water (3:1 v/v, 400 ml) and potassium hydroxide (70 g) for 2 hr, followed by dilution with water and extraction with petroleum ether (bp 40-60 C, 3 × 500 ml).

Preliminary Screening by Thin Layer Chromatography (TLC)

The unsaponifiable matter was analyzed by TLC on silica gel (5 × 20 cm, 0.1 mm thickness, 30% diethyl ether in hexane, visualization by iodine vapor) and the presence of a spot at R_f 0.62 was indicative of oxo triterpenes. The usual triterpene and steroid alcohol fractions were encountered at lower R_f values.

Isolation and Purification of Oxo Triterpenes

The unsaponifiable matter of fats containing oxo triterpenes was chromatographed on silica gel (1:15, w/w) using hexane as eluent. When necessary, the oxo triterpene fraction was further purified on preparative TLC on silica gel (20 × 20 cm, 1 mm thickness, 30% ether in hexane, visualization under UV after spraying with 0.1% dibromofluoroscein in methanol) and the compounds were extracted with 25% methanol in chloroform (v/v).

Instrumental Analysis

IR spectra (neat) were recorded on Perkin-Elmer Model 197 spectrophotometer. Gas chromatography-mass spectrometric (GC-MS) analyses were performed on an LKB Model 2091, electron energy was 70 eV, trap current 50 μA, ion source temperature 290 C and accelerating voltage 3.5 kV. The samples were introduced through the GC inlet using a 25-m WCOT column coated with SE-30. The column temperature was programmed from 220-260 C at 2 C/min.

Preparation of Authentic Samples

β-Amyrin, cycloartenol, lupeol and 24-methylene cycloartenol were isolated from the triterpene fraction of sal unsaponifiable matter. Further fractionation was done as their acetates on argentation preparative TLC (1). α-Amyrin and butyrospermol were isolated as their acetates from shea fat unsaponifiable matter (2). The acetates were hydrolyzed with potassium hydroxide in aq. methanol and free alcohols were oxidized by stirring with pyridinium chlorochromate in methylene chloride (3).

RESULTS AND DISCUSSION

Of the 14 different vegetable fats examined, the unsaponifiable matter of 5, i.e., shea, illipe,

kokum, mango kernel and phulwara, contained the oxo triterpene fraction. This was confirmed by the characteristic IR absorption at 1720 cm^{-1} of the isolated fraction. The unsaponifiable matter of the other 9 fats, i.e., cocoa butter, dhupa, mowrah, palm, peanut, corn, soybean, mustard (*Brassica juncea*) and coconut, did not reveal the presence of oxo triterpenes. All 14 fat samples were of commercial origin except cocoa butter, which was extracted by solvent extraction of cocoa beans grown in Kerala, India.

The oxo triterpenes found in the unsaponifiable matter of the fats by GC-MS analysis is given in Table 1. The GC resolution of different 3-oxo triterpenes was good and the order of elution under the conditions mentioned in the experimental was β-amyrenone, α-amyrenone, butyrospermenone, cycloartenone, lupenone and 24-methylene cycloartanone. The mass spectral fragmentation of different keto triterpenes is discussed later.

β-Amyrenone

The molecular ion was observed at m/e 424 (molecular formula $C_{30}H_{48}O$) and the base peak was observed at m/e 218 (retro Diel's Alder fragmentation) characteristic of Δ^{12} urs or olean compounds. Other characteristic fragments were at m/e (relative intensity) 409 (13), 381 (2), 368 (4), 313 (9), 257 (5), 245 (8), 205 (31), 203 (32), 189 (32), 161 (24), 133 (34).

α-Amyrenone

This also showed molecular ion at m/e 424 (molecular formula $C_{30}H_{48}O$). A base peak was observed at m/e 218 (retro Diel's Alder fragmentation). Other fragments were obtained at m/e 409 (4), 205 (24), 203 (62), 189 (29), 175 (17), 161 (10), 133 (10).

Lupenone

The molecular ion and base peak were observed at m/e 424 (molecular formula $C_{30}H_{48}O$) and m/e 95, respectively. Other fragments were at 409 (24), 381 (5), 313 (18), 245 (17), 218 (30), 205 (72), 189 (40).

Butyrospermenone

The molecular ion was encountered at m/e 424 (molecular formula $C_{30}H_{48}O$) and the base peak at m/e 69. Other principal fragments were observed at 409 (26), 311 (12), 297 (6), 271 (9), 256 (15), 207 (30), 203 (20) and 175 (17).

Cycloartenone

This also had a molecular ion at m/e 424 (molecular formula $C_{30}H_{48}O$) and the base peak

TABLE 1

Constituents of 3-Oxo Triterpenes of Various Fats

Fat	Unsap. matter in fat (%)	3-oxo triterpene; unsap. matter (%)	Composition of 3-oxo triterpenes					
			β-Amyrenone	α-Amyrenone	Lupenone	Butyrospermenone	Cycloartenone	24-Methylene cycloartanone
Shea	2	5	7	12	–	81	–	–
Illipe	0.47	5	38	62	–	–	–	–
Kokum	0.2	12	13	21	–	–	42	24
Mango kernel	0.4	4	24	28	24	–	24	–
Phulwara	0.8	7	–	–	100	–	–	–

at m/e 69. Other fragments were at m/e 409 (13), 365 (5), 339 (7), 313 (8), 286 (8), 257 (9), 205 (18).

24-Methylene Cycloartenone

The molecular ion was found at m/e 438 (molecular formula $C_{31}H_{50}O$) whereas the base peak was at m/e 95. The other fragments were observed at m/e 423 (22), 410 (8), 355 (5), 313 (5), 300 (3), 257 (7), 245 (6), 229 (10), 207 (52), 205 (55), 189 (52) and 121 (84).

The structures assigned for the oxo triterpenes on the basis of mass fragmentation characteristics were confirmed by direct comparison with authentic specimens in respect to GLC retention time and fragmentation pattern.

Further confirmation of the assigned structure of different oxo triterpenes from kokum and mango kernel unsaponifiable matter as illustrative examples was obtained by their reduction to alcohols and comparing the GC-MS pattern of the alcohol acetates with authentic alcohol acetates (1,4).

This investigation indicates that the occurrence of oxo triterpenes, unlike triterpene alcohols, 4-methyl sterols and Δ^5 sterols, is not widespread in vegetable oils. Although the number of oils examined is not large, it is perhaps more than a coincidence that oxo triterpenes occur more widely in those fats which contain substantial proportions of symmetrical triglycerides (5). Cocoa butter is a notable exception. Further work is required to establish if any correlation exists between the presence of oxo triterpenes and the botanical classification of the plants. The practical significance of the present study is that oxo triterpenes could be used to identify some fats and also for the qualitative detection of their presence in cocoa butter and similar fats not containing these compounds.

ACKNOWLEDGMENTS

Dr. K.K.G. Menon gave encouragement and the International Speciality Division, Unilever N.V., Rotterdam, provided the shea and illipe samples.

REFERENCES

1. Kolhe, J.N., Bhaskar, A., and Bringi, N.V. (1981) Lipids 16, 775-776.
2. Itoh, T., Uetsuki, T., Tamura, T., and Matsumoto, T. (1980) Lipids 15, 407-411.
3. Corey, E.J., and Suggs, J.W. (1975) Tetrahedron Lett. 2647-2650.
4. Itoh, T., Tamura, T., and Matsumoto, T. (1974) Lipids 9, 173-184.
5. Swern, D. (1979) in Bailey's Industrial Oil and Fat Products, Vol. 1, 4th Edn., pp. 10-11, John Wiley & Sons, New York, NY.

[Received August 31, 1981]

Absorption and Distribution of Orally Administered Jojoba Wax in Mice

A. YARON, V. SAMOILOFF and **A. BENZIONI**, *Research & Development Authority, Ben-Gurion University of the Negev, PO Box 1025, Beer-Sheva 84110, Israel*

ABSTRACT

The liquid wax obtained from the seeds of the arid-land shrub jojoba (*Simmondsia chinensis*) is finding increasing use in skin treatment preparations. The fate of this wax upon reaching the digestive tract was studied. ^{14}C-Labeled wax was administered intragastrically to mice, and the distribution of the label in the body was determined as a function of time. Most of the wax was excreted, but a small amount was absorbed, as was indicated by the distribution of label in the internal organs and the epididymal fat. The label was incorporated into the body lipids and was found to diminish with time. *Lipids* 17:169-171, 1982.

INTRODUCTION

Jojoba, *Simmondsia chinensis* (Link) Schneider, is a shrub native to the Sonoran desert which has been introduced to Israel in recent years. The beans of the shrub contain about 50% of a liquid wax, which consists mainly of esters of long-chain fatty acids and long-chain fatty alcohols (1).

The wax is reputed to have cosmetic value—both in its natural state and in the form of derivatives. It is therefore important that the fate of wax absorbed in the body be known. Our previous work (2) has shown that ^{14}C-labeled wax that was injected subcutaneously (sc) into mice was absorbed only to a small extent. The label was distributed in the various body organs and was almost completely eliminated by 90 days after application (2).

In this study, the fate of jojoba wax given orally to mice—its absorption patterns and distribution in the body—was investigated.

EXPERIMENTAL PROCEDURES

Animals

Five-week-old male albino mice, each weighing 25-30 g, were used. The mice were housed individually and fed ad lib.

^{14}C-Labeled Jojoba Wax

Randomly labeled jojoba wax was biosynthesized, extracted and refined as described previously (2,3). The labeled wax esters were then purified to remove minor components, i.e., triglycerides, free fatty acids and alcohols, sterols and polar lipids. This was performed as follows. Jojoba wax (0.8 g) in hexane was applied to a column (18 cm × 1 cm) packed with Florisil (60-100 mesh, Sigma). The esters were eluted with 50 ml of hexane while the other components were retained in the column. The recovery was 82%, and the purity as checked by thin layer chromatography (TLC) was 100% esters. This purified wax fraction was used in this study; its sp act was 10.9 μCi/g.

Wax Administration to Mice

To each of 20 mice, 0.1 ml of a 25% solution of ^{14}C-labeled jojoba wax in peanut oil was administered intragastrically by canula. Ten mice were killed one day later and the other 10 were killed 8 days after wax administration. The absorption and distribution of the wax in the body were studied by methods similar to those used in our previous study (2), as described later. The experiment was repeated twice.

Autopsy Procedure

After the mice had been killed by cervical fracture, the internal organs were removed, freed of any connective tissue, washed with 0.25 N sucrose solution, blotted dry, weighed and frozen.

^{14}C Determination in the Organs

The lungs, heart, spleen, testes, kidneys and a piece of muscle (leg) were each digested in Soluene 350 (Packard) and treated as previously described (2). ^{14}C radioactivity in these organs was then counted.

The liver and epididymal fat were subjected to special treatment, as their lipid fractions were not only monitored for radioactivity but their TLC profiles were also examined. These tissues were ground with water (1 ml/organ) in a Turrax homogenizer (Janke & Kunkle, West Germany), and the lipid fractions were extracted by the Folch et al. procedure (4). The radioactivity was counted in a sample of the lipids.

TLC Profiles of Liver and Epididymal Lipids

The chromatograms were run on silica-gel-

coated F254 plates (Merck). The plates were developed with petroleum ether (60-80 C)/ethyl ether/acetic acid (90:10:1, v/v/v). The spots were located with I_2 vapor, and scraped into vials for scintillation counting.

An additional set of chromatograms was run in order to examine the efficiency of separation of the wax esters from the tissue lipids examined. For these chromatograms, a sample of ^{14}C-labeled jojoba wax of a known weight and sp act was added to the tissue lipids. The level of radioactivity in the added wax was of the same order of that expected in the tissue lipids. The samples were separated under the TLC conditions already described. By this method, it was confirmed that there is complete separation of the wax esters from tissue triglycerides, without tailing.

RESULTS AND DISCUSSION

A small amount of the ingested jojoba wax was absorbed and distributed in all the internal organs tested (Tables 1 and 2). The highest accumulation of label that was found 1 day after application was that in the epididymal fat (Table 1). In this tissue and in all the other tissues, the amount of label decreased with time from 1 to 8 days.

From the TLC studies, it can be seen that the label was incorporated into the body lipids (Table 3). The label in the liver lipids was distributed in the triglycerides, the phospholipids and the other lipid groups, whereas that in the epididymal fat tissue was found mainly in the triglycerides (Table 3).

Other studies have shown that the greater part of ingested jojoba wax is not metabolized

TABLE 1

Distribution of ^{14}C in the Body, 1 and 8 Days after Oral Administration of ^{14}C-Labeled Jojoba Wax Esters, Expressed on a Tissue Basis[a]

Tissue	^{14}C sp act in the tissue (dpm/g wet tissue ± SE)			
	1st experiment		2nd experiment	
	1 day	8 days	1 day	8 days
Liver lipids	805 ± 88	136 ± 13	1570 ± 390	776 ± 280
Heart	2140 ± 880	980 ± 78	2080 ± 328	904 ± 248
Lungs	ND	ND	2300 ± 308	1170 ± 296
Spleen	2020 ± 560	685 ± 82	2300 ± 404	1180 ± 330
Testes	1266 ± 360	974 ± 196	1180 ± 224	772 ± 150
Kidneys	2964 ± 674	984 ± 32	3720 ± 544	1404 ± 310
Muscle	1414 ± 290	1346 ± 578	1210 ± 194	882 ± 136
Epididymal fat	3770 ± 430	1740 ± 770	7760 ± 2160	4460 ± 1335

[a]100 µl of 25% jojoba wax solution (500,000 dpm) was applied per mouse.
ND–not determined.

TABLE 2

Distribution of ^{14}C in the Body 1 and 8 Days after Oral Administration of ^{14}C-Labeled Jojoba Wax Esters, Expressed on an Organ Basis and as a Percentage of Administered Dose

Tissue	Radioactivity (dpm/organ[a] ± SE)			
	1st experiment		2nd experiment	
	1 day	8 days	1 day	8 days
Liver lipids	1180 ± 112 (0.24)	750 ± 170 (0.15)	2460 ± 466 (0.49)	760 ± 310 (0.15)
Heart	401 ± 80 (0.08)	230 ± 90 (0.05)	230 ± 52 (0.05)	148 ± 44 (0.03)
Lungs	ND	ND	534 ± 74 (0.11)	140 ± 58 (0.03)
Spleen	230 ± 19 (0.05)	165 ± 45 (0.03)	268 ± 58 (0.05)	56 ± 12 (0.01)
Testes	255 ± 39 (0.05)	175 ± 25 (0.04)	183 ± 36 (0.04)	136 ± 30 (0.03)
Kidneys	630 ± 210 (0.13)	62 ± 28 (0.01)	518 ± 84 (0.10)	236 ± 52 (0.05)

[a]Numbers in parentheses present the amount of ^{14}C incorporated into the organ as a percentage of the administered wax.
ND–not determined.

TABLE 3

Radioactivity TLC Profile of Liver and Epididymal Fat Lipids One Day after Ingestion of ^{14}C-Labeled Jojoba Wax Esters[a]

R_f	Incorporation of ^{14}C into lipid fraction (%)		Lipid standards
	Liver	Epididymal fat	
0.03	27.0 ± 3.1	0	Phospholipids and glycolipids
0.08	5.5 ± 0.5	5.7 ± 3.2	Cholesterol
0.19	5.5 ± 3.2	0	Fatty acids
0.31-0.35	51.3 ± 6.8	92.0 ± 3.2	Triglycerides
0.80	11.6 ± 3.7	4.3 ± 4.1	Wax esters and cholesterol esters

[a]The results are means ± SE. The developing solvent was petroleum ether (60-80 C)/ethyl ether/acetic acid (90:10:1, v/v/v).

in the body but is excreted (5,6) when given either via canula as a liquid oil (5) or incorporated in a solid diet (6). When low concentrations of the wax (1% of their diet) were fed to mice, growth was not impaired (6). Higher concentrations caused diarrhea and retardation of growth, which was assumed to be the result of lubrication of the intestines, causing the elimination of essential nutrients (6).

These studies have shown that the small amounts of ingested jojoba wax that are absorbed are metabolized, incorporated into body lipids, and finally eliminated with time. It is not known at this stage whether the wax is metabolized in the digestive tract and, hence, what proportion is absorbed as esters and what proportion as metabolites. Our findings are in keeping with studies that have shown that other nonglyceride lipids such as fatty alcohols (7) or the paraffin *n*-nonacosane (8), when given orally, were metabolized and incorporated into the body lipids, including triglycerides and phospholipids (7,8).

ACKNOWLEDGMENT

This work was supported by the Negev-Jojoba Co.

REFERENCES

1. Spencer, G.F., Plattner, R.D., and Miwa, T. (1977) J. Am. Oil Chem. Soc. 54, 187-189.
2. Yaron, A., Benzioni, A., and More, I. (1980) Lipids 15, 889-894.
3. Benzioni, A. (1978) New Phytol. 81, 105-109.
4. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
5. Taguchi, M., and Kunimoto, T. (1977) Cosmet. Toilet. 92, 53-61.
6. Verbiscar, A.J., Banigan, T.F., Weber, C.W., Reid, B.L., Trei, J.E., Nelson, E.A., Raffauf, R.F., and Kosersky, D. (1980) J. Agric. Food Chem. 28, 571-578.
7. Friedberg, S.J. (1976) Lipids 11, 587-593.
8. Kollatukudy, P.E., and Hankin, L. (1966) J. Nutr. 90, 167-174.

[Received September 3, 1981]

Hypercholesterolemia in Rats: Combined Effect of High Cholesterol Diet and Female Sex Steroids

G.M. FISCHER* and M.L. SWAIN, *Department of Physiology, University of Pennsylvania, School of Medicine, Philadelphia, 19104*

ABSTRACT

The influence of estradiol and a contraceptive steroid combination on plasma cholesterol was studied in female rats on both normal and high-cholesterol diets which did not contain thiouracil. The high-cholesterol diet resulted in moderate hypercholesterolemia without weight loss, even with prolonged feeding. Hypercholesterolemia was markedly accentuated in the presence of either endogenous or exogenous sex hormones.
Lipids 17:172-175, 1982.

The rat is an animal which is particularly resistant to hypercholesterolemia and atherosclerosis. Various diets used to produce hypercholesterolemia in these animals usually include an antithyroid drug (1-4) such as thiouracil; however, the use of these drugs has 2 distinct disadvantages: (a) the production of hypothyroidism can invalidate many aspects of experimental studies. For example, the study of hormone effects on cholesterol metabolism can be greatly influenced by the thyroid state of the animal. (b) Thiouracil is bitter and makes the diet unpalatable. Consequently, the rats lose weight and long-term mortality is high. This aspect of the diet can invalidate studies such as protein synthesis.

In a study of the effects of sex hormones on connective tissue metabolism and plasma lipid levels in rats, we found that the use of thiouracil would invalidate the studies for both of these reasons: the hypothyroid state and the poor nutrition of the animals. We therefore attempted to produce a diet, without the use of thiouracil, which would result in hypercholesterolemia.

Many studies over the past 3 decades have documented the effect of estrogens and contraceptive steroids on plasma cholesterol and results have often been conflicting (5-12). However, one aspect of the human studies not usually addressed is the amount of cholesterol and/or fat in the diet of patients receiving estrogen.

The following is a description of the effects of female sex hormones on plasma cholesterol levels in rats fed a normal or high-cholesterol diet for 20 weeks. The results document the hypercholesterolemic effect of the special diet, without weight loss, and indicate a marked accentuation of dietary effects on plasma cholesterol by the presence of female sex steroids.

EXPERIMENTAL PROCEDURES

Female rats (CD strain), aged 3 months, half of them ovariectomized, were obtained from Charles River Breeding Laboratories, Wilmington, MA, and divided into groups as described next. The experimental design essentially consisted of 2 separate studies, the first involving the ovariectomized rats and the second the intact rats.

In study 1, the ovariectomized rats were divided into 2 groups, one of which received cottonseed oil (0.1 ml) weekly and the other estradiol (10 μg), in long acting form, weekly (Depoestradiol cypionate, Upjohn).

In study 2, the intact rats were divided into 2 groups, one of which received cottonseed oil (0.1 ml) weekly and the other mestranol (5 μg)/norethynodrel (250 μg) weekly in divided doses. (Mestranol and norethynodrel were kindly supplied by T. Martinez, Searle Laboratories.)

The groups were chosen to represent clinically relevant situations: (a) ovariectomized women with and without estrogen replacement therapy, and (b) women with intact ovaries with and without contraceptive steroid administration.

All injections were administered in 0.1-ml vol, with cottonseed oil as the vehicle. The groups were further divided into 2 subgroups each, one subgroup in each case being maintained on Purina Laboratory rat chow and the other subgroup in each case on Purina Laboratory rat chow to which cholesterol (4%), coconut oil (4%) and cholic acid (1%) were added. In an earlier study, we included thiouracil in the diet but observed that the animals lost weight on this regimen. In the study described here, we omitted the thiouracil and found that the rats would eat this diet freely if whole rat chow pellets were coated with the oil-cholesterol-cholic acid mixture. We added

molasses (Grandma's unsulfered) to the coating mixture to bind it more firmly to pellets and make it more palatable. The special chow thus retained the same physical form as the regular chow. The rats were maintained on this regimen for 20 weeks and were weighed weekly.

Plasma Cholesterol

Rats were fasted overnight and killed by cervical dislocation. Blood was drawn immediately from the heart into tubes containing EDTA; it was centrifuged, and the plasma was frozen for later determination of plasma cholesterol by the method of Pearson et al. (13).

The Student's t-test was used to compare means between groups in each of the 2 studies. Additionally, the intact, oil-treated rats could be compared to the ovariectomized, oil-treated rats.

RESULTS

Table 1 presents the data on final body weight and plasma cholesterol levels after 20 weeks of diet and treatment along with p values when differences were significant.

Body Weight

In ovariectomized rats, the administration of estradiol depressed weight gain in both the normal and high-lipid diet groups. The feeding of the high-lipid diet did not increase the weight gain of the oil-treated rats but did increase the weight gain of the estradiol-treated rats.

In the intact rats, the administration of mestranol/norethynodrel (M/N) depressed weight gain in rats on either the normal diet or high-lipid diet. In both the oil-treated and M/N-treated rats, the feeding of the high-lipid diet increased weight gain.

In all groups, the weight increased progressively over the course of the 20 weeks, so the palatability of the diet was not a problem.

Plasma Cholesterol

In ovariectomized rats, treatment with estradiol resulted in an increase in plasma cholesterol in both normal diet and high-lipid diet groups, with the increase being more marked in the high-lipid group. Estradiol thus accentuated the hypercholesterolemia induced by the high-lipid diet. A synergistic effect can be seen by the fact that the rats fed a high-cholesterol diet and administered estradiol had a larger increase in plasma cholesterol than would have been expected from a merely additive effect of diet and estradiol.

TABLE 1

Final Body Weight and Plasma Cholesterol Levels of Rats after 20 Weeks of Diet and Hormone Treatment

	Final body weight (g ± SE)*		Plasma cholesterol (mg/dl ± SE)		
	Normal diet	High-cholesterol diet	Normal diet	High-cholesterol diet	% Increase**
Ovariectomized rats					
Oil-treated	(24)+ 413 ± 11[a]	(21) 407 ± 11[b]	(16) 115 ± 6[c]	(16) 213 ± 18[d,e]	86
Estradiol-treated	(25) 330 ± 8[a,f]	(23) 365 ± 9[b,f]	(21) 137 ± 8[c]	(18) 378 ± 43[d]	176
Intact rats					
Oil-treated	(25) 373 ± 9[g,h]	(20) 401 ± 8[h,i]	(15) 121 ± 7	(17) 302 ± 35[e]	149
Mestranol/norethynodrel-treated	(23) 339 ± 8[g,j]	(22) 375 ± 12[i,j]	(17) 105 ± 6	(14) 300 ± 40	185

*Mean initial weights were ca. 250 g for each group.

**All increases were significant at $p<0.005$.

+Numbers in parentheses indicate number of animals in each group.

[a] $p<0.0005$. [b] $p<0.005$. [c] $p<0.025$. [d] $p<0.005$. [e] $p<0.025$. [f] $p<0.005$. [g] $p<0.005$. [h] $p<0.025$. [i] $p<0.05$. [j] $p<0.01$.

In intact rats on a normal diet, the administration of M/N was associated with a marked trend toward reduced plasma cholesterol, but the difference was not quite significant. In the group on a high-lipid diet, there was no reduction in plasma cholesterol by the contraceptive steroid.

A comparison of intact, oil-treated rats with ovariectomized, oil-treated rats reveals higher plasma cholesterol in the intact rats when they were fed the high-lipid diet.

It can be seen that, in all treatment groups, the feeding of a high-lipid diet resulted in significant increases in plasma cholesterol; the percentage increase was accentuated when either endogenous or exogenous hormone was present. There were no gross vascular lesions after 20 weeks of the regimen.

DISCUSSION

The results demonstrate an accentuation of dietary hypercholesterolemia by the presence of female sex hormones, because plasma cholesterol levels were disproportionately increased in rats on the high-cholesterol diet when either endogenous or exogenous hormones were present, compared to ovariectomized rats with no hormone present.

The effects of estrogen and contraceptive steroids on plasma lipid levels have received much attention in recent years. Early studies in humans indicated a hypocholesterolemic effect of estrogen (5,6), but more recent studies indicate that estrogen and contraceptive combinations are hypercholesterolemic (7-9). In rats, estrogen has been shown to have a bimodal effect (10,11), high doses depressing plasma cholesterol and lower doses given over a prolonged period of time elevating plasma cholesterol. Our study reported here used the lower level doses over a 20-week period and results are consistent with those of others. It has been shown by others that in the rat mestranol (11) or mestranol/norethynodrel combination (12) can actually lower plasma cholesterol, although the dosage schedule in the second study was different.

We have previously shown that estradiol elevates plasma cholesterol in both normal and castrated young male rats (14), as well as in ovariectomized young female rats (15) fed a normal chow diet. In the second study, M/N had no effect. These previous studies were limited to 3-week periods of hormone administration. The current studies showing that female sex steroids enhance diet-induced hypercholesterolemia suggest that studies in humans on the effect of sex hormones on plasma cholesterol should have the diet defined, in that diet may have a bearing on the degree of alteration produced by the hormone.

It has long been thought that estrogen is protective against atherosclerotic vascular disease, because women before menopause have a lower incidence of myocardial infarction than do men of the same age. However, the more recent reports that women on birth control pills have an increased incidence of myocardial infarction, albeit possibly associated with other risk factors (16), raise doubts as to the "protective" role of estrogen. However, it is possible that female sex steroids protect by some other mechanism, such as effect on arterial wall components (17).

In summary, these studies indicate a synergistic effect of high-cholesterol diet and female sex steroids on plasma cholesterol in rats and suggest that both diet and hormonal status be considered in evaluation of plasma cholesterol levels. The results further indicate that the diet described here, although it does not produce lesions at 20 weeks, can be used for long-term pathophysiological lipid studies without compromising the nutritional state of the rat.

ACKNOWLEDGMENT

This work was supported by U.S.P.H.S. Grant HL 17721.

REFERENCES

1. Fillios, L.C., Andrus, S.B., Mann, G.V., and Stare, F.J. (1956) J. Exp. Med. 104, 539-554.
2. Deming, Q.B., Mosbach, E.H., Bevans, M., Daly, M.M., Abell, L.L., Martin, E., Brun, L.M., Halpern, E., and Kaplan, R. (1958) J. Exp. Med. 107, 581-598.
3. Scott, F.R., Morrison, E.S., Thomas, W.A., Jones, R., and Nam, S.C. (1964) Exp. Mol. Path. 3, 421-443.
4. Weisgraber, K.H., Mahley, R.W., and Assman, G. (1977) Atherosclerosis 28, 121-140.
5. Marmorston, J., Magidson, O., Lewis, J.J., Mehl, J., Moore, F.J., and Bernstein, J. (1958) New Engl. J. Med. 258, 583-586.
6. Robinson, R.W., and Lebeau, R.J. (1965) J. Atheroscler. Res. 5, 120-124.
7. Wynn, V., Doar, J.W.H., Mills, G.H., and Stokes, T. (1969) Lancet 2, 756-760.
8. Rossner, S., Larsson-Cohen, U., Carlson, L.A., and Boberg, J. (1971) Acta Med. Scand. 190, 301-305.
9. Molitch, M.E., Oill, P., and Odell, W.D. (1974) J. Am. Med. Assoc. 227, 522-525.
10. Uchida, K., Kadowaki, M., Miyata, K., and Miyake, T. (1969) Endocrinol. Jpn. 16, 211-214.
11. Ferreri, L.F., and Naito, H.K. (1978) Endocrinology 102, 1621-1627.
12. Aftergood, L., and Alfin-Slater, R.B. (1971) J. Lipid Res. 12, 306-312.
13. Pearson, S., Stern, S., and McGavack, T.H.

(1953) Anal. Chem. 25, 813-814.
14. Fischer, G.M., and Swain, M.L. (1978) Experientia 34, 624-625.
15. Fischer, G.M., and Swain, M.L. (1980) Artery 6, 307-319.
16. Ory, H.W. (1977) J. Am. Med. Assoc. 237, 2619-2622.
17. Fischer, G.M., Cherian, K., and Swain, M.L. (1981) Atherosclerosis 39, 463-467.

[Revision received December 15, 1981]

Role of Steroids and Triterpenoids in the Growth and Reproduction of *Phytophthora cactorum*[1]

W. DAVID NES*, GARY A. SAUNDERS and **ERICH HEFTMANN**, *Plant Physiology and Chemistry Research Unit, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, CA 94710*

ABSTRACT

The life cycle of *Phytophthora cactorum*, a fungal pathogen unable to epoxidize squalene, is controlled by certain combinations and concentrations of polycyclic isopentenoids normally found in the host plant. Data that lend support to this view are given. The fungus is capable of discriminating between various types and amounts of steroids and triterpenoids in terms of their uptake, metabolism, and effects on growth and reproduction. While certain sterols as well as triterpenoids stimulate growth, only sterols induce significant oospore production. Steroidal alkaloids and estradiol are fungistatic and inhibit sterol-induced oospore production. The primary difference in the metabolism of sterols and triterpenoids is that the sterols are converted to both esters and glycosides, whereas the triterpenoids are only esterified. The results demonstrate the importance of sterols and their products, compared to other polycyclic isopentenoids, as promoters of growth and reproduction and suggest that the preference for sterols may have its origin in the adaptation to host-parasite interactions.
Lipids 17:178-183, 1982.

INTRODUCTION

Investigations in several laboratories (1-4) have demonstrated that one role for steroids and triterpenoids as they naturally occur in tracheophytes (5,6) is the control of growth and reproduction of fungal pathogens. There are 2 groups of fungi which parasitize higher plants, viz., those capable of sterol synthesis and those which are not. The only fungi which lack a completed sterol pathway are the Pythiaceae (genera: *Phytophthora* and *Pythium*). While pythiaceous fungi can synthesize squalene, they are unable to epoxidize this hydrocarbon (7,8). In the wild, these fungi assimilate sterols and other nutrients from the host. Depending on environmental conditions and availability of sterols and other polycyclic isopentenoids in the host tracheophyte, vegetative growth may be stimulated and the reproductive cycle turned on, resulting in the induction of asexual zoospores and sexual oospores. From past work on the effects of over 50 polycyclic isopentenoids on growth and reproduction of *Phytophthora cactorum* (Table 1), we may conclude that the fungus prefers the sterols commonly found in tracheophytes to other polycyclic isopentenoids synthesized by fungi, algae and higher plants (9).

No previous studies have correlated the effects of sterol or triterpenoid structure on growth and reproduction of Pythiaceae with their differential metabolism and uptake into the mycelium. In an attempt to shed new light on the physiology and metabolism of polycyclic isopentenoids in *P. cactorum*, we have examined the uptake and metabolism of cholesterol in fungal cultures in the presence and absence of other polycyclic isopentenoids and correlated this with the effects of various combinations and concentrations of steroid and triterpenoid supplements on growth and reproduction.

MATERIALS AND METHODS

The experimental methods for culturing *P. cactorum* on solid and liquid substrates, determining growth rates, counting oospores, and for isolating, identifying, and quantitating radioactive and nonradioactive polycyclic isopentenoids assimilated by the fungus have been described in detail elsewhere (1,9,10). The purity of most radioactive and nonradioactive sterols and triterpenoids supplied to *P. cactorum* was >95%, as determined by gas liquid chromatography (GLC), mass spectrometry (MS) and ^{1}H nuclear magnetic resonance (NMR) (1,9-11). The radiochemical purity of the ^{14}C-labeled compounds was ascertained to be >99% by a combination of thin layer chromatography (TLC), adsorption high performance liquid chromatography (HPLC), and reversed-phase HPLC (11).

RESULTS AND DISCUSSION

Mycelia of *P. cactorum* can grow vegetatively on a synthetic medium devoid of polycyclic isopentenoids. Elliott et al. (12) were first to recognize that *P. cactorum*, grown in batch culture, failed to produce desmethyl sterols. When the fungus was grown to log phase in

[1]Presented at the 72nd Annual Meeting of the American Oil Chemists' Society in New Orleans, LA, May 1981.

TABLE 1

Summary of the Effects of Polycyclic Isopentenoids on Growth and Reproduction in *P. cactorum*

Polycyclic isopentenoids added to culture medium	Effects	
	Stimulate	Inhibit
Sterols	1,2,3[a]	
Polyhydroxylated sterols		1
Tetracyclic triterpenoids	5	5
Polyhydroxylated tetracyclic triterpenoids		1
Pentacyclic triterpenoids	1	1
Steroidal alkaloids (genins or glycoalkaloids)		1,4
Steroidal sapogenins		1
Steryl esters	1,2,3	
Steryl glycosides	1,2,3	
Steroid hormones (pregnane derivatives)		1,4

[a]1—Change in mycelial growth, relative to control, both in terms of dry weight and hyphal extension; 2—oospore production and formation of *antheridia and oogononia*; 3—zoospore development; 4—inhibit sterol-induced sexual reproduction; 5—no significant effect on growth relative to control.

still liquid cultures, no free sterol was detected at a level of 20 μg/20 g dry wt of mycelium (10). However, a compound tentatively identified as a polycyclic isopentenoid by GLC and MS is present at that level. Squalene, originally believed not to be synthesized by *P. cactorum* (13), has more recently been detected in the mycelium at a level of 4 μg/1 g dry weight (8). While there is good reason to believe that squalene is neither expoxidized nor cyclized to a 4,4-dimethyl sterol (7,8), its anaerobic cyclization to a pentacyclic triterpenoid, e.g., tetrahymanol, is mechanistically possible. In the absence of an exogenous source of sterol, such a polycyclic isopentenoid could satisfy the architectual requirements of mycelial membranes without playing a hormonal role. A specific nutritional requirement for sterols would then implicate them or some sterol metabolite as inducers of sexual reproduction (2).

The inability of *Phytophthora* to convert labeled acetate, mevalonate, or squalene to 4,4-dimethyl sterols, such as lanosterol (7,8), indicates that it lacks one or more of the enzyme systems in the isopentenoid pathway. Other studies with labeled isopentenoids have yielded additional detail of the fungal metabolism. For instance, *Phytophthora* incubated in vivo or in vitro with [^{14}C]squalene failed to metabolize it to lanosterol (7,14) and to convert [^{3}H]lanosterol or [^{3}H]cycloartenol to Δ^5-desmethyl sterols (1). Failure to convert the 4,4,14-trimethylsterol, lanosterol, to a Δ^5-sterol (15) may account for the inability of nuclear methylated steroids to stimulate growth and reproduction (1,9). On the other hand, there is good evidence that Δ^7- and $\Delta^{5,7}$-sterols are metabolized to Δ^5-sterols (15) and stimulate both growth and reproduction (1,9). Demethylation of lanosterol by *Phytophthora* has been suggested (16,17) to account for a new peak in GLC, but cycloartenol is unequivocally not demethylated by the fungus, nor is there any evidence for a 9,19-cyclosteroid isomerase or a Δ^{24}-C_1-transferase (1).

Certain enzymes of the sterol pathway which are normally present in other fungi, e.g., nuclear demethylases, Δ^{24}-C_1-transferase and 2,3-epoxidase, are absent from *Phytophthora*. On the other hand, other enzyme systems, e.g., glycosyltransferase, which are not operational in fungi such as *Achlya* (18), have been shown to metabolize cholesterol and sitosterol to steryl glycosides in pythiaceous fungi (11,19, 20). Thus, the ability of *Phytophthora* to assimilate, metabolize, and respond in terms of growth and reproduction to a wide range of polycyclic isopentenoids is unprecedented among fungi.

Phytophthora is also unique among fungi in other respects. For instance, *Saccharomyces* (21) and *Achlya* (22) contain 0.1-0.5% free sterol, whereas no free sterol was found, i.e., <0.0001% (10), in *P. cactorum* cultured on a synthetic medium. *Phytophthora*, like certain bacteria (23), assimilates sterols added to the culture medium, but while sterols stimulate growth and reproduction of *Phytophthora*, they only function to support the growth of bacteria, e.g., mycoplasmas (23). When sterols are assimilated by *Phytophthora*, there is no difference in the uptake during the first 4 hr between cholesterol and sitosterol (16) nor is there any difference in the final accumulation of the 2 compounds, as the free alcohol, into

log phase cultures (ca. 0.01% free sterol based on dry wt of mycelium) (10). Similarly, there is no difference between the 2 sterols in their ability to stimulate growth. Growth stimulation, as measured by changes in mycelial dry wt over an 18-day period in cholesterol- and sitosterol-supplemented still liquid cultures, was found to be due to a shortened lag phase rather than to a change in absolute growth rate (10). While *P. cactorum* does not discriminate between cholesterol and sitosterol in liquid cultures grown at room temperature, there are apparent differences in the colony diameters between the 2 sterols when the fungus is grown on agar at 24 C (24) but not at 20 C.

It is generally assumed that sterol-induced changes in the growth of organisms auxotrophic or heterotrophic for sterols are related to effects on membrane function which results from the stereochemical fit of the sterol molecule with some other architectural components (23). In the case of *Phytophthora*, a relationship between growth response, sterol uptake into the mycelium, and membrane function has been demonstrated. Thus, various sterol supplements are assimilated by subcellular organelles, e.g., mitochondria (16) and plasmalemma (17,25) without undergoing chemical modification (23). The uptake of sterols into membrane structure affects mitochondrial enzyme activities (16,26), alters plasma membrane permeability (25,27,28), and enhances sensitivity to polyene antibiotics (27) and saponins (28). The saponins are known to form complexes with sterols, presumably in the membrane (23).

Sterols are not the only polycyclic isopentenoids that stimulate the growth of *P. cactorum*. Recently, tetrahymanol and α- and β-amyrin were found to stimulate growth, as measured by both changes in dry wt and hyphal extension (1). Moreover, β-amyrin, as the free alcohol, was reisolated from mycelial culture on solid and liquid media in unchanged form (1,11). This implies that β-amyrin was acting in a nonmetabolic role, in analogy to the structural membrane function of sterols.

When polycyclic isopentenoids are assayed for their effect on oospore production, greater specificity is observed than in their effect on growth stimulation. Numerous naturally occurring and synthetic sterols (Table 2) and other isopentenoids (Table 3) were tested, but only certain sterols were found capable of promoting significant oospore formation in *P. cactorum*. The absence or lowered activity was not due to a failure of the various compounds to enter the mycelium, because, in all cases examined, the isopentenoids were assimilated and, in some cases, converted to esters and glycosides by the fungus (1,9-11,24).

TABLE 2

Effects of Sterols on Oospore Production in *P. cactorum*

I.	Sitosterol
	Stigmasterol
	Spinasterol
II.	Chondrillasterol
	Fucosterol
	Sitostanol
	Campesterol
III.	Δ^5-Ergostenol
	Δ^7-Ergostenol
	$\Delta^{7,22}$-Ergostadienol
	$\Delta^{5,22}$-Ergostadienol
	$\Delta^{5,7,22}$-Ergostatrienol
	Campestanol
	Cholesterol
	Lathosterol
	E-17(20)-dehydrocholesterol
	26-Homocholesterol
	22-*cis*-Dehydrocholesterol
	Desmosterol
IV.	20-*iso*-Cholesterol
	Z-17(20)-Dehydrocholesterol
	Ergostanol
	Cholestanol
	5α-Cholestane
	5β-Cholestanol
	Lophenol

Activity decreases from group I (maximal oospore production) to group IV (no significant production). Data from ref. 9 and Nes, W.D., Saunders, G.A., and Heftmann, E., unpublished data.

The quantitative difference in the number of oospores produced by incubation with various sterol supplements suggests that the fungus possesses specific recognition factors (9), perhaps proteins (23), which are capable of discriminating between structural features of the sterol molecule. In principle, structural requirements for oospore induction are: a 3β-hydroxyl group, a branched aliphatic side chain of 8 to 10 carbon atoms, and a planar (A/B-*trans* ring junction) tetracyclic nucleus. Methyl groups at C-4 and C-14 must be absent, the side chain must be *R*- oriented (to the right in the normal view of the molecule), and C-20, when chiral, must posses the *R*-configuration (Table 2). Group I, with the highest activity (500-600 oospores at 21 C), is composed of 24α-alkyl sterols natural to the host plants rather than sterols commonly found in fungi (9). Structural specificity, e.g., the ability of certain sterols to induce vegetative growth but not reproduction, may be the result of a complex evolutionary adaptation to host-parasite interactions (9). Certain triterpenoids also fit this description, as they also stimulate growth and occur naturally in tracheophytes (5). That only certain sterols, but none of the

TABLE 3

Polycyclic Isopentenoids Which Failed to Induce Oospore Production in *P. cactorum*

Antheridiol	Steroids
29-Hydroxyporiferasterol	
20-α-Hydroxycholesterol	
Saringosterol	
Estradiol	
6-Ketocholestanol	
Tetrahymanol	Cyclic triterpenoids
β-Amyrin[a]	
Lupeol	
Betulin	
Oleanolic acid	
Cycloartenol[a]	
Lanosterol	
Cucurbitacin C	
Squalene	Acyclic triterpenoid
15-*aza*-24-Methylene-D-homocholestadienol	Steroidal alkaloids
Jervine	
Muldamine	
Tomatidine	
Solanine	
Solasonine	
Solasodine	
Tigogenin	Steroidal sapogenin

[a]May induce a few oospores at the periphery of the plate at 20 C and 22 C but not at 25 C or higher temperatures.

pentacyclic triterpenoids, can significantly induce both growth and reproduction may be related to the ability of triterpenoids to replace sterols as membrane components in *Phytophthora* (5,23) but not as precursors of the presumed sexual hormones.

Previous studies have shown that sterols such as cholesterol and sitosterol, added separately to cultures, stimulate growth and induce oospore production. Because elevated temperatures (≥ 24 C) (9,24) and pH changes in the liquid culture medium resulting from mycelial growth (29) may affect the reproducibility of the results, we now routinely culture *P. cactorum* on solid (agar) media at 20 C (Table 4). At this temperature, the radial diameters in the control plates continue to vary from one experiment to the next, but the cholesterol-supplemented cultures maintain a constant growth rate and show a reproducible number of

TABLE 4

Effect of Cholesterol on Hyphal Extension and Oospore Production by *P. cactorum* at 20 C in the Dark

Culture no.	Date of measurement for radial diameter	Radial diameter (mm)[a]		Growth stimulation (%)	No. of oospores produced[b]
		Control	Cholesterol		
1	9/10/80	32.8	41.1	25.9	376
2	9/18/80	35.5	43.1	21.4	384
3	10/02/80	36.5	42.9	17.5	ND[c]
4	10/30/80	34.5	41.3	19.7	393
5	3/27/81	36.5	41.5	13.6	321
6	5/01/81	30.3	42.5	40.2	ND
7	6/03/81	37.4	41.8	12.0	331
		34.8[d]	41.9[d]	20.0[d]	

[a]Mean radial diameter of 5 Petri plates. Deviation from the mean rarely exceeded 2%. Mycelia cultured for 6 days on agar plates.

[b]Mean oospore count for 4 radial transects in each of 5 Petri plates. Deviation from the average rarely exceeded 10%: Mycelia cultured for 21 days.

[c]ND, not determined.

[d]Mean value for 7 experiments.

oospores. While the minimal concentration of cholesterol required to stimulate hyphal extension is ca. 1 mg/ℓ, the same concentration of cholesterol induces ca. 10% the number of oospores formed when the concentration is 10 and 20 mg/ℓ cholesterol (Table 5). As 10 mg/ℓ of cholesterol is not a limiting concentration for the induction of maximal growth and reproduction, competition experiments were done with a total concentration of cholesterol and some other polycyclic isopentenoid of <20 mg/ℓ.

When [^{14}C]cholesterol, [^{14}C]β-amyrin, and [^{14}C]cycloartenol were incubated with *P. cactorum*, analysis of the mycelium after 21 days showed that cholesterol is taken up more readily than the triterpenoids (Table 6). Moreover, cholesterol was converted to esters and glycosides, whereas β-amyrin and cycloartenol were converted only to esters. Labeled campesterol and sitosterol were also converted at similar rates to their respective esters and glycosides (Nes, W.D., Saunders, G.A., and Heftmann, E., unpublished data). Thus, unlike the transesterase which esterifies a wide range of polycyclic isopentenoids, the glycosyltransferase is specific for sterols (11).

When labeled cholesterol was incubated at different concentrations in combination with various other steroids and triterpenoids, cholesterol uptake was significantly reduced only in the presence of equal concentrations of estradiol (Table 6). The amount of cholesteryl esters formed in the presence of other isopentenoids was different from the amount formed from cholesterol alone. This may reflect the ability of the transesterase and perhaps the glycosyltransferase to regulate the amount of intracellular free sterol, which may be incorporated into mycelial membranes. Of the various compounds incubated in this study with cholesterol, only estradiol inhibited both cholesterol-induced growth and reproduction (Table 6). None of the compounds tested in combination with cholesterol promoted an increased uptake of cholesterol, number of oospores or radial growth. Moreover, the small change in cholesterol metabolism to esters and glycosides was of little significance to the growth response and oospore production. In other competition studies, the addition of an equal amount of sitosterol to cholesterol did not affect growth and reproduction of *Phytophthora* relative to cholesterol controls (Table 6). Moreover, sitosterol had no significant effect on cholesterol uptake by the mycelium nor on its metabolism to esters and glycosides (Table 6).

In summary, of the wide range of polycyclic isopentenoids incubated with *Phytophthora*, those sterols and triterpenoids which stimulate growth and reproduction are usually found in tracheophytes as membrane constituents (23). After infestation of the host plant by *P. cactorum*, these compounds are undoubtedly assimilated by the mycelia. The loss of sterols and triterpenoids by the host to *Phytophthora* may stimulate the growth and reproduction of the fungus but have deleterious consequences for the infected plant. Of the

TABLE 5

Effects of Sterol and Triterpenoid Structure on Oospore Production and Hyphal Extension in *P. cactorum*

Treatment	Amount of compound added (mg/ℓ)	Percent[a]	Radial[b] diameter (mm)
Cholesterol	1	10	40.3
Cholesterol	10	100	43.1
Cholesterol	20	100	41.8
Cholesterol + sitosterol	10 each	100	43.0
Cholesterol + β-amyrin	10 each	100	39.0
Cholesterol + β-amyrin	1 + 10	10	44.8
Cholesterol + estradiol	10 each	10	30.0
β-Amyrin + estradiol	10 each	0	25.0
β-Amyrin	10	<1	42.4
Cycloartenol	10	<1	42.3
Estradiol	10	0	20.0
Control	0	0	36.5

[a]Mean number of oospores per petri plate divided by the count obtained with cholesterol controls. Cholesterol normally induced ca. 380 oospores per transect. The values are averages of at least 2 experiments. Deviation from the average rarely exceeded 10%.

[b]Mycelia were cultured for 6 days on agar plates at 20 ± 1 C in the dark. The mean radial diameter value for each of the treatments rarely varied by more than 1.78 mm, based on a least significant difference $p \leq 0.05$.

TABLE 6

Uptake and Metabolism of Radioactive Cholesterol, Cycloartenol and β-Amyrin by *P. cactorum*

	Amt. added (mg/l)	Radioactivity recovered from mycelium[a] (%)	Distribution of recovered radioactivity[b]			Ref.
			FP[c]	EP	GP	
[^{14}C]Cholesterol	10	70	72	26	2	11
[^{14}C]β-Amyrin	10	48	92	8[b]	0	11
[^{14}C]Cholesterol + β-amyrin	10 each	70	50	44[b]	6	11
[^{14}C]Cholesterol + β-amyrin	1 + 10	70	80	19	1	d
[^{14}C]Cholesterol + estradiol	10 each	55	88	10	2	d
[^{14}C]Cholesterol + sitosterol	10 each	65	83	16[b]	1	d
[^{14}C]Cycloartenol	10	59	98	2	0	d

[a]Approx. fraction of administered radioctivity recovered from by combining acetone and ethyl acetate extracts, and pyrogallol hydrolyzate of the mycelium.

[b]Amount of esters varied by 2-10% when the experiments were repeated.

[c]FP, EP, and GP refer to zones on the TLC plate of the acetone extract representing the free polycyclic isopentenoids, esterified polycyclic isopentenoids, and glycosides of polycyclic isopentenoids, respectively.

[d]This study.

compounds naturally present in higher plants, steroidal alkaloids appear to possess the greatest capacity for inhibiting the sterol-induced growth and reproduction of *Phytophthora* (24). For instance, jervine at a level of 3 μg/ml plus sitosterol at a level of 10 μg/ml induce ca. 5% of the number of oospores in sitosterol controls. When the jervine level in this experiment is raised to 9 μg/ml, sitosterol-induced oospore production is completely abolished. This effect, observed in vitro, corresponds to the defensive role of the steroidal alkaloids in the field (4). Whether other polycyclic, e.g., polyhydroxylated isopentenoids, may also play a role in plant resistance is unclear and requires field testing. Thus, the ability of sitosterol and related sterols to promote growth and to induce reproduction is controlled by the kind and amount of other polycyclic isopentenoids in the host plant.

REFERENCES

1. Nes, W.D., and Patterson, G.W. (1981) J. Nat. Prod. 44, 215-220.
2. Elliott, C.G. (1977) Adv. Microbiol. Physiol. 15, 121-173.
3. Hendrix, J.W. (1970) Ann. Rev. Phytopathol. 8, 111-130.
4. Kúc, J. (1972) Ann. Rev. Phytopathol. 10, 207-232.
5. Nes, W.R., and Nes, W.D. (1980) Lipids in Evolution, 244 pp., Plenum Press, New York, NY.
6. Nes, W.R., Krevitz, K., Joseph, J., Nes, W.D., Harris, B., Gibbons, G.F., and Patterson, G.W. (1977) Lipids 12, 511-527.
7. Wood, S.G., and Gottlieb, D. (1978) Biochem. J. 170, 355-363.
8. Gottlieb, D., Knaus, R.J., and Wood, S.G. (1978) Phytopathology 68, 1168-1169.
9. Nes, W.D., Patterson, G.W., and Bean, G.A. (1980) Plant Physiol. 66, 1008-1011.
10. Nes, W.D., Patterson, G.W., and Bean, G.A. (1979) Lipids 14, 458-462.
11. Nes, W.D., Saunders, G.A., and Heftmann, E. (1981) Lipids, 16, 744-748.
12. Elliott, C.G., Hendrie, M.R., Knights, B.A., and Parker, W. (1964) Nature 203, 427-428.
13. Richards, J.B., and Hemming, F.W. (1972) Biochem. J. 128, 1345-1352.
14. Wood, S.C., and Gottlieb, D. (1978) Biochem. J. 170, 343-354.
15. Knights, B.A., and Elliott, C.G. (1976) Biochim. Biophys. Acta 441, 341-346.
16. Gonzales, R.A., and Parks, L.W. (1981) Lipids 16, 384-388.
17. Langcake, P. (1975) Trans. Br. Mycol. Soc. 64, 55-65.
18. Hendrix, J.W. (1975) Can. J. Microbiol. 21, 735-737.
19. Wojciechowski, Z.A., and Zimowski, J. (1979) Phytochemistry 18, 39-42.
20. McMorris, T.C., and White, R.W. (1977) Biochim. Biophys. Acta 486, 308-312.
21. Nes, W.R., Adler, J.H. Frasinel, C., Nes, W.D., Young, M., and Joseph, J.M. (1980) Phytochemistry 19, 1439-1443.
22. Popplestone, C.R., and Unrau, A.M. (1973) *Phytochemistry* 12, 1131-1133.
23. Nes, W.D., and Heftmann, E. (1981) J. Nat. Prod. 44, 377-400.
24. Nes, W.D., Hanners, P.H., Bean, G.A., and Patterson, G.W. Phytopathology, in press.
25. Sietsma, J.H., and Haskins, R.H. (1968) Can. J. Biochem. 46, 813-818.
26. Calderone, R.A., and Norman, C. (1976) Mycologia 68, 440-446.
27. Child, J.J., DeFago, G., and Haskins, R.H. (1969) Can. J. Microbiol. 15, 599-603.
28. Olsen, R.A. (1973) Physiol. Plant. 29, 145-149.
29. Parks, L.W., Gonzales, R.A., and Rodriquez, R.J. (1981) Abstr. Intern. Symp. *Phytophthora*, University of California, Riverside.

[Received August 17, 1981]

The Metabolism of Phytosterols in the Insect *Tenebrio molitor:* Utilization of 24-Methylenecholesterol and 24,28-Epoxymethylenecholesterol[1]

FRANCESCO NICOTRA, FIAMMA RONCHETTI and GIOVANNI RUSSO*
Istituto di Chimica Organica dell'Università degli Studi di Milano,
Centro di Studio per le Sostanze Organiche Naturali del C.N.R.,
Via Venezian 21, 20133 Milano, Italy

ABSTRACT

[23,23,25-$^3H_3$] 24-Methylenecholesterol and the corresponding 24,28-epoxide are converted into labeled cholesterol by larvae of *Tenebrio molitor*.
Lipids 17:184-186, 1982.

INTRODUCTION

It is well known (1) that many phytophagous insects need cholesterol for their growth and development; however, they are unable to effect the de novo synthesis of this compound and obtain it from plant sterols through a dealkylation process which essentially involves the elimination of the alkyl group in position 24 (Scheme I).

The sequence of metabolic events to which a typical C-29 sterol, such as sitosterol undergoes (Scheme I: R=CH_3), involves the transformation of the 24-ethyl group into a 24-ethylidene group, generally that of the E-isomer fucosterol; this is then epoxidized to yield a 24,28-epoxide, which then is opened and cleaved to desmosterol; reduction of the 24,25-double bond eventually leads to cholesterol.

In previous studies (2-4), we have shown that the insect *Tenebrio molitor* behaves in a less stereospecific way compared with other insects, in that both fucosterol and its Z-isomer, isofucosterol, are formed from sitosterol in this insect. Recent results indicate that both fucosterol and isofucosterol were identified in the silkworm *Bombix mori* (5). Analogously, in the metabolism of the epoxide intermediate, 3 of the 4 24,28-epoxides, i.e., 24R,28R- and 24S,28S-fucosterol epoxides and 24R,28S-isofucosterol epoxide, are transformed into cholesterol to about the same extent, whereas only the fourth isomer, 24S,28R-isofucosterol epoxide, is used ca. 10 times more readily. As for the metabolism of C-28 phytosterols, little has been done to establish whether the pathway of Scheme I (R=H) is operating, and even less has been done from the stereochemical point of view. Therefore, we decided to study the metabolism of C-28 phytosterols in *T. molitor* and test whether the intermediates represented in Scheme I (R=H) are metabolized by the insect.

MATERIALS AND METHODS

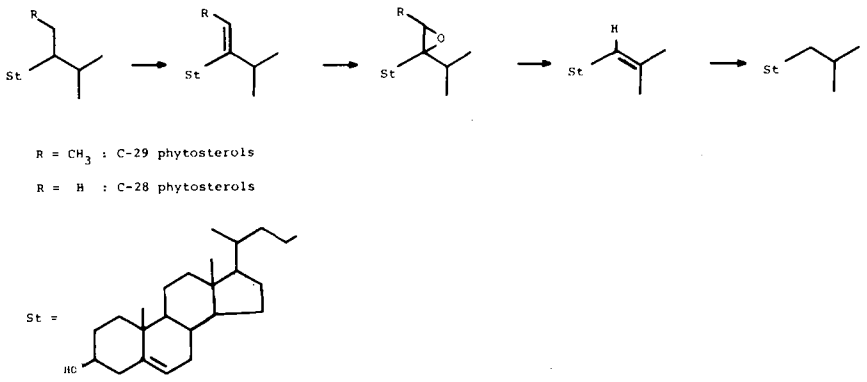
Gas liquid chromatography (GLC) was performed on a Carlo Erba Fractovap 2400 V instrument with flame ionization detector with 2-m analytical or preparative columns packed with 2.5% SE-30 at 220 C. ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Varian XL-100 spectrometer in deuterochloroform solutions with tetramethylsilane as internal reference.

Preparative and analytical thin layer chromatography (TLC) determinations were done on Silica Gel 60 F_{254} plates; the products were detected by spraying with 50% aq. H_2SO_4 and heating at 110 C for 5 min. Radioactive samples were counted with a Packard Tri-Carb 3320 liquid-scintillation counter; the samples were dissolved into 10 ml of a solution consisting of 0.65% (w/v) PPO and 0.013% (w/v) POPOP in toluene/dioxane (1:1, v/v).

24-Ketocholesterol was synthesized following the procedure of Lin and Smith (6). This compound was submitted to $^3H_2O/OH^-$ exchange according to Nicotra et al. (7) to yield [23,23,25-$^3H_3$] 24-ketocholesterol (sp act 2.64 x 10^7 dpm/mg). [23,23,25-$^3H_3$] 24-Methylenecholesterol (sp act 2.72 x 10^7 dpm/mg) was prepared from [23,23,25-$^3H_3$] 24-ketocholesterol with methyltriphenylphosphonium iodide /BuLi by a Wittig reaction (8). The radioactive compounds were analyzed by TLC and GLC and were found to be more than 98% pure.

[23,23,25-$^3H_3$] 24-Methylenecholesteryl acetate (120 mg), obtained from the 3β-ol by the usual acetylation, was dissolved in 50 ml of $CHCl_3$ and treated with *m*-chloroperbenzoic acid (55 mg) at 0 C for 30 min. After the usual work-up, the product was chromatographed on SiO_2 H-60. Elution with hexane/ethyl acetate

[1]Presented at the 72nd Annual Meeting of the American Oil Chemists' Society, New Orleans, LA, May 1981.



SCHEME I. The metabolism of phytosterols by phytophagous insects.

(99:1) afforded 80 mg of starting material. Elution with hexane/ethyl acetate (95:5) afforded 12 mg of the 24,28-epoxide-3-acetate (sp act 2.59 x 10^7 dpm/mg): NMR ($CDCl_3$): 2.60 (s, at C-28), 4.64 (m, 1H at C-3), 5.42 (m, 1H at C-5).

Further elution with hexane/ethyl acetate (95:5) afforded 8 mg of the 5,6-epoxide-3-acetate. [23,23,25-$^3H_3$]24,28-Epoxymethylenecholesterol was obtained from the corresponding 3-acetate by treatment with 0.25% methanolic KOH at 80 C for 1.5 hr.

Experiment 1. A mixture of [23,23,25-$^3H_3$]24-methylenecholesterol (3.75 x 10^7 dpm of ^3H) and [4-^{14}C]sitosterol (3.91 x 10^6 dpm of ^{14}C; the Radiochemical Centre, Amersham; *sp act 54 mCi/mmol*) was deposited onto 350 mg of finely ground oatmeal and fed to 250 young *T. molitor* larvae (3.5 g), which had been starved for 2 days. After 2.5 days, the larvae were sacrificed and processed as previously described (4). The isolated pure (4) cholesteryl acetate was diluted with unlabeled material, crystallized and counted (Table 1).

Experiment 2. A mixture of [23,23,25-$^3H_3$]-24,28-epoxymethylenecholesterol (4.55 x 10^7 dpm ^3H) and [4-^{14}C]sitosterol (3.74 x 10^6 dpm ^{14}C) was administered to young *T. molitor* larvae as described in exp. 1. Cholesteryl acetate was isolated pure (4), from the unsaponifiable fraction, diluted with unlabeled material, crystallized and counted (Table 1).

RESULTS AND DISCUSSION

Each tritiated compound was administered in separate experiments to *T. molitor* larvae, together with [4-^{14}C]sitosterol, for which utilization had already been ascertained (2), as internal control. The radioactivities and the $^3H/^{14}C$ ratios of the administered compounds are reported in Table 1.

After 2 days, the larvae were sacrificed and the unsaponifiable fraction was extracted, acetylated and purified by TLC. The residual tritiated precursor was carefully removed by argentation TLC (hexane/benzene, 1:1, as eluant) from the sterol fraction obtained in the first experiment, whereas the residual tritiated precursor was removed by TLC on Silica Gel

TABLE 1

Total Radioactivities and $^3H/^{14}C$ Ratios of the Administered Precursors and of the Isolated Cholesteryl Acetate

Experiment	Administered precursors			Recovered cholesteryl acetate	
	Compounds	^{14}C (dpm)	$^3H/^{14}C$ ratio	^{14}C (dpm)	$^3H/^{14}C$ ratio
1	[23,23,25-$^3H_3$]24-Methylenecholesterol + [4-^{14}C]Sitosterol	3.91 X 10^6	9.59	5.28 X 10^4	19.2
2	[23,23,25-$^3H_3$]24,28-Epoxymethylenecholesterol + [4-^{14}C]-sitosterol	3.74 X 10^6	11.66	3.90 X 10^4	15.9

60 F_{254} (hexane/ethyl acetate, 8:2, as eluant) in the second experiment.

In both cases, the less polar band, corresponding to cholesteryl acetate was shown by GLC to contain a significant amount of sitosterylacetate due to the unmetabolized sitosterol from the diet and the residual [4-^{14}C]-sitosterol. Cholesteryl acetate was obtained pure from the mixture by preparative GLC (2.5% SE-30, T = 220 C); it was then diluted with unlabeled material and crystallized to constant sp act and $^{3}H/^{14}C$ ratio (see Table I).

As can be seen from Table I, both 24-methylenecholesterol and its 24,28-epoxide are converted into cholesterol. Moreover, they are metabolized more readily than sitosterol, as indicated by the increase of the $^{3}H/^{14}C$ ratio, which doubled in the first precursor and became 1.4 times higher in the second precursor.

This smaller increase of $^{3}H/^{14}C$ ratio in the second experiment compared to the increase found in the first indicates that the 24,28-epoxide is a poorer precursor than 24-methylenecholesterol. This is in contrast, however, to the fact the first compound is a more advanced precursor than the second one. Explanations to justify this discrepancy can be differences in permeability and the fact that the 24,28-epoxide is actually a mixture of 2 stereoisomers (24R- and 24S-) which could be differently used by the insect. In conclusion, the results of these experiments clearly indicate that both 24-methylenecholesterol and its 24,28-epoxide are transformed into cholesterol by the insect *T. molitor*. These findings are in agreement with the metabolic sequence shown in Scheme I (R=H).

REFERENCES

1. Svoboda, J.A., Thompson, M.J., Robbins, W.E., and Kaplanis, J.N. (1978) Lipids 13, 742-753.
2. Nicotra, F.,Ronchetti, F., and Russo, G. (1978) Experientia 34, 699.
3. Nicotra, F., Ronchetti, F., Russo, G., and Toma, L. (1980) J. Chem. Soc. Chem. Commun. 479-480.
4. Nicotra, F., Pizzi, P., Ronchetti, F., Russo, G., and Toma, L. (1981) J. Chem. Soc. Perkin 1, 480-483.
5. Morisaki, M., Ying, B., and Ikekawa, N. (1981) Experientia 36, 336-337.
6. Lin, Y.Y., and Smith, L.L. (1974) J. Lab. Comp. 10, 541-548.
7. Nicotra, F., Ronchetti, F., Russo, G., and Toma, L. (1979) Biochem. J. 183, 495-499.
8. Ohtaka, H., Morisaki, M., and Ikekawa, N. (1973) J. Org. Chem. 38, 1688-1691.

[Received August 17, 1981]

Aspects of Sterol Metabolism in the Yeast *Saccharomyces cerevisiae* and in *Phytophthora*

L.W. PARKS, C. McLEAN-BOWEN, C.K. BOTTEMA, F.R. TAYLOR, R. GONZALES, B.W. JENSEN and **J.R. RAMP**, *Department of Microbiology, Oregon State University, Corvallis, OR 97331*

ABSTRACT

Using 5 wild-type strains of yeast, nonequivalence in the isolation of sterol mutants was observed. Experiments are described on the effects of sterol modifications on growth, physical and enzymic properties of *Saccharomyces cerevisiae* and *Phytophthora cactorum*. Although discontinuities in Arrhenius kinetics were observed by fluorescence anisotropy and enzymic measurements of mutants (but not wild-types) of yeast, evidence based on membrane permeability and differential scanning calorimetry failed to support bulk lipid phase transitions as the cause for the discontinuities.
Lipids 17:187-196, 1982.

INTRODUCTION

Sterols are widely distributed among eucaryotic organisms in nature. Because the synthesis of sterols by an organism represents an expensive investment in carbon intermediates and energy, a strongly selective pressure must exist for their maintenance during evolution and for their distinctive cellular functions. It is the purpose of our studies to investigate experimentally the role of sterols in the general economy of the cell.

Saccharomyces cerevisiae has been selected as the model organism for this study because of the availability of a variety of mutants which do not biosynthesize ergosterol, the predominant sterol found in yeast. Several mutant classes have been isolated and characterized. In this paper, we shall describe some problems of mutant isolation, the feeding of sterols to mutants, and the use of the mutants in enzymic and physical measurements. Furthermore, we have extended our study to include *Phytophthora cactorum*, a member of the Pythiaceous fungi. This group represents a rare instance of eucaryotic organisms which may be cultivated artificially devoid of sterols.

Elucidation of the functional significance of the modification of sterol structures on the organisms is necessary. When an organism accumulates a sterol other than ergosterol, as in the case of many nystatin-resistant mutants, is there a redistribution of membrane components which compensates for the presence of the unusual sterol? If such compensation occurs, are all membrane properties retained, or is there a selective loss or modification of specific membrane functions? In attempting to answer these questions, we have monitored the presence and quantity of lipid moieties in mutant and wild-type strains. In addition, the effect of changes in sterol structure on the mobility of fatty acyl phospholipid chains in membranes from *S. cerevisiae* was determined from the rate of depolarization of a fluorescent probe molecule, and compared to the effect of the sterol on a model membrane system in which the lipid components are defined. These same membranes were examined for bulk lipid transitions by differential scanning calorimetry. The sensitivity of membrane-bound enzymes to alterations of the lipids was examined by comparing Arrhenius kinetics of the activities from different organisms. Finally, changes in proton permeability of membranes were determined.

In this paper, we shall discuss various experimental approaches to our studies on the physiology of fungal sterols. Much of the work is incomplete, and its extension is often speculative. But, it is presented here in keeping with the theme of this symposium to present ongoing research. A comprehensive review of sterols in yeast may be consulted if greater background information is needed (1).

MATERIALS AND METHODS

Organisms

The strains of *S. cerevisiae* used in this study are shown in Table 1. No ergosterol is synthesized by the nystatin-resistant mutants. Strain 3701B-n3 accumulates ergosta-7,22-diene-3β-ol and 8R1 accumulates cholesta-8,24-diene-3β-ol and cholesta-5,7,22,24-tetraene-3β-ol. *P. cactorum* was obtained from E. Hansen, Oregon State University.

Culture Conditions

Yeast were routinely cultured on rich medium (1% tryptone, 0.5% yeast extract, 2% carbon source) or Wickerham's synthetic complete media (2,3). For solid media, 1.5% agar (Difco) was added.

Cultures of *P. cactorum* were grown in a

TABLE 1

Strains of *S. cerevisiae* Used in This Study

Strain	Source	Comment
S288c	S. Fogel	Haploid
3701B	H. Roman	Haploid, ura$^-$
1071-3b	D. Hawthorne	Haploid, trp$^-$
NCYC 366	A. Rose	Haploid
Z008	L. Miller	Haploid
3701B-n3	This laboratory	Nystatin-resistant, derived from 3701B
8R1	L. Miller	Nystatin-resistant, derived from Z008
MCC	This laboratory	Diploid, wild-type
FY3	This laboratory	Sterol auxotroph

New Brunswick Scientific Co. Model MF-114 Microfermentor containing 8 ℓ of basal medium (4).

Mutagenesis

Plates used for mutagenesis were prepared by adding 50 μl of 5, 3, 1 and 0.5% (v/v) ethylmethane sulfonate (EMS) to 4 wells 5 mm in diameter placed 90° apart and 2 cm from the edge of 10-cm petri plates containing 1% tryptone, 0.5% yeast extract and 2% glucose (YTD) agar. Nystatin plates were prepared by adding an ethanolic solution of nystatin at a concentration of 300 units/ml of the synthetic medium. These plates were equilibrated for 16 hr prior to inoculation, permitting the mutagen to diffuse into the agar. The strain to be mutagenized was grown to the early stationary phase in YTD medium, diluted to 10^6 cells/ml, and 0.5 ml of this dilution spread uniformly on mutagenesis plates. Half of the plates were replicated to nystatin after 8 hr and the remaining half after 24 hr. All plates were incubated at 28 C. Suspected resistant clones were streaked for single cells, and the nystatin resistance was confirmed. A minimum of 12 colonies from each parental strain was selected for further study.

Sterol Classification

Cell pellets were subjected to methanolic pyrogallol saponification for 90 min and extracted with hexane (5). The extracted lipids were scanned for UV-absorbing material in a Cary Model 11 recording spectrophotometer from 310 to 210 nm using redistilled hexane as the solvent. Gas liquid chromatography (GLC) of the extract was on a 3% OV-17 column in a Varian Model 2740 chromatograph equipped with a CDS 111 recording integrator. Coupled gas chromatography-mass spectrometry (GC-MS) was performed as previously described (6).

ANALYSES

Techniques for fluorescence anisotropy (7), enzymic assay (8,9), and proton motive force determinations (10) have been described. Procedures for the isolation of mitochondria and the preparation of liposomes have been reported (7). Protein was quantitated by the method of Lowry et al. (11) using bovine serum albumin as the standard. Phospholipids were quantitated by the method of Ames (12). Mitochondrial cytochromes were extracted by the method of Tzagoloff et al. (13). The difference spectra of reduced and oxidized cytochromes were scanned between 500 nm and 650 nm at 25 C using a Beckman Model 25 double beam spectrophotometer with scanning attachments. The instrument employed for high-sensitivity differential scanning calorimetry was an MC-1 (Microcal, Amherst, MA). The liposomes and mitochondria were scanned from 2.5 to 75 C at a rate of 20 C/hr in distilled water. Proteolysis of the mitochondria was performed by adding 1 mg pronase/ml of mitochondrial suspension and incubating the suspension at 25 C for 1 hr prior to scanning.

Glucose uptake and CO_2 evolution rates in *Phytophthora* were determined by the method of Schlosser and Gottlieb (14).

RESULTS AND DISCUSSION

Isolation of Yeast Sterol Mutants

The antimycotic nystatin has been used in fungi to select against prototrophic colonies in mutagenic studies (15) and to select for mutations eliciting altered sterol composition of yeast (16-19). We have attempted to isolate sterol mutants from a variety of different wild-type yeast strains using nystatin to determine whether any previously uncharacterized mutants could be isolated and to increase the number of isogenic mutants available for sterol

studies. Many laboratories, including our own (20-23), have demonstrated that such mutants are useful to identify the sequence of enzyme reactions in the sterol biosynthetic pathway, to isolate labeled precursors for enzymic assays and to examine the physiological role of sterols.

The mutagenesis yielded sterol mutants from each of the 5 parental strains tested (Table 2). The number of mutants obtained per plate and the elapsed time from replication to appearance of identifiable colonies were highly variable. Cells which had been mutagenized for 24 hr showed an average of 5 resistant colonies per 5×10^5 cells plated after 3 days incubation. Following mutagenesis for 8 hr, the earliest colonies appeared at 5 days. Only those colonies which showed relatively unaffected growth on nystatin plates were analyzed completely. Therefore, the mutants characterized in Table 2 reflect analyzed mutants and do not represent the total number of resistant colonies confirmed by plate test.

Our analysis of the sterols accumulated by the nystatin-resistant isolates indicate that mutagenesis of the parental strain generally produces a single mutant class which is characteristic of the parental. The following profiles have been identified in the mutants currently studied. Sterols derived from nystatin-resistant clones of Z008 have conjugated double bonds in positions 5 and 7 ($\Delta^{5,7}$) of the B ring (UV absorption maximum at 282 nm with peaks at 272, 262, 294 nm) and at 22 and 24 ($\Delta^{22,24}$) of the side chain (UV absorption maximum at 238 with a peak at 232 and a shoulder at 247). The 3701B mutants show no conjugated double bonds (UV absorption maximum at 210 nm) with only 3 exceptional isolates showing traces of absorption in the region characteristic of the $\Delta^{5,7}$ conjugation. NCYC 366 is an ergostatetrene accumulator, and the profiles of the resistant mutants show no qualitative sterol alterations. The parental strain is not resistant to 15 unit/ml nystatin as are the derived mutants. When compared to other strains, it neither produces more mutants per plated cell nor accumulates mutants which grow more rapidly. The 1071-3b mutants all show UV spectra with a maximum at 282 nm as does the parental strain. The GLC composition of 3 of the isolates did not show any alteration in sterol composition.

By far the most interesting parental strain, on the basis of the variety of mutants produced, was S288c. UV spectra divided the mutants into 3 classes, one with peaks at 238 and 282 nm, one with only the 282 nm peak, and one with only a peak at 210 nm. The first class could be partitioned further by differences in the ratios of sterols by GLC. The second class could not be distinguished from the parental on the basis of chromatographic profile except that one peak was missing. The third class partitioned into 3 subclasses.

A more complete analysis of one of the S288c mutants showed that it possessed a series of sterols similar to those reported by Sprinson for his SG1 mutant (23). The major GC peak in our mutant was a C_{29} sterol with GC-MS fragmentation patterns similar to that of 14-methylfecosterol. Another C_{29} sterol was present but the extract was not sufficiently pure to show its identity by GC-MS. Two other compounds which demonstrate retention characteristics predicted for the remaining compounds reported by Sprinson's group were seen. As no quantitative data on the SG1 sterol ratios have been published, only circumstantial evidence appears to link it with this newly isolated mutant.

Surprisingly, the mutagenesis of different parental types gave unique mutant isolates. Close identity of the strains is generally accepted, in that genetic compatability is seen. We are intrigued by the results of these mutagenesis results, and are investigating possible differences in the lipid composition of the starting strains. It is our assumption that mutagenesis is equivalent in all of the wild-types, but that the biochemical make-up of the different parental strains will tolerate only certain sterol types. We have observed high mortality among many of the mutants from some strains, in which the mutants may be sub-cloned for only a very few transfers.

As the endogenously synthesized sterols are modified by mutation, is the cell required to compensate for the changes by modification of other lipid components? If this is true, then S288c must be very versatile in its ability to make these adjustments for different sterols. We are currently pursuing this question from several directions. One of these procedures is to determine the effect of feeding different sterols to sterol auxotrophs. This will allow us to define "acceptable" sterols and to follow cellular adjustments on changing the sterols that are available to the organism.

Sterol Feeding Experiments

Sterol auxotrophs have not been readily isolated in yeast. Karst and Lacroute (24) reported that they were only able to obtain sterol auxotrophs by selecting thermolabile mutants. These organisms can grow only if sterol synthesis is still slightly leaky. Tight

TABLE 2

Presumptive Enzyme Defects and Principal Sterol Intermediates Accumulated in Wild-type and Sterol Mutants of *S. cerevisiae*

Strain	Number of isolates	Blocked biosynthetic step	GLC%[a]	Sterol intermediates accumulated
Z008 (wild-type)		–	65	Ergosterol (ergost-5,7,22-trien-3β-ol)
			20	Ergosta-5,7,22,24(28)-tetraen-3β-ol)
Z008 mutants	11	C-24-Transmethylation	75	Zymosterol (cholesta-8,24-dien-3β-ol)
			14	Cholesta-5,7,22,24-tetraene-3β-ol
			3	Cholesta-5,7,24-trien-3β-ol
3701B (wild-type)		–	75	Ergosterol
			1	Fecosterol (ergosta-8,24(28)-dien-3β-ol)
			4	Lanosterol (lanosta-8,24-dien-3β-ol
3701B mutants	12	5(6)-Desaturation	82	Ergosta-7,22-dien-3β-ol Ergosta-8,22-dien-3β-ol
			10	Ergosta-7,22,24(28)-trien-3β-ol Ergosta-8,22,24(28)-trien-3β-ol
			5	Ergosta-7,24(28)-dien-3β-ol Ergosta-8,24(28)-dien-3β-ol
1071-3b (wild-type)		–	77	Ergosterol
			11	Lanosterol (lanosta-8,24-dien-3β-ol
1071 3b mutants	6	22(23)-Desaturation	73	Ergosta-5,7-dien-3β-ol
			10	Zymosterol
NCYC 366 (wild-type)		–	55	Ergosta-5,7,22,24(28)-tetraen-3β-ol
			30	Zymosterol
NCYC 366 mutants	9	?	70	Ergosta-5,7,22,24(28)-tetraen-3β-ol
			20	Zymosterol
S288c (wild-type)		–	58	Ergosterol
			16	Zymosterol
			13	Fecosterol
S288c mutants: JR1,JR2	2	5(6)-Desaturation	75	Ergosta-7,22-dien-3β-ol Ergosta-8,22-dien-3β-ol
			12	Ergosta-7,22,24(28)-trien-3β-ol Ergosta-8,22,24(28)-trien-3β-ol
			8	Ergosta-7,24(28)-dien-3β-ol Ergosta-8,24(28)-dien-3β-ol
JR3	1	8-7 Isomerization	66	Ergosta-8-en-3β-ol
			30	Ergosta-8,22-dien-3β-ol
JR4	1	C-14-Demethylation	78	14-Methyl-ergosta-8,24(28)-dien-3β-ol
			4	4,14-Dimethyl-cholesta-8,24-dien-3β-ol
			12	Lanosterol
JR5-JR11	5	C-24-Transmethylation	30	Zymosterol
			28	Cholesta-5,7,24-trien-3β-ol
			35	Cholesta-5,7,22,24-tetraen-3β-ol

[a]Area percent determined from integration of gas chromatographically separated sterols.

sterol mutants have only been found in heme-deficient backgrounds, and we have reported that one such strain is inhibited by δ-aminolevulinic acid supplementation (25). Therefore, it appears that heme synthesis is only compatable with at least some amount of endogenous sterol synthesis. We are attempting to explore the nature of this relationship. In addition, this discovery has allowed us to begin to select a series of sterol mutants in a heme-deficient background which we hope will develop into an experimental system for investigating the molecular biology of the sterol biosynthetic pathway.

Sterol auxotrophy in yeast, induced by anaerobic growth or by mutational defects, has been utilized by several investigators to study the sterol structural features important for cell growth (26-29). We have previously reported that substituting cholesterol for ergosterol in the sterol auxotroph FY3 narrows the pH range over which growth occurs, particularly at higher pH values (25). This has been shown to apply to a series of sterol supplements; as the sterol approaches ergosterol in structure, the pH range of growth broadens (cholesterol<Δ^5-ergosterol < stigmasterol < brassicasterol < ergosterol). This may be a clue to the aspect of cell physiology most sensitive to changes in sterol structure. In surveying which sterols support the growth of yeast, our results agree with other investigators that a planar ring system (no growth on lanosterol or coprostanol) and a 3β-hydroxyl group (no growth on epicholestanol and cholesta-3,5-diene) are essential for growth. It is unknown whether lack of growth may result from the failure to take up some of these sterols. Growth on sterols lacking the Δ^5-bond (cholestanol, ergostanol, lathosterol and 4-cholestenol) was unexpectedly poor, although poor response to cholestanol has also been reported in *Phytophthora* (29). Some mention has been made in this conference on the different response of organisms to *cis* and *trans* isomers of Δ^{22}-sterols. We find that FY3 grows on both isomers of cholesta-5,22-diene-3β-ol.

Another sterol which does not support the growth of FY3 is ignosterol (8,14-ergostadienol). This is of interest, as ignosterol is the sterol accumulated by yeast in the presence of a very potent antimycotic agent, 15-azasterol (30). Evidence that the accumulation of ignosterol is the cause of growth inhibition has been obtained by demonstrating that a strain of yeast unable to demethylate lanosterol at C-14 (which accumulates 14-methylfecosterol), and therefore cannot produce ignosterol, is not inhibited by azasterol.

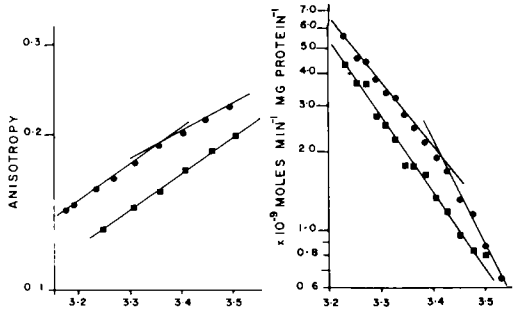


FIG. 1. Enzyme activity and bilayer fluidity comparison of wild-type and mutant. The figure on the left shows the Arrhenius curve of the anisotropy of DPH embedded in mitochondria isolated from 3701B (■) and 3701B-n3 (●). Enzyme activity for L-kynurenine hydroxylase is shown on the right. The abscissa is given in reciprocal absolute temperature X 10^{-3}.

Physical and Enzymatic Properties of Yeast Membranes

Work by our laboratory (8) and by Lees and co-workers (31) has shown that sterol affects the growth characteristics of *S. cerevisiae*. Wild-type and isogenic nystatin mutants which accumulate ergosta-7,22-diene-3β-ol show identical growth characteristics when grown on glucose over a wide temperature range. However, optimal and permissive growth temperatures are altered when a respiratory substrate is supplied. Growth of the wild-type on ethanol was optimal at 29-30 C and was observed to stop above 35 C. The growth of the mutants was optimal at 26-27 C and ceased above 30 C (32). This suggested that the altered sterol composition produced mitochondria defective in energy production at temperatures above 30 C, and has led us to an investigation of mitochondrial membrane properties.

The effect of sterol composition on the physical and enzymic properties of the mitochondrial membranes of mutant and wild-type *S. cerevisiae* has been studied. Using a fluorescence polarization technique with 1,6-diphenyl-1,3,5-hexatriene (DPH), a suspected phase transition was observed in mitochondrial membranes isolated from the mutants but not in the membranes of wild-type cells. Arrhenius kinetics of L-kynurenine-3-hydroxylase exhibited changes in activation energy at temperatures similar to those seen in the fluorescence polarization experiments. These data are shown in Figure 1. A more detailed study of this type has been reported (7). We have also shown that alteration of the sterol accumulated by mutants changes the transition temperatures of

the mitochondrial enzymes, cytochrome c oxidase and sterol methyltransferase (33). Based on these observations, we assumed that a bulk phase transition was occurring in the membranes and was effecting changes in the mitochondrial properties of the mutants.

Such a transition would be expected to alter the permeability of the membrane, and as a result destroy the transmembrane potential necessary for the generation of ATP by oxidative phosphorylation. We have calculated the proton motive force ($\Delta\Psi$) of respirationally competent mitochondria isolated from wild-types and mutants by measuring the accumulation of the lipophylic cation methyltriphenylphosphonium ($TPMP^+$). These ions traverse a lipid bilayer upon generation of a transmembrane potential where there is a net negative charge inside the vesicle. Using the Nerns equation, $\Delta\Psi$ can be calculated from the measured cation accumulated.

A number of respiratory substrates stimulated the accumulation of $TPMP^+$. The uptake of cation was reduced by the addition of respiratory inhibitors and uncouplers and was shown to be a noncarrier mediated process (10), enabling calculation of $\Delta\Psi$. Figure 2 shows the transmembrane potential and respiratory control at 17, 27 and 35 C of mitochondria isolated from the mutant which could not grow on a respiratory substrate above 30 C and from the isogenic wild-type. Even at the nonpermissive growth temperature, the mutant established and maintained a transmembrane potential and coupled phosphorylation to oxidation of the respiratory substrate. The calculated potentials for the wild-type and mutant are very similar. We conclude, therefore, that the inability of the mutant to grow on ethanol above 30 C is not caused by a defect in this mitochondrial function.

Our failure to detect predicted alterations of mitochondrial membrane function of the mutant at elevated temperatures led us to measure more directly the effect of sterol changes on the bulk lipids of the mutant and its isogenic wild-type by differential scanning calorimetry.

No reversible lipid transitions were observed in the mitochondria isolated from either wild-type or mutant yeast within the temperature range examined. Three irreversible transitions at 56, 68 and 63 C (Figs. 3 and 4) appeared in all the mitochondria. The enthalpies of these transitions were reduced by a second scan of the same sample or by treatment with pronase. Therefore, these transitions appeared to be the result of protein denaturation rather than lipid phase transitions. Liposomes formed from the

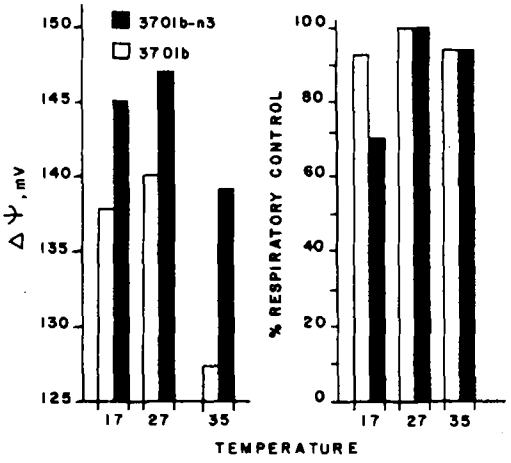


FIG. 2. Transmembrane potential and respiratory competence of yeast. The transmembrane potential ($\Delta\Psi$) was calculated from the uptake of the lipophilic cation ($TMPM^+$). The percentage respiratory control is calculated as the product of the respiratory control ratio at 17 or 35 C and the reciprocal of the respiratory control ratio at 27 C.

extracted lipids of either wild-type or mutant mitochondria also failed to demonstrate a phase transition (Fig. 5).

The inability to detect a transition was not due to a lack of lipid material or to insensitivity of the instrument. The sensitivity of the differential scanning calorimeter was examined using dipalmitoylphosphatidylcholine (DPPC) liposomes of various concentrations. The phase transition at 40.5 C and the pretransition at 35.3 C were clearly demonstrated even at low lipid concentrations (0.5%) (Fig. 5). To insure that any lipid phase transition would be detected, the concentration of phospholipid in all mitochondrial membrane and liposome preparations used in our experiments was at least 1%. To rule out the possibility of DPH influencing or inducing transitions in the fluorescence polarization studies, the probe was added to the mitochondrial preparations and to the liposomes prior to differential calorimetric scans. The presence or absence of the probe had no observable effect.

Because lipid phase transitions are not detected by high-sensitivity differential scanning calorimetry of these mitochondria and because the mitochondria show no loss of membrane function (i.e., no loss of transmembrane potential or coupled phosphorylation to respiration [10]), the total membrane cannot be undergoing a bulk phase transition. We propose that the transitions detected previously by fluorescence anistropy (7) represent

the lateral phase separations of small, isolated domains as described by Lee (34,35). There is no detectable enthalpy increase in the DSC profiles of these membranes, as there is insufficient lipid in the phase-separated domains to detect such a transition by this method. Others (36-38) have reported similar phenomena where phase separations have been observed but no bulk lipid transitions were demonstrated.

The irreversible transitions detected were not influenced by the differences in sterol composition of the various mitochondria examined (Figs. 3 and 4). The transition temperatures did not vary between the mutants and wild-types and the enthalpies of these transitions appeared to be solely dependent on protein concentration.

The lack of a bulk lipid transition in the mitochondria of wild-type yeast had been previously noted and is due to the presence of

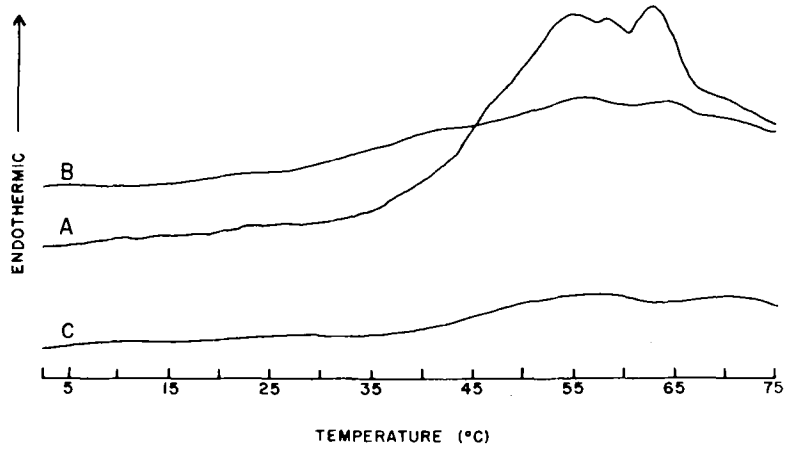


FIG. 3. High sensitivity differential scanning calorimetry heating curves of membranes. (A) 3701B mitochondria (10 mg protein/ml)–first scan. (B) Second scan. (C) 3701B mitochondria (10 mg protein/ml) with pronase (1 mg/ml). Methods as described in text. Scans were conducted in distilled water and 2 buffers: (1) 0.9 M sorbitol, 10 mM TRIS, 0.5 mM EDTA buffer (pH 7.5), and (2) 10 mM TRIS, 50 mM KCl buffer (pH 7.5). No difference between scans of the samples suspended in distilled water or either buffer was observed.

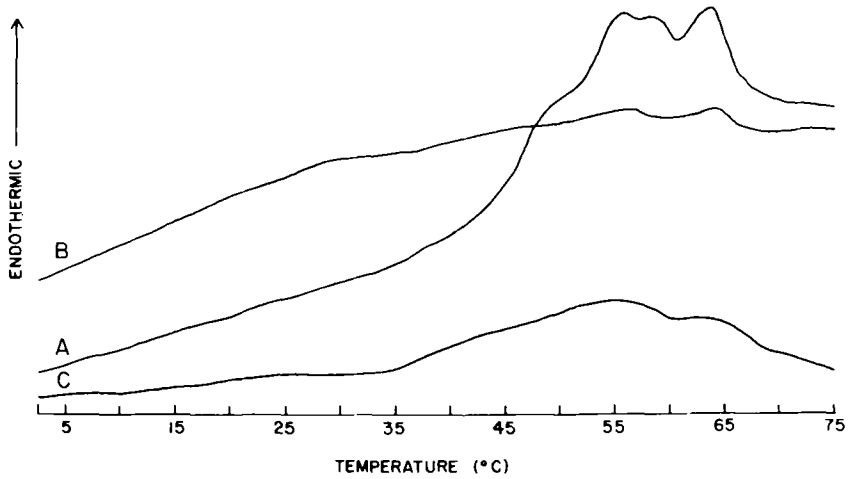


FIG. 4. High sensitivity differential scanning calorimetry heating curves of membranes. (A) 3701B-n3 mitochondria (10 mg protein/ml)–first scan. (B) Second scan. (C) 3701B-n3 mitochondria (10 mg protein/ml) with pronase (1 mg/ml). Methods as described in text. Buffers as described in Fig. 3.

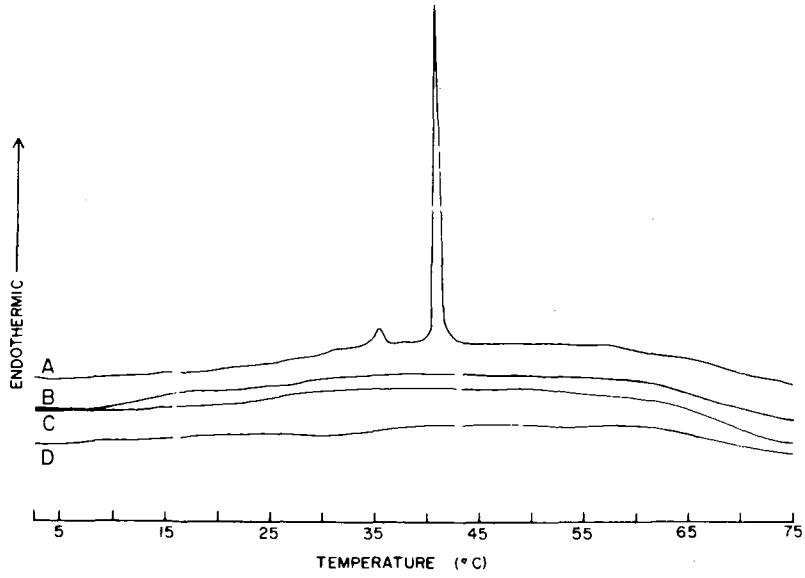


FIG. 5. High sensitivity differential scanning calorimetry heating curves of liposomes. (A) Dipalmitoylphosphatidylcholine (0.5 mg/ml). (B) Lipids extracted from 3701B (1.0 mg/ml). (C) Lipids extracted from 3701B-n3 (1.0 mg/ml). (D) Baseline (no lipid present). Methods as described in text. Buffers as described in Fig. 3.

ergosterol in the mitochondria (39). The presence of sterol intermediates in the mitochondria of yeast sterol mutants possibly precludes a bulk lipid transition in their mitochondrial membranes, as well. Indeed, our experiments are biased, in that the sterol mutants used are viable. It is anticipated that the mutants whose sterols cannot prevent general bulk lipid phase transitions would be inviable and thus, unobserved. The presence of lateral phase-separated domains in the mitochondria of the yeast sterol mutants indicates though, that sterol structure must influence membrane fluidity. We are continuing to examine this influence by differential scanning calorimetry of model membrane systems in an effort to define the role of sterol structure.

Studies with *Phytophthora*

We began studies with *Phytophthora* as it represented a unique experimental system. This organisms can grow vegetatively without sterol but requires sterol for sporulation. A comparison can be made of membranes lacking sterol with membranes of known sterol supplementation, allowing definitive studies of the effect of the presence of sterols on specific physiological functions. As a preliminary to in vitro experiments, we investigated the effect of sterol on the growth of *P. cactorum*. Our experiments were frustrated originally by extremely variable results. However, we observed that the usual culture media used in the growth of *Phytophthora* are inadequately buffered. To circumvent this problem, phthalic acid (pK = 2.95 and 5.41) was added to the growth media to a final concentration of 10 mM.

In basal media unbuffered with phthalate, supplementation of sterol to the growth media decreased the lag phase, but the final cell yield was virtually identical. Excess accumulation of acid had caused a staling effect in the media, adversely affecting growth. The results of growth experiments with the phthalate-buffered and nonbuffered media are shown in Figure 6. In the effectively buffered medium, a 10-fold increase in cell mass was observed for the sterol-supplemented culture, and a 3-fold increase in the cell yield was seen for the culture without sterol.

Glucose consumption and CO_2 production were monitored in log phase cultures; sterol supplementation caused a decreased rate of substrate uptake and a 3-fold higher rate of carbon dioxide production. When glycolytic enzymes were measured, the presence of sterol resulted in little or no change in overall activity. This suggested to us that the presence of sterol in the cell affects the amount of oxidative phosphorylation in the culture.

An extensive literature exists (1) which

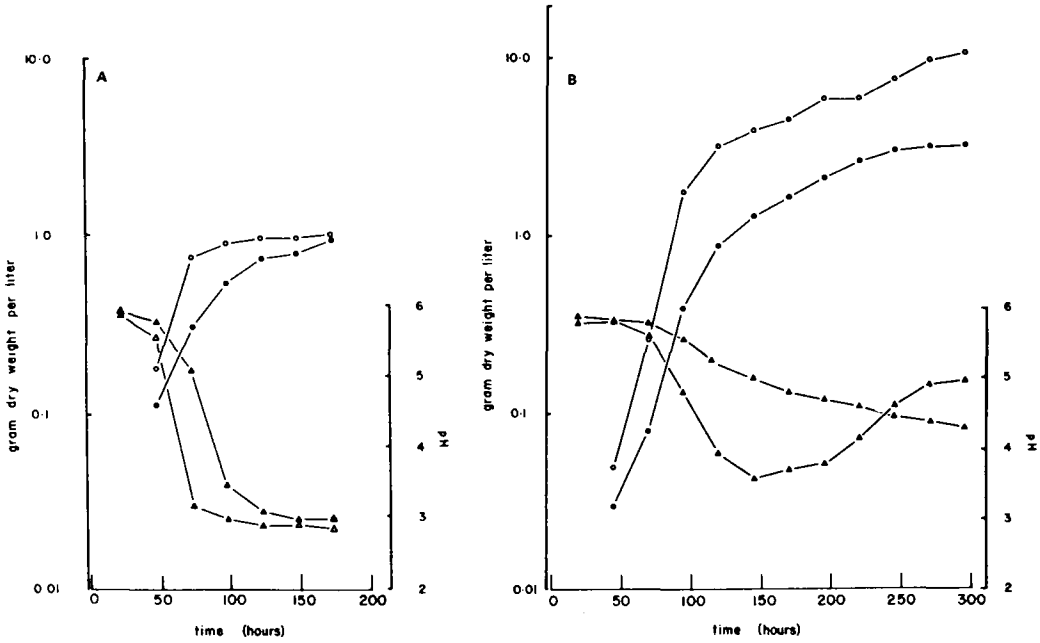


FIG. 6. Growth of *Phytophthora cactorum*. (A) Growth in unbuffered medium. (B) Growth in phthalate-buffered medium. The composition of the medium is as described by Gonzales and Parks (4). ○—○, dry weight in the presence of 10 mg/l sterol (mixture of sitosterol, campesterol, and stigmasterol); ●—●, dry weight in the absence of sterol; △—△, pH in the presence of sterol; ▲—▲, pH in the absence of sterol.

suggests an intimate association of sterols with respiratory competency. This possibility was examined further by examining the cytochrome composition of crude mitochondrial preparations of *Phytophthora* from sterol-grown and nonsterol-grown cultures. The difference spectra of these samples were monitored and are presented in Figure 7. Although cytochromes are present in both cultures of *Phytophthora*, they are present in substantially reduced amounts in those cells incubated without sterols.

We have observed the presence of sterols in the mitochondria of the sterol-supplemented cultures of *Phytophthora*. However, the mechanism of uptake and distribution of sterols by these cells is unclear. The effect of sterol on the synthesis and deposition of respiratory components is yet to be investigated.

Nystatin-resistant mutants and their isogenic parents have been used extensively to provide information on the physiological function of sterols in fungi. We have shown that the replacement of ergosterol with a closely related sterol alters the Arrhenius kinetics of several mitochondrial membrane-bound enzymes, and allows a lateral phase-separation of the lipids of the membrane. However, this does not cause a loss of mitochondrial oxidative phosphorylation. These observations and the results of our mutagenesis and feeding experiments lead us to propose that sterols exist not simply to provide a bulk lipid component that antagonizes major temperature-induced fluidity changes. Rather, we view ergosterol as an evolutionally selected, finely "tuned" sterol that fulfills precise physiological roles. Our

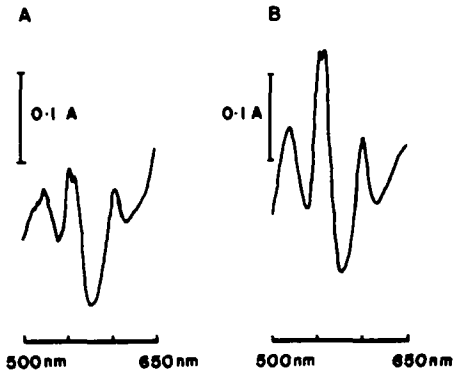


FIG. 7. Cytochrome spectra. Cytochromes were extracted as described by Tzagoloff et al. (13). (A) Difference spectrum of cytochromes from *P. cactorum* grown in the absence of sterol, and (B) grown in the presence of 10 mg/ℓ sterol.

mutant, feeding, in vitro and growth experiments all support this speculation.

In this communication, we have presented several on-going experiments in our laboratory. It is clear that an exciting field for study remains for understanding the physiology of these organisms. Symposia such as these allow investigators from a variety of backgrounds and experiences to share fruitfully in this very diverse effort.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation (PCM-787609) and the National Institutes of Health of the Department of Health and Human Services (AM-05190). C. McLean-Bowen, C.K. Bottema and R.A. Gonzales were the recipients of N.L. Tartar research fellowships. The authors express with sincere appreciation the efforts expended by Dr. H. Kircher in organizing the AOCS Symposium on Sterols. This is Oregon Agricultural Experiment Station Technical Paper no. 6007.

REFERENCES

1. Parks, L.W. (1978) Crit. Rev. Microbiol. 6, 301-341.
2. Hawthorne, D.C., and Mortimer, R.K. (1960) Genetics 45, 1085-1110.
3. Wickerham, J.-L. (1946) J. Bacteriol. 52, 293-301.
4. Gonzales, R.A., and Parks, L.W. (1981) Lipids 16, 384-388.
5. Adams, B.G., and Parks, L.W. (1967) J. Cell. Physiol. 70, 161-168.
6. Hays, P.R., Neal, W.D., and Parks, L.W. (1977) Antimicrob. Agents Chemother. 12, 185-191.
7. McLean-Bowen, C.A., and Parks, L.W. (1981) J. Bacteriol. 145, 1325-1333.
8. Thompson, E.D., and Parks, L.W. (1974) Biochem. Biophys. Res. Commun. 57, 1207-1213.
9. Schott, H., Ellrich, V., and Staudinger, H. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 99-101.
10. McLean-Bowen, C.A., and Parks, L.W. (1981) Chem. Phys. Lipids, 29, 137-145.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Ames, B.N. (1966) Methods Enzymol. 8, 115-118.
13. Tzagoloff, A., Akai, A., and Needleman, R.B. (1975) J. Biol. Chem. 250, 8228-8235.
14. Schlosser, E., and Gottlieb, D. (1968) Arch. Microbiol. 61, 246-253.
15. Snow, R. (1966) Nature 211, 206-207.
16. Ahmed, K.A., and Woods, R.A. (1967) Genet. Res. Camb. 9, 179-193.
17. Bard, M. (1972) J. Bacteriol. 111, 649-657.
18. Gollub, E.G., Trocha, P., Liu, P.K., and Sprinson, D.B. (1974) Biochem. Biophys. Res. Commun. 56, 471-477.
19. Molzahn, S.W., and Woods, R.A. (1972) J. Gen. Microbiol. 72, 339-348.
20. Barton, D.H.R., Corrie, J.E.T., Widdowson, D.A., Bard, M., and Woods, R.A. (1974) J. Chem. Soc. Perkin Trans. 1, 1326-1333.
21. Fryberg, M., Oehlschlager, A.C., and Unrau, A.M. (1974) Arch. Biochem. Biophys. 160, 83-89.
22. Parks, L.W., Bond, F.T., Thompson, E.D., and Starr, P.R. (1972) J. Lipid Res. 13, 311-316.
23. Trocha, P.J., Jasne, S.J., and Sprinson, D.B. (1977) Biochemistry 16, 4721-4726.
24. Karst, F., and Lacroute, F. (1977) Mol. Gen. Genet. 154, 269-277.
25. Taylor, F.R., and Parks, L.W. (1980) Biochem. Biophys. Res. Commun. 95, 1437-1445.
26. Proudlock, J.W., Wheeldon, L.W., Jollow, D.J., and Linnane, A.W. (1968) Biochim. Biophys. Acta 152, 434-437.
27. Hossack, J.A., and Rose, A.H. (1976) J. Bacteriol. 127, 67-75.
28. Lala, A.K., Buttke, T.M., and Bloch, K. (1979) J. Biol. Chem. 254, 10582-10585.
29. Elliott, C.G. (1979) J. Gen. Microbiol. 42, 425-435.
30. Hays, P.R., Parks, L.W., Pierce, H.D., and Oehlschlager, A.C. (1977) Lipids 12, 666-668.
31. Lees, N.D., Lofton, S.L., Woods, R.A., and Bard, M. (1980) J. Gen. Microbiol. 118, 209-214.
32. Thompson, E.D., and Parks, L.W. (1974) J. Bacteriol. 120, 779-784.
33. Thompson, E.D., Bailey, R.B., and Parks, L.W. (1974) Biochim. Biophys. Acta 334, 116-126.
34. Lee, A.G. (1975) Prog. Biophys. Mol. Biol. 29, 3-56.
35. Lee, A.G. (1977) Biochim. Biophys. Acta 472, 285-344.
36. Feo, F., Canuto, R.A., Garcea, R., Avogadro, A., Villa, M., and Celasco, M. (1976) FEBS Lett. 72, 262-266.
37. Hackenbrock, C.R., Hochli, M., and Chau, R.M. (1976) Biochim. Biophys. Acta 544, 466-484.
38. Wunderlich, F., Ronai, A., Speth, V., Seelig, J., and Blume, A. (1975) Biochemistry 14, 3730-3735.
39. Bertoli, E., Chapman, D., Griffiths, D.E., and Sach, S.J. (1974) Br. Biochem. Soc. Trans. 2, 964-967.

[Received August 17, 1981]

Interception of Discrete Oxygen Species in Aqueous Media by Cholesterol: Formation of Cholesterol Epoxides and Secosterols

JERZY GUMULKA[a,1], JAN ST. PYREK[b], and LELAND L. SMITH[a,*],
[a]Division of Biochemistry, University of Texas Medical Branch, Galveston, TX 77550 and [b]Department of Gasteroenterology, University of Texas Health Science Center, Houston, TX 77030

ABSTRACT

The oxidation products of cholesterol oxidized by different dioxygen species, hydroxyl radical, or triatomic species ozone variously implicated in biological oxidations are compared. Some products are unique to the oxygen species involved, whereas others, such as the isomeric cholesterol 5,6-epoxides, are formed broadly by many oxygen species. The resolution of the isomeric cholesterol 5,6-epoxides by capillary column gas chromatography on SE-30 or SE-54 has been achieved.
Lipids 17:197-203, 1982.

INTRODUCTION

Several discrete oxygen species are implicated in specific enzyme oxidations, generalized enzymic lipid peroxidations, endogenous metabolic processes such as phagocytosis, and in nonenzymic peroxidations and autoxidations as well as in the expression of environmental oxygen toxicity. However, demonstration of the unique participation of individual oxygen species is confounded by experimental limitations and by systems dynamics where more than one oxygen species is present.

It has been our interest to devise means of interception of active oxygen species in chemical and biological systems so that from the oxidation products formed one can infer the nature of the oxygen species involved in the oxidations. Of critical importance to applications of these approaches to living systems is full knowledge of the chemistry occurring in aqueous media as well as in conventional organic solvents. Such interceptions of oxidizing species have been probed using the ubiquitous cellular membrane component cholesterol in studies of the monooxygen species hydroxyl radical HO· (1) and the following dioxygen species: ground-state dioxygen $^3O_2$ involved in specific enzymic hydroxylations and in free radical peroxidations (2-5) and autoxidations (6-9), electronically excited (singlet) dioxygen $^1O_2$ implicated in photosensitized oxygenations (10-17) and other cases (17-19), the 1-electron reduced species superoxide radical $O_2^{-\cdot}$ generated in some enzymic oxidations (20), the 2-electron reduced species peroxide $O_2^{=}$ (18,19) and organic hydroperoxides (21), and the dioxygen cation O_2^+ which is not a likely biological species (22).

We have now extended this approach to the triatomic species ozone O_3. Ozonization of cholesterol and its 3β-acetate in nonaqueous media yields poorly characterized ozonides (23-26); in aqueous media, little chemical work has been conducted (27-29). The present report outlines our recent work on cholesterol ozonization and provides a comparison of all results testing the use of cholesterol for the interception of defined oxygen species.

EXPERIMENTAL

Chromatography

Thin layer chromatography (TLC) was conducted on Kieselgel 60, F-254 chromatoplates (E. Merck GmbH, Darmstadt) and on 10-cm-long Alugram Sil G/UV_{254} aluminum-backed chromatostrips (Machery Nagel, Düren) irrigated with benzene/ethyl acetate mixtures. Sterols were detected by ultraviolet light absorption (254 nm), by N,N-dimethyl-*p*-phenylenediamine spray for peroxides (30), and by 50% sulfuric acid spray followed by heating to full color display and/or to charring. High performance liquid chromatography (HPLC) was conducted on 2 3.9 mm x 30 cm μPorasil microparticulate (10 μm diam) adsorption columns in tandem (Waters Associates, Milford, MA) irrigated with hexane/isopropyl alcohol (24:1, v/v) flowing at 2.0 ml/min. Effluent was monitored at 212 nm with a Perkin-Elmer Corp. Model LC-55 variable wavelength spectrophotometric detector and by differential refractive index using a Waters Associates' Model R4-1 detector (31).

Gas chromatography was conducted with a Hewlett-Packard Model 5880A chromatograph equipped with hydrogen flame ionization detector (nitrogen carrier gas) or with a Finnigan Corp. Model 3300 gas chromatograph-mass

[1]On leave from the Department of Chemistry, University of Warsaw, Warsaw, Poland.

spectrometer (GC-MS) (helium carrier gas), using fused silica capillary columns, 0.2-mm id, 5-35-m-long wall coated with SE-30 (Applied Science, State College, PA) or SE-54 (Hewlett-Packard, Palo Alto, CA). Splitless injections of 10-500 ng sterols in 1 μl toluene via a Grob injector were made into the capillary columns. Injector and detector temperature was 285 C; oven temperature was programmed from 100 C held for 1 min to 270 C at 20 C/min. When using the quadrupole mass spectrometer for detection, the capillary column was introduced directly into the ionization source, mass spectra being scanned at 40 scans/min over the range 100-500 amu, using 22 eV ionization.

Quantitation of cholesterol 5,6-epoxides was achieved using cholesterol as an internal standard and integration of peak areas on ion chromatograms constructed with ion m/z 386 for cholesterol and m/z 402 for the cholesterol 5,6-epoxides. A plot of (m/z 402)/(m/z 386) ion abundances vs (cholesterol 5,6-epoxide/cholesterol) weight ratio was linear over the range 10-500 ng for both cholesterol 5α,6α-epoxide (*3*) and cholesterol 5β,6β-epoxide (*4*). Useful mass spectra of both 5,6-epoxides were also obtained over the 50-100 ng range.

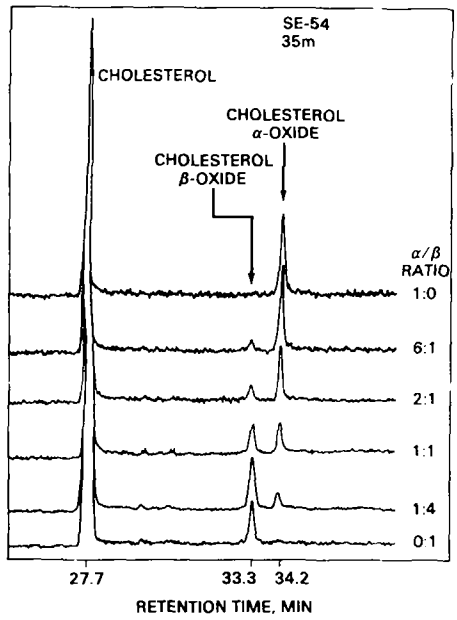


FIG. 1. Capillary column gas chromatography on SE-54 (35 m) of cholesterol and the isomeric cholesterol 5,6-epoxides *3* and *4* in various *3/4* ratios.

Ozonization

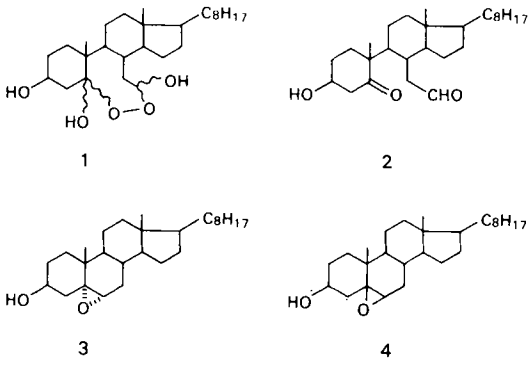
Dispersions of pure cholesterol in water were made by dissolving 100 mg cholesterol in 50 ml acetone, adding the solution under vacuum to 120 ml distilled water, and evaporating the dispersion under vacuum to remove solvent and provide a 1 mg/ml concentration. The dispersion was filtered through sintered glass and used as such. Ozone generated using a Tesla coil leak detector acting on a stream of oxygen flowing at 1 ℓ/min was passed through the cholesterol dispersions at room temperature for 2 hr or until cholesterol was totally destroyed, as evinced by TLC. Sterols were recovered by extraction with equal volumes of benzene and the dried extracts were evaporated under vacuum to yield crude ozonization products. Individual products were isolated by HPLC.

RESULTS

In distinction to cholesterol ozonization in organic solvents where only poorly characterized products are described (23-26), ozonization of cholesterol in water yielded 4 isolable products: a major peroxidic product presumed to be an ozonide hydrate 5ξ,6ξ-epidioxy-5,6-secocholestane-3β,5ξ,6ξ-triol (*1*) and non-peroxidic products 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (*2*) and the isomeric cholesterol 5,6-epoxides 5,6α-epoxy-5α-cholestan-3β-ol (*3*) and 5,6β-epoxy-5β-cholestan-3β-ol (*4*) (Fig. 1). Details of identification of these sterols will be reported elsewhere.

The ozonide hydrate *1* appears to be a product of addition of the elements of water to a putative ozonide initially formed and the secoaldehyde *2* appears to be a transformation product of *1* or of an initially formed ozonide. The mechanism by which the cholesterol 5,6-epoxides are formed is uncertain. It is possible that cholesterol is epoxidized by O_3 or other unrecognized oxidant formed early in the reaction. We have been unable to demonstrate the epoxidation of cholesterol by the ozonide hydrate *1* in the manner cholesterol is epoxidized by sterol hydroperoxides (21).

The 4 products *1-4* can be resolved by TLC and HPLC. We report here for the first time the resolution of the underivatized cholesterol 5,6-epoxides *3* and *4* by GC with capillary columns (cf. Table 1). The ozonide hydrate *1* is characterized by a positive N,N-dimethyl-*p*-phenylenediamine color test, and both 5,6-secosterols *1* and *2* give a characteristic brown color with 50% sulfuric acid on thin layer chromatograms which is diagnostic of destruction of the sterol Δ^5-double bond. Secosterols *1* and *2* are thus readily distinguished from



SCHEME 1

major products of cholesterol autoxidation by $^3O_2$, where intense blue colors with 50% sulfuric acid characterize the initially formed sterol hydroperoxides and their reduction products, the epimeric cholest-5-ene-3β,7-diols (32).

Even though the ozonization of cholesterol involved molecular oxygen (containing O_3), at no time were autoxidation products detected. Clearly, O_3 reacted with cholesterol more rapidly than $^3O_2$. Moreover, in these simple water dispersions, no HO· oxidations of cholesterol were apparent.

Analysis of ozonization products by HPLC known to resolve the 5,6-epoxides *3* and *4* and by capillary column GC established a *3/4* ratio of 1:8. Capillary column gas chromatography of *3* and *4* derived by oxidation of cholesterol in chloroform with *m*-chloroperbenzoic acid and of *3* 3β-acetate and *4* 3β-acetate from cholesterol 3β-acetate similarly oxidized gave other ratios (data incorporated into Table 3).

In the case of the peracid epoxidations, the analysis of products was conducted using a 5-m-long capillary column of SE-54 under the same conditions used with the longer 35-m columns. Using the 5-m column, the retention times were obtained: *3*, 12.28 min; *4*, 12.11 min; *3* 3β-acetate, 13.15 min; *4* 3β-acetate, 12.69 min.

Resolution of the 3β-acetates and 3β-trimethylsilyl ethers of the isomeric 5,6-epoxides *3* and *4* is also readily achieved using capillary columns. Retention data are included in Table 1. Chromatographic resolution of 5,6-epoxides 3β-acetates (33), 3β-benzoates (31,34), and 3β-trimethylsilyl ethers (33) has been previously achieved, and resolution of free sterols *3* and *4* without derivatization by HPLC has been described (31,35). Although small differences in gas chromatographic retention times for *3* and *4* have been noted using packed columns (21,33,36), resolution by that means has not been forthcoming.

Epoxidation of cholesterol by O_3 has not been recognized as occurring, but the 5β,6β-epoxide *4* 3β-acetate was formed in the ozonization of cholesterol 3β-acetate (26). The 5α,6α-epoxide *3* which is interesting as a toxic agent (37,38) has not been recognized as a product of ozonization under any conditions.

DISCUSSION

The successful application of cholesterol or other substrates for interception of active oxygen species depends on the capacity of the interceptor to react with the oxygen species uniquely and at a rate sufficient to compete with other reactions the oxygen species may undergo. The uniqueness of reaction products from cholesterol may now be fairly tested; data in Table 2 summarize the present knowledge.

It is seen that unique products form in several instances. Thus, cholesterol 7-hydroperoxides are uniquely the result of free radical autoxidation or lipid peroxidations. Likewise, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide is found only in systems in which $^1O_2$ is implicated. Furthermore, 5,6-secosterols are unique to O_3 oxidations. The other oxidation products in Table 2 permit no unique inference to be drawn as to the oxidizing species involved.

Table 2 does not include specific enzymic oxidations of cholesterol, but enzymic metabolites may be encountered in systems containing active enzymes. Thus, cholest-5-ene-3β,7α-diol is a hepatic metabolite of cholesterol as well as product of $^3O_2$, O_2^+ and HO· nonenzymic oxidations, and the 5α,6α-epoxide 3 also has enzymic (39,40) and multiple nonenzymic origins.

With exceptions of data in Table 2 for O_2^+ where products listed are for gas-phase oxidations (22) and for $^3O_2$ attack in the sterol side chain and at the 3β-hydroxyl group where products are those of solid-state reactions (7,8), the products listed are those formed in aqueous media. There is no reaction of the defined oxygen species O_2^- with cholesterol, whether it be generated in chemical, photochemical, electrochemical, or enzyme systems, in aqueous or anhydrous organic solvent media (20).

For the highly reactive HO· and O_3 species, the speed of reaction with cholesterol in water insures product formation, and although $^3O_2$ reactions are sluggish at ambient temperature, products do ultimately form, as $^3O_2$ is a stable

TABLE 1

Chromatographic Properties of Cholesterol Ozonization Products

Sterol	Thin layer chromatography[a] (R_f)			Liquid column chromatography[b] (t_R[min])	Gas chromatography[c] (t_R[min])	
	1	2	3		1	2
Cholesterol	0.70	0.77	0.69	6.0 (3.0)	–	22.7
Cholesterol 3β-acetate	–	–	–	–	–	33.5
Cholesterol 3β-trimethylsilyl ether	–	–	–	–	–	29.5
Cholesterol 5α,6α-epoxide (*3*)	0.40	0.44	0.33	13.0 (6.4)	27.1	34.2
Cholesterol 5α,6α-epoxide 3β-acetate	–	–	–	–	29.4	44.1
Cholesterol 5α,6α-epoxide 3β-trimethylsilyl ether	–	–	–	–	–	36.1
Cholesterol 5β,6β-epoxide (*4*)	0.41	0.45	0.35	14.0 (7.3)	26.6	33.3
Cholesterol 5β,6β-epoxide 3β-acetate	–	–	–	–	28.0	39.8
Cholesterol 5β,6β-epoxide 3β-trimethyl silyl ether	–	–	–	–	–	35.1
Cholesterol ozonide hydrate (*1*)	0.20	0.26	0.14	28.0 (14.2)	dec.	–
3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (*2*)	0.43	0.49	0.37	17.2 (8.2)	24.0	–
3β-Hydroxy-5-oxo-5,6-secocholestan-6-oic acid	0.14	0.19	0.10	–	–	–
5β-5,6-Secocholestane-3β,5α,6-triol	0.00	0.02	0.00	–	–	–

[a]System 1, Kieselgel 60, F-254, benzene/ethyl acetate (3:2, v/v); system 2, Alugram Sil G/UV_{254}, benzene/ethyl acetate (3:2, v/v); system 3, Alugram Sil G/UV_{254}, benzene/ethyl acetate (7:3, v/v).

[b]=μPorasil, hexane/isopropyl alcohol (24:1, v/v), 2.0 ml/min. Data in parentheses are for only one column.

[c]System 1, 25-m SE-30 capillary column; system 2, 35-m SE-54 capillary column.

TABLE 2

Cholesterol Oxidation Products Formed by Defined Oxygen Species in Water

Site of attack	Oxidation products	Oxygen species						
		$^3O_2$	$^1O_2$	$O_2^{\cdot-}$	$O_2^{=}$	O_2^{+}	HO•	O_3
C-7	7-Hydroperoxides	+						
C-7	7-Alcohols[a]	+				+[b]	+	
	7-Ketones[a]	+				+[b]	+	
Side chain[c]	Hydroperoxides	+						
	Alcohols, ketones, etc.	+						
	C_{19}-C_{26} sterols[a]	+						
Δ^5	5,6-Epoxides	+			+	+[b]	+	+
	5,6-Secosterols							+
7α-H/Δ^5	Δ^6-5α-Hydroperoxide		+					
3β-Alcohol[c]	3-Ketones	+						

[a]Secondary products formed from initial hydroperoxides.

[b]Gas-phase reaction products.

[c]Solid-state reaction products.

species not altered by water. For $O_2^{=}$ and $^1O_2$ interceptions in aqueous media, the stability of the species must be considered. Whether $O_2^{=}$ is intercepted depends on whether disproportionation dissipates the species before the slower epoxidations occur. In model systems, both disproportionations and epoxidations are observed (18,19). Rapid quenching by water may preclude $^1O_2$ interception by cholesterol, as the reaction is slow. Nonetheless, where an adequate $^1O_2$ flux is maintained, the unique $^1O_2$ oxidation product 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide is formed in aqueous media (10-15).

As the 5,6-epoxides *3* and *4* are formed from cholesterol by attack of several oxygen species (cf. Table 2), their mere presence in a test sample does not imply the oxidizing species involved. The possibility remains that the proportions of *3* and *4* can provide clue to the processes implicated, and data of Table 3 appear to support such a contention. Thus,

TABLE 3

5,6-Epoxides Ratios Obtained in Cholesterol Epoxidations

Epoxidizing conditions	α/β	Ref.
Air (aq dispersions)	1:11	21
Sterol hydroperoxides (aq)	1:10	21
Ozone (aq)	1:8	—[a]
H_2O_2 (aq)	1:8	18,19
Dried egg	ca. 1:5	44
Incubations, liver enzymes	1:3.3 to 1:4.7	5,33,45-47
H_2O_2, Fe (III) acetylacetonate (*aq acetonitrile*)	1:4	48
Incubations, soybean lipoxygenase	1:3.7 to 1:4	5,33
Air, solid [4-^{14}C]cholesterol	1:3.6	33
Rabbit plasma, liver	1:2.1 to 1:3.4	49
HO•, X-radiolysis (methanol)	ca. 1:2	50
Air-aged USP cholesterol	ca. 1:1	8
MoO_5 (dichloroethane)	1.5:1	51
HO• (aq)	3.5:1	1
Organic peracids	6.8:1	—[a,b]
Methylpyridazine oxide photolysis	8:1	52
NO_2-treated rat lung, in vivo	8:1	53
Human serum	1:0	54
Incubations, bovine adrenal cortex mitochondria	1:0	39,40

[a]Present results.

[b]Conditions: *m*-chloroperbenzoic acid in chloroform; product analysis on 5-m SE-54 capillary column. Cholesterol 3β-acetate gave a 7:3 ratio, the same as previously obtained with perbenzoic acid (55).

β-face attack and a predominance of 5β,6β-epoxide *4* occur in systems involving di- or tri-oxygen species in autoxidations, lipid peroxidations and ozonizations, whereas α-face attack and predominance of 5α,6α-epoxide *3* be the case where one oxygen atom oxidants such as N-oxide photolysis, epoxidases, and peracids are involved. However, the *3/4* ratios may be misleading if preferential loss of the less stable 5β,6β-epoxide *4* occurs. The 1:1 ratio observed in naturally air-aged cholesterol appears to be such a case. The predominance of the 5β,6β-epoxide *4* would have been expected a priori.

In biological systems where only the 5α,6α-epoxide *3* is formed, the intervention of specific cholesterol 5α,6α-epoxidases is indicated. In other instances not listed, where formation of the 5α,6α-epoxide *3* is suggested (41-43), available data are inconclusive, as any combination of the 2 isomers might be present.

ACKNOWLEDGMENTS

The financial support of the Robert A. Welch Foundation, Houston, TX, is gratefully acknowledged.

REFERENCES

1. Ansari, G.A.S., and Smith, L.L. (1979) Photochem. Photobiol. 30, 147-150.
2. Teng, J.I., and Smith, L.L. (1973) J. Am. Chem. Soc. 95, 4060-4061.
3. Smith, L.L., and Teng, J.I. (1974) J. Am. Chem. Soc. 96, 2640-2641.
4. Teng, J.I., and Smith, L.L. (1976) Bioorganic Chem. 5, 99-119.
5. Aringer, L. (1980) Lipids 15, 563-571.
6. Smith, L.L., Teng, J.I., Kulig, M.J., and Hill, F.L. (1973) J. Org. Chem. 38, 1763-1765.
7. Smith, L.L. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 119-132, Plenum Press, New York City, NY.
8. Smith, L.L. (1981) Cholesterol Autoxidation, pp. 148-151, 181-199, Plenum Press, New York City, NY.
9. Kimura, M., Jin, Y., and Sawaya, T. (1979) Chem. Pharm. Bull. 27, 710-714.
10. Lamola, A.A., Yamane, T., and Trozzolo, A.M. (1973) Science 179, 1131-1133.
11. Lightner, D.A., and Norris, R.D. (1974) N. Engl. J. Med. 290, 1260.
12. De Geoij, A.F.P.M., and Van Steveninck, J. (1976) Clin. Chim. Acta 68, 115-122.
13. Suwa, K., Kimura, T., and Schaap, A.P. (1977) Biochem. Biophys. Res. Commun. 75, 785-792.
14. Suwa, K., Kimura, T., and Schaap, A.P. (1978) Photochem. Photobiol. 28, 469-473.
15. Foote, C.S., Shook, F.C., and Abakerli, R.A. (1980) J. Am. Chem. Soc. 102, 2503-2504.
16. Kulig, M.J., and Smith, L.L. (1973) J. Org. Chem. 38, 3639-3642.
17. Smith, L.L., and Stroud, J.P. (1978) Photochem. Photobiol. 28, 479-485.
18. Smith, L.L., and Kulig, M.J. (1976) J. Am. Chem. Soc. 98, 1027-1029.
19. Smith, L.L., Kulig, M.J., Miiller, D., and Ansari, G.A.S. (1978) J. Am. Chem. Soc. 100, 6206-6211.
20. Smith, L.L., Kulig, M.J., and Teng, J.I. (1977) Chem. Phys. Lipids 20, 211-215.
21. Smith, L.L., and Kulig, M.J. (1975) Cancer Biochem. Biophys. 1, 79-85.
22. Sanche, L., and van Lier, J.E. (1976) Chem. Phys. Lipids 16, 225-238.
23. Berenstein, M., Georg, A., and Briner, E. (1944) Helv. Chim. Acta 29, 258-271.
24. Cornforth, J.W., Hunter, G.D., and Popják, G. (1953) Biochem. J. 54, 590-597.
25. Lettré, H., and Jahn, A. (1957) Liebigs Ann. 608, 43-53.
26. Tanabe, K., Hayashi, R., and Takasaki, R. (1961) Chem. Pharm. Bull. 9, 1-6.
27. Spanggord, R.J., and McClurg, V.J. (1978) in Ozone/Chlorine Dioxide Oxidation Products of Organic Materials (Rice, R.G., and Cotruvo, A., eds.) pp. 115-125, Ozone Press International, Cleveland, OH.
28. Freeman, B.A., Miller, B.E., and Mudd, J.B. (1979) in Assessing Toxic Effects of Environmental Mutagens (Lee, S.D., and Mudd, J.B., eds.) pp. 151-171, Ann Arbor Science, Ann Arbor, MI.
29. Freeman, B.A., Sharman, M.C., and Mudd, J.B. (1979) Arch. Biochem. Biophys. 197, 264-272.
30. Smith, L.L., and Hill, F.L. (1972) J. Chromatogr. 66, 101-109.
31. Ansari, G.A.S., and Smith, L.L. (1979) J. Chromatogr. 175, 307-315.
32. Smith, L.L., Teng, J.I., Lin, Y.Y., Seitz, P.K., and McGehee, M.F. (1981) in Lipid Peroxide in Biology and Medicine (Yagi, K., ed.) Academic Press, New York City, NY, in press.
33. Aringer, L., and Eneroth, P. (1974) J. Lipid Res. 15, 389-398.
34. Okamura, T. (1977) Bunseki Kagaku 26, 396-401.
35. Tsai, L.S., Ijichi, K., Hudson, C.A., and Meehan, J.J. (1980) Lipids 15, 124-128.
36. Chicoye, E., Powrie, W.D., and Fennema, O. (1968) Lipids 3, 335-339.
37. Kadis, B. (1978) J. Steroid Biochem. 9, 75-81.
38. Black, H.S. (1980) Lipids 15, 705-709.
39. Watabe, T., and Sawahata, T. (1978) Biochem. Biophys. Res. Commun. 83, 1396-1403.
40. Watabe, T., and Sawahata, T. (1979) J. Biol. Chem. 254, 3854-3860.
41. Black, H.S., and Lo, W.-B. (1971) Nature 234, 306-308.
42. Lo, W.-B., and Black, H.S. (1972) J. Invest. Dermatol. 58, 278-283.
43. Kuksis, A., Marai, L., Myher, J.J., and Geher, K. (1976) Lipids 11, 581-586.
44. Tsai, L.S., Ijichi, K., Hudson, C.A., and Meehan, J.J. (1979) Abstracts 39th Ann. Meeting Inst. Food Technol., St. Louis, MO, June 10-13.
45. Watabe, T., Kanai, M., Isobe, M., and Ozawa, N. (1980) Biochem. Biophys. Res. Commun. 92, 977-985.
46. Watabe, T., Kanai, M., Isobe, M., and Ozawa, N. (1980) Biochim. Biophys. Acta 619, 414-419.
47. Watabe, T., Kanai, M., Isobe, M., and Ozawa, N. (1981) J. Biol. Chem. 256, 2900-2907.
48. Tohma, M., Tomita, T., and Kimura, M. (1973) Tetrahderon Lett. 4359-4362.
49. Watabe, T., Isobe, M., and Kanai, M. (1980) J. Pharm. Dyn. 3, 553-556.
50. Coleby, B., Keller, M., and Weiss, J. (1954)

J. Chem. Soc. 66-71.
51. Akhrem, A.A., Timoshchuk, T.A., and Metelitsa, D.I. (1974) Dokl. Akad. Nauk S.S.S.R. 219, 891-894.
52. Tsuchiya, T., Arai, H., and Igeta, H. (1969) Tetrahedron Lett. 2747-2750.
53. Sevanian, A., Mead, J.F., and Stein, R.A. (1979) Lipids 14, 634-643.
54. Gray, M.F., Lawrie, T.D.V., and Brooks, C.J.W. (1971) Lipids 6, 836-843.
55. Tolstikov, G.A., Yur'ev, V.P., Gailyunas, I.A., and Dzhemilev, U.M. (1973) Zh. Obshch. Khim. 44, 215-220.

[Received August 17, 1981]

The Quantitative Analysis of Plant Sterols

B.A. KNIGHTS, *Department of Botany, The University, Glasgow G12 8QQ, Scotland, U.K.*

ABSTRACT

Two methods for the quantitative analysis of plant sterols have been described. In the first, cholesterol is used for control of recovery and gas liquid chromatography (GLC) analysis. When cholesterol is present in the sterol mixture, a radioactive standard (usually cholesterol or sitosterol) is used to control recovery, and coprosterol is used to monitor GLC. The methods are exemplified for nitrogen-fixing root nodules and for chloroplasts, respectively.
Lipids 17:204-208, 1982.

There has been considerable discussion as to the role of sterols in plants (1,2). Three main roles have been postulated: (a) as intermediates, where they are converted into the many other steroidal compounds which are found to occur in plants; (b) as hormones and (c) as membrane components.

The role of sterols as intermediates in the biosynthesis of other compounds can be accepted, there being ample proof for this for a number of different steroids present in plants (3,4). It is hard to divorce any role for sterols as hormones from the likelihood, or the actuality, of their first role (as intermediates). The role of sterols as components of membranes appears to be well established (2).

In plants, the story is rendered complicated by virture of the diversity of structures which is often observed in the sterol mixtures isolated. Selective metabolism is possible and apparently takes place in many cases (5). Thus, different sterols are likely to be relatively different in their performance in the 3 roles which have been postulated.

There have been a number of reports of the quantitative analysis of sterols in plants and fungi and the following examples serve to illustrate the potential for examining the physiological role of such compounds. The analysis of ergosterol in yeast cells indicated a concentration of 6.1×10^{-15} g of sterol/cell, which is equivalent to $\sim 10^7$ molecules/cell (6). It was found that sterols were restricted to the outer of the 2 membranes of mitochondria isolated from *Neurospora crassa* (7) and that the following molar ratios applied: neurosporaxanthin/ergosterol/phospholipid = 1:112:317 (i.e., a molar ratio of $\sim$1:3 for ergosterol/phospholipid). When potato tuber tissue was infected with various races of the late blight organism *Phytophthora infestans*, the relative amounts of sterol, of steroidal glycoalkaloid and of the sesquiterpene rishitin were altered dramatically and in a way which correlated with the type of compatibility reaction between the host and the pathogen (8). In none of these cases was there any report of the use of an internal standard to correct for losses during isolation or during estimation, and no replicate analyses were reported so that there is no indication of the degree of either accuracy or precision. Thus, there exists a need for a method for the reliable, accurate quantitative analysis of sterols in plants, which should then allow a better assessment of the role of a particular sterol fraction to be made based on, e.g., molar ratios, and the deviations from a normal or mean position which may be revealed.

There are a number of reports of the quantitative analysis of steroids, including sterols, which involve the addition of a suitable internal standard to the crude extract. These include radioisotopically labeled substances based on one of the known components of the mixture to be isolated (9,10), a substance of similar structure but known to be absent (or nearly so) from the mixture (11,12) or a substance labeled at 100% isotopic dilution with, e.g., several deuterium atoms. Aspects of these various methods have been reviewed (13,14) and the theory of internal standards for GLC has been discussed (15).

In this paper, I report the use of 2 methods for the analysis of plant sterol mixtures using gas liquid chromatography (GLC). One method is applicable when cholesterol is absent from the extract and the other, which involves a radioactively labeled standard, is of more general application.

MATERIALS AND METHODS

If used unfractionated, plant material was freeze-dried and then ground to a powder before extraction in a Soxhlet apparatus. Chloroplasts were isolated by fractionation of frozen, monofoliate leaves of *Phaseolus vulgaris* (dwarf French bean) using a method described previously (16). Extraction solvents used were chloroform/methanol (2:1, v/v) for most plant

material, or petroleum (bp 40-60 C) followed by acetone for chloroplasts. Thin layer chromatography (TLC) was done on Silica Gel G at 0.5 mm thickness, with chloroform as developing solvent, stigmasterol as zone marker and dichlorofluorescein as locating reagent. Material was recovered by scraping zones into chromatography columns and eluting sterols with diethyl ether. Radioactivity was determined using liquid scintillation counting with AES ratios for quench correction, in a Packard Liquid Scintillation Spectrometer. GLC was undertaken using OV-17 as stationary phase. Two columns were used, a 50-m SCOT glass capillary (Scientific Glass Engineers Pty., Australia) and a 3 m x 3 mm glass column packed with Gas Chrom Q which was coated 2% w/w with stationary phase. Both columns were operated at 285 C but helium, rather than nitrogen, was used as carrier for the SCOT column. The SCOT column was fitted with a packed, heated precolumn (2% OV-17) and was used in a splitless mode. Quantitation was by both triangulation and the use of an Autolab Integrator (Spectra Physics) with coprosterol (5β-cholestan-3β-ol) as internal standard. Trimethylsilyl ethers were prepared using bis-trimethylsilylacetamide, which also served as the injection solvent for GLC. Chloroplasts were suspended in standard volumes of 1% NaCl and numbers assessed using a hemocytometer. After sedimentation, they were then freeze-dried and weighed before extraction. Extraction of chloroplasts was performed in a Soxhlet extractor but was otherwise as described previously (16). Procedures for quantitative analysis were as indicated in Schemes I and II. Because of the large amount of chlorophyll in the acetone extracts, these fractions were subjected to saponification and sterols isolated from the nonsaponifiable material.

RESULTS

Table 1 contains the results for the sterols of root material obtained using Scheme I. Table 2 gives details of the data for chloroplasts isolated from each of the 2 experiments representing separate harvests of dwarf French bean and are the means of at least 2 determinations dependent on separate isolations by thin layer chromatography (TLC) of sterols and using Scheme II. In each case, the replicate sterol isolations were subjected to successive analysis when the SCOT column was used. Table 3 gives an indication of the variation in results obtained, whereas Table 4 summarizes the corrected data for the individual sterols present in the second experiment, i.e., after making allowance for the term F in the calculation.

SCHEME I. Analysis of sterols in mixtures not containing measurable amounts of cholesterol. 1: Freeze-dry and powder plant material; 2: extract aliquots (usually with 2:1, v/v, $CHCl_3$/MeOH); 3: add cholesterol (use 1.0 mg/g dry wt of tissue extracted); 4: concentrate extract and isolate sterol fraction from aliquots of each extract; 5: derivatize and GLC, perform replicate analyses; 6: compare areas with standard curves where sterols are available for comparison. Ratio of area of sterol to that for cholesterol X any correction factor F (see Scheme II) = wt of sterol (mg/g dry wt).

SCHEME II. Procedure used when cholesterol is present in measurable amounts. 1,2: As for Scheme I; 3: add 4-[^{14}C]sitosterol (or cholesterol) in known amount, e.g., 0.1 μCi (3,333 Bq.); 4: as for Scheme I; 5: add GLC standard (e.g., coprosterol) in the range 0.1-1.0 mg/g dry wt of plant material extracted and bring to an exact volume; 6: remove aliquots for radioactivity determinations (2 X 10%); 7: as for 5 Scheme I; 8: as for 6 Scheme I. *Calculation for Scheme II:* amount of sterol/unit wt of tissue is given by $A_2 \times F \times C_1/A_1 \times W \times C_2$ mg when A_1 corresponds to 1.0 mg of GLC standard. A_1 = Peak area for coprosterol (or other standard); A_2 = peak areas for sterols (total or individual); C_1 = cpm (or dpm) for sitosterol added to the total extract; C_2 = cpm (or dpm) in sterol sample isolated; F = the correction factor if, for unit amounts of each $A_1 \neq A_2 = A_1$/unit wt of coprosterol/A_2/unit wt of sterol (step 8). w = The weight of plant material extracted.

DISCUSSION

The range of quantitative data for sterols in plants and fungi which is cited in the literature (1) suggests a reasonable level of reliability for such data. However, in cases where the differences are relatively small but still seem to be both consistent and significant, e.g., the sterols of roots and of nitrogen-fixing root nodules of *Alnus glutinosa* (12), a procedure which is convenient, accurate and precise is necessary before justifiable deductions based on observed differences can be made. In the case of *A. glutinosa*, the method of Scheme I was used.

When the sterol content of the plant material is known and a suitable standard can be found (e.g., when cholesterol is absent), Scheme I represents a simple and convenient method for quantitative analysis of sterols. Sterol standard is added at a predetermined rate, possibly corresponding to 1.0 mg/g dry wt of tissue and this serves as both control for the isolation steps and for the GLC analysis. Providing there is no resolution of individual compounds during isolation and complete separation during quantitations, the criteria for an internal standard are fulfilled. These

TABLE 1

Sterols of Roots and Nitrogen-Fixing Root Nodules (mg/g dry wt)

Sterol	Russell lupin root	Nodules* a	Nodules* b	*Vicia sativa* Root	*Vicia sativa* Nodules
Campesterol	0.13	0.36	0.36	0.088	?
Stigmasterol	0.14	0.14	0.14	0.11	?
Sitosterol	0.29	1.57	1.65	0.35	0.060

*a and b represent data from extractions of replicate samplings of plant material.

TABLE 2

Sterols of Chloroplasts Isolated from *Phaseolus vulgaris*

		1st Experiment	2nd Experiment
No. of chloroplasts extracted		504×10^9	193.2×10^9
Mass (dry) of chloroplasts		2.82 g	1.32 g
Average mass/chloroplast		5.59×10^{-12} g	6.83×10^{-12} g
Desmethyl sterols extracted with petroleum		6.92 mg	3.41 mg
Desmethyl sterols extracted with acetone		152.5 μg	66.3 μg
Desmethyl sterols/chloroplast	(P)	13.7×10^{-15} g	17.6×10^{-15} g
	(A)	0.3×10^{-15} g	0.5×10^{-15} g
No. of sterol molecules/chloroplast	(P)	$\sim 20.0 \times 10^6$	$\sim 27.0 \times 10^6$
	(A)	$\sim 0.45 \times 10^6$	$\sim 0.5 \times 10^6$
Ratio petroleum extract/acetone extract		~44	~54

Results uncorrected for GLC errors.

TABLE 3

Results from Replicate Analyses of Chloroplast Extracts

		TLC replicate	Recovery (%)	GLC* replicate	Sterol yield (mg)
1st Experiment 2.82 g = 504×10^9 chloroplasts	P	1	39	1	6.92
				3	6.3
				4	6.27
		2	17	2	6.91
				3	6.21
				4	6.81
		3	5.7	3	4.87
				4	5.45
	A	1	8.65	1	0.12
		2	24.1	2	0.19
2nd Experiment 1.32 g = 193.2×10^9 chloroplasts	P	1	22.6	5	4.2
				6	3.27
		2	29.5	6	3.6
	A	1	64.3	5	0.050
				6	0.054
		2	8.82	5	0.082
				6	0.079

P = Petroleum extract; A = acetone extract.
*Identical numbers indicate analyses done on the same day.

TABLE 4

Corrected Data for Individual Sterols of *Phaseolus* Chloroplasts

Sterol*	No. of molecules ($\times 10^{-6}$) of each sterol/chloroplast, GLC data corrected		
	Petroleum extract	Acetone extract	Ratio P/A
Cholesterol	0.48	0.226	2.1
24-Methylcholesterol	2.33	0.009	257.7
Stigmasterol*	13.64	0.063	216.5
Sitosterol*	16.73	0.226	74.0
Δ^5-Avenasterol	4.82	0.054	89.2
Total	∿38.0	∿0.58	∿ 65.5

*N.B. The stereochemistry at C-24 has not been investigated in this work and has been presumed to be 'α.'

criteria are the separation characteristics mentioned, plus the closest possible approximation chemically and physically to the substances being analyzed, so that losses and GLC performance are all closely matched.

The use of internal standards for the analysis by GLC of any group of compounds has been well documented (13,14) and several including octacosane, dotriacontane (11), 5α-cholestane, coprosterol and cholesterol have been used for the analysis of sterols. Of these, only cholesterol will meet all of the criteria listed, although it was probably not completely suitable for the analyses of sterols in *N. crassa* (11). Being more stable than ergosterol, its use may be expected to give rise to an underestimate of the ergosterol content. However, many plant sterol fractions contain substantial amounts of cholesterol and are otherwise too complex to allow for any alternative to be used. In these cases, the more complex Scheme II has to be used. The use of radioactively labeled steroid as internal standard during quantitative analyses is a well established procedure (13,14) and has been applied successfully in sterol analyses (10,17).

The principal sources of error in Scheme I are incomplete extractions, inaccurate pipetting of the standard or its lack of purity, plus possible errors due to the GLC. Incomplete extraction usually can only be controlled by reextraction of the plant residue and by replication of the complete procedure, although Sobus and Holmland have made a study of the effectiveness of several methods of extraction (18). Errors arising from the standard should be minimal if routine precautions are adopted, but errors arising in the GLC cannot be controlled by any internal standard after the stage of sample injection. These errors have been reviewed (13,14) but arise mainly as a result of adsorption on the column. In cases when this is observed, there is a decrease in the apparent detector response per unit wt of substance as retention time increases. This phenomenon has been studied generally by Simmonds and Lovelock (19) and was observed for sterols by Bloomfield (20) who found an approximately linear correlation with retention time and used this correlation to calculate correction factors for sterols not available for direct calibration. The same method was used for the SCOT column in the present work. The values for F, the correction factor, were found to vary from day to day and therefore were determined at least once for each batch of analyses performed. While the F values for the packed column were much smaller than those noted for the SCOT column, the resolution and the sensitivity for minor components was less satisfactory with the packed column and the results obtained were consequently also less satisfactory.

The method of Scheme II is clearly more complex, more expensive and slower than that of Scheme I. Also, it is likely to be less easy to make accurate and precise. The first limitation is the radiochemical purity of the chosen standard which will decline with time. In addition, extra steps are added when recovery is being measured, because a GLC standard has to be added (preferably before any sampling for radioactivity counting), the mixture then has to be brought to a standard volume in order to permit precise aliquots (2 × 10%) to be removed and, because color quenching is often observed, unequal quenching effects have to be considered. In spite of these limitations, the results obtained suggest a reasonable level of consistency and reliability. The use of radioactive standards in this way for the quantitative analysis of sterols is well established and, in fact, Scheme II is essentially that of Miettinen and Tarpila (10,17).

With respect to the sterols of chloroplasts,

it was known from earlier work that sterols were present in 2 different forms: one easily extracted by petroleum and the other more tightly bound and requiring acetone extraction. It was also known that the quantitative levels were substantially different (16). For these 2 fractions, the problem of getting the level of standard to correspond to the level of sterol in the extract had to be solved by experience. In the event of one peak going off scale (usually this happens for the standard), analyses can only be considered satisfactory if an integrator is used. In this work, similar results were obtained using triangulation or an integrator, although the integrator usually gave a higher figure for the sterol values. This can probably be attributed to the logic employed in the integrator for determining the baseline for peaks which usually show some tailing.

The numbers of sterol molecules per chloroplast in the fraction *P* compare closely with those for yeast cells which were calculated from the data of Nes et al. (6). However, there is a degree of uncertainty concerning the data for chloroplasts as they are subcellular organelles isolated from disrupted cells and must therefore have some level of contamination with other such organelles (e.g., microsomes and mitochondria). However, the acetone extract *A* probably corresponds to a specialized fraction from within the chloroplasts and with a specialized requirement for cholesterol. The selective metabolism of sterols in this fraction is demonstrated by the data in Table 4, especially by the ratios between the 2 fractions for the individual sterols. Thus, the P/A ratios for sitosterol and Δ^5-avenasterol are similar to those for the total sterol, whereas positive discrimination toward cholesterol is evident in the acetone extract and toward 24-methylcholesterol and stigmasterol in the petroleum extract. It is reasonable to suggest that either a biosynthetic origin exists for cholesterol that is different from other sterols or that a highly selective mechanism operates whereby a specialized structure incorporates cholesterol, but excludes the other compounds during its development.

In the case of the data for roots and their associated nitrogen-fixing nodules, the higher level of sterol observed in the second case is consistent with all the data accumulated previously in corresponding circumstances (12). Although no other consistent pattern has yet emerged, these higher levels are thought to be a reflection of the extensive formation of specialized membranes within such nodules which have to permit the bidirectional flow of metabolites—a feature likely to be observed in many symbiotic associations.

REFERENCES

1. Nes, W.R. (1974) Lipids 9, 596-612.
2. Grunwald, C. (1978) Philos. Trans. R. Soc. London B 284, 541-558.
3. Lockley, W.J.S., Rees, H.H., and Goodwin, T.W. (1976) Phytochemistry 15, 937-939.
4. McMorris, T.C. (1978) Lipids 13, 716-722.
5. Knights, B.A. (1973) Chem. Br. 9, 106-111.
6. Nes, W.R., Adler, J.H., Frasinel, C., Nes, W.D., Young, M., and Joseph, J.M. (1980) Phytochemistry 19, 1439-1443.
7. Hallermayer, G., and Neupert, W. (1974) Z. Physiol. Chem. 355, 279-288.
8. Metliskii, L.V., Ozeretshovskyana, O.L., Vasyukova, N.I., Davydova, M.A., and Segal, G.M. (1976) Chem. Abstr. 85, 59782, and Proc. Acad. Sci. U.S.S.R., Biochem. Sci. 227, 115-119.
9. Collins, W.P., and Sommerville, I.F. (1964) Nature 203, 836-839.
10. Miettinen, T.A., Ahrens, E.H., and Grundy, S.M. (1965) J. Lipid Res. 6, 411-424.
11. Elliott, C.G., Knights, B.A., and Freeland, J.A. (1974) Biochim. Biophys. Acta 360, 339-347.
12. Knights, B.A., Morton, P.J., and Wheeler, C.T. (1981) Phytochemistry 20, 2041-2042.
13. Knights, B.A. (1964) in Steroid Hormone Analysis (Carstensen, H., ed.) Vol. 1, pp. 367-406, Marcel Dekker, New York, NY.
14. Knights, B.A. (1973) in Modern Methods of Steroid Analysis (Heftmann, E., ed.) pp. 103-138, Academic Press, New York, NY.
15. Sanchez, D.W. (1970) Forensic Sci. Soc. J. 10, 23-25.
16. Knights, B.A. (1971) Lipids 6, 215-218.
17. Miettinen, T.A., and Tarpila, S. (1978) Scand. J. Gastroent., 573-576.
18. Sobus, M.T., and Holmlund, C.E. (1976) Lipids 11, 341-348.
19. Simmonds, P.G., and Lovelock, J.E. (1963) Anal. Chem. 35, 1345-1348.
20. Bloomfield, D.K. (1962) Anal. Chem. 34, 737-741.

[Received August 17, 1981]

Response of *Drosophila* to *cis*- and *trans*-22-Dehydrocholesterol: I. A Survey[1]

HENRY W. KIRCHER*, REBECCA L. PHARISS, FUMIKO U. ROSENSTEIN, DAVID BALDWIN and **JAMES C. FOGLEMAN**, *Department of Nutrition and Food Science, University of Arizona, Tucson, AZ 85721*

ABSTRACT

Cis- and *trans*-22-dehydrocholesterol were added to media for 10 species of *Drosophila*. The *cis* isomer prevented normal maturation of 4 species and the *trans* isomer was toxic to 9. These findings were corroborated by tests with 4 representative species on a sterol-deficient medium under axenic conditions. Addition of cholesterol to the latter overcame the toxicity of the *trans* isomer. *Trans*-22-dehydrocholesterol may be acting as a competitive inhibitor in the metabolism of phytosterols to cholesterol or ecdysone by the insects.
Lipids 17:209-214, 1982.

This study was stimulated by observations that *cis*- (*cis*-DHC) but not *trans*-22-dehydrocholesterol (*trans*-DHC) inhibited growth of mouse fibroblast cells in tissue culture; we presumed that this occurred by a change in membrane properties (1). In addition, sporulation of *Phytophtora cactorum* was prevented when *cis*-DHC was the only sterol in its medium (2). These findings suggested that studies of the effect of *cis*- and *trans*-DHC on multicellular organisms would be interesting. We chose *Drosophila* because numerous species in this genus were available to us, methodology for axenic studies had been developed (3,4), and comparisons could be made among species occupying widely different habitats. *Drosophila melanogaster* and *D. hydei* have a cosmopolitan distribution, *D. acanthoptera* is found in southern Mexico, *D. pseudoobscura* in the western U.S., *D. arizonensis* and *D. hamatofila* in the southwestern U.S. and the remainder, *D. mettleri*, *D. mojavensis*, *D. nigrospiracula* and *D. pachea*, are cactophilic species, endemic to the Sonoran Desert (5).

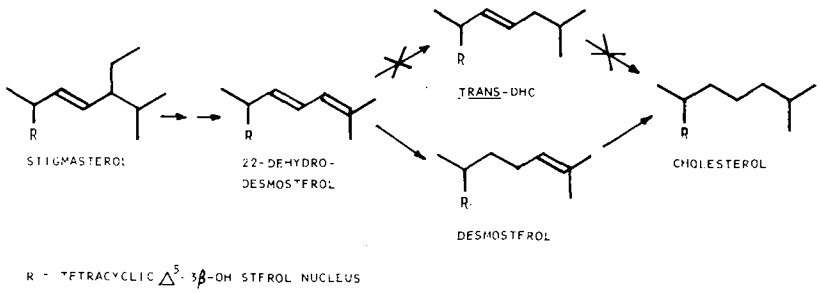
Trans-DHC has been mentioned several times in the insect literature. It was first reported as a metabolite of ergosterol in German cockroaches (6) and then as 4% of the sterols in crickets (7). It could not be used as the sole dietary sterol by the hide beetle (8,9), but sufficed as well as cholestanol as a sparing sterol in the diet of this insect (10). On a medium containing 0.1% *trans*-DHC, 95% of the larvae of the kapra beetle were able to grow to maturity (11). During the dealkylation of stigmasterol to cholesterol by the tobacco hornworm, no *trans*-DHC was detected nor was there any conversion of this sterol to cholesterol by the insect (12) (Scheme I).

In this paper, we report the results obtained when various species of *Drosophila* were reared on standard and sterol-deficient media containing *cis*- and *trans*-DHC.

MATERIALS AND METHODS

Species of *Drosophila* were obtained from the laboratory of W.B. Heed, Ecology Department, University of Arizona. Cholesterol, mp 149-149.5 C, was purified via the dibromide (13); stigmasterol (Upjohn Co.) was crystallized from EtOH, mp 169-170.5 C; lathosterol, mp 127-128 C; and 5,6-dihydroergosterol, mp 180-181 C, were prepared by reduction of

[1]Arizona Agricultural Experiment Station Journal article no. 3469.



SCHEME I. R = Δ^5-3β-OH tetracyclic sterol nucleus.

the corresponding 5,7-dienes over a soluble Rh catalyst (14) and brassicasterol (15) and spinasterol (16) were available from earlier work. All mp values were taken in vacuo and are corrected.

A mixture of *cis*- and *trans*-DHC acetates was prepared by a Wittig synthesis and separated on $AgNO_3$-silica gel columns (17). Impure *cis*-DHC acetate was purified by crystallization from acetone, mp 120-121 C, lit. (17) 116-117 C, and *trans*-DHC acetate by crystallization from 100% EtOH, mp 129.7-130.3 C, lit. (17) 128-129 C. The 2 chromatographically (GLC, $AgNO_3$-TLC) pure acetates were hydrolyzed to *cis*-DHC, mp 140-140.5 C (EtOH), $[\alpha]_D^{25}$ -66° ($C3,CHCl_3$), lit. (18) 137-139 C, -65°, and *trans*-DHC, mp 138.3-139 C (EtOH) $[\alpha]_D^{25}$ -57.1° ($C3,CHCl_3$), lit. (18) 133-135 C, -60°. Structural comparisons of the sterols used in this study are shown in Figure 1.

Early tests were done with a standard medium containing 60 g brewers' yeast, 12 g agar, 60 ml corn syrup, 15 ml malt extract, 1 medium-size banana, 15 ml propionic acid and 1,650 ml water. The dry weight of the ingredients represent 167 g (11%) of the medium. The ingredients (less H_2O and propionic acid) corresponding to 0.8 ℓ of this medium were hydrolyzed in duplicate with alkaline pyrogallol (19) and the sterols (ergosterol, zymosterol and sitosterol) were determined in the nonsaponifiable fraction by triangulation of their respective peaks by GLC (5% OV-101, 250 C). Tests under axenic conditions were done with a sterol-deficient medium (3) from which carboxymethylcellulose was omitted and agar reduced to 8 g/ℓ. Its dry ingredients were also hydrolyzed to determine the sterol content.

Test sterols added to these media were dissolved in ether, made into a slurry with the requisite amounts of yeast and the ether evaporated prior to addition of the yeast to the other ingredients in the media.

Tests with *Drosophila*

In 4 preliminary experiments (Tables 1-3), adults of various species were allowed to oviposit overnight on 30 ml standard medium in small beakers placed in 4-ℓ population jars. The top 2-3 mm of the medium containing the eggs was sliced from the remainder and placed on 200 ml of the test media in 1-ℓ jars. Approximately the same number of eggs were used in each test of a species. In all cases, concurrent controls with no added sterols were run. *D. pachea* required 3-6 days of oviposition to supply enough eggs for tests. Numbers of adult F_1 emerging from each medium were compared. In all cases, sterol concentrations are given as a percentage of the dry ingredients in the standard (11% solids) and sterol-deficient (9% solids) media.

Standard medium (30 ml) containing 0.25% of either *cis*- or *trans*-DHC was poured into 200-ml bottles. Adults of 8 species of *Drosophila* (Table 1) were allowed to oviposit on standard medium as before. The surface of the medium was rinsed with 70% EtOH to inhibit contamination and exactly 50 eggs from each species were transferred to 6 bottles of each medium. Numbers of adults emerging from each bottle were counted and the results expressed as a percentage of the 300 eggs used per test. Bottles that became contaminated with mold were discarded and that test repeated.

Sterol-deficient medium supplemented with various sterols was autoclaved 20 min at 121 C. Eggs collected from *D. pseudoobscura*, *D.*

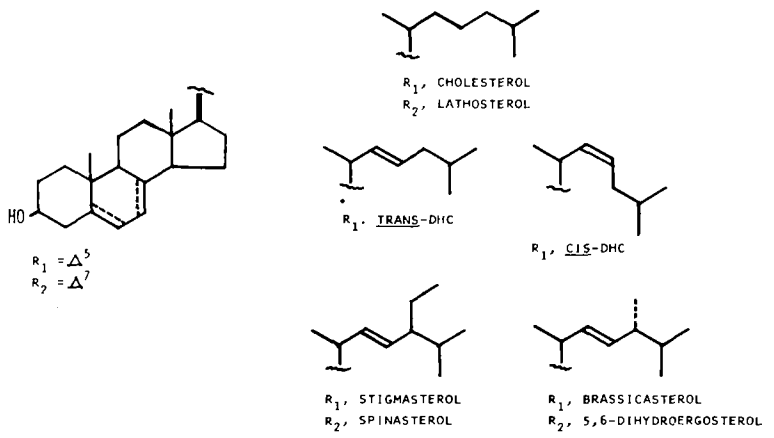


FIG. 1. Structural comparisons of sterols.

TABLE 1

Response of Species of *Drosophila* to 0.25% *cis*- or *trans*-DHC added to Standard Medium

Species	Number of F_1 adults from:			Percentage of 300 eggs which matured to F_1 adults on:		
	Control	*cis*-DHC	*trans*-DHC	Control	*cis*-DHC	*trans*-DHC
D. acanthoptera	916	766	0	–	–	–
D. arizonensis	755	541	2	55	67	0
D. hamatofila	356	30	4	–	–	–
D. hydei	465	269	2	37	60	0
D. melanogaster	1608	1439	54	99	93	0.7
D. mettleri	660	0	0	31	0	0
D. mojavensis	1248	199	0	58	24[a]	0
D. nigrospiracula	470	0	0	11	0	0
D. pseudoobscura	295	270	264	59	71[a]	23[a]
D. pachea	830	32	2	0	0	0

[a]Significantly different from control, $p < 0.01$.

TABLE 2

Response of 2 Species of *Drosophila* to Various Δ^{22}-Sterols Added to Standard Medium

Sterol	% added to medium	Number of F_1 adults of:	
		D. nigrospiracula[a]	*D. mojavensis*[b]
None	–	82	1951
cis- or *trans*-DHC	0.05	122	501
	0.10	67	65
	0.15	58	11
	0.25	0	0
None	–	387	1190
Brassicasterol	0.25	487	1353
Stigmasterol	0.25	456	1469

[a]*D. nigrospiracula* tested with *cis*-DHC.
[b]*D. mojavensis* tested with *trans*-DHC.

mojavensis and *D. mettleri* were sterilized with 2% NaOCl for 20 min, rinsed twice with sterile 0.7% saline and placed on sterile agar plates to hatch. Axenic first instar larvae (100) of each species were aseptically transferred to 30 ml of each sterile medium in 200-ml bottles in 5 replicates (Table 4). Results were expressed as percentages of original larvae that were able to mature to adults on each medium.

Sterilized eggs from the above 3 species and *D. melanogaster* were placed directly on the media in 6 replicates (Table 5). F_1 adults merging from each bottle were axenically transferred to fresh bottles of the same medium from which they had emerged. When F_2 larvae were visible, the F_1 population was removed. Results were expressed by scoring the egg to F_1 and F_2 adult viability for each sterol supplement.

RESULTS AND DISCUSSION

Results of the preliminary experiments are shown in Tables 1-3. Of the 10 species tested, only *D. pseudoobscura* was able to withstand the presence of 0.25% *trans*-DHC in its diet. The standard medium contained ~0.4% ergosterol and zymosterol and ~0.04% sitosterol based on the dry weight of its ingredients. It also benefited from addition of 0.25% *cis*-DHC to its diet (Table 1). The same concentration of *cis*-DHC deleteriously affected 5 species:

TABLE 3

Response of *D. pachea* to 0.25% of Various Δ^{22}-Sterols Added to Standard Medium Supplemented with 0.25% Lathosterol

Sterol added to medium	No. of F_1 adults
None (lathosterol only)	1655
cis-DHC	2858
trans-DHC	844
5,6-Dihydroergosterol	2171
Spinasterol	2063

TABLE 4

Effect of Various Sterols on Maturation of Larvae of 3 Species of *Drosophila*[a,b]

Species	No added sterol	Cholesterol	*cis*-DHC	Cholesterol + *cis*-DHC[c]	*trans*-DHC	Cholesterol + *trans*-DHC[c]
D. pseudoobscura	47	68	56	57	0	52
D. mojavensis	13	46	49	54	0	42
D. mettleri	18	10	7	28	0	42

[a]100 larvae/bottle, 5 bottles/test, all values are ± 2-5 S.E.

[b]Sterol-deficient medium, 0.25% added sterols.

[c]Each sterol added at a concentration of 0.25%.

D. hamatofila, *D. mettleri*, *D. mojavensis*, *D. nigrospiracula* and *D. pachea*. Except for *D. pachea*, which requires a Δ^7 sterol (3), *D. mettleri* and *D. nigrospiracula* were the most sensitive to *cis*- and *trans*-DHC and also showed the least percentage egg-to-adult viability on control. *D. arizonensis*, *D. hydei*, *D. melanogaster* and *D. pseudoobscura* showed the least sensitivity. It is interesting that, of the 10 species, these 4 are the most polyphagous. Transcending the differences between the species, however, is the important observation that the *trans* isomer is the more deleterious to *Drosophila* whereas it was the *cis* that inhibited growth of mammalian cells in culture (1). Alteration of membrane properties are apparently not the controlling factors in all the *cis*,-*trans*-DHC interactions in *Drosophila*.

The concentration of the two 22-dehydrocholesterols required for a significant biological response with 2 species that are sensitive to them is show in the upper part of Table 2. Although the data cannot be treated statistically because the numbers of eggs transferred to each medium are known to be only roughly equal, 0.25% *cis*-DHC and 0.10% *trans*-DHC were required to affect *D. nigrospiracula* and *D. mojavensis*, respectively.

The effects caused by the presence of 2 other *trans*-Δ^{22} sterols in the medium are shown in the lower part of Table 2. They illustrate how a 24-β-methyl (brassicasterol) and a 24-α-ethyl (stigmasterol) group completely nullify the deleterious effect of the *trans*-Δ^{22} double bond. For this unsaturation to be effective as an inhibitor of *Drosophila* growth and maturation, it must be in the cholestane, rather than ergostane or stigmastane, side chain. This is reasonable as the latter 2 sterol types are ubiquitous in the diets of terrestrial phytophagous insects whereas *trans*-Δ^{22} unsaturation in the cholestane series is found principally in marine organisms (1, refs. 1-4).

D. pachea grew well on all sterols except *trans*-DHC (Table 3). The 0.25% lathosterol added to the medium helped overcome the *trans* isomer toxicity to some extent when compared to the results obtained in its absence (Table 1). The 24-alkyl groups in 5,6-dihydroergosterol and spinasterol again rendered the *trans*-Δ^{22} double bond in these 2 sterols innocuous toward development of *D. pachea*.

At this point, we decided to pursue our studies with a sterol-deficient medium under axenic conditions with 4 representative species: *D. pseudoobscura*, able to tolerate both *cis*- and *trans*-DHC; *D. melanogaster*, able to grow as well on 0.25% *cis*-DHC as on control; *D. mojavensis*, able to grow on 0.25% *cis*-DHC but not as well as on the control; and *D. mettleri*, which did not mature on either 22-dehydrocholesterol isomer (Table 1).

The results with axenic larvae of 3 species (Table 4) and with sterilized eggs of all 4 species (Table 5) will be discussed together. In the latter case, sufficient sterol is still present in the sterol-deficient medium (~0.01% of dry wt) to allow some F_2 survival in the absence of added sterols. In both experiments, addition of cholesterol to the medium overcame the toxicity of *trans*-DHC. *Cis*-DHC was unable to do this for the 2 sturdier species (*D. melanogaster* and *D. pseudoobscura*), and, by itself, was inadequate for growth of 2 generations of the other 2. An unanticipated observation was the decreased viability of *D. mettleri* on cholesterol supplemented media compared to control. This species normally feeds and breeds in soil soaked by decaying saguaro cactus, a substrate for which the principal sterol is sitosterol (Fogleman and Kircher, unpublished data). The 2 experiments show that larvae of the 4 species are unable to mature under axenic conditions in a medium containing *trans*-DHC and traces of ergosterol. Most of the larvae died before or during the second instar stage. Survival of *D. pseudoobscura* on standard medium plus *trans*-DHC (Table 1) may be due to its

TABLE 5

Effect of Various Sterol Mixtures on the Maturation of Eggs from 4 Species of *Drosophila* for 2 Generations[a,b]

Species	No added sterol		Cholesterol		*cis*-DHC		Cholesterol +*cis*-DHC[c]		*trans*-DHC	Cholesterol + *trans*-DHC[c]		*cis*-DHC +*trans*-DHC[c]
	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_1	F_2	F_1
D. melanogaster	+	+	+	+	+	+	+	+	0	+	+	0
D. pseudoobscura	+	+	+	+	+	+	+	+	0	+	+	0
D. mojavensis	+	+/0	+	+	+	0	+	+	0	+	+	0
D. mettleri	+	0	+	+/0	W	0	+/0	+/0	0	+	+	0

[a] + = More than 10 adults/bottle, W = 1 to 10 adults/bottle, 0 = no adults observed; +/0 = inconsistent results within a test, some bottles produced adults, others did not.

[b] Sterol-deficient medium, 0.25% added sterols.

[c] Each sterol added at a concentration of 0.25%.

higher sterol content.

Two functions of sterols in insects are their roles as membrane and nerve tissue constituents (20-23) and as precursors to ecdysteroids (24,25). The toxic effect of *trans*-DHC is most likely related to these functions. Its acute toxicity, rather than that of the *cis* isomer as observed earlier (1,2), can be rationalized in a number of ways: (a) the *cis* isomer may be reduced to cholesterol by some of the species of *Drosophila*; (b) *Drosophila* may have a greater capacity to alter membrane components (fatty acids, proteins) than cultures of mouse fibroblast cells and are thus able to tolerate unsuitable sterols (*cis*-DHC) better; (c) the *trans* isomer may be a good membrane sterol but inhibit some metabolic step (e.g., 22-dehydrodesmosterol to desmosterol, Scheme I) in the conversion of phytosterols to cholesterol; (d) the *trans* isomer may inhibit the conversion of cholesterol to ecdysone when concentrations of cholesterol in the insect are very low; (e) when sufficient cholesterol is also present, it may be preferentially absorbed in the gut with relative exclusion of *trans*-DHC or its presence in the diet furnishes enough of a suitable sterol so that the *trans* isomer is not needed to fulfill roles for which it is unsuited, i.e., membrane constitutent or ecdysone precursor.

The last 3 hypotheses are supported by the results (in Tables 4 and 5) where addition of cholesterol to media containing *trans*-DHC overcame the deleterious effect of the latter. Similar results were observed (Table 3) with added lathosterol and *D. pachea*. Furthermore, hypothesis d is supported by the observation that larvae that hatch on *trans*-DHC (Table 5) die as first instars. These hypotheses are currently being tested with sterols other than cholesterol and will be reported in part II. This study of the effect *cis*- and *trans*-DHC on *Drosophila* should provide further insight into the mechanisms involved in sterol assimilation in phytophagous insects.

ACKNOWLEDGMENTS

We thank the National Science Foundation for Grants DEB 74-19149 A03 and DEB-7912924 for support of this work.

NOMENCLATURE

Cholestanol	5α-Cholestan-3β-ol
Cholesterol	Cholest-5-en-3β-ol
Lathosterol	5α-Cholest-7-en-3β-ol
cis-DHC	Cholesta-5,22Z-dien-3β-ol
trans-DHC	Cholesta-5,22E-dien-3β-ol
Desmosterol	Cholesta-5,24-dien-3β-ol
22-Dehydrodesmosterol	Cholesta-5,22E,24-trien-3β-ol
Zymosterol	5α-Cholesta-8,24-dien-3β-ol

NOMENCLATURE, *continued*

Brassicasterol	Ergosta-5,22E-dien-3β-ol
5,6-Dihydroergosterol	5α-Ergosta-7,22E-dien-3β-ol
Ergosterol	Ergosta-5,7,22E-trien-3β-ol
Sitosterol	Stigmast-5-en-3β-ol
Stigmasterol	Stigmasta-5,22E-dien-3β-ol
Spinasterol	5α-Stigmasta-7,22E dien-3β-ol

REFERENCES

1. Kircher, H.W., Rosenstein, F.U., and Kandutsch, A.A. (1981) Lipids 16, 943-945.
2. Elliott, C.G. (1979) J. Gen. Microbiol. 115, 117-126.
3. Goodnight, K.C., and Kircher, H.W. (1971) Lipids 6, 166-169.
4. Kircher, H.W., and Gray, M.A. (1978) J. Insect Physiol. 24, 555-559.
5. Heed, W.B. (1978) in Ecological Genetics: The Interface (Brussard, P.F., ed.) pp. 109-126 and references quoted therein, Springer Verlag, New York, NY.
6. Clark, A.J., and Bloch, K. (1959) J. Biol. Chem. 234, 2589-2594.
7. Martin, M.M., and Carls, G.A. (1968) Lipids 3, 256-259.
8. Clark, A.J., and Bloch, K. (1959) J. Biol. Chem. 234, 2583-2588.
9. Bergmann, E.D., and Levinson, Z.H. (1966) J. Insect Physiol. 12, 77-81.
10. Clayton, R.B., and Bloch, K. (1963) J. Biol. Chem. 238, 586-591.
11. Nair, A.M.G., and Agarwal, H.C. (1977) Indian J. Exp. Biol. 15, 576-578.
12. Thompson, M.J., Svoboda, J.A., Kaplanis, J.N., and Robbins, W.E. (1972) Proc. R. Soc. Lond. B. 180, 203-221.
13. Fieser, L.F. (1953) J. Am. Chem. Soc. 75, 5421-5422.
14. Birch, A.J., and Walker, K.A.M. (1966) J. Chem. Soc. C, 1894-1896.
15. Kircher, H.W., and Rosenstein, F.U. (1973) Lipids 8, 453-458.
16. Kircher, H.W., and Rosenstein, F.U. (1973) J. Org. Chem. 38, 2259-2260.
17. Rosenstein, F.U., Caruso, R., and Kircher, H.W. (1977) Lipids 12, 297-300.
18. Hutchins, R.F.N., Thompson, M.J., and Svoboda, J.A. (1970) Steroids 15, 113-130.
19. Adams, B.G., and Parks, L.W. (1967) Biochem. Biophys. Res. Commun. 28, 490-494.
20. Clayton, R.B. (1964) J. Lipid Res. 5, 3-19.
21. Lasser, N.L., Edwards, A.M., and Clayton, R.B. (1966) J. Lipid Res. 7, 403-412.
22. Lasser, N.L, and Clayton, R.B. (1966) J. Lipid Res. 7, 413-421.
23. Dwivedy, A.K. (1976) J. Insect Physiol. 22, 1093-1100.
24. Galbraith, M.N., Horn, D.H.S., Middleton, E.J., Thomson, J.A., and Wilkie, J.S. (1975) J. Insect Physiol. 21, 23-32.
25. Svoboda, J.A., Kaplanis, J.N., Robbins, W.E., and Thompson, M.J. (1975) Ann. Rev. Entomol. 20, 205-220.

[Received August 17, 1981]

Taxonomic Implication of Sterol Composition in the Genus *Chlorella*[1]

MARCIA J. HOLDEN and **GLENN W. PATTERSON**, *Department of Botany, University of Maryland, College Park, MD 20742*

ABSTRACT

Thirty-five isolates of the genus *Chlorella* were grown under standardized conditions and analyzed for sterol composition. Six different sterol synthetic patterns were found. Two patterns were characterized by nuclear unsaturation at C-5 and one type by nuclear unsaturation at C-7. The remaining isolates all synthesize sterols with a diunsaturated nucleus (C-5 + C-7) with various modifications. Subtype 1 synthesizes only C_{28} sterols, subtype 2 produces C_{28} and C_{29} sterols, and subtype 3 (1 isolate) produces C_{28} sterols with nuclear unsaturation at C-5 + C-8 in addition to the $\Delta^{5,7}$ C_{28} sterols seen in the first 2 subtypes. Comparison of the sterol composition of strains shared in common with taxonomic researchers showed good correlation with the taxonomic scheme of Fott and Novakova which has been confirmed by Kessler and coworkers using other biochemical markers.
Lipids 17:215-219, 1982.

INTRODUCTION

Species of the genus *Chlorella* are nonmotile, unicellular algae belonging to the Chlorophyta or green algae. The genus is characterized by only a few distinguishing structural features including cell morphology (spherical or ellipsoidal), chloroplast size and shape, and presence or absence of pyrenoid. Sexual reproduction is unknown and vegetative reproduction is by autospore production; 2-32 autospores are derived from successive divisions of the nucleus and single chloroplast. With the completion of wall formation, these minatures of adult cells are simultaneously released through rupture of the parent cell wall.

Chlorella is ubiquitous in nature and has been isolated in diverse aquatic and aerial habitats and as symbionts in certain animals. Many members of this genus are highly adaptable to life under marginal conditions. Explosive growth rates under good conditions have earned it a reputation as an algal weed. *Chlorella* was first isolated and the genus described by a microbiologist, Beyerinck. Bold and Wynne suggest that *Chlorella* was probably the first alga to be grown extensively in axenic culture (1). Partly due to the ease of growth and manipulation, *Chlorella* has found extensive use in research in plant physiology. However, due to the minuteness of size where significant subcellular structures are at the limit of resolution of light microscopy and the few available distinguishing features, the identification of an individual isolate is difficult. The result is multiple species designations for the same isolate in different culture collections. The problem of proper identification is particularly acute due to the diversity of biochemical properties and physiological reactions found in different isolates. The latter has been used extensively as a basis for taxonomic schemes.

The algae, as a group, are known for a greater diversity in sterol production than any other group of organisms. It has been suggested that characterization of sterol composition can be used as a tool in biochemical taxonomy and in the establishment of phylogenetic relationships (2). Genera in some algal divisions are united by sterol synthetic patterns, but others, particularly the green algae, are characterized by varied sterol synthesis (3). *Chlorella* is unique in the variety of biosynthetic patterns encountered in members of one genus. Previous work with *Chlorella* species had shown that individual isolates synthesize a Δ^5, Δ^7, or $\Delta^{5,7}$ series of sterols (4-6). It was of interest to investigate both the sterol composition of a large number of isolates and the value of these data in the taxonomy of the genus.

METHODS

Chlorella isolates were grown axenically and heterotrophically in 15-ℓ carboys with glucose as a carbon source. Carboys were bubbled with filtered, compressed air and checked daily for contamination. Cells were harvested at the end of log phase growth with a Sharples Super Centrifuge and freeze-dried. Algal material was stored at -20 C until analysis. Ten g of freeze-dried cells were extracted with chloroform/methanol (2:1, v/v) using a Soxhlet. The crude lipid extract was saponified with alcoholic KOH (20% in 60% EtOH) and the unsaponifiable matter extracted into ether using a liquid-liquid extraction apparatus. Sterols were purified

[1]Scientific article no. A-3030, contribution no. 6093 of the Maryland Agricultural Experiment Station.

using alumina column chromatography. Separation of sterol components was effected by the use of $AgNO_3$/silicic acid columns (12% w/w) with a graded series of ether in hexane (4, 5, 6, 7, 8, 9, 16, 20 and 90%). Steryl acetates, prepared using acetic anhydride/pyridine overnight in the dark, were chromatographed on the $AgNO_3$/silicic acid column and separated according to the number and position of the double bonds.

Sterols were identified and quantitated by gas liquid chromatography (GLC) using a Varian Model 3700 gas chromatograph equipped with a Varian CDS 111 data system. The 6-ft glass column was packed with SE-30 (3%) on 100/120 mesh Gas Chrom Q. Sterols were chromatographed using helium as a carrier gas at 245 C and 20 ml/min. Retention times relative to cholesterol were calculated for unknowns and compared to appropriate authentic standards run in conjunction with the unknown. Mass spectral analyses were conducted on representative samples using an LKB 9000 GC/MS equipped with a Varian SS-100 mass spectra data system. The 0.75% SE-30 column was operated at 230 C. The ionizing energy was 70 eV.

RESULTS AND DISCUSSION

Analysis of the isolates belonging to the genus *Chlorella* showed 6 different patterns of sterol synthesis. Isolates belonging to each of these 6 groups produced component sterols in similar proportions.

Chlorella Group IA

Five of the analyzed isolates (see Table 1) synthesize sterols characterized by a single nuclear unsaturation at C-5. A small but consistent amount of cholesterol was found in these isolates, but the other component sterols were alkylated at C-24, producing both C_{28} and C_{29} sterols. The major sterol produced in this group was the diunsaturated C_{29} sterol, poriferasterol, followed by the C_{28} sterol, 5-ergostenol and the C_{29} sterol clionasterol. These sterols are the 24β epimers of the commonly found series of higher plant sterols, campesterol, stigmasterol and sitosterol.

Group IB

The 2 isolates classified as *Chlorella* IB show a related composition, but one that differs in 2 aspects. The major product of sterol synthesis was 5-ergostenol (69-73% of total sterol) and significant amounts of the C_{29} homolog of ergosterol, 7-dehydroporiferasterol, was also found.

Chlorella Group II

The remaining 28 isolates lack, to some extent, the biosynthetic capacities of the previously discussed isolates. Seven isolates synthesize a series of sterols that are the Δ^7 isomers of the Δ^5-sterols of group IA. There is no Δ^7 equivalent of cholesterol found, however. These isolates alkylate at C-24 exclusively rather than reducing the 24(25) bond of precursor sterols. The Δ^7 sterols produced by these isolates are found in similar proportions to the Δ^5 isomers of group IA, with the major sterol a Δ^{22}, C_{29} sterol, chondrillasterol. The major biosynthetic difference between the group II isolates and those of group I is the inability of group II to introduce the Δ^5 bond, presumably due to the absence of a Δ^5-dehydrogenase.

Chlorella Group IIIA

The largest group, 15 isolates, produced the group IIIA series of sterols. Inspection of their structures shows 2 major biosynthetic differences between these algae and groups I and II. These organisms lack the Δ^7 reductase for the removal of the double bond at C-7 after the introduction of the bond at C-5, the accepted series of biosynthetic events (7). In addition, these isolates lack the ability to introduce a second alkyl group at C-24 resulting in the production, exclusively, of the C_{28} sterols, ergosterol and its mono- and diunsaturated companion sterols, in which ergosterol is the major sterol produced. Biosynthetic studies using the hypocholesteremic drugs Triparanol and AY-9944 result in a build-up of precursors in these isolates. Analysis of AY-9944 inhibition products of treated *C. sorokiniana* (UTEX #1230) showed no 24-methylene intermediates that are believed to be precursors of the 24-ethyl sterols in algae (8). Evidence for the intermediacy of these 24-methylene sterols has been provided by Tomita et al. (9,10) and Tsai and Patterson with the use of labeled compounds in *Chlorella* (11) and by Goad et al. (12) with *Scenedesmus* and *Trebouxia*. However, 24-methylene intermediates have been demonstrated in treated cultures of the C-29-producing *Chlorella* group I and II isolates, *C. emersonii* and *C. ellipsoidea* (13-15). Moreover, the treated *C. sorokiniana* cultures produced 4α,14α-dimenthyl-5α-ergosta-8,25-dienol, indicating the role of 25-methylene sterols in the production of C-28 sterols (8).

Group IIIB

Group IIIB isolates lack the same Δ^7 reductase as group IIIA, resulting again in the production of ergosterol and 5,7-ergostadienol. However, they do have the ability to introduce the

TABLE 1

Component Sterols and Ranges of Sterol Composition in the 6 *Chlorella* Groups

Systematic name (all 3β-ol)	Trivial name	% Composition		
		Group IA	Group IB	
Cholest-5-enol	Cholesterol	1-2	0-1	
24β-Methylcholesta-5,22-dienol	Brassicasterol	1-5	1	
24β-Methylcholest-5-enol	5-Ergostenol	28-34	69-73	
24β-Ethylcholesta-5,22-dienol	Poriferasterol	55-66	10-12	
24β-Ethylcholest-5-enol	Clionasterol	1-8	a	
24β-Ethylcholesta-5,7,22-trienol	7-Dehydroporiferasterol	–	9[a]	
		Group II		
24β-Methylcholesta-7,22-dienol	7,22-Ergostadienol	1-4		
24β-Methylcholest-7-enol	7-Ergostenol	20-28		
24β-Ethylcholesta-7,22-dienol	Chondrillasterol	45-68		
24β-Ethylcholest-7-enol	7-Chondrillastenol	5-27		
		Group IIIA	Group IIIB	Group IIIC
24β-Methylcholesta-5,7,22-trienol	Ergosterol	32-80	23-41	53
24β-Methylcholesta-5,7-dienol	5,7-Ergostadienol	>32-63[b]	>13-35[b]	>13[b]
24β-Methylcholest-7-enol	7-Ergostenol			
24β-Ethylcholesta-7,22-dienol	Chondrillasterol	–	>29-37[c]	–
24β-Ethylcholesta-5,7,22-trienol	7-Dehydroporiferasterol			
24β-Ethylcholest-7-enol	7-Chondrillastenol	–	3-14	–
24β-Methylcholesta-5,8-dienol	5,8-Ergostadienol	–	–	1
24β-Methylcholesta-5,8,22,-trienol	5,8,22-Ergostatrienol	–	–	30

[a]Combined peak—clionasterol seen as a shoulder on the back side of the 7-dehydroporiferasterol.

[b]Combined peak—difficult to separate due to similar retention times; 7-ergostenol is the major component in most cases.

[c]Combined peak, with 7-dehydroporiferasterol the major component.

second alkyl group at C-24, producing the C-29 homolog of ergosterol, 7-dehydroporiferasterol, plus chondrillasterol and 7-chondrillastenol. The presence of C_{29} sterols in ergosterol synthesizing organisms is a rare occurrence. While 7-dehydroporiferasterol has been found in conjunction with ergosterol in *Euglena gracilis* (16), *Ochromonas danica* (17), and one strain of *Chlamydomonas reinhardii* (18), most ergosterol synthesizing species produce only C_{28} sterols as do *Chlorella* group IIIA (7). This is sufficiently widespread that the possibility has been suggested by Nes and Nes (19) of genetic coupling between production of ergosterol and the inability to introduce the second alkyl group.

Group IIIC

The last type of sterol biosynthetic pattern is encountered in only one strain of *Chlorella*. This previously published work (20) was repeated here and the same unique $\Delta^{5,8}$ and $\Delta^{5,8,22}$ sterols were found along with ergosterol, 5,7-ergostadienol, and 7-ergostenol. No second alkylation step occurs in this strain as only C_{28} sterols are produced. But the presence of the $\Delta^{5,8}$ bonds are difficult to explain because the presence of the double bond at C-7 is considered necessary for the introduction of the double bond at C-5 (7). It is either unnecessary in this strain or, perhaps, one is seeing a high degree of reversibility in the $\Delta^8 \rightarrow \Delta^7$ isomerase.

Sterols and Taxonomy

The analysis of a large number of isolates of *Chlorella* uncovered more patterns of sterol synthesis than previously suspected. This suggested more strongly than before the possibility of the use of sterols as a taxonomic marker. Three groups have worked extensively on the taxonomic classification of this genus. All 3 recognized that members of the genus exhibited morphological and physiological diversity between isolates and variability in the behavior of an individual isolate under differing environmental conditions. The 3 groups of researchers accordingly adopted their own standardized conditions, but approaches differed from this point. Shihira and Krauss combined morphological and physiological criteria with the emphasis on physiological (21). Responses to carbon and nitrogen sources were graded and, at times, small differences were considered justification for creation of separate taxa. Observation of 41

isolates led to the creation of 22 species and 8 varieties (21). Fott and Novakova took a more classical morphological approach (22). Fifteen taxa, 9 species and 6 varieties, were defined on the basis of observations of cellular structure. Aspects of the life cycle and reproductive behavior were studied. They agreed with Shihira and Krauss on the necessity for biochemical markers but disagreed with the choice of markers. Kessler and coworkers initially ignored cellular structure and defined groups of related isolates on the basis of biochemical and physiological characters, e.g., hydrogenase activity, formation of secondary carotenoids, pH tolerance and thermophily (23). Agreement was found between Kessler's groups and Fott and Novakova's species, leading to a more complete definition of the various taxa.

In Table 2, the analyzed isolates are grouped according to their sterol composition. Table 3 summarizes the characteristic sterol pattern encountered in those *Chlorella* species defined by Fott and Novakova. Representatives of 6 of the 9 Fott and Novakova species were examined in this study. The *Chlorella* IB and IIIC strains were not studied by Fott and Novakova. The sterol data for these strains, along with other physiological and biochemical work on these isolates, suggest that these strains may comprise yet additional taxa (24 and E. Kessler, personal communication). A subsequent paper will deal in more detail with taxonomic aspects of *Chlorella*. The DNA hybridization studies of Kerfin and Kessler have suggested that *Chlorella* is a genus comprised of biochemically and genetically diverse species united by a common simple morphology (25). The variation in sterol synthetic capacity encountered in this genus underscores this diversity. Sterol composition can serve as a stable marker in the taxonomic definition of an isolate or can serve to set it apart from other defined strains.

TABLE 2

Chlorella Isolates Arranged According to Sterol Composition

	Isolate #
Group IA	
C. saccharophila (Kruger) Nadson[a]	27[b]
C. ellipsoidea Gerneck	247[b]
C. variegata Beij.	257[b]
C. saccharophila (Kruger) Nadson	211/1a[c]
C. saccharophila (Kruger) Nadson	211/1d[c]
Group IB	
C. anitrata S. & K.	1798[b]
C. anitrata var. *minor* S. & K.	1799[b]
Group II	
C. emersonii S. & K.	2[d]
C. pyrenoidosa Chick	251[b]
C. pyrenoidosa Chick[e]	343[b]
C. glucotropha S. & K.	1802[b]
C. pyrenoidosa var. *chick* S. & K.	1806[b]
C. regularis var. *minima* S. & K.	1807[b]
C. emersonii var. *rubescens* Fott et al.	232/1[c]
Group IIIA	
C. pyrenoidosa Chick	26[b]
C. vulgaris var. *viridis* Chodat	30[b]
C. vulgaris Beij.	259[b]
C. vulgaris Beij.	261[b]
C. vulgaris Beij.	265[b]
C. vulgaris var. *viridis* Chodat	396[b]
C. pyrenoidosa Chick	1230[b]
C. infusionum var. *acetophila* S. & K.	1803[b]
C. parva S. & K.	1805[b]
C. vulgaris f. *tertia* F. & N.	211/31[f]
C. salina Butcher	1809[b]
C. sorokiniana var. *pacificiensis* S. & K.	1810[b]
C. vaniellii S. & K.	1811[b]
C. vulgaris f. *tertia* F. & N.	211/40a[f]
C. vulgaris f. *tertia* F. & N.	1/9/30[g]
Group IIIB	
C. vulgaris Beij.	262[b]
C. vulgaris Beij.	397[b]
C. vulgaris Beij.	398[b]
C. vulgaris Beij.	263[b]
C. sp.	580[b]
Group IIIC	
C. ellipsoidea Gerneck	246[b]

[a]Culture names are those assigned by the culture collections.

[b]UTEX # University of Texas at Austin, Culture Collection of Algae.

[c]CCAP # Cambridge Collection of Algae and Protozoa.

[d]MCC # Maryland Culture Collection.

[e]Fott et al. removed this strain from the genus *Chlorella* and suggested it be considered a species of the genus *Scenedesmus* (27).

[f]Gottingen Collection.

[g]High Temperature Strain of Sorokin (26).

TABLE 3

Sterol Composition and *Chlorella* Taxonomy

Sterol biosynthetic pattern	Fott & Novakova species
Group IA	*C. saccharophila*
	C. luteoviridis
Group II	*C. emersonii* var. *vacuolata* (*C. fusca* var. *vacuolata*)[a]
	C. emersonii var. *rubescens* (*C. fusca* var. *rubescens*)[a]
Group IIIA	*C. vulgaris*
	C. sorokiniana (*C. vulgaris* f. *tertia*)[b]
Group IIIB	*C. Kessleri*

[a]Species designation of Shihira and Krauss and adopted by Fott and Novakova in their monograph (22). Species designation determined invalid in 1975 (27).

[b]Species designation assigned by Fott and Novakova later changed.

ACKNOWLEDGMENTS

This research was supported in part by Maryland Sea Grant no. NA-79AA-D-00058. We wish to thank S.R. Dutky for the mass spectra and E. Kessler for several *Chlorella* isolates.

REFERENCES

1. Bold, H.C., and Wynne, M.J. (1978) in Introduction to the Algae, Structure and Reproduction, p. 133, Prentice-Hall, Inc., Englewood Cliffs, NJ.
2. Patterson, G.W. (1971) Lipids 6, 120-127.
3. Patterson, G.W. (1979) 1st International Symposium on Marine Algae of the Indian Ocean Region, Bhavnagar, India.
4. Patterson, G.W., and Krauss, R.W. (1965) Plant Cell Physiol. 7, 211-220.
5. Patterson, G.W. (1967) Plant Physiol. 42, 1457-1459.
6. Patterson, G.W. (1969) Comp. Biochem. Physiol. 31, 391-394.
7. Nes, W.R., and McKean, M.L. (1977) Biochemistry of Steroids and Other Isopentenoids, University Park Press, Baltimore, MD.
8. Chiu, P-L, Patterson, G.W., and Dutky, S.R. (1976) Phytochemistry 15, 1907-1910.
9. Tomita, Y., Uomori, A., and Minota, H. (1970) Phytochemistry 9, 555-560.
10. Tomita, Y., Uomori, A., and Sakurai, E. (1971) Phytochemistry 10, 573-577.
11. Tsai, L.B., and Patterson, G.W. (1976) Phytochemistry 15, 1131-1133.
12. Goad, L.J., Knapp, F.F., Lenton, J.R., and Goodwin, T.W. (1972) Biochem. J. 129, 219-222.
13. Dickson, L.G., and Patterson, G.W. (1973) Lipids 7, 635-642.
14. Doyle, P.J., Patterson, G.W., Dutky, S.R., and Thompson, M.J. (1972) Phytochemistry 11, 1951-1960.
15. Chan, J.T., and Patterson, G.W. (1973) Plant Physiol. 52, 246-247.
16. Anding, C., and Ourisson, G. (1973) Eur. J. Biochem. 34, 345-346.
17. Gershengorn, M.C., Smith, A.R.H., Goultston, G., Goad, L.J., Goodwin, T.W., and Haines, T.H. (1968) Biochemistry 7, 1698-1706.
18. Patterson, G.W. (1974) Comp. Biochem. Physiol. 47B, 453-457.
19. Nes, W.R., and Nes, W.D. (1980) in Lipids in Evolution, p. 105, Plenum Press, New York, NY.
20. Patterson, G.W., Thompson, M.J., and Dutky, S.R. (1974) Phytochemistry 13, 191-194.
21. Shihira, I., and Krauss, R.W. (1963) Chlorella: Physiology and Taxonomy of Forty-One Isolates, Univ. of Maryland, College Park.
22. Fott, B., and Novakova, M. (1969) in Studies in Phycology (Fott, B., ed.), pp. 10-74, Academia, Prague.
23. Kessler, E. (1976) Plant Syst. Evol. 125, 129-138.
24. Gross, R. (1964) A Physiological Examination and Taxonomic Revision on Species of *Chlorella* Isolated from Marine Habitats, Master's Thesis, Univ. of MD, College Park.
25. Kerfin, W., and Kessler, E. (1978) Arch. Microbiol. 116, 97-103.
26. Sorokin, C. (1968) Science 158, 1204-1205.
27. Fott, B., Lochhead, R., and Clemencon, H. (1975) Arch. Protistenk 117, 288-296.

[Received August 17, 1981]

Utilization and Metabolism of Dietary Sterols in the Honey Bee and the Yellow Fever Mosquito

JAMES A. SVOBODA[a,*], MALCOLM J. THOMPSON[a], ELTON W. HERBERT, Jr.,[b] THOMAS J. SHORTINO[a] and PATRICIA A. SZCZEPANIK-VANLEEUWEN[c],
[a]*Insect Physiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705;* [b]*Bioenvironmental Bee Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705; and* [c]*Division of Radiological and Environmental Research, Argonne National Laboratory, Argonne, IL 60440*

ABSTRACT

The honey bee, *Apis mellifera*, does not convert C_{28} and C_{29} phytosterols to cholesterol as found in most previous studies of phytophagous or omnivorous insects, but instead the workers and queens selectively transfer 24-methylenecholesterol, sitosterol and isofucosterol from their endogenous sterol pools to the brood larvae regardless of the sterol in the worker diet. Administering radiolabeled sterols by feeding and injection has made it possible to trace this selective transfer through a second generation of the honey bee. In further comparative sterol metabolism studies, the yellow fever mosquito, *Aedes aegypti*, was shown to be capable of dealkylating and converting a radiolabeled C_{29} dietary sterol ([^{14}C]sitosterol) to cholesterol. Metabolic studies with several radiolabeled dietary sterols and an inhibitor of steroid metabolism in the yellow fever mosquito further verified this capability.
Lipids 17:220-225, 1982.

INTRODUCTION

Research with a number of insect species that undergo various types of development and occupy a diversity of ecological niches has revealed interesting differences in sterol utilization between certain phytophagous and omnivorous species (1). The utilization and metabolism of steroids in insects differ in many respects from those of higher animals and of plants and the uniqueness of this area of insect biochemistry provides an important target system for selective disruption of insect growth and development. Insects are unable to biosynthesize sterols and require a dietary or exogenous source of sterol for normal growth and reproduction (1) and in all but 2 known cases, cholesterol will satisfy this requirement (2,3). Most phytophagous insects are able to meet their cholesterol requirement by converting C_{28} and C_{29} phytosterols to cholesterol which is then available as a precursor for the ecdysteroids or molting hormones as well as for structural purposes (1). The ability of most plant-feeding insects to dealkylate sterols at C-24 is an important biochemical difference between these insects and higher animals.

Desmosterol (24-dehydrocholesterol) was the first intermediate found to be involved in the dealkylation and conversion of sitosterol to cholesterol in insects, and it is the final intermediate in the conversion of all C_{28} and C_{29} phytosterols to cholesterol in those insects capable of this conversion (4). These findings were significant from a comparative biochemical standpoint, as desmosterol is also a terminal intermediate in the de novo biosynthesis of cholesterol in vertebrates (5), and there are Δ^{24}-intermediates in the pathway of plant sterol biosynthesis (6).

Previous studies with the honey bee, *Apis mellifera*, have revealed that this phytophagous social insect possesses some unique ways of utilizing dietary sterols (7,8). This might be expected, considering the complexity of its feeding habits (9). Honey, pollen and royal jelly are all involved in feeding a healthy bee colony in the field, and the honey stomach, mandibular glands and hypopharyngeal glands are involved in the nutritional well-being of the hive. Larvae are fed a glandular secretion consisting partly of a largely protein component from the hypopharyngeal glands and partly of a mostly lipid component from the mandibular glands, together with regurgitations from the honey stomach (9). The relative amounts of these components fed vary with the age of the larvae and whether they are destined to be workers, queens or drones.

When diets containing various sterols were fed to honey bee workers, diets fortified with cholesterol and 24-methylenecholesterol supported the highest level of brood production and worker survival was best on a 24-methylenecholesterol diet (10). Regardless of the dietary sterol, 24-methylenecholesterol was always the predominant sterol found in prepupae, and relatively constant amounts of both sitosterol and isofucosterol were present. Also, there was some increase in the relative concentration of dietary sterol in each sample. Little, if any, conversion of the C_{28} or C_{29} phytosterols to cholesterol occurred in the larvae. A major portion of the sterol made available to

the larvae appeared to be derived from the endogenous sterol pools of the workers (i.e., sterols present in the workers as the tests were begun) rather than from their artificial diet. This ability of the honey bee to selectively utilize 24-methylenecholesterol and transfer it to the next generation was further examined in studies involving injection and feeding of radiolabeled 24-methylenecholesterol, and the results of those studies are discussed in this paper.

Whether the house fly, *Musca domestica*, has the ability to dealkylate the C-24 position of the sterol side chain has been critically examined in metabolic studies with labeled dietary sitosterol and this dipteran species was found to be unable to carry out this conversion (11,12). For many years, we had used the yellow fever mosquito, *Aedes aegypti*, as a test insect in various types of physiological studies, but we had never examined sterol utilization and metabolism in this species. We thus undertook to determine whether mosquito larvae, like a number of previously studied phytophagous and omnivorous insects, could dealkylate C_{28} or C_{29} phytosterols, or whether this mosquito was like the house fly in this regard.

MATERIALS AND METHODS

Honey Bee Tests

The chemically defined diets for the honey bee tests were prepared as previously described (10). In the first experiment of this study, over 2,600 newly emerged "Italian" (a geographical race, *A. mellifera ligustica*, that is widely accepted in beekeeping, 13) workers were each injected with 1 μg [2,4-^{3}H] 24-methylenecholesterol (14) (sp act 4,000 cpm/μg, >98% radiochemical purity). The injected bees were set up in a hive in a screened flight cage (2 × 2 × 2 m) with a mated laying queen, and fed a diet containing 0.1% dry weight of unlabeled cholesterol. A sample of 8 injected workers was weighed and frozen at 4 weeks after the test was begun and a total of 25 prepupae reared by these workers was collected and frozen for later sterol analysis; the queen was also preserved for analysis.

In a second experiment, 5 colonies of newly emerged "Italian" honey bees (ca. 4,000 bees/colony) were set up in flight cages with a mated laying "midnight" queen (a darker colored commercial hybrid, 13) which provided a genetic marker for production of readily identifiable "midnight" or dark-colored progeny. The workers in the original colonies were fed a diet coated with 0.1% dry weight [2,4-^{3}H] 24-methylenecholesterol (sp act 300 cpm/μg). A sample of 8 of the original workers was weighed and frozen 4 weeks after the colony was established and one sample (8 insects) of newly emerged "midnight" worker progeny was weighed and frozen. All other newly emerged "midnight" workers were transferred to another cage and used to establish another colony with a mated laying queen. This colony was fed a cholesterol-fortified diet, and subsequently 3 samples of 25 of these second generation pupae, reared by the "midnight" workers, were weighed and frozen, as were 5 "midnight" queens from the original colonies 5 weeks after the tests were begun.

Yellow Fever Mosquito Tests

A modification of Akov's larval mosquito diet was used to rear yellow fever mosquito larvae (15). Our diet differed from Akov's primarily in the dry vitamin mixture, because we used a standard mixture developed for the house fly in our laboratory which contains inositol (16). The diet ingredients and concentrations included: dry vitamin mixture, 0.2 mg/ml; salt mixture W, 1.4 mg/ml; yeast RNA, 1.0 mg/ml; casein (extracted 6X with $CHCl_3$/MeOH), 9.9 mg/ml. The labeled dietary sterol, in each case, was dissolved in CH_2Cl_2 and coated on the other dietary ingredients at a concentration of 0.1% dry weight. Groups of 1,000 newly hatched larvae each were placed in gallon jars containing 3 ℓ of water (not under aseptic conditions) and were fed as needed. Insects were frozen as pupae when possible to ensure elimination of dietary sterol from the lumen of the gut. Otherwise, larvae were transferred to sterol-free medium for 24 hr prior to being frozen to eliminate dietary sterol from the gut. When possible, at least 1 g of mosquitoes fed each diet was pooled for each sterol analysis. [4-^{14}C] Sitosterol was purchased from Amersham Corp., Arlington Heights, IL; [26-^{14}C] desmosterol was purchased from New England Nuclear, Boston, MA; [2,4-^{3}H] campesterol was prepared by the method used for [^{3}H] 24-methylenecholesterol in the honey bee tests (14). The radiolabeled sterols were purified by column chromatography and examined for radiochemical purity by counting areas of adsorbent scraped from thin layer chromatography (TLC) plates in a Packard liquid scintillation spectrometer and by counting fractions collected from gas liquid chromatograph (GLC) effluent. The radiochemical purity of all sterols was >98% and the sp act were 4,300, 1,000 and 2,500 cpm/μg for [^{14}C] sitosterol, [^{14}C]-desmosterol and [^{3}H] campesterol, respectively. The 25-azacholesterol·HCl used in inhibitor

TABLE 1

Transfer of Radiolabeled ^{3}H-24-Methylenecholesterol by Honey Bees after Injection and Feeding

	Radiolabeling (cpm)/insect
Injection (4,000 cpm/worker)	
Injected workers at 4 weeks	1,740
Prepupae reared by injected workers	2,450
Queen after 6 weeks	7,400
Feeding (300 cpm/μg, 0.1%dry wt of diet)	
Original workers at 4 weeks	5,200
"Midnight" queens after 5 weeks	9,900
Newly emerged "midnight" workers	5,000
Pupae of next generation	3,250

studies was provided by the G.D. Searle Co., Chicago, IL. [1-^{14}C]Sodium acetate (sp act 0.25 mCi/0.35 mg) was purchased from New England Nuclear, Boston, MA.

Sterol Analyses

Samples from both honey bee and yellow fever mosquito tests were homogenized in $CHCl_3$/MeOH (2:1). Crude lipid extracts were saponified and the sterols were isolated by column chromatography on alumina (17). Column fractions were monitored by TLC and the sterols were identified and quantitated by GLC on an 0.75% SE-30 and a 1% OV-17 system. When necessary, the insect sterols were further fractionated, as their acetates, by thin layer and column argentation chromatography (18). Gas chromatography-mass spectral (GC-MS) analyses were done to confirm identities of sterols from certain of the yellow fever mosquito metabolism studies since these conversions were not previously reported in this insect.

RESULTS AND DISCUSSION

The transfer of unchanged [^{3}H] 24-methylenecholesterol to honey bee brood was clearly demonstrated by both the feeding and the injection studies. Table 1 shows comparisons of radioactivity recovered per individual in samples of workers, queens and pupae from both experiments. All radioactivity in these samples is associated with 24-methylenecholesterol as determined by GLC trapping and argentation chromatography of the sterol acetates. Even after 4 weeks, injected workers retained a large part of the labeled sterol. It is obvious that the labeled 24-methylenecholesterol that had been injected into newly emerged workers at the beginning of the test period was incorporated into the brood food for larval rearing, as shown by the radiolabel recovered from the pupae. In the feeding study, the original workers accumulated considerable radiolabeled 24-methylenecholesterol in 4 weeks, and some of this labeled sterol was made available to larvae which developed into the "midnight" worker progeny. They, in turn, transferred it to larvae of the next generation, as indicated by the fact that the label was found in the pupae of the next generation, even though the workers were fed a diet fortified only with unlabeled cholesterol. In addition to this selective utilization or transfer of 24-methylenecholesterol, GLC analyses revealed that these "midnight" workers also transferred a fairly constant amount of both sitosterol and isofucosterol to the larvae. There was a significant amount of labeled 24-methylenecholesterol recovered from queens in both of these experiments: 7,400 cpm/queen from the injection study and 9,900 cpm/queen in the feeding study. All the radioactivity in these queens was associated with 24-methylenecholesterol, and this provides further evidence that this sterol is not metabolized and is transferred unchanged by the honey bee workers.

There are interesting phylogenetic implications related to this utilization of sterols by honey bees. There is good reason to suppose that the first bees developed from some wasplike ancestor, forsaking a carnivorous diet for a vegetarian one (19). It was apparently never necessary for them to develop the ability to convert plant sterols to cholesterol. We have previously noted the small, but persistant, levels of cholesterol found in all stages of honey bees which must originate ultimately from foraging in the field (8). This could perhaps be sufficient for their ecdysteroid production. On the other hand, there may be other as yet unidentified ecdysteroids in the honey bee such as makisterone A, a C_{28}-ecdysteroid found in the milkweed bug (20) and for which campesterol or 24-methylene-

TABLE 2

Relative Percentages of and Distribution of Radioactivity in Sterols of *A. aegypti* Pupae That Had Been Fed [^{14}C]Sitosterol Alone or in Combination with 25-Azacholesterol·HCl (3 ppm) in the Larval Diet[a]

Sterol	Relative % of sterols	Relative % radioactivity in major sterols[b]
	[^{14}C]Sitosterol diet	
Cholesterol	66.1	46.8
Campesterol	1.2	–
Sitosterol	32.7	49.4
	[^{14}C]Sitosterol + 25-azacholesterol·HCl diet	
Cholesterol	27.7	8.0
Desmosterol	18.4	20.6
Campesterol	0.6	–
Sitosterol	53.3	62.9

[a][^{14}C]Sitosterol (4,300 cpm/μg) at 0.1% dry weight of diet in both cases.

[b]Remainder of recovered radioactivity was distributed in pre-peak, intermediate (campesterol), and post-peak elution fractions.

cholesterol could serve as precursors. It will be of interest to see what ecdysteroids are finally isolated and identified from the honey bee.

Table 2 summarizes the fate of the C_{29} sterol [^{14}C]sitosterol fed to yellow fever mosquito larvae. Over 66% of the mass of sterols recovered from these insects was cholesterol, and over 46% of the recovered radioactivity resided with cholesterol. Although there is some obvious dilution of the sp act of cholesterol recovered from these pupae, it is apparent that the major portion of the cholesterol derived from the dietary [^{14}C]sitosterol. The identity of the cholesterol isolated from the pupae was verified by GC-MS analysis.

Feeding certain azasteroids in combination with sitosterol to insects that are able to dealkylate and convert phytosterols to cholesterol results in a reduced production of cholesterol (1). An accumulation of desmosterol, the terminal intermediate in this conversion, also occurs, because Δ^{24}-sterol reductase is inhibited by these azasterols (1). The results from adding 3 ppm 25-azacholesterol·HCl to the medium with [^{14}C]sitosterol in the diet show that, at this concentration, the azasteroid did not significantly inhibit larval development, but sterol metabolism was affected (Table 2). Cholesterol content was reduced to ca. 27% compared to 66% without the inhibitor, and desmosterol (verified by GC-MS) constituted over 18% of the sterols. Considering data from both GLC trapping and chromatographic fractionation, that portion of the radioactivity associated with cholesterol was only about 8% of the total recovered with the sterols. Over 20% of the total radioactivity was associated with desmosterol. These results indicate that the mosquito larvae did produce cholesterol from sitosterol via the expected pathway, but there is a source of unlabeled cholesterol contributing to the total.

When [^{14}C]desmosterol was fed to mosquito larvae, very little residual desmosterol could be found in the insects (Table 3). The larvae grew well and efficiently produced labeled cholesterol, as determined by thorough analysis, including GC-MS. These results further substantiated the presence of Δ^{24}-sterol reductase and the metabolic pathway for converting phytosterols to cholesterol. Nearly 80% of the recovered radioactivity associated with the insect sterols was in the cholesterol fraction.

Results from feeding labeled campesterol, a C_{28} sterol, to yellow fever mosquito larvae were quite unexpected (Table 3). Of those insect species with which we have worked that can convert sitosterol to cholesterol, most were able to convert campesterol to cholesterol about equally as well. In the yellow fever mosquito, however, much less cholesterol was produced from campesterol than from sitosterol. Cholesterol constituted only about 36% of the total sterols in this case and accounted for only 10% of the total radioactivity. So an even larger portion of the cholesterol had come from a source other than the dietary phytosterol in this test with campesterol than in the test with sitosterol. Certainly, campesterol was not as well metabolized by this insect as was sitosterol.

Although we found that this mosquito could convert sitosterol and, to a lesser extent, campesterol, to cholesterol, we have not

TABLE 3

Relative Percentages and Distribution of Radioactivity in Sterols Recovered from *A. aegypti* Fed a Diet Fortified with [^{14}C]Desmosterol or [^{3}H]Campesterol

Sterol	Relative % of sterols	Relative % radioactivity in cholesterol
	[^{14}C]Desmosterol diet[a]	
Cholesterol	97.8	79.0
Desmosterol	2.0	
	[^{3}H]Campesterol diet[b]	
Cholesterol	36.6	10.4
Campesterol	63.4	
Sitosterol	tr	

[a][^{14}C]Desmosterol (1,000 cpm/μg) at 0.1% dry weight of diet.
[b][^{3}H]Campesterol (2,500 cpm/μg) at 0.1% dry weight of diet.

determined the source of unlabeled cholesterol in these tests. The dietary components were analyzed and the cholesterol in the diet could not account for the unlabeled cholesterol found in the insects even through very efficient selective uptake. There was no detectable conversion of [^{14}C]sitosterol to cholesterol when the diet was incubated without mosquito larvae. We are looking at possible sources of de novo biosynthesis of cholesterol such as by microorganisms in the medium. Mosquito larvae do not appear to have symbionts (21), so it is doubtful that any cholesterol biosynthesis occurred within the larvae. We are carrying out studies with [^{14}C]sodium acetate and inhibitors of early steps of de novo biosynthesis to shed some light on the problem. In a single experiment with 0.1 mCi [^{14}C]sodium acetate added to the medium, only 0.013% of the original radioactivity was recovered in the sterols of the pupae. We are also working toward development of a satisfactory aseptic rearing system which should provide more definitive information on the source of unlabeled cholesterol.

Some of the more primitive Diptera, such as the yellow fever mosquito, appear to be able to convert plant sterols to cholesterol, whereas more advanced members of this order, such as house flies, are unable to do so. It is of interest to consider that the yellow fever mosquito larvae are aquatic and omnivorous, feeding on both plant and animal material. Both male and female adults obtain much of their nourishment from nectar and plant juices. The adult female, however, requires a blood meal for successful egg maturation and obtains some cholesterol in this manner. We are therefore interested in determining if both male and female are able to convert plant sterols to cholesterol.

In summary, it is of interest to relate these results from recent honey bee and yellow fever mosquito studies to some of our previous studies with other insects (Fig. 1). With a number of phytophagous Lepidoptera such as the tobacco hornworm (*Manduca sexta*) and omnivorous insects such as several species of cockroaches, we found what was thought to be the "typical" metabolic pathways for utilization and conversion of phytosterols to cholesterol in insects (1). Apparently, however, no one pathway is typical. The confused flour beetle (*Tribolium confusum*), although phytophagous and able to dealkylate, was found to produce about equivalent amounts of 7-dehydrocholesterol and cholesterol from plant sterols (22). Another stored product insect, the khapra beetle (*Trogoderma granarium*), whose metabolism of phytosterols might be expected to be somewhat similar to that of the confused flour beetle, proved unable to dealkylate C_{28} and C_{29} phytosterols and utilizes them unchanged, but it is capable of some selective uptake of cholesterol and campesterol (23). The Mexican bean beetle (*Epilachna varivestis*), which feeds on soybean leaves and other crop plants, produces mostly stanols and significant amounts of lathosterol (24). This coccinelid species may have evolved from a predacious ancestor, and, in fact, the related predacious ladybug beetle, *Coccinella septempunctata*, utilizes the unchanged sterols of its prey (17). The milkweed bug (*Oncopeltus fasciatus*) is another phytophagous insect that we found cannot produce cholesterol from phytosterols (25). This is primarily a seed-feeding insect that utilizes its dietary sterols unchanged, except for some selective uptake of cholesterol, and produces the C_{28} ecdysteroid, makisterone A, most likely from campesterol. The honey bee, although phytophagous, probably evolved from

Tobacco hornworm and other Lepidoptera, omnivorous species	C_{28} and C_{29} Phytosterols→Cholesterol (major)
Confused flour beetle	C_{28} and C_{29} Phytosterols→7-Dehydrocholesterol ⇌Cholesterol
Khapra beetle	C_{28} and C_{29} Phytosterols↛Cholesterol (selective uptake of sterols)
Mexican bean beetle	C_{28} and C_{29} Phytosterols→Cholestanol + Lathosterol
Predacious lady bug beetle	Utilizes sterols of prey
Milkweed bugs	C_{28} and C_{29} Phytosterols↛Cholesterol
Honey bee sterols	C_{28} and C_{29} Phytosterols↛Cholesterol (selective transfer of sterols)
Yellow fever mosquito	C_{29} Phytosterols→Cholesterol

FIG. 1. Comparative summary of utilization of C_{28} and C_{29} phytosterols in various insects.

a predacious, wasp-like insect and cannot dealkylate, but is capable of selectively transferring certain sterols to the next generation. Finally, we discovered that a lower dipteran species, the yellow fever mosquito, can convert plant sterols to cholesterol.

We have obtained sufficient data to allow us to begin discussing phylogenetic relationships of insects in terms of their utilization and metabolism of dietary sterols. However, it is also becoming more apparent that sterol metabolism in insects must often be considered on an individual species basis. Furthermore, studies on neutral sterol metabolism may provide important clues to the ecdysteroid content of certain insect species.

ACKNOWLEDGMENT

We thank William R. Lusby of the Insect Physiology Laboratory, Beltsville, MD, for providing the GC-MS analyses. Mention of a company name or proprietary product does not constitute endorsement by the U.S. Department of Agriculture.

REFERENCES

1. Svoboda, J.A., Thompson, M.J., Robbins, W.E., and Kaplanis, J.N. (1978) Lipids 13, 742-753.
2. Chu, H.M., Norris, D.M., and Kok, L.T. (1970) J. Insect Physiol. 16, 1379-1387.
3. Heed, W.B., and Kircher, H.W. (1965) Science 149, 758-761.
4. Robbins, W.E., Kaplanis, J.N., Svoboda, J.A., and Thompson, M.J. (1971) Annu. Rev. Entomol. 16, 53-72.
5. Steinberg, D., and Avigan, J. (1960) J. Biol. Chem. 235, 3127-3129.
6. Goad, L.J. (1967) in Terpenoids in Plants, pp. 159-190, Academic Press, New York, NY.
7. Svoboda, J.A., Thompson, M.J., Herbert, E.W., Jr., and Shimanuki, H. (1980) J. Insect Physiol. 26, 291-294.
8. Svoboda, J.A., Herbert, E.W., Jr., Thompson, M.J., and Shimanuki, H. (1981) J. Insect Physiol. 27, 183-188.
9. Free, J.B. (1977) in The Social Organization of Honeybees, The Institute of Biology's Studies in Biology No. 81, Edward Arnold Publishers Ltd., London.
10. Herbert, E.W., Jr., Svoboda, J.A., Thompson, M.J., and Shimanuki, H. (1980) J. Insect Physiol. 6, 287-289.
11. Kaplanis, J.N., Monroe, R.E., Robbins, W.E., and Louloudes, S.J. (1963) Ann. Entomol. Soc. Am. 56, 198-201.
12. Kaplanis, J.N., Robbins, W.E., Monroe, R.E., Shortino, T.J., and Thompson, M.J. (1965) J. Insect Physiol. 11, 251-258.
13. Rothenbuhler, W.C., and Kulincevic, J.M. (1968) Annu. Rev. Genetics 2, 413-437.
14. Thompson, M.J., Berngruber, O.W., and Klein, P.D. (1971) Lipids 6, 233-237.
15. Akov, S. (1962) J. Insect Physiol. 8, 319-335.
16. Monroe, R.E. (1960) Ann. Entomol. Soc. Am. 53, 821-824.
17. Svoboda, J.A., and Robbins, W.E. (1979) Experientia 35, 186-187.
18. Svoboda, J.A., and Thompson, M.J. (1974) Lipids 9, 752-755.
19. Butler, C.G. (1975) in The Hive and the Honey Bee (Dadant and Sons, eds.) pp. 39-74, Journal Printing Co., Carthage, IL.
20. Kaplanis, J.N., Dutky, S.R., Robbins, W.E., Thompson, M.J., Lindquist, E.L., Horn, D.H.S., and Galbraith, M.N. (1975) Science 190, 681-682.
21. Brooks, M.A. (1963) in Symposia of the Society for General Microbiology, No. XIII, Symbiotic Association, pp. 200-231.
22. Svoboda, J.A., Robbins, W.E., Cohen, C.F., and Shortino, T.J. (1972) in Insect and Mite Nutrition (Rodriguez, J.G., ed.) pp. 505-516, Northholland, Amsterdam.
23. Svoboda, J.A., Nair, A.M.G., Agarwal, N., and Robbins, W.E. (1980) Experientia 36, 1029-1030.
24. Svoboda, J.A., Thompson, M.J., Robbins, W.E., and Elden, T.C. (1975) Lipids 10, 524-527.
25. Svoboda, J.A., Dutky, S.R., Robbins, W.E., and Kaplanis, J.N. (1977) Lipids 12, 318-321.

[Received August 17, 1981]

A New Route to Steroid Ring C Aromatization from Readily Available Precursors

MARIO ANASTASIA*, PIETRO ALLEVI, ALBERTO FIECCHI and **ANTONIO SCALA,**
Institute of Chemistry, School of Medicine, University of Milan, Via Saldini 50, I-20133, Italy

ABSTRACT

3β-Acetoxy-8α,9α-epoxy-5α-cholest-14-ene (1); 3β-acetoxy-14α,15α-epoxy-5α-cholest-8-ene (2); 3β-acetoxy-5α-cholest-8(14)-ene-9α,15α-diol (3); and 3β-acetoxy-5α-cholesta-8(14),9(11)-dien-15α-ol (4) have been aromatized to a 9:1 mixture of 3β-hydroxy-12-methyl-18-nor-5α,17β(H)-cholesta-8,11,13-triene (5a) and 3β-hydroxy-12-methyl-18-nor-5α,17α(H)-cholesta-8,11,13-triene (5b) in ethanol solution by using hydrochloric acid. The aromatization by action of *p*-toluenesulfonic acid gave mainly the epimer with the natural C-17 configuration as the acetate 5c at the appropriate *p*-toluenesulfonic acid concentration. 3β-Acetoxy-5α-cholesta-7,9(11),14-triene (7a) and 3β-hydroxy-5α-cholesta-8,11,14-triene (8a), 2 intermediary compounds in the aromatization, were isolated and characterized. *Lipids* 17:226-229, 1982.

INTRODUCTION

The synthesis of aromatic ring C steroids is of recent interest (1) because some of them are analogous to naturally occurring estrogens and have potential as a new class of agents useful in the antifertility field (2). We now report a novel molecular rearrangement leading to ring C aromatic steroids from readily available steroids.

MATERIALS AND METHODS

Reagents

3β-Acetoxy-8α,9α-epoxy-5α-cholest-14-ene (1); 3β-acetoxy-14α,15α-epoxy-5α-cholest-8-ene (2); 3β-acetoxy-5α-cholest-8(14)-ene-9α,15α-diol (3); and 3β-acetoxy-5α-cholesta-8(14),9-(11)-dien-15α-ol (4) were prepared as described previously (3).

Reactions with Hydrochloric Acid at Reflux

A solution of the steroid (300 mg) in ethanol (33 ml) and hydrochloric acid (2 ml, 37%) was refluxed under nitrogen for 8 hr. The solution was concentrated under reduced pressure, diluted with water and extracted with diethyl ether. The combined extracts were washed successively with aq. $NaHCO_3$ solution and water, dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield a residue which was then chromatographed (4) on silica (40-63 μm) with hexane/ethyl acetate (80:20, v/v) to separate aromatic sterols (5a) and (5b) from 3β-hydroxy-5α-cholest-8(14)-en-15-one (6a). The aromatic fraction was then rechromatographed on Silica Gel G/Celite/$AgNO_3$ (1:1:.3, w/w) with hexane/ethyl acetate (100:5, v/v) to separate the 2 epimeric aromatic compounds.

Reactions with Hydrochloric Acid at Room Temperature

A solution of the steroid (300 mg) in ethanol (33 ml) and hydrochloric acid (2 ml, 37%) was kept at room temperature for 30 min. At this time, the solvent was reduced to half of its volume under reduced pressure and cooled. Under these conditions, 3β-acetoxy-5α-cholesta-7,9(11),14-triene (7a) crystallized. The mother liquors, after filtration, were evaporated and chromatographed on Silica Gel G/Celite (1:1, v/v, eluting with hexane/ethyl acetate, 90:10, v/v) to afford 3β-acetoxy-5α-cholest-8(14)-en-15-one (6b).

Reactions with Excess *p*-Toluenesulfonic Acid

A solution of *p*-toluenesulfonic acid (0.5 g) in toluene (70 ml) was refluxed and part of the solvent (20 ml) was distilled off. The steroid (800 mg) was added and the solution refluxed for 30 min under nitrogen. The solution was washed successively with aq. $NaHCO_3$ and water, and evaporated to afford a residue which was purified by chromatography as just described for the reactions with hydrochloric acid at reflux.

Reactions with Catalytic Quantity of *p*-Toluenesulfonic Acid

The reaction was done as already described except that a catalytic quantity of *p*-toluenesulfonic acid (30 mg) was used for the same amount of sterol (800 mg).

Analysis of the Steroids

Ultraviolet (UV) spectrometry was performed using a Varian Model 635 UV-visible spectrophotometer. Infrared (IR) spectra were recorded for solutions in chloroform or for

nujol mulls using a Perkin-Elmer Model 157 IR spectrophotometer. Gas liquid chromatographic (GLC) examination of the sterols was on 1% SE-30 on Gas-Chrom Q (80-120 mesh) at 200 C on a Carlo Erba Model Fractovap 2101 gas chromatograph. Mass spectral analysis was performed on a Varian Mat 112 S spectrometer by direct inlet. Proton magnetic resonance (^{1}H NMR) spectra were obtained at 60 MHz at ambient temperature on a Perkin Elmer instrument, Model R-24, in $CDCl_3$ with $Si(CH_3)_4$ as internal standard. Routine optical rotations were recorded with a Perkin-Elmer Model 141 spectropolarimeter for 1% solutions in chloroform. The progress of all reactions was monitored by thin layer chromatography (TLC) on Silica Gel G (HF_{254}) microplates.

RESULTS

Treatment of 3β-acetoxy-5α-cholesta-8(14), 9(11)-dien-15α-ol (4) with hydrochloric acid at reflux after double chromatography gave (Fig. 1): (a) 3β-hydroxy-12-methyl-18-nor-5α,17β-(H)-cholesta-8,11,13-triene (5a) in which the original 3β-acetoxy group has been hydrolyzed, the 15α-hydroxy group has been eliminated, the angular methyl group (C-18) has migrated from C-13 to C-12; and the 17β-configuration of the side-chain has been inverted. Compound (5a) (178 mg from 300 mg of 4) resisted all efforts to crystallize and showed: IR 3500, 3330 cm^{-1}; UV λ_{max} (cyclohexane) 225 nm (log ε 4.07); ^{1}H NMR δ 0.55 (d, 3H, J = 6 Hz, 21-C$\underline{H}_3$), 1.10 (s, 3H, 19-C$\underline{H}_3$), 2.26 (s, 3H, 18-C$\underline{H}_3$), 3.3 (m, 1H, w1/2 ca. 12 Hz, 17β-$\underline{H}$), 3.67 (m, 1H, w1/2 ca. 20 Hz, 3α-$\underline{H}$), 6.94 (s, 1H, 11-$\underline{H}$): MS m/e 382 (M^+). Anal. calcd. for $C_{27}H_{42}O$: C, 84.8; H, 11.1. Found: C, 84.6; H, 11.0.

(b) 3β-Hydroxy-12-methyl-18-nor-5α,17α(H)-cholesta-8,11,13-triene (5b) (20 mg) epimer at C-17 of 5a. The compound crystallized from methanol and showed mp 97-99 C (lit. [5] gum) and showed: IR 3500, 3330 cm^{-1}; UV λ_{max} (cyclohexane) 225 nm (log ε 4.07); ^{1}H NMR δ 1.10 (s, 3H, 19-C$\underline{H}_3$), 2.26 (s, 3H, 18-C$\underline{H}_3$), 3.15 (m, 1H, w1/2 ca. 12 Hz, 17α-$\underline{H}$), 3.67 (m, 1H, w1/2 ca. 20 Hz, 3α-$\underline{H}$), 6.94 (s, 1H, 11-$\underline{H}$); MS m/e 382 (M^+). Anal. calcd. for $C_{27}H_{42}O$: C, 84.8; H, 11.1. Found: C, 84.5; H, 11.0. These chemicophysical properties, apart from the mp, agree well with those reported (5).

(c) 3β-Hydroxy-5α-cholest-8(14)-en-15-one (6a) (25 mg) which, upon crystallization from methanol, melted at 145-146 C and showed identical chemicophysical properties with an authentic sample (6).

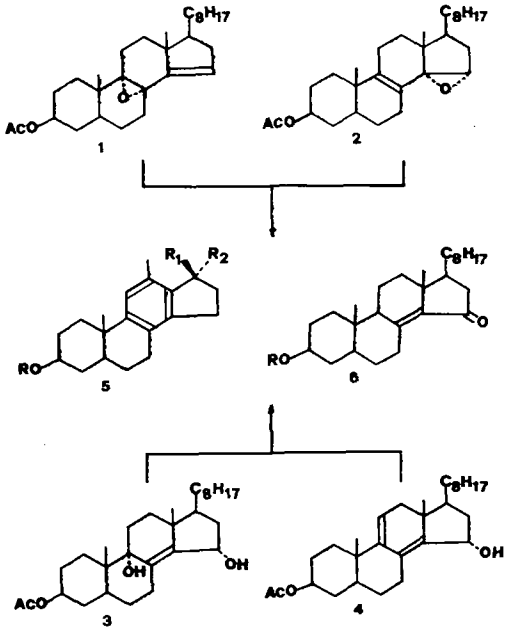


FIG. 1. Formation of compound 5a-e and 6a,b from 1-4. 5a, R = H, R_1 = H, R_2 = C_8H_{17}. 5b, R = H, R_1 = C_8H_{17}, R_2 = H. 5c, R = Ac, R_1 = C_8H_{17}, R_2 = H. 5d, R = Ac, R_1 = H, R_2 = C_8H_{17}. 5e, R = Ac, R_1 = $C_9H_{17}Br_2$, R_2 = H. 6a, R = H. 6b, R = Ac.

When compounds 1, 2 and 3 were subjected to the action of hydrochloric acid at reflux, essentially the same results were obtained. The yields of 5a were 70-80%, those of 5b 6-8%, and those of 6a 7-10%.

In order to isolate possible intermediates of the aromatization, 4 was treated with hydrochloric acid at room temperature. In these conditions, 3β-acetoxy-5α-cholesta-7,9(11),14-triene (7a) was obtained in 70% yield accompanied by a minor amount (10%) of 3β-acetoxy-5α-cholesta-8(14)-en-15-one (6b) (Fig. 2). 7a showed: mp 90-92 C (from methanol); $[\alpha]_D^{20}$ -89; UV λ_{max} (cyclohexane) 228 (log ε 3.99), 236 (4.01), and 268 nm (3.96); ^{1}H NMR δ 2.1 (s, 3H, OCOC$\underline{H}_3$), 4.7 (m, 1H, w1/2 ca. 20 Hz, 3α-$\underline{H}$), 5.5, 5.8, (m, 3H, 7-, 11-, and 15-$\underline{H}$); MS m/e 424 (M^+). Anal. calcd. for $C_{29}H_{44}O_2$: C, 82.0; H, 10.4. Found: C, 81.8; H, 10.2.

When the triene (7a) was refluxed for 30 min in hydrochloric acid, 3β-hydroxy-5α-cholesta-8,11,14-triene (8a) was obtained in 65% yield, accompanied by a trace amount of 5a and 5b (Fig. 2). Triene (8a), and oil, showed: UV λ_{max} 314 nm (log ε 3.8); ^{1}H NMR δ 3.6 (m, 1H, w1/2 ca. 20 Hz, 3α-$\underline{H}$), 6.0 (m, 1H, 15-$\underline{H}$), 6.1 (dd, 2H, J = 11 Hz, 11- and 12-$\underline{H}$); MS m/e 382 (M^+).

When 1, 2, 3, 4 and 7a were treated with excess *p*-toluenesulfonic acid at reflux, after the described work-up, 3β-acetoxy-12-methyl-18-nor-5α,17α(H)-cholesta-8,11,13-triene (5c) was obtained in 70% yield. The compound, oil, showed IR 1730 cm^{-1}; UV λ_{max} (cyclohexane) 225 nm (log ε 4.07); ^1H NMR δ 1.1 (s, 3H, 19-C$\underline{H}_3$), 2.02 (s, 3H, OCOC$\underline{H}_3$), 2.26 (s, 3H, 18-C$\underline{H}_3$), 3.15 (m, 1H, w1/2 ca. 12 Hz, 17α-$\underline{H}$), 4.75 (m, 1H, w1/2 ca. 20 Hz, 3α-$\underline{H}$), 6.92 (s, 1H, 11-$\underline{H}$); MS m/e 424 (M^+). Anal. calcd. for $C_{29}H_{44}O_2$: C, 82.0; H, 10.4. Found: C, 82.2; H, 10.5.

When catalytic amounts of *p*-toluenesulfonic acid were used in the aromatization reaction, a mixture of 5c and 3β-acetoxy-12-methyl-18-nor-5α,17β(H)-cholesta-8,11,13-triene (5d) was obtained in 1:1 ratio in 75% total yield. Compound 5d, oil, showed: UV λ_{max} (cyclohexane) 225 nm (log ε 4.07); ^1H NMR δ 0.56 (d, 3H, J = 6 Hz 21-C$\underline{H}_3$), 1.08 (s, 3H, 19-C$\underline{H}_3$), 2.00 (s, 3H, OCOC$\underline{H}_3$), 2.20 (s, 3H, 18-C$\underline{H}_3$), 3.3 (m, 1H, w1/2 ca. 12 Hz, 17β-$\underline{H}$), 4.8 (m, 1H, w1/2 ca. 20 Hz, 3α-$\underline{H}$), 6.94 (s, 1H, 11-$\underline{H}$); MS m/e 424 (M^+). Anal. calcd. for $C_{29}H_{44}O_2$: C, 82.0; H, 10.4. Found: C, 81.8; H, 10.3.

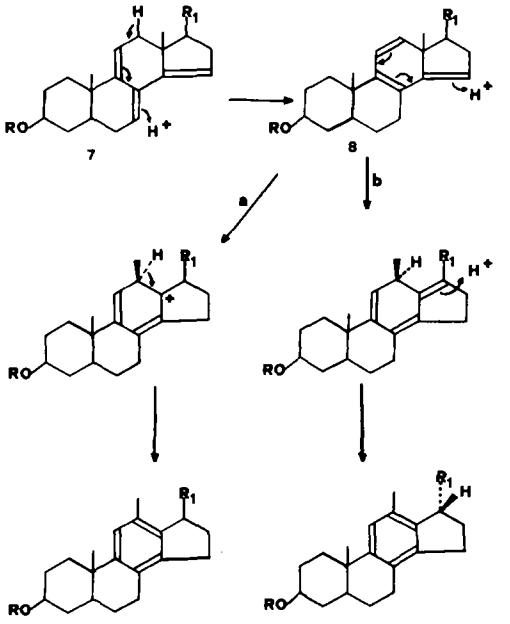


FIG. 2. Intermediates in the formation of ring C aromatic steroids: 7a, R = Ac, R_1 = C_8H_{17}. 7b, R = Ac, R_1 = $C_9H_{17}Br_2$. 8a, R = H, R_1 = C_8H_{17}. 8b, R = Ac, R_1 = C_8H_{17}.

DISCUSSION

Considerable work has been reported on the aromatization of ring A of steroids by molecular rearrangement or by elimination of the C-19 methyl group. On the contrary, relatively few methods have been reported on the aromatization of ring C of steroids having a 17β side chain. The original method of Margulis et al. (7) starting from a 7α,11α-dibromo-8-unsaturated sterol, was improved by Edmunds et al. (5), who demonstrated that, with an acidic catalyst, the dibromo unsaturated sterol is aromatized in high yield. Until now, this method represents the best route to ring C aromatic sterols.

We recently obtained (3) 2 monoepoxidation compounds, 3β-acetoxy-8α,9α-epoxy-5α-cholest-14-ene (1) and 3β-acetoxy-14α,15α-epoxy-5α-cholest-8-ene (2) by action of peracids on 3β-acetoxy-5α-cholesta-8,14-diene. Compound 2 was then transformed by silica in a mixture of 3β-acetoxy-5α-cholest-8(14)-ene-9α,15α-diol (3) and 3β-acetoxy-5α-cholesta-8-(14),9(11)-dien-15α-ol (4).

As it was considered probable that dehydration of the allylic hydroxy group present in compound 4 would yield molecular rearrangement able to produce aromatization of the ring C, we treated compound 4 with hydrochloric acid in ethanol at reflux; under these conditions, we obtained as a major product 3β-hydroxy-12-methyl-18-nor-5α,17β(H)-cholesta-8,11,13-triene (5a, 72%) accompanied by a minor amount of 3β-hydroxy-12-methyl-18-nor-5α,17α-(H)-cholesta-8,11,13-triene (5b) (8%) and 3β-hydroxy-5α-cholesta-8(14)-en-15-one (6a, 10%). The success of this reaction in giving the aromatization of ring C prompted us to subject the other products obtained in the epoxidation of 3β-acetoxy-5α-cholesta-8,14-diene and structurally related to compound 4 to the same acidic treatment. In all cases, the same pattern of reaction products was observed.

In order to clarify the mechanism of this new rearrangement leading to ring C benzenoid steroids, we treated the same sterols with hydrochloric acid at room temperature. Under these conditions, 3β-acetoxy-5α-cholesta-7,9-(11),14-triene (7a) was obtained in crystalline form. The formation of 7a was also observed in the reaction by GLC-mass spectrometry. A similar triene (7b) was also obtained by Edmunds et al. (5) in the ring C aromatization of 7α,11α-dibromo unsaturated steroids. So, it is clear that despite the different starting compound, the aromatization involves a similar intermediate.

Treatment of the triene (7a) with hydrochloric acid at reflux afforded, after 30 min, a new conjugated triene (8a) which was isolated and characterized by its chemicophysical prop-

erties. Pursuing the reflux with hydrochloric acid, the triene (8a) was completely transformed into the aromatic sterols 5a and 5b in a 9:1 ratio.

In view of the fact that Edmunds et al. (5) obtained the corresponding ring C aromatic compound (5e) with the "natural" side chain by treatment of 7b with excess of *p*-toluenesulfonic acid, we considered the possibility of obtaining compound (5c) by treatment of 4 with excess *p*-toluenesulfonic acid. Under these conditions, compound 5c was obtained as a major product, accompanied by a minor amount of 5d and 6b. When a catalytic amount of *p*-toluenesulfonic acid was used, compounds 5c and 5d were obtained in about the same ratio. The same results were obtained when compounds 1, 2 and 3 were subjected to these reactions. Monitoring the reactions by GLC-MS, the intermediary formation of trienes 7a and 8b was observed. From these results, it is clear that the aromatization with hydrochloric acid and *p*-toluenesulfonic acid proceeds through the formation of trienes as 7 and 8. However, hydrochloric acid aromatizes 8 with epimerization of the side chain whereas *p*-toluenesulfonic acid causes aromatization with prevalent retention of the side chain configuration, the extent of the epimerization being dependent on the concentration of *p*-toluenesulfonic acid (5). A reasonable mechanism for the aromatization of a triene as 7 was formulated by Edmunds et al. (5) (Fig. 2). The isolation of triene 8a strongly supports this mechanism, which appears the most reasonable for the formation of the aromatic compound with a natural side-chain stereochemistry. However, formation of the 17-epimeric compound could be more complex. In fact, some years ago, we (8) and Aberhart et al. (9) demonstrated that hydrogen chloride treatment of 3β-acetoxy-5α-cholest-14-ene causes side-chain inversion through the formation of a spirocompound. Accordingly, such a mechanism could be supposed to explain the side-chain inversion in the present aromatization. Work is in progress to evaluate this possibility.

ACKNOWLEDGMENTS

This research was supported by Ministero della Pubblica Istruzione. We thank Professor G. Galli, Institute of Pharmacology and Pharmacognosy, University of Milan, for mass spectra.

REFERENCES

1. Khanapure, S.P., Hazra, B.G., and Das, K.G. (1981) J. Chem. Soc. Perkin Trans. I, 1360-1362.
2. Dalzell, H.C., Manmade, A., Mastrocola, A.R., and Razdan, R.K. (1979) J. Org. Chem. 44, 2457-2461.
3. Anastasia, M., Allevi, P., Fiecchi, A., and Scala, A. (1981) J. Org. Chem. 46, 3265-3267.
4. Still, W.C., Kahn, M., and Mitra, A. (1978) J. Org. Chem. 43, 2923-2925.
5. Edmunds, R., Midgley, J.M., Tagg, L.G., Wilkins, B.J., and Whalley, W.B. (1978) J. Chem. Soc. Perkin Trans. I, 76-80.
6. Anastasia, M., Fiecchi, A., and Scala, A. (1979) J. Chem. Soc. Perkin Trans. I, 1821-1824.
7. Margulis, T.N., Hammer, C.F., and Stevenson, R. (1964) J. Chem. Soc. 4396-4400.
8. Anastasia, M., Fiecchi, A., and Scala, A. (1978) J. Org. Chem. 43, 3505-3508.
9. Aberhart, D.J., Cahn, T.Y., and Caspi, E. (1979) J. Chem. Soc. Perkin Trans. I, 220-224.

[Received August 17, 1981]

The Role of Squalene Synthetase in the Inhibition of Tetrahymanol Biosynthesis by Cholesterol in *Tetrahymena pyriformis*

COLIN F. WARBURG, MARWA WAKEEL and **DAVID C. WILTON***, *Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, SO9 3TU U.K.*

ABSTRACT

The biosynthesis of the triterpenoid alcohol tetrahymanol by *Tetrahymena pyriformis* is rapidly inhibited by the addition of cholesterol to the growth medium. The primary site of this inhibition by cholesterol has been established to be at the level of the enzyme squalene synthetase. The protein synthesis inhibitor cycloheximide produces an identical decline in squalene synthetase activity to that of cholesterol and the half-life of the enzyme is about 50 minutes. No direct inhibition of the enzyme is observed and suggests that cholesterol inhibits the actual synthesis of the enzyme squalene synthetase. Farnesol is accumulated during in vitro incubations derived from cells grown in the presence of cholesterol or cycloheximide.
Lipids 17:230-234, 1982.

The eukaryotic protozoan *Tetrahymena pyriformis* is well established as an excellent biological system for studying various aspects of membrane biochemistry, including membrane biogenesis, membrane structure, membrane adaptation and the properties of membrane-bound enzymes (1). Of particular interest to us is the fact that the membranes of *T. pyriformis* contain a unique triterpenoid alcohol, tetrahymanol (2), which the organism is able to biosynthesize via the mevalonic acid pathway. Tetrahymanol is located primarily in the limiting membranes of this organism (3,4) and the function of tetrahymanol appears to be similar to that of sterols, in particular cholesterol, in the membranes of higher organisms. The similarity in the function of tetrahymanol to that of cholesterol is confirmed by the observation that this otherwise essential membrane component can be replaced by a variety of sterols including cholesterol and ergosterol. In the presence of these sterols, tetrahymanol biosynthesis is completely inhibited (5-8) and, in the case of ergosterol, is replaced quantitatively in the external membranes of the cell by the added sterol (3,9). This regulation of tetrahymanol biosynthesis by added sterols is particularly unusual because, if tetrahymanol is added to the culture medium, it is taken up by the cells and does not inhibit its own biosynthesis (6,10).

Preliminary studies on the precise mechanism of regulation of tetrahymanol biosynthesis by added sterols, in particular cholesterol, has revealed that the inhibition occurs at a step after mevalonic acid in the pathway and possibly at the level of the enzyme squalene synthetase (6). The enzyme β-hydroxy-β-methylglutaryl CoA reductase is not affected by growth of this organism in the presence of cholesterol (6).

The results described in this present paper identify the enzyme squalene synthetase as a primary site of this regulation and strongly suggest that the regulation is the result of the inhibition of synthesis of this enzyme.

MATERIALS AND METHODS

[^{3}H] Squalene or [^{14}C] squalene was prepared by the incubation of sodium [^{3}H] acetate or sodium [^{14}C] acetate with a 10,000 × g supernatant fraction from rat liver under anaerobic conditions and was purified by thin layer chromatography (TLC). [^{3}H] Farnesyl pyrophosphate was prepared by the incubation of [2-^{3}H] mevalonic acid with a 30-60% saturation $(NH_4)_2SO_4$ fraction from rat liver cytosol (11) and was purified by TLC using propanol/ammonia/water (6:3:1, v/v). [^{14}C] Tetrahymanol was prepared by the incubation of [^{14}C]-acetate (100 μCi) with *T. pyriformis* in 100 ml culture for 16 hr followed by extraction and purification (6).

T. pyriformis strain W was grown in a medium containing bacteriological peptone (2%), glucose (0.5%), yeast extract (0.1%) and ethylenediaminetetraacetic acid ferric monosodium salt (0.003%). Cultures were grown in 100 ml of medium in 500-ml Erlenmeyer flasks, maintained at 28 C and shaken at 200 rpm. Stationary phase of growth was reached after 26 hr. Cell numbers were determined with a Coulter counter. Purified cholesterol (15 μg ml^{-1}) was added to cultures dissolved in a minimal

*Address for correspondence.

amount of ethanol. Control cultures received ethanol only. Cycloheximide (5 $\mu g\ ml^{-1}$) was added in a similar manner.

Cultures were harvested by centrifugation and the pellet suspended in 3 ml of 0.1 M phosphate buffer (pH 7.4) containing nicotinamide (30 mM), $MgCl_2$ (4 mM) and 2-mercaptoethanol (5 mM). Cells were disrupted by homogenization in a tight-fitting Teflon-glass homogenizer or by sonication using an ultrasonic cleaning bath. The homogenate was centrifuged at 40,000 x g for 7.5 min and the supernatant used for assays. For some experiments, microsomal and postmicrosomal supernatant fractions were prepared by centrifugation at 105,000 x g for 1 hr.

When incorporation of mevalonate into tetrahymanol was measured, incubations contained $NADP^+$ (0.2 μmol), glucose 6-phosphate (4 μmol), glucose 6-phosphate dehydrogenase (1 unit), ATP (4 μmol), potassium [2-^{3}H] mevalonate (2.5 μCi) and homogenate (0.8 ml) in a final vol of 1 ml. Incubations for the incorporation of isopentenylpyrophosphate contained [1-^{14}C] isopentenylpyrophosphate (0.2 μCi). In both cases, incubation was at 28 C for 30 min (tetrahymanol formation was linear for at least 60 min) and incubations were terminated by the addition of 1 ml of 10% methanolic KOH (v/v). Radioactive tetrahymanol was isolated as described previously (6).

In order to assay squalene synthetase activity, a 10,000 x g supernatant from tetrahymena was prepared as already described. The assay mixture contained [^{3}H] farnesyl pyrophosphate [2 Ci/mmol, 100,000 dpm], $NADP^+$ (0.2 μmol), glucose 6-phosphate (4 μmol), glucose 6-phosphate dehydrogenase (1 unit), [10 μg of carrier squalene] and enzyme 2-20 μl in a total vol of 1 ml. Incubations were at 28 C for 15 min (linear up to 30 min) and terminated by the addition of 1 ml ethanol and 5,000 dpm of [^{14}C] squalene. This was extracted with 3 ml light petroleum (40-60 C)/Et_2O (1:1, v/v). The extract was dried and applied to a Silica Gel G thin layer plate and developed with light petroleum (60-80 C)/acetone (4:1, v/v) and the region of the plate corresponding to authentic squalene was scraped directly into a scintillation vial and assayed for radioactivity.

Because in *T. pyriformis* squalene synthetase activity is located in the cytosolic (105,000 x g supernatant) fraction of the cell (Warburg, C.F., and Wilton, D.C., unpublished data), this fraction was used for some enzyme assays.

RESULTS AND DISCUSSION

The addition of cholesterol to growing cultures of *T. pyriformis* results in about a 90% inhibition of tetrahymanol biosynthesis in vivo from radioactive acetate within 3 hr of addition of the sterol to the growth medium (6). We have therefore investigated in detail the mechanism of inhibition over this 3-hr period using cell-free preparations that are able to readily convert mevalonic acid and other postmevalonic acid precursors into tetrahymanol in order to identify the primary site of the inhibition by cholesterol. Initially, the 10,000 x g supernatants from homogenate derived from control cultures or cells grown in the presence of cholesterol for 3 hr were incubated with [2-$^3H_2$]-mevalonic acid and [1-^{14}C] isopentenylpyrophosphate for 30 min. The extracted lipids were separated by argentation TLC and typical radioscans are shown in Figure 1. These radioscans illustrate in a semiquantitative fashion the effect of growth in the presence of cholesterol on tetrahymanol biosynthesis in vitro. Thus, there is a large decrease in the incorporation of radioactivity into squalene and tetrahymanol and a corresponding increase into a more polar compound. This compound has an identical R_f to that of farnesol after TLC separation in 3 different systems including argentation chromatography. The other minor peaks have not been identified. The tritium-to-carbon (T/C) ratio of radioactivity in the farnesol was identical to that in the squalene and tetrahymanol of the control incubations indicating that the site of the inhibitory effect of cholesterol on tetrahymanol biosynthesis was after isopentenylpyrophosphate in the pathway.

The accumulation of radioactive farnesol in the incubations from cholesterol-grown cells is consistent with an inhibition of the enzyme squalene synthetase and a resulting accumulation of farnesyl pyrophosphate. This farnesyl pyrophosphate is readily hydrolyzed by the high level of phosphate activity that is present in homogenates of *T. pyriformis* (Wakeel, M., and Wilton, D.C., unpublished data).

It has been observed previously (6) that the time course in vivo of the rate of decline of tetrahymanol biosynthesis produced by cholesterol is very similar to the decline of biosynthesis resulting from the addition of cycloheximide to the culture medium and suggested that cholesterol was having its effect by inhibiting protein synthesis. The similarity of the effects of cholesterol and cycloheximide is very clearly shown in Figure 1. Thus, treatment of cells with cycloheximide for 3 hr also resulted in over 90% inhibition of tetrahymanol biosynthesis in vitro and a corresponding accumulation of radioactivity in farnesol from mevalonic acid or isopentenylpyrophosphate. This apparent in-

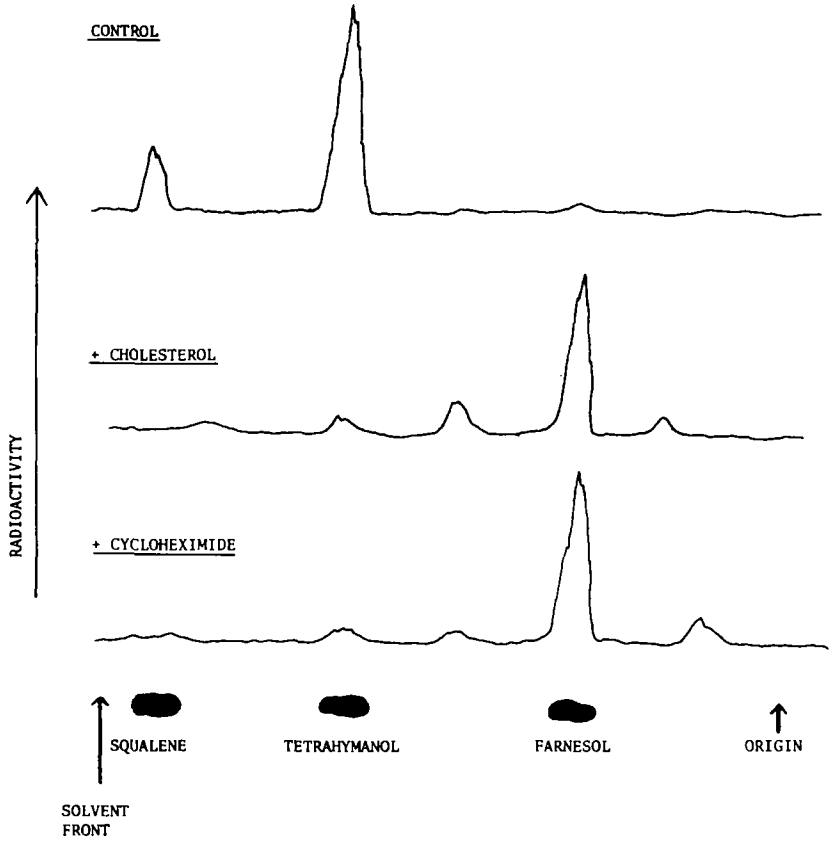


FIG. 1. Thin layer radioscan of intermediates of tetrahymanol biosynthesis obtained from radioactive mevalonic acid and isopentenylpyrophosphate. A 10,000 X g supernatant from homogenates of tetrahymena prepared from cells that had been grown for 3 hr in the absence or presence of either cholesterol (15 $\mu g\ ml^{-1}$) or cycloheximide (5 $\mu g\ ml^{-1}$) was incubated for 30 min with [2-$^3H_2$]mevalonic acid and [1-^{14}C]isopentenylpyrophosphate. The extracted nonsaponifiable lipids were separated by TLC using $AgNO_3$-impregnated silica gel plates and scanned for radioactivity.

hibition of squalene synthetase by over 90% by cycloheximide treatment suggests that this enzyme may have a particularly rapid turnover in *T. pyriformis*.

In order to confirm that the enzyme squalene synthetase is the primary site for the inhibition of tetrahymanol biosynthesis by both cholesterol and cycloheximide, the activity of the enzyme was assayed in parallel to that of the overall pathway from mevalonic acid to tetrahymanol. These in vitro measurements were performed at various time intervals after the addition of either cholesterol or cycloheximide to growing cultures of *T. pyriformis*. The results were plotted in a semilogarithmic form (Fig. 2) as a percentage of the control activity and clearly show that there is no significant difference between the rate of decline of activity produced by either cholesterol or cycloheximide. Moreover, when the rate of decline produced by the 2 inhibitors was calculated (Table 1), it was found that both the squalene synthetase activity and the overall biosynthetic activity of the pathway from mevalonic acid declined at the same rate with half-lives of about 50 min.

These results strongly support the hypothesis that the addition of cholesterol to cultures of *T. pyriformis* cause a rapid cessation of tetrahymanol biosynthesis due, at least in part, to an inhibition of the synthesis of the enzyme squalene synthetase.

If the decline in squalene synthetase activity caused by cholesterol is the result of an inhibition of enzyme synthesis, then cholesterol will produce no further decline in squalene synthe-

tase activity other than that resulting from cycloheximide treatment because cycloheximide concentrations that produce a maximal inhibition of protein synthesis are used. This lack of additive effect of the 2 treatments is demonstrated in Table 2 where squalene synthetase activity was assayed after treatment in vitro for 1 hr with cholesterol and cycloheximide.

In order to exclude the possibility that a metabolite is produced in cells grown in the presence of cholesterol that is directly inhibiting the activity of the enzyme squalene synthetase, the cytosol of control and cholesterol-grown cells was mixed and the activity of the mixture was compared with each individual activity as shown in Table 3. It can be seen that the cytosol from cholesterol-grown cells has no significant effect on the squalene synthetase activity in the cytosol of control cells and, hence, the loss of squalene synthetase activity in the cholesterol-grown cells cannot be due to the presence of a reversible inhibitor.

The results just described clearly establish that the mechanism by which cholesterol is able to specifically regulate tetrahymanol production is primarily at the level of the enzyme squalene synthetase. Moreover, results using the protein synthesis inhibitor cycloheximide strongly suggest that the activity of this enzyme is regulated by controlling its rate of synthesis. This enzyme represents a logical step for control because it immediately follows a branch point in the pathway leading to the formation of other mevalonic-acid-derived compounds such as ubiquinones and dolichol phosphates. An inhibition of squalene synthetase would still allow the biosynthesis of these other essential cellular components. Recent work with mammalian systems (12) suggests that this enzyme may be an important secondary control point in cholesterol biosynthesis after an initial inhibition of the normally rate-limiting enzyme, β-hydroxy β-methylglutaryl CoA (HMG CoA) reductase.

As in the case of HMG CoA reductase in mammalian systems, the precise molecular

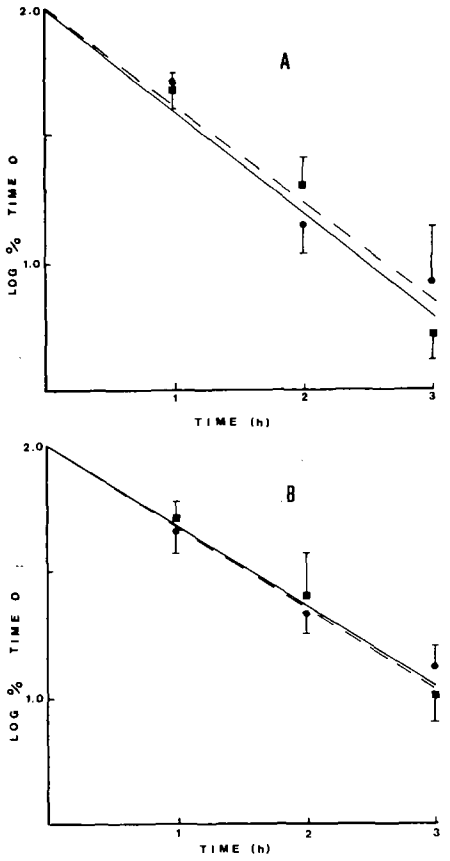


FIG. 2. Time course of the effect of cholesterol or cycloheximide on tetrahymanol biosynthesis and squalene synthetase activity. 10,000 × g supernatants of tetrahymena homogenates were prepared at various times after addition of either cholesterol (15 μg ml^{-1}) or cycloheximide (5 μg ml^{-1}) to cultures (100 ml) in vivo. The activity of the cultures in terms of (A) conversion of [2-$^3H_2$]mevalonic acid to tetrahymanol and (B) squalene synthetase activity was measured. All values are expressed as a percentage of the activity of the time 0 control. There was normally no significant change in the control values over the time course of the experiment. ■——■, + cholesterol; ●– – –●, + cycloheximide.

TABLE 1

Calculated Half-Lives for the Decline of the Conversion of Mevalonic Acid into Tetrahymanol and Squalene Synthetase Activities Resulting from Treatment with Either Cholesterol or Cycloheximide

Activity	*Cholesterol treatment* ($t_{½}$ in min)[a]	*Cycloheximide treatment* ($t_{½}$ in min)
Mevalonic acid → tetrahymanol	45 ± 4	48 ± 7
Squalene synthetase	57 ± 8	59 ± 7

[a]The half-lives were calculated from the data shown in Fig. 2.

TABLE 2

Effects of Cholesterol and Cycloheximide on Squalene Synthetase Activity in *T. pyriformis*[a]

Addition	% Inhibition of squalene synthetase
Cholesterol	62 ± 8
Cycloheximide	71 ± 8
Cholesterol + cycloheximide	67 ± 5

[a]Cultures were grown for 1 hr in the absence or presence of either cholesterol (15 $\mu g\ ml^{-1}$) or cycloheximide (5 $\mu g\ ml^{-1}$) or both. Squalene synthetase activity was measured with 10,000 X g supernatant of homogenate of the cultures and the percentage inhibition as compared to the control cultures was determined.

TABLE 3

Effect of Mixing Cytosols from Control and Cholesterol-Grown Cells[a] on Squalene Synthetase Activity

Incubation	Squalene synthetase activity (% of [a])
Control cytosol (a)	100
Cholesterol cytosol (b)	6 ± 3
(a) + (b)	95 ± 6

[a]Cultures were grown ± cholesterol for 3 hr. Squalene synthetase activity was assayed in the 105,000 X g supernatant (cytosol).

mechanisms of inhibition of squalene synthetase by cholesterol remains to be established. The short half-life of about 50 min is much less than many regulatory enzymes for which activity is apparently controlled by enzyme synthesis and degradation, and suggests the possibility that this loss of activity might be due to a rapid irreversible modification of the enzyme prior to normal protein degradation by intracellular proteolysis. Such a modification could be produced by a covalent change such as phosphorylation of the enzyme or by specific binding of an effector to or loss of an effector from the enzyme. In the case of the effect of cholesterol feeding on HMG CoA reductase activity in rat liver, it would now appear that the initial rapid step is enzyme phosphorylation followed by another as yet unidentified regulatory event (13). More detailed studies of the enzymology of squalene synthetase in *T. pyriformis* are therefore required to evaluate the possible involvement of other types of regulatory mechanisms.

The studies described here have identified a primary role for the enzyme squalene synthetase in the regulation of tetrahymanol biosynthesis by cholesterol. We are, at present, investigating the possible role of this enzyme in the regulation of tetrahymanol biosynthesis in *T. pyriformis* under other physiological conditions that affect cell growth.

REFERENCES

1. Thompson, G.A., and Nozawa, Y. (1977) Biochim. Biophys. Acta 472, 55-92.
2. Mallory, F.B., Gordon, J.T., and Conner, R.L. (1963) J. Am. Chem. Soc. 85, 1362-1363.
3. Conner, R.L., Mallory, F.B., Landrey, J.R., Ferguson, K.A., Kaneshiro, E.S., and Ray, E. (1971) Biochem. Biophys. Res. Commun. 44, 995-1000.
4. Nozawa, Y., and Thompson, G.A., Jr. (1971) J. Cell Biol. 49, 712.
5. Conner, R.L., and Landrey, J.R., Burns, C.H., and Mallory, F.B. (1968) J. Protozool. 15, 600-605.
6. Beedle, A.S., Munday, K.A., and Wilton, D.C. (1974) Biochem. J. 142, 57-64.
7. Conner, R.L., Landrey, J.R., Joseph, J.M., and Nes, W.R. (1978) Lipids 13, 692-696.
8. Rogers, J., Lee, A.G., and Wilton, D.C. (1979) Biochim. Biophys. Acta 552, 23-37.
9. Nozawa, Y., Fukushima, H., and Iida, H. (1975) Biochim. Biophys. Acta 406, 248-263.
10. Mallory, F.B., and Conner, R.L. (1970) Lipids 6, 149-153.
11. Popjak, G. (1969) in Methods in Enzymology (Clayton, R.B., ed.), Vol. 15, pp. 443-448, Academic Press, New York, NY.
12. Faust, J.R., Goldstein, J.L., and Brown, M.S. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5018-5022.
13. Arebalo, R.E., Hardgrave, J.E., and Scallen, T.J. (1981) J. Biol. Chem. 256, 571-574.

[Received August 17, 1981]

A ^{1}H NMR Anomaly for Steroidal Alcohols? Reactions of a Steroidal γ-Ketoacid

DAVID M. PIATAK*, KIRK A. ASHLINE, GEORGE ANGELOS and REBECCA P. SWENSON, *Department of Chemistry, Northern Illinois University, DeKalb, IL 60115,* and ELIAHU CASPI, *Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545*

ABSTRACT

The chemical and spectroscopic properties of several reaction products of 17β-hydroxy-1,3-seco-2-nor-5α-estran-1-oic acid and 17β-hydroxy-1,3-seco-2-nor-4-oxo-5α-estran-1-oic acid were examined and found to be atypical. For instance, the methyl ester of the first acid was resistant to basic hydrolysis conditions but partly hydrolyzed with 100% H_2SO_4. Reduction of the ester by $LiAlH_4$ gave 1,3-seco-2-nor-5α-estrane-1,17β-diol from which diacetate, ditosylate and 17-monotosylate derivatives were prepared. The C-1 methylene protons of each appeared as a singlet in 60, 100 and/or 270 MHz NMR spectra. The methyl ester and the diacetate of the diol were synthesized by alternate methods to verify the assigned structures. A 470-MHz spectrum eventually resolved the C-1 methylene protons of the monotosylate into the AB portion of an ABX pattern, further confirming the assigned structures. Also, 2,3-seco-1-oxo-5α-estran-17β-ol 17-nitrate was synthesized.
Lipids 17:235-240, 1982.

During an investigation (1) of the action of $Pb(OAc)_4$-$BF_3 \cdot Et_2O$-ROH on the steroidal levulinic acid **1a** (2), we had occasion to prepare 4-deoxo acid **1b** by Huang-Minlon reduction of the ketone function of **1a**. Reduction of levulinic acid had been studied by Wolff (3) in his original experiments, so no unusual results were expected. As anticipated, deoxo acid **1b** formed in fair yield accompanied by pyridazone 2 (4,5), a heterocyclic by-product frequently formed from levulinic acids and hydrazines (6). However, while performing additional chemistry with **1b**, it was noted that the chemical and spectroscopic characteristics of some of the various reaction products were rather atypical and further studies were made to examine these observations.

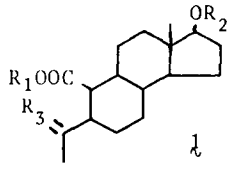
RESULTS AND DISCUSSION

Initially, treatment of deoxo acid **1b** with ethereal CH_2N_2 afforded methyl ester **1c** which was acetylated to ester acetate **1d**. Numerous attempts to base hydrolyze **1d** back to **1b**, even under vigorous conditions (KOH; dioxane; reflux 18 hr), resulted only in removal of the 17-acetate moiety and formation of methyl ester **1c**. On the other hand, the 100% H_2SO_4 procedure (7), normally employed for the hydrolysis of tertiary or highly hindered esters, did give partial hydrolysis to **1b**. A mass spectrum of **1c** revealed the appropriate molecular weight and fragmentations consistent with the proposed structure, and a 270-MHz nuclear magnetic resonance (NMR) spectrum had an appropriate multiplet at δ 2.1 for the C-10 proton. Nevertheless, because the hydrolysis results were typical of a tertiary, rather than a secondary ester, further confirmation of structure **1c** was sought.

Ester **1c** was first reduced by $LiAlH_4$ to diol **3a**, which should have had an NMR complex multiplet for the C-1 methylene protons if the ester structure was as depicted. Diol **3a** was then converted to diacetate **3b**, ditosylate **3c**, and monotosylate **3d** to facilitate solubility of the diol for NMR analysis and to provide a range of data for its C-1 methylene protons. Unexpectedly, NMR spectra (60, 100 and/or 270 MHz) of the 3 derivatives had apparent singlets ($W_{½h} \sim 2$ Hz) for the C-1 methylene protons and even remained as such in a 60-MHz spectrum of diacetate **3b** with pyridine as the solvent. The acetate methyl protons in this latter spectrum did, however, separate into 2 distinct singlets although only an unresolved singlet for the C-1 methylene protons appeared in $CDCl_3$ solution.

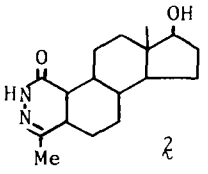
Because these observations, coupled with the hydrolysis information on ester **1d**, did not coincide with what was expected, alternate preparations were undertaken to firmly establish the structures of the various compounds involved. First, the ketoester **1e** (2) was condensed with ethanedithiol to yield thioketal **1f**. An infrared (IR) spectrum of the product was devoid of a band at 1700 cm^{-1}, and an NMR spectrum had singlets at δ 1.80 for the methyl group on C-4 and at δ 3.28 for the 4 protons on the thioketal ring. Reductive removal of the thioketal ring with sponge nickel catalyst then gave ester acetate **1d**, identical in all respects to **1d** prepared via the Huang-Minlon reaction. In addition, it could be hydrolyzed only to ester **1c** and converted to diol diacetate **3b**, both of which were identical

in all respects to those just described. Alternatively, diacetate **3b** was obtained by sponge nickel desulfurization of thioketal derivative **4a**, synthesized by $LiAlH_4$ reduction of thioketal ester **1f**, then by acetylation. Interestingly, the thioketal ring protons of **4a** were split into 2 doublets which was not the case for these protons in ester thioketal **1f**. Inspection of molecular models reveals that the acetate carbonyl group in **4a** can come into close proximity to the thioketal ring, thus influencing the 2 protons of the thioketal ring on the side of the carbonyl group differently than the 2 on the opposite side of the ring. In

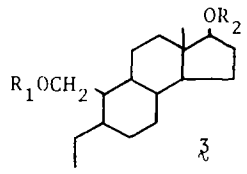


1

a. $R_1=R_2=H;R_3=O$
b. $R_1=R_2=H;R_3=H_2$
c. $R_1=Me;R_2=H;R_3=H_2$
d. $R_1=Me;R_2=Ac;R_3=H_2$
e. $R_1=Me;R_2=Ac;R_3=O$
f. $R_1=Me;R_2=Ac;R_3=$ 
g. $R_1=H;R_2=Ac;R_3=H_2$

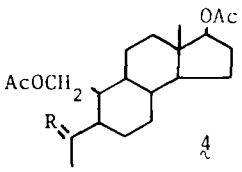


2



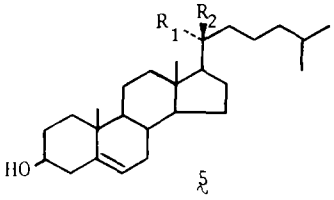
3

a. $R_1=R_2=H$
b. $R_1=R_2=Ac$
c. $R_1=R_2=Ts$
d. $R_1=H;R_2=Ts$



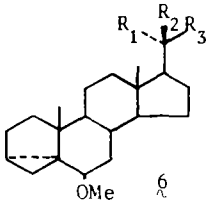
4

a. R= 
b. R=AcO,H



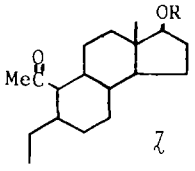
5

a. $R_1=CH_2OH;R_2=H$
b. $R_1=H;R_2=CH_2OH$



6

a. $R_1=Me;R_2=H;R_3=CH_2OH$
b. $R_1=H;R_2=Me;R_3=CH_2OH$
c. $R_1=Me;R_2=H;R_3=CH_2OAc$
d. $R_1=H;R_2=Me;R_3=CH_2OAc$
e. $R_1=Me;R_2=H;R_3=CHO$



7

a. R=H
b. $R=NO_2$

FIG. 1

1f, however, the ester carbonyl moiety is distant enough from the thioketal protons that it has no effect. With all 3 routes yielding diacetate **3b**, it seems unlikely any skeletal rearrangement occurred and ester **1c** has the secondary ester structure as depicted.

The apparent singlet was finally resolved in a 470-MHz spectrum of monotosylate **3d**. The C-1 methylene protons appeared as an AB portion of an ABX system centered at δ 3.703 with $J_{AX} = J_{BX} = 2.1$ Hz and $J_{AB} = 11.5$ Hz. This rather low coupling constant readily explains the inability of lower field instrumentation to thoroughly resolve the apparent singlet. It is not what was anticipated for this structure as the C-1 methylene protons for thioketal diacetate **4a** and for triacetate **4b** had J = 7 Hz, values more consistent with alcohol systems.

The small coupling constants for the C-1 protons on **3b-d** can be best explained with a conformation about the 1(10) bond in which the oxygen moiety on C-1 is situated anti to the axial hydrogen on C-10 or, perhaps, one in which both eclipse one another. These conformers provide a minimal amount of interaction of the C-1 substituents with the C-5 ethyl group and the 11α-hydrogen, and the resultant dihedral angles approach those predicted from the coupling constant by the vicinal Karplus correlation. When a thioketal or acetoxyl group is present on C-4 as in **4a-b**, however, the C-1 acetoxyl moiety is probably forced toward C-11 either by the bulkiness and/or by polar repulsive effects of the substituents on both C-1 and C-4. In this conformation, though, the expected ABX pattern for the C-1 protons, where $J_{gauche} = 3$ Hz and $J_{anti} = 11$ Hz would arise, seems to have been averaged to J = 7 Hz because the chemical shifts of both protons have been equalized.

A similar situation has been noted for (20R)-21-hydroxycholesterol (**5a**) which has a reported singlet at δ 3.70 for the C-21 protons, whereas the 20S isomer **5b** has a multiplet at δ 3.62 for the same protons (8). Again, these observations are quite different for the same set of protons in a differently substituted set of C-20 epimeric alcohols **6a** and **6b** and their corresponding acetates **6c** and **6d**. These had typical ABX coupling with $J_{AX} = 3.5$, $J_{BX} = 7.0$, and $J_{AB} = 11$ Hz for the C-22 methylene protons in all 4 compounds. Each set of epimers had the characteristic chemical shift difference exhibited by C-21 methyl protons of C-20 epimers (9) and a similar chemical shift difference for the C-22 methylene protons.

Analogous dissimilarities in the coupling of primary alcohol protons with the proton of an adjacent asymmetric center have also been reported with 29-hydroxylupane derivatives (10; J.S. Pyrek, private communication). In these instances, one epimer (20S) had the C-29 methylene protons split into a doublet with J = 6.9 Hz and the other (20R) had an ABX pattern with $J_{AX} = 4.4$ and $J_{BX} = 7.0$ Hz.

Exactly what chemical, structural and stereochemical effects contribute to the small differences in coupling constants resulting in an apparent singlet remain to be determined. We hope to better evaluate the effect through additional examples with certain systematic changes in the groups adjacent to the alcohol protons and through NMR temperature studies.

The acetyl analog (**7a**) was also prepared from acid acetate **1g** via its acid chloride and Me_2CuLi. Its structure was verified by an IR band at 1700 cm^{-1} and an NMR singlet at δ2.10 equal to 3 protons. The corresponding 17-nitrate ester **7b** prepared with HNO_3/Ac_2O was exposed to peracids under various conditions of Baeyer-Villiger oxidation in order to secure an acetate moiety at C-10, but only starting material was recovered. Apparently, as with base hydrolysis of ester **1c**, nucleophilic attack of the C-1 carbonyl moiety is sterically hindered.

EXPERIMENTAL

Melting points were determined on a Fisher-Johns mp apparatus and are not corrected. IR spectra were performed with a Perkin-Elmer 237B spectrometer on solids incorporated in KBr discs. A JEOL-106 EC 100 PFT NMR spectrometer was used to record ^{1}H NMR spectra, except as noted. Otherwise, spectra were taken with a Bruker HX-270, a Varian A60-A, or a Nicolet NTC470 instrument. All NMR were performed on $CDCl_3$ solutions with TMS as an internal reference. Elemental analyses were done with a Perkin-Elmer 240 elemental analyzer by P. Rider of Northern Illinois University.

Huang-Minlon Reduction of Acetyl Acid 1a

A mixture of acetyl acid **1a** (2) (1.1 g), KOH (0.55 g), and diethylene glycol (15 ml) was warmed until the solids dissolved. Hydrazine hydrate (100%; 40 ml) was added, and the mixture was refluxed for 3 hr. The temperature of the reaction was then elevated to 200-210 C by distilling off the lower boiling components, and the reaction was refluxed for 12 hr. After cooling, it was diluted with water and extracted with ether. A neutral fraction (0.58 g) was obtained and identified as pyridazinone

2 (4,5).

Acidification of the basic solution and recovery of the acid with EtOAc yielded 0.42 g of acid **1b**. Repeated recrystallization from acetone/pentane gave a pure sample of **1b**, mp 145-153 C (sint); IR 3300, 2610, 1680 cm^{-1}. Anal. calcd. for $C_{17}H_{28}O_3$: C, 72.82; H, 10.06. Found: C, 72.43; H, 9.84.

Methyl Ester 1c

(a) Hydroxy acid **1b** (100 mg) was methylated by etheral CH_2N_2 to give 100 mg of ester **1c**. Recrystallization from EtOAc-pentane gave a pure sample, mp 107-109 C; IR 3540, 1720 cm^{-1}; NMR (270 MHz) δ 0.81 (s, 3H, 18-C$\underline{H}_3$), 0.9 (t, 3H, CH_3CH_2), 2.1 (m, 1H, C$\underline{H}$COO), 3.66 (s, 3H, CH_3O); m/e 294 [M^+], 276 [M-H_2O], 262 [M-CH_3OH], 251 [M-C_2H_3O], 234 [base; M-(CH_3OCO + H)]. Anal. calcd. for $C_{18}H_{30}O_3$: C, 73.43; H, 10.27. Found: C, 73.30; H, 10.21.

Interestingly, ester **1c** crystallized into 2 polymorphic forms—needles and prisms. The needle form converted into prisms while stored in contact with the mother liquor or when heated.

(b) A solution of methyl ester/acetate **1d** (390 mg) in 2 N NaOH (4 ml) and methanol (10 ml) was heated at reflux for 2 hr. The solution was acidified with 2 N HCl and diluted with water. Extraction of the mixture with ether gave ester **1c** (310 mg). Recrystallization from EtOAc/pentane gave a pure sample, mp 107-109 C; IR and NMR spectra were identical to those just reported. Acidification of the aqueous portion and extraction with ether gave a negligible amount of acid **1b**.

Methyl ester acetate 1d

(a) Methyl ester **1c** (100 mg) was acetylated by Ac_2O/pyridine to yield 100 mg of **1d**, mp 73-75 C (from acetone); IR 1730 cm^{-1}; NMR (60 MHz) δ 0.81 (s, 3H, 18-C$\underline{H}_3$), 2.03 (s, 3H, C$\underline{H}_3$CO), 3.67 (s, 3H, C$\underline{H}_3$O). Anal. calcd. for $C_{20}H_{32}O_4$: C, 71.39; H, 9.59. Found: C, 71.41; H, 9.54.

(b) Thioketal **1f** (0.50 g) was desulfurized in methanol (75 ml) with sponge nickel catalyst (50% solids, No. 28; W.R. Grace and Co.; 9.0 g) by refluxing for 3.5 hr. The solids were collected on Celite, and the residue from the filtrate was dissolved in $CHCl_3$ and dried (Na_2SO_4). Removal of the solvent and crystallization of the residue from acetone gave 0.31 g of **1d**, mp 73-75 C; IR and NMR spectra were identical to that of the above material.

Methyl Ester Thioketal 1f

To a warm solution of ester **1e** (2) (800 mg) and $(CH_2SH)_2$ (1.4 ml) in glacial acetic acid (10 ml) was added $BF_3 \cdot Et_2O$ (1.4 ml). The reaction was stored for 1 hr, then diluted with water. The steroids were recovered with ether and washed with dil. $NaHCO_3$ and water. Removal of the solvent gave 800 mg of crystalline thioketal **1f**, which was recrystallized from methanol to mp 116-118 C; IR 1725 cm^{-1}; NMR (60 MHz) δ 0.79 (s, 3H, 18-C$\underline{H}_3$), 1.80 (s, 3H, C$\underline{H}_3$-CS_2), 2.03 (s, 3H, C$\underline{H}_3$CO), 3.28 (s, 4H, SC$\underline{H}_2$C$\underline{H}_2$S), 3.68 (s, 3H, C$\underline{H}_3$O). Anal. calcd. for $C_{22}H_{34}O_4S_2$: C, 61.94; H, 8.04. Found: C, 61.59; H, 7.80.

Diol 3a

A mixture of ester **1c** (500 mg) and $LiAlH_4$ (300 mg) in ether (15 ml) was stirred and heated for 12 hr. It was then decomposed by the addition of small amounts of acetone, followed by 10% aq. HCl. The steroids were extracted into ether/chloroform and washed with water. Removal of the solvent yielded crude material (500 mg) which was recrystallized from chloroform/hexane to diol **3a** (0.32 g), mp 117-119 C; IR 3450 cm^{-1}. Anal. calcd. for $C_{17}H_{30}O_2$: C, 76.64; H, 11.19. Found: C, 76.27; H, 11.19.

Diol Diacetate 3b

(a) A sample of diol **3a** was acetylated in the usual manner to give diacetate **3b**. Recrystallization from EtOAc gave a pure sample, mp 66-69 C; IR 1735 cm^{-1}; NMR δ 0.80 (s, 3H, 18-C$\underline{H}_3$), 2.02 (s, 6H, C$\underline{H}_3$CO), 4.19 (s, 2H, OC$\underline{H}_2$). Anal. calcd. for $C_{21}H_{34}O_4$: C, 71.96; H, 9.78. Found: C, 72.30; H, 9.19.

(b) Thioketal-diacetate **4a** (27 mg) and sponge nickel catalyst (50% solids; 1.8 g) in methanol (50 ml) were refluxed for 3.5 hr. The solids were removed with Celite, and the filtrate residue was dissolved in $CHCl_3$, then dried (Na_2SO_4). Removal of the $CHCl_3$ and crystallization from EtOAc/pentane gave **3b**, mp 68-71 C; IR and NMR spectra identical to those already described.

Tosylation of Diol 3a

A solution of diol **3a** (400 mg) and *p*-TsCl (400 mg) in pyridine (10 ml) was heated at 60 C for 3 hr, then stored at ambient temp. overnight. The reaction mixture was decomposed with water, and the steroids were taken up in ether and washed with dil. HCl and water. Preparative TLC of the reaction product on silica gel with benzene yielded 2 compounds. The material from the more mobile zone (260 mg) was recrystallized from benzene/hexane to yield **3d**, mp 97-100 C, IR 3400, 1600,

1500 cm^{-1}; NMR (270 MHz), δ 0.80 (s, 3H, 18-C$\underline{H}_3$), 2.47 (s, 3H, $C_6H_5C\underline{H}_3$), 3.73 (s, 2H, $OC\underline{H}_2$). Anal. calcd. for $C_{24}H_{36}O_4S$: C, 68.55; H, 8.63. Found: C, 68.11; H, 8.42.

The less mobile product **3c** (310 mg) afforded material melting at 136-138 C (benzene/hexane); IR 1600, 1500 cm^{-1}; NMR (60 MHz) δ 0.77 (s, 3H), 2.47 (s, 6H), 4.08 (s, 2H). Anal. calcd. for $C_{31}H_{42}O_6S_2$: C, 64.77; H, 7.36. Found: C, 65.24; H, 7.38.

Diol Diacetate Thioketal 4a

Thioketal **1f** (215 mg) was reduced with $LiAlH_4$ (1.00 g) in anhyd. ether (30 ml). The reaction mixture was refluxed for 12 hr, then decomposed with acetone and 10% HCl. The steroids were recovered (ether), washed, and acetylated with Ac_2O/pyridine to yield diol diacetate-thioketal **4a** (135 mg). Purification by column chromatography (1% ethyl acetate/benzene) and recrystallization from ethyl acetate/pentane gave homogeneous **4a**; mp 198-201 C; IR 1725, 1250 cm^{-1}; NMR 0.80 (s, 18-C$\underline{H}_3$) 1.66 (s, $C\underline{H}_3CS_2$), 2.04 and 2.07 (s, $C\underline{H}_3CO$), 3.21 and 3.35 (d, J = 4, -$SC\underline{H}_2C\underline{H}_2S$), 4.35 (d, J = 7, $OC\underline{H}_2$). Anal. calcd. for $C_{23}H_{36}O_4S_2$: C, 62.71; H, 8.24. Found: C, 62.87; H, 7.96.

Triacetate 4b

A sample of **1a** (316 mg) in dry THF (15 ml) was added to a 3-fold excess of $LiAlH_4$ in THF (50 ml) and the mixture was refluxed for 12 hr. After decomposition of the excess $LiAlH_4$, the steroids were taken up in EtOAc, then washed with water and saline. The reaction product was acetylated as usual to give triacetate **4b** (257 mg) which had mp 105-108 C (from EtOAc/pentane; IR 1740, 1255 cm^{-1}; NMR δ 0.80 (s, 3H, 18-C$\underline{H}_3$), 1.99, 2.03, 2.07 (s, 9H, $C\underline{H}_3CO$); 4.19 (d, 2H, J = 7 Hz, $CH_3C\underline{H}$-O). Anal. calcd. for $C_{23}H_{37}O_6$: C, 67.62; H, 8.88. Found: C, 67.58; H, 9.32.

i-Steroid Alcohols 6a and 6b

Freshly made aldehyde **6e** (1.43 g) was dissolved in 1% methanolic KOH (15 ml) and refluxed for 30 min. The solution was adjusted to pH 8 with 10% aq. HCl, and $NaBH_4$ (0.5 g) in water (8 ml) was added to the cooled solution. After stirring for 6 hr at ambient temperature, the reaction solution was poured into ice and the steroids were recovered with $CHCl_3$. Preparative TLC (40% EtOAc/hexane) of 750 mg of crude product yielded about equal amounts of **6a** and **6b**.

The 20S-alcohol **6a** crystallized from hexane: mp 39.5-41 C; NMR δ 1.04 (d, J = 6.0 Hz, $C\underline{H}_3CH$), 3.50 (AB of ABX, J_{AX} = 3.5, J_{BX} = 7.5, and J_{AB} = 11 Hz, $C\underline{H}_2$-OH) (reported mp 84.5-86 C [11,12]; reported NMR δ 1.03 [d, J = 6.0], 3.50 [m, 2H] [11,12] and δ 1.01 [d, J = 7 Hz], 3.50 [m] [13]).

The 20R was obtained as a glass: NMR δ 0.96 (d, J = 6.3), 3.60 (AB of ABX, J_{AX} = 3.5, J_{BX} = 7.0, J_{AB} = 11 Hz) (reported NMR δ 0.93 [d, J = 6.0], 3.60 [m, 2H] [11,12]).

i-Steroid Acetates 6c and 6d

A mixture of alcohols **6a** and **6b** (750 mg) obtained above was acetylated as usual to yield a mixture of acetates **6c** and **6d**. Preparative TLC chromatography (10% EtOAc/hexane) gave equal amounts of each. The 20-R acetate **6d** was further purified via another preparative TLC (5% EtOAc/hexane).

The 20S acetate **6c** crystallized from EtOAc/hexane to mp 127-128 C; NMR δ 1.01 (d, J = 6 Hz, 21-C$\underline{H}_3$), 3.93 (AB of ABX, J_{AX} = 3.5, J_{BX} = 7.0, J_{AB} = 11 Hz). Anal. calcd. for $C_{25}H_{40}O$:, C, 77.27; H, 10.38. Found: C, 76.81; H, 10.44 (reported mp 124-125 C; NMR δ 1.00 [d, J = 7 Hz, 21-C$\underline{H}_3$], 3.90 [m, 2H] [13]).

The 20-R acetate **6d** was a glass; NMR δ 0.93 (d, J = 6.6 Hz, 21-C$\underline{H}_3$), 4.02 (AB of ABX, J_{AX} = 3.5, J_{BX} = 7, J_{AB} = 11).

17β-Acetoxy Acid 1g

Acid **1b** was acetylated as usual with Ac_2O/pyridine to **1g**. An analytical sample (from acetone/pentane) had mp 200-204 C (sint); IR 3290, 1725, 1700 cm^{-1}. Anal. calcd. for $C_{19}H_{30}O_4$: C, 70.77; H, 9.38. Found: C, 70.69; H, 9.23.

Methyl Ketone 7a

Acid **1g** (100 mg) and oxalyl chloride (10 ml) were refluxed for 0.5 hr. The excess oxalyl chloride was removed by distillation, and the residue was held under vacuum for 1 hr. The acid chloride was then dissolved in ether, added to a suspension of 1.3 mmol of Me_2CuLi (14) in ether cooled to -20 C and stirred under N_2 for 3 hr. The mixture was allowed to warm to ambient temp., at which time it was treated with dil. HCl. The solid material was removed by filtration, and the organic layer was washed with water and sodium thiosulfate, dried and evaporated. The product **7a** (72 mg), upon recrystallization from EtOAc/pentane, had mp 114-116 C; IR 3530, 1700 cm^{-1}; NMR δ 0.84 (s, 3H, 18-C$\underline{H}_3$), 2.10 (s, 3H, $C\underline{H}_3CO$). Anal. calcd. for $C_{18}H_{30}O_2$: C, 77.65; H, 10.86. Found: C, 77.81; H, 10.95.

17β-Nitrate Methyl Ketone 7b

To a stirred mixture of HNO_3 (d = 1.5; 10 ml) and Ac_2O (7.5 ml) at -5 to -10 C was added ketone **7a** (100 mg). The reaction mixture was stirred a further 20 min, then decomposed with ice. The product was taken up into EtOAc, then washed with dil. $NaHCO_3$, water and saline. Removal of the solvent gave **7b** (100 mg) which was recrystallized from EtOAc; mp 109.5-111 C; IR 1690, 1620 cm^{-1}; NMR δ 0.84 (s, 3H, 18-C$\underline{H}_3$), 2.11 (s, 3H, C$\underline{H}_3$CO). Anal. calcd. for $C_{18}H_{29}O_4N$: C, 66.84; H, 9.04; N, 4.33. Found: C, 67.06; H, 8.61; N, 4.32.

ACKNOWLEDGMENTS

This work was supported in part by USPHS Grants A-5326 and CA-07137 and the NIU Deans' Fund. We wish to thank M. Cushman of Purdue University for the 470 MHz NMR spectrum.

REFERENCES

1. Piatak, D.M., and Caspi, E. (1966) Chem. Commun., 501-502.
2. Caspi, E., Grover, P.K., Piatak, D.M., and Shimizu, Y. (1965) J. Chem. Soc., 3052-3059.
3. Wolff, L. (1912) Justus Liebigs Ann. Chem. 394, 86-108.
4. Piatak, D.M., Dorfman, R.I., Tibbetts, D., and Caspi, E. (1964) J. Med. Chem. 7, 590-592.
5. Caspi, E., Grover, P.K., and Piatak, D.M. (1963) Chem. Ind. (London), 1495-1496.
6. Egmond, G.H., van Gorkon, M., and van Dorp, D.A. (1965) Rec. Trav. Chim. Pays-Bas 84, 648-652.
7. Newman, M.S. (1941) J. Am. Chem. Soc. 63, 2431-2435.
8. Byon, C., Buyuktur, G., Choay, P., and Gut, M (1977) J. Org. Chem. 42, 3619-3621.
9. Piatak, D.M., and Wicha, J. (1978) Chem. Rev. 78, 199-241.
10. Vystrčil, A., Pourar, V., and Křeček, V. (1973) Collect. Czech. Chem. Commun. 38, 3902-3911.
11. Vanderah, D.J., and Djerassi, C. (1977) Tetrahedron Lett. 683-686.
12. Vanderah, D.J., and Djerassi, C. (1978) J. Org. Chem. 43, 1442-1448.
13. Partridge, J.J., Faber, S., and Uskokovic, M.R. (1974) Helv. Chim. Acta 57, 764-771.
14. Rozen, S., Shanhak, I., and Bergmann, E.D. (1972) Synthesis 701-702.

[Received August 17, 1981]

Constituents of Human Meconium: II. Identification of Steroidal Acids with 21 and 22 Carbon Atoms[1]

JAN ST. PYREK, ROMAN STERZYCKI, ROGER LESTER and EUGENE ADCOCK,
Division of Gastroenterology, Department of Internal Medicine and Department of Pediatrics
University of Texas Medical School at Houston, PO Box 20708, Houston, TX 77025

ABSTRACT

Monohydroxylated acid fraction isolated from human meconium was found to contain, in addition to C_{20} and C_{24} acids identified previously, three C_{22} bile acids–(20S)-3α-hydroxy-23,24-bisnor-5β-cholan-22-oic, (20S)- and (20R)-3β-hydroxy-23,24-bisnor-chol-5-en-22-oic, and one C_{21} acid–3β-hydroxypregn-5-en-21-oic. These compounds were identified by capillary gas chromatography-mass spectrometry and by comparison with standards. It is postulated that these C_{22} acids, as well as the two monohydroxylated C_{24} bile acids (lithocholic and 3β-hydroxychol-5-enoic) are produced in the maternal intestine by microbial flora and transferred to the fetus through the placenta.
Lipids 17:241-249, 1982.

Our previous study of the bile acids of human meconium demonstrated the presence of 4 C_{20} 3-hydroxyetianic acids, *1a*, *3a*, *5a* and *9a*, in the sulfate and/or glucuronide conjugated form (part I of this work; also Table 1). With the exception of the acid *3a*, their concentration either exceeded or was comparable to that of the 2 monohydroxylated C_{24} bile acids: lithocholic, *22a*, and 3β-hydroxychol-5-enoic, *23a*, detected previously (1,2).

Two potential metabolic sources of etianic acids in meconium should be considered. One is the degradation of pregnane derivatives, which has precedent in the isolation of acidic products of corticosteroid catabolism (3-5). The second is the degradation of the cholesterol and/or bile acid side chain by the maternal microbial intestinal flora to products which, similarly to secondary bile acids (1), could accumulate in meconium. In relation to the second possibility, however, it should be noted that C_{20} acids were found only in rare cases of microbial degradation (6,7). In view of these hypotheses, further study of other, possibly related acids with the intermediate molecular weight (MW) was undertaken.

RESULTS AND DISCUSSION

Characterization of C_{22} and C_{21} Acids

Gas chromatographic-mass spectrometric (GC-MS) analysis of the total monohydroxylated acid fraction in the form of methyl esters, methyl ester acetates and methyl ester TMS-ethers revealed the presence of several components having R_t intermediate between those of C_{20} and C_{24} derivatives. Thus, 3 components could be detected by monitoring the GC-separation at m/e 342 (1 compound) and 344 (2 compounds), i.e., ions corresponding, respectively, to M-ROH for monounsaturated and saturated C_{22} steroidal acid methyl esters. These 3 substances overlapped with few other components and were preceded by the compound recognized as the methyl ester of a C_{21} steroidal acid. Modifications of the extraction procedure, notably the exclusion of the pre-reduction, had no effect on the appearance of these compounds in the meconium extracts. These methyl esters were found in the medium polarity fraction of monohydroxylated methyl esters obtained by benzene/acetone gradient chromatography together with C_{20} and C_{24} compounds with an equatorial 3-hydroxyl group. GC separation of this fraction, in the form of TMS ethers, is shown in Figure 1. Compounds previously identified are indicated with formulas listed in Table 1. Their distribution among the fractions of the subsequent high performance liquid chromatographic (HPLC) separations is shown inTable 2.

The mass spectrum of the principal unsaturated C_{22} component (R_t 21.70 min) showed a molecular ion at m/e 432 followed by 417 (M-Me), 376 (M-56), 342 (M-TMSOH), 327 (M-TMSOH-Me), 303 (M-129), 215, 213 (ABC ring part), 129 (base peak, TMS-O^+=CH-CH=CH_2). The computer enhanced spectrum of the second C_{22} component (R_t 21.30) displayed the same ions. The presence of an abundant ion at m/e 129, together with the complementary ion M-129, pointed to the probable structures of parent compounds as side chain isomers of 3β-hydroxybisnorchol-5-enoic acids *20a* and *21a*. The same structures were indicated by mass spectra of free alcohols, which showed the abundant molecular ion at

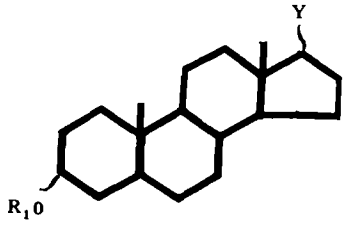
[1]Part I: J. St. Pyrek, R. Lester, E. Adcock and A.T. Sanghvi, manuscript submitted for publication. The "reductive-extraction" of meconium with ethanol containing large excess of sodium borohydride excluded the artifactual formation of C_{20} acids as a result of the degradation of pregnane derivatives with hydroxyketone and hydroxyaldehyde side chains.

m/e 360 and prominent ions due to eliminations of 85 and 111 amu (8). The molecular ion was not observed in the mass spectrum of the major acetate, as is typical for Δ^5-3β-OAc steroids (9) and the spectrum displayed ions at m/e 342 (M-AcOH), 327 (M-AcOH-Me), 283 (M-AcOH-COOMe), 255 (M-AcOH-side chain), 239, (M-H_2O-Me-side chain-H), 234 (M-168 CD-ring part), 221 (M-181 CD ring part), and 213 (ABC part).

The reduction of the carbomethoxyl group with lithium aluminum hydride (LAH) performed on the whole methylated monohydroxylated fraction produced a mixture of diols that was directly analyzed by capillary GC-MS. Among these products, the expected

TABLE 1

Retention Times (R_t) Obtained for C_{20}, C_{21}, C_{22} and C_{24} Monohydroxylated Bile Acid Derivatives by Capillary Gas Chromatography-Mass Spectrometry



a R_1=R_2=H
b R_1=H, R_2=Me
c R_1=Ac R_2=Me
d R_1=TMS R_2=Me

R_t on 12 M 0.25 mm capillary column SP-2100 carrier gas helium, 2 psi

N	3-OR_1	5-H	17-Y	C20	Y	TMG ethers *Nd*	Methyl esters *Nb*
1	α	α	β	–	$COOR_2$	19.70	14.75
2	α	α	α	–	$COOR_2$	19.17	–
3	β	α	β	–	$COOR_2$	20.20	14.80
4	β	α	α	–	$COOR_2$	19.78	–
5	α	β	β	–	$COOR_2$	19.85	14.55
6	α	β	α	–	$COOR_2$	19.16	–
7	β	β	β	–	$COOR_2$	19.70	–
8	β	β	α	–	$COOR_2$	19.14	–
9	β	Δ^5	β	–	$COOR_2$	20.30	14.30
10	β	Δ^5	α	–	$COOR_2$	20.00	–
11	β	Δ^5	β	–	CH_2COOR_2	21.32	15.75
12	α	α	β	S	$CHCH_3COOR_2$	21.20	16.35
13	α	α	β	R	$CHCH_3COOR_2$	21.00	16.08
14	β	α	β	S	$CHCH_3COOR_2$	21.80	16.35
15	β	α	β	R	$CHCH_3COOR_2$	21.60	16.08
16	α	β	β	S	$CHCH_3COOR_2$	21.38	15.37
17	α	β	β	R	$CHCH_3COOR_2$	21.10	15.67
18	β	β	β	S	$CHCH_3COOR_2$	21.20	15.97
19	β	β	β	R	$CHCH_3COOR_2$	20.95	15.67
20	β	Δ^5	β	S	$CHCH_3COOR_2$	21.78	16.48
21	β	Δ^5	β	R	$CHCH_3COOR_2$	21.58	16.25
22	α	β	β	R[a]	$CHCH_3CH_2CH_3COOR_2$	23.13	17.95
23	β	Δ^5	β	R[a]	$CHCH_3CH_2CH_3COOR_2$	23.73	18.20
24	3-oxo	α	β	–	COOMe	–	15.13
25	3-oxo	β	β	–	COOMe	–	14.85
26	3,6-dioxo	Δ^4	β	–	COOMe	–	16.70
27	3,6-dioxo	Δ^4	α	–	COOMe	–	16.10
28	3,6-dioxo	Δ^4	β	–	CH_2COOMe	–	17.63
29	3β-OH	Δ^5	β	–	CH_2CH_2OH	–	–
30	3-oxo	α	β	S	$CHCH_3COOMe$	–	16.70
31	3-oxo	α	β	R	$CHCH_3COOMe$	–	16.40
32	3-oxo	β	β	S	$CHCH_3COOMe$	–	16.40
33	3-oxo	β	β	R	$CHCH_3COOMe$	–	16.10
34	3α-OH	β	β	S	$CHCH_3CH_2OH$	–	–
35	3β-OH	Δ^5	β	S	$CHCH_3CH_2OH$	–	–
36	3,6-dioxo	Δ^4	β	S	$CHCH_3COOMe$	–	18.15
37	3,6-dioxo	Δ^4	β	R	$CHCH_3COOMe$	–	17.88
38	3,6-dioxo	Δ^4	β	R[a]	$CHCH_3CH_2CH_2COOMe$	–	20.72

[a]Note formal reversion of the absolute configuration; compare discussion in ref. 16.

major C_{22} component *35* with a molecular ion at m/e 332 was detected. Moreover, the analogous mixture of reduction products obtained with lithium aluminum deuteride showed the same component with the molecular ion shifted to 334. The comparison of their mass spectra confirmed the presence of the fragmentation processes leading to ions at m/e 247 (M-85) and 221 (M-111) which shifted to 249 and 223 upon deuteration. On the other hand, ions due to the elimination of side chain at m/e 273 and 255 remained unaffected.

The identification of the major C_{22} unsaturated acid as (20S)-3β-hydroxy-23,24-bisnorchol-5-en-22-oic acid, *20a*, was confirmed by the GC-MS comparison with an authentic standard in the form of free alcohol *20b*, acetate *20c* and TMS ether *20d*. The second component was also found to be identical with the derivatives of the "unnatural"–(20R)-3β-hydroxy-23,24-bisnorchol-5-en-22-oic acid, *21a*. The latter was prepared by the equilibration of the (20S)-methyl ester *20b* with potassium *tert*-butoxide in THF to 1:1 mixture of *20b* and *21b*. These compounds showed almost identical mass spectra but could be easily resolved by GC (Table 1) and HPLC (Table 2). Additional confirmation was obtained by GC-MS and HPLC comparison with standards of the chromic acid oxidation products, *36* and *37* 3,6-dioxo-4-enes, isomeric at C-20.

The MS data obtained for the third C_{22} component (R_t 21.17 min, Fig. 1) suggested that the parent compound was one of the possible isomers of 3-hydroxybisnorcholanoic acids *12a-19a*. In the mass spectrum of the TMS ether, a weak ion corresponding to M-15 at m/e 419 was present followed by ions at 344 (M-TMSOH), 323 (M-TMSOH-Me), 255-257 (M-side chain) and 215 (base peak, ABC part). The same ions were observed in spectra of other derivatives, whereas the molecular ions could not be detected. The monitoring of GC-separation, particularly at m/e 215, demonstrated the presence of one principal isomer. The products of the oxidation of the whole monohydroxylated fraction with chromic acid were analyzed directly, as well as after preliminary HPLC separation. Only one principal saturated C_{22} component was detected by a characteristic mass spectrum, indicating it to be a C-20 isomer of 3-oxo-23,24-bisnor-5β-cholan-22-oic acid methyl ester *32* or *33*. Its spectrum showed a molecular ion at m/e 360, followed by 345 (M-Me), 342 (M-H_2O), 328 (M-MeOH), 327 (M-H_2O-Me), 313 (M-MeOH-Me), 290 (M-70, ring BCD fragment), 258 (290- MeOH), 246 (ABC part), 213 (ABC part), 176 (base peak, 246-70, BC part), 161, 162 (BC part).

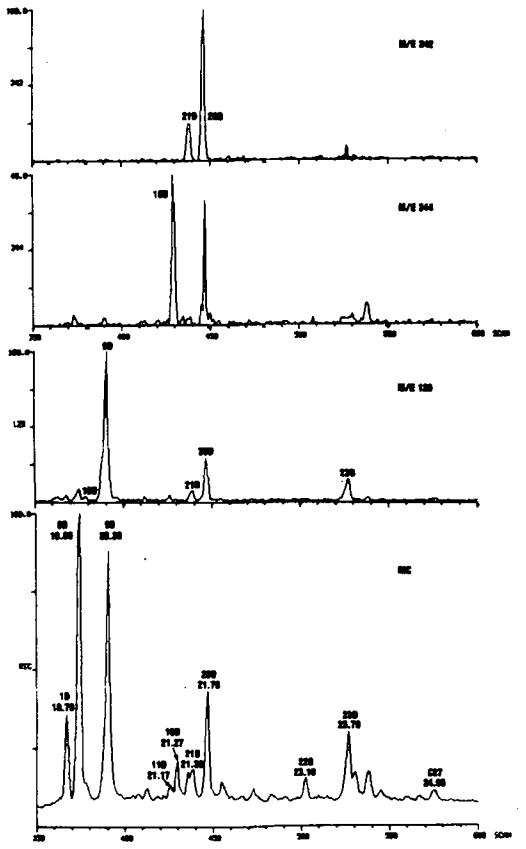


FIG. 1. Capillary GC-MS separation of medium polarity, "3-equatorial" fraction of monohydroxylated bile acids, obtained from human meconium by reductive-extraction, in the form of methyl esters TMS-ethers on 12 M, 0.25-mm fused silica capillary coated with SP2100. Reconstructed ion current (RIC) and mass chromatograms are labeled with R_t (min) and structure numbers (Table 1); temperature program from 50 to 270 C, 10 C/min.

The elimination of 70 amu (Fragment of ring A) was diagnostic for a 5β-H configuration (10). This information, in combination with the thin layer chromatography (TLC) and HPLC behavior of the parent methyl ester-alcohol indicated that the 3-OH group had an equatorial configuration and pointed to 3α-hydroxy-23,24-bisnor-5β-cholan-22-oic methyl ester *16b* with the 20S configuration being most probable.

The appropriate reference compound, *16b*, was prepared from (20S)-3β-hydroxy-23,24-bisnorchol-5-en-22-oic acid methyl ester, *20b*, together with the complete set of C-3,5 and 20 isomers *12a-19a*. Oxidation with Jones' reagent at room temperature yielded (20S)-3,6-dioxo-

TABLE 2

Composition of Fractions Obtained by HPLC Separation of Monohydroxylated Bile Acid Methyl Esters Obtained from Human Meconium after Extraction in the Presence of Sodium Borohydride, Solvolysis and Mild Alkaline Hydrolysis

Elution vol (mo)	R_t of methyl esters (compare Table 1)
15.2-16.4	14.8 (*1b*, major), 18.2 (*23b*), 19.23 (C_{27})
16.4-17.8	14.8 (*1b*), 18.2 (*23b*)
18.8-20.0	14.8 (*9b*, major), 16.5 (*20b*)
20.0-20.8	14.8 (*9b*, major)
20.8-22.8	14.6 (*3b*, major), 15.8 (*11b*)
23.7-25.2	12.9, 14.3, 15.5, 16.4, 18.4 (C_{24} major), 18.9 (C_{24} major)
25.2-26.9	14.6 (*10b*, trace), 16.3 (*21b*, major)
26.9-29.4	14.6 (*10b*, trace), 18.0 (*22b*), 24.00 (major)
30.2-32.5	14.6 (*5b*, major), 14.70, 16.4
35.9-36.0	14.6 (*5b*, major), 17.0
36.0-37.8	12.6 (major), 14.6 (*5b*), 16.1 (*16b*), 17.0
37.8-39.5	16.1 (*16b*, major)
39.5-43.0	15.7 (*17b*, trace), 16.1 (*16b*)+

bisnorchol-4-enoate, *36*, which was submitted to catalytic reduction. The mixture of 5-epimeric products was selectively ketalized at C-3 (11) without separation and the 6-keto group was removed by the Wolff-Kishner method. Under the conditions of the Wolff-Kishner reaction, the equilibration of both C-5 and C-20 configuration occurred (compare discussion below). The resulting mixture of 4 ketones, *30-33*, was resolved by HPLC. The 5α isomers, *30* and *31*, were both identified by characteristic mass spectra in which the ion at m/e 231 dominated, whereas the elimination of 70 amu was absent. These 5α isomers were directly prepared from (20S)-3β-hydroxy-23,24-bisnor-5α-cholan-22-oic acid methyl ester *14a* by equilibration at C-20 with potassium *tert*-butoxide, methylation and oxidation. The subsequent reduction of ketones *30*, *31*, *32* and *33* with sodium borohydride afforded all 8 isomeric alcohols, *12b-19b*, which were characterized at TMS derivatives *12d-19d*. Their GC behavior was parallel to that of the series of 8 lower C_{20} homologs *1d-8d* (Table 1). Under these conditions, the separation and differentiation by GC of all four 3,20 diastereoisomers within the two 5-epimeric groups could be obtained. Of all 8 compounds, only two 3-axial ethers, *12d* and *18d*, had identical retention times. Further distinction could be obtained from MS data. The combined HPLC-GC-MS analysis confirmed the identification of the saturated C_{22} acid as (20S)-3α-hydroxy-23,24-bisnor-5β-cholan-22-oic *16a*. It also permitted the detection of trace amounts of the second 20R isomer, *17a* (Table 2).

The fourth component of the group with intermediate R_t (21.65 min, Fig. 1) could be monitored similarly to the other 3β-Δ^5 compounds, *9d*, *20d*, *21d* and *23d*, at m/e 129. Despite its relatively low concentration, mass spectra could be obtained for all derivatives studied by direct GC-MS analysis of the total fraction, both by background subtraction and computer enhancement. Moreover, double column chromatography followed by HPLC allowed the separation of this minor methyl ester in almost pure form. It was eluted from the column in a fraction immediately following 3β-Δ^5 C_{20} (17β) and C_{22} (20R) methyl esters *9b* and *21b* (Table 2). A mass spectrum of its TMS ether displayed a diagnostic elimination pattern indicating a "Δ^5-3β-OTMS" structure: 418 (M, 10%), 403 (M-Me, 4), 387 (M-MeO, 1), 362 (M-56, 11), 328 (M-TMSOH, 45), 313 (M-TMSOH-Me, 40), 289 (M-129, 95), 257 (35), 253-255 (12), 239 (40), 223 (30), 215 (ABC-part, base peak), 129 (TMS-O^+=CH-CH=CH_2, 90). This spectrum conformed with the structure of the methyl ester of 3β-hydroxypregn-5-en-21-oic acid, *11b*. This structural assignment was corroborated by a spectrum of free alcohol, which showed the molecular ion at m/e 346, prominent ions at 261 and 235 due to the eliminations of 85 and 111 amu, a base peak at 161 (M-side chain-H-111), and a fragment ion at 239 (M-side chain-H-H_2O-Me). The spectrum of the methyl ester acetate showed M-AcOH ion at m/e 328, followed by ions, 255, 254 (M-60-side chain), 239 (as above), 220 (M-168), 207 (M-181) (compare previous data). Other analogies in the fragmentation pattern with those of C_{20} and C_{22} Δ^5-homologs excluded also the alternative "D-homo"-structure. The presence of the carbomethoxyl group in the side chain was confirmed by the observation of a diol with a molecular ion at m/e 318 among LAH reduc-

tion products. Its spectrum displayed ions due to elimination of 85 and 111 amu at 233 and 207. Among the products of Jones' oxidation, the expected C_{21}-3,6-dione *28* with a molecular ion at m/e 358 and base peak at 137 was observed; this compound was localized by GC-MS in polar HPLC fractions together with C_{20}, C_{22} and C_{24} homologs *26*, *36*, *37* and *38*. The final, unambiguous confirmation of the structure of this unique meconium constituent was obtained by direct comparison with the authentic compound synthesized from 3β-hydroxyandrost-5-en-17-one by the Horner-Wittig reaction (12,13). The blocking of 3-hydroxyl groups was omitted without any effect on the yield. Identical mass spectra and R_t were observed for the free alcohol *11b*, its TMS ether *11d* and the oxidation product *28*.

Characterization of C_{20} Acids

The sensitivity for detection of minor components was greatly enhanced with the application of the detailed HPLC separation prior to the final GC-MS analysis. This analysis of the meconium monohydroxylated acid fraction, obtained by "reductive-extraction," confirmed the identification of 3 saturated etianic acids, *1a*, *3a*, *5a*, and unsaturated etianic acid *9a*. R_t and mass spectra of alcohols *1b*, *3b*, *5b*, *9b* and their TMS ethers *1d*, *3d*, *5d* and *9d* were identical to those of authentic standards. The major component, *1b*, was obtained in the amount permitting the registration of an FT PMR spectrum with δ 4.04 bs, (3βH), 3.65 s (OMe), 0.79 s (19-H_3), 0.65 s (18-H_3) which was identical to that of standard *1b*.

This analysis, however, demonstrated the presence of a trace amount of 17α isomer of the unsaturated etianic acid *10a*. It was localized as the methyl ester TMS-ether in a relatively polar HPLC fraction (Table 2). Its presence in the extract prepared with sodium borohydride reduction might indicate partial epimerization and degradation of 21-hydroxypregnenolone during the long period of meconium accumulation in the fetus. 21-Hydroxypregnenolone was previously identified in human meconium (14,15). On the other hand, saturated etianic acids for which 17α-isomers were not found seem to be formed in an enzymatic process.

Metabolic Sources of Short-Chain Bile Acids Present in Human Meconium

The structures of the nuclear part of three C_{22} bile acids detected in human meconium are identical to those of the 2 major monohydroxylated C_{24} bile acids found in this material previously (1,2). Thus, the saturated C_{22} acid can be considered the lower homolog of lithocholic acid, *22a* (1), whereas the 2 unsaturated C_{22} acids *20a* and *21a* are analogous to 3β-hydroxycholenoic acid, *23a* (2).

The detection of the Δ^5 acid *21a* with the "unnatural" 20R configuration is of special interest, especially in view of the fact that saturated counterpart *16b* is accompanied by only a trace amount of 20R isomer *17a*. The stability of the configuration at C-20 in bisnorcholanoic acids has been discussed previously (16). However, old and contradictory data of the position of the equilibrium between 20-epimers has been quoted. We found that the equilibration of 20S methyl esters *14b* and *20b* with potassium *tert*-butoxide in THF produced nearly equimolar amounts of 20R-epimers *15b* and *21b*, respectively. A similar equilibrium was attained under the Wolff-Kishner conditions for 3-ketones *30-33*. Such isomerization, however, would not be expected under the mild alkaline conditions used for the work-up of meconium extracts, provided the C_{22} acids are not esterified. When acid *14a* was submitted to treatment with 1 N methanolic NaOH at 80 C for 24 hr, the formation of its 20-epimer, *15a*, was not observed by capillary GC analysis which would have detected a concentration as minimal as 0.01%. On the other hand, under the same conditions, methyl ester *14b* was isomerized to acid *15a* (9%), indicating that enolization and saponification have comparable rates.

In view of these results, the detection of the (20S) and 20R) acids *20a* and *21a* in a 3:1 proportion could be explained by the occurrence of the (20S) acid *20a* in the esterified form, in contrast to the occurrence of the saturated (20S) acid *16a* in the free form. Alternatively, and more likely, the isomerization of C-20 may occur for a potential intermediate, such as a C_{24} 22-oxo-24-oic acid. This alternative, however, would require the affinity of the side chain degrading enzyme system to be higher for the 3α, 5β intermediate. It could be postulated that, due to the longer half-life and concentration of the C_{24}-Δ^5-22-oxo-24-oic acid intermediate, its isomerization at C-20 is substantial. The demonstration of all three C_{22} acids in extracts of meconium obtained with the sodium borohydride prereduction proves that the artifactual formation of these compounds from α- or β-keto acids does not occur.

In addition to the classical example of sargasterol (17), there are 2 known instances of the natural occurrence of compounds with the "unnatural" C-20 configuration. (20S)-

3β-Hydroxycholest-5,22-diene has been detected among scallop sterols (18), whereas (20S)-3β-hydroxy-5α-chol-22-en-24-oic and (20S)-3β-hydroxychol-5,22-dien-24-oic acids have been isolated as methyl esters from sea pen, *Ptilosarcus gurneyi* (18,19). The authors stressed that the C-20 epimerization did not occur during the isolation and was not reversed by HCl/MeOH.

Although the direct formation of C_{22} bile acids from their higher C_{24} homologs by the process of β-oxidation performed by fetal enzymes would be very attractive, their microbial origin seems to be more likely. There are no examples showing that higher organisms can degrade the cholesterol side chain by β-oxidation beyond C-24. On the other hand, stepwise microbiological scission of sterol (20-22) and bile acid (23,24) side chains is well documented. The ecological significance of this process is illustrated by the detection of C_{22}, together with C_{24} bile acids in petroleum (25,26). The degradation of the bile acid side chain to C_{22} acids with concomitant (or according to Hayakawa [25], prior) oxidation of ring A has been demonstrated with cultures of numerous bacterial strains. These include bacteria isolated from human feces (27-36). It is surprising, however, that the presence of degraded bile acids is not revealed in recent analyses of fecal bile acids. Only relatively old data, overlooked in recent studies, indicate the presence of C_{20} and C_{22} acids in animal feces. Notable is the probable detection of C_{20} and C_{22} acids in elephant and deer feces (37), the formation of 3,12-dioxo-7-hydroxybisnor-cholanoate by cat intestinal flora (38), the formation of 7,12-dioxo-3-hydroxybisnorcholanoate by guinea pig intestinal flora (39), and the formation of bisnordehydrocholate by dog intestinal flora (40). The formation of C_{22} and C_{20} acids in the pig intestine has also been suspected (41).

More recently, labeled cholesterol has been found to be degraded by bacteria present in the rat gut to the C_{24} bile acids lithocholic, *22a*, isolithocholic (3β-hydroxy-5β-cholanoic), and 3β-hydroxychol-5-enic, *23a* (42). In the same study, intestinal flora of the guinea pig has been formed to produce only small amounts of unidentified acids, along with estradiol as the major product. This is an important observation which indicates that lithocholic acid, *22a*, could originate both as a secondary bile acid, by dehydroxylation of chenodeoxycholic acid (43) as well as by the direct degradation of cholesterol with 3β-hydroxychol-5-enoic acid, *23a*, as an intermediate. As discussed previously (28), under specific conditions of the enhanced exposure of bile acids to the action of intestinal flora, the above processes may be pronounced. It is therefore possible to assume that C_{22} bile acids, together with their C_{24} monohydroxylated counterparts, are formed in the maternal intestine. Their subsequent absorption, placental transfer and secretion into the fetal bile results in their accumulation in meconium. This process is entirely analogous to that occurring for deoxycholic acid, a secondary bile acid which is present in meconium in substantial concentration (1,44). In view of this, the fetal synthesis of 3β-hydroxychol-5-enoic acid, considered previously as an indication of the presence of abberant pathway of bile acid biosynthesis (2,44), should be reconsidered. However, it should be stressed that, if C_{22} bile acids *14a*, *20a* and *21a* are of microbial origin, their formation does not follow the order proposed by Hayakawa (23), and ring A remains unoxidized. It is also important to stress that the configurational purity at C-20 of C_{22} bile acids resulting from microbial degradations was not investigated, to the best of our knowledge.

The metabolic origin of the C_{21} acid *11a* seems to be more obscure. Its structure is entirely unique from the biogenetic point of view, and there are no precedents for the isolation of steroids with $-CH_2COOH$ side chain. This compound might be formed as a result of the reduction of C_{21} 20-hydroxy acids present in meconium (J. St. Pyrek and R. Lester, unpublished results). Alternatively, it might indicate the operation of the alternative β-oxidation process of C-21 (in contrast to C-17) in the further (microbial?) degradation of C_{22} bile acids.

The detection of microbially degraded steroids in the entirely sterile fetal intestine proves that products of such degradation, which play a role in the etiology of colon cancer (45), are reabsorbed and spread beyond the intestinal system.

EXPERIMENTAL

Gas Chromatography-Mass Spectrometry

Mass spectra (22 eV) were obtained with a Finnigan 3200 quadrupole instrument connected to a gas chromatograph equipped with a 12 M (or 25 M), 0.25-mm-id, fused silica capillary columns coated with methyl silicone SP2100 (Hewlett-Packard). Spectra were accumulated with an INCOS data system with a repetition time of 1.5 sec in the range 100-600 amu. Samples of methyl esters were injected at an oven temp. of 100 C (injector temp. 270 C), in a splitless mode, with toluene as the

solvent. After 1 min, the oven temp. was programmed to 270 C at the rate 10 C/min. TMS ethers, prepared with *bis*-trimethylsilyltrifluoroacetamide/trimethylchlorosilane (9:1) at 100 C were injected at an oven temp. of 50 C, programmed as just described (Table 1). The GC-MS interphase was modified to introduce the outlet of the capillary column directly to the ion source as described before (part I of this work).

High Pressure Liquid Chromatography

A single-piston pump, loop injector, and UV variable wavelength detector (ISCO, set at 203 nm) were used for the separation of methyl esters on a micro-particulate silica gel column (Vydac) with hexane containing 0.1-0.7% isopropanol.

Extraction of Meconium

Samples of pooled meconium, collected from newborns of both sexes, were extracted with 80% ethanol with and without addition of sodium borohydride as described before. Alternatively, meconium was lyophylized and submitted to solvolysis-extraction with a large excess of acetone/methanol/6 N HCl (9:1:0.01) for 72 hr at room temperature. Further work-up included solvolysis, mild alkaline hydrolysis and ion exchange purification of the acid fraction as described in part I of this study.

The carboxylic acid fraction (70 mg, corresponding to 142 g of fresh meconium) was methylated with diazomethane and separated on a silica gel column (20 × 1 cm) that was prewashed with acetone and benzene. The sample was applied in benzene and elution was performed with 0, 5, 7.5, 10 and 15% of acetone in benzene, 100 ml for each step of the gradient. Fractions (110) were collected and analyzed by TLC in benzene/acetone (8:2) (solvent A) and benzene/hexane/acetone (8:8:2) (solvent B). Acetone in benzene (5%) eluted low polarity fractions with R_f 0.80, 0.85 (solvent A) and 0.20-0.25, 0.35 (solvent B) which constitute less than 5% of the total acid fraction. Most of the material was eluted with higher acetone concentrations, and had R_f of 0.65, 0.55, 0.50, 0.40, 0.35, 0.3-0.1 (solvent A). Methyl esters of lithocholic, deoxycholic and cholic acids had R_f 0.80, 0.35 and 0.10, respectively (solvent A).

The subsequent resolution of substances with R_f 0.20-0.35 (solvent B) was obtained on a silica gel column (20 × 1 cm) in benzene/hexane/acetone (10:10:1). Twenty fractions with partially overlapping substances were obtained. Their composition was examined by GC-MS as TMS ethers. Fractions with R_f 0.32-0.28 were combined and submitted to further fractionation by HPLC and examined by GC-MS with and without silylation (Table 2).

C-20 Epimerization of (20S)-3β-Hydroxy-23,24-bisnorchol-5-en-22-oic and (20S)-3β-Hydroxy-23,24-bisnor-5α-cholan-22-oic Acid Methyl Esters *14b* and *20b*

(a) Methyl esters *14b* and *20b* (Steraloïds, 10-20 mg) in anhydrous THF (2 ml) were treated with solid potassium *tert*-butoxide in Teflon-stoppered tubes for 20 hr at 75-80 C. Products were recovered by dilution with water, acidification and extraction with ethyl ether. GC and GC-MS analysis of methyl esters and methyl ester-TMS-ethers showed, respectively: *14b* (47%), *15b* (47%), and *20b* (42%) and *21b* (54%) along with traces of other products.

(b) Methyl ester *14b* in 1 N methanolic NaOH was heated to 80 C for 24 hr. Products were recovered and analyzed as before to give *14b* (91%) and *15b* (9%).

(c) Free acid *14a* was treated as described in b. GC analysis showed no detectable amount of *15b*. In this case, analysis was performed for methyl ester TMS-ethers on SE-54 12 M, 0.25-mm-id, fused silica capillary column with FID detector (R_t: *14d* 12.34, *15b* 11.97 min).

Methyl 3α- and 3β-Hydroxy-23,24-bisnorcholanoates *12b-19b*

Methyl (20S)-3β-hydroxy-23,24-bisnorchol-5-en-22-oate, *20b* (18.7 mg), was oxidized with the Jones' reagent in acetone to give (20S)-3,6-dioxo-23,24-bisnorchol-4-en-22-oic acid methyl ester, *36* (13.4 mg, recrystallized from hexane/ether, mp 169-171 C, δ ($CDCl_3$): 6.18 s (4-H), 3.66 s (OMe), 1.8-2.8 m (2-H_2, 7-H_2), 1.24 d (21-H_3), 1.17 s (19-H_3), 0.75 (18-H_3), M^+ 472). The diketone *36* (9.0 mg) was hydrogenated over 10% Pd/C in isopropanol followed by Jones' oxidation to give a mixture of 5α- and 5β-dihydroderivatives, 3:1 (8 mg, GC-MS analysis). This mixture was subjected to ketalization with ethylene glycol in benzene in the presence of pyridinium *p*-toluenesulfonate (46). The mixture of 5α- and 5β-3-ketals, 6:1 (4.0 mg, GC-MS analysis), purified by preparative TLC in benzene/acetone (95:5), was reduced with the Nagata procedure of Wolff-Kishner reduction (47). Extraction of the acid fraction, followed by methylation and deprotection with acetone aq HCL, produced a mixture (2.0 mg) of *30* (25%), *31* (25), *32* (16), and *33* (16) accompanied by 4 corresponding bisnorcholanoic acid methyl esters (18%). These ketones were separated on Vydac HPLC column in

hexane containing 0.125% isopropanol. R_V were: *31*-15, *32*-20, *33*-23 and *34*-25 ml. These ketones were reduced with sodium borohydride to give pairs of 3-epimers: *12b-14b*, *13b-15b*, *16b-18b* and *17b-19b*, separated either by HPLC or by GC-MS (Table 1).

3β-Hydroxypregn-5-en-21-oic Acid Methyl Ester, *11*

3β-Hydroxyandrost-5-en-17-one (2.88 g, 10 mmol, Steraloids) and triethylphosphonoacetate (8.45 g, 40 mmol) in ethanol (40 ml) were treated with sodium ethoxide prepared from sodium metal (0.92 g, 40 mmol) in ethanol (25 ml) at 35-40 C. Subsequently, the mixture was refluxed for 12 hr and the solvent was then evaporated in vacuo. The residue was dissolved in water and extracted with ethyl ether. Recrystallization from hexane/ether afforded 3β-hydroxypregna-5,17(20)-dien-21-oic acid ethyl ester (2.98 g, 83%, mp 172-174 C, reported [48] mp 178-180 C). This product was hydrogenated over Adams' catalyst in ethanol. The reaction was stopped after one equivalent of hydrogen was consumed. The ethyl ester of 3β-hydroxypregn-5-en-20-oic acid *11a* was purified by recrystallization from hexane/ether (mp 110-114 C). Its transesterification in boiling methanol/sodium methanolate produced methyl ester *11b* (mp 120-122 C, reported [49] mp 132-133 C); δ $CDCl_3$: 5.36 bd J=4 (5-H), 3.66 s (OMe), 3.50 m (3β-H), 2.30 bd (20-H_2), 1.02 s (18-H_3), 0.62 (18-H_3); M^+ 346.

ACKNOWLEDGMENTS

This work was supported in part by grants from the March of Dimes Foundation and from the National Institute of Child Health and Human Development, grant no. HD14198.

REFERENCES

1. Sharp, H.L., Peller, J., Carey, J.B., Jr., and Krivit, W. (1971) Pediatr. Res. 5, 274-278.
2. Back, P., and Ross, K. (1973) Z. Physiol. Chem. 354, 83-89.
3. Monder, C., and Bardlow, H.L. (1977) J. Steroid Biochem. 8, 897-908.
4. Bradlow, H.L., and Monder, C. (1979) in Steroid Biochemistry (Hobkirk, R., ed.) Vol. 1, pp. 30-82, CRC Press.
5. Bradlow, H.L., Zumoff, B., Monder, C., Lee, H.J., and Hellman, L. (1973) J. Clin. Endocrinol. Metabol. 37, 811-818.
6. Turfitt, G.E. (1948) Biochem. J. 367-383.
7. Furuta, T. (1959) Hiroshima J. Med. Sci. 8, 311-322.
8. Wyllie, S.G., Amos, B.A., and Tokes, L. (1977) J. Org. Chem. 42, 725-732.
9. Partridge, L.G., and Djerassi, C. (1977) J. Org. Chem. 42, 2799-2805.
10. Budzikiewicz, H. and Djerassi, C. (1962) J. Am. Chem. Soc. 84, 1430-1439.
11. Gardi, R., and Ercoli, A. (1972) in Organic Reactions in Steroid Chemistry (Fried, J., and Edwards, J.A., eds.) Vol. 1, pp. 375-422, Van Nostrand Reinhold Company, New York, NY.
12. Bergmann, E.E., and Salomonovici, A. (1976) Steroids 27, 431-437.
13. Wicha, J., and Bal, K. (1978) J. Chem. Soc. I. Perkin, 1282-1288.
14. Gustafsson, J., and Stenberg, A. (1971) Eur. J. Biochem. 22, 246-256.
15. Kinsella, R.A., Jr., and Francis, F.E. (1971) J. Clin. Endocrinol. Metab. 32, 801-818.
16. Piatak, D.M., and Wicha, J. (1978) Chem. Rev. 78, 199-241.
17. Tsuda, K., Hayatsu, R., Kishida, Y., and Akagaki, S. (1958) J. Am. Chem. Soc. 80, 921-925.
18. Vanderah, D.J., and Djerassi, C. (1977) Tetrahedron Lett. 683-686.
19. Vanderah, D.J., and Djerassi, C. (1978) J. Org. Chem. 43, 1442-1448.
20. Martin, C.K.A. (1977) Adv. Appl. Microbiol. 22, 29-58.
21. Smith, L.L. (1974) in Terpenoids and Steroids Specialist Periodical Reports (Overton, K., ed.) Vol. 4, pp. 394-530, The Chemical Society, London.
22. Sih, C.J., Wang, K.G., and Tai, H.H. (1968) Biochemistry 7, 796-807.
23. Hayakawa, S. (1973) in Advances in Lipid Research (Paoletti, R., and Kritchevsky, D., eds.) Vol. 11, pp. 143-192, Academic Press, New York, NY.
24. Hayakawa, S. (1980) Tokyo Tanabe Q. 198-216.
25. Seifert, W.K., Gallegos, E.J., and Teeter, R.T. (1972) J. Am. Chem. Soc. 94, 5880-5887.
26. Seifert, W.K., (1975) Fortschr. Chem. Org. Naturst. 32, 1-49.
27. Bilton, R.F., and Mason, A.N. (1977) Biochem. Soc. Trans. 5, 1717-1719.
28. Tenneson, M.E., Owen, R.W., and Mason, A.N. (1977) Biochem. Soc. Trans. 5, 1758-1760.
29. Owen, R.W., Tenneson, M.E., Bilton, R.F., and Mason, A.N. (1978) Biochem. Soc. Trans. 6, 377-379.
30. Tenneson, M.E., Bilton, R.F., and Mason, A.N. (1978) Biochem. Soc. Trans. 6, 428-430.
31. Tennseon, M.E., Bilton, R.F., and Mason, A.N. (1978) FEBS Lett. 91, 140-143.
32. Tenneson, M.E., Bilton, R.F., and Mason, A.N. (1978) Biochem. Soc. Trans. 6, 975-977.
33. Tenneson, M.E., Baty, J.D., Bilton, R.F., and Mason, A.N. (1979) J. Steroid Biochem. 10, 311-316.
34. Tenneson, M.E., Baty, J.D., Bilton, R.F., and Mason, A.N. (1979) Biochem. J. 184, 613-618.
35. Tenneson, M.E., Bilton, R.F., Drasar, B.S., and Mason, A.N. (1979) FEBS Lett. 102, 311-315.
36. Tenneson, M.E., Baty, J.D., Bilton, R.F., and Mason, A.N. (1979) J.Steroid Biochem. 11, 1227-1232.
37. Ohigashi, T., and Hiroshima, J. (1960) Med. Sci. 9, 83-91 and 125-135; CA 13587 (1961).
38. Hoshita, T., Shimazu, Y., and Matsumura, S. (1959) Hiroshima Igaku 10, 475-479; CA 55, 1844i (1961).
39. Kameo, H. (1958) Hiroshima Igaku 9, 1535-1539; CA 55, 1784i (1961).
40. Shimazu, Y. (1958) Hiroshima Igaku 9, 2243-2250; CA 55, 1785c (1961).
41. Matsumura, S. (1958) Hiroshima Igaku 9, 2235-2242; CA 55, 1785b (1961).

42. Goddard, P., and Hill, M.J. (1974) J. Steroid Biochem. 5, 569-572.
43. Norman, A., and Shorb, M.S. (1962) Proc. Soc. Exp. Biol. Med. 110, 552-555.
44. Back, P., and Walter, K. (1980) Gastroenterology 78, 671-676.
45. Hill, M.J. (1975) Cancer 36, 2387-2400.
46. Sterzycki, R. (1979) Synthesis, 724-725.
47. Nagata, W., and Itazaki, H. (1964) Chem. Inc. 1194-1195.
48. Heusser, H., Eichemberger, K., and Plattner, P.A. (1950) Helv. Chim. Acta 33, 1088-1092.
49. Plattner, P.A., and Schreck, W. (1939) Helv. Chim. Acta 22, 1178-1184.

[Received September 14, 1981]

Microsomal Enzymes of Cholesterol Biosynthesis from Lanosterol: A Progress Report

JAMES M. TRZASKOS[a], WAYNE D. BOWEN[a,b], GARY J. FISHER[a,b], JEFFREY T. BILLHEIMER[a,b] and JAMES L. GAYLOR[a,b,*], [a]*Biochemistry Department, University of Missouri, Columbia, MO 65212;* and [b]*Field of Biochemistry, Cornell University, Ithaca, NY 14853*

ABSTRACT

Our principal goal is the complete resolution and reconstitution of the microsomal enzymes of cholesterol biosynthesis. Elucidation of the enzymology has been achieved primarily through dissection of the membrane-bound, 19-step multienzymic process. This report describes the dissection approach through both interruption of specific steps and reconstitution of enzymes that catalyze oxidation of the 14α-methyl group. In earlier work, 4-demethylation was resolved into 3 component reactions catalyzed by: 4-methyl sterol oxidase (NAD[P] H- and O_2-dependent); steroid 4α-carboxylic acid decarboxylase (NAD-dependent); and 3-ketosteroid reductase (NADPH-dependent). The 3-ketosteroid reductase and decarboxylase have been solubilized with Lubrol WX and deoxycholate, respectively, and characterized. The 4-methyl sterol oxidase (cytochrome b_5-dependent) recently has been solubilized with Renex 690. This study represents successful elucidation of a microsomal enzyme sequence by interruption of the central 10-step segment of the multienzymic formation of cholesterol from lanosterol. The initial C-32 oxidative reaction of 14α-methyl group elimination is catalyzed by a form of cytochrome P-450 that is induced by isosafrole. The induced cytochrome P-450 has been solubilized with Emulgen 913 and purified to homogeneity (17 nmol of cytochrome/mg protein). 24,25-Dihydrolanosterol is oxidized by combination of cytochrome P-450 reductase, hematin, NADPH, glutathione, and the purified, isosafrole-induced cytochrome in an artificial liposome. Oxidation product identification is underway. This study represents successful elucidation of a microsomal multienzymic sequence by solubilization and reconstitution of a segment of the pathway. The remaining enzymes under study are the $\Delta^8 \rightarrow \Delta^7$ isomerase and 3 NADPH-dependent double bond reductases that catalyze reduction of: Δ^7-, Δ^{14}- and Δ^{24}-sterol double bonds. Purification of these nonoxygen-requiring enzymes is in progress. Resolution of the enzymes has demonstrated unequivocally that cholesterol synthesis via this pathway could not have appeared biologically until membranes contained *both* the cytochrome P-450- and cytochrome b_5-electron transport enzymes. Chemically, all enzymic attacks in the formation of cholesterol from lanosterol appear to be initiated on the α-face of the relatively planar steroids. Thus, considerable genetic pressure must have been needed for the stereospecific clearing of the steroidal α-face to form the mature membrane component, cholesterol.
Lipids 17:250-256, 1982.

INTRODUCTION

The biosynthesis of cholesterol from lanosterol is catalyzed by enzymes that are tightly bound to the cellular endoplasmic reticulum and readily isolated in the microsomal fraction as a membrane-bound complex. For investigation of the enzymology of the process, the unique advantage of being able to obtain the entire synthetic enzymic complex is readily offset by 3 difficulties that are presented by affiliation of the enzymes with a membrane (1). First, the steroid product of one reaction becomes the substrate for the next enzyme without either accumulation or diffusion of intermediates from the membrane. Second, although we glibly refer to these membrane-bound enzymes as being associated in a multienzymic complex, direct physical evidence is lacking. However, even within present limits of information, it appears that physical association of the enzymes with each other, as well as with the membrane, can be strongly suggested (2). The third difficulty is that 10 of the 19 plausible reactions in the formation of cholesterol from lanosterol by rat liver microsomal enzymes (Table 1) require molecular oxygen and a source of reducing equivalents (3). These membrane-bound, mixed-function oxidases are also multienzymic; thus, to produce cholesterol enzymatically, one multienzymic system must function within another multienzymic system.

Our goals are: (a) to establish the enzymic pathway from lanosterol to cholesterol; (b) to resolve, purify and characterize each microsomal enzyme; and (c) to reconstitute into artificial membranes a viable and controlled fully enzymic synthesis of cholesterol from lanosterol.

Elimination of C-30 and C-31 as CO_2

For ca. 12 years, our approach to unraveling the multienzymic system has been to prevent the steroidal product of one reaction from

*To whom inquiries should be addressed: Glenolden Laboratory, E.I. du Pont and Co., Glenolden, PA 19036.

TABLE 1

Suggested Intermediates and Reactions of Cholesterol Biosynthesis from Lanosterol

	Number of C-atoms of the substrate	Reaction	Double bonds	Nonsterol substrates	State of oxidation of C-3 oxygen function	State of oxidation of the attacked carbon atom
1	30	Oxidation	8,24	NADPH, O_2	-OH	C-32-CH_3
2		↓	↓	NAD(P)H, O_2	-OH	C-32-OH
3		↓	↓	NAD(P)H, O_2	-OH	C-32=O
4	↓	Decarbonylation	↓	–	-OH	C-?-OH C-32=O
5	29	Reduction	8,14,24	NADPH	-OH	
6	↓	Oxidation	8,24	NADH, O_2	-OH	C-30-CH_3
7	↓	↓	↓	↓	-OH	C-30-OH
8	↓	↓	↓	↓	-OH	C-30=O
9	↓	Decarboxylation	↓	NAD	-OH	C-30≡OOH C-3-OH
10	28	Reduction	↓	NADPH	=O	
11	↓	Oxidation	↓	NADH, O_2	-OH	C-31-CH_3
12	↓	↓	↓	↓	-OH	C-31-OH
13	↓	↓	↓	↓	-OH	C-31=O
14	↓	Decarboxylation	↓	NAD	-OH	C-31≡OOH C-3-OH
15	27	Reduction	↓	NADPH	=O	
16	↓	Isomerization	↓	–	-OH	
17	↓	Oxidation	7,24	NADH, O_2	-OH	
18	↓	Reduction	5,7,24	NADPH	-OH	
19	↓	Reduction	5,24	NADPH	-OH	
20	↓	–	5	–	-OH	

becoming the substrate of the next enzyme to demonstrate the formation of a particular metabolite as well as to anticipate the next enzyme in sequence. Although the overall system is complex, the individual enzymes that catalyze cholesterol synthesis have simple requirements for activity; only NAD(P)H, NAD and O_2 are needed for the 19-step process (Table 1, Fig. 1). Thus, when 4α-methyl-5α-cholest-7-en-3β-ol with ^{14}C-label in C-30, the 4α-methyl group, is attacked by a mixed function oxidase, we expected to generate a 4α-hydroxymethyl sterol in the strict absence of oxidized pyridine nucleotide to prevent further conversion (Fig. 2). The expectation was based on Bloch's suggestion (4) that NADPH-dependent mixed-function oxidative attack would likely be followed by an NAD-dependent alcohol dehydrogenase. For these studies, a detergent treatment (5) was devised to remove entrapped, endogenous microsomal pyridine nucleotide, thus rendering oxidative release of $^{14}CO_2$ from the ^{14}C-labeled 4α-methyl group fully dependent on addition of exogenously oxidized pyridine nucleotide (Fig. 2). Incubation of 4α-methyl-5α-cholest-7-en-3β-ol with NADPH, oxygen, and the detergent-treated rat liver microsomes without the presence of either endogenous or exogenous NAD yielded the 4α-carboxylic acid rather than the expected alcohol (Fig. 2). Miller and Gaylor (6) characterized the carboxylic acid by tedious scaling-up of acid formation, gas liquid chromatographic (GLC) purification of the isolated acid as the stable diester, and mass spectral analysis. Miller and Gaylor and Miller et al. further demonstrated that *only* mixed-function oxidative attack was necessary to generate the carboxylic acid and that the 4α-methyl group of the 4,4-gem-dimethyl sterol substrate (e.g., **6** of Table 1) was oxidized by the same microsomal oxidase (7,8). Subsequent work established the stoichiometry of the oxidase shown in Figure 2 (9). Therefore, by successful interruption of the overall process, we were not only able to eluci-

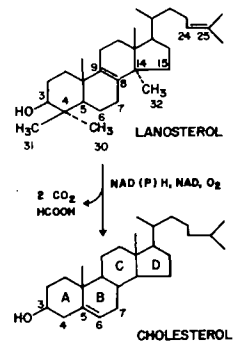


FIG. 1. Structures and overall reaction requirements for the conversion of lanosterol to cholesterol.

date the 6 oxidative steps of the reactions shown in Table 1, **6→7→8→9** and **11→12→13→14**, but because the 4α-carboxylic acid accumulated in the absence of oxidized pyridine nucleotide, oxidative decarboxylation, e.g., **9→10** and **14→15**, was found to be NAD-dependent. Rahimtula and Gaylor partially purified the decarboxylating enzyme after isolation from microsomes with the strong, ionic detergent, deoxycholic acid (10). The decarboxylating enzyme acts on both 4-mono- and 4-disubstituted carboxylic acids, and requires NAD as expected. Reduction of the resulting 3-ketosteroids, i.e., **10→11** and **15→16**, is catalyzed by an NADPH-dependent enzyme that recently has been solubilized and partially purified by Billheimer et al. (11) who treated microsomes with a mixture of detergents, cholate and Lubrol WX. The sequence of reactions elucidated by this process of interruption of microsomal enzymes from **6** to **16** represents the 10 central enzymic steps in the 19-reaction generation of cholesterol from lanosterol (Table 1).

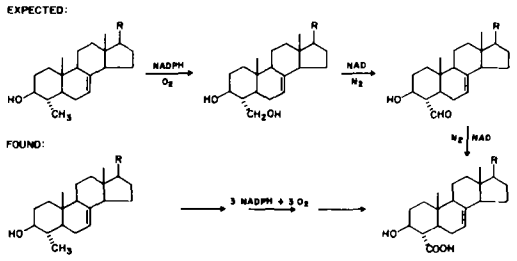


FIG. 2. Reactions in the oxidation of the 4α-methyl group of a synthetic C_{28}-sterol intermediate. The "expected" pathway was proposed by Bloch (4) on the assumption that mixed-function oxidative attack would be followed by an NAD-dependent alcohol dehydrogenase and an NAD-dependent aldehyde dehydrogenase. As described (6), a fully oxidative pathway was found.

Elimination of C-32

With emerging elucidation of the oxidation of the 4-gem dimethyls proceeding well by interruption through specific choices of oxidized and reduced pyridine nucleotides incubated with and without oxygen (e.g., see list of nonsterol substrates in Table 1), in 1975, we initiated analogous studies on the interruption of C-32 oxidation. Microsomal metabolism of lanosterol (1 of Table 1) requires only NAD(P)H and oxygen (Table 2), and when the medium is further supplemented with NAD^+, a C_{27}-sterol product can be obtained chromatographically (Fig. 1). Our attempts to interrupt 14α-demethylation by analogy to the C-30 eliminations already described have been frustratingly unsuccessful. Removal of either NADPH or O_2 led to inactivation of lanosterol attack (Table 2), and although both CO and CN^- were found to be strong inhibitors of microsomal oxidation of lanosterol (Table 2), no oxygenated intermediates accumulated, thus suggesting that the initial oxidation is particularly sensitive to these inhibitors. The 2-step incubations of detergent-treated microsomes with various mixtures of oxidized and reduced pyridine nucleotides that had been so successful in elucidation of steps in the elimination of the 4,4-gem dimethyl group yielded accumulation of no isolable oxygenated steroids.

TABLE 2

Microsomal Oxidation of [^{3}H] Lanosterol[a]

Incubation conditions	Sp act (nmol/30 min·mg protein)	%
Complete	1.79	100
-NADPH	0.24	13
-NAD	1.65	92
-O_2	0.01	0
+CO (90% v/v)[b]	0.70	39
+CN^- (2.67 mM)	0.02	1
+Hematin (5 μM)	1.89	106

[a]All experiments were with rats that had been fed cholestryamine as an inducer of sterol biosynthesis (9). [3α-^{3}H] Lanosterol (100 nmol, 186,000 dpm) was incubated for 30 min at 37 C with 4 mg of microsomal protein, oxygen, a NADPH-generator (consisting of 0.3 mM NADPH, 5 mg of DL-sodium isocitrate, 0.6 mM Mg^{++}, 0.1 mg of isocitric dehydrogenase [Sigma type IV]), and 0.56 mM NAD in a final vol of 2 ml of phosphate buffer (pH 7.4 and containing 1 mM glutathione). Activity was measured by the obligatory loss of the 3α-^{3}H from sterol during oxidative decarboxylation (3). Thus, the assay is a relatively simple and sensitive estimate of combined C_{27} + C_{28}-sterols generated from the C_{30}-sterol substrate. Each value is the result of data from 2 experiments.

[b]10% oxygen.

Fortunately, studies were underway in other laboratories that provided a rational basis for an alternative to our unsuccessful approach of attempted interruption of C-32 oxidation. By 1973, Gibbons and Mitropoulos (12) demonstrated that carbon monoxide, a diagnostic inhibitor of microsomal cytochrome P-450-dependent, mixed-function oxidases, inhibits the oxidation of lanosterol. Because we had earlier established CO-insensitivity of attack of the 4α-methyl group (13) by mixed-function oxidation, and now that we know sterol synthesis after lanosterol attack requires only microsomal cytochrome-b_5 rather than cytochrome P-450 (14,15), cytochrome P-450-dependent attack of lanosterol as the initial step in the 19-step process has been strongly indicated. Penetrating studies in several laboratories (16-18) also showed obligatory cytochrome P-450-dependence of lanosterol oxidation directly, and evidence accumulated that the first reaction of 14α-methyl group elimination is catalyzed by the microsomal cytochrome P-450-containing enzyme. Aoyama and Yoshida were the first workers to isolate an induced lanosterol-metabolizing cytochrome P-450 that was obtained from oxygen-starved, intact yeast; they showed direct dependence of C-32 oxidation upon cytochrome P-450 in yeast isolates (14,18).

Thus, by 1978, with this knowledge about cytochrome P-450 dependence and with no successful interruptions of C-32 oxidation, we changed our approach from interruption to solubilization of the mammalian lanosterol oxidase (i.e., 1→2). We simply set out to purify directly the mammalian, microsomal cytochrome P-450 that would catalyze oxidation of lanosterol.

Most investigations of microsomal cytochrome P-450 isozymes have been done by attempted selective induction in vivo of one form of the hemoprotein relative to the concentrations of the other isozymes. Several types of assays have been used to measure induction. Solubilization, purification, and characterization of the isozyme are then done to demonstrate that a novel form of cytochrome P-450 has been induced. Then, 2 questions are asked: is the isozyme present in uninduced microsomes, and, if so, what is the endogenous substrate for the naturally occurring isozyme? The second question arises because one would not expect to find an enzyme for which there is no known substrate.

Our goal was to isolate and purify the cytochrome P-450 isozyme that catalyzes initial attack of lanosterol. By obtaining pure cytochrome P-450 in the absence of other enzymes that catalyze attack of sterol substrates, we could achieve the equivalence of interruption through reconstitution of active oxidase that might yield the elusive oxygenated intermediate. Accordingly, our study reversed the typical cytochrome P-450 investigation. We know that an endogenous substrate, lanosterol, exists in microsomal membranes. Basal biosynthesis of cholesterol from lanosterol requires the presence of the proper isozyme.

From earlier studies of cytochrome P-450, certain characteristics of the lanosterol-metabolizing isozyme were expected (Table 3). This isozyme of cytochrome P-450 is quite sensitive to inhibition by cyanide (Table 2); accordingly, the isozyme should exhibit a relatively high affinity for cyanide when liganded as the ferri-hemoprotein (19,20). This isozymic form of cytochrome P-450 cochromatographs with NADH-cytochrome b_5-reductase and methyl sterol oxidase (19,21,22). Thus, although the cytochrome P-450 is fully reducible with

TABLE 3

Expected Properties of the Cytochrome P-450 Isozyme That Oxidizes Lanosterol

1. Inhibition by cyanide and relatively high affinity for CN^- binding to Fe^{+3} hemoprotein.
2. Copurifies with NADH-cytochrome b_5 reductase and methyl sterol oxidase; NADH-synergism.
3. Labile. Loses heme group readily with loss of spectral characteristics. Partially reversed to holoenzyme by hematin.

TABLE 4

Induction of Dihydrolanosterol Oxidation by Treatment of Rats with Isosafrole[a]

Pretreatment	Sp act (nmol/30 min/mg protein)	
	Undisplaced	Displaced
None	0.93	0.03
Isosafrole	0.90	2.13[b]
Cholestryamine	2.73	–

[a]Rats were pretreated with isosafrole, and microsomes obtained from control and isosafrole-treated rats were incubated with 2-methylbenzimidazole to displace the endogenous ligand as described previously (24). [24,25-$^3H_2$]24,25-dihydrolanosterol (100 nmol, 200,000 dpm) was incubated for 60 min at 37 C with 4 mg of microsomal protein, oxygen, a 0.3 mM NADPH-generator (Table 2), and 0.56 mM NAD in a final vol of 2 ml. The amount of conversion was calculated by TLC separation of substrate from the mixture of labeled metabolites in the usual manner. Results are the averages of values from 2 comparisons.

[b]2.5 μM hematin.

NADPH alone, modest synergism can be observed by the addition of NADH along with NADPH. Finally, although relatively high lability is not particularly diagnostic, it is a characteristic that had become quite pronounced in our earlier studies and lability certainly mandated stabilization of the isozyme sought.

Obviously, induction of this isozyme by oxygen starvation of living rats, as Aoyama and Yoshida (14,18) had induced the lanosterol-metabolizing cytochrome P-450 in intact yeast, is not possible. Alternatively, we examined literature reports of hepatic, microsomal cytochrome P-450 inductions by drugs, carcinogens and other xenobiotics to find an inducer of the isozyme of cytochrome P-450 with the desired characteristics. In 1978, Dickins and coworkers (23) reported isosafrole-induction of a novel form of rat hepatic cytochrome P-450 that is readily distinguished from the other isozymes by a molecular weight that is intermediate between the weights of rat hepatic isozymes that are induced by phenobarbitol and methylcholanthrene. Upon treatment with isosafrole – (1,2-methylenedioxyl)-4-propenyl benzene–the isozyme of cytochrome P-450 is readily observed by measuring the spectral difference between reduced and oxidized hemoprotein in which the presence of an endogenously generated isosafrole metabolite ligand causes a Soret peak shift to 455 nm (24). (With this simple assay, we have purified the cytochrome P-450 to homogeneity [24].)

We did not want to proceed with studies involving a proven hepatocarcinogen (25) until first showing induction of lanosterol metabolism by isosafrole. Initial studies of 24,25-dihydrolanosterol metabolism were disappointing until investigations were conducted with isosafrole-induced microsomes from which the endogenous metabolite of isosafrole had been displaced from the induced isozyme of cytochrome P-450 by treatment of microsomes with 2-methyl-benzimidazole (24). Thus, with proper metabolite displacement, induction of dihydrolanosterol oxidation of 3- to 7-fold (Table 4) can be observed. The drop in control values with the displacement treatment further emphasizes the extreme lability of even the uninduced isozyme. When optimally fortified with hematin (see Table 2), the control rate of "displaced" microsomal lanosterol oxidation is enhanced maximally to about 0.3 nmol/30 min/mg protein; thus, under identical conditions of displacement and incubation with 2.5 μM hematin, a 7-fold induction by isosafrole is observed. (Lability is associated with loss of the heme group from the hemoprotein; the isosafrole metabolite formed in vivo stabilizes the cytochrome P-450 isozyme during solubilization and purification [24]. See also ref. 26 for heme stimulation.)

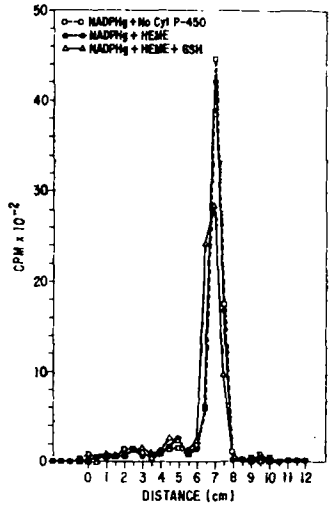


FIG. 5. Reconstitution of vesicular cytochrome P-450 with hematin and glutathione. The conditions were identical to the procedure described in Fig. 4. The final concentrations were 2.5 μM hematin and 5 mM glutathione.

Anticytochrome P-450 immune serum prepared against the pure isozyme of cytochrome P-450 cross-reacted with a protein in control microsomes. Qualitatively (Fig. 3), the amounts of cross-reactive protein were approximately proportional to the relative rates of lanosterol oxidation (Table 4).

Incubation of isosafrole-induced cytochrome P-450, cytochrome P-450 reductase, phospholipid, oxygen and NADPH with isosafrole generates a spectrally determined hemoprotein-bound oxidized metabolite (24). The metabolite difference spectral peak is identical to that

TABLE 5

Components Considered for Reconstitution of Lanosterol Oxidase

Phospholipids
Cytochrome P-450 (after isosafrole induction)
Cytochrome P-450 reductase
Cytochrome b_5
Cytochrome b_5 reductase
Methyl sterol oxidase
Hematin
NADPH, lanosterol, oxygen, NADH
Glutathione
Z-protein (e.g., SCP, fatty acid binding protein)
Cholesterol transfer protein (SCP_2, general lipid exchange protein)

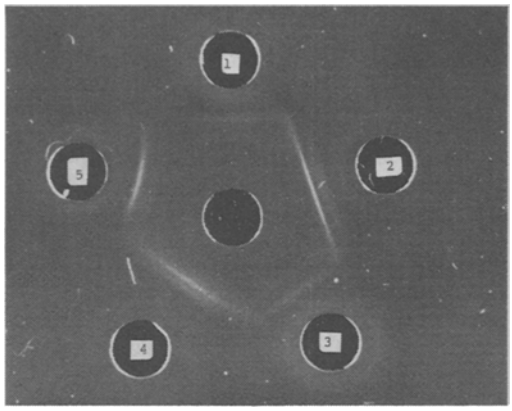


FIG. 3. Ouchterlony diffusions of proteins from solubilized microsomes. Anticytochrome P-450 immune serum was obtained from rabbits immunized with homogeneous cytochrome P-450 from isosafrole-treated rats (24). Microsomes solubilized in 1% Triton X-100 were placed in wells: 1, control; 3, cholestyramine; and 5, isosafrole. Pure cytochrome P-450 was placed in wells 2 and 4.

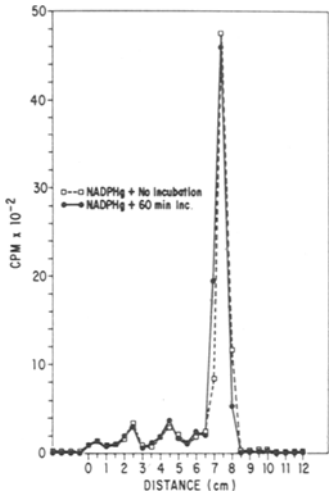


FIG. 4. Reconstitution of vesicular cytochrome P-450. Purified cytochrome P-450 (9.0 nmol) was suspended in 2.2 ml of phosphate buffer to which 3.6 mg of dilaurylphosphatidyl choline and 24 units of cytochrome P-450 reductase were added. The mixture was incubated for 15 min at 37 C with 17 mM 2-methylbenzimidazole and 20% (v/v) of glycerol in phosphate buffer to displace the bound metabolite of isosafrole (24). After cooling on ice, 18 mg of egg phosphatidylcholine in 0.2 ml of a 2% solution of sodium cholate (w/v), 0.2 mM EDTA, and 0.1 mM dithiothreitol were added. The mixture was passed through a Sephadex G-50 column (1.7 × 18 cm) to remove cholate and to generate vesicles. An amount of vesicles containing 1.0 nmol of cytochrome P-450 was incubated in 2 ml for 60 min with 100 nmol of 24,25-dihydrolanosterol by sonic suspension of the steroid with additional phospholipid. Samples of residue from solvent extraction were transferred to TLC plates that were developed with chloroform. TLC mobility was estimated by cutting the plates into 0.5 cm fractions and counting. Final concentrations were 0.3 mM NADPHg (NADPH-generator, see Table 2).

formed in vivo. Substitution of [24,25-^{3}H] dihydrolanosterol for isosafrole in the reconstitution was not as rewarding. No evidence for metabolism of 24,25-dihydrolanosterol was observed in the reconstituted system (Fig. 4). A scan of radioactivity on a TLC plate did not indicate either loss of radioactivity from substrate or formation of a labeled oxidation product that would be expected to chromatograph between the origin and the substrate peak. A more elaborate procedure with 2-methylbenzimidazole pretreatment to remove endogenously bound metabolite, in case lanosterol substrate could not displace the substance, was also negative. However, there was a slight broadening of the peak, particularly when hematin and glutathione were added (Fig. 5), plus a significant reduction in the amount of unchanged substrate was observed when the complete incubation mixture was added. The peak-broadening and substrate loss were associated consistently with metabolic conditions, i.e., requiring the complete reconstitution system plus aerobic incubation at 37 C.

On the basis that we might be generating an analogous cytochrome P-450-bound metabolite that was preventing enzymic turnover by not diffusing from the hemoprotein (24), plus the earlier observation that the high cyanide affinity isozyme copurifies with methyl sterol oxidase (19,21,22 and Table 3), a partially purified source of methyl sterol oxidase (15) was added to the reconstitution. Incubation of the complete system (Fig. 4) with methyl sterol oxidase plus cytochrome b_5, NADH, NADPH, oxygen and 100 nmol of 24,25-dihydrolanosterol yielded significant conversion to a slightly slower-moving sterol on thin layer chromatography (TLC). High resolution mass spectral analysis suggests that a C_{29}-sterol product was formed. Characterization is incomplete at this time. (A large quantity of the pure isosafrole-induced cytochrome P-450 will be needed for each experiment without methyl sterol oxidase addition because a steroidal metabolite does appear to be bound to the hemoprotein, and necessary turnover to generate accumulation of the initial oxidation product of lanosterol does not occur.)

Conditions for reconstitution of the com-

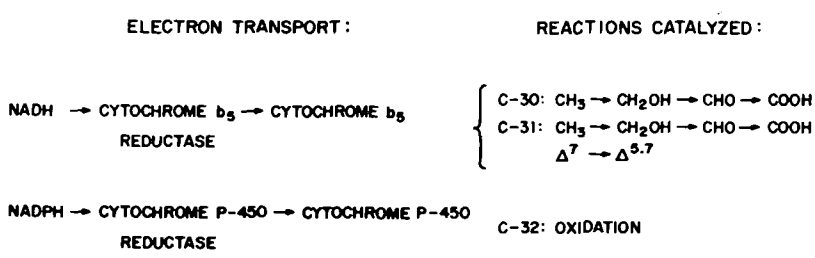


FIG. 6. Microsomal electron transport and sterol biosynthetic reactions catalyzed.

plete 14α-methyl group elimination will be complicated. Fourteen possible components (Table 5) must be checked for optimization of concentration. In addition to the enzymes and substrates, the list includes variations in phospholipids, plus noncatalytic proteins such as Z-protein (26) and a cholesterol (general lipid) transfer protein that Trzaskos has characterized as SCP_2.

Two brief comments should be made. With demonstrated dependence on both cytochrome P-450 and cytochrome b_5 of microsomal electron transport, it is clearly evident why de novo sterol synthesis does not occur in less complex organisms that lack either of these systems (Fig. 6). Second, within limits of present information, all reactions in the process appear to proceed via α-face attack of the relatively planar sterol molecule (3). Thus, in addition to suggesting that the product of one reaction becomes the substrate for the next enzyme in sequence without randomization, the removal of only α-methyl groups to produce cholesterol presumably produces more stable sterol components of biological membranes. Accordingly, enormous biological and chemical pressure may have resulted in selection of stable organisms that have the oxidative machinery to catalyze sterol α-methyl group elimination.

ACKNOWLEDGMENT

This work was supported by Grant AM 21336 from the United States Public Health Service. The authors gratefully acknowledge the technical assistance of A. Terry.

REFERENCES

1. Gaylor, J.L. (1972) Adv. Lipid Res. 10, 89-141.
2. Gaylor, J.L. (1974) in MTP International Review of Science, Biochemistry Section (Lipids) (Goodwin, T.W., ed.) Vol. 1, pp. 1-42, Medical and Technical Publishing Co., Oxford, England.
3. Gaylor, J.L. (1981) in Polyisopropenoid Biosynthesis (Porter, J.W., ed.) Vol. 1, pp. 481-543, John Wiley and Sons, New York, NY.
4. Bloch, K.B. (1965) Science 150, 19-28.
5. Miller, W.L., Kalafer, M.E., Gaylor, J.L., and Delwiche, C.V. (1967) Biochemistry 6, 2673-2678.
6. Miller, W.L., and Gaylor, J.L. (1970) J. Biol. Chem. 245, 5369-5374.
7. Miller, W.L., and Gaylor, J.L. (1970) J. Biol. Chem. 245, 5375-5381.
8. Miller, W.L., Brady, D.R., and Gaylor, J.L. (1971) J. Biol. Chem. 246, 5147-5153.
9. Gaylor, J.L., Miyake, Y., and Yamano, T. (1975) J. Biol. Chem. 249, 1980-1987.
10. Rahimtula, A.D., and Gaylor, J.L. (1972) J. Biol. Chem. 247, 9-15.
11. Billheimer, J.T., Alcorn, M., and Gaylor, J.L. (1981) Arch. Biochem. Biophys. 211, 430-438.
12. Gibbons, G.F., and Mitropoulos, K.A. (1973) Eur. J. Biochem. 40, 267-273.
13. Gaylor, J.L., and Mason, H.S. (1968) J. Biol. Chem. 243, 4966-4972.
14. Aoyama, Y., Yoshida, Y., Sato, R., Susani, M., and Ruis, H. (1981) Biochim. Biophys. Acta 663, 194-202.
15. Fukushima, H., Grinstead, G.F., and Gaylor, J.L. (1981) J. Biol. Chem. 256, 4822-4826.
16. Gibbons, G.F., Pullinger, C.R., and Mitropoulos, K.A. (1979) Biochem. J. 183, 309-315.
17. Trocha, P.J., Jasne, S.J., and Sprinson, D.B. (1974) Biochem. Biophys. Res. Commun. 59, 666-671.
18. Aoyama, Y., and Yoshida, Y. (1978) Biochem. Biophys. Res. Commun. 85, 28-34.
19. Comai, K., and Gaylor, J.L. (1973) J. Biol. Chem. 248, 4947-4955.
20. Jefcoate, C.R.E., and Gaylor, J.L. (1969) Biochemistry 8, 3464-3472.
21. Gaylor, J.L., Moir, N.J., Seifried, H.E., and Jefcoate, C.R.E. (1970) J. Biol. Chem. 245, 5511-5513.
22. Gaylor, J.L., Hsu, S.T., Delwiche, C.V., Comai, K., and Seifried, H.E. (1973) in Oxidases and Related Redox Systems (King, T.E., Mason, H.S., and Morrison, M., eds.) Vol. 2, pp. 431-444, Univ. Park Press, Baltimore, MD.
23. Dickins, M., Bridges, J.W., Elcombe, C.R., and Netter, K.J. (1978) Biochem. Biophys. Res. Commun. 80, 89-96.
24. Fisher, G.J., Fukushima, H., and Gaylor, J.L. (1981) J. Biol. Chem. 256, 4388-4394.
25. Borchert, P., Miller, J.A., Miller, E.C., and Shires, T.K. (1973) Cancer Res. 33, 590-600.
26. Billheimer, J.T., and Gaylor, J.L. (1980) J. Biol. Chem. 255, 8128-8135.

[Received August 17, 1981]

A Comparison of the Biological Properties of of Androst-5-en-3β-ol, a Series of (20R)-*n*-Alkylpregn-5-en-3β-ols and 21-Isopentylcholesterol with Those of Cholesterol

WILLIAM R. NES, JOHN H. ADLER, JEFFREY T. BILLHEIMER, KATHERINE A. ERICKSON, JOHN M. JOSEPH, JOSEPHINE R. LANDREY, ROSEMARIE MARCACCIO-JOSEPH, and KARLA S. RITTER, *Department of Biological Sciences, Drexel University, Philadelphia, PA 19104,* and ROBERT L. CONNER, *Department of Biology, Bryn Mawr College, Bryn Mawr, PA 19010*

ABSTRACT

The Δ^5-sterol, androst-5-en-3β-ol, which has no side chain at C-17, did not permit molting of the insect *Heliothis zea*, growth of either the protozoan *Tetrahymena pyriformis*, or the yeast *Saccharomyces cerevisiae* adapted to anaerobic conditions, nor was the sterol esterified by a mammalian microsomal ACAT preparation. However, the sterol did form a liposome with egg lecithin and, when fed to mice, did inhibit hepatic cholesterol synthesis. 21-Isopentylcholesterol also formed a liposome but neither supported the growth of the yeast nor was metabolized by the protozoan. When sterols, 20(R)-*n*-alkylpregn-5-en-3β-ols, with side chains of varying lengths were added to the medium of the protozoan, maximal esterification with fatty acids occurred with the 20(R)-*n*-pentyl derivative, and maximal inhibition of tetrahymanol formation occurred with the *n*-butyl, *n*-pentyl, and *n*-hexyl derivatives. In all of the assays, cholesterol showed a positive response, either permitting molting or growth, being metabolized, inhibiting sterol or tetrahymanol synthesis, or forming a liposome.
Lipids 17:257-262, 1982.

INTRODUCTION

As pointed out previously (1,2), the reason why the side chains of sterols have their observed structures is not entirely clear. During the biosynthetic process, the number of C_5-units could conceivably be varied, leading to analogs of squalene with, for instance, one C_5-unit more or one less than in squalene, but this never seems to occur. Squalene is the consistent intermediate to sterols which means that the side chain always arises biosynthetically with a carbon skeleton that is the same as the one found in cholesterol. This side chain once formed may then undergo a loss (as in halosterol, a C_{26}-sterol) or a gain of carbon atoms (as in campesterol, a C_{28}-sterol). For a key to the literature on occurrence and metabolism, see Nes and McKean (3), Barbier (4), Goad (5), Khalil et al. (6), and Blanc and Djerassi (7). However, even when such subsequent metabolism does occur, the length of the longest chain on C-17 is rarely altered by more than one carbon atom from the original 6-carbon sequence consisting of C-20, C-22, C-23, C-24, C-25 and C-26. Furthermore, C-21 never seems to be removed nor have additional carbon atoms been found added to it, and the configuration at C-20 is nearly always (8-11) such as to have a 20α-H-atom. It has been suggested (1,2) that, in an evolutionary sense, these similarities in structure result at least in part from analogous structural requirements in the function of sterols.

In order to explore the significance of function further, we have examined the C_{19}-sterol (androst-5-en-3β-ol) with no side chain at all, as well as a series of sterols (20(R)-*n*-alkylpregn-5-en-3β-ols) in which the total length of the side chain varies and also 21-(3-methylbutyl-1-)-cholesterol (21-isopentylcholesterol, "wingsterol"), in which C-20 bears an H-atom and 2 isohexyl groups in a variety of biological situations. The structural variations have permitted us to assess the influence of a shortening of the side chain to zero carbon atoms as well as of an alteration in the length of both substituents on C-20.

The biological systems were chosen for several reasons, one of which was a desire to have representatives of different parts of the evolutionary hierarchy. At the top of the latter are mammals, and 2 parameters were investigated, microsomal esterification in rat liver and feedback regulation of hepatic cholesterol synthesis. Among the lower organisms, we chose 3 which do not biosynthesize sterols but which respond to their presence. The lack of biosynthesis simplified the experiments, because endogenously derived sterol did not have to be considered. The systems used were an insect, *Heliothis zea*, the protozoan *Tetrahymena pyriformis*, and anaerobically grown *Saccharomyces cerevisiae*. In addition, we studied a model membrane system, viz., the formation of liposomes.

MATERIALS AND METHODS

Androst-5-en-3β-ol (12,13) was synthesized by reduction of the tosylhydrazone of dehydroepiandrosterone (17-oxoandrost-5-en-3β-ol) with sodium borohydride (unpublished). The physical properties of 21-isopentylcholesterol ("wingsterol") prepared from 20-oxo-21-norcholesterol and 4-methylpentyl bromide (unpublished) and reisolated from *T. pyriformis* are given in the section on results and serve to define the structure of this new sterol. The 20(R)-*n*-alkylpregn-5-en-3β-ols used in this work were prepared from pregnenolone (2 and unpublished).

For the formation of liposomes, 60 mg of L-α-phosphatidylcholine and 20 mg of sterol were transferred to a sonicator vessel, and 20 ml of 0.1 M Tris HCl buffer at pH 7.4 was added. The mixture was sonicated for 15 min at 85 W in an ice-NaCl water bath with a Sonicator Cell Disruptor Model 185-F, Heat Systems Ultrasonics, Plainview, NY. The resulting sample was centrifuged for 1.0 hr at 40,000 × g. The supernatant was extracted 3 times with 10 ml of a 2:1 $CHCl_3/CH_3OH$ mixture. The amounts of sterol in the combined organic layers and in the pellet fro. centrifugation were determined by gas liquid chromatography (GLC) on 1% XE-60 at 235 C. Data given are averages of 4-8 experiments and represent the percentage of the original sterol sonicated which was found in the supernatant or pellet.

T. pyriformis W cultures were grown in 100 ml of a peptone-based culture fluid supplemented with 1.5 mg of sterol (25 μM) for 21 hr at 28.5 C. The sterols were added in ethanolic solution (0.2 ml). Unsupplemented cultures were grown with an equal amount of ethanol. Cell numbers were estimated with a Model A Coulter Counter equipped with a 200 μm orifice. The morphology and motility of the cells were observed with a Wild-Heerburgh inverted phase contrast microscope at a magnification of 300X. The cells were isolated by centrifugation, rinsed with water and recentrifuged. The lipids were extracted 3 times from the cell pellet with boiling chloroform/methanol (2:1, v/v) and saponified overnight in a nitrogen atmosphere at 37 C with 5 ml of 10% KOH in 80% methanol. The nonsaponifiable fraction, which contained the sterol and tetrahymanol, was not purified further but was analyzed by GLC on a 0.75% SE-52 Gas Chrom P column at 235 C. The peak for tetrahymanol was traced on paper, cut out and weighed. The weights were compared with those from peaks derived from known amounts of tetrahymanol.

For sterol ester content, the total lipid extract was separated on a column of 1.0 g of Unisil silicic acid into neutral and polar lipids. The neutral lipids were analyzed by thin layer chromatography (TLC) on prewashed, activated Silica Gel G plates in C_6H_6/EtOAc (20:1, v/v). Visualization was accomplished with a spray of 10% phosphomolybdic acid in pure ethanol. The sterol ester region, identified by comparison with cholesteryl palmitate, was scraped from the plates and saponified directly overnight at 37 C with 10% KOH in 80% methanol. The resulting nonsaponifiable fraction was analyzed for free sterol by GLC.

The conditions for the incubation of *T. pyriformis* with wingsterol (21-isopentylcholesterol, 120 mg distributed in 20 500-ml batches of medium) were similar to those we have reported for other metabolic studies (14). Procedures for the growth of *H. zea* (15), the assay of the activity of acyl CoA:cholesterol acyltransferase (ACAT) (16), and the anaerobic growth of *S. cerevisiae* well adapted to anaerobiosis (17) were as previously published. Data given are based on visual counts. After 72 hr under anaerobic conditions, 3 ml of sterile air was added, and the cells were counted after an additional 72 hr (fermentative growth). The sterol in the cells was determined for cholesterol after the first 72-hr period and for androst-5-en-3β-ol and wingsterol after the second 72-hr period.

Feedback inhibition of hepatic cholesterol biosynthesis was examined using test sterols which were administered to C57BL/6J mice (Jackson Laboratory) as either 0.25 or 0.5% of the diet for 26 hr. Liver homogenates were prepared in a medium based on that of Bucher and McGarrahan (18) using a volume of medium equal to 2.5 times the wet weight of liver. Homogenates were centrifuged at 800 × g for 10 min at 0-5 C. Incubation mixtures contained 3 ml of the liver homogenate supernatant 0.0016 M NAD, 0.16 M glucose-6-phosphate, 0.001 M NADPH and labeled substrate, [1-^{14}C]acetate (12 μCi, 7 μmol) or D,L-[2-^{14}C]-mevalonate (1.0 μCi, 7.5 μmol) in a total volume of 3.5 ml. Samples were incubated at 37 C for 2 hr in a water bath with shaking at medium intensity. The incubation was terminated by transferring the mixture into 10 ml of 10% potassium hydroxide in methanol (w/w). Carrier sterols were added. The samples were saponified and extracted with diethyl ether. Sterols were isolated from the neutral lipid fraction by TLC on Silica Gel G with C_6H_6/ET_2O (9:1, v/v) and eluted directly into a toluene scintillation cocktail. Radioactivity was quantitated by liquid scintillation counting.

RESULTS

Growth-Support to *H. zea*

The number of molts in a given period of time which the larvae of *H. zea* undergo is dependent on the structure of the sterol in the diet (15,19). During 2 weeks, 95% of 142 larvae grown on dietary cholesterol survived, and 76% of the number of possible molts (5 times the number of larvae) were completed. However, when cholesterol was replaced by androst-5-en-3β-ol, no molts were completed in 2 weeks, and of the 28 animals used, 96% died. Similar results were found with pregn-5-en-3β-ol (19). In the absence of sterol, no molts were completed, and 97% of the 111 larvae died.

Experiments with *T. pyriformis*

When cholesterol was added to the medium, biosynthesis of tetrahymanol was inhibited (20), and as shown in Table 1, when other sterols were used, the closer the length of the side chain was to that of cholesterol, the greater was the inhibition. Maximal reduction in the amount of pentacycle, as well as maximal sterol esterification, occurred with a side chain with the same length as that found in cholesterol, i.e., with 20(R)-*n*-pentylpregn-5-en-3β-ol (27 norcholesterol). Analogously, as the side chain became shorter, although maximal growth was maintained from 20(R)-*n*-dodecyl to 20(R)-*n*-pentyl-pregn-5-en-3β-ol, the second sterol became the point after which growth began to fall, reaching zero with androst-5-en-3β-ol. The C_{19}-sterol was actually lethal to the protozoan at rather low concentrations (Table 2), regardless of whether the sterol was added to freshly inoculated cultures or log phase cultures. The effect could not be reversed by preincubation with either cholesterol or tetrahymanol.

TABLE 1

Effect of the Length of the Sterol Side Chain on Growth of *T. pyriformis*, Tetrahymanol Content of the Cells, and Esterification of the Sterols

Sterol	Cell count[a]	Pentacycle content[b]	Amount esterified[c]
Androst-5-en-3β-ol	0	–	–
Pregn-5-en-3β-ol	62	90	0
20(R)-*n*-Butylpregn-5-en-3β-ol	93	2.0	17
20(R)-*n*-Pentylpregn-5-en-3β-ol	107	2.2	85
20(R)-*n*-Hexylpregn-5-en-3β-ol	115	1.9	32
20(R)-*n*-Heptylpregn-5-en-3β-ol	112	9.9	21
20(R)-*n*-Nonylpregn-5-en-3β-ol	112	29.9	0
20(R)-*n*-Dodecylpregn-5-en-3β-ol	109	88.2	0
None	100	100	–
Cholesterol	106	2.0	100

[a]Expressed as % of the cell count of culture grown without sterol.

[b]Expressed as % of the content/cell in cultures grown without sterol.

[c]Expressed as % of the amount of sterol ester formed in cultures grown with cholesterol.

TABLE 2

Tetrahymena Appearance after Treatment with Androst-5-en-3β-ol

Experiment	Treatment of 100-ml cultures after inoculation	Observations		
		At 5 hr	At 9 hr + 10 min	At 9 hr + 40 min
1	Added 0.5 mg of C_{19} at 2 hr	Nonmotile, lysis	–	–
2	Added 0.5 mg of C_{19} at 9 hr	–	Erratic movement; odd shapes	Nonmotile, lysis
3	Added 1.5 mg of cholesterol at 0 hr and 0.5 mg of C_{19} at 2 hr	Nonmotile, lysis	–	–
4	Added 1.0 mg of tetrahymanol at 0 hr and 0.5 mg of C_{19} at 2 hr	Nonmotile, lysis	–	–

With wingsterol after 24 hr, growth had proceeded well to give 2.7 x 10^9 cells (ca. the same number as with cholesterol supplementation) from which 23.7 mg of sterol and 4.5 mg of pentacycle were isolated. The sterol was a single component by GLC, and various criteria (mp 104-105 C; RRT in GLC on XE-60 C at 235 C 2.10; MW by mass spectroscopy: 454; δ in ^{1}H-NMR 0.670 (C-18), 0.860 (3 H, C-26, C-26', C-27, or C-27'), 0.865 (9 H, C-26, C-26', C-27, or C-27'), 1.004 (C-19) ppm; and less than 0.16% $\Delta^{5,7}$-sterol by UV) indicated the material was unchanged wingsterol.

Growth Support in Anaerobic Yeast

Neither the C_{19}-sterol nor wingsterol permitted growth of *S. cerevisiae* grown anaerobically. In addition, neither of these sterols entered cells grown in the presence of a small supply of air. Although 30 x 10^{-15} g/cell of cholesterol was recovered from 70 x 10^6 cells grown in the presence of cholesterol, less than 0.14 and 0.35 x 10^{-15} g/cell of androst-5-en-3β-ol and wingsterol, respectively, were obtained from the 16.5 and 15.2 x 10^6 cells, respectively, derived from the fermentative growth. The C_{19}-sterol and wingsterol permitted virtually no growth (0.65 and 0.25 x 10^6 cells, respectively) under anaerobic conditions.

Hepatic Esterification Catalyzed by ACAT

When the androst-5-en-3β-ol was added to a microsomal preparation from rat liver, the esterification of either endogenous or added cholesterol was greatly depressed, and little or no esterification of the C_{19}-sterol itself occurred (Table 3).

Inhibition of Hepatic Cholesterol Synthesis

The short-term feeding of androst-5-en-3β-ol to mice resulted in an inhibition of hepatic cholesterol synthesis from acetate but not from mevalonate, although the C_{19}-sterol was somewhat less active than cholesterol (Table 4).

TABLE 3

Effect of Androst-5-en-3β-ol on the Esterification of Cholesterol by ACAT from Rat Liver[a]

Additions to assay medium	Activity[b] (nmol/15 min/mg protein)
None	1.65
20 μg Androst-5-en-3β-ol	0.44
10 μg Cholesterol	14.55
10 μg Cholesterol plus 10 μg androst-5-en-3β-ol	7.68

[a]Microsomes (100 μg) and bovine serum albumin (1 mg) were preincubated for 30 min in the presence or absence of exogenous sterol in 0.1 M K phosphate buffer containing 2 mM glutathione and 0.3% Triton WR-1339 (final vol 200 μl). Incubations were initiated by the addition of [^{14}C]oleoyl CoA (20 nmol).

[b]Each value represents the average of triplicate results from 2 experiments.

TABLE 4

Inhibition of Hepatic Cholesterol Synthesis[a]

Sterol	% of Diet (w/w)	% of Control[b] [^{14}C]Acetate converted to sterols	% of Control[b] [^{14}C]Mevalonate converted to sterols
Cholesterol	0.25	28 (8)	100 (5)
Cholesterol	0.50	18 (7)	–
Androst-5-en-3β-ol	0.25	80 (7)	–
Androst-5-en-3β-ol	0.50	28 (8)	103 (5)

[a]Sterol was fed for 26 hr to mice after which the conversion of the labeled substrate to sterol was examined in an hepatic cell free system as described in the experimental section.

[b]Control animals lacked any sterol in the diet. The number in parentheses is the number of animals studied from which a mean was derived and given in the table for % of control. In each series of experiments, there was a set of controls which the mean count for ^{14}C in sterol from [^{14}C]acetate was typically 118,000 dpm and 259,000 dpm from [^{14}C]MVA, representing conversion of 31 and 877 mμmol of acetate and MVA, respectively, to sterol.

Formation of Liposomes

Not only was approximately as much of the C_{19}-sterol solubilized by egg lecithin as with cholesterol, but the same was true with wingsterol. The percentages of original sterol found in the supernatant after centrifugation were 83% for cholesterol, 75% for androst-5-en-3β-ol and 77% for wingsterol. Except for a few percent unaccounted for, the rest of the sterols was found in the pellets.

DISCUSSION

Enlargement of the Side Chain

Increases in the length of the sterol side chain beyond what is found in cholesterol had a uniformly negative effect on the biological properties examined, except for growth of *T. pyriformis*. However, even the latter can be construed to result from a negative effect if we assume the sterols with the longer side chains simply were too large to fit into appropriate receptor sites. As *T. pyriformis* does not require sterols, it will grow unless inhibited. A negative effect of lengthening the sterol side chain beyond one carbon atom was also found earlier (2) with the ability of *T. pyriformis* to dehydrogenate sterols at C-7(8) and C-22(23). In the 20(R)-*n*-alkylpregn-5-en-3β-ol series, maximal metabolism occurred with the pentyl and hexyl derivatives, and it fell to zero with the dodecyl derivative (2). Similarly, for vitamin D activity (21), the rate of mammalian 7α-hydroxylation (22), the rigidity of bilayers (23), and mammalian ACAT activity (24) extension of the length of the sterol side chain by 1-3 carbon atoms has a negative impact on biological properties. This generalization can also be applied to liposome formation, because as the length of the side chain in the 20(R)-n-hexylpregn-5-en-3β-ol series is increased from hexyl to octyl, the amount of sterol incorporated into liposomes falls from 1.0 to 0.42 as measured by the sterol-to-phospholipid ratio (23). Thus, there is a parallelism between function and the number of C_5-units which are found naturally in the side chain.

When the side chain was enlarged, not by total length, but by total bulk as exemplified in wingsterol, cholesterol-like activity was abolished in 2 (growth support to yeast and metabolism in *T. pyriformis*) of the 3 assays studied. However, in the third, the ability to form liposomes was retained. When coupled with the other known correlations of structure and activity, especially with regard to (E)- and (Z)-17(20)-dehydrocholesterol (see following), this means that receptor sites in yeast and *T. pyriformis* must recognize when the alkyl group on either of the sides of C-20 becomes too large. While an isohexyl group on the right will fit into an active site, one on the left will not.

Shortening of the Side Chain

Shortening of the side chain beyond what is found in cholesterol reduced activity in most, but not all, of the assays discussed. Thus, as with lengthening of the side chain, there is a parallelism between the natural length of the side chain and function. Extrapolation of the existing data indicates that removal of one C_5-unit from the side chain would virtually abolish (a) growth support to the insects *H. zea* and *Musca vicina Macq.* (25) as well as to yeast, (b) sterol metabolism and tetrahymanol inhibition in *T. pyriformis*, (c) rigidification of bilayers (23), (d) hepatic esterification, (e) vitamin D activity (21,26) and (f) 7α-hydroxylation (22,27). However, neither liposome formation nor inhibition of hepatic cholesterol synthesis should be prevented by removal of the terminal C_5-unit (C-23 to C-27). Furthermore, removal of a C_5-unit is known not to abolish suppression of permeability in liposomes (28).

Effect of Configurational Changes

In earlier publications, we have reported on the influence of alterations in the stereochemistry at C-20 (2,14,17,29). Inversion of the chirality at C-20 in cholesterol prevents yeast growth (17) and metabolism by *T. pyriformis* (29). We have extended our studies of chirality to halosterol and 20-epihalosterol, and in *T. pyriformis* only the halosterol is metabolized (14).

In addition to alterations in the chirality at C-20, we fixed C-22 to the right and left (in the usual view of the molecule) by introducing a $\Delta^{17(20)}$-bond into cholesterol (E, C-22 to the right; Z, C-22 to the left). The (E)-sterol was active and the (Z)-sterol was inactive in support of yeast growth (17) and protozoan metabolism (29). These stereochemical problems have also been examined by others (30,31) in terms of the induction of oospore formation in the oomycetous fungus *Phytophthora cactorum*, with the same results, viz., only the (E)-$\Delta^{17(20)}$-sterol or the one with a 20α-H-atom was active.

The discrimination between the (E)- and (Z)-$\Delta^{17(20)}$-sterols implies that C-22 must be to the right in sterols with a single bond between C-17 and C-20. As 20α-hydroxy and 20-methylcholesterol are also inactive in the yeast (17) and protozoan (29) assays, as well as in the oomycete assay with one (20α-hydroxych-

lesterol) of the 2 sterols studied (30), we assume there may be no bulk on the front face of C-20 larger than an H-atom.

The biological activity of (E)- and (Z)-$\Delta^{17(20)}$-sterols and of sterols which are epimeric at C-20 indicates that the natural configuration (20α-H-atom) and the ability to have C-22 to the right with no substituent on C-20 facing forward (14,17,29) is frequently an absolute requirement, e.g., in metabolism in *T. pyriformis*, growth support to anaerobic *S. cerevisiae*, and oospore formation in *P. cactorum* (30,31). However, based on the ability of wingsterol to be solubilized by egg lecithin, the formation of liposomes is insensitive to the positioning of C-22 on the right or left.

Classification of Biological Properties

From the foregoing data and discussion, it appears that there are, at least grossly, 2 types of biological phenomena relating to sterol structure. One is very sensitive to a departure from a side chain with a 20α-H-atom, an unsubstituted C-21, the ability to have C-22 to the right, and the presence of 6 carbon atoms in the longest array from C-17 (5 from C-20). The significance of terminal branching is minimal (2,14), except when there is a functional group at C-25 (20,26). It would appear, even though full documentation is unavailable, that these are generally the requirements for the activity of sterols and that the second category comprises an exception. In this second group of biological properties, exemplified by liposome formation and feedback regulation of cholesterol synthesis, the size and direction of the sterol side chain is of little importance unless, as demonstrated with liposomes, it becomes too long, in which case it can prevent solubilization by lecithin. It has been proposed by Suckling et al. (23) that, in liposome formation, a long sterol side chain penetrates into an adjacent half of a bilayer and increases fluidity too much. The association of feedback regulation of hepatic cholesterol synthesis with liposome formation may be related to the fact that dietary cholesterol in mammals is transported to the liver by lipoproteins.

ACKNOWLEDGMENT

This work was supported in part by NIH Grant no. AM-12172, and the ^{1}H-NMR data were obtained at the NIH-supported Middle Atlantic NMR facility at the University of Pennsylvania.

REFERENCES

1. Nes, W.R. (1980) in Biogenesis and Function of Plant Lipids (Mazliak, P., Benveniste, P., Costes, C., and Douce, R., eds.) pp. 387-394, Elsevier/North-Holland Biomedical Press.
2. Nes, W.R., Joseph, J.M., Landrey, J.R., and Conner, R.L. (1980) J. Biol. Chem. 255, 11815-11821.
3. Nes, W.R., and McKean, M.L. (1977) in Biochemistry of Steroids and Other Isopentenoids, pp. 205-533, University Park Press, Baltimore, MD.
4. Barbier, M. (1981) in Marine Natural Products (Scheuer, P.J., ed.) Vol. IV, pp. 147-186, Academic Press, New York, MY.
5. Goad, L.J. (1978) in Marine Natural Products (Scheuer, P.J., ed.) Vol. II, pp. 75-107, Academic Press, New York, NY.
6. Khalil, M.W., Djerassi, C., and Sica, D. (1980) Steroids 35, 707-716.
7. Blanc, P-A., and Djerassi, C. (1980) J. Am. Chem. Soc. 102, 7113-7114.
8. Nes, W.R., Varkey, T.E., and Krevitz, K. (1977) J. Am. Chem. Soc. 99, 260-262.
9. Joseph, J.M., and Nes, W.R. (1981) J. Chem. Soc. Chem. Commun. 367-368.
10. Boll, P.M. (1974) Acta Chem. Scand. B28, 270-271.
11. Vanderah, D.J., and Djerassi, C. (1977) Tetrahedron Lett. 683-686.
12. Butenandt, A., and Suranyi, I.A. (1942) Ber. Dtsch. Chem. Ges. 75 (B), 591-597.
13. Huang-Minlon (1949) J. Am. Chem. Soc. 71, 3301-3303.
14. Nes, W.R., Joseph, J.M., Landrey, J.R., Behzadan, S., and Conner, R.L. (1981) J. Lipid Res. 22, 770-777.
15. Ritter, K.S., and Nes, W.R. (1981) J. Insect Physiol. 27, 175-181.
16. Billheimer, J.T., Tavani, D., and Nes, W.R. (1981) Anal. Biochem. 111, 331-335.
17. Nes, W.R., Sekula, B.C., Nes, W.D., and Adler, J.H. (1978) J. Biol. Chem. 253, 6218-6225.
18. Bucher, N.L.R., and McGarrahan, K. (1956) J. Biol. Chem. 222, 1-15.
19. Ritter, K.S., and Nes, W.R. (1981) J. Insect Physiol. 27, 419-424.
20. Conner, R.L., Landrey, J.R., Joseph, J.M., and Nes, W.R. (1978) Lipids 13, 692-696.
21. Norman, A.W., Johnson, R.L., Corradino, R., and Okamura, W.H. (1979) J. Biol. Chem. 254, 11445-11449.
22. Arthur, J.R., Blair, H.A.F., Boyd, G.S., Hattersley,N.G., and Suckling, K.E. (1975) Biochim. Soc. Trans. 3, 963-966.
23. Suckling, K.E., Blair, H.A.F., Boyd, G.S., Craig, I.F., and Malcolm, B.R. (1977) Biochim. Biophys. Acta 551, 10-21.
24. Billheimer, J.T., and Tavani, D. (1981) Fed. Proc. 40, 1696.
25. Bergmann, E.D., Rabinowitz, M., and Levinson, Z.H. (1959) J. Am. Chem. Soc. 81, 1239-1250.
26. Holick, M.F., Garabedian, M., Schnoes, H.K., and DeLuca, H.F. (1975) J. Biol. Chem. 250, 226-230.
27. Boyd, G.S., Brown, M.J.G., Hattersley, N.G., and Suckling, K.E. (1974) Biochim. Biophys. Acta 337, 132-135.
28. Nakamura, T., Nishikawa, M., Inoue, K., Mojima, S., Akiyama, T., and Sankawa, U. (1980) Chem. Phys. Lipids 26, 101-110.
29. Nes, W.R., Joseph, J.M., Landrey, J.R., and Conner, R.L. (1978) J. Biol. Chem. 253, 2361-2367.
30. Nes, W.D., Patterson, G.W., and Bean, G.A. (1980) Plant Phys. 66, 1008-1011.
31. Nes, W.D., Saunders, G.A., and Heftmann, E. (1982) Lipids 17, 178-183.

[Received September 10, 1981]

Unusual Urinary Cholesterol Metabolites following Intracerebral Injection of [4-^{14}C]Cholesterol into Rats: I. The Minor ^{14}C-Metabolite

R.B. RAMSEY[1] and **H.J. NICHOLAS**[2], *Institute of Medical Education and Research and the Edward A. Doisy Department of Biochemistry and Department of Neurology, St. Louis University, St. Louis, MO 63104*

ABSTRACT

[4-^{14}C] Cholesterol injected intracerebrally into 10-12-day-old rats becomes localized largely in central nervous system myelin. If sufficient ^{14}C is injected, myelin cholesterol remains labeled for the rest of the rats' lives. In the course of the slow myelin cholesterol turnover that ensues, a unique series of cholesterol metabolites is excreted exclusively in the rats' urine. There is reason to believe that the metabolites are formed in the central nervous system before entering the urine. This manuscript describes separation of the 2 urinary ^{14}C-labeled metabolic types and isolation and identification of the minor ^{14}C-labeled material which consists of cholesterol and 2 other sterols bound covalently to short-chain peptides. The minor sterols have been tentatively identified as 24- and 26-hydroxycholesterol. The sterol-peptide combinations have been isolated from human male urine, also.
Lipids 17:263-267, 1982.

INTRODUCTION

While it is generally accepted that adult central nervous cholesterol undergoes a slow, but measurable, rate of turnover (1), what specifically happens to the sterol in the course of this turnover is unknown. The work described here was designed to answer this question. Upon reproducing the work of Lin and Smith (2) of [4-^{14}C] cholesterol, we found that, after 6 months, ^{14}C was present only in the central nervous system (CNS) (unpublished observations), and, of equal significance, that ^{14}C was being excreted exclusively into rats' urine. It soon became evident that the urine contained no labeled free sterol, sterol glycosides or protein-bound sterol, and it became of interest to determine the exact nature of the urinary ^{14}C. This manuscript describes the separation of the major urinary ^{14}C-component, which we have given the provisional name WS- (for H_2O-soluble)-1, and isolation and partial identification of the minor ^{14}C-constituent, called WS-2.

MATERIALS AND METHODS

Labeling of Brain Cholesterol and Collection of Urine

Sprague-Dawley rats, 10-12 days old, were each injected intracerebrally (ic) with 5 μCi of [4-^{14}C] cholesterol (New England Nuclear Corp., 54.0 μCi/mmol) by the method of Lin and Smith (2). After 6 months, they were placed individually in metabolic cages for the collection of urine uncontaminated by feces. Examination of the feces by solvent extraction showed trace or no ^{14}C material. The urine, collected in flasks under toluene, was either sampled for ^{14}C determination immediately or frozen for future studies. Human urine was collected from a healthy male donor and frozen until examined.

Chromatography

Charcoal columns were prepared from either Fisher Scientific Co. activated charcoal (50-200 mesh) or Darco activated charcoal pH 1-2 (20-40 mesh). Both must be acidified to pH 1-2 with conc. HCl, followed by thorough washing with ion-free H_2O to remove inorganic material. An 8 × 90 cm column can feasibly separate WS-1 and -2 from 20-30 ℓ of urine.

The DEAE cellulose (preswollen microgranular form, D3764) was purchased from Sigma Chemical Co. and was washed and converted to the acetate form (3).

Thin layer chromatography (TLC) was conducted on Silica Gel F-254 plates, 0.25 mm thick for analytical work, 0.5 mm thick for preparatory work. They were washed (ascending chromatography) in the solvent system to be used with multiple irrigations. Reference spots or bands were either visualized under UV (254 nm) light or identified by spraying with 50% H_2O/sulfuric acid and briefly heating the plates. All solvents were distilled before use.

Reference Sterols

24-Hydroxycholesterol (cholest-5-ene-3β,24-diol) was a gift from Dr. Leland Smith, as was 26-hydroxycholesterol (cholest-5-ene-3β,26-diol). Cholesterol from a commercial source

was recrystallized 3 times from methanol. 25-Hydroxycholesterol (cholest-5-ene-3β,25-diol) and other sterols were gifts from Dr. Andrew Kandutsch.

Subcellular Fractionation

Subcellular fractionation of labeled brains was performed according to the method of Ramsey et al. (4), except that each particulate fraction was given a final "wash" in distilled H_2O by centrifugation at the appropriate speed.

Isolation of ^{14}C-Labeled Material from Urine

No ^{14}C could be extracted from labeled rat urine with ethyl ether, benzene or chloroform, with or without acidification to pH 1-2. About 6% of the total activity in the urine could be extracted with acidified butanol, but the extracts contained a huge amount of red, pungent oil which defied attempts to concentrate the ^{14}C. By trial and error, a sharp separation of the major and minor ^{14}C constituents was obtained on a charcoal column. This and subsequent steps leading to partial purification of WS-1 and -2 are shown on the flow sheet, Figure 1. Some comments on this flow sheet with respect to WS-2 follow. These apply to isolation from human urine also, except that changes in solvent are based on changes in the color of the eluting solvents; they are less quantitative than those performed with labeled rat urine.

In a typical isolation, 8,700 ml of ^{14}C-labeled rat urine containing 26,000 dpm of ^{14}C was passed through a 8 x 64 cm charcoal column. After elution of [^{14}C]WS-1 and washing with methanol, 8 ℓ of chloroform/methanol (2:1, v/v) was passed through the column. Further C/M, 2:1, eluted no ^{14}C. Removal of the C/M in vacuo gave 8.89 g of a deep red, pungent and viscous oil, sp act 169 dpm/g. This oil was extracted exhaustively with hot chloroform. Filtration of the extracts through Whatman No. 1 paper and removal of chloroform from the filtrates gave a residue consisting of 0.60 g of orange-colored gum, sp act 2,540 dpm/g. This gum is the starting material for most of the subsequent purification steps discussed in this manuscript, and will be called WS-2 $CHCl_3$-soluble. It contains no carbohydrate, as mea-

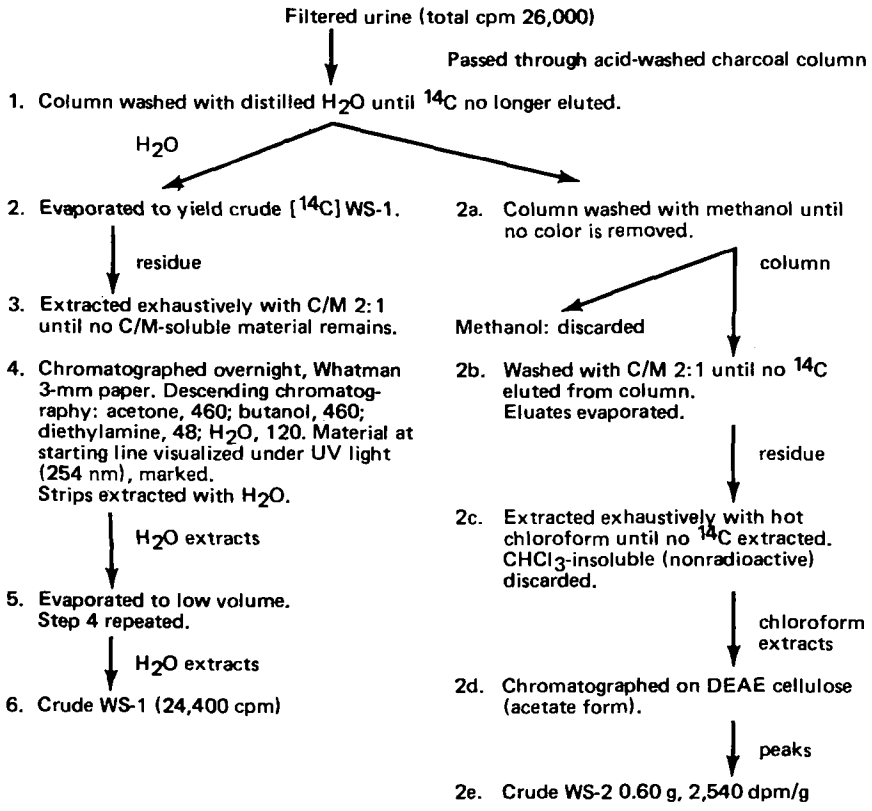


FIG. 1. Flow sheet for the separation and partial purification of WS-1 and WS-2.

sured by 2 sensitive methods (5,6), gives no precipitate with digitonin solution and gives a strong pink reaction with ninhydrin. The material appeared to be sterols bound covalently to amino acids or peptides. The ^{14}C was dialyzable through cellophane, and Aminco filtration procedures indicated that 30% of the material had a molecular weight less than 500; the remainder was between 500 and 2,000 (6,7).

DEAE Cellulose Acetate Chromatography

WS-2 $CHCl_3$-soluble (0.95 g) in 10 ml chloroform was placed on a 2.5 x 25 cm DEAE cellulose (acetate) column and eluted with $CHCl_3$, 2:1 and 1:1, and ammonium acetate (20 mM) in 99% methanol. Twenty-ml samples were collected for analysis.

Amino Acid Analyses

Samples (0.02-0.05-g) were placed on the steam bath in sealed vessels containing 6 N HCl overnight. The cooled hydrolysates were extracted with ether. No recognizable sterol could be identified in ether extracts by TLC. The aqueous fractions were analyzed for amino acids in a Beckman Model 120 C amino acid analyzer, courtesy of Dr. Karl Dus and associates.

Solvent Fractionation of WS-2

By partition of [^{14}C]WS-2 $CHCl_3$-soluble between chloroform and 1 N KOH, about 20% ^{14}C was found to be soluble in the alkaline fraction, suggesting that this portion of the mixture must contain a terminal carboxyl group.

Hydrolysis of WS-2: The Neutral Sterol Moiety

WS-2 $CHCl_3$-soluble was heated on the steam bath in HCl and KOH solutions of varied concentration. The cooled mixtures were extracted thoroughly with ethyl ether freshly distilled from $FeSO_4$. With higher concentrations of hydrolyzing agent, it became evident (with ^{14}C preparations) that an acidic steroid is a constant component of WS-2, representing about 30% of the ^{14}C.

When followed by TLC, no cholesterol could be detected after hydrolyses by concentrations up to 4 N HCl (steam bath overnight), although more polar sterols were evident. Accordingly, a hydrolytic procedure with alkali was developed.

WS-2 $CHCl_3$-soluble (4 g) containing 600 dpm ^{14}C was dissolved in 100 ml 4 N KOH. The container was flushed with N_2, sealed and heated on a steam bath overnight. The cooled mixture was extracted thoroughly with ethyl ether. The ether extract was back-washed with 1 N KOH and then to neutrality with H_2O. The residue was dissolved in acetate/ethanol (1:1) and digitonides precipitated by the Sperry-Webb procedure (7). The free sterol obtained by treatment of the digitonide with pyridine, followed by extraction with ether, weighed 20 mg and contained 350 dpm ^{14}C as a light-colored solid. This was crystallized 3 times from methanol, giving 4 mg white crystals, mp 147-150 C (cholesterol mp 149 C [8]) containing 200 dpm ^{14}C. Upon TLC, this material gave the R_f and color with 50% H_2SO_4 characteristic of cholesterol. The mother liquor from these 2 crystallizations contained 140 dpm.

The filtrate from the original solution and first crystallization contained 245 dpm ^{14}C. Thus, all of the ^{14}C associated with digitonin-precipitable material was accountable. TLC with $CHCl_3$/MeOH (24:1) (9) indicated the presence of 24-, 25- and 26-hydroxycholesterol. The solid in the second and third crystallizations (10 mg) was spread on a 14-cm starting line of a Silica Gel F-254 TLC plate and chromatographed with the above sterols as markers. The 3 sterols traveled together as a band well separated from marker cholesterol. The combined dihydroxysterol band was marked, scraped and extracted with C/M 2:1, giving 6 mg of solid containing 200 dpm.

RESULTS

Urinary Excretion Pattern of ic-Injected Rats

A typical ^{14}C-urinary excretion pattern for rats injected ic with [4-^{14}C] cholesterol at 10-12 days of age is shown in Figure 2. The day-to-day variation shown by this curve is representative of many such urines examined.

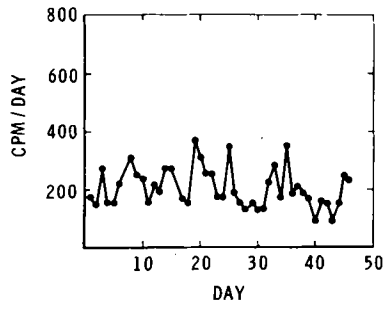


FIG. 2. Daily urinary ^{14}C-excretion pattern of a normal 6-month-old rat which had been injected intracerebrally at age 12 days with 5 μCi of [4-^{14}C] cholesterol. For ^{14}C-determination, 0.5 ml urine was added to 10 ml Instaged (Packard Instrument Co.) and counted 100 min for statistical accuracy.

Distribution of ^{14}C in Subcellular Fractions of ic-Injected Rats

Characteristic distribution of ^{14}C in the subcellular fractions of rats injected ic as described is shown in Table 1. The relatively high concentration in the synaptosome-mitochondrial fraction (but insignificant compared to brain and spinal cord myelin) may deserve further investigation.

TABLE 1

Distribution of ^{14}C in Subcellular Fractions of a Normal Rat Brain from a Rat Injected One Year Previously with [4-^{14}C]Cholesterol[a]

Subcellular fraction	Total cpm[b]
Brain myelin	650,000
Spinal cord myelin	70,000
Brain 105,000 X g supernate	4,000
Spinal cord 105,000 X g supernate	18,000
Brain microsomes	200
Spinal cord microsomes	0
Brain synaptosomes + mitochondria	1,500
Spinal synaptosomes + mitochondria	0

The tissues were suspended by homogenization in H_2O and an aliquot was taken for ^{14}C-determination.

[a]This rat received 5 μCi of [4-^{14}C]cholesterol ic at age 10 days.

[b]Fractions prepared as reported by Ramsey et al. (4) except that each cellular preparation was given an additional "wash" by centrifugation at the appropriate speed in distilled H_2O.

Fractionation of WS-2 Concentrate: The Amino Acid Moiety

When the chloroform extract at step 2c (Fig. 1) was chromatographed on a DEAE cellulose (acetate) column, the fractionation shown in Figure 3 was obtained. The 4 early peaks were named A-D. When peak B was subjected to high performance liquid chromatography (courtesy of Dr. Marvin McMasters, Beckman Instrument Co.), 6 components were indicated using both UV and refractive index detectors. The components were marked by UV light after TLC (Table 2). This fraction from rat urine is nearly identical to an analogous fraction from human urine when compared under the same conditions. Each band is radioactive (band 2 is a possible exception) due to the sterol moiety (Table 2). On rechromatography, each band can be obtained free from adjacent bands, and after acid hydrolysis, the amino acid composition of each band was obtained. Each of the other 3 early peaks (A,C,D) and the later fractions from the DEAE column also gave many bands on TLC.

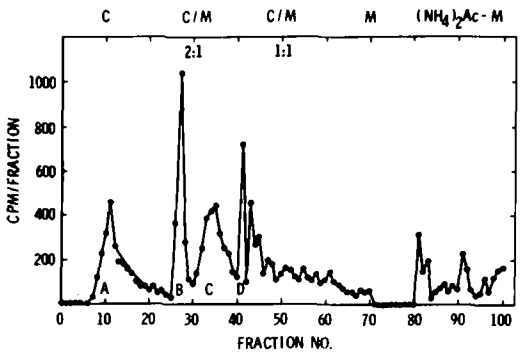


FIG. 3. DEAE acetate elution pattern of partially purified [^{14}C] WS-2 concentrates (Fig. 1, step 2d). The more polar peaks have not been given alphabetical terminology. C = chloroform; C/M = chloroform/methanol; M = methanol; $(NH_4)_2$Ac-M = 20 mM ammonium acetate in methanol/H_2O, 99:1.

Crude WS-2: The Sterol Moiety

Cholesterol and a fraction which contained 24- and 26-hydroxycholesterol appear to be the major sterols of the neutral sterol fraction. 7α-Hydroxycholesterol was conspicuously absent

TABLE 2

Amino Acid Composition of Selected Fractions of WS-2

Band[a]	R_f	Total dpm	Sp act (dpm/g)	Amino acid composition
1	0.03	1,540	1,540	Lysine, histidine
3	0.3	720	120	Proline, glycine, unidentified (close to lysine)
4	0.6	560	33	Proline, glycine, alanine
5	0.7	1,480	296	Proline, glycine, leucine, phenylalanine, aspartic acid
6	0.8	1,300	650	Glycine, serine, glutamic acid, aspartic acid

[a]All bands indicated considerable ammonia, suggesting the presence of asparagine and/or glutamine.

in all neutral fractions (TLC), strong evidence that the more polar compounds than cholesterol are not autoxidation products. Designation of 24- and 26-hydroxycholesterol as the primary sterols of this fraction is based on TLC analysis, the fact that 25-hydroxycholesterol is generally considered an artifact in brain sterol extracts (10), and that both 24- and 26-hydroxycholesterol have been identified in brain (11,12). Conclusive evidence for the presence of these sterols as constituents of urinary fraction WS-2 will depend on the results obtained by gas chromatography-mass spectrometry (GC-MS).

DISCUSSION AND CONCLUSIONS

Six months after ic injection of [4-^{14}C]cholesterol into immature rats, a unique series of metabolites is excreted exclusively into the urine of rats. We assume that these metabolites are formed in the CNS itself and are associated with CNS myelin because of the following 2 observations: (a) they are labeled with ^{14}C, and only rat CNS myelin contains sufficient ^{14}C to explain their source (Table 1); and (b) administration of triethyl tin chloride to drinking H_2O of rats with their brain labeled as described causes an enormous excretion of ^{14}C into the rats' urine (13). The presence of this drug in drinking H_2O is known to have a markedly disintegrating effect on CNS myelin (14,15).

The presence of 24- and 26-hydroxycholesterol in the urine is not unexpected in view of their detection in brain, and of metabolic studies conducted on the 24-diol by Lin and Smith (2). We have presumed that they are conjugated to peptides at the 3β-position. It seems important that the complete structures of the brain-derived urinary sterol-peptides described here be established. It is unclear at this time how much of the label in the urine can be recovered as cholesterol and the hydroxycholesterols. There is no precedent for peptide-linked neutral sterols.

Work on WS-1 is continuing and will be the subject of a future publication.

REFERENCES

1. Ramsey, R.B., and Nicholas, H.J. (1972) Adv. Lipid Res. 10, 143-167.
2. Lin, Y.Y., and Smith, L.L. (1975) J. Neurochem. 25, 659-665.
3. Rouser, O., Kritchevsky, G., and Yamamoto, A. (1967) in Lipid Chromatographic Analysis (Marinetti, G.V., ed.) Vol. 1, pp. 120-122.
4. Ramsey, R.B., Jones, J.P., Nagvi, S.H.M., and Nicholas, H.J. (1971) Lipids 6, 154-161.
5. Scott, T.A., Jr., and Melvin, E.H. (1953) Anal. Chem. 25, 1656-1660.
6. Sperry, N.M., and Webb, M.J. (1950) Biol. Chem. 187, 97-106.
7. Dialog (1979) Vol. 1, No. 1, p. 3, Amnicon Corp., Lexington, Mass.
8. Steroids (1959) Fieser, L.F., and Fieser, M., eds.) Reinhold Pub. Corp., New York, NY, pp. 44-45.
9. Van Lier, J.E., and Smith, L.L. (1967) Biochemistry 6, 3269-3278.
10. Van Lier, J.E., and Smith, L.L. (1969) Tex. Rep. Biol. Med. 17, 167-176.
11. Smith, L.L., Wells, J.D., and Pandya, N.L. (1972) Tex. Rep. Biol. Med. 31, 38-46.
12. Smith, A.G., Gilbert, J.D., and Harland, W.A. (1974) Biochem. J. 139, 793-795.
13. Nicholas, H.J. (1981) Fed. Proc. 40, 1843.
14. Eto, Y., Suzuki, K., and Suzuki, I. (1971) J. Lipid Research 12, 570-579.
15. Smith, M.E. (1973) J. Neurochem. 21, 357-372.

[Received September 28, 1981]

Influence of Vitamin E and Nitrogen Dioxide on Lipid Peroxidation in Rat Lung and Liver Microsomes

ALEX SEVANIAN[1], **ALLEN D. HACKER** and **NABIL ELSAYED**,
University of California-Los Angeles, Schools of Medicine and Public Health, Los Angeles, CA 90024

ABSTRACT

Rat lung and liver microsomes were used to examine the effects of dietary vitamin E deficiency on membrane lipid peroxidation. Microsomes from vitamin-E-deficient rats displayed increased lipid peroxidation in comparison to microsomes from vitamin-E-supplemented controls. The extent of lipid peroxidation, as determined by measurement of thiobarbituric acid reacting materials, was enhanced by addition of reduced iron and ascorbate (or NADPH). Rats fed a vitamin-E-supplemented diet and exposed to 3 ppm NO_2 for 7 days did not exhibit increases in microsomal lipid peroxidation compared to air-breathing controls. However, increases were found in microsomes prepared from rats fed a vitamin-E-deficient diet and exposed to NO_2. Lung microsomes from vitamin-E-fed rats contained almost 10 times as much vitamin E as liver microsomes when expressed in terms of polyunsaturated fatty acid content. The extent of lipid peroxidation was, in turn, considerably less in lung than in liver microsomes. Lipid peroxidation in lung microsomes from vitamin-E-deficient rats was comparable to liver microsomes from vitamin-E-supplemented rats as was the content of vitamin E in these respective microsomal samples. A combination of vitamin E deficiency and NO_2 exposure resulted in the greatest increases in lung and liver microsomal lipid peroxidation with the largest relative increases occurring in lung microsomes. An inverse relationship was found between the extent of lipid peroxidation and vitamin E content. Most of the peroxidation in lung microsomes appeared to proceed nonenzymatically whereas peroxidation in liver was largely enzymatic. Vitamin E appears to be assimilated by the lung during oxidant inhalation, but with dietary vitamin E deprivation, the margin for protection in lung may be less than in liver.
Lipids 17:269-277, 1982.

INTRODUCTION

Considerable attention has been devoted to the role of vitamin E as a membrane antioxidant. Previous studies have shown that the ability of tissue homogenates to undergo lipid peroxidation is related to the vitamin E status of the animal (1,2). Lipid peroxidation in tissues or membranes also appears to result from vitamin E deprivation (2-4), and normally the uptake of vitamin E or its content in tissues is related to polyunsaturated fatty acid (PUFA) content (5). This relationship may be complicated in that the requirement for vitamin E in different tissues or subcellular fractions is quite variable (6). This variability has been proposed to arise from differences in the assimilation and/or dependency for vitamin E among tissues (7,8) which is due, in part, to their PUFA content. Kornburst and Mavis (9) recently reported that the degree of peroxidation in microsomes, prepared from organs such as heart, lung, liver, brain and testis, was related to the content of vitamin E. They demonstrated that lung and heart were least susceptible to lipid peroxidation which could be accounted for by the high ratios of vitamin E to PUFA.

The measurement of lipid peroxidation by the thiobarbituric acid test has been widely used in biological systems. Metal salts, such as reduced iron, are useful in this assay in that they appear to accelerate release of thiobarbituric acid reacting materials from lipid hydroperoxides (10). Accordingly, Terao and Matsushita (11) demonstrated that the thiobarbituric acid test can accurately represent the amounts of fatty acid hydroperoxides, either as malonaldehyde or other thiobarbituric acid reacting materials which originate from hydroperoxides. The formation and subsequent decomposition of hydroperoxides has been described to proceed by both enzymatic and nonenzymatic mechanism. In the first case, it is known that microsomes display an iron- and NADPH-dependent lipid peroxidation (12) which probably uses NADPH-cytochrome c reductase (13). Nonenzymatic peroxidation, or autoxidation, of PUFA may also be facilitated by metals (12), and in the presence of ascorbate (14), through the formation of nonlipid (15) and lipid-free radicals (16).

In this study, we examined the relationship between microsomal lipid peroxidation and vitamin E content in lungs and livers of rats subjected to varied dietary vitamin E and/or exposure to low levels of nitrogen dioxide (NO_2). The decision to study the effects of NO_2 was based partly on earlier findings that vitamin E affords protection against NO_2-

[1]Present address: Institute for Toxicology, University of Southern California, 1985 Zonal Ave., Los Angeles, CA 90033.

induced injury in animals (17).

METHODS

Specific pathogen-free pregnant Sprague Dawley rats were purchased from Hilltop Labs (Hilltop, MA) and were received on the 14th day of gestation. The rats were housed in laminar flow cabinets and placed on a specially prepared diet, as described by Mohrhauer and Holman (18), which was formulated to contain either 0 or 50 IU/kg diet of vitamin E ((dl,α-tocopherol). The main components of this diet are listed in Table 1. The mothers and their litters were maintained on these diets and the litters were weaned onto these diets at 20 days of age. After 1 month, serum vitamin E levels were measured by drawing a 1-ml sample of blood by transthoracic cardiac puncture. Vitamin E was analyzed as described later. Significantly reduced serum vitamin E levels were found by 8 weeks of age.

On the 8th week, the rats raised on either diet were divided into 2 groups each consisting of 8 rats. One group was comprised of rats exposed to filtered air and the other group was exposed to 3 ± 0.1 ppm NO_2 continuously for 7 days. Exposures were done in specially designed chambers at 22 C, 40-60% rel humidity, as described by Hinners et al. at an air exchange rate of 20 vol/hr (19). During this time, the animals had free access to food and water which were provided fresh daily during a 15-min cessation of exposure. After exposure, the rats were immediately sacrificed by injection of sodium pentobarbital (60 mg/kg, IP) and exsanguinated via cardiac puncture. The lungs and liver were excised, trimmed free of connective tissues and major vessels, and homogenized in 0.1 M phosphate-buffered normal saline, pH 7.4, at 4 C using a polytron homogenizer (Brinkman Instruments). The homogenates were centrifuged for 5 min at 500 x g to remove intact cells and large debris. The resulting supernates were centrifuged for 15 min at 18,500 x g and the pellets discarded. The supernates were centrifuged for 1 hr at 100,000 g for a preliminary isolation of microsomes. The microsomal pellets were resuspended in fresh buffer containing 0.05 mM EDTA and recentrifuged as before. The resulting microsomal pellets were suspended in phosphate buffer devoid of EDTA by mild sonication and were adjusted to a concentration of 0.375 mg protein/ml buffer. Protein content was determined by the method of Gornall et al. (20).

Four groups of lung and liver microsomes were prepared as experimental study groups.

TABLE 1

Composition of Experimental Diet

Dietary components	% by wt
Sucrose	57.7
Casein (vitamin-free)	28.8
Corn oil (tocopherol stripped)	4.8 (12%, 18:1; 60%, 18:2)
Cellulose	3.8
Salt mixture (minerals)	3.8
Vitamin mixture[a]	1.0

[a]Prepared using standard dietary composition of vitamins but excluding vitamins A and E. Vitamins A and E were added by dissolution in ether and addition of this solution to the diet during its preparation. Ether was subsequently allowed to evaporate under vacuo. Vitamin-E-deficient diet was prepared by addition of an ethereal solution of vitamin A only.

The first group representing controls were rats raised on vitamin-E-supplemented diet which were exposed to air. The second group consisted of rats fed a vitamin-E-supplemented diet and exposed to 3 ppm NO_2. The third group was raised on a 0 vitamin E diet and exposed to air and the last group was fed the 0 vitamin E diet and exposed to NO_2. Aliquots of lung and liver microsomes were used to measure vitamin E content as well as lipid composition, particularly the fatty acid profile. Microsomal vitamin E content was determined by high pressure liquid chromatography (HPLC) according to the method of Vatassary and Hagen (21) using a Varian Model 8500 liquid chromatograph fitted with a Unimetrics Corp. RP-18, 10-μm reversed-phase column. The elution solvent was absolute methanol at a flow rate of 80 ml/hr and measurements were made using a Varichrome photometric detector set at 292 nm. α-Tocopherol acetate was used as an internal standard. Standard curves were constructed with known amounts of α-tocopherol.

Total lipids were extracted from microsomes by the method of Bligh and Dyer (22). The total lipid extract was evaporated to dryness under a stream of nitrogen and dissolved in 0.5 M HCl in methanol to effect transmethanolysis. The resulting fatty acid methyl esters were extracted into hexane and injected into a Hewlett-Packard Model 5830A gas chromatograph equipped with an 8-ft Silar 10C column. The chromatograph was operated over a temperature range of 170-210 C and programmed at a rate of 1 C/min. Fatty acid methyl ester composition was estimated using methyl heptadecanoate as an internal standard. Total lipid content was determined gravimetrically using a Cahn electrobalance.

The extent of lipid peroxidation was determined by the thiobarbituric acid test described by Ottolenghi (23) as modified by Hunter et al. (24). The thiobarbituric acid test reagent was prepared as described by Waravdekar and Saslaw (25). This analysis included addition of $FeSO_4$ or $FeCl_3$ when determinations in the presence of iron salts were desired. To some of these samples, reducing equivalents were added in the form of either ascorbic acid or NADPH with a final assay volume of 0.75 ml in 0.1 M phosphate buffer, pH 7.4. Measurements with no additions to microsomes served as controls. The preparations contained final microsomal protein concentrations of 0.25 mg/ml, whereas Fe^{+2} or Fe^{+3} concentrations were 1 mM and ascorbate or NADPH were 0.25 and 0.5 mM, respectively. These mixtures were combined in 15 x 150 mm glass screw-capped tubes and incubated for 1 hr in a 37 C water bath. Spectrophotometric measurements were made with a Varian Model S634 spectrophotometer set a 532 nm. The amount of thiobarbituric acid-reacting materials (designated as malonaldehyde) was calculated using an extinction coefficient of 1.6×10^5 M^{-1}, cm^{-1}. The relative degrees of lipid peroxidation were calculated on the basis of mg protein content, but the results were also computed and are expressed as nmol malonaldehyde formed/μmol PUFA/hr. Statistical analysis among study groups was made using Students' t-test and data are expressed as mean ± one standard deviation.

RESULTS

Serum vitamin E content was monitored at intervals over the 8 weeks during which rats were raised on the prepared diets. A gradual decrease in serum vitamin E was noted in the rats fed the 0 vitamin E diet and, by the eighth week, vitamin E concentrations were found to be 1.02 ± 0.14 μg compared to 1.54 ± 0.18 μg/ml serum for the controls. Although this does not appear to be a remarkable decrease, it is statistically significant. Previous experience with Sprague-Dawley rats has shown that it is very difficult to achieve serum vitamin E concentrations below 1 μg/ml even when the rats are raised and maintained on a deficient diet for periods in excess of 2 months. We therefore selected the 8-week interval as the starting point for the experiments.

After 7 days of exposure to 3 ppm NO_2 or air, the microsomal content of vitamin E was immediately analyzed and the results for the 4 study groups are presented in Table 2. Vitamin E content is expressed on the basis of protein and PUFA content of the microsomes.

TABLE 2

Microsomal Vitamin E Content in Lung and Liver[a]

	Lung				Liver			
	Vitamin-E-supplemented		Vitamin-E-deficient		Vitamin-E-supplemented		Vitamin-E-deficient	
	Control	NO_2 Exp.	Control	NO_2 Exp.	Control	NO_2 Exp.	Control	NO_2 Exp.
Protein (μg/mg)	1.72 ± 0.46	2.15 ± 0.64	0.36 ± 0.09	0.19 ± 0.02	0.17 ± 0.05	0.18 ± 0.03	0.04 ± 0.01	0.03 ± 0.01
PUFA (μg/μmol)	0.96 ± 0.19	1.20 ± 0.35	0.18 ± 0.04	0.10 ± 0.01	0.13 ± 0.04	0.14 ± 0.03	0.05 ± 0.01	0.04 ± 0.01
Protein (mg TL/0.1 G)	285 ± 10.1	277 ± 11.9	289 ± 16.0	266 ± 9.9	275 ± 12.7	264 ± 8.4	222 ± 14.1	237 ± 17.6

[a] Values are given for preparations from control and NO_2-exposed rats. Data represent means and one standard deviation for triplicate measurements of pooled samples from microsomes prepared from lungs and livers obtained from each experimental group.

Dietary vitamin E deficiency produced a 5-fold decrease in vitamin E content in lung and a 3- to 4-fold decrease in liver microsomes after 9 weeks. Exposure to NO_2 produced a 25% increase in vitamin E content in the supplemented group (not significant) but a 44% decrease ($p < 0.05$) in lung microsomes of the vitamin-E-deficient group. A similar trend resulting from NO_2 exposure was found for liver; however, the changes were much smaller than in the lung, and the differences were not statistically significant.

The fatty acid composition of microsomal lipids is shown in Table 3. The proportion of major fatty acid methyl esters (FAME) is indicated. Vitamin E deficiency resulted in relative increases for all lung PUFA with exception of eicosapentaenoic (20:5), docosatetraenoic (22:4) and arachidonic (20:4) acids, the last of which decreased significantly relative to vitamin-E-fed controls. Exposure to NO_2 resulted in an increase in linolenic (18:3) and eicosatrienoic acids (20:3), and a decrease in 20:5 in rats fed the supplemented diet. In contrast, vitamin-E-deficient rats exposed to NO_2 showed relative increases in palmitoleic acid (16:1) and all 18 carbon PUFA including linoleic acid (18:2), but decreases in all 20 carbon PUFA with the exception of 20:3. In contrast to the lung, liver microsomes showed few changes in FAME composition. The only differences noted were decreased oleic acid (18:1) and an increase in 22:4 in deficient rats exposed to air, and a decrease in 16:1 and 18:2 in deficient rats exposed to NO_2.

The peroxide content in microsomes was determined as described by Buege and Aust (26). Measurement of peroxide values and formation of malonaldehyde was examined for intervals up to 1 hr and is shown in Figure 1. Peroxide values increased rapidly over the first 5 min of incubation and were maximal by 5 and 20 min for vitamin-E-deficient and vitamin-E-supplemented liver microsomes, respectively. Lung microsomes attained maximal values by 20 min in both treatment groups. During this period, malonaldehyde levels rose gradually, reaching a maximum by 60 min. Moreover, by 60 min, the peroxide levels had decreased significantly in lung microsomes but remained at maximal levels in liver microsomes. Significant differences in peroxides between vitamin-E-deficient and control microsomes were found only during the first 10 to 20 min of incubation for lung and the first 5 min for liver.

The extent of PUFA peroxidation in lung and liver microsomes is presented in Tables 4 and 5, respectively. The formation of malonaldehyde for each study group was also examined in microsomes preheated at 90 C for 5 min. This treatment was used as an alternate means for determining the degree of nonenzy-

TABLE 3

Fatty Acid Composition of Microsomal Total Lipid Extracts[a]

	Lung				Liver			
	Vit.-E-supplemented		Vit.-E-deficient		Vit.-E-supplemented		Vit.-E-deficient	
	Control (n = 8)	NO_2 Exp. (n = 6)	Control (n = 7)	NO_2 Exp. (n = 6)	Control (n = 8)	NO_2 Exp. (n = 6)	Control (n = 7)	NO_2 Exp. (n = 6)
14:0	1.1	0.8	2.3[a]	2.9[d]	0.3	0.3	0.3	0.6
16:0	37.7	34.7	33.0	38.8	22.0	18.4	19.9	24.2
16:1	6.3	4.3	3.9	7.7[c]	2.2	1.6	1.5	1.3[d]
18:0	13.3	10.1	9.8	12.9	20.5	23.5	25.8	24.1
18:1	23.6	22.8	26.4	20.0	13.1	13.1	9.2[a]	12.5
18:2	5.6	7.2	6.8	7.3[d]	12.8	13.7	11.6	9.4[c]
18:3	0.4	1.8[b]	2.0[a]	4.5[c,d]	0.2	0.3	0.2	0.2
20:3	0.8	1.6[b]	1.6[a]	1.2	0.2	0.2	0.2	0.2
20:4	5.5	4.6	3.1[a]	2.4[d]	21.9	19.1	21.1	17.5
20:5	0.8	0.2[b]	0.4	0.2[d]	0.8	0.7	1.0	0.7
22:4	2.4	2.0	1.9	0.2[c,d]	4.6	3.6	8.1[a]	6.0
22:6	1.2	1.2	3.4[a]	0.2[c,d]	1.7	1.8	1.8	1.4

[a]Results are expressed as percentage composition of fatty acid methyl esters prepared from total microsomal lipids (content of microsomal total lipids shown in Table 2).

n = Number of animals examined in each study group.

[a]$p < 0.05$, control supplemented vs control deficient.

[b]$p < 0.05$, control supplemented vs exposed supplemented.

[c]$p < 0.05$, control deficient vs exposed deficient.

[d]$p < 0.05$, control supplemented vs exposed deficient.

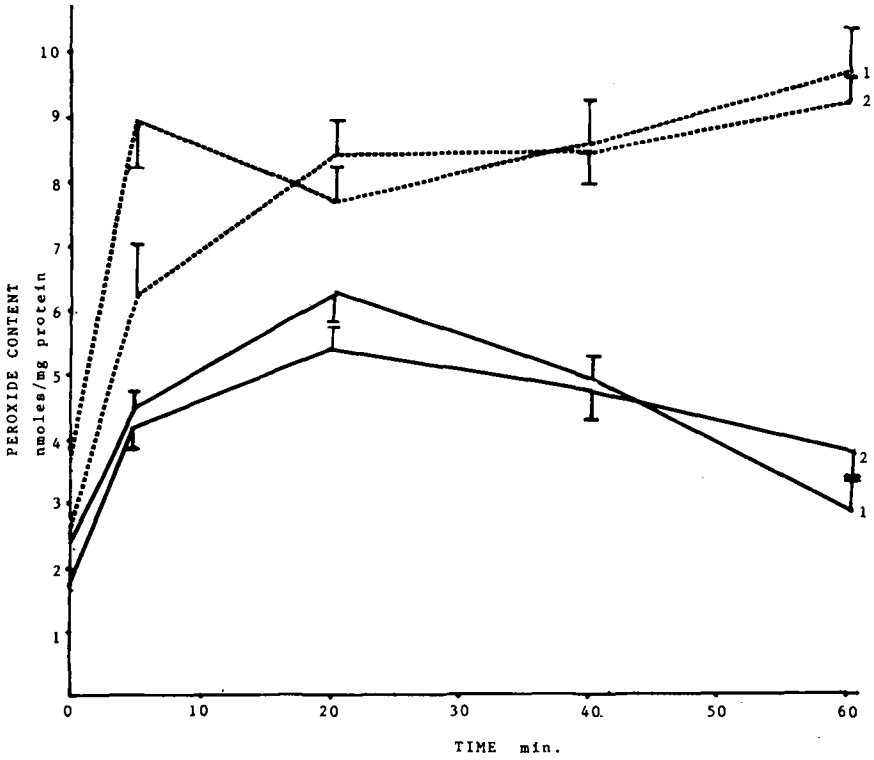


FIG. 1. Lipid peroxide levels in lung (——) and liver (– – –) microsomes measured over 1-hr incubation. Samples were incubated in 0.15 M phosphate buffer, pH 7.4, at 37 C. Values at designated intervals represent the mean and one standard deviation of triplicate determinations. 1: microsomes prepared from vitamin-E-deficient rats. 2: microsomes prepared from vitamin-E-supplemented rats. The amounts of peroxides are calculated using an extinction coefficient of 1.73×10^4 M^{-1} for cumene hydroperoxide. Zero time values represent the content of peroxides measured immediately following isolation of microsomes and prior to their incubation.

matic peroxidation. An assumption was made that heating of the microsomes did not contribute appreciably to the overall formation of lipid peroxidation products; thus, on the basis of the above observations, we assumed that malonaldehyde measurement was a reflection of hydroperoxide decomposition and that these hydroperoxides were either preformed or produced during the initial phase of incubation. This assumption appears to be supported by examining the data, e.g., rows 1 and 2 in Tables 4 or 5.

The results indicate that malonaldehyde formation occurs to a limited extent in the absence of added iron (row 1, Tables 4 and 5), but this may result from catalysis due to trace amounts of free iron or other metals including heme compounds (27,28). Under most conditions, formation of malonaldehyde was unchanged in vitamin-E-supplemented rats exposed to NO_2. There was a significant increase in malonaldehyde formation in microsomes from vitamin-E-deficient rats when compared to supplemented controls. The largest increases were usually found in the vitamin-E-deficient group exposed to NO_2. Increases were noted in both lung and liver microsomes; however, vitamin E deficiency appeared to be associated with greater and more consistent changes in lung microsomes. For example, the formation of malonaldehyde in lung microsomes incubated with Fe^{+2} and NADPH (row 7) increased in vitamin-E-deficient rats, but this effect was not found in liver microsomes. Addition of Fe^{+3} alone also produced an increase in malonaldehyde formation compared to samples with no additions. It is unclear whether this was due to hydroperoxide decomposition in the presence of Fe^{+3}, an ambient reduction of Fe^{+3} to Fe^{+2} or reaction of Fe^{+3} with vitamin E resulting in its destruction.

The results for enzymatic vs nonenzymatic

TABLE 4

Malonaldehyde Formation in Lung Microsomes
(nmol μmol PUFA^{-1} hr^{-1})[a]

Additions	Vitamin-E-supplemented		Vitamin-E-deficient	
	Control	NO_2 Exp.	Control	NO_2 Exp.
None	0.13 ± 0.009	0.12 ± 0.031	0.22 ± 0.048	0.32 ± 0.024
Heated	0.12 ± 0.008	0.05 ± 0.029	0.16 ± 0.033	0.29 ± 0.020
+Fe^{+3}	0.56 ± 0.154	0.72 ± 0.278	0.68 ± 0.216	1.02 ± 0.252
Heated	0.38 ± 0.080	0.24 ± 0.059	0.56 ± 0.086	0.80 ± 0.035
+Fe^{+3}	0.19 ± 0.009	0.36 ± 0.022	0.29 ± 0.015	0.50 ± 0.025
Heated	0.20 ± 0.019	0.31 ± 0.030	0.21 ± 0.010	0.36 ± 0.041
+Fe^{+3} +NADPH	0.80 ± 0.021	0.86 ± 0.148	0.88 ± 0.024	1.29 ± 0.044
Heated	0.39 ± 0.012	0.31 ± 0.013	0.59 ± 0.014	0.83 ± 0.017
+Fe^{+3} +NADPH	0.54 ± 0.022	–	0.73 ± 0.040	–
Heated	0.43 ± 0.039	–	0.60 ± 0.039	–
+Fe^{+3} +ascorbate	1.02 ± 0.144	1.46 ± 0.228	1.69 ± 0.369	2.41 ± 0.237
Heated	1.00 ± 0.029	0.96 ± 0.070	1.59 ± 0.045	1.99 ± 0.048

[a]Values are expressed as means ± one standard deviation. Sampling is identical to that shown in Table 2.

TABLE 5

Malonaldehyde Formation in Liver Microsomes
(nmol μmol PUFA^{-1} hr^{-1})[a]

Additions	Vitamin-E-supplemented		Vitamin-E-deficient	
	Control	NO_2 Exp.	Control	NO_2 Exp.
None	0.26 ± 0.020	0.49 ± 0.042	0.41 ± 0.022	0.37 ± 0.008
Heated	0.15 ± 0.024	0.24 ± 0.040	0.23 ± 0.029	0.29 ± 0.022
+Fe^{+2}	0.81 ± 0.138	0.89 ± 0.095	0.96 ± 0.346	1.65 ± 0.320
Heated	0.31 ± 0.008	0.19 ± 0.023	0.25 ± 0.024	0.25 ± 0.045
+Fe^{+3}	0.49 ± 0.038	0.34 ± 0.022	0.52 ± 0.086	0.37 ± 0.080
Heated	0.35 ± 0.024	0.19 ± 0.038	0.38 ± 0.060	0.31 ± 0.044
+Fe^{+2}+NADPH	1.81 ± 0.232	1.86 ± 0.400	1.86 ± 0.252	2.64 ± 0.588
Heated	0.31 ± 0.028	0.21 ± 0.023	0.18 ± 0.026	0.37 ± 0.015
+Fe^{+3}+NADPH	0.82 ± 0.161	–	1.02 ± 0.047	–
Heated	0.31 ± 0.046	–	0.26 ± 0.033	–
+Fe^{+2}+ascorbate	2.00 ± 0.206	1.84 ± 0.315	2.72 ± 0.316	2.42 ± 0.213
Heated	1.43 ± 0.108	1.68 ± 0.091	2.40 ± 0.111	2.16 ± 0.039

[a]Values are expressed as means ± one standard deviation. Sampling is identical to that shown in Table 2.

lipid peroxidation indicate that enzymatic peroxidation in the presence of Fe^{+3} (row 6, Tables 4 and 5) was relatively small in lung compared to liver in vitamin-E-supplemented rats. The proportion of enzymatic peroxidation in lung microsomes increased as a result of NO_2 exposure or vitamin E deficiency. Addition of Fe^{+2} + NADPH (row 7) resulted in a 20-40% increase in malonaldehyde formation, whereas a 100% increase was found in liver microsomes when compared to samples incubated in the presence of Fe^{+2} alone (row 3). Addition of NADPH to samples containing Fe^{+3} enhanced malonaldehyde formation (row 5 vs 9) with the amounts of total malonaldehyde approximating that measured in the samples containing Fe^{+2} alone (row 3). This suggests that the microsomes were capable of enzymatically reducing Fe^{+3} to Fe^{+2}. This was not found in heated microsomes on addition of NADPH.

Addition of Fe^{+2} + ascorbate resulted in the largest amounts of malonaldehyde generated among all microsomal samples. It also appears that lipid peroxidation in the presence of Fe^{+2}-ascorbate was largely nonenzymatic for both lung and liver microsomes (row 11 vs 12). These results differ from the other additions involving Fe^{+2} or Fe^{+3}. With the exception of Fe^{+2}-ascorbate and Fe^{+3} alone, the proportion of nonenzymatic lipid peroxidation was over twice as large in lung as in liver microsomes. Increases in lipid peroxidation resulting from vitamin E deficiency and/or NO_2 exposure were commonly found in lung microsomes and

clearly enhanced by the addition of Fe^{+2}-ascorbate to both lung and liver microsomes. Increased lipid peroxidation resulting from NO_2 exposure was not observed in liver microsomes with the exception of those samples deficient in vitamin E and incubated in the presence of Fe^{+2} (rows 3 and 7). Comparing these results with the preheated samples (rows 4 and 8) indicates that the increases could be accounted for by enzymatic peroxidation.

Liver microsomes produced nearly twice as much malonaldehyde as lung microsomes, corresponding with approximately twice the proportion of PUFA in liver. The overall levels of lipid peroxidation appear to be related to the proportion or content of PUFA in microsomes from these 2 organs whereas the relative increases in malonaldehyde formation appear to be influenced by vitamin E content. Furthermore, the extent of lipid peroxidation attributable to vitamin E deficiency was more pronounced in lung microsomes.

DISCUSSION

The lung is a primary target for oxidizing air pollutants, many of which are damaging to tissues with which they react. Because much of this damage may originate by lipid peroxidation initiated by free radical mechanisms, the lung may require particularly effective protection mechanisms in order to minimize these damaging reactions. Recently, Kornburst and Mavis (9) demonstrated that the high content of vitamin E in lung microsomes may serve to protect the membrane lipid constituents. Oxidant damage may extend to other organs and vitamin E may serve a protective role in this case, as well, because vitamin E deficiency has been shown to produce increased lipid peroxidation in liver following ozone exposure (29). It was proposed (29) that the ozone effect was able to reach the liver in the vitamin-E-deficient animals with the original effect being elicited in the lung as demonstrated by Fletcher and Tappel (30). The present study indicates that lipid peroxidation can be correlated with membrane vitamin E content which is influenced by a dietary vitamin E regimen. This correlation has been reported previously (31). The protective effect of vitamin E in natural and artificial membranes has been well demonstrated. Tinberg and Barber (32) observed an inhibition of lipid peroxidation when vitamin E was added to structural protein-lipid micelles derived from rat liver microsomes. Evidence of increased lipid peroxidation was reported in erythrocyte membranes derived from vitamin-E-deficient rats was well as in phospholipid model systems (33).

Dietary vitamin E deficiency produced significant decreases in the vitamin E content of lung and liver microsomes. It is notable that the levels of the vitamin are ca. 10 times higher in lung than in liver and that this difference holds whether the values are expressed on either protein or PUFA content. Exposure to NO_2 appears to have no effect on liver microsomal vitamin E content in animals receiving dietary vitamin E; however, the levels in lung show a consistent increase. This tendency was not found with dietary vitamin E deficiency. Instead, NO_2 exposure reduced lung vitamin E content whereas no changes were found in liver microsomes. These results suggest that the lung is capable of assimilating vitamin E during oxidant exposure, provided that it is available in body stores or in the diet. This may be a response to an increased antioxidant demand, a condition which cannot be satisfied in the vitamin-E-deficient state. Hence, exposure to NO_2 results in a further depletion of the vitamin in the lung and is accompanied by increased lipid peroxidation.

The data for microsomal fatty acid composition appears to be consistent with the above hypothesis. Only minor changes were found in the liver, despite a significantly reduced vitamin E content, as in the case of combined vitamin E deprivation and NO_2 exposure. The effects on lung microsomes were more pronounced as evidenced by significant changes for a number of PUFA. Mino (34) reported that vitamin E deficiency produced significant decreases in phospholipids and linoleic acid content in mouse lung. Although phospholipid data are not reported in this study, no changes were found for total lipids and the results for linoleic acid contrast with the findings of Mino (34). This discrepancy may be rationalized on the basis of the composition of dietary lipids. Mino used 10% refined lard, whereas the diet in this study used 5% corn oil which is significantly enriched in linoleic acid.

Malonaldehyde formation was found to be maximal when microsomes were incubated with Fe^{+2}-ascorbate. It also appears that this process is largely independent of an enzymatic component as contrasted to those samples incubated with Fe^{+2} or Fe^{+3} and NADPH. The effects of vitamin E deficiency or NO_2 exposure are magnified in lung or liver microsomes when Fe^{+2}-ascorbate is added. Our findings with Fe^{+2}-ascorbate differ from those of Wright et al. (35) who found that addition of Fe^{+2}-ascorbate reduced the amounts of malonaldehyde generated from microsomes in comparison to additions of Fe^{+2} alone. The concen-

trations of Fe^{+2} and ascorbate in our studies were similar; thus, this could not explain the observed differences. The discrepancy may instead be due to the microsomal source itself. Wright et al. used guinea pig lung microsomes which may have different requirements for ascorbate than those of rats. The difference in the ascorbate effect could arise from the fact that rats, but not guinea pigs, are ascorbate auxotrophs and may not be subject to limitations in ascorbate availability or its utilization. Possible differences in lipid composition may be another factor influencing the course of peroxidation.

Malonaldehyde production correlates well with microsomal vitamin E content. An inverse relationship between vitamin E concentrations and the extent of lipid peroxidation in tissues has been reported previously (31,36). The lower amounts of peroxidation in lung appear to relate to its high vitamin E content and, perhaps, to its affinity for the vitamin, as demonstrated by others (7,8,37). Our results show that the disparity in the extent of lipid peroxidation between lung and liver cannot be entirely rationalized on the basis of PUFA and vitamin E content. For example, when the extent of malonaldehyde formation in the presence of Fe^{+2}-ascorbate is compared between lung and liver, it appears that similar amounts are measured when vitamin E concentrations are also similar. By expressing malonaldehyde production on the basis of PUFA content, lung microsomes from vitamin-E-deficient/NO_2-exposed rats produced 2.4 nmol compared to 2.0 nmol/μmol PUFA for liver microsomes from control rats. The corresponding values for vitamin E for these 2 groups were 0.10 and 0.13 μg/μmol PUFA, respectively.

An increase in lipid peroxidation with the addition of Fe^{+3} was found only in lung microsomes. Although it is possible that Fe^{+3} may be directly destroying vitamin E (38), this effect should have been more apparent in liver microsomes which contain much less vitamin E. It is also possible that the basis of the differences between lung and liver may depend on the nonenzymatic vs enzymatic mechanisms for peroxidation, which prevail in these respective tissues. Eliminating the enzymatic component by heat inactivation result in no changes in malonaldehyde formation in relation to vitamin E deficiency for liver, whereas lung microsomes continue to display increased malonaldehyde formation with vitamin E deficiency. The results of this study indicate that the lung uses vitamin E as a membrane antioxidant to a greater extent than does liver. This is demonstrated by the tendency of lung, but not liver, vitamin E concentrations to increase following oxidant challenge, provided dietary vitamin E is available. This also suggests that the liver may be less dependent on vitamin E and may be more capable of using alternate mechanisms for protection against lipid peroxidation.

ACKNOWLEDGMENTS

The authors wish to thank Joanne Nakagawa and Christine L. Quinn for their valuable assistance. This study was supported by a grant from the California Research and Medical Education Fund, E800328, of the American Lung Association of California and by a grant, R8806642, from the U.S. Environmental Protection Agency.

REFERENCES

1. Bieri, J.G., and Anderson, A.A. (1960) Arch. Biochem. Biophys. 90, 105-110.
2. Tappel, A.L. (1972) Ann. N.Y. Acad. Sci. 203, 12-28.
3. Tappel, A.L., and Zalkin, H. (1960) Nature 185, 35-37.
4. McCay, P.B., Poyer, J.L., Pfeifer, P.M., May, H.E., and Gilliam, J.M. (1971) Lipids 6, 297-306.
5. Witting, L.A. (1974) Am. J. Clin. Nutr. 27, 952-959.
6. Taylor, S.L., Lamden, M.P., and Tappel, A.L. (1976) Lipids 11, 530-538.
7. Gallo-Torres, H.E., and Miller, O.N. (1971) Int. J. Vit. Nutr. Res. 41, 339-354.
8. Kelleher, J., Davies, T., Smith, C.L., Walker, D.E., and Losowsky, M.S. (1972) Int. J. Vit. Nutr. Res. 42, 394-402.
9. Kornburst, D.J., and Mavis, R.D. (1980) Lipids 15, 315-322.
10. Asakawa, T., and Matsushita, S. (1980) Lipids 15, 137-140.
11. Terao, J., and Matsushita, S. (1981) Lipids 16, 98-101.
12. Hochstein, P. (1981) Isr. J. Chem. 21, 52-53.
13. Pederson, T.C., Buege, J.A., and Aust, S.D. (1973) J. Biol. Chem. 248, 7134-7141.
14. Kornburst, D.J., and Mavis, R.D. (1980) Mol. Pharmacol. 17, 400-407.
15. Thomas, M.J., Mehl, K.S., and Pryor, W.A. (1978) Biochem. Biophys. Res. Commun. 83, 927-932.
16. Pryor, W.A., and Stanley, J.P. (1975) J. Org. Chem. 40, 3615-3617.
17. Menzel, D.B. (1979) Environ. Health Perspect. 29, 105-113.
18. Mohrhauer, H., and Holman, R.T. (1963) J. Lipid Res. 4, 151-159.
19. Hinners, R.G., Burkart, J.K., and Punte, C.L. (1968) Arch. Environ. Health 16, 194-201.
20. Gornall, A.G., Bardawill, C.J., and David, M.W. (1949) J. Biol. Chem. 177, 751-766.
21. Vatassary, G.T., and Hagen, D.F. (1977) Anal. Biochem. 79, 129-134.
22. Bligh, E.C., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
23. Ottolenghi, A. (1959) Arch. Biochem. Biophys. 79, 355-361.
24. Hunter, F.E., Gebicki, J.M., Hoffstein, P.E., Weinstein, J., and Scott, A. (1963) J. Biol. Chem.

238, 828-835.
25. Waravdekar, V.S., and Saslaw, L.D. (1959) J. Biol. Chem. 234, 1945-1950.
26. Buege, J.A., and Aust, S.D. (1978) in Methods in Enzymology (Fleisher, S., and Packer, L., eds.) Vol. 52, pp. 302-310, Academic Press, New York, NY.
27. Kaschnitz, R.M., and Hatefi, Y. (1975) Arch. Biochem. Biophys. 171, 292-304.
28. Hochstein, P., Kumar, K.S., and Forman, S.J. (1980) Ann. N.Y. Acad. Sci. 355, 240-248.
29. Sato, S., Shimura, S., Hirose, T., Maeda, S., Kawakami, M., Takishima, T., and Kimura, S. (1980) Tohoku J. Exp. Med. 130, 117-128.
30. Fletcher, B.L., and Tappel, A.L. (1973) Environ. Res. 6, 165-175.
31. Krishnamurthy, S., and Bieri, J.G. (1962) J. Nutr. 77, 245-252.
32. Tinberg, H.M., and Barber, A.A. (1970) J. Nutr. 100, 413-418.
33. Barker, M.O., and Brin, M. (1975) Arch. Biochem. Biophys. 166, 32-40.
34. Mino, M. (1973) Acta Paediatr. Jpn. 15, 1-8.
35. Wright, J.R., Colby, H.D., and Miles, P.R. (1980) Biochim. Biophys. Acta 619, 374-384.
36. Chen, L.H., Thacker, R.R., and Chow, C.K. (1980) Nutr. Rep. Int. 22, 873-881.
37. Krishnamurthy, S., and Bieri,J.G. (1963) J. Lipid Res. 4, 330-336.
38. Frampton, V.L., Skinner, W.A., and Bailey, P.B. (1952) Science 116, 34-35.

[Received August 26, 1981]

Studies on the Substrate Specificity of Purified Human Milk Lipoprotein Lipase

C.-S. WANG[a,*], A. KUKSIS[b] and F. MANGANARO[b], [a]*Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation, Oklahoma City, OK; and* [b]*Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada*

ABSTRACT

The fatty acid specificity of purified human milk lipoprotein lipase was studied using the C_{18} to C_{54} (total acyl carbon number) saturated and the C_{54} mono-, di- and triunsaturated monoacid triacylglycerols. Kinetic determinations indicated that the medium-chain triacylglycerols were better substrates than long- or very short-chain saturated triacylglycerols. The unsaturated triacylglycerols were hydrolyzed at rates comparable to that of tricaprylin with triolein having the highest rate of hydrolysis of the unsaturated species tested. The enzyme attacked the primary ester bond much more readily than the secondary ester bond. The purified human milk lipoprotein lipase showed a preferential stereospecific lipolysis of the *sn*-1-position of the triacylglycerol molecule.
Lipids 17:278-284, 1982.

INTRODUCTION

Lipoprotein lipases (LPL, EC 3.1.1.34) catalyze the hydrolysis of emulsified long-chain triacylglycerols at maximal rates in the presence of the cofactor apolipoprotein C-II or serum (1,2). Their physiological site of action is at the luminal surface of the capillary endothelium and the hydrolysis of blood triglyceride by LPL facilitates their uptake by extrahepatic tissue (3,4). The presence of LPL in milk is considered to reflect a leakage of enzyme from the mammary tissue (5,6). Milk fat is not a good substrate for LPL and LPL activity is inhibited by bile salts; thus, it appears unlikely that the enzyme has any physiological function in infant intestinal fat digestion (3).

Because of the functional importance of LPL, its substrate specificity has received much experimental attention. Early studies by Korn (7), using an acetone-ether powder of adipose tissue as the enzyme source, indicated that LPL had no marked positional specificity. However, Greten et al. (8) observed that the fatty acid in the *sn*-2-position was released at a higher rate than that in the *rac*-1-position when the corresponding acyldialkylglycerols were incubated with rat adipose and heart tissue LPL. More recently, Nilsson-Ehle et al., using purified LPL from human post-heparin plasma (9) and bovine milk (10), have reported that the enzyme exhibits an absolute specificity for the primary ester bonds. On the basis of stereospecific analyses of the hydrolysis products of triolein by bovine milk LPL, Morley and Kuksis (11) demonstrated a preferential attack at the *sn*-1-position of the triacylglycerol molecule. A similar observation has been made by Paltauf et al. (12) and by Akesson et al. (13) using the *sn*-1- and *sn*-3-alkyldiacylglycerols as substrates. Except for a report by Morley and Kuksis (14) that LPL does not exhibit acyl specificity for triacylglycerols containing mainly mono- and diunsaturated fatty acids, there have been no systematic studies on the acyl group specificity of the enzyme.

Because LPL from post-heparin plasma is heterogeneous with respect to tissue origin, we have used LPL from human milk in the substrate specificity study using a series of saturated and unsaturated monoacid triacylglycerols. The results show that LPL activity is markedly influenced by the fatty acid chain length and degree of unsaturation. In contrast to the previously claimed absolute specificity of LPL for the primary ester (9,10), we have also detected measurable reactivity for the secondary ester bond of the triacylglycerol molecule.

MATERIALS AND METHODS

Substrates

The *rac*-1-oleoyl-2,3-dipalmitoylglycerol and the *sn*-2-monopalmitoylglycerol were obtained from Applied Science Laboratories, State College, PA. The *rac*-1,3-dipalmitoyl-2-oleoylglycerol was obtained from Supelco, Inc., Bellefonte, PA. The other synthetic triacylglycerols along with *rac*-1-palmitoylglycerol were purchased from Sigma Chemical Co., St. Louis, MO. The [2-^{3}H-glycerol] triolein (1-2 Ci/mmol) and the *sn*-1,2-dipalmitoylphosphatidylcholine (50-60 mCi/mmol) were purchased from Amersham Co., Amersham, England. These compounds were greater than 95% single molecular weight species and were not purified further.

Enzyme

LPL was prepared from the acetone-diethyl ether powder of human milk cream (15) and purified by heparin-Sepharose affinity chromatography (16,17). The acetone-diethyl ether powder (40 mg, containing 7.1 mg of protein) was dispersed with a pestle and solubilized by stirring for 10 min at room temperature in 4 ml of 40 mM HCl-NH_4OH buffer, pH 8.5, containing 0.1% Triton X-100. The mixture was centrifuged at low speed for 2 min and the supernatant fluid was collected and applied to a small heparin-Sepharose column (0.7 × 2 cm). After loading the sample, the column was washed first with 4 ml of 50 mM HCl-NH_4OH buffer, pH 8.5, containing 0.3 M NaCl and then with 4 ml of the same buffer containing 0.72 M NaCl. Following the washes, the enzyme was eluted with 3 ml of 50 mM HCl-NH_4OH buffer containing 10 mg/ml of heparin (beef lung, Upjohn Co.).

The heparin-Sepharose affinity chromatography was performed at 4 C in a period of 1.5-2 hr. A unit of enzyme activity was defined as 1 μmol of fatty acid released/hr at 37 C. A standard assay procedure for determining the lipase activity was described recently (16). The LPL was purified 2,500-fold from human milk with a specific activity (sp act) of 5120 units/mg protein (17). The LPL activity in the heparin fraction was in the range of 60-100 units/ml. The purified enzyme was free of the major lipolytic activity of human milk: the bile-salt-activated lipase (18). For the present study, the enzyme was freshly prepared for each experiment.

Lipolysis of Synthetic Triacylglycerols

The various triacylglycerol substrates were emulsified with Triton X-100 as described by Schotz et al. (19). The final mixture contained the amounts and types of substrate specified in the legends, 60 mg/ml bovine serum albumin, 0.6 ml human serum and 1 ml of the purified human milk LPL, brought to a final vol of 6 ml with 50 mM HCl-NH_4OH, pH 8.5, buffer solution and incubated at 37 C. At various time intervals, aliquots (0.1-0.5-ml) were taken in duplicate and transferred to tubes containing 4 ml of *n*-heptane/isopropanol (3:7, v/v) for neutral lipid or fatty acid analysis by gas liquid chromatography (GLC).

Gas Liquid Chromatography

For analysis of intact triacylglycerols, each heptane-isopropanol extract, containing 50 μg of cholesterol butyrate as internal standard, was acidified with 2.5 ml of 0.033 N H_2SO_4. After mixing for 30 sec, the organic phase containing the triacylglycerols and the internal standard was transferred to a 3-ml conical tube and the solvent was evaporated under nitrogen. The residue was redissolved in 100 μl of *n*-hexane and 2-μl aliquots were injected into the gas chromatograph (20). Figure 1 shows the resolution and the relative hydrogen flame ionization response of the various triacylglycerols and the internal standards in an equimolar mixture.

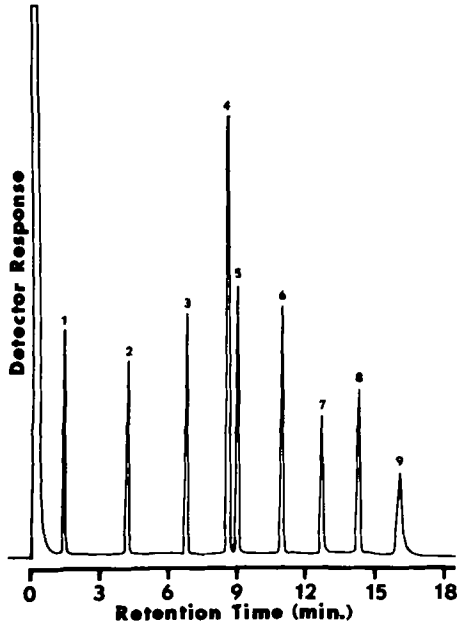


FIG. 1. The separation of monoacid-triacylglycerols by GLC with cholesterol butyrate as internal standard. The peaks are: 1, tributyrin; 2, tricaproin; 3, tricaprylin; 4, cholesterol butyrate; 5, tricaprin; 6, trilaurin; 7, trimyristin; 8, tripalmitin; 9, tristearin.

The partial acylglycerols were acetylated before the gas chromatographic analysis. Each heptane-isopropanol extract containing the internal standard was centrifuged to eliminate the protein precipitate. The supernatant fraction was transferred to a 25-ml, round-bottom flask and evaporated to dryness. The sample was redissolved in 5 ml of acetic anhydride/pyridine 1:1, heated for 10 min at 80 C, and immediately placed on ice. The excess reagent was removed by evaporation on a rotatory evaporator under vacuum at 30 C. The sample was redissolved in 4 ml of *n*-heptane/isopropanol (3:7, v/v) and extraction was compleated as for triacylglycerols. The final preparation, in hexane, contained the triacylglycerols and the acetylated partial acylglycerols.

Free fatty acids were extracted from the reaction mixture with heptane-isopropanol and

determined following methylation by GLC as described by McConathy et al. (21).

Stereospecific Analysis

The incubations of trioleoyl [^{3}H]glycerol with LPL, the subsequent isolation of diacylglycerols, and the stereospecific analyses of the diacylglycerols via the x-1,2-diacylphosphatidylcholines and phospholipase C were performed as described by Manganaro et al. (22). The stereospecific hydrolysis with phospholipase C (*Clostridium welchii* type 1) was performed in the presence of *sn*-1,2-dipalmitoylphosphatidyl[^{14}C]choline as internal standard. Molar ratios of the *sn*-1,2- and *sn*-2,3-diacylglycerols were calculated from the radioactivity in the glycerol moiety of the appropriate reaction products. Where necessary, corrections were made for the incompleteness of the digestion with phospholipase C on the basis of the extent of hydrolysis of the radioactive phosphatidylcholine internal standard.

The corrected *sn*-1,2-/*sn*-2,3-diacylglycerol ratio was calculated to be equal to (100 a + bx) : (100 a - bx), where a and b are the uncorrected relative values of the *sn*-1,2- and *sn*-2,3-enantiomers and x is the percentage of the undigested internal standard. This part of work was performed in Toronto.

Other Methods

Protein content of the samples was determined by a previously described modification (23) of the procedure of Lowry et al. (24). Bovine serum albumin was used as the standard. The vicinal hydroxyl groups of monoacylglycerols were determined by the periodate-chromotropic acid procedure, as described by Korn (25).

RESULTS

Fatty Acid Specificity

The use of mixtures of monoacid triacylglycerols as substrates and the kinetic analysis of the lipolysis reaction permitted the examination of the fatty acid specificity of the purified human milk LPL. Starting with an equimolar mixture (0.17 mM each) of tricaproin (trihexanoylglycerol), tricaprylin (trioctanoylglycerol), tricaprin (tridecanoylglycerol), trilaurin (tridodecanoylglycerol), trimyristin (tritetradecanoylglycerol), tripalmitin (trihexadecanoylglycerol) and tristearin (trioctadecanoylglycerol) as substrates, the relative rates of lipolysis were measured by the disappearance of the various triacylglycerol species from the incubation medium by means of high temperature GLC of the intact triacylglycerols. Table 1 gives the degree of lipolysis as a function of time and the fatty acid acyl chain length. The relative rates of hydrolysis were: 8:0 > 10:0 > 12:0 > 6:0 > 14:0 > 16:0 > 18:0. During lipolysis, the generated partial glycerides from long-chain triacylglycerols did not overlap with the medium- or short-chain triacylglycerols when separated by GLC. The serum used as cofactor contained tributyrinase with a high activity and precluded the testing of triacylglycerols shorter than tricaproin in this study. In a control experiment, it was shown that the serum esterase had no reactivity toward the tricaproin and longer chain triacylglycerols under the present experimental conditions.

TABLE 1

Hydrolysis of Medium-Chain and Long-Chain Triacylglycerol Mixtures by Human Milk Lipoprotein Lipase[a]

Substrate	Length of hydrolysis (min)		
	20	40	60
	% digested		
Tricaproin	0	25	68
Tricaprylin	50	90	100
Tricaprin	30	65	88
Trilaurin	20	45	75
Trimyristin	0	14	23
Tripalmitin	0	16	14
Tristearin	0	4	8

[a]The incubation was performed at 37 C in duplicate as described in Materials and Methods with 0.17 mM of each triacylglycerol.

The effect of unsaturation of the fatty acids on the rate of hydrolysis by LPL was determined by examining the kinetics of the fatty acids released from an equimolar mixture of trimyristin, tripalmitin, tristearin, triolein (tri-*cis*-9-octadecenoylglycerol), trilinolein (tri-*cis*, *cis*-9,12-octadecadienoylglycerol) and trilinolenin (tri-*cis*,*cis*,*cis*-9,12,15-octadecatrienoylglycerol). The time course of the fatty acid release is given in Figure 2. The relative order of appearance was 18:1 > 18:3 > 18:2 > 14:0 > 16:0 > 18:0. Triolein was degraded at rates comparable to that of tricaprylin with the first order rate constants giving a ratio of 0.96:1.00.

Positional Specificity

The positional specificity of the purified human milk LPL was assayed using mixed diacid-triacylglycerols of known composition and of the primary and secondary positions as substrates. Table 2 shows the release of the diacyl- and monoacyl-glycerols from *rac*-1,3-dipalmi-

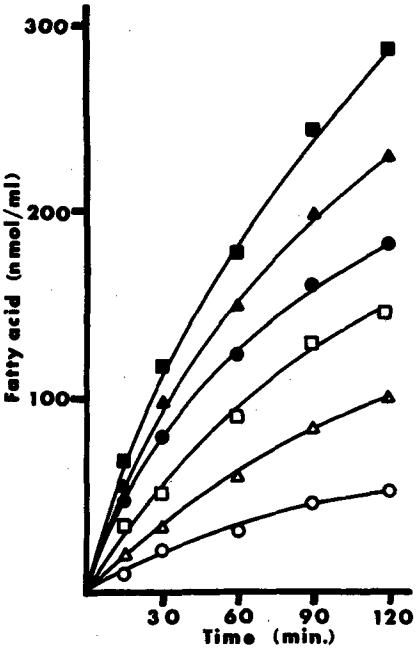


FIG. 2. The kinetics of the hydrolysis of an equimolar mixture (0.5 mM) of trimyristin, tripalmitin, tristearin, triolein, trilinolein and trilinolenin with human milk lipoprotein lipase. The released myristic acid (□), palmitic acid (△), stearic acid (○), oleic acid (■), linoleic acid (●) and linolenic acid (▲) were measured by GLC. Experiments were performed at 37 C in duplicate.

TABLE 2

Hydrolysis of *rac*-1,3-Dipalmitoyl-2-oleoylglycerol by Human Milk Lipoprotein Lipase[a]

	Length of hydrolysis (min)			
	0	20	40	60
	Concentration (nmol/ml)			
TG-50[b]	393	350	302	267
DG-32	0	3.4	4.6	4.7
DG-34	0	13.7	16.4	15.3
MG-16	0	6.7	6.8	10.1
MG-18	0	15.6	47.7	52.6

[a]The incubation was performed at 37 C in duplicate as described in Materials and Methods.

[b]TG: triacylglycerol; DG: diacylglycerol; MG: monoacylglycerol. The glycerides were identified by their acyl-carbon number by GLC.

toyl-2-oleoylglycerol as a function of time. It is seen that diacylglycerols with acyl carbon number 34 and the monoacylglycerols with acyl carbon number 18 are the major partial acylglycerols released. This indicates that the

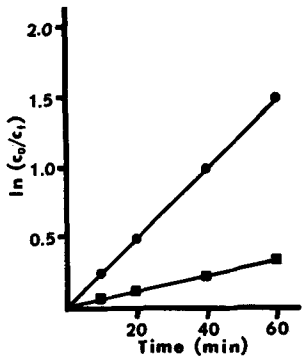


FIG. 3. The hydrolysis of *rac*-1-palmitoyl-2,3-diacetylglycerol (●) and *sn*-2-palmitoyl-1,3-diacetylglycerol (■) by human milk lipoprotein lipase, where C_t represents the concentration of the substrate at each time point and C_0 (0.3 mM) represents the substrate concentration prior to lipolysis. The slopes of the linear plots represent the first order rate constant for the degradation of these substrates. Experiments were performed at 37 C in duplicate.

LPL preferentially attacks the primary positions of the triacylglycerol molecule. However, the detection of diacylglycerols with acyl carbon number 32 and monoacylglycerol with acyl carbon number 16 in the hydrolyzate indicates that the enzyme also has significant reactivity with the secondary ester bond of the triacylglycerol molecule. The relative rates of hydrolysis of the primary and secondary ester bonds containing the same fatty acid was further investigated by means of *rac*-1-monopalmitoylglycerol and *sn*-2-monopalmitoylglycerol. In order to prevent acyl migration, the monoacylglycerols were acetylated prior to LPL digestion. Figure 3 gives the relative rates of hydrolysis of the 2 monoacylglycerol acetates. By measuring the disappearance of the diacetylmonopalmitoylglycerols, it was found that the first order rate constant for the degradation of the acetyl derivatives of *rac*-1-monopalmitoylglycerol and *sn*-2-monopalmitoylglycerol was $25 \times 10^{-3}min^{-1}$ and $5.8 \times 10^{-3}min^{-1}$, respectively. Thus, the rate of hydrolysis of the primary ester bond was 4.3 times that of the secondary ester bond. During this lipolysis, no vicinal hydroxyl groups were detected as determined by the periodate-chromotropic acid method (25); this finding suggests that the acetyl ester group was not released by the LPL.

Stereospecificity

Because 2 primary ester bonds of triacylglycerols have been shown to be attacked at different rates by crude bovine milk LPL (11), we examined the stereospecificity of the purified

human milk LPL. Table 3 gives the relative proportions of the *sn*-1,2- and *sn*-2,3-diacylglycerol intermediates recovered during the lipolysis of [^{3}H-glycerol] triolein in the presence of human or rat serum as LPL activators. The ratio of the *sn*-2,3-diacylglycerols/*sn*-1,2-diacylglycerols ranged from 2.8 to 3.8, suggesting that the human milk LPL preferentially attacked the *sn*-1-position in the triacylglycerol molecule.

As several laboratories had demonstrated (26,27) a resynthesis of di- and triacylglycerols from *sn*-2-monoacylglycerols and free fatty acids by LPL, we also determined the stereochemical course of this reaction. Table 4 gives the yields and enantiomer proportions of the diacylglycerols arising from incubation of 2 different ratios of triacylglycerols with *sn*-2-monooleoyl[^{3}H]glycerol. Despite an extensive hydrolysis of the triacylglycerol and of the diacylglycerols released from it, a measurable quantity (5,000-7,000 cpm) of radioactive x-1,2-diacylglycerols accumulated which obviously originated via acylation of the added radioactive monoacylglycerol. The ratios of the radioactive *sn*-1,2-/*sn*-2,3-diacylglycerols ranged from 56/44 to 74/26 with an average of 65/35. Because the radioactive x-1,2-diacylglycerols made up less than 1% of the total x-1,2-diacylglycerol recovered from the incubation mixture, it is unlikely that the reverse reaction could have contributed significantly to the enantiomeric diacylglycerol ratio resulting from the forward reaction.

TABLE 3

Relative Content of *sn*-1,2- and *sn*-2,3-Diacylglycerols in Hydrolyzates of Glycerol [1-^{14}C] Trioleate after Digestion by Human Milk Lipoprotein Lipase[a]

Activator	% Diglyceride	
	sn-1,2-	*sn*-2,3-
Rat serum	25 ± 2[b]	75 ± 2
Human serum	21 ± 1	79 ± 1

[a]The incubations were performed at 37 C as described by Morley and Kuksis (11) using glycerol [1-^{14}C] trioleate as substrate. The results obtained represented the average of 2 experiments using 2 LPL concentrations (4 units/ml and 2 units/ml) performed in duplicate.

[b]Mean ± SD (n = 4).

DISCUSSION

The present experiments show that purified human milk LPL exhibits a marked fatty acid specificity when assayed both with monoacid and diacid triacylglycerols. The medium-chain saturated and the long-chain unsaturated species are hydrolyzed in preference to the long-chain saturated species. The rate of hydrolysis, however, is not directly related to water solubility of the substrate because tricaproin, which is more soluble in water, was found to be degraded more slowly than the tricaprylin, which is relatively less soluble in water. It appears that the decrease in the chain length from C_8 to C_6 is critical for the hydrophobic interaction between the substrate and the LPL. However, the lower reactivity of long-chain triacylglycerols due to an incomplete emulsification was not ruled out in this study. Rapp and Olivecrona (28) have shown that the tributyrin-LPL complex must be stabilized with gum-arabic in order to achieve rates of hydrolysis comparable to those obtained for long-chain triacylglycerols (Intralipid). Furthermore, triolein was hydrolyzed more readily than tri-

TABLE 4

Biosynthesis of Diacyl- and Triacylglycerols from 2-Monoacylglycerols during Active Lipolysis[a]

Substrates	% Yield[b]		Enantiomer ratio	
	Diacylglycerols	Triacylglycerols	*sn*-1,2-	*sn*-2,3-
Triolein + 2-monooleoylglycerol (2:1)	1.0 ± 0.1[c]	0.5 ± 0.1	66 ± 8	34 ± 8
Triolein + 2-monooleoylglycerol (1:1)	0.8 ± 0.1	0.4 ± 0.05	64 ± 8	36 ± 8

[a]Each incubation mixture contained 5 mg triacylglycerol, 2.8 mg egg yolk phosphatidylcholine and either 2.5 mg or 5 mg of 2-monooleoylglycerol along with its radioactive tracer (1 μCi, 2-monooleoyl[^{3}H]glycerol). The system was buffered at pH 8.4.

[b]As percentage of total dpm recovered.

[c]Mean ± SD (n = 3).

linolein or trilinolenin. An isolated *cis*-double bond at C_9 appeared to promote the substrate-enzyme interaction more effectively than a double bond at C_9 in association with double bonds at C_{12} and C_{15}. Morley and Kuksis (14) had concluded that crude bovine milk LPL does not exhibit acyl specificity when emulsions of soybean and rearranged cod liver or peanut oils are hydrolyzed. These oils, however, contained the mono- and diunsaturated acids in the form of mixed acid triacylglycerols with small amounts of saturated fatty acids. The relative rates of lipolysis of the unsaturated monoacid triacylglycerols, however, agree with the results of lipolysis of *rac*-1-monoacylglycerols reported by Miller et al. (29). These workers observed that lipoprotein lipase was more active on unsaturated than on saturated *rac*-1-monoacylglycerols. It has been observed that chylomicrons rich in saturated fatty acid esters had a slower turnover rate (30); whether this is related to the LPL acyl-specificity remains to be established.

To account for both the fatty acid chain-length and double bond effect on LPL reactivity, we suggest that for optimal interaction of LPL with its substrate the first 8 carbons (C_1-C_8) are most important. An increase in acyl chain length beyond 8 carbons decreases the interaction, as does a decrease in chain length below 8 carbons. The presence of a double bond at C_9 (possibly with *cis*-configuration) may reduce the steric hindrance effect. With additional double bonds at C_{12} and C_{15}, as in the case of linoleoyl- and linolenoyl-glycerols, some hindrance is again reintroduced. In view of the single acid triacylglycerols in these studies, it is not possible to determine which one of the three fatty acid residues in the triacylglycerol molecule is involved in the fatty acid recognition, the one that is released or the one that is retained. On the basis of studies with mixed alkylacylglycerols, Paltauf and Wagner (31) have proposed that LPL may interact specifically with the fatty chain and carbonyl group of the *sn*-2-position. It remains to be determined whether the fatty acid specificity observed in the lipolysis of the single acid triacylglycerols is retained in the mixed-acid triacylglycerols.

The relative rates of release of fatty acids from the primary and secondary positions of the triacylglycerol molecule as estimated from the degradation of the *rac*-1,3-dipalmitoyl-2-oleoylglycerol as substrate indicated a greater preference for the attack at the primary positions, but some hydrolysis also is seen at the *sn*-2-position. The production of x-1,3-dipalmitoylglycerol as the intermediate indicated beyond doubt that the *sn*-2-position was hydrolyzed prior to the primary positions. Based on a failure to demonstrate the presence of x-1,3-diacylglycerols as intermediates in the lipolysis mixture, Nilsson-Ehle et al. had concluded that both human post-heparin plasma (9) and bovine milk (10) LPL have an absolute specificity for the primary ester bond of the triacylglycerols. In view of the generally recognized high reactivity of the LPL toward the intermediary diacylglycerols (32,33), the amount of the x-1,3-diacylglycerols may have been too low to be detected by the TLC methods used. The relative rates of release of palmitic acid from the primary and secondary positions of the diacetylpalmitoylglycerols by the purified human milk LPL gave a ratio of 4.3:1.0. Greten et al. (8) reported a ratio of 6:10 for the hydrolysis of the primary and secondary ester bonds from incubations of monoacyldialkylglycerols and LPL. Hülsmann et al. (34) have reported that the 2-monopalmitoylglycerol is used, as well as the *rac*-1-isomer, by the heparin-releasable LPL from rat liver. In this study, however, acyl migration was neither controlled nor estimated and could have provided a sufficient amount of the *rac*-1-isomer to be present to account for the observed high reactivity.

This demonstration of a preferential hydrolysis of the *sn*-1-position of the triacylglycerol molecule by the purified human milk LPL confirms previous observations on crude preparations of bovine milk (11,32) and rat post-heparin plasma LPL (12,13). An absolute specificity for the *sn*-1-position, however, was not obtained after the enzyme purification. This suggests that the same enzyme attacks both primary and secondary positions, although at unequal rates. Interestingly, the minor reversal of the reaction via reacylation of the *sn*-2-monoacylglycerols also appears to proceed preferentially via the *sn*-1-position; however, considerable reacylation of the *sn*-3-position would also appear to occur. In view of the minor quantitative contribution to diacylglycerol formation, the reverse reaction cannot significantly affect the ratio of the enantiomers generated by the forward reaction.

In conclusion, purified human milk LPL exhibits both fatty acid and positional specificity for its triacylglycerol substrate. Although this specificity is of great interest for the elucidation of the mechanism of the enzyme reaction and possibly its metabolic regulation, it does not reveal its physiological significance, which remains to be explored.

ACKNOWLEDGMENTS

This study was supported in part by grant HL23705 from the U.S. Public Health Service and by the resources of the Oklahoma Medical Research Foundation. The work in Toronto was supported by the Medical Research Council of Canada, Ottawa, Canada, and the Ontario Heart Foundation, Toronto, Canada. We gratefully acknowledge the expert technical assistance of Helen Bass, Randy Whitmer and Deborah Downs.

REFERENCES

1. Krauss, R.M., Herbert, P.M., Levy, R.E., and Fredrickson, D.S. (1973) Circ. Res. 33, 403-411.
2. Havel, R.J., Fielding, D.J., Olivecrona, T., Shore, V.G., Fielding, P.E., and Egelrud, T. (1973) Biochemistry 12, 1828-1833.
3. Hamosh, M. (1981) in Textbook of Gastroenterology and Nutrition in Infancy (Lebenthal, E., ed.) pp. 473-482, Raven Press, New York, NY.
4. Nilsson-Ehle, P., Garfinkel, A.S., and Schotz, M.C. (1980) Annu. Rev. Biochem. 49, 667-693.
5. Korn, E.D. (1962) J. Lipid Res. 3, 246-250.
6. Hamosh, M., and Scow, R.O. (1971) Biochim. Biophys. Acta 231, 283-289.
7. Korn, E.D. (1961) J. Biol. Chem. 236, 1638-1642.
8. Greten, H., Levy, R.I., Fales, H., and Fredrickson, D.S. (1970) in Atherosclerosis (Jones, R.J., ed.) pp. 200-204, Springer Verlag, New York, NY.
9. Nilsson-Ehle, P., Belfrage, P., and Borgström, B. (1971) Biochim. Biophys. Acta 248, 114-120.
10. Nilsson-Ehle, P., Egelrud, T., Belfrage, P., Olivecrona, T., and Borgström, B. (1973) J. Biol. Chem. 248, 6734-6737.
11. Morley, N., and Kuksis, A. (1972) J. Biol. Chem. 247, 6389-6393.
12. Paltauf, F., Esfandi, F., and Holasek, A. (1974) FEBS Lett. 40, 119-123.
13. Akesson, B., Gronowitz, S., and Herslof, B. (1976) FEBS Lett. 71, 241-244.
14. Morley, N., and Kuksis, A. (1977) Biochim. Biophys. Acta 487, 332-342.
15. Hernell, O., and Olivecrona, T. (1974) J. Lipid Res. 15, 367-374.
16. Wang, C.-S., Bass, H.B., Downs, D., and Whitmer, R.K. (1981) Clin. Chem. 27, 663-668.
17. Wang, C.-S., Weiser, D., Alaupovic, P., and McConathy, W.J. (1982) Arch. Biochem. Biophys., in press.
18. Wang, C.-S. (1981) J. Biol. Chem. 256, 10198-10202.
19. Schotz, M.C., Garfinkel, A.S., Huebotter, R.J., and Stewart, J.E. (1970) J. Lipid Res. 11, 68-69.
20. Kuksis, A., Myher, J.J., Marai, L., and Geher, K. (1975) J. Chromatogr. Sci. 13, 423-430.
21. McConathy, W.J., Blackett, P.B., and Kling, O.R. (1981) Clin. Chim. Acta 111, 153-162.
22. Manganaro, F., Myher, J.J., and Kuksis, A. (1982) Anal. Biochem., in press.
23. Wang, C.-S., and Smith, R.L. (1975) Anal. Biochem. 63, 414-417.
24. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
25. Korn, E.D. (1962) Methods Enzymol. 5, 552-545.
26. Borgström, B., and Carlsson, L.A. (1957) Biochim. Biophys. Acta 24, 638-639.
27. Bengtsson, G., and Olivecrona, T. (1980) Eur. J. Biochem. 106, 557-562.
28. Rapp, D., and Olivecrona, T. (1978) Eur. J. Biochem. 91, 379-385.
29. Miller, C.H., Parce, J.W., Sisson, P., and Waite, M. (1981) Biochim. Biophys. Acta 665, 385-392.
30. Massaro, E.R., Green, J.B., and Green, M.H. (1981) Fed. Proc. 40, 350.
31. Paltauf, F., and Wagner, E. (1976) Biochem. Biophys. Acta 431, 359-362.
32. Morley, N., Kuksis, A., Buchnea, D., and Myher, J.J. (1975) J. Biol. Chem. 250, 3414-3418.
33. Fielding, C.J., and Fielding, P.E. (1980) Biochim. Biophys. Acta 620, 440-446.
34. Hülsmann, W.C., Oerlemans, M.C., and Jansen, H. (1980) Biochim. Biophys. Acta 618, 364-369.

[Received November 5, 1981]

Hypolipidemic Effects of Gossypol in Cynomolgus Monkeys (*Macaca fascicularis*)

L.N. SHANDILYA* and **T.B. CLARKSON**, *Arteriosclerosis Research Center, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103*

ABSTRACT

The effect of gossypol acetic acid (gossypol) on plasma lipid concentrations was studied in adult male cynomolgus monkeys consuming a diet containing 0.19 mg cholesterol/Kcal. Gossypol was administered orally at 5 (n=4) or 10 (n=3) mg/kg/day for 6 months. A significant decrease in total plasma cholesterol (TPC) and low density lipoprotein and very low density lipoprotein-cholesterol (LDL + VLDL-chol) concentrations was observed without any significant decrease in plasma high density lipoprotein-cholesterol (HDL-chol) levels among 10 mg/kg/day gossypol-treated animals. This is a new therapeutic property of gossypol that has not been previously reported. No appreciable differences were observed in plasma levels of TPC and LDL + VLDL-chol among 5 mg/kg/day gossypol-treated animals when compared to controls until the gossypol dosage was increased to 10 mg/kg/day, thus suggesting that hypolipidemic effect of gossypol is dose-dependent. In general, no adverse clinicopathological findings were noted except a temporary diarrhea and loss of appetite among 10 mg/kg/day gossypol-treated animals during the initial stages of treatment. In conclusion, it is tempting to speculate that gossypol might possibly reduce the intestinal absorption of dietary cholesterol or it may reduce the hepatic synthesis of low density lipoproteins. These results also suggest that gossypol may be a particularly useful drug in lowering plasma cholesterol concentrations in addition to its previously demonstrated antifertility properties in males.
Lipids 17:285-290, 1982.

Gossypol is a yellowish phenolic compound isolated from the seeds, stems and roots of cotton plants. It has been found to be effective in decreasing sperm concentration in experimental animals and human beings (1-4).

There is evidence that the extent of atherosclerosis exacerbation in vasectomized monkeys may be related to the degree of continued exposure to sperm antigens resulting from continued spermatogenesis post-vasectomy (5,6). For that reason, we undertook the study reported here to determine the possibility of using gossypol as a therapeutic intervention to decrease sperm concentration and thereby reduce the sperm antigen load of vasectomized animals. An unexpected finding was the lowering of plasma cholesterol concentrations among the monkeys consuming a cholesterol-containing diet and treated with gossypol. In this report, we describe the effects of orally administered gossypol acetate on the plasma lipid concentrations of male cynomolgus macaques and present evidence that the hypolipidemic effects of the drug are not due to its toxicity.

MATERIALS AND METHODS

Animals

Adult male cynomolgus monkeys (*Macaca fascicularis*) of Malayan origin were obtained from Primate Imports, Inc., Port Washington, NY, and were maintained in individual cages under controlled environmental conditions (temperature 24 C, light/dark, 10/14-hr schedule). The animals were provided food and water ad libitum. All animals were fed the diet shown in Table 1.

Dosage and Treatment Schedule

Gossypol acetate (hereafter referred to as gossypol) was obtained from the Southern Regional Research Center of the U.S. Department of Agriculture. We suspended the gossypol in a small quantity of corn oil (50 mg/ml). Initially, gossypol was administered orally with a syringe; but subsequently, we were able to administer the drug in a small slice of apple. The animals were divided into 3 groups: the first group consisted of 4 monkeys which received vehicle only and served as controls; the second group of 4 monkeys received gossypol at a dose level of 5 mg/kg/day for 3 months and later the dose was increased to 10 mg/kg/day for the next 3 months; the third group consisted of 3 animals who were treated with 10 mg/kg/day of gossypol. The study was divided into 2 phases: a pretreatment phase of 2 months and treatment phase of 6 months.

Laboratory Determinations

Animals were fasted overnight prior to collection of blood samples at monthly intervals during the pretreatment and treatment phases of the study. Total plasma cholesterol (TPC) and triglyceride (TG) concentrations were measured by the Auto-Analyzer II Method of Rush et al. (7). The heparin-manganese precipitation method as described in the Manual of

TABLE 1

Composition of the Experiment Diet[a]

Ingredients	g/100 g	Protein (g)	Lipid (g)	Carbohydrate (g)	Cal/100 g of diet	Chol (mg)
Casein, USP	8.0	8.0	–	–	32.0	–
Lactalbumin	8.0	8.0	–	–	32.0	–
Wheat flour	36.0	3.77	0.36	30.6	140.7	–
Dextrin	6.0	–	–	6.0	24.0	–
Sucrose	5.0	–	–	5.0	20.0	–
Applesauce	4.5	0.02	Trace	1.08	4.4	–
Lard	6.0	–	6.0	–	54.0	6.0
Butter	3.0	0.01	2.43	0.01	21.95	9.0
Beef tallow	7.0	–	7.0	–	63.0	7.0
Dried egg yolk	3.0	0.66	1.98	0.08	20.76	66.0
Safflower oil	4.0	–	4.0	–	36.0	–
Complete vitamin mixture (devoid of vitamin D)	2.5	–	–	2.0	8.0	–
Alphacel	1.0	–	–	–	–	–
Hegsted salts mixture	4.0	–	–	–	–	–
D_3 in corn oil	b	–	–	–	–	–
NaCl (table salt)	2.0	–	–	–	–	–
Total (Calories)	100.0	20.46 (81.84)	21.77 (195.93)	44.77 (179.08)	456.81	88.0

[a]The diet contains 456.81 Kcal/100 g. The cholesterol content of all diets was 0.19 mg/Kcal and 43% of calories were provided from a mixture of fat, 18% of calories as protein and 39% of calories as carbohydrates.
[b]To provide 2.5 IU/g.

Laboratory Operations of the Lipid Research Clinics Program (8) was used for high density lipoprotein-cholesterol (HDL-chol) determinations. Low density lipoprotein and very low density lipoprotein-cholesterol (LDL + VLDL-chol) concentrations were calculated as the difference between TPC and HDL-chol. All plasma lipids were determined in our Lipid Analytic Laboratory that is in complete compliance with the Cooperative Lipid Standardization Program of the Center for Disease Control. Determinations of serum concentrations of GOT, GPT, γGT, total protein, creatinine, urea nitrogen, and enumerations of erythrocytes, leukocytes and hematocrit were done in our Comparative Clinical Pathology Laboratory according to standard procedures.

Statistical analyses of the data were done by Student's t-test and multivariate analysis of variance.

RESULTS

Treatment of male cynomolgus monkeys with gossypol at 5 and 10 mg/kg/day resulted in a decrease in sperm concentration and sperm motility without any significant decrease in plasma levels of testosterone. The details of the effects of gossypol on reproductive and endocrine functions will be reported elsewhere.

In general, no adverse clinical findings were noted among gossypol-treated animals except the transient diarrhea and anorexia among the animals treated at 10 mg/kg/day of gossypol during the beginning of the treatment. The animals recovered when the medication was discontinued for a short time. The average body weight of all animals did not change from month to month throughout the study ($F_{6,4} = 2.10$, $p = 0.25$; see Table 2).

Assessment of possible liver injury due to gossypol was done by determination of SGOT, SGPT and serum γGT concentrations among gossypol-treated animals and these measurements showed no significant deviation from controls. Results of kidney function tests (blood urea nitrogen and serum creatinine) showed no marked changes from controls. Results of hematological studies (hematocrit, erythrocytes and leukocytes) are also within normal range. Erythrocyte counts among the control and 10 mg/kg/day gossypol-treated animals were significantly lower ($p<0.025$) before the beginning of the treatment and after 6 months of treatment the erythrocyte counts had not declined.

Effect of Gossypol on Plasma Lipids

Total plasma cholesterol concentrations. Figure 1 shows data on the plasma cholesterol concentrations for each group. It can be seen that, in the control and 5-mg/kg/day gossypol-treated animals, mean TPC concentrations

TABLE 2

Effect of Gossypol Treatment on Body Weight (kg)[a]

Group	Baseline	Months of treatment					
		1	2	3	4	5	6
I Control	5.24 ± 0.29	5.56 ± 0.23	5.55 ± 0.27	5.59 ± 0.31	5.54 ± 0.29	5.53 ± 0.27	5.74 ± 0.30
II Gossypol-treated (5 mg/kg/day)	5.52 ± 0.23	5.54 ± 0.21	5.64 ± 0.33	5.91 ± 0.25	5.41 ± 0.22[b]	5.47 ± 0.21[b]	5.40 ± 0.33[b]
III Gossypol-treated (10 mg/kg/day)	4.60 ± 0.10	4.55 ± 0.18	4.43 ± 0.19	4.88 ± 0.17	4.33 ± 0.22	4.55 ± 0.14	4.35 ± 0.27

[a] All values are means for the group ± SEM.
[b] Dose increased to 10 mg/kg/day.

increased from 154 to 277 mg/dl and from 219 to 304 mg/dl, respectively, over 3 months of the experimental period. When the dose of gossypol was increased to 10 mg/kg/day among group II (5-mg/kg/day) animals, a significant decrease ($p<0.01$) in TPC concentration was observed after 3 months of treatment at increased dose level. Among the animals treated with 10 mg/kg/day of gossypol, TPC concentrations, in fact, decreased from 187 to 146 mg/dl. The difference in the mean TPC concentrations of the control and 10-mg/kg/day gossypol-treated animals was statistically significant ($F_{6,4} = 11.79$, p. = 0.016).

Plasma HDL-chol and LDL + VLDL-chol concentrations. As there were significant decreases in TPC concentrations among gossypol-treated animals, it was important to us to determine whether the decreases were in HDL-chol or in the cholesterol concentrations of LDL + VLDL. In Figure 2, the plasma levels of HDL-chol before and during gossypol treatment are shown. Although there were slight decreases in mean plasma HDL-chol levels among gossypol-treated animals compared to controls, the differences were not statistically significant at any given time of treatment.

No appreciable differences could be seen in plasma levels of LDL + VLDL-chol among group II (5-mg/kg/day gossypol-treated) animals up to 3 months of treatment when compared to controls. However, a significant decrease ($p<0.025$) in mean LDL + VLDL-chol concentration was observed after 3 months following an increase in gossypol dose to 10 mg/kg/day. Among the group III (10-mg/kg/day gossypol-treated) animals, a significant decrease ($p<0.001$) in plasma LDL + VLDL-chol concentration compared to controls was seen after 2 months of treatment and these differences remained statistically significant throughout the study.

Plasma triglyceride concentrations. Table 3 shows the mean plasma TG concentrations among gossypol-treated animals throughout the study period. As can be seen, the mean plasma TG concentrations were slightly higher among group II (5-mg/kg/day gossypol-treated) animals compared to controls, but the differences were not statistically significant. Among group III (10-mg/kg/day gossypol-treated) monkeys, a significant increase ($p<0.025$) in mean plasma TG concentrations was observed following 2 months of treatment, but by the sixth month of treatment, mean plasma TG concentrations decreased to control levels.

Total plasma cholesterol/high density lipoprotein-cholesterol ratio. The ratios of TPC to HDL-chol among gossypol-treated and con-

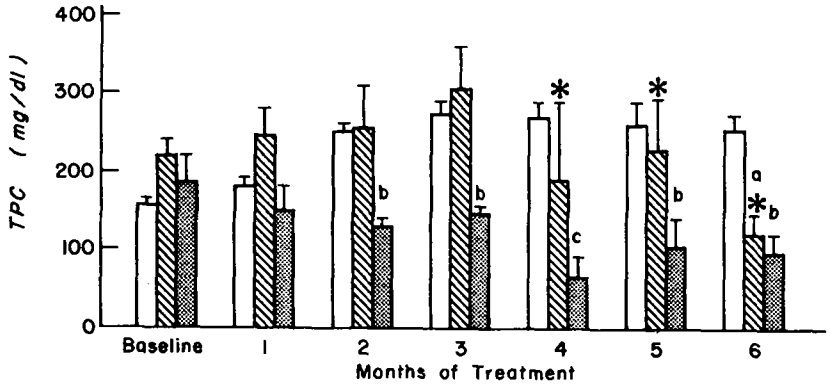


FIG. 1. Influence of gossypol on total plasma cholesterol concentrations (mean ± SEM). □ Control; ■ gossypol-treated (5 mg/kg/day); ⊠ gossypol-treated (10 mg/kg/day); * dose increased to 10 mg/kg/day. (a) $p<0.01$; (b) $p<0.005$; (c) $p<0.001$.

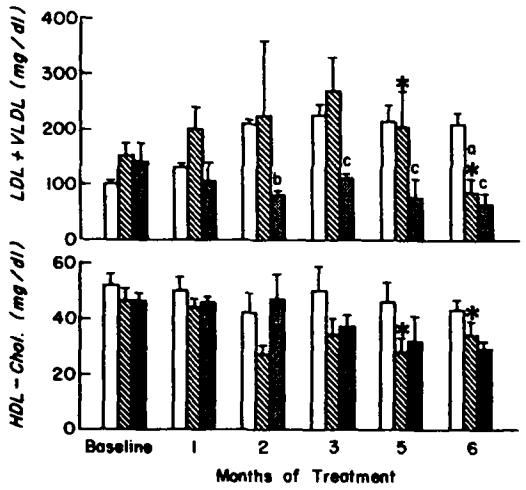


FIG. 2. Effect of gossypol on plasma concentrations of HDL and LDL + VLDL-chol (mean ± SEM). □ Control; ■ gossypol-treated (5 mg/kg/day); ⊠ gossypol-treated (10 mg/kg/day); * dose increased to 10 mg/kg/day. (a) $p<0.025$; (b) $p<0.001$; (c) $p<0.005$.

trol monkeys are presented in Table 4. TPC/HDL-chol ratio increased from 2.96 to 6.78 and from 5.05 to 10.92 among control and group II (5-mg/kg/day gossypol-treated) animals, respectively. Following an increase in dose of gossypol to 10 mg/kg/day among group II (5-mg/kg/day gossypol-treated) animals, the TPC/HDL-chol ratio started declining. Among group III (10-mg/kg/day gossypol-treated) animals, TPC/HDL-chol ratio never exceeded 4.05 throughout the period of experiment and this ratio was significantly lower ($p<0.05$) than control values.

DISCUSSION

Consistent with results from previous studies in laboratory animals and humans (1-4), gossypol decreases sperm concentration and sperm motility without any significant decrease in plasma testosterone levels in cynomolgus monkeys.

These studies also demonstrate that daily oral administration of gossypol at a dose of 10 mg/kg/day effectively lowers the TPC concentration. This is a therapeutic property of the compound that has not been reported previously. The difference in HDL-chol concentrations between the control and gossypol-treated animals is not statistically significant, whereas the differences in plasma concentrations of LDL + VLDL-chol are highly significant, suggesting that the hypolipidemic effect of gossypol is primarily due to a reduction in LDL + VLDL-chol. The results of this study show further that the hypolipidemic effect of gossypol is dose-dependent, i.e., no decreases in TPC or LDL + VLDL-chol were noted among group II (5-mg/kg/day gossypol-treated) animals until the gossypol dosage was increased to 10 mg/kg/day.

Because the hypocholesterolemic effect of gossypol is due to a reduction in LDL + VLDL-chol, the TPC/HDL-chol ratio remained low among group III (10-mg/kg/day gossypol-treated) animals. Based on the suggestive evidence from the Framingham study (9) that the ratio of TPC to HDL-chol is of critical importance in predicting increased risk of atherosclerosis (ratios greater than 4 being atherogenic), it is of interest to note that the ratio differences in this experiment are 6.30 for control animals and 4.05 for the animals treated with gossypol at the dose of 10 mg/kg/day.

In addition to the hypolipidemic effects, gossypol treatment at the 10-mg/kg/day dose level resulted in an increase in plasma TG concentrations, though there were large individual

TABLE 3

Effect of Gossypol Treatment on Plasma Triglyceride Concentrations (mg/dl)[a]

Group	Baseline	Months of treatment				
		2	3	4	5	6
I Control	7.75 ± 2.21	7.50 ± 1.55	10.50 ± 2.06	11.50 ± 2.40	13.50 ± 1.94	34.00 ± 8.37
II Gossypol-treated (5 mg/kg/day)	8.00 ± 2.12	11.00 ± 3.11	19.25 ± 8.35	28.25 ± 14.26[b]	29.66 ± 17.67[b]	42.00 ± 13.25[b]
III Gossypol-treated (10 mg/kg/day)	10.70 ± 3.18	17.70 ± 2.40[c]	65.30 ± 26.93[d]	80.00 ± 32.00[d]	53.00 ± 19.00[d]	34.36 ± 11.05

[a]All values are means for the group ± SEM.
[b]Dose increased to 10 mg/kg/day.
[c]$p<0.025$.
[d]$p<0.05$.

TABLE 4

Effect of Gossypol Treatment on Total Plasma Cholesterol/High Density Lipoprotein-Cholesterol Ratio[a]

Group	Baseline	Months of treatment					
		1	2	3	4	5	6
I Control	2.96 ± 0.10	3.71 ± 0.24	6.74 ± 1.41	6.42 ± 1.42	7.78 ± 0.89	6.15 ± 1.39	6.01 ± 0.56
II Gossypol-treated (5 mg/kg/day)	5.05 ± 0.78	5.82 ± 1.23	10.41 ± 3.43	10.74 ± 3.54	10.92 ± 4.24[b]	8.98 ± 2.67[b]	3.74 ± 1.02[b]
III Gossypol-treated (10 mg/kg/day)	4.04 ± 0.70	3.25 ± 0.65	2.93 ± 0.55	4.05 ± 0.42	2.94 ± 1.18[c]	3.27 ± 0.44[c]	3.20 ± 0.57[c]

[a]All values are means for the group ± SEM.
[b]Dose increased to 10 mg/kg/day.
[c]$p<0.05$.

variations among animals in different groups, and by 6 months posttreatment, mean plasma TG concentrations decreased to the control levels. We sought to determine if this increase was any indication of liver injury. Results of liver enzyme concentrations (SGOT, SGPT and serum γGT) among gossypol-treated monkeys showed no deviation from controls, thus excluding the likelihood of liver damage. We are unable to explain this transient increase in plasma TG concentrations.

Although a formal nutritional study was not done and thus the diet intake was not measured, it was possible that gossypol-treated monkeys were eating less of the diet and hence their plasma cholesterol levels were lower than the controls. We have 3 kinds of indirect evidence that this was not the case. First, all animals were given the same amount of diet each day and judging from the amount of uneaten diet, there was no indication that certain groups of animals consumed markedly different amounts. Second, because body weight remained fairly constant throughout the experiment, each animal must have eaten a relatively constant number of calories. Third, a dose increase among group II animals resulted in a significant decrease in TPC. Despite experimental design limitations, the original conclusion seems quite plausible, though a continued mild anorectic effect with only slight reduction in food intake and maintenance of a lower body weight cannot be excluded. However, our data suggest that the hypocholesterolemic effect is much greater than would be expected from simple reduction in food intake.

From these experiments, one can speculate on the possible mechanism(s) of the hypolipidemic effect of gossypol. The most likely effect is that gossypol reduced the intestinal absorption of dietary cholesterol. Another possible mode of action is reduced hepatic synthesis of the low density lipoproteins.

In conclusion, gossypol appears to be an interesting pharmacologic agent, because it lowers total plasma cholesterol (LDL + VLDL-chol) concentrations in addition to its previously demonstrated antifertility properties in males. It is fairly well tolerated and has shown only minimal side effects at the dosage used in our experiment. However, a further period of observation will be necessary to confirm the long-term safety of this compound, as gossypol has been reported to be toxic at high dose levels to dogs, cats, swine, chickens, rats, mice, rabbits, guinea pigs and other nonruminant animals (10). In ruminant animals, oral gossypol is relatively nontoxic, probably because of bacterial metabolism in the rumen which results in the binding of gossypol to protein (11). Among humans, 10,000 healthy men in China have been administered gossypol acetic acid or gossypol formic acid for more than 6 months and more than half of them have been clinically observed for 2 years (12). Some men initially experienced fatigue, increased or decreased appetite, gastrointestinal complaints or decreased libido, but these symptoms gradually disappeared without discontinuation of therapy (1,12). More work is required to understand the mechanism(s) by which the hypolipidemic effects of gossypol are mediated.

ACKNOWLEDGMENTS

We thank Dr. Angelo V. Graci of Southern Regional Agricultural Research Center, New Orleans, for providing us with gossypol acetic acid. We also thank L. Trust and C. Lavender for typing the manuscript. This work was supported by a grant from R.J. Reynolds Industries, Inc.

REFERENCES

1. National Coordinating Group on Male Infertility Agents (1979) Chinese Med. J. 4, New Series, 417. Cited in Res. Reprod. (1979) 11(3), 3.
2. Nadakavukaren, M.J., Sørensen, R.H., and Tone, J.N. (1979) Cell Tissue Res. 204, 293-296.
3. Lin, Y.C., Hadley, M.A., Klingener, D., and Dym, M. (1980) Soc. for Study of Reprod. 13th Annual Meeting, Ann Arbor, Michigan, Abstract #150.
4. Chang, M.C., Gu, Z., and Saksena, S.K. (1980) Contraception 21, 461-469.
5. Alexander, N.J., and Clarkson, T.B. (1978) Science 201, 538-541.
6. Clarkson, T.B., and Alexander, N.J. (1979) Fed. Proc. 38(3), Part II, 1346.
7. Rush, R.L., Leon, L., and Turrell, J. (1971) in Advances in Automated Analysis-Technicon International Congress (Barton, E.C., DuCros, M.J., Erdrich, M.M., Golin, J.E., Levine, J.B., Mansberg, H., Mayfield, D.W., Ohringer, P., Reed, D.N., Roesch, C.R., Roth, K.L., Saunders, A.M., Smythe, W., and Weldon, W.A., eds.) Vol. 1, pp. 503-507, Futura Publishing Co., Mt. Kisco, NY.
8. Manual of Laboratory Operations. Lipid Research Clinics Program, (1974) DHEW Publication No. (NIH) 75-628.
9. Castelli, W.P. (1980) in Atherosclerosis V (Gotto, A.M., Jr., Smith, L.C., and Allen, B., eds.) pp. 478-483, Springer-Verlag, New York, NY.
10. Dai, R-X., Pang, S-N., and Liu, Z-L. (1978) Acta Biol. Sinica 11, 27-30.
11. Abou-Donia, M.B. (1976) in Residue Reviews, Vol. 61, pp. 126-160, Springer-Verlag, New York, NY.
12. Maugh, T.H., II (1981) Science 212, 314.

[Received July 14, 1981]

The Synthesis in vivo of Choline and Ethanolamine Phosphoglycerides in Different Brain Areas during Aging

ALBERTO GAITI[a], MARINA BRUNETTI[a], GIAN LUIGI PICCININ[b], HELMUT WOELK[c] and GIUSEPPE PORCELLATI[a,*], [a]*Istituto di Chimica Biologica, Università di Perugia, 06100 Perugia, Italy;* [b]*Clinica Neurologica, Villa Massari, 06100 Perugia, Italy; and* [c]*Psychiatrisches Krankenhaus, 63 Giessen, BRD*

ABSTRACT

The biosynthesis of choline and ethanolamine phosphoglycerides was tested in vivo in different brain areas of the rat during aging. Mixtures of [2-^{3}H] glycerol and [*Me*-^{14}C] choline or [2-^{3}H] glycerol and [2-^{14}C] ethanolamine were injected into lateral ventricle of the brain as lipid precursors and their incorporation into corresponding phospholipid was examined. A significant decrease of synthesis of both phosphoglycerides takes place in cerebral cortex and in the striatum, and is already apparent at 9 months of age with no further decrease or change thereafter. No significant change takes place in the cerebellum. The unchanged absorption of injected water-soluble precursors, together with the lack of any significant change of phospholipid/protein ratio in all examined brain areas, suggests that the incorporation of both glycerol and nitrogen bases are affected by aging.
Lipids 17:291-296, 1982.

INTRODUCTION

It has been clearly demonstrated that the enzymatic synthesis of choline and ethanolamine phosphoglycerides (CPG and EPG) are decreased in brain during aging (1-3). The rate of both ethanolamine and choline phosphotransferases (EC 2.7.8.1. and 2.7.8.2.) is, in fact, significantly decreased in vitro in rat brain microsomes during aging (1). The same enzymic activities are differently affected when tested in vitro with neuronal- and glial-enriched fractions. The rate of CPG synthesis in neurons decreases significantly with age up to 18 months, and does not further change after this time (3). Similar results were obtained for EPG synthesis in the same cells (3), with a further decrease after 18 months of age. On the contrary, no significant change in either choline- or ethanolamine phosphotransferase takes place in glia during aging.

The aim of this work is to verify these findings by investigating CPG and EPG biosynthesis from injected glycerol and free bases during aging in vivo. The rat brain was divided into 4 defined parts, including the cortex, striatum, cerebellum and the rest of brain.

The results indicate that a decrease of the rate of synthesis of both lipids takes place during aging in the brain areas. Preliminary results have appeared elsewhere (4).

EXPERIMENTAL

Animals

Wistar rats (male, 60-day-old) kept in our animal house were used throughout, as adult animals. For aging studies, the rats were raised in the same hygienic and ambient environments to the desired time intervals. Water was given ad libitum. All animals were sacrificed 12 hr after fasting.

Injection

Rats were lightly anesthesized by diethyl ether and treated as described elsewhere (4). The precursors were injected into the lateral ventricle of the brain in a total vol of 10 μl of 0.9% NaCl containing either 36.5 μCi [2-^{3}H] glycerol (sp act, 500 mCi/mmol) and 3.8 μCi [2-^{14}C] ethanolamine (sp act, 60 mCi/mmol) or 37.5 μCi [2-^{3}H] glycerol (sp act, 500 mCi/mmol) and 4.6 μCi [*Me*-^{14}C] choline (sp act, 60 mCi/mmol) to obtain double labeled phospholipids. Each radioactive compound was tested for chemical and radiochemical purity before injection. All animals, adult and aged, were sacrificed by decapitation 60 min (see Discussion) after injection.

Extraction of Lipid and Water-Soluble Components

Brain areas were quickly removed, weighed and treated as follows. Cortex and the rest of the brain (called the rest) were homogenized first with 7 ml of methanol. After complete homogenization in a Teflon-glass Potter-Elvehjem homogenizer, 14 ml of chloroform was added, and the mixture was again homogenized. After centrifugation, the upper and organic phases were transferred into different test tubes. The pellet was washed twice with 4.5 ml of chloroform/methanol (2:1, v/v) and twice with 4 ml of ethanol/water (1:4, v/v). The organic washings were added to organic lower phase and the aqueous washings to upper phase. The pellet was dried in a heater at 60 C overnight and dissolved in 2 N NaOH for protein content assay.

The organic phase was washed with 4 ml of water and twice with 4 ml of methanol/water (1:1, v/v), and the washings were added to upper phase. The extract was taken to dryness under N_2, dissolved in a known vol of chloroform/methanol (2:1, v/v) and used for lipid separation by 2-dimensional thin layer chromatography (TLC), lipid analysis and counting of labeled, separated lipid as described elsewhere (1). The upper phase was equally concentrated and analyzed as reported previously (5).

The extraction of lipid and water-soluble components from cerebellum and striatum was similarly done, except that vol of solvents were 2/3 and 1/10, respectively, of those previously used.

Analyses

Protein was determined according to Lowry et al. (6), by using crystalline bovine serum albumin as the standard. Phospholipid P content was assayed as reported elsewhere (7).

Chemicals

Radioactive products were from Radiochemical Centre, Amersham, England. Reagents used were pure quality compounds.

RESULTS AND DISCUSSION

The patterns of specific activities (sp act, nCi/μg of phospholipid P) of labeled lipid in different brain areas at different ages are reported in Figures 1-4. The statistical evaluation of the results indicates that all the changes reported in Figures 1-3 are significant, whereas poor variation of sp act occurs in lipids extracted from the cerebellum (Fig. 4). The highest decrease of the sp act due to age was found in the striatum (Fig. 2), both for [2-^{3}H]glycerol- and [*Me*-^{14}C]choline- or [2-^{14}C]ethanolamine-containing lipids (about 50%), whereas the lowest (about 20-25%) was observed in the cerebral cortex (Fig. 1).

The reported changes of the sp act are due, in our opinion, to a variation in incorporation of the 2 simultaneously injected precursors into the corresponding phospholipids. This opinion is supported by previous results (8,9), which indicated that the turnover of the phospholipid fraction during 1 hr interval from the time of injection and the sacrifice was found negligible. It is further supported by the forthcoming observations: (a) Aging does not affect the phospholipid P/protein ratio of all examined areas, as reported in Table 1. Therefore, the decreases of the sp act found in this work are essentially due to a lower incorporation of precursors into phospholipids (b). Table 2 shows the [^{3}H]/[^{14}C] ratio of synthesized CPG and EPG determined in different brain areas at different ages after the injection in the lateral ventricle of brain of labeled precursors. No significant variation of this ratio occurs in the areas with age. This result indicates that aging does not affect the incorporation of one precursor more than that of the other. In addition, the same isotopic ratio of water-soluble precursors extracted from the different brain areas was similarly unchanged during

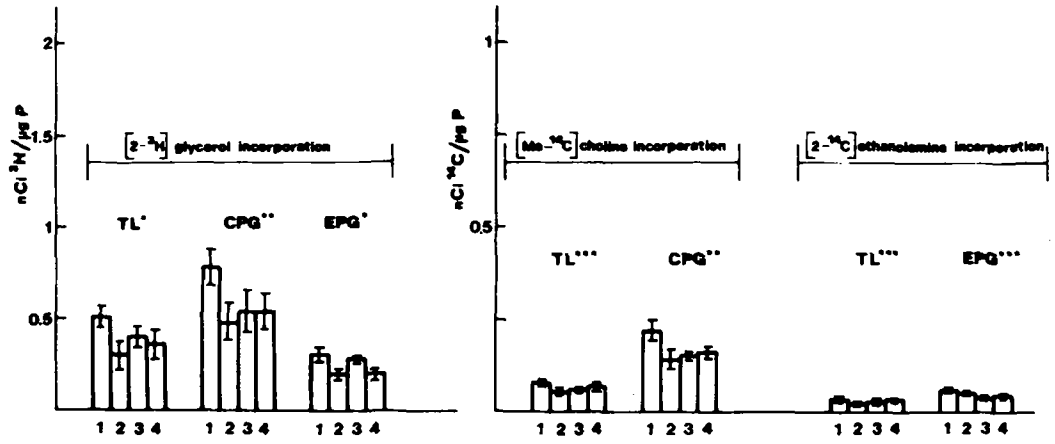


FIG. 1. Brain cortex. Sp act (nCi/μg phospholipid P) of total lipid (TL), CPG and EPG in brain cortex of rats at different ages after 1 hr from injection. [2-^{3}H]Glycerol incorporation is on the left; [*Me*-^{14}C]choline incorporation is in the middle; [2-^{14}C]ethanolamine incorporation is on the right. The bars represent mean value from 4 (^{14}C-containing compounds) to 8 experiments (^{3}H-containing compounds), with standard deviations. The p is obtained from analysis of variance of results. See the text for other details. *$p < 0.01$; **$p < 0.05$; ***not significant, except for EPG synthesis at 18 months of age ($0.05 > p > 0.025$).

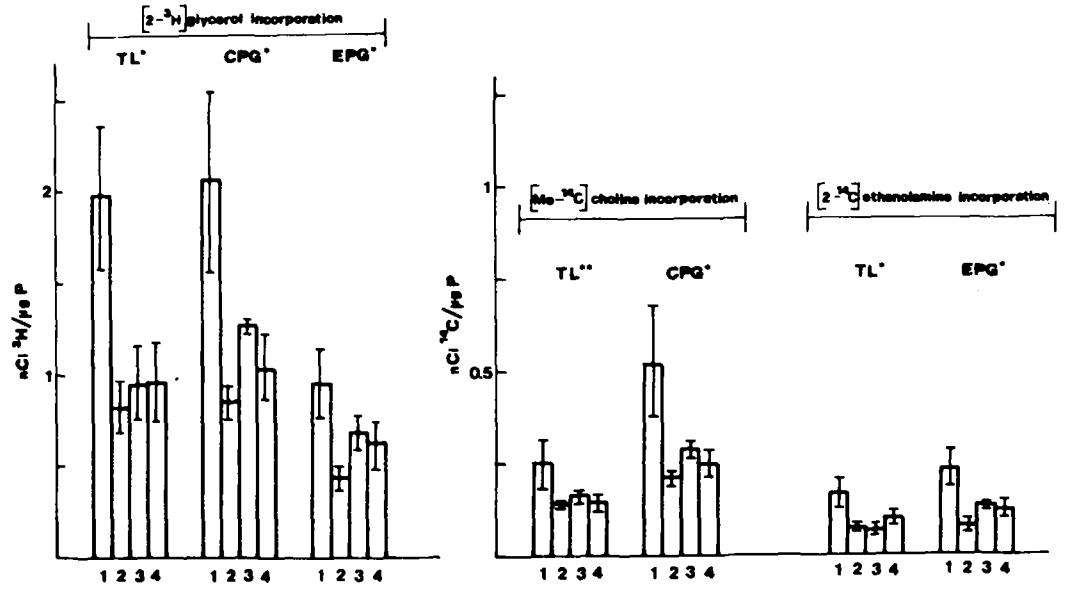


FIG. 2. Striatum. Sp act (nCi/µg phospholipid P) of total lipid (TL), CPG and EPG in striatum. See Fig. 1 for further explanation, and see text for other details. *$p < 0.01$; **$p < 0.05$.

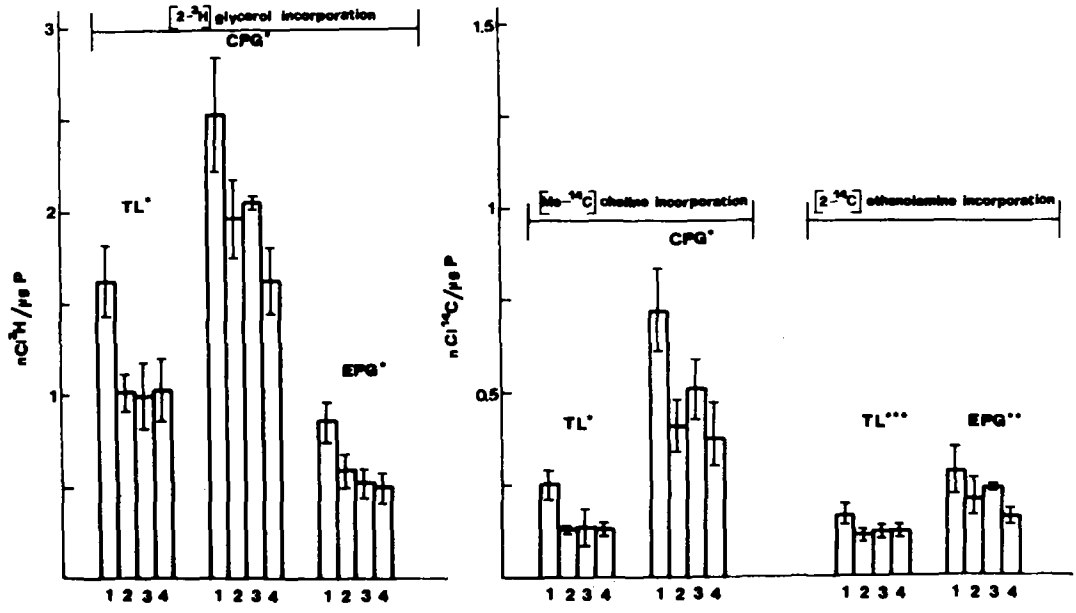


FIG. 3. Rest of brain. Sp act (nCi/µg phospholipid P) of total lipid (TL), CPG and EPG in the rest of brain (see text). See Fig. 1 for further explanation, and the text for other details. *$p < 0.01$; **$p < 0.05$; ***not significant.

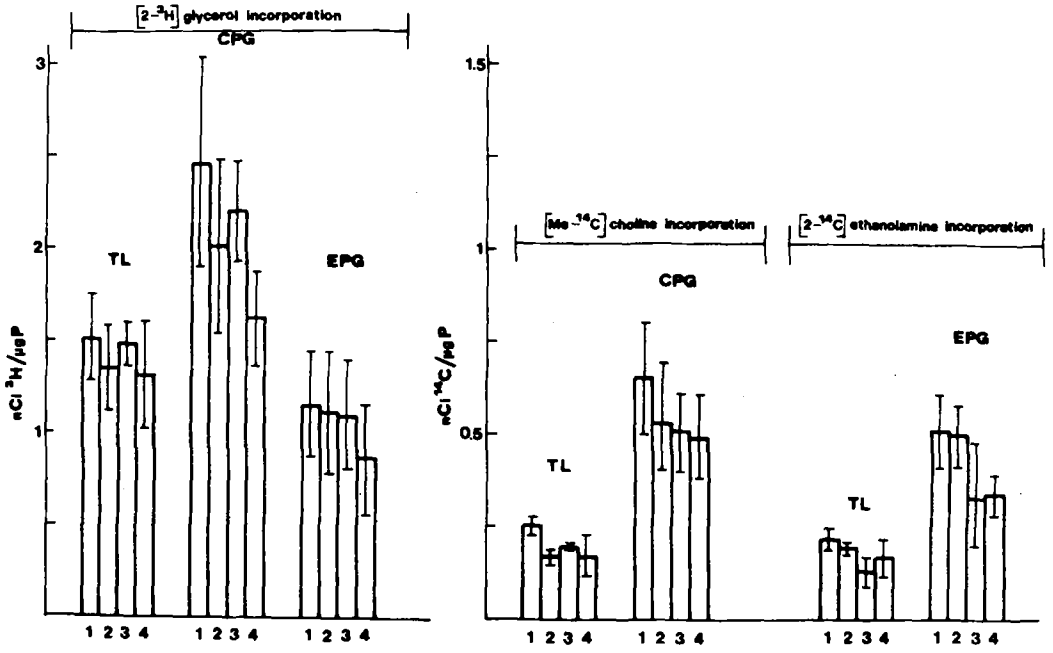


FIG. 4. Cerebellum. Sp act (nCi/µg phospholipid P) of total lipid (TL), CPG and EPG in cerebellum. See Fig. 1 for further explanation, and the text for other details. All values are not significant, except for: glycerol incorporation into CPG (analysis of variance, $p < 0.05$); glycerol incorporation into EPG at 24 months of age ($0.01 > p > 0.005$); and choline incorporation into total lipid at 9 months of age ($0.01 > p > 0.005$).

TABLE 1

Phospholipid/Protein Ratio in Different Brain Areas of the Rat during Aging[a]

Brain areas	Lipid	Age of animals (months) 2	9	18	24
Cortex	Total	15.95 ± 2.95 (12)	15.76 ± 1.78 (7)	14.40 ± 2.83 (11)	15.97 ± 1.81 (10)
	CPG	4.85 ± 0.80 (12)	5.05 ± 0.78 (7)	4.50 ± 0.75 (11)	4.78 ± 1.35 (10)
	EPG	4.76 ± 0.56 (12)	4.42 ± 0.70 (7)	4.17 ± 0.48 (11)	4.91 ± 0.52 (10)
Striatum	Total	14.84 ± 2.83 (11)	17.08 ± 2.88 (8)	14.95 ± 1.30 (9)	16.90 ± 3.36 (10)
	CPG	4.85 ± 0.53 (10)	4.87 ± 0.73 (8)	4.68 ± 0.54 (9)	5.89 ± 1.46 (9)
	EGP	4.57 ± 0.48 (10)	4.85 ± 0.85 (7)	4.64 ± 0.91 (8)	5.69 ± 1.25 (9)
Rest	Total	18.72 ± 1.50 (11)	19.36 ± 2.35 (8)	18.45 ± 2.11 (9)	21.09 ± 1.39 (10)
	CPG	5.89 ± 0.83 (11)	5.67 ± 0.80 (8)	5.24 ± 0.80 (8)	6.00 ± 0.61 (10)
	EPG	6.94 ± 0.60 (11)	6.76 ± 1.31 (8)	6.70 ± 0.67 (8)	7.77 ± 0.82 (10)
Cerebellum	Total	15.81 ± 3.00 (12)	16.26 ± 2.65 (8)	15.48 ± 1.72 (11)	15.95 ± 2.41 (10)
	CPG	4.77 ± 0.91 (11)	5.06 ± 0.85 (8)	4.78 ± 0.74 (10)	5.02 ± 0.81 (9)
	EPG	4.98 ± 0.71 (11)	5.00 ± 0.78 (8)	5.16 ± 1.07 (10)	5.18 ± 1.17 (9)

[a]Data are represented as µg phospholipid P/mg protein ± SEM. Number of estimations is in parentheses.

TABLE 2

[^{3}H]/[^{14}C] Ratio of Choline and Ethanolamine Phosphoglycerides in Different Brain Areas of the Rat at Different Ages[a]

Brain areas	Age of the animals (months)							
	2		9		18		24	
	A	B	A	B	A	B	A	B
Cortex	4.21 ± 0.61	4.57 ± 0.62	3.68 ± 0.60	4.19 ± 1.19	4.14 ± 0.71	4.49 ± 0.20	4.33 ± 1.04	4.18 ± 0.51
Striatum	3.70 ± 0.49	5.08 ± 1.62	3.40 ± 0.92	3.40 ± 0.93	4.26 ± 0.74	4.73 ± 0.10	3.88 ± 0.47	4.27 ± 0.42
Rest	4.01 ± 0.54	3.05 ± 0.69	4.26 ± 0.33	2.83 ± 0.33	4.41 ± 0.66	3.25 ± 0.09	4.57 ± 0.26	3.15 ± 0.25
Cerebellum	3.48 ± 0.29	2.23 ± 0.32	3.47 ± 0.57	2.22 ± 0.20	3.85 ± 0.56	2.90 ± 0.72	3.64 ± 0.79	2.38 ± 0.25

[a]A = [2-^{3}H]Glycerol/[*Me*-^{14}C]choline ratio in choline phosphoglyceride fraction. Zero time ratio: 8.15. B = [2-^{3}H]Glycerol/[2-^{14}C]ethanolamine ratio in ethanolamine phosphoglyceride fraction. Zero time ratio: 9.60.

aging (data not shown), giving additional support and significance to the results of Table 2. (c) The level of the total radioactivity of ^{3}H- and ^{14}C-containing compounds (obtained by summing the lipid and water-soluble radioactivity content from the 4 regions studied and dividing by brain weight) is practically the same at all examined ages after 1 hr from the injection of precursors (about 4.5 μCi/g for [2-^{3}H]-glycerol, 1 μCi/g for [*Me*-^{14}C]choline and 1.5 μCi/g for [2-^{14}C]ethanolamine). On the contrary, the radioactive content of the lipid fraction in the 4 regions from either [2-^{3}H]-glycerol, [*Me*-^{14}C]choline or [2-^{14}C]ethanolamine significantly decreases with age (data not shown). These results indicate that aging does not affect the absorption process of water-soluble precursors injected into the lateral ventricle of brain, but rather affects their incorporation into lipid. (d) Finally, the areas which have been chosen possess similar activity in regard to the utilization of lipid precursors diffusing from the site of injection, as tested by the analysis of the percentage of labeling of total lipid over total recovered radioactivity in the different areas. The values have been found similar for all areas (for the 2-month-old rats, about 70% for [2-^{3}H]glycerol and 45% for [*Me*-^{14}C]choline utilization), with a little difference for [2-^{3}H]ethanolamine utilization (about 15% in cortex and striatum and 25% in the rest and cerebellum of the 2-month-old-rats). Because these levels of total radioactivity of the different areas are the same at all ages examined (see c), these percentages decrease with age according to the decreases found for sp act values (Figs. 1-4).

In our opinion, all these considerations give more significance to the hypothesis that the variations of sp act observed in the present work are caused by a decrease of the synthesizing activity of the areas examined.

It has been found previously (1-3) that aging produces a significant decrease of phospholipid synthesis in rat brain subcellular fraction in vitro. The decrease was essentially due to the neuronal fraction, because biosynthesis in glia is almost unaffected (3). We expected to find, therefore, with these experiments, the highest decrease of sp act in the brain areas containing the highest neuronal concentration. This explains the data of brain cortex and striatum (where the neuronal concentration is even higher than in cortex), but is in contrast with the data reported in the cerebellum (Fig. 4), where the neuronal concentration is also very high. To explain this discrepancy, we postulate that the biosynthetic activity of different neuronal cell types is differently affected by

aging. This hypothesis is supported in part by the particular neuronal composition of the cerebellum (10).

The decrease of the lipid synthesis is not further changed after 9 months of age. This result contrasts with previous observations (1-3) which indicated a longer time interval, up to 18 months of age or more, for further decreases of the rate of CPG and EPG synthesis in vitro in rat brain microsomes and neuronal cell types. This discrepancy is probably explained in terms of different incorporation rates into various cell types. More precisely, the decrease of neuronal activity may be masked in vivo by the unchanged synthesis in other cell types, so that we observe after 9 months of age only an average of different contributions.

Aging processes affect the degree of incorporation of simple water-soluble precursors (glycerol, nitrogen bases) into brain lipids. The decrease is similar for both precursors in all brain areas, indicating a comparable loss of utilization for both glycerol and bases. This suggests that aging affects the rate of assembly mechanism of both the hydrophobic tail and hydrophilic head of phospholipids rather than other biosynthetic steps or interconversion reaction (11). This hypothesis agrees with previous findings (1-3) which indicated the inhibition of choline and ethanolamine phosphotransferases as the more probable cause for the decrease of CPG and EPG synthesis.

ACKNOWLEDGMENTS

This work was supported by a grant from the Consiglio Nazionale delle Ricerche, Rome (contribution no. 80.00543.04/115). Technical assistance by G. Marinacci and E. Giovagnoli is gratefully acknowledged.

REFERENCES

1. Brunetti, M., Gaiti, A., and Porcellati, G. (1979) Lipids 14, 925-931.
2. Gaiti, A., Brunetti, M., and Porcellati, G. (1979) Bull. Mol. Biol. Med. 4, 24-36.
3. Gaiti, A., Sitkiewicz, D., Brunetti, M., and Porcellati, G. (1981) Neurochem. Res. 6, 11-20.
4. Calderini, G., Aldinio, C., Crews, F., Gaiti, A., Scapagnini, V., Algeri, S., Ponzio, F., and Toffano, G. (1981) in Apomorphine and other Dopaminomimetics, Clinical Pharmacology (Corsini, G.U., and Gessa, G.L., eds.) Vol. 2, pp. 235-242, Raven Press, New York, NY.
5. Arienti, G., Corazzi, L., Woelk, H., and Porcellati, G. (1976) J. Neurochem. 27, 203-210.
6. Lowry, O.H., Rosebrough, M.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
7. Binaglia, L., Roberti, R., Michal, G., and Porcellati, G. (1973) Int. J. Biochem. 4, 577-611.
8. Goracci, G., Francescangeli, E., Piccinin, G.L., Binaglia, L., Woelk, H., and Porcellati, G. (1975) J. Neurochem. 24, 1181-1186.
9. Francescangeli, E., Goracci, G., Piccinin, G.L., Mozzi, R., Woelk, H., and Porcellati, G. (1977) J. Neurochem. 28, 171-176.
10. Gombos, G., Ghandour, M.S., Vitiello, F., Longley, O.K., Zanetta, J.P., and Vincedon, G. (1980) in Multidisciplinary Approach to Brain Development (Di Benedetta, C., Balazs, R., Gombos, G., and Porcellati, G., eds.) pp. 15-28, Elsevier/North-Holland,Amsterdam.
11. Porcellati, G. (1981) Trans. Am. Soc.Neurochem. 12, 191.

[Received June 3, 1981]

Prostaglandin and Acyl Chain Effects on Glutamate Dehydrogenase Activity

PAUL T. SHAFER and **A. MAX FISKIN**, *Medical Research Service, Veterans Administration Medical Center, 4801 Linwood Boulevard, Kansas City, MO 64128*

ABSTRACT

Prostaglandins A_1 (PGA_1), A_2, B_1, B_2, E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, and 19 esterified natural fatty acids were tested as effectors of beef liver glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase [deaminating], EC 1.4.1.3). All prostaglandins tested are found to activate the enzyme initially, but only $PGA_2 > PGB_2 \geq PGA_1$ cause a subsequent time-dependent loss (not inhibition) of NADH oxidation activity. Both PGA_1 and PGA_2 desensitize glutamate dehydrogenase to allosteric activation by ADP, whereas PGA_2 and PGB_2 desensitize to allosteric inactivation by GTP. Preincubation of enzyme with diethylstilbestrol prevents the initial activation by the PG. Of the methyl esters, only prostaglandin precursors inactivated the enzyme. Simultaneous desensitization to the ADP and GTP allosteric effects resulted. Multiple esterification to glycerol or phospholipids enhanced the action of linoleoyl and diminished the action of linolenoyl chains. Preincubation of the PGA with glutathione or cysteine prevents the inactivation; i.e., the sulfhydryl binding region of the prostaglandin must be free for enzyme to be inactivated. Sulfhydryl reagents also protect the enzyme from the effects of the unsaturated acyl chains, and pHMB mimics acyl protection against GTP allosteric inactivation. Where the lipid effector is active against sulfhydryl groups, the desensitizations to the ADP and GTP allosteric effectors are reciprocal. The initial activation, subsequent inactivation and desensitization to ADP and GTP are all characteristic of binding in the estrogen-specific effector site, suggesting this site as the target for PG and acyl action. In the PGA_2 activation, the effect is found to be amplified by the cooperativity of the enzyme at 1 PG molecule/6 molecules of GDH. We conclude from the action of the PG and structural analogs that the initial activation of glutamate dehydrogenase is caused by α,β-unsaturated monoketo cyclopental structures. GTP inhibition is blocked primarily by diketo structures which eventually inactivate the enzyme. ADP activation is blocked by sulfhydryl binding of the unsaturated cyclopental keto structure of the PG. Appearance of a 270 nm absorbance simultaneous to the acyl effects on the enzyme suggests that conjugated unsaturations are responsible for the precursor's qualitatively similar action to that of the PG.

Lipids 17:297-306, 1982.

INTRODUCTION

Glutamate dehydrogenase (GDH) activity is affected by such a wide variety of substances, including purines and steroids, that LeJohn et al. (1) concluded GDH to be a sensitive target for the evolution of regulatory mechanisms. As such, it might be considered a receptor for regulatory messengers.

In higher eukaryotic organisms, the enzyme is reversibly bound to the mitochondrial inner membrane, evidently via formation of enzymatically inactive complexes with the phospholipid cardiolipin (CL) (2). However, the enzyme is reportedly assembled in the cytoplasm (3), while bound to endoplasmic reticulum via phosphatidylserine. Dodd (2) has presented nuclear magnetic resonance (NMR) data implicating both head groups and acyl chains in the binding of these phospholipids to GDH. Godinot observed that an inhibitory effect of CL on the enzyme was dependent on the unsaturation in the acyl chains, and was lost if the acyl chains were removed (4). We have encountered problems in preparing complexes of GDH bound to monolayers (for studies relating structure and function) that require a detailed understanding of the role of acyl chains in the formation of lipid-GDH complexes. Exploratory studies indicated that prostaglandin precursors altered GDH function significantly.

Johnson and Ramwell (5) provided evidence that PGE_1 or PGE_2 acts on a phospholipid-protein complex to viscotropically regulate membrane-associated enzymes, pointing to the possibility that GDH may be affected by prostaglandins. If so, the solubility of the prostaglandins could provide an interpretative advantage for exploring the stereochemical requirements for phospholipid effectors of the enzyme. Most prostaglandin synthesis takes place in the endoplasmic reticulum (6). Possibly only PGD_2 is produced in mitochondria (7), but the enzyme may be exposed to prostaglandins during both assembly and transport into the organelle.

The competitive binding experiments of Attallah and Lee (8) suggest the existence of specific binding sites in the rabbit kidney for PGA_1. It has been proposed that Na^+-K^+ ATPase acts as a PGA_2 receptor, and a PGE_1 receptor has been reported in the plasma membranes of rat liver cells (9). The PGE_1

receptor of bovine corpus luteum cell membrane is a phospholipid-requiring protein (10). Modification of specific residues indicates that tyrosine, but not sulfhydryl, groups are involved in the binding. The PGE_1 receptor of lipocyte suspensions (11) is inactivated by trypsin, *p*-chloromercuribenzoate or heat. The authors concluded the receptor to be a sulfhydryl-containing lipoprotein. Contractions induced by prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ in excised rat uterus and gerbil colon can be prevented by prior treatment of the organ with dithiothreitol (12), again indicating the possible involvement of a sulfhydryl group at prostaglandin receptor sites.

We therefore studied the action of 8 commercially available prostaglandins and 19 esterified natural fatty acids on the enzyme activity of GDH, and we additionally tested the hypothesis that lipid effectors bind the enzyme through a sulfhydryl group.

The concentration of GDH within the mitochondrial matrix is at least 2 mg/ml (13), an extraordinary level for an enzyme. Our use of GDH at biologically relevant concentrations often predisposed our use of PG, as well, at extraordinary levels.

MATERIALS AND METHODS

Prostaglandins A_1, A_2, B_1, B_2, E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ were obtained from Sigma Chemical Co., St. Louis, MO. Triethanolamine hydrochloride, reduced glutathione (GSH), DL-dithiothreitol (DTT), L-cysteine (CYS), *p*-hydroxymercuribenzoate (pHMB), reduced β-nicotinamide adenine dinucleotide (NADH) and dinucloetide phosphate (NAPDH), adenosine 5′-diphosphate (sodium salt) (ADP), guanosine 5′-triphosphate (GTP), and diethylstilbestrol (DES), N-ethylmaleimide (NEM), 4-cyclopentene-1,3-dione, 2-cyclopentenone and cyclopentanone were also obtained from Sigma. Malonaldehyde bis(dimethyl acetal) was obtained from Aldrich Chemical Co., Milwaukee, WI. It was distilled once then converted to malonaldehyde by the method of Kwon and Watts (14). Sepharose CL-43 was obtained from Pharmacia, Uppsala, Sweden. Bovine liver glutamate dehydrogenase (GDH) purified by passage over activated charcoal–at about 15 mg/ml in 100 mM potassium phosphate buffer (pH 7.6)–was obtained from Dr. H.F. Fisher, VA Medical Center, Kansas City, MO. It was dialyzed before use against 75 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 100 μm sodium azide. All other materials were of reagent grade, and solutions were prepared with deionized water doubly redistilled in a quartz apparatus.

L-α-Dipentadecanoyl, L-α-dihepatadecanoyl, L-α-dioleoyl and L-α-dilinoyl phosphatidylcholine were obtained from Supelco, Bellefonte, PA. L-α-Dilauroyl, L-α-dimyristoyl, L-α-dipalmitoyl, L-α-distearoyl and L-α-1-oleoyl-2-palmitoylphosphatidylcholine were obtained from Sigma. L-α-Dimyristoyl, L-α-dioleoyl, L-α-dilinoleoyl, L-α-dilinolenoyl, L-α-1-palmitoyl-2-palmitoleoyl, and L-α-I-steryl-2-oleoylphosphatidylcholine were obtained from Applied Science Laboratories, State College, PA.

L-α-Dioleoylphosphatidylethanolamine was obtained from Sigma, and L-α-disteroylphosphatidylethanolamine was obtained from Applied Science Laboratories.

1,3-Diolein, 1,2-diolein, trimyristin, triolein, trilinolein, trilinolenin and trinervonin were obtained from Sigma.

Methyl erucate, methyl docosahexaenoate, methyl nervonate and methyl 12-methyltetradecanoate were obtained from Supelco. Methyl myristate, methyl myristoleate, methyl palmitoleate, methyl oleate, methyl elaidiate, methyl vaccenate, methyl petroselenate, methyl ricinoleate, methyl DL-12-hydroxystearate, methyl linoleate, methyl linoelaidiate, methyl α-linolenate, methyl γ-linolenate, methyl eicosanoate, and methyl docosahexaenoate were obtained from Sigma. Methyl α-linolenate, methyl arachidonate and methyl docosahexaenoate were obtained from Applied Science Laboratories.

Linoleic acid, linolenic acid, arachidonic acid and docosahexaenoic acid were obtained from Applied Science Laboratories.

Each of the prostaglandins was dissolved in 75 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 100 μM sodium azide and purged with N_2. Interaction between GDH and the PG was done in the same buffer, in the dark and under N_2 (to reduce peroxidation), using 500 μg/ml GDH and from 30 ng/ml to 1 mg/ml PG or 1 mg/ml of all other lipids. GDH was assayed for enzymatic activity as reported by Arnold and Maier (15) using 10 μl of enzyme-containing solution and 3 ml assay solution. Oxidation of NADH was monitored spectroscopically at 340 nm, and the slope of the resulting trace between 15 and 45 sec after mixing was used to determine activity.

Purity determinations and purification of phospholipids were done on Silica Gel G thin layer chromatography (TLC) plates by elution with chloroform/methanol/acetic acid/acetone/water (10:2:2:4:1, v/v/v/v). Purity determinations of fatty acids and their esters, diglycerides and triglycerides were carried out on Silica Gel G 15% silver nitrate thin layer plates by elution

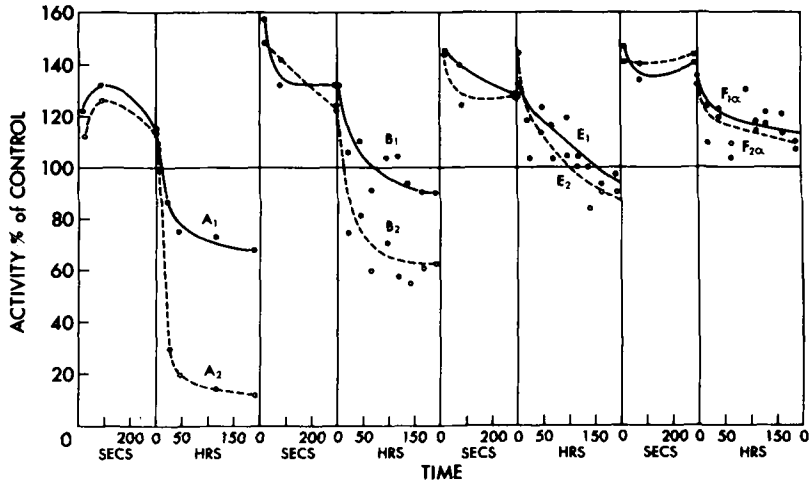


FIG. 1. Glutamate dehydrogenase activity (reverse, or NADH oxidation reaction) following addition of 500 μg of enzyme to 1 mg of prostaglandin in 1 ml of 75 mM potassium phosphate (pH 7.5) at 25 C. Ten μl of the mixture was added to 3 ml of assay solution, and enzyme activity was determined from the disappearance of NADH which was monitored at 340 nM between 20 and 45 sec after the mixing of enzyme with the prostaglandin.

with hexane/diethyl ether (2:1, v/v). Plates were developed with iodine vapor or osmium tetroxide vapor (for unsaturated acyl chains), then with 50% sulfuric acid saturated with potassium chromate and heated (for saturated acyl chains). Phospray (Supelco) was applied to visualize phospholipids.

Each of the lipids was sonicated from the solvent-free state into 75 mM phosphate buffer (pH 7.5) containing 1 mM EDTA and 100 μM sodium azide at 4 C and under N_2 immediately before use.

Extraction of lipid from GDH was done with 2 vol of chloroform/methanol (2:1, v/v) to 1 vol of aq sample. After 1 hr of agitation, the solvent was removed and washed with 1 vol of water.

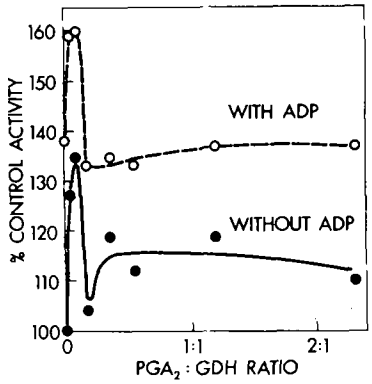


FIG. 2. Comparison of GDH activity when assayed with and without 2 mM ADP 30 sec after addition of PGA_2 at 360 nM to 19 μM levels. Ratios are given as PGA_2 molecules to GDH 56,000 Dalton subunit.

RESULTS

The enzyme activity of GDH was tested beginning 20 sec after mixing with prostaglandin. Figure 1 shows that, in all cases, GDH activity was initially elevated above the controls with no added prostaglandin. Subsequently, a time-dependent loss of activity was elicited by PGA_1, A_2, and B_2, but not by PGE_1, $F_{1\alpha}$, $F_{2\alpha}$, or B_1. A slight elevation of activity by $PGF_{1\alpha}$ and $F_{2\alpha}$ was sustained for 200 hr. The GDH control samples showed no decline in activity during this period. In all cases, the more unsaturated PG yielded the least active combination, but only for the PGA and B does the difference appear significant. The extent of these differences varied slightly with lot, but both the rate and extent of inactivation was greatest for $PGA_2 > B_2 \simeq A_1$.

The loss of enzyme activity decreased with the concentration of the PGA, but is still significant at 1.5 μM (22% loss in both cases).

Figure 2 shows the effect of PGA_2 (at 360 nM to 19 μM levels) on the reverse reaction activity of GDH for different ratios of the 2 components. The large activation at 0.17:1, which represents a stoichiometry of 1:6 between the PGA_2 and GDH 56,000 molecular weight monomer molecules, respectively, appeared initially and was not found after 24 hr.

These results suggest a cooperative effect relating to an attack on one member of the GDH heximeric structure, thus modifying the activity of all 6 members. It is notable that PGA_2 did not block the ADP allosteric activation of the enzyme at the short time of this experiment. However, the initial activation and that which was sustained in the presence of the PGF was prevented by preincubating the enzyme for 1 hr with 5 mg of DES/mg of GDH before addition of the prostaglandin.

Figure 3a, b and c, show the results of tests for inactivation of GDH by lipids. Figure 3a summarizes that, out of a number of methyl esters of naturally occurring fatty acids, only highly unsaturated chains or prostaglandin precursors inactivate the enzyme, but that the most highly unsaturated methyl docosahexaenoate has only a minor effect.

In Figure 3b, we can see that when acyl chains are combined by mutual attachment to a glycerol group, the more unsaturated chains are slightly diminished as inactivators, but as part of trilinolein there is a significant enhancement of the action of the linoleoyl chain against the enzyme. When phospholipids, which are diacylated with like chains, were tested for inactivation of GDH (Fig. 3c), those with the linolenyl chains were ineffective whereas those with linoleoyl chains were active, initially activating the enzyme, but subsequently inactivating it incompletely. If the acyl chains have a specific effect on the enzyme, the presence of the glyceryl or phosphorylcholine appears to be able to modify that effect.

To test possible sulfhydryl involvement,

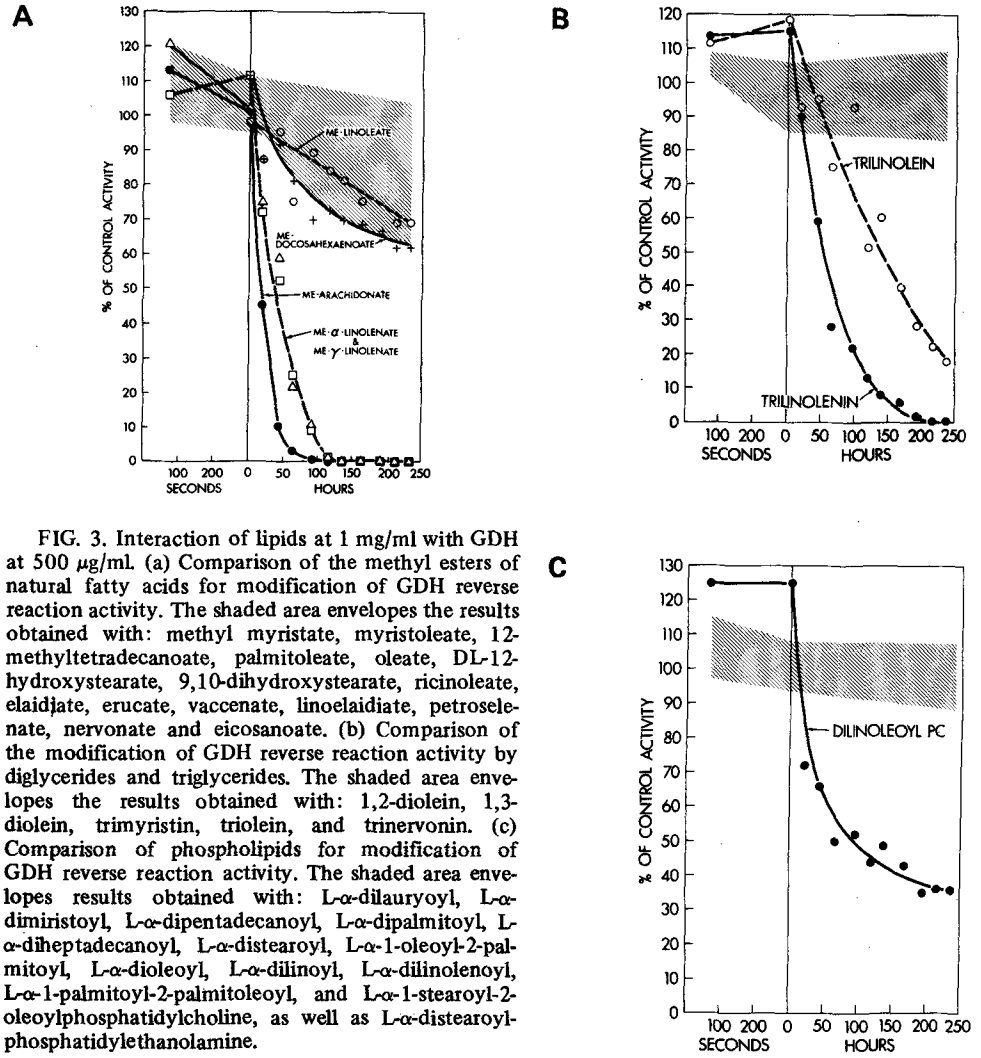


FIG. 3. Interaction of lipids at 1 mg/ml with GDH at 500 μg/ml. (a) Comparison of the methyl esters of natural fatty acids for modification of GDH reverse reaction activity. The shaded area envelopes the results obtained with: methyl myristate, myristoleate, 12-methyltetradecanoate, palmitoleate, oleate, DL-12-hydroxystearate, 9,10-dihydroxystearate, ricinoleate, elaidjate, erucate, vaccenate, linoelaidiate, petroselenate, nervonate and eicosanoate. (b) Comparison of the modification of GDH reverse reaction activity by diglycerides and triglycerides. The shaded area envelopes the results obtained with: 1,2-diolein, 1,3-diolein, trimyristin, triolein, and trinervonin. (c) Comparison of phospholipids for modification of GDH reverse reaction activity. The shaded area envelopes results obtained with: L-α-dilauryoyl, L-α-dimiristoyl, L-α-dipentadecanoyl, L-α-dipalmitoyl, L-α-diheptadecanoyl, L-α-distearoyl, L-α-1-oleoyl-2-palmitoyl, L-α-dioleoyl, L-α-dilinoyl, L-α-dilinolenoyl, L-α-1-palmitoyl-2-palmitoleoyl, and L-α-1-stearoyl-2-oleoylphosphatidylcholine, as well as L-α-distearoylphosphatidylethanolamine.

each of the PGA was premixed for 1 hr with 0.5 mg/ml of each of the sulfhydryl-containing reagents, glutathione, cysteine and dithiothreitol. GDH was added to each of these samples as well as to PGA (control) samples without the protective sulfhydryl reagent. Background samples containing only GDH were also prepared. The protection afforded by pretreatment of the PGA is represented as the percentage of the prostaglandin-free (background) GDH activity in Figure 4. The percentage of activity preserved in the presence of PGA_2 was generally higher than that in the presence of PGA_1. Glutathione and cysteine prevented inactivation more effectively than did dithiothreitol. When preincubated for 1 hr with 2 mg/ml glutathione, all prostaglandins produced an increase in the activity of the enzyme at 30 sec after addition which was subsequently sustained for more than 43 hr.

The sulfhydryl reagent N-ethylmaleimide has all the characteristics of PGA_2 action on the enzyme except the initial activation. It appears from Table 1 that the α,β-unsaturated keto group on the cyclopentane ring is required for the initial activation. The slow inactivation of GDH that parallels the blockage of GTP inhibition may be the result of the conformation change associated with GTP site binding (16).

A sample of GDH was incubated for 1 hr with pHMB, an agent known to block sulfhydryl groups on this enzyme, and to inhibit GDH activity (17). Equal aliquots were then added to samples containing either prostaglandins or buffer only. Figure 5 traces the percentage activity of the PGA-containing samples with respect to the buffer sample over a period of 166 hr. Two features of these data are notable: the pHMB-treated enzyme was further inactivated by PG, to the same extent as untreated enzyme, i.e., pHMB and PG effects are additive; and the initial activation by PG is evident for the pHMB-treated enzyme.

ADP is normally an activator of GDH in the reverse reaction used in the present work to test enzyme activity. It was present at 2 mM in all routine assays of the enzyme. A duplicate set of assays was done without ADP during inactivation of the enzyme by the PGA. Interestingly, as shown in Figure 6, ADP appears to

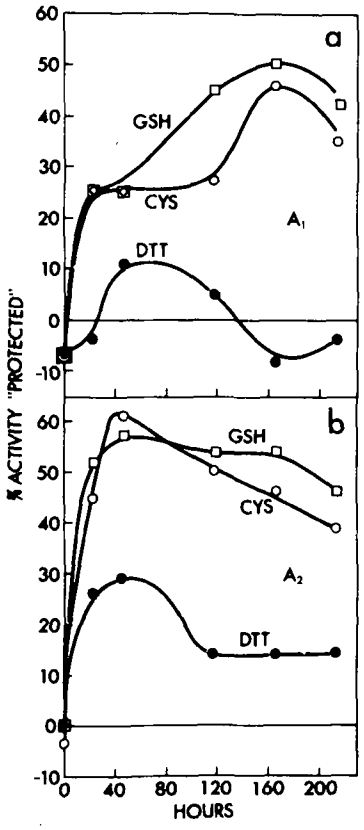


FIG. 4. Preservation of glutamate dehydrogenase activity in the presence of prostaglandins A_1 and A_2 by 500 μg/ml sulfhydryl reagents. DTT, CYS, GSH and GDH were at 500 μg/ml. Data calculated from differences in percentage of control activity between samples with GDH and PGA, and those with GDH, PGA and sulfhydryl reagent.

TABLE 1

Effect on GDH Reverse Reaction Activity of Cyclopental Keto Structure and Malonaldehyde in the 0.3-3 mM Concentration Range

Substance	Causes initial activation	Inactivates enzyme	Blocks ADP activation	Blocks GTP inhibition
PGA_2	+	+	+	+
N-Ethylmaleimide	-	+	+	+
Malonaldehyde	-	+	-	+
4-Cyclopentene-1,3-dione	-	+	-	+
2-Cyclopentenone	+	-	-	-
Cyclopentanone	-	-	-	-

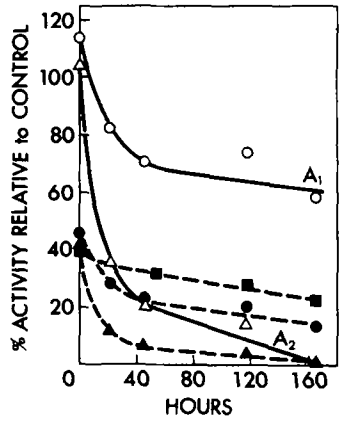


FIG. 5. The time-dependent loss of activity with pHMB + PGA_1 (○) and pHMB + PGA_2 (△) is shown in solid lines as percentage activity relative to control (pHMB only, ■), and relative to untreated enzyme (solid points, dashed lines).

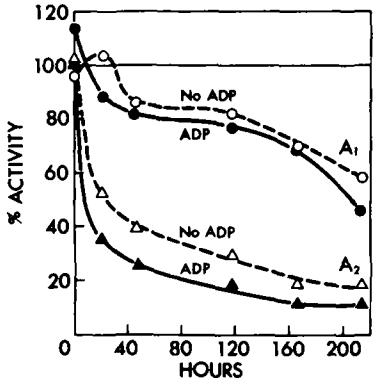


FIG. 6. Comparison of the activity of PGA incubated glutamate dehydrogenase in assay mixtures with and without 2 mM ADP.

have no effect, if not a slight inhibitory action, on the enzyme when PGA are present.

Figure 7 shows a replication of the experiment in Figure 6 but with 20 μg/ml pHMB present in place of the PGA. The inactivation of the GDH is not unlike that caused by PGA_2. It was initially rapid, but stopped short of complete inactivation. The inhibitory effect of ADP in the enzyme assay was more pronounced than that caused by the PGA.

When the products of incubation of GDH with each of the prostaglandins for 45 hr were assayed for enzyme activity in the presence of 500 μM GTP (enough to fully inactivate the enzyme), the combination with PGA_2 was not completely inhibited. The results shown in Table 2 indicate that protection against GTP

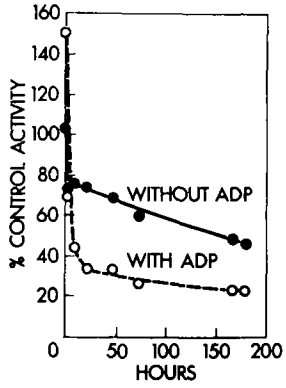


FIG. 7. Comparison of the activity of GDH assayed with and without 2 mM ADP upon incubation with 20 μg/ml pHMB.

inhibition corresponds to the loss of activity caused by the prostaglandins.

Figure 8 demonstrates a similar reciprocal relationship between the ADP and GTP allosteric effects when GDH (calculated as the 56,000 molecular weight subunit) has been incubated with increasing ratios of PGA_2 for 192 hr.

Table 1 compares these reciprocal changes in the ADP and GTP allosteric effects caused by chemical structures related to the cyclopental ring of PGA_2. Except for PGA_2, only diketo compounds block GTP inhibition. Because short term initial activation is caused by PGA_2 and 2-cyclopentenone, it appears that the α,β-unsaturated cyclopental keto group is involved.

Figure 9 shows the protection afforded GDH by the sulfhydryl reagent dithiothreitol suggesting sulfhydryl involvement as a common element to the inactivation of the enzyme by unsaturated-acyl lipids. As there are no sulfhydryl binding groups on any of the lipids, the dithiothreitol must either act on the enzyme or on a modified form of each lipid. Additionally, both cysteine and reduced glutathione offered protection (not shown).

As the arachidonate and linolenate increasingly protect the enzyme against inhibition by GTP, the NADH oxidation activity is simultaneously decreased. Figure 10 compares this relationship for methyl linolenate and methyl arachidonate with the sulfhydryl binding pHMB and PGA_2. pHMB inactivated the enzyme more rapidly than the lipids, and only a small amount of protection against GTP inhibition was achieved. The pHMB effect paralleled the lower (initial) part of the curves for acyl chains, but failed to offer the progressively increasing protection relative to inhi-

TABLE 2

Comparison of Inhibition by Prostaglandins and Subsequent Inhibition by GTP of GDH Reverse Reaction Activity after 43 hr of Incubation with the Prostaglandins

PG	% of Control activity remaining	% Inhibition of remaining activity by GTP
A_2	21	-3
B_2	34	33
A_1	71	90
B_1	92	97
$F_{2\alpha}$	111	95
E_1	113	100
$F_{1\alpha}$	118	100
E_2	124	100

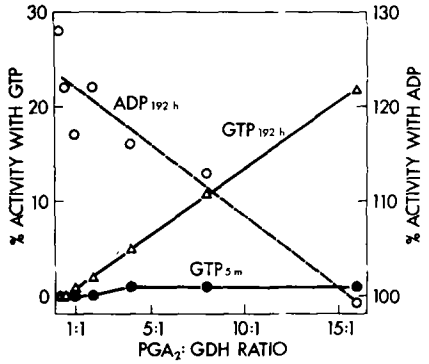


FIG. 8. Comparison of GDH reverse reaction activity assayed in the presence of 2 mM ADP or 100 μm GTP at 5 min and 192 hr after addition of PGA_2 at 3 μM to 300 μM. Ratios are given as PGA_2 molecules to GDH 65,000 Dalton subunit.

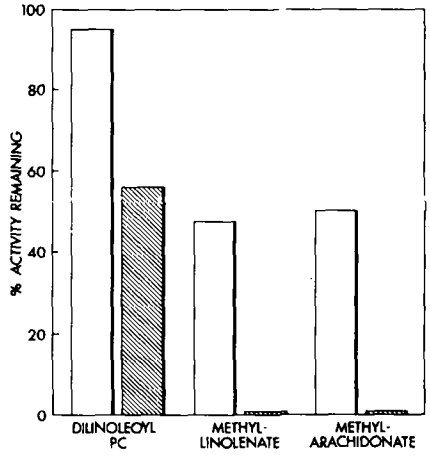


FIG. 9. Diethiothreitol protection of GDH against inactivation by lipids. Dilinoeoyl phosphatidylcholine at 143 hr with 1.2 mM DTT, methyl linolenate at 115 hr with 3 mM DTT, and methyl arachidonate at 115 hr with 3 mM DTT.

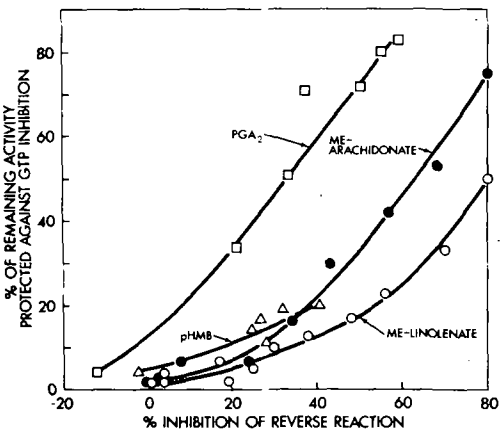


FIG. 10. Comparison of methyl arachidonate (3.1 mM) and methyl linolenate (3.4 mM) with PGA_2 (3 mM) and pHMB (5.5 μM) for protection of the remaining enzyme activity against GTP inactivation.

bition. Gel filtration of glutamate dehydrogenase partially inactivated by methyl arachidonate yields separate, well resolved bands of the lipid and of enzyme for which activity is almost fully restored, but not the sensitivity to ADP or GTP.

Chloroform/methanol extraction of mixtures in which GDH has been 77% inactivated by methyl arachidonate yields an ethanol-soluble residue. When its UV absorption is compared to that of extract from a like sample protected with cysteine (Fig. 11), a significant difference is found at 270 nM. Comparison of the protected sample extracts with the extracts of control samples, which contained only the methyl arachidonate, yielded the same difference at 270 nm. Comparison of extracts of GDH with GDH plus cysteine showed no difference in absorption between 230 and 300 nM. These results indicate that the modification which took place in the lipid was not caused

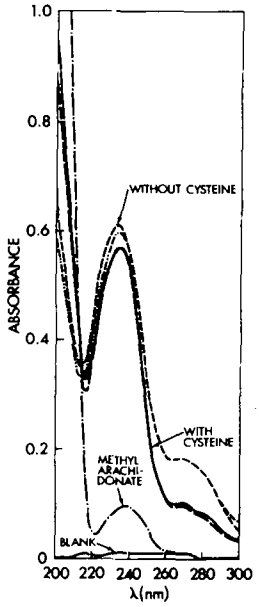


FIG. 11. UV difference spectrum of chloroform/methanol (2:1) extracted methyl arachidonate inactivated (77%) GDH and duplicate samples protected with 2 mg/ml cysteine (6% inactivated). Chloroform/methanol extracts were washed once with an equal volume of water then blown dry with N_2. The residues were redissolved in ethanol for spectral analysis.

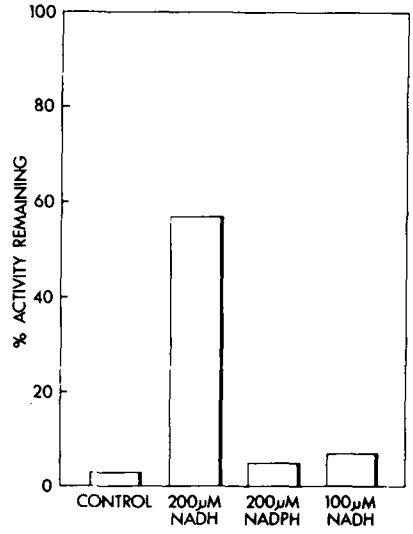


FIG. 12. Effect of preincubation of GDH with different concentrations of NADH and NADPH 1 hr prior to and during 141 hr of incubation with methyl arachidonate.

by the enzyme. Additionally, when the pH values of the extracts were reduced to below pH 3, no change in the absorbance spectrum took place as would be expected for malonaldehyde.

The results of adding NADH at inhibitory and noninhibitory levels to the enzyme 1 hr before addition of methyl arachidonate are presented in Figure 12. The presence of NADH at inhibitory levels (18) prevented much of the arachidonate inhibition, but NADPH at the same concentration was not inhibitory and did not offer any significant protection. The implication is that occupation of the secondary binding site for NADH where it inhibits the enzyme also blocks the attack by methyl arachidonate. Similar preaddition of ADP was found to protect the enzyme from arachidonate inactivation, as well (not shown). Both ADP and the NADH acting as inhibitor share, in part, a common site (19).

DISCUSSION

GDH has 6 sulfhydryl groups per primary chain of 56,000 molecular weight (19). It does not have an essential sulfhydryl group in the active site, and it has no disulfide bonds. However, it is conceivable that lipid effectors binding to sulfhydryl groups associated with the catalytic or allosteric sites could cause the observed inactivation of the enzyme. The proximity of a nonessential cysteine to an enzyme active site has been found to allow the binding of agents which regulate enzyme activity through hindering enzyme conformational changes (20), sterically restricting the size of substrates (21), or by extending into the active site (22).

It has been demonstrated that PGA_1 and A_2 but not PGB_1, E_2, or $F_{2\alpha}$ are capable of reacting covalently with the sulfhydryl group of reduced glutathione (23). PGA_1 also binds cysteine but not DTT. PGA_1 is both enzymatically as well as nonenzymatically conjugated to the sulfhydryl group of GSH through the double bond of its cyclopentenone moiety (24). This Michael-type addition may occur generally between the double bonds of α,β-unsaturated keto groups and the sulfhydryl groups of GSH (25). The nonenzymatic adduct of PGA_1 (at 1.2 mg/ml) with GSH (at 6 mg/ml) at pH 7.4 has been shown to be identical to the products of their conjugation by red cells (PGA_1 at 100 μg/ml, pH 7.4) (26). We have found that inactivation of GDH is associated with the presence of cyclopentenone moieties in the A_1, A_2 and B_2 prostaglandins. We interpret this to be an indication that the inactivation involves covalent linkage with sulfhydryl groups on the enzyme, which can be prevented by pretreating PGA with GSH or cysteine. However, 2-cyclopentenone does

not cause inactivation. It is, therefore, likely that other parts of the PGA and PGB are also involved in the activation.

PGB_1 has a cyclopentenone moiety, but it had no appreciable effect in reducing the activity of GDH. Chaudhari et al. (25) similarly found that PGA_1, but not PGB_1 serves as a substrate for GSH S-transferase. The differences were suggested to be their dissimilar 3-dimensional structures.

GDH inhibited by noncovalently bound steroid hormones is reactivated by ADP (17), but it is desensitized to ADP when covalent binding to cysteine-89 (residue 89) in the estrogen-specific site is involved (27). ADP in the ADP regulatory site can also exclude GTP from the separate GTP regulatory site (17). The DES-preventable initial activation that we found was caused by all of the tested PG, but the subsequent decline in GDH activity resulted from the presence of only PG that are expected to bind sulfhydryl groups. Activation of GDH by PG could be similar in mechanism to that produced by ADP. Methyl mercuric hydroxide, which activates the enzyme and desensitizes it to the inhibition by GTP and activation by ADP, is suggested to stabilize the enzyme conformation induced by ADP (17). Because GTP inhibition is prevented by the sulfhydryl-binding prostaglandins, it is possible that the subsequent inactivation also involves the reactive cysteine at the estrogen-specific site and the stabilization of the same conformation.

Methyl mercuric derivatives and pHMB desensitize GDH to DES and high-concentration NADH inhibition, as well as to ADP activation (17). Methyl mercuric iodide has been found to bind one specific -SH group on GDH up to a molar ratio of 1:1 with the enzyme primary chains, causing an increase in glutamate catalytic activity. Further binding of methyl mercuric iodide causes a decrease in activity (28).

Methyl mercuric hydroxide also increases GDH activity in the forward (NAD) reaction maximally when one equivalent mercurial is bound per primary chain (29). It blocks inactivation by DES, raises the concentration of NADH required to inhibit GDH and, at increased concentration, reduces the ADP activation to the point at which ADP inhibition takes place. As our assays were performed using NADH concentrations at which it would be inhibitory (30), it is possible that at least part of the initial increase in activity caused by the PGA is a reduction of NADH inhibition.

Both the additive effects and the similarities of modification produced by pHMB and the PGA suggests them as effectors for the steroid binding site.

It appears that all of the PG tested can enter the estrogen-specific binding site and activate the enzyme, but that only PGA_1, PGA_2 and PGB_2 can interact with a cysteine residue at the site to inactivate GDH.

The modification of the reverse reaction enzyme activity of GDH by PGA_2 at a stoichiomeric ratio of 1 PGA_2/6 GDH molecules (56,000 molecular weight) indicates the remarkable power of the prostaglandin, and suggests that an enzyme conformational change is involved.

The modification of the reverse (NADH) reaction of glutamate dehydrogenase, at low oxygen tension, by esterified acyl chains of biological significance closely parallels those caused by prostaglandin A_1, A_2 and B_2. Arachidonate and linolenate, the precursors to the prostaglandins, were more effective than linoleate, and their inhibitory effect also appears to involve sulfhydryl binding.

Incubation of methy linoleate with glutamate dehydrogenase yields products with absorption maxima at 232 nM and 277 nM in ethanol, indicating that conjugation by peroxidation and formation of conjugated dienone has occurred (31-36). Yet we found that methyl linoleate did not inactivate GDH. Both ethyl linoleate and ethyl linolenate produce the conjugated diene and dienone chromophores upon autoxidation (37).

The differences in the isomerism of the conjugated dienone structures formed from methyl linoleate and methyl linolenate appear critical in our elucidation of the action of only the methyl linolenate on glutamate dehydrogenase. Linolenate differs from linoleate by additionally forming hydroperoxides at the 12- and 13-positions, internal to the methylene interrupted double bond system, with *cis-cis* conjugates being formed (38). Neither would allow the resulting chain to fold or bend upon itself in the manner of a prostaglandin as well as the *cis-cis* conjugated structures. It is possible, therefore, that prostaglandin might have evolved as a more stable (covalently folded), more reactive (strained 5 member ring, e.g., cyclopentene is more reactive than cyclohexene [39]) substitute in higher yield for a substance already spontaneously formed in low yield from linolenate and arachidonate.

ACKNOWLEDGMENT

This work was supported in part by the Veteran's Administration.

REFERENCES

1. Lejohn, H.B., Stevenson, R.M., and Meuser, R.

(1970) J. Biol. Chem. 245, 5569-5576.
2. Dodd, G.H. (1973) Eur. J. Biochem. 33, 418-427.
3. Godinot, C., and Lardy, H.A. (1973) Biochemistry 12, 2051-2060.
4. Godinot, C. (1979) Biochemistry 12, 4029-4032.
5. Johnson, M., and Ramwell, P.W. (1973) Prostaglandins 3, 703-719.
6. Kingston, W.P., and Graves, H.W. (1976) Prostaglandins 12, 51-69.
7. Parkes, D.G., and Eling, T.E. (1974) Biochemistry 13, 2598-2604.
8. Attallah, A.A., and Lee, J.B. (1973) Prostaglandins 4, 703-709.
9. Smigel, M., and Fleischer, S. (1973) Fed. Proc. 32, 454.
10. Rao, C.V. (1974) J. Biol. Chem. 249, 7203-7209.
11. Kuehl, F.A., Humes, J.L., Ham, E.A., and Cirillo, V.J. (1972) Intra-Sci. Chem. Rep. 6, 85-95.
12. Johnson, M., Jessup, R., and Ramwell, P.W. (1974) Prostaglandins 5, 125-136.
13. Nemat-Gorgani, M., and Dodd, G. (1977) Eur. J. Biochem. 74, 139-147.
14. Kwon, T., and Watts, B.M. (1963) J. Food Sci. 28, 627-630.
15. Arnold, H., and Maier, K.P. (1971) Biochim. Biophys. Acta 251, 133-140.
16. Huang, C., and Frieden, C. (1969) Proc. Natl. Acad. Sci. 64, 338-344.
17. Goldin, B.R., and Frieden, C. (1971) Curr. Top. Cell Regul. 4, 77-117.
18. Pal, P.K., and Colman, R.F. (1976) Eur. J. Biochem. 68, 437-443.
19. Eisenberg, H., Josephs, R., and Reisler, E. (1976) Adv. Protein Chem. 30, 101-181.
20. Duggan, D.E., and Noll, R.M. (1965) Arch. Biochem. Biophys. 109, 388-396.
21. Singer, T.P. (1948) J. Biol. Chem. 174, 11-21.
22. Holbrook, J.J., Liljas, A., Steindel, S.J., and Rossmann, M.G. (1975) in The Enzymes II (Boyer, P.D., ed.) Part A, p. 258, Academic Press, New York, NY.
23. Ham, E.A., Oien, H.G., Ulm, E.H., and Kuehl, F.A., Jr. (1975) Prostaglandins 10, 217-229.
24. Cagen, L.M., Pisano, J.J., Ketley, J.N., Habig, W.H., and Jakoby, W.B. (1975) Biochim. Biophys. Acta 398, 205-208.
25. Chaudhari, A.,Anderson, M.W., and Eling, T.E. (1978) Biochim. Biophys. Acta 531, 56-64.
26. Cagen, L.M., Fales, H.M., and Pisano, J.J. (1976) J. Biol. Chem. 251, 6550-6554.
27. Michel, F., Pons, M., Descomps, B., and Orastes de Paulet, A. (1978) Eur. J. Biochem. 84, 267-274.
28. Nishida, N., and Yielding, K.L. (1970) Arch. Biochem. Biophys. 141, 409-415.
29. Bitensky, M.W., Yielding, K.L., and Tomkins, G.M. (1965) J. Biol. Chem. 240, 663-667.
30. Pal, P.K., and Colman, R.F. (1976) Eur. J. Biochem. 68, 437-443.
31. O'Brien, P.J. (1969) Can. J. Biochem. 47, 485-492.
32. McMurray, W.C. (1973) in Form and Function of Phospholipids, BBA Library (Ansell, G.B., Hawthorne, J.N., and Dawson, R.M.C., eds.) Vol. 3, Elsevier Scientific Publishing Co., Amsterdam.
33. Garssen, G.J., Vleigenthart, J.F.G., and Boldingh, J. (1971) Biochem. J. 122, 327-332.
34. Hamberg, M. (1975) Lipids 10, 78-92.
35. Holman, R.T. (1954) in Progress in Fats and Other Lipids (Holman, R.T., Lundberg, W.O., and Malkin, T., eds.) Vol. 2, p. 60, Academic Press, Oxford.
36. Vioque, E., and Holman, R.T. (1962) Arch. Biochem. Biophys. 99, 522-528.
37. Holman, R.T., and Burr, G.O. (1946) J. Am. Chem. Soc. 68, 562-566.
38. Lundberg, W.O. (1962) in Lipids and Their Oxidation, (Schultz, H.W., Day, E.A., and Sinnhuber, R.C., eds) p. 68, AVI Publishing Co., Inc., Westport, CT.
39. Walling, C., and Halmreich, W. (1959) J. Am. Chem. Soc. 81, 1144-1148.

[Received August 27, 1981]

Effects of Dietary 9-*trans*,12-*trans* Linoleate on Arachidonic Acid Metabolism in Rat Platelets

D.H. HWANG*, P. CHANMUGAM and **R. ANDING**, *Louisiana Agricultural Experiment Station, Human Nutrition, Home Economics Building, Louisiana State University, Baton Rouge, LA 70803*

ABSTRACT

In order to determine the minimal amount of dietary 9-*trans*,12-*trans*-linoleate which can decrease endoperoxide metabolites synthesized and their precursor in rat platelets, graded amounts (0, 0.1, 0.5, 1.0, 2.5%) of the *trans*-linoleate were fed to rats with a constant amount of all-*cis*-linoleate (2.5%) for 12 weeks. Arachidonic acid levels in platelet phospholipids of groups receiving the *trans*-linoleate at 2.5 and 1.0% were significantly ($p<0.01$) lower than that of the control receiving no *trans*-linoleate. Concentrations of TXB_2 and $PGF_{2\alpha}$ in sera of the group receiving 2.5% *trans*-linoleate were significantly ($p<0.05$) lower than those of the control; however, there was no difference between the group receiving 1.0% *trans*-linoleate and the control. To determine whether the difference in serum concentrations of endoperoxide metabolites could be manifested if rats were fed for longer period of time, 2 groups of rats were again fed diets containing 0 and 1.0% *trans*-linoleate, respectively, for 16 weeks. Arachidonic acid in platelet phospholipids of the group receiving the *trans*-linoleate was again significantly ($p<0.01$) lower than that of the control group. Concentrations of TXB_2 and $PGF_{2\alpha}$, and 12-hydroxyeicosatetraenoic acid formed in platelets, were smaller in the group receiving *trans*-linoleate than the control group; however, the difference was not statistically significant. These results indicated that all-*trans*-linoleate can reduce arachidonic acid metabolites formed in rat platelets when its dietary level is equal to or exceeds the level of all-*cis*-linoleate.
Lipids 17:307-313, 1982.

INTRODUCTION

Geometric and positional isomerization of double bonds of unsaturated fatty acids occurs during the hydrogenation of vegetable oil (1,2). The presence of geometric and positional isomers of unsaturated fatty acids in vegetable shortening, hydrogenated, winterized oil and margarines has been demonstrated (3-5). The presence of *trans* fatty acids was also demonstrated in human tissue (6). It has been generally suggested that *trans* fatty acids have no adverse effects in animal models if adequate amounts of essential fatty acids (EFA) are provided. This suggestion has been largely based on the data for weight gain, longevity and reproduction performance (7).

The difference in the metabolism of *trans*-linoleate and *cis* linoleate in animal tissues has been well documented. 9-*trans*,12-*trans*-Linoleate does not possess EFA activity, and actually aggravated EFA deficiency when fed to animals as a sole dietary fat (8,9). The aggravation of EFA deficiency was, in part, due to the inhibition of the conversion of *cis*-linoleate to arachidonic acid by the *trans*-linoleate. Because the *trans*-linoleate inhibited the desaturation of 9-*cis*,12-*cis*-linoleate (9,10), diets containing inhibitory amounts of the *trans*-linoelate might cause reduction in tissue level of arachidonic acid. In a previous study, it was shown that feeding rats with a diet containing equal amounts of *cis*-linoleate and 9-*trans*,12-*trans*-linoleate (5 cal % each) caused significant reduction in the tissue level of arachidonic acid compared to rats receiving *cis*-linoleate alone (11).

It is generally recognized that availability of direct precursor acids (free fatty acids) is one of the limiting factors in the biosynthesis of endoperoxide metabolites in animal tissues. The amount of the precursor acids (i.e., free arachidonic acid) released from tissue phospholipids by the action of phospholipase would depend on the fatty acid composition of tissue lipids, which, in turn, can be affected by kinds of dietary fat. This indicates that dietary factors affecting tissue levels of precursor acids could enhance or decrease the biosynthesis of endoperoxide metabolites in tissues. In previous studies, it was demonstrated that reduction in tissue arachidonic acid level caused by feeding 9-*trans*,12-*trans*-linoleate (11) or linolenate (12) resulted in concomitant decrease in serum levels of endoperoxide metabolites.

In light of the previous observation, the objective of this study was to determine the minimal amount of dietary 9-*trans*,12-*trans*-inoleate which can decrease arachidonic acid content and the formation of its metabolites through lipoxygenase and cyclooxygenase pathways in rat platelets.

MATERIALS AND METHODS

Animal Care and Diet

Weanling male Sprague-Dawley rats (Gibco

Animal Research Lab., Madison, WI) weighing 35-50 g were individually housed in stainless steel cages in a room maintained at constant temperature (23 ± 3 C). Fresh diets were provided daily ad libitum. All the diets except that for EFA-deficient (EFAD) group were mixed weekly and stored at -20 C. No noticeable change occurred in the ratio of *cis*-linoleic acid to stearic acid of the dietary fat during 24 hr at room temperature or 1 week at -20 C. The composition of the basic diet is shown in Table 1. Weight gain of rats was monitored weekly.

Design of the Experiment

Experiment 1. Rats were randomly assigned to each group (13 rats/group). They were fed diets containing graded amounts of purified 9-*trans*,12-*trans*-linoleate as triglyceride (hereafter designated as *trans*-linoleate, 99% pure as determined by gas chromatography, Nu-Chek-Prep, Elysian, MN) in presence of a constant amount of safflower oil as a source of *cis*-linoleate (except EFAD group) as shown in Table 2. An appropriate amount of hydrogenated coconut oil was added to each diet to give a constant level of fat. Because in the previous study (11), an equal mixture of *trans*-linoleate (2.5% wt) and *cis*-linoleate (2.5%) caused reduction in serum PG, the maximal level of *trans*-linoleate in this study was 2.5%. Group A was the control group receiving no *trans*-linoleate.

Experiment 2. The result of experiment 1 showed that *trans*-linoleate at 1% (group D) caused significant reduction of arachidonic acid in platelet phospholipids, but serum concentrations of TXB_2 and $PGF_{2\alpha}$ in group D were not significantly different from those of the control group. In order to determine whether the difference in serum concentrations of TXB_2 and $PGF_{2\alpha}$ between group D and the control could be manifested if rats were fed longer period of time, 2 groups of rats were fed the same diets for group A and group D of experiment 1 for 16 weeks.

Sampling Procedures

Experiment 1. After 12 weeks, rats chosen randomly from each group were lightly anesthetized with diethyl ether, and blood was withdrawn from the abdominal aorta using a butterfly infusion set (Abbott Hospital Products, North Chicago, IL). The first 5 ml of whole blood collected into a plastic syringe was incubated at 37 C for 1 hr to optimize the PG formation during clotting as determined in the

TABLE 1

Composition of the Basic Diet Fed to Experimental Rats

Ingredient	Wt %
Fat[a]	5.0
Casein (vitamin-free)	20.0
Salt mix[b]	4.0
Vitamin mix[c]	2.0
Cellulose	4.0
Choline chloride	0.2
Sucrose	30.0
Corn starch	to 100.0

[a]See Table 2.

[b]Jones, J.H., and Foster, C. (1942) J. Nutr. 24, 245. This salt mix was supplemented with $ZnCl_2$ (518 mg/kg), $Na_2SeO_3 \cdot 5H_2O$ (4.4 μg/kg) and $Na_2CrO_4 \cdot 4H_2O$ (56.5 mg/kg).

[c]ICN Pharmaceuticals, Inc., Cleveland, OH; g/1 kg; vitamin A, 900,000 IU; vitamin D, 100,000 IU; dl-α-tocopherol, 10 (additional 5 g were added to give 10 g); ascorbic acid, 45; inositol, 1.0; choline chloride, 75.0; menadione, 2.25; paraminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine hydrochloride, 1.0; thiamine hydrochloride, 1.0; calcium pantothenate, 3.0; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 1.35 mg.

TABLE 2

Effects of Dietary 9-*trans*,12-*trans* Linoleate on Growth of Rats[a]

Group	Dietary fat (%)			Body wt (g)
	HCO	Safflower oil	*trans* Linoleate	
EFAD	5			345 ± 8[b]
A	2.5	2.5		370 ± 5
B	2.4	2.5	0.1	385 ± 9
C	2.0	2.5	0.5	378 ± 6
D	1.5	2.5	1.0	383 ± 4
E		2.5	2.5	349 ± 9

[a]Values for body wt are mean ± SEM of 10-13 rats.

[b]Significantly less than that of the control group (group A), $p < 0.05$ (Duncan's multiple range test).

previous report (11). Plasma samples were stored at -20 C until assayed. The remaining blood was withdrawn into another plastic syringe containing 1 ml of anticoagulant solution (3.8% trisodium citrate; 5 mM mepacrine HCl) in order to harvest intact platelets. In the previous study (13), it was shown that preincubation of mepacrine HCl (0.5 mM) with platelet-rich plasma (PRP) not only inhibited the aggregation of rat platelet by adenosine diphosphate (ADP) or collagen suspension but also reversed the aggregation already induced by ADP or collage. If platelets aggregate during preparation, then arachidonic acid in platelet lipids would be released into the medium. This could affect fatty acid composition of intact phospholipids in platelets. Platelet-rich plasma was prepared by centrifuging the citrated blood at 100 g for 10 min. The PRP isolated was further centrifuged at 1000 x g for 10 min. Platelet pellets were washed once with the same anticoagulant solution, and stored at -20 C until they were assayed for fatty acid composition. Livers were quickly removed and frozen in liquid nitrogen and stored at -20 C until they were assayed.

Experiment 2. The sampling procedure was modified from that of experiment 1. Citrated (without mepacrine HCl) blood samples withdrawn from the abdominal aorta were diluted 5 times in phosphate buffered saline (PBS, pH 7.0) containing collagen suspension, and incubated with constant stirring rate of 1,200 rpm in a platelet aggregometer. After 20 min of incubation, the blood sample was centrifuged for 5 min at 1,000 x g. Concentrations of TXB_2 and $PGF_{2\alpha}$ in plasma samples reached the maximum within 10 min. During the standardization experiments, it was found that variability in concentrations of arachidonic acid metabolites in the plasma of diluted blood samples was considerably smaller than that of serum samples obtained from undiluted clotting blood.

Concentrations of TXB_2 and $PGF_{2\alpha}$ were also determined in plasma obtained from PRP aggregated by collagen suspension. Platelet aggregation and sampling procedures were described in a previous report (14).

Analyses

Experiment 1: radioimmunoassay (RIA) for TXB_2 and $PGF_{2\alpha}$. Preparation and characterization of $PGF_{2\alpha}$ antisera and assessment of the validity of the assay system were reported in a previous report (15). The preparation and characterization of TXB_2 antiplasma were also reported in previous reports (12,14); the highest cross-reactivity of TXB_2 antiplasma was shown by PGD_2 (0.5%) among arachidonic acid and its various metabolites. When various amounts of standard TXB_2 (400 pg, 800 pg, 1.6 ng, 3.2 ng and 6.4 ng) were added to a diluted serum sample, recoveries were 97 ± 1.4, 98 ± 2.3, 98 ± 2.5 and 100 ± 2.6%, respectively. The concentration of TXB_2 in rat serum was ca. 10 times greater than that of PGE_2 or $PGF_{2\alpha}$ (13). Therefore, dilution of samples was necessary. All serum samples were analyzed at once for $PGF_{2\alpha}$ or TXB_2 to avoid interassay variation. Radioimmunoassay data were analyzed using the RIA computer program (16). The principle of the computer program is based on the linearity of the logit transformation of the dose-response curve and least square regression analysis with weighing procedure due to the inherent nonhomogenous distribution of error along the regression line.

Precursors of endoperoxides in platelet phospholipids. Lipids of platelets were extracted by Folch et al. (17). Platelets from 2-3 rats were pooled before the extraction due to limited amounts of platelets per rat. The total phospholipids were fractionated by thin layer chromatography (TLC; Silica Gel G, Kontes, Vineland, NJ) using diethyl ether/petroleum ether/acetic acid (30:70:1, v/v/v). The phospholipid fraction remained at the origin was scraped off and eluted using 20 ml of chloroform/methanol/acetic acid (2:1:0.2, v/v/v). Fatty acid composition of phospholipids with emphasis on PG precursor acids was determined as described in a previous report (11).

Experiment 2. In addition to TXB_2 and $PGF_{2\alpha}$, the concentrations of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) in plasma from diluted blood samples and PRP were determined by radioimmunoassay using 12-HETE antiplasma (gift from Dr. Laurence Levine, Brandeis University, Waltham, MA). The highest cross-reactivity (0.3%) for the antiplasma was shown by 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) among compounds tested (arachidonic acid, 15-HETE, HHT, TXB_2, PGE_2, $PGF_{2\alpha}$, 6-K-$PGF_{1\alpha}$, 5-HETE). This value was considerably lower than that (8.6%) reported by Levine et al. (18) in a solid-phase radioimmunoassay system using the same antiplasma.

RESULTS AND DISCUSSION

Fatty acid composition of platelet phospholipids shown in Table 3 indicated that increasing the dietary *trans*-linoleate enhanced the content of *cis*-linoleate, whereas it resulted in gradual reduction of longer chain polyunsaturated fatty acids (PUFA) derived from *cis*-

linoleate. Particularly, arachidonic acid in platelet phospholipids decreased as the dietary *trans*-linoleate increased in a dose-dependent fashion (Table 4). These results are consistent with the well known inhibitory effect of the *trans*-linoleate on desaturation of *cis*-linoleate in animal tissues (10). Platelet lipids contained an unknown compound which was not separable from the *trans*-linoleate in Silar-10C phase in the packed column used in this study. The unknown compound was not derived from the preparation of dietary *trans*-linoleate because it also appeared in platelet lipids of EFAD group and the control group (group A) receiving no *trans*-linoleate. This unknown compound was tentatively identified as 18:1 dimethyl acetal (DMA) based on its retention time. Presence of 18:1 DMA in human platelet lipids was also reported (19). The level of *trans*-linoleate in platelet phospholipids, therefore, could not be quantitated in this study. The fatty acid composition of liver phospholipids, in which 18:1 DMA was not found, showed a dose-dependent increase in *trans*-linoleate:

TABLE 3

Fatty Acid Composition of Platelet Phospholipids of Rat Receiving Graded Amount of 9-*trans*,12-*trans* Linoleate[a,b]

Fatty acids	EFAD	A	B	C	D	E
			Wt %			
16:0	31.0 ± 0.4	32.3 ± 0.3	31.6 ± 0.6	32.4 ± 0.7	31.6 ± 0.4	30.6 ± 1.3
16:DMA	4.5 ± 0.8	4.4 ± 1.5	6.4 ± 0.4	5.0 ± 0.7	6.0 ± 0.3	5.5 ± 0.5
16:1	3.8 ± 0.8	2.0 ± 0.01	2.2 ± 0.1	2.3 ± 0.07	2.3 ± 0.1	2.3 ± 0.2
18:0	15.0 ± 1.8	16.0 ± 2.6	13.2 ± 0.9	16.3 ± 1.4	15.2 ± 1.3	15.0 ± 1.6
18:DMA	4.5 ± 0.7	4.9 ± 0.6	5.1 ± 0.2	4.6 ± 1.1	5.2 ± 0.2	5.8 ± 0.9
18:1ω9	11.9 ± 0.2	7.4 ± 0.2	7.4 ± 0.1	7.8 ± 0.2	8.0 ± 0.2	8.6 ± 0.4
T, 18:2ω6 + unknown	5.0 ± 0.6	3.4 ± 0.5	4.2 ± 0.6	3.8 ± 0.4	6.0 ± 0.6	8.3 ± 0.3
18:2ω6	2.5 ± 0.2	5.3 ± 0.4	5.8 ± 0.3	5.9 ± 0.4	6.5 ± 0.5	7.8 ± 0.5
18:3ω6	–	0.2 ± 0.04	0.3 ± 0.05	0.2 ± 0.06	0.2 ± 0.05	0.2 ± 0.04
18:3ω3	0.4 ± 0.1	0.2 ± 0.02	0.1 ± 0.07	0.2 ± 0.03	0.2 ± 0.02	0.3 ± 0.03
20:1ω9	0.1 ± 0.01	–	–	–	–	–
20:2ω6	0.1 ± 0.01	0.4 ± 0.05	0.4 ± 0.06	0.5 ± 0.05	0.5 ± 0.07	0.6 ± 0.1
20:3ω9	8.3 ± 0.6	–	–	–	–	–
20:3ω6	0.7 ± 0.1	0.2 ± 0.09	0.2 ± 0.03	0.2 ± 0.06	0.2 ± 0.04	0.5 ± 0.1
20:4ω6[c]						
20:5ω3	–	0.2 ± 0.1	0.2 ± 0.06	0.2 ± 0.03	0.2 ± 0.05	0.2 ± 0.1
22:3ω9	1.6 ± 0.14	–	–	–	–	–
22:4ω6	0.2 ± 0.05	2.4 ± 0.4	2.0 ± 0.2	2.2 ± 0.08	1.9 ± 0.1	1.5 ± 0.1
22:5ω6	0.2 ± 0.05	0.3 ± 0.08	0.3 ± 0.05	0.3 ± 0.08	0.2 ± 0.01	0.1 ± 0.04

[a]See Table 2 for the composition of dietary fat of each group.

[b]Values are mean ± SEM of 4-5 samples; each sample consisted of platelets pooled from 2-3 rats.

[c]Values are reported in Table 4.

TABLE 4

Effects of Dietary 9-*trans*,12-*trans* Linoleate on Serum Concentrations of Endoperoxide Metabolites and Their Precursor in Platelets[a]

Group	Arachidonic acid in platelet phospholipids (wt %)	TXB_2 (ng/ml)	$PGF_{2\alpha}$ (ng/ml)
EFAD	11.0 ± 1.0**	53.8 ± 11.8*	3.9 ± 0.9
A	21.9 ± 0.8	148.1 ± 28.5	15.5 ± 4.0
B	21.5 ± 1.2	147.9 ± 31.1	14.1 ± 4.2
C	19.0 ± 1.4	113.1 ± 33.6	11.7 ± 4.0
D	16.6 ± 0.6**	119.8 ± 20.2	7.6 ± 1.1
E	12.7 ± 0.9**	62.5 ± 9.5*	3.5 ± 0.4*

[a]Values are mean ± SEM of 5 observations for arachidonic acid; 10-13 observations for TXB_2 and $PGF_{2\alpha}$.

**Significantly different from the value of group A ($p < 0.01$).

*$p<0.05$.

trace, trace, 0.5, 1.9, 3.0 and 5.7% for EFAD group, groups A, B, C, D and E, respectively.

Serum concentrations of TXB_2 and $PGF_{2\alpha}$ are shown in Table 4. Serum concentrations of endoperoxide metabolites reflect amounts of these compounds synthesized by platelets during blood clotting. Although arachidonic acid levels in platelet phospholipids of groups D and E, and EFAD group were significantly lower than that of the control (group A), there was no significant difference between the control and the group receiving 1.0% *trans*-linoleate (group D) in concentrations of TXB_2 and $PGF_{2\alpha}$. This indicated that levels of TXB_2 and $PGF_{2\alpha}$ were unaffected by the *trans*-linoleate as markedly as was arachidonic acid in platelet phospholipids. Arachidonic acid in phospholipids is a potential precursor for endoperoxides. It is unknown whether amounts of free arachidonic acid released from platelet phospholipids by phospholipase during blood clotting is proportional to the levels of arachidonic acid in platelet phospholipids at the range found in this study. This result also indicated that the *trans*-linoleate itself may not inhibit the formation of endoperoxide metabolites in platelets.

When 2 groups of rats were fed the same diets as those for groups A and D for longer periods of time (16 weeks compared to 12 weeks in the first experiment), the level of arachidonic acid in platelet phospholipids of group D was significantly lower than that of the control (Table 5). The difference between 2 groups appeared slightly greater than the difference found in the first experiment.

The major arachidonic acid metabolites in rat platelets derived from cyclooxygenase are TXB_2 and HHT, and that derived from lipoxygenase is 12-HETE. Concentrations of endoperoxide metabolites and 12-HETE in PRP and diluted whole blood samples were lower in group D receiving *trans*-linoleate than those in the control group (Table 5). However, the differences were not statistically significant. There was no significant difference in the degree of platelet aggregation induced by collagen suspension between 2 groups. The concentrations of 12-HETE in PRP were ca. 3 times greater than those of TXB_2, indicating that endogenous arachidonic acid is metabolized mainly through lipoxygenase pathway in rat platelets when aggregated by collagen suspension. It was shown that the concentration of TXB_2 reached the maximum within 5 min of incubation after the aggregation of rat platelet was initiated by collagen suspension (13). However, the concentration of 12-HETE continued to increase even after 60 min of the

TABLE 5

Effects of Dietary 9-*trans*,12-*trans* Linoleate on Arachidonic Acid Metabolism and Platelet Aggregation[a]

Group[b]	Arachidonic acid in platelet phospholipids (wt %)	TXB_2		$PGF_{2\alpha}$		12-HETE		Platelet aggregation (%)
		PRP (ng/ml)	Diluted whole blood (ng/ml)	PRP (ng/ml)	Diluted whole blood (ng/ml)	PRP (μg/ml)	Diluted whole blood (μg/ml)	
A	19.9 ± 1.2	151.8 ± 15.4	75.7 ± 3.1	3.8 ± 0.4	2.9 ± 0.3	0.48 ± 0.09	11.7 ± 0.8	63.4 ± 2.3
D	13.7 ± 0.9**	128.6 ± 11.1	68.9 ± 2.6	3.2 ± 0.2	2.6 ± 0.2	0.44 ± 0.07	9.1 ± 1.1	60.4 ± 1.2

[a]Values are mean ± SEM of 9-10 observations.

[b]The dietary compositions of group A and D is the same as those for group A and D in Table 2; however, rats were fed for 16 weeks in this study instead of 12 weeks (see text).

**Significantly different from the value of group A ($p<0.01$).

incubation (Hwang, D.H., unpublished data). The concentrations of 12-HETE in diluted whole blood samples incubated for 20 min in presence of collagen suspension were ca. 150 times greater than those of TXB_2. This might be because the formation of 12-HETE by platelet lipoxygenase was time-dependent over the incubation period, whereas cyclooxygenase was rapidly inactivated. Another possibility was that 12-HETE might be formed by leukocytes in the diluted blood sample. However, when leukocyte-rich preparation (buffy coat after harvesting PRP) was challenged with collagen suspension, the concentration of 12-HETE was not greater than that of PRP preparation, suggesting that leukocyte lipoxygenase could not be stimulated by collagen suspension. The concentration of 12-HETE in PRP incubated for 60 min after the initiation of aggregation was comparable to those in diluted whole blood samples in this study (Hwang, D.H., unpublished data).

The *trans*-isomer (9-*trans*,12-*trans*) of linoleate used in this study is one of geometric isomers of linoleate found in partially hydrogenated vegetable oil. Normally, the level of this isomer does not exceed 0.5% (5). It appeared from this study that such a level of 9-*trans*,12-*trans*-linoleate may not be enough to reduce arachidonic acid content in platelet lipids and subsequently to decrease the formation of arachidonic acid metabolites through cyclooxygenase and lipoxygenase in rat. 9-*cis*,12-*trans*-Linoleate, which is another kind of geometric isomer of linoleate found in partially hydrogenated oil, can be desaturated and elongated to longer chain PUFA (20). Therefore, the animal receiving this fatty acid is expected to have arachidonic acid and perhaps longer chain PUFA with *trans* double bonds in its tissue lipids. Based on gas chromatographic analysis using a capillary column, Anderson et al. (21) reported increased formation of arachidonic acid with *trans* double bonds in tissue lipids of rats fed a mixture of 9-*cis*,12-*trans*- and 9-*trans*,12-*cis*-linoleate. It was demonstrated that monoconjugated *trans*-isomrs of eicosatrienoic acid and eicosatetraneoic acid inhibited the formation of prostaglandins from all-*cis*-arachidonic acid (22).

Geometric and positional isomers of octadecenoate comprise the major portion of fatty acid isomers present in partially hydrogenated oil (5). Recently, Mahfouz et al. (23) and Pollard et al. (24) showed that positional isomers of *trans*-octadecenoate are desaturated in rat liver microsomal preparations, and some of these isomers also can be elongated (25). It is conceivable that products of the desaturation of the isomers of octadecenoate can be further desaturated and elongated to form longer chain PUFA with *trans* double bonds. Quantitation of arachidonic acid and longer chain PUFA with *trans* double bonds in human tissue lipids has not yet been reported. Therefore, in order to evaluate effects of hydrogenated vegetable oil on arachidonic acid metabolism via cyclooxygenase and lipoxygenase in animal tissues, effects of desaturation and elongation products of isomers of octadecenoate and 9-*cis*,12-*trans*-linoleate need to be studied.

ACKNOWLEDGMENT

This work was supported by USDA competitive human nutrition research grant (5901-0410-0311-0), American Heart Association-La., Inc., and the Lousiana Agricultural Experiment Station. The authors thank E. Tolle for typing the manuscript.

REFERENCES

1. Allen, R.R., and Kies, A.A. (1956) J. Am. Oil Chem. Soc. 33, 355-359.
2. Markley, K.S. (1961) in Fatty Acids, Part 2 (Markley, K.S., ed.), pp. 1187-1305, Interscience Publishers, Inc., New York, NY.
3. Jones, E.P., Schofield, C.R., Davison, V.L., and Dutton, H.J. (1965) J. Am. Oil Chem. Soc. 42, 727-730.
4. Carpenter, D.L., and Slover, H.T. (1973) J. Am. Oil Chem. Soc. 50, 372-376.
5. Lanza, E., and Slover, H.T. (1981) Lipids 16, 260-267.
6. Kumerow, F.A. (1974) J. Am. Oil Chem. Soc. 51, 255-259.
7. Alfin-Slater, R.B., and Aftergood, L. (1979) in Geometric and Positional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.), pp. 53-74, Am. Oil Chem. Soc., Champaign, IL.
8. Holman, R.T., and Aaes-Jorgensen, E. (1956) Proc. Soc. Exp. Biol. Med. 93, 175-179.
9. Privett, O.S., and Blank, M.L. (1964) J. Am. Oil Chem. Soc. 41, 292-297.
10. Privett, O.S., Phillips, F., Shimasaki, H., Nozawa, T., and Nickell, E.C. (1977) Am. J. Clin. Nutr. 30, 1009-1017.
11. Hwang, D.H., and Kinsella, J.E. (1979) Prostaglandins 17, 543-559.
12. Hwang, D.H., and Carroll, A. (1980) Am. J. Clin. Nutr. 33, 590-597.
13. Hwang, D.H. (1980) Prostaglandins Med. 5, 163-173.
14. Hwang, D.H., LeBlanc, P., and Chanmugam, P. (1981) Lipids 16, 583-588.
15. Hwang, D.H., Mathias, M., Dupont, M., and Meyer, D.L. (1975) J. Nutr. 105, 995-1002.
16. Duddleson, W.G., Midgley, A.R. Jr., and Niswender, G.D. (1972) Comput. Biomed. Res. 5, 205-217.
17. Folch, J., Lees, M., and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497-509.
18. Levine, L., Iftekhar, A., Gjika, H., Carty, T.J., and Goetzl, E.J. (1980) Prostaglandins 20, 923-934.
19. Marcus, A.J., Ullman, H.L., and Safier, L.B. (1969) J. Lipid Res. 10, 108-114.

20. Privett, O.S., Stearns, E.M., Jr., and Nickell, E.C. (1967) J. Nutr. 92, 303-310.
21. Anderson, R.L., Curtis, S.F., Jr., and Hollenbach, E.J. (1975) J. Nutr. 105, 393-400.
22. Nugteren, D.H. (1970) Biochim. Biophys. Acta 210, 171-176.
23. Mahfouz, M.M., Valicenti, A.J., and Holman, R.T. (1980) Biochim. Biophys. Acta 618, 1-12.
24. Pollard, M.R., Gunstone, F.D., James, A.T., and Morris, L.J. (1980) Lipids 15, 306-314.
25. Kameda, K., Valicenti, A.J., and Holman, R.T. (1980) Biochim. Biophys. Acta 618, 13-17.

[Received November 2, 1981]

METHODS

Preparation of *cis,cis,cis*-5,8,11-Eicosatrienoic Acid from Arachidonic Acid

AMITABHA GHOSH, MALATI KOLEY and **J. DUTTA**, *Department of Chemistry, Bose Institute, 93/1 A.P.C. Road, Calcutta 700 009, India*

ABSTRACT

Arachidonic acid was reduced by hydrazine to yield isomeric eicosatrienoic acids with other products. Methyl *cis,cis,cis*-5,8,11-eicosatrienoate was isolated from the products by silver ion chromatography and preparative gas liquid chromatography in 8% yield. The structure was confirmed by spectral studies and oxidative degradation.
Lipids 17:314-316, 1982.

INTRODUCTION

It was first shown by Mead and Salton and Fulco and Mead (1,2) that eicosa-5,8,11-trienoic acid (20:3ω9) was derived from oleic acid (18:1ω9), sometimes called Mead's acid. The structure of this eicosatrienoic acid (20:3ω9) was also established by Mead and Salton (1). It is well established that, under essential fatty acid (EFA)-deficient conditions, the arachidonic acid (20:4ω6) level decreases and there is a considerable increase of 20:3ω9 in different tissues of rat (3-5), which normally contains a negligible amount of the latter acid. Although it is quite clear that EFA deficiency is responsible for accumulation of 20:3ω9 acid, very little is known about the effect of this acid on various metabolic functions, because of the very limited availability of this acid. The significance of 20:3ω9 acid in EFA deficiency has been currently reviewed (6).

A novel, general and flexible method for the synthesis of some EFA was described by Osbond et al. (7). Their method consisted of stereospecific partial reduction of the corresponding polyacetylenic acids. These were prepared by the condensation of various propargyl bromides with the di-Grignard derivatives of ω-acetylenic acids. Subsequently, various polyethenoid fatty acids were synthesized for metabolic studies, notably by Sprecher (8-10), with some modification of the original method of Osbond et al. (7). Synthesis of 20:3ω6 has also been reported (9). For the purpose of some metabolic studies with 20:3ω9, we have developed a simple and very short route to its preparation, by the partial reduction of 20:4ω6, which is easily available.

MATERIALS AND METHODS

Arachidonic acid (20:4ω6) and methyl eicosa-8,11,14-trienoate (20:3ω6) were purchased from Nu-Chek-Prep. Inc., Elysian, MN. All the reagents and solvents were of analytical grade. Solvents were dried and distilled before use.

Hydrazine Hydrate Reduction

To 100 mg of arachidonic acid in 50 ml of aq (50%) ethanol, 1 ml of hydrazine hydrate (99%, BDH, England) was added and stirred vigorously at 50 C (11). Five-ml aliquots were withdrawn at intervals of 1 hr up to 7 hr. From each of the aliquots, fatty acids were extracted with diethyl ether, methylated with diazomethane (12) and analyzed by gas liquid chromatography (GLC). For a preparative scale, 500 mg of arachidonic acid was dissolved in 250 ml of aq ethanol, 3 ml of hydrazine hydrate was added and the mixture was stirred vigorously for 5 hr at 50 C. The fatty acids were extracted and methylated by diazomethane.

Argentation Column Chromatography

The mixed methyl esters obtained by the partial reduction of 20:4ω6 acid was charged in a glass column (50 cm × 3 cm) containing silver nitrate (20%) coated silicic acid (150 g) and eluted with a solvent system of light petroleum (40-60 C) containing increasing amounts of diethyl ether (13). The column chromatography was monitored by GLC, using a 10% DEGS column (6 ft × 0.25 in.).

Isolation of 20:3ω9 by Preparative GLC

The pooled triene fraction obtained from argentation column chromatography was subjected to preparative GLC using glass columns (2.1 m × 8 mm) packed with 15% DEGS on Diatomite C (AW, DMCS-treated, 60-70 mesh) obtained from Pye Unicam Ltd., England. The purity of 20:3ω9 acid isolated was checked by analytical GLC. The overall yield was 8% of arachidonic acid.

Determination of Double Bond Position of 20:3ω9

Thirty mg of 20:3ω9 was reduced by hydrazine hydrate (11) for 3.5 hr. After extraction and methylation of the reduced products, the monoenes were isolated by silver ion TLC (14) and were subjected to permanganate-periodate oxidation (15). The fatty acids were recovered and methylated. The mono- and dicarboxylic acids were separated on thin layer chromatographic (TLC) plate, recovered, and analyzed separately for their chain lengths (16).

Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS) and Elemental Analysis

The ^{1}H NMR ($CHCl_3$, $\underline{d}$) 0.9 ppm (t, 3H, $C\underline{H}_3$ CH_2), 1.3 (broad, 10H, $C\underline{H}_2$), 1.7 (m, 4H, $C\underline{H}_2$ CH_2 CH = CH) 2.0 (d, 4H, $C\underline{H}_2$ CH = CH) 2.25 (t, 2H, $C\underline{H}_2COO$), 2.8 (t, 4H, = $CHC\underline{H}_2CH$ =) 3.7 (s, 3H, $C\underline{H}_3OCO$), 5.3 (t, 6H, $C\underline{H}$ = $C\underline{H}$). The MS had a parent ion peak at m/e 320. Elemental analysis of methyl eicosatrienoate, $C_{21}H_{36}O_2$, requires C, 78.69; H, 11.32 and 0, 9.98%; found C, 78.42; H, 11.20 and 0, 9.94%.

RESULTS AND DISCUSSION

The GLC analyses of the reduction product of 20:4ω6 at 5 hr, which showed maximal production of 20:3ω9 along with other products, are presented in Table 1.

It is known that hydrazine randomly reduces polyethenoid fatty acids without double bond movement or stereomutation (17). Theoretically, 15 products are possible altogether, of which there should be 4 trienes, 6 dienes, 4 monoenes and 20:0. In the present study, traces of 20:0 were found along with some unreduced 20:4ω6. The main peaks in the GLC tracing had shoulders, indicating the presence of other compounds. With the help of published equivalent chain length (ECL) values (18,19) on the DEGS column and using authentic 20:3ω6, it was possible to identify 20:3ω9 and 20:3ω6. The triene band isolated by silver ion column chromatography contained primarily 20:3ω9 and 20:3ω6 with some contaminants, probably the other triene isomers. Isolation of 20:3ω9 by preparative GLC produced a perfect Gaussian peak with an ECL of 21.2 by analytical GLC on a 10% DEGS column.

The NMR data support the presumption that the FAME in question contains 3 double bonds, interrupted by 2 methylene groups. The MS of methyl arachidonate, its fully reduced product, and of the purified 20:3ω9 gave molecular ion peaks at 318, 326 and 320, respectively, and confirmed the presence of 3 double bonds in the purified 20:3ω9.

The GLC of the mono- and dicarboxylic acid methyl esters obtained by the permanganate-periodate oxidation of 20:3ω9 produced peaks due to 9-, 12- and 15-carbon chain monocarboxylic acids and 5-, 8- and 11-carbon chain dicarboxylic acids. These facts unambiguously indicate that 20:3ω9 contained double bonds at the 5-, 8- and 11-positions.

Table 1 shows that, under optimal conditions, the percentages of 20:3ω9 and 20:3ω6 are 31 and 37, respectively. Although low, the overall 8% yield of 20:3ω9 is encouraging in the sense that the production of 20:3ω9 through animal experiments or by total synthesis are long and expensive routes.

ACKNOWLEDGMENT

Authors are thankful to Prof. S.C. Bhattacharyya, Director, and Prof. A. Sen, Chairman, Department of Chemistry, for providing research facilities and to Indian Council of Medical Research for providing a fellowship to M. Koley.

TABLE 1

Peak	1	2	3	4	5
Components	20:0	20:1 20:2	20:3ω9	20:3ω6	20:4ω6
% (w/w)	1	11	31	37	20
ECL	20.0	20.5	21.2	21.8	22.5

REFERENCES

1. Mead, J.F., and Salton, W.H., Jr., (1956) J. Biol. Chem. 219, 705-709.
2. Fulco, A.J., and Mead, J.F. (1959) J. Biol. Chem. 234, 1411-1416.
3. Holman, R.T. (1960) J. Nutr. 70, 405-410.
4. Holman, R.T. (1968) in Progress in the Chemistry of Fats and Other Lipids (Holman, R.T., ed.) Vol. 9, Part 2, p. 275, Pergamon Press, London.
5. Sun, G.Y., Winniczek, H., Go, J., and Sheng, S.L. (1975) Lipids 10, 365-373.
6 Lundberg, W.O. (1980) Nutr. Rev. 38, 233-235.
7. Osbond, J.M., Philpott, P.G., and Wickens, J.C. (1961) J. Chem. Soc. 2779-2787.
8. Sprecher, H. (1967) Biochim. Biophys. Acta 144, 296-304.
9. Sprecher, H. (1968) Biochim. Biophys. Acta 152, 519-530.
10. Sprecher, H. (1971) Biochim. Biophys. Acta 231, 122-130.
11. Aylward, F., and Rao, C.V.N. (1956) J. Appl. Chem. 6, 248-252.
12. Schlenk, H., and Gellerman, J.L. (1960) Anal. Chem. 32, 1412-1414.
13. Ghosh, A., Hoque, M., and Dutta, J. (1972) J. Chromatogr. 69, 207-208.
14. Morris, L.J., Marshall, M.O., and Hammond, E.W. (1968) Lipids 3, 91-95.
15. Chang, T.C.L., and Sweeley, C.C. (1962) J. Lipid Res. 3, 170-176.
16. Wu, J., Ghosh, A. and Beal, J.L. (1980) J. Natural Prod. 43, 360-364.
17. Gunstone, F.D. (1967) An Introduction to the Chemistry and Biochemistry of Fatty Acids and Their Glycerides, p. 90, Chapman and Hall, London.
18. Ackman, R.G. (1963) J. Am. Oil Chem. Soc. 40, 558-564.
19. Ackman, R.G., and Burgher, R.D. (1965) J. Am. Oil Chem. Soc. 42, 38-42.

[Received August 11, 1981]

COMMUNICATIONS

The Lipid Composition of Human Liver Microsomes

LUCY WASKELL*[a], DONALD KOBLIN[b] and ELEANOR CANOVA-DAVIS[a],
Department of Anesthesia, Veterans Administration Medical Center[a] and [b]University of California, San Francisco, CA 94143

ABSTRACT

The lipid composition of human liver microsomes isolated from liver biopsy samples obtained at abdominal surgery has been determined. Human liver microsomal phospholipid is composed of 49% phosphatidylcholine, 31% phosphatidylethanolamine, 14% phosphatidylserine + phosphatidylinositol and 6% sphingomyelin, very similar to the phospholipid composition of rat liver microsomes. The fatty acid composition of human liver microsomes is remarkable only for its content of polyunsaturated fatty acids, with 20% of the fatty acids consisting of arachidonic, docosatetraenoic, docosapentaenoic and docosahexaenoic acids. This value contrasts with 33% in rats and 9% in rabbits. The molar cholesterol/phospholipid ratio in human liver microsomes is 0.069, similar to the ratio in rat and rabbit microsomes.
Lipids 17:317-320, 1982.

The chemical composition and physical nature of the lipid matrix of a membrane often influence the activity of membrane-bound enzymes. For example, in various species of laboratory animals, differences in the phospholipid and/or fatty acid composition of the microsomal membrane are associated with differences in the enzymatic activity of S-adenosyl methionine microsomal phospholipid methyl transferase (1), drug metabolizing enzymes (1), carbonic anhydrase (2), and the microsomal calcium pump (2). Because of the relationship in experimental animals between lipid composition and the activities of several microsomal enzymes, we determined the phospholipid, fatty acid, and cholesterol composition of microsomes isolated from human liver. In particular, we thought the markedly lower levels of the mixed-function oxidases in human, compared to animal liver microsomes, might be related to the lipid composition of microsomal membranes (3).

MATERIALS AND METHODS

Liver biopsies were obtained at abdominal surgery which was being performed for therapeutic or diagnostic reasons. The University of California's San Francisco Human Experimentation Committee has approved our protocol for obtaining liver biopsies. Within 1-2 min of removal, the biopsies were placed in a 4 C solution of 1.15% KCl and weighed. The liver biopsy was homogenized in 5 vol of a 1.15% KCl and 5 mM EDTA solution with a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 12,000 rpm in a type 30 rotor. This supernatant fluid was centrifuged at 30,000 rpm for 60 min in a solution of 0.1 M Na pyrophosphate and 5 mM EDTA and centrifuged at 30,000 rpm for 60 min in a type 30 rotor. The final pellet was resuspended in a solution of 0.1 M Tris-Cl, pH 7.4 at 25 C, and 5 mM EDTA. A stream of nitrogen was blown over the microsomes and they were immediately placed at -80 C.

One hundred μl of a microsomal membrane suspension (containing ca. 1 mg of protein) was extracted once with 0.5 ml of methanol and twice with 0.5 ml of a 1:1 chloroform/methanol ($CHCl_3$/MeOH) solution. The MeOH and $CHCl_3$/MeOH solutions contained 50 mg of butylated hydroxytoluene (BHT)/ℓ. Sephadex G-25 (ca. 100 mg) was added to the pooled supernatants, and the mixture was taken to dryness with a rotary evaporator. The sample was resuspended in $CHCl_3$ and the Sephadex G-25 removed by passage through a Pasteur pipet packed with silane-treated glass wool. The $CHCl_3$ was evporated under an inert gas and the lipids were resuspended in 40 μl of $CHCl_3$.

Membrane phospholipid components and cholesterol were separated with a 1-dimensional TLC system on 0.25-mm Silica Gel 60 plates (EM Labs) activated at 120 C for 30 min. The solvent phase consisted of $CHCl_3$/MeOH/acetic acid/H_2O (65:50:5:3) and contained 50 mg BHT/ℓ. Greater than 90% of the phosphate applied to the plate could be recovered in the phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and

sphingomyelin fractions. Phospholipids and cholesterol were detected under an ultraviolet (UV) light after spraying with 2',7'-dichlorofluorescein (0.2% in methanol). The components were quantitatively scraped from the plates and dried under vacuum.

Phospholipid fatty acid groups were transesterified to their methyl esters by a procedure that closely followed that of Morrison and Smith (4), except that 12% (w/v) BCl_3/MeOH was used instead of BF_3/MeOH (5). Fatty acid methyl esters were separated using a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector. A 6-ft x 1/8 in. id glass column packed with 10% SP-2300 cyanosilicone was used over a 180-230 C temperature range, increasing at a rate of 2 C/min. Acyl groups with 15 or more carbon atoms were quantitated.

Phospholipid content was quantitated by measuring the amount of inorganic phosphate after perchloric acid digestion (5,6). Cholesterol was measured using gas chromatography following the conversion of cholesterol to its trimethylsilyl derivative, and employing 5-α-cholestane as the internal standard (5).

Standards for fatty acid methyl esters and phospholipids were obtained from Supelco, Bellefonte, PA. All solvents used in the lipid analyses were distilled and saturated with inert gas.

RESULTS

Table 1 provides information about the experimental human subjects. Table 2 provides the data on the fatty acid composition of each phospholipid class in human liver microsomes and the total fatty acid composition of the microsomes.

Table 3 provides the data on the phospholipid composition of the human microsomes.

The mean molar cholesterol/phospholipid ratio of the 5 human microsomal preparations is 0.069 with a range from 0.047 to 0.112. The serum cholesterol was measured in 4 of our 5 patients and was found to be normal in all cases (see Table 1).

Because these studies were performed with microsomes isolated only from males, we could not determine if the lipid composition of human microsomes varies with sex as it does in rats, but not in rabbits (2).

DISCUSSION

Our results reveal that the lipid composition of human liver microsomes is quantitatively and qualitatively similar to the lipid composition of rat and rabbit liver microsomes. For example, the phospholipid composition of rat liver microsomes has been reported by several investigators (see Table 3 for a partial list of the range of values) (1,7,8). These previously published data in rats do not differ by more than a few percent from the values we obtained in our human liver microsomes. In contrast to the almost identical phospholipid composition of human and rat liver microsomes, the fatty acid compositions are more variable between species. One of the major differences is in the content of peroxidizable polyunsaturated fatty acids (9). The data from humans reveal that phosphatidylcholine, which accounts for ca. 50% of the phospholipid in the liver microsomal membrane, contains ca. 13% arachidonic,

TABLE 1

Information on Patients from Whom the Liver Biopsies Were Obtained

Age (yr) Race Sex	Operation	Serum cholesterol (mg/dl)	Microscopic pathology on intraoperative liver biopsy
63 Black Male	Partial liver resection	ND	Grossly normal; no microscopic
77 White Male	Partial colectomy	175	Normal
64 White Male	Radical nephrectomy	177	Normal
60 White Male	Cholecystectomy	135	Moderate fatty metamorphosis
45 Chinese Male	Partial colectomy	118	Normal

TABLE 2

Fatty Acid Composition of the Phospholipids in Human Liver Microsomes

Fatty acid	Total fatty acids of phosphatidylcholine (%)	Total fatty acids of phosphatidylethanolamine (%)	Total fatty acids of phosphatidylserine + phosphatidylinositol (%)	Total fatty acids of sphingomyelin (%)	Total fatty acid in total phospholipids (%)
15:0	0.25 ± 0.18	0.52 ± 0.26	0	0	0.2
16:0	33.1 ± 2.3	17.4 ± 2.4	6.3 ± 1.3	25.8 ± 4.3	26.0
16:1	1.8 ± 0.38	0.38 ± 0.15	0.52 ± 0.24	1.0 ± 1.0	1.15
17:0	0.43 ± 0.05	0.3 ± 0.09	0.33 ± 0.08	0.4 ± 0.13	0.35
18:0	10.3 ± 1.6	20.8 ± 0.9	41.5 ± 1.8	14.2 ± 1.4	15.5
18:1	13.5 ± 1.2	9.3 ± 0.44	7.37 ± 1.08	6.7 ± 1.6	13.4
18:2	23.8 ± 0.8	14.8 ± 2.9	7.82 ± 1.65	0.73 ± 0.16	18.5
18:2ω6 or 20:0	0.42 ± 0.25	0.15 ± 0.06	0.3 ± 0.19	(20:0) 5.5 ± .55	0.25
20:1	0.1 ± 0	0.10 ± 0	0	0	0.1
20:2	0.2 ± 0.07	0.22 ± 0.12	0	0	0.1
20:3ω6	2.2 ± 0.6	0.92 ± 0.19	2.5 ± 0.33	0	1.7
20:4	9.7 ± 0.89	20.1 ± 0.98	29.5 ± 2.22	0	14.6
20:5	0.35 ± 0.19	0.32 ± 0.07	0	0	0.25
22:0	0	0	0	15.6 ± 2.3	0
22:4	0.25 ± 0.05	0.93 ± 0.16	0.58 ± 0.3	0	0.6
22:5ω6	0.32 ± 0.1	1.05 ± 0.26	0.45 ± 0.26	0	0.4
22:5ω3	0.42 ± 0.04	1.1 ± 0.21	0.63 ± 0.23	0	0.7
22:6	2.7 ± 0.50	11.7 ± 1.26	2.25 ± 1.4	0	5.75
24:0	0	0	0	12.3 ± 1.6	0.2
24:1	0	0	0	16.5 ± 1.77	0

The percentage composition of total fatty acid of each phospholipid class is presented as the mean ± SD of 5 human microsomal preparations. The percentage composition of total fatty acids of all phospholipid classes is the mean of 2 of the human microsomal preparations. Note that sphingomyelin is a very minor component of human liver microsomal lipids.

TABLE 3

Phospholipid Composition of Human Liver Microsomes

Phospholipid	Content (% of total phospholipid)	
	Human	Rats[a]
Sphingomyelin	5.93 ± 2.37	3.7 – 6.3
Phosphatidylcholine	48.8 ± 2.9	53 – 54
Phosphatidylethanolamine	31.3 ± 1.9	20 – 25
Phosphatidylserine + phosphatidylinositol	14.0 ± 1.6	12.6 – 14

[a]Range of representative previously published values for phospholipid composition of male rat liver microsomes is also given for comparison (1,2,6).

The results are expressed as the mean ± SD of the molar percentage of the total phospholipids of 5 human microsomal preparations.

docosatetraenoic, docosapentaenoic and docosahexaenoic acids. This compares to 30% in rats (8,9) and 5% in rabbits (2). Phosphatidylethanolamine, which accounts for 25-30% of the phospholipid in microsomes, contains 35% of these polyunsaturated fatty acids in humans, 35% in rats (8,9), and 16% in rabbits (2). Phosphatidylserine and phosphatidylinositol, which account for ca. 14% of the phospholipids in the microsomal membrane, contain 33% polyunsaturated fatty acids in humans, 40% in rats (7,9), and 13% in rabbits (2). Furthermore, the determination of the overall fatty acid composition of the phospholipid fraction from liver microsomes reveals that in rats 33% of the fatty acids are polyunsaturated (8), in humans 20%, and in rabbits 9% (2). Thus, rat liver microsomes contain the greatest percentage of polyunsaturated, peroxidizable (10) fatty acids, humans have intermediate levels and rabbits have the least. These substantial differences in the amount of peroxidizable, polyunsaturated fatty acids in liver microsomes probably explain the increased lipid peroxidation observed with in vitro lipid peroxidation by rat in contrast to rabbit liver microsomes (11). Our data suggest that in vitro lipid peroxidation by human liver microsomes will be less than that observed in the rat but more than that observed in rabbits. Chelating agents such as EDTA (ethylenediaminetetraacetic acid) should probably be added to human in vitro microsomal reactions to decrease lipid peroxidation and increase the stability of the mixed-function oxidases (12).

The cholesterol/phospholipid molar ratio in human liver microsomes of 0.069 is of the same order of magnitude as determined in rats by 2 investigators (7,13) but one-half of that reported in a third publication (8). The reason for this discrepancy is unknown. Unpublished data from our laboratory reveal that uninduced rabbit and rat liver microsomes exhibit approximately the same molar cholesterol/phospholipid ratio as do human microsomes.

ACKNOWLEDGMENTS

Financial support from the Norris Foundation, the Academic Senate Committee on Research, The Research Evaluation and Allocation Committee of the University of California, San Francisco, NIGMS grant number 15571 & 27791, Anesthesia Research Center Grant and the Anesthesia Research Foundation is gratefully acknowledged. We are indebted to our surgical colleagues, Drs. L. Way, C. Pelligrini, R. Williams, G. Zsigmond and their surgical residents for obtaining the liver biopsies.

REFERENCES

1. Belina, H., Cooper, S.D., Farkas, R., and Feuer, G. (1975) Biochem. Pharmacol. 24, 301-303.
2. Moore, L., Knapp, H.R., and Landon, E.C. (1977) Endocrinology 100, 1516-1520.
3. VonBahr, C., Groth, C. Jansson, H., Lundgren, G., Lind, M., and Glauman, H. (1980) Clin. Pharm. Ther. 27, 711-725.
4. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
5. Koblin, D.D., Dong, D.E., and Eger, E.I., II (1979) J. Phar. Exp. Ther. 211, 317-325.
6. Rouser, G., Siakotos, A.N., and Fleisher, S. (1966) Lipids 1, 85-86.
7. Davison, S.C., and Willis, E. (1974) Biochem. J. 140, 461-468.
8. Suzuki, Y., Depierre, J., and Ernster, L. (1980) Biochim. Biophys. Acta 601, 532-543.
9. Moore, L., Chen, T., Knapp, R., and Landon, E. (1975) J. Biol. Chem. 250, 4562-4568.
10. May, H., and McCay, P.B. (1968) J. Biol. Chem. 243, 2288-2295.
11. Jacobson, M., Levin, W., Lu, H., Conney, A.H., and Kuntzman, R. (1973) Drug Metab. Disp. 1, 766-774.
12. Levin, W., Lu, A., Jacobson, M., Kuntzman, R., Poyer, J.L., and McCay, P. (1973) Arch. Biochem. Biophys. 158, 842-852.
13. Colbeau, A., Nachbaur, J., and Vignais, P.M. (1971) Biochim. Biophys. Acta 249, 462-492.

[Received August 17, 1981]

Essential Fatty Acid-Supplemented Diet Decreases Renal Excretion of Immunoreactive Arginine-Vasopressin in Essential Fatty Acid-Deficient Rats

HARALD S. HANSEN, *Biochemical Laboratory, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen, Denmark*

ABSTRACT

Essential fatty acid (EFA)-deficient rats have been reported to have very concentrated urine and low urinary prostaglandin E_2 (PGE_2) excretion. Both parameters were normalized by feeding an EFA-supplemented diet (H.S. Hansen [1981] Lipids 16, 849-854). The urinary excretion rate of immunoreactive arginine-vasopressin (iAVP) has been determined in these rats. The iAVP excretion rate was high: ca. 4.8 mU/24 hr, during the EFA-deficient period compared to the controls, 0.7-1.3 mU/24 hr. One day after the dietary change, iAVP excretion rate was still high, but it decreased significantly ($p < 0.05$) at the second measurement 7 days later. It is suggested that the water-conserving effect of vasopressin 1 day after the dietary change was suppressed by the very high PGE_2 production, resulting in normal renal water excretion. PGE_2 and water excretion data were published in the paper just cited. *Lipids* 17:321-322, 1982.

Decreased urine output is one of the well known EFA deficiency symptoms (1). We recently reported that the decreased urine output in rats was mainly due to an efficient water-conserving mechanism in the kidney, resulting in a highly concentrated urine (2). The urine from these rats has been stored at -20 C for 1 year. Thus, to investigate whether this effect could be due to increased production of antidiuretic hormone, we have measured iAVP in the urine from these rats.

MATERIALS AND METHODS

Animals and Collection

Ten 21-day-old male Wistar rats (K. Møllegaard-Hansens Avlslaboratorium A/S, Ll. Skensved, Denmark) were divided into 2 groups of 5 animals each. Group 1 was reared on a semi-synthetic fat-free diet. Group 2 was reared on a diet with 28% arachis oil, i.e., the fat-free diet with 28% by wt of the sucrose replaced by 28% by wt of arachis oil. Thus, linoleic acid contributed 14 cal of the diet (3). After 25 weeks of feeding, the diets were switched between the 2 groups and fed for 3 weeks. Diets and water were supplied ad libitum throughout the experiment. The animals were housed in wire mesh cages, 2 animals per cage. Once a week, the rats were housed individually in metabolic cages for 24 hr with free access to water and food. The 24-hr urine was collected in a polyethylene tube packed in Dry Ice which ensured immediate freezing of the urine after voiding. The collected urine samples were kept at -20 C for 1 year before iAVP determinations were performed. The change of diet between the 2 groups was made 1 day before the weekly urine collection. The experimental feeding period started in June and ended in December. The humidity decreased from around 60% to around 40% within this period. Throughout the experiment, the temperature was 23 ± 1 C. The rats were kept in a 12-hr/12-hr light-dark period.

AVP-Radioimmunoassay

^{125}I-AVP, ca. 1215 μCi/μg was obtained from New England Nuclear, Boston, MA, and unlabeled AVP 345 U/mg was from Ferring AB, Malmø, Sweden. The AVP antiserum was kindly provided by Dr. Jens Dencker Christensen, Biological Laboratory, Royal Danish School of Pharmacy, Denmark. The RIA procedure was performed as described by Christensen and Jensen (4), with the exception that an initial ion-exchange column purification was omitted. Each urine sample was serially diluted with assay buffer in duplicate (1/256, 1/128, 1/64, 1/32, 1/16, 1/8) and 200 μl was used for the RIA incubation.

Statistical analysis was done using the paired and unpaired Student's t-test for intraindividual and interindividual comparison, respectively.

RESULTS AND DISCUSSION

iAVP was determined in the 24-hr urine obtained during the period 3 weeks before and 2 weeks after the dietary change (Fig. 1). The EFA-deficient rats (group 1) had significantly ($p < 0.002$) higher iAVP excretion rates than the controls (group 2).

Although the urine samples have been stored at -20 C for ca. 1 year, this should not affect

Abbreviations: EFA = essential fatty acid; PGE_2 = prostaglandin E_2; iAVP = immunoreactive arginine vasopressin; RIA = radioimmunoassay.

the iAVP levels as vasopressin at acid pH values is very stable (5,6). The pH value in the urine from both groups was ca. 5.6. The iAVP excretion rates of 0.7-1.3 mU/24 hr obtained in the present control rats are within the range of values reported by others: 0.4-0.6 mU/24 hr (7), 0.8-1.0 mU/24 hr (8), ca. 2.2 mU/24 hr (9), and 3.7 mU/24 hr (10). The EFA-deficient rats had a very concentrated urine, ca. 2,700 mOsmol/kg (2), and a ca. 4.8-fold higher iAVP excretion as compared to the controls. Moses and Miller (10) have shown that the maximal vasopressin excretion during dehydration in rats was ca. 5-fold higher than in the controls.

It cannot be ruled out that the high excretion rates of iAVP in the EFA-deficient rats were due to changes in vasopressin metabolism. However, if it is assumed that changes in urinary iAVP excretion reflect changes in plasma vasopressin concentrations, this could explain the very high urine osmolality seen in the same rats (2). Furthermore, this could also be the reason for the increased prostaglandin E-synthetase activity in the kidney of EFA-deficient rats, suggested from in vitro (11,12) and in vivo data (2). Beck and Dunn (13) have suggested that chronic high levels of vasopressin in vivo stimulate the activity of the cyclooxygenase in collecting tubule cells.

One day after the dietary change (measurement at week 25) urinary iAVP excretion was still high whereas—as reported earlier (2)—the urine output and urine osmolality was normalized, and urinary PGE_2 excretion was increased 8-fold being twice the values seen in the controls. It is suggested that the rapid increase in renal water excretion following EFA feeding to the EFA-deficient rats was caused by the concomitant increase in renal PGE_2 synthesis, which counteracted the effect of vasopressin. At the second measurement after the dietary change, the iAVP excretion was significantly decreased ($p < 0.05$), but it was still higher than that of the controls ($p < 0.001$). At week 27, the iAVP excretion rates were the same in the 2 groups.

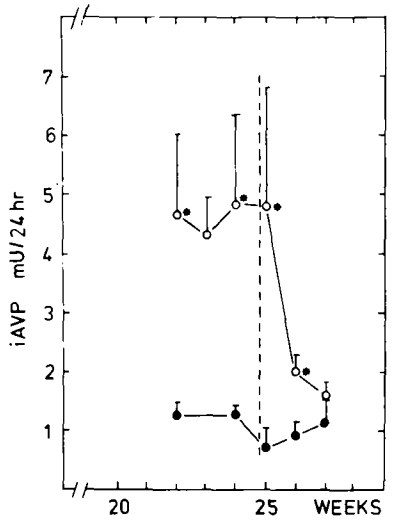


FIG. 1. iAVP urinary excretion of rats fed diets with or without linoleate. Twenty-one-day-old male rats were fed either a fat-free diet (group 1) o—o, or the same diet in which 28% by wt of sucrose was replaced by 28% by wt of arachis oil (group 2) •—•. The dashed line indicates that 1 day before the measurement at week 25, the diets were switched over between the 2 groups for the next 3 weeks. Once a week, the rats were housed individually in metabolic cages, and the 24 hr-urine was collected in a tube packed in Dry Ice. The bars indicate 1 SD for n=5. *Statistically significant difference ($p < 0.002$) compared to group 2.

ACKNOWLEDGMENTS

Drs. P. Bie and J.D. Christensen are thanked for helpful discussions. Birgit Nielsen provided skillful technical assistance. Vitamins were a gift from Hoffmann-La Roche and Co., Basel, Switzerland. Dietary lipids were generously supplied by Aarhus Oliefabrik Ltd., Aarhus, Denmark. The work has been supported by a research grant from the Danish Fat Research Foundation.

REFERENCES

1. Aaes-Jørgensen, E. (1961) Physiol. Rev. 41, 1-51.
2. Hansen, H.S. (1981) Lipids 16, 849-854.
3. Jensen, B. (1976) Lipids 11, 179-188.
4. Christensen, J.D., and S.E. Jensen (1978) Acta Endocrinol. 87, 283-291.
5. Nielsen, A.T. (1964) Posterior Pituitary Hormones. Assay, Standard Preparations, and Stability, Doctoral Thesis. Royal Danish School of Pharmacy, Kandrup & Wunsch Bogtrykkeri, Copenhagen.
6. British Pharmacopoeia (1980) Vol. II, pp. 676, London Her Majesty's Stationary Office, London.
7. Crofton, J.T., Share, L., Shade, R.E., Allen, C., and Tarnowski, D. (1978) Am. J. Physiol. 235, H361-H366.
8. Marchetti, J., Burlet, A., and Boulangé, M. (1978) Acta Endocrinol. 87, 292-302.
9. Jones, R.M., LaRochelle, F.T., Jr., and Tenney, S.M. (1981) Am. J. Physiol. 240, R182-R186.
10. Moses, A.M., and Miller, M. (1974) in Handbook of Physiology. The Pituitary Gland. Part 1. (Greep, R.O., and Astwood, E.B., section eds.) pp. 225-242, American Physiological Society, Washington DC.
11. Kaa, E. (1976) Lipids 11, 693-696.
12. Dunham, E.W., Balasingham, M., Privett, O.S., and Nickell, E.C. (1978) Lipids 13, 892-897.
13. Beck, T.R., and Dunn, M.J. (1981) Miner. Electrolyte Metab. 6, 46-59.

[Received November 9, 1981]

Sedimentation Equilibrium of Human Low Density Lipoprotein Subfractions[1]

TALWINDER S. KAHLON*, GERALD L. ADAMSON, MASON M.S. SHEN and FRANK T. LINDGREN, *Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720*

ABSTRACT

The molecular weights of low density lipoprotein (LDL) subfractions were determined precisely by meniscus depletion sedimentation equilibrium. Equilibrium speeds ranged from 9743 to 5896 rpm. The average molecular weights of various LDL subfractions of S_f^o values 9.49, 7.94, 6.42, 5.17, and 3.71 determined by sedimentation equilibrium were 2.97×10^6; 3.13×10^6; 2.89×10^6; 2.45×10^6; and 2.61×10^6 daltons, respectively; and their respective densities were 1.0267, 1.0306, 1.0358, 1.0422, and 1.0492 g/mL Minimal hydrated molecular weights for these fractions determined by flotation velocity at 37,020 rpm were 2.57×10^6; 2.37×10^6; 2.09×10^6; 1.94×10^6; and 1.81×10^6 daltons; whereas similar molecular weights determined at 52,640 rpm were 2.53×10^6; 2.27×10^6; 1.99×10^6; 1.86×10^6; and 1.74×10^6 daltons for the respective LDL subfractions. Higher molecular weights of fractions 2 and 5 compared to their adjacent fractions 1 and 4 by sedimentation equilibrium are of great interest. The calculated frictional ratio f/f^o from sedimentation equilibrium and flotation velocity data ranges from 1.10 to 1.31, suggesting complexity and asymmetry of LDL subfraction molecules. There is also evidence that compressibility of LDL molecules may be different than that for the salt solution under high g-force. Assuming that redistributed LDL molecules at equilibrium under low g-force are spherical, it is possible that the shape of LDL molecules undergoing flotation velocity determinations may be distorted in high g-force conditions. Such distortion may be consistent with the high f/f^o values obtained and may also be a basis for structural rearrangement and/or lipoprotein degradation with prolonged preparative ultracentrifugation at high g-force and pressure.

Lipids 17:323-330, 1982.

Particle diameters of low density lipoprotein (LDL) subfractions have previously been determined by electron microscopy (1), by laser light scattering (2), and by calculation from flotation-velocity MW determinations. In each case, LDL molecules have been assumed to be spherical. Such sphericity, or 1/1 axial ratio, needs to be investigated by studying LDL particles under low g-force at equilibrium, and by comparing the results with these earlier data.

Sedimentation equilibrium for the determination of MW is an accurate procedure because of its fundamental thermodynamic basis (3). The sedimentation or flotation velocity procedures, however, lead to inherent uncertainty because of such factors as frictional coefficient, high salt concentration, possible changes in both shape and partial specific volume ($\bar{v}$) under substantial centrifugal force (4). However, meniscus depletion sedimentation equilibrium results in consistent and accurate values, provided $\bar{v}$ (ml/g) is precisely known and true equilibrium is attained (5). This condition may not be present for fringe shifts observed over short (3-4-hr) intervals, and such observations may lead to wide discrepancies in values.

This study was undertaken to determine MW of LDL subfractions by sedimentation equilibrium at different rotor speeds between 5,000 and 10,000 rpm and to compare these values with those obtained by sedimentation velocity at 37,020 and 52,640 rpm (corrected for the discrepancies inherent in sedimentation velocity data).

MATERIALS AND METHODS

Lipoprotein Preparation

Four healthy normolipoproteinemic individuals (2 males and 2 females) were selected for this study. EDTA plasma was obtained from the fresh blood samples (0.1 mg EDTA/ml). After removing VLDL, LDL was isolated in sequence by ultracentrifugation (density 1.019 to 1.063 g/ml) at 17 C (6). Five LDL subfractions were obtained by density gradient ultracentrifugation in a swinging bucket SW 45 rotor (SW 45 Beckman special order rotor: 2.5 × 0.5 in.; 7-ml buckets; r min, 6.664 cm; and r max, 12.689 cm). This procedure is an approach to equilibrium after 40 hr of centrifugation at 40,000 rpm. Buoyant densities of

[1]MW = molecular weight; $\bar{v}$ = partial specific volume ($1/\rho$) using corresponding salt gradient density obtained from the background tube in LDL fractionation; R = molar gas constant; T = absolute temperature (degrees Kelvin); ρ = density of dialysate (1.0063 g/ml); ω^2 = angular velocity; ln c/r^2 = slope of ln concentration c vs r^2; r = distance from axis of rotation (cm).

LDL subfractions are close to equilibrium values. Density cuts for isolation of subfractions were measured by precision refractometry (7). Each lipoprotein fraction was dialyzed overnight at 4 C against ρ_{20} 1.063 g/ml and 1.203 g/ml (ρ1.0063 NaCl solution containing 10 mg EDTA/100 ml adjusted to appropriate densities by adding NaBr) (8) for flotation velocity measurements. Evaluation by salt background overnight dialysis with 3 changes showed results accurate to less than one part at the fourth decimal place. Refractive indices were determined at 26.0 ± 0.02 C with calibration at 20.0 C with a Mettler/Paar DMA-46 density meter. Sedimentation equilibrium was performed at 20.0 ± 0.05 C (RTIC-Beckman).

Sedimentation Equilibrium

Beckman Model E ultracentrifuges with Rayleigh interference optical systems were used for the sedimentation equilibrium procedure. LDL subfractions were dialyzed overnight at 4 C against ρ_{20} NaCl, 1.0063 g/ml, η_{20} 1.0226, pH 7.2-7.4 solution, for sedimentation equilibrium measurements. In the interference cells, 0.20 ml of the dialyzed lipoprotein was placed adjacent 0.21 ml ρ_{20} 1.0063 g/ml background solution. The air above the sample in the interference cell was displaced by nitrogen. LDL subfractions were run at 20 C between 5,000 and 10,000 rpm to achieve meniscus depletion sedimentation equilibrium (9), using An-D and An-J rotors. As soon as a rotor attained equilibrium speed, the first photographs correcting baseline optical distortion were taken. The rotor speed was increased by 20% over the equilibrium speed for 3 hr and then decreased to this equilibrium speed. Equilibrium fringes were obtained after 72 hr. True equilibrium was confirmed by photographs after 24-hr intervals to verify that there was no further change in the ln c vs r^2 plot. Zero fringe pictures were taken using a clear glass filter to properly align the Gaertner Model M2001RSP microcomparator. At equilibrium, a set of photographs was taken with variable exposure time to achieve the best equilibrium photographs. A second equlibrium experiment was performed at either a lower or higher speed, depending on the position of the initial equilibrium fringes. The fringe displacements (x_i,y_i) were measured with the Gaertner microcomparator. Molecular weight was determined by the equation[1]:

$$MW = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \ln c/r^2$$

Sedimentation Velocity

Sedimentation velocity ηF^o vs ρ data were obtained at 26 C at densities ρ_{26} 1.061 and 1.200 g/ml at 2 rotor speeds (37,020 and 52,640 rpm) in Beckman Model E ultracentrifuges equipped with schlieren optics. Schlieren patterns were analyzed by the procedure explained in detail by Lindgren et al. (8). Flotation velocities were determined at concentrations ranging from 2.5 to 3.0 mg LDL/ml. Flotation coefficients were further corrected for the concentration dependence factor (1-KC where K = 0.888 × 10^{-4} ml/g) in order to correct for any discrepancies due to concentration dependence. Any discrepancies in the concentration dependence factor (determined on total LDL) would be minimal as the samples were analyzed at nearly constant concentration. Unpublished observations from this laboratory (Lindgren, F.T., unpublished experiments) confirm linearity between $\rho^{20}_{1.063}$ and $\rho^{20}_{1.203}$, so adding a third point is not essential.

RESULTS

Following swinging bucket ultracentrifugation, the 1-g densities in the gradient corresponding to the LDL subfractions obtained from one background tube run in each fractionation are shown in Table 1. The highly reproducible gradient densities were: 1.0267 ± 0.0001; 1.0306 ± 0.0001; 1.0358 ± 0.0001; 1.0422 ± 0.0002; and 1.0492 ± 0.0006 g/ml for fractions 1, 2, 3, 4 and 5, respectively.

Dependence of peak flotation rates on g-force for 5 LDL subfractions is shown in Table 2. Peak $S^o_{f\ 1.063}$ ranged from 9.79 for fraction 1 to 3.74 for fraction 5 at 37,020 rpm; whereas at 52,640 rpm, it ranged from 9.19 to 3.68 for the same respective fractions. However, these consistent differences between the 2 speeds are statistically significant ($p \leq 0.05$) only for fraction 2, for which peak $S^o_{f\ 1.063}$ rate at 37,020 rpm is 8.11 ± 0.06 and that at 52,640 rpm is 7.74 ± 0.11. Also, there were no significant differences in $S^o_{f\ 1.063}$ in various LDL subfractions for male or female plasma with the 2 g-forces used (104,283 × g or 210,849 × g). However, it is of considerable interest that all mean S^o_f and $F^o_{1.20}$ values were higher at the lower g-force.

Table 3 shows dependence of peak $F^o_{1.20}$ flotation rate on g-force for the 5 LDL subfractions. Peak $F^o_{1.20}$ ranges from 36.99 for fraction 1 to 25.21 for fraction 5 at 37,020 rpm, and it ranges from 36.10 to 24.59 for the respective fractions at 52,640 rpm. On the average, 37,020 rpm results were 0.88 $F^o_{1.20}$ units higher compared to the 52,640 rpm

TABLE 1

1-g Gradient Densities[a] Corresponding to LDL Subfractions[b]

Sample no.	Fraction 1	2	3	4	5
5911[c]	–	1.0306	1.0355	1.0427	1.0499
5947[c]	1.0267	1.0303	1.0357	1.0418	–
5989[d]	1.0269	1.0308	1.0361	1.0423	1.0480
6015[d]	1.0264	1.0307	1.0356	1.0420	1.0497
Mean density	1.0267	1.0306	1.0358	1.0422	1.0492
± SEM	± 0.0001	± 0.0001	± 0.0001	± 0.0002	± 0.0006
Density at 40,000 rpm	1.0317	1.0403	1.0496	1.0592	1.0680
Pressure in atm at 40,000 rpm	106	223	353	496	653

[a]1-g represents 981 dynes pressure/g/cm/sec^2.

[b]The densities were determined by 5th-decimal-place precision refractometry, checked by pycnometry, and considered to be accurate to 2 parts at the 5th-decimal place.

[c]Female.

[d]Male.

values. These consistent differences were statistically significant ($p \leq 0.05$) only in fraction 5.

MW of the LDL subfractions determined by sedimentation equilibrium are shown in Table 4. Partial specific volume (reciprocal of buoyant density is given as best estimate of $\bar{v}$) for the fractions 1, 2, 3, 4 and 5 are 0.9740, 0.9703, 0.9654, 0.9595, and 0.9531 ml/g, respectively. Mean MW are $2.97 \pm 0.06 \times 10^6$; $3.13 \pm 0.10 \times 10^6$; $2.89 \pm 0.09 \times 10^6$; $2.45 \pm 0.07 \times 10^6$; and $2.61 \pm 0.17 \times 10^6$ daltons for fractions 1, 2, 3, 4 and 5, respectively. The optimal speed to reach equilibrium decreased as the fraction density increased; thus, equilibrium speed ranged from 9,743 to 5,896 rpm. In general, a higher equilibrium speed resulted in somewhat lower MW, but these differences were not statistically significant. The MW of LDL subfractions from male or female plasma were similar for fractions 1, 2, 3 and 4, whereas the statistical significance of the differences in fraction 5 could not be tested due to limited data.

Table 5 shows minimal anhydrous MW assuming a f/f^o ratio of 1.00, from ηF^o vs ρ flotation velocity data. At 37,020 rpm, MW are 2.57×10^6; 2.37×10^6; 2.09×10^6; 1.94×10^6; and 1.81×10^6 daltons for fractions 1, 2, 3, 4 and 5, respectively. At 52,640 rpm, the values for these respective fractions are 2.53×10^6; 2.27×10^6; 1.99×10^6; 1.86×10^6; and 1.74×10^6 daltons. However, these consistent differences in MW for each fraction at these 2 speeds are not statistically significant. Also, there are no significant differences in LDL subfraction MW from male or female plasma.

Table 6 shows densities of fractions 1-5 obtained by ρ intercept from ηF^o vs ρ flotation velocity data. These densities are 1.0178, 1.0233, 1.0277, 1.0331 and 1.0409 g/ml for fractions 1, 2, 3, 4 and 5 at 37,020 rpm. In contrast, these densities at 52,640 rpm are 1.0201, 1.0241, 1.0286, 1.0339 and 1.0407 g/ml for the respective fractions. These estimated hydrated densities obtained at 2 different g-forces were significantly higher at 52,640 rpm for fraction 1 ($p \leq 0.01$) and fraction 2 ($p \leq 0.05$) compared to the densities at 37,020 rpm.

Figure 1 provides a comparison of the mean particle diameters obtained by different techniques. Electron microscopy, as well as laser light scattering, give apparently significantly higher LDL subfraction particle diameters, whereas flotation velocity data give lower particle diameters at corresponding $S^o_{f\ 1.063}$ rates than our sedimentation equilibrium data.

Individual LDL subfractions in relation to the $S^o_{f\ 1.063}$ distribution for all male and female subjects are shown in Figure 2. In both females, the dominant subfraction was #2 (S^o_f 7.90 and 7.53). In contrast, the dominant subfractions in the males were fractions 3 and 4, with corresponding $S^o_{f\ 1.063}$ values of 6.32 and 4.29, respectively.

DISCUSSION

This study shows that the MW of LDL subfractions determined by sedimentation equilibrium are significantly higher than those determined by flotation velocity. MW determinations by sedimentation equilibrium do not fall into the pattern obtained by earlier flota-

TABLE 2

Dependence of $S^{\circ}_{f\ 1.063}$ Rate on g-Force

Fraction:	1		2		3		4		5	
rpm:	37020	52640	37020	52640	37020	52640	37020	52640	37020	52640
Sample no.										
5911[a]	–	–	8.15	7.90	6.71	6.31	5.43	5.12	3.86	3.79
5947[a]	9.55	9.01	8.00	7.53	6.69	6.36	5.56	5.20	–	–
5989[b]	10.15	9.49	8.28	8.00	6.84	6.32	5.67	5.52	3.77	3.61
6015[b]	9.67	9.06	7.99	7.53	6.20	5.89	4.61	4.29	3.59	3.63
Mean S°_{f}										
± SEM	9.79	9.19	8.11	7.74	6.61	6.22	5.32	5.03	3.74	3.68
	± 0.18	± 0.15	± 0.06[c]	± 0.11[d]	± 0.14	± 0.11	± 0.22	± 0.23	± 0.08	± 0.06
Overall mean										
S°_{f}	9.49		7.94		6.42		5.17		3.17	
ΔS°_{f}	0.60		0.37		0.39		0.29		0.07	

[a]Female.

[b]Male.

[c>d]($p < 0.05$).

TABLE 3

Dependence of $F^{o}_{1.20}$ Rate on g-Force

Fraction:	1		2		3		4		5	
rpm:	37020	52640	37020	52640	37020	52640	37020	52640	37020	52640
Sample no.										
5911[a]	–	–	34.31	33.48	31.06	29.95	27.92	27.43	24.98	24.57
5947[a]	36.14	35.46	33.20	33.02	29.80	28.86	28.42	27.84	–	–
5989[b]	38.69	37.40	34.82	33.66	31.18	29.36	29.53	27.80	25.46	24.68
6015[b]	36.13	35.45	32.92	31.95	29.04	28.13	25.41	24.49	25.18	24.51
Mean $F^{\circ}_{1.20}$	36.99	36.10	33.81	33.03	30.27	29.07	27.82	26.89	25.21	24.59
± SEM	± 0.85	± 0.65	± 0.40	± 0.34	± 0.52	± 0.39	± 0.78	± 0.72	± 0.14[c]	± 0.05[d]
Overall mean										
$F^{\circ}_{1.20}$	36.54		33.42		29.67		27.36		24.90	
$\Delta F^{\circ}_{1.20}$	0.89		0.78		1.20		0.92		0.62	

[a]Female.

[b]Male.

[c>d]($p < 0.05$).

TABLE 4

Molecular Weights of LDL Subfractions by Sedimentation Equilibrium (MW X 10^{-6})

Fraction:	1		2		3		4		5	
Avg. speed (rpm):	9141	9743	8718	9471	8165	9439	7851	8994	5896	7512
Sample no.										
5911[a]	–	–	3.22	3.16	3.16	–	2.58	–	3.14	–
5947[a]	2.90	2.84	3.02	3.22	2.58	2.62	2.46	2.65	–	–
5989[b]	3.10	3.07	3.60	3.27	3.12	2.91	2.56	2.43	2.61	2.35
6015[b]	3.10	2.80	2.81	2.69	2.87	2.65	2.23	2.11	2.21	2.23
Overall mean MW	3.03	2.90	3.16	3.09	2.93	2.73	2.46	2.40	2.65	2.29
± SEM	± 0.07	± 0.08	± 0.17	± 0.13	± 0.13	± 0.09	± 0.08	± 0.16	± 0.27	± 0.06
Overall mean MW	2.97 ± 0.06		3.13 ± 0.10		2.89 ± 0.09		2.45 ± 0.07		2.61 ± 0.17	
$S^{\circ}_{f\ 1.063}$	9.49		7.94		6.42		5.17		3.71	

SEM ± 35,000 daltons between 2 speeds
SEM ± 14,000 daltons within different fringe measurements (same plate)

[a]Female.
[b]Male.

TABLE 5

Minimum Anhydrous Molecular Weight (MW X 10^{-6}) of LDL Subfractions from ηF^{o} vs ρ Data ($f/f^{o} = 1.0$)

Fraction:	1		2		3		4		5	
rpm:	37020	52640	37020	52640	37020	52640	37020	52640	37020	52640
Sample no.										
5911[a]	–	–	2.43	2.34	2.18	2.09	1.94	1.92	1.77	1.72
5947[a]	2.48	2.46	2.29	2.20	2.02	1.94	1.99	1.96	–	–
5989[b]	2.76	2.67	2.48	2.36	2.18	2.01	2.12	1.91	1.84	1.76
6015[b]	2.46	2.46	2.26	2.19	1.98	1.90	1.72	1.65	1.83	1.74
Mean MW	2.57	2.53	2.37	2.27	2.09	1.99	1.94	1.86	1.81	1.74
± SEM	± 0.10	± 0.07	± 0.05	± 0.04	± 0.05	± 0.04	± 0.08	± 0.07	± 0.02[c]	± 0.01[d]
Mean $S^{\circ}_{f\ 1.063}$	9.49		7.94		6.42		5.17		3.71	
Δ MW	0.04		0.10		0.10		0.08		0.07	
f/f^{o}[e]	1.10	1.11	1.20	1.24	1.24	1.28	1.17	1.20	1.28	1.31

Mean f/f^{o} 37020 = 1.20 ± 0.03
Mean f/f^{o} 52640 = 1.23 ± 0.03
SEM ± 4100 daltons within duplicate schlieren film measurements (52640 rpm)

[a]Female.
[b]Male.
[c]>[d] ($p < 0.05$).
[e]f/f^{o} are calculated as (sedimentation equilibrium MW ÷ flotation velocity MW)$^{2/3}$.

TABLE 6

σ-Densities of LDL Subfractions Obtained from ηf^{o} vs ρ Data

Fraction:	1		2		3		4		5	
rpm:	37020	52640	37020	52640	37020	52640	37020	52640	37020	52640
Sample no.										
5911[a]	–	–	1.0238	1.0241	1.0282	1.0293	1.0324	1.0340	1.0398	1.0399
5947[a]	1.0179	1.0202	1.0230	1.0243	1.0265	1.0273	1.0323	1.0339	–	–
5989[b]	1.0183	1.0203	1.0237	1.0238	1.0275	1.0284	1.0329	1.0317	1.0409	1.0413
6015[b]	1.0172	1.0199	1.0227	1.0242	1.0287	1.0295	1.0349	1.0361	1.0419	1.0409
Mean σ	1.0178	1.0201	1.0233	1.0241	1.0277	1.0286	1.0331	1.0339	1.0409	1.0407
± SEM	± 0.0004[d]	± 0.0001[c]	± 0.0003[e]	± 0.0001[c]	± 0.0005	± 0.0005	± 0.0006	± 0.0009	± 0.0006	± 0.0004
Δ σ	0.0023		0.0008		0.0009		0.0008		0.0002	

[a]Female.
[b]Male.
[c]>[d]($p < 0.01$).
[c]>[e]($p < 0.05$).

tion velocity data (1,11,12); i.e., MW do not consistently decrease with decreasing flotation rate. MW of fraction 2 (ρ 1.0306 and S^{o}7.94) are higher than those of fraction 1 (ρ 1.0267 and S_f^{o} 9.49). Also, the MW of fraction 5 (ρ 1.0492 and S_f^{o} 3.71) are higher than those of fraction 4 (ρ 1.0422 and S_f^{o} 5.17). Similar observations were made by Fless and Scanu (13) in rhesus monkey LDL. This discrepancy is of great interest and may relate to the possible metabolic interconversion or noninterconversion of LDL subfractions during LDL catabolism. One sample was run at both 20 and 26 C, and MW determinations by sedimentation equilibrium were not affected over this temperature range. Adams and Schumaker (14) and Fisher (15) determined that an f/f^{o} factor of ca. 1.11 must be used to correct estimated MW obtained by flotation velocity; these molecular weights must be multiplied by $1.11^{3/2}$. Our results indicate that larger values of f/f^{o} than found by Adams and Schumaker (14) and Fisher (15) are required to bring MW determined from flotation velocity and from sedimentation equilibrium into agreement. Sedimentation equilibrium, because of its fundamental thermodynamic principles, provides a more accurate MW determination, independently of the shape of the molecule. The $\bar{v}$ were obtained from the corresponding background salt gradient. These densities are quite reproducible, as shown in Table 1. It took 48 min for the SW 45 rotor to decelerate from 40,000 rpm. The effect of salt diffusion while the rotor was slowing down was checked by accelerating and decelerating the rotor several times, and it did not change the salt density significantly (± 0.0001 g/ml). We assume that diffusion was occurring in both background and sample tubes essentially at the same rate during such deceleration. The frictional ratio f/f^{o} calculated from sedimentation equilibrium and flotation velocity (sedimentation equilibrium MW ÷ flotation velocity MW)$^{2/3}$ in this study result in values ranging from 1.10 for fraction 1 (ρ 1.0267 and S_f^{o} 9.49) to 1.31 for fraction 5 (ρ 1.0492 and S_f^{o} 3.71). Mean f/f^{o} for flotation velocity data at 37,020 and 52,640 rpm is 1.20 ± 0.03 and 1.23 ± 0.03, respectively. Although MW of LDL subfractions can be determined quite precisely at low g-forces (9,743 to 5,896 rpm), there exists wide variability in MW of fraction 5. This may be due to the potentially high Lp(a) in this one female subject. These results are not in conflict with previously published data showing differences in S_f^{o} rate between the sexes (8), but they do confirm the data (1) pointing to differences in lipoprotein concen-

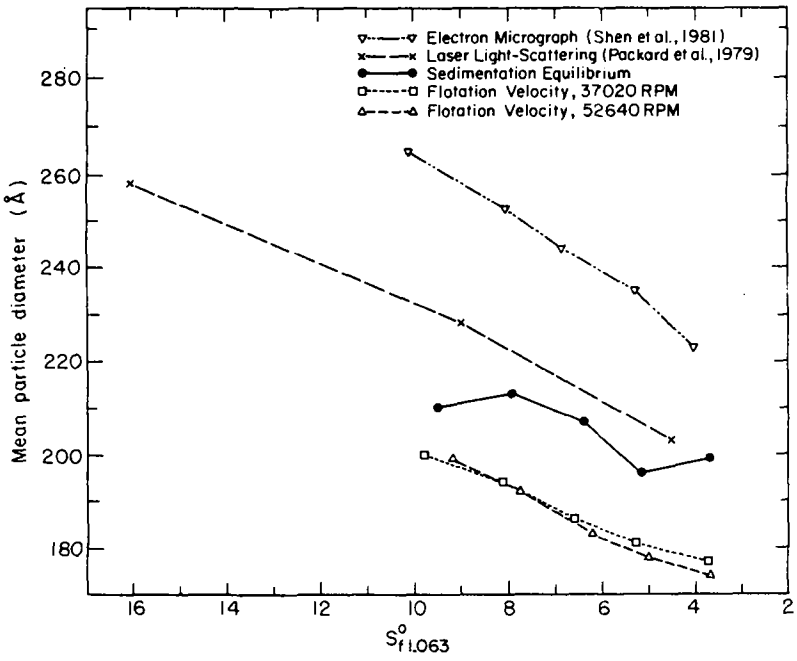


FIG. 1. Comparison of mean particle diameters, assuming spheres, by different techniques with flotation $S^{\circ}_{f\ 1.063}$ values. Particle diameters were obtained by the equation: molecular weight (daltons) = 0.3153 d^3 σ, where σ is the hydrated density and d is the diameter in Å (10). Data obtained by others (11) have not been compared because methods to obtain and define LDL subfractions were different, i.e., sequential preparative flotation as compared with equilibrium density gradient isolation.

tration among various subfractions. At equilibrium, ln c vs r^2 plots for all subfractions were extremely linear with correlation coefficients ranging from 0.9962 to 0.9998.

Densities of LDL subfractions obtained from ηF^{o} vs ρ flotation velocity data show that when g-force increased from 104,283 × g to 210,849 × g, the density increased 0.0023 density units for fraction 1 and 0.0002 density units for fraction 5. However, all density values from ηF^{o} vs ρ data are lower than corresponding 1-g densities of LDL subfractions. Flotation data using the 1-g densities ($\bar{v}$) would result in much lower MW; conversely, using ηF^{o} vs ρ densities for $\bar{v}$ in sedimentation equilibrium would make MW values much higher. In other words, such a correction would only further complicate analysis and would exaggerate these LDL MW discrepancies. Densities in Table 6 are derived from ηF^{o} vs ρ data extrapolated to zero migration rate and may not be as accurate at 1-g densities which could be measured precisely (Table 1). Adams (16) suggested that the compressibility of LDL is similar to that of the solvent under 200,000 × g force. Hence, 1-g densities may be the best estimate to be used for $\bar{v}$ in sedimentation equilibrium analysis. Nowhere in the literature has the true buoyant density of LDL been defined unequivocally.

Sedimentation equilibrium MW are calculated using $\bar{v}$ obtained from 1-g densities of corresponding salt gradient fractions. Values of $\bar{v}$ should be corrected for the discrepancies between the values of 1-g vs those existing under sedimentation equilibrium conditions. Discrepancies in 1-g densities vs those obtained from ηF^{o} vs ρ data might be due to differences in compressibility of LDL molecules as compared to the compressibility of salt solution, although, as suggested (16), such a difference may not exist. However, our data would indicate this may not be the case. LDL molecules redistributed under low g-force (2.5 atm) at equilibrium may be considered essentially spherical. Thus, particle diameters obtained on similar LDL subfractions (1) by electron microscopy gave LDL molecules with axial ratios ranging from 2.21/ 1.00 to 1.30/1.00 for S°_{f} 9.5 to S°_{f} 3.5, respectively. The average axial ratio obtained by electron microscopy, assuming sedimentation equilibrium to be correct, was ca. 1.65/1.00. Similarly, laser light scattering (2) data represented axial ratios of 1.52/ 1.00 to 1.03/1.00 for the respective S°_{f} rates of

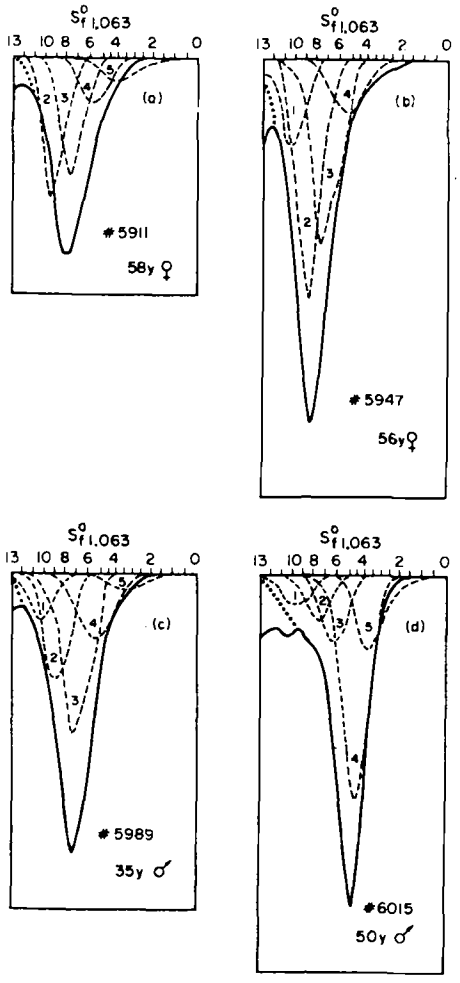


FIG. 2. $S^{o}_{f\ 1.063}$ schlieren plots of individual LDL subfractions (-----) and total LDL distribution (——) for all male and female subjects.

LDL molecules of corresponding normal individuals.

In this study, significant lower MW obtained by flotation velocity data, compared to sedimentation equilibrium data, suggest substantial molecular distortion of these LDL molecules under high g-force (104,283 × g and 210,849 × g) in high salt solutions and/or differences in compressibility between the lipoprotein molecules and the solvent.

ACKNOWLEDGMENTS

This research was supported by NIH Training Grant HL07279 and Program Project Grant HL18574. We wish to thank Dr. Virgie Shore, Dr. Ying Yang, Tony Silva and Tjet Sun for helpful suggestions. Special thanks to Dr. Verne Schumaker for extensive discussion.

REFERENCES

1. Shen, M.M.S., Krauss, R.M., Lindgren, F.T., and Forte, T.M. (1981) J. Lipid Res. 22, 236-244.
2. Packard, C.J., Shepherd, J., Joerns, S., Gotto, A.M., Jr., and Taunton, O.D. (1979) Biochim. Biophys. Acta 572, 269-282.
3. Svedberg, T., and Pedersen, K.O. (1940) The Ultracentrifuge, pp. 5-66, Clarendon Press, Oxford.
4. Lindgren, F.T. (1980) Ann. N.Y. Acad. Sci. 348, 1-15.
5. Yphantis, D.A. (1964) Biochemistry 3, 297-317.
6. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353.
7. Lindgren, F.T. (1975) in Analysis of Lipids and Lipoproteins (Perkins, E.G., ed.), pp. 204-224, J. Am. Oil Chem. Soc., Champaign, IL.
8. Lindgren, F.T., Jensen, L.C., Wills, R.D., and Freeman, N.K. (1969) Lipids 4, 337-344.
9. Chervenka, C.H. (1973) A Manual of Methods for the Analytical Ultracentrifuge, Beckman Instruments Co., Palo Alto, CA.
10. Nelson, G. (1972) Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism, p. 211, Wiley-Interscience, New York, NY.
11. Lee, D.M., and Alaupovic, P. (1974) Biochem. J. 137, 155-167.
12. Lindgren, F.T., Jensen, L.C., Wills, R.D., and Stevens, G.R. (1972) Lipids 7, 194-201.
13. Fless, G.M., and Scanu, A.M. (1979) J. Biol. Chem. 254, 8653-8661.
14. Adams, G.H., and Schumaker, V.N. (1969) Anal. Biochem. 29, 117-129.
15. Fisher, W.R. (1970) J. Biol. Chem. 245, 877-884.
16. Adams, G.H. (1966) A New Approach to the Study of Serum Lipoproteins, Ph.D. Thesis, University of Pennsylvania, Philadelphia, pp. 60-64.

[Received September 10, 1981]

Copper(II)-Catalyzed Lipid Peroxidation in Liposomes and Erythrocyte Membranes

PHILLIP C. CHAN*, OWEN G. PELLER and **LEO KESNER**, *Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, NY 11203*

ABSTRACT

Cu^{++} was uniquely capable of catalyzing the peroxidation of rat erythrocyte membrane lipid in the presence of 10 mM H_2O_2, whereas several other transition metal ions were without significant effect. In contrast, peroxidation of soybean phospholipid liposomes could be catalyzed with decreasing efficiency by Co^{++}, Cu^{++}, Pb^{++}, or Cr^{+++} also in the presence of H_2O_2. The effect of imidazole on Cu^{++}-catalyzed lipid peroxidation was stimulatory in liposomes and inhibitory in membrane preparations, whereas EDTA, histidine, citrate and alanine inhibited peroxidation in both systems. EDTA could stop the peroxidation after initiation, but catalase could not, indicating that Cu^{++} alone was necessary for the propagation of the chain reaction. Competitive inhibition studies with various scavengers of hydroxyl radicals or singlet oxygen and the absence of significant reaction enhancement by D_2O indicated that neither of these reactive oxygen species was a major mediator in the Cu^{++}-H_2O_2 oxidative system. A copper-oxygen complex may be directly involved in the initiation of peroxidation. Normal erythrocyte membranes and phospholipid liposomes also differ in their sensitivities toward external oxidative stress. In the absence of H_2O_2, Cu^{++} (0.2 mM) was capable of catalyzing lipid peroxidation in liposomes, aged erythrocyte membranes and membranes from vitamin-E-deficient rats; however, freshly prepared membranes from control rats and liposomes containing α-tocopherol required H_2O_2 greater than 2 mM for the catalytic effect of Cu^{++} to be observed.
Lipids 17:331-337, 1982.

Various reactive oxygen species have been implicated as mediators of biological membrane lipid peroxidation (1-6). Among them, hydroxyl radicals ($OH\cdot$) and singlet molecular oxygen ($^1O_2$) are considered to be the most reactive, and the most likely immediate oxidants of the polyunsaturated fatty acid side-chains of phospholipids. Other less reactive species, such as superoxide anion radicals ($O_2^{-}\cdot$) and hydrogen peroxide, have been postulated to be the precursors of either $OH\cdot$ (1,6-8), $^1O_2$ (3) or both (9,10). Metal ions have been suggested as catalysts for the eventual production of either $OH\cdot$ or $^1O_2$ (1,3,6-8,11,12). Alternatively, metal ion-oxygen complexes have also been proposed as the proximate reactive species for oxidation of lipid, protein and nucleic acids (12-16).

In earlier reports, we have shown that Cu^{++} was capable of catalyzing the oxidation of NADH by H_2O_2 (15), and the degradation of lysozyme by H_2O_2 (16) without detectable involvement of either $^1O_2$ or $OH\cdot$.

In this study, we examine the initiation of lipid peroxidation by Cu^{++}-H_2O_2 and the difference between the peroxidation of phospholipid liposomes and erythrocyte membranes.

MATERIALS AND METHODS

Asolectin, a phospolipid mixture extracted from soybeans, was purchased from Associated Concentrates (Woodside, NY); catalase (beef liver) from Boehringer Mannheim Biochemicals; 99.8% D_2O from Bio-Rad Laboratories; DABCO from Matheson, Coleman and Bell; 2,5-dimethylfuran from the Aldrich Chemical Co., and vitamin E (α-tocopherol) from Sigma Chemical Co. All solutions were prepared in glass-distilled water.

Preparation of Liposomes

Asolectin was suspended in water at a concentration of 5 mg/ml with a Teflon-pestle tissue grinder. The suspension was then treated for 5 min in a Branson bath-type ultrasonifier, Model 452E. The absorbance of the resulting suspension, measured at 650 nm in a 1-cm cell, was between 0.10 and 0.25. In the experiments in which vitamin E was incorporated into the liposomes, vitamin E and Asolectin were dissolved in 1-2 ml of chloroform, and then evaporated to dryness under nitrogen. The lipid mixture was then suspended in water and sonicated as just described.

Preparation of Erythrocyte Membranes

Male Wistar rats were fed a Purina rat chow (vitamin E content = 38 IU/kg) and provided with distilled water ad lib. Four-to 6-month-old animals were used for normal erythrocyte membranes. Experimental rats were fed a vitamin-E-deficient diet (ICN National Biochemical, catalog no. 904640) for 6 weeks prior to use. Blood

Abbreviations: TBA = 2-thiobarbituric acid, DABCO = 1,4-diazabicyclo[2.2.2]octane.

was withdrawn by cardiac puncture with heparin as an anticoagulant.

Erythrocyte membranes were prepared according to the procedure of Dodge et al. (17) with some modification (4). A 7-mM sodium phosphate, pH 7.8, buffer was used for washing and final suspension of membranes instead of the Tris-EDTA buffer previously described (4). The membrane suspension was stored at -20 C and used within 1 week.

Lipid Peroxidation Determination

The standard incubation mixture containing either liposomes or membranes was suspended in 5 mM sodium phosphate, pH 7.5, $CuSO_4$ and H_2O_2 as indicated. A typical reaction mixture contained either 2.5 mg of Asolectin or 0.6 mg membrane protein in a total vol of 1.0 ml. $CuSO_4$ was preincubated with either membranes or Asolectin for 10 min prior to addition of H_2O_2, and then the total mixture was incubated at 37 C for 1 hr in a constant-temperature shaking water bath. The reaction was stopped by addition of 20 μl 0.5 M EDTA and 20 μl catalase (1 mg/ml). Lipid peroxidation products reactive with TBA (18) were determined according to the procedure of Bidlack and Tappel (19) and expressed as the equivalent amount of malonaldehyde. All data presented, with the exception of those in Figure 3, are from representative experiments. Each experiment was repeated in 3 or more different preparations with consistent results.

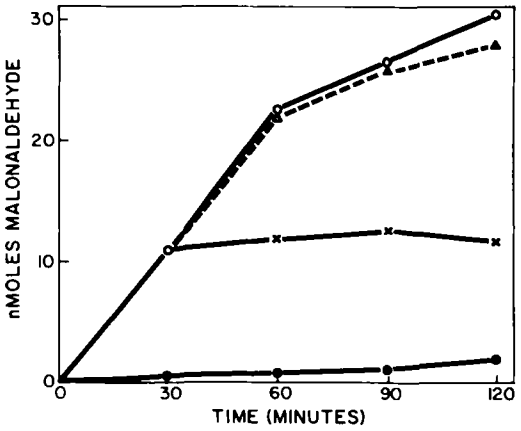


FIG. 1. Effect of Cu^{++} and H_2O_2 on lipid peroxidation in erythrocyte membranes. Lipid peroxidation was determined by TBA-reactive products, expressed as equivalents of malonaldehyde/sample. The procedures were as described in Methods. (○), Complete incubation mixture containing 0.2 mM $CuSO_4$ and 10 mM H_2O_2; (△), complete system followed by addition of 20 μl of catalase (1 mg/ml) at 30 min; (X), complete system followed by addition of 20 μl 0.5 M EDTA at 30 min; (●), incubation with 0.2 mM $CuSO_4$, without H_2O_2.

RESULTS AND DISCUSSION

When rat erythrocyte membranes were incubated with Cu^{++} and H_2O_2, lipid peroxidation was observed as indicated by the increase of TBA-reactive products in Figure 1. When H_2O_2 was omitted, no significant peroxidation was detected in the presence of Cu^{++} alone. The chelation of Cu^{++} by EDTA after 30 min of incubation prevented any further net formation of TBA-reactive material. On the other hand, when H_2O_2 was decomposed by the addition of catalase at 30 min, peroxidation continued at a rate similar to that of the control, indicating that although both Cu^{++} and H_2O_2 were required for the initiation of peroxidation, Cu^{++} alone was necessary for the propagation of the peroxidation. When the reaction was done anaerobically, under N_2, no significant peroxidation was detected (data not shown), suggesting the dependence on O_2 as the terminal electron acceptor in the propagation of the peroxidation. This is in agreement with the often described chain mechanism of autoxidation of unsaturated lipids (18,20-22), in which molecular oxygen serves as an oxidant in the propagation steps.

Metal Ion Specificity

Several other transition metal ions were compared with Cu^{++} for their ability to catalyze lipid peroxidation in both the Asolectin liposomes and erythrocyte membranes. Table 1 indicates that there are substantial differences between the 2 systems. With liposomes, H_2O_2-induced peroxidation was observed in the presence of Co^{++}, Cu^{++}, Pb^{++}, or Cr^{+++}. In erythrocyte membranes, however, Cu^{++} was unique in its ability to catalyze the initiation of oxidation by H_2O_2. No significant activity was observed with any of the other metal ions tested. The difference may be attributed to the presence of membrane proteins with exposed functional groups capable of selectively binding Cu^{++} ions in such a way as to promote catalysis.

Fe^{++} catalyzed peroxidation in both liposomes and membranes, but peroxidation was not enhanced by addition of H_2O_2. Decomposition of H_2O_2 by Fe^{++} to form $OH^{\cdot}$ has often been called Fenton's reaction, which has been shown to promote oxidation of microsomal phospholipid liposomes (6):

$$H_2O_2 + Fe^{++} \longrightarrow OH^{\cdot} + OH^{-} + Fe^{+++}.$$

The excess H_2O_2 present in our system probably served as a scavenger for $OH^{\cdot}$ according to the following reaction (23):

$$H_2O_2 + OH^{\cdot} \longrightarrow HO_2 + H_2O.$$

TABLE 1

Metal Ion-Catalyzed Lipid Peroxidation[a]

Metal ion	Asolectin		Membranes	
	$-H_2O_2$	+8 mM H_2O_2	$-H_2O_2$	+8 mM H_2O_2
(0.2 mM)	(nmol malonaldehyde)			
$CuSO_4$	4.0	7.8 (100)[b]	1.8	18.8 (100)[b]
$FeCl_3$	0.6	0.8 (10)	0.8	0.6 (3)
$FeCl_3$ + 1 mM ADP	1.0	0.9 (12)	0.7	0.6 (3)
$FeSO_4$	7.8	7.6 (97)	7.5	3.7 (20)
$FeSO_4$ + 1 mM ADP	7.5	7.1 (91)	7.1	6.6 (35)
$CoCl_2$	0.8	11.8 (151)	0	0.4 (2)
$Cr(NO_3)_3$	0.1	3.8 (50)	0.1	0.4 (2)
$Pb(NO_3)_2$	0.1	4.7 (60)	0.5	0.2 (1)
$ZnSO_4$	0	0	0.8	0.4 (2)
$MnSO_4$	0	0	0.8	0.2 (1)
$CdCl_2$	0	0	0	0.2 (1)

[a]The reaction mixture contained either Asolectin liposomes (2.5 mg) or erythrocyte membranes (0.6 mg protein). Lipid peroxidation was determined as described in Methods.

[b]The numbers within parentheses compare the catalytic activities of all metal ions in the presence of H_2O_2 to that of Cu^{++}, which is arbitrarily designated as 100. In the absence of added metal ions, the malonaldehyde equivalent in Asolectin liposomes and membranes were 0.9 and 1.2 nmol, respectively. No significant change was observed with H_2O_2 addition. These control values were subtracted from all the samples with added metal ions.

TABLE 2

Effects of Ligands on Cu^{++}-Catalyzed Peroxidation[a]

Addition	Asolectin		Membranes	
	Malonaldehyde (nmol)	Inhibition (%)	Malonaldehyde (nmol)	Inhibition (%)
Control	7.18	–	25.6	–
1 mM EDTA	0.78	89	0	100
1 mM L-Histidine	2.67	63	4.1	84
1 mM Citrate	0.51	93	7.3	72
1 mM L-Alanine	3.77	47	16.9	34
1 mM Imidazole	6.89	4	1.4	94
5 mM Imidazole	10.59	(+47)[b]	0.5	98

[a]The reaction mixture contained either Asolectin liposomes (2.5 mg) or erythrocyte membranes (0.6 mg protein), 0.2 mM $CuSO_4$, 8 mM H_2O_2, 5 mM sodium phosphate, pH 7.5, and addition as indicated, in a final vol of 1.0 ml. Lipid peroxidation was determined as described in Methods.

[b]Percentage of enhancement.

Consequently, no potentiation of lipid peroxidation was detected by addition of H_2O_2 in the presence of either Fe^{++} or ADP-Fe^{++} (Table 1). Under physiological conditions, however, there are only trace amounts of H_2O_2, which may be expected to react with Fe^{++} to promote lipid peroxidation.

Metal Ion Ligands

In addition to EDTA, other metal ion chelators having strong affinities for copper, including histidine, citrate, and alanine, were able to inhibit Cu^{++}-H_2O_2-catalyzed lipid peroxidation, presumably by competing for Cu^{++} (Table 2). At high concentrations, complete inhibition was achieved by each of these chelators (data not shown).

Imidazole can serve as a ligand for Cu^{++}, forming complexes of 1, 2, 3, or 4 ligands per Cu^{++} ion (24), some of which can catalyze decomposition of H_2O_2 (24) and oxidation of NADH by H_2O_2 (15) at much higher rates than can Cu^{++} alone. Five mM imidazole stimulated the peroxidation of liposomes by 47%; however, in contrast, most lipid peroxidation of erythrocyte membranes was abolished by either 1 or 5 mM of imidazole. This difference may also be attributed to the membrane proteins, which may hinder the accessibility of imidazole-Cu^{++} complexes to the sites of peroxida-

tion.

Scavengers for $OH^{\cdot}$ and $^1O_2$

Benzoate, formate, mannitol and methanol are wellknown scavengers for $OH^{\cdot}$ radicals which can react with the radical at diffusion-controlled rates (25). Table 3 shows that, at 20 mM, none of these compounds inhibited peroxidation of either liposomes or erythrocyte membranes, apparently excluding the hydroxyl radical as an intermediate in the process. In contrast, when lipid peroxidation was initiated by Fenton reaction-generated $OH^{\cdot}$ radicals, Tien et al. (6) observed that over 95% inhibition was caused by 10 mM mannitol; and, in another study on lipid peroxidation by ionizing radiation (26), over 65 and 55% inhibition was found with 10 mM formate and 10 mM ethanol, respectively.

DABCO and dimenthylfuran are often used as quenchers for singlet oxygen. As can be seen from Table 3, in the membrane system, DABCO and dimethylfuran inhibited lipid peroxidation by only 27 and 10%, respectively. In order to further test the possible involvement of $^1O_2$, the reaction was done in 96% D_2O, which did not potentiate membrane peroxidation (Table 3). Because the mean lifetime of $^1O_2$ in D_2O is ca. 10 times longer than that in H_2O (27), a significant potentiation of peroxidation would be expected if $^1O_2$ were the reactive species. With Asolectin liposomes, neither DABCO nor dimethylfuran caused any inhibition of peroxidation, and although 26% potentiation was observed in D_2O, such stimulation is not significant when compared with other systems where $^1O_2$ is known to be involved. As an example, more than a 5-fold increase in the oxidation of NADPH by photochemically generated $^1O_2$ was observed when H_2O was replaced with D_2O (28).

These results do not support a role for $OH^{\cdot}$ or $^1O_2$ as major mediators in the peroxidation process catalyzed by Cu^{++}-H_2O_2 in our systems. This differs from various other systems of lipid peroxidation (1-3,6-8) in which either $^1O_2$ or $OH^{\cdot}$ or both have been implicated as the primary mediators. The possibility that either $^1O_2$ or $OH^{\cdot}$ may be produced at the actual site of peroxidation, thus preventing interference by scavengers, cannot be excluded. Because the superoxide radical is rapidly dismutated in the presence of copper complexes (11), it is also an unlikely participant in this peroxidation process.

Ingraham (13) has proposed $Cu^{+}OOH$ as a possible intermediate in the Cu^{++}-catalyzed peroxidation of catechol:

$$Cu^{++} + H_2O_2 \longrightarrow Cu^{+}OOH + H^{+}.$$

He has also indicated that $Cu^{+}OOH$ can serve as a reactive oxidant for both 1-electron and 2-electron reactions. Other species such as $Cu^{+}O$ and CuO_2OH have also been suggested as possible oxidizing species (13). The present study does not suggest which, if any, of these intermediates is the actual proximate reactant.

Difference in Sensitivity toward Oxidative Stress

One distinct difference between Asolectin liposomes and freshly prepared erythrocyte membranes is in their sensitivities toward external oxidative stress. As seen in Table 4, H_2O_2 alone did not cause any detectable lipid peroxidation in either system. When 0.2 mM $CuSO_4$ without H_2O_2 was used, significant peroxidation was observed with liposomes, but not with fresh membrane preparations. In the presence of $CuSO_4$, 2 mM H_2O_2 further stimulated peroxidation of liposomes, but did not cause significant oxidation of the fresh membrane. Peroxidation of the fresh membrane was observed only at high levels of H_2O_2 (10 and 20 mM) in the presence of Cu^{++}.

Table 4 also reveals that when the membrane preparation was stored for 5 months at -20 C, its resistance to low levels of oxidative stress was lost. The response of aged membranes to external oxidative stress was similar to that of liposomes in that both were now susceptible to oxidation by 0.2 mM $CuSO_4$. This may be a reflection of some degree of autoxidation in the stored membranes. Hydroperoxide determinations (29) revealed only 2 nmol of hydroperoxide/mg of membrane protein in the fresh preparation in contrast with 30 nmol/mg of protein in a 9-month-old preparation. The Asolectin liposome contained 6 nmol of hydroperoxide/mg phospholipid.

Effect of Vitamin E

One possible explanation for the different response to external oxidative stress exhibited by liposomes and erythrocyte membranes may be the presence of endogenous antioxidants in the erythrocyte membrane. Because vitamin E is considered the major biological antioxidant for chain breaking in lipid peroxidation (30), this hypothesis was tested by evaluating the susceptibility to oxidation of vitamin-E-containing liposomes. Figure 2 shows that incorporation of vitamin E into the liposome could increase its resistance to peroxidation by Cu^{++} and low levels of H_2O_2. At 20 μg of vitamin E/mg phospholipids, no significant peroxidation was observed until H_2O_2 was increased to 10 mM, a situation similar to that seen in the fresh membrane preparation (Table 4).

On the basis of in vivo studies, Tappel (30)

TABLE 3

Effects of Scavengers on Reactive Oxygen Species and D_2O on Cu^{++}-Catalyzed Peroxidation[a]

Addition	Asolectin		Membranes	
	Malonaldehyde (nmol)	Inhibition (%)	Malonaldehyde (nmol)	Inhibition (%)
Control	7.18	–	25.6	–
20 mM Na Benzoate	8.18	–	24.0	6
20 mM Na Formate	8.23	–	24.4	5
20 mM D-Mannitol	8.28	–	26.0	–
20 mM Methanol	7.11	1	26.7	–
20 mM DABCO	7.45	–	18.7	27
9.3 mM 2,5-Dimethylfuran	7.45	–	22.9	10
In 96% D_2O	9.08	(+26)[b]	24.3	5

[a]Similar procedure as in Table 2.

[b]Percentage of enhancement.

TABLE 4

Effect of Cu^{++} and H_2O_2 on Asolectin and Membrane Lipid Peroxidation[a]

Addition	Asolectin	Membranes[b]	
		Fresh	5-Month
	(nmol of malonaldehyde)		
20 mM H_2O_2	0	0	0.3
0.2 mM $CuSO_4$	2.8	0.4	17.5
0.2 mM $CuSO_4$ + 2 mM H_2O_2	6.1	0.5	20.0
0.2 mM $CuSO_4$ + 10 mM H_2O_2	6.0	11.0	22.5
0.2 mM $CuSO_4$ + 20 mM H_2O_2	5.0	26.8	17.0

[a]The procedure was as described in Table 1 and in Methods. The results were corrected for malonaldehyde in the control tubes without additions. The malonaldehyde values of controls for Asolectin, fresh and 5-month-old membrane preparations were 0.6, 0.6, and 0.5 nmol, respectively.

[b]Erythrocyte membrane preparations were stored at -20 C. Fresh preparations refer to the samples used within one week.

has postulated that lipid peroxidation in the body is inversely proportional to tissue vitamin E content, which is itself proportional to the log of dietary vitamin E. Brownlee et al. (9) have reported that the erythrocytes of vitamin-E-deficient rats were more sensitive than controls to external oxidant stress, resulting in increased lipid peroxidation and hemolysis. From Figure 3, it can been seen that at all concentrations of H_2O_2 tested, erythrocyte membranes from vitamin-E-deficient rats were more susceptible to lipid peroxidation than those from rats receiving normal diets. The effect was more significant at low levels of H_2O_2 (0-6 mM). In vitamin-E-deficient membranes, lipid peroxidation was detected when incubated with Cu^{++} alone in the absence of H_2O_2, similar to the aged membrane preparation (Table 4), indicating the presence of hydroperoxide prior to incubation.

Liposomes of varying compositions and structures are frequently used as models for biological membranes in studies involving lipid peroxidation (3,5,7,8,31). Our study has shown that these 2 systems do not always behave similarly, which may be due to differences in the lipid and fatty acid compositions and the presence of proteins and other endogenous components in biological membranes. Metal ion-catalyzed lipid peroxidation by H_2O_2 was apparently specific for Cu^{++} in the membrane, but not in the liposome preparations (Table 1). Membrane peroxidation was inhibited by imidazole ligands, whereas liposome peroxidation was enhanced (Table 2). This may be due to interaction of protein functional groups with either metal ions or their complexes.

The relative resistance of fresh membranes to external oxidative stress, compared to liposomes, was probably attributable to endogenous antioxidants such as vitamin E. This hypothesis was supported by the experiments in which vitamin-E-containing liposomes were

found to be resistant to peroxidation (Fig. 2).

In the physiological situation, a complex mixture of toxic oxygen species, their scavengers, metal ions and their ligands exists. Lipid peroxidation may be accelerated by a relative change in concentration of any one of more of these factors. This becomes obvious in certain disease states and in aging phenomenon (32). Thus, in Wilson's disease (33), primary biliary cirrhosis (34) and rheumatoid arthritis (35), abnormal distribution of copper may interact with endogenously generated H_2O_2 to damage membrane lipids and other biological components. Degradation of nucleic acids (36) and proteins (16,37) by Cu^{++} and H_2O_2 have also been reported.

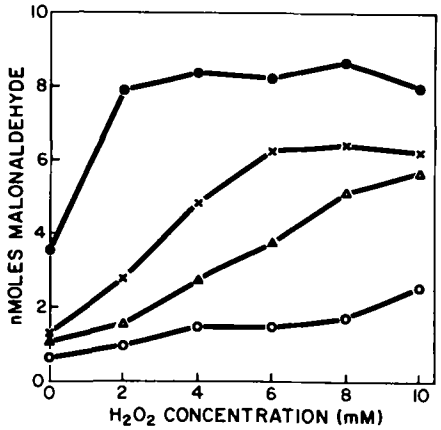


FIG. 2. Effect of α-tocopherol on Cu^{++}-H_2O_2-induced lipid peroxidation in Asolectin liposomes. Liposomes were prepared as described in Methods with varying amount of vitamin E/mg Asolectin: (•), none; (X), 2 μg; (△), 6 μg; and (○), 20 μg. Peroxidation in the presence of 0.2 mM $CuSO_4$ and varying amounts of H_2O_2 was determined as described in Methods.

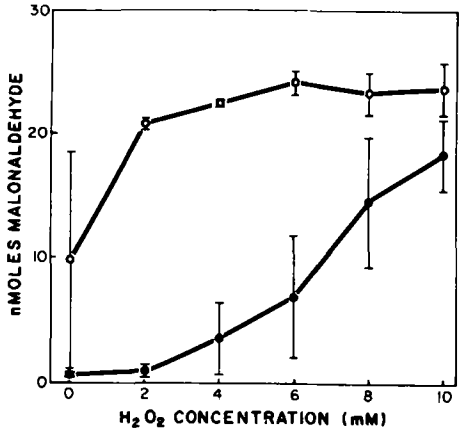


FIG. 3. Effect of H_2O_2 concentration on Cu^{++}-catalyzed peroxidation in membranes from vitamin-E-deficient and control rats. Membrane lipid peroxidation in the presence of 0.2 mM $CuSO_4$ was determined as described in Methods. The average values for membranes from 3 rats are presented: (•), control; (○), vitamin-E-deficient. The vertical bars indicate ± SEM. The p values at 2, 4 and 6 mM of H_2O_2 were < 0.001, 0.01, and 0.05, respectively (Student's t-test).

ACKNOWLEDGMENTS

This work was supported in part by a grant from the State University of New York Research Foundation. We are grateful to Wanshang Yu for excellent technical assistance.

REFERENCES

1. Fong, K.L., McCay, P.B., Poyer, J.E., Keele, B.B., and Misra, H.(1973) J. Biol. Chem. 248, 7792-7797.
2. King, M.M., Lai, E.K., and McCay, P.B. (1975) J. Biol. Chem. 250, 6496-6502.
3. Kellogg, E.W., III, and Fridovich, I. (1977) J. Biol. Chem. 252, 6721-6728.
4. Kesner, L., Kindya, R.J., and Chan, P.C. (1979) J. Biol. Chem. 254, 2705-2709.
5. Logani, M.K., and Davis, R.E. (1980) Lipids 15, 485-495.
6. Tien, M., Svingen, B.A., and Aust, S.D. (1981) Fed. Proc. 40, 179-182.
7. Gutteridge, J.M.C., Richmond, R., and Halliwell, B. (1979) Biochem. J. 184, 469-472.
8. Fridovich, S.E., and Porter, N.A. (1981) J. Biol. Chem. 256, 260-265.
9. Brownlee, N.R., Huttner, J.J., Panganamala, R. V., and Cornwell, D.G. (1977) J. Lipid Res. 18, 635-644.
10. Witting, L.A. (1980) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. 4, pp. 295-319, Academic Press, New York, NY.
11. Fee, J.A. (1980) in Metal Ion Activation of Dioxygen (Spiro, T.G., ed.) pp. 209-237, John Wiley and Sons, Inc., New York, NY.
12. Brawn, K., and Fridovich, I. (1981) Arch. Biochem. Biophys. 206, 414-419.
13. Ingraham, L.L. (1959) Arch. Biochem. Biophys. 81, 309-318.
14. Hodgson, E.K., and Fridovich, I. (1975) Biochemistry 14, 5294-5299.
15. Chan, P.C., and Kesner, L. (1980) Biol. Trace Element Res. 2, 159-174.
16. Chung, M.H., Kesner, L., and Chan, P.C. (1981) Fed. Proc. 40, 1613.
17. Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119-130.
18. Pryor, W.A., Stanley, J.P., and Blair, E. (1976) Lipids 11, 370-379.
19. Bidlack, W.R., and Tappel, A.L. (1973) Lipids 8, 177-182.
20. Dahle, L.K., Hill, E.G., and Holman, R.T. (1962) Arch. Biochem. Biophys. 98, 253-261.
21. Mead, J.F. (1976) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. 1, pp. 51-68, Academic Press, New York, NY.
22. Frankel, E.N. (1980) Prog. Lipid Res. 19, 1-22.
23. Walling, C. (1975) Acc.Chem. Res. 8, 125-131.
24. Sharma, V.S., and Schubert, J. (1969) J. Am. Chem. Soc. 91, 6291-6296.

25. Anbar, M., and Neta, P. (1967) Int. J. Appl. Radiat. Isot. 18, 493-523.
26. Raleigh, J.A., and Kremers, W. (1978) Int. J. Radiat. Biol. 34, 439-447.
27. Merkel, P.B., and Kearns, D.R. (1972) J. Am. Chem. Soc. 94, 7244-7253.
28. Bodaness, R.S., and Chan, P.C. (1977) J. Biol. Chem. 252, 8554-8560.
29. Buege, J.A., and Aust, A.D. (1978) Methods Enzymol. 52, 302-310.
30. Tappel, A.L. (1980) Ann. N.Y. Acad. Sci. 355, 18-29.
31. Ryman, B.E., and Tyrrell, D.A. (1980) Essays Biochem. 16, 49-98.
32. Harman, D., Hendricks, S., Eddy, D.E., and Seibold, J. (1976) J. Am. Geriatr. Soc. 24, 301-307.
33. Dobyns, W., Goldstein, N., and Gordon, H. (1979) Mayo Clin. Proc. 54, 35-42.
34. Mason, K.E. (1979) J. Nutr. 109, 1979-2066.
35. Underwood, E.J. (1962) in Trace Elements in Human and Animal Nutrition, pp. 48-99, Academic Press, New York, NY.
36. Massie, R., Samis, H.V., and Baird, M.B. (1972) Biochim. Biophys. Acta 272, 539-548.
37. Phelps, R.A., Neet, K.E., Lynn, L.T., and Putnam, F.W. (1961) J. Biol. Chem. 236, 96-105.

[Received September 10, 1981]

Interaction of Discoidal Complexes of Dimyristoyl Phosphatidylcholine-Cholesterol-Apolipoprotein A-I with Human Plasma High Density Lipoprotein HDL_3

RICHARD L. JACKSON, ALAN D. CARDIN, ROGER L. BARNHART, MUHAMMAD ASHRAF and **J. DAVID JOHNSON**, *Division of Lipoprotein Research, Departments of Pharmacology and Cell Biophysics, Biological Chemistry, Medicine, and Pathology, University of Cincinnati Medical Center, Cincinnati, OH 45267*

ABSTRACT

The interaction of human plasma high density lipoproteins (HDL_3) with discoidal complexes of apolipoprotein A-I (apoA-I) and dimyristoyl phosphatidylcholine (DMPC) containing 0, 10, 20 or 30 mol % cholesterol was investigated. Discoidal complexes containing various amounts of cholesterol were prepared by incubating apoA-I and DMPC-cholesterol liposomes for 12 hr at 25 C; the protein-lipid complexes were isolated by gel filtration chromatography on Bio-Gel A15m. Increasing the cholesterol content from 0 to 30 mol % caused a decrease in the fluidity of the discoidal complexes as determined by fluorescence polarization with 1,6-diphenyl-1,3,5-hexatriene; a reduced phase-transition amplitude; a decrease in the ratio of apoA-I to DMPC; and an increase in the width of the discoidal complexes as determined by electron microscopy after negative staining. Incubation of the apoA-I-lipid complexes with HDL_3 resulted in a complete breakdown of the discoidal structures and a transfer of DMPC and cholesterol to HDL_3. As a result of lipid transfer, there was an increase in the size of HDL_3. These in vitro results may be of significance as they relate to the interconversion of HDL subfractions during lipoprotein-lipase-induced lipolysis of triglyceride-rich lipoproteins.
Lipids 17:338-344, 1982.

INTRODUCTION

Human plasma high density lipoproteins (HDL) are a polydisperse mixture of spherical-shaped particles of hydrated density 1.063-1.210 g/ml (1). Patsch et al. (2) have fractionated HDL by rate-zonal ultracentrifugation into 2 major subclasses, HDL_2 ($F^o_{1.20}$ = 3.5-9.0) and HDL_3 ($F^o_{1.20}$ = 0-3.5). The lipoprotein precursors for the HDL subfractions are not known for certain. It is known that discoidal HDL are synthesized in the intestine (3) and liver (4) or are produced from the catabolism of triglyceride-rich lipoproteins (5,6). The conversion of discoidal structures to spherical HDL occurs in the circulation by the action of lecithin:cholesterol acyltransferase (LCAT); patients with hereditary LCAT deficiency (7) or with low LCAT activity due to alcoholic hepatitis (8) contain discoidal lipoproteins and decreased amounts of HDL-cholesterol.

The metabolic interrelationship between HDL subfractions and the mechanism for the transfer of lipids and proteins between HDL subclasses is not well understood. It is known that hormonal, dietary and pharmacological agents which affect HDL are mainly reflected by changes in HDL_2 (9). It is also known from studies in vivo (10-14) and in vitro (15) that chylomicron and very low density lipoprotein (VLDL) lipid and protein surface constituents are transferred to HDL_3 during catabolism of triglyceride-rich lipoproteins. Tall and Small (16) have proposed that discoidal structures are formed during lipolysis and are acted on by LCAT to produce spherical HDL. It is also possible that discoidal structures combine directly with HDL generating larger HDL particles.

The purpose of this study was to investigate the interaction of discoidal lipoproteins with HDL_3. Discoidal complexes of apoA-I, the major apoprotein of HDL, and dimyristoyl phosphatidylcholine (DMPC) containing 0, 10, 20 or 30 mol % cholesterol were prepared and incubated with HDL_3. The results of these studies show that discoidal lipids are transferred quantitatively to HDL_3 giving rise to larger lipoprotein particles.

MATERIALS AND METHODS

Preparation of HDL_3

Plasma was obtained by plasmapheresis of a normal healthy male volunteer; final concentrations of 0.01% sodium azide and 10^{-3} M phenylmethyl sulfonyl fluoride were immediately added to the plasma. Total plasma HDL were isolated between d 1.063-1.210 g/ml by ultracentrifugal flotation in salt solutions of KBr. The ultracentrifugations were performed in a Beckman Model L5-65 centrifuge operating at 48,000 rpm for 24 hr at 8 C using a Beckman type Ti 50.2 rotor. The HDL

were removed by aspiration and dialyzed against a standard buffer containing 10 mM Tris-HCl, 0.9% NaCl, 1 mM EDTA, 0.01% NaN_3. Total HDL were further fractionated into HDL_2 and HDL_3 by rate-zonal ultracentrifugation in a salt gradient of NaBr (d 1.00-1.40 g/ml) as described by Patsch et al. (2). Zonal ultracentrifugation was performed in a Beckman Ti-14 rotor operating at 41,000 rpm for 24 hr at 10 C. The HDL subfractions were removed from the rotor with a Beckman gradient pump and the appropriate fractions corresponding to HDL_3 were pooled and dialyzed against standard buffer.

Preparation of DMPC-ApoA-I-Cholesterol Complexes

ApoA-I was isolated from total HDL as described previously (17). The protein was homogeneous by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by amino acid analysis and the absence of isoleucine. Prior to incubation with lipid, lyophilized apoA-I was dissolved in 0.05 M Tris-HCl, pH 8.0, containing 6 M guanidine-HCl to give 5 mg/ml and incubated at 37 C for 2 hr. The protein solution was then dialyzed 4 hr against standard buffer and incubated with DMPC as described later.

DMPC (Sigma) and di[1-^{14}C]palmitoyl phosphatidylcholine (100 Ci/mol) (New England Nuclear) gave single species after thin layer chromatography (TLC) on silica gel ($CHCl_3$/CH_3OH/H_2O, 65:25:4, v/v). [7-^{3}H(N)]Cholesterol (11 Ci/mol) was purchased from New England Nuclear andwas repurified by TLC on Silica Gel G in hexane/ether/acetic acid (86:16:1). To prepare the lipid-protein complexes, DMPC (5 mg), di[1-^{14}C]palmitoyl phosphatidylcholine (0.5 μCi) and the appropriate mol % of cholesterol (relative to DMPC) were dissolved in 1 ml of $CHCl_3$, evaporated to dryness with a stream of ultrapure nitrogen and then placed under vacuum for 1 hr. ApoA-I (2.9 mg) was added to the lipids and the volume was adjusted, as indicated, with standard buffer; the mixtures were incubated 12 hr at 25 C with gentle stirring. The lipid-protein complexes were isolated by column chromatography on Bio-Gel A15m (Bio Rad). The columns (1.5 x 30 cm) were operated at room temperature and equilibrated with standard buffer. The flow rates were 10 ml/hr and 1.0-ml fractions were collected. The apoA-I-lipid complexes were reisolated by ultracentrifugation at d 1.21 g/ml.

Experimental Procedures

HDL_3 and the lipid-protein complexes of DMPC-cholesterol-apoA-I were incubated at 37 C. At the appropriate time, as indicated, the mixtures were subjected to chromatography on Bio-Gel A15m. The molecular weights of the isolated complexes were determined by gradient gel electrophoresis according to Anderson et al. (18). A Pharmacia electrophoresis apparatus was used with premade gradient gels containing 4-30% acrylamide (Pharmacia). α_2-Macroglobulin (MW 820,000), apoferritin (MW 460,000), catalase (MW 240,000), ceruloplasmin (MW 150,000) and albumin (MW 67,000) were used as known protein standards. Lipid-protein complexes were examined with a Phillips EM 300 microscope after negative staining with 2% phosphotungstic acid, pH 7.4. Preparations were initially observed at a magnification of 42,000; particle size was determined by examination of the original negative and of the prints processed to a final magnification of x 105,000.

ApoA-I concentration was determined by amino acid analysis. Phospholipid phosphorus analysis was performed according to Bartlett (19). Unesterified cholesterol concentrations were determined by enzymic procedures (Mannheim Boehringer). Flourescence polarization measurements were determined on a Perkin-Elmer MPF-44A ratio recording spectrofluorometer. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was incorporated into discoidal complexes (1:200 probe to DMPC molar ratio) by bath sonication at 37 C; the incorporation of DPH was followed by monitoring the increase in its fluorescence intensity. The polarization did not change as a function of fluorescence intensity, indicating that no fluorescence depolarization occurred as a consequence of radiative or nonradiative energy transfer between dye molecules. Corrected fluorescence polarization values, P, were determined from the formula

$$P = \frac{Vv - Lv\,(V_H/L_H)}{Vv + Lv\,(V_H/L_H)},$$

where Vv and Lv are the fluorescence intensities measured with polarizers parallel and perpendicular to the vertically polarized excitation beam, respectively. V_H and L_H are the fluorescence intensities measured with polarizers perpendicular and parallel to the horizontally polarized excitation beam, respectively. The correction factor for unequal dispersion of the components of the polarization was $V_H/L_H = 0.80$.

RESULTS

Chromatography on Bio-Gel A15m of DMPC-apoA-I complexes containing either 0, 10, 20 or

30 mol % cholesterol is shown in Figure 1. Incorporation of unesterified cholesterol into the DMPC-apoA-I complexes caused an increase in the size of the complexes as shown by their elution profile (Fig. 1) and by electron microscopy after negative staining (Fig. 2). ApoA-I-DMPC complexes appeared as discoidal particles 100 Å in diameter and packed into rouleaux structures. ApoA-I-DMPC complexes containing various amounts of unesterified cholesterol also appeared as discoidal structures with diameters up to 600 Å. The lipid-protein complexes were pooled as indicated in Figure 1 and were reisolated by ultracentrifugation in KBr at d 1.21 g/ml. The molar ratios of DMPC to apoA-I of the isolated complexes containing 0, 10, 20 or 30 mol % cholesterol were 60, 88, 142 and 102, respectively. The DMPC-cholesterol molar ratios of the complexes were nearly identical to those of the initial apoA-I-DMPC-cholesterol mixtures.

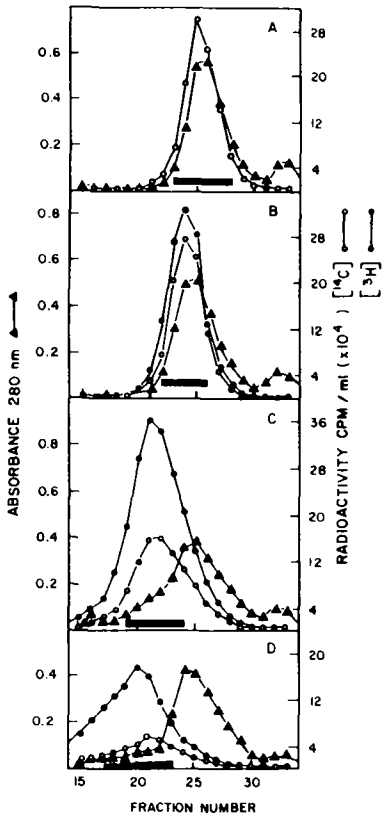


FIG. 1. Chromatography of DMPC-cholesterol-apolipoprotein A-I discoidal complexes on Bio-Gel A15m. (A) The lipid-protein complex was prepared with DMPC (5 mg), di[1-^{14}C] palmitoyl phosphatidylcholine (0.5 μCi) and apoA-I (2.9 mg) in 1.0 ml of a standard buffer of 10 mM Tris-HCl, pH 7.4, containing 0.9% NaCl, 1 mM EDTA and 0.01% NaN_3. After incubation for 12 hr at 30 C, the complexes were dialyzed against standard buffer and applied to a column (1.5 X 30 cm) of Bio-Gel A15m. The column was operated at room temperature and was equilibrated with standard buffer; the flow rate was 10 ml/hr and 1-ml fractions were collected. (B) Same as (A) plus 10 mol % cholesterol and [^{3}H] cholesterol (1.44 X 10^6 cpm total). (C) Same as (A) plus 20 mol % cholesterol and [^{3}H] cholesterol (6.68 X 10^4 cpm total). (D) Same as (A) plus 30 mol % cholesterol and [^{3}H] cholesterol (4.97 X 10^6 cpm total). The mol % cholesterol is based on 100 mol % DMPC. Fractions were assayed for protein absorbance at 280 nm (▲—▲) and for phospholipid (●—●) and cholesterol (○—○) by radioactivity. The lipid-protein complexes were pooled as indicated. The void volume of the column was at fraction 14 and the included volume, fraction 35.

The differences in lipid-protein composition of the isolated discoidal complexes were reflected in their physical properties. The temperature dependence of the fluorescence polarization of DPH incorporated into either sonicated DMPC vesicles or into DMPC:apoA-I complexes containing various amounts of cholesterol was determined (Fig. 3). Compared to DMPC vesicles, the discoidal complexes containing cholesterol exhibited a markedly decreased phase-transition amplitude. Both the phase transition temperatures and the fluorescence polarization values at all temperatures were significantly increased relative to the DMPC vesicles. The phase-transition temperatures were as follows: DMPC vesicles (≅22 C); 0% cholesterol:DMPC:apoA-I (≅28 C); 10% cholesterol:DMPC:apoA-I (≅32 C). The complexes containing 20 and 30 mol % cholesterol did not show a definite phase-transition temperature.

Interaction of lipid-apoA-I discoidal complexes with HDL_3 is illustrated in Figures 4-6. Incubation of complexes of apoA-I and DMPC containing either 0 (Fig. 4B) or 10 mol % (Fig. 4C) cholesterol with HDL_3 caused degradation of the discoidal structures and transfer of lipid to HDL_3. As a result of the incorporation of lipids into HDL_3, the molecular weight of HDL_3 increased from ≅170,000 to ≅200,000 as determined by gradient gel electrophoresis. It was not possible by chromatography on Bio-Gel A15m to demonstrate intermediate complexes between the discoidal structures containing 0 or 10 mol % cholesterol and HDL_3. However, as is shown in Figure 5, an intermediate complex was formed between HDL_3 and the discoidal complex containing 20 mol % cholesterol. After 1 hr at 37 C, ca. 50% of the DMPC and cholesterol were transferred to

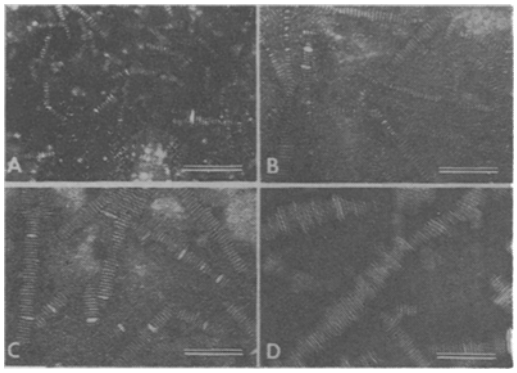


FIG. 2. Electron micrographs of DMPC-cholesterol-apoA-I complexes isolated by gel filtration chromatography (Fig. 1). (A) DMPC-apoA-I; (B) plus 10 mol % cholesterol; (C) plus 20 mol % cholesterol; (D) plus 30 mol % cholesterol. Bar marks represent 1000 Å.

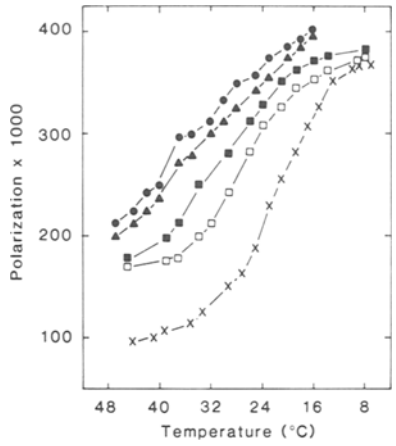


FIG. 3. Temperature dependence of the fluorescence polarization values of DMPC-cholesterol-apoA-I discoidal complexes labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH). Each lipid-protein complex was labeled with DPH at a probe-to-phospholipid ratio of 1:200. The incorporation of DPH was followed by monitoring the increase in DPH fluorescence intensity at 37 C. After the maximal intensity was reached, each sample was rechromatographed on Bio-Gel A15m, the lipid-protein complex pooled and fluorescence polarization determined as described in the text. DMPC (–X–X–); DMPC-apoA-I (–□–□–); DMPC-apoA-I plus 10 mol % cholesterol (–■–■–); plus 20 mol % cholesterol (–▲–▲–); plus 30 mol % cholesterol (–●–●–).

HDL_3 (Fig. 5B); complete transfer of lipids was observed at 17 hr (Fig. 5C). The complex containing 30 mol % was also completely degraded after 17 hr incubation with HDL_3 (Fig. 6B). The molecular weights of the lipoprotein after transfer of the complexes containing 20 and 30 mol % cholesterol into HDL_3 were ca. 180,000.

Electron micrographs of the isolated complexes of mixtures of apoA-I-lipids and HDL_3 are shown in Figure 7. The particles appeared as spherical structures with no evidence of discoidal or rouleaux structures.

DISCUSSION

The purpose of the present study was to prepare discoidal complexes of lipid and apoA-I, to characterize the complexes with respect to composition and physical properties, and to then determine whether these complexes were incorporated into HDL_3. The results of this study show that human HDL_3 take up discoidal particles containing apoA-I, DMPC and unesterified cholesterol.

The advantage of the discoidal system, as opposed to the liposome or sonicated vesicle systems used by others (20-30), is that the complexes contained apoprotein, thus avoiding the possible loss of apoprotein from HDL_3. The molar ratio of DMPC and apoA-I in the isolated discoidal complexes was similar to those described by Pownall et al. (31). Incorporation of cholesterol into discoidal particles increased the phase-transition temperature of the lipid-protein complexes. Further experiments are required to determine the effect which cholesterol has on the transfer of the complexes into HDL_3. However, the results of the present study suggest that the incorporation of cholesterol into the discoidal structures decreased the rate of lipid transfer and are consistent with those of Kirby et al. (32) and Allen (33) who showed that the incorporation of cholesterol into unilamellar vesicles of phospholipid reduced the loss of phospholipids to HDL.

The mechanism for the transfer of lipids into HDL is not well established. With the exception of this study and one by Nichols et al. (34), most investigators (20-30) have used phospholipid liposomes or sonicated vesicles to study the transfer of lipid into HDL. Tall et al. (20) proposed that apoA-I transfers from HDL to phospholipid liposomes forming discoidal particles; apoA-I-depleted HDL then fuse with other spherical particles forming larger HDL particles. Nichols et al. (21) studied the interaction of single bilayer vesicles of DMPC with HDL_{2b}. These investigators showed that the presence of discoidal structures was dependent on the ratio of DMPC to HDL_{2b} and occurred at only high levels of DMPC. Nichols et al. (21) suggested that DMPC transfers directly into HDL_{2b} by unknown mechanisms causing release of apoA-I. With excess DMPC, Nichols et al. (21) proposed that released apoA-I then inter-

acts with the lipid forming discoidal complexes. The studies of Jonas (22) also showed that sonicated vesicles of egg phosphatidylcholine and cholesterol are taken up directly by HDL without the formation of discoidal complexes. Nichols et al. (21) used a similar system to the present one and reported that discoidal complexes of DMPC and apoA-I were transferred to HDL_2; the number of discoidal complexes which were degraded was dependent directly on the molar ratio of HDL_2.

The physiologic significance of these results as it relates to lipoprotein metabolism is only speculative. However, it is possible that the catabolism of triglyceride-rich lipoproteins and the transfer of VLDL lipid constituents to HDL_3 may depend on the relative content of unesterified cholesterol in discoidal structures. At high concentrations of plasma cholesterol, i.e., cholesterol-fed animals, the rate of lipid transfer from cholesterol-rich discoidal structures to HDL or of apoA-I from HDL to the discoidal particles may be reduced and, as a result, there may be decreased conversion of HDL_3 to HDL_2. Conversely, discoidal structures may not be present under conditions of normal plasma cholesterol because they are rapidly incorporated into HDL. Experiments

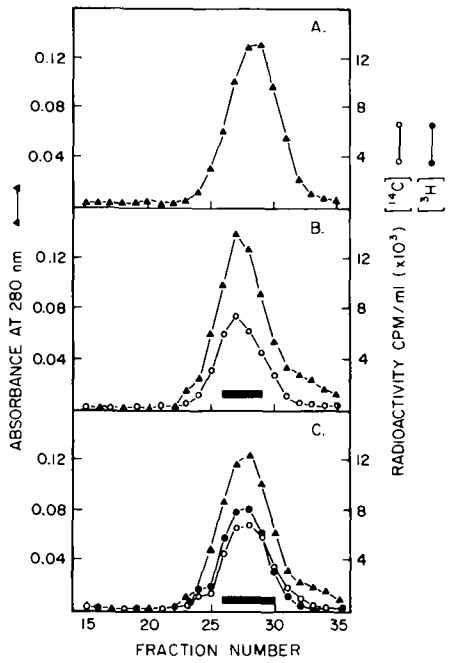


FIG. 4. Chromatography on Bio-Gel A15m of HDL_3 (A) and HDL_3 incubated with discoidal complexes of DMPC-apoA-I (B) or with DMPC-apoA-I containing 10 mol % cholesterol (C). The incubation mixtures in a total volume of 1.0 ml of standard buffer were chromatographed on a column (1.5 X 30 cm) of Bio-Gel A15m as described in Fig. 1. (A) HDL_3 (1 mg protein; 1.5 mg phospholipid). (B) HDL_3 (1 mg protein; 1.5 mg phospholipid) and 0.7 ml of the discoidal complex of DMPC-apoA-I (Fig. 1A) (0.22 mg protein; 0.31 mg DMPC). (C) HDL_3 (1 mg protein; 1.5 mg phospholipid) and 0.5 ml of the discoidal complex of DMPC-apoA-I containing 10 mol % cholesterol (Fig. 1B) (0.16 mg protein; 0.32 mg DMPC). After incubation for 1 hr at 37 C, the samples were applied to Bio-Gel A15m and eluted as described in Fig. 1. Fractions were analyzed for protein by absorbance at 280 nm (▲–▲) and for cholesterol (●–●) and phospholipids (○–○) by radioactivity.

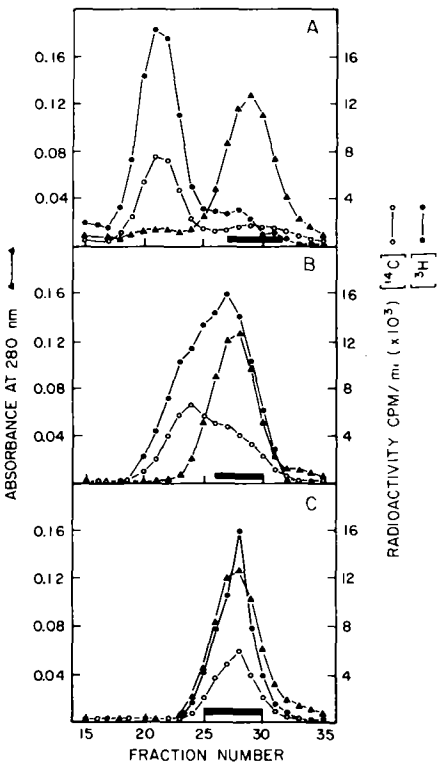


FIG. 5. Chromatography on Bio-Gel A15m of HDL_3 incubated with discoidal complexes of DMPC-apoA-I containing 20 mol % cholesterol. The incubation mixture contained 0.3 ml of HDL_3 (3.0 mg protein; 4.5 mg phospholipid) and 3.75 ml of the discoidal complex of DMPC-apoA-I containing 20 mol % cholesterol (Fig. 1C) (0.21 mg protein; 0.75 mg DMPC). At zero time, 1.35 ml of the incubation were applied directly to the column (A). The remaining portion was incubated at 37 C. After 1 hr of incubation, 1.35 ml were applied (B). After 12 hr of incubation, 1.0 ml was applied (C). The samples were eluted and analyzed as described in Fig. 1. Fractions were analyzed for protein at 280 nm (▲–▲) and for cholesterol (●–●) and phospholipid (○–○) by radioactivity.

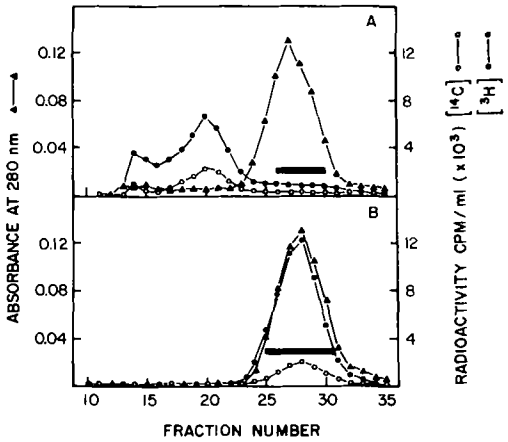


FIG. 6. Chromatography on Bio-Gel A15m of HDL_3 incubated with discoidal complexes of DMPC-apoA-I containing 30 mol % cholesterol. The incubation mixture contained 0.2 ml of HDL_3 (2.0 mg protein; 3.0 mg phospholipid) and 2.8 ml of a discoidal complex of DMPC-apoA-I containing 30 mol % cholesterol (Fig. 1D) (0.06 mg protein; 0.14 mg DMPC). After mixing, 1.5 ml of the incubation mixture was applied directly to Bio-Gel A15m. The remaining portion was incubated at 37 C for 17 hr and then applied to the column. Fractions were analyzed for protein by absorbance at 280 nm (▲–▲) and for cholesterol (●–●) and phospholipid (○–○) by radioactivity.

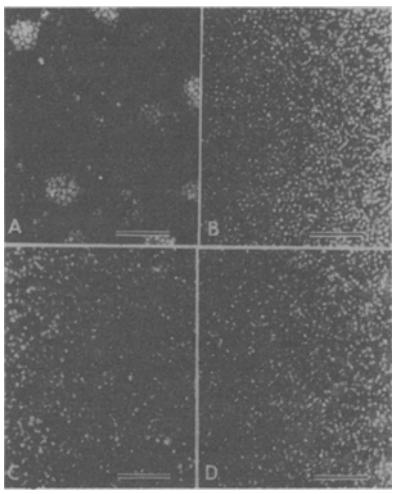


FIG. 7. Electron micrographs of isolated complexes after incubation of HDL_3 with discoidal complexes of DMPC-apoA-I containing various amounts of cholesterol. (A) HDL_3 plus DMPC-apoA-I (Fig. 3B). (B) HDL_3 plus DMPC-apoA-I containing 10 mol % cholesterol (Fig. 3C). (C) HDL_3 plus DMPC-apoA-I containing 20 mol % cholesterol (Fig. 4C). (D) HDL_3 plus DMPC-apoA-I containing 30 mol % cholesterol (Fig. 5B). Bar marks represent 1000 Å.

are currently in progress to determine the effect of cholesterol on the rate of lipid transfer between lipoprotein particles and its role in HDL interconversion.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grants HL-22619 and 23019, by the American Heart Association (J.D. Johnson), by the Muscular Dystrophy Association (J.D. Johnson), and by General Clinical Research Center grant RR-00068. A.D. Cardin is supported by NIH Training Grant HL-07382. We gratefully acknowledge the assistance of B. Menkhaus and J. Simons in preparing the manuscript for publication and of G. Kraft in preparing the figures.

REFERENCES

1. Anderson, D.W., Nichols, A.V., Pan, S.S., and Lindgren, F.T. (1978) Atherosclerosis 29, 161-179.
2. Patsch, W., Schonfeld, G., Gotto, A.M., Jr., and Patsch, J.R. (1980) J. Biol. Chem. 255, 3178-3185.
3. Green, P.H.R., Tall, A.R., and Glickman, R.M. (1978) J. Clin. Invest. 61, 528-534.
4. Hamilton, R.L., Williams, M.C., Havel, C.J., and Havel, R.J. (1976) J. Clin. Invest. 58, 667-680.
5. Chajek, T., and Eisenberg, S. (1978) J. Clin. Invest. 61, 1654-1665.
6. Deckelbaum, R.J., Eisenberg, S., Fainaru, M., Barenholz, Y., and Olivecrona, T. (1979) J. Biol. Chem. 254, 6079-6087.
7. Forte, T., Nichols, A., Glomset, J., and Norum, K. (1974) Scand. J. Clin. Lab. Invest. 33 (Suppl. 137), 121-132.
8. Ragland, J.B., Bertram, P.D., and Sabesin, S.M. (1978) Biochem. Biophys. Res. Commun. 80, 81-88.
9. Nikkila, E.A. (1978) in High Density Lipoproteins and Atherosclerosis (Gotto, A.M., Jr., Miller, N.E., and Oliver, M.F., eds.) p. 177-192, Elsevier/North Holland Biomedical Press, Amsterdam.
10. Havel, R.J., Kane, J.P., and Kashyap, M.L. (1973) J. Clin. Invest. 52, 32-38.
11. Forte, T.M., Krauss, R.M., Lindgren, F.T., and Nichols, A.V. (1979) Proc. Natl. Acad. Sci. USA 76, 5934-5938.
12. Redgrave, T.G., and Small, D.M. (1979) J. Clin. Invest. 64, 162-171.
13. Tall, A.R., Green, P.H.R., Glickman, R.M., and Riley, J.W. (1979) J. Clin. Invest. 64, 977-989.
14. Schaefer, E.J., Jenkins, L.L., and Brewer, H.B., Jr. (1978) Biochem. Biophys. Res. Commun. 80, 405-412.
15. Patsch, J.R., Gotto, A.M., Jr., Olivecrona, T., and Eisenberg, S. (1978) Proc. Natl. Acad. Sci. USA 75, 4519-4523.
16. Tall, A.R., and Small, D.M. (1978) N. Engl. J. Med. 299, 1232-1236.
17. Baker, H.N., Jackson, R.L., and Gotto, A.M. (1973) Biochemistry 12, 3866-3871.
18. Anderson, D.W., Nichols, A.V., Forte, T.M., and Lindgren, F.T. (1977) Biochim. Biophys. Acta 493, 55-68.
19. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
20. Tall, A.R., Hogan, V., Askinazi, L., and Small,

D.M. (1978) Biochemistry 17, 322-326.
21. Nichols, A.V., Gong, E.L., Forte, T.M., and Blanche, P.J. (1978) Lipids 13, 943-950.
22. Jonas, A. (1979) J. Lipid Res. 20, 817-824.
23. Jonas, A., and Maine, G.T. (1979) Biochemistry 18, 1722-1728.
24. Gong, E.L., and Nichols, A.V. (1979) Lipids 15, 86-90.
25. Scherphof, G., Roerdink, F., Waite, M., and Parks, J. (1978) Biochim. Biophys. Acta 542, 296-307.
26. Damen, J., Waite, M., and Scherphof, G. (1979) FEBS Lett. 105, 115-119.
27. Chobanian, J.V., Tall, A.R., and Brecher, P.I. (1979) Biochemistry 18, 180-187.
28. Tall, A.R., and Small, D.M. (1977) Nature 265, 163-164.
29. Tall, A.R., and Lange, Y. (1978) Biochem. Biophys. Res. Commun. 80, 206-212.
30. Tall, A.R. (1980) J. Lipid Res. 21, 354-363.
31. Pownall, H.J., Massey, J.B., Kusserow, S.K., and Gotto, A.M., Jr. (1979) Biochemistry 18, 574-579.
32. Kirby, C., Clarke, J., and Gregoriadis, G. (1980) FEBS Lett. 111, 324-328.
33. Allen, T.M. (1981) Biochim. Biophys. Acta 640, 385-397.
34. Nichols, A.V., Gong, E.L., Blanche, P.J., and Forte, T.M. (1980) Biochim. Biophys. Acta 617, 480-488.

[Revision received February 6, 1982]

Comparison of Bile Acid Synthesis Determined by Isotope Dilution versus Fecal Acidic Sterol Output in Human Subjects

WILLIAM C. DUANE*, DAVID E. HOLLOWAY, SCOT W. HUTTON, PATRICIA J. CORCORAN and **NANCY A. HAAS,** *Department of Medicine, Veterans Administration Medical Center, Minneapolis, MN 55417,* and *Department of Medicine, University of Minnesota, Minneapolis, MN 55417*

ABSTRACT

Fecal acidic sterol output has been found to be much lower than bile acid synthesis determined by isotope dilution (*J. Lipid Res.* 17: 77, 1976). Because of this confusing discrepancy, we compared these 2 measurements done simultaneously on 13 occasions in 5 normal volunteers. In contrast to previous findings, bile acid synthesis by the Lindstedt isotope dilution method averaged 16.3% lower than synthesis simultaneously determined by fecal acidic sterol output (95% confidence limit for the difference -22.2 to -10.4%). When one-sample determinations of bile acid pools were substituted for Lindstedt pools, bile acid synthesis by isotope dilution averaged 5.6% higher than synthesis by fecal acidic sterol output (95% confidence limits -4.9 to 16.1%). These data indicate that the 2 methods yield values in reasonably close agreement with one another. If anything, fecal acidic sterol outputs are slightly higher than synthesis by isotope dilution.

Lipids 17:345-348, 1982.

INTRODUCTION

Bile acid synthesis is a major component of overall cholesterol homeostasis accounting for roughly 40% of daily cholesterol loss. There are 2 major methods for measuring synthesis rate of bile acid: the isotope dilution technique pioneered by Lindstedt (1) and the fecal sterol balance technique in which daily output of acidic sterols in the steady-state is taken to be equivalent to synthesis (2). It would be reassuring if values for bile acid synthesis by these 2 techniques agreed. However, Subbiah et al. recently reported wide discrepancies between these 2 measurements (3). They found that synthesis determined by fecal acidic sterol output was 18-44% lower than synthesis by isotope dilution in the same human subjects. Because there is no way of knowing the true synthesis rate, it is impossible to determine which of these 2 methods gave the more accurate value. Davidson et al. have provided evidence that discrepancies between the 2 measurements exist in patients with hyperlipidemia, but not in healthy subjects (4).

We have recently had the opportunity to perform simultaneous measurements of bile acid synthesis by isotope dilution and by fecal acidic sterol balance in 5 normal human subjects on 13 occasions. Our findings support the validity of both techniques in such subjects.

METHODS

We studied 5 normal volunteers, free from disease by previously published criteria (5) in addition to biliary ultrasonography. All were hospitalized for at least 4 weeks on our metabolic ward prior to study. All were studied during a period of normal nutrition and again after a period of ascorbic acid depletion. In addition, 3 of the subjects were studied in a second period of normal nutrition. During these various periods, many other measurements were made which are either in press or being prepared for submission separately.

Bile acid synthesis by isotope dilution was performed exactly as described in previous publications (5). Briefly, at 9:00 p.m., about 4 hr after a light supper, known amounts of ^{14}C cholic acid and ^{14}C chenodeoxycholic acid were administered via a duodenal tube. The subjects were permitted nothing except water by mouth until bile sampling was completed the following morning between 8:00 and 9:00 a.m. Bile was obtained via the duodenal tube after stimulation of gallbladder contraction by intraduodenal infusion of a protein hydrolyzate solution (Amigen, Baxter Laboratories, Deerfield, IL). Bile was collected on ice and a 1-5-ml aliquot (depending on depth of color) was saved by analysis. The remainder was returned to the subject by slow drip into the duodenal tube. After sampling, the tube was removed and the subjects ate normally. This same sampling procedure was repeated on 4 consecutive days. Specific activity of cholic and chenodeoxycholic acids were determined using enzymatic assay of bile acid mass following careful thin layer chromatographic (TLC) separation of individual bile acids as previously described (5). To ensure that chenodeoxycholic completely

separated from deoxycholic acid, an aliquot from each chenodeoxycholic acid band was analyzed by gas liquid chromatography (5).

For both cholic acid and chenodeoxycholic acid, we performed linear regression analysis of the natural logarithm of specific activity vs time. Fractional turnover rate is the slope of this line. Pool size of each of the 2 primary bile acids was calculated by dividing the amount of isotope administered by the specific activity at time zero, obtained by extrapolating the regression line to time zero. Synthesis rate was calculated by multiplying this pool size by the fractional turnover rate.

In addition, for both cholic and chenodeoxycholic acids, we calculated a one-sample pool size as described previously (6) by dividing the amount of isotope administered by the specific activity in the first sample of duodenal bile obtained 12 hr after isotope administration. A second isotope dilution synthesis rate was calculated by multiplying this one-sample pool size by the appropriate fractional turnover rate.

Fecal acidic sterol output was determined by a method similar to that used by Subbiah (7). Subjects were given 100 mg of chromic oxide 3 times each day for a total of 20 days. All stools were collected for the last 10 days of chromic oxide administration. The stool collection for each day was homogenized in a Waring blender with an equal volume of water. Chromic oxide was determined in duplicate aliquots of this homogenate by the method of Davignon et al. (8). Acidic sterols were determined on an aliquot of homogenate to which a known amount of hyocholic acid had been added as an internal standard. This homogenate was hydrolyzed at 125 C and 15 psi for 2 hr. After acidification, the bile acids were extracted into chloroform/methanol, methylated and acetylated as previously described (5). Final analysis of these derivatives was performed on a Hewlett-Packard 5830A gas-chromatograph with a column of SP-2250 (Supelco, Bellefonte, PA).

RESULTS

Table 1 summarizes our findings. Bile acid synthesis rate obtained by the method of Lindstedt averaged 16.3% lower than synthesis by acidic sterol output. The 95% confidence limit for this difference was -22.2 to -10.4%, indicating that this 16.3% difference was not a result of random variation in the measurements. The Lindstedt method, therefore, systematically provided a lower value for bile acid synthesis than did measurement of fecal acidic sterol output.

When synthesis was calculated using one-sample pools, rather than Lindstedt pools, the isotope dilution method provided a value that averaged 5.6% higher than fecal acidic sterol outputs. Because the 95% confidence limits of this difference (-4.9 to +16.1%) included zero, it is possible that there was no actual systematic difference in synthesis rates between the one-sample isotope dilution method and acidic sterol output measurements.

DISCUSSION

In the present study, bile acid synthesis by fecal acidic sterol output was on average 16.3% higher than synthesis by isotope dilution (Table 1). This similarity between values obtained by the 2 methods is in agreement with the findings of Davidson et al. in normolipidemic subjects (4). However, it stands in sharp contrast to the findings of Subbiah et al. (3), who reported that fecal acidic sterol output rates were much lower than rates of synthesis measured by isotope dilution. Davidson et al. also found marked disagreement between the 2 methods in subjects with hyperlipidemia. The explanation for the findings of Subbiah et al. may be that all their subjects except one were hyperlipidemic; however, the reason that hyperlipidemia should be associated with such discrepancies between these 2 methods of measuring bile acid synthesis remains unclear. It is also possible that their use of generally labeled [^{3}H] chenodeoxycholic acid, which is known to overestimate bile acid synthesis by about 2-fold in isotope dilution studies (9), contributed to the disparate results reported by Subbiah et al.

Although the 16% difference in synthesis by these 2 methods is relatively small, it nevertheless indicates that one or both methods must be subject to some systematic error. Because ^{14}C labeled bile acids and chromic acid are both quantitatively recovered in feces (2,8), there is no obvious source of such systematic error in the fecal sterol measurement. Synthesis by isotope dilution is a product of fractional turnover and pool size for the 2 primary bile acids, cholic and chenodeoxycholic. Fractional turnover is equivalent to the slope of the linear regression relating natural logarithm of specific activity to time. The correlation coefficients of these regressions in the present study and in our previous studies (5,6) almost always exceed 0.98 and were usually 0.99, indicating a high degree of linearity. This slope, therefore, seems to provide a valid estimate of mean daily fractional turnover of bile acid. The accuracy of pool size measured by the Lindstedt technique has been a matter of debate. We have previously pointed out (6) that when the isotope is administered in

TABLE 1

Bile Acid Synthesis by Fecal Acidic Sterol Output and Isotope Dilution

Acidic sterols (μmol/day)	Lindstedt method[a]		One-sample method[b]	
	μmol/day	% difference[c]	μmol/day	% difference[c]
1268	1014	-20.0	1303	+2.8
1502	1091	-27.4	1400	-6.8
963	882	-8.4	1287	+33.6
921	824	-10.5	1111	+20.6
1010	951	-5.8	1321	+30.8
1617	1110	-31.4	1186	-26.6
1457	1131	-22.4	1646	+13.0
810	632	-22.0	674	-16.8
848	813	-4.1	863	+1.8
941	950	+1.0	1095	+16.4
1525	1215	-20.3	1488	-2.4
1086	902	-16.9	1122	+3.3
1554	1185	-23.8	1611	+3.7
Mean		-16.3		+5.6
SD		9.8		17.3
95% confidence limits		-22.2 to -10.4		-4.9 to +16.1

[a](Cholic fractional turnover) (cholic Lindstedt pool) + (chenodeoxycholic fractional turnover) (chenodeoxycholic Lindstedt pool).

[b](Cholic fractional turnover) (cholic one-sample pool) + (chenodeoxycholic fractional turnover) (chenodeoxycholic one-sample pool).

[c][(Isotope dilution value) − (acidic sterol output value)] × 100 ÷ (acidic sterol output value)].

the evening, 12 hr prior to the first sampling, extrapolation of the linear regression to time zero may not be perfectly valid because the subjects are fasting during this time. As fasting is known to inhibit bile acid synthesis (10), the rate at which unlabeled bile acid is produced to dilute the administered isotope may be lower than the average daily synthesis rate. If so, extrapolation across this 12-hr period would yield a falsely high estimate of specific activity at time zero and a falsely low pool size of bile acid. This, in turn, would yield a falsely low synthesis rate, perhaps explaining at least part of the discrepancy between synthesis by isotope dilution vs fecal acidic sterol output noted in this study.

As an alternative to extrapolating the specific activity-time relationship, we have used a so-called one-sample method for determination of pool size (6). In this technique, the specific activity of bile acid 12 hr after isotope administration is simply divided into the amount of isotope administered. Because there is undoubtedly some synthesis of bile acid between sampling and administration of isotope, this technique probably overestimates pool size to some extent. It is notable that when synthesis is calculated as the product of this one-sample pool and fractional turnover, the result is, on average, about 6% higher than synthesis by fecal acidic sterol output (Table 1). One interpretation of these data is that fecal acidic sterol outputs, falling in between synthesis calculated by an isotope dilution technique which slightly underestimates pool size and synthesis calculated by a technique that slightly overestimates pool size, is probably an accurate measure of bile acid synthesis. But whichever of the 3 is the most accurate, the fact that they all lie within a few percentage points of one another suggests that none of them is off the mark by much.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant #AM25811 and the Veterans Administration. The dietetic and nursing staff of the Special Diagnostic and Treatment Unit of the Minneapolis Veterans Administration Medical Center provided invaluable care of the subjects. V. Wesley carefully prepared the manuscript.

REFERENCES

1. Lindstedt, S. (1957) Acta Physiol. Scand. 40, 1-9.
2. Grundy, S.M., Ahrens, E.H., Jr., and Miettinen, T.A. (1965) J. Lipid Res. 6, 397-410.
3. Subbiah, M.T.R., Tyler, N.E., Buscaglia, M.D., and Marai, L. (1976) J. Lipid Res. 17, 78-84.
4. Davidson, N.O., Samuel, P., Lieberman, S., Shane, S.P., Crouse, J.R., and Ahrens, E.H., Jr. (1981) J. Lipid Res. 22, 620-631.
5. Duane, W.C. (1978) J. Lab. Clin. Med. 91, 969-

978.
6. Duane, W.C., Adler, R.D., Bennion, L.J., and Ginsberg, R.L. (1975) J. Lipid Res. 16, 155-158.
7. Subbiah, M.T.R. (1973) J. Lipid Res. 14, 692-697.
8. Davignon, J., Simmonds, W.J., and Ahrens, E.H., Jr. (1968) J. Clin. Invest. 47, 127-138.
9. Panveliwalla, D.K., Pertsemlidis, D., and Ahrens, E.H., Jr. (1974) J. Lipid Res. 15, 530-532.
10. Duane, W.C., Ginsberg, R.L., and Bennion, L.J. (1976) J. Lipid Res. 17, 211-219.

[Received December 10, 1981]

Metabolism of Malonaldehyde in vivo and in vitro

G.M. SIU and H.H. DRAPER*, *Department of Nutrition, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

ABSTRACT

The metabolism of malonaldehyde (MA) was investigated in vivo using male Wistar rats and in vitro using rat liver mitochondria. Twelve hr after intubation with [1,3-^{14}C] MA, 60-70%, 5-15% and 9-17% of administered radioactivity was recovered in expired CO_2, feces and urine, respectively. In rats intubated with [1,2-^{14}C] acetate, the corresponding values were 68-82%, 1-2% and 2-3%. $^{14}CO_2$ evolution was initially slower after ^{14}C-MA administration than after ^{14}C-acetate administration and more radioactivity was excreted in the feces and urine. In vitro experiments using [1,3-^{14}C] MA showed that MA is metabolized primarily in the mitochondria via reactions involving O_2 utilization and $^{14}CO_2$ production. The apparent K_m and V_{max} were 0.5 mM and 9.3 nmol/min/mg protein for O_2 uptake, respectively, and 2.0 mM and 2.4 nmol/min/mg protein for $^{14}CO_2$ production. Addition of malonic acid to mitochondrial incubates at concentrations inhibitory to succinate dehydrogenase did not affect MA-induced O_2 uptake but enhanced $^{14}CO_2$ production from ^{14}C-MA. ^{14}C-Acetate appeared to be the major accumulating metabolite in rat liver mitochondrial preparations following a 120-min incubation with ^{14}C-MA. A probable biochemical route for MA metabolism involves oxidation of MA by mitochondrial aldehyde dehydrogenase followed by decarboxylation to produce CO_2 and acetate.

Lipids 17:349-355, 1982.

INTRODUCTION

Malonaldehyde (MA) is a product of lipid peroxidation which is found in foods and which has been reported to occur in animal tissues, especially under conditions of antioxidant deficiency (1,2). Recent studies have confirmed the presence of significant quantities of MA in foods of animal origin (3,4) where it apparently arises mainly from the oxidation of arachidonic acid in cell membranes. Malonaldehyde is reactive toward sulfhydryl and amino groups of proteins (5). It produces intramolecular and intermolecular linkages which can lead to inactivation and polymerization of enzymes such as ribonuclease (6). Its reactivity toward amino groups can result in interactions with the nitrogenous bases of deoxyribonucleic acid (DNA) (7,8) and inhibition of DNA, ribonucleic acid (RNA) and protein synthesis (9).

Malonaldehyde has been reported to be mutagenic in bacteria (10,11) and genotoxic in cultured mammalian cells (12). It is a liver carcinogen when applied to the skin (13) or administered chronically in the diet of mice (14).

Previous studies indicate that MA is readily metabolized in vivo and in vitro. Holtkamp and Hill (15) showed that incubation of MA with rat liver homogenate induced increases in the rate of oxygen uptake. Recknagel and Ghoshal (16) found that incubation of MA with rat liver homogenate or mitochondria led to disappearance of MA as measured by the thiobarbituric acid (TBA) assay. Placer et al. (17) demonstrated the disappearance of TBA-reactive material in the tissues of rats injected intraperitoneally with MA. These observations may be attributed to oxidation of MA by the low specificity mitochondrial aldehyde dehydrogenase (18) and/or to reactions of MA with amino or sulfhydryl groups which can result in a decrease in TBA reactivity. In this study, the metabolism of MA was further investigated using ^{14}C-labeled material.

MATERIALS AND METHODS

[1,3-^{14}C] Malonaldehyde Generation

1,1,3,3-Tetramethoxy [1,3-^{14}C] propane (TMP) (sp act = 15.0 mCi/mmol) was purchased from Amersham Searle Co. (Arlington Heights, IL) as a solution in benzene/methanol (1:1) in sealed borosilicate glass ampoules. The solvent was removed and the residue was treated with 1 N HCl for 25 min at room temperature to generate ^{14}C-MA (19). These conditions were found to produce optimal hydrolysis as determined by monitoring MA production as a function of time using the ultraviolet (UV) method of Kwon and Watts (20).

in vivo Experiments

Two young male Wistar rats (150-200 g) were given [1,3-^{14}C] MA (1 μCi in 1 ml aq solution) by intubation 2-3 hr after their morning feeding. Immediately after dosing, the animals were placed in metabolic chambers and air was pumped through at the rate of $\sim$ 2 ℓ/min. $^{14}CO_2$ was collected in Oxifluor-CO_2 (New England Nuclear, Montreal) using 3 traps in series containing 70 ml/trap. At 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 hr, the trapping solutions were removed and 10-ml aliquots were counted using a Nuclear Chicago Mark I

counter.

After 12 hr, the animals were killed, blood samples were collected in EDTA, and 50-μl aliquots of plasma were counted in 10 ml scintillation fluid containing 120 ml Bio-Solv (Beckman), 0.3 g POPOP, 5 g PPO and 880 ml toluene/ℓ. Gastrointestinal tract contents and feces were combined and counted using the method of Case and Benevenga (21). Urine collected from the chamber and bladder was made up to 50 ml and 0.5 ml aliquots were counted. All radioactivity determinations were corrected for background and for counting efficiency using the external standard ratio method.

A companion experiment was done using [1,2-^{14}C]acetate (∿5 μCi in 1 ml aq solution) as a reference compound.

in vitro Experiments

The metabolism of MA was studied in vitro using male Wistar rat liver homogenate, mitochondria and postmitochondrial supernate. Livers were homogenized in a medium containing 250 mM sucrose, 3.4 mM Tris-HCl and 1.0 mM EGTA at pH 7.0 using a Potter-Elvejhem homogenizer. Mitochondria were isolated by the method of Chappell and Hansford (22). After sedimenting the mitochondria, the supernatant fraction was removed and recentrifuged at 9,000 x g for 10 min to ensure removal of mitochondrial contaminants. The postmitochondrial fraction from the second centrifugation was used as the supernatant fraction. The protein content of each fraction was determined by the method of Lowry et al. (23).

Standard incubation mixtures contained 3.2 ml incubation medium (sucrose 150 mM, KCl 50 mM, $MgSO_4$ 5 mM, Na phosphate 1 mM and Tris-HCl 50 mM, final pH 7.4) to which were added 0.75 mM ADP and 3-5 mg protein from homogenate, mitochondria and supernate. Substrates were added at appropriate concentrations. MA was added as a sodium enol salt after preparation by the method of Protopopova and Skoldinov (19). Incubations were done at 37 C with continuous agitation. MA metabolism was monitored by measuring disappearance of MA, O_2 uptake (mitochondria) and $^{14}CO_2$ production from ^{14}C-MA.

The reactions were terminated at appropriate times by addition of 1 ml of 0.6 N perchloric acid. The protein precipitates were removed by filtration using millipore filters (Millipore Corporation, Bedford, MA) and MA in the filtrates was determined using the colorimetric thiobarbituric acid (TBA) assay (24). Oxygen consumption was measured polarographically using a Clark's oxygen electrode (Yellow Springs Instruments, Model 53, OH). Endogenous O_2 uptake was subtracted to obtain substrate-induced O_2 consumption. Other substrates used in this study included Na succinate, acetaldehyde and pyruvaldehyde (Sigma Chemical Co., Milwaukee, WI) which were added as free aldehydes.

Incubations of radioactive substrates were done in 25-ml flasks with center wells. The $^{14}CO_2$ produced was trapped in hyamine hydroxide (1 M solution in methanol, New England Nuclear, Lachine, Quebec) contained in glass culture tubes located in the center wells. Rubber stoppers (sleeve type) fitted with hypodermic needles (plugged except during additions) were used to stopper reaction flasks and provide a closed system for $^{14}CO_2$ collection. Reactions were terminated with addition of 1 ml $HClO_4$ (0.6 M). Shaking was continued for 30 min, whereupon $^{14}CO_2$ traps were quantitated by liquid scintillation counting.

For the investigation of accumulated metabolites, mitochondrial incubations were done for 120 min in open flasks. After the reactions were terminated by addition of 1 ml $HClO_4$ (0.6 M), the deproteinized samples were filtered through millipore filters and neutalized with KOH. The $HClO_4$ precipitate was removed by centrifugation; 0.5-ml aliquots of filtrate were applied to a Dowex-1-formate column (0.9 x 14 cm) using a procedure similar to that described by LaNoue et al. (25) except that the gradient was formed by running 100 ml of 3 N formic acid into 100 ml H_2O. ^{14}C-Labeled metabolites were identified on the basis of elution volume using ^{14}C-labeled reference compounds.

RESULTS AND DISCUSSION

in vivo Metabolism

The rates of $^{14}CO_2$ production from [1,3-^{14}C]MA and [1,2-^{14}C]acetate in vivo are shown in Figure 1. Table 1 summarizes the percentage of radioactivity recovered in $^{14}CO_2$, plasma, urine and feces. The data show that ^{14}C-MA was extensively metabolized to $^{14}CO_2$ within 12 hr. A lower initial rate of $^{14}CO_2$ production was observed and a greater percentage of radioactivity was recovered in the excreta of the ^{14}C-MA-dosed animals than of the ^{14}C-acetate-dosed animals.

in vitro Oxidation

Incubation of MA (3×10^{-2} mM) with rat liver homogenate or mitochondria resulted in loss of TBA-reactive material with time. There

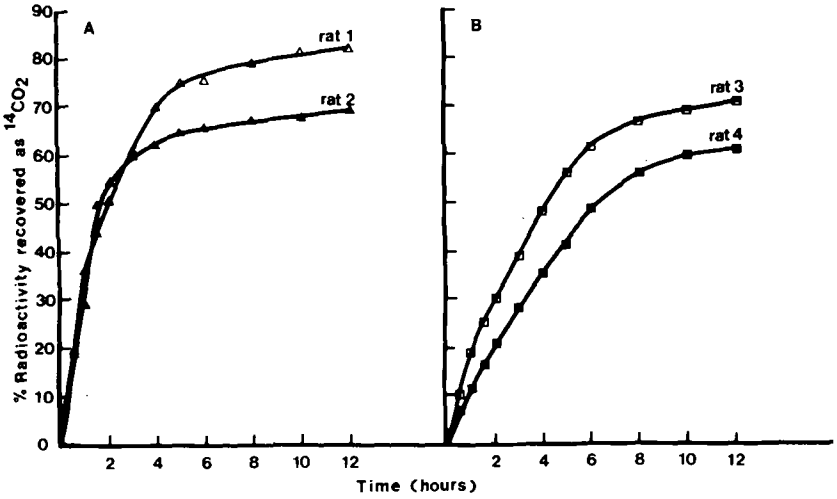


FIG. 1. Rates of in vivo $^{14}CO_2$ production from rats intubated with [1,2-^{14}C] acetate (A) or [1,3-^{14}C] MA (B).

TABLE 1

Recovery of Radioactivity from Rats 12 hr after Intubation with [1,3-^{14}C] MA or [1,2-^{14}C] Acetate

	^{14}C-Acetate		^{14}C-Malonaldehyde	
	% of total radioactivity recovered			
Rat no.	1	2	3	4
$^{14}CO_2$	82	68	70	60
Urine	2	3	9	17
Feces	1	2	5	15
Plasma	0.1	0.1	0.1	0.1

was essentially no utilization of MA by the postmitochondrial supernate. The rate of MA utilization by mitochondria exhibited pseudo-first order kinetics. After 70 min, 50% of the MA present initially had been converted to a form which was not TBA-reactive. This finding is in agreement with previous reports that MA is metabolized by the mitochondrial enzyme aldehyde dehydrogenase (Ald-DH) (18,26). Background absorbance at 450 nm was $\sim$ 0.02 OD units. Lack of utilization of MA by the postmitochondrial supernate suggests that microsomes and soluble enzymes are not involved in the initial step(s) of MA utilization, but does not exclude their possible participation in subsequent reactions involving MA metabolites.

The rates of substrate induced O_2 uptake by rat liver mitochondria for "state 3" respiration (i.e., in the presence of ADP) (27) are plotted as a function of MA concentration in Figure 2A. This figure shows that the rate of O_2 uptake increased with increasing MA concentrations up to ca. 3.5 mM, then progressively decreased. This apparent substrate inhibition of Ald-DH also was observed when acetaldehyde or pyruvaldehyde was used as substrate. Dietrich (28) reported a similar substrate inhibition in rat liver homogenate metabolizing indole-3-acetaldehyde. The incubation time and protein concentrations used in the present study were within the linear range for measuring initial velocities (Fig. 3, A and B).

The apparent K_m and V_{max} for MA-induced O_2 uptake by mitochondria, estimated from a Lineweaver-Burk plot (Fig. 2B), were 0.5 mM and 9.3 nmol/min/mg protein, respectively. The rate of $^{14}CO_2$ production from [1,3-^{14}C]-MA was measured over a range of MA concentrations and the apparent K_m and V_{max} were estimated (Fig. 4). The corresponding values for $^{14}CO_2$ production were 2.0 mM and 2.4 nmol/

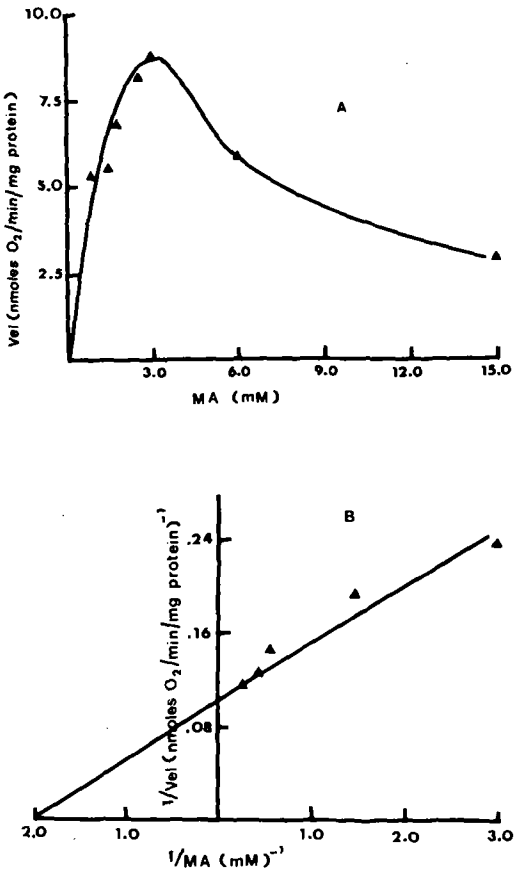


FIG. 2. (A) Effect of substrate concentration on rate of MA-induced O_2 uptake by rat liver mitochondria. (B) Lineweaver-Burk plot of the rate of O_2 uptake by rat liver mitochondria oxidizing MA as substrate. Apparent K_m = 0.5 mM; V_{max} = 9.3 nmol/min/mg protein.

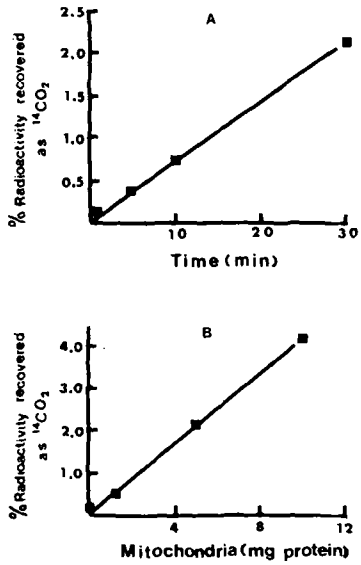


FIG. 3. (A) $^{14}CO_2$ production from [1,3-^{14}C]MA as a function of incubation time in rat liver mitochondria. (B) $^{14}CO_2$ production from [1,3-^{14}C]MA as a function of mitochondrial protein present in the incubates.

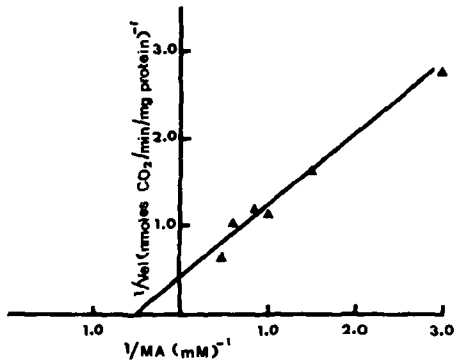


FIG. 4. Lineweaver-Burk plot of the rate of $^{14}CO_2$ production by rat liver mitochondria oxidizing ^{14}C-MA as substrate. Apparent K_m = 2.0 mM; V_{max} = 2.4 nmol/min/mg protein.

min/mg protein.

Intermediary Metabolites

Complete oxidation of MA to CO_2 involves the overall reaction:

$$OHC{\cdot}CH_2{\cdot}CHO + 3\ O_2 \longrightarrow 2\ H_2O + 3\ CO_2$$

Because MA is a 3-carbon compound, $^{14}CO_2$ production from ^{14}C-MA may proceed either via oxidation and decarboxylation of MA to a residual 2-carbon metabolite which is further oxidized through the tricarboxylic acid (TCA) cycle, or via carboxylation of MA to form a 4-carbon compound which can be oxidized to CO_2 via the TCA cycle. Conversion of malonic semialdehyde (MSA) to succinyl-CoA via β-hydroxypropionate, propionyl-CoA and methylmalonyl-CoA has been reported (29). These 2 possibilities were examined by adding malonic acid at concentrations which inhibit succinate dehydrogenase. A summary of the effect of malonic acid addition on MA-induced O_2 uptake and CO_2 production from ^{14}C-MA is presented in Table 2. The lack of inhibition by malonic acid of MA oxidation indicates that, under the assay conditions used (10 min incubations), O_2 uptake and CO_2 production from MA occurred independently of the TCA cycle, and that the metabolism of MA proceeds via

TABLE 2

Effect of Malonic Acid on Malonaldehyde-Induced O_2 Uptake and $^{14}CO_2$ Production from ^{14}C-Malonaldehyde

Substrate	Concentration (mM)	Malonic acid (mM)	O_2 uptake (% of control)	$^{14}CO_2$ production (% of control)
Succinate	3.0	–	100	–
Succinate	3.0	3.0	15	–
Malonaldehyde	3.0	–	100	–
Malonaldehyde	3.0	3.0	111	–
^{14}C-Malonaldehyde	2×10^{-3}	–	–	100
^{14}C-Malonaldehyde	2×10^{-3}	3.0	–	198

oxidation and decarboxylation to form a 2-carbon metabolite which is subsequently metabolized via the cycle.

The increased $^{14}CO_2$ production from ^{14}C-MA associated with addition of unlabeled malonic acid cannot be adequately explained on the basis of available data. However, a possible speculation is that this increase may be an effect of malonic acid inhibition of oxidation of other endogenous substrates which compete for available O_2 and other cofactors in the medium.

Based on these data, it appeared that, under the in vitro conditions used, ^{14}C-MA metabolism probably involves oxidation to malonic semialdehyde (MSA) and decarboxylation to acetaldehyde which is rapidly converted to acetate with equimolar amounts of O_2 consumed and $^{14}CO_2$ produced. Detection of accumulating metabolites was attempted by fractionating deproteinized mitochondrial incubates and enzyme blanks on an anion exchange column. Four peaks (A, B, C and D) were obtained from the mitochondrial incubates (Fig. 5a), of which 3 (A, B and D) were also present in the enzyme blank incubates (Fig. 5b). From chromatographed radioactive standards, peaks C and D were identified as acetate and malonaldehyde, respectively.

Because MA has been reported to polymerize readily as well as to be reactive toward amino groups (5,6), peaks A and B were suspected of being products of MA polymerization or binding to amino groups in ADP or to the amino moiety of Tris. Column separation of MA incubated in H_2O in the absence mitochondria and ADP revealed one major peak corresponding to the MA standard (Fig. 5c). A similar elution profile was observed when ^{14}C-MA was incubated in H_2O in the absence of mitochondria but with added ADP, indicating that neither peak A nor peak B is a product of MA binding with ADP. However, the chromatograph from incubates of ^{14}C-MA in Tris-HCl medium with no added mitochondria

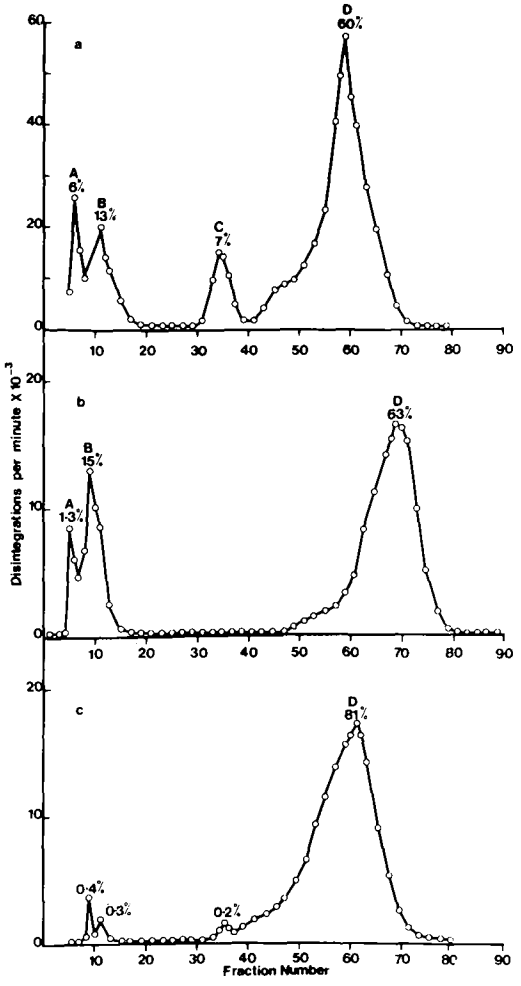


FIG. 5. Separation of ^{14}C-MA and metabolites by anion exchange chromatography. Column size 0.9 cm X 24 cm and elution rate ca. 2 ml/min. One-ml fractions were collected. (a) ^{14}C-MA incubated for 2 hr in Tris HCl buffer in the presence of mitochondria and ADP. (b) ^{14}C-MA incubated for 2 hr in Tris-HCl buffer with equal volume of buffer added in place of mitochondria and with added ADP. (c) ^{14}C-MA incubated for 2 hr in H_2O with no added mitochondria or ADP.

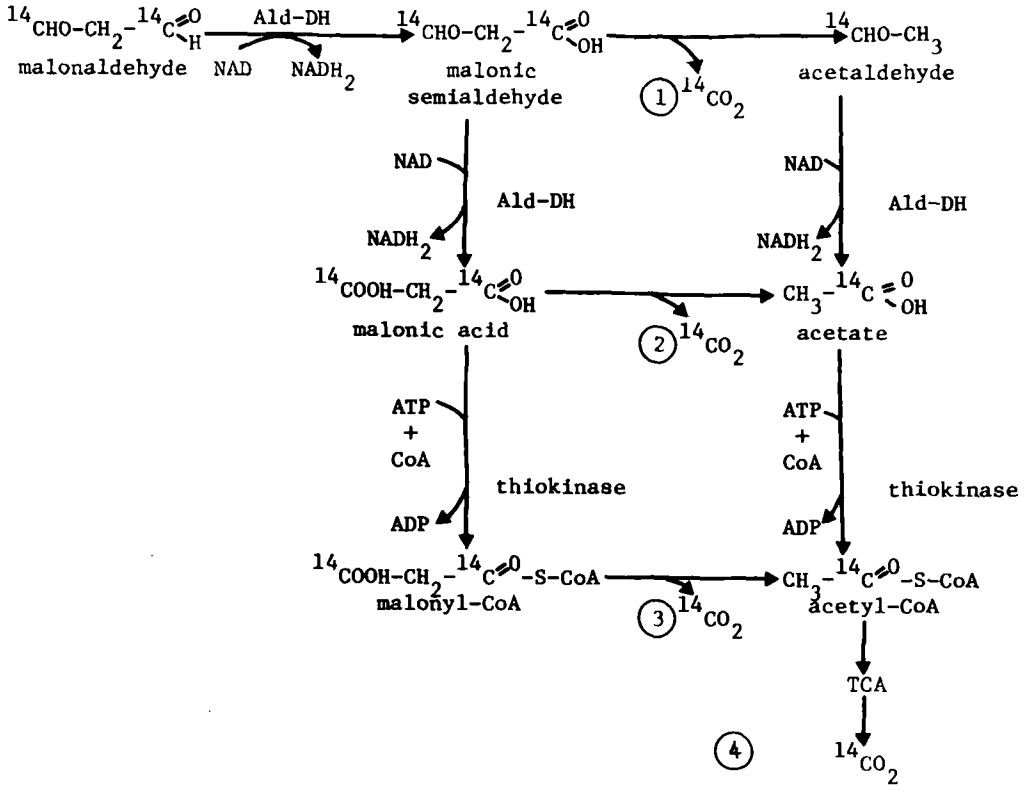


FIG. 6. Schematic representation of the possible routes of MA metabolism and sites of $^{14}CO_2$ production from [1,3-^{14}C]MA. Ald-DH-aldehyde dehydrogenase.

or ADP showed the presence of 2 peaks corresponding to peaks B and D. Thus, peak B appears to be the product of a reaction between MA and the amino group of Tris. Peak A could not be positively identified, but it may arise from incomplete hydrolysis of the parent compound TMP used to generate MA (30).

Figure 6 shows schematically the possible routes of MA metabolism and sites of $^{14}CO_2$ production from [1,3-^{14}C]MA. Based on the observations that the K_m for O_2 uptake was less than the K_m for CO_2 production, and that substrate inhibition was observed for O_2 uptake with MA concentrations greater than 3.5 mM whereas no substrate inhibition was observed for $^{14}CO_2$ production from ^{14}C-MA, it can be suggested that substrate-induced O_2 uptake by MA is not rate limiting for the production of CO_2 from MA. Also, the apparent K_m for acetaldehyde oxidation (0.1 mM) is less than the apparent K_m for MA-induced oxidation (0.5 mM) and decarboxylation (2.0 mM), indicating that any acetaldehyde formed by route 1 (Fig. 6) would be converted rapidly to acetate. When ^{14}C-malonic acid was used as substrate, the apparent K_m for $^{14}CO_2$ production (50.2 mM) was much greater than that for $^{14}CO_2$ production from ^{14}C-MA, signifying that malonic acid is not a major intermediate in the formation of $^{14}CO_2$ from ^{14}C-MA (Fig. 6, routes 2 and 3).

As the V_{max} for $^{14}CO_2$ production was less than that for O_2 uptake, it is probable that MSA accumulates. Because of the structural similarity between MA and MSA, as well as the lack of available MSA standards, it could not be determined whether MSA was inadequately resolved from MA in the chromatographic system or whether no significant MSA accumulation occurred at the low substrate concentrations used.

The isolation of acetate from the deproteinized incubates supports the contention that MA can be metabolized via oxidation to MSA followed by decarboxylation to acetaldehyde, which is readily oxidized to acetate. Under the in vitro conditions used, acetate accumulated and was not significantly oxidized via the TCA

cycle. However, in vivo acetate formed from MA would not be expected to accumulate. This view is consistent with the observation that ^{14}C-MA can be extensively oxidized in vivo to $^{14}CO_2$ but that the rate of $^{14}CO_2$ production from ^{14}C-MA is initially slower than that from ^{14}C-acetate (Fig. 1).

The difference between ^{14}C-MA and ^{14}C-acetate utilization in vivo, indicated by the difference in kinetics of $^{14}CO_2$ production and in radioactivity recovered in the excreta, may be related to the readiness with which MA reacts with amino groups, as illustrated by the binding of MA to Tris during the 2-hr in vitro incubations. The MA-amino reaction product(s) formed in vitro appear to be susceptible to acid-heat hydrolysis, because TBA assays on post-mitochondrial supernates incubated with MA showed little loss of TBA-reactive material following the 2-hr incubations.

ACKNOWLEDGMENTS

This work was supported by the Ontario Ministries of Health and of Agriculture and Food, and by the U.S. National Cancer Institute.

REFERENCES

1. Dahle, L.K., Hill, E.G., and Holman, R.T. (1962) Arch. Biochem. Biophys. 98, 253-261.
2. Pryor, W.A., and Stanley, J.P. (1975) J. Org. Chem. 40, 3615-3617.
3. Shamberger, R.J., Shamberger, B.A., and Willis, C.E. (1977) J. Nutr. 107, 1404-1409.
4. Siu, G.M., and Draper, H.H. (1978) J. Food Sci. 43, 1147-1149.
5. Shin, B.C., Huggins, J.W., and Carraway, K.L. (1972) Lipids 7, 229-233.
6. Chio, K.S., and Tappel, A.L. (1969) Biochemistry 8, 2827-2832.
7. Brooks, B.R., and Klamerth, O.L. (1968) Eur. J. Biochem. 5, 178-182.
8. Reiss, U., Tappel, A.L., and Chio, K.S. (1972) Biochem. Biophys. Res. Commun. 48, 921-926.
9. Bird, R.P., and Draper, H.H. (1980) J. Toxicol. Environ. Health 6, 811-823.
10. Mukai, F.H., and Goldstein, B.D. (1976) Science 191, 868-869.
11. Shamberger, R.J., Corlett, C.L., Beaman, K.D., and Kasten, B.L. (1979) Mutation Res. 66, 349-355.
12. Bird, R.P., and Draper, H.H. (1979) Fed. Proc. 38, 709.
13. Shamberger, R.J., Andreone, T.L., and Williss, C.E. (1974) J. Natl. Cancer Inst. 53, 1771-1773.
14. Bird, R.P., Draper, H.H., and Valli, V.E.O. (1981) Proc. Can. Fed. Biol. Soc., p. 301 (abstract).
15. Holtkamp, D.E., and Hill, R.M. (1951) Arch. Biochem. Biophys. 34, 216-219.
16. Recknagel, R.O., and Ghoshal, A.K. (1966) Lab Invest. 15, 132-146.
17. Placer, Z., Veselkova, A., and Rath, R. (1965) Experientia 21, 19-20.
18. Horton, A.A., and Packer, L. (1970) J. Gerontol. 25, 199-204.
19. Protopopova, T.V., and Skoldinov, A.P. (1958) J. Gen. Chem. U.S.S.R. 28, 341-243.
20. Kwon, T.W., and Watts, B.M. (1963) J. Food Sci. 28, 627-630.
21. Case, G.L., and Benevenga, N.J. (1976) J. Nutr. 106, 1721-1736.
22. Chappell, J.B., and Hansford, R.G. (1972) in Subcellular Components (Birnie, G.D., ed.) p. 74, Butterworth & Co. Ltd., Woburn, MA.
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
24. Sinnhuber, R.O., and Yu, T.C. (1958) Food Technol. 12, 9-12.
25. LaNoue, K., Nicklas, W.J., and Williamson, J.R. (1970) J. Biol. Chem. 245, 102-111.
26. Horton, A.A., and Barrett, M.C. (1975) Arch. Biochem. Biophys. 167, 426-436.
27. Chance, B., and Williams, G.R. (1955) J. Biol. Chem. 217, 383-393.
28. Deitrich, R.A. (1966) Biochem. Pharmacol. 15, 1911-1922.
29. Menon, G.K.K., Stern, J.R., Kupiecki, F.P., and Coon, M.J. (1960) Biochim. Biophys. Acta 44, 602-604.
30. Marnett, L.J., and Tuttle, M.A. (1980) Cancer Res. 40, 276-282.

[Received September 25, 1981]

Effect of Ethanol on Transport from Rat Intestine during High and Low Rates of Oleate Absorption

D.R. SAUNDERS*, J. SILLERY and **G.B. MCDONALD**, *Department of Medicine, University of Washington, Seattle, WA 98195*

ABSTRACT

Long-chain fatty acids (LCFA) are transported predominantly in the intestinal lymph when rates of LCFA absorption are high, and oral ethanol has been shown to enhance this lymphatic transport. A greater proportion of absorbed LCFA is transported via portal blood when rates of LCFA absorption are low. We tested the hypothesis in unanesthetized lymph-fistula rats that ethanol might also enhance the mucosal absorption and lymphatic transport of oleic acid when oleate absorption rates were low. The results did not support this hypothesis. Ethanol enhanced oleate absorption and transport from the intestine when 360 μmol, but not when 8 μmol of [^{14}C] oleate was infused intraduodenally over 4 hr. There were major differences in intestinal mucosal metabolism of high and low loads of oleic acid. After the high load, the proportion of intestinal [^{14}C] phospholipid to [^{14}C] neutral lipid was 8:92. This ratio changed to 37:63, and the percentage of neutral ^{14}C as triglyceride decreased from 87 to 68% when the low load of oleate was infused. We suggest that a portion of absorbed LCFA is incorporated into phospholipid and transported as high-density lipoproteins in portal blood. This portal pathway for LCFA was uninfluenced by ethanol in the present experiments.

Lipids 17:356-360, 1982.

INTRODUCTION

Ethanol has profound effects on lipid metabolism throughout the body (1). The liver is consistently affected, but acute doses of ethanol alter the absorption of both dietary and endogenous lipids. Intraduodenal ethanol causes increased intestinal lymphatic output of endogenous lipid (2,3), and of a simultaneously administered intraduodenal bolus of 25 μmol of palmitic acid (3).

We reexamined the effects of ethanol on intestinal lipid transport because of recent work which suggests that an appreciable portion of unsaturated long-chain fatty acid (LCFA) is transported in portal venous blood rather than in intestinal lymph (4). Portal transport of LCFA was proportionally greater when LCFA was being absorbed at low rates. We tested the hypothesis, therefore, that ethanol might alter the transport of oleic acid when oleate was being absorbed at such a rate that one-third of absorbed oleate was being transported via portal blood. The experimental model was the thoracic duct fistula rat. A feeding duodenostomy was used to avoid delays in gastric emptying caused by ethanol (5). Oleic acid was infused at high (360 μmol/4 hr) or at low (8 μmol/4 hr) rates. A concentration of 5 g of ethanol/100 ml was used because duodenol concentrations of 1-5 g/100 ml are observed when a man drinks 4 oz of 80-proof spirits (6).

METHODS

Materials

Sodium taurocholate was synthesized (7); the product contained a trace (<5%) of taurine. Its $[\alpha]_D^{23}$ was 21.9 (c = 5 in water) (reported, 21.7 ± 3). Oleic acid and 2-monoolein (Serdary Research Lab., London, Ontario) were pure by thin layer chromatography (TLC). [1-^{14}C] Oleic acid (New England Nuclear, Boston, MA) was purified by radiochromatography. Reference standards for thin layer chromatography were obtained from Supelco, Bellefonte, PA.

Homogeneous mixtures of oleic acid (30 mM, or 0.67 mM) and 2-monoolein (2:1 molar ratio) were prepared in 5 mM taurocholate, 118 mM NaCl, 6 mM KCl, 10 mM $NaHCO_3$, and 10 mM glucose by sonication for 2 min at one-third of maximal output (Biosonik III, Bronwill Scientific, Rochester, NY). Eighteen rats had ethanol, 5 g/100 ml, included in the infusion mixture, whereas 20 rats did not.

Procedures

Male Sprague-Dawley rats weighing 200-300 g (Tyler Labs, Seattle, WA) were given free access overnight to a solution of glucose, 100 g, NaCl, 5 g, and KCl, 0.4 g/ℓ of water. The following morning, thoracic duct cannulation and feeding duodenostomy were performed under pentobarbital sodium (50 mg/kg) and halothane anesthesia. Postoperatively, rats were placed in restraining cages, and they were infused intraduodenally with an amino acid-carbohydrate mixture (Vivonex, 0.5 strength, Eaton Laboratories, Norwich, NY) at 1 ml/hr (Gilson Minipuls Pump, Middleton, WI). The next day, a lipid infusate was pumped intraduodenally at 3 ml/hr for 4 hr. Animals whose lymph flow was <1 ml/hr were discarded. The rat was then

anesthetized, and clamps were placed at the gastro-esophageal junction, the pylorus, the terminal ileum, and the distal rectum. The small intestine was removed and its contents flushed out with 30 ml of NaCl, 9 g/ℓ and air. Small intestinal washings, stomach, large intestine, and feces were combined and homogenized (Polytron PT20ST, Brinkman Instruments, Westbury, NY). The remaining small intestine was analyzed separately.

Homogenates and thoracic duct lymph were made up to volume and their ^{14}C was measured in 2 ways so that water-soluble, as well as lipid-soluble, radioactivity could be known. [^{14}C] lipid in portions of homogenate and lymph was extracted with chloroform/methanol (8). Water-soluble plus lipid-soluble ^{14}C in 0.5-ml portions of unextracted homogenate were mixed with 1 ml of Protosol (New England Nuclear), bleached with benzoyl peroxide (1 g/10 ml toluene), and counted in a toluene-based scintillant. [^{14}C] Toluene was used as an internal standard.

The recoveries of [^{14}C] lipid as a percentage of [^{14}C] oleate infused were incomplete: 89% after 360 μmol, 75% after 360 μmol plus ethanol, 64% after 8 μmol, 61% after 8 μmol plus ethanol (Table 1). These incomplete recoveries were not because water-soluble ^{14}C was being lost during the extraction procedures. No disparity existed in counting ^{14}C from the small intestinal lumen in either chloroform methanol extractions or in total washings. Total homogenates contained 1.6% more small intestinal wall ^{14}C, and 2.2% more lymph ^{14}C than did chloroform methanol extracts. We inferred that the missing [^{14}C] oleate was transported from the intestine via portal venous blood. The validity of this inference was proven previously (4).

[^{14}C] Lipid in chloroform was applied to a column of silica gel (CC-4, Mallinckrodt Inc., St. Louis, MO). Neutral lipids were eluted with chloroform/diethyl ether (5:1, vol/vol), phospholipids with methanol/chloroform/water (9:1:1). Recovery of [^{14}C] lipid from the column was 101 ± 2% for small intestinal wall lipid and 97 ± 4% for lymph lipid.

[^{14}C] Neutral lipid was chromatographed on thin layers (250-μm) of Silica Gel G (Analtech, Newark, DE) using the solvent system *n*-hexane/diethyl ether/acetic acid (5:1:0.1). Reference standards were run in lateral lanes of the chromatoplate which was stained with iodine. Separated bands, after deiodinating, were scraped into vials so that ^{14}C could be counted in 10 ml of Aquasol (New England Nuclear). Recovery of ^{14}C from chromatoplates was 92 ± 6% for small intestinal wall neutral lipid and 93

TABLE 1

Absorption and Transport of [^{14}C] Oleic Acid[a]

[^{14}C] Oleic infused (μmol)	Ethanol infused (μmol)	No. of rats	^{14}C in gut lumen (μmol)	^{14}C in small gut lipids			^{14}C in lymph lipid		
				Total (μmol)	NL (%)	PL (%)	Total (μmol)	NL (%)	PL (%)
360	0	10	37 ± 3	141 ± 32	92 ± 4	8 ± 2	144 ± 17	98 ± 1	2 ± 1
360	13000	9	14 ± 7[b]	93 ± 14[b]	85 ± 3[b]	15 ± 3[b]	162 ± 58	98 ± 0.4	2 ± 0.4
8	0	10	0.1 ± 0.1	2.1 ± 0.7	63 ± 7[c]	37 ± 7[c]	2.9 ± 0.7	98 ± 1	2 ± 1
8	13000	9	0.2 ± 0.2	1.8 ± 0.4	59 ± 11[c]	41 ± 11[c]	2.9 ± 1.1	98 ± 1	2 ± 1

[a]Thoracic-duct fistula rats were infused intraduodenally at 3 ml/hr for 4 hr with 10 μCi [1-^{14}C] oleic acid, 30 mM, or 0.67 mM, with or without ethanol, 1.08 M. [^{14}C] Lipid was extracted with chloroform/methanol.

[b]Significantly different ($p<0.01$) from controls fed 360 μmol without ethanol.

[c]Significantly different ($p<0.01$) from animals fed 360 μmol.

TABLE 2

Distribution of ^{14}C in Gut Wall Lipid[a]

[^{14}C] Oleic infused (μmol)	Ethanol infused (μmol)	[^{14}C] Neutral lipid (%) in:					[^{14}C] Phospholipid (%) in:				
		MG	DG	FFA	TG	CE	SPH	LPC	PC	PS	PE
360	0	2 ± 1	2 ± 1	3 ± 1	87 ± 3	1 ± 0.3	1 ± 1	2 ± 1	64 ± 7	7 ± 3	16 ± 3
360	13000	2 ± 1	2 ± 0.3	3 ± 1	85 ± 3	1 ± 0.2	1 ± 0.3	4 ± 3	54 ± 14	9 ± 3	18 ± 3
8	0	11 ± 5[b]	4 ± 2	4 ± 2	68 ± 5[b]	5 ± 2[b]	1 ± 1	2 ± 0.4	61 ± 10	12 ± 5	16 ± 2
8	13000	13 ± 5[b]	4 ± 1	4 ± 2	60 ± 8[b]	5 ± 4[b]	1 ± 0.3	2 ± 0.3	62 ± 6	10 ± 3	19 ± 7

[a]Lipids were separated on a column of silicic acid so that neutral lipids and phospholipids could be fractionated further by TLC. The numbers are mean percentage ± SD of the total ^{14}C recovered from chromatoplates. MG = monoglyceride, DG = diglyceride, FFA = free fatty acid, TG = triglyceride, CE = cholesteryl ester, SPH = sphingomyelin, LPC = lysophosphatidylcholine, PC = phosphatidylcholine, PS = phosphatidylserine, PE = phosphatidylethanolamine.

[b]Significantly different ($p<0.01$) from [^{14}C] neutral lipid distribution after 360 μmol of [^{14}C] oleate was infused.

± 4% for lymph neutral lipid.

[^{14}C] Phospholipid was chromatographed on LK-5 silica gel plates (Whatman Co., Clifton, NJ) using acetone/chloroform/methanol/acetic acid/water (4:3:1:1:0.5). Reference standards were run in lateral lanes of the chromatoplate which was stained with Phospray (Supelco, Bellefonte, PA). The chromatoplate was then sprayed with distilled water and the separated bands were scraped into vials so that ^{14}C could be counted in 10 ml of Aquasol. Recovery of ^{14}C from chromatoplates was 89 ± 5% for lymph phospholipid.

Results are expressed as means ± SD. Statistical significance of mean differences among groups of observations was assessed by Student's t-test.

RESULTS

Infusion of Oleate at 90 μmol/hr

Without ethanol. Ninety percent of the ^{14}C was absorbed from the small intestinal lumen over the 4-hr infusion (Table 1). Of the ^{14}C absorbed, 44% remained in the small intestinal wall, and 45% was recovered in the lymph; by subtraction, 11% was transported in portal venous blood.

Ninety-two percent of intestinal wall ^{14}C was in neutral lipid (Table 1) of which 87% was in the triglyceride fraction (Table 2); 98% of [^{14}C] lymph lipid was in neutral lipid (Table 1) of which 93% was in the triglyceride fraction (Table 3).

With ethanol. Ethanol increased the absorption of [^{14}C] oleate to 96% and it decreased the amount of ^{14}C in the small intestinal wall to 29% of the ^{14}C absorbed. Therefore, significantly more ^{14}C was transported from the intestine during the ethanol infusion (Table 1), although the amount of ^{14}C carried in the lymph was not significantly different from control animals without ethanol.

Ethanol altered the distribution of ^{14}C in intestinal wall lipids so that 85% was in the triglyceride fraction. The percentage of ^{14}C in intestinal wall phospholipids doubled to 15% compared with control animals (Table 1).

No changes were observed in the distribution of [^{14}C] neutral lipid or [^{14}C] phospholipid in intestinal wall or in lymph with ethanol (Tables 2 and 3).

Infusion of Oleate at 2 μmol/hr

Without ethanol. Ninety-nine percent of the infused ^{14}C was absorbed from the lumen of the small bowel. Of the ^{14}C absorbed, 27% remained in the intestinal wall, and 37% appeared

in the lymph; by subtraction, 36% was transported in portal venous blood (Table 1).

The distribution of ^{14}C in intestinal wall lipids was distinctly different from the distribution after the high rate of oleate absorption: 63% of ^{14}C was in neutral lipid, whereas 37% was in phospholipid after the low dose of [^{14}C] oleate (Table 1).

The low rate of oleate absorption was associated with a change in the distribution of ^{14}C in the neutral lipids of the intestinal wall: 68% of ^{14}C was in triglyceride, 11% was in monoglyceride, and 5% was in the cholesterol ester fraction (Table 2).

In the lymph, there was relatively more [^{14}C] neutral lipid in cholesterol ester (6%) after the low rate of [^{14}] oleate infusion than after the high rate of infusion (Table 3).

With ethanol. Ethanol had no effect on the absorption or transport of [^{14}] oleate during infusions of 2 μmol/hr. As in the control animals, 98% of the 8 μmol of [^{14}C] oleate infused was absorbed. Of the ^{14}C absorbed, 23% remained in the intestinal wall, whereas 37% appeared in the lymph; by subtraction, 40% was transported in portal venous blood (Table 1).

The distribution of ^{14}C in intestinal wall lipids was also distinctly different from the distribution after the high rate of oleate absorption: 59% of ^{14}C was in neutral lipid, and 41% was in phospholipid after the low dose of [^{14}C] oleate (Table 1).

The low rate of oleate absorption with ethanol was also associated with a change in the distribution of ^{14}C in the neutral lipids of the intestinal wall: 60% of ^{14}C was in triglyceride, 13% was in monoglyceride, and 5% was in cholesteryl ester (Table 2).

In the lymph, there was relatively more [^{14}C] neutral lipid in cholesteryl ester and relatively less in triglyceride compared to lymph lipid after the high dose of [^{14}C] oleate (Table 3).

TABLE 3

Distribution of ^{14}C in Lymph Lipid[a]

[^{14}C] Oleic infused (μmol)	Ethanol infused (μmol)	[^{14}C] Neutral lipid (%) in:					[^{14}C] Phospholipid (%) in:				
		MG	DG	FFA	TG	CE	SPH	LPC	PC	PS	PE
360	0	0.4 ± 0.4	2 ± 1	1 ± 1	93 ± 5	1 ± 1	0.5 ± 0.3	2 ± 1	72 ± 4	4 ± 4	7 ± 4
360	13000	0.2 ± 0.2	1 ± 0.3	1 ± 1	95 ± 1	1 ± 0.2	0.4 ± 0.9	2 ± 2	76 ± 7	2 ± 1	9 ± 4
8	0	0.6 ± 0.6	2 ± 1	1 ± 1	88 ± 2	6 ± 2[b]	0.5 ± 0.5	2 ± 2	67 ± 9	5 ± 3	9 ± 3
8	13000	1 ± 2	2 ± 0.5	2 ± 2	87 ± 3[b]	5 ± 2[b]	0.5 ± 0.5	2 ± 2	70 ± 12	3 ± 3	10 ± 4

[a]Lymph lipids were separated on a column of silicic acid so that neutral lipids and phospholipids could be fractionated further by TLC. The numbers are mean percentages ± SD of the total ^{14}C recovered from chromatoplates. Abbreviations are as in Table 2.

[b]Significantly different ($p<0.01$) from animals fed 360 μmol.

DISCUSSION

The small intestine of a 200-g rat may receive 150 μmol of dietary fatty acid/hr if the rat consumes 15 g of 4%-fat chow over a 10-12 hr feeding period. The hourly load of oleic acid may be 50 μmol because oleate is 35% of the fatty acid in laboratory chow (Allied Mills, Chicago, IL). We showed previously that the route of transport of absorbed LCFA is influenced by the rate of fatty acid absorption: proportionally more of absorbed LCFA is transported via portal venous blood, rather than via the intestinal lymph, when rates of absorption are low (4).

There have been several studies on the effect

of ethanol on lipid metabolism in intestinal absorptive cells and on the transfer of endogenous and exogenous lipid into lymph. Ethanol enhances triglyceride synthesis in rat intestinal slices (9) and increases transport of endogenous triglycerides in lymph (2,3). When rats are fed with lipid, ethanol increases the lymphatic delivery of [^{14}C]palmitic acid (3) and of [^{14}C] cholesterol (10). In the present study, we hypothesized that ethanol would increase the lymphatic transport of exogenous LCFA when the rate of LCFA absorption was low, and therefore, when a greater percentage of absorbed LCFA was being transported via the portal vein (4).

The data did not support the hypothesis, although ethanol enhanced the absorption and transport of the high load of oleic acid. Less oleic acid remained in the intestinal lumen, and in the small intestinal wall, and the distribution of intestinal wall lipids was changed when ethanol was fed with 360 μmol of oleic acid. Ethanol did not increase the absorption of the low load of oleic acid for which absorption was virtually complete without ethanol, and ethanol did not enhance the recovery of absorbed oleic acid in lymph.

The more interesting finding concerned differences in the processing of high and low loads of oleic acid by rat small intestinal mucosa. The proportion of [^{14}C]phospholipid to [^{14}C]neutral lipid in the gut wall lipids changed from 8:92 after the high oleate load to 37:63 after the low oleate load. Gut-wall neutral lipid consisted of monoglycerides and cholesteryl esters in greater proportions at the expense of triglyceride after the lower load of oleate. Similarly, relatively more of absorbed linolenate was incorporated into monoglycerides plus phospholipids than into triglycerides by the human small intestinal mucosa when linolenic acid was being absorbed at low rates (11). These combined observations can be interpreted in the light of Wu and Windmueller's experiments in which portal blood and intestinal lymph were collected. The synthesis of apolipoproteins A-I and A-IV by small intestine appeared to be constant during fasting and during fat absorption. When fat was not being absorbed, however, a greater proportion of the synthesized apoproteins A-I and A-IV entered the portal blood rather than the intestinal lympth (12). These apoproteins could serve as vehicles for the small amounts of lipophilic substances exiting from the intestinal absorptive cells. Conceivably, some LCFA may exit from the intestine as phospholipid in a high density lipoprotein. Such a macromolecular structure originating from the small intestine has been identified in portal venous blood (13).

ACKNOWLEDGMENTS

This study was supported in part by Grant AM-16059 from the National Institutes of Health and by the Veterans Administration. We thank M. Weidman for helping with the thoracic duct cannulations.

REFERENCES

1. Baraona, E., and Lieber, C.S. (1979) J. Lipid Res. 20, 289-315.
2. Mistilis, S.P., and Ockner, R.K. (1972) J. Lab. Clin. Med. 80, 34-40.
3. Baraona, E., and Lieber, C.S. (1975) Gastroenterology 68, 495-502.
4. McDonald, G.B., Saunders, D.R., Weidman, M., and Fisher, L. (1980) Am. J. Physiol. 239, G141-G150.
5. Bouquillon, M. (1976) Lipids 11, 848-852.
6. Halsted, C.H., Robles E.A., and Mezey, E. (1973) Am. J. Clin. Nutr. 26, 831-834.
7. Tserng, K.-Y., Hachey, D.L., and Klein, P.D. (1977) J. Lipid Res. 18, 404-407.
8. Nelson, G.J. (1975) in Analyses of Lipids and Lipoproteins (Perkins, E.G., ed.) pp. 1-35, American Oil Chemists' Society, Champaign, IL.
9. Carter, E.A., Drummey, G.D., and Isselbacher, J. (1974) Science 174, 1245-1247.
10. Middleton, W.R.J., Carter, E.A., Drummey, G.D., and Isselbacher, K.J. (1971) Gastroenterology 60, 880-887.
11. Surawicz, C.M., Saunders, D.R., Sillery, J., and Rubin, C.E. (1981) Am. J. Physiol. 240, G157-G162.
12. Windmueller, H.G., and Wu, A.-L. (1981) J. Biol. Chem. 256, 3012-3016.
13. Wu, A.-L., and Windmueller, H.G. (1978) J. Biol. Chem. 253, 2525-2528.

[Revision received February 16, 1982]

Phospholipid Synthesis in *S. cerevisiae* Strain GL7 Grown without Unsaturated Fatty Acid Supplements[1]

THOMAS M. BUTTKE[a,*], **REBECCA REYNOLDS**[b] and **AMY L. PYLE**[a], [a]*Department of Microbiology, University Medical Center, 2500 North State Street, Jackson, MS 39216;* and [b]*Department of Biochemistry, Harvard University, Cambridge, MA 02138*

ABSTRACT

In the absence of exogenous unsaturated fatty acids (UFA), *Saccharomyces cerevisiae* strain GL7 synthesizes low levels of UFA and large amounts of decanoic, dodecanoic and tetradecanoic fatty acids. Supplementation with hemin leads to slightly higher levels of UFA, but synthesis of the medium-chain saturated fatty acids (SFA) continues. Under these conditions of limited UFA availability, strain GL7 incorporates most of its UFA into phosphatidylethanolamine (PE), whereas phosphatidylcholine (PC) and phosphatidylserine + phosphatidylinositol (PS+PI) are enriched with the medium-chain SFA. The association of specific fatty acids with the various phospholipids is not accompanied by changes in the proportions of newly synthesized phospholipids, demonstrating that the fatty acid composition of PE can be modulated independently of the other phospholipids. The effect of sterol structure on the fatty acid composition of cells grown with limiting UFA was also examined. Yeast cells grown with either ergosterol or stigmasterol contained less UFA and more medium-chain SFA in their phospholipids than did cholesterol-grown cells, suggesting that the former sterols allow strain GL7 to grow with a lower UFA content.

Lipids 17:361-366, 1982.

INTRODUCTION

Most organisms display considerable diversity in their membrane lipid composition. Eukaryotic membranes generally consist of several different phospholipids, a broad spectrum of fatty acids, and at least one type of sterol molecule. Although the reasons for this diversity are not completely understood, recent studies suggest that each particular lipid may perform a specific function. For example, arachidonic acid is used for prostaglandin synthesis (1), cholesterol modulates membrane fluidity (2), and PE induces membrane fusion (3). The specific roles of many other membrane lipids are less well defined, but their ubiquitous presence in a wide variety of organisms implies that lipid molecules have been "phylogenetically tailored" to perform discrete, critical functions.

The membranes of *Saccharomyces cerevisiae*, like those of other eukaryotic cells, contain sterols, several different phospholipids, and a variety of fatty acids. *S. cerevisiae* is particularly valuable for delineating the lipid requirements of eukaryotic cells, because it can be made auxotrophic for specific lipids either by growing the organism anaerobically (4,5), or by readily induced mutations (6-8). As such, numerous workers have used either wild-type *S. cerevisiae* or lipid-deficient mutants to test the suitability of assorted fatty acids (9-14), sterols (15-19), and phospholipid precursors (17-22) as growth supplements for yeast. This approach has provided much useful information regarding the general lipid requirements of *S. cerevisiae*, but the specific functions of the individual lipid molecules are less well resolved.

S. cerevisiae strain GL7, deficient in both 3-oxidosqualene cyclase and heme biosynthesis, requires exogenous unsaturated fatty acids and sterols for growth (23). We have recently reported that strain GL7 alters its phospholipid fatty acid composition in response to different sterol supplements (24). Cells grown with cholesterol contained more unsaturated fatty acids in their phospholipids than did cells grown with either ergosterol or phytosterols. Further, the suitability of cholesterol as a growth supplement for strain GL7 was dependent on the specific UFA provided in the medium (24). Based on those results, we proposed that an important relationship exists between membrane fatty acid composition and sterol structure in yeast. In order to examine this relationship in greater detail, we have now tested several sterols for their effects on the membrane fatty acid composition of strain GL7 during growth in the absence of UFA supplements.

MATERIALS AND METHODS

S. cerevisiae strain GL7 (*erg*12, *heme*3) was kindly provided by D.B. Sprinson (23). The mutant was grown on a synthetic medium supplemented with methionine and a sterol supplement (10 μg/ml). Oleic acid, when added, was

*To whom correspondence regarding this manuscript should be addressed.

[1]Portions of this paper were presented at the AOCS 72nd Annual Meeting, New Orleans, May 1981.

Abbreviations used: UFA, unsaturated fatty acid; SFA, saturated fatty acid; PE, phosphatidylethanolamine; PC phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid.

present at a concentration of 100 μg/ml. Lipids were added to the medium as solutions in Brij 58-ethanol (24). Where indicated, the medium was supplemented with hemin chloride at a concentration of 13 μg/ml (23). Cultures were grown aerobically at 30 C, and growth was monitored by measuring the absorbance at 540 nm. Studies involving UFA deprivation were performed by growing yeast cells overnight with oleic acid to serve as an inoculum. The cells were collected by centrifugation, washed twice with medium, and resuspended in medium minus UFA to the desired optical density.

The procedures for phospholipid extraction, separation of phospholipids, and fatty acid methylation have been described (24). Fatty acid analyses were performed using a Perkin-Elmer gas liquid chromatograph (series 3B) interfaced to a Sigma 10 data station. The fatty acid methyl esters were separated on a 6-ft column of 10% SP-2330 on 100/120 Chromosorb W AW (Supelco), with a temperature program from 130 to 175 C, at 5 C/min. The effect of UFA depletion on phospholipid synthesis was determined by growing cells in the presence of ^{32}P-Pi (1.25 μCi/ml for 4 hr) at various times after the removal of UFA supplements. Labeled phospholipids were separated by 2–dimensional chromatography (25), localized by autoradiography, and quantitated by liquid scintillation counting.

Sterols, fatty acids, hemin chloride and Brij 58 were obtained from the Sigma Chemical Co. [^{32}P]Orthophosphate was a product of New England Nuclear.

RESULTS

We have recently reported that *S. cerevisiae* strain GL7 alters its fatty acid composition in response to sterol supplements (24). In order to eliminate differences in UFA uptake as the basis for the sterol-induced fatty acid changes, strain GL7 was grown with hemin chloride in place of exogenous UFA. The medium was further supplemented with either cholesterol or stigmasterol, 2 sterols which differ only in their side chains. Under these conditions, the mutant grew with a doubling time of about 4 hr. Table 1 shows the fatty acid compositions of the individual phospholipids isolated from the heme-grown cells. Substantial amounts of decanoic and dodecanoic acids were present in the phospholipids, and the UFA content was considerably lower than in cells grown with exogenous UFA (24). This suggests that the hemin chloride was only partly restoring UFA synthesis, and that the heme-supplemented cells were compensating for the reduced supply of UFA

TABLE 1

Fatty Acid Composition of Strain GL7 Grown with Exogenous Hemin Chloride

Sterol supplement	Phospholipid	Fatty acid composition (mol %)[a]								
		10:0	12:0	14:0	16:0	16:1	18:0	18:1	UFA[b]	≤12:0
Cholesterol	PE	8.3 ± 2.5	3.8 ± 0.1	8.2 ± 4.8	29.1 ± 8.8	37.8 ± 1.3	1.6 ± 0.7	9.0 ± 0.9	48.9	12.1
	PC	20.8 ± 0.5	8.6 ± 0.5	13.7 ± 3.6	32.5 ± 5.8	15.9 ± 2.2	3.4 ± 1.4	3.3 ± 0.6	20.8	29.4
	PS+PI	22.7 ± 0.9	10.7 ± 1.1	10.8 ± 5.2	27.9 ± 2.3	17.9 ± 1.9	5.5 ± 4.2	3.2 ± 0.6	22.4	33.4
Stigmasterol	PE	15.1 ± 3.3	9.0 ± 2.1	14.5 ± 6.5	34.3 ± 5.1	19.7 ± 5.1	2.0 ± 0.6	4.2 ± 1.2	25.0	24.1
	PC	24.1 ± 4.6	15.8 ± 2.2	17.1 ± 4.5	29.5 ± 2.6	8.2 ± 3.5	3.1 ± 1.8	1.6 ± 0.7	10.3	39.9
	PS+PI	23.5 ± 4.5	16.5 ± 2.1	13.1 ± 4.7	31.2 ± 1.7	9.1 ± 3.2	4.9 ± 1.6	1.2 ± 0.6	10.2	40.0

[a]Data are presented as the mean of 3 experiments ± standard deviation.
[b]All samples contained 1-2% 14:1.

by synthesizing medium-chain SFA. Of additional interest was the finding that PE maintained a high level of UFA compared to PC and PS+PI which were more enriched with the medium-chain SFA. These results demonstrate a preferential utilization of UFA for PE biosynthesis. It is also apparent from Table 1 that heme-grown cells alter their phospholipid fatty acid composition in response to different sterol supplements. Growth with stigmasterol led to lower levels of UFA and increased levels of medium-chain SFA as compared to growth with cholesterol.

In subsequent studies, it was found that strain GL7 was slightly leaky for UFA biosynthesis, which allowed the mutant to grow for extended periods of time in the absence of either heme or UFA supplements. The finding that strain GL7 is slightly leaky for unsaturated fatty acid synthesis implies that it contains cytochrome b_5. This may explain the ability of the mutant to demethylate cholesterol precursors (19). The phospholipid fatty acid composition of these UFA-starved cells was similar to that of the heme-grown cells, except that the former contained less UFA and more of the long-chain SFA (Table 2). The UFA-starved cells also displayed a preferential incorporation of UFA into PE as well as sterol-dependent fatty acid changes. Cells grown with ergosterol contained lower levels of UFA in all phospholipids than did cholesterol-grown cells. These results are analogous to the results shown in Table 1, and demonstrate that strain GL7 replaces a greater percentage of its UFA with medium-chain SFA when it is grown with either ergosterol or stigmasterol. This suggests that these 2 sterols allow the mutant to grow with a lower level of UFA than is required for growth with cholesterol.

Figure 1 shows the fatty acid compositions of the individual phospholipids at various times after removal of the UFA supplement. Little change was observed during the first 4 hr, a time equal to about one cell doubling. Between 4 hr and 24 hr, however, there was a rapid decline in the UFA content of PC and PS+PI. The loss of UFA from these phospholipids was accompanied by increased levels of both medium- and long-chain SFA. This trend continued for up to 48 hr, at which time PC and PS+PI each contained about 5% UFA. By contrast, the UFA content of PE declined more slowly. After 24 hr, PE still contained 35% UFA, as compared to 10 and 15% for PC and PS+PI, respectively. The slower rate of UFA loss in PE led to a delayed incorporation of medium-chain SFA, but long-chain SFA accumulated at the same rate as in PC and PS+PI. Thus, PE differed from

TABLE 2

Fatty Acid Composition of Strain GL7 Grown in the Absence of UFA Supplements[a]

Supplement	Phospholipid	Fatty acid composition (mol %)								
		10:0	12:0	14:0	16:0	16:1	18:0	18:1	UFA	⩽12:0
Cholesterol + oleic acid	PE	–	1.5	11.6	23.8	1.2	1.2	60.5	61.7	1.5
	PC	–	2.9	11.1	22.3	0.6	3.7	59.3	59.9	2.9
	PS+PI	–	2.7	9.9	31.4	1.3	4.4	50.2	51.5	2.7
Cholesterol	PE	4.2	4.5	3.6	42.7	33.7	2.3	8.9	42.7	8.7
	PC	17.8	12.2	4.7	45.2	7.9	8.0	4.1	12.0	30.0
	PS+PI	18.4	12.9	3.1	34.9	12.0	15.2	3.5	15.5	31.3
Ergosterol	PE	22.0	14.0	6.3	44.0	9.5	2.0	2.3	11.8	36.0
	PC	25.5	17.3	7.0	43.3	0.9	5.4	0.6	1.5	42.8
	PS+PI	19.9	18.0	6.2	41.4	3.4	10.0	1.0	4.4	37.9

[a]Cells were grown in the absence of UFA supplements for 30 hr.

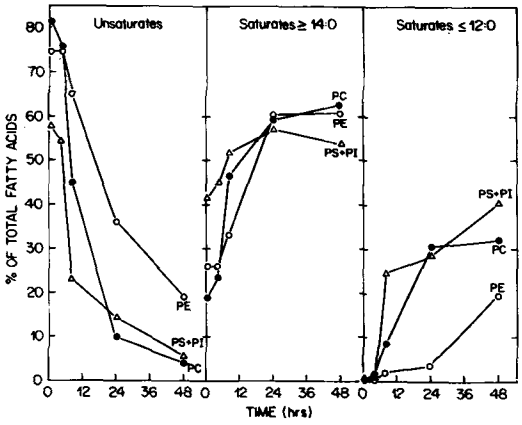


FIG. 1. Effect of UFA starvation on the phospholipid fatty acid composition of strain GL7. Yeast cells were grown into log phase with oleic acid, collected by centrifugation and resuspended in media without UFA. At the indicated times, samples were removed and the phospholipid fatty acid compositions were determined. •, PC; ○, PE; △, PS+PI.

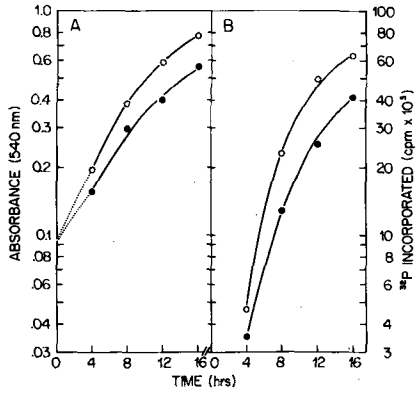


FIG. 2. Effect of UFA starvation on the incorporation of ^{32}Pi into total phospholipids of strain GL7. At various times (0, 4, 8, 12 hr), aliquots were removed and the cells were labeled for 4 hr with ^{32}Pi (1.25 μCi/ml). Points on the graph represent the end of each 4-hr labeling period. (A), growth of strain GL7; (B), ^{32}Pi incorporation into phospholipids. ○, plus oleic acid; •, minus oleic acid.

the other phospholipids only with regard to the incorporation of UFA vs medium-chain SFA. It is worth noting that each phospholipid contained a maximum of 50-60% long-chain SFA.

Our results clearly show that strain GL7 is able to regulate the fatty acid composition of PE independently of the other phospholipids. To determine if the preferential utilization of UFA for PE is accompanied by changes in phospholipid synthesis, strain GL7 was labeled for 4 hr with ^{32}Pi at various times after UFA removal. As shown in Figure 2, UFA-starved cells incorporated less ^{32}Pi into total phospholipids than UFA-supplemented cells. The growth rate of UFA-starved cells was also decreased, however, which resulted in similar ^{32}Pi/optical density ratios for both populations. The labeled phospholipids were subsequently separated and quantitated as a function of time (Figure 3). Although there were some quantitative differences between starved and supplemented cells, in general, the phospholipid compositions of the 2 populations were similar. In repetitive experiments, the only difference consistently observed was increased PA and decreased PI in the UFA-starved cells. These results demonstrate that the preferential incorporation of UFA into PE is not due to major changes in phospholipid biosynthesis.

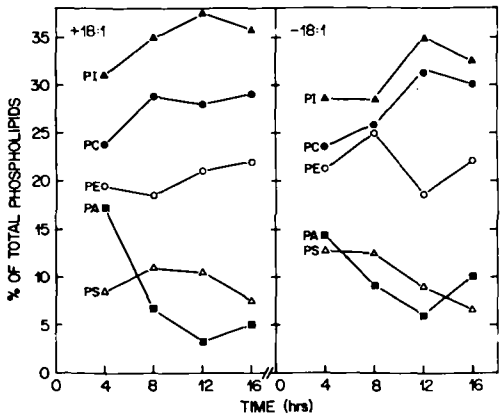


FIG. 3. Effect of UFA starvation on the phospholipid composition of strain GL7. Phospholipids were labeled as described in the legend to Fig. 2, and were separated by TLC.

DISCUSSION

The initial purpose of this study was to determine if strain GL7 displays sterol-induced fatty acid changes during growth in the absence of exogenous unsaturated supplements. Previous studies have shown that growth of strain GL7 with cholesterol leads to more UFA being incorporated into phospholipids than when cells are grown with either ergosterol or phytosterols (24). Our previous experiments, however, were performed with UFA-supplemented cells and it was possible that the observed fatty acid changes reflected sterol effects on UFA uptake. To eliminate this possibility, we supplemented strain GL7 with hemin chloride to re-

store endogenous UFA biosynthesis, and examined the cells for sterol-induced fatty acid changes. Under these conditions, as well, cholesterol-grown cells contained more UFA in their phospholipids than cells grown with stigmasterol. Based on these results, it seems unlikely that the sterol-induced changes reported previously (24) were due to differences in UFA uptake. We therefore conclude that membrane sterol composition directly affects phospholipid biosynthesis in *S. cerevisiae* strain GL7.

Our results also demonstrate that strain GL7 synthesizes increased levels of decanoic, dodecanoic and tetradecanoic acids when the supply of UFA is limited. In this respect, strain GL7 is similar to anaerobically grown yeast (9,10) and to UFA auxotrophs (26). Meyer and Bloch have shown that anaerobically grown yeast incorporate the medium-chain SFA into the β-position of PC (10). Because this is the position normally occupied by UFA, the authors proposed that medium-length SFA could partly replace olefinic acids as β-linked phospholipid constituents (10). Although we have not performed similar phospholipase analyses, our results with strain GL7 are nevertheless in good agreement with the findings of Meyer and Bloch. Assuming that tetradecanoic acid is evenly distributed between the α- and β-positions (10), the level of long-chain SFA approaches 50 mol %, as does the sum of medium-length SFA and UFA (Tables 1 and 2; Fig. 1). Extrapolating from the results of Meyer and Bloch, it therefore seems likely that the long-chain SFA are occupying the α-position, whereas the medium-chain SFA and UFA are localized to the β-position.

By separating the phospholipids of UFA-starved or heme-grown cells and analyzing their fatty acid compositions, we have detected an additional degree of specificity in phospholipid biosynthesis. When the supply of UFA is limiting, strain GL7 esterifies UFA primarily into PE while incorporating medium-chain SFA into the other phospholipids. This trend is particularly noticeable 24 hr after removal of the UFA supplement. At that time, PC and PS+PI contain 10-15% UFA and 35-40% medium-chain SFA whereas the situation is reversed in PE. This pattern cannot merely be due to reduced turnover of PE for several reasons. First, hexadecanoic and octadecanoic fatty acids accumulate in PE at the same rate as in other phospholipids (Fig. 1). Second, the PE of UFA-starved cells contains large amounts of palmitoleic acid despite the fact that the inoculum was grown with oleic acid (Tables 1 and 2). Third, there were no major differences in phospholipid synthesis between UFA-supplemented and UFA-starved cells (Fig. 3). Thus, strain GL7 preserves the UFA composition of PE while maintaining a normal bulk phospholipid composition. This could occur by the selective utilization of specific phospholipid biosynthetic pathways. In *S. cerevisiae*, PE may be formed via a decarboxylation of PS, or by the addition of CDP-ethanolamine to diacylglycerol (27). Similarly, PC can result from either the sequential methylation of PE, or by the addition of CDP-choline to diacylglycerol (27). It is therefore conceivable that strain GL7 could attain different fatty acid compositions in PE and PC by directing the flow of intermediates through specific biosynthetic pathways. Future studies with strain GL7 should provide useful information regarding the regulation of phospholipid biosynthesis in *S. cerevisiae*.

The preferential incorporation of UFA into PE by strain GL7 suggests that PE performs a unique, critical function in this organism. Of particular interest is the possibility that during normal yeast growth, PE may function both as a bulk membrane component and as a membrane fusogen (28). In *S. cerevisiae*, cell wall biosynthesis is believed to involve cytoplasmic vesicles which transport enzymes and cell wall precursors from their site of synthesis (endoplasmic reticulum or Golgi) to the plasma membrane (29-31). After the vesicles come in contact with the plasma membrane, it is likely that a fusion event must occur to transfer the contents of the vesicle across the plasma membrane. In this process, PE could be required to initiate membrane fusion between the vesicles and the plasma membrane. Alternatively, PE may be required to initiate cell fusion during mating. Recent studies with a yeast mutant deficient in PS biosynthesis have correlated an absence of PE or PS with an inability to undergo cell fusion (21). This reduced capacity for fusion may either reflect a requirement for PE in the membrane to be fused, or it may be due to altered surface properties resulting from unbalanced cell wall biosynthesis.

It has been suggested that the amount of PE required for membrane fusion may be quite small, on the order of 5-10% of the total phospholipids (21). When the supply of PE within the cell is limited (as in the PS mutants), the PE may be used primarily as a fusogen whereas other phospholipids are able to substitute for PE as a bulk membrane component. This would allow the organism to grow with a PE composition that was only 1/3 to 1/2 of the level in wild-type cells. Molecules acting as fusogens appear to do so by promoting the formation of hexagonal (H_{II}) phases (3). The ability of PE to form this phase is related to its

acyl chain composition; PE molecules containing UFA will readily form a hexagonal phase (28), but enrichment of the molecule with medium- or long-chain SFA severely hinders this transition (32-34). If the same situation holds true for yeast, then at least 10% of the total phospholipid composition must be UFA-containing PE, a level which closely agrees with the level of unsaturated-PE preserved during UFA starvation (Table 2; Fig. 1). Thus, by preferentially coupling UFA synthesis with PE biosynthesis, *S. cerevisiae* may be able to ensure an ample supply of unsaturated PE for use as a fusogen, even when the supply of UFA is extremely limited.

ACKNOWLEDGMENTS

We thank Dr. Konrad Bloch for his interest and support during the initial stages of this study. This work was supported by grants S07RR 05386 awarded by the Biomedical Research Support Grant Program and HL28468 from N.I.H. to T.M. Buttke.

REFERENCES

1. Kuehl, F.A., and Egan, R.W. (1980) Science 210, 978-984.
2. Demel, R.A., and Dekruijff, B. (1976) Biochim. Biophys. Acta 457, 109-132.
3. Cullis, P.R., and Hope, M.J. (1978) Nature 271, 672-674.
4. Andreason, A.A., and Stier, T.J.B. (1954) J. Cell. Comp. Physiol. 41, 23-35.
5. Andreason, A.A., and Stier, T.J.B. (1954) J. Cell. Comp. Physiol. 43, 271-282.
6. Resnick, M.A., and Mortimer, R.K. (1966) J. Bacteriol. 92, 597-600.
7. Karst, F., and Lacroute, F. (1977) Molec. Gen. Genet. 154, 269-277.
8. Atkinson, K.D., Jensen, B., Kolat, A.I., Storm, E. M., Henry, S.A., and Fogel, S. (1980) J. Bacteriol. 141, 558-564.
9. Light, R.J., Lennarz, W.J., and Bloch, K. (1962) J. Biol. Chem. 237, 1794-1800.
10. Meyer, F., and Bloch, K. (1963) J. Biol. Chem. 238, 2654-2659.
11. Alterthum, F., and Rose, A.H. (1973) J. Gen. Microbiol. 77, 371-382.
12. Barber, E.D., and Lands, W.E.M. (1973) J. Bacteriol. 115, 543-551.
13. Walenga, R.W., and Lands, W.E.M. (1975) J. Biol. Chem. 250, 9121-9129.
14. Vandenhoff, G., Gunstone, F.D., Barve, J., and Lands, W.E.M. (1975) J. Biol. Chem. 250, 8720-8727.
15. Nes, W.R., Sekula, B.C., Nes, W.D., and Adler, J. H. (1978) J. Biol. Chem. 253, 6218-6225.
16. Hossack, J.A., and Rose, A.H. (1976) J. Bacteriol. 127, 67-75.
17. Taylor, F.R., and Parks, L.W. (1980) Biochem. Biophys. Res. Commun. 95, 1437-1445.
18. Buttke, T.M., and Bloch, K. (1980) Biochem. Biophys. Res. Commun. 92, 229-236.
19. Buttke, T.M., and Bloch, K. (1981) Biochemistry 20, 3267-3272.
20. Atkinson, K., Fogel, S., and Henry, S.A. (1980) J. Biol. Chem. 255, 6653-6661.
21. Kovác, L., Gbelská, I., Poliachová, V., Subik, J., and Kovácova, V. (1980) Eur. J. Biochem. 111, 491-501.
22. Ratcliffe, S.J., Hossack, J.A., Wheeler, G.E., and Rose, A.H. (1973) J. Gen. Micro. 76, 445-449.
23. Gollub, E.G., Lin, K., Doyan, J., Adlersberg, M., and Sprinson, D. (1977) J. Biol. Chem. 252, 2846-2854.
24. Buttke, T.M., Jones, S.D., and Bloch, K. (1980). J. Bacteriol. 144, 124-130.
25. Yavin, E., and Zutra, A. (1977) Anal. Biochem. 80, 430-437.
26. Marzuki, S., and Linnane, A.W. (1979) Methods Enzymol. 56, 568-577.
27. Rattray, J.B.M., Schibeci, A., and Kidby, D.K. (1975) Bacteriol. Rev. 39, 197-231.
28. Cullis, P.R., and Dekruijff, B. (1979) Biochim. Biophys. Acta 559, 399-420.
29. Matile, P.H., Moor, H., and Rabinow, C.F. (1969) in The Yeasts (Rose, A.H., and Harrison, J.S., eds.) Vol. 1, pp. 220-302, Academic, New York, NY.
30. Cabib, E., Ulane, R., and Bowers, B. (1973) J. Biol. Chem. 248, 1451-1458.
31. Novick, P., and Schekman, R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1858-1862.
32. Cullis, P.R., and Dekruijff, B. (1978) Biochim. Biophys. Acta 513, 31-42.
33. Harlos, K., and Eibl, H. (1981) Biochemistry 20, 2888-2892.
34. Mantsch, H.H., Martin, A., and Cameron, D.G. (1981) Biochemistry 20, 3138-3145.

[Received November 16, 1981]

Tissue Culture of Cocoa Beans (*Theobroma cacao* L.): Incorporation of Acetate and Laurate into Lipids of Cultured Cells

C.H. TSAI and J.E. KINSELLA, *Institute of Food Science, Cornell University, Ithaca, NY 14853*

ABSTRACT

Suspension cultures of cocoa bean tissue readily incorporated exogenous acetate into lipids. The distribution of radioactivity from acetate in individual lipid classes after 48 hr was 20, 5, 1, 15, 25, and 35% in triglycerides, diglycerides, free fatty acids, sterol esters, sterols and polar lipids, respectively. The labeled acetate was rapidly incorporated into various fatty acids within 2 hr. The [1-^{14}C] saturated fatty acids declined slightly after 4 hr, whereas [1-^{14}C] oleate declined significantly after 2 hr. There was a concomitant increase in [1-^{14}C] linoleate. The radioactivity associated with linolenate was relatively high up to 4 hr, declined by 24 hr, and then increased again. The kinetics of fatty acid labeling suggested that biosynthesis of linolenic acid in cocoa bean suspension culture may occur via the desaturation of linoleic acid and the chain elongation of dodecatrienoic acid. The patterns of fatty acid radiolabeling following incubation of cells with [1-^{14}C] laurate was consistent with this mechanism.

Lipids 17:367-371, 1982.

INTRODUCTION

Because of their inherent advantages and the ease of control of experimental conditions, plant cell cultures have been exploited extensively for studies of plant cell metabolism (1-6). The fact that cell cultures often exhibit a characteristic fatty acid composition similar to that of the original plant (7,8) supports their use as a model system for studying plant lipid metabolism. Investigations of lipid metabolism in plant suspension cultures have been reported (3,5,6,8,9).

Tissue culture (callus and cell) has been initiated from cocoa bean leaf (10-14) and recently, we established cell cultures of cocoa bean cotyledons (15) and studied the effect of culture conditions on lipid content and composition of cultured cells (16). A long-term objective of this research is to study the control of lipid metabolism and determine if these cells can produce a fat typical of cocoa butter. In order to select optimal conditions for the synthesis of cocoa butter, information concerning the pathways of lipid biosynthesis is needed. This paper reports the use of acetate for fatty acid and lipid synthesis in cocoa bean suspension cultures.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C] Sodium acetate (56.0 mCi/nmol) was purchased from New England Nuclear, Boston, MA, and [1-^{14}C] lauric acid (32 mCi/mmol) was from Amersham, Arlington Heights, IL. PCS (Phase Combining System) liquid scintillant was obtained from Amersham, Chicago, IL. Precoated thin layer chromatography (TLC) plates (Silica Gel 60H) were purchased from Applied Science Labs (State College, PA).

Cell Culture

Suspension cultures of cocoa beans were initiated from cocoa bean calli as described previously (16). Cultures were grown in 125-ml flasks containing 40 ml of MS basal medium (17,18) supplemented with 2,4-D (0.5 ppm) and kinetin (0.1 ppm). Cultures were grown in darkness on a Lab-Line Orbit Eviron shaker (Melrose, IL) shaking at 120 rpm at 28 C. Cultures were subcultured every 12-14 days. Cells were harvested by filtration using Miracloth (Calbiochem, San Diego, CA). The fresh weight of cells was obtained by blotting dry with Kimwipes and weighing immediately.

Incubation with [1-^{14}C] Acetate

Cells of the third subculture were grown for 10 days in 40 ml to obtain a concentration of 100 mg cells/ml. Then [1-^{14}C] acetate (1.0 mCi) was added to each flask and various concentrations (0-100 μM) of unlabeled acetate were added to each flask. The cells were incubated at 28 C for a given period of time. At the appropriate time, 5-ml aliquots were removed from each flask. The reaction was terminated by immersing the test tube containing the cells in boiling water for 10 min. The cell suspensions were then centrifuged in a Precision clinical centrifuge (Chicago, IL) at full speed for 1 min. The clear supernatant was removed, the pellet was washed with 5 ml of distilled water, and centrifuged again. Five ml of chloroform/methanol (2:1, v/v) was added to the pelleted cells

and the mixture was agitated for 30 min. One ml of distilled water was added, mixed, and then centrifuged 1 min to separate the 2 phases. The lower chloroform layer containing the lipids was removed and evaporated under N_2 gas. Lipid extraction was repeated. The extracted lipids were dissolved in 1 ml of chloroform/methanol (2:1, v/v). The radioactivity in aliquots (0.1 ml) of the lipid extracts was determined in a scintillation counter (Packard Tri-Carb, Downers Grove, IL). The remaining lipids were stored at -10 C and used for further analysis of lipids.

Lipid analyses. An aliquot (0.5-ml) of the lipid extract was used for separation of lipid classes by TLC (15). Silica Gel 60H TLC plates were developed with a solvent system of petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v). Spots corresponding to each lipid class (authentic lipid standards, phospholipids, monoglycerides, diglycerides, fatty acids, triglycerides, Nu-Chek-Prep, Elysian, MN) were located using a 2',7'-dichlorofluorescein (0.2%) spray and viewing under ultraviolet (UV) light or by exposure to I_2 vapor. The appropriate zones were scraped into counting vials for determination of radioactivity in the lipid classes.

Fatty acid analyses. An aliquot (0.3-ml) of the lipid solution was used for preparation of fatty acid methyl esters using boron trifluoride as described previously (19). The methyl esters of saturated, mono-, di- and trienoic fatty acids were cochromatographed with authentic standards of methyl esters on Silica Gel 60H TLC plates impregnated with 5% $AgNO_3$. The TLC plates were then developed using a solvent system of chloroform/ethanol/acetic acid (99:1:0.1, v/v/v). The separated methyl esters of saturated, mono-, di- and trienoic acids were located following spraying with 2',7'-dichlorofluorescein (0.2%) spray. The spots corresponding to the fatty acids were located, scraped into scintillation vials and the radioactivity was determined.

Metabolism of [1-^{14}C] Laurate

The incubation system and conditions were the same as used for acetate except 100 nmol [1-^{14}C] lauric acid (1 μCi) was used. The radioactive lipids were extracted and analyzed as just described. The data presented are means from triplicate studies.

RESULTS

Suspension Culture of Cocoa beans

The growth rate of suspension cultures of cocoa beans was rapid, i.e., the fresh weight of cells increased over 20-fold in 14 days.

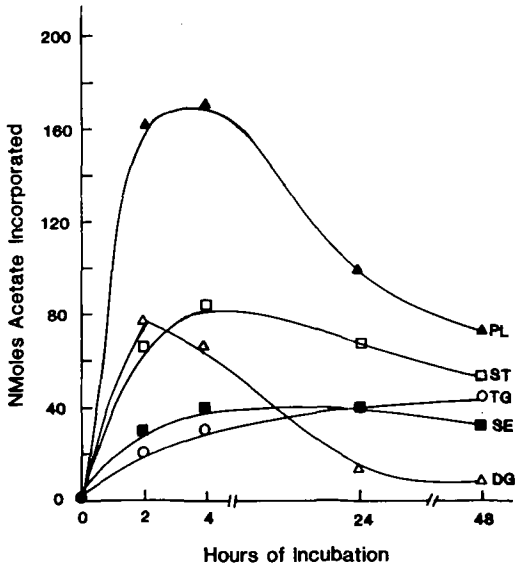


FIG. 1. Incorporation of [^{14}C] label into the various lipid classes of cocoa bean suspension cells following incubation with [^{14}C] acetate (100 μM; 1.0 μCi). Results are given as nmol of [^{14}C] acetate in individual lipids. TG = triglycerides; DG = diglycerides; SE = sterol esters; ST = sterols; PL = polar lipids.

The total lipid content of cell suspensions averaged 6.5% of the dry cell weight. The total lipids were composed of triglyceride (12.6%), sterols (9.2%), phospholipids (22.0%), and glycolipids (56.0%), respectively (15). Neither the lipid content nor composition changed during the incubation periods used in the studies described next. The total lipids contained 28.3% palmitic, 2.4% stearic, 11.5% oleic, 48.6% linoleic and 7.7% linolenic acid, respectively. The fatty acid composition of the total lipids resembled that of immature cocoa beans harvested at very early stages of maturation. The lipid composition of cocoa bean suspension culture was similar to that reported in our previous paper (15).

Incorporation of [^{14}C] Acetate into Lipids

Acetate was readily incorporated into cellular lipids. Incorporation was linear with acetate concentration up to 25 μM and leveled off above 50 μM. Thus, to ensure saturation levels of acetate, a concentration of 100 μM was used in all subsequent studies. Acetate incorporation into lipids was maximal, ca. 15% after 4 hr, and then declined slightly.

Most of the radioactivity was initially associated with the polar lipids, but after 4 hr, this declined (Fig. 1). The diglycerides reached max-

imal radioactivity after 2 hr and then declined. The radioactivity associated with the triglycerides continued to increase over the experimental period. A significant amount of the acetate was incorporated into the sterol and sterol ester components.

The distribution of radiolabel after 48 hr was 34% in polar lipids, 25% in sterols, 20% in triglycerides, 15% in steryl esters, 5% in diglycerides and 1% in free fatty acids.

Labeling of Fatty Acids

The incorporation and distribution of [^{14}C] acetate in various fatty acids as a function of time is summarized in Figure 2. The radioactive acetate was rapidly incorporated into various fatty acids within 2 hr. Radiolabeled saturated fatty acids declined gradually after 2 hr whereas the monoenoic acids declined rapidly between 2 and 4 hr and then gradually for the remaining time (Fig. 2). The [^{14}C]dienoic acid progressively increased up to 24 hr and then declined. The [^{14}C]trienoic fatty acids revealed a fluctuating pattern showing a maximum at 3 hr, declining to 24 hr and then increasing up to 48 hr.

Analyses by GC indicated that the saturated, monounsaturated, diunsaturated and triunsaturated fatty acids of these cocoa bean cell cultures were composed mostly of palmitic (25%), stearic (2.5%), palmitoleic (1.2%), oleic (15%), linoleic (45%), and linolenic acid (10.5%), respectively (15).

The pattern of the radiolabeling in trienoic fatty acids, predominantly linolenic acid, may have reflected the operation of a dual pathway for the synthesis of linolenic acid in cocoa beans, i.e., from linoleic acid and from lauric acid, as reported in chloroplast (20). Therefore, we incubated the cells with [^{14}C]lauric acid. There was a progressive uptake of [^{14}C]laurate with time (Fig. 3). Most of the ^{14}C was associated with saturated fatty acids; however, there was a significant amount of ^{14}C in the trienoic species after 2 hr. This declined slightly by 4 hr and then remained constant. The patterns of radioactivity in the monoenoic and dienoic fatty acids were consistent with a precursor-product relationship.

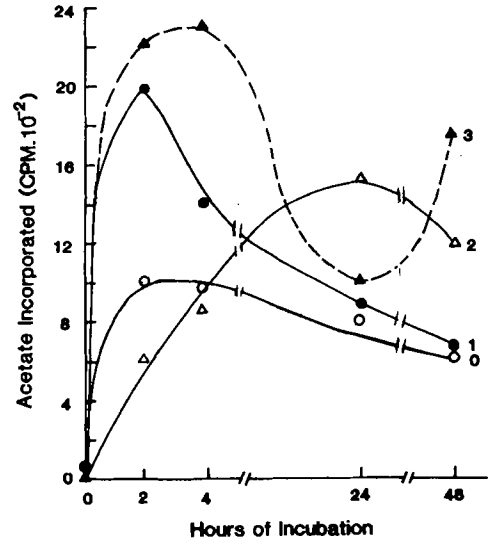


FIG. 2. Incorporation of [^{14}C]acetate into fatty acids of cocoa bean suspension cells following incubation with [^{14}C]acetate (100 μM; 1.0 μCi). Fatty acids were separated as methyl esters by argentation TLC. 0 = saturated; 1 = monoenoic; 2 = dienoic; and 3 = trienoic fatty acids, respectively.

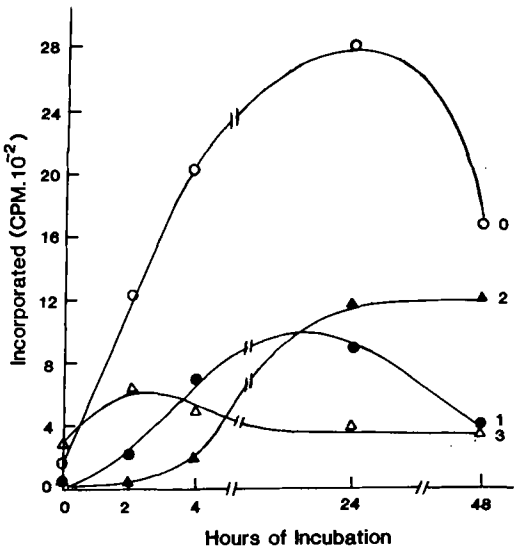


FIG. 3. Distribution of radioactivity in fatty acids of cocoa bean suspension cells following incubation with [^{14}C]lauric acid (20 μM; 1.0 μCi). 0 = saturated; 1 = monoenoic; 2 = dienoic; and 3 = trienoic fatty acids, respectively.

DISCUSSION

The absorption and incorporation of acetate into total lipids of cocoa suspensions was rapid up to 2 hr and reached a maximum uptake of about 12% of the added acetate after 4 hr. Rapid uptake of [^{14}C]acetate was observed in soybean suspensions by many investigators (3, 8,9). The initial pattern of acetate incorporation into lipids of cocoa bean cell suspensions was similar to that observed in soybean cell suspensions (3,8,9). The slight drop in [^{14}C] lipids after 4 hr was not caused by depletion of [^{14}C]

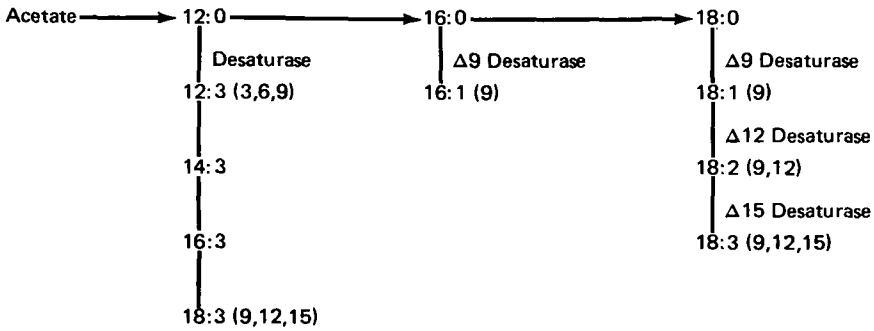


FIG. 4. Scheme for showing proposed desaturation sequence of fatty acids in cocoa bean suspension cultures (adapted in part from Stumpf [20] and Harwood [23]).

acetate in the reaction mixture as this was still well above saturation level.

A large proportion of [^{14}C]acetate was incorporated into polar lipids by cocoa suspensions. About 25, 18 and 45% of [^{14}C]acetate was incorporated into sterols, triglycerides and polar lipids, respectively. These data were comparable to those reported for soybean suspensions (8). [^{14}C] Diglycerides were relatively high early in the incubation and then declined. This decline was accompanied by a gradual increase in [^{14}C] triglycerides. This relationship between DG and TG was compatible with a precursor-product relationship between DG and TG as reported by a number of investigators (21,22).

The kinetics of conversion of acetate into fatty acids were consistent with those reported for soybean suspension cultures (8,9). The relationship between saturated and monoene, monoene and diene, and diene and triene fatty acids in this study, as well as those reported by Stearns and Morton (9) was consistent with the sequential desaturation of 18:0 → 18:1 → 18:2 → 18:3.

However, the pattern of ^{14}C labeling in the trienoic acid species, composed predominantly of linolenic acid, was consistent with the operation of 2 pathways for linolenic acid synthesis. Thus the initial maximum in labeling of trienoic species (Fig. 2) may have reflected rapid desaturation and elongation of lauric acid. This is supported by the data from the [^{14}C]laurate study where there was an early pulse of ^{14}C from laurate in the trienoic species (Fig. 3). The second increase observed in ^{14}C trienoate (Fig. 2) probably reflects the pathway involving the sequential desaturation of stearic acid. This putative pathway has been consistently observed in cocoa bean cells in our studies using [1-^{14}C]oleic and [1-^{14}C]linoleic acid as precursors (Tsai and Kinsella, 1981 manuscript).

The dual pathway for the synthesis of linolenic acid has been described for chloroplasts (20,23), though the elongation pathway for linolenic acid synthesis from hexadecatrienoic acid has been questioned recently (24). Our observations tentatively indicate that it may exist in cocoa bean cells. The relative importance of the 2 pathways may depend on the conditions of culture, such as illumination and concentration of sugar, or mode of carbon supply (25). Studies are in progress to assess the effect of growing conditions on the relative importance of both pathways in the formation of linolenic acid in cocoa bean suspension cultures.

This study shows that cocoa bean cell cultures can be useful for studying lipid synthesis in plant tissue. In a subsequent paper, the metabolism of the major fatty acids occurring in cocoa bean will be reported.

REFERENCES

1. Street, H.E. (1975) in Genetic Manipulation with Plant Materials (Ledoux, L., ed.) pp. 211-229, Plenum Press, New York, NY.
2. Radwan, S.S., and Mangold, H.K. (1976) in Advances in Lipid Research (Paoletti, R., and Kritchevsky, D., eds.) pp. 171-211, Academic Press, New York, NY.
3. Stumpf, P.K., and Weber, N. (1977) Lipids 12, 120-124.
4. Radwan, S.S., Mangold, H.K., Barz, W., and Hüseman, W. (1979) Chem. Phys. Lipids 25, 101-109.
5. MacCarthy, J.J., and Stumpf, P.K. (1980a) Planta 147, 384-388.
6. MacCarthy, J.J., and Stumpf, P.K. (1980b) Planta 147, 389-395.
7. Tattrie, N.H., and I.A. Veliky (1973) Can. J. Bot. 51, 513-516.
8. Wilson, A.C., Kates, M., and de La Roche, A.I. (1978) Lipids 13, 504-510.
9. Stearns, E.M., and Morton, W.T. (1975) Lipids 10, 597-601.
10. Archibald, J.F. (1954) Nature 173, 351-352.
11. Hall, T.R.H., and Collin, H.A. (1975) Ann. Bot. 39, 555-570.
12. Prior, C. (1977) J. Gen. Microbiol. 99, 219-222.
13. Pence, V.C., Hasegawa, P.M., and Janick, J. (1979) J. Am. Soc. Hort. Sci. 104(2), 145-148.
14. Pence, V.C., Hasegawa, P.M., and Janick, J.

(1980) Z. Pflanzenphysiol. Bd. 98, 1-14.
15. Tsai, C.H., and Kinsella, J.E. (1981) Lipids 16, 577-582.
16. Tsai, C.H., and Kinsella, J.E. (1981) Ann. Bot. 48, 549-557.
17. Murashige, T., and Skoog, F. (1962) Physiol. Plant. 15, 473-497.
18. Gamborg, O.L., and Wetter, L.R. (1975) Plant Tissue Culture Methods, NRCC, Saskatoon, Saskatchewan, Canada.
19. Kinsella, J.E., Shimp, J.L., Mai, J., and Weihrauch, J.L. (1977) J. Am. Oil Chem. Soc. 54, 61-65.
20. Stumpf, P. (1975) in Recent Advances in the Chemistry and Biochemistry of Plant Lipids (Gailliard, T., and Mercer, E.I., eds.) pp. 95-114, Academic Press, New York, NY.
21. Wilson, R.F., and Rinne, R.W. (1976) Plant Physiol. 57, 556-559.
22. Taylor, F.R., and Parks, L.W. (1979) Biochim. Biophys. Acta 575, 204-214.
23. Harwood, J.L. (1975) in Recent Advances in the Chemistry and Biochemistry of Plant Lipids (Gailliard, T., and Mercer, E.I., eds.) pp. 44-48, Academic Press, New York, NY.
24. Murphy, D., and Stumpf, P.K. (1979) Plant Physiol. 64, 428.
25. Radwan, S.S., Mangold, H.K., Barz, W., and Hüseman, W. (1979) Chem. Phys. Lipids 25, 101-109.

[Received July 27, 1981]

Reduction of Myocardial Necrosis in Male Albino Rats by Manipulation of Dietary Fatty Acid Levels[1]

J.K.G. KRAMER[a,*], E.R. FARNWORTH[a], B.K. THOMPSON[b], A.H. CORNER[c] and H.L. TRENHOLM[a], [a]*Animal Research Centre and* [b]*Engineering and Statistical Research Institute, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada; and* [c]*Animal Diseases Research Institute, Agriculture Canada, Nepean, Ontario K2H 8P9, Canada*

ABSTRACT

A comprehensive statistical analysis had shown a significant correlation between the incidence of myocardial lesions in male albino rats and the concentration of certain dietary fatty acids. To test this result under controlled conditions, male rats were fed for 16 weeks diets containing 20% by weight soybean oil or a low erucic acid rapeseed (LEAR) oil. Both dietary oils contained substantial amounts of linolenic acid, and both groups developed a high incidence of myocardial necrosis. The addition of dietary saturated fatty acids to the oil in the form of cocoa butter significantly lowered the incidence of heart lesions in both groups. The addition of cocoa butter resulted in increased absorption of saturates and increased growth. Replacement of the cocoa butter by at least an equal amount of synthetic triolein resulted in no significant changes in the cardiopathogenic response compared to the original oils, thus ensuring that the reduction in heart lesions associated with the addition of cocoa butter was not due to dilution of cardiopathogenic compounds in the original vegetable oils. These results support the hypothesis that myocardial lesions in male rats are related to the balance of dietary fatty acids and not to cardiotoxic contaminants in the oils. Changes in the dietary fatty acids did not appear to influence the proportion of the cardiac phospholipids, but their fatty acid composition was markedly influenced. Dietary linolenic acid affected the C22 polyunsaturated fatty acids (PUFA) and dietary saturates increased the level of saturates in cardiac phospholipids. The level of arachidonic acid and total C22 PUFA did not appear to be affected by diet.

Lipids 17:372-382, 1982.

INTRODUCTION

Evidence of necrosis affecting the heart muscle (myocardial necrosis) of male rats after feeding diets rich in fat was first reported by Roine et al. (1) in 1960. These workers attributed the pathological finding to erucic (*cis*-13-docosenoic) acid (22:1) which was present at high levels in the older varieties of rapeseed oil. Subsequent studies showed that male rats fed either the new varieties of rapeseed oils which are practically devoid of 22:1 (2) or other vegetable oils (3) for at least 4 months also developed myocardial necrosis. The occurrence of myocardial necrosis has been demonstrated repeatedly in male albino rats fed corn oil (4-7), coconut oil (8), olive oil (4,9-11), peanut oil (12-14), poppyseed oil (15), safflower oil (8,10), soybean oil (4,5,8,9,16-19), sunflower oil (11,15,20), lard (21), and lard-corn oil mixtures (9,15,22).

The etiology of myocardial necrosis in male rats is still not completely understood. Of the several hypotheses advanced to explain this phenomenon, the one suggesting solely the presence of 22:1 is not supported by experimental evidence (4,7,8,11,21). The one suggesting the presence of cardiotoxic compounds in vegetable oils is highly improbable, because highly purified triglycerides from soybean oil (18) and low erucic acid rapeseed (LEAR) oil (10,18,23,24) are as cardiopathogenic as the corresponding vegetable oil. These findings led to a third hypothesis that the dietary fatty acids per se may give rise to myocardial necrosis in male rats (3,4,8,11,21). This hypothesis was strengthened by the results of a comprehensive statistical analysis of most published data on heart lesions which showed a significant correlation between the level of certain dietary fatty acids and the incidence of myocardial necrosis in male rats (25). In this statistical study, most of the variations in incidence of heart lesions among diets within experiments (59.5%) was explained by the level of saturated fatty acids (16:0 and 18:0) and linolenic acid (18:3); their effect was similar in magnitude but opposite in sign (Table 1).

The statistical analysis (25) suggests a model for the fatty acid imbalance hypothesis which was tested experimentally in this study. The present communication gives the results of this study in which dietary oils were prepared containing different levels of specific fatty acids that were fed to male rats for 16 weeks. In addition to cardiopathology, growth measurements and cardiac lipid were investigated to determine if, and to what extent they reflect changes in myocardial necrosis. Detailed nutri-

[1]Contribution no. 1004 from the Animal Research Centre and No. I-272 from the Engineering and Statistical Research Institute.

TABLE 1

A Summary of a Regression Analysis[a] of Aggregate Data Comparing Levels of Dietary Fatty Acids with Observed Incidence of Myocardial Lesions in Male Albino Rats

	Fatty acids					
	16:0 + 18:0	18:1	18:2	18:3	20:1	22:1
Overall regression coefficient	-0.013	0.002	0.001	0.016	-0.003	0.014
Partial correlation	-0.72	0.40	-0.23	0.61	0.45	0.42

[a]The regression coefficients and partial correlations were reported previously (25) except for the sum of 16:0 + 18:0 which was obtained by reevaluating the data set used in that publication. The regression coefficients are expressed as incidence of lesions/% by wt of fatty acid in test oil. Incidence is defined as number of rats affected over number of rats examined. The correlations between incidence and dietary fatty acid levels take into account experimental differences.

tional aspects have been published elsewhere (26).

MATERIALS AND METHODS

Rationale for Test Oil Mixtures

Two vegetable oils were selected which contained substanial amounts of 18:3, soybean oil and LEAR oil (Table 2). The presence of 18:3 in these oils should produce, based on the statistical evaluation (25), a relatively high incidence of myocardial necrosis in male albino rats. Based on the model, an increase in the level of saturated fatty acids in these oils should lower the incidence of myocardial necrosis. This increase in saturated fatty acids was accomplished by mixing the vegetable oils with cocoa butter (56% saturated; 26% 16:0; 29% 18:0; 1% 20:0; 0.5% 16:1; 38.5% 18:1; 5% 18:2) in a ratio of 16:4. To guarantee that the results of cardiopathology were not due to dilution of toxic substances in the 2 vegetable oils, synthetic triolein (91% 18:1; 6% 18:2; 2% 18:0; 1% 16:0) was mixed with the oils in at least an equal proportion as was the saturated fat. Oleic acid was selected because it showed a relatively low correlation to myocardial necrosis in the statistical analyses (Table 1). Soybean oil was mixed with the synthetic triolein in a ratio of 1:1 because the resultant mixture would

TABLE 2

Composition of Dietary Oils and the Fatty Acid Analysis of These Oils

	% by wt of the diet						
LEAR oil (cv. Tower)	20	16	16	–	–	–	–
Soybean oil	--	–	–	20	16	9.6	9.5
Cocoa butter	–	4	–	–	4	–	–
Triolein	–	–	4	–	–	9.6	9.5
Linseed oil	--	–	–	–	–	0.8	0.8
X:1 (20:1, 22:1 and 24:1)	–	–	–	–	–	–	0.2
Fatty acids (% by wt)							
14:0	0.1	0.1	0.1	0.1	0.1	tr	tr
16:0	4.4	8.2	3.4	12.1	17.9	5.8	5.8
18:0	1.5	7.5	1.2	3.5	9.2	2.6	2.7
20:0	0.8	0.6	0.5	0.4	0.4	0.3	0.5
22:0	0.3	0.2	0.2	0.1	tr	tr	0.1
24:0	0.1	tr	tr	tr			
Total saturates	7.2	16.6	5.4	16.2	27.6	8.7	9.1
16:1	0.4	0.2	0.2	0.2	0.3	0.1	0.1
18:1	57.5	55.8	66.9	24.6	26.9	56.1	55.3
20:1	1.9	1.2	1.2	0.3	0.2	0.1	1.0
22:1	0.6	0.4	0.4	0.1	tr	tr	0.4
24:1	0.1	tr	tr				tr
Total monounsaturates	60.5	57.6	68.7	25.1	27.4	56.3	56.7
18:2	22.0	17.8	17.9	51.9	40.1	28.3	27.5
18:3	10.3	7.9	7.8	6.7	4.9	6.7	6.7

have a fatty acid composition similar to that of the LEAR oil. The addition of a small amount (14.9 g/kg test oil) of long-chain monounsaturated fatty acids (79% 20:1; 20% 22:1 and 1% 24:1) to the latter mixture provided an even closer resemblance to LEAR oil. An oil that mimicked the fatty acid composition of a LEAR oil would be expected to give a similar incidence of heart lesions. The 18:3 content of the 2 test oils containing the mixture of soybean oil and triolein was restored to that of the original soybean oil by the addition of an appropriate amount of linseed oil (49%, 18:3).

Experimental Design

The diets were formulated to contain 20% oil and 20% casein as described previously (3, 10,26). All test oils were added to the diet at a level of 20% by weight (Table 2). The caloric content of the diets was measured by bomb calorimetry (27). Male Sprague-Dawley rats (50-60 g) were randomly allocated among 7 groups, each consisting of 50 animals, and fed ad libitum the semisynthetic diets for 16 weeks. All rats were weighed individually at the beginning and at 2-week intervals throughout the experiment. Apparent digestibilities were calculated from consumption, excretion, and composition data measured at 4, 8, and 12 weeks (28,29). Consumption data were measured from 5 groups of 10 rats per diet for the period of the whole experiment.

Analytical Procedures

Six rats from each dietary group were killed after 16 weeks by exsanguination while under CO_2 anesthesia. The hearts were removed immediately and the total lipids extracted according to a procedure intended to minimize lipolysis (30).

The cardiac lipid classes were quantitated following separation on thin silica-coated quartz rods (Chromarod S) using an Iatroscan (Technical Marketing Associates, Mississauga, Ont.) equipped with a flame ionization detector (H_2 flow rate, 175 ml/min; air flow rate, 1,850 ml/min), a scanner (scanning speed, 0.47 cm/sec), and an integrator and recorder (sensitivity, 10 mV; chart speed, 0.42 cm). The chromarods (type S) were successively developed using the following solvents: (a) hexane/diethyl ether/formic acid (85:15:0.04), (b) acetone, and (c) $CHCl_3/CH_3OH/H_2O$ (67:29:4). After each development, the chromarods were partially burned (31) to determine, in turn, neutral lipids, cholesterol and polar lipids. The phospholipids were isolated by thin layer chromatography (TLC) according to Rouser et al. (32). The fatty acid composition of cardiac lipid classes was determined by gas chromatography as described previously (7).

The hearts from the remaining 44 rats per diet group were removed and fixed in 10% neutral buffered formalin. Three histological sections were prepared from each heart as described previously (33).

Statistical Methods

The various measurements by the analytical procedures just described were analyzed using the analysis of variance. An approximate χ^2 method, described by Fienberg (34), was used to analyze the incidence data, i.e., the frequency of rats showing evidence of myocardial lesions in any of their 3 heart sections.

The data set and the statistical methods described by Trenholm et al. (25) were used to estimate a prediction equation involving levels of saturates (16:0 and 18:0) and 18:3. The original study used levels of 16:0 or 18:0 separately because they were highly correlated but, because cocoa butter contains considerable amounts of both fatty acids, it was felt that the sum of the saturate levels would be more appropriate for the analysis of this paper.

Dietary Test Oils

The proportion of fats and oils in the experimental diets and the corresponding fatty acid compositions are shown in Table 2. The addition of cocoa butter increased the percentage of saturated fatty acids of both LEAR and soybean oils by ca. 10%. The addition of triolein to soybean oil resulted in a mixture with a fatty acid composition much like that of LEAR oil. The further addition to this mixture of small amounts of long-chain monounsaturated fatty acids (20:1, 22:1 and 24:1) provided an even closer resemblance. Linseed oil was added to the mixtures containing soybean oil and triolein (1:1) to restore the level of 18:3 close to that in the original soybean oil. All diets were found to be isocaloric as determined by bomb calorimetry.

RESULTS

Cardiopathology

A high incidence of myocardial necrosis was observed in male Sprague-Dawley rats fed diets containing both LEAR and soybean oils; the difference in lesion incidence between these diets was not significant (Table 3). A 10% increase in the level of dietary saturated fatty acids of these oils, achieved by the addition of

TABLE 3

Myocardial Lesions in Male Rats Fed the Experimental Diets for 16 Weeks and Lesion Incidence Predicted

	Incidence (%)[a]		Severity[c]				
	Observed (n = 44)	Predicted[b]	1	2	3	4	>4
LEAR (cv. Tower)	61	64	10	10	3	3	1
LEAR + cocoa butter	36	47	8	3	2	2	1
LEAR + triolein	55	62	11	9	3	1	0
Soybean	57	46	10	7	7	1	0
Soybean + cocoa butter	34	27	11	1	1	1	1
Soybean + triolein	59	55	16	4	1	4	1
Soybean + triolein + X:1[d]	55	55	16	3	2	2	1

Comparisons	d.f.	χ^2[e] Incidence
All diets	6	13.0*
Diets with cocoa butter vs original oils	1	10.2**
Diets with triolein vs original oils	1	0.1

[a]The incidence of myocardial lesions is the number of rats affected over the number of rats examined per diet (44 rats).

[b]The predicted incidence of heart lesions was calculated using the following equation: $Z_i = \bar{Y} - 0.013\ (X_{1i}-\bar{X}_1) + 0.016\ (X_{2i}-\bar{X}_2)$ where Z_i is the predicted incidence of heart lesions, $\bar{Y}$ the average observed incidence of heart lesions for all diets (0.51), -0.013 and 0.016 the correlation coefficients of 16:0 + 18:0 and 18:3, respectively, X_i and X_2 the dietary concentration of 16:0 + 18:0 and 18:3 in the ith diet, and $\bar{X}$ the overall mean concentration of the specific fatty acid(s) from all diets.

[c]Severity of myocardial necrosis represents the number of rats with 1, 2, 3, 4 and >4 lesions per heart (3 sections).

[d]X:1 (20:1, 22:1, 24:1).

[e]The χ^2 analysis was according to Fienberg (34); d.f., degrees of freedom, and significance at the 5% (*), and 1% (**) level.

cocoa butter, resulted in a major decrease in the incidence of myocardial necrosis. On the other hand, using triolein in the mixture, rather than cocoa butter, gave levels of incidence in heart lesions not significantly different from those of the original oils.

The severity of myocardial necrosis, expressed as the total number of lesions per set of 3 heart sections, was relatively low in this study for rats fed soybean oil or LEAR oil compared to previous results from this laboratory (4,7, 18). Most of the affected rats had only 1 or 2 lesions per heart; those with more than 4 lesions per heart were very few. Hence, no attempt was made to analyze the severity data.

Table 3 includes the incidence of heart lesions as predicted by the regression equation (Table 1) using the fatty acid composition of the test oils (Table 2). It can be seen that the observed and predicted incidence of myocardial necrosis is in fairly close agreement.

Growth Performance and Fat Consumption

The rats fed the LEAR diet consumed less feed overall and weighed less at the end of the experiment than did rats fed the soybean diet (Table 4). The fact that only the weight data showed a significant difference here was perhaps because the consumption data, unlike the weight data, were not measured on individuals. Growth and feed consumption increased with the addition of cocoa butter to the test oils; the increase in growth was significant for the addition of saturates to LEAR oil. Substitution of triolein for the cocoa butter in the mixture with the LEAR oil appeared to have little influence on either variable. The dietary oils formulated to mimic LEAR oils seemed to reduce body weight and feed consumption, relative to soybean oil, the differences being appreciable only for the weight data.

Estimates of the consumption of 16:0 and 18:0 by the dietary groups are also given in Table 4. Rats fed soybean oil consumed much more saturated fatty acids than did those fed LEAR oil. The consumption of 16:0 and 18:0 increased markedly in rats fed diets with cocoa butter, whereas the addition of triolein reduced

TABLE 4

Body Weight, Feed Consumed and Consumption of Saturated Fatty Acids

Diets	Body weight (g)[a]	Feed consumed (g/rat)[b]	Consumption (g/rat)[c] 16:0	18:0
LEAR (cv. Tower)	416	1636	14	4
LEAR + cocoa butter	466	1684	24	17
LEAR + triolein	427	1654	12	5
Soybean	496	1709	34	10
Soybean + cocoa butter	508	1733	41	23
Soybean + triolein	461	1712	19	8
Soybean + triolein + X:1[d]	464	1681	20	8
LSD ($p<0.01$)[e]	26	70		

[a]Body weights are the mean of 50 rats per diet.

[b]Feed consumption is the total feed consumed by the rats over the entire experimental period (16 weeks) divided by the number of rats per group. Five groups of 10 rats were used for each diet.

[c]The total amount of 16:0 and 18:0 consumed was calculated by: average feed consumed (per 16 weeks) X % oil in diet X % composition of fatty acid in oil X apparent digestibility of fatty acid (average of week 4, 8 and 12).

[d]X:1, (20:1, 22:1 and 24:1).

[e]LSD, least significant difference.

TABLE 5

Weight, Lipid Content and Lipid Class Composition of the Hearts of Rats Fed the Experimental Diets for 16 Weeks

	Heart weight (g)	Lipid weight (mg/heart)	Lipid classes (mg/g wet heart)[a] CE	TG	C	DPG	PE	PS & PI	PC	SP
LEAR (cv. Tower)	1.02	31.9	0.4	8.9	2.1	2.6	6.0	0.8	9.0	0.7
LEAR + cocoa butter	1.03	34.5	0.3	11.2	2.0	2.4	6.0	0.9	8.9	0.8
LEAR + triolein	1.18	43.3	0.3	14.2	2.3	2.5	5.7	0.9	9.0	0.9
Soybean	1.09	36.1	0.3	9.3	2.2	2.7	6.6	0.8	9.8	0.7
Soybean + cocoa butter	1.25	41.2	0.4	8.9	2.2	2.7	7.1	0.9	9.3	0.8
Soybean + triolein	1.07	36.2	0.3	9.0	2.3	3.0	6.9	0.9	9.8	0.8
Soybean + triolein + X:1[b]	1.11	35.0	0.3	7.9	2.3	2.8	6.5	0.8	9.5	0.9
LSD ($p<0.01$)[c]	0.19	9.3	0.2	4.2	0.5	0.7	1.3	0.4	1.3	0.3

[a]All values are the mean of 6 rats per diet. The lipid classes are: cholesterol ester (CE), triacylglycerol (TG), cholesterol (C), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC) and sphingomyelin (SP). Trace amounts of lysophosphatidylcholine, cerebroside, diacylglycerol and free fatty acid were found but were not included in the table.

[b]X:1 (20:1, 22:1 and 24:1).

[c]LSD, least significant difference.

the absorption of these fatty acids.

Cardiac Lipid Changes

The heart and lipid weights, including the cardiac lipid class composition, are shown in Table 5. Mixing either cocoa butter or triolein with LEAR oil resulted in increased levels of cardiac lipids in the form of triacylglycerol compared to rats fed LEAR oil. The addition of cocoa butter or triolein to soybean oil appeared to have no effect on total cardiac lipids or its composition. The phospholipids were remarkably similar among all diets.

The fatty acid composition of the major cardiac phospholipids are summarized in Table 6. An analysis of variance incorporating the data from 3 phospholipids were calculated and are included in Table 6 (diphosphatidylglycerol was excluded because the composition of this polar lipid was so different). It was evident

from the statistical analysis that rats fed soybean oil showed significantly higher levels of saturated and total PUFA, and significantly lower levels of monounsaturated fatty acids in all phospholipids compared to rats fed LEAR oil. In contrast to the significant differences between the soybean and LEAR oil groups, there was a remarkable similarity in the fatty acid composition of the phospholipids of rats fed LEAR and soybean + triolein mixtures (the latter intended to mimic LEAR), there being but one significant difference in fatty acid levels. Enrichment of dietary saturated fatty acids by the addition of cocoa butter resulted in a significant increase in cardiac saturated fatty acids, apparently at the expense of monounsaturated fatty acids and linoleic acid. The most noticeable changes arising from the addition of triolein were in the increased levels of the monounsaturated fatty acids and C22 (n-3) PUFA, and in the decreased levels of saturated and (n-6) PUFA. The differences here were more pronounced in those diets with the higher proportion of triolein. The level of cardiac arachidonic acid and the total C22 PUFA did not appear to be influenced by the modifications to the dietary oils.

It should be noted that the interactions between phospholipid classes and diets were significant for most fatty acids, except saturates. However, the interactions were generally of a much smaller magnitude than the overall differences. The trends among diets for the phospholipid classes were similar; the reason for the interactions seemed to be the differences in degree of change. Transformations of the data did not remove these interactions.

Cardiac sphingomyelin consisted almost exclusively of saturated and monounsaturated fatty acids (Table 7). Rats fed soybean oil showed significantly more saturated (particularly 18:0, 22:0 and 24:0) and less monounsaturated (particularly 18:1 and 24:1) fatty acids than did rats fed LEAR oil. The addition of cocoa butter to the 2 oils had little effect on the relative proportion of saturates to monounsaturates; however, there was an increase of the C18 fatty acids at the expense of the longer chain fatty acids. The addition of triolein to LEAR oil resulted only in minor changes. On the other hand, the composition in rats fed the soybean oil triolein mixtures approached the composition of rats fed LEAR oil in the proportion of saturates and unsaturates, but there were significant differences between the kind of fatty acids within each group.

DISCUSSION

An earlier study (25) showed a relationship between levels of certain dietary fatty acids and the incidence of myocardial necrosis in male albino rats. In particular, in the presence of appreciable amounts of dietary 18:3, increases in the levels of dietary saturates were associated with a lower incidence of lesions. The nature of the study, however, precluded conclusions relating to cause and effect. The lower incidence of lesions might, e.g., be due to the type of oil, the fatty acid levels characteristic of that oil, or some other related factors. To test the "cause-effect" hypothesis properly, one must control the levels of the dietary fatty acids specifically. To this end, in this study, the level of saturated fatty acids in 2 vegetable oils, viz., soybean and LEAR oils, were increased by the addition of cocoa butter. It might still be argued that any observed change in incidence was due, not to the addition of the saturates, but to the dilution of other fatty acids or even some cardiotoxic compounds. Hence, additional mixtures were included with a synthetic triacylglycerol replacing the cocoa butter. This material was composed almost entirely of oleic acid, a fatty acid which the original study suggested was not closely related to the incidence of lesions (Table 1).

The results from this study provide convincing experimental evidence to support the hypothesis suggested by the earlier study (25). Addition of the saturates to both vegetable oils led to similar reductions (about 25%) in incidence of lesions. Furthermore, the addition of oleic acid had no discernible effect whatsoever. The fact that there are some discrepancies between the observed and predicted incidence of heart lesions (Table 3) is, indeed, not surprising. It is well established that differences in lesion incidence will occur between (35) and within (18) experiments even though all parameters were kept as nearly identical as possible. The fact is, the results of this study fit remarkably well into the continuum of points derived from the regression equations of the aggregate data of heart lesions and dietary fatty acids (25) as seen in Figure 1. Although the etiology may not be clear, it seems apparent that the manipulation of the saturate levels in the dietary oils will lead to changes in the incidence of myocardial necrosis.

The improved growth observed in rats fed the diets enriched with saturated fatty acids provided additional evidence that myocardial necrosis may be related to an improper balance of dietary fatty acids. In fact, some earlier studies had established a maximal growth in rats, provided the level of saturated fatty acids was 20-40% (36-38). It may, therefore, not be coincidental that when the level of saturates in

TABLE 6

Fatty Acid Composition (%) of the Major Cardiac Phospholipids[a]

Diets	DMA	Saturated	Monounsaturated	Polyunsaturated						
				Total	n-6	n-3	18:2	20:4	C22	C22 n-3
Phosphatidylethanolamine										
LEAR	5	33	12	50	25	25	4	19	26	24
LEAR + cocoa butter	7	34	10	48	24	25	4	19	26	24
LEAR + triolein	4	32	14	49	24	25	3	19	27	25
Soybean	7	34	7	52	31	21	8	19	24	21
Soybean + cocoa butter	9	35	7	49	30	20	6	20	23	19
Soybean + triolein	5	32	12	51	26	26	5	19	27	25
Soybean + triolein + X:1	4	33	13	51	26	26	5	19	27	26
LSD ($p<0.01$)	2	2	2	2	3	3	2	2	3	3
Phosphatidylcholine										
LEAR	0.8	40	14	45	38	7	7	30	7	7
LEAR + cocoa butter	0.9	42	12	45	38	7	6	31	8	7
LEAR + triolein	1.1	39	15	44	36	8	6	30	8	8
Soybean	1.0	42	8	49	44	5	11	32	6	5
Soybean + cocoa butter	0.8	43	7	49	44	5	8	34	6	5
Soybean + triolein	1.0	39	13	47	39	8	9	30	8	8
Soybean + triolein + X:1	1.0	39	13	47	40	8	8	31	8	8
LSD ($p<0.01$)	0.4	1	1	1	2	2	2	2	2	2
Phosphatidylserine and phosphatidylinositol										
LEAR	0.8	45	12	42	30	12	4	23	13	12
LEAR + cocoa butter	1.1	47	10	42	30	13	3	24	14	12
LEAR + triolein	1.0	45	12	42	30	12	4	23	13	12
Soybean	1.0	46	7	45	34	11	7	23	14	11
Soybean + cocoa butter	1.0	47	7	45	34	10	6	23	14	10
Soybean + triolein	1.0	44	11	44	31	13	5	23	15	13
Soybean + triolein + X:1	1.0	44	11	44	32	12	5	24	15	12
LSD ($p<0.01$)	0.3	2	1	2	2	2	1	1	2	2
Diphosphatidylglycerol										
LEAR	0.8	7	15	77	70	7	64	4	6	6
LEAR + cocoa butter	0.9	7	14	78	71	8	64	4	7	6

LEAR + triolein	0.8	7	19	74	66	8	59	4	6	7		
Soybean	0.8	6	6	88	86	3	82	2	2	2		
Soybean + cocoa butter	0.6	6	7	87	84	3	80	3	3	2		
Soybean + triolein	1.0	7	11	82	76	5	71	3	5	5		
Soybean + triolein + X:1	1.0	6	11	81	78	4	73	3	4	3		
LSD ($p<0.01$)	0.4	2	2	3	5	2	6	1	2	2		
Analysis of variance (d.f.)					Mean square							
Soybean vs LEAR (1)	5.8*	18*	247*	83*	261*	49*	111*	0.6	5.3	48*		
LEAR vs soybean-triolein mixtures (1)	0.1	3.7	6.8	27*	8.6	5.1	14	2.2	11	6.3		
Oils vs added cocoa butter (1)	7.3*	29*	20*	8.1	5.1	0.3	29*	8.1	0.1	0.1		
Oils vs added triolein (1)	3.1	32*	138*	17*	136*	57*	41*	7.9	29	60*		
Error (30)	0.4	2.1	0.9	2.1	4.1	4.4	2.1	1.9	4.6	4.5		

[a]Polyunsaturated fatty acids were grouped into those derived from the linoleic (n-6) and linolenic (n-3) acid families. The column designated DMA are the dimethyl acetals produced during acid-catalyzed transesterification of the alkenyl ethers. X:1 (20:1, 22:1 and 24:1). LSD, least significant difference at the 1% level, based on each phospholipid class separately. Analyses of variance pooled over 3 phospholipids (excluding diphosphatidylglycerol) is given at the bottom; significance is indicated at the 1% level (*).

LEAR and soybean oils was increased to the levels suggested, it was accompanied by a marked reduction in heart lesions. This appears to be consistent with the results of Hulan et al. (21) who showed that diets rich in saturates are associated with a low incidence of heart lesions in male rats. Conversely, the lack of sufficient amount of saturates may explain why previous studies (9) failed to conclude (39) any ameliorating effects when the level of saturates was raised from 6.7 or 7.7% to 11% (9).

The reasons for the limited addition of saturates to the dietary oil in a previous study (9) to test for the effect of saturates (LEAR oil to palm oil, 18 to 2) was based in part on the assumption that LEAR oils contain cardiopathogenic compounds, the effect of which would be diluted by the addition of supposedly noncardiopathogenic oil or fat. This assumption, however, is no longer tenable, as extensive studies provided no experimental evidence of the presence of cardiotoxic compounds in soybean oil (18), LEAR oils (10,18,24) or rapeseed oils high in erucic acid (24). The highly purified triglycerides from soybean oil (18) and LEAR oils (10,18) retained their cardiopathogenic properties. Furthermore, the results of this study provide additional evidence that the heart lesions were not caused by trace contaminants in the oils. Mixing either LEAR or soybean oils with cocoa butter or triolein did not result in a similar response in heart lesions as would have been expected by dilution of a toxin. From this new perspective, the data presented in Tables 3 and 4 of reference (9) can be interpreted in a different manner, i.e., LEAR oils were mixed with different proportions of saturated fat or fat-oil mixtures. If the data are thus viewed together, the results show a remarkable similarity with those presented in the present study, the correlation between levels of saturates and incidence of heart lesions being -0.76, as opposed to -0.72 in Table 1.

If dietary fatty acids are related in some way to myocardial necrosis, one might expect differences in cardiac lipid classes and/or their fatty acid composition to reflect the cardiopathological state. In general, the cardiac lipid class composition was not found to be affected by the different diets tested for heart lesions except triacylglycerol. However, the changes in the level of triacylglycerol could not be used as an indicator of cardiopathogenicity because the response to the addition of cocoa butter or triolein was unique to each oil and unlike the directional changes observed with heart lesions. Previous attempts to relate cardiac triacylglycerol levels resulting from erucic acid to heart lesions (40) were equally unsuccessful. A level of di-

TABLE 7

Fatty Acid Composition of Cardiac Sphingomyelin in Male Rats Fed the Experimental Diets for 16 Weeks[a]

Fatty acids	LEAR (cv. Tower)	LEAR + cocoa butter	LEAR + triolein	Soybean	Soybean + cocoa butter	Soybean + triolein	Soybean + triolein + (X:1)	LSD ($p<0.01$)
14:0	2.1	1.2	0.5	0.9	2.0	1.3	0.5	0.5
15:0	1.3	0.8	0.2	0.7	1.5	1.0	0.2	0.5
16:0	13.7	12.0	13.0	11.8	12.4	12.4	11.5	1.0
17:0	1.0	0.4	0.5	0.6	0.7	0.9	0.6	0.4
18:0	9.1	22.9	11.8	14.2	18.4	19.3	16.6	2.1
19:0	1.7	2.8	1.9	2.9	3.0	4.4	3.6	0.9
20:0	17.2	14.3	16.4	13.1	13.2	9.9	11.5	2.0
21:0	1.3	0.9	1.1	1.9	1.3	1.3	1.6	0.6
22:0	18.5	15.4	18.8	23.6	19.4	15.7	17.4	1.8
23:0	2.5	2.0	2.9	5.1	4.0	4.3	4.5	1.3
24:0	7.2	6.6	8.2	11.0	9.6	8.8	8.4	1.0
Total	75.6	79.3	75.3	85.8	85.5	79.3	76.4	2.7
16:1	1.9	0.9	0.9	1.3	1.3	1.3	1.1	0.5
18:1	3.5	5.7	4.6	2.9	3.9	7.1	5.8	1.1
20:1	0.3	0.3	0.4	0.2	0.2	0.3	0.5	0.2
22:1	1.2	0.7	1.0	0.7	0.1	0.7	0.9	0.3
23:1	0.3	0.2	0.4	0.2	0.2	0.2	0.2	0.2
24:1	13.4	10.8	14.5	6.3	5.2	8.0	11.3	1.5
Total	20.6	18.6	21.8	11.6	10.9	17.6	19.8	1.8
CN22.6	0.9	0.4	0.5	0.4	0.7	0.5	0.7	0.4
CN23.6	2.4	1.2	1.9	1.6	2.3	2.2	2.5	0.6

[a]All values are expressed as percent by wt and are the mean of 6 rats per diet. X:1 (20:1, 22:1 and 24:1). LSD, least significant difference. CN, carbon number.

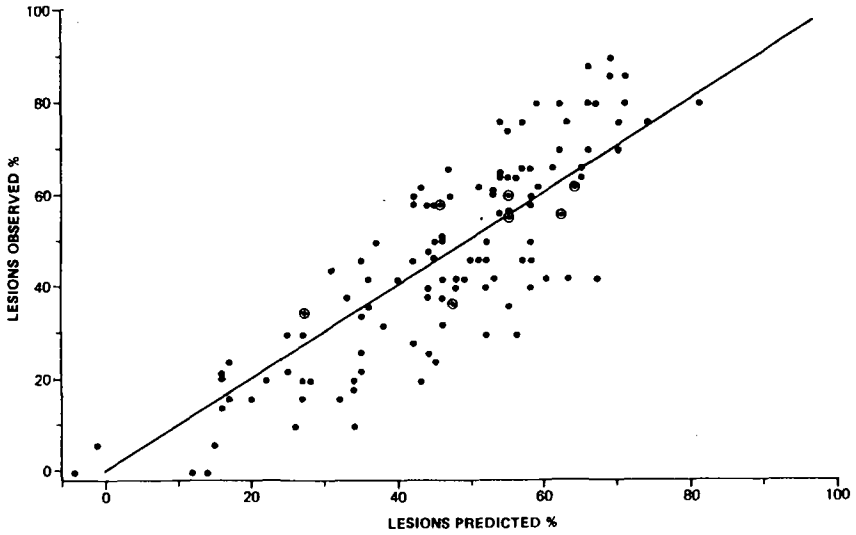


FIG. 1 Observed vs predicted incidence of myocardial lesions in male rats. The observed incidence of heart lesions was taken from published data for which regression coefficients were calculated and used to determine the predicted incidence of lesions (25). The results of each of the 7 diets of the present study (◉) are included in the previous plot (•) (25).

etary erucic acid which gave a positive cardiac triacylglycerol response (3,41) did not result in a corresponding increase in heart lesions (4,21). Furthermore, despite a similar accumulation of cardiac triacylglycerols between sexes (3) and strains (7) of rats, the lesion response was widely different.

In contrast to the cardiac lipid class composition, marked changes were observed in the fatty acid composition of the lipid classes. There was a consistent increase in the level of saturates in cardiac phospholipids with the addition of saturates to either soybean or LEAR oils. This increase parallels an increase in the consumption of saturates (Table 4) and a decrease in incidence of heart lesions (Table 3). The addition of triolein marked an increase in the level of monoenoic fatty acid as expected, as 18:1 is readily incorporated into cardiac phospholipids (42,43). The consumption of saturates was only slightly reduced by mixing triolein with LEAR oil, but a major reduction in the consumption of saturates occurred by feeding the triolein-soybean oil mixtures. The incidence in heart lesions, however, showed no significant change. Little change in heart lesion incidence was expected with the addition of triolein to LEAR oil because the level of dietary saturates was not greatly affected and 18:1 showed a low correlation to heart lesions (Table 1). On the other hand, the addition of triolein to soybean oil was expected to increase the incidence of heart lesions because the level of dietary saturates was significantly lowered. The results of this study suggest that the observed incidence of heart lesions in rats fed the soybean oil diet was relatively high—higher than expected based on previous studies (4,18). In fact, an average incidence of about 44% (4,18) would be consistent with both the consumption and fatty acid composition data. As stated previously in the discussion, the incidence of heart lesions is known to vary both within (18) and between (35) experiments, and the present value of soybean oil (57%) is within the observed variation (Fig. 1).

Dietary 18:3 resulted in a relatively high level of C22 PUFA of the linolenic acid (n-3) family with virtual exclusion of the C22 PUFA of the linoleic acid (n-6) family as seen in Table 6 and as observed previously (15,42,44). The level of (n-3) family acids appeared to be little influenced by the addition of saturates in the diet, despite the observed significant decrease in lesion incidence by the addition of saturates. This suggests that saturates are not undoing the cardiopathogenic effect of 18:3, but appear to act independently. Therefore, dietary fats or oils which contain at least 20-25% saturates, irrespective of dietary 18:3, will be associated with a low incidence of heart lesions in male rats.

It should be noted, however, that the apparent requirement of a balance of dietary fatty acids for the male albino rat is critical only when the level of fat in the diet is high, i.e., 15

or 20%. Myocardial necrosis was observed to decrease significantly in male rats when the same oil was fed at a lower level, i.e., at 5 or 10% of the diet (13,14,45,46). This would not seem unreasonable, because the de novo synthesis of saturates by the rat would be significantly reduced when a diet rich in fat was fed (47,48). Therefore, the rat might be under a nutritional stress to synthesize the required amount of saturates for its phospholipids. Proper membrane function and stability may well require a fatty acid composition within a certain range.

ACKNOWLEDGMENTS

We acknowledge the advice of Drs. S. Mahadevan and F.D. Sauer, and the skillful technical assistance of R.C. Fouchard, A.M. Honkanen, F. Muldoon, D. Robillard and B. Ullett.

REFERENCES

1. Roine, P., Uksila, E., Teir, H., and Rapola, J. (1960) Z. Ernährungsw. 1, 118-124.
2. Rocquelin, G., and Cluzan, R. (1968) Ann. Biol. Anim. Biochim. Biophys. 8, 395-406.
3. Kramer, J.K.G., Mahadevan, S., Hunt, J.R., Sauer, F.D., Corner, A.H., and Charlton, K.M. (1973) J. Nutr. 103, 1696-1708.
4. Hulan, H.W., Kramer, J.K.G., and Corner, A.H. (1977) Lipids 12, 951-956.
5. Slinger, S.J. (1977) J. Am. Oil Chem. Soc. 54, 94A-99A.
6. Umemura, T., Slinger, S.J., Bhatnager, M.K., and Yamashiro, S. (1978) Res. Vet. Sci. 25, 318-322.
7. Kramer, J.K.G., Hulan, H.W., Trenholm, H.L., and Corner, A.H. (1979) J. Nutr. 109, 202-213.
8. McCutcheon, J.S., Umemura, T., Bhatnager, M. K., and Walker, B.L. (1976) Lipids 11, 545-552.
9. Beare-Rogers, J.L., Nera, E.A., and Heggtveit, A.H. (1974) Nutr. Metab. 17, 213-222.
10. Kramer, J.K.G., Hulan, H.W., Mahadevan, S., Sauer, F.D., and Corner, A.H. (1975) Lipids 10, 511-516.
11. Vles, R.O., Bijster, G.M., and Timmer, W.G. (1978) Arch. Toxicol. Suppl. 1, 23-32.
12. Ackman, R.G. (1974) Lipids 9, 1032-1035.
13. Cluzan, R., Suschetet, M., Rocquelin, G., and Levillain, R. (1979) Ann. Biol. Anim. Biochim. Biophys. 19, 497-500.
14. Vles, R.O., Timmer, W.G., and Zaalberg, J. (1979) Ann. Biol. Anim. Biochim. Biophys. 19, 501-508.
15. Beare-Rogers, J.L., Gray, L., Nera, E.A., and Levin, O.L. (1979) Nutr. Metab. 23, 335-346.
16. Vogtmann, H., Christian, R., Hardin, R.T. and Clandinin, D.R. (1975) Int. J. Vit. Nutr. Res. 45, 221-229.
17. Ilsemann, K., Reichwald, I., and Mukherjee, K.D. (1976) Fette Seifen Anstrichm. 78, 181-187.
18. Kramer, J.K.G., Hulan, H.W., Corner, A.H., Thompson, B.K., Holfeld, N., and Mills, J.H.L. (1979) Lipids 14, 773-780.
19. Clandinin, M.T., and Yamashiro, S. (1980) J. Nutr. 110, 1197-1203.
20. Vles, R.O., Bijster, G.M., Kleinekoort, J.S.W., Timmer, W.G., and Zaalberg, J. (1976) Fette Seifen Anstrichm. 78, 128-131.
21. Hulan, H.W., Kramer, J.K.G., Mahadevan, S., Sauer, F.D., and Corner, A.H. (1976) Lipids 11, 9-15.
22. Beare-Rogers, J.L., and Nera, E.A. (1977) Lipids 12, 769-774.
23. Kramer, J.K.G., Hulan, H.W., Mahadevan, S., and Sauer, F.D. (1975) Lipids 10, 505-510.
24. Bijster, G.M., Hudalla, B., Kaiser, H., Mangold, H.K., and Vles, R.O. (1979) in Proceedings of the 5th International Rapeseed Conference, Malmö, Sweden, June 12-16, 1978, Vol. 2, pp. 141-143, Lagerblads Tryckeri AB, Karlshamn, Sweden.
25. Trenholm, H.L., Thompson, B.K., and Kramer, J.K.G. (1979) Can. Inst. Food Sci. Technol. J. 12, 189-193.
26. Farnworth, E.R., Kramer, J.K.G., Thompson, B.K., and Corner, A.H. (1982) J. Nutr. 112, 231-240.
27. Schneider, B.H., and Flatt, W.P. (1975) in The Evaluation of Feeds through Digestibility Experiments, pp. 100-107, University of Georgia Press, Athens, GA.
28. Van de Kamer, J.H., Huinink, H.B., and Weyers, H.A. (1949) J. Biol. Chem. 177, 347-355.
29. Lloyd, L.E., McDonald, B.E., and Crampton, E. W. (1978) in Fundamentals of Nutrition, 2nd edn., pp. 319-333, W.E. Freeman and Co., San Francisco, CA.
30. Kramer, J.K.G., and Hulan, H.W. (1978) J. Lipid Res. 19, 103-106.
31. Ackman, R.G., and Woyewoda, A.D. (1979) J. Chromatogr. Sci. 17, 514-517.
32. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Lipids 5, 494-496.
33. Charlton, K.M., Corner, A.H., Davey, K., Kramer, J.K.G., Mahadevan, S., and Sauer, F.D. (1975) Can. J. Comp. Med. 39, 261-269.
34. Fienberg, S.E. (1970) Ecology 51, 419-433.
35. Hulan, H.W., Thompson, B., Kramer, J.K.G., Sauer, F.D., and Corner, A.H. (1977) Can. Food Sci. Technol. J. 10, 23-26.
36. Hopkins, C.Y., Murray, T.K., and Campbell, J.A. (1955) Can. J. Biochem. Physiol. 33, 1047-1054.
37. Murray, T.K., Beare, J.L., Campbell, J.A., and Hopkins, C.Y. (1958) Can. J. Biochem. Physiol. 36, 653-657.
38. Beare, J.L., Campbell, J.A., Youngs, C.G., and Craig, B.M. (1963) Can. J. Biochem. Physiol. 41, 605-612.
39. Beare-Rogers, J.L. (1977) Prog. Lipid Res. 15, 29-56.
40. Abdellatif, A.M.M., and Vles, R.O. (1973) Nutr. Metab. 15, 219-231.
41. Kramer, J.K.G., and Hulan, H.W. (1978) Lipids 13, 438-445.
42. Kramer, J.K.G. (1980) Lipids 15, 651-660.
43. Wood, R. (1979) in Geometrical and Positional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 213-281, Am. Oil Chem. Soc., Champaign, IL.
44. Rocquelin, G. (1979) Nutr. Metab. 23, 98-108.
45. Hulan, H.W., Kramer, J.K.G., and Corner, A.H. (1977) Can. J. Physiol. Pharmacol. 55, 258-264.
46. Hulan, H.W., Kramer, J.K.G., Corner, A.H. and Thompson, B. (1977) Can. J. Physiol. Pharmacol. 55, 265-271.
47. Romsos, D.R., and Leveille, G.A. (1974) Adv. Lipid Res. 12, 97-146.
48. Iritani, N., and Fukuda, E. (1980) J. Nutr. 110, 1138-1143.

[Received October 20, 1981]

Studies on the Biosynthesis of the Oogoniols

SUSAN E. BARROW and **TREVOR C. MCMORRIS***, *Department of Chemistry D-006, University of California-San Diego, La Jolla, CA 92093*

ABSTRACT

In feeding experiments with *Achlya heterosexualis*, [3-^{3}H] fucosterol was efficiently incorporated into oogoniols possessing an unsaturated side chain as well as those with a saturated side chain (clionasterol skeleton). [23,25-^{3}H]-29-Hydroxyfucosterol was also efficiently incorporated into the oogoniols and its role as an intermediate in the biosynthesis was confirmed by a trapping experiment. This indicated the presence of a small pool of endogenous 29-hydroxyfucosterol in the mycelium. [23,25-^{3}H]-29-Oxofucosterol was also well incorporated into the oogoniols and it is probably an intermediate in the biosynthesis. It was found to be converted to 29-hydroxyfucosterol in a trapping experiment. Reduction of the C-24(28) double bond make take place after all the functional groups have been introduced, at C7, C-11 and C-15, in the tetracyclic structure.

Lipids 17:383-389, 1982.

INTRODUCTION

Several years ago, we undertook an investigation of the biosynthesis of the oogoniols, a group of steroid hormones which are involved in sexual reproduction in the water mold *Achlya* (1). These compounds are secreted by hermaphroditic strains of *Achlya*, e.g., *A. heterosexualis*, or by the male strain *A. ambisexualis* E87, when stimulated by the male-activating steroid antheridiol. The oogoniols induce the formation of oogonial initials or female sex organs in the mold (2).

The investigation revealed that the major sterol in *Achlya* was fucosterol (**1**, Fig. 1) and that it was a precursor of the oogoniols. In the transformation of fucosterol to the oogoniols, oxidation at C-29 appeared to proceed to the level of aldehyde. Thus, when *Achlya* was grown in the presence of [CD_3]methionine, fucosterol containing 4 deuterium atoms (at C-28, C-29) could be isolated. However, oogoniols produced in this experiment contained only 2 deuterium atoms, one at C-28 and the other at C-29 (3).

At the time this work was done, it was believed that only oogoniols possessing a saturated side chain existed. Subsequently, it was discovered that oogoniol-1 was actually a mixture of the saturated side-chain steroid **7** (R = $[CH_3]_2CHCO$) and its C-24(28)-dehydro analog **6** (R = $[CH_3]_2CHCO$) which chould be separated only by high pressure liquid chromatography (HPLC). Oogoniol-2 (**7**, R = CH_3CH_2CO) and oogoniol (**5**) consisted, similarly, of mixtures of saturated and unsaturated side-chain steroids (4).

Dehydrooogoniol-1 was found to induce formation of oogonial initials in *A. ambisexualis* 734 (♀) at a concentration of 50 ng/ml whereas oogoniol-1 was inactive at concentrations lower than 5 μg/ml (4). Thus, the presence of the double bond on the side chain is an important condition for high biological activity.

This study was undertaken in an attempt to demonstrate that both saturated and unsaturated side-chain oogoniols are derived from fucosterol. Information was also sought about the sequence in which the various functional groups are introduced on the fucosterol skeleton, and the timing of the reduction of the C-24,C-28 double bond.

RESULTS AND DISCUSSION

Demonstration of precursor-product relationships with μg quantities of difficultly separable polar metabolites would not have been possible without the availability of HPLC. Baseline separation of, e.g., oogoniol-1 and 24(28)-dehydrooogoniol-1, oogoniol-2 and 24(28)-dehydrooogoniol-2, was achieved and μg quantities of individual steroids could be accurately collected for measurements of radioactivity.

A. heterosexualis 8-6 was found to be the best producer of oogoniols and was used in all the feeding experiments. [3-^{3}H] Fucosterol (sp act 7.33 × 10^9 dpm/mmol) prepared as described earlier was added to sterile production medium (5 ℓ), which was then inoculated with the organism. After 5 days' growth at 25 C with aeration, the culture medium was separated from the mycelium and the steroids were isolated by preparative thin layer chromatography (TLC) followed by HPLC. The results are summarized in Table 1. About 5% of the radioactivity was recovered in the fucosterol whose specific activity indicated that the added substrate had been diluted considerably by endogenous fucosterol. The pool of labeled fucosterol plus endogenous fucosterol appeared to be the source of all the oogoniols because the specific activities of the oogoniols were the same and equal to that of the recovered fucosterol (within experimental error).

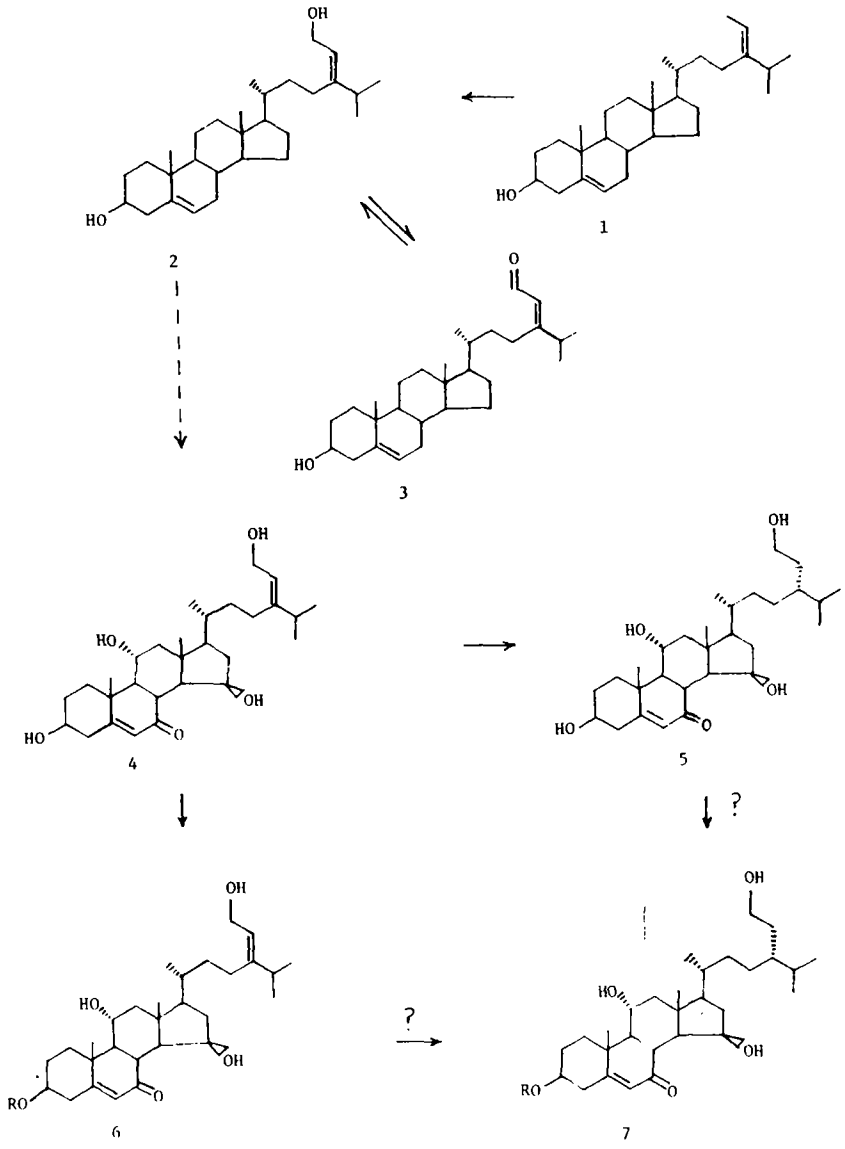


FIG. 1

In the biosynthesis of antheridiol, evidence has been reported which indicates that fucosterol is first converted to the C-22,23 dehydroderivative, which is then oxidized at C-29 (5). The possibility that similar oxidation at C-29 is an early event in the formation of the oogoniols has been investigated by feeding experiments with labeled 29-hydroxy fucosterol (2). Tritium atoms were introduced at C-23 and C-25 in this compound in the following way. Phosphorus pentachloride was added to $^{3}H_2O$ (sp act 4.0 x 10^{10} dpm/mmol) followed by a solution of 24-oxocholesterol acetate in tetrahydrofuran. The product (sp act 4.44 x 10^{10} dpm/mmol) was reacetylated, then subjected to a Wittig reaction with the anion of diethyl cyanomethylphosphonate to give 3β-hydroxy stigmasta-5, 24(28)-dien-29-nitrile 3β-acetate as a mixture of C-28 isomers (ratio of E:Z, 3:1). Reduction of the mixture (after diluting 10 times with unlabeled nitrile) with diisobutylaluminum hydride (Dibal) gave the corresponding aldehyde

TABLE 1

Sterols Isolated after Incubation of *A. heterosexualis* with Fucosterol (1.14 mg; sp act = 7.33×10^9 dpm/mmol)

	k[a] (HPLC)	Wt (μg)	Activity (dpm)	Sp act (dpm/mmol)	Fraction of total activity (%)
Fucosterol	7.0[b]	16300	1.0×10^6	2.6×10^7	5.000
Oogoniol	9.2[c]	35.0	2.1×10^3	2.8×10^7	0.015
Dehydrooogoniol	8.2[c]	10.4	5.0×10^2	2.3×10^7	0.003
Oogoniol-1	14.1[d]	7.6	4.2×10^2	3.0×10^7	0.002
Dehydrooogoniol-1	12.2[d]	tr[e]	–	–	–
Oogoniol-2	10.9[d]	4.2	2.3×10^2	2.9×10^7	0.001
Dehydrooogoniol-2	9.2[d]	tr	–	–	–
Unknown	8.7[d]	142.2	7.9×10^3		0.040

[a]k = exclusion volume of column.
[b]Solvent system, methanol (100%).
[c]Solvent system, methanol/water (70%:30%).
[d]Solvent system, methanol/water (75%:25%).
[e]tr = trace.

TABLE 2

Sterols Isolated after Incubation of *A. heterosexualis* with 29-Hydroxyfucosterol (1.07 mg; sp act = 4.92×10^9 dpm/mmol)

	k[a] (HPLC)	Wt (μg)	Activity (dpm)	Sp act (dpm/mmol)	Fraction of total activity (%)
29-Oxofucosterol	–	None	–	–	–
29-Hydroxyfucosterol	–	None	–	–	–
Fucosterol	7.0[b]	3700	None	None	None
Oogoniol	9.2[c]	115	1.9×10^5	7.6×10^8	1.6
Dehydrooogoniol	8.2[c]	110	1.8×10^5	7.6×10^8	1.5
Oogoniol-1	14.1[d]	19	3.4×10^4	9.8×10^8	0.3
Dehydrooogoniol-1	12.2[d]	16	2.6×10^4	8.7×10^8	0.2
Oogoniol-2	10.9[d]	21	3.6×10^4	9.0×10^8	0.3
Dehydrooogoniol-2	9.2[d]	20	3.0×10^4	8.0×10^8	0.2

[a]k = exclusion volume of the column.
[b]Solvent system, methanol (100%).
[c]Solvent system, methanol/water (75%:25%).
[d]Solvent system, methanol/water (70%:30%).

(3, mixture of C-28 isomers) but the acetate group was lost. The product was reacetylated and the C-28 isomers could then be readily separated by chromatography. The pure E-aldehyde (3, sp act 5.67×10^9 dpm/mmol) was further reduced with dibal to give 29-hydroxyfucosterol (2, sp act 4.92×10^9 dpm/mmol).

The results of a feeding experiment with 29-hydroxyfucosterol are given in Table 2. Although the growth of mycelium was not good (as reflected in the small amount of fucosterol isolated), more of the oogoniols could be isolated than in the previous experiment. They all possessed similar specific activity (~8×10^8 dpm/mmol), which was about 16% that of the substrate. Therefore, 29-hydroxyfucosterol can clearly be readily converted to the oogoniols by the mold. The conversion does not proceed via fucosterol because the fucosterol isolated in this experiment was not radioactive. The ratios of oogoniol:dehydrooogoniol, oogoniol-1:dehydrooogoniol-1 and oogoniol-2:dehydrooogoniol-2 were about the same, i.e., ~1:1, but differed from the ratio obtained in the experiment with labeled fucosterol as substrate.

Evidence confirming the intermediacy of 29-hydroxyfucosterol in the biosynthesis of the oogoniols was obtained by repeating the feed-

ing experiment with [3-^{3}H]fucosterol. Unlabeled 29-hydroxyfucosterol (10 mg) was added to the mycelial extract to act as carrier for endogenous 29-hydroxyfucosterol which would be expected to contain a tritium label. It was reisolated after the usual saponification procedure and rigorously purified. Only 10% of the 29-hydroxyfucosterol was recovered and it had sp act 3.9 x 10^4 dpm/mmol. Fucosterol isolated in this experiment had sp act 5.8 x 10^7 dpm/mmol. If one assumes that endogenous 29-hydroxyfucosterol had the same specific activity as the recovered fucosterol, then the amount of the 29-hydroxyfucosterol which was present in the mycelial extract was only about 66 μg. As most of this would have been lost in the saponification step, it is not surprising that we have been unable to detect 29-hydroxyfucosterol in the mycelium of *Achlya* by methods not involving radioactive labeling.

We next addressed the question of the role of 29-oxofucosterol in the biosynthesis. As mentioned earlier, 2 deuteriums were lost from C-29 of [28,29-$^2H_4$]fucosterol during the conversion to the oogoniols. This might be explained if an enzyme system is present which will permit reversible oxidation of alcohol to aldehyde. When labeled 29-oxofucosterol (sp act 5.67 x 10^9 dpm/mmol) was used as substrate in a feeding experiment, incorporation of radioactivity into the oogoniols was observed (Table 3). As in previous cases, the level of radioactivity was the same in all the oogoniols isolated. The specific activity was about 1.5% that of the substrate. Thus, the aldehyde was not as good a substrate as 29-hydroxyfucosterol. To check the possibility that the lower sp act might be the result of a bigger pool of endogenous aldehyde than alcohol, extracts of the mycelium (and also the culture medium) were examined carefully for the presence of the aldehyde, but none was found. The analytical method was capable of detecting as little as 10 μg of the aldehyde so the amount, if any, of endogenous aldehyde cannot be greater than that of 29-hydroxyfucosterol.

If 29-oxofucosterol is, indeed, a biosynthetic intermediate, reduction at C-29 to give back an alcohol may occur before or after other functional groups are introduced, at C-11, C-15 and C-7, Evidence about the timing of the reduction was obtained from an experiment with the labeled aldehyde as substrate (0.72 mg, sp act 5.67 x 10^9 dpm/mmol). Unlabeled 29-hydroxyfucosterol (10 mg) was added to the crude mycelial extract and, after re-isolation, it was found to be radioactive (sp act 9.4 x 10^6 dpm/mmol), indicating that the aldehyde is converted to 29-hydroxyfucosterol in the mold. The results of experiments with 29-oxofucosterol (taken together with the result of the [CD_3]methionine experiment) suggest that this compound is a biosynthetic intermediate and that it is reduced to the corresponding alcohol which is then further metabolized to give the oogoniols.

Another interesting question about the biosynthesis is the timing of the reduction of the C-24(28) double bond. From the data in the tables, it appears that this reduction may occur after all the functional groups (at C-7, C-11, C-15, C-29) have been introduced because

TABLE 3

Sterols Isolated after Incubation of *A. heterosexualis* with 29-Oxofucosterol (0.72 mg; sp act = 5.67 X 10^9 dpm/mmol)

	k[a] (HPLC)	Wt (μg)	Activity (dpm)	Sp act (dpm/mmol)	Fraction of total activity (%)
29-Oxofucosterol	–	None	–	–	–
29-Hydroxyfucosterol	–	None	–	–	–
Fucosterol	7.0[b]	31700	None	None	None
Oogoniol	9.2[c]	950	1.9 X 10^5	9.5 X 10^7	2.00
Dehydrooogoniol	8.2[c]	113	2.4 X 10^4	9.9 X 10^7	0.25
Oogoniol-1	14.1[d]	43	8.2 X 10^3	10.4 X 10^7	0.08
Dehydrooogoniol-1	12.2[d]	5	9.0 X 10^2	9.8 X 10^7	0.01
Oogoniol-2	10.9[d]	155	3.2 X 10^4	11.2 X 10^7	0.33
Dehydrooogoniol-2	9.2[d]	20	4.6 X 10^3	11.1 X 10^5	0.05
Unknown-1	8.7[d]	10	2.1 X 10^3	–	0.02

[a]k = exclusion volume of HPLC column.

[b]Solvent system, methanol (100%).

[c]Solvent system, methanol/water (70%:30%).

[d]Solvent system, methanol/water (75%:25%).

oogoniol and dehydrooogoniol possess similar specific activities. If reduction occurs at an earlier stage, this requires subsequent reactions to take place on 2 substrates, one with a fucosterol skeleton and the other with a clionasterol skeleton implying parallel pathways. Taking into consideration the likelihood of different pool sizes for intermediates in that event, one would not expect the end products to have the similar specific activities as were actually found.

If oogoniol is a metabolite of 24(28)-dehydrooogoniol, it seemed possible that the reduction might be the way in which the hormone is "deactivated" in the process of exercising its function in the mold. To test this idea, the heterothallic strain *A. ambisexualis* E87 (♂) was grown in the presence of antheridiol. This "strong" male secretes oogoniols only after simulation by antheridiol. We reasoned that, if our idea was correct, only dehydrooogoniols would be produced because no female would be present to metabolize dehydrooogoniols to oogoniols. Analysis of the culture extracts indicated, however, the presence of both types of steroid, in a ratio of 13:16, so we still do not understand why the saturated side-chain steroid is elaborated in the organism.

The ratio of oogoniol to dehydrooogoniol was approximately the same as that of oogoniol-1 to dehydrooogoniol-1 and oogoniol-2 to dehydrooogoniol-2 in each experiment, although the actual value differed from one experiment to another. This observation may be interpreted in 2 ways. Either dehydrooogoniol is first partly esterified to the propionate and isobutyrate derivatives and each is then reduced at C-24(28) to the same extent, or dehydrooogoniol is first partly reduced at C-24(28) and the unsaturated steroid plus saturated steroid are partly esterified in the same way. Both alternatives require the occurrence of enzyme reactions which are not structure-specific. The esterification step presumably involves participation of isobutyryl- and propionyl-coenzyme A. The significance of this step is unknown at present. It should be noted that the biological activity of dehydrooogoniol is about the same as that of dehydrooogoniol-1.

EXPERIMENTAL

Melting points were determined on a Kofler hot-stage microscope and are uncorrected. Spectra were obtained on the following instruments: Varian EM 390 (^{1}H NMR), Perkin-Elmer spectrophotometer 550 (UV), Beckman IR 18-X (IR), LKB 9000 (low-resolution mass spectra). Nuclear magnetic resonance (NMR) spectra were taken in $CDCl_3$ with Me_4Si as internal standard. Ionizing voltage for mass spectra (MS) was 70 eV. Infrared (IR) spectra were taken of KBr pellets.

Column chromatography was done with Silica Gel G (E. Merck, Darmstadt, Germany) and TLC with Silica Gel 60 F254. Final purification of steroids was achieved by HPLC with a reverse-phase μBondapak C-18 semipreparative column (300 × 7.8 mm id). Methanol/water systems were used (isocratic elution) and a flow rate of 2.0 ml/min. For radioactivity measurements, samples of known weight were dissolved in toluene (10 ml) containing 2,5-diphenyl oxazole (5 g/ℓ) and 2,2-phenylene bis-(5-phenyl)oxazole (100 mg/ℓ). Counts obtained on the scintillation counter were corrected for quenching, by means of a calibration curve, and for background activity.

[3-^{3}H] Fucosterol

This compound was prepared as described earlier (1). The specific activity of the newly prepared sample which was purified by HPLC with methanol/water (95:5) and ultraviolet (UV) detector, 215 nm, was 7.33×10^9 dpm/mmol.

[23,25-^{3}H] -24-Oxocholesterol 3β-acetate

The method was adapted from a general exchange procedure described in *Methods in Enzymology* (6). A solution of $[^3H]^+$ was made by carefully adding phosphorus pentachloride (225 mg) to ^3H_2O (1 ml, sp act 4.0×10^{10} dpm/mmol) in a dry, screw-capped test tube under nitrogen and was cooled in ice. A solution of 24-oxocholesterol 3β-acetate (5) (440 mg, 1 mmol) in anhyd tetrahydrofuran (THF) (5 ml) was added, the tube was tightly capped, the contents thoroughly mixed (vortex mixer) then stored in the dark at room temperature for 72 hr. The contents of the test tube, after removal of the THF in a stream of N_2, were extracted with ethyl acetate (3 × 10 ml) and the extract was washed with saturated sodium chloride solution (2 × 15 ml), and dried (K_2CO_3). The solution was filtered through a short column of K_2CO_3 and the solvent was removed, leaving a crystalline residue. Analysis of the residue by gas chromatography indicated that partial hydrolysis of the acetate group had occurred. The mixture (358 mg) was therefore dissolved in pyridine (10 ml) and acetic anhydride (5 ml) and stored at 5 C for 24 hr. The solution was added to ice-water and the acetylated product was extracted into ethyl acetate. The ethyl acetate solution was washed with 1 N hydrochloric acid, saturated sodium bicarbonate solution, saturated sodium chloride solu-

tion and dried ($MgSO_4$). Removal of the solvent gave the crystalline acetate (339 mg) which was 100% pure as indicated by gas chromatography (1% SE 30, 200 C), sp act 4.3 x 10^{10} dpm/mmol.

[23,25-^{3}H]-3β-Hydroxystigmasta-5,24(28)-dien-29-nitrile 3β-acetate

About 227 mg (8.1 mmol) 57% NaH/oil dispersion was added to a solution of diethylcyanomethyl phosphonate (1.6 g, 13.9 mmol) in 2 ml of dry THF (N_2 atmosphere). After stirring for 30 min, the temperature was lowered to -78 C and a solution of [23,25-^{3}H]-24-oxocholesterol-3β-acetate (330 mg, 0.8 mmol) in THF (3 ml) was added. The mixture was allowed to warm to room temperature, was then stirred for 3 hr; water was added and the mixture was extracted with ether. The combined extracts were washed with saturated sodium chloride solution, dried over $MgSO_4$ and the solvent was removed, yielding an oil which crystallized upon standing. Chromatography with hexane/ethyl acetate (5:1) gave the nitrile (282 mg, 81%) mp 103-105 C (mixture of E and Z isomers). Upon gas chromatography, 1% SE 30, 200 C (2 C/min program), *n*-alkanes standard, 2 peaks MU 37.3 and 37.7 (E and Z isomers) were obtained. The estimated purity was 99% and the ratio of E isomer:Z isomer was 71:29. This material was diluted with unlabeled nitrile to give a sample with sp act 5.5 x 10^9 dpm/mmol.

[23,25-^{3}H]-3β-Hydroxystigmasta-5,24(28)-dien-29-aldehyde

To a solution of the above nitrile (100 mg, 0.22 mmol) in 4 ml of dry toluene was added 0.67 ml of 20% diisobutyl aluminum hydride in hexane solution (0.89 mmol). The mixture was stirred at room temperature for 150 min then cooled to 0 C and a mixture of 7% aq acetic acid/THF/methanol (1:1:1, 15 ml) was added by drops over a period of 20 min with vigorous stirring. The mixture was then allowed to warm to room temperature and chloroform (2 ml) was added; stirring was continued overnight. Two clear phases formed. Water (20 ml) was added and the product was extracted with chloroform (3 x 20 ml). The combined extract was washed with sodium bicarbonate solution, saturated sodium chloride solution and dried ($MgSO_4$). Removal of the solvent gave crystalline aldehyde (95 mg, 94%) mp 89-91 C; NMR δ 0.69 (s, 3), 0.98 (s, 3), 1.11 (d J = 7 Hz, 6), 1.15 (d J = 7 Hz, 3), 3.2-3.8 (m, 1), 5.34 (m, 1), 5.77 (d J = 8.4 Hz, Z isomer, 0.25 H), 5.81 (d J = 8.4 Hz, E isomer, 0.75 H), 9.97 (d J = 8 Hz, E isomer, 0.75 H), 10.06 (d J = 8 Hz, Z isomer, 0.25 H); sp act 5.1 x 10^9 dpm/mmol.

Acetylation of the aldehyde with acetic anhydride/pyridine at room temperature overnight gave the crystalline acetate in almost quantitative yield, mp 117-121 C. Upon TLC with hexane/ethyl acetate (4:1), the E isomer had R_f = 0.33 and Z isomer had R_f = 0.30. Separation by preparative TLC gave pure E isomer, mp 124.5-125.5 C, and pure Z isomer, mp 122.5-124 C, in a ratio of about 3:1.

For feeding experiments, the free alcohol, rather than acetate, was used. Deacetylation was effected by adding a few drops of 10% potassium carbonate solution to the E isomer (13.2 mg) in methanol/THF. After 4 hr, the organic solvent was removed in a stream of nitrogen and water was added to the residue followed by chloroform. The chloroform layer was dried ($MgSO_4$) and the solvent was removed leaving the product which was purified by preparative TLC with ethyl acetate/hexane (1:2); sp act 5.67 x 10^9 dpm/mmol.

[23,25-^{3}H]-3β,29-Dihydroxystigmasta-5,24(28)E-diene, (29-hydroxyfucosterol)

To a cooled (0 C) solution of the above aldehyde (E isomer, 3β-acetate, 29 mg, 0.06 mmol) in dry toluene (1 ml) was added diisobutylaluminum hydride (400 μl, 0.54 mmol, 20% solution in hexane). The solution was stirred for 1 hr, then water was added and the product was extracted with chloroform. Work-up in the usual way followed by preparative TLC with hexane/ethyl acetate (1:1) gave 29-hydroxyfucosterol (15 mg, 60%), mp 138-141 C; sp act 4.92 x 10^9 dpm/mmol; NMR δ 0.68 (s, 3), 0.98 (d, J = 7 Hz, 3), 1.00 (s, 3), 1.01 (d, J = 7.5 Hz, 6), 3.3-3.8 (m, 1), 4.15 (broad d, J = 7.5 Hz, 2), 5.31 (m + t, J = 7.5 Hz, 2); MS m/z (rel. intensity) 428 (M^+, 15), 410 (19), 384 (16), 314 (100), 299 (36), 271 (67).

Procedure for Feeding Experiments

For feeding experiments, *Achlya heterosexualis* 8-6 was found to be the most suitable strain. It was used to inoculate petri dishes 90 mm in diameter containing agar in Barksdale's medium A 25 ml (1). After 4 days' growth at 25 C, the mycelium from 2 plates was cut into 4-mm squares and was distributed aseptically between four 250-ml Erlenmeyer flasks, each containing 100 ml of Barksdale's sporulation medium. Sporulation was induced by shaking the flasks gently for 3 days at 25 C, The resulting suspension of zoospores and agar plugs was transferred aseptically to a 6-ℓ Erlenmeyer flask containing 5 ℓ of production medium. This consisted of Barksdale's production medium

with 2 additional ingredients: edamin (hydrolyzed lactalbumin, 400 mg/ℓ) and calcium glycerophosphate (80 mg/ℓ).

Radioactive sterols were dissolved in acetone (2 ml) and added to the production medium at the time of inoculation. The medium was aerated with sterile air for 5 days and the resulting mycelium was then separated from the culture liquid by filtration and air-dried. The culture liquid was extracted with dichloromethane (1 ℓ) in a continuous liquid-liquid extractor for 3 days. The extract was first chromatographed on a short column of silica gel with ethyl acetate/hexane (2:1) to elute oogoniol-1 and oogoniol-2 with their dehydro analogs, followed by ethyl acetate alone to elute oogoniol and dehydrooogoniol. Fractions containing oogoniol-1 and -2 were subjected to preparative TLC with ethyl acetate/hexane (2:1) whereas those containing oogoniol were chromatographed with ethyl acetate. Bands corresponding to the different oogoniols were collected and the steroids were eluted with ethyl acetate. After removal of the solvent, the residues were each dissolved in methanol (100 μl). Separation of the oogoniols from dehydrooogoniols was effected by HPLC. Oogoniol-1, oogoniol-2 and their dehydro analogs were chromatographed with methanol/water (3:1) and the more polar oogoniol and dehydrooogoniol with methanol/water (7:3). Compounds were detected with a UV absorbance detector at 254 nm. As a check of the identity of each peak, an aliquot (1% of the sample) was combined with an appropriate known steroid and reinjected onto the column to see if the compounds would cochromatograph. The remainder of the sample (99%) was then injected onto the column for quantitation. The sample was collected from the column and blank samples were collected, in each case, before and after elution of the steroid to check for background radioactivity. The weights of the steroids were determined by measuring peak areas on the chromatograms and comparing them with a calibration curve which had been constructed for each steroid.

After solvent removal, each steroid collected from the column and the blank samples were used for radioactivity measurements. Background activity was usually 50-70 dpm.

The mycelium from each feeding experiment was ground to a fine powder in a mortar containing liquid nitrogen, mixed with an equal weight of anhyd Na_2SO_4 and the mixture was extracted with chloroform in a Soxhlet apparatus for 6-8 hr. After removal of the solvent, a solution of potassium hydroxide (5 g) in methanol (75 ml) was added and the mixture was refluxed for 5 hr. Water (100 ml) was added and most of the methanol was removed under reduced pressure. Sterols were then extracted from the aqueous solution with hexane in a continuous liquid-liquid extractor for 6 hr. The hexane phase was dried (Na_2SO_4) and the solvent was removed under reduced pressure. The residue was chromatographed (TLC) with ethyl acetate/hexane (1:2), individual bands were collected and organic material was eluted with chloroform. Each sample (in methanol, 100 μl) was subjected to HPLC. Fucosterol and 29-hydroxyfucosterol were detected by their UV absorbance at 215 nm and 29-oxofucosterol by its absorbance at 254 nm.

ACKNOWLEDGMENT

We thank D.L. Villanueva for technical assistance. This work was supported by National Institutes of Health Grants GM 21350 and RR 08135.

REFERENCES

1. McMorris, T.C., and White, R.H. (1977) Phytochemistry 16, 359-362.
2. McMorris, T.C. (1978) Lipids 13, 716-722.
3. White, R.H., and McMorris, T.C. (1978) Phytochemistry 17, 1800-1802.
4. Preus, M.W., and McMorris, T.C. (1979) J. Am. Chem. Soc. 101, 3066-3071.
5. Popplestone, C.R., and Unrau, A.M. (1974) Can. J. Chem. 52, 462-468.
6. Nadeau, R.G., and Hanzlik, R.P. (1969) in Methods in Enzymology, Vol. 15, pp. 346-351, Academic Press, New York, NY.

[Received November 9, 1981]

COMMUNICATIONS

Hepta- and Nonadecene from Marine Fish

NOBUHIKO HOSHITA* and **YOUTARO KODAMA**, *Division of Chemistry, Hiroshima Prefectural Institute of Public Health, 1-5-70 Ujinakanda, Minamiku, Hiroshima 734, Japan*

ABSTRACT

Two monoalkenes were detected in gizzard shad (*Clupanodon punctatus*) and sardine (*Sardinops melanosticta*) by gas chromatography and were identified by combined gas chromatography-mass spectrometry of their cyclic boronate and dioxolane derivatives as *cis*-6-*n*-heptadecene and *cis*-8-*n*-nonadecene.

Lipids 17:390-392, 1982.

INTRODUCTION

Since squalene was identified from shark liver oil (1), hydrocarbons have been found in various animals and plants. It is now clear that hydrocarbons are ubiquitous in nature and their occurrence has been recently reviewed (2,3). Most marine organisms contain a hydrocarbon series ranging in chain length from C_{13} to C_{33} with odd-chain predominance. In fish, the n-C_{15}, n-C_{17}, n-C_{19} alkanes and pristane are generally the major hydrocarbons (4-6). On the other hand, polyunsaturated hydrocarbons, particularly 21:6, are dominant in many marine algae (7,8). The other alkanes containing between 1 and 5 double bonds have been identified in marine animals and algae (8-12), but the alkenes in fish are little known except for the isoprenoids squalene and zamene (2,15).

Lambertsen and Holman (4) and Nagy et al. (16) assumed that paraffinic hydrocarbons might originate from decarboxylation of fatty acids because a homologous series of predominantly odd-numbered *n*-alkanes has been observed in living organisms. This assumption was supported by the fact that labeled palmitate and stearate were mainly converted into n-C_{15} and n-C_{17} in a culture of blue-green algae (17). Even-numbered unsaturated fatty acids, as well as saturated fatty acids, occur widely in living organisms, and therefore, alkenes could also result during production of hydrocarbons.

The results of this investigation show that *cis*-6-*n*-heptadecene and *cis*-8-*n*-nonadecene are included in the hydrocarbons of gizzard shad (*Clupanodon punctutas*) and sardine (*Sardinops melanosticta*).

EXPERIMENTAL PROCEDURES

Extraction and Isolation of the Hydrocarbon Fraction

Minced samples of fresh fish muscle (10 g) were refluxed with 30 ml of ethanolic 2 N KOH for 2 hr. The mixture was extracted with 100 ml of hexane 3 times. The combined hexane phases were washed twice with 50 ml of water and dried over anhyd Na_2SO_4. The solution was evaporated to a volume of ca. 1 ml under reduced pressure at 50 C. The hydrocarbon fraction was obtained by column chromatography on silica gel (60/80 mesh). The eluent was hexane and the ratio of silica gel to sample was about 50:1. The alkanes and alkenes (except polyenes) were completely eluted within the first 10 column volumes.

Identification of Hydrocarbons

Hydrocarbons were analyzed by gas chromatography on a 3 m x 3 mm id glass column packed with 30% eutectic salts (18) on Chromosorb-WAW (60/80 mesh) programmed from 90 to 300 C at a rate of 7 C/min, with nitrogen as carrier gas at a flow rate of 10 ml/min. The flame ionization detector (FID) and injector were kept at 300 C. Identifications were achieved by comparison with a standard mixture of alkanes and alkenes (n-C_{12} to n-C_{30}). Mass spectra were obtained by GC-MS using a JEOL JMS D-300 double focusing mass spectrometer, operating at an ionization energy of 70 eV, accelerating voltage 3.0 kV, ion source and separator temperature 250 C. The gas chromatographic conditions were the same as those just described, using helium as the carrier gas.

Derivative Formation

2-Methyl-4,5-dialkyl-1,3-dioxolane (2MD) and O-isopropylidene (OIP) derivatives were prepared by oxidation of alkenes with OsO_4

and condensation with acetaldehyde and acetone as described by Wolff et al. (19). Cyclic *n*-butylboronates were synthesized according to the methods of Brooks and Maclean (20) by reacting the diols derived from oxidation of alkenes with *n*-butylboronic acid. Analyses were done by GLC on a 1.5% SE-30 column programmed from 150 to 250 C at 10 C/min, with a carrier gas flow rate of 30 ml/min. The detector and the injector temperature were kept at 250 C. GC-MS conditions were the same as those already described.

Synthesis of Alkenes

6-*n*-Hepta- and 8-*n*-nonadecene were synthesized by the Wittig reaction between *n*-undecyltriphenyl phosphonium bromide and *n*-hexanal and *n*-octanal, respectively (21), and were purified by silica gel column chromatography. The isomers were separated into *cis* and *trans* fractions according to the procedure of Chapman and Kuemmel (22).

RESULTS AND DISCUSSION

Paraffinic hydrocarbons in gizzard shad (*Cl. punctatus*) and sardine (*S. melanosticta*) were identified by retention times and GC-MS analysis. The chromatograms illustrated in Figure 1 show some complexity in the area corresponding to n-C_{17} and n-C_{19}, and 2 interesting peaks, designated A and B, were found in both samples; they showed a retention time slightly longer than that of n-C_{17} and n-C_{19}, respectively. These components were inseparable from n-C_{17} and n-C_{19} on SE-30, OV-17 and Dexsil 400 GC columns. MS of the components A and B showed molecular ions at m/z 238 and 266 with larger intensities than those of *n*-alkanes, and these spectra consisted almost exclusively of 2 series of alkyl and alkenyl ions. Furthermore, A and B disappeared on the chromatograms after oxidation with $KMnO_4$ or hydrogenation catalyzed with palladium carbon. These results suggested that A and B were mono-*n*-alkenes.

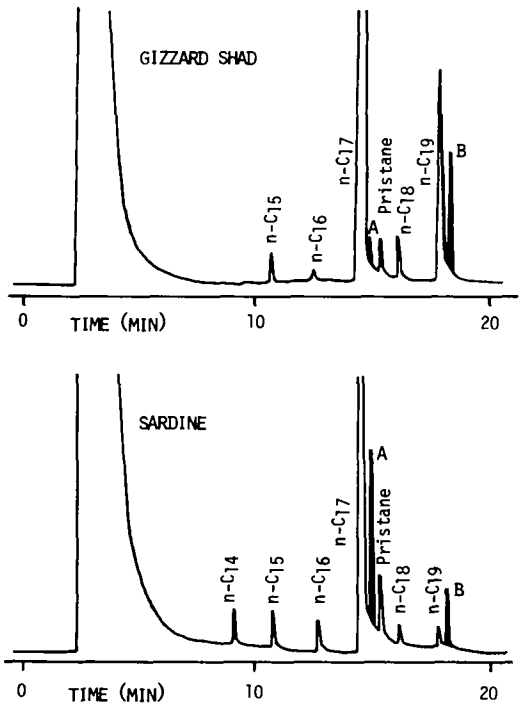


FIG. 1. Gas chromatograms of the hydrocarbon fraction in gizzard shad and sardine. For conditions, see Experimental Procedures.

MS of the cyclic boronate derivatives showed molecular ions at m/z 388(A) and 366(B). The fragmentation gave significant peaks at m/z 267 (M-71), 197(M-141)(A) and at m/z 267(M-99), 255(M-141)(B) attributable to α-cleavage of alkyl side-chain (C_5H_{11}, $C_{10}H_{21}$, C_7H_{15} and $C_{10}H_{21}$, respectively). These fragments appeared to be more predominant than the corresponding ions from acetaldehyde and acetone condensation products. Mass spectral examination of boronate esters that were derived from diols showed that component A was 6-*n*-heptadecene and B was 8-*n*-nonadecene. However, the fragmentations of *n*-butylboronates of *cis* and *trans* isomers were not clearly distinguishable. In the mass spectra of 2MD and OIP derivatives, the molecular ions were indicated at m/z 297 for the A and at m/z 325 for the B due to loss of a proton (2MD) and a methyl group (OIP). The mass spectral fragmentations of both derivatives showed significant peaks at m/z 255(A) and 283(B) by further elimination of ketene, and that of 2MD derivatives at m/z 238(A) and 311(B) due to loss of a methyl group. Moreover, the spectra gave the peaks attributable to α-cleavage of alkyl side-chain similar to those of the boronate esters. The influence of stereochemistry is evident from a comparison of spectra derived from *cis* and *trans* isomers, which are characterized by the loss of a methyl group and a ketene from the dioxolane ring attributable to α-cleavage of alkyl side-chain as mentioned by Wolff et al. (19). The ratios [M-43]/ [M-15] of 2MD and [M-57]/[M-15] of OIP of both components were 0.40:0.50, which were almost the same as those reported for *cis* isomers. These ratios were very different for *cis* and *trans* isomers, which when derived from *cis* structures were 0.40:0.46, or 3- to 5-fold greater than when derived from *trans*

structures (0.09:0.14). In the case of 2MD derivatives of both components, the %Σ of [M-15] ion were 1.8%(A) and 2.1%(B), and they were one-third lower than those of *trans*. On the other hand, in the case of OIP of *cis* isomers, the peaks attributable to α-cleavage of the alkyl side chain were 2-fold greater than those of *trans* isomers. These results confirm that component A is *cis*-6-*n*-heptadecene and that B is *cis*-8-*n*-nonadecene. It is known that some marine organisms such as algae, fungi and urchins contain mono-*n*-alkenes in which double bond is located in odd-number positions (2,8,9,11-13). The present result is the first evidence for the occurrence of *n*-alkenes with double bonds in even-numbered positions in fish. Recently, C_{20} hydrocarbons with a double bond in the 6-position were detected in the hydrocarbon portions of aplasmomycin isolated from a marine Actinomycetes (23). They have interesting structural characteristics, being multibranched and non-isoprenoid and this dissimilar to isoprenoids such as pristane, phytane and phytene, but their origin is as yet unproved.

The enzymatic decarboxylation of fatty acids has been previously found in a culture of algae; labeled palmitate and stearate were mainly converted into n-C_{15} and n-C_{17} in blue-green algae (17), and n-C_{17} was synthesized from [^{14}C]acetate via stearate in *Anacystis nidulans* (24). Murray et al. (25) reported that n-$C_{17:1}$ was synthesized in marine algae grown on medium containing $Na_2{}^{14}CO_3$. Some produced n-$C_{17:1}$ as the major hydrocarbon component. *n*-Alkenes with *cis* configuration of the double bonds were also detected in cultures of some bacteria (26). These results suggested that *n*-alkenes, as well as *n*-alkanes, are specifically biosynthesized from fatty acids by enzymatic decarboxylation in living organisms. Definite conclusions as to the origin of hydrocarbons in fish must await further experimentation.

ACKNOWLEDGMENT

The authors thank Dr. H. Umisa and Dr. K. Morimoto for their helpful advice.

REFERENCES

1. Tsujimoto, M. (1916) Ind. Eng. Chem. 8, 889-891.
2. Scheuer, P.J. (1973) Chemistry of Marine Natural Products, Academic Press, New York, NY.
3. Kolattukudy, P.E. (1976) in Chemistry and Biochemistry of Natural Waxes (Kolattukudy, P.E., ed.) pp. 1-15, Elsevier, Amsterdam.
4. Lambertsen, G., and Holman, R.T. (1963) Acta Chem. Scand. 17, 281-282.
5. Law, R.F. (1978) Mar. Pollut. Bull. 9, 321-324.
6. Saito, Y., Takeda, M., and Uchiyama, M. (1979) J. Assoc. Off. Anal. Chem. 62, 1327-1332.
7. Lee, R.F., and Leoblich, A.R. III (1971) Phytochemistry 10, 593-602.
8. Blumer, M., Guillard, R.R.L., and Chase, T. (1971) Mar. Biol. 8, 183-189.
9. Youngblood, W.W., Blumer, M., Guillard, R.R.L., and Fiore, F. (1971) Mar. Biol. 8, 190-201.
10. Winter, K., Parker, P.L., and van Baalen, C. (1969) Science 163, 467-468.
11. Lytle, J.S., Lytle, T.F., Gearing, J.N., and Gearing, P.J. (1979) Mar. Biol. 51, 279-288.
12. Okano, M., and Aratani, T. (1979) Bull. Jpn. Soc. Sci. Fish. 45, 389-393.
13. Yasuda, S., and Fukamiya, N. (1977) Bull. Jpn. Soc. Sci. Fish. 43, 1175-1180.
14. Shaw, D.G., and Wiggs, J.N. (1980) Mar. Pollut. Bull. 11, 297-300.
15. Sargent, J.R., Lee, R.F., and Nevenzel, J.C. (1976) in Chemistry and Biochemistry of Natural Waxes (Kolattukudy, P.E., ed.) pp. 50-91, Elsevier, Amsterdam.
16. Nagy, B., Modzeleski, V.E., and Scott, W.M. (1969) Biochem. J. 114, 645-648.
17. Han, J., Chan, H.W.-S., and Calvin, M. (1969) J. Am. Chem. Soc. 91, 5156-5159.
18. Snowdon, L.R., and Peake, E. (1978) Anal. Chem. 50, 379-381.
19. Wolff, R.E., Wolff, G., and McCloskey, J.A. (1966) Tetrahedron 3093-3101.
20. Brooks, C.J.W., and Maclean, I. (1977) J. Chromatogr. Sci. 9, 18-24.
21. Hauser, C.F., Brooks, T.W., Miles, M.L., Raymond, M.A., and Butler, G.B. (1963) J. Org. Chem. 28, 372-379.
22. Chapman, L.R., and Kuemmel, D.F. (1965) Anal. Chem. 37, 1598-1600.
23. Barrick, R.C., and Hedges, J.I. (1981) Geochim. Cosmochim. Acta 45, 381-392.
24. McInnes, A.G., Walter, J.A., and Wright, J.L.C. (1980) Lipids 15, 609-615.
25. Murray, J., Thomson, A.B., Stagg, A., Hardy, R., Whittle, K.J., and Mackie, P.R. (1977) Rapp. P.V. Réun. Cons. Int. Explor. Mer. 171, 84-90.
26. Albro, P.W., and Dittmer, J.C. (1969) Biochemistry 8, 394-405.

[Received December 14, 1981]

Effect of Diethyl Maleate and Glutathione on Linoleate Peroxidation

NANCY FERNANDEZ[a], ALFONSO VALENZUELA[a,*], VIRGINIA FERNANDEZ[b] and LUIS A. VIDELA[b], [a]*Unidad de Bioquímica, Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile, Casilla 15138, Santiago 11; and* [b]*Unidad de Bioquímica, División de Ciencias Médicas Occidente, Facultad de Medicina, Universidad de Chile, Casilla 10455, Correo Central, Santiago, Chile*

ABSTRACT

The antioxidant effect of diethyl maleate and reduced glutathione was studied in an in vitro peroxidizing system. Linoleate peroxidation, measured as conjugated diene formation, was not altered by diethyl maleate (2 or 4 mM), whereas the addition of reduced glutathione (1, 2 or 4 mM) elicited a marked and progressive reduction. This effect of glutathione is not modified by diethyl maleate. The inhibition of linoleate peroxidation by glutathione was found concomitantly with a decrease in the concentration of its reduced form and a corresponding increase in glutathione disulfide levels, so that the total equivalents of reduced glutathione in the system remained constant. It is concluded that diethyl maleate does not have antioxidant properties in a peroxidizing system, as found for reduced glutathione.

Lipids 17:393-395, 1982.

Recently, a metabolic interrelationship between reduction in the concentration of reduced glutathione (GSH) and the stimulation of lipoperoxidative processes in the liver cell was observed following acute ethanol intoxication in rats (1). The lipoperoxidative pressure induced by ethanol was suggested to be a consequence of both an increased generation of oxygen-related free radicals (2) and the low GSH levels (1), thus impairing the protective systems of the hepatocyte against peroxide toxicity.

Diethyl maleate is known to be a powerful GSH-depleting agent in the cell (3). Its mechanism of action has been proposed to be exerted by a conjugation with the tripeptide, forming a complex that is released to the extracellular medium (3). Studies carried out in rats have revealed that the administration of diethyl maleate in vivo produces a faster and greater effect of liver GSH levels than ethanol ingestion (4) without a concomitant enhancement of lipid peroxidation (4-6). It is known that unsaturated compounds such as polyunsaturated fatty acids are susceptible to hydrogen abstration by free radicals, leading to the production of fatty acyl radicals and to an autoxidation reaction in the presence of oxygen (7). Thus, it is conceivable that the lack of effect of diethyl maleate on hepatic lipid peroxidation (4-6) may be a consequence, in part, of an antioxidant action related to its unsaturated structure that could scavenge the free radicals involved in the stimulation of lipid peroxidation. In view of these observations, the possible antioxidant effects of diethyl maleate were evaluated in an in vitro peroxidizing system formed by linoleic acid and Fe^{2+} (8), and were compared to those elicited by GSH.

MATERIALS AND METHODS

The peroxidizing system was prepared by emulsifying 1 ml of linoleic acid with 10 ml of distilled water containing 0.5 ml of Tween-60 (90% w/v) with agitation. The emulsion obtained was neutralized with 1 N KOH, resuspended in 100 ml of 0.05 M potassium phosphate buffer, pH 7.0, and made up to a final volume of 150 ml with distilled water. This system was incubated at 37 C and peroxidation was initiated in all experiments by adding $FeSO_4$ to a final concentration of 500 μM Fe^{2+} (8). In the nonperoxidizing system, linoleic acid was replaced by water. Diethyl maleate and/or GSH were added at time zero as indicated in Figure 1.

Linoleate peroxidation was assessed by conjugated diene formation. Aliquots of 0.2 ml of the incubation medium were taken every 30 min and were added to 7.8 ml of 50% v/v ethanol at room temperature. Conjugated dienes were measured at 233 nm according to Hasse and Dunkley (8). Results were expressed as mmol of peroxide/ml of incubation medium by using the $\epsilon = 2.52 \times 10^4\ M^{-1} \times cm^{-1}$ (9).

Glutathione measurements were done enzymatically in aliquots of 40 μl of the incubation medium taken every hour. GSH was measured using methylglyoxal and glyoxalase I at 240 nm and glutathione disulfide (GSSG) was determined with NADPH and glutathione reductase at 340 nm in the same aliquots (10).

All the reagents used were obtained from Sigma (St. Louis) except for diethyl maleate (Aldrich, Milwaukee, WI).

RESULTS AND DISCUSSION

Data presented in Figure 1 show that the

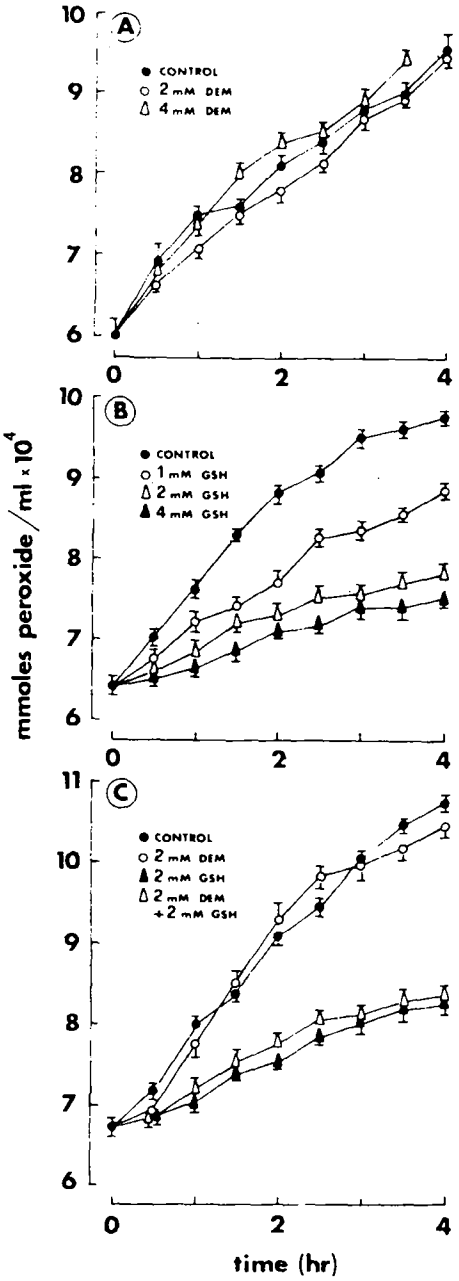


FIG. 1. (A) Effect of diethyl maleate (DEM) on linoleate peroxidation induced in vitro by Fe^{2+}. Control (●) peroxidizing system with no addition. Addition of (○) 2 mM or (△) 4 mM DEM. Each point represents the mean ± SEM for values obtained in duplicate from 5 separate experiments. (B) Effect of reduced glutathione (GSH) on linoleate peroxidation induced in vitro by Fe^{+2}. Control (●) peroxidizing system with no addition. Addition of (○) 1 mM; (△) 2 mM or (▲) 4 mM GSH. Each point represents the mean ± SEM for values obtained in duplicate from 5 separate experiments. (C) Effect of diethyl maleate (DEM) and reduced glutathione (GSH) on linoleate peroxidation induced by Fe^{+2}. Control (●) peroxidizing system with no additions. Addition of (○) 2 mM DEM; (▲) 2 mM GSH and (△) 2 mM DEM + 2 mM GSH. Each point represents the mean ± SEM for values obtained in duplicate from 5 separate experiments.

addition of Fe^{2+} to a final concentration of 500 μM induces the peroxidation of linoleate as evidenced by the enhancement of conjugated diene formation during the incubation period of 4 hr. A similar lipoperoxidative response is obtained when diethyl maleate is added to the peroxidizing system at a final concentration of 2 or 4 mM (Fig. 1A). This indicates that the GSH-depleting agent diethyl maleate does not have any protective effect on linoleate peroxidation induced by Fe^{2+} in the in vitro conditions used. These results suggest that the lack of stimulation of hepatic lipid peroxidation by diethyl maleate when given in vivo (4,6) or added to isolated rat liver cells (5) does not seem to be related to an antioxidant property of the agent due to its unsaturated structure.

The extent of lipoperoxidative processes in the cell depends on the balance between peroxidant and antioxidant systems (11,12). Among the antioxidant systems, GSH has been postulated as the most important one (13). The antioxidant actions of GSH can be visualized in its participation with either the catabolism of cellular peroxides formed by free-radical-induced lipid peroxidation (11,12) or with the direct interception of free radical species (14). In both mechanisms, GSSG is formed (11,12, 14).

The addition of GSH to the peroxidizing system elicited a drastic and progressive inhibition of linoleate peroxidation when added to a final concentration of 1, 2 or 4 mM, respectively (Fig. 1B). This antioxidant effect of GSH was observed concomitantly with a decrease in its concentration in the peroxidizing system as a function of time (Fig. 2A), suggesting an interaction between GSH and the free radicals generated in this condition that could form glutathionyl radicals (GS°) and GSSG by homologous binding (14). This view is further supported by the fact that the decrease in GSH concentration is accompanied by a corresponding increase in GSSG levels (Fig. 2A), so that the concentration of total GSH equivalents (GSH + 2GSSG) of the peroxidizing system remains constant throughout the incubation period (Fig. 2A). The possibility of a spontaneous oxidation of GSH in the in vitro condi-

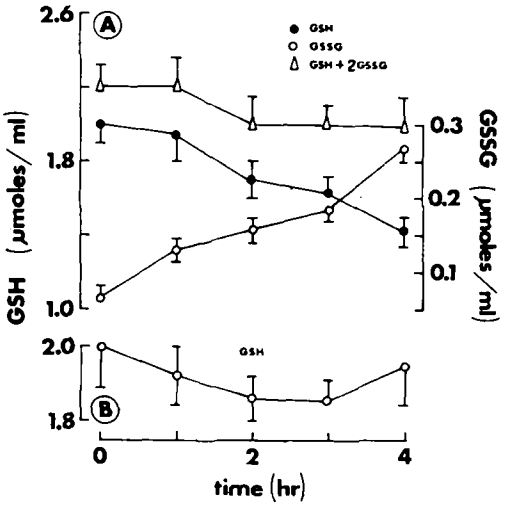


FIG. 2. (A) Time course of the changes in the concentration of reduced glutathione (GSH), glutathione disulfide (GSSG) and total GSH equivalents (GSH + 2GSSG) in the peroxidizing system. (B) Time course of the changes in GSH concentration in the nonperoxidizing system. Each point represents the mean ± SEM for values obtained in duplicate from 5 separate experiments.

tions used can be discarded as its concentration is not altered when added to a nonperoxidizing system (Fig. 2B).

Diethyl maleate was unable to modify the antioxidant effect of GSH in the in vitro peroxidizing system (Fig. 1C), indicating that an interaction between diethyl maleate and GSH does not seem to occur in this situation. In the cell, however, this interaction seems to occur mediated by an enzymatic conjugation catalyzed by the glutathione-S-alkenetransferase system (15).

ACKNOWLEDGMENTS

This work was supported in part by Grant B-1162-811-3 from the Servicio de Desarrollo Científico, Artístico y de Cooperación Internacional, Universidad de Chile, and by the Research Associateship Program (L.A. Videla) from the Natural Sciences and Engineering Research Council of Canada. The technical assistance of R. Guerra and C. Almeyda is acknowledged. The secretarial assistance of F. Rodríguez is appreciated.

REFERENCES

1. Videla, L.A., Fernández, V., Ugarte, G., Valenzuela, A., and Villanueva, A. (1980) FEBS Lett. 111, 6-10.
2. Valenzuela, A., Fernández, N, Fernández, V., Ugarte, G., Videla, L.A., Guerra, R., and Villanueva, A. (1980) FEBS Lett. 111, 11-13.
3. Chasseaud, L.F. (1976) in Glutathione: Metabolism and Function (Arias, I.M., and Jakoby, W.B., eds.) pp. 77-114, Raven Press, New York, NY.
4. Juanet, A., Tabak, M., Videla, L.A., and Valenzuela, A. (1981) IRCS Med. Sci. 9, 388.
5. Hogberg, J., and Kristoferson, A. (1977) Eur. J. Biochem. 74, 77-82.
6. Wendel, A., Feuerstein, S., and Kong, K.H. (1979) Biochem. Pharmacol. 28, 2051-2055.
7. Mead, J.F. (1976) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. 1, pp. 51-68, Academic Press, New York, NY.
8. Hasse, G., and Dunkley, W. (1969) J. Lipid Res. 10, 55-58.
9. Buege, J.A., and Aust, S.D. (1978) Methods Enzymol. 52, 302-310.
10. Bernt, F., and Bergmeyer, H.U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) Vol. 4, pp. 1643-1647, Academic Press, New York, NY.
11. Chance, B., Sies, H., and Boveris, A. (1979) Physiol. Rev. 59, 527-605.
12. Sies, H., Wahllander, A., Waydhas, C., Soboll, S., and Haberle, D. (1980) Adv. Enz. Reg. 18, 303-320.
13. Meister, A. (1975) in Metabolic Pathways (Greenberg, D.M., ed.) Vol. 7, pp. 101-188, Academic Press, New York, NY.
14. Kosower, N.S., and Kosower, E.M. (1979) Int. Rev. Cytol. 54, 109-160.
15. Chasseaud, L.F. (1973) Drug Metab. Rev. 2, 185-220.

[Received October 1, 1981]

ERRATA

Please note the following correction to the article "Reactivity of Key Metabolic Sterols in Standard Colorimetric Assays for Cholesterol," by C.P. Sarkar and R.J. Cenedella (*Lipids* 17: 46-49 [1982]). On page 49, second column, line 1, "but only with the $FeSO_4$ method of Zlatkis et. al. (4)." should read "but only with the $FeSO_4$ method of Searcy and Bergquist (8)."

Please note the following addition to the article "Effects of Dietary 9-*trans*,12-*trans* Linoleate on Arachidonic Acid Metabolism in Rat Platelets," by D.H. Hwang, P. Chanmugam and R. Anding (*Lipids* 17:307 [1982]). On page 310, Table 4, the first line of numbers in the last column should have an asterisk to denote $p < 0.05$ (3.9 ± 0.9*).

Stearoyl-Coenzyme A Desaturase Activity in the Mammary Gland and Liver of Lactating Rats

MICHAEL A. CALABRO, M. RENUKA PRASAD, SALIH J. WAKIL and **VASUDEV C. JOSHI***, *Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030*

ABSTRACT

Stearoyl-CoA desaturase activity in microsomes from lactating rat mammary gland is very low (0.05-0.15 nmol/min/mg of protein) regardless of lactating time. In such microsomes, reductase activities and content of cytochrome b_5 are several-fold lower than in normal rat liver microsomes. Preincubation of the mammary microsomes with purified terminal desaturase gives a 55-fold stimulation of stearoyl-CoA desaturase activity, whereas preincubation with cytochrome b_5 has no effect. However, preincubation of mammary microsomes with both cytochrome b_5 and terminal desaturase results in a 200-fold stimulation of overall desaturation. These observations suggest that negligible stearoyl-CoA desaturase activity in lactating rat mammary microsomes is due to a low cytochrome b_5 content and the absence of terminal enzyme. The hepatic stearoyl-CoA desaturase activity increases 9-fold during lactation. There is little or no change in the NADH-cytochrome c reductase activity or in the concentration of cytochrome b_5 during this period, but the activity of the terminal desaturase increases with the increase of overall desaturation. These results suggest that liver is one of the more important sources of oleic acid for milk triglycerides.
Lipids 17:397-402, 1982.

The conversion of stearoyl-CoA to oleoyl-CoA is catalyzed by the stearoyl-CoA desaturase system in the presence of NADH and oxygen. This system is located in the endoplasmic reticulum of the cell, and consists of 3 membrane-bound proteins: NADH-cytochrome b_5 reductase (flavoprotein), cytochrome b_5, and a terminal desaturase enzyme (1-8). In addition to these protein components, the desaturase activity is dependent on the presence of lipids (9).

Stearoyl-CoA desaturase activity is high in the lactating mammary glands of many species. For example, the enzyme activity in the glands of cows (10,11), sheep (12), and mice (13) is comparable to that in normal rat liver (14-16). On the other hand, stearoyl-CoA desaturase activity in the mammary gland microsomes of lactating goats and sows is low, but can be stimulated by glycerol-3-phosphate and the particle-free supernatant cell fraction (17). In the special cases of the lactating rat and rabbit mammary glands, however, such desaturase activity has been reported to be low or absent (11,17-20) and is not affected by glycerol-3-phosphate (20). Despite the absence of desaturase activity in rat mammary gland, oleic acid is one of the major fatty acids of milk triglycerides (21,22). In the rabbit, oleic acid for milk triglycerides is absorbed by the mammary gland from the serum (23).

Because the stearoyl-CoA desaturase is multicomponent in nature, absence of mammary desaturase activity may be due to lack of one or more components. The present results indicate that the absence of stearoyl-CoA desaturase activity in lactating rat mammary glands is due to a low cytochrome b_5 content and an absence of the terminal desaturase protein. We also find a 9-fold induction of stearoyl-CoA desaturation in the liver during the lactation period, which suggests that the liver may serve as one of the important sources of oleic acid for milk triglycerides. In the liver, the major cause of the increase in stearoyl-CoA desaturation is an increase in the activity of the terminal enzyme.

MATERIALS AND METHODS

Preparation of Microsomes

In our experiments, Sprague-Dawley female rats (170 g) are bred, and are fed a diet ad libitum of water and Lab-blox rat pellets (Allied Mills, Inc., Chicago). The first full day after parturition is termed day-one of lactation. At the indicated lactation times, the nursing dam is separated from her pups and killed by decapitation. The 2 abdominal mammary glands and the liver are removed, washed repeatedly in cold 0.25 M sucrose, minced and homogenized in 3 times their weight of 0.25 M sucrose using seven 15-sec bursts of a Polytron PT-10 homogenizer. Preparation of the microsomal fraction by differential centrifugation is done according to an earlier report (17). The microsomal pellets are resuspended in fresh sucrose solution by Dounce homogenization (Teflon pestle) and then stored at -50 C up to several days. Under these conditions, the

stearoyl-CoA desaturase activity is stable for at least 6 months.

Enzyme Assays

Desaturase activity is assayed by the method of Jones et al. (9). Microsomes (0.10-0.50 mg protein) are incubated in a final volume of 0.5 ml with [1-^{14}C]stearoyl-CoA (10-15 nmol) and NADH (100 nmol) in 160 mM potassium phosphate buffer, pH 7.2 for 10 min at 37 C. The methyl esters of [1-^{14}C]oleate and [1-^{14}C]-stearate are isolated by thin layer chromatography and the radioactivity is quantitated using a liquid scintillation counter. The NADH-ferricyanide reductase and NADH-cytochrome c reductase activities are determined as described previously (24) using a Gilford Model 240 recording spectrophotometer. Cytochrome b_5 contents are measured from the NADH-reduced minus oxidized spectra between 424 and 409 nm (6,24) using an Aminco DW-2 spectrophotometer. Because cytochrome b_5 content in mammary microsomes is low, a full-scale calibration of 0.02 absorbance is used. The terminal component of the liver desaturase system is assayed spectrophotometrically (6,24) at 25 C, and the rate of oxidation of reduced cytochrome b_5 is calculated from the extrapolated linear rates as described by Strittmatter et al. (25).

Reconstitution of Desaturase Activity

Purified cytochrome b_5 (detergent-extracted from rabbit liver) was a gift from Dr. B.S.S. Masters. Terminal desaturase protein is isolated by the method of Strittmatter et al. (25) from starved-refed rat liver and the purification procedure is followed up to the DEAE-cellulose column step (fraction 8).

Reconstitution of desaturase activity using microsomes of lactating rat mammary glands or liver and purified cytochrome b_5 and terminal desaturase is accomplished according to the procedure of Joshi et al. (26). Thus, cytochrome b_5 and/or terminal enzyme is added to microsomes (400 μg protein) in a final volume of 50 μl containing 1% Triton X-100 and 0.06% sodium deoxycholate. These components are preincubated for 10 min at room temperature. The desaturation is then assayed using NADH (100 nmol) and [1-^{14}C]stearoyl-CoA (15 nmol) and the mixture is incubated for either 2 or 10 min in a final volume of 0.5 ml. The rate of desaturation is unaffected by increasing the preincubation time from 10 to 60 min, so the shortest time is chosen for use in our experiments. The reaction time is determined by the ability of the particular assay mixture to respond in a linear fashion.

RESULTS

Stearoyl-CoA Desaturase Activity of Rat Mammary Gland and Liver during Lactation and Weaning

Desaturase activity of lactating rat mammary gland microsomes is found to be low during early lactation (about 120 pmol/min/mg protein) and further decreases as the lactation continues. This is in contrast to the case of corresponding liver microsomes wherein the desaturase activity is low during early lactation (about 150 pmol/min/mg) but increases about 9-fold during later lactation (Table 1).

The desaturase activity of mammary gland is very high 10 days after weaning (1.9 nmol/min/mg), presumably because of the increase in the fat cell content of the tissue. This may indicate that the very low desaturase activity found in the mammary gland during lactation may even be attributable to a small amount of invasive fatty tissue. In the corresponding weaned rat liver, the desaturase activity decreases slightly (to 1.0 nmol/min/mg) from optimum levels due to the end of demand for milk fat production.

NADH-ferricyanide and NADH-cytochrome c Reductase Activities and Cytochrome b_5 Content of Lactating Mammary Gland and Liver

The NADH-ferricyanide reductase activity is a measure of the activity of the flavoprotein, NADH-cytochrome b_5 reductase, whereas the NADH-cytochrome c reductase activity is a measure of the flavoprotein and cytochrome b_5 (27). Although the NADH-ferricyanide reductase activity in the mammary gland increases by about 2-fold during lactation, the activity of NADH-cytochrome c reductase and the content of cytochrome b_5 remain almost the same regardless of the lactation time (Table 1). The increase in NADH-ferricyanide reductase activity does not seem to play any role in the regulation of stearoyl-CoA desaturation, as during the lactation time no increase in overall desaturation was observed. Although the average activity of mammary gland NADH-ferricyanide reductase is about 9 times lower than that reported for normal rat liver (15), the reductase activity cannot be rate-limiting because its activity is 3 to 4 orders of magnitude greater than the desaturase activity (Table 1). However, th activity of the NADH-cytochrome c reductase and the content of cytochrome b_5 are about 15-fold lower than those of normal rat liver microsomes (15). These results suggested that the low activity of stearoyl-CoA desatura-

TABLE 1

Activities of the Components of Stearoyl-CoA Desaturation in Microsomes from Lactating Rat Mammary Glands and Livers[a]

Tissue	Lactation time (days)	Stearoyl-CoA desaturation (pmol/min/mg protein)	NADH-ferricyanide reductase (nmol/min/mg protein)	NADH cytochrome c reductase (nmol/min/mg protein)	Cytochrome b_5 (pmol/min/mg protein)
Mammary gland	2	120 ± 30	282 ± 24	49 ± 9	18 ± 2
	8	80 ± 20	380 ± 17	41 ± 2	20 ± 2
	15	50 ± 10	600 ± 93	45 ± 7	20 ± 5
Liver	1	150 ± 40	3060 ± 98	860 ± 41	290 ± 18
	8	1170 ± 180	ND[b]	ND	ND
	15	1300 ± 90	3039 ± 102	995 ± 62	317 ± 21

[a]Values are reported as the mean ± SEM for 4 separate experiments. Litter size is 10-13 pups.
[b]ND = not determined.

tion in mammary microsomes may be due to the low content of cytochrome b_5. In addition, the activity of the terminal desaturase in microsomes from lactating mammary gland was negligible (data not shown) which is consistent with the low overall stearoyl-CoA desaturation.

The measurement of component activities in liver show that, on days 1 and 15 of lactation, there is little or no change either in the content of cytochrome b_5 or in the activities of reductases. These results indicate that the increase in hepatic microsomal stearoyl-CoA desaturation during lactation is not due to the regulation of the activity of the first 2 electron-transfer components, and that the terminal desaturase may be involved in the induction of overall stearoyl-CoA desaturation. Direct support for the latter suggestion is obtained by measuring the terminal component activity as reflected in the rate of stearoyl-CoA stimulated oxidation of NADH-reduced cytochrome b_5 (6,24). The rate of stearoyl-CoA-dependent oxidation of cytochrome b_5 in liver microsomes of 1-day lactating rats is not detectable (i.e., less than 15 pmol/min/mg protein), whereas that in microsomes of 15-day lactating rats is 172 pmol/min/mg protein. The rate of stearoyl-CoA-stimulated oxidation of reduced cytochrome b_5 in liver microsomes is proportional to the activity of the Δ^9 terminal desaturase (6,14,15,24). The results lead us to conclude that the increase of hepatic stearoyl-CoA desaturation during lactation is due to an increase in the activity of the terminal desaturase component.

Reconstitution of Stearoyl-CoA Desaturase Activity

The mammary gland microsomes are supplemented with either cytochrome b_5 or terminal desaturase, or both, to determine which of these components may be lacking and thereby responsible for the lack of overall stearoyl-CoA desaturation. Active desaturation comparable to liver microsomes is generated only by the addition of both cytochrome b_5 and terminal desaturase, suggesting that the low levels of NADH-cytochrome b_5 reductase are not limiting the system (Table 2). Addition of cytochrome b_5 alone causes a negligible increase in stearoyl-CoA desaturation indicating that mammary microsomes have insignificant terminal desaturase activity. On the other hand, addition of purified terminal desaturase by itself could increase desaturation, demonstrating that some endogenous, functional cytochrome b_5 is present. Because this activity can be further increased 4-fold by simultaneous supplementation of microsomes with terminal desaturase and cytochrome b_5, this suggests that the

TABLE 2

Reconstitution of Stearoyl-CoA Desaturation by Cytochrome b_5 and Terminal Desaturase Enzyme

Microsomes[a]	Component added: Cytochrome b_5 (nmol)	Component added: Terminal desaturase (μg)	Oleate formed (nmol/min)
1) Lactating rat mammary[b]	0	0	0.01
	0.14	0	0.01
	0	10.4	0.55
	0.14	10.4	2.01
2) 1-Day lactating rat liver[c]	0	0	0.02
	0	2	0.36
	0	6	1.26
	0	10	2.20
	0	15	2.50
3) 12-Day lactating rat liver[d]	0	0	0.79[e]
	0	2	1.37
	0	4	2.13
	0	6	2.38

[a]In each assay, 400 μg of microsomal protein was reconstituted with other components of the desaturation system as described in Methods.

[b]Microsomes were prepared from 14-15-day lactating rats and their cytochrome b_5 content was 23-37 pmol/mg protein.

[c]Microsomes were prepared from 1-day lactating rat livers which contained 215 pmol of cytochrome b_5/mg.

[d]Microsomes were prepared from 15-day lactating rat livers which contained 358 pmol of cytochrome b_5/mg.

[e]Because Triton X-100 in the reconstituted system inhibits stearoyl-CoA desaturation, the value is 40% of that observed in the absence of Triton X-100.

endogenous level of mammary cytochrome b_5 is low compared to liver, and that this also is partly responsible for the low stearoyl-CoA desaturase activity in mammary microsomes.

It has been shown earlier that neither NADH-cytochrome b_5 reductase nor the content of cytochrome b_5 limits the hepatic stearoyl-CoA desaturase activity in various other physiological conditions (6,14,15,24,28, 29). Because the liver microsomes from lactating rats contain similar amounts of NADH-ferricyanide reductase activity and cytochrome b_5 as compared to rats under various other physiological conditions, it is likely that the observed overall stearoyl-CoA desaturation in lactating rat liver reflects the activity of the terminal enzyme. Indeed, supplementation of liver microsomes from rats early or late in lactation with increasing amounts of terminal desaturase results in a progressive increase in overall desaturation (Table 2). Furthermore, the microsomes from 1-day lactating rat liver require much higher amounts of the exogenous terminal enzyme than microsomes from 12-day lactating rat liver to attain the same level of stearoyl-CoA desaturation. The 2 preparations of hepatic microsomes contain equal amounts of cytochrome b_5 and NADH-ferricyanide reductase activities and, therefore, identical maximal stearoyl-CoA desaturation representing the same amount of functional complex is attained in the presence of saturating amounts of terminal desaturase in the 2 preparations of microsomes. As the microsomes from 12-day lactating rat liver require much lower amounts of exogenous terminal enzyme than microsomes from 1-day lactating rat liver to reach the same saturating stearoyl-CoA desaturation activity, the results indicate that the late lactating rat liver has more of the endogenous terminal desaturase than that of early lactating rat liver.

DISCUSSION

This study examined the reasons for the lack of stearoyl-CoA desaturase activity in lactating rat mammary gland microsomes. Changes in stearoyl-CoA desaturation activity can be effected, in principle, by modulation of any one of the individual components of the desaturase system. These results show that the lack of stearoyl-CoA desaturase activity in the mammary gland microsomes is mainly due to the lack of activity of the terminal enzyme. Furthermore, we observe that the terminal

desaturase activity is involved in the induction of hepatic desaturase activity during lactation. This report, then, provides 2 new examples demonstrating the general regulatory role of the terminal enzyme in the overall desaturase system. This is consistent with earlier reports studying the chicken and rat hepatic desaturase system under various dietary and hormonal conditions (6,14,15,28-30) and during the development of neonatal chicks (24) where it is also found that the overall desaturase system is regulated by the activity of the terminal enzyme.

In the liver immediately after parturition, desaturase activity is depressed below even normal levels. Stearoyl-CoA desaturase activity of normal rat liver has been found in our laboratory (15) to be about 0.59 nmol/min/mg. Shortly after the onset of lactation, however, hepatic activity rises rapidly, suggesting that the liver may serve as one of the important sources of oleic acid for the milk triglycerides via the blood lipoproteins. This induction of the hepatic terminal desaturase activity during lactation may result from the increased production of insulin during lactation (31). A role for insulin (15,29) and other supportive hormones, such as triiodothyronine and hydrocortisone (29,30), in the induction of hepatic Δ^9 terminal desaturase has been demonstrated in our laboratory.

It is notable that the rat mammary gland is responsive to hormonal induction of other lipogenic enzymes. For example, during pregnancy, in vitro studies have shown that prolactin in the presence of insulin and a glucocorticoid raises the overall rate of lipogenesis in rat mammary gland explants as measured by [^{14}C]-acetate incorporation into total fatty acids (32). In addition, under similar hormonal conditions, short- and medium-chain-length fatty acids are produced in preference to longer-chain-length fatty acids. Such effects have been confirmed in our own laboratory. We find, however, that such hormonal manipulations in the presence of [^{14}C]acetate do not induce stearoyl-CoA desaturase activity as measured by appearance of labeled oleate in mid-pregnant rat mammary gland explant culture during several days (data not shown).

There have been proposals for why the rat mammary gland shows no desaturase activity. One suggestion was that, in comparison to ruminants, the fatty acids found in circulating plasma are already sufficiently unsaturated and can be used directly to produce the necessary fluid (33) and highly digestible (12) milk fat, so that an active desaturase is unnecessary. It has been suggested that the presence of such polyunsaturated fatty acids may inhibit the desaturase (12). Our reconstitution experiments indicate that there is not a large amount of inhibition present in the mammary microsomes, because the activity can be restored by the addition of cytochrome b_5 and terminal desaturase. Thus, it is likely that any inhibitory effect of polyunsaturated fatty acids on the stearoyl-CoA desaturase is mediated by changes in the amount of terminal desaturase (34).

ACKNOWLEDGMENTS

V.C. Joshi is the recipient of United States Public Health Service Research Career Development Award AM-00397. This investigation was supported by the United States Public Health Service grants HD 07516 and AM 21286.

REFERENCES

1. Oshino, N., Imai, Y., and Sato, R. (1966) Biochim. Biophys. Acta 128, 13-28.
2. Holloway, P.W., and Wakil, S.J. (1970) J. Biol. Chem. 245, 1862-1865.
3. Holloway, P.W. (1971) Biochemistry 10, 1556-1560.
4. Shimakata, T., Mihara, K., and Sato, R. (1972) Biochem. (Tokyo) 72, 1163-1174.
5. Enoch, H.G., Catala, A., and Strittmatter, P. (1976) J. Biol. Chem. 251, 5095-5103.
6. Prasad, M.R., and Joshi, V.C. (1979) J. Biol. Chem. 254, 6362-6369.
7. Joshi, V.C., Prasad, M.R., and Sreekrishna, K. (1981) Methods Enzymol. 71, 252-258.
8. Sreekrishna, K., Prasad, M.R., Wakil, A.S., and Joshi, V.C. (1981) Biochim. Biophys. Acta 665, 427-433.
9. Jones, P.D., Holloway, P.W., Peluffo, R.O., and Wakil, S.J. (1969) J. Biol. Chem. 244, 744-754.
10. Kinsella, J.E. (1972) Lipids 7, 349-355.
11. McDonald, T.M., and Kinsella, J.E. (1973) Arch. Biochem. Biophys. 156, 223-231.
12. Wahle, K.W.J. (1974) Comp. Biochem. Physiol. 48B, 87-105.
13. Rao, G.A., and Abraham, S. (1974) Lipids 9, 269-271.
14. Oshino, N., and Sato, R. (1972) Arch. Biochem. Biophys. 149, 369-377.
15. Prasad, M.R., and Joshi, V.C. (1979) J. Biol. Chem. 254, 997-999.
16. Prasad, M.R., and Joshi, V.C. (1979) Lipids 14, 413-415.
17. Bickerstaffe, R., and Annison, E.F. (1970) Comp. Biochem. Physiol. 35, 653-665.
18. Bu'Lock, J.D., and Smith, G.N. (1965) Biochem. J. 96, 495-499.
19. Lossow, W.J., and Chaikoff, I.L. (1958) J. Biol. Chem. 230, 149-156.
20. Jarasch, E.D., Bruder, G., Keenan, T.W., and Franke, W.W. (1977) J. Cell Biol. 73, 223-241.
21. Smith, S., Watts, R., and Dils, R. (1968) J. Lipid Res. 9, 52-57.
22. Kinsella, J.E. (1974) Int. J. Biochem. 5, 417-421.
23. Jones, C.S., and Parker, D.S. (1978) Biochem. J. 174, 291-296.
24. Wilson, A.C., Wakil, S.J., and Joshi, V.C. (1976) Arch. Biochem. Biophys. 173, 154-161.
25. Strittmatter, P., Spatz, L., Corcoran, D., Rogers,

M.J., Setlow, B., and Redline, R. (1974) Proc. Natl. Acad. Sci. USA 71, 4565-4569.
26. Joshi, V.C., Wilson, A.C., and Wakil, S.J. (1977) J. Lipid Res. 18, 32-36.
27. Oshino, N., and Omura, T. (1973) Arch. Biochem. Biophys. 157, 395-404.
28. Oshino, N. (1972) Arch. Biochem. Biophys. 149, 378-387.
29. Joshi, V.C., and Aranda, L.P. (1979) J. Biol. Chem. 254, 11779-11782.
30. Joshi, V.C. (1981) Methods Enzymol. 72, 743-747.
31. Sutter-Dub, M.Th., Leclercq, R., Sutter, B.Ch.J., and Racquot, J. (1974) Horm. Metab. Res. 6, 297-300.
32. Hallowes, R.C., Wang, D.Y., Lewis, D.J., Strong, C.R., and Dils, R. (1973) J. Endocrinol. 57, 265-276.
33. Kinsella, J.E. (1970) J. Dairy Sci. 53, 1757-1765.
34. Holloway, C.T., and Holloway, P.W. (1975) Arch. Biochem. Biophys. 167, 496-504.

[Received December 21, 1981]

Autoxidation of Phosphatidylcholine Liposomes

GUEY-SHUANG WU, ROBERT A. STEIN and **JAMES F. MEAD***, *Laboratory of Biomedical and Environmental Science, 900 Veteran Avenue, University of California, Los Angeles, CA 90024; and Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024*

ABSTRACT

Autoxidation of pure soybean phosphatidylcholine liposomes at 40 C was found to proceed without an observed induction period, but otherwise, the rates of disappearance of the linoleic acid (70% of total) and linolenic acid (6% of total) followed typical autocatalytic kinetics. Incorporation of 0.05 mol % of tocopherol into the liposomes produced an induction period of about 7 hr under the condition used for the incubation. The products formed from the autoxidation of pure soybean phosphatidylcholine liposomes were mostly 9- and 13-hydroperoxyoctadecadienoates (isolated as hydroxy esters). The yield of hydroperoxides with *cis,trans* configuration was about the same as those with *trans,trans* configuration throughout incubation period. After extensive autoxidation, a large quantity of trihydroxyoctadecenoate was also produced. When a large quantity of dipalmitoyl phosphatidylcholine was incorporated into soybean phosphatidylcholine liposomes, the rate of autoxidation decreased and was found to conform to apparent first-order kinetics. In this system, the yield of *trans, trans* hydroperoxides was much greater than that of *cis,trans* isomers at all stages of autoxidation. Late in the autoxidation of the mixed liposomes, both trihydroxyoctadecenoate and hydroxyepoxyoctadecenoate were produced in substantial quantities.
Lipids 17:403-413, 1982.

INTRODUCTION

The oxidation of membrane phospholipid has been recognized to be one of the primary events leading eventually to the destruction of cell membranes. For example, an increase in permeability (1) and hemolysis of erythrocytes (2) were found to be direct consequences of phospholipid oxidation. Clinically, the results of phospholipid oxidation have been observed in degenerative and other diseases.

In light of the potential importance of phospholipid peroxidation, it seems surprising that little effort has been expended in this area. There is only scattered information available on the autoxidative reactions of phospholipid either in a neat film, in an aqueous emulsion or in an ordered arrangement, as in liposomes. Moreover, the available reports seem to deal mainly with the rates and infrequently with the products of the reaction. In an emulsified system, phosphatidylethanolamine isolated from egg and soybean lipids was found to oxidize at a greater rate than did phosphatidylcholine (PC) from the same sources (3). Using both vitamin-E-deficient erythrocyte membranes and a phospholipid micellar system, arachidonic acid was found to be the most susceptible fatty acid to peroxidation induced by glucose oxidase-glucose or dialuric acid (4). The extent of autoxidation of phospholipids in liposomes under different conditions of sonication has been investigated (5,6). PC liposomes have been autoxidized using various heme compounds as initiators. Among many heme compounds tested, hematin appears to be most active for this purpose (7). Other peroxide-splitting metal ions have also been effectively used as initiators in ox brain phospholipid liposomes (8) and in lyophilized red blood cell bilayer membranes (9). Recently, the autoxidation products from synthetic 1-palmitoyl-2-linoleoyl PC and 1-stearoyl-2-arachidonoyl PC in neat film have been isolated and characterized. The major products were found to be a mixture of hydroperoxides (10).

Previously in our laboratory, we have studied extensively the autoxidation of membrane lipids using a simple model system, unsaturated fatty acid monolayers supported on silica gel (11-14). We found that unsaturated fatty acids in a monolayer arrangement oxidize at a considerably greater rate than in neat films and that the major products are not hydroperoxides, but epoxides and other more polar material (12). When the surface coverage was reduced to only 5% of the available adsorption sites, or saturated fatty acid was incorporated into linoleic acid monolayers, the rate decreased and the major products formed were hydroxyepoxyoctadecenoic acids.

In extending our investigation of autoxidation of model membrane systems, we undertook a study using a more complex system, phospholipid liposomes. The close structural similarities between biomembranes and microvesicles have been demonstrated by numerous studies of the physical properties of these 2 structures. In the research reported here, we investigated primarily the rates and major prod-

ucts of autoxidation of soybean PC liposomes to see whether the findings in monolayers prevail in this system. Soybean PC was initially chosen for this purpose because the possible product pattern would be derived mostly from linoleic acid (soybean PC contains up to 70% of linoleic acid). The amount of linolenic acid is small compared to linoleic acid, and oleic acid is known to autoxidize slowly. We also investigated the autoxidation of liposomes containing soybean PC intermixed with a large quantity of dipalmitoyl PC. This particular system was designed to simulate to some extent the known composition of pulmonary surfactant lipid which contains dipalmitoyl PC (15).

EXPERIMENTAL PROCEDURES

Materials and Methods

Pure soybean PC was isolated from crude vegetable lecithin (Mann Research Laboratories, Inc.) by chromatography on alumina (Matheson, Coleman and Bell, chromatographic grade) (16). After less polar material was removed by chloroform, PC was eluted with chloroform/methanol (9:1, v/v). Thin layer chromatography (TLC) showed that the PC was pure except for contamination by a small amount of column material, which was subsequently removed by passing PC through a silicic acid column (J.T. Baker Chemical Co., Phillipsburg, NJ). The fatty acid content determined after hydrolysis was as follows: 16:0, 7.9%; 18:0, 4.4%; 18:1, 11.9%; 18:2, 69.8% and 18:3, 6.0%. The principal molecular species of soybean PC has been determined by Terao and coworkers to contain 16:0-18:2, 25.2%; 18:1-18:2, 13.2% and di-18:2, 31.3% (17). Dipalmitoyl PC purchased from Sigma Chemical Co. (St. Louis, MO) is 99% pure PC as determined by TLC and was used directly. After hydrolysis, the fatty acid content was found to be 14:0, 1.2%; 16:0, 95.5% and 18:0, 3.3%. D-α-Tocopherol (Mann Research Laboratories, Inc.) was found to be pure on TLC and gave ultraviolet (UV) absorption maxima at 292 nm (ϵ 3,430).

Gas liquid chromatography (GLC) was done using a Hewlett Packard Gas Chromatograph Model 5830A. Methyl esters were separated on a 0.20 x 300 cm metal coiled column packed with 10% Silar 10C on 100/120 mesh Gas Chrom Q, and the autoxidation products were chromatographed on a 0.20 x 180 cm glass column packed with 3% OV-101 on 100/120 mesh Gas Chrom Q. For the quantitation of the autoxidized products, GLC (OV-101 column) was run with temperature programmed starting at 175 C to elute the unchanged methyl esters and then raising the temperature quickly to 190 C to elute other oxygenated methyl esters. The relative yield of the product was expressed as the ratio of the product peak area vs that of either methyl stearate or methyl palmitate present in the mixture. TLC was done using precoated Silica Gel G plates (0.25 mm thick, Analtech, Inc., Newark, DE). The purity of PC was checked with the solvent system chloroform/methanol/water (65:25:4, v/v/v) and the analyses of methyl esterified autoxidation products were done using the solvent system petroleum ether/diethyl ether/acetic acid (80:20:1 or 60:40:1, v/v/v). The spots on the plates were revealed by dipping in a solution of 3% cupric acetate in 8.5% phosphoric acid and subsequently charring at 140 C. For preparative TLC, samples were spotted on prescored plates, one segment of the plate was broken off for dipping and charring to locate the spots, and the adsorbent on corresponding areas on the other part of the plate was scraped off and extracted with ether. UV spectra were recorded using 1-cm path quartz cells in a Cary Model 14 spectrophotometer. Mass spectroscopic analyses were performed on a Finnigan Model 300 quadrupole spectrometer coupled with a Varian Aerograph Series 1400 gas chromatograph. The column used was a 0.20 x 180 cm metal coiled column packed with 3% OV-1 on 100/120 mesh Chromosorb WHP. The trimethylsilyl (TMS) derivatives were prepared by treating the hydroxy compounds with the silylating reagent, TRI-SIL (Pierce Chemical Co., Rockford, IL). High pressure liquid chromatography (HPLC) was done on a LiChrosorb RP-18 column (4.6 mm x 25 cm) (Unimetric, Anaheim, CA) using methanol as eluting solvent for α-tocopherol and soybean PC. For separation of the hydroxy esters, a Partisil 10 column (4.6 mm x 25 cm) (Whatman, Inc., Clifton, NJ) with solvent system 2-propanol/hexane (0.75:100, v/v) and flow rate of 80 ml/hr was used. Equipment for HPLC was a Varian Model 8500, a Varian Fluorichrom and a Hewlett Packard Model 3380A Integrator.

Preparation and Autoxidation of PC Liposomes

The established methods of forming small unilamellar vesicles have been followed with slight modifications (18,19). In a typical preparation, 0.136 g of pure soybean PC dissolved in a small quantity of chloroform was dried down to a very thin film on the walls of a sonication tube. The lipid was suspended by adding 20 ml of buffer solution (0.1 M KCl in a 0.01 M Tris buffer adjusted to pH 7.4), and then sonicated with a Branson Sonifier (Model S110) at power level 2. During sonication, nitrogen was flushed over the surface of the solution, and the

temperature was kept below 35 C by immersing in a cold water bath. It usually required 15-20 min of sonication for the original milky suspension to become translucent. After sonication, the titanium fragments released from the probe and any undispersed multilamellar vesicles were removed by centrifugation at 105,00 x g for 60 min. The resulting supernatant was divided into portions and was usually used immediately for the subsequent incubation. Autoxidation was done at 40 C by exposing the samples to air in an incubator-shaker for the desired length of time.

After autoxidation, the liposome samples were extracted twice with chloroform/methanol (2:1, v/v), the solvent evaporated under nitrogen and the residual PC, usually 10-15 mg in weight, was saponified using 4 ml of diethyl ether and 0.6 ml of KOH solution (3 g of KOH in 10 ml of methanol). The saponification mixture was stirred vigorously at room temperature for 3 hr, neutralized with acetic acid/water (1:3, v/v), and the ether layer was washed several times with water. (Methyl 9,10-epoxystearate was used to test the method to insure the complete preservation of the epoxide ring during hydrolysis.) The resulting fatty acids were then esterified with diazomethane. The methyl esters were quantitated by GLC.

Purity of PC before and after sonication were checked both by TLC and HPLC, and after hydrolysis, the methyl esters were checked by GLC and TLC. The separation of pure and oxidized PC has also been done by others (20). It was found that, with sonication under nitrogen atmosphere and at a low power level, the losses of both linoleic and linolenic acid due to oxidation were usually below 1% of the total amount. Only a small amount of phosphatidic acid was detected on TLC as the result of degradation by sonication, but no lyso PC or free fatty acid was detected as previously reported by others (6).

Preparation of Soybean PC-Dipalmitoyl PC Liposomes and Soybean PC-α-Tocopherol Liposomes

Dipalmitoyl PC, 0.073 g (9.099 mmol), and soybean PC, 0.020 g (0.026 mmol), were co-dissolved in a small amount of chloroform. After thorough mixing, the solvent was removed. The mixture was sonicated in 14 ml of 0.1 M KCl in 0.01 M Tris buffer at 45 C.

Soybean PC, 0.217 g (0.278 mmol), was dissolved in 1 ml of α-tocopherol solution (0.0139 mmol of α-tocopherol in 50 ml of chloroform). The concentration of α-tocopherol incorporated in the liposomes is 0.05 mol % of total fatty acid.

The procedures used for the preparation, autoxidation and work-up of soybean PC-dipalmitoyl PC and soybean PC-α-tocopherol liposomes were exactly the same as those described for the pure soybean PC liposomes.

Preparation of Trihydroxyoctadecenoates

A mixture of hydroxyepoxyoctadecenoates (about 1 mg) (13) was stirred with 1 ml of 7% perchloric acid in tetrahydrofuran/water (4:1, v/v) at room temperature for 21 hr. After the reaction, the mixture was evaporated down to a small volume and was then extracted 3 times with ether. The ether layer was washed with water several times before the ether was removed. Diazomethane was added to the residue. GLC revealed that the conversion was nearly quantitative under the condition used.

RESULTS

The rate of autoxidation of pure soybean PC liposomes at 40 C is shown in Figure 1. In this figure, the remaining quantity of each substrate expressed as a percentage of the control was plotted against the incubation period. The autoxidation of both linoleic and linolenic acids commenced immediately without a detectable induction period. For oleic acid, however, there seemed to exist an induction period which continued for about 17 hr of incubation. As expected, the rate of disappearance of linolenic acid was faster than that of linoleic acid. At the end of the 22 hr incubation period, the remaining linoleic and linolenic acids were 46 and 23%, respectively. The loss of oleic acid to autoxidation eventually reached 30% at the end of the 68-hr incubation period.

The autoxidation of liposomes which contained 0.05 mol% of α-tocopherol resulted in an

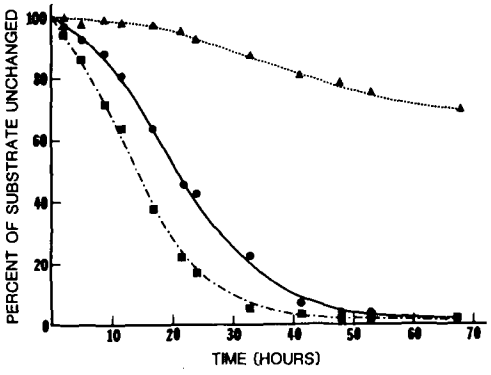
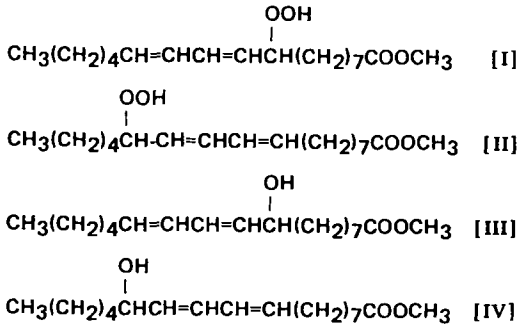


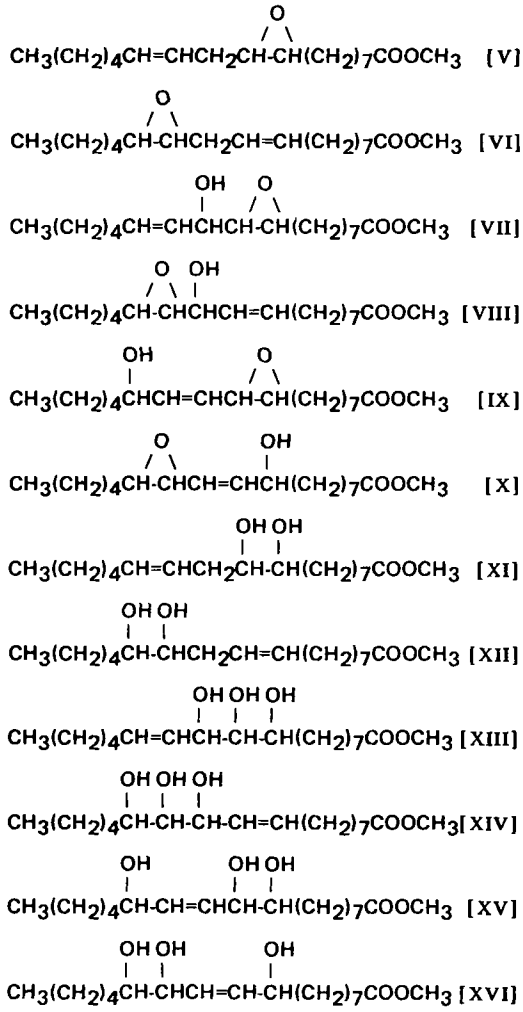
FIG. 1. Rate of autoxidation of unsaturated fatty acids in soybean PC liposomes. ---▲---, Disappearance of oleic acid; —●—, disappearance of linoleic acid; —■—■—, disappearance of linolenic acid.

induction period for both linoleic acid and linolenic acid as shown in Figure 2. The end of the induction period is defined as the time at which the straight line drawn for the early oxidation curve intercepts the straight line drawn to represent the succeeding rapid oxidation curve. The induction periods for linoleic and linolenic acid are both ca. 7 hr, with more loss of linolenic acid than linoleic acid to autoxidation (7 vs 2%) during the induction period. The induction period for oleic acid is not obvious, as the overall loss for the entire 28-hr incubation period measured is only about 10%. At the end of the induction period, the rates of autoxidation for both linoleic and linolenic acids seem to be slightly faster than those from the liposomes without added tocopherol.

Several attempts to determine the amount of remaining α-tocopherol at various stages of the induction period were unsuccessful. Without prior isolation, the routine methods of assaying tocopherols were found to be unsuitable in quantitating small amounts of α-tocopherol in a mass of lipid and its autoxidized product. In Emmerie and Engle's method using ferric chloride and bathophenanthroline (21), the presence of both PC and tris(hydroxymethyl)aminomethane in the mixture interferes with the color reaction. HPLC equipped with spectrofluorometer can be successfully used for the determination of α-tocopherol present in the control sample. However, as the incubation proceeds, the oxidized PC formed in the sample interferes progressively with the tocopherol determination.

Soybean PC can be successfully hydrolyzed with a mild 2-phase system, methanolic potassium hydroxide and ether, a procedure specially developed in this laboratory to preserve epoxides and hydroxyepoxides, during hydrolysis. The conditions used for the hydrolysis of PC also convert the hydroperoxide to alcohol, presumably through a nucleophilic, substitution-type reaction reported previously (22). Similar conversion has been observed in saponification of fatty ester hydroperoxides (23).





With the exposure of soybean PC liposomes to air at 40 C for 22 hr or less, the major product observed on TLC, after hydrolysis and esterification, was a mixture of 9- and 13-hydroxyoctadecadienoates (III and IV). On GLC, without derivatization, the major products were resolved into 3 components with retention times 14.02, 14.83 and 17.06 min. On conversion to the TMS ether, as shown in Figure 3, a partial resolution is now seen for the slow-moving compound (Fig. 3, peaks C and D). The faster-moving twin peak in the underivatized compound, after silylation, coalesced to form peak B. The major products obtained from autoxidation of neat methyl linoleate behaved similarly on TLC and GLC.

The mass spectra of the TMS ether of both III and IV have been reported (24). The major fragmentation ions obtained from III are shown in Table 1, A. In this case, however, the same

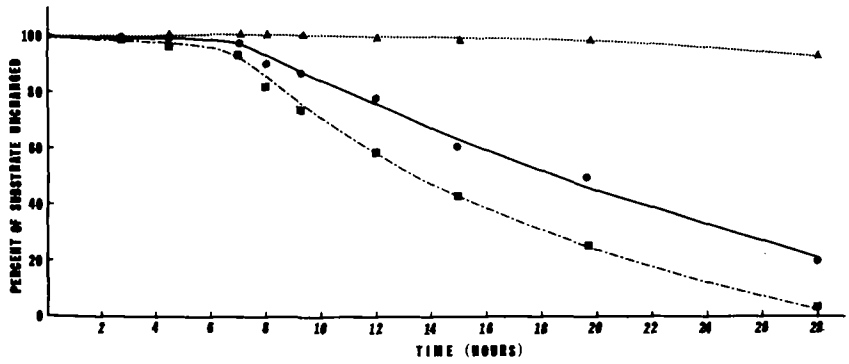


FIG. 2. Effect of 0.05 mol % α-tocopherol on autoxidation of soybean PC liposomes at 40 C. ---▲---, Disappearance of oleic acid; —●—, disappearance of linoleic acid; --■---■---, disappearance of linolenic acid.

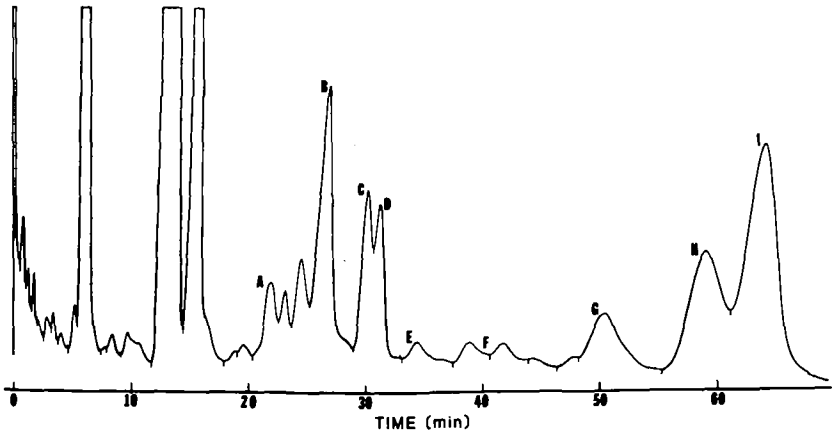


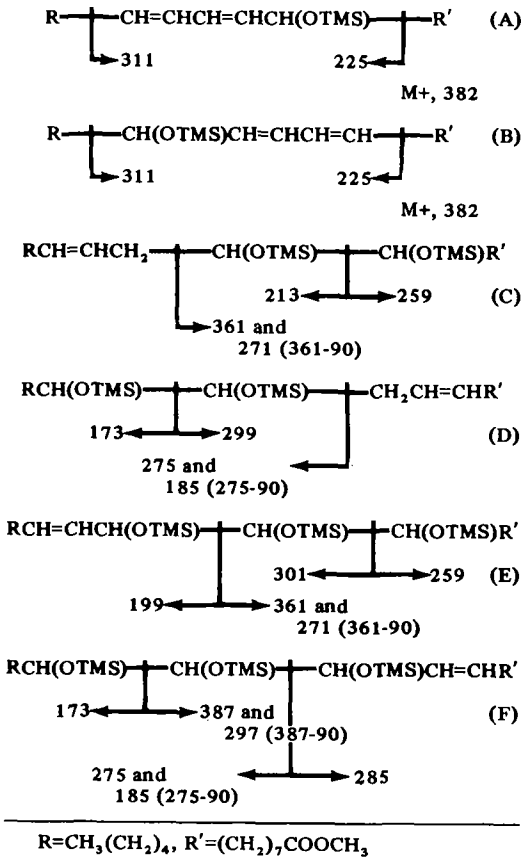
FIG. 3. Gas chromatography of silylated autoxidized products from soybean PC liposomes at high level of conversion. A, mixture of V and VI; B, mixture of III (10-*trans*,12-*cis*) and IV (9-*cis*,11-*trans*); C and D, mixture of III (10-*trans*,12-*trans*) and IV (9-*trans*,11-*trans*); E, mixture of VII and VIII; F, mixture of XI and XII; G, unidentified product; H and I, mixture of trihydroxyoctadecenoates.

set of major ions is also obtained from IV as shown in Table 1, B, with differences only in the intensities of 2 major ions. Therefore, gas chromatography/mass spectroscopy (GC/MS) is not entirely suitable for distinguishing between the positional isomers of these hydroxy fatty acids.

With HPLC, the 4 isomers of hydroxy ester, III (10-*trans*,12-*cis*), III (10-*trans*,12-*trans*), IV (9-*cis*,11-*trans*) and IV (9-*trans*, 11-*trans*) can be successfully separated on a micro-silica column (25,26). A typical HPLC chromatogram of these 4 hydroxy esters obtained from soybean PC liposomes is presented in Figure 4. The identity of these 4 peaks on HPLC chromatograms has been well established in the past (25,26), IV (9-*cis*,11-*trans*) being the least polar, followed by IV (9-*trans*,11-*trans*), III (10-*trans*, 12-*cis*) and III (10-*trans*,12-*trans*) (corresponding to peaks A, B, C and D in that order in Fig. 4). For the identification of the hydroxy ester GLC peaks (peaks B, C and D in Fig. 3), all 4 isomers (peaks A, B, C and D in Fig. 4) were collected separately from the HPLC runs and were injected into GLC. By this method, the peak B (Fig. 3) is positively identified as a mixture of III (10-*trans*,12-*cis*) and IV (9-*cis*,11-*trans*) and peak C and D as a mixture of III (10-*trans*,12-*trans*) and IV (9-*trans*,11-*trans*). This confirms the assignment of GLC peaks of monohydroxy esters published earlier by other workers using different methods of identifica-

TABLE 1

Mass Spectral Data on Major Autoxidation Products



R=$CH_3(CH_2)_4$, R'=$(CH_2)_7COOCH_3$

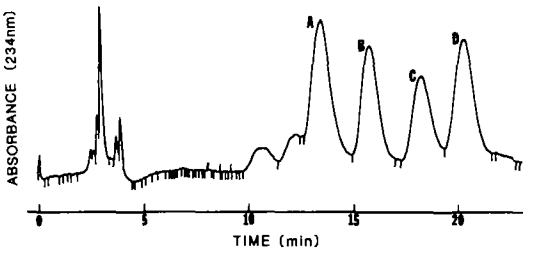


FIG. 4. HPLC elution pattern of hydroxy fatty esters from autoxidation of soybean PC liposomes. A, IV (9-*cis*, 11-*trans*); B, IV (9-*trans*, 11-*trans*); C, III (10-*trans*,12-*cis*) and D, III (10-*trans*,12-*trans*).

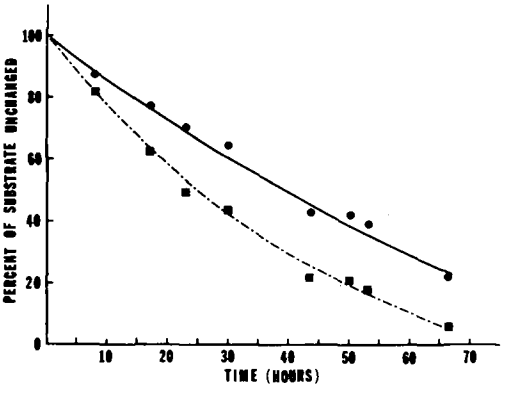


FIG. 5. Rate of autoxidation of linoleic and linolenic acid in soybean PC-dipalmitoyl PC mixed liposomes (1:4). —●—●—, Disappearance of linoleic acid; --■--■--, disappearance of linolenic acid.

tion (27,28). No attempt was made to characterize the small amount of autoxidized products formed from linolenic and oleic acids.

When liposomes containing 4 mol of dipalmitoyl PC and 1 mol of soybean PC were autoxidized, the rate of the reaction at 40 C was decreased considerably and the shape of the disappearance curve was changed (Fig. 5). After 23 hr of incubation, the loss of linoleic acid was only 30% and that of linolenic acid was 51%. The conversion of a large proportion of substrate was accomplished after close to 70 hr of incubation. Throughout the entire incubation period, the disappearance of oleic acid was negligible.

Both liposome systems, pure soybean PC and soybean PC-dipalmitoyl PC, at a high level of conversion, produce considerable amounts of other products in addition to the hydroxy fatty acids derived from hydroperoxides. On TLC, these more polar products are seen as a series of spots ranging in R_f values from 0.13 to the origin. Gas chromatograms of hydrolyzed, esterified (diazomethane) and silylated total autoxidation product from both pure soybean PC and soybean PC-dipalmitoyl PC liposomes are shown in Figures 3 and 6, respectively. Small quantities of relatively pure substances corresponding to peaks A, E, F, G, H and I (Figs. 3 and 6) were isolated individually by preparative TLC for identification. According to the behaviors in GLC, TLC and GC/MS, the structures of these products in both Figures 3 and 6 were identified as peak A, epoxides V and VI; peak E, hydroxyepoxides VII and VIII; peak F, dihydroxy esters XI and XII; peak G, an unidentified compound and peaks H and I, a mixture of trihydroxy esters. Peaks B, C and D are geometrical isomers of III and IV as described earlier.

The structures of epoxides V and VI and hydroxyepoxides VII and VIII from liposomes

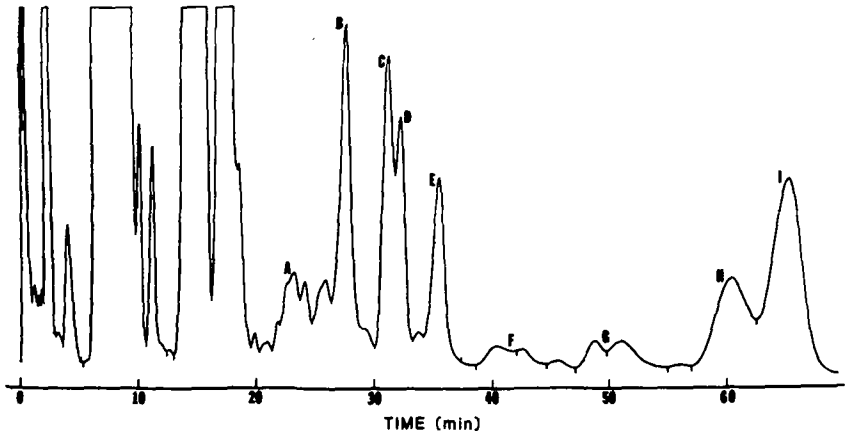


FIG. 6. Gas chromatography of silylated autoxidized products from soybean PC-dipalmitoyl PC mixed liposomes (1:4). A, mixture of V and VI; B, mixture of III (10-*trans*,12-*cis*) and IV (9-*cis*,11-*trans*); C and D, mixture of III (10-*trans*,12-*trans*) and IV 9-*trans*,11-*trans*); E, mixture of VII and VIII; F, mixture of XI and XII; G, unidentified product; H and I, mixture of trihydroxyoctadecenoates.

were confirmed by comparing GLC retention times and mass spectra with those of authentic samples isolated from monolayer autoxidation (12,13). The details of the mass spectra of methoxy trimethylsilyloxy derivatives obtained from epoxides V and VI (12) and epoxy trimethylsilyloxy compounds obtained from hydroxyepoxides VII and VIII have been reported (13).

The isomeric dihydroxy esters XI and XII are inseparable on TLC and gave an R_f value identical to that of methyl 9,10-dihydroxystearate. On GLC, the mixture appears as 2 overlapping peaks (Fig. 6, peak F) and the retention time (25.29 min) is close to that of dihydroxystearate (25.53 min). Mass spectra of a vicinal dihydroxy fatty acid with similar structural features have been reported (29). Compound XI, as the TMS derivative, cleaved as shown in Table 1, C, and similarly, XII gave ions shown in Table 1, D. Sampling of different areas of the 2 peaks (Fig. 6, peak F) gave all the ions expected from both structures XI and XII and there is no indication of the partial resolution of the positional isomers.

Samples of trihydroxy esters were prepared from hydroxyepoxides VII and VIII isolated from monolayer autoxidation of linoleic acid (13). The products (Fig. 6, peaks H and I) from liposome oxidation were found to be similar to the mixture of trihydroxy ester obtained from VII and VIII in GLC, TLC and GC/MS behavior. The fragmentation pattern of 1,2,3-trihydroxy fatty acids in MS has been reported in the past (30). The major cleavage occurs between 2 adjacent carbon atoms bearing trimethylsilyloxy groups. The TMS derivative of compound XIII cleaved in the manner shown in Table 1, E. Similarly, XIV cleaved as shown in Table 1, F. From GC/MS data alone, however, it was not possible to exclude the presence of 1,2,5-trihydroxy esters XV and XVI. The mixture of TMS derivatives of XV and XVI give major ions, 301, 259, 387 and 173, cleaving between 2 trimethylsilyloxy groups, and 387, 173 and 259, cleaving between the trimethylsilyloxy group and the double bond (31). All of these ions are also present in the 1,2,3-trihydroxy ester XIII and XVI. In the case of hydroxyepoxides, we were able to exclude the presence of isomeric compounds IX and X. In GC/MS, compounds IX and X give a series of major ions, 327, 259, 241, 199 and 173 (31), which are distinguishable from the fragments obtained from compounds VII and VIII.

The structure of peak G (Fig. 6) is not yet identified. On TLC, this compound has an R_f value ranging between dihydroxy esters XI and XII and trihydroxy esters XIII and XIV. The overall features of the mass spectrum of this compound are similar to those of XI and XII, except that the ions from cleavage between the trimethylsilyloxy group and the adjacent methylene group, i.e., 361 and 275, are much more intense than those from XI and XII. The presence of a third type of functional group, such as a keto group in the molecule might make this bond more labile, but none of the major ions obtained could account for the presence of such a functional group.

The production of the 4 major products including the combined yield of III (10-*trans*,12-*cis*) and IV (9-*cis*,11-*trans*), the combined yield of III (10-*trans*,12-*trans*) and IV (9-*trans*,11-*trans*). hydroxyepoxy ester VII and VIII and trihydroxy ester at different stages of autoxidation in soybean PC liposomes without and with added dipalmitoyl PC are shown in Figures 7 and 8, respectively. In both figures, the yield of each product is expressed as the ratio of GLC peak area of the product vs that of methyl stearate present in the mixture. Therefore, the yields of the products shown in both figures are on a comparable scale. Similarly, the estimation of yields using methyl palmitate as the internal standard (corrected for the amount added in the soybean PC-dipalmitoyl PC liposomes) gave the same results.

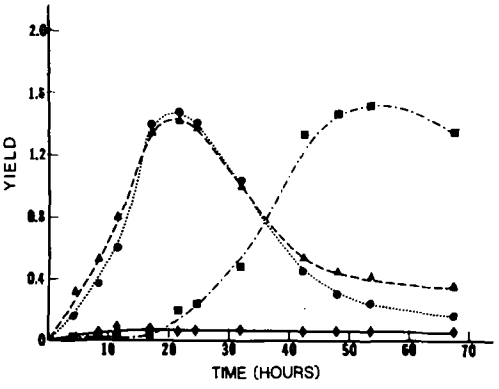


FIG. 7. Rate of production of 4 major products from autoxidation of pure soybean PC liposomes. ---▲---, Mixture of III (10-*trans*,12-*cis*) and IV (9-*cis*, 11-*trans*); ---●---●---, mixture of III (10-*trans*,12-*trans*) and IV (9-*trans*,11-*trans*); —◆—◆—, mixture of VII and VIII and —■---■-—, mixture of trihydroxyoctadecenoates. Yield of the product is expressed as the ratio of GLC peak area vs that of methyl stearate present in the mixture. (Methyl stearate amounts to 6.3% of methyl linoleate.)

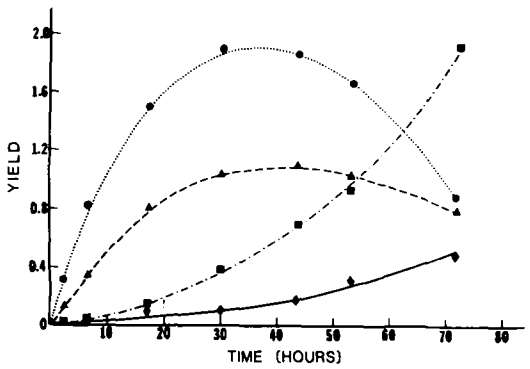


FIG. 8. Rate of production of 4 major products from autoxidation of soybean PC-dipalmitoyl PC mixed liposomes (1:4). ---▲---▲---, Mixture of III (10-*trans*,12-*cis*) and IV (9-*cis*,11-*trans*); ---●---●---, mixture of III (10-*trans*,12-*trans*) and IV (9-*trans*,11-*trans*); —◆—◆—, mixture of VII and VIII and —■---■-—, mixture of trihydroxyoctadecenoates. Yield of the product is expressed as the ratio of GLC peak area vs that of methyl stearate present in the mixture. (Methyl stearate amounts to 6.3% of methyl linoleate.)

In the autoxidation of pure soybean PC liposomes, the production of hydroperoxide (analyzed as hydroxy esters) rose rapidly and reached the maximum when about 55% of linoleic acid was consumed (at 22-hr incubation). After the maximum, the yields of hydroxy esters fell quickly to a low level after 45 hr of incubation. In soybean PC-dipalmitoyl PC liposomes, both the rise and fall in the production of hydroxy esters were more gradual than with soybean PC, and the yield also reached the maximum when about half of the linoleic acid was consumed. In pure soybean PC liposomes, the combined yield of the 2 *trans,trans* hydroxy esters at some stages was slightly lower than that of the 2 *cis,trans* isomers, but generally the yields of 2 geometric isomers were fairly close. However, in soybean PC-dipalmitoyl PC liposomes, the yields of the 2 *trans,trans* isomers greatly exceeded that of *cis,trans* isomers at all incubation periods measured. This difference was largest when the yield of the hydroxy esters was at its maximum. Similar quantitative differences in the yields of geometrical isomers in 2 systems were also obtained from HPLC, after the peak areas of 4 hydroxy esters, III (10-*trans*,12-*cis* and 10-*trans*,12-*trans*) and IV (9-*cis*,11-*trans* and 9-*trans*,11-*trans*) were corrected by the corresponding molar extinction coefficient (25).

The production of trihydroxy esters in pure soybean PC liposomes appears to accelerate rapidly after the level of hydroxy esters reaches the maximum, whereas in the soybean PC-dipalmitoyl PC system, the production of trihydroxy esters started earlier and eventually reached a level that was slightly higher than that in the pure soybean PC system. A considerable amount of hydroxyepoxides VII and VIII was also formed in the mixed liposome system, but only a very low level persisted throughout the entire incubation period in the pure soybean PC system. As shown in Figures 7 and 8, the combined yield of all major monomeric products in pure soybean PC liposomes is much smaller than that in the soybean PC-dipalmi-

toyl PC liposomes, presumably because of the increasing possibility of radical addition and/or termination reactions in the pure soybean system leading eventually to dimers and polymers which were not observable by GLC.

DISCUSSION

The autoxidation of linoleic and linolenic acids in pure soybean PC liposomes at 40 C is reasonably fast and the rate curve (Fig. 1) is similar to those of autocatalytic reactions previously reported for the autoxidation of linoleic acid in solution (32). The induction period seen in the neat autoxidation is either nonexistent or too short to be detected for both linoleic and linolenic acid. For oleic acid, however, there is a visible discontinuity in the disappearance curve which can be taken as an induction period at the early stage of autoxidation. The point of inflection here lies at about 17 hr of incubation. The reason for the existence of an induction period for oleic acid specifically could be that the autoxidation of oleic acid can be effective only after accumulation of a certain amount of linoleic acid hydroperoxide. Pure oleic acid itself is known to be relatively resistant to autoxidation.

The incorporation of 0.05 mol % of α-tocopherol into soybean PC liposomes produced an induction period of about 7 hr for both linoleic and linolenic acid. The similar concentration of α-tocopherol in linoleic acid monolayers produced about 5 hr of induction period at 60 C (14). Efficient protection by α-tocopherol has also been reported for blood components (9, 33) and isolated microsomal and mitochondrial membranes (34). As was encountered in the case of monolayers, the efficient protection by a small number of molecules of α-tocopherol in a highly ordered system is not explainable without ascribing some kind of mobility to either α-tocopherol, the substrate (35) or a chain carrier. Further experiments are needed to substantiate this assumption. Unlike the case with the monolayers, however, in α-tocopherol-containing PC liposomes, at the termination of the induction period, the oxidation for both linoleic and linolenic acids occurs with rates significantly faster than those for PC liposomes without added α-tocopherol. Because the overall rate of an autocatalytic oxidation is proportional to the amount of hydroperoxide generated during the earlier stages of the reaction, it seems likely that the accelerated rate is due to an accumulation of hydroperoxide or other initiators during the time the α-tocopherol is decreasing. When the α-tocopherol is depleted, the reaction is accelerated by the increased concentration of initiators to a greater extent than would have occurred if α-tocopherol had not been present originally.

When a large quantity of dipalmitoyl PC was incorporated into soybean PC liposomes, the rate of autoxidation of linoleic and linolenic acids at 40 C was decreased considerably and the kinetics of the disappearance of both substrates changed to an apparent first order in substrate concentration. The loss of oleic acid to the autoxidation was found to be negligible throughout the entire incubation period.

As shown in Figure 7, in pure soybean PC liposomes, the production of hydroperoxides (isolated as hydroxy esters) increases rapidly and after a narrow maximum drops sharply. The interspersing of a large number of saturated acyl chains in the liposomes slows effectively both the formation and degradation of hydroperoxides (Fig. 8); a broad maximum of hydroxy esters in soybean PC-dipalmitoyl PC liposomes is now centered at about 40 hr of incubation instead of 20 hr. However, in both cases, the maximal yield in hydroxy esters was reached when about half of the linoleic acid was consumed.

The point of interest in considering the formation of hydroxy esters in the 2 systems is the striking difference in the yields of geometrical isomers (*cis,trans* vs *trans,trans*) isolated. A difference in the *cis,trans/trans,trans* hydroperoxide ratio at the initial stages of oxidation (smaller than 2% conversion) has been determined for the following systems (26). In the autoxidation of linoleic acid in benzene solution, the concentration of linoleic acid and the ratio of the diene configuration of hydroperoxides, *cis, trans/trans, trans*, are proportional, whereas an increase in temperature lowers the ratio. In dipalmitoyl PC-dilinoleoyl PC mixed aqueous emulsion, the ratio of *cis,trans/trans, trans* hydroperoxide decreased as the molar ratio of dipalmitoyl PC in the system increased. The rationale for these observations has been well elaborated by Porter et al. (26).

Our observation suggests that, because the palmitate is a poor hydrogen atom donor, the dilution of soybean PC with dipalmitoyl PC suppresses the hydrogen atom abstraction by the peroxy radical which would result in yielding mostly the *cis,trans* isomer, and instead, favors an alternative reaction, β-scission of the peroxy radical to regenerate the pentadienyl system. One of the possible configurations of the pentadienyl radical leads to the formation of the *trans,trans* isomer. The details of these 2 pathways have been presented schematically by Porter and coworkers (26). The Porter et al. results (26) were for the initial stages of autoxi-

dation (less than 2% of conversion) only. Our observations indicate that the β-scission pathway that occurs in the presence of palmitate is the primary course of the reaction until more than 70% of the substrate has reacted. There is good evidence that the yields of *cis,trans* and *trans,trans* isomers shown in Figure 8 represent the kinetic control of the process and do not represent any significant isomerization of hydroperoxides (36). It has been shown recently that the rate of interconversion of hydroperoxide isomers decreases as the polarity of the solvent increases, and no significant interconversion was noted in ethanol after heating for 16 hr at 40 C (37).

The major products from decomposition of hydroperoxides I and II under various conditions have been studied recently. The anaerobic thermal decomposition of II at 100 C yields at least 4 major products. One of the major products was identified as hydroxyepoxide VIII. From experiments using ^{18}O-labeled hydroperoxide and ^{18}O-enriched water, it was concluded that the epoxy oxygen in VIII came from hydroperoxide and the hydroxy group came from the solvent (38). At a lower temperature (37 C) and with catalysis by hemoglobin, II decomposed to 5 major compounds including IV, 2 stereoisomers of VIII, X and 13-keto-9, 11-octadecadienoic acid (39). Aerobic decomposition of II with ferrous ion in aq ethanol was found to give a complex series of products, including keto, ketoepoxy, ketohydroxy, dihydroxyethoxy, VIII, X and XVI (40-42). Among these products formed, X and 9-oxo-12,13-epoxy-10-octadecenoic acid are postulated to arise from a common intermediate, 12,13-epoxy-9-hydroperoxy-10-octadecenoic acid (41). Recently, the major products from decomposition of II in the presence of cysteine-$FeCl_3$ have been quantitated to give 23% isomeric 12,13-epoxy-9-oxo-octadecenoate, 7% X and 7% VIII (43). Thus, the decomposition of hydroperoxides in solution invariably gave a complex mixture of products, including simple or bifunctional keto fatty acids among major products.

In the PC liposome system, the major products formed from the degradation of hydroperoxides are extremely simple, including only trihydroxy esters from pure soybean PC and trihydroxy esters and hydroxyepoxides (VII and VIII) from the soybean PC-dipalmitoyl PC mixed system. The epoxides V and VI and presumably their hydrolysis products XI and XII are formed only in very small quantities in both systems. These findings suggest that a matrix or solvent-oriented course of the reaction is responsible for the differences in these cases. The conversion of II to VIII (in solution) in particular has been studied in detail recently (43). The hydroxyepoxide VIII has been postulated to arise from an intramolecular cyclization of alkoxy radicals formed by the decomposition of II. The resulting epoxyallylic radical is converted to VIII by undergoing a sequence of steps involving a second peroxidation, a cleavage of the hydroperoxide group and a disproportionation of the resulting alkoxy radical. In our liposome system, the fact that the incorporation of saturated PC into the soybean PC liposomes apparently facilitates the formation of hydroxyepoxy ester suggests that when the intermolecular reactions are blocked, the hydroperoxides or peroxy radicals proceed eventually to the intramolecular reactions. A similar increase in the production of VII and VIII has been observed in the fatty acid monolayers in which linoleic acid molecules are separated by the interspersed palmitic acids (13). The hydroxyepoxy fatty esters formed in soybean PC-dipalmitoyl PC liposomes were found to be exclusively VII and VIII; no significant intensity of fragment ions originating from the structures IX and X were noted in the mass spectra. The hydroxyepoxy ester X, in particular, has been demonstrated to undergo a facile transformation to 13-oxo-9-hydroxy-octadecenoate with a Lewis acid, BF_3-etherate (42). The same rearrangement can also be brought about, but to a lesser extent (about 20% yield), by treatment of X with cysteine-$FeCl_3$ in methanol/water (30%) (43). In our case, no significant amount of the presumed rearranged product, 13-oxo-9-hydroxy- or 9-oxo-13-hydroxy-octadecenoate was found. Therefore, the evidence at hand, although inconclusive, suggests that the formation of hydroxyepoxides VII and VIII (particularly the origin of the hydroxy group) arises from a pathway different from those 2 processes previously reported. It has been shown that the hydroxy group can come from solvent (38) or from molecular oxygen in the atmosphere (43,44).

The formation of trihydroxy esters in the autoxidation was thought previously to result from hydrolysis of the corresponding hydroxyepoxy esters (32,42). For example, the hydrolysis of X in methanol/water with $FeCl_3$-cysteine as catalyst gives 3 major products of comparable yields, dihydroxymethoxy, trihydroxy and ketohydroxy esters (42). However, we have been forced to abandon the possibility of the hydrolysis route as the major pathway because of the following two reasons: (a) in the pure soybean PC liposomes, the amount of the presumed precursor VII and VIII was extremely small compared to that of the

trihydroxy esters at all incubation periods; and (b) even in the soybean PC-dipalmitoyl PC liposomes, in which the combined yield of VII and VIII is larger, there is no indication that the onset of the formation of the trihydroxy ester is triggered by the accumulation of VII and VIII. Therefore, although some of the trihydroxy ester might arise by the hydrolysis route, another pathway probably exists to which the major part of the trihydroxy ester production can be ascribed.

The structures XIII and XIV that were tentatively assigned to trihydroxy esters were based on the behaviors identical to a mixture of trihydroxy ester prepared from the reaction of a mixture of VII and VIII with perchloric acid. We did not, however, exclude the possibility that the ring-opening reaction with perchloric acid could also result in a rearrangement of VII and VIII; therefore, the products from this reaction might not be limited to the trihydroxy esters of structures XIII and XIV. The fragmentation pattern in GC/MS does not distinguish between 2 sets of the structures XIII, XIV and XV, XVI. It will not be possible to consider the mechanism of the formation of trihydroxy esters until the position of the 3 hydroxy groups has been determined unequivocally. This task is being done in this laboratory using chemical degradation methods.

ACKNOWLEDGMENTS

These studies were supported by Contract DE-AMO3-76-SF00012 between the Department of Energy and the University of California and by U.S. Public Health Service Research Career Award No. GM K-6-A, 177 from the Division of General Medical Sciences, National Institutes of Health.

REFERENCES

1. Smolen, J.E., and Shohet, S.B. (1974) J. Lipid Res. 15, 273-280.
2. Brounlee, N.R., Huttner, J.J., Panganamala, R.V., and Cornwell, D.G. (1977) J. Lipid Res. 18, 635-644.
3. Corliss, G.A., and Dugan, L.R., Jr. (1970) Lipids 5, 846-852.
4. Barker, M.O., and Brin, M. (1975) Arch. Biochem. Biophys. 166, 32-40.
5. Klein, R. (1970) Biochim. Biophys. Acta 210, 486-489.
6. Hauser, H.O. (1971) Biochem. Biophys. Res. Commun. 45, 1049-1055.
7. Kaschnitz, R.M., and Hatefi, Y. (1975) Arch. Biochem. Biophys. 171, 292-304.
8. Gutteridge, J.M. C. (1977) Anal. Biochem. 82, 76-82.
9. Porter, W.L., Henick, A.S., Murphy, F., Colgan, R., and Porfert, G. (1977) Lipids 13, 137-146.
10. Porter, N.A., Wolf, R.A., and Weenen, H. (1980) Lipids 15, 163-167.
11. Wu, G.-S., and Mead, J.F. (1977) Lipids 12, 965-970.
12. Wu, G.-S., Stein, R.A., and Mead, J.F. (1977) Lipids 12, 971-978.
13. Wu, G.-S., Stein R.A., and Mead, J.F. (1978) Lipids 13, 517-532.
14. Wu, G.-S., Stein, R.A., and Mead, J.F. (1979) Lipids 14, 644-650.
15. Clements, J.A. (1973) Respiratory Distress Syndrome, pp. 77-98, Academic Press, New York, NY.
16. Singleton, W.S., Gray, M.S., Brown, M.L., and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53-56.
17. Terao, J., Hirota, Y., Kawakatsu, M., and Matsushita, J. (1981) Lipids 16, 427-432.
18. Huang, C.-H. (1969) Biochemistry 8, 344-352.
19. Tyrrell, D.A., Heath, T.D., Colley, C.M., and Ryman, B.E. (1976) Biochim. Biophys. Acta 457, 259-302.
20. Crawford, C.G., Plattner, R.D., Sessa, D.J., and Rackis, J.J. (1980) Lipids 15, 91-94.
21. Tsen, C.C. (1961) Anal. Chem. 33, 849-851.
22. O'Brien, P.J. (1969) Can. J. Biochem. 47, 485-492.
23. Frankel, E.N., Evans, C.D., McConnell, D.G., and Jones, E.P. (1961) J. Am. Oil Chem. Soc. 38, 134-137.
24. Kleiman, R., and Spencer, G.F. (1973) J. Am. Oil Chem. Soc. 50, 31-38.
25. Chan, H.W.S., and Levett, G. (1977) Lipids 12, 99-104.
26. Porter, N.A., Weber, B.A., Weenen, H., and Khan, J.A. (1980) J. Am. Chem. Soc. 102, 5597-5601.
27. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weeden, B.C.L. (1977) Lipids 12, 908-913.
28. Gardner, H.W., and Weisleder, D. (1970) Lipids 5, 678-683.
29. Sessa, J.J., Gardner, H.W., Kleiman, R., and Weisleder, D. (1977) Lipids 12, 613-619.
30. Streckert, G., and Stan, J. H. (1975) Lipids 10, 847-854.
31. Graveland, A. (1970) J. Am. Oil Chem. Soc. 47, 352-361.
32. Cillard, J., Cillard, P., Cormier, M., and Girre, L. (1980) J. Am. Oil Chem. Soc. 57, 252-255.
33. Shimasaki, H., and Privett, O.S. (1975) Arch. Biochem. Biophys. 169, 506-512.
34. McCay, P.B., Pfeifer, P.M., and Stipe, W.H. (1972) Ann. N.Y. Acad. Sci. 203, 62-73.
35. Devaux, P., and McConnel, H.M. (1972) J. Am. Chem. Soc. 94, 4475-4481.
36. Chan, H.W.S., Costaras, C.T., Prescott, F.A.A., and Swoboda, P.A.T. (1975) Biochim. Biophys. Acta 398, 347-350.
37. Chan, H.W.S., Levett, G., and Matthew, J.A. (1979) Chem. Phys. Lipids 24, 245-256.
38. Hamberg, M., and Gotthammar, B. (1973) Lipids 8, 737-744.
39. Hamberg, M. (1975) lipids 10, 87-92.
40. Gardner, H.W., Kleiman, R., and Weisleder, D. (1974) Lipids 9, 696-706.
41. Gardner, H.W., Weisleder, D., and Kleiman, R. (1978) Lipids 13, 246-252.
42. Gardner, H.W., and Kleiman, R. (1979) Lipids 14, 848-851.
43. Gardner, H.W., and Kleiman, R. (1981) Biochim. Biophys. Acta 665, 113-125.
44. Gardner, H.W., and Crawford, C.G. (1981) Biochim. Biophys. Acta 665 126-133.

[Revision received March 12, 1982]

Cyclic Fatty Esters: Synthesis and Characterization of Methyl ω -(6-Alkyl-3-Cyclohexenyl) Alkenoates[1]

R.A. AWL and **E.N. FRANKEL**, *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604.*

ABSTRACT

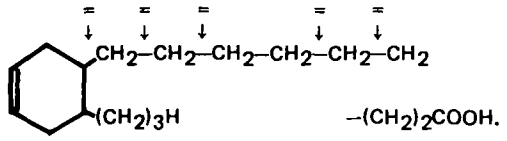
Diunsaturated C_{18} cyclic fatty acid methyl esters of known structure and configuration were synthesized as model derivatives of cyclic fatty acids formed in heat-abused vegetable oils for characterization and further biological evaluation. The Wittig reaction was used to prepare 5 pure methyl esters: (a) 12-(3-cyclohexenyl)-11-dodecenoate, (b) 11-(6-methyl-3-cyclohexenyl)-10-undecenoate, (c) 10-(6-ethyl-3-cyclohexenyl)-9-decenoate, (d) 9-(6-propyl-3-cyclohexenyl)-8-nonenoate and (e) 8-(6-butyl-3-cyclohexenyl)-7-octenoate. Diels-Alder cycloaddition reactions between 1,3-butadiene and appropriate (E)-2-alkenals produced 3-cyclohexenal intermediates. The appropriate methyl ω-bromoesters and their triphenylphosphonium bromides were made and converted to their respective ylids with $NaOCH_3$ in DMF. The appropriate 3-cyclohexenals and phospho-ylids were reacted, and the desired cyclic ester products were isolated in crude yields of 30-83% as liquids and fractionally distilled. The crude cyclic esters were purified either by preparative TLC or by saponification-esterification. Double bonds in purified cyclic esters were *trans*-isomerized and hydrogenated. Each derivative was characterized by IR, ^{1}H-NMR, ^{13}C-NMR, capillary GLC and GC-MS. On the basis of these analyses, no positional isomers were detected, Z-unsaturated isomers were produced in better than 90% purity, and the alkyl and ester ring substituents were predominantly *trans* to each other.

Lipids 17:414-426, 1982.

The formation of cyclic fatty acids in thermally abused cooking oils has been well documented, and investigations concerning their toxicity and that of heat-abused vegetable oils have been reviewed (1-3). According to previous investigations, monomeric cyclic acids caused quick deaths of experimental animals. The monomeric cyclic acids, unlike the dimeric or polymeric, have the greatest potential for harm because they are absorbed more readily by the digestive and lymphatic systems (4) and may be included in body fat along with natural fatty acids.

During the last 20 years, many studies indicated that 1,2-disubstituted, 6-membered ring compounds were the principal, cyclic components of heat-abused oils (1-3). Even with the most abused oils, however, the concentration of cyclic acids was too low and the isomeric distribution too large to permit practical isolation of any pure compound for direct characterization and definitive feeding studies (2,3).

The specific structures of the toxic, monomeric cyclic acids found in heat-abused vegetable oils are still unknown. On the basis of gas chromatographic studies, McInnes et al. (5) proposed the generalized cyclohexene structure, which included a complicated mixture of isomers:



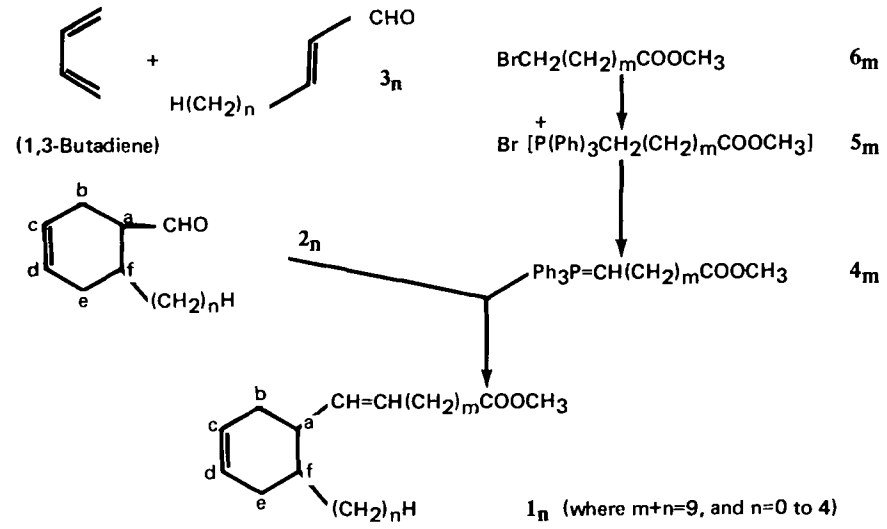
Possible double bond positions are indicated by arrows. The unsaturation in the ring and the chain was not conjugated, according to McInnes. Later investigators (3,6,7) noted that the 6-membered ring was not formed exclusively, and the respective length of the substituent chains and position of the double bonds varied. Other questions that remain unanswered include: what biological effects are produced by the pure cyclic compounds, and can specific, unsaturated cyclic acids or esters be determined in heated oils. A synthetic program was initiated to address these questions. This paper reports the synthesis and characterization of a family of diunsaturated, C_{18} fatty esters having the 1,6-disubstituted-3-cyclohexenyl ring. These synthetic compounds will be evaluated biologically later.

RESULTS AND DISCUSSION

Synthesis and Stereochemistry

One monosubstituted- and four 1,6-disubstituted-3-cyclohexenyl methyl esters (1_n, where n = 0 to 4) with unsaturation α to the ring on the ester substituent were prepared as new compounds according to Scheme 1. The chain

[1]Presented in part at the AOCS meeting, New Orleans, LA, May 1981.



SCHEME 1. Subscript n refers to a specific cyclic ester (1_n) or cyclohexenal (2_n); e.g., 1_0 has no alkyl substituent.

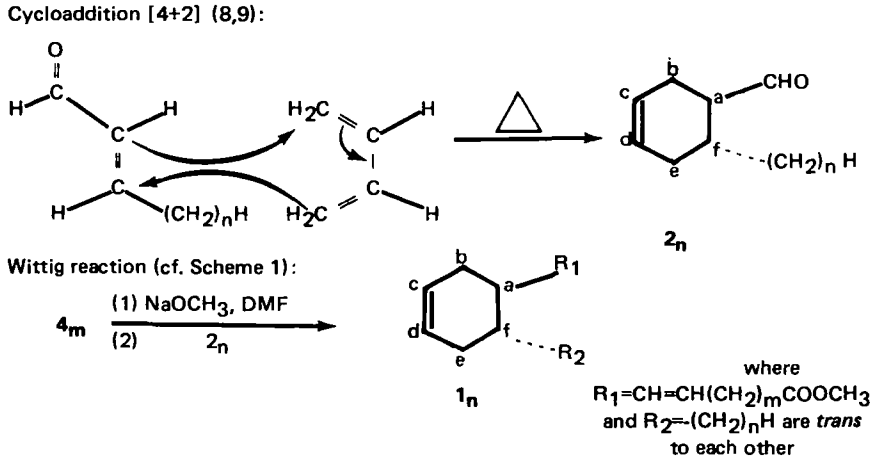
lengths of the ester and alkyl substituents were varied to determine their effect on physicochemical properties and analytical separations. Although the monosubstituted cyclohexenyl ester 1_0 is not expected in heated fats, it was synthesized as a reference compound.

Methyl 9-(6-propyl-3-cyclohexenyl)-8-nonenoate (cyclic ester 1_3) was our principal synthetic target for later feeding studies because previous work (3) indicated the ester with (n = 3) to be the most abundant isomer. The corresponding monounsaturated cyclohexenyl ester was another synthetic target. A monounsaturated cyclic ester was recently synthesized by Graille et al. (8) by a different route, which included a Diels-Alder cycloaddition step. According to the "Alder rules," the cycloaddition of E-unsaturated aldehydes 3_n with butadiene will give a *trans*-adduct (9). Therefore, the cyclic esters expected from our synthesis have ring substituents *trans* to each other (Scheme 2). To avoid confusion in this paper, *cis* and *trans* refer to the disubstituted cyclohexene ring isomers and the geometric double bond isomers are called Z (*cis*) or E (*trans*) isomer. After the final Wittig reaction step, the double bond in the side chain would be predominantly Z configuration under our reaction conditions, and the ring substituents would retain their *trans* relationship as indicated in Scheme 2.

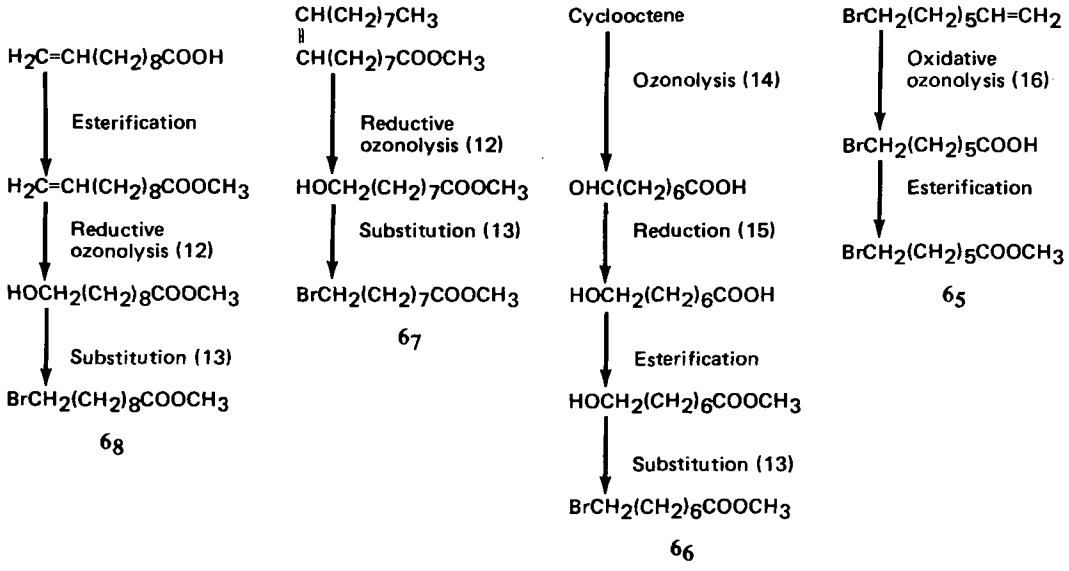
Diels-Alder cycloaddition (9) of butadiene and (E)-2-alkenals gave the cyclohexenal intermediates 2_n, which were reacted with the appropriate phospho-ylid 4_m in a Wittig reaction (10) to produce Z-unsaturated cyclic esters 1_n predominantly (Scheme 1). Both reactions as used are stereoselective. By using NaOEt in dimethylformamide (DMF) to generate the ylid from its phosphonium halide and then adding an appropriate aldehyde, Bergelson et al. (11) previously showed that the Wittig reaction formed Z-unsaturated isomers of fatty acids in better than 90% isomeric purity.

Those ω-bromoacids or -bromoesters 6_m (Scheme 1) that were not commercially available were made according to Scheme 3. The phosphonium bromides 5_m were prepared in better than 97% yields by refluxing an acetonitrile solution of Ph_3P and bromoester 6_m for ca. 36 hr. The phospho-ylids 4_m generated from their phosphonium bromides 5_m were reacted directly with the cyclohexenals 2_n by the method of Bergelson et al. (11).

When crude, cyclic ester 1_0 (Scheme 1) was isolated by the method of Bergelson et al. (11), which called for treatment with Al_2O_3, the product was still contaminated with Ph_3P or similar P-containing compound. This P impurity was removed completely by saponification-esterification (17), but the yield was reduced considerably. The colored, purified product 1_0 became clear after a short-path distillation. Gas chromatography-mass spectrometry (GC-MS) and capillary GLC indicated that it was composed of 2 isomers (M^+, m/z 292.3) in the ratio



SCHEME 2



SCHEME 3

93.2:6.2 (Fig. 1, curve I). IR showed only Z couble bonds. To confirm this double bond configuration, cyclic ester 1_0 was *trans*-isomerized with *p*-toluenesulfinic acid (p-TSIA) catalyst (18). This treatment changed the GLC peak ratio to 21.6:77.2 (Fig. 1, curve II). Therefore, the predominant isomer of 1_0 had Z unsaturation. After hydrogenation of 1_0, capillary GLC indicated 96% saturated cyclic ester and ca. 2% of 1_0 remaining (Fig. 1, curve III). The results were confirmed by IR, NMR and GC-MS.

Cyclic esters $1_{1\text{-}4}$ were adequately purified by TLC or on a larger scale by saponification-esterification, and the resulting products showed no evidence of isomerization by spectroscopic or chromatographic analyses. In contrast, when Graille et al. (8) purified their intermediate keto ester by saponification with KOR and re-esterification, they reported positional isomerization. Apparently, their keto ester was susceptible to isomerization under their conditions.

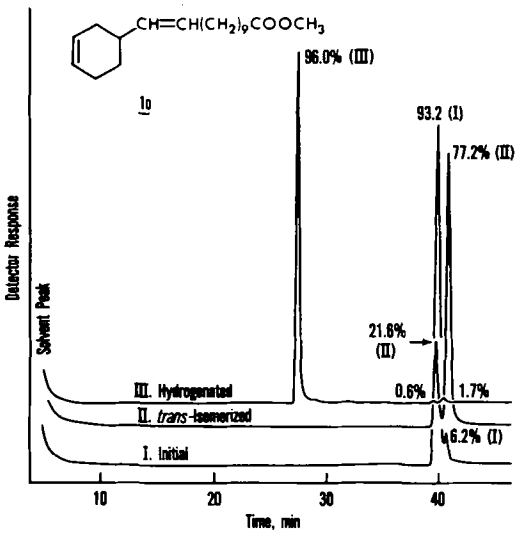


FIG. 1. Capillary GLC of cyclic fatty ester 1_0.

Capillary GLC Characterization

An equivalent chain length (ECL) for each cyclic ester was determined by the capillary GLC method of Scholfield (19) to determine how they would be resolved from other fatty acids in heated fats. On the basis of ECL (Table 1), cyclic isomers 1_0 and 1_1 corresponded to conjugated methyl octadecadienoates (E,E- or E,Z-9,11-; E,Z- or E,E-10,12- or E,E-11,13-); cyclic isomers 1_3 corresponded to methyl 9,12, 15-octadecatrienoates, and cyclic isomers 1_4 corresponded to nonconjugated E,E-12,15- and E,Z-12,15-octadecadienoates in 2 instances and to a Z,E,E,-9,12,15-octadecatrienoate in the other instance (19). The cyclic isomers 1_2 were intermediate between the nonconjugated octadecatrienoates and the conjugated octadecadienoates. For the monosubstituted cyclic ester 1_0, the E unsaturated ester had longer retention than the Z, but with the disubstituted ester 1_3 this order was reversed. As expected with a polar Silar 10C column, the saturated derivatives had lower retention times than the unsaturated cyclic esters. Also, for both the saturated and unsaturated cyclic esters, retention increased as the length of alkyl chain decreased.

Spectral Characterization

IR spectra of the cyclohexenals 2_n and cyclic esters 1_n displayed absorption bands consistent with the expected structures (Scheme 1): Z unsaturation at 1660 and 660 cm^{-1} (5,20); aldehyde (1728 cm^{-1}) or methyl ester (1747 cm^{-1}), respectively; and chain methylenes (725 cm^{-1}). An absorption band for E CH=CH (965 cm^{-1}) was observed only after cyclic esters 1_0 and 1_3 were isomerized with *p*-toluenesulfinic acid (18). Although the conversion of methyl oleate into methyl elaidate with this reagent was ca. 80%, the isomerization of cyclic ester 1_3 from Z to E unsaturation under the same conditions was only ca. 12%, according to capillary GLC (Fig. 2, curves I and II). The double bond in the side chain of 1_3 would be expected to isomerize with much difficulty because of steric hindrance by the α-alkyl substituent. This steric hindrance is confirmed by the greater degree of isomerization (ca. 77%) observed under the same conditions for the monosubstituted cyclic ester 1_0 (Fig. 1, curves I and II).

^{1}H-NMR data (Table 2) were consistent with those expected from structures of cyclohexenals 2_n and cyclic esters 1_n (Scheme 1). Our assignments for the diunsaturated cyclic ester 1_3 were in good agreement with those of Graille et al. (8) for their monounsaturated cyclic ester. ^{1}H-NMR of cyclohexenals $2_{1\text{-}3}$ (Table 2) showed evidence for mixtures of conformational isomers, e.g., 2 different -CHO resonances (δ 9.66 and 9.74) for 2_1 in a ratio of 2:1 (axial:equatorial). Apparently, the longer the alkyl branch, the more favored was the axial -CHO conformation. Examination of a molecular model of 2_1 demonstrated that the *trans* configuration can exist either as a half-chair form (conformer) with axial-axial (a_{CHO} a_R) and equatorial-equatorial (e_{CHO} e_R) substituents, or as a boat form with a_{CHO} e_R and e_{CHO} a_R substituents. Similarly, the *cis* configuration can assume either a half-chair form with a_{CHO} e_R and e_{CHO} a_R, or a boat form with a_{CHO} a_R and e_{CHO} e_R substituents. With either *trans* or *cis* configuration, the axial and equatorial CHO substituent would seem equally probable if conformational interconversions were purely random and not influenced by other factors. Thermodynamic considerations (21) and NMR data (8) strongly suggest that the half-chair form is much preferred over the boat form. The literature is controversial regarding the stability and preference of an axial CHO over an equatorial CHO in the half-chair conformation. For example, Kugatova-Shemyakina et al. (22,23) concluded from reactivity and spectral studies that axial CHO interacts with the ring double bond (a "supra-annualar effect") forming an intramolecular pi-complex that favors axial CHO. However, Zefirov et al. (24) concluded from heteronuclear double resonance studies that the equatorial CHO is favored.

^{13}C-NMR data for the cyclohexenals 2_n and

TABLE 1

Capillary Gas Liquid Chromatography[a]

Compound[b]	Peak (stereoisomer)	Relative area (%)[c]	ECL
1_0	1 (Z)	93.2	21.50
	2 (E)	6.2	21.57
1_1	1	2.1u	20.60
	2	61.1u	20.66
	3	2.8	20.75
	4	30.5	21.11
1_2	1	3.3	20.49
	2	70.5	20.59
	3	24.3	20.89
1_3	1	0.8	19.94
	2	77.1	20.07
	3	21.1	20.42
1_4	1	0.8	19.67
	2	75.2	19.81
	3	20.8	20.13
trans-Isomerized 1_0	1 (Z)	21.6	21.48
	2 (E)	77.2	21.58
Hydrogenated 1_0	1	96.0	20.09
	2	0.6	–
	3	0.6	21.46
	4	1.7	21.55
Hydrogenated 1_1	1	1.6u	19.44
	2	61.5u	19.55
	3	28.0	20.10
	4	1.9	20.19
	5	1.4u	20.34
trans-Isomerized 1_3	1 (E)	12.9	19.94
	2 (Z)	77.7	20.08
	3 (Z)	9.4	20.37
Hydrogenated 1_3	1	0.9u	18.76
	2	13.6	18.82
	3	60.9u	19.04
	4	0.8u	19.26
	5	21.6u	19.35
	6	0.9	19.53

[a]Ref. 19.

[b]See Scheme 1.

[c]u = unresolved.

the cyclic esters 1_n are given in Tables 3 and 4. Only cyclohexenal 2_1 gave a spectrum with resonances attributable to a *cis* ring isomer (designated by [*c*] in Table 3) showing a mixture of the *cis* and *trans* ring isomers. The commercial source of cyclohexenal 2_1 could explain this isomeric mixture. Reaction conditions different than ours, such as acid catalysis, higher temperatures or longer reaction times, generated mixtures of the *cis*- and *trans*-disubstituted-3-cyclohexene isomers (27,28). In the present work, all cyclohexenals showed one peak on GLC, except the commercial cyclohexenal 2_1 which gave 2 peaks in the ratio of 2:1. As expected, a mixture of *cis* and *trans* ring isomers was also indicated by ^{13}C-NMR for cyclic ester 1_1 derived from 2_1; more ring resonances were observed for 1_1 than for the other cyclic esters. The resonances for the *cis* ring were not observed in the spectra of the other cyclohexenals and cyclic esters and, therefore, supported our previous assessment that the *trans* ring isomer was formed by the Diels-Alder cycloaddition (Scheme 2).

Chemical shift assignments for the ester chain moiety A and the alkyl moiety D in Table 4 are based on the literature (29). The assignments for moiety B and ring moiety C are ten-

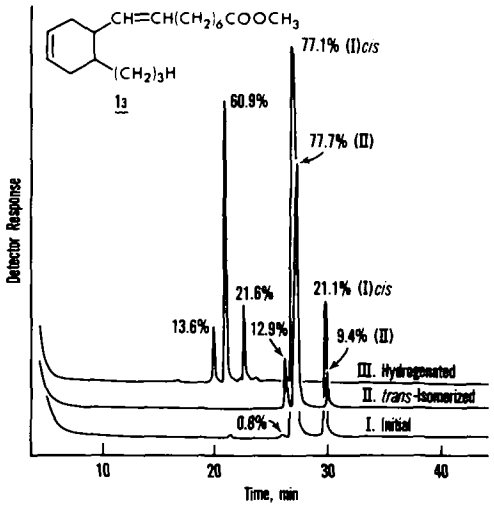


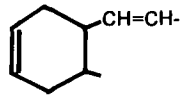
FIG. 2. Capillary GLC of cyclic fatty ester 1_3.

tative and based on comparisons with similarly substituted cyclic compounds (25,26), and our cyclohexenals 2_n. The assignments for E double bond in moieties B and C are based on its appearance after the cyclic esters were isomerized with *p*-TSIA.

GC-MS Characterization

After hydrogenation, cyclic esters 1_0, 1_1 and 1_3 showed characteristic MS fragments (Fig. 3 and Table 5) conforming to those reported in the literature (30,31). The skeletal structures of 1_0, 1_1 and 1_3 were thus confirmed. In addition to the fragments listed in Table 5, characteristic aliphatic methyl ester fragments (m/z 59, 74 and 87) and chain/ring fragments (m/z 41, 43, 55, 69, 83 and 97) were recorded with relative intensities exceeding 10% (32).

The diunsaturated cyclic esters 1_n gave more intense molecular ion (M^+) peaks than their saturated derivatives. Because of the double bond alpha to the cyclohexenyl ring in cyclic esters 1_n, the B-type fragmentation of Figure 3 was very weak or absent as would be expected between a saturated and unsaturated carbon. Otherwise, the characteristic A-D fragmentations were observed (Table 6). However, several new relatively intense fragmentations (m/z 238, 206 and 164) were characteristic of the diunsaturates 1_n. The fragment at m/z 238 and one at m/z 54 were attributed to a homolytic, retro Diels-Alder reaction, as illustrated in Figure 3, characteristic of cyclic olefins (32). Loss of CH_3OH (m/z 32) from m/z 238 would account for m/z 206. A McLafferty rearrangement on m/z 238 and resulting loss of m/z 74 would explain the m/z 164 fragment. In addition to the characteristic aliphatic methyl ester fragments and alkyl fragments, relatively intense peaks due to m/z 79-82 were noted due to fragmentation of the cyclohexenyl ring. Moderately intense peaks at m/z 107-110 were attributed to the



fragment and its rearrangements.

The cyclic esters $1_{0\text{-}4}$ and cyclohexenals $2_{2\text{-}4}$ are new compounds. The study of their selective reactions (e.g., isomerizations and hydrogenations), isolation and identification of specific geometric or stereoisomers is the subject of another paper.

EXPERIMENTAL

Materials and Methods

Commercial products included: 6-methyl-3-cyclohexene carboxaldehyde (cyclohexenal 2_1: bp, 77 C/25 mm; lit. [33] 75 C/22 mm) (K&K labs, Plainview, NY), 1,2,3,6-tetrahydrobenzaldehyde (cyclohexenal 2_0; 99%: bp, 63.5-65.5 C/23 mm; lit. [34] 58 C/17 mm), (E)-2-hexenal (3_3; 99%: bp, 54 C/23 mm; lit. [35], 50.5-51.5/20 mm), 11-bromoundecanoic acid (99+%), undecylenic acid (99%), cyclooctene (95%), triphenylphosphine (99%) and sodium methoxide (anhydrous powder) (Aldrich Chemical Co., Milwaukee, WI); (E)-2-pentenal (3_2: bp, 50-51 C/23 mm; lit. [36], 56 C/65 mm), (E)-2-heptenal (3_4: bp, 71-72 C/21 mm; lit. [37], 61-62 C/15 mm), 8-bromo-1-octene, sodium borohydride (98%) and 6-bromohexanoic acid (Alfa Products, Danvers, MA); 1,3-butadiene (99.5% min) (Matheson, East Rutherford, NJ) and bromine (J.T. Baker Chem. Co., Phillipsburg, NJ). All aldehydes were kept under N_2 and freshly distilled before use. Carboxylic acids were esterified in methanol containing conc. H_2SO_4 or HCl. The cyclic esters 1_0, 1_1 and 1_3 (ca. 50 mg) were hydrogenated with PtO_2 in 2-5% EtOH solution under ambient conditions. The cyclic esters 1_0 and 1_3 (ca. 250 mg) were isomerized with *p*-TSIA catalyst (18).

IR spectra were recorded on a Perkin-Elmer 621 spectrometer. ^{1}H-NMR spectra were determined on a Varian XL-100 Spectrometer using $CDCl_3$ as the solvent with Me_4Si as internal standard. ^{13}C-NMR was run on a Bruker WH-90 Fourier Transform spectrometer at 22.63 MHz, and $CDCl_3$served as the internal deuterium lock

TABLE 2

^{1}H-NMR Chemical Shifts (δ, ppm from TMS), in $CDCl_3$

Group proton	n = 0	n = 1	n = 2	n = 3	n = 4
			3-Cyclohexenals 2_n (Scheme 1):		
$-CH_3$	–	1.02 (m, 3)[a]	0.93 (m, 3)	0.92 (m, 3)	0.89 (m, 3)
$-(CH_2)_n-$	–	–	ca. 1.07-1.57 (m, 2)	1.37 (m, 4)	1.36 (m, 6)
-CH-CH- (a,f)	ca. 1.4-2.0 (m, 2)	ca. 1.5-2.0 (m, 2)	ca. 1.5-2.0 (m, 2)	ca. 1.5-2.0 (m, 2)	ca. 1.5-2.0 (m, 2)
$-CH_2-$ (b,e)	ca. 2.0-2.4 (m, 4)	ca. 2.0-2.4 (m, 4)	ca. 2.0-2.7 (m, 5)	ca. 2.0-2.6 (m, b)	ca. 2.0-2.6 (m, 5)
-CH=CH- (c,d)	5.71 (m, 2)	5.68 (m, 2)	5.67 (m, 2)	5.67 (m, 2)	5.66 (m, 2)
-CHO[b]	9.69 (d, 1)	9.66 (d, 0.67)[b]	9.64 (d, 0.75)[b]	9.64 (d, 0.9)[b]	9.64 (d, 1)
		9.74 (d, 0.33)[b]	9.75 (d, 0.25)[b]	9.75 (d, 0.1)[b]	
			Cyclic fatty methyl esters 1_n (Scheme 1):		
$-CH_3$	–	0.90 (m, 3)[c]	0.88 (m, 3)	0.87 (m, 3)	0.87 (m, 3)
$-(CH_2)_x$	1.29 (m, ca. 12)	1.29 (m, ca. 12)	1.32 (m, 9)	1.32 (m, ca. 10)	1.32 (m, 10)
CH-CH (a,f)	1.62 (m, ca. 4)	1.60 (m, ca. 4)	1.61 (m, 4)	1.64 (m, ca. 4)	1.64 (m, 5)
$-CH_2-$ (b,e)	2.05 (m, 4-5)	2.00 (m, 4-5)	2.00 (m, 4)	2.00 (m, ca. 4)	2.00 (m, 5)
$-CH_2COO-$	2.29 (t, 2)	2.30 (t, ca. 3)	2.30 (t, ca. 3)	2.29 (t, ca. 3)	2.29 (t, ca. 3)
CH_3O-CO-	3.65 (s, 3)	3.66 (s, 3)	3.66 (s, 3)	3.66 (s, 3)	3.66 (s, 3)
-CH=CH-	5.28 (m, 2)	5.26 (m, 2)	5.26 (m, 2)	5.26 (m, 2)	5.28 (m, 2)
-CH=CH- (c,d)	5.67 (d, 2)	5.65 (d, 2)	5.66 (m, 2)	5.64 (m,2) 5.66	5.64 (m, 2)

[a]Most likely a doublet overlapping a doublet, because a mixture of 2 ring isomers is indicated (by gas chromatography).

[b]The 2 aldehyde resonances (axial and equatorial -CHO) suggest a mixture of conformational isomers.

[c]Most likely a doublet overlapping a doublet.

TABLE 3

^{13}C-NMR Chemical Shifts (ppm, rel. TMS) for 3-Cyclohexenals 2_n (Scheme 1), in $CDCl_3$

Carbon	n = 0	n = 1[a]	n =2	n = 3	n = 4
-CHO	203.7	204.6	204.9	204.6	204.9
a	46.1	50.0 (*c*) 52.5 (*t*)	50.4	50.8	50.7
b	23.9[b]	22.8 (*c*) 23.9 (*t*)	23.3	23.3	23.2
c	125.0	124.1 (*c*) 124.6 (*t*)	124.0	124.1	124.0
d	127.3	126.0 (*c*) 126.4 (*t*)	126.3	126.3	126.3
e	24.6[b]	32.1 (*c*)[b] 32.2 (*t*)[b]	34.3	32.4	32.5
f	22.4	27.8 (*c*) 28.1 (*t*)	28.3	28.8	28.8
$-CH_2-$	–	–	26.5	36.1	33.5
$-CH_2-$	–	–	–	20.0	29.0
$-CH_2-$	–	–	–	–	22.8
$-CH_3$	–	16.2 (*c*) 19.7 (*t*)	11.2	14.2	14.0

[a]Resonances attributed to either: (*c*) = *cis*-1,6-disubstituted, -3-cyclohexene ring or (*t*) = *trans*-1,6-disubstituted-3-cyclohexene ring (cf. refs. 25,26 and text).

[b]These assignments are tentative and may be reversed.

with Me_4Si as internal reference.

Analyses by GLC were run on a Hewlett-Packard Model 5710A gas chromatograph (FID, 300 C; injection port 260 C or on-column) with a 6 ft × 1/8 in. s.s. column of 10% SP2330 (Supelco, Inc. Bellefonte, PA) on 100-120 mesh Chromosorb WAW. Methyl cyclic fatty esters, ω-hydroxyesters and ω-bromoesters were analyzed isothermally at 190 C, and the ω-aldehyde esters at 150 C. For other aldehydes, however, the column temperature was held at 80 C for 16 min and then programmed at 8 C/min to 130 C. The procedure for capillary GLC of the fatty methyl esters was described by Scholfield (19).

GC-MS was run on a Nuclide 12-90 DF mass spectrometer with 70 eV impact ionization and equipped with an all-glass jet separator (source temperature, 200 C). Output of the MS was to a Finnigan INCOS 2000 computer system repeatedly scanning masses 15-370 every 8 sec. A Bendix 2600 gas chromatograph interfaced with the MS was used with a 6 ft × 2 mm glass column packed with 3% JXR on Gas Chrom Q, 100-120 mesh. The column was held at 190 C for 4 min, then programmed at 2 C/min to 250 C, with a 20 ml/min flow of carrier gas (He); injection temperature was 210 C and detector temperature was 235 C.

Precoated plates of silica gel with fluorescent indicator, 0.25 mm thick, were used for analytical TLC. For preparative TLC, plates of silica gel, 0.50 mm thick, with fluorescent indicator but no binder, were used. The developing solvent was either 1:5 (v/v) ether/hexane for the cyclic fatty esters or 1:2 (v/v) ether/hexane for the intermediates. Developed spots were generally visualized by charring with 50% H_2SO_4 after UV detection for phenylphosphine impurities.

Methyl 10-Hydoxydecanoate

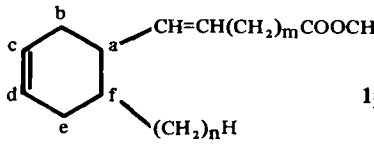
Methyl 10-undecenoate was prepared by conventional esterification of undecylenic acid. The distilled ester (99.9% by GLC) was ozonized in MeOH and the products were reduced with $NaBH_4$ (12) to give 145.3 g (68% yield) of the crude hydroxyester (93.7% by GLC), which was distilled through a Vigreux column (bp 152-156 C/11 mm; lit. [38], 154 C/17 mm). The distillate (96-97% purity by GLC) was purified further by low-temperature crystallization to yield clear, colorless liquid (99+% hydroxyester by GLC). IR (KBr neat): 3650-3100 (-OH), 1742 (ester C=O), (1250, 1197 and 1170 [Me ester C-O-C]) and 720 ($-[CH_2]_n-$) cm^{-1}.

Methyl 9-Hydroxynonanoate

Methyl oleate (99% by GLC, 40.0 g, 0.1439

TABLE 4

^{13}C-NMR Chemical Shifts (δ, ppm) for Cyclic Fatty Methyl Esters 1_n (cf. Scheme 1) in $CDCl_3$



1_n (where m+n=9; n=0 to 4)

Compound	Assignments					
A.	CH_3	OOC	CH_2	CH_2	$(CH_2)_{m-3}$	
1_{0-4}	51.3	174.2	34.2	25.0	(28.9-29.5)[a]	
B.	CH_2	CH=CH				
1_0	27.5 32.6 (E)[b]	128.9 128.6 (E)[b]	135.2 135.4 (E)[b]			
1_1	27.6	129.5	134.3			
1_2	27.5	129.2	134.4			
1_3	27.5 32.4 (E)[b]	129.2 129.8 (E)[b]	134.6 134.9 (E)[b]			
1_4	27.4	129.0	134.7			
C.	Ring Carbons:					
	a	b	c	d	e	f
1_0	32.2 36.7[b]	30.0	126.4	126.9	24.8	31.9
1_1	35.2[c] 38.9	30.0	125.4[c] 126.3	126.7[c] 130.9	32.4	–[a]
1_2	37.2	30.1	126.0	126.6	32.3	–[a]
1_3	37.5 42.8[b]	30.7	126.0 126.6[b]	126.7 129.2[b]	32.3	–[a]
1_4	37.5	30.7	125.9	126.6	32.3	–[a]
D.	CH_2	CH_2	CH_2	CH_3		
1_0	–	–	–	–		
1_1	–	–	–	17.3[c] 20.2		
1_2	–	–	26.8	11.0		
1_3	–	36.7	19.8	14.4		
1_4	37.4	–[a]	23.0	14.1		
	Unassigned resonances:					
1_1	31.3, 32.9, 33.9					
1_2	29.9, 39.2, 125.5, 126.5, 130.3					
1_3	27.7, 30.5[b], 31.1, 32.0, 125.6, 126.5, 130.6					
1_4	29.6, 31.9, 32.6, 36.8, 130.1, 130.6					

[a]Assignment to chain methylenes. Individual assignments are not possible.

[b]Resonances observed only after reaction with *p*-toluenesulfinic acid (conversion of acyclic [Z]-CH=CH to [E]-CH=CH) (18).

[c]The *cis* ring isomer (i.e., *cis*-1,6-disubstituted-3-cyclohexene) was indicated by these resonances.

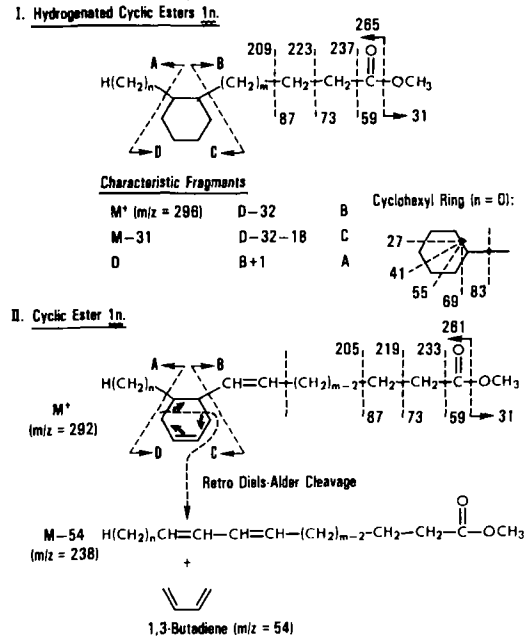


FIG. 3. Characteristic mass fragmentations of hydrogenated (I) and nonhydrogenated (II) cyclic esters 1_n (Scheme 1).

TABLE 5

GC-MS Fragmentations of Hydrogenated Cyclic Fatty Methyl Esters 1_n (Scheme 1)

Compound	Ion fragment (cf. Fig. 3):m/z (% rel. intensity)									
	M+	M-31	D	D-32	D-32-18	B+1	B	C	A	(Base)
1_0	296 (27)	265 (4)	296 (27)	264 (<3)	246 (<1)	214 (<5)	213 (<5)	83 (41)	–	74 (100)
1_1	296 (19)	265 (5)	281 (0)	249 (0)	231 (0)	200 (17)	199 (14)	97 (97)	15 N.D.	55 (100)
1_3	296 (11)	265 (3)	253 (22)	221 (17)	203 (11)	172 (16)	171 (3)	125 (43)	43 (29)	69 (100)

TABLE 6

GC-MS Fragmentations of Diunsaturated Cyclic Fatty Methyl Esters 1_n (Scheme 1)

Compound	Ion fragment (cf. Fig. 3):m/z (% rel. intensity)										
	M+	M-31	D	D-32	D-32-18	B+1	B	C	A	(Base)	M-54
1_0	292 (32)	261 (18)	292 (32)	260 (3)	242 –	212 –	211 –	81 (67)	– –	94 (100)	238 (11)
1_1	292 (46)	261 (5)	277 (1)	245 –	227 –	197 –	196 (2)	96 (31)	15 –	94 (100)	238 (45)
1_2	292 (33)	261 (9)	263 (14)	231 (13)	213 (2)	183 (1)	182 (5)	110 (32)	29 –	67/79 (100)	238 (72)
1_3	292 (16)	261 (3)	249 (5)	217 (5)	199 –	169 –	168 (3)	124 (7)	43 (29)	67 (100)	238 (38)
1_4	292 (39)	261 (3)	235 (6)	203 (6)	185 (3)	155 (1)	154 (3)	138 (3)	57 (11)	67 (100)	238 (83)

mol) was reductively ozonized (12) to yield a mixture (39.70 g) of crude hydroxyester and nonanol. Vacuum distillation (28.77 g) through a Vigreux column gave the hydroxyester (17.71 g; bp 89-95 C/0.05 mm) in 95.4% purity (<3% nonanol by GLC); lit. (39), bp 82-95 C/0.05 mm (90% purity). IR (KBr neat): 3650-3100 (-OH), 1742 (ester C=O), (1242, 1198 and 1172 [Me ester C-O-C]) and 722 (-$[CH_2]_n$-) cm^{-1}.

Methyl 8-Hydroxyoctanoate

Cyclooctene (99.0 g, 0.853 mol) was ozonized in cyclohexane (1150 g) and glacial HOAc (145 g), and the ozonide was treated with acetic anhydride and sodium acetate (14) to obtain crude 8-oxooctanoic acid, 114.9 g. The fractionally distilled aldehydic acid (bp 125-127 C/0.08 mm; 97+%), 41.3 g (0.261 mol), was selectively reduced with $NaHCO_3$ and $NaBH_4$ (15) to give 27.3 g of crude 8-hydroxyoctanoic acid as white solid. The hydroxyacid was then esterified ($CH_3OH+H_2SO_4$) and distilled to give a clear, colorless liquid (bp 93-95 C/0.22 mm; 98+%); lit. (40), bp 137-138 C/8 mm. IR (KBr neat): 3650-3100 (-OH), 1742 (ester C=O), (1250, 1200 and 1172 [Me ester C-O-C]) and 727 (-$[CH_2]_n$-) cm^{-1}.

Methyl ω-Bromoalkanoates (6_m)

11-Bromoundecanoic acid was converted to its methyl ester **69** by conventional esterification. Methyl 10-bromodecanoate (**68**), methyl 9-bromononanoate (**67**) and methyl 8-bromooctanoate (**66**) were prepared from their respective ω-hydroxyesters by bromination of the -OH group, using $Ph_3P{\cdot}Br_2$ reagent (13).

An oxidative ozonolysis procedure for olefins (17) was adapted to synthesize methyl 7-bromoheptanoate (**65**). 8-Bromo-1-octene (13.37 g, 0.070 mol) in MeOH (450 ml) was ozonized at 5-10 C; then N_2 was bubbled through the stirred solution as it warmed to room temperature (RT). After removing the MeOH, the residue was transferred with 91% formic acid (225 ml) and cooled to 15 C. Cold 30% H_2O_2 (35 ml) was added by drops to the stirred solution, which was then allowed to warm to RT and heated gradually in 3 hr to 75 C. The cooled reaction mixture was extracted with petroleum ether, washed, dried, filtered and stripped of solvent. Esterification with MeOH and H_2SO_4 gave the crude bromoester **65** (5.0 g). Short-path distillation with dimethyl sebacate as chaser gave the bromoester (bp 65-71 C/0.19 mm; lit. [41], bp 112 C/5 mm) in 92.4% purity.

Boiling points for the other methyl ω-bromoesters were (11-) 106-108 C/0.10 mm; (10-) 104-114 C/0.20 mm; (9-) 92 C/0.25 mm; and (8-) 83-84 C/0.20 mm. Literature boiling points (41): 176 C/14 mm, 165 C/12 mm, 131 C/2 mm and 124 C/6 mm, respectively.

IR (KBr, neat): 1742 (ester C=O), (ca. 1250, 1200 and 1172 [Me ester C-O-C]), 725 (-$[CH_2]_m$-), 641 (C-Br) and 560 (C-Br) cm^{-1}. A mixture of these homologous C_6-C_{11} ω-bromo-esters showed on GLC the expected linear relationship between carbon number and log of retention time.

(ω-Carbomethoxyalkyl)triphenylphosphonium Bromides (5_m)

The following procedure for 10-methoxycarbonyldecyl)triphenylphosphonium bromide (**50**) is generally representative of that used for the other phosphonium bromides (**58-5**). However, phosphonium bromides (**57-5**) could not be crystallized and were isolated as viscous, transparent gums.

A mixture of Ph_3P (82.8 g, 0.316 mol), bromoester **60** (75.4 g, 0.270 mol) and CH_3CN (300 ml) was stirred magnetically and heated under N_2. After 36 hr reflux, the solution was concentrated on a rotary evaporator and crystallized from ether after 4 repetitive extractions by kneading it in the ether (10 vol) and decanting. Final weight of **59** was 139.9 g (95.9% yield). IR (KBr disc): 1740 (ester C=O), (1248, 1190 and 1170 [Me ester C-O-C]), 725 (-$[CH_2]_9$-), 691 (C-Br) cm^{-1}.

By the same procedure, bromide **58** (144.3 g; 92% yield) was obtained from bromoester **68** (85.6 g); bromide **57** (13.60 g, 98.1% yield) from bromoester **67** (8.00 g); bromide **56** (40.1 g, 100.3% yield) from bromoester **66** (18.8 g) and bromide **55** (12.4 g, 95.0% yield) from bromoester **65** (6.00 g).

6-Alkyl-3-Cyclohexenals (2_{3-4}).

The following procedure for 6-propyl-3-cyclohexenal (**23**) from 1,3-butadiene and (E)-2-hexenal (**33**) was typical.

A 250-ml Hastelloy autoclave (rocker type) was evacuated and charged with (E)-2-hexenal (18.3 g, 0.186 mol) through the inlet tube and attached syringe needle. The autoclave was then chilled in Dry Ice/acetone and re-evacuated. The inlet tube needle was inserted through a 2-hole, crown cap and gasket into a tared pressure bottle (Lab Glass, Inc., Vineland, NJ) containing liquefied 1,3-butadiene (cooled in Dry Ice/CCl_4). The valve on the inlet tube was opened, and the butadiene (33.6 g, 0.521 mol) was transferred into the autoclave. After standing

overnight at RT, the autoclave was agitated and heated to 165 C for 5 hr. The cooled contents were transferred with ether, and the solution was concentrated on a rotary evaporator to a clear, pale-yellow liquid (39.4 g; 61% cyclohexenal, 33% hexenal, and 6% unknown byproducts by GLC). Distillation of the concentrate (28.3 g) with a Vigreux column (4.5 x 0.5 in.) afforded a main fraction 11.6 g; bp 94-97 C/13 mm; 94+% cyclohexenal 2_3 by GLC.

Similarly prepared were: 6-butyl-3-cyclohexenal (2_4, 5.21 g, bp 47-54 C/0.04 mm, 98% purity by GLC; crude yield, 43.7%) from (E)-2-heptenal; and 6-ethyl-3-cyclohexenal (2_2, 11.03 g, bp 33-40 C/0.24-0.20 mm, 96+% by GLC) from (E)-2-pentenal. Cyclohexenals 2_{2-4} are new compounds.

IR (neat) for cyclohexenals 2_n: 3025 (CH=CH), 1728 (aldehyde C=O), 1660 and 660 (Z CH-CH); except 2_0, 1650 and 652 (Z CH=CH) cm^{-1}. NMR (cf. Table 2). All commercial E-2-alkenals were freshly distilled before use and showed high E-purity by GLC and IR. Any isomerization would not be expected before cycloaddition because no thermal isomerization of E-crotonaldehyde was observed even at 240 C (27).

Methyl ω-(6-Alkyl-2-Cyclohexenyl) Alkenoates (1_n)

The preparation of methyl 9-(6-propyl-3-cyclohexenyl)-8-nonenoate (1_3) was typical of the other cyclic ester 1_n syntheses.

Phosphonium bromide 5_6 (39.5 g, 0.0791 mol) in dry DMF (100 ml) was stirred magnetically under N_2, cooled (ca. 5 C) in an ice bath, and $NaOCH_3$ (4.83 g, 0.0894 mol) was added quickly. The initially colorless solution turned orange-brown. After 45 min, a solution of cyclohexenal 2_3 (10.59 g, 0.0695 mol) in DMF (20 ml) was added by drops (ca. 10 min). The color of the reaction became light tan or cream, and the mixture was stirred overnight under N_2 after removing the ice bath. The mixture was concentrated (at 40 C/1.0-0.5 mm) on a rotary evaporator to a brown residue (56.3 g), which was slurried in ether (100 ml), filtered and concentrated. The resulting brown residue was chromatographed through neutral alumina (48 g) in hexane (100 ml) followed by ether (100 ml). GLC indicated that the hexane eluate contained mainly cyclic ester 1_3 (96+% pure; crude yield, 79.8%), and the ether eluate contained mostly cyclohexenal 2_3 (ca. 83% pure). A short-path distillation gave a clear, nearly colorless fraction (10.80 g; bp 126-133 C/0.04 mm; 98.5% 1_3 by GLC), which was still contaminated wih PH_3P according to NMR and TLC. The impurity was completely removed by preparative TLC. The saponification-esterification procedure of Bergelson et al. (17) was used to purify larger quantities of 1_3. From 3.0 g of distilled 1_3, we obtained by the saponification-esterification a clear, pale-yellow liquid (1_3, 2.12 g; 98+% by GLC), free of phenylphosphines according to TLC. Boiling points of the purified cyclic esters were: 1_0, 125-128 C/0.02 mm; 1_1, 127-132 C/0.05 mm; 1_2, 125-127 C/0.04 mm; 1_3, 125-129 C/0.05 mm; 1_4, 124-130 C/0.09 mm.

IR (KBr, neat) for cyclic esters 1_n: 3020 (CH=CH), 1748 (ester C=O), 1660 (Z CH=CH), (1250-1255, 1198 and 1172 [Me ester C-O-C]), 725 (-$[CH_2]_4$-) and 660 (Z CH=CH) cm^{-1}; except 1_0, which had 1653 and 655 cm^{-1} for Z CH=CH.

ACKNOWLEDGMENT

The authors thank E. Selke for GC-MS spectra, D. Weisleder and L.W. Tjarks for ^{13}C-NMR and ^1H-NMR spectra, J.P. Friedrich for pressure reactions and W.J. DeJarlais for seed crystals of 5_0 and its preparative procedure.

REFERENCES

1. Artman, N.R. (1969) Adv. Lipid Res. 7, 245-329.
2. Perkins, E.G. (1976) Rev. Fr. Corps Gras 23, 257-262 and 313-322.
3. Potteau, B., Dubois, P., and Rigaud, J. (1978) Ann. Technol. Agri. 27, 655-679.
4. Combe, N., Constantin, M.J., and Entressangles, B. (1981) Lipids 16, 8-14.
5. McInnes, A.G., Cooper, F.P., and MacDonald, J.A. (1961) Can. J. Chem. 39, 1906-1914.
6. Hutchinson, R.B., and Alexander, J.C. (1963) J. Org. Chem. 28, 2522-2526.
7. Michael, W.R. (1966) Lipids 1, 365-368.
8. Graille, J., Bonfand, A., Perfetti, P., and Naudet, M. (1980) Chem. Phys. Lipids 27, 23-41.
9. Holmes, H.L. (1948) in Organic Reactions (Adams, R. ed.), Vol. 4, pp. 60-173, John Wiley and Sons, New York, NY.
10. Gosney, I., and Rowley, A.G. (1980) in Organophosphorous Reagents in Organic Synthesis (Cadogan, J.I.G., ed.), pp. 17-53, Academic Press, Inc. New York, NY.
11. Bergelson, L.D., Vaver, V.A., YuKovtun, V., Senyavina, L.B., and Shemyakin, M.M. (1962) Zh. Obsshch. Khim. 32, 1802-1807; C.A. (1963) 58, 4415g.
12. Diaper, D.G.M., and Mitchell, D.L. (1960) Can. J. Chem. 38, 1976-1982.
13. Schaefer, J.P., Higgins, J.G., and Shenov, P.K. (1968) Org. Synth. 48, 51-53.
14. Siclari, F. (1974) U.S. Patent 3,856,833.
15. Siclari, F. (1980) U.S. Patent 4,192,813.
16. Ackman, R.G., Retson, M.E., Gallay, L.R., and Vandenheuvel, F.A. (1961) Can. J. Chem. 39, 1956-1963.
17. Bergelson, L.D., Vaver, V.A., Bezzubov, A.A., and Shemyakin, M.M. (1962) Zh. Obshch. Khim. 32, 1807-1811; C.A. (1963) 58, 4416c.
18. Gibson, T.W., and Strassburger, P. (1976) J. Org. Chem. 41, 791-793.

19. Scholfield, C.R. (1981) J. Am. Oil Chem. Soc. 58, 662-663.
20. MacDonald, J.A. (1956) J. Am. Oil Chem. Soc. 33, 394-496.
21. Eliel, E.L. (1962) Stereochemistry of Carbon Compounds, McGraw-Hill Book Company, Inc., New York, NY.
22. Kugatova-Shemyakina, G.P., Nikolaev, G.M., and Andreev, V.M. (1967) Tetrahedron 23, 2721-2733.
23. Kugatova-Shemyakina, G.P., and Nikolaev, G.M. (1967) Tetrahedron 23, 2987-2995.
24. Zefirov, N.S., Sergeev, N.M., and Gurvich, L.G. (1970) Dokl. Akad. Nauk SSSR 190, 345-347.
25. Johnson, L.F., and Jankowski, W.C. (1972) Carbon-13 NMR Spectra, Spectrum No. 272, John Wiley & Sons, Inc., New York, NY; and Sadtler Standard Carbon-13 NMR Indexes (1977), Vol. 5, Spectrum No. 896, Sadtler Research Laboratories, Inc., Philadelphia, PA.
26. Nakagawa, K., Sawai, M., Ishii, Y., and Ogawa, M. (1977) Bull. Chem. Soc. Jpn. 50, 2487-2488.
27. Kugatova-Shemyakina, G.P., and Nikolaev, G.M. (1967) Zh. Org. Khim. 3, 1448-1455.
28. Miyajima, S., and Inukai, T. (1972) Bull. Chem. Soc. Jpn. 45, 1553-1554.
29. Bus, J., Sies, I., and Lie Ken Jie, M.S.F. (1976) Chem. Phys. Lipids 17, 501-518.
30. Friedrich, J.P. (1967) J. Am. Oil Chem. Soc. 44, 244-248.
31. Oshima, M., and Ariga, T. (1975) J. Biol. Chem. 250, 6963-6968.
32. Silverstein, R.M., Bassler, G.C., and Morrill, T.C. (1974) Spectrometric Identification of Organic Compounds, 3rd edn., pp. 5-71, John Wiley & Sons, Inc., New York, NY.
33. Diels, O., and Alder, K. (1929) Justus Liebigs Ann. Chem. 470, 62-103.
34. Sobecki, W. (1910) Chem. Ber. 43, 1038-1041.
35. Winter, M., and Goutschi, F. (1962) Helv. Chim. Acta 45, 2567-2575.
36. Prevost, C. (1944) Bull. Soc. Chim. Fr. 11, 218-225.
37. Delaby, R., and Guillot-Allegre, S. (1931) Compt. Rend. 192, 1467-1469.
38. Lycan, W.H., and Adams, R. (1929) J. Am. Chem. Soc. 51, 625-629.
39. Pryde, E.H., Theirfelder, C.M., and Cowan, J.C. (1976) J. Am. Oil Chem. Soc. 53, 90-93.
40. Chuit, P., and Hausser, J. (1929) Helv. Chim. Acta 12, 463-492.
41. Hunsdiecker, H., and Hunsdiecker, C. (1942) Chem. Ber. 75, 291-297.

[Received December 4, 1981]

Effects of Phosphatidylcholines on de novo Synthesis and Excretion of Sterol by L-929 Fibroblasts

GEORGIOS THOMAIDIS and **CHESTER E. HOLMLUND***, *Department of Chemistry, University of Maryland, College Park, MD 20742*

ABSTRACT

The effects on [^{14}C] sterol synthesis and excretion by exposure of L-929 cells to several phosphatidylcholines (PC) has been examined. No significant effects were noted on either parameter during a 6-hr period if exposure of cells to the phospholipid preceded the addition of [1-^{14}C] acetate by just 30 min. However, if cultures were grown in media containing delipidized serum and 2×10^{-5} M PC through 2 or more subculturings prior to adding [1-^{14}C] acetate, the amount of [^{14}C] sterol increased in both cells and medium by 70-200% when saturated or monounsaturated PC were used. Dilinoleylphosphatidylcholine (18:2 PC) at the same concentration did not stimulate synthesis or excretion of newly synthesized sterol. Total cellular sterol was determined by gas chromatography, and was only marginally affected by long-term exposure to dipalmitylphosphatidylcholine (16:0 PC), whereas the total sterol of the medium increased by 4-fold over a 19-hr period. Cultures which had been exposed to 16:0 PC through 3 subculturings continued to display enhanced de novo sterol synthesis, but not excretion, for up to 5 hr after replacement with fresh medium lacking 16:0 PC. The disparity in response to 2×10^{-5} M levels of 16:0 PC and 18:2 PC may relate to differences in metabolism of the PC. Exposure to 18:2 PC resulted in about 3-fold increases in the 18:2-to-18:0 plus 18:1 ratio of cellular fatty acids, whereas relatively small changes in the cellular fatty acid composition were noted with 16:0 PC-treated cells. The results indicate that extracellular PC can promote sterol synthesis and excretion by L-929 cells, and that the magnitude of this response is influenced by the time of exposure to the phospholipid and by its fatty acid composition.

Lipids 17:427-433, 1982.

INTRODUCTION

Numerous studies have indicated that plasma lipoproteins play an important role in regulating cellular sterol synthesis and flux (1-6). Exogenous sterol may be assimilated by cells, resulting in a decrease in the rate of endogenous sterol synthesis (5,9,12,14). Conversely, it has been demonstrated that cellular sterol can be excreted into the cellular medium, and that this process is stimulated by the presence in the medium of apolipoproteins and phospholipids (5,9,12). The type of apolipoprotein and phospholipid affected the extent of sterol excretion (6). Concomitantly, endogenous sterol synthesis is stimulated as a result of the enhanced sterol excretion (5,9,12).

This study was conducted to compare the influence of saturated and unsaturated phosphatidylcholine (PC) on sterol synthesis and excretion as a function of 2 extremes in the length of time the cells were exposed to delipidized serum to which phospholipid had been added.

MATERIALS AND METHODS

Materials

Phospholipids were purchased from Supelco (Bellefonte, PA) and were 98-99% pure, with the exception of 1,2-dilinoleoyl PC (~90% pure). Coprostanol and desmosterol (~92% pure) were also from Supelco. Cholesterol (~98% pure) was purchased from Sigma (St. Louis, MO). All lipids were tested for purity by thin layer chromatography (TLC) on Whatman analytical TLC precoated plates (LK5DF Linear-I silica gel). The solvent system, hexane/diethyl ether/glacial acetic acid (70:30:1 by vol) was used for the sterols, and the system chloroform/methanol/glacial acetic acid/water (150:28:8:4) was used for the phospholipids. All tested lipids appeared as single spots without tailing.

[1-^{14}C] Acetate (2.4 mCi/mmol) and [1a,2a (n)-^{3}H] cholesterol (58 Ci/mmol) were purchased from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL), respectively.

All solvents were distilled prior to use.

Cell Cultures

A culture of L-929 cells was kindly provided by Dr. Frank Hetrick, University of Maryland, and was maintained on L-15 medium (Flow Labs, Rockville, MD) supplemented with 10% calf serum (Flow Labs), delipidized, after heating to inactivate any lecithin-cholesterol acyl transferase present (17), by the method of Cham and Knowles (18). Less than 1% of the cholesterol content of serum remained in the delipidized serum (DLS). Penicillin and streptomycin also were added to the medium at

50,000 units/ml. Cultures were grown to 60-80% confluency (about 3×10^6 cells/flask) in 25 cm^2 Corning flasks before subculturing, replacement of medium, or initiation of an experiment. PC were dissolved in ethanol, diluted with delipidized serum (DLS) to a maximal ethanol concentration of 5% (v/v), and were shaken at 37 C for 1 hr before addition to cultures. The medium for control was treated similarly, but lacked any added phospholipid. The physical state of the added phospholipid was not determined.

"Short-term" studies were conducted by rinsing monolayer cultures with 2 ml buffered salt solution (BSS) (19), followed by addition of 3 ml of fresh medium containing 10% DLS supplemented with the test lipid. Cultures were incubated for 30 min prior to addition of 4×10^{-4} M [1-^{14}C]acetate (2.4 μCi/μmol). Incorporation of label into digitonin-precipitable sterol (20) was linear for at least 4 hr and is an accurate measure of sterol synthesis in this system (19). The amount of labeled sterol in the cells and medium reflected sterol synthesis, whereas the amount in the medium represented the excreted sterol.

In the "long-term" studies, the cells were subcultured 3 times (1:2) in the presence of the PC prior to the addition of [1-^{14}C]acetate in fresh medium. Experiments were terminated after 2 hr incubation by transferring the media to other tubes and by removal of cells with a 1-min treatment with 1 ml of 0.25% trypsin (Gibco), followed by the addition of 1 ml fresh 0.25% trypsin solution and incubation with shaking for 3-4 min. The cell suspension was then diluted with 4 ml L-15 medium and the cells were pelleted by centrifugation at 1,000 x g. The pelleted cells were rinsed twice with BSS and were resuspended in 0.8 ml of 1 N NaOH.

Analyses

Extraction and determination of medium and cellular sterol was performed essentially as described by Bates and Rothblat (12). After alkaline digestion of the cells, 0.1 ml of the cell lysate was used for determination of protein (21). To the remaining cell lysate, 30-60 μl of [1a,2a(n)-^{3}H]cholesterol in hexane (2 μCi/ml) was added as an internal standard for experiments wherein de novo synthesis of sterol was to be followed, whereas 5 μg coprostanol was added to lysates which were to be analyzed for total sterol by gas liquid chromatography (GLC). Lipids were extracted from the alkali-digested cell pellet by the method of Bligh and Dyer (22) and, after saponification (23), the nonsaponifiables were either subjected to digitonin precipitation (20) subsequent to the addition of 0.4 mg cholesterol as a carrier, or were analyzed by GLC. The digitonin precipitate was washed, dissolved in methanol and counted in 5 ml scintillation fluid consisting of 2,5-diphenyloxazole (4.0 g/ℓ), bis-(0-methyl styryl)-benzene (1.1 g/ℓ), 660 ml toluene and 340 ml Cellosolve. Double label counting was effected with an Intertechnique SL30 liquid scintillation spectrometer. Losses were corrected for by the recovery of the added [^{3}H]cholesterol.

Analysis of total sterol in the nonsaponifiables was conducted with a Hewlett-Packard 5830 gas liquid chromatograph equipped with a hydrogen flame ionization detector and an electronic integrator. A 6 ft x 1/8 in. glass column packed with 100-120 mesh Gas Chrom Q, coated with 1.4% Silar 10-C, was used. The column was maintained at 250 C and an N_2 carrier gas flow rate of 22 ml/min was used. These conditions readily resolved coprostanol (internal standard) from desmosterol, the major sterol produced by L-929 cells (24), and traces of cholesterol occasionally present from the DLS.

Cellular fatty acid composition was determined by preparation of the methyl esters (25) from the dry lipid residue obtained from the Bligh and Dyer extract. The fatty acid methyl esters were resolved and quantified with the chromatograph just described containing a 6 ft x 1/8 in. glass column packed with 100-120 mesh Gas Chrom Q coated with 15% DEGS and operated at 160 C with an N_2 carrier gas flow rate of 20-22 ml/min. An internal standard of nonadecanoic acid methyl ester was included with each sample.

Analysis of medium sterol (1 ml) was performed as described by Bates and Rothblat (12), and involves essentially the same sequence of steps already outlined for cellular sterol subsequent to, and including, the addition of an internal standard and extraction of lipid by the Bligh-Dyer procedure (22).

RESULTS

Figures 1 and 2 reveal the effects of 4 PC, each at 2×10^{-5} M concentration, on the amount of de novo synthesized sterol in cells and medium over a 5-hr period. These experiments are considered to represent short-term exposure to the phospholipids, because only a 30-min preincubation to the phospholipid occurred before addition of [1-^{14}C]acetate. Although there appeared to be a decrease of incorporation of label into the sterol of cells and medium when phospholipid was present, these results are not significant at the 5% level.

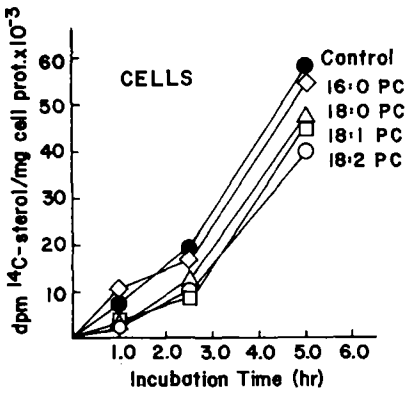


FIG. 1. Effect of "short-term" exposure to PC on [1-^{14}C] acetate incorporation into digitonin-precipitable sterol extracted from cells. Cultures were grown to 60-80% confluency on L-15 medium, supplemented with 10% delipidized calf serum. At this time, the spent medium was removed, the cell monolayer was rinsed with 2 ml BSS, and 3 ml fresh medium was added containing delipidized serum with or without the indicated PC at 2 X 10^{-5} M. After 30 min preincubation in the fresh medium, [1-^{14}C] acetate was added and the incorporation of label over a 5-hr period into digitonin-precipitable sterol obtained by extraction of cells was determined. Each point represents the mean of duplicate samples.

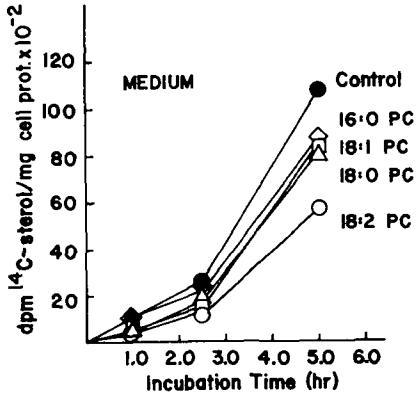


FIG. 2. Effect of "short-term" exposure to PC on the excretion of digitonin-precipitable sterols by L-929 cells. The procedure described in Fig. 1 was followed. At the indicated analysis times, the medium was removed from the cell culture and 1 ml was extracted by the Bligh-Dyer method (22) and analyzed for its content of digitonin-precipitable sterol. Each point represents the mean of duplicate samples.

However, when cells were exposed to phospholipid-containing medium for 2-3 subculturings prior to the addition of [1-^{14}C] acetate, it is evident that the presence of phospholipids caused enhanced sterol synthesis and excretion into the medium compared to the control conditions. The effect was more pronounced in the presence of PC containing saturated and monounsaturated fatty acids than with diunsaturated fatty acids (Figs. 3 and 4).

The influence of concentration of dipalmitylphosphatidylcholine (16:0 PC) on sterol synthesis and excretion is shown in Table 1. The stimulatory effect of 16:0 PC continued to increase over the concentration 7 X 10^{-6} M to 8 X 10^{-5} M. On the other hand, dilinoleylphosphatidylcholine (18:2 PC), which was comparable to the control from 2 X 10^{-5} M to 8 X 10^{-5} M, showed progressively greater stimulatory effects on synthesis and excretion of sterol as the concentration of the PC decreased to 7 X 10^{-6} M. This unexpected finding was not observed under altered conditions of incubation (Y.S. Sun and C.E. Holmlund, unpublished data), and is being explored further.

The effect of 16:0 PC on the total cellular and medium sterol content was determined by GLC, and the results are presented in Table 2.

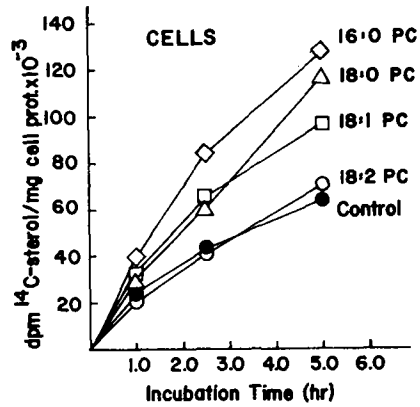


FIG. 3. Effect of "long-term" exposure to PC on [1-^{14}C] acetate incorporation into digitonin-precipitable sterols by L-929 cells. Cells were subcultured (1:2) times in the presence or absence of the added PC (2 X 10^{-5} M). After the third subculture, replacement media were added and the culture were incubated for 30 min before adding [1-^{14}C] acetate. Incorporation of label into the digitonin-precipitable sterol extracted from cells was determined over a 5-hr period. Each point represents the mean of duplicate samples.

It is evident that the medium withdrawn from cell cultures which had been exposed to 16:0 PC-containing medium had over 4 times as much sterol as medium from control cells. After addition of replacement media, sterol

analyses were subsequently made at 3, 7 and 19 hr on both cells and media. The results reveal a progressive increase in medium sterol content with time, with little change in the cellular sterol content from the control over the 19-hr period.

It was of interest to determine whether the stimulation of 16:0 PC on synthesis and excretion of newly synthesized sterol would persist after removal of 16:0 PC from the medium. Table 3 presents the results of an experiment designed to provide a response to this question. It appears that cells which have undergone a long-term exposure to 16:0 PC continue to display enhanced de novo sterol synthesis (compared to the control) irrespective of whether the 16:0 PC continues to be present in the medium for up to 5 hr after adding the replacement medium. However, the medium content of newly synthesized sterol is elevated only with cells which remain in contact with 16:0 PC-containing medium.

The influence of long-term exposure to media containing PC on cellular fatty acid composition is shown in Table 4. Only small changes were noted with cultures exposed to 16:0 PC, whereas exposure to 18:2 PC caused about a 3-fold increase in the relative cellular content of linoleic acid, a 40-50% increase in the relative amount of stearic acid, and a decrease in the relative amount of oleic acid.

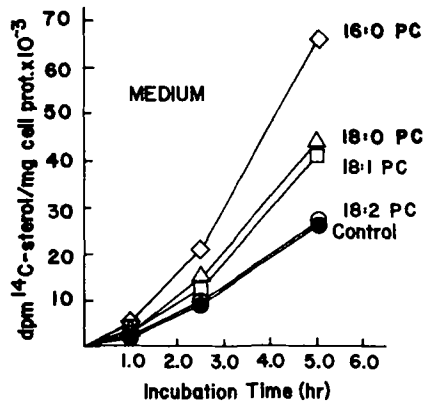


FIG. 4. Effect of "long-term" exposure to PC on the excretion of digitonin-precipitable sterols by L-929 cells as a function of time. The protocol for Fig. 3 was followed. At each time point, one ml of the medium was extracted by the Bligh-Dyer method and analyzed for labeled sterol that was precipitable by digitonin. Each point represents the mean of duplicate samples.

DISCUSSION

The results of this study indicate that the extent of the effect of external PC on de novo synthesis and excretion of sterol by L-cells grown in delipidized medium is dependent on the length of exposure of the cells to the PC-containing medium and on the concentration and fatty acid composition of the PC. Burns and Rothblat (6) found that L-cells that were grown in a medium supplemented with delipidized serum and prelabeled by growth in medium containing [2-^{14}C]acetate excreted sterol during a 6-hr incubation period. This process was stimulated by either delipidized calf serum, egg lecithin or whole calf serum. The combination of egg lecithin and delipidized calf serum promoted more release of sterol than was observed with either component alone. In the same study (6), Burns and Rothblat also used L-51784 cells which were prelabeled by exposure to [4-^{14}C]cholesterol by overnight

TABLE 1

"Long-Term" Effects on Sterol Metabolism by L-929 Cells as a Function of 16:0 PC Concentration

Treatment	16:0 PC concentration (M)	dpm [^{14}C]Sterol/mg cell protein/2 hr ± SD[a]	
		Cells[b]	Medium[b]
Control	–	39587 ± 1507	2062 ± 14
16:0 PC	7×10^{-6}	60756 ± 756	3966 ± 198
	2×10^{-5}	70130 ± 2208	4634 ± 10
	4×10^{-5}	76976 ± 940	6133 ± 428
	8×10^{-5}	90290 ± 2787	8311 ± 166

[a]Two samples were analyzed for each treatment.

[b]Cells were subcultured (1:2) 3 times in the presence of the indicated concentration of 16:0 PC. After the third subculture, replacement media were added with the above concentration of 16:0 PC and the cultures were incubated for 30 min before adding [1-^{14}C]acetate.

TABLE 2

Effect of "Long-Term" Exposure to 16:0 PC on Sterol Cellular Content and Excretion into the Medium

Treatment[a]	Incubation time (hr)[b]	Sterol (μg)/mg cell prot. ± SD[c]	
		Cells	Medium
Control	0	–	3.20 ± 0.78
	3	14.34 ± 0.08	2.04 ± 0.26
	7	16.04 ± 0.23	3.00 ± 0.00
	19	14.28 ± 0.36	2.90 ± 0.13
16:0 PC	0	–	12.95 ± 0.31[d]
	3	12.33 ± 0.41[d]	4.79 ± 0.59[d]
	7	14.00 ± 0.40[d]	8.55[e]
	19	16.60 ± 0.28[d]	14.16 ± 1.19[d]

[a]Cells were subcultured (1:2) 3 times in the presence of 16:0 PC (2×10^{-5} M). After the third subculture, replacement media were added and the cultures were incubated from 3 to 19 hr. Sterols were extracted and quantified by GLC.

[b]The 0-hr values represent the amount of sterol excreted between the initiation of the third subculture and addition of replacement medium (a period of 36 hr).

[c]Two samples were analyzed for each treatment.

[d]Statistically significant ($p < 0.05$).

[e]Missing value.

TABLE 3

Persistence of the Effect of 16:0 PC on Sterol Metabolism in L-929 Cells

Treatment[a]			dpm [^{14}C] Sterol/mg cell prot./2 hr ± SD[b]	
Three subcultures	Replacement medium–addition of 16:0 PC	Incubation time (hr)	Cells[c]	Medium
Control	No	2.5	20478 ± 229	776 ± 5
		5	21380 ± 768	674 ± 58
		24	7640 ± 577	854 ± 28
16:0 PC	Yes	2.5	37156 ± 388	1646 ± 385
		5	37566 ± 921	1868 ± 704
		24	2366 ± 323	3250 ± 1772
16:0 PC	No	2.5	31889 ± 847	842 ± 54
		5	36450 ± 1002	994 ± 9
		24	1823 ± 24	682 ± 42

[a]Cells were subcultured 3 times in the presence (2×10^{-5} M) or absence of 16:0 PC. After the third subculture, replacement media (with or without 16:0 PC) were added and the cultures were incubated for 0.5 to 22 hr before adding [1-^{14}C] acetate.

[b]Two replicates were analyzed for each treatment.

[c]All the lipid treatments were statistically significant ($p < 0.05$).

growth in 5% heat-inactivated fetal bovine serum containing the [4-^{14}C] cholesterol. Such cells also readily released the prelabeled sterol upon exposure to delipidized serum or to mixtures of delipidized serum and phospholipid. The apparent difference between our results and those of Burns and Rothblat concerning the time of exposure of PC-containing medium required before enhanced sterol excretion is observed may indicate the involvement of different sterol pools. Our study followed the fate of newly synthesized sterol, whereas Burns and Rothblat measured the excretion of prelabeled sterols.

More recently (Y.S. Sun and C.E. Holmlund, unpublished data), we have followed by GLC the effect of 16:0 PC on excretion of cellular sterol as a function of time and have observed

TABLE 4

Effects of "Long-Term" Exposure to 16:0 PC and 18:2 PC on Cellular Total Fatty Acid Composition

Treatment[a]	Fatty acid					
	14:0	16:0	16:1	18:0	18:1	18:2
Control-						
% ± SD	2.15 ± 0.37	20.69 ± 0.69	4.72 ± 0.42	24.40 ± 0.27	43.26 ± 1.22	4.76 ± 0.02
16:0 PC-						
% ± SD	1.37 ± 0.01	18.70 ± 0.01	3.38 ± 0.11[b]	27.41 ± 0.39[b]	45.20 ± 0.10	3.94 ± 0.49
18:2 PC-						
% ± SD	1.80 ± 0.28	19.26 ± 0.36	1.23 ± 0.07[b]	35.96 ± 0.16[b]	27.84 ± 0.15[b]	13.90 ± 0.26[b]

[a]Cells were subcultured (1:2) 3 times in the presence or absence of the added PC at 2×10^{-5} M. After the third subculture, the cultures were allowed to grow to 50-60% confluency, and the cellular fatty acids were analyzed by GLC. Duplicate samples were analyzed for each treatment.

[b]Statistically significant ($p < 0.05$).

a significant increase after just 30 min exposure to 16:0 PC. It appears, therefore, that, although enhanced sterol excretion is rapidly initiated by exposure to a medium containing 16:0 PC in addition to delipidized serum, stimulation of sterol synthesis and of the excretion of newly synthesized sterol requires several hours' exposure to the 16:0 PC-containing medium. This conclusion is supported by the work of Edwards et al. (26), who found that cholesterol losses from rat hepatocytes as a result of exposure to media containing albumin and egg lecithin were followed by increases in β-hydroxy-β-methylglutaryl CoA reductase (HMG-CoA reductase). In each instance, sterol loss preceded the increase in enzyme activity. Because HMG-CoA reductase is considered to be the rate-limiting enzyme in sterol synthesis (27), increases in its activity can be presumed to lead to increases in the rate of sterol synthesis.

From the data of Figures 3 and 4, together with the analyses of total cellular and medium sterol shown in Table 2, it appears that the addition of 16:0 PC to medium containing delipidized serum stimulates the excretion and synthesis of sterol by L-cells in such a fashion as to maintain a fairly constant level of cellular sterol, even though sterol excretion continues over a 19-hr period. However, the data of Table 3 suggest that the stimulatory effect of external 16:0 PC on the rate of de novo synthesis by L-cells continues for at least an additional 5 hr after replacement of 16:0 PC-containing medium with fresh medium lacking the phospholipid. Under these circumstances, the enhanced rate of synthesis occurs in the absence of any increase in sterol secretion. This finding is also supported by Edwards et al. (26). They exposed hepatocytes to varying concentrations of lecithin for 45 min, after which the cells were washed and resuspended in fresh standard medium for an additional 2.25 hr. It was found that, although the sterol excretion rate dropped to control levels after removal of lecithin from the medium, the level of HMG-CoA reductase continued to rise.

Burns and Rothblat (6) compared the effect of several phospholipids on cholesterol excretion by L-51784 cells. Excretion was dependent on the concentration and type of phospholipid mixed with the delipidized serum. Egg PC was more effective in causing excretion of sterol than was egg phosphatidylethanolamine when both were compared at equivalent μg/ml concentrations. No attention was given, however, to possible affects of differences in fatty acyl composition of the phospholipids. Jackson et al. (13) have reported on the removal of cellular lipids from Lanschutz ascites cells by mixtures of various PC and apoproteins, and by isolated PC-apoprotein complexes. In agreement with our observations, they found that, in the mixture experiments, the disaturated PC caused a greater removal of cholesterol than did the mono- or diunsaturated phospholipids. Our finding (Table 4) that the fatty acid composition of the cells is more drastically altered upon exposure to 18:2 PC than to 16:0 PC probably bears on the different degree of sterol excretion caused by these PC. Because we do not have quantitative information regarding whether either 16:0 PC or 18:2 PC is taken up by the cells as the initial phospholipid or merely undergoes fatty acyl group exchange processes, we cannot distinguish between explanations which invoke differences in membrane fluidity or differences in extracellular apolipoprotein-phospholipid composition. Such studies are now in progress.

Two mechanisms have been proposed to

account for the transfer of cellular sterol to medium constituents. One involves a collision mechansim and has been discussed by Gurd (28). The other, first suggested by Hagerman and Gould (29), postulates the formation of a water-soluble intermediate. Phillips et al. (30) and Backer and Davidowicz (31) have convincingly demonstrated that cholesterol exchange between phospholipid vesicles does not require a collision process between vesicles. In addition, Phillips et al. (30) studied cholesterol exchange in cell-vesicle systems, and proposed a model which involves desorption of cholesterol from the cell membrane as the rate-limiting step followed by collision with acceptor vesicles. An additional factor suggested by these authors is diffusion of the desorbed cholesterol through an unstirred water layer surrounding cells which adhere as a monolayer. Interaction between acceptor and cell would influence the penetration of the acceptor into the unstirred water layer and thus influence the rate of cholesterol transfer. The fatty acid composition of the phospholipid compound of the acceptor may affect the extent to which this process occurs.

Bartholow and Geyer (J. Biol. Chem. [1982] 257, 3126-3130) have reported that the synergistic effect of saturated PC on sterol release in the presence of human serum albumin was greater than that of unsaturated PC.

ACKNOWLEDGMENTS

We wish to extend our gratitude for the support of the research by Biomedical Research Support Grant no. RR-07042 to the University of Maryland from the Division of Research Resources, National Institutes of Health, Public Health Services; by the University of Maryland Chapter of Sigma Xi; and by the Specific Cooperative Agreement no. 58-3204-9-101 between the U.S. Department of Agriculture and the Department of Chemistry, University of Maryland, College Park.

REFERENCES

1. Bailey, J.M. (1961) Proc. Soc. Exp. Biol. Med. 107, 30-35.
2. Bailey, J.M. (1964) Am. J. Physiol. 207, 1221-1225.
3. Bailey, J.M. (1965) Exp. Cell Res. 37, 175-182.
4. Bailey, J.M. (1967) Wistar Inst. Symp. Monogr. 6, 85-109.
5. Rothblat, G.H., and Kritchevsky, D. (1968) Exp. Mol. Pathol. 8, 314-329.
6. Burns, C.H., and Rothblat, G.H. (1969) Biochim. Biophys. Acta 176, 616-625.
7. Werb, Z., and Cohn, Z.A. (1971) J. Exp. Med. 134, 1545-1569.
8. Williams, C.D., and Avigan, J. (1972) Biochim. Biophys. Acta 260, 413-423.
9. Bailey, J.M., Howard, B.V., Dunbar, L.M., and Tillman, S.F. (1972) Lipids 7, 125-134.
10. Brown, M., Dana, S., and Goldstein, J. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2162-2166.
11. Stein, Y., and Stein, O. (1972) in Ciba Foundation Symposium 12, Atherogenesis: Initiating Factors, pp. 165-183, Elsevier, Exerpta Medica, North-Holland, Amsterdam.
12. Bates, S.A., and Rothblat, G.H. (1974) Biochim. Biophys. Acta 360, 38-55.
13. Jackson, R.L., Gotto, A.M., Stein, O., and Stein, Y. (1975) J. Biol. Chem. 250, 7204-7209.
14. Goldstein, J.L., and Brown, M.S. (1977) Ann. Rev. Biochem. 46, 897-930.
15. Chen, R.M., and Fischer-Dzoga, K. (1977) Atherosclerosis 28, 339-353.
16. Henriksen, T., Evensen, S.A., Blomhoff, J.P., Torsvik, H., and Carlander, B. (1979) Biochim. Biophys. Acta 574, 312-320.
17. Federoff, S. (1958) Tex. Rep. Biol. Med. 16, 31-47.
18. Cham, B.E., and Knowles, B.R. (1976) J. Lipid Res. 17, 176-181.
19. Bates, S., and Rothblat, G. (1972) J. Virol. 9, 883-890.
20. Sperry, W.M., and Webb, M. (1950) J. Biol. Chem. 187, 97-106.
21. Lowry, O.H., Rosebrough, N., Farr, A., and Randall, R. (1951) J. Biol. Chem. 193, 265-275.
22. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. 37, 911-917.
23. Sokoloff, L., and Rothblat, G.H. (1972) Biochim. Biophys. Acta 280, 172-181.
24. Rothblat, G.H., Burns, C.H., Conner, R.L., and Landrey, J.R. (1970) Science 169, 880-882.
25. Stoffel, W., Chu, F., and Ahrens, E.H. (1959) Anal. Chem. 31, 307-308.
26. Edwards, P.A., Fogelman, A.M., and Popjak, G. (1976) Biochem. Biophys. Res. Commun. 68, 64-69.
27. White, L.W., and Rudney, H. (1970) Biochemistry 9, 2725-2731.
28. Gurd, S.N. (1976) in Lipid Chemistry (Hanahan, D.J., ed.) p. 283, John Wiley & Sons, New York, NY.
29. Hagerman, J.S., and Gould, R.G. (1951) Proc. Soc. Exp. Biol. Med. 78, 329-332.
30. Phillips, M.C., McLean, L.R., Stoudt, G.W., and Rothblat, G.H. (1980) Atherosclerosis 36, 409-422.
31. Backer, J.M., and Davidowicz, E.A. (1981) Biochemistry 20, 3805-3810.

[Received August 17, 1981]

Development of the Diurnal Rhythm of Chick 3-Hydroxy-3-methylglutaryl-CoA Reductase

H. RAMIREZ, M.J. ALEJANDRE and **E. GARCIA-PEREGRIN***, *Department of Biochemistry, University of Granada, Granada, Spain*

ABSTRACT

Chick liver and intestine 3-hydroxy-3-methylglutaryl-CoA reductase did not show diurnal rhythm at hatching. Differences in activity between light and dark periods appeared during the first week and remained more or less constant between 10-14 days after hatching. Hepatic and intestinal reductase activities were maximal during the light period and minimal during the dark period. Amplitude of the rhythm was practically similar in both tissues (about 3-fold) although specific activities and differences between peak and nadir values were always higher in liver. Chick brain reductase did not show significant diurnal variations in the age range assayed.

Lipids 17:434-436, 1982.

INTRODUCTION

Neonatal manipulation of cholesterol metabolism has recently been postulated as a way of improving cholesterol handling capacity during the adult life (1). Reiser and Sidelman (2) proposed a hypothesis suggesting that cholesterol intake during the suckling period could determine the developmental feature of enzymes concerned with cholesterol synthesis and degradation.

The perinatal development of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34) has been studied in mouse brain (3), rat liver (4) and rat lung, liver and brain (5). Recently (6), we have studied the developmental pattern of the enzyme in liver, intestine and brain of neonatal chicks, showing that hepatic reductase sharply increased between 5 and 9 days after hatching, whereas intestinal reductase increased immediately after hatching and brain reductase did not change within the age assayed (1-15 days).

The rate of cholesterol biosynthesis in rat liver has been shown to possess a diurnal rhythm (7). Similarly, cholesterol synthesis from acetate in intestinal mucosa of the rat has a diurnal rhythm that parallels that of the liver, but has a lower amplitude (8). These rhythmic changes are associated with changes in the amount of HMG-CoA reductase, the rate-limiting enzyme for sterol synthesis in developing (9) as well as in adult rat (10). The existence of the diurnal rhythm in HMG-CoA reductase from different rodent tissues has been demonstrated in many laboratories (11). However, the developmental pattern of these rhythmic changes has been less studied and their existence in other species has not yet been properly established.

In view of these considerations, we have studied the existence of diurnal variations in different neonatal chick tissues and the changes in the amplitude of these variations throughout postnatal development.

MATERIALS AND METHODS

Newborn white Leghorn male chicks were obtained from a commercial hatchery and maintained fed ad libitum on a commercial diet in a chamber with a light cycle from 0700 to 1900 hr and controlled temperature. The chicks were killed by decapitation and tissues rapidly removed, weighed, minced and then homogenized with a motor-driven, all-glass Potter-Elvehjem homogenizer in 3 vol of 50 mM phosphate buffer, pH 7.4, containing 30 mM EDTA, 250 mM NaCl and 1 mM DTT. Microsomes were obtained as previously described (12). Protein concentration was determined by the method of Lowry et al. (13), using albumin as a standard.

HMG-CoA reductase activity was measured essentially as described by Shapiro et al. (14). This method measured the formation of radioactive mevalonate from [^{14}C]HMG-CoA using [^{3}H]mevalonate as an internal standard. Reductase activity was expressed as pmol of mevalonic acid (MVA) synthesized/min/mg protein.

RESULTS

One- to 14-day-old chicks were killed at the middle of dark and light periods (0100 and 1300 hr, respectively). Liver, brain and intestine were assayed for HMG-CoA reductase activity. The reductase from 1-day-old chicks did not exhibit significant variations (Table 1). However, some differences were observed between 0100 and 1300 hr in hepatic and intestinal reductase activity from 4 days on. The magnitude of these differences increased during the first week after hatching, remaining more or

TABLE 1

Diurnal Differences of HMG-CoA Reductase from Neonatal Chick Liver, Brain and Intestine[a]

	Specific activity (pmol/min/mg protein)					
	Liver		Brain		Intestine	
Age (days)	Time of day 0100	1300	Time of day 0100	1300	Time of day 0100	1300
1	20.9 ± 3.8	22.5 ± 1.7	398.3 ± 29.5	348.6 ± 51.0	14.6 ± 2.4	15.3 ± 0.5
4	14.7 ± 1.3	23.4 ± 0.5	354.6 ± 31.2	332.4 ± 8.8	24.8 ± 8.8	50.3 ± 7.2
7	121.1 ± 3.0	426.4 ± 19.5	308.5 ± 18.7	326.1 ± 3.9	118.4 ± 2.4	145.6 ± 4.3
10	527.5 ± 4.1	1064.7 ± 14.3	390.5 ± 9.8	296.5 ± 49.5	176.3 ± 3.7	264.0 ± 5.9
14	577.2 ± 12.5	1152.7 ± 36.3	328.2 ± 17.6	311.0 ± 24.4	101.5 ± 22.7	173.7 ± 4.4

[a]Microsomal HMG-CoA reductase activity was measured at the indicated time. Results are given as means ± SEM of 4 experiments with pools of 5 animals.

less constant between 10 and 14 days. No significant diurnal differences were observed in brain reductase in the age range assayed.

In order to pinpoint the diurnal variations in the neonatal chick HMG-CoA reductase, liver, brain and intestine of 9-day-old chicks fed ad libitum were assayed for reductase activity at 3-hr intervals. Figure 1 shows the diurnal rhythm of the microsomal reductase of liver and intestine. In both tissues, maximal HMG-CoA reductase was observed ca. 5-6 hr after the start of the light period. The amplitude of the rhythm was similar in both tissues (about 3-fold), although specific activities and differences between peak and nadir values were always higher in liver. On the other hand, in intestine, a slight increase seemed to appear between 3 and 6 hr after the start of the dark period, although its magnitude was smaller than that observed during the light period.

Figure 1 also shows that HMG-CoA reductase from 9-day-old chick brain did not show significant diurnal variations in its activity. The levels of cerebral activity were always higher than those obtained in intestine and were similar to those found in the dark period for the hepatic reductase.

Age-related changes of the microsomal reductase activity in the whole liver were studied at the middle of dark and light periods. Both total microsomal activity and differences in this activity between peak and nadir increased between 4 and 14 days (Fig. 2). The peak total activity 14 days after hatching was about 90-fold that obtained at 4 days whereas peak specific activity was about 50-fold.

Table 2 shows that microsomal activity in the whole brain, measured at the middle of

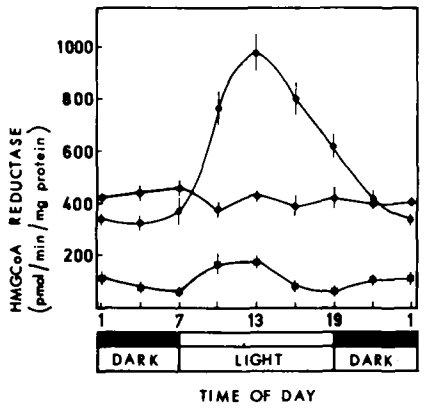


FIG. 1. Diurnal variations of microsomal HMG-CoA reductase of 9-day-old chick liver (•), brain (♦) and intestine (■). Each point represents the mean of 6 experiments ± SEM.

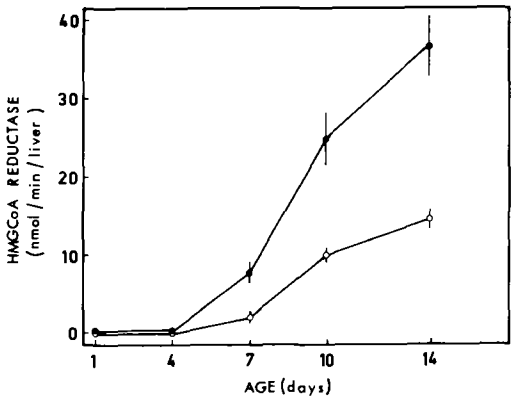


FIG. 2. Microsomal HMG-CoA reductase activity per liver at 1300 hr (•) and 0100 hr (○) as a function of age. Each point represents the mean of 4 experiments ± SEM.

TABLE 2

HMG-CoA Reductase Activity in the Whole Chick Brain as a Function of Age[a]

Age (days)	Total activity (pmol/min/brain) 0100	1300
1	756.7 ± 63.2	631.9 ± 125.1
4	780.1 ± 81.5	687.7 ± 69.7
7	660.2 ± 51.3	770.7 ± 93.3
10	741.9 ± 29.7	768.4 ± 89.5
14	600.7 ± 77.0	726.2 ± 53.5

[a]Reductase activity was measured at the indicated time. Results are given as means ± SEM of 4 experiments with pools of 5 animals.

dark and light periods, did not change with age. Comparison of data in Table 2 and those in Figure 2 also shows that the microsomal activity in the whole organ was higher in brain than in liver only during the first days after hatching, and was clearly lower afterwards.

DISCUSSION

Exogenous factors, such as lighting period and food intake time, should be considered as synchronizing agents of the rhythm, although the food intake parameter, normally influenced by the lighting, is the main exogenous synchronizer of the diurnal rhythm of liver HMG-CoA reductase (15,16). In rat, this rhythm in the hepatic activity was detected as early as 6 days of age, but was inverted relative to that of the adult, perhaps reflecting the fact that suckling pups ingest most of their food during the day (4).

No information is available on the existence of a similar rhythm in nonmammalian species. Variations observed in chick liver HMG-CoA reductase as a function of time of day are in agreement with feeding conditions. Chicks feed mainly during daytime, whereas rats are reluctant to feed during the day. Thus, reductase activity in chick liver was minimal during the dark phase, in contrast to the results reported in rat liver for which reductase activity was maximal during the dark period and minimal during the light period (10,17). However, during the first days after hatching, energy in the chick is supplied by both the diet and the yolk sac, for which regression occurs at about 5 days of age. These facts may explain the developmental pattern of diurnal rhythm in the hepatic reductase.

Likewise, diurnal variations of HMG-CoA reductase in chick intestine coincided with the diurnal rhythm of the hepatic reductase. However, differences between peak and nadir activities of intestinal reductase were found already before the regression of yolk sac. On the other hand, the small increase found in the dark period in intestinal reductase may be also related to feeding. In fact, the food content was large in the chick crop at the beginning of the dark period and disappeared during the first hours of this phase, so that the food intake from the crop may be responsible for this small increase in the intestinal HMG-CoA reductase. The absence of rhythm in brain reductase agrees with the lack of response in brain to the dietary factors that operate in other tissues.

ACKNOWLEDGMENTS

This work was supported in part by a Grant from the "Comisión Asesora de Investigación Científica y Técnica" (4089-79). The authors gratefully acknowledge the technical assistance of D. Franco. We thank Avigrana S.A. for the generous supply of newborn male chicks.

REFERENCES

1. Subbiah, M.T.R. (1979) Artery 6, 82-88.
2. Reiser, R., and Sidelman, Z. (1972) J. Nutr. 102, 1009-1016.
3. Kandutsch, A.A., and Saucier, S.E. (1969) Arch. Biochem. Biophys. 135, 201-208.
4. McNamara, D.J., Quackenbush, F.W., and Rodwell, V.W. (1972) J. Biol. Chem. 247, 5805-5810.
5. Ness, G.C., Miller, J.P., Moffler, M.H., Woods, L.S., and Harris, H.B. (1979) Lipids 14, 447-450.
6. Alejandre, M.J., Ramirez, H., Suarez, M.D., and Garcia-Peregrin, E. (1981) Biol. Neonate 40, 232-236.
7. Kandutsch, A.A., and Saucier, S.E. (1969) J. Biol. Chem. 244, 2299-2305.
8. Edwards, P.A., Muroya, H., and Gould, R.G. (1972) J. Lipid Res. 13, 369-401.
9. Wrobel, J., Boguslawski, W., Michalska, L., and Niemiro, R. (1973) Int. J. Biochem. 4, 565-568.
10. Shapiro, D.J., and Rodwell, V.W. (1971) J. Biol. Chem. 246, 3210-3216.
11. Rodwell, V.W., Nordstrom, J.L., and Mitschelen, J.J. (1976) Adv. Lipid Res. 14, 1-74.
12. Ramirez, H., Alejandre, M.J., Segovia, J.L. and Garcia-Peregrin, E. (1981) Lipids 16, 552-554.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
14. Shapiro, D.J., Nordstrom, J.L., Mitschelen, J.J., Rodwell, V.W., and Schimke, R.T. (1974) Biochim. Biophys. Acta 370, 369-377.
15. Dugan, R.E., Slakey, L.L., Briedis, A.V., and Porter, J.W. (1972) Arch. Biochem. Biophys. 152, 21-27.
16. Gregory, K.W., Smith, C.Z., and Booth, R. (1972) Biochem. J. 130, 1163-1165.
17. Hamprecht, B., Nüssler, C., and Lynen, F. (1969) FEBS Lett. 4, 117-121.

[Received November 12, 1981]

Positional Distribution of Fatty Acids in Triglycerides from Milk of Several Species of Mammals

PETER W. PARODI, *Fats Research Laboratory, The Butter Marketing Board, Hamilton Central, 4007, Queensland, Australia*

ABSTRACT

Milk triglycerides from the echidna, koala, Tammar wallaby, guinea pig, dog, cat, Weddell seal, horse, pig and cow were subjected to fatty acid and stereospecific analysis to determine the positional distribution of the fatty acids in the triglycerides. The samples presented a wide range of fatty acids, most of which varied in content among species. The compositions of the acids at the 3 positions also varied among species, reflecting the content of these acids in the triglycerides. However, there was a general similarity in fatty acid positional distribution patterns for all the species with the exception of the echidna. The echidna exhibited a completely different fatty acid positional distribution pattern. The saturated acids were preferentially esterified at the *sn*-1-position whereas the unsaturated acids were selectively esterified at the *sn*-2-position. The triglyceride carbon number distribution of milk from the above species (with the exception of the Weddell seal) was determined by gas liquid chromatography and compared to that predicted by the 1-random-2-random-3-random fatty acid distribution hypothesis. Agreement was excellent between observed and predicted composition for echidna, koala, Tammar wallaby, guinea pig and pig milk, and agreement was reasonable for dog, cat, horse and cow milk. Results are discussed in relation to biochemical mechanisms.
Lipids 17:437-442, 1982.

Milk triglycerides are synthesized from fatty acids derived from plasma triglycerides (TG) and from de novo synthesis in the mammary gland (1). While the *sn*-glycerol-3-phosphate pathway is considered to be the major synthetic pathway, others, such as the monoglyceride pathway, may also make a significant contribution (2). The positional distribution of fatty acids in milk triglycerides has been determined for the cow (3), sheep (4), goat (4), human (5), pig (6) and rat (7). These studies indicate that milk triglycerides are asymmetrical with the short-chain fatty acids preferentially esterified at the *sn*-3-position.

To determine if the positional distribution of fatty acids in milk triglycerides from all species followed a uniform pattern, 8 other species, representing 5 different orders, were studied. The pig and cow, species which have been examined previously, were included in the study. The TG carbon number distribution of the milks was compared with that calculated from the 1-random-2-random-3-random fatty acid distribution hypothesis.

MATERIALS AND METHODS

Samples

Milk samples were obtained from the koala (*Phascolarctus vinereus*), guinea pig (*Cavia porcellus*), dog (*Canis familiaris*), cat (*Felis domesticus*), Weddell seal (*Leptonychotes weddelli*), horse (*Equus caballus*), pig (*Sus scrofa*) and cow (*Bos taurus*). Lipids were extracted with diethyl ether and petroleum ether (boiling range 30-60 C) by the Roese-Gottlieb method (8). Milk lipids from the echidna (*Tachyglossus aculeatus*) and Tammar wallaby (*Macropus eugenii*) were extracted from milk using chloroform and methanol by CSIRO, Division of Wildlife Research. Samples were stored under nitrogen at -20 C until they were required for analysis. Triglycerides were obtained from milk lipid by column chromatography using 7% hydrated Florisil (9).

Stereospecific Analysis

The *sn*-1,2(2,3)-diglyceride method of Brockerhoff (10), adapted for mg quantities by Christie and Moore (11) was used with modification. This method, together with the pancreatic lipase deacylation procedure used to obtain monoglycerides, was reported by Parodi (12). In the current study, diglycerides for stereospecific analysis were generated by the Grignard reagent, ethyl magnesium bromide. In the case of cow milk, diglycerides were obtained using a pancreatic lipase deacylation. A sample of interesterified cow milk fat was used to obtain optimal conditions for pancreatic lipase deacylation. Results for the *sn*-1-position were obtained by analysis of the lysophosphatide, those for the *sn*-2-position were obtained from monoglycerides by pancreatic lipase deacylation and those for the *sn*-3-position were calculated by difference from the known triglyceride composition. The composition of the *sn*-2,3-diacyl-1-phosphatidyl phenols pro-

vided a check for the *sn*-3-position.

Fatty Acid and Triglyceride Analysis

Triglycerides and partial glycerides were transesterified to methyl esters (13). Phospholipids were transesterified by the addition of 5 μl of 2.0 N methanolic sodium methoxide and 50 μl of hexane. Methyl esters and TG carbon number distribution were analyzed by gas liquid chromatography (GLC) as previously reported (12,14).

RESULTS

Triglyceride fatty acid composition and the positional distribution of the fatty acids in the triglycerides from the milk of the 10 species of mammals is given in Table 1. The milk that was studied presented a wide range of fatty acids, most of which varied in content among species. The compositions of the acids at the 3 positions also varied among species, reflecting the content of these acids in the triglycerides. However, there was a general similarity in fatty acid positional distribution patterns for all the species with the exception of the echidna. The 4:0 and 6:0 acids were exclusively esterified at the *sn*-3-position whereas 8:0 was preferentially esterified at this position. For the horse, 10:0 was selectively associated with the *sn*-3-position buf, in the cow, there was slightly more of this acid at the *sn*-2-position than at the *sn*-3-position. The 12:0, 14:0 and 16:0 acids were preferentially associated with the *sn*-2-position except in the cow, where there was a little more 16:0 at the *sn*-1-position than at the *sn*-2-position. In all species, 18:0 was selectively esterified at the *sn*-1-position. For the seal and horse, 18:1 was preferentially associated with the *sn*-1-position but, for the other species, it was preferentially associated with the *sn*-3-position. The 18:2 and 18:3 acids were always preferentially esterified at the *sn*-3-position.

The echidna exhibited a completely different fatty acid positional distribution pattern. The 14:0, 16:0 and 18:0 acids were preferentially esterified at the *sn*-1-position whereas the unsaturated acids 18:1, 18:2 and 18:3 were selectively esterified at the *sn*-2-position.

A computer program was devised to calculate the amounts of the triglycerides predicted by the 1-random-2-random-3-random fatty acid distribution hypothesis. The program also allowed for the predicted triglycerides to be summed into groups according to their carbon number. Data from the stereospecific analysis of echidna, koala, Tammar wallaby, guinea pig, dog, cat, horse, pig and cow milk triglycerides were used to generate the TG carbon number distribution predicted by the 1-random-2-random-3-random fatty acid distribution hypothesis. This was compared to carbon number distribution determined experimentally by GLC. The difference between the observed and calculated composition for each carbon number distribution was determined. As the specificity in utilization of particular fatty acids in certain triglycerides must be balanced by discrimination in others, the sum of either the positive or negative differences (D mol %) was used to test deviation from the 1-random-2-random-3-random fatty acid distribution hypothesis.

Results for the 9 species of mammals are presented in Table 2. Agreement was excellent between TG carbon number distribution, calculated by the 1-random-2-random-3-random fatty acid distribution hypothesis, and that determined experimentally for echidna, koala, Tammar wallaby, guinea pig and pig milk. For these animals, the value of D was less than 5 mol %. It was difficult to assess the effect of experimental error from TG carbon number distribution and stereospecific analyses on the value of D. A subjective estimate considered that D values above 5 mol % may indicate deviation from the distribution hypothesis. Dog, cat, horse and cow milk had D values between 5 and 7.5 mol % and it may be considered that there was reasonable agreement for TG carbon number distribution determined experimentally and that calculated from the 1-random-2-random-3-random hypothesis.

The major triglycerides in most milk samples were C 50, C 52 and C 54. In general, C 54 was present in amounts greater than that predicted by the fatty acid distribution hypothesis whereas the C 52 and C 50 triglycerides occurred in less-than-predicted amounts.

DISCUSSION

The species of mammals selected for study provided a wide range of fatty acid types and compositions for a comparison of fatty acid positional distributions in triglycerides. There has been a number of stereospecific analyses of bovine milk triglycerides from butter or individual cows (3,12,15-17). The results show that 4:0 and 6:0 are always almost exclusively esterified at the *sn*-3-position. The 12:0 and 14:0 acids are always preferentially esterified at the *sn*-2-position whereas 18:0 is always preferentially associated with the *sn*-1-position. In some samples, 18:1 is selectively esterified at the *sn*-1-position and in others at the *sn*-3-position. The 8:0 and 10:0 acids are selectively esterified

TABLE 1

Positional Distribution of Fatty Acids in Triglycerides from Milk of Several Mammals

Species	Position	Fatty acid composition (mol %)																
		4:0	6:0	8:0	10:0	12:0	14:0	14:1	15:0	15:1	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	Other
Echidna	TG	–	–	–	–	–	1.0	0.7	0.4	0.2	22.8	7.4	1.1	0.7	11.1	43.5	9.1	2.0	–
	sn-1	–	–	–	–	–	1.7	1.3	0.8	0.4	31.5	7.1	1.5	0.7	16.8	33.1	4.1	1.0	–
	sn-2	–	–	–	–	–	0.9	0.7	0.2	0.1	9.0	7.0	0.4	0.8	2.1	57.6	18.3	2.9	–
	sn-3	–	–	–	–	–	0.4	0.2	0.1	0.2	27.9	8.0	1.6	0.6	14.3	39.8	4.9	2.0	–
Koala	TG	–	–	–	–	0.1	3.9	0.2	0.8	–	25.7	4.6	1.1	0.8	4.9	16.1	10.3	31.5	–
	sn-1	–	–	–	–	0.1	3.9	0.2	1.0	–	25.3	5.8	2.5	1.2	14.4	20.7	7.4	20.5	–
	sn-2	–	–	–	–	0.1	8.6	0.3	1.6	–	50.4	6.1	0.7	0.6	1.0	4.8	5.3	20.5	–
	sn-3	–	–	–	–	0.1	-0.7	–	0.1	–	1.5	1.8	0.2	0.6	2.2	22.7	18.1	53.4	–
Wallaby	TG	–	–	–	0.1	0.1	1.7	0.2	0.5	0.1	19.6	6.0	0.6	0.7	2.7	51.6	8.8	7.3	–
	sn-1	–	–	–	0.1	0.2	1.9	0.4	0.7	0.2	21.2	6.1	0.8	0.7	5.3	52.8	6.6	3.0	–
	sn-2	–	–	–	0.1	0.3	3.8	0.3	0.7	0.2	34.8	9.9	0.8	0.9	2.3	33.3	6.6	6.0	–
	sn-3	–	–	–	–	–	-0.6	–	–	–	3.0	2.1	0.1	0.6	0.4	68.4	13.1	12.9	–
Guinea pig	TG	–	–	–	–	0.1	2.5	0.2	0.3	0.1	30.9	3.0	0.6	0.5	2.8	33.8	20.3	4.9	–
	sn-1	–	–	–	–	–	2.7	0.2	0.5	0.1	24.1	4.1	1.0	0.6	6.9	40.8	15.6	3.4	–
	sn-2	–	–	–	–	0.1	4.6	0.3	0.5	0.1	68.1	3.7	0.6	0.4	0.6	7.3	11.6	2.1	–
	sn-3	–	–	–	–	–	0.2	–	–	–	0.4	1.3	0.2	0.6	1.0	53.3	33.9	9.1	–
Dog	TG	–	–	–	0.2	0.7	4.9	1.1	0.7	0.2	26.5	7.8	0.9	1.1	3.7	40.9	9.2	2.1	–
	sn-1	–	–	–	0.1	0.3	3.4	0.6	0.9	0.2	34.5	7.3	1.2	0.9	6.3	39.2	4.8	0.4	–
	sn-2	–	–	–	0.2	1.1	10.3	1.7	1.1	0.3	42.6	9.8	0.8	1.0	1.2	20.1	8.8	1.0	–
	sn-3	–	–	–	0.3	0.6	1.0	1.1	–	0.1	2.4	6.5	0.7	1.5	3.6	63.4	14.1	4.7	–
Cat	TG	–	–	–	0.4	0.9	5.4	0.9	0.8	0.2	26.8	5.1	1.2	0.8	10.1	40.3	5.8	1.3	–
	sn-1	–	–	–	0.1	0.3	2.8	1.1	0.7	0.2	20.5	4.8	1.4	0.7	16.4	45.5	4.4	1.1	–
	sn-2	–	–	–	0.4	1.5	12.4	0.9	1.8	0.3	51.1	6.3	1.1	0.7	2.4	15.5	4.6	1.0	–
	sn-3	–	–	–	0.7	1.0	0.9	0.7	-0.2	0.1	8.9	4.1	1.1	1.0	11.7	59.5	8.6	1.9	–
Seal	TG	–	–	–	–	0.2	11.5	1.3	0.4	0.2	15.0	13.7	0.5	0.9	2.1	39.5	2.1	0.5	12.1
	sn-1	–	–	–	–	0.2	7.3	1.9	0.6	0.1	13.1	10.2	0.3	0.8	4.5	53.8	1.3	0.3	5.6
	sn-2	–	–	–	–	0.3	23.6	1.9	1.1	0.3	31.0	16.8	0.6	0.7	0.7	19.4	2.3	0.5	0.8
	sn-3	–	–	–	–	0.2	3.8	0.1	0.1	0.3	1.0	14.1	0.6	1.2	1.0	45.4	2.8	0.7	28.7
Horse	TG	–	0.3	2.3	3.6	4.3	5.0	0.9	0.3	0.2	16.4	9.0	0.1	0.5	1.0	15.2	9.3	29.8	1.8
	sn-1	–	–	0.2	0.7	3.0	5.1	1.1	0.6	0.2	23.5	8.4	0.4	0.5	2.7	20.6	7.2	25.6	0.2
	sn-2	–	–	3.0	4.6	6.3	9.0	1.1	0.7	0.2	25.4	10.9	0.1	0.5	0.6	7.5	7.2	20.1	2.8
	sn-3	–	0.9	3.6	5.5	3.7	0.8	0.5	-0.2	0.1	0.2	7.6	-0.1	0.4	-0.3	17.4	13.6	43.7	2.6
Pig	TG	–	–	–	–	0.1	2.4	ND[a]	0.2	ND	26.9	4.0	0.6	ND	6.8	30.3	27.3	1.4	–
	sn-1	–	–	–	–	0.1	1.8	ND	0.3	ND	26.4	3.5	0.8	ND	11.2	32.7	21.8	1.4	–
	sn-2	–	–	–	–	0.2	5.1	ND	0.5	ND	48.1	5.1	0.5	ND	1.8	15.5	21.9	1.2	–
	sn-3	–	–	–	–	-0.1	0.4	ND	-0.2	ND	6.2	3.4	0.5	ND	7.5	42.8	38.0	1.6	–

Cow[b]	TG	8.8	5.0	2.5	5.3	5.2	13.8	1.7	1.0	0.2	28.0	2.3	0.6	0.2	8.3	14.1	1.1	0.9	1.0
	sn-1	–	–	0.2	1.4	3.4	12.7	1.4	1.5	0.3	41.2	2.7	1.0	0.3	15.2	16.2	1.2	0.7	0.6
	sn-2	–	0.1	2.9	7.8	8.1	23.9	2.4	1.8	0.4	36.7	3.3	0.5	0.2	3.0	7.2	0.6	0.3	0.8
	sn-3	26.4	14.8	4.4	6.7	4.0	4.9	1.2	-0.3	–	6.1	1.0	0.2	0.2	6.7	19.0	1.6	1.7	1.4

[a]Not determined.

[b]The 14:1, 15:1, 16:1 and 17:1 acids also contain 15:0 br, 16:0 br, 17:0 br and 18:0 br, respectively; 18:3 also contains 18:2*c*,*t* conj.

TABLE 2

Observed Triglyceride Carbon Number Distribution Composition and the Composition Calculated Using the 1-Random-2-random-3-random Fatty Acid Distribution Hypothesis for Milk from 9 Species of Mammals

Carbon number	Composition (mol %)																	
	Echidna		Koala		Wallaby		Guinea pig		Dog		Cat		Horse		Pig		Cow	
	Obs[a]	Calcd[b]	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.0	0.1
26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.4	0.3
28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.9	1.1
30	–	–	–	–	–	–	–	–	–	–	–	–	0.1	0.1	–	–	1.7	2.6
32	–	–	–	–	–	–	–	–	–	–	–	–	0.3	0.2	–	–	3.8	4.7
34	–	–	–	–	–	–	–	–	–	–	–	–	0.7	0.4	–	–	8.4	8.4
36	–	–	–	–	–	–	–	–	–	–	–	–	1.7	0.9	–	–	13.3	12.5
38	–	–	–	–	–	–	–	–	–	–	–	–	2.9	1.6	–	–	13.6	12.0
40	–	–	–	–	–	–	–	–	0.0	0.1	0.1	0.1	3.7	2.8	–	–	10.1	8.3
42	–	–	–	–	–	–	–	–	0.2	0.2	0.4	0.4	5.8	5.3	–	–	8.3	6.8
44	–	–	0.0	0.1	0.1	0.1	0.2	0.0	1.2	0.9	1.6	1.2	8.4	8.7	–	–	7.6	7.2
46	0.6	0.7	1.2	0.9	1.0	0.6	1.3	0.4	4.3	3.2	4.3	3.5	9.4	10.3	1.2	0.6	7.5	8.1
48	5.0	5.6	7.0	7.1	4.6	3.9	6.2	4.9	12.2	12.5	10.8	11.6	10.3	11.2	6.0	4.9	8.0	10.1
50	22.7	23.6	27.4	27.9	19.7	19.5	25.0	26.8	27.8	32.5	24.6	29.8	15.4	18.2	22.1	23.8	8.4	10.6
52	41.1	42.9	45.5	45.1	42.6	44.8	53.0	53.9	34.9	37.1	38.9	40.4	24.7	25.5	43.4	46.2	5.8	6.0
54	30.6	27.2	18.9	18.9	32.0	31.1	14.3	14.0	19.4	13.5	19.3	13.0	16.6	14.8	27.3	24.5	2.3	1.2
D(mol %)	3.4		0.7		2.1		2.7		7.3		7.5		5.7		4.5		7.2	

[a]Observed.

[b]Calculated.

at either the *sn*-2- or the *sn*-3-position. In many samples, there is little difference in fatty acid composition at the 2 positions competing for preferential esterification. The interspecies differences in fatty acid positional distribution noted in Table 1 are of the same nature as the intraspecies differences noted for the cow.

Stereospecific analysis of triglycerides from the sheep and goat (4,18), human (5) and rat (7) show that these species also exhibit a similar fatty acid distribution pattern to that just outlined. In human and rat triglycerides, 12:0 is preferentially esterified at the *sn*-3-position rather than at the *sn*-2-position.

Factors which influence the specific fatty acid distribution pattern in milk triglycerides include: acyl-CoA concentrations, acyltransferase specificity and activity and the biochemical pathways used for triglyceride synthesis. Studies with the bovine (19-22) and with the rat (7,23,24) demonstrate that the fatty acid specificities of mammary *sn*-glycerol-3-phosphate acyltransferases and 1-acyl-*sn*-glycerol-3-phosphate acyltransferases are related to the fatty acid composition at the *sn*-1- and *sn*-2-positions of milk triglycerides. The fatty acid composition at the *sn*-3-position is not determined to any great extent by the specificities of bovine mammary diacylglycerol acyltransferases (25).

Gross and Kinsella (20) and Kinsella (21) found that the specific activity of palmitoyl-CoA:*sn*-glycerol-3-phosphate acyltransferase from the mammary tissue of different cows varied widely. It can be assumed that the activities of other acyltransferases will also vary among animals and this may explain why a particular fatty acid is preferentially esterified at different positions in some samples.

It is now generally accepted that milk triglycerides are mainly synthesized by the *sn*-glycerol-3-phosphate pathway, however, in the pig, Bickerstaffe and Annison (2) showed that a monoglyceride pathway was as active as the *sn*-glycerol-3-phosphate pathway. The positional distribution of fatty acids in pig milk triglycerides reported in Table 1 and by Christie and Moore (6) is similar to other species, including the cow, in which the *sn*-glycerol-3-phosphate pathway is known to be the major, if not the only, synthetic pathway. The *sn*-glycerol-3-phosphate pathway is also the major pathway for adipose tissue triglyceride synthesis (26). These triglycerides have a fatty acid distribution pattern different from milk triglycerides, indicating that other factors such as acyltransferase specificity are more important than the synthetic pathway in determining fatty acid distribution.

The positional distribution of fatty acids in echidna milk triglycerides is different from the distribution in other species. Grigor (27) also has recently shown, by pancreatic lipase deacylation, that the proportional distribution of fatty acids at the *sn*-2-position of milk triglycerides from this species is different from other animals. His results are comparable to data in Table 1. The echidna, along with the platypus, is the most primitive surviving mammal. Although the structure of monotreme mammary glands is similar to those of marsupials and eutherians (28), the glands may be unspecialized (29).

Of the various animal tissues, the depot fat of mammals most closely resembles echidna milk triglycerides in the positional distribution of fatty acids (30). However, the symmetrical nature of echidna milk triglycerides is more akin to those of common vegetable oils (31). Tissue and organ microsomal fractions may contain *sn*-glycerol-3-phosphate and 1-acyl-*sn*-glycerol-3-phosphate acyltransferases with different fatty acid specificities from those of the mammary gland (24). For the diacylglycerol acyltransferases, this may not be the case (25). It is thus possible that echidna mammary tissue contains acyltransferases with different specificities than those of other mammals, although other factors may be involved. Stokes and Tove (32) presented evidence that pig adipose tissue contained a specifier factor which, by interacting with acyltransferases, appeared to direct the acylation of 16:0 to the *sn*-2-position.

Most past studies, as reviewed by Litchfield (33), used triglyceride class composition to evaluate 1,3-random-2-random or 1-random-2-random-3-random fatty acid distribution hypotheses. Although this approach allows for the chain length of the fatty acids, it does not distinguish between type and degree of unsaturation. Recently, Managanaro et al. (34) have applied a more sophisticated approach to determining enantiomeric structures of peanut oil triglycerides. Using chromatographic techniques and stereospecific analysis detailed analyses of the molecular species of generated *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diglycerides led these workers to conclude that the fatty acids in the 3 positions of the glycerol molecule were combined with each other solely on the basis of their relative molar concentrations. As a result, it was possible to calculate the composition of the molecular species of the peanut oil triglycerides using the 1-random-2-random-3-random hypothesis.

For pig milk, Christie and Moore (6) found excellent agreement between triglyceride class composition and that calculated by a 1-random-

2-random-3-random distribution hypothesis. In the current study, agreement was also excellent for pig milk when TG carbon number distribution was compared to that expected by the 1-random-2-random-3-random hypothesis. In Table 2, the milks with the highest D values were those with the largest range of fatty acids (Table 1). The magnitude of D in these milks may be related to specificity due to the chain length of the fatty acids.

Biochemical evidence to support a 1-random-2-random-3-random fatty acid distribution in milk triglycerides is very limited. For the bovine, Marshall and Knudsen (22) found that the chain-length specificity of the acyltransferases was unaffected by the nature of the fatty acid (palmitic or oleic acid) at the *sn*-1-position of 1-acyl-*sn*-glycerol-3-phosphate. This is an example of noncorrelative acylation (35). Lin et al. (7) found, with lactating rat mammary gland, that acyl-CoA specificity was affected by the type of 1,2-diglyceride acceptor offered. However, although dilaurin was the best acceptor and *sn*-1,2-dilaurin > *sn*-1,2-dimyristin > *sn*-1,2-dipalmitin > *sn*-1,2-distearin, the authors could not say unequivocally that the apparent preference for shorter-chain diglycerides did not result at least partially from the greater solubility of these substrates.

TG carbon number distribution is a necessary, put perhaps insufficient, test of the 1-random-2-random-3-random fatty acid distribution hypothesis. Although care must be exercised when using the hypothesis to calculate triglyceride composition, the procedure does provide information quickly. This information would perhaps take years to obtain using other analytical techniques. Kuksis (36), however, has cautioned that obtaining data from distribution hypotheses should not stifle the development of analytical procedures which will provide such data without making a priori hypotheses.

ACKNOWLEDGMENTS

Thanks to C. Beck for technical assistance and Dr. R.E. Timms, CSIRO, Division of Food Research, Dairy Research Laboratory, for the computer program to test the fatty acid distribution hypothesis. Horse and pig milk was provided by B.E. Wilson, echidna and Tammar wallaby by Dr. C.H. Tyndale-Biscoe, koala by R. Beeby, guinea pig by Dr. D.P. Henry, dog and cat by Dr. C.C. Kratzing, and Weddell seal by Dr. R.A. Tedman. This work was supported in part by a grant from the Australian Dairy Research Committee.

REFERENCES

1. Moore, J.H., and Christie, W.W. (1979) Prog. Lipid Res. 17, 347-395.
2. Bickerstaffe, R., and Annison, E.F. (1971) Int. J. Biochem. 2, 153-162.
3. Pitas, R.E., Sampugna, J., and Jensen, R.G. (1967) J. Dairy Sci. 50, 1332-1336.
4. Marai, L., Breckenridge, W.C., and Kuksis, A. (1969) Lipids 4, 562-570.
5. Breckenridge, W.C., Marai, L., and Kuksis, A. (1969) Can. J. Biochem. 47, 761-769.
6. Christie, W.W., and Moore, J.H. (1970) Biochim. Biophys. Acta 210, 46-56.
7. Lin, C.Y., Smith, S., and Abraham, S. (1976) J. Lipid Res. 17, 647-656.
8. Official Methods of Analysis (1970) 11th Edn., sec. 16.052, AOAC, Washington, DC.
9. Kates, M. (1972) in Techniques of Lipidology (Work, T., and Work, E., eds.) pp. 402-405, North Holland Publishing, Amsterdam.
10. Brockerhoff, H. (1965) J. Lipid Res. 6, 10-15.
11. Christie, W.W., and Moore, J.H. (1969) Biochim. Biophys. Acta 176, 445-452.
12. Parodi, P.W. (1979) J. Dairy Res. 46, 75-81.
13. Shehata, A.Y., de Man, J.M., and Alexander, J.C. (1970) Can. Inst. Food Technol. J. 3, 85-89.
14. Parodi, P.W. (1979) J. Dairy Res. 46, 633-639.
15. Taylor, M.W., and Hawke, J.C. (1975) N.Z. J. Dairy Sci. Technol. 10, 49-57.
16. Morrison, I.M., and Hawke, J.C. (1977) Lipids 12, 1005-1011.
17. Parodi, P.W. (1982) J. Dairy Res. 49, 73-80.
18. Mills, S.C., Cook, L.J., Scott, T.W., and Nestel, P.J. (1976) Lipids 11, 49-60.
19. Kinsella, J.E., and Gross, M. (1973) Biochim. Biophys. Acta 316, 109-113.
20. Gross, M., and Kinsella, J.E. (1974) Lipids 9, 905-912.
21. Kinsella, J.E. (1976) Lipids 11, 680-684.
22. Marshall, M.O., and Knudsen, J. (1977) Biochim. Biophys. Acta 489, 236-234.
23. Tanioka, H., Lin, C.Y., Smith, S., and Abraham, S. (1974) Lipids 9, 229-234.
24. Cooper, S.M., and Grigor, M.R. (1980) Biochem. J. 187, 289-295.
25. Marshall, M.O., and Knudsen, J. (1979) Eur. J. Biochem. 94, 93-98.
26. Vernon, R.G. (1980) Prog. Lipid Res. 19, 23-106.
27. Grigor, M.R. (1980) Comp. Biochem. Physiol. 65B, 427-430.
28. Griffiths, M., Elliott, M.A., Leckie, R.M.C., and Schoefl, G.I. (1973) J. Zool. London 169, 255-279.
29. Parker, T.J., and Haswell, W.A. (1964) in Textbook of Zoology, 7th edn., Vol. II, p. 688, MacMillan and Co. Ltd., London.
30. Brockerhoff, H., Hoyle, R.J., and Wolmark, N. (1966) Biochim. Biophys. Acta 116, 67-72.
31. Brockerhoff, H., and Yurkowski, M. (1966) J. Lipid Res. 7, 62-64.
32. Stokes, G.B., and Tove, S.B. (1975) J. Biol. Chem. 250, 6315-6319.
33. Litchfield, C. (1972) Analysis of Triglycerides, p. 263, Academic Press, New York, NY.
34. Manganaro, F., Myher, J.J., Kuksis, A., and Kritchevsky, D. (1981) Lipids 16, 508-517.
35. Lands, W.E.M., Pieringer, R.A., Slakey, P.M., and Zschocke, A. (1966) Lipids 1, 444-448.
36. Kuksis, A. (1972) in Progress in the Chemistry of Fats and Other Lipids (Holman, R.T., ed.) Vol. 12, pp. 1-163, Pergamon Press, New York, NY.

[Received December 16, 1981]

METHODS

Digitonide Precipitable Sterols: A Reevaluation with Special Attention to Lanosterol

RICHARD J. CENEDELLA*, *Department of Biochemistry, Kirksville College of Osteopathic Medicine, Kirksville, MO 63501*

ABSTRACT

The ability of digitonin to precipitate lanosterol from prepared mixtures and biological sources was evaluated. Commercially available lanosterol was determined to be composed of about 60% lanosterol and 40% dihydrolanosterol. Both sterols were only partially precipitated by digitonin under all conditions examined. The presence of cholesterol increased the precipitation of lanosterol, but never to completion. About 40% of the lanosterols from saponified sheep's-wool fat was not precipitated by digitonin. Also ^{14}C-labeled lanosterol recovered from rat brain following intracerebral injection of 2-[^{14}C]mevalonate was only 70% precipitated by digitonin. Steric hinderance by the methyl groups at carbon -4 is suggested to explain the poor precipitability of this sterol. In conclusion, lanosterol can not be considered to be a digitonide-precipitable sterol equivalent to cholesterol. Caution should be exercised in situations where digitonin-precipitable sterols are being prepared from sources containing significant concentrations of lanosterol (i.e., mass and/or radiolabel).
Lipids 17:443-447, 1982.

INTRODUCTION

Measurement of incorporation of ^{14}C-labeled precursors or tritiated water into digitonin-precipitable sterols (DPS) is a standard method for assessing cholesterol biosynthesis (1-3). Cholesterol is assumed to be the main sterol recovered; however, cholesterol precursors possessing a 3β-hydroxy group are also assumed to be precipitated. As explained by Lakshmanan and Veech (1), precipitation of methylated sterols such as lanosterol can complicate the calculation of cholesterol biosynthesis from ^{14}C-labeled substrates. They state that "lanosterol which also has a 3β-hydroxy group is precipitated along with cholesterol by digitonin." An earlier observation in our laboratory that lanosterol gave no reaction with digitonin prompted us to examine critically the precipitability of lanosterol by digitonin. In this study, we compare the precipitation of lanosterol from prepared mixtures and biological sources. The results show that, in contrast to cholesterol, 7-dehydrocholesterol and desmosterol, lanosterol is only partially, and often poorly, precipitated by digitonin.

MATERIALS AND METHODS

Chemicals

Cholesterol, desmosterol, 5-α-cholestane and squalene were purchased from Sigma Chemical Co., St. Louis; 7-dehydrocholesterol was from Aldrich Chemical Co., Milwaukee, and lanosterol was from Sigma Chem. Co. and Serdary Research Labs, London, Ontario, Canada. J.T. Baker, Phillipsburg, NJ, was the source of digitonin. All sterol standards except lanosterol gave a single peak when analyzed by gas chromatography (GC). Lanosterol from both sources was composed of 2 distinct substances. The substance that eluted first in the GC analysis comprised about 40% of the total and was identified by GC-mass spectrometry (GC-MS) as dihydrolanosterol (see Appendix).

Formation of Digitonide Precipitates

Digitonide precipitates were usually formed as described by Sperry and Webb (4). With this method, the sterols were dissolved in 3 or 4 ml of acetone/ethanol (1:1, v/v), the solution was acidified with 50 μl of 10% acetic acid and the sterols were precipitated by addition of 1 or 2 ml of 1% digitonin (1 g/100 ml ethanol/H_2O, 1:1). These amounts of digitonin were in excesses of that required for complete precipitation of several mg of sterol. Precipitates were allowed to form for 20-24 hr at 5 C. In an initial experiment, the recovery of digitonides following reaction overnight at 22 C was also measured, as markedly different conditions of precipitation are reported in the literature. For example, Igarashi and Suzuki (5) precipitated

cholesterol by reaction with digitonin for only 1 hr presumably at room temperature, whereas Sperry and Webb (4) conducted their reaction overnight at 5 C. Precipitates were quantitated gravimetrically after being washed twice with acetone and once with diethyl ether. Precipitates were then dried at room temperature to constant weight. Treatment of the supernatant recovered from the digitonin precipitates with additional digitonin resulted in no additional precipitate being formed. In some cases, unprecipitated sterols were quantitated in aliquots of supernatant separated from the digitonide precipitates by low-speed centrifugation (2,000 rpm for 10 min). One- or 2-ml aliquots of the supernatant were evaporated to dryness under nitrogen at 40 C and 200 μl of CS_2 containing 20 μg of 5-α-cholestane (internal standard) was added to the residue. Sterols that were soluble in the carbon disulfide were separated at 265 C on a 6-ft coiled glass column (2 mm id) packed with 3% OV-17 on 100/120 Gas-Chrom Q using a Packard Model 417 gas chromatograph and flame ionization detection. This procedure is identical to that detailed before (6) except that the column was packed with 3% OV-17 rather than 3% QF-1. The OV-17 appeared to yield better resolution of the sterols. The approximate retention time of 5-α-cholestane, cholesterol, 7-dehydrocholesterol, desmosterol, dihydrolanosterol and lanosterol were 3, 8, 9.5, 9.5, 11.5 and 14 min, respectively. The area under the peaks was estimated by triangulation and the concentration of sterols was determined by comparison with the area produced by known amounts of 5-α-cholestane. The area under the peaks obtained with equal masses of 5-α-cholestane, cholesterol and lanosterol (dihydrolanosterol plus lanosterol) were 1.0 to about 0.8 and 0.5, respectively. The response ratios were determined with each GC run and were used to correct the measured apparent concentrations to the true concentrations. Lanosterol and dihydrolanosterol were assumed to give equal signals per unit weight in flame ionization detection.

Precipitation of Lanosterol from Biological Sources

Sheep's wool. The precipitability of lanosterol from 2 natural sources, sheep's wool and rat brain, was evaluated. Total lipid from a 2-g sample of fresh unwashed wool was extracted overnight into 70 ml of chloroform/methanol (2:1, v/v). Aliquots (1/23 or 1/92) of the total lipid was saponified in alcoholic KOH (7). Wool fat was saponified because a major sterol ester band was present in thin layer chromatography (TLC) of samples of this fat (Silica Gel G was used with a solvent of *n*-hexane/diethyl ether/glacial acetic acid, 73:25:2, v/v/v.) One-half of the recovered nonsaponifiable fraction was reacted with digitonin at 5 C. Sterols in the other half of the nonsaponifiable fraction (not treated with digitonin) and those recovered from the supernatant of the digitonide precipitates were quantitated by the GC procedures as already described.

Rat brain. Cholesterol and cholesterol precursors in rat (130-g, Sprague-Dawley) brain were radiolabeled by intracerebral injection of 2.2 μCi of 2-[^{14}C]DL-mevalonate (New England Nuclear Corp.) dissolved in 40 μl of saline. The [^{14}C]mevalonate as the dibenzylethylene diamine salt was diluted to a specific activity of 0.5 mCi/mmol and then converted to the free acid by treatment with bicarbonate immediately prior to injection. One hr after injection, the rat was sacrificed by decapitation; the brain was removed, weighed and homogenized in 20 vol of chloroform/methanol as described before (8). Aliquots of the whole brain total lipids containing ca. 400 μg of cholesterol were precipitated with digitonin in the cold. Because sterol esters are only trace components of rat brain (9,10) and only trace amounts of [^{14}C] mevalonate are incorporated into sterol esters by rat brain (11), saponification of brain total lipids prior to treatment with digitonin was unnecessary. The digitonide precipitates were centrifuged and the supernatants were recovered. The precipitates were washed consecutively with two 5-ml portions of acetone and once with diethyl ether. The precipitates were air-dried, dissolved in 2 ml of methanol and the ^{14}C content of the solutions measured by scintillation counting using a Beckman LS 7000 instrument with internal standardization. Maximal counting error (σ) was about 4%. The supernatant plus the combined washes of the precipitates were evaporated to dryness under nitrogen, the residue was suspended in chloroform, the suspension was filtered through a fritted glass funnel and the chloroform extract (containing unprecipitated sterol) was evaporated. This extract, plus aliquots of the brain total lipid that were identical to those precipitated with digitonin, were analyzed for [^{14}C] squalene, [^{14}C]cholesterol, [^{14}C]desmosterol and [^{14}C]lanosterol contents. After addition of unlabeled squalene and sterol carriers, the lipids were fractionated by 2 consecutive TLC runs, the second using Silica Gel G containing 7% silver nitrate (11). The TLC zones containing the separated lipids were extracted with diethyl ether and the recovered sterol fractions were prepared for scintillation counting as we have recently described in detail (11).

TABLE 1

Precipitation of Sterols with Digitonin[a]

Sterol[b]	Sterol added (mg)	Sterol precipitated with digitonin (mg)	
		Room temp.	5 C
Cholesterol	1	1.00 ± 0.04	1.14 (1.13,1.16)
Cholesterol	2	2.13 ± 0.05	–
7-Dehydrocholesterol	1	0.88 ± 0.03	0.98 (0.99,0.96)
7-Dehydrocholesterol	2	1.85 ± 0.05	–
Desmosterol	1	1.13 ± 0.06	1.08 (1.08,1.08)
Desmosterol	2	2.15 ± 0.03	–
Lanosterol[c]	1	0-tr	0-tr
Lanosterol[c]	2	1.01 ± 0.04	1.11 ± 0.04

[a]One or 2 ml of 1% digitonin in ethanol/water (1:1) was added to the stated amount of sterol dissolved in 3 or 4 ml of acetone/ethanol (1:1). After 20-24 hr at room temperature or 5 C, the digitonide precipitates were washed twice with acetone and once with diethyl ether and dried to constant weights. The weights of the precipitates were determined gravimetrically. Values are the mean ± SEM of 3 or 4 separate assays or the average of 2 determinations (individual values in parentheses).

[b]Analysis of the supernatants from the digitonide precipitates of cholesterol, 7-dehydrocholesterol and desmosterol revealed no unprecipitated sterol. In contrast, lanosterol remained in the supernatant after reaction of this sterol with digitonin.

[c]Precipitates were allowed to react for 2 days.

RESULTS AND DISCUSSION

Cholesterol, 7-dehydrocholesterol and desmosterol at a level of 1 or 2 mg were precipitated by reaction with digitonin overnight either at room temperature or at 5 C (Table 1). No clear differences were apparent between the recoveries of these sterols at the 2 temperatures. Examination of the supernatants from the precipitates formed at 22 C by GC analysis revealed no unprecipitated sterols. Cholesterol at all concentrations examined (to as low as 50 μg) was completely precipitated. By contrast, 1 mg of lanosterol formed little or no precipitate with digitonin, even after standing for 2 days, and only about 50% of the 2-mg samples formed a recoverable digitonide precipitate after this time either at room temperature or at 5 C (Table 1). Digitonin precipitation of lanosterol, particularly at low concentrations of lanosterol, was increased by conducting the reaction in the presence of 1 mg of cholesterol (Fig. 1A). Increasing the cholesterol concentration to above 1 mg had little additional effect on the amount of free lanosterol remaining in solution from reaction of a given amount of lanosterol with digitonin (Fig. 1B). On the basis of these studies, it seems clear that lanosterol is, at best, only partially precipitated by digitonin. The extent to which these results with artificial mixtures apply to lanosterol present in natural lipid mixtures was investigated by measuring the precipitation of lanosterol present in sheep's-wool

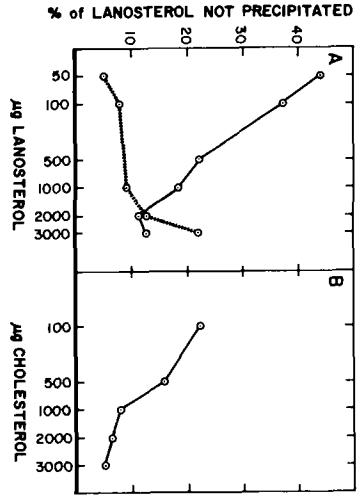


FIG. 1 (A) Precipitation of varying concentrations of lanosterol in the presence and absence of cholesterol. Varying amounts of lanosterol (from Serdary Labs) were dissolved in 3 ml of acetone/ethanol (1:1) either in the absence (——) or presence (-----) of 1000 μg of cholesterol. After acidification with 50 μl of 10% acetic acid, 2 ml of 1% digitonin was added and the samples were precipitated overnight at 5 C. Aliquots of the supernatant that were recovered following centrifugation of the precipitates were evaporated, dissolved in CS_2 and the CS_2 was analyzed for free sterol content by gas liquid chromatography. Each point represents one determination or the average of 2. (B) Precipitation of 1000 μg of lanosterol overnight at 5 C in the presence of varying amounts of cholesterol.

TABLE 2

Precipitation of Sheep's Wool Sterols with Digitonin

Sterol	mg Sterol/g wool (fresh wt)	
	Total[a]	In supernatant of digitonin precipitate[b]
Cholesterol	10.4 ± 0.6	0
7-Dehydrocholesterol-desmosterol	0.4 ± 0.16	0
Dihydrolanosterol	6.66 ± 0.26	3.36, 3.08
Lanosterol	1.84 ± 0.27	0.74, 0.51

[a]Aliquots of freshly extracted sheep's wool fat were saponified and the total nonsaponifiable lipids recovered. Individual sterols were quantitated by gas chromatography. Values are means ± SEM of 3 determinations.

[b]Aliquots of sheep's wool fat, containing either 0.08 or 1.59 mg of sterol, were dissolved in 3 ml of acetone/ethanol (1:1), and precipitated with digitonin overnight at 5 C following acidification. Sterols remaining in the supernatant after exposure to digitonin were quantitated by GC assay.

fat and of radiolabeled lanosterol recovered from rat brain following injection of 2-[^{14}C] mevalonate.

Sheep's wool was found to contain about 20 mg of total sterol per g of fresh wool, of which 55% was cholesterol and 44% was lanosterols (Table 2). About 0.5% of the total sterol possessed a GC retention time equal to 7-dehydrocholesterol and desmosterol; both sterols elute at the same time on the GC column. Lanosterols were identified as dihydrolanosterol and lanosterol by comparison of column retention times with those of the standard lanosterol mixture that was subjected to MS analysis. The cholesterol and 7-dehydrocholesterol/desmosterol in nonsaponifiable wool fat completely precipitated when treated with digitonin. On the other hand, between 40-50% of the lanosterols were not precipitated.

When [^{14}C] mevalonate is used to follow synthesis of brain sterols in vitro, lanosterol can account for about one-quarter of the total ^{14}C label incorporated into sterols (11). Intracerebral injection of 2-[^{14}C] mevalonate at low specific activity can also result in significant incorporation of radioisotope into precursors of cholesterol, in addition to cholesterol itself. For example, Ramsey and Fredericks (12) found that lanosterol contained about one-third of the total ^{14}C label in free sterols of brain following intracerebral injection of 2-[^{14}C] mevalonate.

Aliquots of the total lipid extracted from whole brain were directly separated by TLC into squalene, lanosterol, cholesterol and desmosterol fractions. Other aliquots were treated with digitonin and the lipid present in the supernatant of the digitonide precipitates was recovered and identically fractionated. Ramsey and Fredericks (12) demonstrated that ^{14}C label recovered from the lanosterol region on the silver nitrate TLC plates was about 80% [^{14}C] lanosterol and the remainder was radiolabeled 4α-methyl-5α-cholesta-8,24-dien-3β-ol. Prior to treatment with digitonin, the squalene and lanosterol zones of the TLC plates contained similar amounts of radiolabel, both possessing about 3 times the level found in cholesterol (Table 3). Radioactivity essentially disappeared from the cholesterol zone following reaction with digitonin and 80-95% of the ^{14}C in squalene was recovered; however, between 25 and 30% of the ^{14}C label originally present in the lanosterol region of the TLC plate was retained in this area after treatment with digitonin. Therefore, the radiolabeled lanosterol or a closely related ^{14}C-labeled, methylated sterol in brain appeared to be only partially precipitated by digitonin. It is notable that the combined radiolabel in lanosterol, cholesterol and desmosterol which disappeared upon treatment with digitonin accounted for only about one-half of the total ^{14}C label recovered in the DPS. Possibly other sterols besides those examined contributed significantly to the total ^{14}C label in the DPS of rat brain radiolabeled by this procedure.

In conclusion, our results indicate that lanosterol is not a digitonin-precipitable sterol comparable to cholesterol. The precipitability of lanosterol is only partial, is variable and is affected by the presence of other sterols. One can speculate that the poor reactivity of digitonin with lanosterol could be due to structural hindrance of the 3β-hydroxy group by the presence of methyl groups at position 4. We recommend caution when interpreting results in situations where sterols are to be quantitatively isolated as DPS and where lanosterol is

TABLE 3

Digitonin Precipitation of Brain Sterols Labeled in vivo from 2-[^{14}C]Mevalonate[a]

Lipid fraction	dpm/Aliquot (1/16th of whole brain)			
	Total lipid		In supernatant of digitonin precipitate	
	Assay 1	Assay 2	Assay 1	Assay 2
Squalene	1089	1081	867	1028
Lanosterol	1037	1081	346	270
Cholesterol	337	325	11	11[b]
Desmosterol	55	49		21[b]
Digitonide ppt.	–	–	2247	2127

[a]Total lipids were extracted from the intact brain of a rat (130-g, Sprague-Dawley) injected intracerebrally with 2-[^{14}C]mevalonate (2.2 μCi, 0.5 mCi/mmol) 1 hr earlier. Aliquots of the recovered total lipid were either directly fractionated by 2 separate thin layer chromatographies (the second using $AgNO_3$-impregnated plates) into squalene and individual sterols or precipitated with digitonin overnight at 5 C. Aliquots of the supernatant recovered from the digitonide precipitates were evaporated and identically fractionated by TLC. Radioactivity values are dpm above background (about 40 dpm).

[b]Estimate 25% low due to a loss during recovery.

likely to comprise a significant fraction of the total sterols (as either mass or radiolabel).

APPENDIX

Lanosterol obtained from Serdary Laboratories was subjected to GC-MS analysis. The lanosterol sample showed 2 well separated peaks in the GC trace, the first peak representing about 40% of the total sample and the second 60%. The MS spectra for both peaks gave a base ion of m/E 76. The first peak had a high mass ion at m/E 395 and m/E 410, whereas the second peak had high mass ions at m/E 393, 408, 424 and 426. This pattern was consistent with lanosterol and dihydrolanosterol, losing water at the GC/MS interface. A direct probe spectrum of the lanosterol sample gave the expected ionization patterns which were: m/E 428 molecular ion dihydrolanosterol; m/E 413 molecular ion-methyl group; m/E 395 molecular ion-methyl group-water; m/E 426 molecular ion lanosterol; m/E 411 molecular ion-methyl group; m/E 393 molecular ion-methyl group-water. GC-MS and direct probe spectra of purified dihydrolanosterol and lanosterol confirmed our identification of the first peak as dihydrolanosterol and the second peak as lanosterol.

ACKNOWLEDGMENTS

The author thanks D. Schroeder and S. Gearhart for technical assistance and Prof. C.W. Gehrke and Dr. K. Gerhardt of the Experiment Station Chemical Laboratories of the University of Missouri-Columbia for the mass spectrometric analysis of the commercially available lanosterol. This work was supported by grants from the NIH (EY02568 and NS14446).

REFERENCES

1. Lakshmanan, M.R., and Veech, R.L. (1977) J. Biol. Chem. 252, 4667-4673.
2. Andersen, J.M., and Dietschy, J.M. (1979) J. Lipid Res. 20, 740-752.
3. Jeske, D.J., and Dietschy, J.M. (1980) J. Lipid Res. 21, 364-376.
4. Sperry, W.M., and Webb, M. (1950) J. Biol. Chem. 187, 97-106.
5. Igarashi, M., and Suzuki, K. (1974) Exp. Neurol. 45, 549-553.
6. Belis, J.A., and Cenedella, R.J. (1979) Cancer 43, 1840-1846.
7. Cenedella, R.J., and Belis, J.A. (1981) J. Lipid Res. 22, 122-130.
8. Cenedella, R.J., Galli, C., and Paoletti, R. (1975) Lipids 10, 290-293.
9. Eto, Y., and Suzuki, K. (1972) J. Neurochem. 19, 109-115.
10. Bierkamper, G.G., and Cenedella, R.J. (1978) Epilepsia 19, 155-167.
11. Cenedella, R.J. (1980) Biochem. Pharmacol. 29, 2751-2754.
12. Ramsey, R.B., and Fredericks, M. (1977) Biochem. Pharmacol. 26, 1161-1167.

[Received August 31, 1981]

Use of Radiolabeled Hexadecyl Cholesteryl Ether as a Liposome Marker

G.L. POOL[a,*], M.E. FRENCH[b], R.A. EDWARDS[a], L. HUANG[b] and R.H. LUMB[a],
[a]Biochemistry Group, Western Carolina University, Cullowhee, NC 28723; and
[b]Department of Biochemistry, University of Tennessee, Knoxville, TN 37916

ABSTRACT

Radiolabeled hexadecyl cholesteryl ether can serve as an effective marker for liposomes in a variety of studies. This paper demonstrates the use of a cholesteryl ether marker in the assay of phospholipid transfer protein activity and in assessing phospholipid vesicle-cell interactions. The cholesteryl ether has the advantages of ease of synthesis, metabolic inertness, lipid solubility and nonexchangeability. *Lipids* 17:448-452, 1982.

INTRODUCTION

Phospholipid bilayers have been used as model membranes in a variety of systems (1). They have the advantage of providing a well-defined structure that can be used to investigate a number of membrane dynamics, including phospholipid exchange and transfer (2), phospholipid hydrolysis (3), asymmetry and flip-flop (4,5), and phospholipid-cell interactions (6,7). Closed phospholipid bilayer vesicles are also finding increasing use as a vehicle to transport substances targeted to certain cell types in vivo (8). In many of these investigations, it is essential to use a metabolically inert marker to provide information about liposome interactions and recovery. Radiolabeled triolein and cholesteryl esters have been used as lipid markers (9,10) but have the major difficulty of being substrates for naturally occurring hydrolytic enzymes; the products released on hydrolysis may spontaneously equilibrate between membranes and lead to uninterpretable results. The use of water-soluble, trapped markers has also received favor in some investigations but this approach suffers from the low trapping volumes of liposomes and the tendency for leakage (11,12). We describe here the use of radiolabeled hexadecyl cholesteryl ether as a nonexchanged, nonhydrolyzed marker that is readily synthesized and provides reliable information about liposome behavior. We demonstrate the use of hexadecyl [^{3}H]cholesteryl ether in determining protein-catalyzed transfer of phosphatidylcholine (PC) between microsomes and phospholipid vesicles and in determining the interactions between phospholipid vesicles and V-79 cells in tissue culture. While this work was in progress, Stein et al. (13) reported the use of cholesteryl ether as an in vivo marker for very low density lipoproteins in the rat.

MATERIALS AND METHODS

Synthesis of Hexadecyl [^{3}H]Cholesteryl Ether

Hexadecylmethane sulfonate was prepared from hexadecanol by the procedure of Baumann and Mangold (14). Infrared analysis showed the loss of the O-H stretch in the 3300 cm^{-1} region and the appearance of bands at 1325 cm^{-1} and 1160 cm^{-1} characteristic of covalent sulfonates.

Hexadecyl cholesteryl ether was synthesized by a modification of the Paltauf procedure (15) from [7-^{3}H(N)]cholesterol (21.6 Ci/mmol, New England Nuclear Corp., Boston, MA). Hexadecylmethane sulfonate, [^{3}H]cholesterol, and potassium metal in dry benzene were heated at 70 C overnight in a 2-ml sealable reaction vial. The crude product was purified by chromatographing on a silicic acid column successively eluted with petroleum ether, petroleum ether/benzene (9:1), petroleum ether/benzene (8:2) and chloroform/methanol (3:1); the hexadecyl cholesteryl ether eluted in the 8:2 fraction. This step removes the majority of the unreacted hexadecylmethane sulfonate and dihexadecyl ether by-product. Final purification was effected by preparative thin layer chromatography (TLC) on silica gel plates developed in hexane/diethyl ether/acetic acid (80:20:1); the ether product migrated slightly ahead of cholesteryl oleate. The overall product yield was typically 60-80% of theoretical. The product was routinely greater than 95% pure by radioactivity and was resistant to acid hydrolysis in 7% HCl in methanol for 4 hr at 60 C, conditions that resulted in 93% hydrolysis of cholesteryl oleate.

Assay of Phospholipid Transfer Protein

Transfer of [^{14}C]PC from rat liver microsomes to unilamellar phospholipid vesicles was assayed according to Kamp and Wirtz (16).

Sheep lung soluble fraction prepared according to Robinson et al. (17) was the source of transfer protein activity. Unilamellar phospholipid vesicles were prepared by sonicating 98 mol % egg PC, 2 mol % dicetylphosphate and a trace of [^{3}H]cholesteryl ether (0.01 μCi/μmol of phospholipid) or [^{3}H]cholesteryl ester for 30 min in 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris/HCl, pH 7.4. Transfer of PC was calculated from the ^{14}C/^{3}H ratio of the recovered liposomes.

Assay of Vesicle-Cell Interactions

Transfer of [^{14}C]PC from phospholipid vesicles to V-79 cells and the fusion of the vesicles with the cells were assayed according to Huang et al. (7). Unilamellar vesicles were prepared by sonicating egg PC, [^{14}C]dioleoylphosphatidylcholine (Applied Science, State College, PA), hexadecyl [^{3}H]cholesteryl ether and [^{131}I]lysozyme (18) in Ca-Mg free Gey's medium (6). The sonicate was chromatographed on a Sephadex G75 column (1.5 cm × 34 cm) eluted with Ca-Mg free Gey's medium to separate the untrapped [^{131}I]lysozyme. The void volume peak containing the vesicles was pooled, diluted to 12 ml and the divalent cation concentration was restored. This preparation was immediately incubated with V-79 cells (10^7 cells/dish) in 60-mm dishes. After 60 min at 4 C or 37 C, the cells were washed 3 times with the Gey's medium, trypsinized for 15 min at 37 C and harvested. The cells were washed twice with the Gey's medium by centrifugation and the final pellet was dissolved in 1.5 ml 1% Triton X-100 containing 10 mM Tris/HCl, pH 7.8. This solution was transferred to a liquid scintillation vial and radioactivity was determined; raw counts for the 3 labels were corrected for background and channel efficiencies.

Miscellaneous Methods

For some experiments, 1-acyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine was prepared by the method of Waite and van Deenen (19). Hydrolysis of cholesteryl ether and ester was monitored on thin layer chromatograms and by determining radioactivity in the cholesteryl ester and free cholesterol regions. Total protein was determined by the procedure of Lowry et al. (20) using bovine serum albumin as a standard. Experimental details are given in the figure legend and in the footnotes to the tables.

RESULTS AND DISCUSSION

In many of the types of investigations which utilize lipid vesicles, it is essential to include a vesicle marker which will incorporate randomly into the lipid layer(s), will remain metabolically inert under the conditions of the experiment, will not freely exchange between membranes and thus will report the disposition of the vesicles and not behavior unique to individual vesicle constituents.

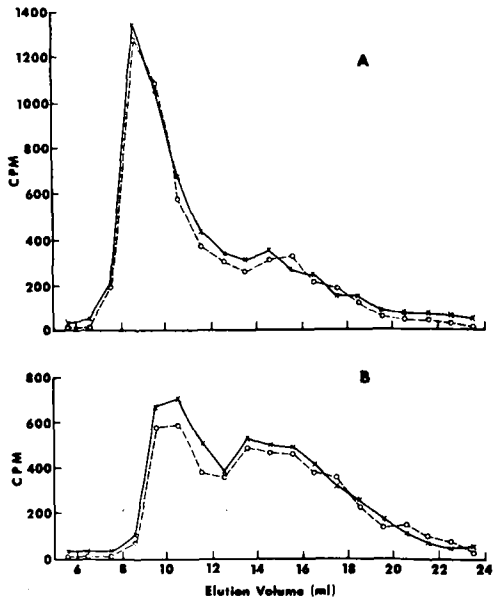


FIG. 1. Chromatography of sonicated liposomes on a Sepharose 4B column (1.0 cm × 32 cm). Liposomes were prepared with 98 mol % [^{14}C]phosphatidylcholine, 2 mol % dicetylphosphate and a trace (0.01 μCi/μmol phospholipid) of [^{3}H]hexadecyl cholesteryl ether by sonication for 5 min (panel A) or 30 min (panel B) in 0.25 M sucrose, 1 mM EDTA and 10 mM Tris/HCl, pH 7.4, and were eluted from the column with the same solvent. (×–×), [^{14}C]phosphatidylcholine; (o–o), [^{3}H]cholesteryl ether.

Chromatography of vesicles containing [^{14}C]PC and [^{3}H]cholesteryl ether on Sepharose 4B (Fig. 1) results in coelution of the 2 labeled lipids as evidenced by the constant ^{14}C/^{3}H ratio of the eluent. Sonication for 5 min (panel A) shows most of the radioactivity in the void volume (multilamellar liposomes) whereas, after 30 min of sonication, a significant portion of both labels enters the column as single bilayer liposomes. This demonstrates the homogeneous distribution of the cholesteryl ether throughout the range of vesicle sizes, paralleling the PC and showing that the ether is effectively reporting the distribution of vesicle sizes.

In another set of experiments, vesicles prepared by sonication were incubated with varying amounts of rat liver microsomes and the

TABLE 1

Effect of Increasing Microsome Concentration on Recovery of Vesicle Lipids[a]

Microsomal protein (mg)	Phosphatidylcholine recovery (%)	Cholesteryl ester recovery (%)	Cholesteryl ether recovery (%)
1.25	83	77	77
2.50	73	67	70
3.75	69	63	66

[a]Unlabeled rat liver microsomes were incubated with 1 μmol vesicle phospholipid (98 mol % [^{14}C]phosphatidylcholine, 2 mol % dicetylphosphate and 0.01 μCi of either [^{3}H]-cholesteryl ester or [^{3}H]cholesteryl ether). Incubation was in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris/HCl, pH 7.4, at a total volume of 2.5 ml for 30 min at 37 C. The incubation was stopped by the addition of 0.5 ml of 0.2 M sodium acetate, pH 5.0, containing 0.25 M sucrose and centrifugation at 15,000 X g for 10 min to pellet the microsomes. Vesicle lipids were recovered from the supernatants by Bligh and Dyer extraction (23) and radioactivity was determined.

TABLE 2

Protein-Catalyzed Transfer of [^{14}C]Phosphatidylcholine from Rat Liver Microsomes to Sonicated Liposomes Containing [^{3}H]Cholesteryl Ether or [^{3}H]Cholesteryl Ester Markers[a]

Vesicle marker	Apparent % of microsomal phosphatidylcholine transferred[b]	% of vesicle marker recovered as free cholesterol[c]
[^{3}H]cholesteryl ether	36.3	1.0
[^{3}H]cholesteryl ester	45.3	17.5

[a]Assay system consisted of phosphatidyl[N-methyl-^{14}C]choline-labeled rat liver microsomes (1.25 mg protein), 1 μmol vesicle phospholipid (98 mol % egg phosphatidylcholine, 2 mol % dicetylphosphate and 0.01 μCi of either [^{3}H]cholesteryl ether or ester) and sheep lung soluble fraction (2.3 mg protein). Incubation was in 2.5 ml containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris/HCl pH 7.4 for 30 min at 37 C. Incubation was stopped with the addition of 0.5 ml of 0.2 M acetate buffer pH 5.0 containing 0.25 M sucrose and centrifugation at 15,000 X g to pellet the microsomes. Vesicle lipids were extracted from the supernatants by the method of Bligh and Dyer (23) and radioactivity was determined.

[b]The apparent percentage of microsomal phosphatidylcholine transferred from microsomes to acceptor vesicles corrected for controls run in the absence of soluble fraction was calculated according to Kamp and Wirtz (16):

$$\%\ T = \frac{^{14}\text{C dpm}}{^{3}\text{H dpm}}\ \text{sample} \times \frac{^{3}\text{H dpm original vesicles}}{^{14}\text{C dpm original microsomes}} \times 100.$$

[c]Determined by thin layer chromatography as described in Materials and Methods.

recoveries of the [^{14}C]PC and [^{3}H]cholesteryl ether or ester in the vesicle fraction were monitored and results are shown in Table 1. The parallel recoveries of all 3 lipids demonstrates the lack of selective exchange or transfer of any one of the lipids from the vesicles to the microsomes under these conditions. This shows that the ether is not subject to selective diffusional or contact exchange with the microsomes; the modest loss of vesicles reflected in the incomplete recovery of all labels can be attributed to an apparent coprecipitation of intact vesicles with microsomes.

Table 2 shows the results obtained for the protein-catalyzed transfer of [^{14}C]PC from microsomes to unilamellar vesicles by sheep lung soluble fraction using either [^{3}H]cholesteryl ether or ester as the vesicle marker. Under these conditions, TLC showed that 17.5% of the cholesteryl ester was hydrolyzed to free cholesterol and fatty acid, whereas the cholesteryl ether marker was not altered upon incubation. The larger calculated percentage transfer of PC for the cholesteryl-ester-marked incubation is consistent with the observed hydrolysis of the ester and the subsequent diffusional

TABLE 3

Uptake of Radiolabeled Vesicle Markers by V-79 Cells[a]

Temperature (C)	Theoretical uptake (%)[b]		
	[^{131}I]	[^{3}H]	[^{14}C]
37	100 (3.9)	111 (24.2)	94 (3.2)
4	100 (0.73)	102 (4.14)	192 (1.21)

[a]Confluent monolayer cultures (about 10^7 cells/dish) of Chinese hampster cells were incubated with sonicated liposomes (1.46 mg [^{14}C]phosphatidylcholine, [^{131}I]lysozyme, and [^{3}H]cholesteryl ether) by gentle rocking for 60 min. Cells were washed, trypsinized and harvested as described in Methods.

[b]Percentage theoretical uptake was determined by comparison of radiolabels recovered in the cells with the radiolabels in the applied vesicles and using the [^{131}I]lysozyme trapped marker as reference (^{14}C/^{131}I=0.87 and ^{3}H/^{131}I=5.58 in applied vesicles). Numbers in parentheses are the actual cpm X 10^{-3}.

$$\% \text{ theoretical uptake } {}^{14}C = \frac{{}^{14}C \text{ cells}/{}^{131}I \text{ cells}}{0.87} \times 100$$

$$\% \text{ theoretical uptake } {}^{3}H = \frac{{}^{3}H \text{ cells}/{}^{131}I \text{ cells}}{5.58} \times 100.$$

redistribution of free labeled cholesterol to the microsomes (21). This redistribution of marker label results in a lower recovery of the radiolabeled marker in the vesicle fraction, giving an erroneously lower calculated recovery and, consequently, an elevated apparent transfer of PC. This effect becomes of even greater consequence in transfer experiments involving incubations for longer periods of time or requiring greater concentrations of soluble fraction.

The suitability of the cholesteryl ether as an inert marker for lipid vesicles in studies of the uptake of intact vesicles during interaction with whole cells was also assessed. The use of water-soluble radioactive markers trapped in the included volume of the vesicles is an alternative; however, the small trapping volume of liposomes necessitates the use of markers of high specific radioactivity and the leakage of the trapped marker during incubation with cells complicates the interpretation of the experimental results (11,12). The results of a triple-label experiment are shown in Table 3. Single bilayer vesicles containing [^{131}I]lysozyme as trapped marker with [^{14}C]PC and [^{3}H]cholesteryl ether as two independent lipid markers were incubated with V-79 cells at 37 C and the uptakes of both lipid markers were very close to that of the trapped marker, as indicated by the near theoretical values of the predicted uptake. This result is in complete agreement with those reported previously, in that the predominant mechanism of uptake at 37 C of small, sonicated liposomes by V-79 cells is fusion (7,22). At 4 C, however, the uptake of [^{14}C]PC was almost twice that of the trapped marker, whereas the uptake of the [^{3}H]cholesteryl ether was still the same as the trapped marker. It has been shown previously that the uptake of liposomal PC by cells at low temperature is predominantly by lipid exchange, whereas neither fusion nor endocytosis of intact liposomes occurs (7,22). The residual uptake of vesicles by cells at low temperature probably is due to adsorption of intact vesicles to the cell. The ability of the [^{3}H]hexadecyl cholesteryl ether to report the fusion events at 37 C and its lack of exchange with cellular lipids at 4 C directly indicate the suitability of the ether as a faithful marker for vesicles with advantages over the trapped markers.

The variety of data presented here demonstrates that radiolabeled cholesteryl ether can serve as an effective liposomal marker. It is readily synthesized from radiolabeled cholesterol and a stable stock of hexadecylmethane sulfonate or other desired sulfonates. Because cholesterol of high specific radioactivity (^{3}H or ^{14}C) is commercially available, the specific activity of the synthesized cholesteryl ether can be adjusted to the requirements of the particular investigation. The cholesteryl ether has the advantages of metabolic inertness, lipid solubility and nonexchangeability; these ensure an efficient and faithful representation of the liposomes, and it therefore has potential use in a variety of both in vivo and in vitro investigations.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants HL 21176, CA 24553 and GM 23473.

REFERENCES

1. Papahadjopoulos, D. (1978) Ann. N.Y. Acad. Sci. 308, 1-462.
2. Zilversmit, D.B., and Hughes, M.E. (1976) Methods Membr. Biol. 7, 211-259.
3. DeHaas, G.H., Slotboom, A.J., and Verheij, H.M. (1977) Expo. Annu. Biochim. Med. 33, 191-211.
4. Johnson, L.W., Hughes, M.E., and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176-185.
5. DiCorleto, P.E., and Zilversmit, D.B. (1979) Biochim. Biophys. Acta 552, 114-119.
6. Huang, L., and Pagano, R.E. (1975) J. Cell Biol. 67, 38-78.
7. Huang, L., Ozato, K., and Pagano, R.E. (1978) Membr. Biochem. 1, 1-25.
8. Pagano, R.E., and Weinstein, J.N. (1978) Annu. Rev. Biophys. Bioeng. 7, 435-468.
9. DiCorleto, P.E., and Zilversmit, D.B. (1977) Biochemistry 16, 2145-2150.
10. Wirtz, K.W.A., Vriend, G., and Westerman, J. (1979) Eur. J. Biochem. 94, 215-221.
11. Szoka, F.C., Jacobson, K., and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 551, 295-303.
12. Van Renswoude, A.J.B.M., Westenberg, P., and Scherphof, G.L. (1979) Biochim. Biophys. Acta 558, 22-40.
13. Stein, O., Halperin, G., and Stein, Y. (1980) Biochim. Biophys. Acta 620, 247-260.
14. Baumann, W.J., and Mangold, H.K. (1964) J. Org. Chem. 29, 3055-3057.
15. Paltauf, F. (1968) Monatsh. Chem. 99, 1277-1280.
16. Kamp, H.H., and Wirtz, K.W.A. (1974) Methods Enzymol. 32, 140-146.
17. Robinson, M.E., Wu, L.N.Y., Brumley, G.W., and Lumb, R.H. (1978) FEBS Lett. 87, 41-44.
18. Adrian, G., and Huang, L. (1979) Biochemistry 18, 5610-5614.
19. Waite, M., and van Deenen, L.L.M. (1967) Biochim. Biophys. Acta 137, 498-517.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
21. Bloj, B., and Zilversmit, D.B. (1977) Biochemistry 16, 3943-3948.
22. Pagano, R.E., and Huang, L. (1975) J. Cell Biol. 67, 49-60.
23. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.

[Received November 20, 1981]

An Improved Procedure for the Synthesis of Choline Phospholipids via 2-Bromoethyl Dichlorophosphate[1]

WILLIAM J. HANSEN, RAMASWAMY MURARI, YURI WEDMID and **WOLFGANG J. BAUMANN***, *Section of Bio-Organic Chemistry, The Hormel Institute, University of Minnesota, Austin, MN 55912*

ABSTRACT

Choline phospholipids can be conveniently synthesized by reaction of a lipophilic alcohol, such as diacylglycerol, with 2-bromoethyl dichlorophosphate followed by nucleophilic displacement of the bromine with trimethylamine. We found that the low yields often encountered in the initial phosphorylation step are particularly due to exchange of both chlorines for alkoxy functions (triester formation) and to chlorination of the alcohol by 2-bromoethyl dichlorophosphate. However, these drawbacks can be overcome by proper choice of the reaction medium and by optimizing other reaction conditions. The procedure described is efficient and most versatile, and it lends itself to the preparation of a wide range of choline phospholipids containing a glycerol, diol, or long-chain alkyl backbone and bearing various aliphatic functions. Proton and carbon-13 nuclear magnetic resonance spectroscopy proved useful in establishing the homogeneity and structures of the synthetic intermediates and byproducts and of the choline phospholipids synthesized.
Lipids 17:453-459, 1982.

Studies on the structures, interactions and dynamics in biological and model membranes are of much current interest and have created considerable demand for defined synthetic phospholipids. Phosphatidylcholines (PC; 1,2-di-*O*-acyl-*sn*-glycero-3-phosphocholine) are often prepared semisynthetically by reacylation (1-10) of *sn*-glycero-3-phosphocholine (11-15) whereas the synthesis of specific ether analogs of PC (16-22) or of choline phospholipids containing a long-chain alkyl group or a substituted diol (23-29) as backbone usually require total chemical synthesis. This also holds true for insertion of specific labels into the polyol backbone or into the polar headgroup of choline phospholipids.

In this study, we have reinvestigated the choline phospholipid synthesis via 2-bromoethyl dichlorophosphate as originally outlined by Hirt and Berchtold (30). The procedure involves phosphorylation of a lipophilic alcohol, such as diacylglycerol, with 2-bromoethyl dichlorophosphate, followed by exchange of the 2-halogen function with trimethylamine (20-22, 28-31). In principle, this procedure is convenient and most versatile. Yet, we found that phosphorylation of the backbone alcohol with bromoethyl dichlorophosphate, which usually is done in chloroform (20,28,30) in the presence of a base, tends to lead to triester formation, i.e., nucleophilic substitution of both chlorines by alkoxy functions. We observed, in fact, that triester formation is actually favored unless the reaction is sterically hindered by the bulkiness of the lipophilic backbone alcohol. We also found that the excess amount of phosphorylating reagent required and the elevated temperatures and long reaction times commonly used to bring the condensation to completion do cause chlorination of the backbone alcohol unless certain precautions are taken.

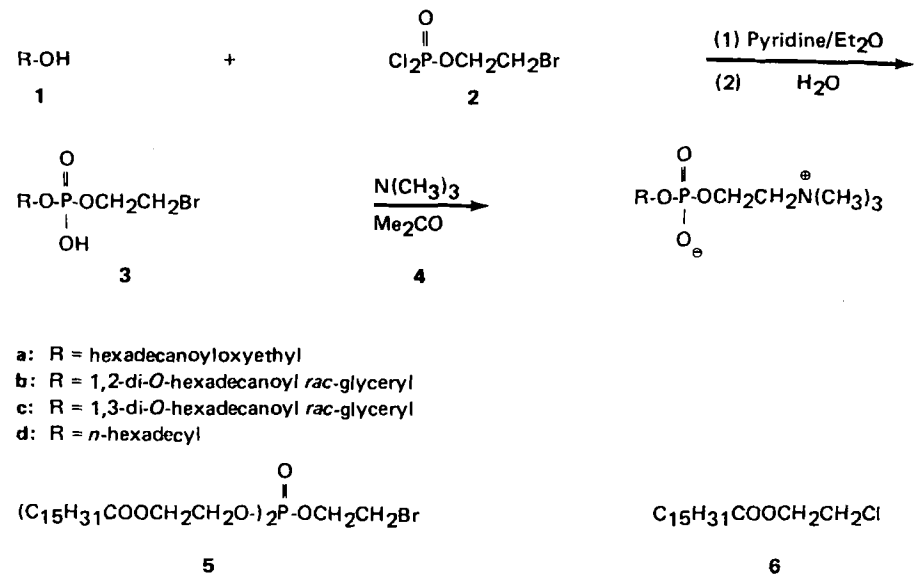
The synthesis presented here avoids triester formation as well as chlorination through proper choice of reaction conditions. The method is convenient and versatile and permits the efficient synthesis of a wide variety of choline phospholipids bearing 1 or 2 long-chain moieties. Proton and, in particular, carbon-13 nuclear magnetic resonance (NMR) spectroscopy of the synthetic phospholipids in solution proved useful for verifying structural assignments (32,33) and to ascertain the homogeneity of preparations. Assignments of ^{13}C NMR signals were aided by specific deuteration, and by measuring ^{13}C-^{14}N and ^{13}C-^{31}P couplings (33).

RESULTS AND DISCUSSION

Condensation of various lipophilic alcohols (**1a-1d**) with 2-bromoethyl dichlorophosphate (2) and subsequent hydrolytic cleavage of the remaining phosphochloride bond affords the respective 2-bromoethyl phosphate intermediates **3a-3d** which, in turn, are reacted with trimethylamine to form choline phospholipids **4a-4d** (see Scheme 1).

We found that the condensation proceeds smoothly when the alcohol (1) is reacted in the presence of pyridine with three equivalents of

[1]Preliminary data were presented at the ISF/AOCS World Congress, New York, NY 1980; Baumann, W.J., Parthasarathy, S., Wedmid, Y., and Murari, R. (1980) *J. Am. Oil Chem. Soc.*, 57 (2), abstract no. 292.



a: R = hexadecanoyloxyethyl
b: R = 1,2-di-*O*-hexadecanoyl *rac*-glyceryl
c: R = 1,3-di-*O*-hexadecanoyl *rac*-glyceryl
d: R = *n*-hexadecyl

SCHEME 1

the phosphorylating reagent 2 using absolute diethyl ether as solvent. With *O*-hexadecanoyl ethanediol (1a), 1,2-di-*O*-hexadecanoyl glycerol (1b) or hexadecanol (1d), the reaction is essentially quantitative (as judged by thin layer chromatography [TLC]) after 4 hr at reflux temperature (isolated yields, 76-85%). However, the condensation of the sterically hindered secondary alcohol 1,3-di-*O*-hexadecanoyl glycerol (1c) produced only 18% of 3c under these conditions; a longer reaction time (45 hr) was required to afford acceptable yields (62%).

Condensations of this type usually have been done in chloroform (20,28,30) which adequately dissolves the reaction components 1 and 2, as well as the reaction product. We observed, however, that use of chloroform as a reaction medium not only required 3-4 times longer reaction times (20,30) but, more importantly, produced substantial quantities of byproducts. Thus, condensation of the ethanediol monoester 1a with 2-bromoethyl dichlorophosphate (2) in chloroform predominantly formed the phosphoric acid triester 5 (55% yield), and similarly, the reaction of hexadecanol (1d) with 2 in chloroform caused exchange of both chlorines by the respective alkoxy groups. This type of triester formation is also known to be favored when single-chain alcohols, such as ethanediol monoester 1a, are reacted in chloroform with other phosphorylating reagents, e.g., with phenyl dichlorophosphate (26). We now have shown that such double substitution at the phosphorus and triester formation are actually favored in chloroform unless they are hindered by the bulkiness of R, as is the case with diacyl glycerols 1b and 1c, e.g.

We furthermore found that the reaction of alcohol 1 with the phosphorylating reagent 2 in chloroform tends to cause chlorination of the alcohol; thus, ethanediol monoester 1a was chlorinated to produce 6. Chlorination was favored at higher temperature, with extended reaction times, and with increasing quantities of phosphorylating reagent 2 used. 1,2-Diacylglycerol 1b was particularly susceptible to chlorination unless mild reaction conditions were chosen.

Ease and specificity of the phosphorylation in diethyl ether appears to be largely due to the fact that diethyl ether readily dissolves the reagents 1 and 2, but that the phosphorylation product is precipitated from the reaction mixture. Low solubility of the phosphate diester in diethyl ether, but not in chloroform, apparently prevents exchange of the second chlorine.

Final displacement of the bromine of 3a-3d by the quaternary amine group is readily accomplished by reaction with trimethylamine in anhydrous acetone for 4 hr, and the resulting choline phospholipids 4a-4d are purified by preparative TLC (isolated yields, 62-85%). Use of chromatography for the isolation of the final products and avoidance of more traditional

complexing, precipitation and recrystallization procedures greatly simplifies the purification and renders the procedures generally applicable.

Use of a phosphorylating reagent, such as 2-bromoethyl dichlorophosphate (2), which already contains the headgroup skeleton, proved, in our hands, less laborious and more efficient than initial introduction of the phosphoric acid moiety followed by coupling to the choline base (17-19,26,27,34-40). The procedure described has proven applicable to the preparation of a wide range of choline phospholipids which contained a glycerol, diol or long-chain alkyl backbone and which bore saturated or unsaturated *O*-acyl or *O*-alkyl moieties of various chain lengths (33).

EXPERIMENTAL

Melting points (mp) were determined on a Kofler hot stage and are corrected. Elemental analyses were done by M-H-W Laboratories, Phoenix, AZ. Phosphorus was determined using standard digestion (41) and assay (42) procedures. Solvents were prepared as follows: diethyl ether was refluxed over and distilled from $LiAlH_4$, then stored over Na wire; acetone was refluxed over and distilled from anhyd $CaSO_4$, then stored over anhyd Na_2SO_4; pyridine was refluxed over, distilled from, and stored over fresh KOH pellets.

Analytical and preparative TLC was done on layers of Silica Gel H (Merck), 0.3 and 3 mm thick, respectively. For analytical purposes, plates were developed in tanks lined with filter paper, and fractions were made visible by spraying with a solution prepared from 6 g potassium dichromate in 1 ℓ of 55% aq sulfuric acid followed by charring; phosphorus-containing fractions were made visible also with molybdenum spray reagent (43). In preparative work, ca. 200-250 mg of material was applied per 20 × 20 cm plate, and after developing, lipid fractions were visible as opalescent bands; these were scraped off, and the lipids were eluted.

Infrared (IR) spectra were recorded with a Perkin-Elmer Model 21 spectrophotometer using CS_2 and C_2Cl_4 (2400-2000 cm^{-1} and 1650-1400 cm^{-1}) or KBr pellets. Relative intensities of IR absorption bands are given: s, strong; m, medium; sh, shoulder; weak bands are without designation. The following bands associated with vibrations of the aliphatic chains occur in all the spectra quoted and are not listed individually: 2925-2905 (s) and 2850-2835 cm^{-1} (s), νCH of CH_2; 1485-1465 (m), δCH of CH_2; 1448-1445 (sh or weak) and 1380-1375 (m), asymmetrical and symmetrical δCH of CCH_3, respectively; 719-717 (m), CH rocking vibration of $(CH_2)_n$.

Carbon-13 NMR spectra were recorded at 20 MHz, proton NMR spectra at 79.54 MHz, using Varian CFT-20 and FT-80A pulse Fourier transform instruments. ^{13}C spectra were measured on 30-100 mg/ml samples (8- or 10- mm sample tubes) at the ambient probe temperature of 37 ± 1 C; $CDCl_3/CD_3OD/D_2O$ (50:50:15) (32,33), $CDCl_3/CD_3OD$ (4:1), or $CDCl_3$ served as solvent and for field frequency locking purposes. ^{13}C NMR spectra were obtained with broadband proton decoupling using 8K data points for a spectral width of 4000 Hz. All chemical shifts are expressed in ppm downfield from Me_4Si used as internal standard.

1-*O*-Hexadecanoyl ethanediol (**1a**) was prepared by acylation of 1,2-ethanediol with hexadecanoyl chloride (Nu-Chek-Prep, Elysian, MN) (44). 1,2-Di-*O*-hexadecanoyl-*rac*-glycerol (**1b**) was synthesized by acylation of 1-*O*-tetrahydropyranyl-*rac*-glycerol (45) with hexadecanoyl chloride followed by acid-catalyzed hydrolytic removal of the protective group (46). 1,3-Di-*O*-hexadecanoyl-*rac*-glycerol (**1c**) was purchased from Nu-Chek-Prep. Hexadecanol (**1d**) was prepared by $LiAlH_4$ reduction (47) of methyl hexadecanoate (Nu-Chek-Prep).

2-Bromoethyl dichlorophosphate 2 (30) was prepared by dropwise addition of freshly distilled 2-bromoethanol (0.5 mol; Eastman, Rochester, NY) to an ice-cooled solution of freshly distilled phosphorus oxychloride (0.5 mol; Eastman) in dry chloroform over a 1-hr period followed by 5 hr at reflux, and vacuum distillation (bp 66-68 C at 0.4-0.5 mm Hg). The reagent was stored (-20 C) under nitrogen in small sealed ampoules prior to use.

1-*O*-Hexadecanoyl 2-*O*-(2′-Bromoethyl)phosphoryl Ethanediol (3a)

2-Bromoethyl dichlorophosphate (3.63 g, 15 mmol) in 75 ml of anhyd Et_2O was placed into a 500-ml, 3-necked flask equipped with heating mantle, magnetic stirrer, reflux condenser and drying tube, dropping funnel, and an inlet tube for dry N_2. After cooling in an ice bath, 6 ml of dry pyridine was added with stirring followed by dropwise addition of 1.5 g (5 mmol) of 1-*O*-hexadecanoyl 1,2-ethanediol (44) in 75 ml anhyd Et_2O over a 30-min period. The mixture was allowed to come to room temperature (30 min) and then was refluxed gently for 4 hr. After cooling in an ice bath, 6 ml of water was added by drops and stirring was continued for 1 hr. The solvent was removed on a rotary evaporator, the residue was taken up in 100 ml of $CHCl_3$/MeOH (2:1, v/v), and the solution was extracted with 20 ml of water while the pH of the organic phase was kept at 5 by addition of

2 N H_2SO_4. The aqueous phase was re-extracted with 2 portions of 50 ml of $CHCl_3$/MeOH, (2:1, v/v) and the combined organic phases were evaporated. The freeze-dried residue (3.6 g) was purified on 3-mm layers of Silica Gel H (developing solvent, $CHCl_3$/MeOH/H_2O, 55:33:6, v/v/v), and the fraction (R_f 0.45-0.80) was eluted from the adsorbent with $CHCl_3$/MeOH/H_2O (50:48:2, v/v/v). Evaporation of the solvent afforded 1.84 g of **3a** (76%); mp 61-62 C (coalesces; contracts at 45-46 C). IR (KBr) 1727(s), 1246(s), 1179(m), 1110(s), 1088(s), 961(m), 906, 789 cm^{-1}. ^{13}C NMR ($CDCl_3$/CD_3OD, 4:1, v/v), 174.70 (C=O), 65.86 (*d*, $^2J_{CP}$ 5.5 Hz, $PO\underline{CH_2}CH_2Br$), 64.34 (*d*, $^3J_{CP}$ 7.7 Hz, CH_2OCOR), 64.06 (*d*, $^2J_{CP}$ 3.6 Hz, CH_2OP of glycol), 34.56 (C-2′), 32.32 (ω-2 CH_2), 31.20 (*d*, $^3J_{CP}$ 6.6 Hz, CH_2Br), 29.97 (-$[CH_2]_n$-), 25.29 (C-3′), 22.96 (ω-1 CH_2), 14.15 (ωCH_3). ^1H NMR ($CDCl_3$/CD_3OD, 4:1, v/v), CH_2Br signal at 3.53 (*t*,*J* 5.6 Hz). Anal. Calcd for $C_{20}H_{40}O_6PBr$: C, 49.29; H, 8.27; P, 6.35. Found: C, 48.98; H, 8.71; P, 6.60.

1-*O*-Hexadecanoyl 2-[2′-(*N,N,N*-Trimethylamino)-ethyl] phosphoryl Ethanediol (4a)

Bromide **3a** (1.84 g, 3.78 mmol) was transferred in chloroform into a 100-ml, thick-walled glass bulb, the solvent was removed with N_2, and the sample was dried in vacuo. Anhyd acetone (80 ml) was added, the bulb was cooled in dry ice, and 8 ml (90 mmol) of anhyd trimethylamine (Eastman) was added with a pre-cooled syringe. (This is readily done in a -20 C cold room.) The bulb was briefly flushed with N_2, sealed, and was placed in a shaking water bath (caution: use protective shield) at 75 C for 4 hr. After cooling in dry ice, the bulb was *carefully* opened, and the solvent was removed with N_2. The residue was taken up in 200 ml of $CHCl_3$/MeOH (2:1, v/v) and extracted with 40 ml water, the pH of the organic phase was adjusted to 5 with 2 N H_2SO_4, the aqueous phase was separated and re-extracted with 2 portions of 100 ml of $CHCl_3$/MeOH (2:1, v/v), the organic phases were combined, and the solvent was removed under reduced pressure. The residue was purified by preparative TLC (developing solvent, $CHCl_3$/MeOH/H_2O, 55:33:6, v/v/v) and the fraction (R_f 0.09-0.18) was eluted with $CHCl_3$/MeOH/H_2O (50:40:10, v/v/v). Removal of the solvent and lyophilization of the residue from benzene/MeOH (99:1, v/v) gave 1.26 g (2.7 mmol) of **4a** in 71% yield. The product was free of bromide as judged by a silver nitrate test; mp 242-243 C (coalesces; contracts and becomes translucent above 65 C). IR (KBr) 1723(s), 1247(s), 1178(m), 1090(s), 966(s), 925, 873, 789(m). ^{13}C NMR ($CDCl_3$/CD_3OD/D_2O, 50:50:15, v/v/v), 174.97 (C=O), 66.83 (*dt*, $^3J_{CP}$ 7.2 Hz, J_{CN} 3.5 Hz, CH_2N), 64.26 (*d*, $^3J_{CP}$ 7.3 Hz, CH_2OCOR), 63.95 (*d*, $^2J_{CP}$ 4.9 Hz, CH_2OP of glycol), 59.73 (*d*, $^2J_{CP}$ 4.7 Hz, $POCH_2$ of choline), 54.50 (*t*, J_{CN} 3.5 Hz, $N[CH_3]_3$), 34.38 (C-2′), 32.26 (ω-2 CH_2), 30.01 (-$[CH_2]_n$-), 25.22 (C-3′), 22.98 (ω-1 CH_2), 14.18 (ωCH_3). The ^{13}C NMR spectrum of acyl diol phosphocholine synthesized from d_4-ethanediol, was essentially identical to that of **4a** except that the signals at 64.26 (CH_2 OCOR) and 63.95 (CH_2OP of glycol) were absent. Anal. Calcd for $C_{23}H_{48}O_6NP \cdot H_2O$: C, 57.12; H, 10.42; N, 2.90; P, 6.40. Found: C, 57.12; H, 10.56; N, 2.78; P, 6.60.

Side Products of the Phosphorylation Reaction

Reaction of ethanediol monoesters **1a** with 2-bromoethyl dichlorophosphate (**2**) in chloroform leads to 2 major side-products **5** and **6**. Chloroform as reaction medium favors formation of *bis*(hexadecanoyloxyethyl) 2-bromoethyl phosphate **5**. Excess phosphorylating reagent **2** favors formation of 1-*O*-hexadecanoyl 2-chloroethanol **6**. Both side products were isolated from the reaction mixture by TLC; chloro compound **6** was also prepared by chlorination of ethanediol monoesters **1a** with thionyl chloride.

Bis(hexadecanoyloxyethyl) 2-Bromoethyl Phosphate (5)

The compound was isolated by TLC (R_f 0.5) using diethyl ether as developing solvent; mp 48.5-49.5 C. IR (CS_2/C_2Cl_4) 1737(s), 1352, 1291(s), 1273(s), 1236(sh), 1167(s), 1114, 1064(s), 1019(s), 982(s), 850, 796. ^{13}C NMR ($CDCl_3$) 173.48 (C=O), 66.97 (*d*, $^2J_{CP}$ 5.2 Hz, $PO\underline{CH_2}CH_2Br$), 65.91 (*d*, $^2J_{CP}$ 5.7 Hz, CH_2OP of glycol), 62.62 (*d*, $^3J_{CP}$ 7.1 Hz, CH_2OCOR), 34.10 (C-2′), 31.94 (ω-2 CH_2), 29.68 (-$[CH_2]_n$-, and CH_2Br), 24.87 (C-3′), 22.70 (ω-1 CH_2), 14.10 (ωCH_3). ^1H NMR ($CDCl_3$) 4.28 (*m*, CH_2OP and CH_2OCOR), 3.51 (*t*, 2H, *J* 6.2 Hz, CH_2Br), 2.32 (*t*, 2H, *J* 7.0 Hz, CH_2CO), 1.25 (broad band, -$[CH_2]_n$-), 0.87 (t, *J* 4.9 Hz, CH_3). Anal. Calcd for $C_{38}H_{74}O_8PBr$: C, 59.28; H, 9.69; P, 4.02. Found: C, 59.06; H, 10.04; P, 3.90.

1-*O*-Hexadecanoyl 2-Chloroethanol (6)

The product was isolated by TLC (R_f = 0.44) using hexane/diethyl ether (95:5, v/v) as developing solvent. The compound was also prepared by reaction of 1-*O*-hexadecanoyl ethanediol (**1a**) with thionyl chloride at reflux temperature for 3 hr (48) followed by TLC purification; mp 40-41 C. IR (CS_2/C_2Cl_4) 1735(s),

1342, 1296(m), 1161(s), 1116(m), 1027, 673. ^{13}C NMR ($CDCl_3$) 173.44 (C=O), 63.81 (CH_2-OCOR), 41.62 (CH_2Cl), 34.13 (C-2′), 31.94 (ω-2 CH_2), 29.68 (-[CH_2]$_n$-), 24.93 (C-3′), 22.71 (ω-1 CH_2), 14.10 (ωCH_3). ^1H NMR ($CDCl_3$) 4.29 (*t*, 2H, *J* 5.5 Hz, CH_2OCOR), 3.63 (*t*, 2H, *J* 6.2 Hz, CH_2Cl), 2.32 (*t*, 2H, *J* 6.9 Hz, C-2′), 1.25 (-[CH_2]$_n$-), 0.87 (*t*,*J* 5.2 Hz, CH_3). Anal. Calcd for $C_{18}H_{35}O_2Cl$: C, 67.79; H, 11.06. Found: C, 67.70; H, 11.05.

1,2-Di-*O*-hexadecanoyl 3-*O*-2′-Bromoethyl)phosphoryl *rac*-Glycerol (3b)

2-Bromoethyl dichlorophosphate (0.73 g, 3 mmol) in 30 ml of anhyd Et_2O, 1.5 ml of dry pyridine, and 0.57 g (1 mmol) of 1,2-di-*O*-hexadecanoyl *rac*-glycerol (**1b**) in 30 ml of anhyd Et_2O were reacted as described for the preparation of **3a**, except that diglyceride **1b** was added over a 30-min period without cooling to avoid its precipitation. After stirring for another 30 min at room temperature followed by reflux for 4 hr, the mixture was cooled and the intermediate was hydrolyzed by addition of 3 ml of water. Solvent removal and extraction (see preparation of **3a**), and preparative TLC (developing solvent, $CHCl_3$/MeOH, 90:20, v/v; R_f 0.4-0.8) afforded 0.60 g of **3b** (79% yield); mp 164-166 C (coalesces; translucent at 50-52 C). IR (KBr) 1733(s), 1246(s), 1174(m), 1113(*s*), 1091(*s*), 1021(m), 953, 908, 829. ^{13}C NMR ($CDCl_3$/CD_3OD/D_2O, 50:50:15, v/v/v) 174.58 (C=O), 71.01 (*d*, $^3J_{CP}$ 8.5 Hz, CHOCOR), 65.89 (*d*, $^2J_{CP}$ 4.5 Hz, $POCH_2CH_2Br$), 64.13 (*d*, $^2J_{CP}$ 4.4 Hz, CH_2OP of glycerol), 63.17 (CH_2OCOR), 34.68 and 34.54 (C-2′), 32.29 (ω-2 CH_2), 31.25 (*d*, $^3J_{CP}$ 8.1 Hz, CH_2-Br), 30.03 (-[CH_2]$_n$-), 25.30 (C-3′), 23.00 (ω-1 CH_2), 14.21 (ωCH_3). Anal. Calcd for $C_{37}H_{72}$-O_8PBr: C, 58.80; H, 9.60; P, 4.10. Found: C, 59.08; H, 9.84; P, 4.00.

1,2-Di-*O*-hexadecanoyl 3-*O*-[2′-(*N,N,N*-Trimethylamino)ethyl] phosphoryl *rac*-Glycerol (4b)

Bromide **3b**(0.36 g, 0.47 mmol), 30 ml of anhyd acetone, and 1 ml (11.3 mmol) of trimethylamine were reacted in a sealed 50-ml ampoule at 75 C for 4 hr as described for the preparation of **4a**. Extraction and purification by TLC (R_f 0.30-0.45) gave 0.22 g of **4b** (62% yield); mp 224-225 C (coalesces; translucent globules at 80 C); lit. (34), mp 227-229 C; lit. (30), mp 220 C. IR (KBr) 1728(s), 1252(s), 1200, 1174(m), 1093(s), 1067(s), 968(m). ^{13}C NMR ($CDCl_3$/CD_3OD/D_2O, 50:50:15, v/v/v) 174.66, 174.37 (C=O), 70.97 (*d*, $^3J_{CP}$ 7.7 Hz, CHOCOR), 66.85 (*dt*, $^3J_{CP}$ 7.2 Hz, J_{CN} 3.5 Hz, CH_2N), 64.08 (*d*, $^2J_{CP}$ 4.5 Hz, CH_2OP of glycerol), 63.30 (CH_2OCOR), 59.71 (*d*, $^2J_{CP}$ 3.9 Hz, $POCH_2$ of choline), 54.52 (*t*, J_{CN} 3.5 Hz, $N[CH_3]_3$), 34.55 (br, C-2′), 32.29 (ω-2), 30.03 (-[CH_2]$_n$-), 25.30 (C-3′), 23.00 (ω-1 CH_2), 14.20 (ωCH_3). Anal. Calcd for $C_{40}H_{80}$-$O_8NP \cdot H_2O$: C, 63.88; H, 10.99; N, 1.86; P, 4.12. Found: C, 63.70; H, 10.71; N, 1.59; P, 4.30.

1,3-Di-*O*-hexadecanoyl 2-*O*-(2′-Bromoethyl)phosphoryl *rac*-Glycerol (3c)

2-Bromoethyl dichlorophosphate (0.47 g, 1.95 mmol) in 50 ml of anhyd Et_2O, 2 ml of dry pyridine, and 0.37 g (0.65 mmol) 1,3-di-*O*-hexadecanoyl *rac*-glycerol (**1c**) in 50 ml of anhyd Et_2O were reacted as described for **3a**, except that diglyceride **1c** was added without cooling and that the reflux period was extended from 4 to 45 hr. After hydrolysis and extraction, preparative TLC (developing solvent $CHCl_3$/MeOH, 90:20, v/v; R_f 0.4-0.8) gave 0.30 g of **3c** (62% yield); mp 158-160 C (coalesces; translucent droplets at 50 C). IR (KBr) 1729(s), 1417, 1400, 1247(s), 1199, 1180(m), 1118(m), 1091(s), 1015(m), 953, 908, 801. ^{13}C NMR ($CDCl_3$) 174.00 (C=O), 71.16 (CHOP), 65.91 ($POCH_2$), 63.46 (CH_2OCOR), 34.29 (C-2′), 32.02 (ω-2 CH_2), 31.44 (*sh*, CH_2Br), 29.79 (-[CH_2]$_n$-), 25.00 (C-3′), 22.74 (ω-1 CH_2), 14.08 (ωCH_3). Anal. Calcd for $C_{37}H_{72}O_8PBr$: C, 58.80; H, 9.60; P, 4.10. Found: C, 58.62; H, 9.80; P, 3.90.

1,3-Di-*O*-hexadecanoyl 2-*O*-[2′-(*N,N,N*-Trimethylamino)ethyl] phosphoryl *rac*-Glycerol (4c)

Bromide **3c** (0.30 g, 0.4 mmol), 20 ml anhyd acetone, and 0.9 ml (10 mmol) of anhyd trimethylamine were reacted in a sealed 50-ml ampoule at 75 C for 4 hr as described for the preparation of **4a**. Extraction and purification by TLC (R_f 0.23-0.45) gave 0.25 g of **4c** (85% yield); mp 213-214 C (coalesces; translucent droplets at 75 C). IR (KBr) 1728(s), 1252(s), 1201, 1177(m), 1091(s), 1062(s), 971(m), 804. ^{13}C NMR ($CDCl_3$/CD_3OD/D_2O, 50:50:15, v/v/v) 174.64 (C=O), 71.20 (*d*, $^2J_{CP}$ 4.9 Hz, CHOP), 66.95 (*dt*, $^3J_{CP}$ 7.9 Hz, J_{CN} 3.5 Hz, CH_2N), 63.31 (*d*, $^3J_{CP}$ 3.9 Hz, CH_2OCOR), 59.72 (*d*, $^2J_{CP}$ 4.6 Hz, $POCH_2$), 54.50 (*t*, J_{CN} 3.5 Hz, $N[CH_3]_3$), 34.46 (C-2′), 32.30 (ω-2 CH_2), 30.02 (-[CH_2]$_n$-), 25.28 (C-3′), 23.02 (ω-1 CH_2), 14.20 (ωCH_3). Anal. Calcd for $C_{40}H_{80}O_8NP \cdot H_2O$: C, 63.88; H, 10.99; N, 1.86; P, 4.12. Found: C, 63.66; H, 10.92; N, 1.54; P, 3.90.

Hexadecyl 2-Bromoethyl Phosphate (3d)

2-Bromoethyl dichlorophosphate (3.65 g, 15 mmol) in 80 ml of anhyd Et_2O, 5 ml of dry pyridine, and 1.2 g (5 mmol) of hexadecanol (**1d**)

in 80 ml of anhyd Et_2O were reacted in analogy to the preparation of 3a. Hydrolysis of the intermediate and extraction (see 3a) followed by preparative TLC (developing solvent $CHCl_3$/MeOH/H_2O, 65:25:4, v/v/v; R_f 0.25-0.70) produced 1.68 g of 3d (85% yield); mp 98-99 C (coalesces; translucent at 84-86 C). IR (KBr) 1228(s), 1084(s), 1015(m), 954, 912. ^{13}C NMR ($CDCl_3$) 66.58-65.84 (*m*, both CH_2OP), 32.00 (ω-2 CH_2), 31.63-30.70 (m, CH_2Br and C-2'), 29.85 (-[CH_2]$_n$-), 26.02 (C-3'), 22.73 (ω-1 CH_2), 14.09 (ωCH_3). Anal. Calcd for $C_{18}H_{38}O_4PBr$: C, 50.35; H, 8.92; P, 7.21. Found: C, 50.41; H, 8.62; P, 7.30.

Hexadecyl 2-(*N,N,N*-Trimethylamino)ethyl Phosphate (4d)

Bromide 3d (1.0 g, 2.5 mmol), 40 ml of anhyd acetone, and 5.4 ml (60 mmol) of trimethylamine were reacted in a sealed 100-ml ampoule at 75 C for 4 hr as described for 4a. Extraction and purification by TLC (R_f 0.1-0.25) produced 0.64 g of 4d (67% yield); mp 232-234 C (decomposes; discoloration at 225 C). IR (KBr) 1243(s), 1083(s), 1063(s), 968(s), 921, 872, 821(m). ^{13}C NMR ($CDCl_3$/CD_3OD/D_2O, 50:50:15, v/v/v) 66.66 (*m*, CH_2N), 66.50 (*d*, $^2J_{CP}$ 5.8 Hz, CH_2OP of alkyl), 59.43 (*d*, $^2J_{CP}$ 4.7 Hz, $POCH_2$ of choline), 54.46 (*t*, J_{CN} 3.5 Hz, $N[CH_3]_3$), 32.24 (ω-2 CH_2), 31.10 (*d*, $^3J_{CP}$ 7.5 Hz, C-2'), 30.01 (-[CH_2]$_n$-), 26.11 (C-3'), 22.96 (ω-1 CH_2), 14.16 (ωCH_3). Anal. Calcd for $C_{21}H_{46}O_4NP \cdot H_2O$: C, 59.26; H, 11.37; n, 3.29; P, 7.28. Found: C, 58.75; H, 11.61; N, 3.17; P, 7.10.

ACKNOWLEDGMENTS

This investigation was supported in part by U.S. Public Health Service Research Grant HL 08214 from the Program Project Branch, Extramural Programs, National Heart, Lung and Blood Institute, by USPHS Research Grants CA 12150 from the National Cancer Institute and NS 14304 from the National Institute of Neurological and Communicative Disorders and Stroke, and by the Hormel Foundation. We also thank J.M. Hawkins and T.H. Madson for help in some of the experiments.

REFERENCES

1. Baer, E., and Buchnea, D. (1959) Can. J. Biochem. Physiol. 37, 953-959.
2. Kögl, F., de Haas, G.H., and van Deenen, L.L.M. (1960) Rec. Trav. Chim. 79, 661-674.
3. Cubero Robles, E., and van den Berg, D. (1969) Biochim. Biophys. Acta 187, 520-526.
4. Pugh, E.L., and Kates, M. (1975) J. Lipid Res. 16, 392-394.
5. Boss, W.F., Kelley, C.J., and Landsberger, F.R. (1975) Anal. Biochem. 64, 289-292.
6. Warner, T.G., and Benson, A.A. (1977) J. Lipid Res. 18, 548-551.
7. Gupta, C.M., Radhakrishnan, R., and Khorana, H.G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4315-4319.
8. Lammers, J.G., Liefkens, Th. J., Bus, J., and van der Meer, J. (1978) Chem. Phys. Lipids 22, 293-305.
9. Patel, K.M., Morrisett, J.D., and Sparrow, J.T. (1979) J. Lipid Res. 20, 674-677.
10. Mason, J.T., Broccoli, A.V., and Huang, C. (1981) Anal. Biochem. 113, 96-101.
11. Baer, E., and Kates, M. (1948) J. Am. Chem. Soc. 70, 1394-1399.
12. Tattrie, N.H., and McArthur, C.S. (1955) Can. J. Biochem. Physiol. 33, 761-766.
13. Maurukas, J., and Holland, C.V. (1961) J. Org. Chem. 26, 608-610.
14. Brockerhoff, H., and Yurkowski, M. (1965) Can. J. Biochem. 43, 1777.
15. Chadha, J.S. (1970) Chem. Phys. Lipids 4, 104-108.
16. Baumann, W.J. (1972) Ether Lipids. Chemistry and Biology (Snyder, F., ed.) pp. 51-79, Academic Press, New York, NY.
17. Stanacev, N.Z., Baer, E., and Kates, M. (1964) J. Biol. Chem. 239, 410-413.
18. Chacko, G.K., and Hanahan, D.J. (1968) Biochim. Biophys. Acta 164, 252-271.
19. Brockerhoff, H., and Ayengar, N.K.N. (1979) Lipids 14, 88-89.
20. Eibl, H., Arnold, D., Weltzien, H.U., and Westphal, O. (1967) Liebigs Ann. Chem. 709, 226-230.
21. Arnold, D., Weltzien, H.U., and Westphal, O. (1967) Liebigs Ann. Chem. 709, 234-239.
22. Paltauf, F. (1969) Biochim. Biophys. Acta 176, 818-827.
23. Bergelson, L.D., Vaver, V.A., Prokazova, N.V., Ushakov, A.N., Rozynov, B.V., Stefanov, K., Ilukhina, L.I., and Simonova, T.N. (1972) Biochim. Biophys. Acta 260, 571-582.
24. Baumann, W.J., Schupp, E., and Lin, J.T. (1975) Biochemistry 14, 841-847.
25. Yabusaki, K.K., and Wells, M.A. (1973) Biochim. Biophys. Acta 296, 546-548.
26. Baer, E. (1953) J. Am. Chem. Soc. 75, 5533-5539.
27. Baer, E., Duke, A.J., and Buchnea, D. (1968) Can. J. Biochem. 46, 69-74.
28. Eibl, H., and Westphal, O. (1967) Liebigs Ann. Chem. 709, 244-247.
29. Kozhukov, V.I., Molotkovskii, Y.G., and Bergelson, L.D. (1969) Izv. Akad. Nauk SSSR, Ser. Khim. 7, 1559-1562.
30. Hirt, R., and Berchtold, R. (1958) Pharm. Acta Helv. 33, 349-356.
31. Eibl, H., and Nicksch, A. (1978) Chem. Phys. Lipids 22, 1-8.
32. Murari, R., and Baumann, W.J. (1981) J. Am. Chem. Soc. 103, 1238-1240.
33. Murari, R., Abd El-Rahman, M.M.A., Wedmid, Y., Parthasarathy, S., and Baumann, W.J., (1982) J. Org. Chem., in press.
34. Baer, E., and Kates, M. (1950) J. Am. Chem. Soc. 72, 942-949.
35. Baer, E., and Maurukas, J. (1952) J. Am. Chem. Soc. 74, 158-160.
36. Baer, E., Buchnea, D., and Newcombe, A.G. (1956) J. Am. Chem. Soc. 78, 232-237.
37. Baer, E., and Mahadevan, V. (1959) J. Am. Chem. Soc. 81, 2494-2498.
38. De Haas, G.H., and van Deenen, L.L.M. (1961) Rec. Trav. Chim. 80, 951-970.
39. Baer, E., and Kindler, A. (1962) Biochemistry 1,

518-521.
40. Aneja, R., and Chadha, J.S. (1971) Biochim. Biophys. Acta 248, 455-457.
41. Ames, B.N., and Dubin, D.T. (1960) J. Biol. Chem. 235, 769-775.
42. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
43. Dittmer, J.C. and Lester, R.L. (1964) J. Lipid Res. 5, 126-127.
44. Baumann, W.J., Schmid, H.H.O., Ulshöfer, H.W., and Mangold, H.K. (1967) Biochim. Biophys. Acta 144, 355-365.
45. Barry, P.J., and Craig, B.M. (1955) Can. J. Chem. 33, 716-721.
46. Krabisch, L., and Borgström, B. (1965) J. Lipid Res. 6, 156-157.
47. Brown, W.G. (1951) Org. Reactions 6, 469-509.
48. Fieser, L.F., and Fieser, M. (1967) Reagents for Organic Synthesis, Vol. 1, p. 1160, John Wiley and Sons, New York, NY.

[Received January 27, 1982]

High Performance Reversed-Phase Chromatography of the Triglycerides from Human Plasma Lipoproteins

E.G. PERKINS[a,*], DAVID J. HENDREN[a], NICHOLAS PELICK[b] and JOHN E. BAUER[c]
[a]Department of Food Science, Burnsides Research Laboratory, 1208 West Pennsylvania, University of Illinois, Urbana, IL 61801; [b]Supelco Inc., Supelco Park, Bellefonte, PA 16823; and [c]Department of Metabolism, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610

ABSTRACT

The triglycerides of human plasma lipoproteins were separated with high performance reversed-phase liquid chromatography. An octadecyl bonded 5-μ silica column was used with a mobile phase of acetonitrile/acetone. Individual triglyceride types and critical pairs may be easily separated and identified.
Lipids 17:460-463, 1982.

INTRODUCTION

Natural triglyceride mixtures, present in vegetable fats and oils, can be separated with varying degrees of efficiency using high performance reversed-phase chromatography (HPRC). Columns used in these separations use packing materials with varying characteristics, such as particle size, particle type, bonded chain length, percentage of coverage of the particle and uniformity of particle size. Each of these parameters has either positive or negative effects on the final separations.

Triglyceride mixtures have been separated with column packings exhibiting all of the above characteristics (1-5). Interacting ions such as silver have been added to mobile phases to enhance triglyceride separations (6). The relatively recent availability of very high efficiency column packings with octadecyl-bonded particles has allowed the separation of natural triglyceride mixtures, including critical pairs, by HPRC using nonaqueous solvent systems (7,8). The triglyceride mixtures of natural fats are less complex than those of biological origin, because triglycerides from animals and humans reflect the composition of dietary fats (8). The presence, then, of fatty acids with both positional and geometrical isomerism may complicate separations. Demonstrated here is the application of HPRC to the separation and identification of triglycerides from human plasma lipoproteins.

EXPERIMENTAL

Equipment used included a Tracor 995 isochromatographic pump (Tracor Inst., Austin, TX), a Rheodyne loop injector (20-μl) (Rheodyne Model 7120, Berkeley, CA), and a Waters Model R 401 differential refractometer detector (Waters Assoc., Milford, CT) connected to a Hewlett-Packard Model 3385A electronic integrator, that recorded the separations and retention times. One Supelcosil LC-18 column, 250 mm x 4.6 mm packed with 5 μ octadecyl-bonded spherical silica (Supelco Inc., Bellefonte, PA) was used, with the mobile phase consisting of a mixture of analytical grade acetone and distilled acetonitrile (63.6:36.4). As standards, triglycerides were obtained from Supelco or else previously identified natural mixtures were used.

The triglycerides were solubilized in tetrahydrofuran at 200 mg/ml and sample volumes of 5-10 μl were injected onto the column. Human plasma lipoproteins were isolated from pooled, whole blood or plasma of undetermined patient source obtained from the Shands Teaching Hospital of the University of Florida's College of Medicine. The lipids were then extracted from the isolated lipoprotein and the corresponding triglycerides were isolated as previously described (9). Components eluting from the column were converted to the corresponding methyl esters, and these were identified by gas liquid chromatography, also as described (9). The triglyceride structure of the eluting component was then ascertained. HPRC currently is incapable of resolving triglyceride isomers such as POO, OPO and OOP, so peak labels indicate the corresponding isomer mixture. The following coding was used for fatty acids: P–palmitic, S–stearic, O–oleic, Lo–linolenic and Ln–linolenic.

TABLE 1

Triglyceride Compositions Which Can Have the Same Equivalent Carbon Number[a]

36.0	38.0	40.0	42.0	44.0	46.0	48.0	50.0	52.0	54.0
54:9	54:8	54:7	54:6	54:5	54:4	54:3	54:2	54:1	54:0
52:8	52:7	52:6	52:5	52:4	52:3	52:2	52:1	52:0	
50:7	50:6	50:5	50:4	50:3	50:2	50:1	50:0		
48:6	48:5	48:4	48:3	48:2	48:1	48:0			
46:5	46:4	46:3	46:2	46:1	46:0				
44:4	44:3	44:2	44:1	44:0					
42:3	42:2	42:1	42:0						
40:2	40:1	40:0							
38:1	38:0								
36:0									

[a]Equivalent carbon no. = (total no. of carbon atoms in triglyceride fatty acids) - (2X total number of bouble bonds in triglyceride). Data are from El-Hamdy and Perkins (8).

[b]Indicates a triglyceride–54:9 with 54 carbon atoms and 9 double bonds (disregarding glycerol moiety), e.g., trilinolenin.

RESULTS AND DISCUSSION

We have recently shown the separation of complex mixtures of natural triglycerides from vegetable oils using HPRC on highly efficient, octadecyl-bonded, reversed-phase packings (8). These separations allow the collection of fractions of triglycerides of sufficient purity to be used as standards themselves. This allows facile identification of eluted components when the triglyceride composition of tissue lipids is studied.

The composition of blood plasma lipoprotein fractions reflects the composition of dietary fat. It is reasonable to expect that appropriate separations devised for these triglycerides will be applicable to those from other tissues. Critical pairs of triglycerides are those triglycerides which have differing numbers of double bonds and carbon chain length fatty acids, but the same equivalent carbon numbers. Combinations of these triglycerides are listed in Table 1. It is evident that those triglycerides containing the greatest degree of unsaturation will form critical pairs with certain saturated triglycerides. The greatest number anticipated are those triglycerides with equivalent carbon number of 36:0, and these are the most difficult to separate. Such separations require very efficient columns.

The separations of 2 different standard mixtures of known triglycerides are illustrated in Figures 1 and 2. Separation within critical pairs, such as LoLoO and LoLoP, may be obtained with one column. Efficiency is considerably increased by using 2 columns, but

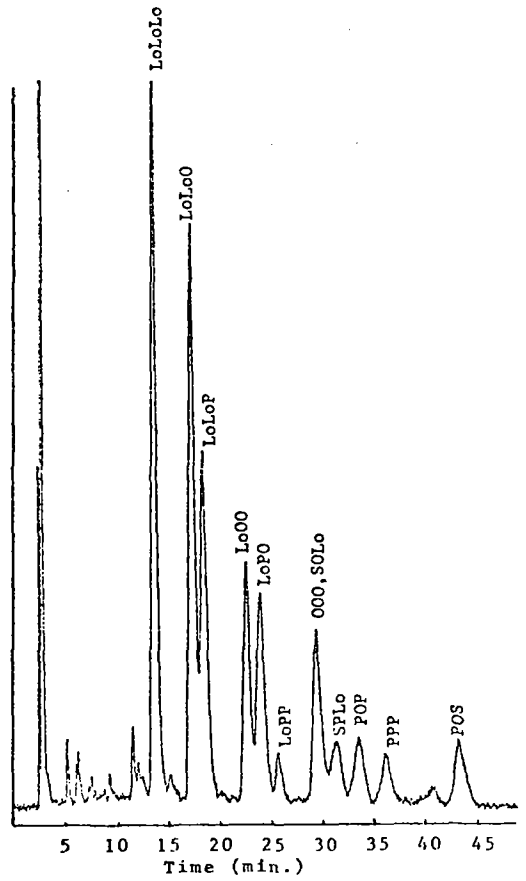


FIG. 1. Separation of triglyceride standards on 250 X 4.6 mm Supelcosil-18 octadecyl bonded packing. Mobile phase, acetone/acetonitrile, 63.6:36.4; flow rate=1 ml/min. Abbreviations indicate, e.g., POS, a triglyceride composed of stearic, oleic and palmitic acids.

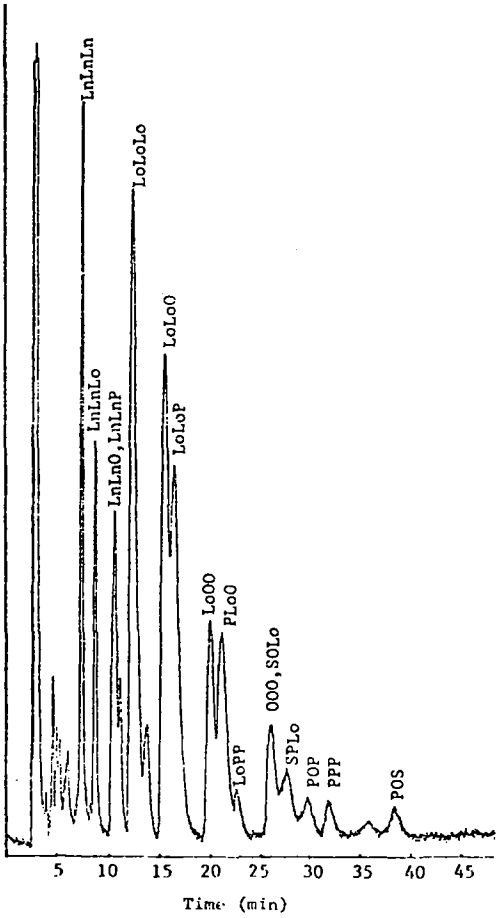


FIG. 2. Separation of triglyceride standards; conditions and identifications are as indicated in Fig. 1.

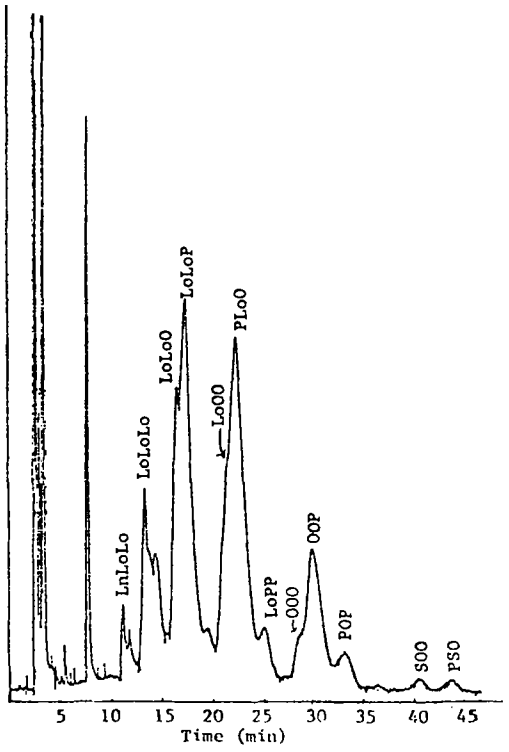


FIG. 3. Separation of human plasma VLDL triglycerides; conditions are as in Fig. 1.

analysis time is longer. Figure 2 illustrates the separation of a standard mixture, with critical pairs, containing linolenic, linoleic, oleic and palmitic acids. Tissue and blood lipids of land animals generally do not contain large amounts of either highly unsaturated or highly saturated triglycerides. These are more likely to occur as mixed triglycerides in critical pair mixtures. As indicated in Figure 2, they are adequately separated.

Application of this powerful technique to lipoprotein triglycerides and the separations achieved for very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) triglycerides are shown in Figures 3-5.

The triglyceride profiles were predictably very similar because they were obtained from

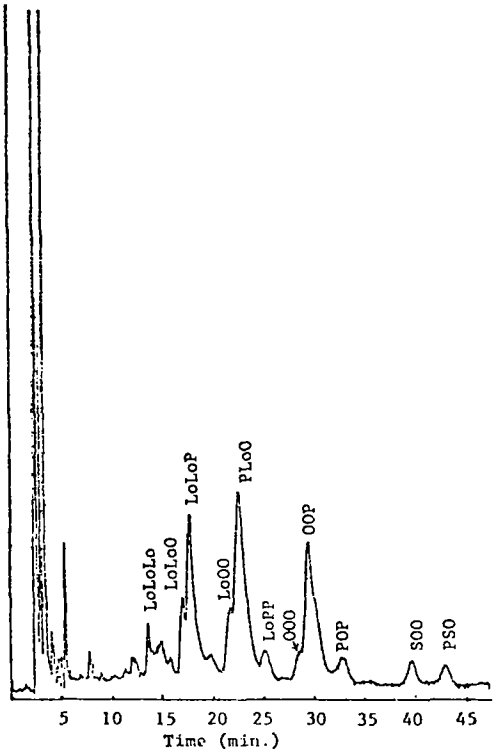


FIG. 4. Separation of human plasma LDL triglycerides; conditions are as in Fig. 1.

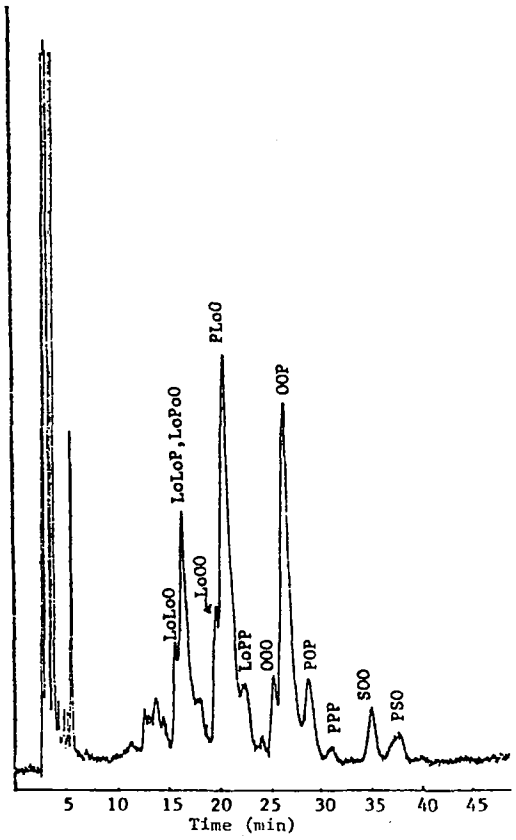


FIG. 5. Separation of human plasma HDL triglycerides; conditions are as in Fig. 1.

pooled blood samples and were not representative of an average sample of normo-triglyceridemic blood. One would expect to observe differences in individual triglyceride profiles, primarily in samples from individuals. The present work indicates that the methodology applied can be used to investigate the effects of diet and disease states on such lipoprotein triglyceride profiles.

ACKNOWLEDGMENT

Partial support was obtained from the Illinois Agricultural Experiment Station, University of Illinois.

REFERENCES

1. Plattner, R.D., Wase, K., and Kleiman, R. (1978) J. Am. Oil Chem. Soc. 55, 381-382.
2. Plattner, R.D., Spencer, G.F., and Kleiman, R. (1977) J. Am. Oil Chem. Soc. 54, 511-515.
3. Spencer, G.F., Plattner, R.D., and Miwa, T. (1977) J. Am. Oil Chem. Soc. 54, 187-189.
4. Payne-Wahl, K., Plattner, R.D., and Spencer, G.F. (1979) Lipids 14, 601-605.
5. Herslof, B., Podlaha, O., and Toregard, B. (1979) J. Am. Oil Chem. Soc. 56, 864-866.
6. Vonach, B., and Schomberg, G. (1979) J. Chromatogr. 149, 417-430.
7. Parris, N.A. (1978) J. Chromatogr. 149, 615-624.
8. El-Hamdy, A.H., and Perkins, E.G. (1981) J. Am. Oil Chem. Soc. 58, 867-872.
9. Perkins, E.G., Hendren, D.J., Bauer, J.E., and El-Hamdy, A.H. (1981) Lipids 16, 609-613.

[Received December 7, 1981]

Determination of Cholesteryl Esters and of Cholesteryl and Epicholesteryl Silyl Ethers by Capillary Gas Chromatography

NORMAN B. SMITH, *Biophysics Department, Health Sciences Centre, The University of Western Ontario, London, Ontario, Canada N6A 5C1*

ABSTRACT

The capillary gas chromatography of cholesteryl esters after splitless injection into a 25-m, OV-1-coated, fused silica WCOT column and a 7-m Silar 10C-coated glass WCOT column is reported. The nonpolar OV-1 column separated the cholesteryl esters principally on the basis of carbon number, but separation of the saturated esters from the unsaturated esters was also achieved. The polar Silar 10C column separated the esters mainly according to the degree of unsaturation. Thus, the 2 column types complement each other in the analysis of nanogram quantities of cholesteryl esters from small samples, such as those from plasma or single arterial atherosclerosis lesions. This technique therefore obviates some of the difficulties of analyzing such cholesteryl ester samples in the form of methyl esters (incomplete transmethylation, and contamination by solvent impurities and/or plasticizer esters). Both columns were also found to be useful for the separation and quantitaiton of the *t*-BDMS ethers of cholesterol and epicholesterol in mixtures containing various proportions of these epimers.
Lipids 17:464-468, 1982.

INTRODUCTION

In most studies of individual CE from mammalian tissues, GLC analyses are performed on the corresponding methyl esters (1-3). Direct transmethylation methods (4,5) have been generally preferred for methyl ester preparation over saponification-re-esterification procedures because of greater efficiency and milder conditions of the former (5). Cholesteryl esters saponify only very slowly (6) and polyunsaturated fatty acids tend to be oxidized and/or isomerized during prolonged incubations (4,6). Direct transmethylation of CE under acidic conditions can result in the generation of artifact peaks due to formation of cholesterol derivatives (7).

Quantitative transmethylation of CE samples > 1 mg can be achieved by base-catalyzed methanolysis with sodium or potassium methoxide, but these reagents must be used with care and excessively long incubations must be avoided to prevent alterations in the fatty acids (4). The methoxide reagent prepared from metallic sodium or potassium rather than the corresponding hydroxides is generally preferred, because methoxide reagent prepared from the hydroxide contains water and significant saponification always occurs with its use. With both reagents, however, the recommended procedure following the base-catalyzed incubation is to acidify the reaction mixture and incubate again to re-esterify any released fatty acids (8,9).

Unpublished studies in this laboratory have shown that very small CE samples (< 200 μg) could be transesterified quantitatively only if 2,2-dimethoxypropane (DMP) was added to the methanol prior to the addition of sodium. However, the use of DMP in transesterification procedures (10) has been criticized because polymers may form and give rise to spurious peaks (4). Another difficulty with the analysis of minute samples of fatty acid methyl esters is the possibility of sample contamination by the ubiquitous presence of plasticizers (most notably the phthalate esters) and of solvent impurities (11,12).

Earlier, this author analyzed the total free cholesterol and CE content of individual atherosclerotic lesions of various sizes from the aortas of cholesterol-fed rabbits (13). The analyses were performed by GLC on a pair of packed 3% OV-1 columns by the method of Kuksis et al. (14). The smallest of the lesions had to be pooled to provide enough total ester, and analysis of the individual constituent CE was out of the question. Capillary GLC is noted for its superior resolution and sensitivity compared to that of conventional GLC (15). Therefore, because of the limitations imposed by the small sample sizes, separation of intact CE by GLC on WCOT capillary columns was studied.

Abbreviations: CE, cholesteryl ester; FC, cholesterol, cholest-5-en-3-β-ol; EFC, epicholesterol, cholest-5-en-3-α-ol; GLC, gas liquid chromatography; *t*-BDMS ether, tertiary-butyldimethylsilyl ether; TZ, Trennzahl, separation number; μ, average linear velocity; WCOT, wall-coated open tubular.

MATERIALS AND METHODS

All solvents (reagent grade) were purchased from Fisher Scientific Ltd. (Toronto, Ont.). All lipids except EFC were obtained from Sigma (St. Louis, MO) or Serdary Research Laboratories (London, Ont.). EFC was supplied by Research Plus Steroid Laboratories (Denville, NJ). Tertiary-butyldimethylchlorosilane/imidazole reagent and a 25-m Quadrex glass WCOT column which was coated with Silar 10C were purchased from Applied Sciences Division, Milton Roy Co. Lab Group (State College, PA).

t-BDMS ether was prepared (16) by dissolving 100 μg or less of FC or EFC in 50 μl CH_2Cl_2 and adding an equal volume of the *t*-butyldimethylchlorosilane/imidazole reagent. The presence of CH_2Cl_2 in the reagent mixture was required to prevent the precipitation of the sterol and its ether derivative (17). After 1 hr at room temperature (22-28 C), the reaction was stopped with 1 ml H_2O. The ethers were extracted with CH_2Cl_2, dried with anhyd Na_2SO_4 and evaporated to dryness under a stream of N_2. The silyl ethers were then redissolved in CH_2Cl_2 to give a final concentration of each component of about 30 ng/μl.

Chromatography of CE and silyl ethers was performed with a Hewlett-Packard Model 5830A gas chromatograph equipped with a Hewlett-Packard (Model 18835B) capillary inlet and a flame ionization detector, on nonpolar (OV-1), and polar (Silar 10C) WCOT columns.

The nonpolar column was a 25-m Hewlett-Packard fused silica WCOT column (0.2 mm id) coated with OV-1. The injection port temperature was 340 C, initial column temperature, 130 C, and detector temperature, 350 C. The carrier gas was H_2 at an inlet pressure of 20 psi (linear velocity 45 cm/sec at 130 C). The H_2 flow rate through the detector was 48 ml/min and the makeup gas (N_2) flow rate was 58 ml/min. The splitless injection technique was used. A 1-μl sample, containing 15-50 ng/component, was drawn up entirely into the barrel of the syringe. Purge of carrier gas through the inlet system was stopped. The needle was then inserted into the injector and allowed to heat for 10 sec. The sample was injected and the temperature program was begun.. The needle was held in position for another 10 sec before being rapidly removed. Carrier gas purge of the inlet then resumed 0.6 min after injection. The oven temperature was held at 130 C for 0.5 min, after which it was rapidly increased (30 C/min) to 285 C; the temperature was increased thereafter at 1.5 C/min to 330 C.

For chromatography of the CE on the polar phase, a 7-m Quadrex glass capillary column made from a 25-m column (0.25 mm id) coated with Silar 10C was used, with He as the carrier gas (inlet pressure 20 psi, linear velocity 56 cm/sec at 150 C). The injector and detector temperatures were 300 and 260 C, respectively, and splitless injection was used. The oven temperature, initially at 150 C for 0.5 min, was increased at 30 C/min to 219 C, after which the temperature was increased at 0.5 C/min to 230 C and held there until all components were eluted. For the separation of the silyl ethers, the oven temperature was increased quickly from 100 to 210 C and held at that temperature thereafter. The injector port and detector temperatures were both set at 220 C. the H_2 back pressure was 3 psi (linear velocity 56 cm/sec at 100 C).

RESULTS AND DISCUSSION

Figure 1 shows the separation of the CE of (A) a homologous series in a standard mixture, (B) a large atherosclerotic fatty lesion from a rabbit aorta, and (C) hypercholesterolemic rabbit plasma. Excellent separation of the component esters in the mixtures were obtained as evidenced in Table 1, which gives the TZ for saturated homolog pairs. Separation of the unsaturated CE from the corresponding saturated esters of the same carbon number was also achieved, with the unsaturated species having shorter retention times than the saturated species. Analogous separation of unsaturated triglycerides from the corresponding saturated species has been reported by Grob et al. (20). Cholesteryl oleate (CE-18:1) and cholesteryl linoleate (CE-18:2) were not separated by the capillary column. A peak corresponding in retention time to that of cholesterol linolenate (CE-18:3) eluted between the CE-18:1 + CE-18:2 and the cholesteryl stearate (CE-18:0) peaks. These separations represent a significant improvement over those obtained from packed columns coated with OV-1 (10) and allow more detailed analyses of neutral lipids with the nonpolar liquid phase. Chromatography of the hypercholesterolemic plasma and aortic lesion CE revealed similar patterns, detailed in Table 2. These data agree well with those of earlier studies, similarly grouped (1-3).

Because the analysis of the components of the monoene-diene peak with OV-1 column is difficult, the ability of a Silar 10C column to separate CE according to degree of saturation was investigated. Silar 10C in packed columns has been shown to separate CE according to degree of unsaturation (21). Figure 2 shows that this phase can also be used in a capillary

system to achieve a similar separation. The separation of the saturated and monoene species was incomplete, as shown by the TZ value in Table 3. There was a tendency for the saturated ester (CE-18:0) peak to trail slightly into the monoene peak (CE-18:1), with a resulting loss of about 10% of the saturated area and a corresponding increase in the monoene peak area. However, in samples containing a relatively small amount (5-10%) of CE-18:0, such as those from aortic lesions or plasma (Table 3) (1-3), the tailing effect is negligible. Also, reverse overlap from the leading edges of following peaks was rarely a problem.

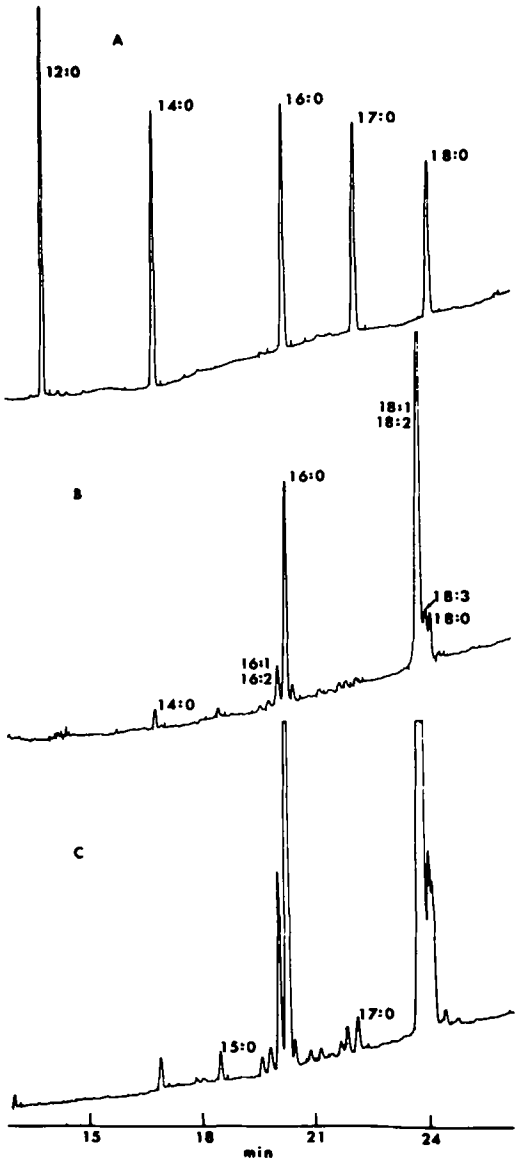


FIG. 1. Chromatogram of CE from (A) a synthetic homologous series; (B) a rabbit aortic atherosclerotic lesion; (c) hypercholesterolemic rabbit plasma; on an OV-1 fused silica capillary column: 25 m long, 0.2 mm id. 0.1 μm film thickness; hydrogen as carrier gas at 20 psi, μ = 45 cm/sec; splitless injection, 1 μl in petroleum ether at 130 C, temperature program 1.5 C/(285-330 C); injection temperature, 340 C, FID temperature, 350 C.

The suitability of cholesteryl decanoate (CE-10:0) and cholesteryl eicosanoate (CE-20:0) as internal standards for quantitating other CE in a mixture was investigated. The detector response was found to decrease with increasing molecular weight of the esters, probably mostly due to losses in the injector port (22). The data in Figure 3 show an example of the relationship between the molecular weight of CE present in a sample in equal amounts and their peak areas, as normalized to that of CE-10:0. This relationship was independent of the quantity of ester injected. The slope of the discrimination curve varied from one chromatographic analysis to another, but data scatter in most analyses was similar to that in Figure 3. Occasionally, the shape of the curve also varied. However, the peaks in a biological sample could be quantitated by adding an internal standard mixture containing both CE-10:0 and CE-20:0 and then applying appropriate corrections to the peak areas of the esters by interpolation of the discrimination data based on recent calibrations.

The separation of the epimers FC and EFC by the 2 capillary columns was also investigated. Figure 4 shows typical chromatograms of the epimers in the 2 systems. The OV-1 column was more efficient than the Silar 10C column: the TZ values for the peak separations were

TABLE 1

Separation of Saturated Cholesteryl Esters on an OV-1 Fused Silica Capillary Column[a]

Cholesteryl esters	Carbon number	TZ[b]
CE-18:0/CE-17:0	45/44	9.1
CE-17:0/CE-16:0	44/43	8.9
CE-18:0/CE-16:0	45/43	19.1
CE-16:0/CE-14:0	43/41	19.9
CE-14:0/CE-12:0	41/39	20.8

[a]For conditions, see Fig. 1.

[b]$TZ = \frac{D}{b^{(1)}_{0.5} + b^{(2)}_{0.5}} - 1$, where D is the distance between the peak maxima, and $b^{(1)}_{0.5}$ and $b^{(2)}_{0.5}$ are the widths of the peaks at half-height. The use of TZ has been discussed in detail recently (18,19).

TABLE 2

Quantitation of Cholesteryl Esters from a Rabbit Atherosclerotic Lesion and Hypercholesterolemic Plasma[a]

Cholesteryl ester	Total cholesteryl esters (mol %)[b]	
	Atherosclerotic aorta lesion	Hypercholesterolemic plasma
CE-14:0	1.0	0.5
CE-15:0	–	0.5
CE-16:0	17.3	16.3
CE-16:1 + 16:2	3.5	5.1
CE-17:0	0.7	1.0
CE-18:0	5.4	4.1
CE-18:1 + 18:2	61.3	60.7
CE-18:3	7.3	7.8
Minor, unknown peaks[c]	3.5(5)	4.1(9)

[a]Chromatography conditions as in Fig. 1.

[b]Calculated from peak area percentage data appropriately corrected for detector response as described in text.

[c]Percent (no. of peaks).

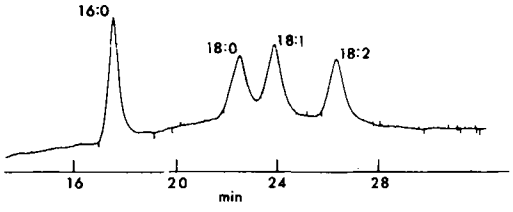


FIG. 2. Chromatogram of saturated, monoene and diene CE on a Silar 10C glass capillary column: 7 m long, 0.25 mm id, 0.1 μm film thickness; helium as carrier gas at 20 psi, μ = 56 cm/sec, splitless injection, 1 μl in petroleum ether at 150 C, temperature program 0.5 C/min (219-230 C); injector temperature 300 C, FID temperature 260 C.

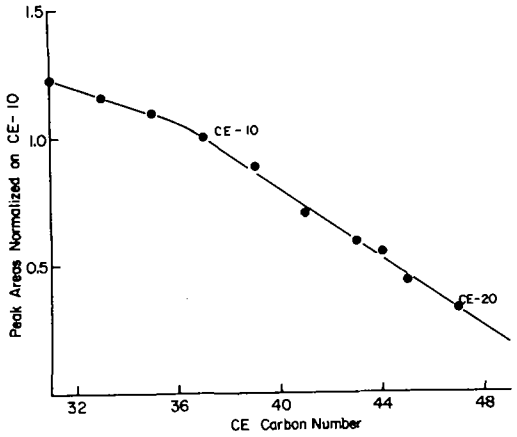


FIG. 3. Relationship between peak area and molecular weight of CE chromatographed on OV-1 fused silica column. For conditions, see Fig. 1.

TABLE 3

Separation of Saturated, Monoene and Diene Cholesterol Esters on a Silar 10 C Glass Capillary Column[a]

Cholesteryl esters	TZ
CE-16:0/CE-18:0	2.68
CE-18:0/CE-18:1	-0.17
CE-18:0/CE-18:2	1.38
CE-18:1/CE-18:2	0.49

[a]For conditions, see Fig. 2.

11.3 and 1.6, respectively. However, with both systems, analysis of samples containing varying amounts of each epimer from zero to 100% gave the correct proportions, as shown in Figure 5 for the Silar 10C column. Thus, these capillary columns, originally selected for CE analysis, are also suitable for estimating the epimers of cholesterol in a mixture.

ACKNOWLEDGMENTS

Grants to Dr. Margot R. Roach from the following agencies in support of this work are gratefully acknowledged: "The Provincial" lottery fund of Ontario and the Medical Research Council of Canada provided funds for the purchase of the gas chromatograph and the capillary accessories, respectively. The Ontario Heart Foundation supported other aspects of the research.

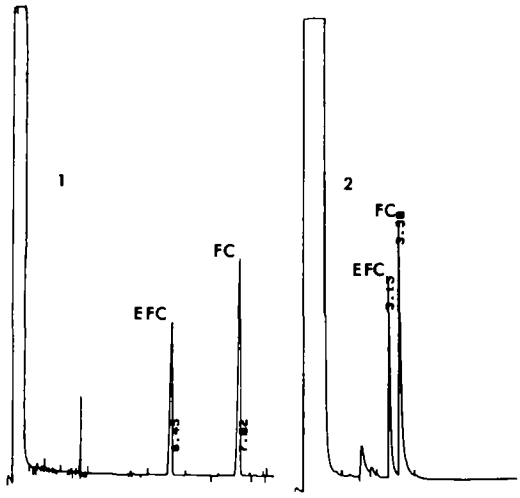


FIG. 4. Chromatograms of a mixture of FC and EFC (1) OV-1 fused silica capillary column. The chart speed was increased from 0.5 cm/min to 4 cm/min about 6 min after injection to allow TZ measurements. For conditions, see Fig. 1; (2) Silar 10C glass capillary column. Splitless injection with injector and detector at 220 C. The oven temperature, initially at 100 C with hydrogen carrier flow at μ = 56 cm/sec, was increased rapidly to 210 C.

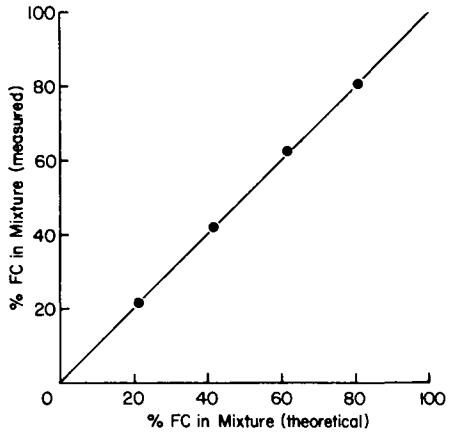


FIG. 5. Quantitation of FC in a mixture of FC and EFC by chromatography on Silar 10C glass capillary column. The same results were obtained using an OV-1 fused silica column. For conditions, see Fig. 4.

REFERENCES

1. Zilversmit, D.B., Sweeley, C.C., and Newman, H.A.I. (1961) Circulation Res. 9, 235-241.
2. Tuna, N., and Mangold, H.K. (1963) In Evolution of the Atheromatous Plaque (Jones, R.I., ed.) pp. 85-108, University of Chicago Press, Chicago, IL.
3. Swell, L., Law, M.D., and Treadwell, C.R. (1962) J. Nutr. 76, 429-434.
4. Christie, W.W. (1972) Topics Lipid Chem. 3, 171-197.
5. Sheppard, A.J., and Iverson, J.L. (1975) J. Chromatogr. Sci. 13, 448-452.
6. Christie, W.W. (1973) in Lipid Analysis, pp. 85-90, Pergamon Press, Oxford.
7. Kramer, J.K.G., and Hulan, H.W. (1976) J. Lipid Res. 17, 674-676.
8. Glass, R.L. (1971) Lipids 6, 919-925.
9. Tuckey, R.C., and Stevenson, P.M. (1979) Anal. Biochem. 94, 402-408.
10. Mason, M.E., and Walter, G.R. (1964) Anal. Chem. 36, 583-586.
11. Rouser, G., Kritchevsky, G., Whatley, M., and Baxter, C.F. (1966), Lipids 1, 107-112.
12. Ishida, M., Suyma, K., and Adachi, S. (1980) J. Chromatogr. 189, 421-424.
13. Smith, N.B. (1980) Atherosclerosis 36, 491-504.
14. Kuksis, A., Myher, J.J., Marai, L., and Geher, K. (1975) J. Chromatogr. Sci. 13, 423-430.
15. Schomburg, G. (1979) J. High Resolut. Chromatogr. Commun. 2, 461-474.
16. Corey, E.J., and Venkateswarlu, A. (1972) J. Am. Chem. Soc. 94, 6190-6191.
17. Quillam, M.A., and Westmore, J.B. (1978) Anal. Chem. 50, 59-68.
18. Grob, K., Jr., Grob, G., and Grob, K. (1978) J. Chromatogr. 156, 1-20.
19. Grob, K., Jr., and Grob, K. (1981) J. Chromatogr. 207, 291-297.
20. Grob, K., Neukom, H.P., and Battaglia, R. (1980) J. Am. Oil Chem. Soc. 57, 282-286.
21. Takagi, T., Sakai, A., Itabashi, Y., and Hayashi, K. (1977) Lipids 12, 228-232.
22. Grob, K. (1979) J. Chromatogr. 178, 387-392.

[Received October 14, 1981]

Improved Methods for the Isolation and Study of the C_{18}, C_{20} and C_{22} Monoethylenic Fatty Acid Isomers of Biological Samples: Hg Adducts, HPLC, $AgNO_3$-TLC/FID, and Ozonolysis[1]

J-L. SEBEDIO, T.E. FARQUHARSON and **R.G. ACKMAN***, *Technical University of Nova Scotia, Fisheries Research & Technology Laboratory, PO Box 1000, Halifax, Nova Scotia, B3J 2X4*

ABSTRACT

The monoethylenic isomers of C_{18}, C_{20} and C_{22} chain lengths of the depot fat of a nonhominid primate (cynomolgus monkeys, *Macaca fascicularis*), fed a partially hydrogenated herring oil (IV=76.0) for 30 months, were examined by 2 different approaches. The first isolation method involved preparative gas liquid chromatography and argentation thin layer chromatography (TLC). The second sequence involved a chain-length fractionation system based on the TLC of the methoxy-bromomercuri adducts of the total methyl esters to isolate groups of acids of common degrees of unsaturation, and then high performance liquid chromatography on a reverse-phase column. In both cases, the monoethylenic isomer distribution was determined by ozonolysis in BF_3/MeOH. Comparable results were obtained with the 2 methods. The second approach is recommended for small biological samples, especially for those containing a relatively high proportion of di- and other polyethylenic isomers which might interfere.
Lipids 17:469-475, 1982.

INTRODUCTION

Different techniques must be combined in the study of the geometric and positional isomers of monoethylenic fatty acids of partially hydrogenated oils (1-9). These often include preparative gas liquid chromatography (GLC) for isolation of the chain length of interest (10), the detection of the acids being effected with a thermal conductivity detector. The collected sample is then usually further fractionated into *cis* and *trans* isomers by thin layer chromatography (TLC) with silver nitrate (11), and the isomer distribution is determined by ozonolysis (12), or by GLC on wall-coated, open-tubular columns (13,14). The amount of *trans* fatty acid can be determined by infrared (IR) spectroscopy (10,11), as material is usually plentiful, or by GLC on nonpolar columns (15, 16).

To study the metabolic effects of various *trans* fatty acids such as those encountered in margarine and shortening, feeding studies have been effected on different animal species. To carry out detailed studies on monoethylenic fatty acids in those biological samples where the amount of material is often very small, it was necessary to develop preparative and analytical techniques that are more economical and sensitive than preparative GLC and IR spectroscopy. The authors propose a method based on the fractionation of the total fatty acid methyl esters via the formation of methoxy-bromomercuri adducts, followed by regeneration of each group of esters of common degree of unsaturation. Chain length fractionation of the regenerated esters is by high performance liquid chromatography (HPLC) on a reverse-phase column. Preparative separation of *cis/trans* monoene isomers is still executed by silver nitrate TLC, but the actual *trans* acid content is determined accurately by a sensitive silver nitrate Iatroscan thin layer chromatography/flame ionization detection (TLC/FID) method. The monoenoic isomer distribution can then be determined as usual by oxidative ozonolysis.

[1]Presented in part at the AOCS annual meeting, New Orleans, May 1981.

EXPERIMENTAL

Preparation of Methyl Esters

The lipids from the animal tissues were extracted by the Bligh and Dyer method (17). In the case of nonhominid primate tissue, the composition of the lipid extract was checked by Iatroscan (18) and, if necessary, the triglyceride was freed of minor amounts of phospholipid and free fatty acid by TLC on silica gel (Prekotes Adsorbosil-5, Applied Science Laboratories, State College, PA) using a mixture of hexane/diethyl ether/acetic acid (80:20:1) as the solvent system. The triglyceride (R_f=0.49)

was recovered and the acyl moieties were converted to methyl esters by refluxing in a 7% BF_3/MeOH solution (19).

Analytical and Preparative GLC Analysis

All analytical GLC was performed on stainless-steel, open-tubular columns, 47 m in length and 0.25 mm id, coated with SILAR-7CP, Apiezon-L or BDS (butanediol succinate) and operated in a Perkin Elmer series 900 apparatus with FID. Preparative GLC was executed on a stainless-steel column, 1 m in length and 4 mm id, packed with 10% SE-30 on Chromosorb W/100-120 mesh, operated at 180 C and 40 psig helium in a Varian Autoprep with thermal conductivity detector. For collection of methyl esters, the effluent gas was passed into glass tubes containing 20 ml of chloroform.

Determination of Monoethylenic Isomers by a Combination of Methoxy-mercuri-adducts, HPLC and Ozonolysis

The total methyl esters of the fatty acids of tissue triglycerides were converted to the methoxy–acetoxy-mercuri-adducts in a 10-ml, screw-capped centrifuge tube by heating at 100 C for 2 hr with an excess (100%) of a mercuric acetate solution in methanol (0.25 M) as described elsewhere (20). The reaction mixture was then dissolved in 10 ml of chloroform in a 50-ml, screw-capped tube. A 10% excess of sodium bromide in methanol (0.5 M) was added and the tube contents were mixed for 2 min. After addition of water, the methoxy-bromomercuri-adducts were extracted 3 times into chloroform. The adducts were fractionated into groups of equal numbers of ethylenic bonds on TLC plates (Prekotes, Adsorbosil-5, Applied Science Laboratories) using a mixture of hexane/dioxane (60:40) as solvent system (20). The 2 principal fatty acid classes, monoene (R_f =0.61) and diene (R_f=0.51), were recovered and the methyl esters were regenerated from the adducts by addition of HCl (21).

The monoene band containing the C_{16}, C_{18}, C_{20} and C_{22} *cis* and *trans* isomers was further fractionated by HPLC on a reverse-phase column (Waters Bondapak C_{18}, 10μ 200 x 3.9 mm id). The samples were run on a Waters' Scientific instrument (6000A solvent delivery system) using a mixture of methanol/water (90:10) at 1.7 ml/min as solvent and a refractive index detector (R 401). Each collected methanol/water solution was extracted 3 times with chloroform to recover the original methyl esters. The collected chain length groups of monoethylenic isomers (C_{16}-C_{22}) were uncontaminated as baseline separation was complete for samples up to 5 mg.

The monoethylenic acids of each chain length were then separated into their respective sets of *cis* (R_f=0.69) and *trans* (R_f=0.60) isomers by TLC with silver nitrate (5,22), and were then ozonized in BF_3/MeOH (23,24). The mixed ozonolysis reaction products, including both the mono- and diesters, were analyzed by GLC. A BDS column was used at 2 different temperatures, 165 C for the long-chain diesters and 110 C for the short-chain ones (dimethyl malonate, succinate and glutarate).

Determination of *trans* Fatty Acid Content by $AgNO_3$-Iatroscan Chromarods

The rods were first cleaned of any organic compounds by passing them through the Iatroscan flame, and were then immersed in a solution (2.5%) of silver nitrate in acetonitrile for 15 min (25). After activation at 120 C for 3 hr, the rods were spotted with the sample (ca. 10^{-2} mg), developed for 25 min in a mixture of hexane/benzene (1:1), then scanned with an FID in the Iatroscan TH-10 apparatus. A Linear Instruments Corp. (Costa Mesa, CA) Model 252A recorder was operated at 10 mV full-scale deflection to record the signal. Peak areas were measured by either the recorder stepping-pen integrator or on a Technicon (Chauncey, NY) Model AAG integrator/calculator. Response factors were based on standards (25). After use, the rods were cleaned for re-use by soaking in concentrated nitric acid for several hours.

RESULTS AND DISCUSSION

The monoethylenic fatty acids of the depot fat of a monkey fed a partially hydrogenated herring oil (PHHO) for 30 months were studied by the 2 methods summarized in Figure 1. The first method (Fig. 1A) is the most widely used technique for studying partially hydrogenated oils and the second (Fig. 1B) is the method proposed by the authors for studying small biological samples.

The first step of the "conventional" method (Fig. 1A) is a chain-length fractionation of the total methyl esters by preparative GLC. The main disadvantages of this technique are the low sensitivity of the thermal conductivity detector and the poor recovery of the eluted samples. It is often hardly applicable to biological material due to the small sample size available. In the second step, the *cis* and *trans* monoethylenic isomers are then isolated by $AgNO_3$-TLC. The migration of the methyl ester on an $AgNO_3$-TLC plate depends on 4 factors: the number of ethylenic bonds, the geometry and position of the ethylenic bonds, and the chain

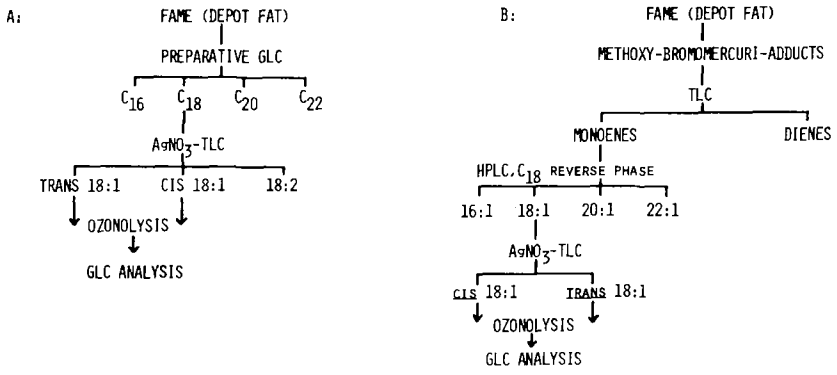


FIG. 1. (A) Conventional analytical procedures for the isolation of the monoethylenic fatty acids of partially hydrogenated oils. (B) Analytical procedure for the isolation and study of the monoethylenic fatty acids of the depot fat of a monkey fed a partially hydrogenated herring oil (IV=76.0) for 30 months.

length (26-28). However, partially hydrogenated oils can contain acids with conjugated diethylenic bonds, and also some non-methylene-interrupted diethylenic isomers of *cis,trans* or *trans,trans* structures. As a result of differences in the migration of these diethylenic acids on $AgNO_3$-TLC plates (29,30), it is possible to foresee contamination of the *cis* and/or *trans* monoethylenic TLC bands for partially hydrogenated oils containing appreciable quantities of the artifact polyunsaturated acids such as those obtained by partial hydrogenation of the "highly unsaturated" marine oils. In order to avoid this possible contamination (20), we have transformed the total fatty acids, as methyl esters, into the methoxy-bromomercuri-adducts which can be specifically fractionated into the different fatty acid classes (e.g., monoene, diene) by TLC (Fig. 1B). In the solvent system used, migration of the adducts depends only on the degree of unsaturation, and not on the geometry or the position of the ethylenic bonds (20).

The esters regenerated from the monoethylenic band were then fractionated into the different chain lengths (C_{16}-C_{22}) by HPLC on a C_{18} reverse-phase column. With the conditions used for chain-length fractionation, only a partial separation of *cis* and *trans* isomers was obtained, especially for the C_{16} and C_{18} acids (Fig. 2). However, in each chain length, a recovery of > 94% was obtained. Therefore, the total peak (*cis* + *trans*) was collected for each chain length and these were further fractionated into the *cis* and *trans* isomers by $AgNO_3$-TLC (Fig. 1B). In order to quantify the ozonolysis results for the $AgNO_3$-TLC bands, it was necessary to determine the percentage of the short-chain diesters recovered in the chloroform extraction, and also to apply the appropriate FID correction factor for the GLC analysis. A known mixture (GLC analysis) of dimethyl esters (C_3-C_{10}) was submitted to a chloroform extraction carried out as described for the ozonolysis process. For the dimethyl esters of C_7 and longer chains, a recovery of 100% was achieved (Table 1). How-

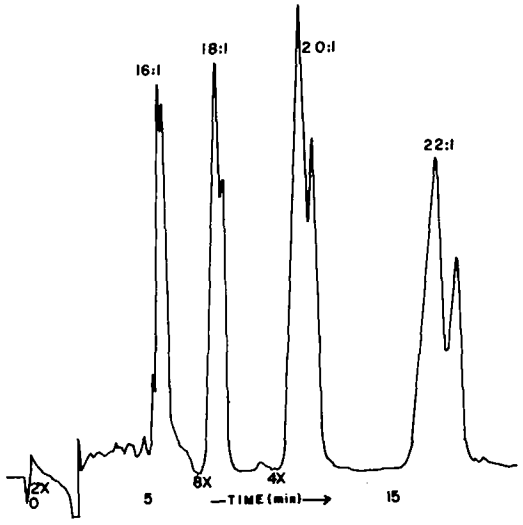


FIG. 2. HPLC analysis of the monoethylenic isomers of the depot fat of a monkey fed a partially hydrogenated herring oil for 30 months; column, Waters Bondapak C_{18}, 10 μ, 200 × 3.9 mm id, MeOH/H_2O (90:10), 2 ml/min; refractive index detector.

TABLE 1

Recovery (%) of Short-Chain Dimethyl Esters by Extraction with $CHCl_3$, and Flame Ionization Correction Factors for the GLC Analysis (BDS) of Dimethyl Esters (Relative to Methyl Stearate as 1.00)

Dimethyl esters	Wt % recovery	FID correction factors
C_3	83.5	2.91
C_4	90.5	2.13
C_5	91.7	1.91
C_6	96.5	1.60
C_7	100	1.55
C_8	100	1.41
C_9	100	1.31
C_{10}	100	1.32
C_{11}	–	1.38
C_{15}	–	1.31

ever, only partial recovery was effected for diesters having less than 7 carbon atoms. The diester FID correction factors relative to methyl stearate (18:0) were also determined from a weighed mixture and the results are listed in Table 1. For practical purposes, the dimethyl esters of C_9 and higher have similar correction factors. The relatively large correction factor for dimethyl malonate (Table 1) has been explained by its decomposition in the injection port of the GLC (31), but interaction with some polar liquid phases is known (32). Other quantitatively accurate ozonolysis procedures would be suitable (33-35).

The monoethylenic isomer distributions of the depot fats of the monkey fed PHHO, determined by the 2 methods described in Figure 1, are reported in Tables 2 and 3. For the *cis* C_{18}, C_{20} and C_{22} monoethylenic isomers, generally good agreement was obtained between the 2 methods (Table 2, cf. 18:1Δ7 and Δ9, 20:1Δ9 and Δ11, 22:1Δ11 and 22:1Δ13). Larger differences due to the compounded experimental errors were observed for some isomers present in minor proportions (less than 0.5%). A good agreement (Table 3) was also observed for the *trans* acids except for 20:1Δ9 and 22:1Δ9 for which the mercuri-adduct method gave higher values. The sample size was, unfortunately, too small to repeat the experiment, but actual results for ozonolysis products were verified on 2 GLC columns. Although this larger amount of the long-chain Δ9 isomers could be due to an impurity having the same GLC retention time as dimethyl nonanoate on both SILAR-5CP and BDS columns, the concurrent presence of more Δ8 isomers suggests a genuine difference due to technique.

The distribution of the *cis* monoethylenic isomers, especially 20:1Δ11, 20:1Δ9, 18:1Δ7 (Table 2), revealed an important chain shortening process of the long-chain monoethylenic fatty acids (C_{20} and C_{22}) by 2 carbons at once to the C_{18} isomers.

One of the more interesting results of the recent accelerated research on dietary C_{22} monoethylenic fatty acids (36) is confirmation that a substantial proportion of such acids may be degraded stepwise, reentering lipid pools as C_{18} acids, instead of being totally catabolized for energy (36-39). Most demonstrations have mixture of *cis* 18:1Δ9 and *trans* 18:1Δ9 was submitted to an HPLC analysis using a solvent mixture of $MeOH/H_2O$ (90:10) at 0.7 ml/min. No difference was observed between the actual isomer weight percentage and the chart area percentage given by the HPLC analysis, indicating no difference in the detector response factor for the *cis* and *trans* 18:1Δ9 isomers. The quantitation of *trans* fatty acids by the 2 other methods ($AgNO_3$-TLC/GLC on SILAR-7CP; $AgNO_3$-Iatroscan) was not influenced by the chain lengths. These two methods can therefore be applied to any of the common chain lengths (C_{16}-C_{22}). An advantage of the $AgNO_3$-Iatroscan method over GLC methods is the small sample size (10 μg) and the short time needed for an analysis (Fig. 3A) (24). An advantage of the HPLC quantitation using a refractive index detector is the possibility of recovering the sample after analysis. The main disadvantage with HPLC is the lack of sensitivity of the refractive index detector.

The approach to the study of monoethylenic fatty acids, based on the HPLC fractionation of the monoenoic fraction recovered from the methoxy-bromomercuri-adducts, is especially useful for biological samples and/or also for partially hydrogenated oils containing significant amounts of interfering conjugated diethy-

TABLE 2

Comparisons of Distributions of the *cis* Monoethylenic Isomers (mol %) from the Depot Fat of a Monkey Fed a Partially Hydrogenated Herring Oil for 30 Months and Isolated by the 2 Techniques Shown in Figure 1

Ethylenic bond position (Δ)	C_{18} GLC	C_{18} Hg + HPLC	C_{20} GLC	C_{20} Hg + HPLC	C_{22} GLC	C_{22} Hg + HPLC
3	–	–	–	–	tr[a]	–
4	0.4	0.4	0.4	0.2	0.5	0.5
5	1.5	0.7	1.6	0.9	0.2	0.3
6	2.0	1.5	1.1	0.6	0.4	0.6
7	14.1	14.3	1.1	0.7	0.3	0.8
8	5.5	5.8	2.0	2.4	0.3	0.8
9	61.9	62.5	30.5	33.4	0.6	2.1
10	2.3	2.4	5.1	5.2	4.7	7.3
11	8.5	8.7	50.9	49.9	83.1	76.8
12	1.6	1.7	3.2	3.0	4.6	4.9
13	1.5	1.4	2.6	2.3	4.3	5.0
14	0.3	0.2	0.6	0.5	0.5	0.3
15	0.3	0.3	0.5	0.4	0.4	0.5
16	0.1	0.1	0.2	0.2	0.1	0.1
17	–	–	0.2	0.3	<0.1	<0.1
18	–	–	tr	tr	<0.1	ND[b]

[a]tr: trace, ≪0.1%

[b]ND: not detected under analytical conditions.

TABLE 3

Distribution of the *trans* Monoethylenic Isomers (mol %) from the Depot Fat of a Monkey Fed a Partially Hydrogenated Herring Oil for 30 Months and Isolated by the 2 Techniques Shown in Figure 1

Ethylenic bond position (Δ)	C_{18} GLC	C_{18} Hg + HPLC	C_{20} GLC	C_{20} Hg + HPLC	C_{22} GLC	C_{22} Hg + HPLC
3	tr[a]	–	–	–	tr	–
4	0.7	0.8	0.3	0.5	0.2	0.1
5	1.5	1.0	0.7	1.0	0.4	0.2
6	4.0	3.1	0.6	0.9	0.4	0.2
7	9.1	8.8	0.7	0.8	0.5	0.5
8	14.8	14.4	2.2	2.8	0.5	0.9
9	23.0	22.8	8.3	17.1	1.5	4.4
10	17.7	17.8	18.8	17.0	18.1	17.0
11	14.4	15.7	37.7	35.3	48.0	46.4
12	7.9	8.8	18.6	15.2	18.5	17.6
13	3.5	3.7	5.3	4.8	6.4	7.0
14	1.7	2.0	2.6	2.1	2.4	2.5
15	1.3	0.9	2.0	1.4	1.3	1.5
16	0.4	0.2	1.3	0.9	0.7	0.9
17	–	–	0.7	0.2	0.6	0.6
18	–	–	0.2	–	0.5	0.2

[a]tr: trace, ≪0.1%.

lenic fatty acids. Moreover, although this method will be more time-consuming than the method using preparative GLC as a technique been based on radioactively labeled acids, but isomer distributions offer an alternative if one or more of the dietary acids have unusual structures (in this case, the 22:1Δ11) compared to those normally found in animal biochemistry

TABLE 4

Trans Content (wt %) of Monoethylenic Fatty Acids of a Depot Fat of a Monkey Fed a Partially Hydrogenated Herring Oil for 30 Months

	Direct GLC on AP-L	TLC-$AgNO_3$, GLC on SILAR 7CP internal standard	Direct quantitation from HPLC run	Iatroscan ($AgNO_3$) Chromarod TLC/FID after HPLC fractionation
18:1	ND[a]	33.4	ND	29.2
20:1	31.8	33.3	33.6	31.8
22:1	28.6	32.3	32.5	29.1

[a]ND: not determined.

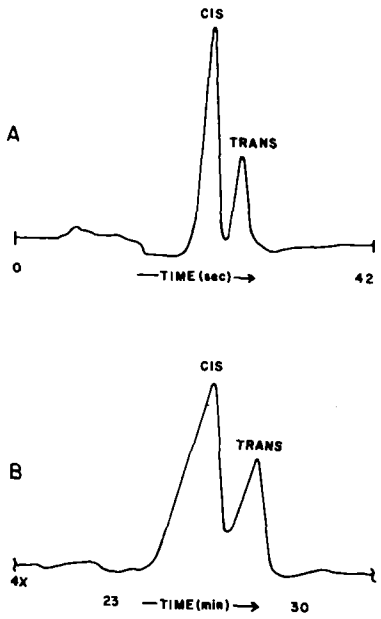


FIG. 3. (A) Silver nitrate-Iatroscan Chromarod TLC/FID analysis of the 22:1 isomers of the depot fat of a monkey fed a partially hydrogenated herring oil for 30 months (conditions in text). (B) HPLC analysis of the decosenoic acid isomers of the depot fat of a monkey fed a partially hydrogenated herring oil for 30 months; column, Waters Bondapak, C_{18}, 10 μ, 300 X 3.9 mm id, MeOH:H_2O (90:10), 1.7 ml/min, refractive index detector.

which are mostly of the oleic family.

The proportions of *trans* isomers in the C_{18}, C_{20} and C_{22} chain lengths were studied by 4 different methods (Table 4): a direct GLC analysis on a nonpolar column (AP-L), a GLC analysis on SILAR-7CP with an internal standard following the fractionation of the total monoene fraction by $AgNO_3$-TLC, the direct quantitation of the HPLC charts, and by $AgNO_3$-Iatroscan analysis of the HPLC fractions (Fig. 3A). As reported earlier (15), the direct GLC analysis on an AP-L column can only be applied to the long-chain fatty acids (C_{20} and C_{22}) because of the inadequate resolution of the *cis* and *trans* isomers of the shorter-chain acids (C_{16} and C_{18}). The same phenomenon was observed for the HPLC analysis. A good separation of the *cis* and *trans* isomers was obtained for the C_{20} and C_{22} chain lengths (Fig. 3B), but only by reduction of the solvent flow rate; a slight overlap between the *cis* and *trans* occurred for the C_{16} and C_{18} chain lengths. To determine if the chart areas using the refractive index detector corresponded to the amount of *trans* acid, a known to separate the different chain lengths, the stability of the methoxy-bromomercuri-adducts facilitates sample handling. As is often the case in lipid research, the choice of alternatives must be made on an individual laboratory basis.

ACKNOWLEDGMENTS

This work was funded in part by a contract from the U.S. Department of Commerce, National Marine Fisheries Service and was also partially supported by the Natural Sciences Engineering and Research Council of Canada.

REFERENCES

1. Conacher, H.B.S., Page, B.D., and Beare-Rogers, J.L. (1973) Lipids 8, 256-258.
2. Hølmer, G., and Aaes Jorgensen, E. (1969) Lipids 4, 507-514.
3. Conacher, H.B.S., Page, B.D., and Chadha, R.K. (1972) J. Am. Oil Chem. Soc. 49, 520-523.
4. Koritala, S., and Dutton, H.J. (1969) J. Am. Oil Chem. Soc. 46, 245-248.

5. Sebedio, J-L., Langman, M.F., Eaton, C.A., and Ackman, R.G. (1981) J. Am. Oil Chem. Soc. 58, 41-48.
6. Scholfield, C.R., Davidson, V.L., and Dutton, H.J. (1967) J. Am. Oil Chem. Soc. 44, 648-651.
7. Strocchi, A., and Bonaga, G. (1979) Rev. Fr. Corps Gras 1, 9-16.
8. Lambertsen, G., Myklestad, H., and Braekkan, O.R. (1971) J. Am. Oil Chem. Soc. 48, 389-391.
9. Dutton, H.J. (1978) J. Am. Oil Chem. Soc. 55, 806-808.
10. Conacher, H.B.S., and Page, B.D. (1972) J. Am. Oil Chem. Soc. 49, 283-286.
11. Scholfield, C.R. (1979) in Geometrical and Positional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 17-52, American Oil Chemists' Society, Champaign, IL.
12. Ackman, R.G., Sebedio, J-L., and Ratnayake, W.M.N. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) Vol. 72, pp. 253-276, Academic Press, New York, NY.
13. Ojanpera, S.H. (1978) J. Am. Oil Chem. Soc. 55, 290-292.
14. Lanza, E., and Slover, H.T. (1981) Lipids 16, 260-267.
15. Ackman, R.G., Hooper, S.N., and Hingley, J. (1972) J. Chromatogr. Sci. 10, 430-438.
16. Conacher, J.B.S., and Iyengar, J.R. (1978) J. Assoc. Off. Anal. Chem. 61, 702-708.
17. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. 37, 911-917.
18. Ackman, R.G. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) Vol. 72, pp. 205-252, Academic Press, New York, NY.
19. Metcalfe, L.D., and Schmitz, A.A. (1961) Anal. Chem. 33, 363-364.
20. Sebedio, J-L., and Ackman, R.G. (1981) Lipids 16, 461-467.
21. White, H.B. (1966) J. Chromatogr. 21, 213-222.
22. Sebedio, J-L., and Ackman, R.G. (1979) J. Am. Oil Chem. Soc. 56, 15-21.
23. Ackman, R.G. (1977) Lipids 12, 293-296.
24. Sebedio, J-L., and Ackman, R.G. (1978) Can. J. Chem. 56, 2480-2485.
25. Sebedio, J-L., and Ackman, R.G. (1981) J. Chromatogr. Sci. 19, 552-557.
26. Lie Ken Jie, M.S.F. (1980) in Advances in Chromatography (Giddings, J.C., Grushka, E., Cazes, J., and Brown, P.R., eds.) Vol. 18, pp. 35-43, Marcel Dekker, New York, NY.
27. Morris, L.J. (1966) J. Lipid Res. 7, 717-732.
28. Gunstone, F.D., Ismail, I.A., and Lie Ken Jie, M. (1967) Chem. Phys. Lipids 1, 376-385.
29. Vigneron, P.Y., and Spicht, P. (1973) Rev. Fr. Corps Gras 11, 631-636.
30. Pelloquin, A., and Ucciani, E. (1974) Rev. Fr. Corps Gras 10, 545-551.
31. Binder, H., and Linder, W. (1973) J. Chromatogr. 77, 175-180.
32. Ackman, R.G., Bannerman, M.A., and Vandenhuevel, F.A. (1960) Anal. Chem. 32, 1209.
33. Davidson, V.L., and Dutton, H.J. (1966) Anal. Chem. 38, 1302-1305.
34. Stein, R.A., and Nicolaides, N. (1962) J. Lipid Res. 3, 476-478.
35. Johnston, A.E., and Dutton, H.J. (1972) J. Am. Oil Chem. Soc. 49, 98-100.
36. Sauer, F.D., and Kramer, J.K.G. (1980) in Advances in Nutrition Research (Draper, H.H., ed.) Vol. 3, pp. 207-230, Plenum Press, New York, NY.
37. Craig, B.M., and Beare, J. (1967) Can. J. Biochem. 45, 1075-1079.
38. Boucrot, P., and Bezard, J. (1973) Arch. Sci. Physiol. 27, 1-24.
39. Norseth, J. (1980) Biochim. Biophys. Acta 617, 183-191.

[Received October 29, 1981]

The Metabolism of Lithocholic Acid and Lithocholic Acid-3-α-sulfate by Human Fecal Bacteria

S.P. BORRIELLO[a,1], and R.W. OWEN[b,2], [a]*Bacterial Metabolism Research Laboratory, Central Public Health Laboratory, Colindale Avenue, Colindale, London NW9 5HT;* and [b]*Department of Chemistry and Biochemistry, Liverpool Polytechnic, Liverpool L3 3AF, England*

ABSTRACT

Both lithocholic acid and lithocholic acid-3α-sulfate are metabolized by mixed fecal bacteria and by pure strains of the genus *Clostridium*. Mixed fecal bacteria metabolized lithocholic acid to 3-keto-lithocholic acid; lithocholic acid-3α-sulfate was metabolized to isolithocholic acid, 5β-cholanic acid and Δ^3-cholenic acid under both aerobic and anaerobic conditions. The results indicate that a specific genus, the *Clostridium*, has a primary role in the metabolism of lithocholic acid-3α-sulfate to Δ^3-cholenic acid.

Lipids 17:477-482, 1982.

INTRODUCTION

Lithocholic acid is formed in the distal intestine by the activity of bacterial 7α-dehydroxylase on the primary bile acid chenodeoxycholic acid. This major fecal bile acid may be present in above-average concentrations in the stools of subjects from populations at high risk for the development of colonic cancer (1). It has also been shown to enhance chemically induced tumorigenesis in the intestine (2) and liver (3) of rats, to enhance the mutagenicity of suboptimal amounts of 2-amino-anthracene (4) and benzo(a)pyrene (5) in the Ames mutagenicity test, and to transform Syrian hamster embryo cells (6). Lithocholic acid is conjugated in the liver to taurine or glycine (about 40%) (7) and also exists as the water-soluble 3α-sulfate, which can account for up to 80% of the biliary lithocholate (8). Sulfation occurs in the liver and is a detoxifying process that also apparently facilitates excretion, as the sulfate is poorly absorbed by the intestinal mucosa. Both lithocholic acid and lithocholic acid-3α-sulfate can escape the small bowel enterohepatic cycle and seep into the large bowel where they are available for metabolism by the gastrointestinal flora. Little is known about the further metabolism of this bile acid in the large bowel. This study was designed to investigate this question and to try and delineate the role of various components of the fecal flora in these activities. Metabolism of lithocholic acid-3α-sulfate probably represents a retoxifying process. The roles that these microbially produced metabolites may play in gastrointestinal disorders, such as colorectal cancer, are discussed.

MATERIALS AND METHODS

Synthesis of Lithocholic Acid-3α-Sulfate

Lithocholic acid-3α-sulfate ($LASO_4$) was synthesized from lithocholic acid (Koch-Light Laboratories, Colnbrook, Bucks) as described by Tserng and Klein (9).

The product, mp 232 C, lit. mp 233-235 C; IR (KBr disc) 3450 (broad), 1560 (C=O), 1220, 1060 and 960 cm^{-1} was obtained as a white crystalline solid. Thin layer chromatography (TLC) on 20 x 20 cm DC-Fertig Platten Kieselgel F 254 0.25 mm plastic backed plates (E. Merck, Darmstadt) in the solvent systems *n*-butanol/acetic acid/water (10:1:1, v/v) and methanol/dichloromethane (1:19, v/v) revealed that the product was free of residual lithocholic acid.

Fermentation Medium

$LASO_4$ and lithocholic acid (LA) were both maintained as 1% (w/v) sterile stock solutions in distilled water. The LA was added to water as the sodium salt to overcome solubility problems. Aliquots of these stock solutions were added to brain-heart infusion broth (BHI) with added reducing agents (10) to a final concentration for $LASO_4$ and LA of 0.05% (w/v) to yield BHI-$LASO_4$ and BHI-LA, respectively ("Fermentation media").

Metabolism of $LASO_4$ and LA by Mixed Cultures of Human Fecal Bacteria (MHFB)

Freshly voided feces were passed into an anaerobic chamber and suspended in BHI-

[1]Present address: Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, England.

[2]Present address: Bacterial Metabolism Research Laboratory, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, England.

$LASO_4$ and BHI-LA to yield a 1:10 dilution (w/v). Serial 10-fold dilutions of these stock stool solutions were made over 10 steps in the fermentation media. This was performed in triplicate. Controls consisted of uninoculated BHI, BHI-$LASO_4$ and BHI-LA broths, and a 1:10 dilution (w/v) of stool in BHI broth. Incubations were performed under both aerobic and anaerobic conditions. Aerobic incubations consisted of 5-ml aliquots in loose capped bijoux bottles incubated on an orbital shaker. Anaerobic incubation was performed under an atmosphere of 90% hydrogen and 10% CO_2 in the presence of a cold 'D' catalyst (Englehard Industries, Cinderford, Gloucestershire, England). All broths were screened for the presence of metabolites after 16, 48 and 72 hr incubation. Fecal flora analysis was performed as described elsewhere (10) at 0 and 72 hr in an attempt to correlate metabolism with the presence of the major genera of bacteria.

Large-Scale Fermentation

The procedure just described was repeated. However, in this case, 5 g of freshly voided stool was inoculated into 1 ℓ of BHI-$LASO_4$ and incubated under anaerobic conditions for 72 hr. This large-scale fermentation process was to enable sufficient quantities of the metabolites to be isolated for more precise spectroscopic identification. After incubation, the medium was extracted twice with an equal volume of methanol/dichloromethane (1:19, v/v). The resultant extract was dried in vacuo at 50 C overnight yielding 110 mg of solid residue. The residue was dissolved in 5.0 ml of methanol/dichloromethane (1:19, v/v) and preparative TLC was performed on 20 × 20 cm glass plates coated with Silica Gel G (Anachem) to a thickness of 0.5 mm. The plates were irrigated in the solvent system methanol/dichloromethane (1:19, v/v). Observation of the plates under ultraviolet (UV) light (254 nm) did not reveal any products; however, spraying with anisaldehyde (11) followed by heating showed 4 distinct bands. The 2 most polar products (metabolites A and B) were well resolved whereas the relatively nonpolar products were poorly resolved. Elution of metabolite A with dichloromethane yielded white spikey crystals (1 mg) after evaporation of the solvent. This product was identical in its TLC, gas liquid chromatographic (GLC) and spectroscopic properties to authentic isolithocholic acid (3β-hydroxy-5β-cholan-24-oic acid). Metabolite B gave white crystals (3 mg). This metabolite was found to be identical to 3-keto-lithocholic acid (5β-cholan-3-oxo-24-oic acid) using the same criteria as above. Reseparation of the poorly resolved nonpolar metabolites in the solvent system chloroform/methanol (200: 1, v/v) gave 2 distinct bands, designated metabolites C and D. Elution of metabolite C followed by crystallization gave 10 mg of a white powder. Metabolite C was identical in its TLC, GLC and spectroscopic properties to authentic 5β-cholanic acid. Metabolite D could not be crystallized after elution. This metabolite gave only one spot on TLC and one peak on GLC by the methods described previously. Similar GLC results were obtained on a Pye Series 204 chromatograph (isothermal, 260 C) fitted with a 3% OV-17 column (2-mm id). However, using a temperature gradient (140-260 C) at 4 C/min metabolite D gave rise to a well-defined doublet of peaks. The major peak (90%) of the doublet had a GLC mobility, relative to 5α-cholestane, of 1.10 which was identical to methyl-5β-cholanoate, whereas the minor peak (10%) had a GLC mobility of 1.12.

Study of metabolite D by GLC-MS revealed that the major peak gave a molecular ion at m/e 372, with significant fragment ions at m/e 201, 215, 230, 257, 318 and 357 which is typical of methyl Δ^3-cholenate (12).

Metabolism of $LASO_4$ and LA by Pure Strains of Facultative and Strict Anaerobes

Ten-ml aliquots of BHI-$LASO_4$ and BHI-LA media in universal bottles were inoculated with 2-3 drops of an actively growing culture in Robertson's cooked meat medium. Anaerobic isolates, which were all fresh fecal isolates, were incubated under anaerobic conditions. Facultative bacteria were incubated under both aerobic and anaerobic conditions. Controls consisted of uninoculated fermentation media and inoculated BHI broth. All broths were screened for the presence of metabolites after 16, 48 and 72 hr incubation.

Metabolism of $LASO_4$ by MHFB after Treatment with Alcohol, Heat or Vancomycin

The suspension of MHFB was treated with alcohol or heat for the selection of clostridial spores (13) or with vancomycin at a final concentration of 7.5 μg/ml to inhibit Gram-positive organisms. The treated MHFB suspensions were then added to BHI-$LASO_4$ fermentation medium prior to incubation under anaerobic conditions. Aliquots of all dilutions were tested for the presence of products of $LASO_4$ metabolism at 16, 48 and 72 hr.

Thin Layer Chromatography of $LASO_4$ and LA Metabolites

Metabolites of $LASO_4$ and LA were detected by TLC on 25 × 25 cm plastic-backed 0.25 mm plates. Ten μl from each fermentation was

applied directly to the plates along with the standards lithocholic acid, isolithocholic acid, 3-keto-5β-cholanoic acid (3KLA) and 5β-cholanic acid (5βC). The plates were developed in the solvent system methanol/dichloromethane (1:19, v/v). Metabolites and standards were visualized by spraying the plates with anisaldehyde reagent (11), followed by heating at 110 C for 15 min in an oven.

Gas Liquid Chromatography

After TLC analysis, the fermentation broths were extracted twice with an equal volume of methanol/dichloromethane (1:19, v/v). The pooled extracts were dried over anhyd $MgSO_4$, and the solvent was finally removed by evaporation under a stream of nitrogen gas. The dried extracts were methylated prior to GLC using an ethereal solution of diazomethane (14). GLC analysis was carried out at 250 C on a Pye 105 gas chromatograph fitted with a 3% OV-1 column. Identification of peaks was achieved by reference to the retention times of the methyl esters of known standards. Retention times were calculated relative to the internal standard, 5α-cholestane.

Mass Spectrometry

Mass spectra were obtained on a Dupont 21-491 series mass spectrometer, equipped with a 21-0948 data system, and coupled to a Varian Aerograph 2700 gas chromatograph. The column effluent was split, and one portion was diverted to a hydrogen flame ionization detector, while the remainder entered the ion source via a heated transfer line and jet separator. The chromatographic conditions follow. The column (2M-4 mm) packing was 3% OV-1 on 100/120 mesh Supelcoport with helium flow rate of 20 ml min^{-1}; the heated injector was maintained at 200 C, and the column oven was temperature-programmed from 150-270 C at 10 C min^{-1}. The FID oven and transfer line were maintained at 260 C. Mass spectra were obtained at an ionization energy of 70 eV. The calibration range was 31-617 atomic mass units and the resolution was 600. The magnet was scanned at a rate of 2 sec/decade. The source temperature was 200 C throughout. Bile acid methyl esters were analyzed as their trimethylsilyl ethers. Identifications were made by comparison to the spectra of authentic standards obtained under identical conditions.

RESULTS AND DISCUSSION

Several workers have demonstrated that the incubation of lithocholic acid (LA) or its 3α-sulfate ($LASO_4$) with fecal suspensions will result in the metabolism of these substrates (15-17). The results presented here confirm those observations, and evidence as to which components of the flora are important in these metabolic activities has been provided.

The taurine or glycine conjugates of LA and $LASO_4$ were not used in these in vitro experiments, even though they are known to exist in vivo (7), because the ability to hydrolze these conjugates is fairly widespread (18,19) and much free LA and $LASO_4$ would be expected to be released within the large bowel from these conjugates. The interest here was to investigate the further metabolism of these compounds.

Metabolism of LA

Incubation of LA with fecal suspension yielded 3-keto lithocholic acid (3KLA) after either aerobic or anaerobic incubation. This product was only found in the lowest dilution and then only after 72 hr incubation under anerobic conditions whereas, under aerobic conditions, 3KLA could be detected over the dilution range 10^{-1} to 10^{-4} at both 48 and 72 hr. These results indicate that the metabolism of LA is favored under aerobic incubation conditions, a finding also noted by Kelsey and Sexton with rat intestinal microflora (16). Kelsey et al. (15), found 3KLA, isolithocholic acid (ILA) ethyl lithocholic acid (EtLA) and 5β-cholanic acid (5βC) produced from the metabolism of LA by human fecal flora. The inability to detect EtLA in this study is not surprising as the addition of ethanol to the incubation medium is required.

The inability to detect ILA and 5βC may be an indication of the relative sensitivities of the 2 methods. Here, TLC was used as an initial screen for metabolites whereas Kelsey et al. (15) used Sephadex LH-20 column chromatography and glass fiber paper analysis coupled with liquid scintillation for the detection of radiolabeled metabolites. It is also possible that the different metabolic profiles in these 2 studies reflect the differing capability of the fecal flora of different subjects to metabolize LA. Norman and Palmer (20) found labeled ILA, 3KLA and an unidentified, unsubstituted, unsaturated bile acid in the stools of volunteers fed lithocholic acid-24-C (14). However, these metabolites are possibly the result of microbial metabolism of (^{14}COOH)-$LASO_4$ that would be formed by the liver and not necessarily of unsubstituted LA.

Having shown that the fecal flora can metabolize LA, the obvious question is which components of that flora are responsible. Most of

TABLE 1

Products of Lithocholic Acid 3α-Sulfate Metabolism by Pure Strains of the Genus *Clostridium*

	Products		
	16 hr	48 hr	72 hr
C. sporogenes	ILA, 5βC and Δ^3	ILA, 5βC and Δ^3	ILA, 5βC and Δ^3
C. butyricum	5βC and Δ^3	ILA, 5βC and Δ^3	Δ^3
C. perfringens	ILA, 5βC and Δ^3	ILA, 5βC	Δ^3
C. bifermentans	ILA, 5βC and Δ^3	ILA, 5βC and Δ^3	5βC and Δ^3
C. paraputrificum	5βC and Δ^3	5βC and Δ^3	Δ^3
C. tertium	5βC and Δ^3	5βC and Δ^3	Δ^3
C. difficile	ILA, 5βC and Δ^3	5βC and Δ^3	5βC and Δ^3
C. sordellii	ILA, 5βC and Δ^3	ILA, 5βC and Δ^3	5βC and Δ^3

the previous work on LA metabolism by isolated bacteria has been restricted to soil microorganisms (21,22) and isolates from the rat intestine (23). Other than the side-chain degradation of LA by *Escherichia coli* yielding C22 and C19 products (24), no strains of bacteria isolated from the human gastrointestinal tract has been shown to metabolize LA, and none have been shown to metabolize LA in vitro to products found in vivo. Our findings show that facultative organisms can metabolize LA under aerobic conditions as part of a mixed fecal flora to yield 3KLA. In addition, 14 of 57 isolates of facultative bacteria (25%) had the ability to metabolize LA to 3KLA when they were incubated under anaerobic conditions. It was interesting to note that, of the more common fecal aerobes, only 1 to 14 *E. Coli* strains and none of 18 *Streptococcus sp* metabolized LA. Of 157 strains of anaerobic bacteria tested, the ability to metabolize LA was found to be absent among the *veillonella, bacteroides, bifidobacteria* and *lactobacilli*. It was, however, present in 2 of the 8 known strains of clostridia listed in Table 1 and 12 of 29 randomly selected, unidentified clostridial strains isolated from feces. In all cases, there had been active growth of the organism under test. In contrast to the facultative organisms, the active clostridia produced a range of metabolites. *C. Sporogenes* produced ILA, 5βC and Δ^3-cholenic acid at all times of testing, and *C. sordellii* produced 5βC and Δ^3-cholenic acid at both 48 and 72 hr. The ability of *C. sordellii* and the apparent inability of *C. bifermentans* to metabolize LA indicate that this test may be of use in differentiating these 2 very similar microorganisms.

The evidence presented here indicates that the ability to metabolize LA is not widespread among bacteria isolated from the human gastrointestinal (GI) tract. This contrasts with the known ability of many GI tract microorganisms to deconjugate conjugated LA (19). Of all the individual microorganisms tested other than the clostridia, only a few facultative bacteria, and then rarely the more common GI tract isolates, could metabolize LA. It would appear that the clostridial flora is a potentially important component of that part of the human fecal flora that has the ability to metabolize LA. The possible relevance of these metabolites to human disease are discussed later.

Bacterial metabolism of $LASO_4$ was more extensive, and was favored under anaerobic conditions. Products detected were 5βC and Δ^3 cholenic acid, and ILA which was only detected under anaerobic conditions.

Of 57 facultative anaerobic organisms tested, only 26% could metabolize $LASO_4$ (Table 2). Of these, only 1 of 14 strains of *E. coli* and none of 18 strains of *Streptococci* were active. Of 157 unspecified strains of anaerobic bacteria tested, the ability to metabolize $LASO_4$ was restricted to the genus *Clostridium* (20/37); 30 strains each of the genera *veillonella, lactobacillus, bifidobacterium* and *bacteroides* failed to metabolize $LASO_4$.

Under anaerobic incubation conditions with fecal dilutions, clostridia were isolated from each dilution at which metabolism of $LASO_4$ occurred. Members of the genus *Bacteroides* were also found at higher dilutions in which metabolism was not detected, whereas *veillonella* and *lactobacilli* were absent at dilutions in which metabolism occurred. Additionally, $LASO_4$ metabolism was carried out by 8 speciated strains of clostridia (Table 1).

The addition of vancomycin to the incubation mixtures of MHFB resulted in the absence of $LASO_4$ metabolism. This indicated that the Gram-positive organisms were involved in its metabolism. Further studies using selective methods (heat shock and alcohol treatment) for *Clostridium* spores revealed that the fecal

TABLE 2

Metabolism of Lithocholic Acid and Its 3α-Sulfate by Facultative Microorganisms under Anaerobic Conditions

Organism (no.)	No. + ve for metabolism of:		
	$LASO_4$	LA	Both
E. Coli (14)	1	1	0
Bacillus sp (7)	4	4	4
Serrattia sp (2)	1	1	1
Chromobacterium sp (2)	0	1	0
Proteus sp (6)	3	3	3
Streptococcus sp (18)	0	0	0
Erwinia sp (3)	2	1	1
Enterobacter sp (4)	3	2	2
Klebsiella sp (1)	1	1	1

flora were still able to metabolize $LASO_4$. These observations indicated that the genus *Clostridium* was primarily responsible for $LASO_4$ metabolism. These results substantiate the studies of Kelsey et al. (15), in that the mixed fecal flora of the human are capable of extensive metabolism of LA and $LASO_4$. Additionally, our data indicate that, of the fecal flora, the genus *Clostridium* is the most important regarding LA and $LASO_4$ metabolism.

In the light of these results, it is interesting to speculate on the possible pathways for the metabolism of $LASO_4$ (Fig. 1). Our data suggest that the most probable pathway involves cleavage of the sulfate ester at the C-O bond to yield ILA. C-O bond cleavage with subsequent inversion of configuration has been demonstrated with aryl sulfatases and soil isolates of detergent-degrading microorganisms (25). The production of Δ^3-cholenic acid can be explained by 2 mechanisms: first by dehydroxylation at C3 of ILA or by *trans*-elimination of the sulfate ester group from $LASO_4$. Dehydroxylation at C3 could be analogous to the re-

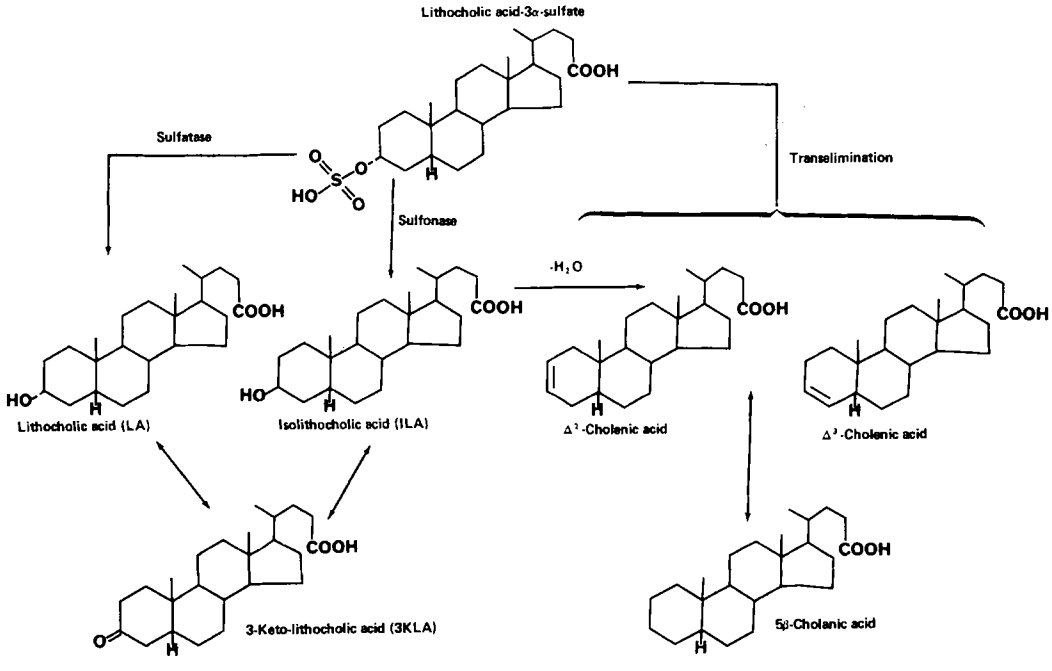


FIG. 1. Metabolism of lithocholic acid-3α-sulfate.

moval of the C7-hydroxyl group from primary bile acids giving a Δ^6-cholenic acid compound which is subsequently reduced to form the secondary bile acids (26). It is also possible that Δ^3-cholenic acid is produced via a bacterially mediated *trans*-elimination of the sulfate ester group. However, the presence of ILA indicates this to be a less likely mechanism.

The metabolism of the 3α-sulfate of lithocholic acid probably represents a retoxifying process and it is possible that some of these metabolites may be potential carcinogens. Recent studies suggest that LA derivatives should not necessarily be considered as innocuous, endogenous compounds as LA enhances the mutagenicity of carcinogens of different chemical classes and transforms mammalian cells. Palmer (27) has suggested that more unsubstituted and degraded bile acids are more potent pharmacological agents than di- and trihydroxy compounds, and the cholenic acid produced resembles the unsaturated ring-A bile acids postulated as potential carcinogens by Hill (28). Kelsey et al. (15) have produced some evidence to suggest that the fecal flora of patients at high risk of contracting colon cancer more readily convert $LASO_4$ to unsubstituted, unsaturated metabolites such as Δ^2/Δ^3-cholenates. The results presented here further substantiate the ability of the fecal flora to perform the reactions and, in addition, for the first time implicate a specific genus, the *Clostridium*, as having a primary role in this metabolism. Further, it has been shown that a number of known individual strains can produce ILA, 5βC and Δ^3-cholenic acid. Most important of all, it would appear that those clostridia that are thought to be important in the introduction of a double bond at the Δ^4 position of the ring A of bile acids and have an association with colon cancer (29-31) are also those that are most active in metabolizing $LASO_4$ to the unsaturated cholenates. If further substantiated evidence is forthcoming on an association between high fecal levels of Δ^2- or Δ^3-cholenate and colorectal cancer, then there is obviously a good case to be made for a primary etiological role for the clostridial component of the fecal flora.

ACKNOWLEDGMENTS

R.W. Owen received a Liverpool Education Authority grant during this study. We are grateful to P.R. Wait of the Bacterial Metabolism Research Laboratory for the mass spectra.

REFERENCES

1. Reddy, B.S., and Wynder, E.L. (1977) Cancer 39, 2533-2539.
2. Narisawa, T., Magadia, N.E., Weisburger, J.H., and Wynder, E.L. (1974) J. Natl. Cancer Inst. 53, 1093-1097.
3. Hiasa, Y., Konishi, Y., Kamamoto, Y., Watanabe, T., and Ito, N. (1971) Gann 62, 239-245.
4. Silverman, S.J., and Andrews, A.W. (1977) J. Natl. Cancer Inst. 59, 1557-1559.
5. Kawalek, J.C., and Andrews, A.W. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 844.
6. Kelsey, M.I., and Pienta, R.J. (1979) Cancer Lett. 6, 143-149.
7. Cowan, A.E., Korman, M.G., Hofmann, A.F., and Cass, O.W. (1975) Gastroenterology 69, 59-66.
8. Palmer, R.H. (1972a) in Bile Acids in Human Disease, (Back, P., and Gerok, W., eds.) p. 65, Shattauer, Stuttgart.
9. Tserng, K.Y., and Klein, P.D. (1977) Lipids 13, 479-486.
10. Boriello, S.P., Hudson, M.J., and Hill, M.J. (1978) Clin. Gastroenterol. 7, 329-349.
11. Kirtchevsky, D., Martak, D.S., and Rothblat, G.H. (1963) Anal. Biochem. 5, 388-392.
12. Child, P., Kuksis, A., and Marai, L. (1978) Can. J. Biochem. 57, 216-225.
13. Koransky, J.R., Allen, S.D., and Dowell, V.R. (1978) Appl. Environ. Microbiol. 35, 762-765.
14. De Boer, T.J., and Backer, H.J. (1954) Tec. Trav. Chim. Pay-Bas. 73, 229-234.
15. Kelsey, M.I., Molina, J.E., and Hwang, K.K. (1979) Front. Gastrointest. Res. 4, 38-50.
16. Kelsey, M.I., and Sexton, S.A. (1976) J. Steroid Biochem. 7, 641-647.
17. Kelsey, M.I., Muschik, G.M., and Sexton, S.A. (1978) Lipids 13, 152-157.
18. Bremmelgaard, A., and Bremmelgaard, A. (1974) Acta. Path, Microbiol. Scand. Sect. B. 82, 537-540.
19. Kelsey, M.I., and Thompson, R.J. (1976) J. Steroid Biochem. 7, 117-124.
20. Norman. A., and Palmer, R.H. (1964) J. Lab. Clin. Med. 63, 986-1001.
21. Tenneson, M.E., Bilton, R.F., and Mason, A.N. (1978) FEBS Lett. 91, 140-143.
22. Hayakawa, S., Kanematsu, Y., and Fujiwara, T. (1969) Biochem. J. 115, 249-256.
23. Dickinson, A.B., Gustafsson, B.E., and Norman, A. (1971) Acta Pathol. Microbiol. Scand. 79B, 691-698.
24. Tenneson, M.E., Owen, R.W., and Mason, A.N. (1977) Biochem. Soc. Trans. 5, 1758-1760.
25. Bartholomew, B., Dodyson, K.S., Matcham, G.W.J., Shaw, D.J., and White, G.F. (1977) Bio-Chem. J. 165, 575-580.
26. Samuelsson, B. (1960) J. Biol. Chem. 235, 361-366.
27. Palmer, R.H. (1972b) Arch. Intern. Med. 130, 606-617.
28. Hill, M.J. (1971) in Some Implications of Steroid Hormones in Cancer (Williams, D.C., and Briggs, M.H., eds.) p. 94, Heinemann, London.
29. Goddard, P., Fernandez, F., West, B., Hill, M.J., and Barnes, P. (1975) J. Med. Microbiol. 8, 429-435.
30. Borriello, S.P., Drasar, B.S., and Tomkins, A. (1978) Proc. Nutr. Soc. 37, 40A.
31. Hill, M.J., Drasar, B.S., Williams, R.E.O., Meade, T.W., Cox, A.G., Simpson, J.E.P., and Morson, B.C. (1975) Lancet 1, 535-539.

[Received January 12, 1982]

Effect of Garlic (*Allium sativum* Linn) on Serum Lipoproteins and Lipoprotein Cholesterol Levels in Albino Rats Rendered Hypercholesteremic by Feeding Cholesterol

V.S. KAMANNA and **N. CHANDRASEKHARA**, *Discipline of Biochemistry and Applied Nutrition, Central Food Technological Research Institute, Mysore 570 013, India*

ABSTRACT

The hypocholesteremic activity of garlic was tested by incorporating freeze-dried garlic powder at 0.5, 1.0, 2.0 and 3.0% levels in an atherogenic diet fed to rats. It was observed that 0.5 and 1.0% levels were not effective whereas the other 2 levels were. The group fed 2.0% garlic powder had much lower serum cholesterol level than the one fed 3%. The increased levels of low density lipoproteins (LDL) and LDL-cholesterol in rats fed the atherogenic diet were partly reversed in rats receiving a supplement of 2% garlic powder. On a cholesterol-containing diet, high density lipoproteins (HDL) and HDL-cholesterol levels were decreased. Inclusion of garlic powder in the atherogenic diet enhanced the percentage of HDL whereas no change was observed in HDL cholesterol levels. Commercial garlic pearls (equivalent to 0.15% garlic powder in the diet) produced a significant decrease in serum and liver cholesterol levels in rats fed the atherogenic diet. On the other hand, asafoetida at 1.5% level failed to reduce the serum cholesterol levels in the cholesterol-fed rats.

Lipids 17:483-488, 1982.

INTRODUCTION

Garlic (*Allium sativum* Linn) has long been used as a popular remedy for various physiological disorders such as hemorrhoids, cough, abdominal pain, loss of appetite, arthritis, pneumonia and rheumatism (1). Recent studies have indicated that garlic exhibits antibacterial properties (2,3). Its use in neurological manifestations of leprosy (4), alloxan-induced diabetes (5) and hypercholesterolemia (6,7) have also been reported. Among these, particular attention has been focused on the cholesterol-lowering activity of garlic in recent years.

It has been observed that ingestion of garlic juice, alcoholic extract or essential oil of garlic by cholesterol-fed rabbits resulted in inhibition of hypercholesterolemia and atheroscelerosis (8-11). "Acute clinical studies" in human volunteers have shown that crushed garlic bulbs or the essential oil of garlic can prevent alimentary hyperlipemia (12,13). It has also been reported that garlic can lower the serum cholesterol level in normal human volunteers (14,15). Aqueous extract of garlic is also known to have hypocholesterolemic activity in human beings (6) and in rats (16).

Although many references have been made in the literature to the hypocholesteremic activity of garlic and its different fractions, the mechanism by which it lowers the serum cholesterol level is still unclear. To gain further insight into the action of garlic, experiments were carried out on the response to the dose of garlic and its effect on tissue cholesterol levels, serum lipoproteins and lipoprotein cholesterol levels in rats. The efficacy of garlic pearls and asafoetida (*Ferula asafoetida* Linn), another sulfur containing spice, as cholesterol-lowering agents was also tested. Results of these studies are summarized in this communication.

MATERIALS AND METHODS

Female albino rats of the Wistar strain in the weight range 60-65 g were used as experimental animals. They were grouped on a body weight basis and kept in individual cages with raised screen bottoms.

The composition of the control diet was in g/100 g: casein, 12; corn starch, 65; fat (hydrogenated groundnut oil), 10; cane sugar, 10; salt mix (17), 2; vitaminized starch (18), 1; and fat-soluble vitamins A, D and E were added to the diet in a proportion such that each rat received daily 20, 10 and 1 IU, respectively. Cholesterol, sodium tauroglycocholate (mixed bile salts), garlic powder, asafoetida powder and the contents of garlic pearls were supplemented in the required proportion to the control diet at the expense of corn starch. Diets were prepared by mechanically mixing the ingredients.

At the end of the experimental period (8-9 weeks), rats were anesthetized under ether, blood was collected by heart puncture and centrifuged to obtain serum. The liver was excised, washed in cold isotonic saline, blotted well and weighed. Both serum and liver were preserved in a deep-freezer at -20 C till they were used for analysis.

Garlic and garlic pearls were purchased from

the local market. Asafoetida powder was supplied by the Discipline of Plantation Products and Flavour Technology, CFTRI, Mysore.

Preparation of Freeze-Dried Garlic Powder

Garlic was deskinned manually and the cloves were frozen at -30 to -35 C and dried at 30 C. Dried flakes were powdered in an Apex mill through a sieve (140 holes/in^2).

Estimation of Serum Cholesterol

Both total and free cholesterol in serum were estimated by the method of Searcy and Bergquist (19).

Liver Cholesterol

One g of liver was homogenized in a Potter Elvehjem homogenizer with a Teflon pestle in about 2.0 ml of 0.74% KCl solution. Twenty ml of the chloroform/methanol (2:1, v/v) mixture was then added and rehomogenized. The mixture was kept overnight and filtered through Whatman No. 1 filter paper. The extract was washed with 0.74% KCl solution according to the method of Folch et al. (20). Total and free cholesterol were determined as already described.

Serum Lipoproteins

Serum lipoproteins, mainly VLDL, LDL and HDL, were separated by polyacrylamide gel electrophoresis. The conditions were: gel concentration, 7.5%; Tris-glycine buffer (0.05 M), pH 8.5; current, 2.5 mA/tube. Fresh serum was prestained with Sudan Black B solution (21) by the method of Ressler et al. (22). Fifty to 75 μl prestained serum was applied on top of the gel and electrophoresis was performed for 40-45 min. The gels were removed immediately from the tubes and scanned in a Chromoscan 200 densitometer equipped with a 620 filter. The relative percentages of HDL and LDL (including VLDL) were calculated.

Serum LDL- and HDL-Cholesterol

Serum HDL-cholesterol was determined in the supernatant after precipitation of the apolipoprotein B containing lipoproteins with heparin-Mn^{2+} reagent according to the procedure of Warnick and Albers (23). The LDL precipitate was extracted with chloroform/methanol (2:1, v/v) mixture and an aliquot was taken for cholesterol determination.

RESULTS

Serum cholesterol levels of rats fed atherogenic diet and atherogenic diet containing different levels of garlic powder (0.5, 1.0, 2.0 and 3.0%) are given in Table 1. Total, free and cholesteryl ester levels were not significantly reduced in groups fed an atherogenic diet supplemented with garlic powder at 0.5 and 1.0% level (groups 3 and 4), when compared with animals fed the atherogenic diet (group 2). On the other hand, garlic powder at 2.0 and 3.0% levels in the atherogenic diet caused a reduction in cholesterol levels of 52 and 33%, respectively.

The influcence of garlic on liver total, free and cholesteryl ester levels is shown in Table 2. It was observed that inclusion of garlic in the control diet significantly reduced the total cholesterol; the decreases in free as well as cholesteryl ester levels were not so marked. The beneficial effect of garlic supplementation was more predominant in rats fed the atherogenic diet; total and cholesteryl ester levels were 33-36% lower in the garlic-fed rats.

Except during the first week, diet consumption was not adversely affected by the addition of garlic. Among the 4 groups of rats (Table 2 and 3), the average daily intake per rat was 8.4 ±0.4, 8.9±0.4, 8.3±0.1 and 8.8±0.3 g (SEM), respectively.

The relative percentage of HDL and serum lipoprotein cholesterol levels of rats fed the various diets are given in Table 3. In rats fed the control diet, HDL formed 79% of the total lipoproteins and addition of garlic powder did not alter the percentage distribution of HDL and LDL significantly; however, a slight increase in HDL was observed. When cholesterol was added to the diet, the percentage of HDL was markedly reduced and addition of garlic to the atherogenic diet increased HDL to 49%.

There was a significant increase in the serum HDL-cholesterol levels in rats receiving garlic powder in the control diet. On a cholesterol-containing diet, there was a significant reduction in HDL-cholesterol but addition of garlic at 2% level to this atherogenic diet did not alter the HDL-cholesterol level (Table 3). Garlic feeding lowered the LDL-cholesterol levels in animals fed the control diet. The influence of garlic was more marked in rats which were on the atherogenic diet; here the reduction in LDL-cholesterol was 42% on the basis of the group fed cholesterol.

Results of a comparative study on the effect of garlic powder, garlic pearls and asafoetida on serum and liver total cholesterol levels in rats are shown in Figure 1. It was found that garlic powder and garlic pearls reduced the serum cholesterol levels by 52 and 31%, respectively, whereas asafoetida did not alter it significantly (only 7%) in rats fed the atherogenic diet. How-

TABLE 1

Hypocholesterolemic Activity of Garlic Powder at Different Levels

Group	Supplements to control diet*	Serum cholesterol (mg/dl) Total cholesterol	Free cholesterol	Cholesteryl ester	Cholesteryl ester/ free cholesterol
1		66.8 ± 2.8 (6)	19.9 ± 0.61 (6)	47.0 ± 2.3 (6)	2.36
2	1% cholesterol + 0.15% bile salt	700.9 ± 43.3[a] (4)	216.2 ± 22.9[a] (4)	483.3 ± 33.9[a] (4)	2.24
3	1% cholesterol + 0.15% bile salt + 0.5% garlic powder	694.2 ± 116.8 (3)	227.4 ± 50.6 (3)	466.9 ± 68.9 (3)	2.05
4	1% cholesterol + 0.15% bile salt + 1% garlic powder	658.0 ± 44.7 (6)	228.9 ± 24.4 (6)	430.1 ± 35.0 (6)	1.88
5	1% cholesterol + 0.15% bile salt + 2% garlic powder	331.6 ± 40.6[a] (6)	100.8 ± 11.3[b] (6)	231.2 ± 30.3[a] (6)	2.29
6	1% cholesterol + 0.15% bile salt + 3% garlic powder	462.9 ± 63.0[c] (5)	149.8 ± 12.6[d] (5)	291.7 ± 40.9[b] (5)	1.94

*The composition of the control diet is given in materials and methods; number of rats is given in parentheses. Comparisons made between groups 2 and 1 and between groups 3, 4, 5 or 6 and group 2.

[a]$p<0.001$; [b]$p<0.01$; [c]$p<0.02$; [d]$p<0.05$.

TABLE 2

Effect of Garlic Powder on Liver Cholesterol Levels*

Group	Diet	Total cholesterol (mg/g)	Free cholesterol (mg/g)	Cholesteryl ester (mg/g)	Cholesteryl ester/ free cholesterol (mg/g)
I	Control (C)	4.67 ± 0.15 (7)	2.37 ± 0.11 (7)	2.30 ± 0.09 (7)	0.97
II	C + 2% garlic powder	4.12 ± 0.16[c] (7)	2.16 ± 0.11 (6)	2.03 ± 0.13 (6)	0.94
III	C + 1% cholesterol + 0.15% bile salt	52.44 ± 3.14[a] (5)	10.45 ± 0.84[a] (7)	41.40 ± 2.30[a] (5)	3.96
IV	C + 1% cholesterol + 0.15% bile salt + 2% garlic powder	35.24 ± 4.46[b] (5)	9.33 ± 0.88 (7)	26.76 ± 3.70[a] (5)	2.87

*Number of rats is given in parentheses. Comparisons are made between groups II and I, III and I, and IV and III.

[a]$p<0.001$; [b]$p<0.02$; [c]$p<0.05$.

TABLE 3

Influence of Garlic Powder on Serum HDL and Lipoprotein Cholesterol Levels*

Group	Diet	HDL (rel. %)	Total cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	LDL-cholesterol/ HDL-cholesterol
I	Control (C)	78.6 ± 1.9 (5)	53.7 ± 2.7 (7)	31.2 ± 1.1 (7)	30.8 ± 3.8 (7)	0.99
II	C + 2% garlic powder	83.1 ± 2.0 (5)	56.3 ± 2.4 (7)	37.2 ± 1.7[c] (7)	25.9 ± 1.8 (7)	0.70
III	C + 1% cholesterol + 0.15% bile salt	13.4 ± 0.9[a] (5)	490.7 ± 25.7[a] (6)	20.2 ± 2.1[a] (5)	446.0 ± 25.9[a] (6)	22.1
IV	C + 1% cholesterol + 0.15% bile salt + 2% garlic powder	49.4 ± 5.1[a] (5)	268.0 ± 27.9[a] (6)	18.5 ± 1.3 (7)	259.7 ± 37.8[b] (7)	14.0

*Number of rats is indicated in parentheses. Comparisons were made between groups II and I, III and I, and IV and III.
[a]$p<0.001$; [b]$p<0.01$; [c]$p<0.02$.

ever, liver cholesterol levels decreased by 34, 25 and 20%, respectively, in the animals fed garlic powder, garlic pearls and asafoetida.

DISCUSSION

Experiments with different levels of garlic powder in the diet indicated that 0.5 and 1% doses were ineffective whereas 2 and 3% doses caused a marked reduction in the serum cholesterol levels (53 and 34%, respectively). Thus, 2% garlic powder in the diet equivalent to 6-7% fresh garlic was optimal. This dose of 1.2-1.6 g/100 g body wt is closely comparable to the dosage of 1.7-2.5 g/100 g body wt found to be effective in rabbits (8). The cholesterol-lowering activity of garlic powder was also noticed in livers of rats fed both control and atherogenic diets. The effectiveness of garlic powder against hypercholesterolemia was also corroborated by histological examination of liver sections. The serum cholesteryl ester:free cholesterol ratio did not vary much among the various groups.

Much attention has been given to the serum lipoprotein profiles of experimental animals in recent years because of the finding that the hypercholesterolemia commonly present in coronary heart disease is correlated with an increase in the plasma concentration of LDL or LDL-cholesterol and a decrease in the HDL or HDL-cholesterol concentrations (24-26). Further, a depression in serum cholesterol caused by administered antihypercholesteremic agents such as thyroxine analogs or curcumin is usually accompanied by the reduction of serum LDL with a concomitant increase in the HDL (27, 28). In conformity with these observations, inclusion of garlic powder in the atherogenic diet reduced the percentage of LDL and LDL-cholesterol levels. On the other hand, HDL level increased in rats fed the atherogenic diet to which garlic powder was added, but no change was observed in HDL-cholesterol.

In in vitro studies, Carew et al. (29) and Stein et al. (30) demonstrated that HDL inhibits the LDL uptake by the arterial wall and also facilitates the transport of cholesterol from peripheral tissues to the liver, where it is catabolized or discarded from the body. Other in vitro studies using human fibroblasts, arterial smooth muscle cells and erythrocytes suggest that a nonhepatic cell controls the over-accumulation of cholesterol by controlling its own synthesis via the so-called LDL pathway (31). Because garlic partly corrects the imbalance in the lipoprotein profile produced by cholesterol feeding, either or both of these mechanisms may be operating to lower the serum cholesterol levels.

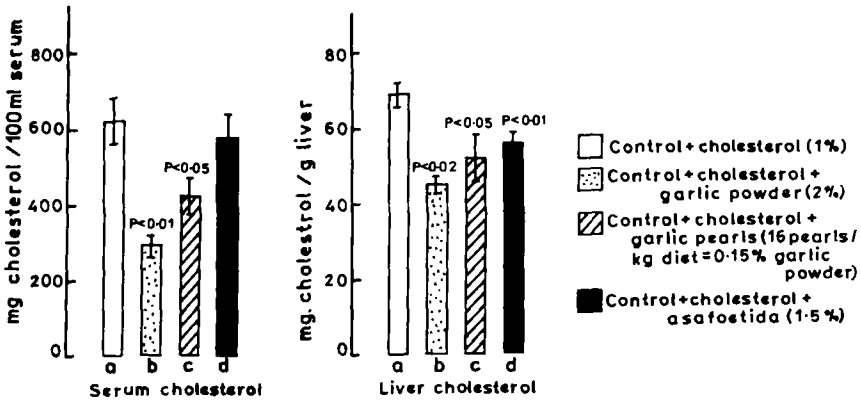


FIG. 1. Effect of garlic powder, garlic pearls and asafoetida on serum and liver cholesterol. Values are mean ± SEM of 6 rats. Comparisons are made between b, c or d and a.

Commercially available garlic pearls were also examined for their efficacy against hypercholesteremia. Compared on the basis of the volatile oil mentioned on the label of the product, the calculated efficacy of garlic pearls was found to be 6 times that of garlic powder.

Unlike garlic powder and garlic pearls, asafoetida at 1.5% could not reduce the hypercholesterolemia in rats. However, all of these agents, i.e., garlic powder, garlic pearls and asafoetida, have the same tendency to reduce the accumulation of cholesterol in liver. This report on asafoetida is in agreement with Bordia's findings on human alimentary lipemia (32).

In common with the other spices like turmeric (28) and ginger (33), inclusion of garlic in the atherogenic diet partly corrects the alteration in the serum lipoprotein profile produced by cholesterol feeding. Further studies in relation to the influence of garlic on cholesterol metabolism are in progress.

ACKNOWLEDGMENTS

The authors thank H.P. Ramesh for help in the histological examination of the liver sections. V.S. Kamanna is indebted to the CSIR for a Senior Research Fellowship.

REFERENCES

1. Nadkarni, A.K., (1976) in Indian Materia Medica (Nadkarni, K.M., ed.) 3rd revised edn., pp. 65-71, Popular Prakashan (P) Ltd., Bombay, India.
2. Subrahmanyan, V., Krishnamurthy, K., Sreenivasamurthy, V., and Swaminathan, M. (1957) J. Sci. Ind. Res. 16C, 173-174.
3. Mantis, A.J., Pr. Karaioannoglou, G., Spanos, G.P., and Panetsos, A.G. (1978) Food Sci. Technol. 11, 26-28.
4. Chaudhury, D.S., Sreenivasamurthy, V., Jayaraj, P., Sreekantiah, K.R., and Johar, D.S. (1962) J. Ind. Med. Assoc. 39, 517-520.
5. Jain, R.C., and Vyas, C.R. (1975) Am. J. Clin. Nutr. 28, 684-685.
6. Augusti, K.T. (1977) Ind. J. Exp. Biol. 15, 489-490.
7. Bordia, A.K., Joshi, H.K., Sanadhya, Y.K., and Bhu, N. (1977) Atherosclerosis 28, 155-159.
8. Jain, R.C. (1976) Ind. J. Med. Res. 64, 1509-1515.
9. Jain, R.C. (1975) Lancet 1, 1240.
10. Jain, R.C. (1978) Am. J. Clin. Nutr. 31, 1982-1983.
11. Bordia, A., Verma, S.K., Vyas, A.K., Khabya, B.L., Rathore, A.S., Bhu, N., and Bedi, H.K. (1977) Atherosclerosis 26, 379-386.
12. Sharma, K.K., Sharma, A.L., Dwivedi, K.K., and Sharma, P.K. (1976) Ind. J. Nutr. Dietet. 13, 7-10.
13. Bordia, A., and Bansal, H.C. (1973) Lancet 2, 1491-1492.
14. Sainani, G.S., Desai, D.B., Gorhe, N.H., Natu, S.M., Pise, D.V., and Sainani, P.G. (1979) Ind. J. Med. Res. 69, 776-780.
15. Jain, R.C. (1977) Am. J. Clin. Nutr. 30, 1380-1381.
16. Augusti, K.T., and Mathew, P.T. (1973) Ind. J. Exp. Biol. 11, 239-241.
17. Hubbel, R.B., Mendel, L.B., and Wakeman, A.J. (1937) J. Nutr. 14, 273-285.
18. Chapman, D.G., Castillo, R., and Campbell, J.A. (1959) Can. J. Biochem. Physiol. 37, 679-686.
19. Searcy, R.L., and Bergquist, L.M. (1960) Clin. Chim. Acta 5, 192-199.
20. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
21. McDonald, H.J., and Ribeiro, L.P. (1959) Clin. Chim. Acta 4, 458-459.
22. Ressler, N.R., Springgate, R., and Kaufman, J. (1961) J. Chromatogr. 6, 409-415.
23. Warnick, G.R., and Albers, J.J. (1978) J. Lipid Res. 19, 65-76.
24. Nikkila, E. (1953) Scand. J. Clin. Lab. Invest. 5, Suppl. 8, 5-101.
25. Barr, D.P., Russ, E.M., and Eder, H.A. (1951) Am. J. Med. 11, 480-493.
26. Jencks, W.P., Hyatt, M.R., Jetton, M.R., Mattingly, T.W., and Durrum, E.L. (1956) J. Clin. Invest.

35, 980-990.
27. Boyd, G.S., and Oliver, M.F. (1960) J. Endocrinol. 21, 25-32.
28. Subba Rao, D., Chandrasekhara, N., Stayanarayana, M.N., and Srinivasan, M. (1970) J. Nutr. 100, 1307-1315.
29. Carew, T.E., Hayes, S.B., Koschinsky, T., and Steinberg, D. (1976) Lancet 1, 1315-1317.
30. Stein, Y., Glangeaud, M.C., Fainaru, M., and Stein, O. (1975) Biochim. Biophys. Acta 380, 106-118.
31. Goldstein, J.L., and Brown, M.S. (1977) Ann. Rev. Biochem. 46, 897-930.
32. Bordia, A., and Arora, S.K. (1975) Ind. J. Med. Res. 63, 707-711.
33. Gujral, S., Bhumra, H., and Swaroop, M. (1978) Nutr. Rep. Int. 17, 183-189.

[Received December 30, 1981]

Plasma High Density Lipoprotein Subgroup Distribution in Rats Fed Diets with Varying Amounts of Sucrose and Sunflower Oil

ARNE T. HØSTMARK[*,a], **ØYSTEIN SPYDEVOLD**[b] and **EINAR LYSTAD**[a], with the technical assistance of **EVA KRISTENSEN**[a] and **IDA GOFFENG BAY**[a], [a]*Institute of Preventive Medicine, University of Oslo, Gydas vei 8, Oslo 3, Norway;* and [b]*Institute for Medical Biochemistry, University of Oslo, Sognsvannsveien 9, Oslo 3, Norway*

ABSTRACT

The effect of varying the dietary sunflower oil/sucrose (SO/SU) ratio on rat plasma lipid concentration and lipoprotein distribution was studied. Four groups of 10 rats were fed for 4 weeks diets with varying SO/SU ratios. Lipoprotein components were then estimated in whole plasma and after cumulative density ultracentrifugation. Whole plasma triacylglycerol (TG), total cholesterol (TC) and free cholesterol (FC) decreased with increasing SO/SU ratio; the CE/FC ratio increased, because CE remained virtually unaltered. Plasma TG-lowering was due to a decrease in VLDL and LDL-TG. Protein, CE and FC in d=1.063-1.100 g/ml (HDL_{2b}) and d=1.100-1.125 g/ml (HDL_{2a}) lipoproteins decreased upon increasing the SO/SU ratio. In contrast, in d=1.125-1.200 g/ml (HDL_3) lipoproteins, there was a concomitant increase in these components. Although increasing the SO/SU ratio effected more protein and CE transportation in HDL_3 and less in HDL_2, the total amount of these components in high density lipoproteins (d=1.063-1.200 g/ml) remained constant. Apo A-I and apo C-III decreased in HDL_2 but increased in HDL_3 upon increasing the SO/SU ratio. Also, HDL_2 apo E, and the apo C-II/apo C-III and small apo B/large apo B ratios in VLDL and LDL were lowered by increasing the SO/SU ratio. The hepatic VLDL-TG output during isolated liver perfusion was lowest in rats fed the diet with the highest SO/SU ratio. In perfusate, like in plasma, the VLDL and LDL apo C-II/apo C-III ratio, as well as the small apo B/large apo B ratio, decreased upon increasing the dietary SO/SU ratio. The results indicate that there can be appreciable diet-dependent variations in plasma HDL subgroup distribution in spite of unchanged total HDL levels.
Lipids 17:489-499, 1982.

INTRODUCTION

Epidemiological studies have shown that there is a positive correlation between the incidence of coronary heart disease and the blood level of total cholesterol, triacylglycerol and low density lipoprotein (LDL) cholesterol, but an inverse relationship between coronary risk and high density lipoprotein (HDL) cholesterol levels (1-4). Among the dietary components affecting the plasma lipids are the amount and kind of dietary fat and carbohydrates. It has been shown that polyunsaturated fatty acids (PUFA) act hypolipemic compared to saturated fat (5-8). Similarly, keeping the amount and type of fat constant, sucrose and fructose have been reported to be hyperlipemic when compared to glucose or starch (9); furthermore, when some of the carbohydrate in the diet is replaced by PUFA, there is a reduction in the plasma triacylglycerol concentration (5,9). As shown by Dumaswala et al. (6), the hypocholesterolemic effect of safflower seed oil was abolished by the presence of an excess of sucrose (54% by wt) in the diet. While there are some reports on the carbohydrate-PUFA antagonism on plasma total cholesterol and triacylglycerol levels, their relative effects on the plasma lipoprotein distribution seem less well explored. The lipoprotein classes currently referred to are very low density lipoprotein (VLDL) (d<1.006 g/ml), LDL (d=1.006-1.063 g/ml) and HDL (d=1.063-1.210 g/ml). It has been reported recently (10) that human HDL consists of the subgroups HDL_{2b} (d=1.063-1.100 g/ml), HDL_{2a} (d=1.100-1.125 g/ml) and HDL_3 (d=1.125-1.210 g/ml). Moreover, it appears that HDL subgroups might have antagonistic actions; HDL_2 recently was shown (11) to inhibit whereas HDL_3 stimulated fibroblast 3-hydroxy 3-methyl glutaryl coenzyme A reductase, the rate-limiting enzyme in cellular cholesterol synthesis.

The aim of this work was to investigate the influence of varying sunflower oil/sucrose ratio on rat plasma lipoproteins of several density classes, adopting human lipoprotein density limits. The currently used procedure to isolate lipoproteins—sequential ultracentrifugation—has been reported to cause considerable loss of lipoprotein components (12-15) and this might be substantial during the prolonged ultracentrifugations which would be necessary to

isolate lipoproteins in 5 density classes (VLDL, LDL, HDL_{2b}, HDL_{2a}, HDL_3). To avoid extensive loss of lipoprotein components, in this work, we have used cumulative density ultracentrifugation to assess the lipoprotein distribution. At the end of the diet period, isolated liver perfusion was done to assess hepatic VLDL output. The liver is essential for VLDL production; furthermore, it has been reported that sucrose-PUFA antagonism occurs in the liver (16).

PROCEDURES

Four groups of 10 male Wistar rats (one month of age, weight 60 g) were fed for 4 weeks diets in which the sunflower oil/sucrose (SO/SU) ratio was either 0.03 (group 1), 0.17 (group 2), 0.46 (group 3) or 1.01 (group 4) kJ/kJ (Table 1). The diets were fed ad libitum as dry mixtures. The rats had free access to water. They were kept under light from 06.00 to 18.00 hr and the room temperature was 22±1 C. Mean body weights at the end of the diet period were: 343±10, 337±9, 365±7 and 373±10 g in groups 1, 2, 3 and 4, respectively. The slight group differences in body weight suggest that the feeding was not strictly isocaloric; some differences in energy intake possibly contribute to the plasma lipoprotein effects of the various diets. Blood (5-7 ml) was collected from the aorta under Nembutal (30 μg/g body wt) anesthesia, using heparin-moistened syringes. Plasma samples from 3-4 rats in each group were pooled. One-ml samples from each pooled plasma were transferred to ultracentrifuge tubes and the density was adjusted according to Havel et al. (17) to make the 5 densities—1.006, 1.063, 1.100, 1.125 and 1.200 g/ml. Ultracentrifugation was done at 105,000 × g for 46 hr at 5 C in a Beckman L8-80 ultracentrifuge using a Ti 50 fixed-angle rotor. Thus, for each diet group, 3 plasma pools (together representing 10 rats) were subjected to ultracentrifugation at 5 densities. By this procedure, a cumulative flotation of the various lipoproteins was obtained. In a separate experiment, samples of a common plasma pool from rats fed a high-sucrose diet were subjected to ultracentrifugation at 11 different densities. Polyacrylamide gel electrophoresis of each supernatant, followed by densitometry, was

TABLE 1

Diets

	Diet 1	Diet 2	Diet 3	Diet 4
		Composition (g/100 g)		
Casein	20.0	As in	As in	As in
DL-methionine	0.3	group 1	group 1	group 1
Mineral mixture[a]	4.0			
Vitamins[b]	0.3			
Choline chloride	0.2			
Sunflower oil[c]	1.0	5.0	12.0	22.0
Sucrose	70.2	66.2	59.2	49.2
		Energy distribution (%)		
Protein	22	21	19	17
Carbohydrate	76	68	56	41
Fat	2	12	25	42
		Sunflower oil/sucrose ratio (kJ/kJ)		
	0.03	0.17	0.46	1.01
		Energy density (mJ/100 g)		
	1.55	1.63	1.78	1.99

[a]Composition of mineral mixture (g/100 g): NaCl 10.81, potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$) 23.65, K_2HPO_4 7.73, $CaHPO_4 \cdot 2H_2O$ 35.51, $CaCO_3$ 16.36, $MgCO_3$ 4.09, ferricitrate ($FeC_6H_5O_7 \cdot 3H_2O$) 1.60, $CuSO_4 \cdot 5H_2O$ 0.16, $MnSO_4 \cdot H_2O$ 0.14, KI 0.004, $ZnCO_3$ 0.04.

[b]Amount of vitamins/100 g diet: thiamin HCl 2.2 mg, riboflavin 2.2 mg, Ca-pantothenate 6.6 mg, pyridoxine HCl 2.2 mg, biotin 0.06 mg, folic acid 0.4 mg, *p*-aminobenzoic acid 11.0 mg, menadion 5 mg, cobalamine 0.03 mg, inositol 10 mg, ascorbic acid 20 mg, niacin 10 mg, α-tocopheryl acetate 10 mg, retinyl acetate 2000 IU, cholecalciferol 300 IU.

[c]Fauser Vitaquellwerk K6, 2 Hamburg 54, Germany.

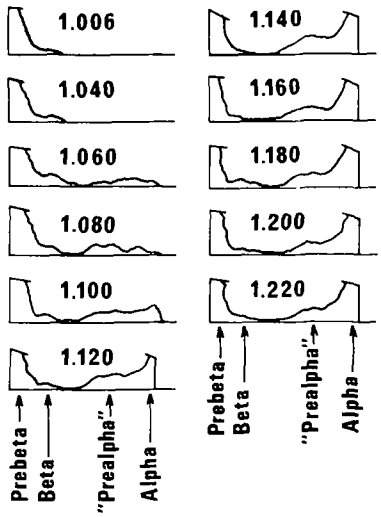


FIG. 1. Densitograms of lipoprotein electrophoretograms. Lipoproteins present in supernatants after cumulative density ultracentrifugation (at the 11 densities indicated) of pooled plasma from rats fed a high-sucrose diet were separated using polyacrylamide gel electrophoresis (kits from Labo International, Holland; acrylamide conc = 3% in separation gel, 2.5% in spacer and sample gels; densitometry at 620 nm; prestrained lipoproteins). "Prealpha" refers to poorly resolvable HDL_2 lipoproteins found between beta and alpha (18).

then performed using methods previously described (18). As shown in Figure 1, at d<1.060 g/ml, only prebeta (VLDL) and beta (LDL) are present. We previously reported that the beta band is reduced in rats fed a high-sucrose diet compared to rats fed stock diet (18). Above d=1.060 g/ml, lipoproteins of intermediate mobility appear, previously called "prealpha" (18), i.e., HDL_2. The presence of some "prealpha" (HDL_2) lipoproteins at d=1.060 g/ml indicates that LDL (d=1.006-1.063 g/ml) is overestimated. Also, some previous work suggests that both HDL and LDL appear in the 1.040-1.063 g/ml density fraction of rat plasma (19); others, however, recommend density limits 1.006-1.063 g/ml for quantitative LDL recovery (19). In any instance, the fact that d=1.006-1.063 g/ml lipoproteins contained very low levels of protein compared to HDL_2 (Fig. 5, vide infra) indicates that the contamination must have been small. Finally, above d=1.100-1.120 g/ml, the fastest migrating lipoproteins, alpha (HDL_3), also are present. By immunodiffusion in oxoid agar (Behringwerke, Germany) against rabbit anti-rat albumin (ICN, USA), no albumin contamination could be detected in the top layers after ultracentrufugation. However, sodium dodecylsulfate polyacylamide gel electrophoresis showed that some albumin was present (discussed later).

The top layer in each centrifuge tube was pipetted off and dialyzed 3 times against 0.9% NaCl 1 mM EDTA, pH 7.4. Each of the 40 whole plasma samples and each of the dialyzed supernatants after ultracentrifugation were analyzed enzymatically for free cholesterol, esterified cholesterol and triacylglycerols using Boehringer kit reagents. Total protein concentration in the dialyzed supernatants was determined with the Xylene brillant Cyanin G micromethod (20,21). The amount of lipoprotein components in VLDL (d<1.006 g/ml), LDL (d=1.006-1.063 g/ml), HDL_{2b} (1.063-1.100 g/ml), HDL_{2a} (1.100-1.125 g/ml) and HDL_3 (1.125-1.200 g/ml) was calculated by subtraction.

Apolipoprotein Determination

Estimation of apolipoprotein distribution in the cumulated lipoproteins was done for groups 1 and 4 by sodium dodecylsulfate polyacrylamide gel electrophoresis. For each group, lipoproteins in a pool of all 3 supernatants (representing 10 rats) in a given density class was precipitated with perchloric acid (0.4 M final concentration) without prior delipidation. By appropriate dilutions, about the same amount of protein was obtained from pooled supernatants in each density class. After centrifugation at 3,000 rpm in a table centrifuge, the lipoprotein precipitates from d<1.063 g/ml supernatants—containing large amounts of lipid—were floating in the tubes, whereas the precipitated lipoprotein was d>1.100 g/ml supernatants was found in the bottom of the tube. The protein-lipid complexes were then dissolved in electrophoresis buffer, containing 3% SDS and 40 mmol/ℓ dithiothreitol and incubated for 30 min at 37 C. Five to 10 μg protein was then subjected to SDS-polyacrylamide gel electrophoresis according to Fairbanks et al. (22), with the following modification: slab gels were used, the electrophoresis buffer contained 0.2% SDS, and the proteins were stained with 0.01-0.001% Xylene brillant Cyanin G. The various apoproteins were fairly well separated (Fig. 2). The separation pattern is in complete agreement with that obtained previously; molecular weights calculated from R_f values correspond to those reported by Swaney and Kuehl for rat apoproteins (23). From top to bottom, the bands in VLDL represent: high molecular weight apo B (apo B_h), low molecular weight

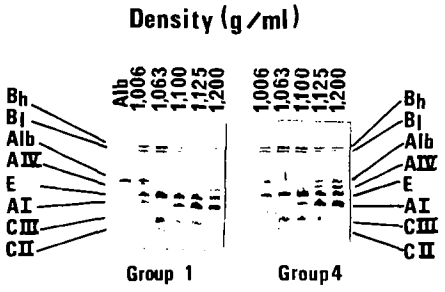


FIG. 2. Electropherograms of apolipoproteins present in supernatants after cumulative density ultracentrifugation. The apoproteins were separated on sodium dodecylsulfate polyacrylamide gel electrophoresis and stained with Xylene brillant Cyanin G. Groups 1 and 4 represent rats fed diets with the lowest and highest sunflower oil/sucrose ratios, respectively.

apo B (apo B_I), albumin, apo E, apo A-I, apo C-III and apo C-II. The relative intensity of the various bands was greatly altered from d=1.006 to d=1.200 g/ml. At the lower densities, apo B and apo E were the major bands. At higher densities, apo A-I was especially prominent, and the relative amount of apo E decreased. Apo A-IV was only visible above d=1.125 g/ml. This picture suggests that apo E is a major HDL_{2b} protein and apo A-I is the major HDL_3 protein. The various bands were cut off the gel, transferred to tubes containing 1 ml of extraction solution (methanol/H_2O/NH_3, 33:17:1, v/v/v) for the dye (Xylene brillant Cyanin G), and left overnight at room temperature to obtained complete extraction of the dye. Optical densities at 620 nm were read on a Vitatron digitial filter photometer. A standard curve was obtained with bovine serum albumin, run in parallel on the gel. The curve was linear over the range tested (0.5-10 μg protein). Using the method, 95-102% of the protein subjected to electrophoresis was recovered. Optical density corresponding to 1 μg protein/ml (extinction coefficient) was 0.060. Based on optical densities, a relative (%) distribution of apoproteins was calculated. Multiplying apoprotein percentages by total lipoprotein protein in the supernatants allows estimates of the cumulated amount of apoprotein. By subtraction, the amount of a given apoprotein in each density class can be estimated. Experiments currently are being performed to further investigate this approach of apolipoprotein determination. Also, densitometry (Vitatron densitometer, Hewlett-Packard recorder) was used after electrophoresis to assess apolipoprotein distribution; however, by this method, it is not easy to evaluate the contribution of the two B apoproteins, as also indicated by the results of others (24,25).

Liver Perfusion

The livers were perfused in a recirculating system for 2 hr as described earlier (7). Eight livers (2 from each of the 4 diet groups) were perfused during 1 day, between 09.00 and 14.00 hr. During this period, there are no major diurnal alterations in rat plasma lipoproteins (26). Mean weights of the livers were 15.1±0.5, 14.6±0.5, 15.0±0.4 and 15.1±0.6 g in groups 1, 2, 3 and 4, respectively. Perfustates were first centrifuged at 500 × g for 10 min to remove blood cells. Then, ultracentrifugation at d= 1.063 g/ml was done for 20 hr, as described for plasma. Total VLDL protein, triacylglycerol, free cholesterol, cholesteryl ester and apoproteins were determined in the dialyzed supernatants, as described for plasma. Student's t-test was used to estimate significance of differences between means.

RESULTS

Whole Plasma Triacylglycerol, Total Cholesterol, Free Cholesterol and Cholesteryl Ester Concentration

When increasing the relative amount of energy fom fat in the diet at the expense of sucrose (from groups 1-4, Fig. 3), there was a progressive decrease in plasma total triacylglycerol (TG) concentration. The TG level in plasma of rats fed the highest fat diet (group 4) was 43% lower than in plasma of rats fed diet with the lowest SO/SU ratio. A decreasing trend was also noted in plasma free cholesterol (FC) concentration in response to increasing the SO/SU ratio. The concentration of total cholesterol (TC) and cholesteryl ester (CE) remained fairly constant. This means that when sunflower oil replaced sucrose in the diet, a larger fraction of plasma total cholesterol was transported in esterified form, as also illustrated by the progressive increase in the cholesteryl ester/free cholesterol (CE/FC) ratio in response to increasing the SO/SU ratio (Fig. 3, right).

Cumulative Density Distribution of Plasma Lipoprotein Components

Only small amounts of lipoprotein *protein* were floating at d<1.063 g/ml (Fig. 4), and there were no significant group differences below this density. In contrast to this, the amount of lipoprotein protein floating at d= 1.100 g/ml was appreciably group-dependent, being gradually smaller from group 1 (lowest SO/SU ratio) to group 4 (highest SO/SU ratio).

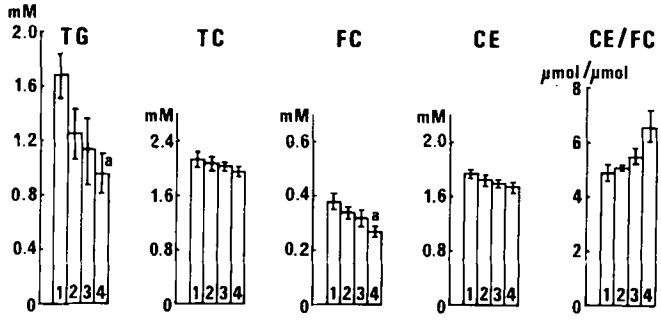


FIG. 3. Whole plasma triacylglycerol (TG), total cholesterol (TC), free cholesterol (FC) and cholesteryl ester (CE) concentrations. Groups 1-4: increasing sunflower oil/sucrose ratios (see Procedures). Mean values ± SEM (n=10); (a) $p<0.05$ vs group 1.

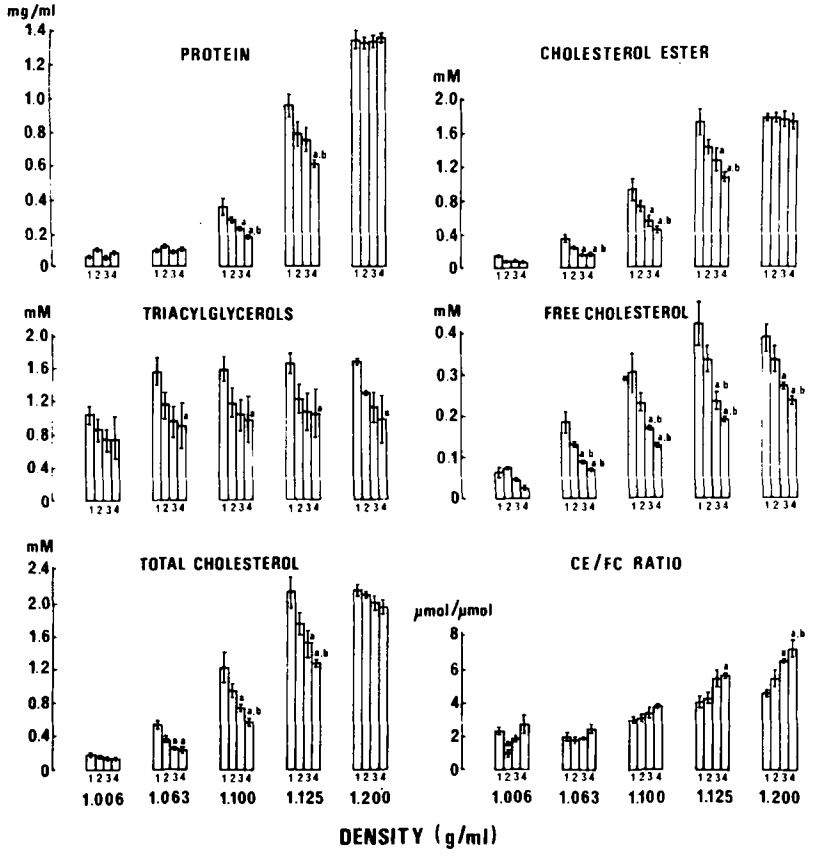


FIG. 4. Cumulative density distribution of plasma lipoprotein components. Plasma pools from each diet group were ultracentrifuged at densities indicated in Procedures. Diet groups 1, 2, 3 and 4 are indicated below the columns. Cholesteryl ester/free cholesterol (CE/FC) ratio was calculated for each pool. Mean values of the 3 pools (each representing 3-4 rats) ± SEM are indicated (a) $p<0.05$ vs group 1; (b) $p<0.05$ vs group 2.

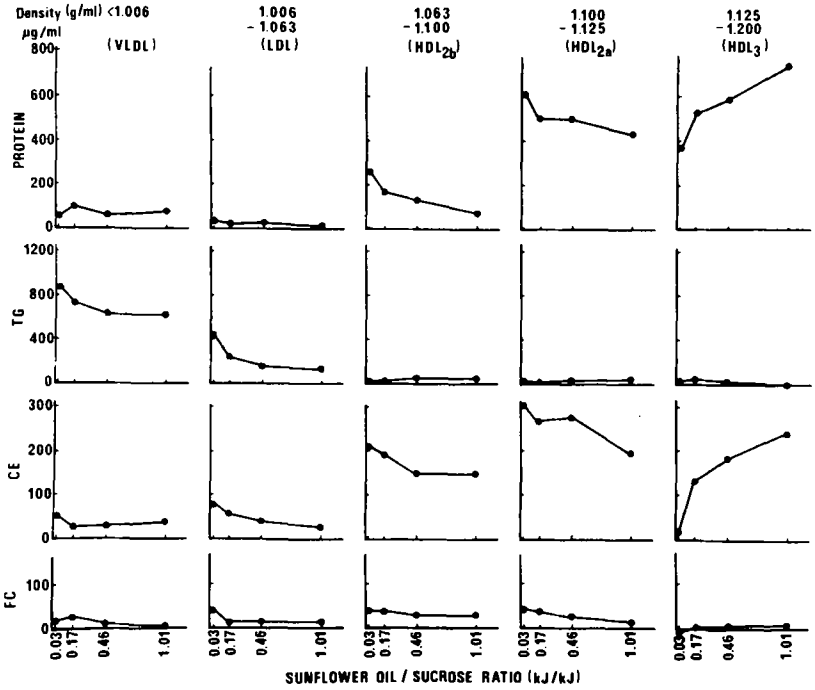


FIG. 5. Distribution of lipoprotein components on 5 density classes. Amount of lipoprotein component in each density class was calculated from the cumulative density ultracentrifugation values.

Thus, when the dietary SO/SU ratio was increased, there was a marked reduction of protein of d=1.063-1.100 g/ml lipoproteins. The group relationship was maintained at d=1.125 g/ml. However, at the highest density, 1.200 g/ml, there were again no group differences. This means that the group order of d=1.100-1.125 g/ml lipoproteins is quite opposite that of d=1.063-1.100 and d=1.100-1.125 g/ml lipoproteins. The figure also shows that the major part of lipoprotein protein is associated with HDL. The major part of plasma lipoprotein TG was, as expected, floating at d<1.063 g/ml. At all densities, there was a progressive TG decrease with increasing SO/SU ratio. The mean cumulative density lipoprotein TG values at d=1.200 g/ml were in excellent agreement with the corresponding ones determined in whole plasma, indicating full recovery by the cumulative density ultracentrifugation procedure. There were no significant group differences in TC or CE concentrations at d=1.006 g/ml. At d=1.063, d=1.100 and d=1.125 g/ml, however, there was a decrease in both TC and CE with increasing SO/SU ratio. However, as with protein, there were no group differences in TC or CE at 1.200 g/ml. Lipoprotein FC decreased with increasing SO/SU-ratio at all densities. The CE/FC ratio was higher at high than at low densities, and a trend toward a higher such ratio with increasing SO/SU ratio. Figure 5 illustrates the responses in each lipoprotein class to increasing the SO/SU ratio. The low lipoprotein protein values in VLDL and LDL were unaffected by increasing the SO/SU ratio, whereas the concentration of HDL_{2b} and HDL_{2a} protein decreased, and that of HDL_3 greatly increased. Lipoprotein VLDL and LDL TG levels fell appreciably in response to increasing the amount of sunflower oil at the expense of sucrose, whereas the diet change had little influence on the low HDL_2 and HDL_3 TG values. CE was higher in HDL than in VLDL and LDL, and seemed to decrease in HDL_2 and LDL, but increased in HDL_3. A similar trend was also noted in free cholesterol. The close inverse relationship between HDL_2- and HDL_3-protein, and between HDL_2- and HDL_3-CE, is illustrated in Figure 6. To estimate the dietary influence on the total amount of lipoprotein in each density class, the weight sum of all measured components (protein+TG+FC+CE) in

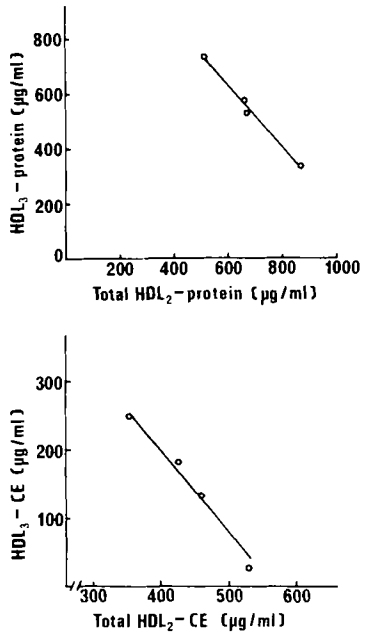


FIG. 6. Relationship between HDL_2 and HDL_3 components.

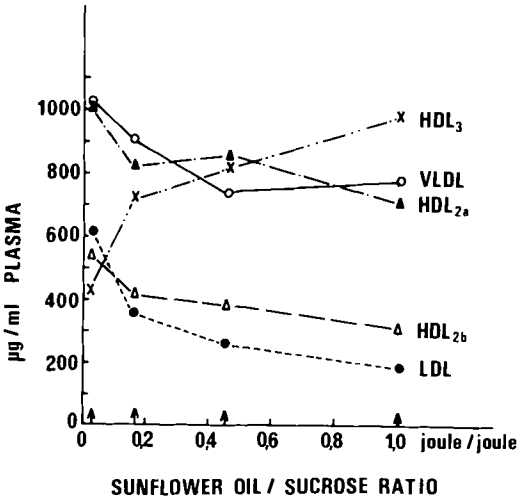


FIG. 7. Lipoprotein amount in relation to diet. Total weight, i.e., sum of the measured components (protein+FC+CE+TC) was calculated for each density class.

each lipoprotein class was determined (Fig. 7). VLDL, LDL, HDL_{2b} and HDL_{2a} decreased progressively in response to increasing the SO/SU ratio. In contrast, there was an appreciable increase in HDL_3 concentration accompanying this diet alteration.

Apoproteins in Plasma Lipoproteins

Apoproteins in pools of plasma lipoproteins in groups 1 and 4 (lowest and highest SO/SU ratio, respectively) were assessed by cumulative density ultracentrifugation followed by SDS-PAGE as described in Procedures. There were only small amounts of the high and low molecular weight apo B (apo B_h and apo B_l, Fig. 8) with no clear group differences, except for VLDL-apo B_h which was higher in group 4 than in group 1. Apo A-IV was solely associated with HDL_{2a} and HDL_3, and was higher in group 4 than in group 1. Apo E was more evenly distributed among the density classes. Group order for this apoprotein was: VLDL 1<4, LDL 1>4, HDL_{2b} and HDL_{2a} 1>4, HDL_3 1=4. Apo A-I was associated only with HDL. There was an appreciable decrease in HDL_{2b} and HDL_{2a} apo A-I, but an increase in HDL_3 apo A-I in response to increasing the SO/SU ratio. A similar pattern was noted in apo C-III. There were only small amounts of apo C-II present

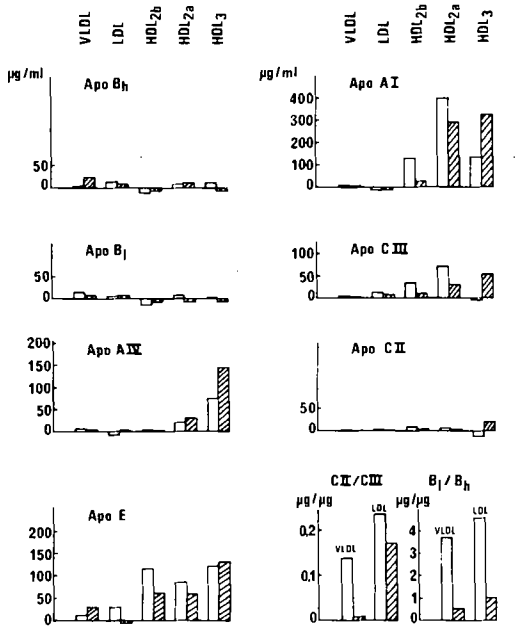


FIG. 8. Apoproteins in plasma lipoproteins. Lipoproteins were isolated by cumulative density ultracentrifugation and the apoproteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The apoproteins in the gel were stained; the dye was then extracted and apoprotein amount was estimated from optical densities. Open column: group 1; striped column: group 4.

in the 5 density classes. VLDL and LDL apo C-II/apo C-III ratio was higher in group 1 than in group 4 (Fig. 7, bottom right). Also, the VLDL and LDL apo B_l/apo B_h ratio was much higher in group 1 than in group 4.

Hepatic Output of VLDL

The output of VLDL-TG was lowest from livers of rats fed the highest relative amount of sunflower oil (Fig. 9), but was not significantly different in the other groups. No significant group differences were observed in the hepatic output of VLDL protein and free cholesterol. VLDL CE was highest in group 3. The CE/FC ratio in perfusate VLDL was lowest in group 1; there were no significant differences in CE/FC ratio between the other groups.

Apoproteins in Perfusate VLDL

Hepatic VLDL apoproteins were well separated (Figs. 10 and 11). There was a trend toward higher relative amount of apo B_h, apo A-IV and apo A-I accompanying the replacement of dietary sucrose by sunflower oil (Fig. 12). In contrast, apo C-II levels decreased with increasing SO/SU ratio, whereas there

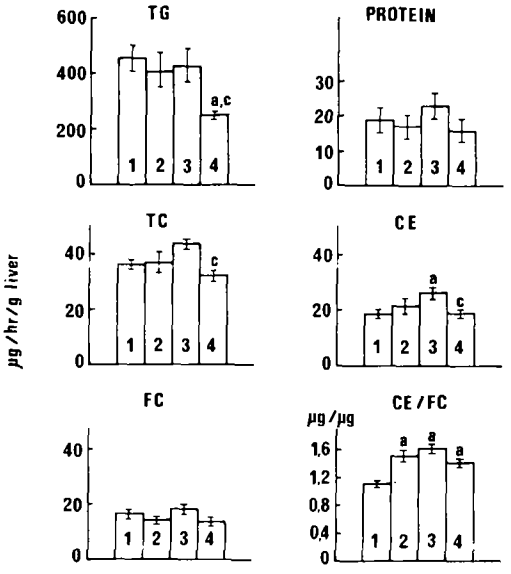


FIG. 9. Hepatic output of VLDL components. Livers of rats in the 4 diet groups were perfused for 2 hr. Then, perfusate VLDL was isolated as described in Procedures. TG=triacylglycerols, TC=total cholesterol, CE=cholesteryl ester, FC=free cholesterol. Mean values±SE, (a) $p<0.05$ vs group 1; (c) $p<0.05$ vs group 3.

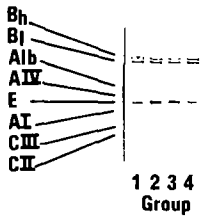


FIG. 10. Electropherogram of perfusate VLDL apoproteins. Perfusate VLDL was isolated by ultracentrifugation. A pool was made for each of the 4 diet groups and apoproteins were determined as described for plasma apoproteins.

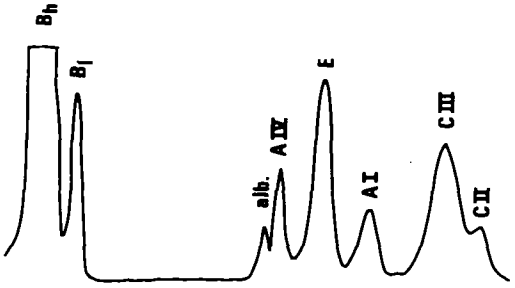


FIG. 11. Densitogram of perfusate VLDL apoproteins.

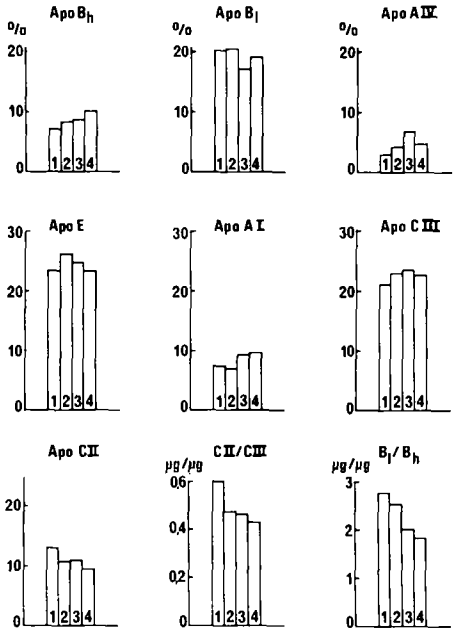


FIG. 12. Relative distribution of perfusate VLDL apoproteins.

were no consistent diet-dependent alterations in apo B_l, apo E or apo C-III. The apo C-II/apo C-III and apo B_l/apo B_h ratios decreased in response to increasing the amount of dietary sunflower oil at the expense of sucrose.

DISCUSSION

The results of this work strongly suggest that the plasma HDL subgroup distribution can be appreciably influenced by diet alteration. It seems, furthermore, that diet-dependent changes in protein and cholesterol distribution among HDL subgroups can occur, although total HDL protein and CE concentration—when estimated in the density interval 1.063-1.200 g/ml—remain virtually unaffected by diet manipulation.

It has been suggested that HDL_2 is produced from HDL_3 during lipoprotein lipase-catalyzed breakdown of TG-rich lipoproteins (27). During this process, components of VLDL and chylomicrons are taken up by HDL_3, resulting in formation of the less dense HDL_2. The close inverse relationship between HDL_2 and HDL_3 observed in this work is in accordance with this hypothesis. Lipoprotein lipase is stimulated by apo C-II and inhibited by apo C-III (28). The apo C-II/apo C-III ratio should, accordingly, be an indicator of lipoprotein lipase activation. In keeping with this, there was a concomitant increase in plasma HDL_2/HDL_3 ratio (Fig. 7) and in the apo C-II/apo C-III ratio of plasma and perfusate VLDL (Figs. 8 and 12) when the relative amount of sucrose in the diet was increased. The observation that the apo C-II/apo C-III ratio both in plasma and perfusate VLDL decreased in response to replacing sucrose by sunflower oil might imply that the primary diet effect was in the liver. Diet-dependent alterations in the hepatic VLDL apo C-II/apo C-III ratio could secondarily govern lipoprotein lipase activity and plasma lipoprotein interconversions related to the action of this enzyme.

The increase in the plasma CE/FC ratio with increasing density during ultracentrifugation might be related to the concomitant increase in apo A-I, because this apoprotein is an activator of lecithin cholesteryl acyl transferase (LCAT) which catalyzes cholesterol esterification (28). There was also a positive correlation between apo A-I and HDL_2 CE concentration. It would appear that LCAT activity increases in response to feeding polyunsaturated fat, as suggested by the dietary sunflower-oil-associated increase in plasma CE/FC ratio observed in this and in previous studies (8).

As mentioned earlier (23), a high-sucrose diet leads to an increase in the low molecular weight apo B/high molecular weight apo B ratio. This finding is supported by the present study: there was a higher such ratio with increasing relative amounts of sucrose in the diet. The significance of this diet-dependent alteration for binding of LDL to the receptor remains to be elucidated. It is likely that the liver caused the observed diet-related changes in plasma VLDL B-apoproteins, as similar alterations were found in plasma and perfusate VLDL apo B. It has been reported recently that both the low and high molecular weight B-apoproteins are of hepatic origin in the rat (29).

It has been previously reported that feeding cholesterol-rich diets leads to hyperlipemia and increased formation of apo E containing HDL_2 (HDL_C) in man, dog, swine and rat (30). This lipoprotein has a high affinity for the LDL receptor (31). Hamilton has suggested that some apo E-containing lipoproteins might be associated with transport of cholesterol from peripheral cells to the liver for cholesterol excretion (32). This suggestion was recently supported by the finding that the hepatic uptake of HDL rich in apo E was about 10 times higher than the uptake of the main type of HDL rich in apo A-I (33). Our results also suggest that high-sucrose diets elicit increased plasma apo-HDL_2 levels. This suggestion agrees with our previous report in which it was shown that rats fed high-sucrose diets had an appreciable increase in lipoproteins migrating between alpha and beta on polyacrylamide gel electrophoresis (18,34,35); subsequent studies showed that these lipoproteins, like apo E-HDL_2, could be precipitated with polyanions (36). We also recently observed that dietary fructose and glucose, but not starch, increased rat plasma HDL_2 in comparison to stock diet (37).

It would appear that the study of dietary effects on HDL subgroup distribution is of particular interest. In the HDL range (d=1.063-1.200 g/ml), lipoproteins seem to exist that have antagonistic effects; it was recently reported (11) that the less dense HDL_2 inhibited, and the more dense HDL_3 stimulated fibroblast HMG-CoA reductase, the rate-limiting enzyme in cellular cholesterol synthesis. Furthermore, a low HDL_2/HDL_3 ratio has been associated with increased coronary risk (38). Moreover, endurance exercise, which is considered beneficial with regard to reducing the coronary risk, seems to increase the HDL_2/HDL_3 ratio (39-41). In addition, premenopausal women, who are relatively protected against atherosclerotic diseases, have a higher such ratio than men (42,43). These epidemiological studies suggest that an increased HDL_2/HDL_3 ratio might be

indicating coronary protection.

On the other hand, hyperlipemic diets also seem to increase the HDL_2/HDL_3 ratio (30). We previously reported that feeding high-sucrose diets increased both plasma lipids and the amount of a less dense type of HDL (34-37). Also, the results of this study strongly suggest that the HDL_2/HDL_3 ratio increases in parallel with the increase in whole plasma TG and cholesterol concentration. How, then, can the HDL_2/HDL_3 ratio be an indicator of coronary protection when it increases in response to hyperlipemic, and presumably, atherogenic, diets? It is tempting to speculate, like others (32,42,43), that increased HDL_2 formation is a mechanism to counteract cholesterol accumulation. Accordingly, individuals (women, persons performing endurance exercise) possessing a high potential for HDL_2 formation might be protected from vascular damage due to cholesterol deposition.

The results of this work indicate that replacing sucrose by sunflower oil greatly affects the plasma lipoprotein distribution. Assessment of HDL subgroup distribution seems to be essential in the study of dietary effects on plasma HDL; in this work, total HDL protein and CE (d=1.063-1.200 g/ml) were virtually unaffected by the diet manipulation, in spite of an appreciable diet-dependent variation in the HDL subgroups. It would appear that cumulative density ultracentrifugation is a useful approach to estimate lipoprotein distribution among HDL subgroups.

REFERENCES

1. Carlson, L.A., and Böttiger, L.E. (1972) Lancet 1, 865-868.
2. Miller, G.J., and Miller, N.E. (1975) Lancet 1, 16-19.
3. Miller, N.E., Førde, O.H., Thelle, D.S., and Mjøs, O.D. (1977) Lancet 1, 965-970.
4. Castelli, W.P., Doyle, J.T., Gordon, T., Hames, C.G., Hjortland, M.C., Hulley, S.B., Kagan, S., and Zukel, W.J. (1977) Circulation 55, 767-772.
5. Lewis, B. (1976) in The Hyperlipidaemias: Clinical and Laboratory Practice, pp. 133-155, Blackwell Scientific Publications, Oxford.
6. Dumaswala, U.J., Dumaswala, R.U., and Venkataraman, A. (1976) Ital. J. Biochem. 25, 289-303.
7. Høstmark, A.T., Spydevold, Ø., and Eilertsen, E. (1980) Artery 7, 367-383.
8. Høstmark, A.T., Spydevold, Ø., Eilertsen, E., and Lystad, E. (1981) Nutr. Rep. Int. 24, 371-377.
9. MacDonald, I. (1973) in Progress in Biochemical Pharmacology (Paoletti, R., ed.) Vol. 8, pp. 216-241, S. Karger, München.
10. Anderson, D.W., Nichols, A.V., Pari, S.S., and Lindgren, F.T. (1978) Atherosclerosis 29, 161-179.
11. Daerr, W.H., Gianturco, S.H., Patsch, J.R., Smith, L.C., and Gotto, A.M. (1980) Nutr. Metab. 24 (Suppl. 1), 26-33.
12. Herbert, P.N., Forti, T.M., Shulman, R.S., La Piana, M.J., Gong, E.L., Levy, R.I., Fredrickson, D.S., and Nichols, A.V. (1975) Prep. Biochem. 5, 93-129.
13. Fainaru, M., and Eisenberg, S. (1978) Artery 3, 472-488.
14. Bar-On, H., Roheim, R.S., and Eder, H.A. (1976) J. Clin. Invest. 57, 714-721.
15. Mahley, R.W., and Holcombe, K.S. (1977) J. Lipid Res. 18, 314-324.
16. Jeffcoat, R., Roberts, P.A., and James, A.T. (1979) Eur. J. Biochem. 101, 447-453.
17. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353.
18. Høstmark, A.T., and Askevold, R. (1978) Artery 4, 129-143.
19. Calvert, G.D. (1976) in Low Density Lipoproteins (Day, C.E., and Levy, R.S., eds.) pp. 281-319, Plenum Press, New York, NY.
20. Bramhall, S.N., Noack, M., Wu, M., and Loewenberg, J.R. (1969) 31, 146-148.
21. Høstmark, A.T., Sørensen, L., and Askevold, R. (1977) Anal. Biochem. 83, 782-784.
22. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
23. Swaney, J.B., and Kuehl, K.S. (1976) Biochim. Biophys. Acta 446, 561-565.
24. DeLamatre, J.G., and Roheim, P.S. (1981) J. Lipid Res. 22, 297-306.
25. Ladislav, D., and Roheim, P.S. (1981) J. Lipid Res. 22, 287-296.
26. Kalopissis, A.D., Girard, A., and Griglio, S. (1978) Horm. Metab. Res. 11, 118-122.
27. Patsch, J.R., Gotto, A.M., Jr., Olivecrona, T., and Eisenberg, S. (1978) Proc. Natl. Acad. Sci (USA) 75, 4519-4523.
28. Lewis, B. (1976) in The Hyperlipidaemias: Clinical and Laboratory Practice, pp. 56-66, Blackwell Scientific Publications, Oxford.
29. Sparks, C.E., Hnatiuk, O., and Marsh, J.B. (1981) Can. J. Biochem. 59, 693-698.
30. Mahley, R.W. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M., Jr., and Ontko, J.A., eds.) pp. 181-197, Am. Physiol. Soc., Bethesda, MD.
31. Innerarity, T.L., Kempner, E.S., Hui, D.Y., and Mahley, R.W. (1981) Proc. Natl. Acad. Sci. USA 78, 4378-4382.
32. Hamilton, R.L. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M., Jr., and Ontko, J.A., eds.) pp. 155-171, Am. Physiol. Soc., Bethesda, MD.
33. Quarfordt, S., Hanks, J., Jones, R.S., and Shelburne, F. (1981) J. Biol. Chem. 255, 2934-2937.
34. Høstmark, A.T., Rasmussen, E.W., and Askevold, R. (1978) J. Nutr. 108, 1823-1829.
35. Høstmark, A.T., Eilertsen, E., and Grønnerød, O. (1979) J. Nutr. 109, 1073-1078.
36. Grønnerød, O., and Høstmark, A.T. (1979) Artery 5, 144-156.
37. Høstmark, A.T., Spydevold, Ø., Lystad, E., and Eilertsen, E. (1982) Nutr. Rep. Int. 25, 151-167.
38. Miller, N.E. (1980) Postgrad. Med. J. 56, 575-578.
39. Krauss, R., Lindgren, F.T., Wood, P.D., Haskell, W.L., Albers, J.J., and Cheung, M.C. (1977) Circulation 56 (Suppl. 3), 4.
40. Miller, N.E., Rao, S., Lewis, B., Bjørsvik, G., Myhre, K., and Mjøs, O.D. (1979) Lancet 1, 111.
41. Heiss, G., Johnson, N.J., Reiland, S., Davis, C.E., and Tyroler, H.A. (1980) Circulation 62 (Suppl. IV), 116-136.

42. Cheung, M.C., and Albers, J.J. (1977) J. Clin. Invest. 60, 473-485.
43. Fellin, R., Baggio, G., Baiocchi, M.R., Martini, S., Baldo, G., Manzato, E., and Crepaldi, G. (1980) in Diets and Drugs in Atherosclerosis (Noseda, G., Lewis, B., and Paoletti, R., eds.) pp. 85-91, Raven Press, New York, NY.

[Received January 4, 1982]

METHODS

A Simple Method for Labeling Lipids in the Bile Lipoprotein

GILLES NALBONE*, JEAN-LOUIS VIGNE, HUGUETTE LAFONT, MONIQUE AUGEIRE-CHARBONNIER, CHRISTIANE CHABERT, DENIS LAIRON and **JACQUES C. HAUTON**, *I.N.S.E.R.M. U 130, Unité de Recherches sur le Transport des Lipides, 10, ave. Viton, 13009 Marseille, France*

ABSTRACT

A simple method is proposed for the specific radioactive labeling of phosphatidylcholines and cholesterol in the bile lipoprotein complex. It can be used for human and animal bile samples and results in labeling with the desired specific radioactivity and position. Experiments which determined the intermicellar concentration of lipid constituents suggested that incorporation of radioactive lipids could occur through small dialyzable structures termed mixed premicelles in thermodynamic equilibrium with the bile lipoprotein complex.
Lipids 17:500-503, 1982.

INTRODUCTION

Bile lipids, primarily bile salts, phosphatidylcholines (PC) and cholesterol, are organized in bile in the form of a water-soluble macromolecular complex (1). An apoprotein fraction is combined with this complex (2,3). The most widespread method for studying bile lipid metabolism is that of radioisotopic labeling. In rats, bile PC are labeled by intravenously injecting a radioactive fatty acid (4,5). After several hours of draining, bile containing the radioactive PC can be collected.

Bile cholesterol can be labeled by either injecting the radioactive precursor (6) or by the addition of radioactive cholesterol to rat feed (7). In these 2 cases, however, bile salts are also labeled.

We describe a method for the specific labeling of the bile lipoprotein complex. It is relatively simple and has the advantage of being applicable to human gallbladder bile as well as animal bile.

MATERIALS AND METHODS

Bile Samples

Human gallbladder bile was obtained by preoperative puncture and was processed as described elsewhere (2). Rat bile was recovered for 4 hr after catheterization of the common bile duct. In all samples, sodium azide, 0.02% was added.

Radioactive Products

The [^{14}C (U)]PC were purchased from New England Nuclear (specific radioactivity $4.26 \cdot 10^9$ dpm/mg of P). [C-4 ^{14}C]Cholesterol was supplied by the C.E.A. (France) (specific radioactivity 50 mCi/mmol).

Solutions of Mixed Micelles

Eight hundred mg of PC (Sigma egg phosphatidylcholines, 98% pure) and 135 mg of cholesterol (Sigma 99% pure) were dissolved in choloroform with 2.5 μl of [^{14}C]cholesterol. After drying under a nitrogen stream, lipids were taken up with 1.41 g of glycodeoxycholate (Calbiochem A grade), previously dissolved in 50 ml of buffer as has been described (8). Undissolved cholesterol was eliminated by centrifuging at 100,000 × g for 30 min. A clear mixed micellar solution was obtained in which the molar ratio of bile salts/PC/cholesterol was 7.2:3:1.

Glycodeoxycholate-[^{14}C]cholesterol micelles were prepared by an identical procedure. The postcentrifugation supernatant showed that 70% of the cholesterol had been solubilized with a molar ratio of glycodeoxycholate/cholesterol of 18:1.

Labeling of Bile Phosphatidylcholines

Twenty to 30 μl of the radioactive PC in organic solvent were evaporated to dryness under vacuum in a glass tube. Human gallbladder bile (6-7 mg of PC and 20-25 mg of bile salts) or

rat hepatic bile (3-4 mg of PC and 10-12 mg of bile salts) was then added to the tube. Incubation was overnight at ambient temperature with slight agitation, followed by dialysis against 0.01 M Tris-HCl (pH 7.0) plus 1 mM glycodeoxycholate for 6 hr at 4 C. The final preparation was then used for gel filtration, ultracentrifugation and agarose gel electrophoresis.

Labeling of Bile Cholesterol

Five μl of [^{14}C]cholesterol in organic solvent were evaporated to dryness under vacuum in a glass tube and incubated as already described. Undissolved cholesterol was removed by centrifuging at 100,000 × g for 30 min. The clear supernatant was removed for ultracentrifugation studies.

Intermicellar Concentration of Lipid Constituents (Dialysis Space)

This determination was performed using a method recently described (9). It was used with mixed micellar solutions to demonstrate the dialysis not only of bile salts, but of PC and cholesterol, as well. A perforated Tygon or Solvaflex tube was placed in a dialysis sack (Visking with an MW exclusion limit of 13,000 daltons or Spectra-por/3 with an exclusion limit of 3,500 daltons) to avoid collapse induced by osmotic pressure. The sack was filled with 0.5 ml of buffer, which constituted a small dialysis space in which lipidic structures with molecular weights lower than the exclusion limit of the sack could penetrate. The sack was closed and placed in 50 ml of the micellar solution in a stoppered flask, which was agitated by rotation for 4 days at ambient temperature. After carefully rinsing the exterior of the dialysis sack, its contents were removed and analyzed for bile salts, PC and [^{14}C]cholesterol.

Assays

Previously described methods were used for the assays of PC (10) and bile salts (11). Samples containing labeled molecules were diluted with Ready Solve MP (Beckman) scintillation liquid and radioactivity was determined with an LS-9000 (Beckman) liquid scintillation spectrometer connected to a Silent 700 terminal (Texas Instruments).

RESULTS AND DISCUSSION

In the case of human gallbladder bile, the labeling of the PC of the bile lipoprotein complex showed that ca. 85% of the radioactivity that eluted during gel filtration chromatography was associated with this complex (Fig. 1). The complex was eluted in a peak whose MW was estimated as about 120,000 daltons, in agreement with prior results (12).

After ultracentrifugation, the bile complex sedimented in a zone with a density of 1.16 g/ml (Fig. 2) (3). About 90% of the [^{14}C]PC recovered were present with the bile complex.

After performing agarose gel electrophoresis

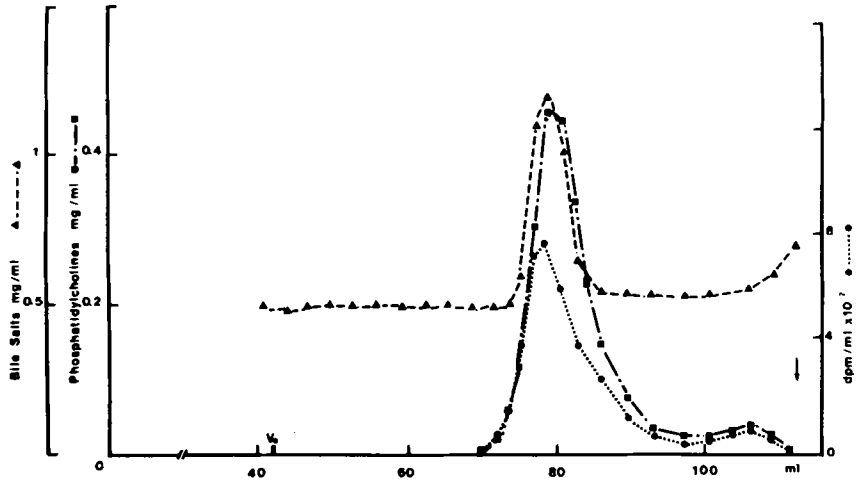


FIG. 1. Gel chromatography on Ultrogel ACA 34 (I.B.F. -France) of human gallbladder bile incubated with [^{14}C]phosphatidylcholine. Column: 1.5 × 90 cm. Eluting buffer: Tris 0.01 M, NaCl 0.1 M, $CaCl_2$ 10^{-3} M, sodium azide 0.02% and glycodeoxycholate 10^{-3} M, pH 7.0. Flow rate: 11-12 ml/hr. The arrow indicates the elution volume of [^{14}C]phosphatidylcholine alone.

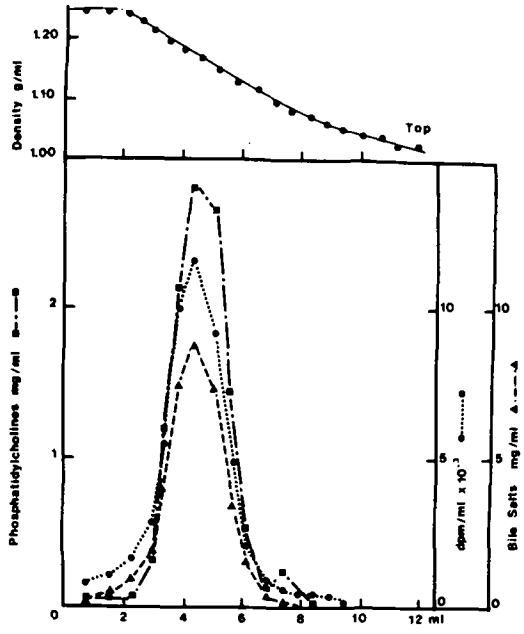


FIG. 2. Ultracentrifugation of human gallbladder bile incubated with [^{14}C]phosphatidylcholine; A NaCl-KBr density gradient (upper part) was obtained according to the method described by Chapman et al. (13). The centrifugation was at 160,000 × g for 60 hr at 10 C in an SW 40 rotor placed in a L5-75B ultracentrifuge (Beckman). The gradient was pumped out at a rate of 0.5-0.6 ml/min (total volume: 12.5 ml).

as previously described (3), 85-90% of the radioactivity migrated toward the anode with the bile complex.

Comparable results were obtained with rat hepatic bile. After gel filtration, 60-65% of the radioactivity was eluted with the bile complex, the remainder emerging in later fractions. After ultracentrifugation, 90% of the radioactivity in the gradient sedimented with the bile complex.

Labeling of the bile complex with [^{14}C]cholesterol furnished results similar to those obtained with radioactive PC (data not shown). After ultracentrifugation, 80-85% of the radioactivity sedimented with the bile complex formed from human bile and 65% sedimented with the rat bile. Nonincorporated cholesterol sedimented at a density lower than that of the complex, d ≈ 1.09 g/ml.

These results show that this simple labeling method furnished satisfactory labeling of bile complex lipids with the desired specific radioactivity.

The mechanism by which the labeled molecules are incorporated into the complex could occur by a simple phenomenon of intermolecular collision. It is equally possible that it could occur through small structures (MW ≈ 3,000 daltons), termed mixed premicelles, in thermodynamic equilibrium with the mixed micelles (9). These structures were demonstrated by the use of the dialysis space for measuring the intermicellar concentration of lipid constituents. In the case of mixed tertiary glycodeoxycholate/PC/cholesterol micelles (Table 1), the technique showed that a considerable proportion of PC and cholesterol crossed the wall of the dialysis sack. This was true for both Visking and Spectra-por/3. With the Spectra-por/3, the lipid concentration in the sack was about 4 times higher than with the Visking. This had already been described for phospholipid-containing micellar solutions (9) and is explained by the polymerization of mixed premicelles within the sack. The size of the resulting structures prohibits them from diffusing out of the Spectra-por/3 dialysis space toward the exterior. In the case of Visking dialysis sack, however, this diffusion remains possible as a result of the higher exclusion limit of this material.

TABLE 1

Concentration of Glycodeoxycholate (GCD), Phosphatidylcholine (PC) and Cholesterol (CH) in the Dialysis Bag*

	GDC mM	PC mM	CH mM
Visking[a]	10.7	3.08	0.59
Spectra-por[a]	28	12.4	3.7
Visking[b]	43	–	0.82
Spectra-por[b]	40	–	0.81

*For dialysis space, see Materials and Methods.

[a]External micellar solution: GDC = 52 mM; PC = 21.5 mM; CH = 7.1 mM in buffer Tris 0.01 M; NaCl = 0.15 M; NaN_3 = 0.02%, pH 9.4.

[b]External micellar solution: GDC = 52 mM; CH = 2.92 mM. Buffer was the same as in a.

Based on theoretical deductions, the mixed premicelle would be composed of 4 molecules of bile salts for one molecule of PC (9). Bile salt/cholesterol mixed premicelle can also form, because it was found (Table 1) that a nonnegligible fraction of cholesterol traversed the Spectra-por/3 dialysis sack. The mixed premicelle model apparently can be extrapolated to bile, as we observed (unpublished results) that a nonnegligible proportion of PC traversed the wall of the dialysis sack.

The method of labeling the bile lipoprotein complex described here seems to be a simple

and rapid method which is applicable to human samples. It can be used for double-labeling PC and cholesterol with the desired specific radioactivity and labeling position.

ACKNOWLEDGMENTS

The authors thank Mrs. Roussey for typing the manuscript and Miss Roux and Dr. Vilani for furnishing human bile samples. Financial support was provided by the Institut National de la Santé et de la Recherche Médicale (C.R.L. No. 79.5.477.7).

REFERENCES

1. Small, D.M., Bourges, M.C. and Dervichian, D.G. (1966) Biochim. Biophys. Acta. 125, 563-580.
2. Nalbone, G., Lafont, H., Vigne, J.L., Domingo, N., Lairon, D., Chabert, C., Lechene, P., and Hauton, J.C. (1979) Biochimie 61, 1029-1041.
3. Vigne, J.L., Nalbone, G., Lafont, H., Lairon, D., Chabert, C., Domingo, N., and Hauton, J.C. (1981) Biochimie 63, 735-742.
4. Boucrot, P. (1972) Lipids 7, 282-288.
5. Robins, S.J. (1975) Am. J. Physiol. 229, 598-602.
6. Gregory, D.H., Vlahcevic, Z.R., Schatzki, P., and Swell, L. (1975) J. Clin. Invest. 55, 105-114.
7. Chevallier, F., and Lutton, C. (1966) Bull. Soc. Chim. Biol. 48, 507-524.
8. Montet, J.C., and Dervichian, D.G. (1971) Biochimie 53, 751-754.
9. Hauton, J.C., Vigne, J.L., Nalbone, G., Lafont, H., Lairon, D., and Augeire-Charbonnier, M. (1982) in Exposés Annuels de Biochimie Médicale, (Polonovski, J., ed.) pp. 39-73, Masson.
10. Amic, J., Lairon, D., and Hauton, J.C. (1972) Clin. Chim. Acta 40, 107-114.
11. Domingo, N., Amic, J. and Hauton, J.C. (1972) Clin. Chim. Acta 37. 399-404.
12. Lafont, H., Lairon, D., Domingo, N., Nalbone, G., and Hauton, J.C. (1974) Biochimie 56, 465-468.
13. Chapman, M.J., Goldstein, S., Lagrange, D., and Laplaud, P.M. (1981) J. Lipid Res. 22, 339-358.

[Received February 2, 1982]

COMMUNICATIONS

Saturated Fatty Acids > C_{20} Are Not Activated by Acid:CoA Ligase in Rat Brain or Liver?

MARY G. MURPHY[1] and **MATTHEW W. SPENCE***, *Departments of Biochemistry and Pediatrics, and the Atlantic Research Centre for Mental Retardation, Dalhousie University, 5849 University Ave., Halifax, Nova Scotia B3H 4H7, Canada*

ABSTRACT

The formation of long-chain saturated acyl-[^{3}H]CoA and [1-^{14}C]acyl-[^{3}H]CoA by rat brain microsomes and rat liver was examined. Acyl-CoA formation was markedly decreased as fatty-acid chain length increased from C_{16} to C_{20}. No biosynthesis of behenyl-[^{3}H]CoA or [1-^{14}C]lignoceryl-[^{3}H]CoA was observed. The results suggest that long-chain saturated fatty acids >20 carbons in length are not activated by acid:CoA ligase to form acyl-CoA.
Lipids 17: 504-506, 1982.

Although it is generally accepted that activation of fatty acids to fatty acyl-CoA (acid:CoA ligase AMP, EC 6.2.1.3) is the initial step in metabolic processing of fatty acids in mammals, evidence suggests that there are reactions for which acyl-CoA is not the required substrate. Using rat brain preparations, Kishimoto and coworkers (1-5) demonstrated that α-hydroxylation of lignoceric acid (tetracosanoic acid) to cerebronic acid (α-hydroxytetracosanoic acid) and the subsequent formation of cerebronyl sphingosine, and the oxidation of lignoceric acid do not require exogenous coenzyme A. Other examples include the formation of palmitoyl sphingosine from palmitic acid (6) and the synthesis of cholesterol esters from oleic acid (7). One tentative conclusion from these studies is that there are acyl-CoA-dependent, and non-CoA-dependent routes of fatty acid metabolism, and that the latter may be particularly important for the metabolism of long-chain saturated fatty acids (>C_{20}).

As the initial difference in these 2 routes of metabolism would be the formation of either acyl-CoA or acyl-X and as the identity of acyl-X is unknown, we have studied the formation of lignoceryl-CoA by acid:CoA ligase. We have used a method recently developed in this laboratory that is both very sensitive (≥0.02 nmol of product) and fatty-acid specific (8). The results indicate that lignoceryl-CoA is not formed by acid:CoA ligase in rat brain or liver, and that the non-CoA dependent route of metabolism may predominate for this fatty acid.

*To whom correspondence should be addressed.

[1]Present address: Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada.

MATERIALS AND METHODS

[1-^{14}C]Lignoceric acid was a generous gift from Dr. Yasuo Kishimoto, the John F. Kennedy Institute and Johns Hopkins School of Medicine, Baltimore, MD. Other [1-^{14}C]-labeled fatty acids, and [^{3}H(G)]coenzyme A were purchased from New England Nuclear, Lachine, Canada, or Applied Sciences, State College, PA. Remaining reagents were obtained as described previously (8). Wistar rats of either sex, aged 20-30 days, were from our own inbred colony. Immediately following decapitation, cerebral hemispheres were homogenized in 0.32 M sucrose and a microsomal fraction was isolated (9). Protein was determined by the method of Lowry et al. (10), with bovine serum albumin as the standard.

The acid:CoA ligase assay, which is based on the formation of [^{14}C]fatty acyl-[^{3}H]CoA, or product containing either isotope alone, has been described in detail elsewhere (8). Except where indicated otherwise, the final reaction mixture contained in 0.2 ml: 0.19 M Tris-chloride buffer, pH 7.4, 8 mM $MgCl_2$, 10 mM ATP, 1 mM dithiothreitol, 45-55 μM coenzyme A (±[^{3}H]coenzyme A, 10 μCi/μmol), 45-55 μM fatty acid (±[1-^{14}C]fatty acid, 7 μCi/μmol), 0.2 mg Triton WR-1339, and 0.04-0.06 mg rat-brain protein as enzyme source. Incubation was for 5 min at 30 C. Ligase activities (nmol fatty acyl-CoA formed/min/mg protein) were calcu-

lated from the sp act (dpm/nmol) of either [^{14}C] fatty acid or [^{3}H] coenzyme A.

RESULTS AND DISCUSSION

Formation of [1-^{14}C] fatty acyl-CoA by rat brain microsomes (Fig. 1A) and liver homogenates (Fig. 1B) was markedly decreased by increases in fatty-acid chain length from 16 to 20 carbons. [1-^{14}C] Behenic acid (22:0) is not commercially available; however, examination of the reaction with nonradioactive 22:0 and [^{3}H] coenzyme A indicated that, like lignocerate (24:0), this fatty acid is not activated by the ligase. Thus, ligase activity with long-chain saturates 22:0 and 24:0 was not demonstrable in 2 tissues where it might be expected. For example, if activation of C_{24} were a function of a specific ligase, one would anticipate measurable activity in brain because of its relatively high content of long-chain saturates and their derivatives (11). On the other hand, if activation of all long-chain saturates (C_{12} to C_{24}) were due to a single ligase, then activity with C_{24} might be detected more readily in liver where rates are high (12).

To ensure that the measurements of ligase activity were not being altered by variations in substrate concentration due to difficulties in suspending the lipid substrates, radioactivity was measured in portions of the incubation mixture prior to and after incubation. Highly reproducible triplicate measurements indicated that solubility of fatty acid from 16:0 to 24:0 consistently exceeded 90%, and that the acids were uniformly dispersed throughout the aqueous detergent-containing mixtures. Using this method of solubilization, a broad range of long-chain fatty acids are activated to their acyl-CoA esters by both brain and liver (13).

To rule out the possibility that reaction rates with 24:0 were being underestimated due to incomplete extraction of lignoceryl-CoA, the chloroform/methanol extracts of the incubation mixtures with [^{14}C] lignoceric acid were analyzed by thin layer chromatography (TLC) for free fatty acid and fatty acyl-CoA (14). Little radioactivity (<0.02% of the total in the extract) cochromatographed with fatty acyl-CoA and there was no difference between blanks and regular incubated assay mixtures.

We have no evidence that the lack of acyl-CoA formation with 24:0 is due to the suboptimal reaction conditions. Assays were carried out at variable concentrations of ATP [2.5-20 mM], coenzyme A [12.5-100 μM] and fatty acid [25-100 μM], and with other neutral detergents, including Triton X-100 and Miranol-HR and at varied detergent concentrations. Activities did not change under any of these conditions, and did not differ from those observed under blank conditions (Fig. 1A). Ligase activity with 16:0 or 18:0 was markedly reduced when the fatty acids were suspended by ultrasonication in the absence of detergent, or coated on Celite (15).

Additional indirect evidence that 24:0 is not activated by a CoA-dependent mechanism was obtained from competition experiments in which the formation of [^{14}C] oleoyl-[^{3}H] CoA by rat brain microsomes was measured in the presence of increasing concentrations of nonradioactive palmitic (16:0) and lignoceric (24:0) acids (Fig. 2). Incorporation of [^{14}C]-oleic acid into product decreased with increasing 16:0, whereas the amounts of fatty acyl-

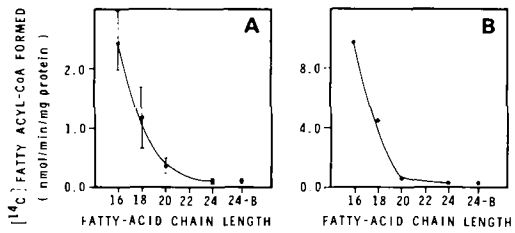


FIG. 1. The effect of chain length of saturated fatty acids on the formation of fatty acyl-CoA by rat brain microsomes (A) and rat liver homogenate (B). Experimental conditions are described in Materials and Methods. The point indicated 24-B refers to data obtained after incubating 24:0 under blank conditions ([^{14}C] fatty acid solution added to the incubation mixture after chloroform/methanol is added to stop the reaction). Vertical bars indicate standard deviation (n = 5). Single points are the mean of 2 experiments.

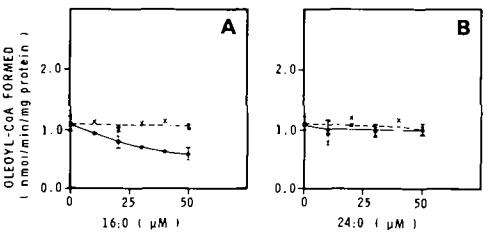


FIG. 2. The effect of increasing concentrations of nonradioactive palmitic (16:0) (A) and lignoceric (24:0) (B) acids on the formation of [^{14}C] oleoyl-[^{3}H] CoA by rat-brain microsomes. [^{14}C] Oleic acid was held constant at 25 μM, and [^{3}H] coenzyme A at 50 μM. Other conditions are as described in Materials and Methods. •—•, incorporation of ^{14}C into product; X—X, incorporation of ^{3}H into product. Vertical bars indicate standard deviation (n = 5). Single points are means of 2 experiments.

[^{3}H]CoA did not change appreciably, indicating an increasing proportion of palmitoyl-[^{3}H]-CoA in the product formed (Fig. 2A). In distinct contrast, increasing 24:0 did not significantly alter rates of incorporation of either isotope (Fig. 2B), strongly suggesting that lignoceric acid is not a substrate for the ligase.

To our knowledge, this is the first study to directly examine the activation of long-chain saturated fatty acids using a sensitive, specific and direct measurement. Our conclusion that long-chain saturated acyl-CoA esters are not formed in mammalian tissues is supported by chain-length specificity studies of partially purified (16) and purified (17) rat liver ligase and by the observations of a CoA-independent synthesis of 2-hydroxy fatty acids from a saturated precursor of the same chain length (1-5,18).

What is the basis for the impression that long-chain saturated fatty acyl-CoA is formed in mammalian tissues? For the most part, this has been a peripheral observation of very low activity for C_{22} and C_{24} in studies where indirect methods, such as acyl-hydroxamates, were used to show acyl-CoA formation (19-22) or where conditions were such that activation of endogenous shorter chain fatty acids could account for the observed activity (19,23,24). The acyl-CoA products formed were not characterized with respect to fatty-acid composition, and the long-chain fatty acids were not radiolabeled. Thus, these reports (19-24) of very low levels of activation of C_{22} and C_{24} saturated fatty acids probably do not, in fact, represent enzymatic activity specific to these acids.

The results of this study provide strong additional support for an alternative non-CoA-dependent route of long-chain saturated fatty acid metabolism. Clearly, further knowledge of this route is essential to our understanding of complex lipid formation and turnover in mammalian tissues.

ACKNOWLEDGMENTS

The technical assistance of L. Ragan is gratefully acknowledged. This work was supported in part by grants and a Career Investigatorship (to M.W. Spence) from the Medical Research Council of Canada.

REFERENCES

1. Hoshi, M., and Kishimoto, Y. (1973) J. Biol. Chem. 248, 4123-4130.
2. Murad, S., Chen, R.H.K., and Kishimoto, Y. (1977) J. Biol. Chem. 252, 5206-5210.
3. Akanuma, H., and Kishimoto, Y. (1979) J. Biol. Chem. 254, 1050-1056.
4. Singh, I., and Kishimoto, Y. (1979) J. Biol. Chem. 254, 7698-7704.
5. Uda, M., Singh, I., and Kishimoto, Y. (1981) Biochemistry 20, 1295-1300.
6. Yavin, E., and Gatt, S. (1969) Biochemistry 8, 1692-1698.
7. Eto, Y., and Suzuki, K. (1971) Biochim. Biophys. Acta 239, 293-311.
8. Murphy, M.G., and Spence, M.W. (1980) J. Neurochem. 34, 367-373.
9. Spence, M.W., and Wolfe, L.S. (1967) Can. J. Biochem. 45, 671-688.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
11. O'Brien, J.S., and Sampson, E.L. (1965) J. Lipid Res. 6, 545-551.
12. Polokoff, M.A., and Bell, R.M. (1977) J. Biol. Chem. 252, 1167-1171.
13. Murphy, M.G., and Spence, M.W. (1982) J. Neurochem. 38, 675-679.
14. Ullman, M.D., and Radin, N.S. (1972) J. Lipid Res. 13, 422-423.
15. Kishimoto, Y. (1978) in Methods in Enzymology (Fleischer, S., and Packer, L., eds.), Vol. 52, pp. 310-318, Academic Press, New York, NY.
16. Bar-Tana, J., Rose, G., and Shapiro, B. (1971) Biochem. J. 122, 353-362.
17. Tanaka, T., Hosaka, K., Hoshimaru, M., and Numa, S. (1979) Eur. J. Biochem. 98, 165-172.
18. Kishimoto, Y., Akanuma, H., and Singh, I. (1979) Mol. Cell. Biochem. 28, 92-105.
19. Kornberg, A., and Pricer, W.E., Jr. (1953) J. Biol. Chem. 204, 329-343.
20. Lippel, K. (1973) J. Lipid Res. 14, 102-109.
21. Wender, M., Zorzalewicz, B., and Talkowska, D. (1976) Folio Biol. (Krakow) 24, 393-400.
22. Normann, P.T., Thomassen, M.S., Christiansen, E.N., and Flatmark, T. (1981) Biochim. Biophys. Acta 664, 416-425.
23. Brophy, P.J., and Vance, D.E. (1976) Biochem. J. 160, 245-251.
24. Polokoff, M.A., and Bell, R.M. (1975) J. Lipid Res. 16, 397-402.

[Received February 23, 1982]

Effect of Age and Cholestyramine Feeding on Rat Liver 3-Hydroxy-3-methyl Glutaryl CoA Reductase, Sterol Carrier Protein 1 and Sterol Carrier Protein 2 Activities

ROBERT J. MORIN*, MICHAEL J. BRUN and **M.V. SRIKANTAIAH,** *Department of Pathology, Harbor-UCLA Medical Center, 1000 West Carson Street, Torrance, CA 90509*

ABSTRACT

The rate of formation of sterol from squalene in livers from suckling rats was less than one-third that of adults. This difference was due to a lesser activity of microsomal enzymes in the suckling rat livers, and not to any difference in cytosolic sterol carrier protein 1. The microsomal enzymes and sterol carrier protein 2 of the cytosol required for the conversion 7-dehydrocholesterol to cholesterol were both lower in suckling rats compared to adults. Both those activities paralleled the differences in HMG-CoA reductase activities between suckling and adult rats. Feeding of cholestryamine to adult rats increased the activities of the microsomal enzymes, sterol carrier protein 1 and sterol carrier protein 2 involved in the conversion of squalene to cholesterol.
Lipids 17: 507-510, 1982.

INTRODUCTION

Conversion of squalene to cholesterol is a complex, multistep process which is as yet incompletely characterized. Several well documented studies have demonstrated an interaction of one or more cytosolic noncatalytic proteins with microsomal enzymes in the conversion of the water-insoluble intermediates (1-13). Two of these noncatalytic proteins were discovered in the 105,000 x g soluble supernatant (S_{105}), and are called sterol carrier proteins (SCP). The first of these, SCP_1, is required for the enzymatic conversion of squalene to lanosterol. A second protein, SCP_2 is required for the conversion of lanosterol to cholesterol. Both SCP_1 and SCP_2 have been isolated in a highly purified state (6,13). This study deals with the effect of age and cholestryamine feeding on the rates of conversion of squalene to cholesterol. These activities were also compared to that of 3 hydroxy-3-methyl glutaryl (HMG) CoA reductase.

MATERIALS AND METHODS

DL-[3-^{14}C]HMG-CoA (sp act 18.5 μCi/μmol) and DL-[5-^{3}H]mevalonic acid were purchased from New England Nuclear. The following compounds were purchased from Sigma Chemical Co.: 7-dehydrocholesterol, NAD, NADPH, bovine brain phosphatidylserine and FAD. Cholestryamine (Questran) was obtained from Mead Johnson. [^{3}H]Squalene was prepared by anaerobic incubation of DL-[5-^{3}H]mevalonic acid with a 20,000 x g rat liver supernatant according to the method of Tchen (14). The radioactive squalene was extracted into petroleum ether and purified by silicic acid column chromatography twice. Tritiated squalene and phosphatidylserine liposomes were prepared as previously described (15).

Litter mate male Sprague Dawley rats were allowed to grow with the mother for 25 days and were given solid food (rat chow) after 25 days. All the rats were maintained in a reversed light cycle (dark 4 a.m. to 4 p.m.). Rats of different ages were sacrificed at 10 a.m., the mid-point of the dark phase at which time HMG-CoA reductase activity is expected to be maximal.

To study the effect of cholestryamine feeding, 2- to 3-month-old male rats (250-300 g) were used. One group of rats was fed ground rat chow and the second group was given 2% cholestryamine mixed with the ground rat chow. Feeding was continued for 2 weeks, after which they were sacrificed at the mid-point of the dark phase.

Microsomes and S_{105} (the 105,000 x g supernatant fraction) containing SCP_1 and SCP_2 were prepared as described in our earlier publication (15). Microsomes for study of HMG-CoA reductase were prepared according to the method of Srikantaiah et al. (16), and HMG-CoA reductase activity was assayed according to the method of Ackerman et al. (17).

SCP_1 Activity

The activity of SCP_1 is proportional to its capacity to enhance the microsomal formation of sterols from squalene. This was assayed in a

TABLE 1

Conversion of Squalene to Sterols and 7-Dehydrocholesterol to Cholesterol by Rat Livers as a Function of Age

Source of S_{105}	Source of microsomes	pmol sterols/mg S_{105}/min[a]	pmol cholesterol/mg S_{105}/min[b]
Sucklings	Sucklings	6.9 ± 2.4	25 ± 3.8
Adults	Sucklings	9.3 ± 2.1	45 ± 4.6
Sucklings	Adults	18.6 ± 2.05	63 ± 8.0
Adults	Adults	24.6 ± 0.5	120 ± 20
40-45 days old	40-45 days old	26.4 ± 1.6	112 ± 14

For suckling rats, 3 groups of 10 rats each were used. For 40-45-day-old rats, 3 groups of 3 rats each were used. Three individual adult rats were used. S_{105} is the 105,000 × g supernatant fraction.

[a]Squalene substrate.

[b]7-Dehydrocholesterol substrate.

reaction mixture of one ml buffer, microsomes (2 mg protein), NADPH (1.2 mM), NAD^+ (3 mM), FAD (0.1 mM), S_{105} as a source of SCP_1 (2.0 mg protein) and 100 μl [^{3}H]squalene-phosphatidylserine vesicles (20 μM). The incubations were conducted for 1 hr in a shaking water bath under an oxygen atmosphere. The remainder of the procedure was the same as described by Scallen et al. (1). Specific activity was defined as pmol of sterols formed in 1 min/mg S_{105} protein, with microsomes present in excess.

SCP_2 Activity

The activity of SCP_2 is proportional in its capacity to enhance microsomal 7-dehydrocholesterol reductase activity. This activity was assayed by our previously described method (18). This involved measuring the unreduced 7-dehydrocholesterol remaining at the end of the reaction. One-ml incubation mixtures contained 1.3 mM NADPH, 2 mg of microsome protein, S_{105} as a source of SCP_2 (2.0 mg) and 150 nmol of 7-dehydrocholesterol added in 10 μl dioxane, propylene glycol, 2:1. After 1 hr incubation with shaking at 37 C under nitrogen, the reaction was stopped by adding 1 ml 15% alcoholic KOH, and extracted twice with 2 ml of hexane each time. The combined extracts were evaporated under nitrogen, dissolved in 2 ml of heptane and scanned between 260-320 nm in a Hitachi dual-beam spectrophotometer. The amount of 7-dehydrocholesterol reduced is proportional to the decrease in optical density at 280 nm. SCP_2 activity is expressed as pmol of cholesterol formed/mg S_{105} protein/min. Protein was determined by the method of Bradford (19).

RESULTS AND DISCUSSION

Conversion of squalene to sterols in suckling rats was very low compared to adult rats when suckling rat liver microsomes and suckling rat S_{105} (source of SCP_1) were incubated together (Table 1). This appeared to be due to a lower level of microsomal enzymes rather than SCP_1, because when adult S_{105} was used in conjunction with suckling rat microsomes, the activity was comparable to that observed when both S_{105} and microsomes were from suckling rats. Conversely, when microsomes from adult rats were used in combination with suckling rat S_{105}, the activity was ca. 3-fold higher than the activity with suckling rat microsomes and S_{105}. After the weaning period, microsomal enzymes reached normal levels and SCP_1 remained constant. The lower cholesterol synthesis in suckling rats was primarily due to low HMG-CoA reductase activity (Table 2). The 7-dehydrocholesterol reductase involved in the conversion of squalene to cholesterol also seemed to be age-dependent (Table 1). Of the cytosolic factors, SCP_1 did not seem to vary with age, whereas SCP_2 was age-dependent (Table 1). Other investigators also have reported age regulation of HMG-CoA reductase (20,21) and other enzymes between mevalonate and cholesterol (22). McNamara et al. (20) reported that HMG-CoA reductase activity in suckling rats was very low and that it rose rapidly after weaning. Shah (22) showed that incorporation of mevalonate into nonsaponifiable lipids and digitonin-precipitable sterols was low in suckling rats and increased rapidly after weaning. From these results, he postulated that the steps in cholesterol synthesis between mevalonate and cholesterol were age-dependent. Takeuchi et al. (12) found that, in aged rats,

TABLE 2

HMG-CoA Reductase Activity in Liver Preparations from Rats of Varying Ages

Age of rats	Number used	Enzyme activity (pmol/mg/min protein)
17-22-day-old rats (suckling rats)	3 groups of 10 rats each	28.3 ± 16
40-45-day-old rats (weaned)	3 groups of 3 rats each	717 ± 90
120-130-day-old rats (adult)	3 individual rats	659 ± 116

TABLE 3

Conversion of Squalene to Sterols and 7-Dehydrocholesterol to Cholesterol by Livers from Control and Cholestyramine-Fed Rats

Source of S_{105}	Source of microsomes	pmol sterols/mg S_{105}/min[a]	pmol cholesterol/mg S_{105}/min[b]
Control	Control	24 ± 3.6	115 ± 12
Cholestyramine	Control	38 ± 4.2	144 ± 18
Control	Cholestyramine	36 ± 2.1	191 ± 28
Cholestyramine	Cholestyramine	51 ± 6.2	269 ± 40

Results are means ± SD of 4 rats each. S_{105} is the 105,000 × g supernatant.

[a]Squalene substrate.

[b]7-Dehydrocholesterol substrate.

the inactivation of HMG-CoA reductase by ATP and Mg^{++} was decreased, whereas activation by cytosol was increased. They also reported that the conversion of squalene to cholesterol using microsomes and cytosol decreased about 9-fold in 60-65-week-old rats (aged rats) compared to rats that were 4-8 weeks old (young rats). They both found the microsomal enzymes and the sterol carrier protein to be decreased in the aged rats. Cholestryamine feeding to adult rats in the present study increased the rate of conversion of squalene to sterols (Table 3). This increased activity was due to an increase in SCP_1, as well as in microsomal enzymes. Similar increased activity was observed in SCP_2 and the microsomal enzymes responsible for the reduction of 7-dehydrocholesterol to cholesterol (Table 3). HMG-CoA reductase activity increased from 414±46 in the control group to 863±236 in the group fed 2% cholestryamine.

Our studies show that whenever there is age-dependent regulation of cholesterol synthesis, changes in the microsomal enzymes responsible for conversion of squalene to cholesterol or the sterol carrier proteins or both of these factors are involved in addition to HMG-CoA reductase.

ACKNOWLEDGMENTS

This work was supported by American Heart Association Greater Los Angeles Affiliate Award No. 697G2-2.

REFERENCES

1. Scallen, T.J., Schuster, M.W., and Dhar, A.K. (1971) J. Biol. Chem. 246, 224-230.
2. Ritter, M.C., and Dempsey, M.E. (1970) Biochem. Biophys. Res. Commun. 38, 921-929.
3. Scallen, T.J., Srikantaiah, M.V., Skrdlant, H.B., and Hansbury, E. (1972) FEBS Lett. 25, 227-233.
4. Scallen, T.J., Srikantaiah, M.V., Seetharam, B., Hansbury, E., and Gavey, K.L. (1974) Fed. Proc. 33, 1733-1746.
5. Scallen, T.J., Seetharam, B., Srikantaiah, M.V., Hansbury, E., and Lewis, M.K. (1975) Life Sci. 16, 853-874.
6. Srikantaiah, M.V., Hansbury, E., Loughran, E.D., and Scallen, T.J. (1976) J. Biol. Chem. 251, 5496-5504.
7. Tai, H.H., and Bloch, K. (1972) J. Biol. Chem. 247, 3767-3773.
8. Johnson, R.C., and Shah, S.N. (1973) Biochem. Biophys. Res. Commun. 53, 105-111.
9. Gaylor, J.L., and Delwiche, C.V. (1976) J. Biol. Chem. 251, 6638-6645.
10. Ritter, M.C., and Dempsey, M.E. (1971) J. Biol.

Chem. 246, 1536-1539.
11. Ritter, M.C., and Dempsey, M.E. (1973) Proc. Natl. Acad. Sci. 70, 265-269.
12. Takeuchi, N., Koga, M., Kukita, H., Uchida, K., and Yamamura, Y. (1978) in Liver and Aging, (Kitani, K., ed.) pp. 177-191, Elsevier, Amsterdam.
13. Noland, B.J., Arebalo, R.E., Hansbury, E., and Scallen, T.J. (1980) J. Biol. Chem. 255, 4282-4289.
14. Tchen, T.T. (1963) Methods Enzymol. 6, 509-512.
15. Morin, R.J., and Srikantaiah, M.V. (1980) J. Lipid Res. 21, 1143-1147.
16. Srikantaiah, M.V., Tormanen, C.D., Redd, W.L., Hardgrave, J.E., and Scallen, T.J. (1977) J. Biol. Chem. 252, 6145-6150.
17. Ackerman, M.E., Redd, W.L., Tormanen, C.D., Hardgrave, J.E., and Scallen, T.J. (1977) J. Lipid Res. 18, 408-413.
18. Srikantaiah, M.V., Assef, S., and Morin, R.J. (1978) Biochem. Biophys. Res. Commun. 81, 1087-1090.
19. Bradford, M.M. (1976) Anal. Biochem. 72, 248-251.
20. McNamara, D.J., Forrest, W.D., and Rodwell, V.W. (1972) J. Biol. Chem. 247, 5805-5810.
21. Ballard, R.J., and Hanson, R.W. (1967) Biochem. J. 102, 952-958.
22. Shah, S.N. (1973) Lipids 8, 284-288.

[Received March 10, 1982]

Antioxidant Activities of Tocopherols on Fe^{2+}-ascorbate-Induced Lipid Peroxidation in Lecithin Liposomes

KENJI FUKUZAWA, AKIRA TOKUMURA, SETSUHIRO OUCHI and **HIROAKI TSUKATANI**, *Faculty of Pharmaceutical Sciences, Tokushima University, Shomachi-1, Tokushima, 770, Japan*

ABSTRACT

The antioxidant activities of 4 tocopherols, tocol, and a water-soluble model analog of α-tocopherol were compared. Egg lecithin liposomes were used and oxidation was catalyzed by Fe^{2+}-ascorbate. The activities decreased in the order α->β->γ->δ-tocopherol>tocol, in agreement with their potencies in vivo. The water-soluble analog was the least effective. Activity depended on the molar ratio of antioxidant to unsaturated lipid, with one molecule each of the α-, β-, γ-, δ-tocopherol and tocol capable of protecting, respectively, 220, 120, 100, 30 and 20 molecules of polyunsaturated fatty acid. The mechanism of possible antioxidant effect of the compounds used is discussed.
Lipids 17: 511-513, 1982.

It is widely believed that vitamin E functions in vivo mainly as an antioxidant by protecting unsaturated lipids from peroxidation (1-3). This function is assisted by the location of the various tocopherols, which make up the vitamin, in the lipid regions of plasma and subcellular membranes (2). There is, however, a discrepancy in the relative activities of the tocopherols in vitro and in vivo. When dissolved in edible oil and subjected to photolytic or autoxidizing conditions, the antioxidant activities increase in the order α-<β-<γ-<δ-tocopherol (4,5). This is the exact reverse of their vitamin E potency sequence in vivo (6). In this study, we have re-examined the antioxidant activities of different tocopherols incorporated into liposomes under conditions close to physiological.

The tocopherols (dl-α-, d-β-, d-γ- and d-δ-), designated α-Toc, β-Toc, γ-Toc and δ-Toc, together with tocol and an α-tocopherol model compound, 3-(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl) propionic acid, were supplied by Eisai Co., Tokyo, and purified by the method of Abe et al. (7). The final purity, measured by gas liquid chromatography (GLC), was over 98%. Egg-yolk lecithin was prepared by the method of Pangborn (8). Dimyristoyl lecithin (99% pure) came from Sigma Chemical Co. The concentrations of phospholipids were determined by the method of Chalvadjian and Rudnicki (9) and their fatty acid composition by GLC after acid methanolysis was determined as described previously (10). For egg-yolk lecithin, mol % composition was 45.8 palmitate, 1.4 palmitoleate, 12.6 stearate, 18.7 oleate, 8.1 linoleate, 4.0 linolenate and 9.5 arachidonate.

Liposomes were prepared as previously described (10), with a stock solution of lipid in chloroform evaporated under nitrogen and dispersed in 0.01 M Tris-HCl buffer, pH 7.5, followed by ultrasonic irradiation in a Branson Sonifier (Model W-185) for 6 min using 15-sec intervals. Lipid peroxides were measured by the thiobarbituric acid (TBA) colorimetric method (10) and the results were expressed in terms of malondialdehyde (MDA) present. The oxidation was initiated by addition of $FeSO_4$ and ascorbic acid to final concentrations 5 μM and 100 μM, respectively. Each sample had a volume of 4 ml and contained 2 μmol of lecithin. After incubation at 30 C, the reaction was stopped by addition of 1 ml of 5 mM EDTA. Then, 1 ml of the suspension was mixed with 2.5 ml of 20% trichloroacetic acid and 1 ml of 0.67% aq TBA solution. After heating for 10 min in a boiling water bath, the pink pigment was extracted with 4 ml of *n*-butanol and its absorbance was measured at 535 nm. Standard MDA solutions were prepared by hydrolysis of 1,1,3,3-tetraethoxypropane. Concentration of tocopherols were measured fluorometrically by the method of Abe and Katsui (11). One ml of liposome suspension was mixed with 1 ml of ethanol and 5 ml of hexane and the fluorescence intensity of the organic phase was measured using excitation and emission wavelengths of 295 and 320 nm, respectively. The relative intensities were 100, 80, 91 and 168 for the α-, β-, γ- and δ-tocopherols, respectively.

The time-course of lipid peroxidation and disappearance of α-tocopherol in egg lecithin liposomes exposed to Fe^{2+}-ascorbate is shown in Figure 1. Two concentrations of α-tocopherol were used. At 0.25 mol %, the antioxidant completely prevented lipid oxidation without undergoing any measurable oxidation itself. How-

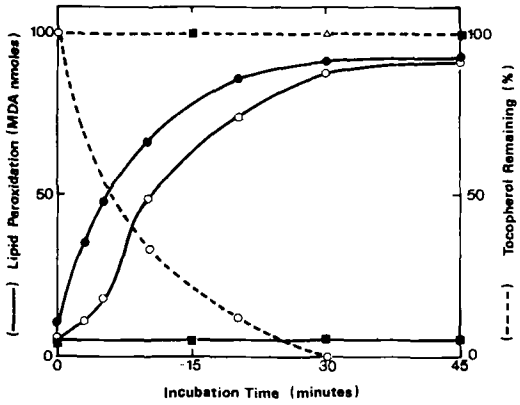


FIG. 1. Time courses of Fe^{2+}-ascorbate-induced lipid peroxidation (solid line) and α-Toc decomposition (dotted line) in egg lecithin and dimyristoyl-lecithin liposomes. The reaction mixture (4 ml) consisted of 5 μM $FeSO_4$, 0.1 mM ascorbic acid, 0.01 M Tris-HCl buffer (pH 7.5) and liposomes containing 2 μmol of lecithin. Incubation was done at 30 C. Liposome composition (molar ratio) was as follows: (•) egg lecithin; (○) egg lecithin, α-Toc (1000:1); (▪) egg lecithin, α-Toc; (1000:2.5); (△) dimyristoyl-lecithin, α-Toc (1000:1).

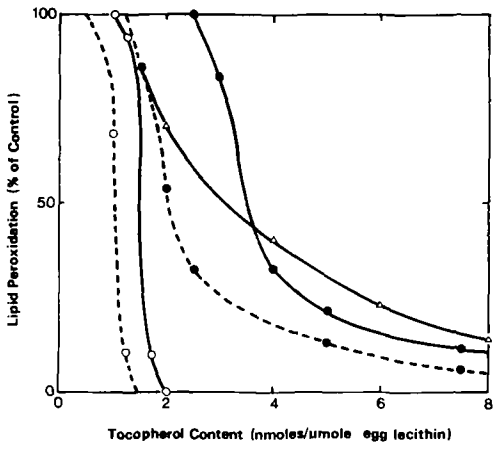


FIG. 2. Dose-dependent antioxidant effects of tocopherols and a model compound of α-Toc on Fe^{2+}-ascorbate-induced lipid peroxidation in liposomes prepared from egg lecithin (solid line) and an equimolar mixture of egg lecithin and dimyristoyl-lecithin (dotted line). Incubation conditions were as for Fig. 1, except that α-Toc model was dissolved in 50% ethanol and 20 μl of the solution was added to 4 ml of reaction mixture. The incubation time was 30 min; 100% peroxidation estimated as the amount of MDA produced by liposomes not containing tocopherols were 83.1 ± 4.9 (solid line) and 35.5 ± 2.4 (dotted line) nmol/4 ml of reaction mixture/30 min, respectively. (○) α-Toc, (•) δ-Toc, and (△) α-Toc model.

TABLE 1

Concentrations of Tocopherols Needed for 100% Inhibition of Fe^{2+}-Ascorbate-Induced Lipid Peroxidation in Egg Lecithin Liposomes[a]

Tocopherols	(nmol/μmol egg lecithin)
α-Tocopherol	2.0
β-Tocopherol	3.8
γ-Tocopherol	4.2
δ-Tocopherol	14
Tocol	20

[a]Experimental conditions were the same as for Fig. 2.

ever, at 0.1 mol %, the α-tocopherol disappeared in 30 min and was only able to delay the onset of rapid lipid oxidation by about 6 min. When it was incorporated into dimyristoyl-lecithin liposomes at 0.1 mol % and exposed to the Fe^{2+}-ascorbate system, most of the α-Toc remained unoxidized for 30 min. This indicated that loss of antioxidant depended on active peroxidation of unsaturated lipids.

The concentrations at which tocol and the tocopherols acted as effective inhibitors of peroxidation were compared next (Fig. 2). All compounds gave sigmoid, dose-dependent responses but with varying steepness. Their antioxidant capacities are compared in Table 1. We calculated from these results that ca. 220, 120, 100, 30 and 20 molecules of polyunsaturated fatty acids (PUFA) in the membranes were completely protected by one molecule of α-, β-, γ-, δ-Toc and tocol, respectively. For α-Toc, this molar ratio of about 200:1 is found in the membranes of microsomes of highly oxygenated tissues of heart and lung (12). Figure 2 also shows the good correlation between the PUFA content of liposomes and the ability of 2 of the tocopherols to prevent their oxidation. When the liposomes were prepared from equimolar mixtures of egg and dimyristoyl lecithin, the amounts of α- and δ-Toc required for complete inhibition was halved. In all cases, the water-soluble α-tocopherol model compound proved to be the least effective, in agreement with the suggestion that the potency of the antioxidants depends on their molecular environment, as well as on the steric roles of the chromanol ring and the isoprene chain (13).

Results shown in Figures 2 and 3 show that the threshold concentrations at which the compounds lost their antioxidant activity were similar to those at which they were rapidly lost themselves. This is not surprising, but the close correspondence in the slopes of the curves for each particular substance is remarkable.

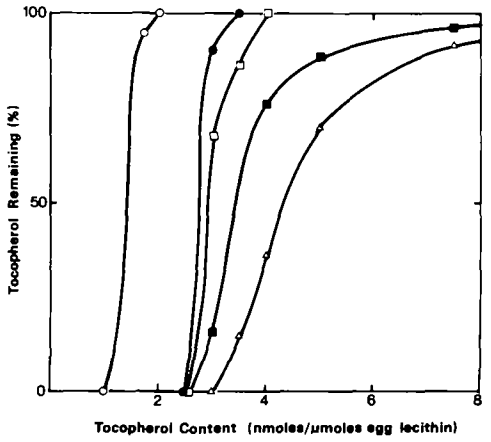
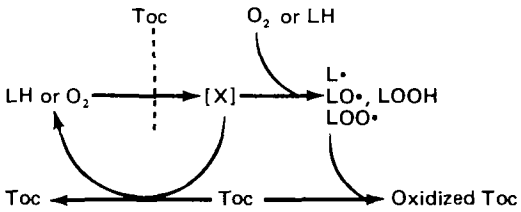


FIG. 3. Dose-dependence of tocopherol loss during Fe^{2+}-ascorbate-induced lipid peroxidation in egg lecithin liposomes. Incubation conditions were as for Fig. 2. (○) α-Toc, (●) β-Toc, (□) γ-Toc, (■) δ-Toc and (△) tocol.

The mechanism of Fe^{2+}-ascorbate-induced lipid peroxidation which might operate in biological systems is unknown. Singlet oxygen, hydrogen peroxide or superoxide and hydroxyl free radicals are not thought to be involved (10). Our results can be explained by the assumption, outlined in Scheme 1, that the peroxidation process occurs in 2 steps:



SCHEME 1

LH and LOOH indicate PUFA and its hydroperoxide, with L•, LO• and LOO• as free radical intermediates. The reactive transient [X] generated in the first step may be an activated oxygen, probably bound to the iron-ascorbate complex (14,15) such as perferryl ion (16) or activated PUFA observed in a photosensitizing process (17). In this scheme, high concentrations of tocopherols would inhibit the reaction leading to formation of [X], or would inactivate [X] itself by quenching. In these reactions, the antioxidant remains unchanged. At low tocopherol concentrations, the quenching may be less complete. The antioxidant then reacts with the lipid free radicals formed in the second step, becoming irreversibly oxidized in the process.

It appears that several variables may determine the relative antioxidant capacities of different tocopherols. One could be the mode by which the oxidation is initiated. Because catalysis by metal ion complexes is believed to play a major role in lipid oxidation in biological systems (16), the relative quenching abilities of different tocopherols may be dominant in determining their effectiveness. Such factors may account for the differences in the sequence of oxidation of tocopherol and unsaturated lipids in our system and in some biological membranes (18).

ACKNOWLEDGMENTS

The authors thank Dr. J.M. Gebicki for helpful suggestions and A. Kusunoki for technical assistance. Thanks also to Eisai Co. Ltd. for their generous gift of tocopherols and their analogs.

REFERENCES

1. Tappel, A.L. (1962) Vitam. Horm. 20, 493-510.
2. Tappel, A.L. (1972) Ann. N.Y. Acad. Sci. 203, 12-28.
3. Hafeman, D.G., and Hoekstra, W.G. (1977) J. Nutr. 107, 666-672.
4. Stern, M.H., Robeson, C.D., Weisler, L., and Baxter, J.G. (1947) J. Am. Chem. Soc. 69, 869-874.
5. Lea, C.H., and Ward, R.J. (1959) J. Sci. Food Agric. 10, 537-548.
6. Century, B., and Horwitt, M.K. (1965) Fed. Proc., Fed. Am. Soc. Exp. Biol. 24, 906-916.
7. Abe, K., Yuguchi, Y., and Katsui, G. (1974) Jpn. J. Food Nutr. 27, 329-332.
8. Pangborn, M.C. (1951) J. Biol. Chem. 188, 471-476.
9. Chalvadjian, A., and Rudnicki, E. (1970) Anal. Biochem. 36, 225-226.
10. Fukuzawa, K., Chida, H., Tokumura, A., and Tsukatani, H. (1981) Arch. Biochem. Biophys. 206, 173-180.
11. Abe, K., and Katsui, G. (1975) Jpn. J. Food Nutr. 28, 277-280.
12. Kornbrust, D.J., and Mavis, R.D. (1980) Lipids 15, 315-322.
13. Fragata, M., and Bellemare, F. (1980) Chem. Phys. Lipids 27, 93-99.
14. Hamilton, G.A. (1964) J. Am. Chem. Soc. 86, 3391-3392.
15. Khan, M.M.T., and Martell, A.E. (1967) J. Am. Chem. Soc. 89, 4176-4185.
16. Tien, M., Svingen, B.A., and Aust., S.D. (1981) Fed. Proc. 40, 179-182.
17. Logani, M.K., and Davies, R.E. (1980) Lipids 15, 385-495.
18. McCay, P.B., Pfiefer, P.M., and Stipe, W.H. (1972) Ann. N.Y. Acad. Sci. 203, 62-73.

[Received February 2, 1982]

ERRATUM

ERRATUM

In the article "Effects of Phosphatidylcholines on de novo Synthesis and Excretion of Sterol by L-929 Fibroblasts" (*Lipids* 17:427-433 [1982]), the first author's name should read "Georgios N. Thomaidis, Ph.D.," instead of "Georgios Thomaidis." A footnote to the article should read: "This work was a part of the doctoral dissertation that Georgios N. Thomaidis submitted to the faculty of the Graduate School of the University of Maryland."

Isolation and Determination of Cholesterol Glucuronide in Human Liver

ATSUSHI HARA and **TAMOTSU TAKETOMI**, *Department of Biochemistry, Institute of Adaptation Medicine, Shinshu University School of Medicine, Matsumoto 390, Japan*

ABSTRACT

Separation of the acidic lipid fraction from human liver led to the identification of cholesterol-β-glucuronide for the first time from this organ. Cholesterol glucuronide was purified by DEAE-Sephadex column chromatography and preparative silica gel thin-layer chromatography. The content in normal human liver was about 33 nmol/g wet tissue. It must be emphasized that cholesterol glucuronide cannot be distinguished readily from ganglioside GM4 by thin-layer chromatography.
Lipids 17:515-518, 1982.

INTRODUCTION

In the course of a study of gangliosides in human liver, we found a substance which reacted with both anthrone and resorcinol reagents on thin-layer plates. However, it contained neither sialic acid nor fatty acids. It seemed important to identify this substance, not only for its intrinsic interest, but also because it is liable to be misunderstood as a ganglioside. This paper describes the chemical analysis and identification of the compound as cholesterol glucuronide.

MATERIALS AND METHODS

A normal liver and a liver from a patient with GM1-gangliosidosis type II were obtained by autopsy. The tissue was homogenized with chloroform/methanol (C/M) according to Suzuki (1). Forty g of liver was homogenized with 800 ml each of C/M (2:1, v/v) and C/M (1:2) containing 5% water. The filtrate was evaporated to dryness under vacuum and the lipid residue was dissolved in 240 ml of C/M (2:1). To the solution was added 5 ml of 5 N NaOH in methanol and the reaction mixture was stirred 1 hr at room temperature. The mixture was concentrated under vacuum to half volume and dialyzed against water. The contents of the dialysis bag were evaporated to dryness and the acidic lipids were isolated by a modification of the method of Ledeen et al. (2). The solvent used in this method to dissolve the sample (C/M/W, 30:60:8) yielded a cloudy mixture due to the fatty acid methyl esters, so it was changed to C/M/W (60:60:8). The sample in 200 ml of the solvent was applied to a column of DEAE-Sephadex A25 (Pharmacia, 3.5 meq/g dry wt, 15 × 280 mm). The nonacidic lipids were eluted with 100 ml of the same solvent, then the acidic lipids were eluted with 800 ml of C/M/0.8 M NaOAc (30:60:8). The latter effluent was evaporated to a small volume and dialyzed against water. The free fatty acids in the dialyzate were removed by silica gel chromatography (2). The resulting acidic lipids were then separated by preparative thin-layer chromatography (TLC) on plates precoated with Silica Gel 60 (0.25 mm thick, E. Merck), developed with C/M/0.25% KCl (60:35:8). The bands were located with bromthymol blue (3), scraped from the plate, suspended in chloroform, and applied to a column of silica gel 60 (10 g, 10 × 300 mm). The indicator was eluted with 100 ml of C/M (80:20), then cholesterol glucuronide or ganglioside was eluted with 100 ml of C/M (50:50).

A portion of the isolated cholesterol glucuronide, about 1 mg, was methanolyzed with 3% HCl in dry methanol at 80 C for 3 hr, with or without mannitol as internal standard. To the methanolyzate was added 3 × 3 ml of hexane and the two solvent layers (hexane and methanol) were analyzed as the trimethylsilyl derivatives by gas-liquid chromatography (GLC) using a silicone column (2% OV-1, 3 mm × 2 m) (4). For the quantitative analysis of cholesterol, cholesterol benzoate (Wako Chemical Co., Japan) was added to the hexane layer as internal standard and analyzed as just described. The column was programmed from 140 to 220 C at 2 C/min. The silylation reactions were carried out according to Cater and Gaver (5) with a mixture of trimethylchlorosilane (Pierce Chemical Co.), hexamethyldisilazane (Gas-Chro Kogyo Co., Japan) and dry pyridine (Nakarai Chemicals, Japan) which was distilled in our laboratory.

A portion of the isolated cholesterol glu-

curonide, about 15 μg, was incubated with 10 U of *Escherichia coli*-β-D-glucuronidase, EC 3.2.1.31 (Boehringer Mannheim, W. Germany), in 10 mM acetate buffer, pH 7.0 for 48 hr in a total vol of 1 ml (6). The reaction was stopped by adding C/M (2:1) and evaporated to dryness, then analyzed by TLC (C/M/W, 65:25:4 or hexane/ether, 80:20).

The infrared spectrum was prepared with a KBr disk.

RESULTS

The acidic lipids obtained by silica gel column chromatography were analyzed by TLC (Fig. 1) and found to contain two major bands and one minor band. One of the major bands was ganglioside GM3, which appeared as a double band due to the different fatty acid chain lengths. The other major band was cholesterol glucuronide, as shown by subsequent analysis. Its R_f value was 0.61 (1.35 relative to GM3). The minor band had the same R_f value as ganglioside GD3. All of these bands were positive to anthrone (7) and resorcinol (8) reagents.

Analysis of the sugar components by GLC showed that GM3 and GD3 contained glucose and galactose in the molar ratio, 1:1.1. The methanol layer from cholesterol glucuronide yielded two major peaks corresponding to the ones produced by glucuronic acid (Fig. 2). The hexane layer from the new lipid also yielded a peak pattern corresponding to cholesterol (Fig. 3). When the whole methanolyzate was neutralized with silver carbonate, trimethylsilylated, and analyzed by GLC, peaks were seen (Fig. 4) for glucuronic acid (peaks A and B) and trimethylsilyl cholesterol (peak G). The

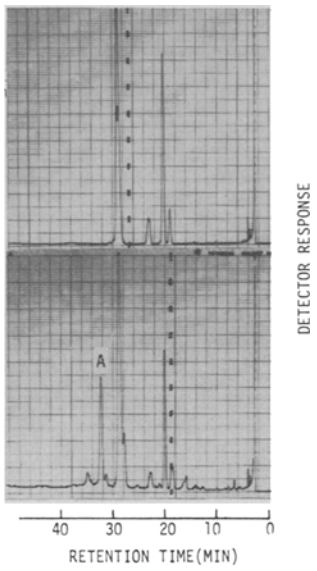


FIG. 2. Gas chromatogram of the methanol layer obtained by treatment of glucuronic acid (upper) and cholesterol glucuronide (lower) with methanolic HCl. A = mannitol standard. The contents of the methanol layer were converted to the trimethylsilyl derivatives.

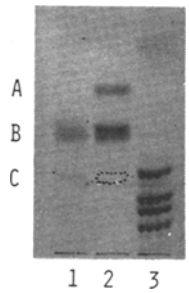


FIG. 1. Thin layer chromatogram of acidic lipids, developed with C/M/0.25% KCl (60:35:8). (1) Ganglioside GM3 standard. (2) Acidic lipids from normal liver. (3) Gangliosides from human brain. A = cholesterol glucuronide, B = ganglioside GM3, C = ganglioside GD3.

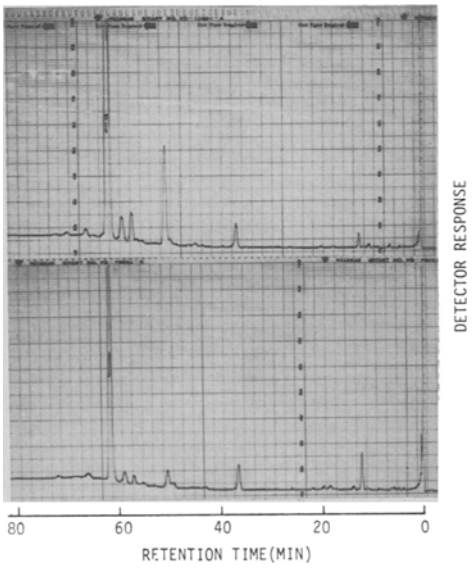


FIG. 3. Gas chromatogram obtained by treatment of cholesterol (upper) and cholesterol glucuronide (lower) with methanolic HCl. The contents of the hexane layer were reacted with silylating reagent. The GLC column was operated at 170-280 C, at 2 C/min.

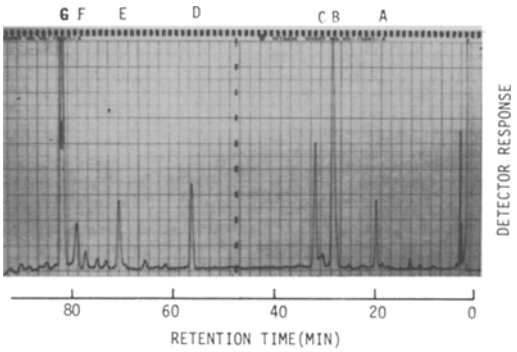


FIG. 4. Gas chromatogram obtained with the methanolysis mixture obtained from cholesterol glucuronide, without separating into two fractions. A lower starting temperature was used for this run: 140-280 C at 2 C/min. A and B are the major peaks from glucuronic acid, C represents mannitol, D is an impurity from the silylating reagent, E is cholesta-3,5-diene, F is 3-O-methyl cholesterol, and G is obtained from cholesterol.

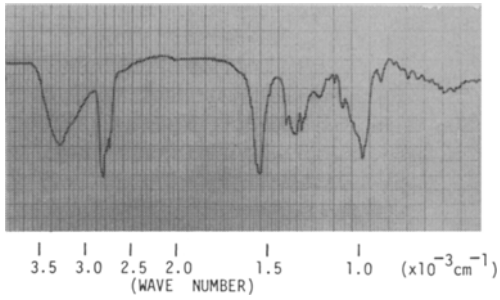


FIG. 5. Infrared spectrum of cholesterol glucuronide, supported in a KBr disk.

molar ratio of glucuronic acid to cholesterol, determined by area comparisons with internal standard, was 1:1.3. It is confirmed that one molecule of glucuronic acid binds to one molecule of cholesterol. The same result was obtained from Figure 4. Some of the smaller peaks (E and F) were identified as being formed from cholesterol during methanolysis: cholesta-3,5-diene and 3-O-methyl cholesterol. These artifacts were identified by their retention times in GLC analysis as reported previously by Kawamura and Taketomi (9). It seemed that the amounts of the artifacts varied among tubes. This may be due to the losses of the artifacts while drying because of their nonpolarity and small sample weight.

The infrared spectrum of the glucuronide (Fig. 5) showed specifically absorbing groups: 2800-3000 cm^{-1} due to methylene, 1600 cm^{-1} due to the carboxyl salt (of Na^+), and 1000-1100 cm^{-1} due to hydroxy groups.

The linkage between cholesterol and glucuronic acid was examined with β-D-glucuronidase. The condition described in Materials and Methods gave complete cleavage of the linkage. Thus, it is confirmed that the compound is cholesterol-β-D-glucuronide.

The content of cholesterol glucuronide was 32.5 nmol/g wet tissue in normal liver and 89.8 nmol/g in the liver from the ganliosidosis patient. The patient's brain and spleen contained no detectable cholesterol glucuronide although the brain contained much ganglioside GM1 (4.87 μmol/g). This finding confirmed the diagnosis of the type II disorder. The GM3 and GD3 contents in the patient's liver were 210 and 15.5 nmol/g, respectively.

DISCUSSION

The problem of misidentification of cholesterol glucuronide as ganglioside GM4 arose only when the resorcinol spray reagent was used on a TLC plate. When we incubated cholesterol (20, 100, or 200 μg) or glucuronic acid (20 or 100 μg) with resorcinol reagent which was the same one used in the spray in a test tube in the usual way for sialic acid determination (8), the typical blue-purple color was not obtained. The highest absorbance seen was 0.020 for 100 μg of glucuronic acid, measured at 580 nm. Yet, on a TLC plate, glucuronic acid showed the characteristic color and cholesterol showed a more bluish color.

This paper describes, for the first time, the occurrence of cholesterol-β-glucuronide in liver. It has previously been identified in human urine and plasma (10,11). In our study, the compound was identified chemically, physically and enzymatically. The content of cholesterol glucuronide in the normal liver we analyzed was higher than the reported value for plasma (18 μg/g vs 6 μg/ml). Presumably, cholesterol glucuronide is synthesized in the liver and some of it enters the bloodstream. Probably the rest goes into the bile, like the other glucuronides.

The content of cholesterol glucuronide in the liver from the ganglioside patient was distinctly higher than in the normal one, but more samples are needed to establish the normal range of values.

ACKNOWLEDGMENT

The authors thank Dr. Norman Radin (The University of Michigan, USA) for helpful suggestion and for his help in the preparation of this manuscript.

REFERENCES

1. Suzuki, K. (1964) Life Sci. 3, 1227-1233.
2. Ledeen, R.W., Yu, R.K., and Eng, L.F. (1973) J. Neurochem. *21*, 829-839.
3. Yarmosh, D.F., and Ferniany, R.C. (1977) Clin. Chem. 23, 614-615.
4. Hara, A., and Taketomi, T. (1975) J. Biochem. 78, 527-536.
5. Cater, H.E., and Gaver, R.C. (1967) J. Lipid Res. 8, 391-395.
6. Nambara, T., Numazawa, M., and Akiyama, S. (1971) Chem. Pharm. Bull. 19, 153-158.
7. Roe, J.H. (1955) J. Biol. Chem. 212, 335-343.
8. Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604-611.
9. Kawamura, N., and Taketomi, T. (1973) Jpn. J. Exp. Med. 43, 157-162.
10. Winter, J.S.D., and Bongiovanni, A.M. (1968) J. Clin. Endocrinol. Metab. 28, 297-390.
11. Wade, A.P. (1970) Clin. Chim. Acta 27, 109-116.

[Received January 6, 1982]

Uptake and Oxidation of Malonaldehyde by Cultured Mammalian Cells

R.P. BIRD and **H.H. DRAPER**, *Department of Nutrition, College of Biological Science, University of Guelph, Guelph, Ontario, Canada, N1G 2W1*

ABSTRACT

Primary cultures of rat skin fibroblasts were used as a model system to investigate the cellular uptake and oxidation of malonaldehyde (MA). The cells were grown in a medium containing 10^{-5} M, 10^{-4} M or 10^{-3} M concentrations of [1,3-^{14}C]MA. There was a limited, concentration-dependent uptake of MA by 24 hr (~4% at all concentrations). The uptake of [1,2-^{14}C]acetate by 24 hr was ~24%; 83-89% of the ^{14}C in the MA taken up was oxidized to $^{14}CO_2$ by 24 hr and ~5% was recovered in the major lipids. Despite its low uptake and rapid oxidation to CO_2, pretreatment of the cells with 10^{-3} M MA for 24 hr produced a latent inhibition of [^{14}C]glucose oxidation. Limited cellular uptake of MA may explain the tolerance of cells grown in culture to relatively high MA concentrations.
Lipids 17:519-523, 1982.

INTRODUCTION

Malonaldehyde (MA) is a well known product of lipid peroxidation which occurs in increased concentrations in animal tissues as a result of vitamin E deficiency, radiation damage, exposure to chlorinated hydrocarbons and oxygen toxicity. It has been reported to be a normal side-product of prostaglandin synthesis, and it is ingested in variable quantities in a number of food products, mainly of animal origin.

Malonaldehyde is mutagenic in bacterial systems (1-4) and in a mammalian cell culture system (5). It is also a carcinogenic initiator when applied to the skin of mice in large amounts (12 mg/day) (6). MA has been observed to inhibit DNA, RNA and protein synthesis in primary cultures of rat skin fibroblasts (7) and to induce DNA repair synthesis at concentrations as low as 10^{-6} M (Bird and Draper, unpublished results). The cytotoxicity of MA has stimulated interest in its metabolism. MA has been shown to stimulate oxygen uptake by rat liver mitochondria (8) and to be actively metabolized to acetate and CO_2 in these organelles (9). This study was designed to investigate the uptake and oxidation of MA by rat skin fibroblasts and its effect on the capacity of the cells to oxidize glucose.

MATERIALS AND METHODS

Materials

Cell culture medium HB 597 was purchased from Connaught Laboratories (Willowdale, Ont.). Fetal bovine serum, phosphate-buffered saline (PBS, pH 7.2) and trypsin solution (0.25% in Hanks' balanced salt solution) were obtained from GIBCO (Grand Island, NY). D-[U-^{14}C]Glucose (>230 mCi/mmol), NCS (a solution of quaternary ammonium base in toluene), OCS (Organic Counting Scintillant) and PCS (Phase Combining Scintillant) were purchased from Amersham Corporation (Arlington Heights, IL). Aquasol and [1,2-^{14}C]-acetic acid sodium salt (52 mCi/mmol) were purchased from New England Nuclear Canada (Lachine, Que.). 1,1,3,3-Tetramethoxy [1,3-^{14}C]propane (15 mCi/mmol) was obtained from Amersham Corporation. The radiochemical purity of the compound was >95% and no chemical impurity was detectable.

Preparation of a Primary Culture of Rat Skin Fibroblasts

Primary cultures of skin fibroblasts were prepared from newborn rat pups by a conventional method consisting of trypsinization, collection of the cells by centrifugation and suspension in the medium (7). The cells were seeded into Leighton tubes and grown as a monolayer (7). The number of cells used to start the cultures was ~1 x 10^5/tube.

Preparation of [^{14}C]MA

[1,3-^{14}C]Tetramethoxypropane (0.1 ml) in benzene and methanol (1:1, v/v) was evaporated to dryness and the residue was shaken with 0.1 ml of 1 N HCl for 30 min at room temperature. The hydrolyzate then was made up to 1.0 ml with distilled water (9).

Preparation of the Sodium Salt of MA (Na-MA)

Na-MA was synthesized by the method of Protopopova and Skoldinov (10). The purity of the salt was >98% by sodium analysis and by colorimetric determination using the thiobarbituric acid (TBA) reaction (11). Chroma-

tography on an LH-20 column as described by Marnett and Tuttle (3) revealed no detectable impurity. Reaction with TBA yielded a single peak for the TBA-MA complex by a high pressure liquid chromatography procedure (Bird et al., unpublished results). The recovery of Na-MA as TBA-MA complex was quantitative by this procedure. The nuclear magnetic resonance (NMR) spectrum of Na-MA revealed proton shifts consistent with those of hydrated malonaldehyde.

Treatment of Cell Cultures

Four-day-old cultures were divided into two groups. One was used to measure the uptake of [^{14}C]MA and its incorporation into the chloroform/methanol (2:1, v/v) soluble fraction and the other was used to measure the oxidation of [^{14}C]MA to $^{14}CO_2$. Those culture tubes which were used to measure $^{14}CO_2$ released were fitted with serum stoppers and a center well. For the uptake and oxidation study, MA was used at three levels (10^{-5} M, 10^{-4} M or 10^{-3} M) and observations were made at 6, 12 and 24 hr. Reference cultures were exposed to 10^{-3} M [1,2-^{14}C]acetate. There were five replicate cultures for each treatment for each time period, except that for the 10^{-4} M and 10^{-3} M MA-treated groups, five additional cultures were used for each time period to provide enough ^{14}C-labeled cellular lipids for thin-layer chromatography (TLC). Each tube contained 0.5 μCi of [^{14}C]MA or 1.0 μCi of [^{14}C]acetate in 1 ml of medium.

Measurement of ^{14}C Uptake

During incubation at 37 C, each culture vessel was unstoppered for 1-2 min every 3-6 hr to release accumulated $^{14}CO_2$. The uptake of ^{14}C-substrate (MA or acetate) was measured by counting the radioactivity in the medium at the beginning and after 6, 12 and 24 hr of incubation. Before sampling, the medium was decanted and exposed to air at 4 C for 2 hr to allow dissolved CO_2 to escape. The cells were washed twice with 10 ml of PBS.

Measurement of ^{14}C-Substrate Oxidation

$^{14}CO_2$ evolved was trapped in CO_2-absorbing fluid placed in center wells attached to the tubes using serum stoppers. After incubation, the tubes were placed vertically in crushed ice to stop the reaction and 0.25 ml of concentrated perchloric acid was injected into the medium through the stopper to release any dissolved $^{14}CO_2$. Then 0.50 ml of NCS was injected into the center well and the tubes were shaken at low speed for 12 hr. The center wells were removed from the stoppers and placed directly into scintillation vials. Twenty ml of OCS was added to each vial and radioactivity was determined as described previously.

Oxidation of [U-^{14}C] Glucose

Four-day-old cultures were treated with MA or acetaldehyde (AA) at one of two concentrations (10^{-4} M or 10^{-3} M) for 24 hr. After removing the incubation medium, the cells were washed twice with 5 ml of PBS at 37 C. One ml of fresh medium containing 10.17×10^5 dpm [^{14}C]glucose per ml was added to each tube and the uptake and oxidation of [^{14}C]glucose were determined after 12 hr as described for [^{14}C]MA or [^{14}C]acetate, using four replicate cultures.

Extraction of Lipid

The cells were scraped from the glass surface using a wire probe and suspended in 2 ml of distilled water. One ml of cell suspension was transferred to a tube containing 10 ml of chloroform/methanol (2:1, v/v), shaken at maximal speed on a Vortex mixer for 2 min and allowed to stand overnight at 4 C. The chloroform phase was removed with a pipet and the solvent was evaporated under nitrogen at low heat (37-40 C). The cellular lipid was kept at -20 C under nitrogen. The radioactivity in the chloroform/methanol extract of the remaining 1 ml of cell suspension was determined after addition of 15 ml of PCS.

TLC of Cellular Lipids

The cellular lipid from 5 replicate cultures was dissolved in 50 μl of chloroform with 0.5 mg of rat liver lipid as carrier and applied to 0.5 mm layers of Silica Gel G using a 50-μl Hamilton syringe. The plates were developed in a glass chamber lined with filter paper in a solvent system consisting of heptane/isopropyl ether/acetic acid (60:40:3, v/v) (12). The developed plates were dried under nitrogen, sprayed with 2',7-dichlorofluorescein (0.2% in 95% ethanol), and visualized under ultraviolet light. Bands representing the various lipid classes were scraped into scintillation vials, 15 ml of Aquasol was added and the radioactivity was determined by scintillation counting.

Statistical Analysis

The data were analyzed by analysis of variance as outlined by Steel and Torrie (13). Duncan's multiple range test was used to test for the significance ($p<.05$) of differences between treatment means.

TABLE 1

Uptake and Oxidation of [1,3-^{14}C]Malonaldehyde (MA) by Cultured Rat Skin Fibroblasts

Treatment	Incubation time (hr)	Uptake (nmol)	% uptake	Oxidation to $^{14}CO_2$	
				nmol	% of uptake
	6	0.32 ± 0.01	3.2	0.22 ± 0.09	68.2 ± 3.1
10^{-5} M MA	12	0.36 ± 0.02	3.6	0.25 ± 0.02	69.8 ± 0.5
	24	0.44 ± 0.04	4.4	0.39 ± 0.05	88.9 ± 1.1
	6	3.60 ± 0.12	3.6	2.2 ± 0.8	62.5 ± 2.9
10^{-4} M MA	12	3.70 ± 0.54	3.7	2.3 ± 0.3	61.9 ± 1.3
	24	4.30 ± 0.92	4.3	3.9 ± 1.1	89.8 ± 1.2
	6	38.0 ± 3.0	3.8	23.0 ± 2.4	61.5 ± 1.2
10^{-3} M MA	12	37.0 ± 6.0	3.7	24.0 ± 1.0	62.7 ± 2.7
	24	42.0 ± 1.9	4.2	36.0 ± 1.3	83.1 ± 3.0
	6	77.0 ± 3.7	7.7	66.0 ± 2.0	85.1 ± 2.9
10^{-3} M Acetate	12	88.0 ± 3.6	8.3	69.0 ± 4.0	81.6 ± 2.5
	24	245.0 ± 23.8	24.5	147.0 ± 2.8	60.4 ± 3.0

Each value is the mean of 5 observations ± SEM.

TABLE 2

Incorporation of [^{14}C] into the Lipid Fraction of Cells Exposed to [^{14}C]Malonaldehyde or [^{14}C]Acetate

Treatment	Time (hr)	Uptake (nmol)	Incorporation into lipids	
			nmol	% of Uptake
	6	3.6 ± 0.12	0.08 ± 0.10	2.2
10^{-4} M MA	12	3.7 ± 0.54	0.15 ± 0.10	4.0
	24	4.3 ± 0.92	0.25 ± 0.10	5.8
	6	38 ± 3.0	0.85 ± .33	2.2
10^{-3} M MA	12	37 ± 6.0	1.34 ± .23	3.6
	24	42 ± 1.9	2.24 ± .13	5.3
	6	77 ± 3.7	4.34 ± .30	5.6
10^{-3} M Acetate	12	88 ± 3.6	8.21 ± .34	9.3
	24	245 ± 23.8	27.42 ± .50	11.2

Each value is the mean of 5 observations ± SEM.

RESULTS

As shown in Table 1, a linear, concentration-dependent uptake of MA by cells was observed. However, the total uptake of MA was limited (~4% at all concentrations). By comparison, the uptake of 10^{-3} M acetate after 12 hr was 24.5%.

Of the [^{14}C]MA taken up, 83-89% was oxidized to $^{14}CO_2$ by 24 hr. The amount of $^{14}CO_2$ recovered increased with time but the increase was not linear (Table 1). The amount of acetate taken up at 10^{-3} M markedly exceeded the uptake of 10^{-3} M MA but the fraction converted to CO_2 was lower. Radioactivity in the chloroform/methanol extractable fraction increased with time and by 24 hr represented ca. 5 and 10% of the [^{14}C]MA and [^{14}C]acetate taken up, respectively (Table 2). In the MA-treated groups, there was no obvious effect of the concentration of the test compound or duration of exposure on the distribution of radioactivity among different lipid classes (Table 3). However, [^{14}C]MA differed from [^{14}C]acetate in that there were relatively fewer counts in the diacylglycerol (DC) and cholesterol (C) and free fatty acid (FFA) fractions, and more counts in the triacylglycerol (TG) and cholesterol esters (CE) fractions.

The uptake of [^{14}C]glucose was unaffected by 24-hr pretreatment with 10^{-4} M or 10^{-3} M MA or AA (Table 4). However, those cells which were pretreated with 10^{-3} M MA oxidized significantly ($p<0.05$) less glucose to CO_2.

TABLE 3

Percentage Distribution of [^{14}C] among Different Lipid Classes of Cells Exposed to [^{14}C] Malonaldehyde or [^{14}C] Acetate

Treatment	Incubation time (hr)	PL	DG + C	FFA	TG	CE
10^{-4} M MA		60.4 ± 1.5	13.6 ± 0.7	8.9 ± 0.3	10.9 ± 1.0	6.9 ± 0.2
10^{-3} M MA	6	61.0 ± 1.0	12.4 ± 0.7	9.1 ± 0.3	9.6 ± 0.1	7.4 ± 0.3
10^{-3} M Acetate		60.7 ± 0.5	16.1 ± 0.3	11.2 ± 0.3	7.8 ± 0.1	3.8 ± 0.6
10^{-4} M MA		58.2 ± 0.9	13.6 ± 0.7	11.4 ± 0.6	9.1 ± 0.5	7.3 ± 0.4
10^{-3} M MA	12	59.3 ± 0.6	11.9 ± 0.2	12.4 ± 0.2	9.1 ± 0.5	7.5 ± 0.5
10^{-3} M Acetate		57.7 ± 0.3	14.8 ± 0.9	15.1 ± 0.29	8.0 ± 0.4	4.3 ± 0.1
10^{-4} M MA		57.2 ± 1.6	14.9 ± 0.6	7.1 ± 0.7	10.7 ± 0.3	8.5 ± 0.2
10^{-3} M MA	24	61.0 ± 1.6	13.0 ± 0.8	9.8 ± 0.5	9.2 ± 0.5	6.8 ± 0.3
10^{-3} M Acetate		59.0 ± 1.1	15.7 ± 0.2	12.7 ± 0.3	8.2 ± 0.5	4.2 ± 0.6

Each value is the mean±SEM of five observations. PL = phospholipids, DG = diacylglycerol, C = cholesterol, FFA = free fatty acids, TG = triacylglycerol, CE = cholesterol esters.

TABLE 4

Effect of Pretreatment with Malonaldehyde on the [^{14}C] Glucose Oxidizing Capacity of Rat Skin Fibroblasts

Pretreatment	Uptake of glucose (nmol)	Oxidation of glucose (nmol)
10^{-4} M MA	529 ± 7.9	40.6 ± 2.6
10^{-3} M MA	533 ± 6.4	24.6 ± 1.5[a]
10^{-4} M AA	532 ± 7.1	41.1 ± 2.1
10^{-3} M AA	528 ± 11.2	47.3 ± 3.0
Control	526 ± 12.5	39.2 ± 2.0

Each tube contained 5.5 μmol of glucose. Each value is the mean±SEM of 4 observations.
[a]Significantly different from the control value ($p<0.05$).

DISCUSSION

The reasons for the limited cellular uptake of [^{14}C] MA relative to [^{14}C] acetate were not investigated. However, following the incubation of [^{14}C] MA in a culture medium containing 10% serum, a portion of the radioactivity was recovered in the TCA-precipitable fraction. It is probable that TCA precipitation released some [^{14}C] MA from the protein fraction. TCA extraction of foods of animal origin yields higher values for MA than extraction with neutral solutions. MA has been shown to bind with albumin (14) and amino acids (15). It is therefore probable that the limited uptake of MA observed in this study was due in part to its interaction with proteins and other constituents of the medium.

The cells took 24 hr to oxidize ∿90% of the [^{14}C] MA taken up. The longer time required to recover $^{14}CO_2$ derived from [^{14}C] MA than from [^{14}C] acetate could be due to the time required for metabolic conversion of the former to the latter. The plateau in MA or acetate oxidation between 6 and 12 hr of incubation may reflect a metabolically inactive stage of the cell cycle during which there is depressed activity of mitochondrial enzymes of the TCA cycle. Fluctuations in the activity of several mitochondrial enzymes at different stages of the cell division cycle have been reported (16, 17) with maximal activities observed during the synthetic phase.

Increased incorporation of radioactivity into cholesterol esters and the TG fraction of ^{14}C-treated cells was observed at both MA concentrations. A further experiment demonstrated that the incorporation of [^{14}C] acetate into different lipid classes was unaffected by exposure to 10^{-3} M MA (data not shown). Hence,

the difference in incorporation of ^{14}C from MA and acetate is not attributable to MA toxicity. The difference in rates of labeling of the acetate pool from [^{14}C]MA and [^{14}C]acetate or in the position of the labeled carbon entering the pool may have resulted in the shift in the incorporation of [^{14}C]acetate into different lipid fractions.

The reduced capacity of the cells pretreated with 10^{-3} M MA for 24 hr to oxidize glucose indicates a damaging effect of MA on energy yielding reactions. Since MA is known to inactivate enzymes in vitro (18), bind with proteins (14) and amino acids (15) and interact with mitochondria, one or more of these reactions could be involved in decreasing the metabolic capacity of the cells. Acetaldehyde, an inhibitor of DNA, RNA and protein synthesis (7), did not appear to affect the glucose-oxidizing capacity of the cells at any concentration used.

This study demonstrates that MA is absorbed and rapidly oxidized to CO_2 by mammalian cells in culture; however, the cellular uptake of MA is markedly lower than that of acetate. This may explain why MA is tolerated by mammalian cells at relatively high concentrations (7). Despite the fact that MA was inefficiently absorbed by fibroblasts and that 83-89% of the MA taken up was recovered as CO_2 by 24 hr, it is apparent that sufficient MA can accumulate in the cells to exert toxic effects such as inhibition of glucose oxidation and of DNA, RNA and protein synthesis (7).

ACKNOWLEDGMENT

This work was supported by a grant from the U.S. National Cancer Institute and by the Ontario Ministry of Agriculture and Food.

REFERENCES

1. Mukai, F.H., and Goldstein, B.D. (1976) Science 19, 868-869.
2. Shamberger, R.J., Corlett, C.L., Beaman, K.D., and Kasten, B.L. (1979) Mutation Res. 66, 349-355.
3. Marnett, L.J., and Tuttle, M.A. (1980) Cancer Res. 40, 276-282.
4. Yonei, S., and Hidefumi, F. (1981) Mutation Res. 88, 23-32.
5. Yau, T.M. (1979) Mech. Ageing Dev. 11, 137-144.
6. Shamberger, R.J., Andreone, T.L., and Willis, C.E. (1974) J. Natl. Cancer Inst. 53, 171-174.
7. Bird, R.P., and Draper, H.H. (1980) J. Toxicol. Environ. Health 6, 811-823.
8. Horton, A.A., and Packer, L. (1970) J. Gerontol. 25, 199-204.
9. Siu, G.M., and Draper, H.H. (1982) Lipids 17, 349-355.
10. Protopopova, T.V., and Skoldinov, A.P. (1958) J. Gen. Chem. USSR 28, 241-243.
11. Sinnhuber, R.O., and Yu, T.C. (1958) Food Res. 23, 626-633.
12. Holub, B.J. (1974) Biochim. Biophys. Acta 369, 111-122.
13. Steel, R.G.D., and Torrie, J.H. (1960) in Principles and Procedures of Statistics, pp. 194-253, McGraw Hill Book Co. Inc., New York, NY.
14. Crawford, D.L., Yu, T.C., and Sinnhuber, R.O. (1967) J. Food Sci. 32, 332-335.
15. Chio, K.S., and Tappel, A.L. (1969) Biochemistry 8, 2821-2827.
16. Bosmann, H.B. (1971) J. Biol. Chem. 246, 3817-3823.
17. Vendrely, C., Lageron, A., and Tournier, P. (1970) Exp. Cell Res. 62, 249-253.
18. Chio, K.S., and Tappel, A.L. (1969) Biochemistry 8, 2827-2832.

[Received January 27, 1982]

Selective Deposition of *trans*-8- and *cis*-9-Octadecenoates in Egg and Tissue Lipids of the Laying Hen

A.C. LANSER, *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604*

ABSTRACT

The deposition of *trans*-8-octadecenoate-8(9)-^{3}H (8*t*-18:1-^{3}H) was compared to *cis*-9-octadecenoate-10-^{14}C (9*c*-18:1-^{14}C) in the major egg yolk neutral lipids and phospholipids and in organ lipids from the laying hen. *trans*-8-Octadecenoate was preferentially incorporated into only the phosphatidylethanolamines (PE), whereas discrimination against 8*t*-18:1-^{3}H occurred in the phosphatidylcholines (PC), triglycerides (TG) and cholesteryl esters (CE). The 1-acyl position of both PE and PC contained three times more 8*t*-18:1-^{3}H than 9*c*-18:1-^{14}C. Almost total exclusion of the 8*t*-18:1-^{3}H from the 2-acyl position of these phospholipids was found. Preferential incorporation of 9*c*-18:1-^{14}C occurred at the combined 1- and 3-acyl positions and at the 2-acyl position of yolk TG. Tissue lipid analyses indicated that there was preferential deposition of 9*c*-18:1-^{14}C into all organs. Individual liver lipid classes displayed the same relative order of discrimination against 8*t*-18:1-^{3}H as did egg yolk lipids (CE>TG>PC>PE).

Lipids 17:524-528, 1982.

INTRODUCTION

Partial hydrogenation of vegetable oils causes positional and geometrical isomerization of the normal *cis*-unsaturated fatty acids. Analyses of hydrogenated oils show that the largest percentage of isomerized fatty acids is in the octadecenoic acid component. Double bond positions range from Δ5 to Δ15 in fatty acids with the *cis* configuration, and from Δ4 to Δ16 in the *trans* fraction (1). *trans* Fatty acids can comprise more than one-third of the total acids in hydrogenated products (2).

Our knowledge of the metabolic fate of these isomerized fatty acids is limited and comes mostly from feeding studies of hydrogenated fats that contain the whole spectrum of isomers in which isomer enrichment is examined. At this laboratory, the metabolism of individual fatty acid isomers has been studied using a dual-isotope-labeling technique in which the ^{3}H-labeled object isomer is fed together with a ^{14}C-labeled "internal standard" fatty acid to laying hens. The egg from the laying hen serves as a biological trap. Lipid deposited into the egg yolk provides an automatic daily biopsy of ingested fats. The metabolism of *cis* octadecenoates (Δ8 to Δ12) and of *trans*-9- and *trans*-12-octadecenoates has been compared to that of *cis*-9-octadecenoate in the laying hen using this technique (3-5).

The *trans*-8-octadecenoate (8*t*-18:1) content in some soybean salad oils and margarines ranges from zero to 4.0% (wt) of the total fatty acids (6,7). Since this corresponds to up to 22% of the *trans*-octadecenoates in these products, 8*t*-18:1 is one of the major isomeric fatty acids. Isomer enrichment studies with rats fed partially hydrogenated vegetable oils (8-11) indicate that the 8*t*-18:1 content in tissue lipids varies with the tissue, lipid class and lipid acyl position examined.

This study uses the dual labeling technique to determine differences in the metabolism of *trans*-8- and *cis*-9-octadecenoates in the laying hen by examining their deposition into the major egg yolk lipids. Lipids from various organs were also examined for radioisotope content 6 hr after administration of labeled esters.

MATERIALS AND METHODS

Radioactive Esters

Methyl oleate-10-^{14}C was synthesized via a Wittig coupling of nonyl-1-^{14}C-triphenylphosphonium iodide and methyl 8-formyloctanoate following the general procedure of Adlof and Emken (12). Nonanoic-1-^{14}C acid was prepared by $^{14}CO_2$ addition to octyl bromide. The acid was converted to the alcohol and then to the iodide before preparing the triphenylphosphonium salt. *cis*-9-Octadecenoate-10-^{14}C (1.02 g, 25 mol %; 5.71 mCi) was purified by silver resin chromatography (13) (Rohm and Haas, XN1010 sulfonic acid resin; 80-100 wet mesh, saturated with $AgNO_3$; 2 cm x 93 cm; elution with methanol).

Previously prepared *cis*-8-octadecenoate-8(9)-^{3}H (3) was isomerized to the *trans*-8-isomer with *p*-toluene sulfinic acid catalysis (14). The resulting mixture of Δ8-octadecenoate isomers was separated by silver resin column chromatography as just described.

Purity of both the *cis*-9-octadecenoate-10-^{14}C (9*c*-18:1-^{14}C) and *trans*-8-octadecenoate-

8(9)-^{3}H (8*t*-18:1-^{3}H) was 98% by gas chromatography (GC; Aerograph gas chromatograph; 8 ft x ¼ in. aluminum column packed with 15% EGSS-X on 100/120 mesh Gas Chrom P; 190 C; helium flow was 58 ml/min). The double bond position was confirmed by ozonolysis (15). Radiochromatogram scans (Packard 7201) of the *cis*- and *trans*-octadecenoates after argentation-TLC using a modified solvent system of Von de Vries and Jurriens (16) (benzene/petroleum ether/acetic acid, 8:2:0.01) showed radiochemical purities to be 99%. Gas liquid chromatography followed by liquid scintillation counting of the serially collected GC column effluent (17) confirmed these purities.

Experimental Design

Mixtures of fatty acid methyl esters containing 202 μCi 8*t*-18:1-^{3}H (S.A. 10.4 mCi/mmol) and 212 μCi 9*c*-18:1-^{14}C (S.A. 1.7 mCi/mmol) (^{3}H/^{14}C = 0.95) were prepared and placed in gelatin capsules for administration as a single dose to each of three laying hens. The dual isotope experimental design (3,18) used in this study allows the metabolism of the labeled fatty acids to be compared without confusion with endogenous or other exogenous fatty acids (18) by noting differences in ^{3}H/^{14}C ratios from that fed.

Hens (DeKalb XL; 31-wk-old) were fed ad libitum a standard laying ration (Purina CF 6501, Ralston Purina, St. Louis, MO), which contained 2.6% hexane extractable fat. Fatty acid composition of the feed was 14:0, 0.6%; 16:0, 17.2%; 16:1, 0.8%; 18:0, 5.7%; 18:1, 27.9%; 18:2, 44.5%; 18:3, 2.9%. No detectable *trans* acids (<0.1%) were found.

Methods used for administration of radioactive methyl esters, for collection of eggs after feeding and for lipid extraction have been described previously (19). Maximal incorporation of radioactive labels into yolk lipids occurred in the fourth and fifth eggs laid after administration of the isomers. Lipid analyses were performed on individual egg yolks collected from each hen on those days. Yolks from the third eggs from each hen were combined into one sample, as were those from the sixth eggs. Neutral lipid components (cholesteryl esters and triglycerides) were isolated by preparative thin layer chromatography (prep-TLC) using benzene/petroleum ether/ethyl ether/acetic acid (8:2:1:0.25); major phospholipids (phosphatidylethanolamines and phosphatidylcholines) were isolated with chloroform/methanol/petroleum ether/water (8:8:6:1) (20).

Another mixture of 8*t*-18:1-^{3}H (423 μCi) and 9*c*-18:1-^{14}C (489 μCi) (^{3}H/^{14}C = 0.87) was prepared and administered in a gelatin capsule to one hen. The hen was killed by decapitation after 6 hr. Organs were quickly removed and frozen in ethanol/dry ice, then stored in ethanol at -30 C overnight. Organs/tissue taken for examination included blood, crop, gizzard, intestine, liver, heart and adipose tissue. Organ contents were analyzed with the organ. Tissue samples were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) and lipids were extracted using the method of Hara and Radin (21).

Enzymatic hydrolysis of egg yolk triglycerides was accomplished with pancreatic lipase (Calbiochem, San Diego, CA) (22) to yield free fatty acids from the 1- and 3-positions and a 2-monoglyceride. Hydrolysis products were separated by prep-TLC using ethyl ether/petroleum ether/acetic acid (1:1:0.02). Phospholipase A_2 (*O. hannah*, Ross Allen Reptile Inst., Inc., Silver Springs, FL) was used for fatty acid positional analysis of phospholipids (23). Products were isolated by prep-TLC using the phospholipid solvent system just described. Hydrolysis products were converted to methyl esters with HCl-methanol prior to liquid scintillation counting.

Radiochemical assays were performed using a Beckman three-channel LS-250 liquid scintillation counter with previously described parameters (19). Output from the counter was interfaced to a Mod-Comp computer (Modular Computer System, Inc., Ft. Lauderdale, FL) programmed to calculate disintegrations per minute (dpm) and ^{3}H/^{14}C ratios. All dpm were corrected by external standard ratios and previously established quench correction curves. Specific activities were determined by counting aliquots of weighed fractions after converting fatty acids to methyl esters. Selectivity values were calculated as the logarithm of the quotient of the ^{3}H/^{14}C ratio found in the yolk lipids divided by the ^{3}H/^{14}C ratio in the fed mixture.

RESULTS AND DISCUSSION

Egg Yolk Lipids

Selectivity values (Table 1) were negative in the phosphatidylcholines (PC), triglycerides (TG) and cholesteryl esters (CE), indicating that more 9*c*-18:1-^{14}C than 8*t*-18:1-^{3}H was incorporated into these major yolk lipid components. When compared to 9*c*-18:1-^{14}C, the 8*t*-18:1-^{3}H was preferentially incorporated into only the phosphatidylethanolamines (PE).

Cholesteryl esters. The CE fraction displayed a large negative selectivity, indicating a strong preference for the 9*c*-18:1-^{14}C over the 8*t*-18:1-

TABLE 1

Radiochemical Analysis of Egg Yolk Lipids after Feeding 8t-18:1-^{3}H and 9c-18:1-^{14}C (^{3}H/^{14}C = 0.95)

Lipid component[a]	Lipid acyl positions	Specific activity[b] ^{3}H	Specific activity[b] ^{14}C	^{3}H/^{14}C in yolk lipids	Selectivity
CE		342	1406	0.24 ± 0.02(7)[c]	-0.60
TG		5206	8474	0.67 ± 0.01(7)	-0.15
	TG 1+3	6223	8452	0.75 ± 0.02(7)	-0.10
	TG 2	2211	7710	0.29 ± 0.01(7)	-0.51
PE		3941	2807	1.23 ± 0.04(8)	0.11
	PE 1	8628	2601	2.94 ± 0.09(7)	0.49
	PE 2	312	4503	0.03 ± 0.01(7)	-1.50
PC		4342	5659	0.73 ± 0.04(8)	-0.11
	PC 1	8201	2806	3.08 ± 0.18(6)	0.51
	PC 2	401	9348	0.03 ± 0.01(6)	-1.50

[a]Abbreviations: CE = cholesteryl esters; TG = triglycerides, PE = phosphatidylethanolamines; PC = phosphatidylcholines.

[b]^{3}H or ^{14}C dpm/mg methyl oleate.

[c]Average ± standard error determined on egg yolk samples from three hens. The number of samples analyzed is given in parentheses and includes at least one egg yolk from each hen collected during peak incorporation of radioactivity.

^{3}H isomer for esterification to cholesterol. This result is not unexpected since *cis* acids have been shown to selectively esterify cholesterol over *trans* acids. Acyl CoA:cholesterol-*O*-acyltransferase from rat liver microsomes esterified 9*c*-18:1 to cholesterol almost three times faster than 9*t*-18:1 (24). When Sgoutas et al. (25) incubated 9*t*-18:1-^{3}H and 9*c*-18:1-^{14}C with rat liver microsomes, the ^{3}H/^{14}C ratio of 3.92 in the incubated mixture dropped to 1.22 in the recovered CE. Human plasma CE gave large negative selectivities for 9*t*- and 12*t*-18:1 when compared to 9*c*-18:1 (26,27).

Esterification of cholesterol by lecithin:cholesterol acyltransferase would also contribute to the large negative selectivity of CE, since the 2-acyl position of PC contains practically no 8*t*-18:1-^{3}H as shown later.

Triglycerides. TG were the only lipid class to show negative selectivities in the total fraction and at each of the acyl positions analyzed (TG 1+3 and TG 2), which indicates incorporation of more 9*c*-18:1-^{14}C than 8*t*-18:1-^{3}H at each position (Table 1). Bickerstaffe and Annison (28) have shown that 9*c*-18:1 is incorporated into triglycerides to a greater extent than 9*t*- or 11*t*-18:1 in perfused chicken liver. Human plasma triglycerides displayed this same selectivity for 9*c*-18:1 over 12*t*-18:1 (27). Incorporation of more 9*c*-18:1-^{14}C than 8*t*-18:1-^{3}H into yolk TG may result from a real enzymatic selectivity for 9*c*-18:1 during TG synthesis or from a reduced concentration of 8*t*-18:1-^{3}H in fatty acid pools because of the selectivity shown for this isomer for deposition into yolk phospholipids.

Incorporation of *cis* fatty acids at the 2-position of yolk triglycerides is more selective than at the combined 1- and 3-positions. These data agree with previous acyl positional analyses of triglycerides which show that *trans* octadecenoates are preferentially esterified at the 1- and 3-positions and discriminated against at the 2-position (28-30).

Phospholipids. The PE fraction had a positive selectivity for the 8*t*-18:1-^{3}H isomer in total PE and a more positive selectivity at the 1-acyl position. Almost total exclusion of the *trans* isomer at the 2-position was indicated. PC displayed the same exclusion of 8*t*-18:1-^{3}H at the 2-position and the same selectivity for 8*t*-18:1-^{3}H at the 1-position as did PE, but had an overall negative selectivity value. These data are consistent with phospholipid data for 9*t*- and 11*t*-18:1 in perfused chicken liver (28).

Specific activities (dpm/mg methyl oleate) are nearly identical for PE-1 and PC-1. One possible explanation for this is that these fatty acids may come from the same pool or compartment during lipid synthesis (31). The specific activity at PC-2 is twice that at PE-2. Examination of specific activities as ^{3}H dpm/mg and ^{14}C dpm/mg at each acyl position (Table 1) reveals only small differences in incorporation of

TABLE 2

Radiochemical Analysis of Tissue Lipids after Feeding 8*t*-18:1-^{3}H and 9*c*-18:1-^{14}C (^{3}H/^{14}C = 0.87)

Tissue	Radioactivity in tissue		Specific activity[a]	Selectivity
	^{3}H/^{14}C	^{3}H + ^{14}C (μCi)		
Crop	0.88	0.9	7769	0.0
Gizzard	0.84	14.0	17416	-0.01
Intestine	0.68	32.6	17019	-0.11
Blood	0.64	0.7	87134	-0.13
Liver	0.56	18.0	41597	-0.19
Adipose	0.54	3.1	1273	-0.21
Heart	0.59	0.2	1875	-0.17
Egg yolk	0.72		9879	-0.12

[a]Total ^{3}H + ^{14}C dpm/mg methyl oleate.

8*t*-18:1-^{3}H into PE and PC and of 9*c*-18:1-^{14}C into PE-1 and PC-1. The difference in selectivity values of PE and PC lies in the greater incorporation of 9*c*-18:1-^{14}C into the 2-position of PC. Yolk PE contains more long-chain polyunsaturated fatty acids than PC. These acids are preferentially esterified at PE-2, thus reducing the 9*c*-18:1-^{14}C concentration at this position and lowering the specific activity.

Hexadecenoates. Radio-gas chromatograms (17) of yolk lipid methyl esters after feeding 8*t*-18:1-^{3}H and 9*c*-18:1-^{14}C to the hens indicated that up to 30% of the ^{3}H-label was associated with methyl hexadecenoate (presumably 6*t*-16:1 via a single β-oxidation of the *trans* isomer). Only the 8*t*-18:1-^{3}H was chain shortened to a large extent. PE, PC and TG contained 7, 24 and 32%, respectively, of the ^{3}H label found in these lipid classes in the hexadecenoate ester. These data are consistent with those of Wood (32), which reported Δ7 to Δ14 positional *trans*-hexadecenoate isomers in tissue triglycerides of rats fed a diet containing 15% partially hydrogenated safflower fatty acids. No 6*t*-16:1 was reported. From 5 to 7% of the ^{14}C-label was found in methyl hexadecanoate; no radioactivity was detected in any shorter or longer chain fatty acids.

In previous monoene feeding studies with the laying hen (4 and unpublished data), 90% or more of the recovered isotopic label (when within the acyl chain) remained associated with the fatty acid moiety fed. Only small percentages of the radioactivity were seen in products assumed to be derived from chain-shortening or biosynthesis using acetate fragments from fatty acid degradations.

No explanation for the amount of 16:1 produced by chain-shortening or for the cessation of oxidative cleavage after loss of two carbon atoms is known.

Tissue Lipids

Specific activities determined on the total lipid extract from various tissues 6 hr after feeding (Table 2) indicate the labeled esters had entered the intestinal tract and were partially absorbed and distributed into various tissues. Approximately 8% of the labeled esters was recovered in the organs. Selectivity values determined on the crop and gizzard lipids were zero and provide evidence that no discrimination for either 8*t*- or 9*c*-18:1 occurred during transport to the intestine. Different absorption rates for these esters (8*t*-18:1-^{3}H>9*c*-18:1-^{14}C) are indicated because intestinal content contained more ^{14}C-labeled than ^{3}H-labeled ester. Selectivity values from other tissues analyzed were all negative and ranged from -0.11 to -0.21. Since we could find no site of preferential deposition of 8*t*-18:1-^{3}H, it appears that 8*t*-18:1-^{3}H is catabolized at a faster rate than 9*c*-18:1-^{14}C.

The difference in selectivity values for adipose and egg yolk lipids (Table 2) is consistent with the suggestion that different mechanisms control deposition of fatty acids in hen adipose tissue and egg yolk (33). Gornall and Kuksis (34) proposed that specific lipoproteins are synthesized by the hen's liver, secreted into the plasma, and selectively deposited in the yolk. Nearly identical selectivity values from blood and yolk lipid analyses support the theory that the yolk is a depository for plasma lipids. In contrast, selectivity values for adipose and heart lipids suggest either that these organs exert similar discriminations on these fatty acids during uptake or that discriminations which occur in the liver are only slightly modified during fatty acid uptake by the organs.

Analyses of individual liver lipid classes show the same relative order of selectivity values as

those determined in the egg yolk (CE<TG<PC<PE). Liver CE displays marked exclusion of 8*t*-18:1-^{3}H (selectivity = -0.86). Liver TG and PC discriminate against 8*t*-18:1-^{3}H to a lesser extent (-0.28 and -0.14, respectively), and PE contains slightly more 8*t*-18:1-^{3}H compared to 9*c*-18:1-^{14}C than was fed (0.01).

These data are consistent with liver CE and TG data from rats fed partially hydrogenated soybean oil (9). Rat liver PC, however, showed an increase in 8*t*-18:1 compared to 9*c*-18:1. This relative increase in 8*t*-18:1 was apparently due to preferential incorporation of dietary Δ11 to Δ14 positional *trans*-octadecenoate isomers at the expense of 9*c*-18:1.

ACKNOWLEDGMENTS

The author thanks Dr. DeJarlais and Mr. Adlof for technical advice and synthetic intermediates.

REFERENCES

1. Elson, C.E., Benevenga, N.J., Canty, D.J., Grummer, R.H., Johnston, A.E., Lalich, J.J., Porter, J.R., Rapacz, J., and Shrago, E.S. (1977) in Food Research Institute Annual Report–1976, University of Wisconsin, Madison, WI, pp. 401-414.
2. Sgoutas, D., and Kummerow, F.A. (1970) Am. J. Clin. Nutr. 23, 1111-1119.
3. Mounts, T.L. (1976) Lipids 11, 676-679.
4. Mounts, T.L., Emken, E.A., Rohwedder, W.K., and Dutton, H.J. (1971) Lipids 6, 912-918.
5. Lanser, A.C., and Emken, E.A. (1981) Lipids 16, 15-19.
6. Carpenter, D.L., and Slover, H.T. (1973) J. Am. Oil Chem. Soc. 50, 372-376.
7. Carpenter, D.L., Lehmann, J., Mason, B.S., and Slover, H.T. (1976) J. Am. Oil Chem. Soc. 53, 713-718.
8. Reichwald-Hacker, I., Ilsemann, K., and Mukherjee, K.D. (1979) J. Nutr. 109, 1051-1056.
9. Reichwald-Hacker, I., Grosse-Oetringhaus, S., Kiewitt, I., and Mukherjee, K.D. (1979) Biochim. Biophys. Acta 575, 327-334.
10. Wood, R. (1979) Lipids 14, 975-982.
11. Høy, C.-E., and Hølmer, G. (1979) Lipids 14, 727-733.
12. Adlof, R.O., and Emken, E.A. (1978) Labeled Compd. Radiopharm. 15, 97-104.
13. Emken, E.A., Scholfield, C.R., and Dutton, H.J. (1964) J. Am. Oil Chem. Soc. 41, 388-390.
14. Gibson, T.W., and Strassburger, P. (1976) J. Org. Chem. 41, 791-793.
15. Wood, R., Falch, J., and Wiegand, R.D. (1974) Lipids 9, 987-992.
16. Von de Vries, B., and Jurriens, G. (1963) Fette Seifen Anstrichm. 65, 725-727.
17. Thomas, P.J., and Dutton, H.J. (1969) Anal. Chem. 41, 657-660.
18. Ono, K., and Fredrickson, D.S. (1964) J. Biol. Chem. 239, 2482-2488.
19. Mounts, T.L., and Dutton, H.J. (1976) Biochim. Biophys. Acta 431, 9-15.
20. Peter, H.W., and Wolf, H.U. (1973) J. Chromatogr. 82, 15-30.
21. Hara, A., and Radin, N. (1978) Anal. Biochem. 90, 420-426.
22. Mattson, F.H., and Volpenheim, R.A. (1961) J. Lipid Res. 2, 58-62.
23. Robertson, A.F., and Lands, W.E.M. (1962) Biochemistry 1, 804-810.
24. Sgoutas, D.S. (1970) Biochemistry 9, 1826-1833.
25. Sgoutas, D., Jones, R., Befanis, P. and Szlam, F. (1976) Biochim. Biophys. Acta 441, 14-24.
26. Emken, E.A., Rohwedder, W.K., Dutton, H.J., Dougherty, R., Iacono, J.M., and Mackin, J. (1976) Lipids 11, 135-142.
27. Emken, E.A., Dutton, H.J., Rohwedder, W.K., Rakoff, H., Adlof, R.O., Gulley, R.M., and Canary, J.J. (1980) Lipids 15, 864-871.
28. Bickerstaffe, R., and Annison, E.F. (1970) Biochem. J. 118, 433-442.
29. Duncan, W.R.H., and Garton, G.A. (1967) J. Sci. Food Agric. 18, 99-102.
30. Duncan, W.R.H., and Garton, G.A. (1969) J. Sci. Food Agric. 20, 39-42.
31. Wood, R., and Chumbler, F. (1978) Lipids 13, 75-84.
32. Wood, R. (1979) in Geometrical and Positional Fatty Acid Isomers, (Emken, E.A., and Dutton, H.J., eds.) American Oil Chemists' Society, Champaign, IL. pp. 213-281.
33. Sell, J.L., Choo, S.H. and Kondra, P.A. (1968) Poult. Sci. 47, 1296-1302.
34. Gornall, D.A., and Kuksis, A. (1973) J. Lipid Res. 14, 197-205.

[Received November 23, 1981]

Uptake and Metabolism of Free Fatty Acids by the Morris 7777 Hepatoma and Host Rat Liver

RICHARD E. MORTON[a,1], **MOSELEY WAITE**[a], **V. LYNN KING**[a] and **HAROLD P. MORRIS**[b], [a]*Department of Biochemistry, Bowman Gray School of Medicine, Winston Salem, NC 27103*, and [b]*Department of Biochemistry, Howard University College of Medicine, Washington, DC 20059*

ABSTRACT

The relative capacity of Morris 7777 hepatomas and livers of tumor-bearing rats to take up and subsequently metabolize intravenously injected radiolabeled free fatty acids was investigated. The objective was to determine differences in lipid metabolism which may affect the lipid composition previously observed in this tumor. Both tissues demonstrated comparable selectivity in the uptake of palmitate, linoleate and arachidonate from blood, although the hepatoma took up one-tenth as much free fatty acid per g wet wt as liver. A much greater percentage of fatty acid taken up by the hepatoma was converted to aqueous soluble radioactivity, perhaps the result of oxidation. In the hepatoma, palmitate was incorporated into phospholipid molecular species in a pattern similar to that observed for diglyceride, which suggested that phospholipid synthesis occurred predominantly de novo. On the other hand, in liver, a large percentage of palmitate was incorporated into polyunsaturated phospholipid molecular species that were not present in the diglyceride pool, which suggested significant incorporation by the acylation of monoacyl phosphoglycerides. These studies indicate that the specificity for the uptake of fatty acids was not different in the two tissues; however, the subsequent metabolic processes are markedly different.
Lipids 17:529-537, 1982.

INTRODUCTION

When compared to that of normal tissue, the fatty acid composition of lipids isolated from various tumors has been shown to be significantly altered, hallmarked by an increased monoene and decreased polyene content (1-3). It is reasonable to suggest that this abnormal lipid composition may result from alterations in one or more of the pathways involved in the uptake and intracellular metabolism of fatty acids. In recent studies with an experimental, transplantable hepatoma, we have identified significant changes in the activities of enzymes involved in the synthesis of polyunsaturated fatty acids and in the formation of cellular phospholipids (3,4). Similar findings by other laboratories studying different tumors (5-7) suggest that these abnormal enzymatic activities may play a key role in inducing the altered fatty acid composition observed in many neoplasms.

In this study, we have further defined the pathways of fatty acid metabolism in tumor cells by monitoring the in vivo uptake and subsequent metabolic fate of free fatty acids from circulation. Although these processes have been studied in considerable detail in the Ehrlich ascites tumor (8), the absence of a suitable normal or control tissue in these studies prevented the comparison of noncancerous and cancerous tissues. Therefore, it was desirable for our purpose to study a tumor tissue of well-defined origin. Using the Morris 7777 hepatocarcinoma, we have contrasted the uptake and short-term metabolic fates of isotopic palmitate, linoleate and arachidonate in the liver and hepatoma of the same animal.

EXPERIMENTAL PROCEDURES

Materials

BSA was purchased from Sigma Chemical Co., St. Louis, MO [1-^{14}C]Linoleic acid (60 mCi/mmol) and [1-^{14}C]palmitic acid (56 mCi/mmol) were obtained from Amersham Searle, Arlington Heights, IL. [9,10-^{3}H(n)]Palmitic acid (23.5 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H(n)]arachidonic acid (98.5 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Silica Gels G and H were purchased from Merck, Darmstadt, Germany. Ready-Solv GP (Beckman Instruments, Inc., Atlanta, GA) was used for double-label counting on silica gel impregnated with silver nitrate.

Abbreviations: BSA, bovine serum albumin; BHT, butylated hydroxytoluene; TLC, thin layer chromatography; GLC, gasliquid chromatography; PC, diacyl glycerophosphatidylcholine; PE, diacyl glycerophosphatidylethanolamine; PS, diacyl glycerophosphatidylserine; PI, diacyl glycerophosphatidylinositol; TG, triacylglycerol(s); DG, diacylglycerol(s); PL, phospholipid(s); x:y, fatty acids where x is the hydrocarbon chain length and y is the number of double bonds.

[1]Current address: Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853.

Methods

Animal maintenance and diet. Buffalo strain male rats (2.5-3 months old) were used throughout the experiments. The Morris 7777 hepatocarcinoma (generations 163-170) was bilaterally transplanted subcutaneously into the hind leg region of host animals. All animals were maintained on Purina rat chow ad libitum and were used for experiments 18-20 days after tumor implantation. Animals bearing 18-20 day transplants (tumors 3-13 g in wt) were eating well and maintaining body weight. This is in contrast to the poor health status of host animals 24-26 days postimplantation. Sixteen to 20 hr prior to an experiment, animals were placed on a liquid diet containing 10% glucose and 0.45% NaCl ad libitum (9).

Radiolabel injections and tissue isolation. [^{3}H]16:0 (21.4 x 10^6 cpm) and [^{14}C]18:2 (5.5 x 10^6 cpm) or [^{14}C]16:0 (4.5 x 10^6 cpm) and [^{3}H]20:4 (9.7 x 10^6 cpm) dissolved in 0.3 ml of 0.9% NaCl containing 5% BSA were injected into the exposed jugular vein of rats that were anesthetized with diethyl ether. The initial specific activities in the blood were calculated (based on free fatty acid composition of serum and an assumed whole blood volume of 8% of body wt) to be: [^{3}H]16:0, 2,830 cpm/nmol; [^{14}C]18:2, 1,150 cpm/nmol; [^{3}H]20:4, 38,500 cpm/nmol. At various times after injection, the livers and tumors were quickly removed from the animals and placed in cold water (0-2 C). Approximately 45-60 sec elapsed between the time that liver and tumor tissues were removed. The results for the tumor were not influenced appreciably by the order in which the two tistues were removed; therefore, the liver was routinely removed first. Although these tissues were not perfused, it was calculated that, based on the reported blood content of these tissues (10,11), contaminating blood accounted for <5% of tissue radioactivity 1 min after the injection and <2% after 2 min.

The tumors were rapidly dissected in the cold to remove necrotic and connective tissues; then both tumor and liver tissues were blotted dry and placed in 10 ml and 20 ml of dry-ice-cooled methanol, respectively. The tissues were minced and then homogenized in a Waring blender after the addition of chloroform (containing 25 mg/ℓ BHT) and water to yield a final chloroform/methanol/H_2O (+tissue) ratio of 1:2:1, v/v. The lipids were extracted from the homogenate by adding 2 vol of chloroform and 1 vol of water. The extraction was repeated by adding 3 vol of chloroform to the methanol/water phase. All radioactivity remaining in the aqueous methanol phase is subsequently referred to as "nonlipid" radioactivity, which presumably reflects β-oxidation products of the labeled fatty acids.

Lipid fractionation. The chloroform extracts from all tissues were separated into total PL and individual neutral lipid classes by TLC (12). The total PL fraction was eluted from the silica gel and further resolved by TLC in a developing system of chloroform/methanol/acetic acid/water (50:32:8:3, v/v). The radioactivity of each isolated lipid fraction was determined by liquid scintillation counting (12). When necessary, PL fractions were extracted from silica gel by washing the gel three times with 4-ml aliquots of chloroform/methanol/water (1:2:0.0, v/v). Neutral lipids were eluted in a similar fashion using chloroform/methanol/diethyl ether (1:1:1, v/v).

The total PL fraction was hydrolyzed with phospholipase C isolated from *Bacillus cereus* using the procedure of Burstein et al. (13). Molecular species of the resulting diglycerides, and the DG fraction originally isolated by TLC, were separated by chromatography on Silica Gel G plates impregnated with 4% $AgNO_3$ in a developing system of chloroform/absolute ethanol (94:6, v/v). The desaturation of [^{14}C]16:0 was analyzed by separating methyl esters prepared from total lipid extracts (described later) on Silica Gel H plates containing 4% $AgNO_3$ in a developing system of petroleum ether/diethyl ether/absolute ethanol (95:5:2, v/v). The radioactivity of compounds separated on $AgNO_3$-containing gels was determined by scintillation counting in glass vials containing 10 ml Ready-Solv GP, 0.5 ml 20% NaCl, and 2 drops of 2 M H_2SO_4.

Chemical analysis. Isolated free fatty acids were methylated by the method of Rogozinski (14) and the resulting methyl esters were separated and quantitated by GLC (3). Glyceride determinations were performed according to the method of Sardesai and Manning (15). The method of Chalvardjian and Rudnicki (16) was used to quantitate total lipid phosphorus. Protein was quantitated according to the method of Lowry et al. (17).

RESULTS

Chemical Analysis

In these studies were compared the livers of control animals with those from the tumor-bearing animals. In general, very few differences were found; those are noted in the appropriate sections. The cellular pools of free fatty acid in liver and hepatoma were measured to determine the relative dilution of the injected radiolabeled

TABLE 1

Free Fatty Acid Composition of Host Liver and the Morris 7777 Hepatoma[a]

Tissue	Fatty acid							
	16:0	16:1	18:0	18:1	18:2	18:3	20:4	Total
Host liver[b]	4.71 ± 0.25	0.61 ± 0.13	0.66 ± 0.08	1.50 ± 0.36	0.86 ± 0.22	ND	0.16 ± 0.13	8.50
Hepatoma[b]	3.81 ± 0.35	1.05 ± 0.12	3.82 ± 0.37	3.91 ± 0.64	1.98 ± 0.14	0.27 ± 0.09	1.49 ± 0.09	16.33

[a]Free fatty acids were isolated by TLC, methylated, and then quantitated by GLC. Values are mean ± SE of single determinations on tissues isolated from 4 host animals. ND = none detected, i.e., <1% of total; fatty acid designations are chain length:number of double bonds.

[b]Values are nmol fatty acid/mg protein.

fatty acid. As shown in Table 1, the hepatoma contained about twice as much total free fatty acid as liver when related to the protein content of the tissue. This was true for all fatty acids with the exception of palmitate, which was slightly higher in the host liver. By comparison, control livers had about the same amount of free fatty acid as the host liver, 6.04 nmol/mg protein. When the total free fatty acid concentration was expressed relative to the wet wt of tissue (217 and 161 mg protein/g wet wt for liver and hepatoma, respectively), the concentration of free fatty acid in the tumor was about 40% higher than that in liver.

Fatty Acid Uptake

In initial studies, we observed significant variation in the amount of radioactivity taken up per g of nonnecrotic tumor tissue. An analysis of these data indicated that the radioactivity taken up per g of tumor tissue was inversely related to the tumor size (Fig. 1), with values ran-

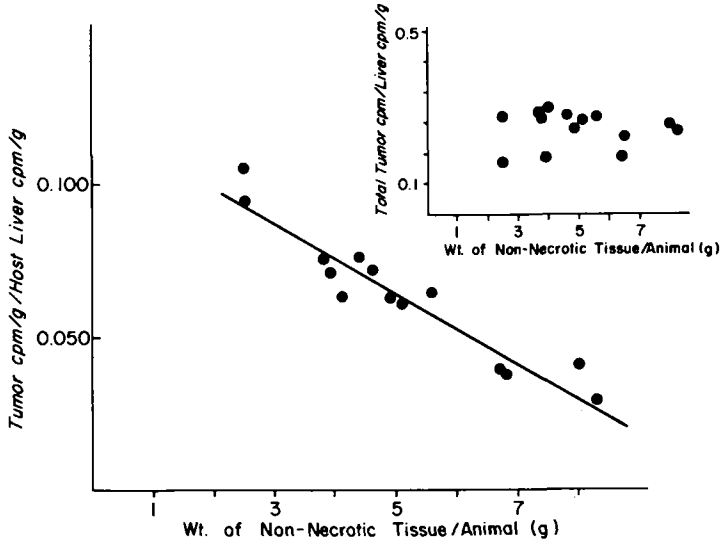


FIG. 1. Uptake of [^{3}H]16:0/g tumor tissue (nonnecrotic) vs weight of nonnectotic tumor tissue. The values, from individual animals sacrificed 0.5-2 min after isotope injection, are plotted relative to the uptake into host liver so that small variations in the injected radioactivity will not affect the results. The line, determined by linear regression analysis, had a correlation coefficient of 0.94. Inset–values were similarly calculated but are expressed as the cpm taken up into the total tumor tissue (nonnecrotic) of an animal vs weight of nonnecrotic tissue/animal. The nonnecrotic portion of the tumor contained ~70% of the label taken up by the whole tumor. On a wet weight basis, the nonnecrotic tissue took up 5-fold more label than the necrotic regions.

ging from one-tenth to one-twentieth of the uptake observed per g of liver. Over the size range studied, the uptake of radiolabeled free fatty acids into the total tumor was rather constant, indicating that the uptake process occurs independently of changes in tumor size (see Fig. 1, inset). Consequently, we intentionally kept tumor size relatively constant within a given experiment.

The uptake of coinjected [^{3}H]16:0 and [^{14}]18:2 is shown in Figure 2A. Approximately 85-90% of the labeled fatty acid taken up by the liver and tumor was present in the tissues within 0.5 min. This corresponded well with the rapid removal of radioactivity from the circulation (12.5% remained at 1 min). In part due to its larger mass, the liver took up 15 times more of the two fatty acids than did the tumor tissue. The uptake of [^{3}H]16:0 and [^{14}C]18:2 per g of liver tissue was 9-fold higher than the uptake by hepatoma. Initially, the isotopic ratio of [^{14}C]/[^{3}H] was 0.296 in both liver and tumor (injected ratio = 0.257). This deviation of the isotopic ratio from the injected ratio indicates that the uptake process was not solely dependent on the relative concentrations of the free fatty acid in plasma. In this instance, both tissues demonstrated a slight preferential uptake of [^{14}C]18:2 relative to its concentration in plasma. The isotopic ratio in the liver varied little over the 6-min study period, whereas the ratio in the hepatoma decreased to 0.221 due to a decrease in the total amount of [^{14}C] (Fig. 2A).

[^{14}C]Palmitate was then coinjected with [^{3}H]arachidonate (Fig. 2B). Again, the initial isotopic ratios of [^{14}C]/[^{3}H] were comparable for both tissues (0.354 vs 0.378) and were lower than that of the ratio of the injected fatty acid, 0.464. The isotopic ratio in the liver increased slightly at 5 min, in contrast to the sharp decrease in the tumor isotopic ratio to 0.238. The decrease in the [^{14}C]/[^{3}H] ratio of the hepatoma appeared to be in part due to a decrease in the amount of [^{14}C]. The pattern of [^{14}C]16:0 uptake in these studies was similar to that seen with [^{3}H]16:0 (Fig. 2A). The uptake by whole liver was 13 times the uptake

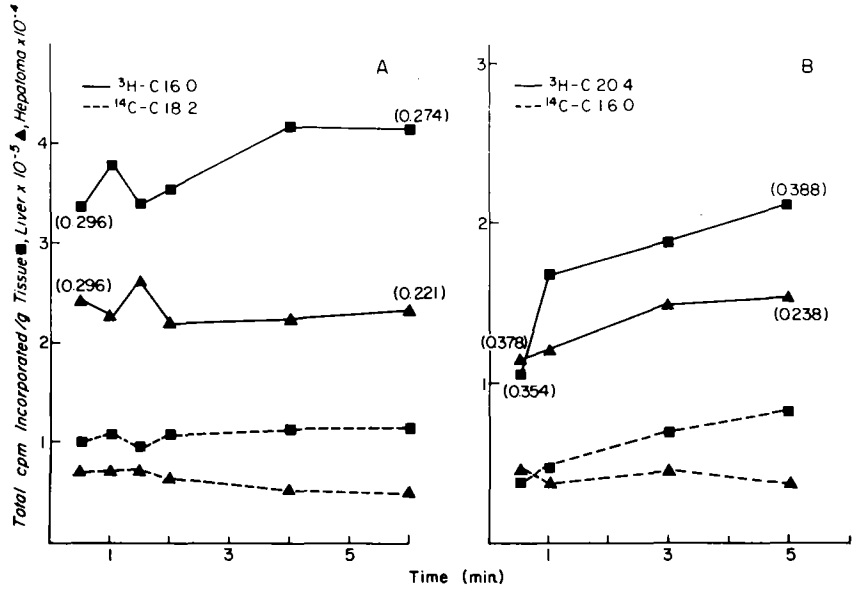


FIG. 2. The uptake of radiolabeled fatty acid into liver and hepatoma. Values at each time point are expressed as the total cpm taken up/g of tissue (wet wt) and are the mean of single determinations on 3-4 separate experimental animals. A–uptake of [^{3}H]16:0 (——) and [^{14}C]18:2 (-----); B–uptake of [^{3}H]20:4 (-----) and [^{14}C]16:0 (——). Values in parentheses are the [^{14}C]/[^{3}H] ratios of each tissue at the time point indicated. The injected [^{14}C]/[^{3}H] ratio was 0.257 for panel A, and 0.464 for panel B studies. The average tumor weight was 4.7 g in panel A and 6.6 g in panel B studies (nonnecrotic tissue only). The average liver weight was 8.4 g for both studies. See the Methods for the amount of each isotope injected. The standard error values calculated for each point were <15% of the mean, except for those indicated by an asterisk (*) which were <20%.

by the total tumor tissue. On an average, the liver took up 10-fold more [^{3}H]20:4 than the hepatoma per g wet wt.

Fatty Acid Metabolism

The metabolism of the [^{3}H]16:0 taken up by both tissues is shown in Figure 3. The data, presented as the percentage distribution of the incorporated radioactivity, evaluate the relative importance of the metabolic pathways utilizing plasma free fatty acid within a given tissue. At 6 min after injection, the TG of host liver contained 48% of the [^{3}H]16:0, whereas 30% was in PL. The percentage of label in PL was about the same in the hepatoma whereas TG contained only 15-20% of the [^{3}H] label. The DG pool of host liver was initially more highly labeled than the hepatoma (30% vs 18% at 0.5 min), although the label in both pools decreased with time to similar values. Another notable difference between the two tissues was the high initial level of labeled free fatty acid found in the tumor. Perhaps the most striking difference between the two tissues was the proportion of the radioactivity recovered in the nonlipid fraction of the tumor extract (40% at 6 min). The liver, on the other hand, contained a low percentage of label in both free fatty acid and nonlipid fractions over the study period.

One min after injection, the distribution of [^{14}C]18:2 and [^{3}H]16:0 into TG and PL of host liver was comparable (cf. Figs. 3A and 4A). However, as shown in panel B of these figures, the tumor incorporated a higher percentage of [^{14}C]18:2 into PL (50%) than [^{3}H]16:0 (30%). The initial percentage of [^{14}C]18:2 in the DG fraction was lower in both tissues when compared to [^{3}H]16:0. Labeled free [^{14}C]18:2, almost absent in the liver, accounted for 23% of the label in the hepatoma initially. Radioactivity in the nonlipid fraction of the hepatoma increased linearly from 0.5-2 min, then increased slowly to a maximum of 16.5% at 6 min. Interestingly, if the initial rate of radioactivity accumulation in the nonlipid fraction was assumed to continue (see dashed line, Fig. 4B), as it did for [^{3}H]16:0 (Fig. 3B), then the estimated total [^{14}C]18:2 taken up by the hepatoma would be constant over the 6-min period (Fig. 2A), as was the case for the liver. Furthermore, the recalculated [^{14}C]/[^{3}H] isotopic ratio in the tumor at 6 min would be similar to the value observed at 0.5 min in this tissue (Fig. 2A). The rationale for correcting for this "lost" [^{14}C] radioactivity is discussed later.

The incorporation of arachidonate into cellular lipids was markedly different from that of palmitate and linoleate (Fig. 5). Approximately 70% of the [^{3}h]20:4 was incorporated in the PL fraction of both liver and hepatoma. TG comprised 15-24% of the [^{3}H]20:4 radioactivity in host liver and 6-11% of that in hepatoma. Both DG and free fatty acid fractions represented less than 2-3% of the radioactivity in either tissue. The nonlipid fraction constituted only 3-4% of the label from [^{3}H]20:4 in host liver,

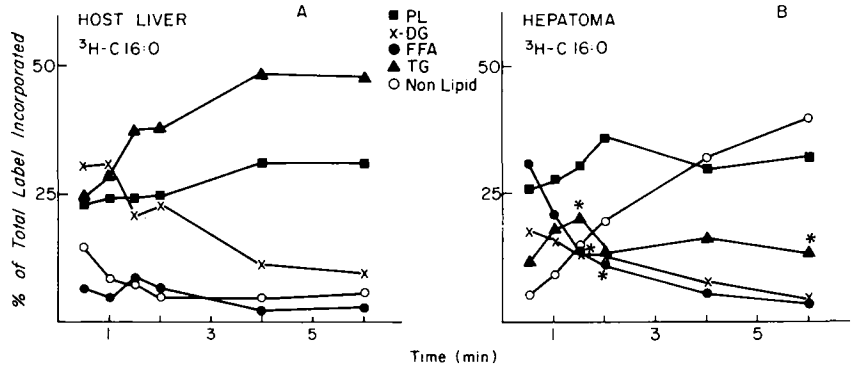


FIG. 3. Distribution of [^{3}H]16:0 radioactivity in host liver and hepatoma. Host liver (A); hepatoma (B). Abbreviation and symbols are: PL, phospholipid (▲); DG, diacylglycerol (X); FFA, free fatty acid (●); TG, triacylglycerol (■); and nonlipid, aqueous phase of tissue extraction (○). Monoacylglycerol and cholesterol ester values were low and thus are not shown. Values at each time point are the mean of single determinations on 3-4 separate host animals. In all cases, except those indicated, the calculated standard errors at each time point were ≤10% of the mean or the standard error value was ≤ ±1.3 for fractions containing less than 10% of the total radioactivity. Asterisk (*), standard error ≤15% of the mean.

TABLE 2

Distribution of Radioactivity among Phospholipid Classes Isolated from Host Liver and the Morris 7777 Hepatoma[a]

	Phospholipid					
	PC		PE		PS-PI	
Fatty acid	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma
16:0	66.9 ± 1.8	72.9 ± 2.4	29.0 ± 1.6	18.4 ± 1.3[b]	4.2 ± 0.3	8.7 ± 1.2[b]
18:2	76.5 ± 2.4	76.9 ± 1.9	19.8 ± 1.9	13.9 ± 0.8[c]	3.7 ± 0.8	9.2 ± 1.1[b]
20:4	79.2 ± 3.5	67.4 ± 1.6[c]	17.1 ± 3.3	21.5 ± 0.6	3.4 ± 0.5	11.1 ± 1.6[c]

[a]Determined on tissue extracts from 0.5 and 1 min injection times. Percentage distributions are shown of radiolabeled fatty acids within the total phospholipid fraction of host liver and hepatoma. Values are the mean ± SE for single determinations on phospholipid fractions isolated from 3-6 separate host animals. See Methods for details. PC = diacyl glycerophosphatidylcholine; PE = diacyl glycerophosphatidylethanolamine; PS-PI = diacyl glycerophosphatidylserine-diacyl glycerophosphatidylinositol.

[b]Significantly different from host liver, as determined by Student's t-test, $p < 0.01$.

[c]Significantly different from host liver, as determined by Student's t-test, $p < 0.05$.

whereas in the hepatoma, 28% of the [^{3}H] was in this fraction at 5 min. The metabolism of the coinjected [^{14}C]16:0 (data now shown) was comparable to that seen with [^{3}H]16:0 (Fig. 3), except that a lower percentage of [^{14}C] was recovered in the nonlipid fraction. In the hepatoma, the incorporation of [^{14}C]16:0 radioactivity into the nonlipid fraction followed a time course similar to that observed for [^{14}C] 18:2 (Fig. 4B).

The phospholipid fraction of liver and hepatoma from rats injected with [^{3}H]16:0 and [^{14}C]18:2 or with [^{3}H]20:4 was fractionated to determine the distribution of the radiolabeled fatty acids among the major phospholipid classes (Table 2). At least two-thirds of the [^{3}H]16:0 and [^{14}C]18:2 incorporated into PL was present in the PC fraction of both tissues. Less [^{3}H] 16:0 and [^{14}C]18:2 was found in the PE fraction of the hepatoma than in the liver. Conversely, [^{3}H]20:4 was incorporated into the PE fraction of the hepatoma and liver to a similar extent; the amount in PC, however, was lower in the hepatoma. A higher percentage of the three fatty acids studied was found in the PS-PI fraction of the hepatoma. Control livers were similar to liver from tumor-bearing rats except that the percentage of 18:2 incorporated into PC was about 8% higher, concomitant with a decrease in PE.

Recent studies have indicated that the pathway for de novo PL synthesis, in which DG is a precursor, forms primarily mono- and diene species of PL. On the other hand, the deacylation-reacylation cycle plays a major role in the formation of tetraene and hexaene (polyene) species of PL (18). We studied these two pathways indirectly by contrasting the percentage distribution of [^{3}H]16:0 radioactivity among the molecular species of DG and PL in each tis-

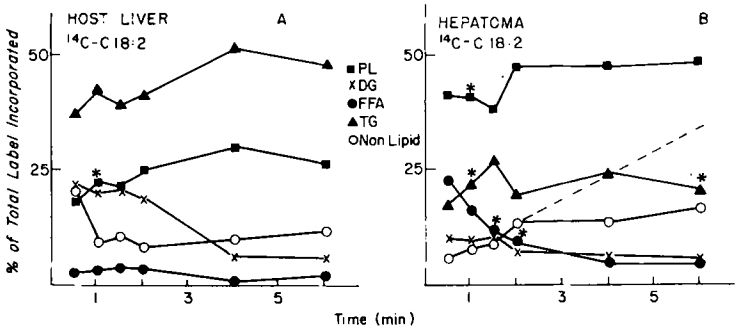


FIG. 4. Distribution of [^{14}C]18:2 radioactivity in host liver and hepatoma. Host liver (A); hepatoma (B). See Fig. 3 for abbreviations and details. See text for explanation of the dashed line in panel B.

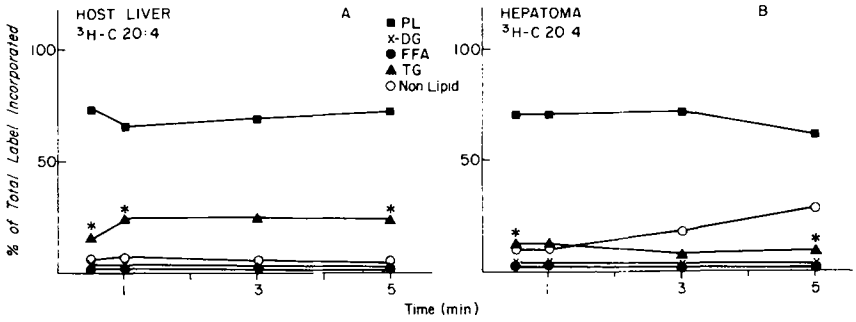


FIG. 5. Distribution of [^{3}H]20:4 radioactivity in host liver and hepatoma. Host liver (A); hepatoma (B). See Fig. 3 for abbreviations and details.

sue (Table 3). In the liver, a much smaller percentage of [^{3}H] label from 16:0 was incorporated into the monoene pool of PL than was found in the pool of DG (21.9% vs 50.8%, respectively). There was, however, a 6.0-fold enrichment in the total polyene fraction of PL relative to that in DG (33.4% vs 5.6%, respectively). In the hepatoma, the percentage of label in the monoene fractions of PL and DG did not differ as greatly (45.1% vs 54.7%). Here, the major differences in the distribution of [^{3}H] were a higher amount of diene species (27.7% vs 18.8%), a 3-fold decrease in triene species (6.7% vs 18.2%), and only a 2.4-fold enrichment in polyenoic species of PL (17.9% vs 7.6%), as compared to hepatoma DG. This lower enrichment of PL in polyenoic species, relative to that observed in the liver, suggests that the contribution of the deacylation-reacylation pathway is less in the tumor than in liver.

In order to determine whether these differences were the result of fatty acid desaturation, the desaturation of [^{14}C] 16:0 was studied in host liver and the hepatoma. Moderate conversion of palmitate to the monoenoic species was observed in the hepatoma (4.2% at 0.5 min) whereas little desaturase activity was detected in host liver (0.9% at 0.5 min). This was similar to our findings when the Δ^9 desaturase was assayed in vitro (4). This finding indicates that the differences in the distribution of palmitate radioactivity observed above for liver and hepatoma can be explained by desaturation to only a minor extent.

TABLE 3

Percentage Distribution of [^{3}H] 16:0 Radioactivity among Phospholipid and Diglyceride Molecular Species Isolated from Host Liver and the Morris 7777 Hepatoma[a]

Fraction	Tissue	Total double bond content						Total polyene[b]
		0	1	2	3	4	5-6	
DG	Host liver	3.6 ± 0.9	50.8 ± 3.1	23.9 ± 3.2	14.1 ± 3.3	5.6 ± 0.4	Tr	5.6
	Hepatoma	5.7 ± 0.3[c]	54.7 ± 1.5	13.8 ± 1.3[c]	18.2 ± 2.0	7.6 ± 0.6[d]	Tr	7.6
PL	Host liver	Tr	21.9 ± 1.4	38.4 ± 3.3	5.6 ± 0.9	24.2 ± 2.1	9.2 ± 0.7	33.4
	Hepatoma	2.6 ± 0.7	45.1 ± 2.3[c]	27.7 ± 1.8[d]	6.7 ± 0.5	12.6 ± 0.9[c]	5.3 ± 0.5[c]	17.9

[a]Determined on tissue extractions from 0.5 and 1 min injection times. The original diglyceride fraction and the diglyceride products of phospholipid phospholipase C hydrolysis were fractionated as described in Methods. Values are the mean ± SE of single determinations on lipids extracted from tissues from 6 separate host animals. The tissue concentrations of PL in liver and hepatoma were 171.4 ± 1.9 and 118.8 ± 3.8 nmol lipid phosphorus/mg protein, respectively. DG concentrations were 1.9 ± 0.3 and 2.2 ± 0.2 nmol/mg protein in liver and hepatoma, respectively. The pool size of DG in control livers was 10.6 nmol/mg protein. DG = diacylglycerol; PL = phospholipid; Tr = trace (<1%).

[b]Percentage as 4-6 double bonds.

[c]Statistically different from host liver, as determined by Student's t-test, $p < 0.01$.

[d]Statistically different from host liver, as determined by Student's t-test, $p < 0.05$.

DISCUSSION

The results of this study established the following points on tumor lipid metabolism, relative to that of the liver of the tumor-bearing rat: (a) the relative selectivity for the uptake of the three fatty acids studied was comparable; (b) less free fatty acid was taken up from circulation per g of tissue; (c) the pathways of complex lipid synthesis were significantly altered; and (d) the results presented suggest that the conversion of fatty acids to nonlipid products (presumably by oxidation) represented a major fate of fatty acids taken up by the hepatoma. Also, we found that, under the conditions of these studies, the tumor had little effect on the liver of the host rat.

The capacity of the hepatoma to take-up polyunsaturated fatty acids from circulation is of significant interest since alterations in this process could contribute to the low amount of these polyunsaturated fatty acids found in tumors (1-3). This capacity was evaluated by contrasting the relative uptake of three free fatty acids by both tissues. Assuming that both the liver and tumor encounter the same plasma free fatty acid pool, we observed that liver and hepatoma have similar selectivity in the removal of free fatty acids from circulation. This observation might be expected since fatty acid uptake in normal tissues has been reported to proceed via passive diffusion (19). However, our experiments do demonstrate that the altered composition of the membrane barrier in cancer cells has not influenced their interaction with circulating fatty acids. We conclude that the selectivity of fatty acid uptake does not account for the lack of polyunsaturated fatty acids in the tumor; rather, it appears that the altered fatty acid composition reflects changes in the metabolic pathways of the hepatoma.

The reduced uptake of free fatty acids by the tumor, relative to the liver, was evident when expressed on a wet wt basis (9- to 10-fold lower) or when related to the protein content of the two tissues (7-fold lower). This may reflect differences in the vascularization of liver and subcutaneously transplanted hepatomas since the relative uptake of free fatty acids determined here for liver and hepatoma is similar to the reported relative blood flow for liver and rapidly growing carcinomas, roughly 10 to 1 (11). Moreover, the decrease in free fatty acid uptake (per g wet wt) with increasing tumor size (Fig. 1) is consistent with the findings of Cataland et al. (20) which suggest that subcutaneously transplanted tumors proliferate more rapidly than their blood supply.

Both palmitate and linoleate were preferentially esterified into TG by liver (Figs. 3A and 4A). In the hepatoma, on the other hand, these free fatty acids were incorporated into PL primarily (Figs. 3B and 4B), demonstrating a qualitative shift in glyceride metabolism. This preferential incorporation of free fatty acids into tumor PL, which has been reported in other tumor tissues (8,21), does not appear to reflect a decreased tumor TG content (data not shown) but may reflect an elevation in the relative turnover of tumor PL.

Differences in the pathways of incorporation of fatty acids into the PL of liver and hepatoma were suggested by analysis of the PL molecular species formed. The data in Table 3 describe the isotopic composition of DG, which is the precursor pool for de novo PL biosynthesis. This composition, when compared to the isotopic composition of the PL pool, reflects the contribution of de novo synthesis to the PL pool, assuming there was no selectivity in the utilization of DG species (22). Using this comparison, it was found that the deacylation-reacylation cycle contributed much less to the hepatoma PL pool than that in liver. This observation supports our previous studies in vitro that identified dysfunctions in the deacylation-reacylation cycle and associated catabolic enzymes in the hepatoma (3). The consequence of such an alteration would be the synthesis of PL that is deficient in polyunsaturated fatty acids, consistent with the PL molecular species found in many tumors.

Free fatty acids taken up by the hepatoma were rapidly converted to nonlipid products (Figs. 3-5). Studies by Hepp et al. (23) suggest that these nonlipid products reflect not only complete fatty acid oxidation products but also incomplete oxidation products, such as acetate, which were shown to accumulate in tumor cells during fatty acid oxidation. The correction for nonlipid [^{14}C] radioactivity in Figure 4B dashed line), therefore, reflects the potential loss of [^{14}C]O_2 from [^{14}C] 18:2 as the result of oxidation. The labeled oxidation products from [^{3}H] 16:0 and [^{3}H] 20:4, including [^{3}H]$_2O$, should be retained in the methanol/H_2O layer during the extraction procedure. Indeed, we observed that the total [^{3}H] from 16:0 in the hepatoma was rather constant over the 6-min period (Fig. 2A), even though the nonlipid radioactivity during this period increased to 50% of the total radioactivity in the tumor (Fig. 3B). This indicates that the majority of the nonlipid [^{3}H] radioactivity in the hepatoma originated in, and most it has remained associated with, the tumor itself.

These studies do not permit a quantitative comparison of the purported oxidation of fatty

acids in liver and hepatoma. Since the amount of free fatty acid taken up by the liver was 10-fold higher than that taken up by the hepatoma (Fig. 2), it would appear that the liver oxidized as much or more fatty acid than the hepatoma. Nonetheless, it can be concluded that fatty acid oxidation appears to be of quantitatively greater importance in the hepatoma, relative to the other metabolic fates of free fatty acid. This finding is not completely unexpected in light of two recent studies. Wu-Rideout et al. (24) demonstrated that the concentration of the cytoplasmic free fatty acid-binding protein participates in defining the metabolic fate of bound free fatty acids. Low concentrations of binding protein were shown to increase mitochondrial acyl-CoA synthetase and β-oxidation activities and to diminish microsomal esterification. Additionally, Mishkin and Halperin (25) have shown that the binding protein concentration of this tumor is 20% of control liver. Taken together, these studies offer a plausible explanation for the higher percentage of fatty acids oxidized by the hepatoma.

It seems reasonable to speculate that the decreased uptake of circulating free fatty acids and a relatively high proportion of fatty acid oxidation (including essential and polyunsaturated fatty acids), coupled with a diminished capacity to synthesize polyunsaturated fatty acids (4), may lead to a depletion of polyunsaturated fatty acids in the tumor. Although it is consistent with the data at hand, this conclusion has yet to be verified.

ACKNOWLEDGMENTS

This work was supported by a grant from the Forsyth Cancer Service and by grants AM-11799, CA-10729, CA-14318 and HL-14164 from the National Institutes of Health. The authors wish to thank Charity Jackson and Louise Lawson for the maintenance and transplantation of the Morris hepatoma, and Tommye Campbell for editorial assistance in preparing this manuscript.

REFERENCES

1. Bergelson, L.D., and Dyatlovitskaya, E.V. (1973) in Tumor Lipids: Biochemistry and Metabolism (Wood, R., ed.) pp. 111-125, American Oil Chemists' Society, Champaign, IL.
2. Reitz, R.C., Thompson, J.A., and Morris, H.P. (1977) Cancer Res. 37, 561-567.
3. Waite, M., Parce, B., Morton, R., Cunningham, C., and Morris, H.P. (1977) Cancer Res. 37, 2092-2908.
4. Morton, R.E., Hartz, J.W., Reitz, R.C., Waite, B.M., and Morris, H.P. (1979) Biochim. Biophys. Acta 573, 321-331.
5. Chiappe, L.E., DeTomás, M.E., and Mercuri, O. (1974) Lipids 9, 489-490.
6. Chiappe, L., Mercuri, O., and DeTomás, M.E. (1974) Lipids 9, 360-362.
7. Tso, W.W., Haldar, D., and Pullman, M.E. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 1724.
8. Spector, A.A. (1975) Prog. Biochem. Pharmacol. 10, 42-75.
9. Olivecrona, T. (1962) Acta Physiol. Scand. 54, 295-305.
10. Sharpe, L.M., Culbreth, G.C., and Klein, J.R. (1950) Proc. Soc. Exp. Biol. Med. 74, 681-685.
11. Gullino, P.M., and Grantham, F.H. (1961) J. Natl. Cancer Inst. 27, 1465-1491.
12. El-Maghrabi, M.R., Waite, M., Rudel, L.L., and Sisson, P. (1978) J. Biol. Chem. 253, 974-981.
13. Burstein, C., Kandrach, A., and Racker, E. (1971) J. Biol. Chem. 246, 4083-4089.
14. Rogozinski, M. (1964) J. Gas Chromatogr. 2, 328-329.
15. Sardesai, V.M., and Manning, J.A. (1968) Clin. Chem. 14, 156-161.
16. Chalvardjian, A., and Rudnicki, E. (1970) Anal. Biochem. 36, 225-226.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
18. van den Bosch, H. (1974) Ann. Rev. Biochem. 43, 243-277.
19. van Golde, L.M.G., and van den Bergh, S.C. (1977) in Lipid Metabolism in Mammals (Snyder, F., ed.) pp. 35-149, Plenum Press, New York, NY.
20. Cataland, S., Cohen, C., and Sapirstein, L.A. (1962) J. Natl. Cancer Inst. 29, 389-394.
21. DeTomás, M.E., and Mercuri, O. (1977) Adv. Exp. Med. Biol. 83, 119-125.
22. De Kruyff, B., van Golde, L.M.G., and Van Deenen, L.L.M. (1970) Biochim. Biophys. Acta 210, 425-435.
23. Hepp, D., Prüsse, E., Weiss, H., and Wieland, O. (1966) Biochem. Z. 344, 87-102.
24. Wu-Rideout, M.Y.C., Elson, C., and Shrago, E. (1976) Biochem. Biophys. Res. Commun. 71, 809-816.
25. Mishkin, S., and Halperin, M.L. (1978) Adv. Exp. Med. Biol. 92, 583-605.

[Received October 12, 1981]

Changes in Liver Lipids after Administration of 2-Decanoylamino-3-morpholinopropiophenone and Chlorpromazine

ASHOK V. HOSPATTANKAR[1], RANGA R. VUNNAM[2] and NORMAN S. RADIN*,
Mental Health Research Institute (Department of Psychiatry) and Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109

ABSTRACT

The enzyme which forms glucocerebroside, ceramide:UDP-glucose glucosyltransferase, is inactivated in vitro by a cationic analog of cerebroside, 2-decanoylamino-3-morpholinopropiophenone. A study of the inhibitor using intraperitoneal injection into young mice showed that the level of the enzyme activity in liver was appreciably lowered between 3 and 6 hr after injection. The activity increased subsequently, overshooting the normal level within 24 hr by about 20%, then returning to normal within the next 24 hr. Additional effects observed in liver were an increase in lipid content (primarily in the triglyceride fraction and ceramides) and a decrease in the glucocerebroside level. Body temperature dropped rapidly. Markedly similar effects were produced by injecting chlorpromazine, which was tried in order to reduce the hyperirritability and inhibitory effects on monoamine oxidase previously demonstrated by the glucosyltransferase inhibitor. Chlorpromazine did indeed block the hyperirritability and resulted in enhancement of the keto amine's effects on the enzyme and lipids. It is possible that the two drugs in combination would be helpful in ameliorating the symptoms due to the cerebroside accumulation that occurs in Gaucher disease. Diazepam also produced a reduced level of glucosyltransferase. A color reaction for chlorpromazine, possibly suitable for quantitative determination in tissues, was accidentally discovered.
Lipids 17:538-543, 1982.

Glucocerebroside (glucosyl ceramide) is synthesized from ceramide and UDP-glucose by a glucosyltransferase, EC 2.4.1.80 (1,2). It is also hydrolyzed by a glucosidase to ceramide and glucose (3). In the human genetic disorder Gaucher disease, the glucosidase is inefficient and glucocerebroside accumulates to a great extent. If the manifold symptoms of the disorder are due primarily to the accumulation of the lipid, it may be possible to ameliorate the symptoms by administration of a drug that inhibits the synthesis of the lipid. Hopefully, after an initial period of depletion, the rate of synthesis can be matched to the individual's rate of hydrolysis. This laboratory has described several such inhibitors which work in vitro (4,5) and we now describe some of the effects produced by one of the compounds in young mice. The inhibitor used here, DMP, is an analog of the enzyme product glucocerebroside. The phenyl group in the inhibitor resembles the long aliphatic chain of sphingosine, the decanoylamino group resembles the much longer fatty acid amide found in the naturally occurring lipid, the ketone group resembles the 3-OH of sphingosine, and the morpholine resembles the glucose moiety. Because the ketone group has a beta relationship to the morpholine nitrogen atom, DMP is chemically reactive and it is likely that it inactivates the glucosyltransferase by direct, covalent reaction with the active site.

MATERIALS AND METHODS

Ceramide:UDP-glucose glucosyltransferase was assayed by a 2-phase liquid scintillation method, in which 5 or 6 mg of homogenized liver was incubated in a plastic counting vial with octanoyl sphingosine as the glucose acceptor (6,7).

Male ICR mice (Harlan Industries, Indianapolis, IN), 24 days old, were maintained for 6 days in our animal room on mouse chow in wire-bottomed cages and 12 hr light/dark cycles. They were divided into groups of 6-10, matched in average weight and weight distribution, and injected intraperitoneally (ip) with 0.2 ml of saline or 0.2 ml of saline containing CPZ·HCl (10 mg/kg body wt). After 1 hr, half the mice were injected with 0.2 ml saline and the other half were injected with 0.2 or 0.4 ml of DMP·HCl (250 or 500 mg/kg), freshly dis-

[1]Present address: Molecular Disease Branch, Natl. Heart, Lung and Blood Institute, Bethesda, MD.

[2]Present address: Technicon Instruments Corp., Tarrytown, NY.

Abbreviations: DMP, 2-decanoylamino-3-morpholino-propiophenone; CPZ, chlorpromazine.

solved in saline. Thus, each mouse received 2 injections (e.g., the controls received 2 saline injections 1 hr apart, the CPZ-DMP mice received CPZ first, then DMP 1 hr later). At a later point, the livers were removed, washed with cold saline, wiped dry, weighed, cooled on Dry Ice, and stored at -70 C in groups of 3, 4 or 5.

For enzyme assays, the pooled livers were homogenized in water. For lipid analyses, they were homogenized with hexane/isopropanol 3:2 (8) and processed as described separately (9,10). Total lipids were determined gravimetrically with the extract from 0.4 g liver, washed with conc aq Na sulfate to remove nonlipids (11).

RESULTS

The initial studies with DMP were carried out with mice from Spartan Research and only the glucosyltransferase activities in liver were measured. Injection of DMP (250 mg/kg) produced distinct decreases in the enzyme activities, with a maximal decrease in the specific activities of about 48% in 2-6 hr. Sacrifice at later time-points showed smaller reductions, evidently due to loss of the inhibitor and, probably, to resynthesis of the enzyme. However, by 24 hr after the injection, the enzyme specific activity had risen to 20% above normal. It was back to normal after a second day.

It was noticed that, shortly after injection, the mice exhibited symptoms of hyperirritability, body tremors and head twitching, as well as abduction of the hind legs. They appeared to be normal after a period of about 3 hr. Tests of DMP in vitro and after injection showed (12) that it possesses the ability to inhibit monoamine oxidases, apparently by a covalent inactivation similar to that seen with the glucosyltransferase. To reduce the symptoms of central nervous system stimulation, and to enable us to administer higher doses of DMP, we tried injecting CPZ 1 hr before the DMP. The CPZ did indeed eliminate the hyperactivation effect of DMP without affecting the recovery period (3 hr). The unexpected finding (Table 1) was made that CPZ (10 mg/kg) was as effective as DMP (250 mg/kg) in lowering the level of glucosyltransferase, even showing the same rebound phenomenon at 25 hr. Particularly satisfying was the observation that the two drugs produced additive effects.

Tests with sedating drugs, using mice killed 3 hr after injection, showed 0.997 nmol/hr of cerebroside synthesized by 6 mg of liver from control mice, a 25% decrease with CPZ, a 10% decrease with reserpine (2.5 mg/kg), and a 14% decrease with diazepam (10 mg/kg). Stimulating drugs (8 mg/kg D-amphetamine, 8 mg/kg metrazol) had no effect.

The Spartan mice became unavailable, so we tested several strains of mice for their enzyme levels and reaction to DMP. It was found that none of the strains tested had as high a specific activity as the Spartan mice, nor was the variability between mice lower, even with inbred mice (C3H). The observed specific activities for cerebroside synthesis by 5 mg of liver were 443 ± 50 pmol with Charles River CD-1 outbred albinos, 490 ± 70 pmol with Charles River C3H mice, 691 ± 50 pmol with Charles River CF-1 mice, and 496 ± 60 pmol with ICR mice. Although the ICR mice did not have the highest specific activity, they responded a little better to DMP and were used for further tests.

Trials of various doses of DMP with these mice, without CPZ, showed that they appeared normal within 3 hr after injection of 500

TABLE 1

Effect of Chlorpromazine and Decanoylaminomorpholinopropiophenone on Liver Glucosyltransferase of Spartan Mice

	Drug injected			
	Controls	CPZ	DMP	CPZ + DMP
2-hr groups:[a]				
Cerebroside formed (nmol/6 mg)	0.91	0.49	0.48	0.30
Inhibition (% below controls)	–	47	48	67
24-hr groups:[a]				
Cerebroside formed (nmol/6 mg)	0.75	0.88	0.90	1.06
Increase (% above controls)	–	18	19	41

[a]Times shown are for animals injected with DMP. All animals were injected 1 hr previously with saline or CPZ. Values shown are averages of 2 values obtained by pooling 2 groups of 3 livers (i.e., 6 mice/group) and assaying for glucocerebroside synthetase.

mg/kg; at 750 mg/kg, recovery took 6 hr and, at 1 g/kg, they recovered within 24 hr. All mice seemed quite normal for the next 3 wk.

Trials with trifluoperazine, a drug related to CPZ but more potent as an antipsychotic, showed only a 7% decrease in glucosyltransferase at 10 mg/kg and the additive effect with DMP was slight. Haloperidol, an antipsychotic drug resembling DMP in structure, produced little or no decrease 3 hr after injecting 5 mg/kg.

An experiment with CPZ and DMP was carried out with the ICR mice, which were sacrificed 2 hr after DMP injection at a dosage of 500 mg/kg (sacrificed 3 hr after CPZ injection). The reduction in glucosyltransferase was not as large as with Spartan mice, but the two drug effects were still additive (Table 2). The body weights were unaffected in this short period of exposure to the drugs, whereas the liver became distinctly enlarged in all three drug-treated groups. However, there was no sign of additivity here.

The effects of CPZ and DMP on glucosyltransferase were found also with female ICR mice, possibly to a greater degree than in males. The depression in enzyme activity (about 42% for DMP and DMP+CPZ) appeared within about 3 hr and persisted about 3 hr, then the overshoot effect (about 41%) was visible 23 hr after the initial injection.

Another trial in which the drugs were allowed to act for 5 hr (for DMP) showed that CPZ could produce a marked increase in liver weight, but here the effect of DMP seemed to be absent (Table 3). Determination of the total lipids in the livers showed that part of the weight increase was due to an increase in the weight of the lipids. The ceramide concentrations also increased, more so than the total lipids, but the cerebroside level decreased. All the lipid changes, like the cerebroside synthetase changes, showed additivity between the two drugs. It was evident that the changes in cerebroside levels showed a parallelism with the drug-induced reduction in glucosyltransferase. The relatively large loss of cerebroside, up to 32%, in just 5-6 hr shows that this lipid undergoes a relatively rapid turnover.

A shorter term study, in which the DMP was allowed to act only 2 hr (Table 4), showed similar, but smaller, changes in ceramide and cerebroside concentrations.

A semiquantitative examination of the liver lipids by thin layer chromatography (TLC), with visual evaluation of the charred spots, revealed that the increased level in liver lipids was due almost entirely to an increase in triglyceride level. Evidently, the two drugs stimulate a very rapid biosynthesis of triglyceride or mobilization from the body to the liver. (However, the solvent that was used for chromatography could not discriminate between triglyceride and dialkyl acyl glycerol, an appreciable component of normal liver.)

It was also noticed, during handling of the mice, that the two drugs produced a marked lowering of body temperature in the mice. CPZ has previously been noted to weaken the body's ability to control its temperature, in animals and in human patients (13), and the toxicity of the drug depends on the ambi-

TABLE 2

Glucosyltransferase in Livers of ICR Mice after Injection of Chlorpromazine or Decanoylaminomorpholinopropiophenone

	Body wt (g)	Liver wt (g)	Cerebroside formed (pmol/5 mg)	Inhibition (%)
Controls	26.1 25.9 (26.1) 26.4	1.07 1.08 (1.09) 1.12	591 506 (540) 523	–
CPZ-injected	25.4 26.9 (26.1) 26.1	1.27 1.40 (1.33) 1.33	514 328 (412) 395	24
DMP-injected	25.3 26.2 (26.1) 26.8	1.23 1.28 (1.21) 1.11	506 372 (398) 316	26
CPZ + DMP	25.1 26.5 (26.0) 26.3	1.15 1.23 (1.21) 1.24	289 251 (263) 249	51

Values shown are averages from 3 mice, 2 hr after injection of DMP (3 hr after injection of CPZ). Values in parentheses are the averages of the 3 measurements.

TABLE 3

Liver Lipids after Injection of Chlorpromazine and Decanoylaminomorpholinopropiophenone

	Body wt (g)	Liver wt (g)	Lipids (mg/g)	Lipid increase (%)	Ceramide level µg/g	Ceramide level Increase	Cerebroside level µg/g	Cerebroside level Decrease
Controls	22.2	1.03	51.3	–	103	–	38	–
	22.3	1.06	48.8		105		37	
CPZ-injected	22.6	1.19	59.8	17	140	25%	35	6%
	22.3	1.17	57.3		120		36	
DMP-injected	22.4	1.05	58.5	15	194	69%	31	20%
	22.4	1.03	56.3		159		30	
CPZ + DMP-injected	22.7	1.18	80.5	53	192	72%	26	32%
	22.4	1.15	72.5		167		25	

Values shown are averages from 5 mice, injected 5 hr prior to sacrifice with DMP (or 6 hr with CPZ).

TABLE 4

Ceramide and Cerebroside Levels in the Livers of Control and Treated Groups of ICR Mice

	Liver wt[a] (g)	Ceramide level[b] µg/g	Ceramide level[b] Increase	Cerebroside level[b] µg/g	Cerebroside level[b] Decrease
		150		33	
Controls	1.09	117	–	33	–
		101		32	
		154		33	
CPZ-injected	1.33	132	20%	27	8%
		155		22	
		171		33	
DMP-injected	1.21	203	46%	29	7%
		164		29	
		196		29	
CPZ + DMP	1.21	203	54%	24	21%
		167		24	

[a]Liver weights are the averages from 9 mice.

[b]Values shown are the averages from groups of 3 mice. All were sacrificed 2 hr after injecting DMP (3 hr after CPZ).

ent temperature (14). Measurements of rectal temperature in the mice (Fig. 1) revealed that both drugs induced hypothermia in 30 min or less and that it persisted for at least 5 hr. Here the additive effect of the two drugs was apparent only during the first 90 min.

The CPZ effect on glucosyltransferase, unlike that produced by DMP, could not be demonstrated in incubations with liver homogenate, even at a concentration of 0.3 mM. Thus, it is possible that the enzyme is inhibited by one or more of the several known metabolites of CPZ. Mixing the liver homogenates from control and CPZ-injected mice gave only the expected intermediate values, indicating an absence of soluble inhibitors in the CPZ-injected mice. This might mean that a CPZ metabolite had combined irreversibly with some of the glucosyltransferase, and the amount of the free metabolite was too low to affect the added normal enzyme.

An interesting side-observation was the finding that CPZ could be seen in the liver extracts after TLC of the cerebroside fraction (using silica gel with chloroform/methanol/water 24:7:1). The CPZ was visible just above the cerebroside pair of bands as a pink spot immediately after spraying with cupric acetate and aq phosphoric acid (15). The color deepened upon heating, instead of charring to the usual gray band. Two µg or less of CPZ could be seen readily and it is possible that the

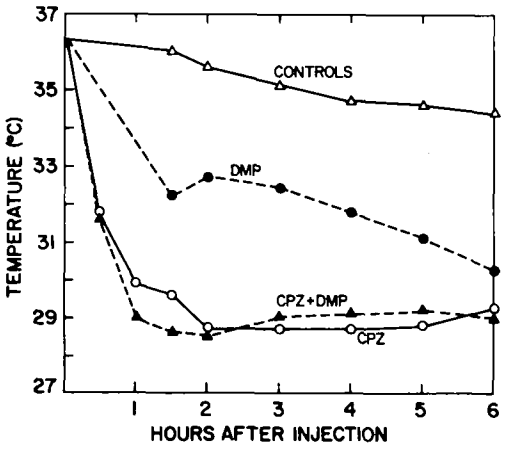


FIG. 1. Rectal temperatures of mice injected with chlorpromazine or decanoylaminomorpholinopropiophenone, or both. Data points are averages from three mice.

method—which seems to show no interference at all—could be useful for quantitative analysis of tissues or blood. A similar color reaction between CPZ and phosphoric acid has been reported for paper chromatograms (16).

DISCUSSION

The ability of DMP to lower liver cerebroside concentration, through its known effect on ceramide:UDP-glucose glucosyltransferase, is encouraging for our goal of treating Gaucher disease by chemotherapy. The current approach to treatment, injection of normal cerebroside glucosidase obtained at great cost from human placentas, has not proved effective in reversing the progress of the disorder (17). Although the interference with regulation of body temperature by DMP and CPZ is a drawback, this problem is readily handled by CPZ-consuming patients by the use of appropriate clothing and control of body activity.

The effects of DMP and CPZ in mice on glucosyltransferase and cerebroside could be attributed to some indirect effect of body cooling, but this explanation is improbable because the additivity of the drugs in their biochemical effects is not paralleled by their nonadditivity in reduction of body temperature.

Remarkably many effects on enzyme reactions and physiological phenomena have been demonstrated for CPZ and we can now add the observed reduction in glucosyltransferase and cerebroside, and the accumulation of ceramide. The accumulation of fat in liver has been previously noted in rats (18) and in patients (19) given CPZ chronically. However, ours seems to be the first demonstration that this accumulation appears very quickly, after a single dose of the drug. Experiments with rats given two single (but heavy) does of CPZ have shown a 41% increase in total phospholipid weight in the liver microsomes within 48 hr after the initial dose (20). Since CPZ is being given to patients over a relatively long period of time, sometimes at dosage levels as high as ours, one might ask whether there is any effect in these patients on sphingolipid metabolism.

The accumulation of ceramide, seen with both drugs, is presumably due to reduction in its rate of utilization for glucocerebroside synthesis without an accompanying decrement in its rates of synthesis or hydrolysis. This kind of accumulation is seen in the human genetic disorder ceramidosis or Farber's lipogranulomatosis, where the defect is in the rate of hydrolysis. The second major synthetic conversion route for liver ceramide is the reaction with lecithin to form sphingomyelin (21), but this reaction appears to be much slower than ceramide glucosylation, at least in vitro. The rapid rise in ceramide level suggests that it has a relatively high turnover rate in liver. Alternatively, one could postulate a rapid mobilization from the peripheral organs, like triglycerides. Ceramides and triglycerides do resemble one another in their low polarity and in their derivation from fatty acids.

The overshoot effect observed 24 hr after administering the two drugs is not unknown with other inhibitors and is presumably the result of imprecise regulation of enzyme levels—in this case, a temporary overrapid synthesis of glucosyltransferase. This lability in the enzyme level suggests that it has a high turnover rate and is relatively responsive to exogenous influences. This interpretation is consistent with the results of experiments on the effects of protein synthesis inhibitors on the enzyme (22).

It is possible that the large reduction in glucosyltransferase we produced would not be necessary in treating Gaucher disease since the accumulation of cerebroside is relatively slow and it might suffice to produce only a small decrement in its rate of synthesis. In general, it seems that organisms possess somewhat more hydrolase than the corresponding synthetase; that is, there is surplus hydrolase activity. The large reduction in cerebroside glucosidase seen in Gaucher disease probably does not signify so large a decrease in the actual rate of cerebroside breakdown.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health, HD 07406. We are indebted to Inez Mason for her assistance. Trifluoperazine was kindly furnished by Smith Kline & French Labs., Haldol (haloperidol), by McNeil Laboratories.

REFERENCES

1. Basu, S., Kaufman, B., and Roseman, S. (1973) J. Biol. Chem. 248, 1388-1394.
2. Morell, P., Costantino-Ceccarini, E., and Radin, N.S. (1970) Arch. Biochem. Biophys. 141, 738-748.
3. Brady, R.O., Kanfer, J., and Shapiro, D. (1965) J. Biol. Chem. 240, 39-43.
4. Vunnam, R.R., and Radin, N.S. (1980) Chem. Phys. Lipids 26, 265-278.
5. Radin, N.S., and Vunnam, R.R. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) Vol. 72, pp. 673-684, Academic Press, New York, NY.
6. Vunnam, R.R., and Radin, N.S. (1979) Biochim. Biophys. Acta 573, 73-82.
7. Hospattankar, A.V., and Radin, N.S. (1981) Lipids 16, 764-766.
8. Hara, A., and Radin, N.S. (1978) Anal. Biochem. 90, 420-426.
9. Radin, N.S., Deshmukh, G.D., Selvam, R., and Hospattankar, A.V. (1982) submitted for publication.
10. Selvam, R., and Radin, N.S. (1981) Anal. Biochem. 112, 338-345.
11. Radin, N.S. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) Vol. 72, pp. 5-7, Academic Press, New York, NY.
12. Vunnam, R.R., Bond, D., Schatz, R.A., Radin, N.S., and Narasimhachari, N. (1980) J. Neurochem. 34, 410-416.
13. Borison, H.L., and Clark, W.G. (1967) Adv. Pharmacol. 25, 129-212.
14. Dandiya, P.C., Johnson, G., and Sellers, E.A. (1960) Can. J. Biochem. Physiol. 38, 591-596.
15. Fewster, M.E., Burns, B.J., and Mead, J.F. (1969) J. Chromatogr. 43, 120-126.
16. Vignoli, L., Gouezo, F., and Morel, M.C. (1962) Bull. Soc. Pharm. Marseille 11, 311-314.
17. Gatt, S., Grabowski, G.A., and Desnick, R.J. (1982) Gaucher Disease: A Century of Delineation and Research, Alan R. Liss, New York, NY.
18. Hormia, M., Hormia, A., and Hakola, P. (1957) Ann. Med. Exp. Biol. Fenniae 35, 316-323.
19. Waitzkin, L., and MacMahon, H.E. (1962) Ann. Intern. Med. 56, 220-232.
20. Ide, H., and Nakazawa, Y. (1980) J. Pharm. Dyn. 3, 612-619.
21. Ullman, M.D., and Radin, N.S. (1974) J. Biol. Chem. 249, 1506-1512.
22. Shah, S.N., and Peterson, N.A. (1971) Biochim. Biophys. Acta 239, 126-131.

[Received February 26, 1982]

^{13}C Nuclear Magnetic Resonance Spectroscopic Analysis of Seed Oils Containing Conjugated Unsaturated Acids[1,2]

A.P. TULLOCH, *National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada S7N 0W9*

ABSTRACT

^{13}C Nuclear magnetic resonance spectroscopy has been used in a nondestructive investigation of conjugated unsaturated acids in seed oil triacylglycerols. Spectra of seven seed oils, from *Punica granatum, Cucurbita palmata, Jacaranda mimosifolia, Centranthus ruber, Catalpa bignonioides, Chilopsis linearis* and *Calendula officinalis*, containing among them six isomeric trienoic acids, *cis,trans,cis-* and *trans,trans,cis*-8,10,12-, *cis,trans,cis-*, *cis,trans,trans-*, *trans,trans,cis-* and *trans,trans,trans*-9,11,13-octadecatrienoic acids, and of the oil of *Impatiens balsamina* containing *cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid, have been examined. Structures of component acids were derived from shifts of double bond carbons and of carbons close to the double bond systems. Compositions of the oils were obtained from signal intensities. Results were similar to those obtained by older methods. Only oil of *Centranthus ruber* contained more than one major conjugated acid; both *cis,trans,trans-* and *trans, trans,trans*-9,11,13-octadecatrienoic acids were found. The latter acid is now thought to occur naturally.

Lipids 17:544-550, 1982.

INTRODUCTION

In an earlier investigation of a seed oil containing two conjugated trienoic acids, it was found that the acids could be identified and the oil composition determined by ^{13}C nuclear magnetic resonance (NMR) spectroscopy (1). Examination of seed oils or fatty acid mixtures by ^{13}C NMR has several advantages over gas liquid chromatographic (GLC) and mass spectrometric (MS) methods, since in these methods derivatives have to be prepared and high temperatures are involved; both procedures may cause decomposition, or isomerization, of sensitive compounds. Using MS, it is difficult to distinguish double bond isomers or to analyze them quantitatively. NMR, however, is nondestructive, requires no derivatives, and is reasonably quantitative.

Five conjugated octadecatrienoic acids have been reported in seed oils with the double bond systems *cis,trans,cis-* and *trans,trans,cis*-8,10,12- and *cis,trans,cis-*, *cis,trans,trans-* and *trans,trans, cis*-9,11,13 (2). Acids were identified mainly by older methods such as ultraviolet (UV) spectroscopy and isolation of the trienoic acid by crystallization and, as a result, mixtures of acids would have been hard to identify.

Because of the finding of *cis,trans,cis-* and *cis,trans,trans*-9,11,13-octadecatrienoic acids, together, in oil of *Fevillea trilobata* (1), other oils known to contain conjugated trienoic acids have been investigated to determine whether they, too, contain more than one conjugated triene. Also, it was useful to examine oils containing isomers other than those investigated earlier (1) so that these could be recognized and distinguished by ^{13}C NMR.

^{13}C NMR spectra of oils from *Calendula officinalis*, containing the *t,t,c*-8,10,12-acid (3), *Jacaranda mimosifolia*, with the *c,t,c*-8,10,12-acid (4), *Punica granatum* (2) and *Cucurbita palmata* (5), with *c,t,c*-9,11,13-acid, *Centranthus ruber*, with the *c,t,t*-9,11,13-acid (6), *Chilopsis linearis* (7) and *Catalpa bignonioides* (8), with the *t,t,c*-9,11,13-acid, have been obtained. Oil of *Impatiens balsamina*, which contains a conjugated *c,t,t,c*-9,11,13,15-octadecatetraenoic acid (9), has also been examined. The compositions of the oils have been determined from the intensities of the signals.

MATERIALS AND METHODS

Oil Extraction

Seeds of *C. officinalis, C ruber* and *I. Balsamina* were obtained from local seed suppliers; *P. granatum* seeds were removed from fresh commercial pomegranates, washed with water and dried at 20 C. Seeds of *C. bignonioides* were from F.W. Schmumacher Company, Sandwich, MA 02563, and those of *J. mimosifolia* were from Etablissments Versepuy, Le Puy (H.L.), France. Seeds of *C. linearis* and *C. pal-*

[1]Presented in part at the International Society for Fat Research/American Oil Chemists' Society World Congress, New York, 1980.

[2]NRCC no. 20405.

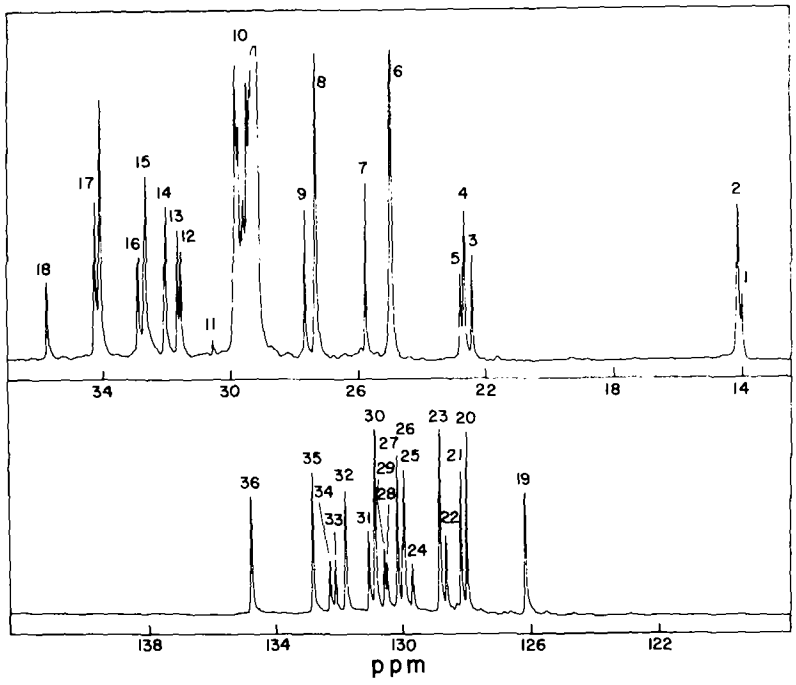


FIG. 1. ^{13}C NMR spectrum of oil from *Chilopsis linearis*, 13-38 ppm and 122-138 ppm regions. Spectrum was obtained with a spectral width of 5 KHz and horizontally expanded 5 times, no pulse delay was used.

mata were gifts from the Rancho Santa Ana Botanic Gardens, Claremont, CA. Seeds were extracted with hexane by the method of Troeng (10), hexane was removed below 30 C and at 0.1 mm. The oil contents were: *P. granatum*, 15.0%; *C. palmata*, 28.7%; *J. mimosifolia*, 28.3%, *C. ruber*, 16.7%; *C. bignonioides*, 15.6%; *C. linearis* 28.3%; *C. officinalis*, 16.5% and *I. balsamina*, 22.7%.

Nuclear Magnetic Resonance (NMR)

The ^{13}C NMR spectra of 1 g of the oils in 2 ml $CDCl_3$ were measured immediately after extraction at a probe temperature of 32 C. A Varian XL-100-15 spectrometer in the Fourier transform mode at 25.1603 MHz with a Nicolet 1180 data system with proton noise decoupling was used. The number of data points was 16,384; spectral width was 5 KHz and the acquisition time was 1.64 sec. Chemical shifts were determined using 10,000 acquisitions, the solvent provided the internal deuterium lock signal and shifts are expressed in ppm downfield from TMS. For quantitative analysis, 45° pulses and a pulse delay of 25 sec were used, the number of acquisitions was 2,000; the proton decoupler was on only during acquisition. The spectrum was expanded horizontally to 5 Hz/cm and the relative intesities of the peaks measured by triangulation. Compositions were calculated by averaging intensities of the same carbon as previously described (1); acids could be detected at a level of 1% and estimated at a level of 2%. Longitudinal relaxation times were measured by the Freeman-Hill procedure.

RESULTS AND DISCUSSION

Satisfactory spectra were obtained for each oil and the relevant parts of the spectrum of oil from *C. linearis* are shown, as an example, in Figure 1. Chemical shifts and relative intensities are listed in Table 1. Assignments of saturated carbon signals were made using the previously established long-range α, β, γ and δ effects of the conjugated triene system (1), assuming them to affect the shifts of stearate carbons additively (11). Thus, signal 9, at 27.58 ppm, is due to C-15 of the *t,t,c* system (if *c,t,t* had been present, instead, a signal due to C-8 would have appeared at 27.87 ppm) and signal 16, at 32.81, is due to C-8 of *t,t,c*. The signals of the double

TABLE 1

^{13}C Chemical Shifts of Seed Oil from *Chilopsis linearis*[a]

Signal Number	Chemical shift	Assignment[b]	Relative intensity
1	13.98	C-18 *t,t,c*	94.1
2	14.09	C-18 S, O, L, *t,t*-9,12-and 10,12	
3	22.37	C-17 *t,t,c*	23.5
4	22.61	C-17 L,*t,t*-9,12-and 10,12	57.8
5	22.73	C-17 S,O	23.0
6	24.90	C-3 all	96.6
7	25.69	C-11 L	28.9
8	27.24	C-8,C-11 O, C-8,C-14 L	89.2
9	27.58	C-15 *t,t,c*	22.5
10	29.10-29.76	Unassigned	–
11	30.46	?	3.0
12	31.49	C-16 *t,t*-9,12-and 10,12	23.0
13	31.58	C-16 L	23.0
14	31.95	C-16, S, O, *t,t,c*	49.0
15	32.58	C-8,C-14 *t,t*-9,12, C-9,C-14 *t,t*-10,12	60.3
16	32.81	C-8 *t,t,c*	20.1
17	34.02, 34.18	C-2 all	109.9
18	35.68	C-11 *t,t*-9,12	15.2
19	126.12	*t,t,c*	25.0
20	127.94	L	32.8
21	128.12	L	31.4
22	128.59	*t,t*-9,12	23.5
23	128.79	*t,t*-9,12, *t,t,c*	44.1
24	129.66	O	11.3
25	129.90	L	48.5
26	129.97	O	
27	130.12	L	30.9
28	130.45	*t,t*-10,12	9.3
29	130.53	*t,t*-10,12	8.8
30	130.82	*t,t*-9,12, *t,t,c*	45.6
31	131.07	*t,t*-9,12	15.7
32	131.75	*t,t,c*	24.5
33	132.06	*t,t*-10,12	9.8
34	132.24	*t,t*-10,12	10.3
35	132.77	*t,t,c*	28.4
36	134.70	*t,t,c*	23.0

[a]Signals of glycerol and carboxyl carbons omitted.

[b]Abbreviations: S, saturated; O, oleate; L, linoleate; *t,t,c*, *trans,trans,cis*-9,11,13-octadecatrienoate; *t,t*-9,12, *trans,trans*-9,12-octadecadienoate; *t,t*-10,12, *trans,trans*-10,12-octadecadienoate.

bond carbons of the conjugated system are discussed later.

The presence of *trans,trans*-9,12- and *trans,trans*-10,12-octadecadienoic acids, previously detected in this oil (7), were shown by signals at 31.49 and 32.58 ppm (assigned in Table 1). The signal at 35.68 ppm, due to C-11 of *t,t*-9,12, confirmed that this acid was present. The double bond carbon signals of these acids appeared in characteristic pairs (12), those of *t,t*-9,12 at 128.59, 128.79, 130.82 and 131.07 ppm and those of *t,t*-10,12 at 130.45, 130.53, 132.06 and 132.24 ppm.

Table 2 lists the shifts of the saturated carbons of the conjugated trienoic acids and of the tetraenoic acid, but only signals which are most affected by the conjugated system and which can be readily seen in the spectra are shown. The *c,t,c*-8,10,12- and -9,11,13-systems and the *cis* end of *c,t,t*- and *t,t,c*-systems all have quite similar effects on neighboring carbons. These effects are characterized by an upfield α effect of *c.* -1.8, markedly less than that of an isolated *cis* double bond, a very small β effect and a moderate γ effect of *c.*-0.35. Only the α and one of the β effects of the *c,t,t,c*- system could be observed: on C-8, the former was the same as in the above groups but on C-17 it was only -1.43, smaller than the effect on the same carbon in linolenate (13). The β effect on the terminal CH_3 was unexpectedly downfield (+0.15); effects on the last two carbons of a chain are often differ-

TABLE 2

^{13}C Chemical Shifts of Some Saturated Carbons of Conjugated Acids as Triacylglycerols in Seed Oils and Effects of the Conjugated Systems

Carbon	*c,t,c*-9,11,13[a] (*P. granatum*)	*c,t,t*-9,11,13[b] (*C. ruber*)	*t,t,c*-9,11,13[c] (*C. bignonioides*)	Conjugated acid (seed oil) *t,t,t*-9,11,13[d] (*C. ruber*)	*c,t,t,c*-9,11,13,15[e] (*I. balsamina*)	Carbon	*c,t,c*-8,10,12[f] (*J. mimosifolia*)	*t,t,c*-8,10,12[g] (*C. officinalis*)
–						5	28.89γ-0.42	28.85γ-0.46
8	27.89α-1.80	27.83α-1.86	32.84α+3.15	32.80α+3.11	27.94α-1.75	7	27.81α-1.83	32.78α+3.14
–						14	27.89α-1.82	27.89α-1.82
15	27.65α-1.76	32.53α+3.12	27.58α-1.83	32.53α+3.12		15		
16	31.94β-0.03	31.58β-0.03	31.95β-0.02	31.58β-0.39		16	31.57γ-0.40	31.56γ-0.41
17	22.39γ-0.34	22.28γ-0.45	22.38γ-0.35	22.28γ-0.45	21.30α-1.43	17	22.62δ-0.11	22.62δ-0.11
18	14.02δ-0.13	13.95δ-0.20	14.00δ-0.15	13.95δ-0.20	14.30β+0.15	18	14.10ε-0.05	14.11ε-0.04

[a] *cis, trans, cis*-9,11,13-octadecatrienoic acid.
[b] *cis, trans, trans*-9,11,13-octadecatrienoic acid.
[c] *trans, trans, cis*-9,11,13-octadecatrienoic acid.
[d] *trans, trans, trans*-9,11,13-octadecatrienoic acid.
[e] *cis, trans, trans, cis*-9,11,13,15-octadecatetraenoic acid.
[f] *cis, trans, cis*-8,10,12-octadecatrienoic acid.
[g] *trans, trans, cis*-8,10,12-octadecatrienoic acid.

ent from those on the interior carbons (13,14). The *trans* ends of the *t,t,c*-8,10,12- and -9,11,13-systems and the *c,t,t*- and *t,t,t*-9,11,13-systems were also similar to each other. These have a large downfield α effect, *a.* +3.1, and a β effect almost as large as the γ effect both being significantly upfield. The effects of the *t,t*-9,12 and *t,t*-10,12 systems were as reported previously (13).

The difference between the α and β effects of the *cis* end and of the *trans* end of the double bond systems makes it possible to distinguish *c,t,t* and *t,t,c* isomers unambiguously. The β effect on C-16 is the most useful in this respect. The position of the double bond system at either 8,10,12 or 9,11,13 is also clearly shown. In 9,11,13-systems, C-16, C-17 and C-18 are subject to β, γ and δ effects, but in the 8,10,12-system these carbons are affected by γ, δ and ε effects. C-17, in particular, is affected by either a γ effect or a δ effect and these are appreciably different no matter in which double bond the system ends. The presence of an 8,10,12-system is confirmed by a signal slightly upfield of 29.00 ppm. This is due to C-5 which in this system experiences a γ effect of *c.*-0.4 ppm.

Table 3 lists the signals of the double bond carbons of the trienes and the tetraene. Signals have not been assigned to particular carbons, though this was done before for three of the acids (1), because assignments have not been confirmed and some, at least when the shifts are close together, may be incorrect. It was not possible to assign the signals from the longitudinal relaxation times (T_1) though this has been done for the double bond signals of linoleate and linolenate (15). In these nonconjugated acids, carbons closer to the methyl end of the chains have appreciably larger T_1 values. It is supposed that introduction of further double bonds decreases the restriction on segmental motion (15). Values close to those reported for linoleate and linolenate were obtained with sunflower and linseed oils, respectively, using the conditions of the present investigation. The double bond carbons of *c,t,c*-9,11,13 in oil of *P. granatum*, and of *c,t,t*-9,11,13 in tung oil, however, all had T_1 values close to 1 sec so that no reliable assignments could be made. These T_1 values are very short, presumably because, in conjugated triene systems, motion is more restricted, due to π orbital interaction, than it is in stearate and linolenate.

Listing the double bond carbon shifts makes it easier to compare the different systems. The two *c,t,c*-acids are similar to each other as are the *c,t,t*- and *t,t,c*-acids; little difference within these two groups would be expected since carbons 8 to 14 of stearate have very similar shifts

TABLE 4

Composition of Seed Oils Determined by ^{13}C NMR[a]

Acid	Plant Species							
	P. granatum	*C. palmata*	*J. mimosifolia*	*C. ruber*	*C. bignonioides*	*C. linearis*	*C. officinalis*	*I. balsamina*
Saturated	8	4	8	18	10	7	8	14
Oleic	3	34	12	4	5	17	2	18
Linoleic	3	45	44	36	45	28	30	13
Linolenic	–	–	–	–	–	–	–	25
t,t-9,12	–	–	–	–	9	16	–	–
t,t-10,12	–	–	–	–	–	10	–	–
c,t,c 9,11,13	84	17	–	–	–	–	–	–
c,t,c 8,10,12	–	–	36	–	–	–	–	–
c,t,t-9,11,13	2	–	–	25	–	–	–	–
t,t,c-9,11,13	–	–	–	–	31	22	–	–
t,t,c-8,10,12	–	–	–	–	–	–	60	–
t,t,t-9,11,13	–	–	–	17	–	–	–	–
c,t,t,c-9,11,13,15	–	–	–	–	–	–	–	30

[a]See footnotes to Tables 1 and 2.

(14). To identify the type of conjugated triene system, the following two observations appear to be sufficient: (a) symmetrical systems *c,t,c* and *t,t,t* have signals at intervals; (b) for each *cis* double bond present, there are two signals at higher field than those of the double bond carbons of oleate. Thus, in *c,t,c* and *c,t,t,c*, there are four signals at higher field than 130 ppm but, in *c,t,t* and *t,t,c*, there are only two such signals and in *t,t,t*, all signals are at lower field than 130 ppm. The structure of an acid is thus derived by consideration of shifts of both saturated and unsaturated carbons.

The compositions of the oils determined from signal intensities are listed in Table 4 and, in general, they are close to those previously reported. The structures of the major conjugated acids were originally determined, relatively laboriously, by chemical degradation after isolation. Amounts of conjugated components were determined by UV spectroscopy (2). Complete compositions were reported for some oils (7,8). Later, all the oils in Table 4 containing conjugated trienoic acids were analyzed by GLC, using a packed column, though the isomeric dienes and trienes were not distinguished (16). Except for *C. ruber* and *C. linearis*, discussed later, agreement between the GLC results (regarding the amount of unspecified triene at least) and Table 4 is fairly close; the larger amount of oleic acid in *C. palmata* in Table 4 may be due to biological variation. When this investigation was complete, GLC analysis of a number of conjugated trienoic acid-containing oils, including *P. granatum* and *C. officinalis*, using a 54-m, open-tubular glass column, was reported (17). In this analysis, different isomers were distinguished and decomposition and isomerization was probably avoided. The composition of oils from *P. granatum* and *C. officinalis* found by this method and by ^{13}C NMR (Table 4) were very close, including the finding of a very minor amount of *c,t,t*-9,11,13-acid in *P. granatum*. Only one of the six double bond carbon signals of this acid has the same shift as one of the *c,t,c*-9,11,13 signals (128.90 ppm, Table 3), the *t,t,c*-acids have similar signals but the presence of the *c,t,t* isomer was indicated by a very small signal (at 32.55 ppm) due to C-15 (Table 2).

The ^{13}C NMR method also clearly showed the presence of the two unusual dienes *t,t*-9,12 and *t,t*-10,12 (Fig. 1, Tables 1 and 4) in *C. linearis*. There is remarkably good agreement between the present composition and that obtained when the two dienoic acids were first found in the oil (7). The *t,t*-9,12-acid was also present in oil from *C. bignonioides*, agreeing with an earlier report (8). No evidence for the presence of

TABLE 3

^{13}C Chemical Shifts of Double Bond Carbons of Conjugated Trienoic and Tetraenoic Acids as Triacylglycerols in Seed Oils[a]

c,t,c-9,11,13	*c,t,c*-8,10,12	*c,t,t*-9,11,13	*t,t,c*-9,11,13	*t,t,c*-8,10,12	*t,t,t*-9,11,13	*c,t,t,c*-9,11,13,15
127.87	127.81	125.98	126.12	126.21	130.60	128.12
128.02	128.07	128.91	128.79	128.71	130.73	128.15
128.86	128.86	130.73	130.82	130.85	130.81	128.25
128.96	129.07	131.52	131.75	132.04	130.95	128.91
132.38	132.09	132.91	132.77	132.71	133.98	132.69
132.62	132.57	134.90	134.70	134.66	134.19	132.87
						132.87
						134.38

[a]See footnotes to Table 2.

appreciable amounts of isomeric trienoic acids was obtained, except for oil of *C. ruber* which had 17% of *t,t,t*-9,11,13-acid as well as 25% of *c,t,t*-9,11,13-acid. Previously only the latter acid was reported, though the isolated acid was difficult to purify (6). The all *trans* acid has usually been considered an artifact, formed by isomerization of one of the other trienoic acids (16,18), and not a naturally occurring acid. It is now believed that this acid occurs naturally in tung oil (17) and it is also most probably a natural constituent of oil of *C. ruber* (Table 4) (unless the seed was very old and isomerization had occurred before extraction). It is of interest that a related species, *Valeriana officinalis* (in the same family Valerianaceae) also contained 10-15% of the all *trans* acid (16). No evidence was found for the presence of hydroxy acids in oil of *C. officinalis*, where 9-hydroxy-10,12-*trans,cis*-octadecadienoic acid has been reported to occur to the extent of 5% (19).

Oil from *I. balsamina* has not been completely analyzed before but another species, *I. edgeworthii*, had a moderately similar composition but with more tetraene and less oleic and linoleic acids (20). The configuration of the double bonds as *c,t,t,c* has been established for acids from both species (9,20). No conjugated trienoic acids were detected in this oil by ^{13}C NMR; they have not been reported for members of this genus, but have been found in oils from *Parinarium* species which also contain the *c,t,t,c*-acid (21).

Thus, it has been shown that six isomeric conjugated trienoic acids and also a conjugated dienoic acid and a conjugated tetraenoic acid can be detected in seed oils, in the presence of oleic, linoleic and linolenic acids, by ^{13}C NMR. The complete composition, except for individual saturated acids, can also be determined without any chemical modification of the oil. Except for oil of *C. ruber*, only one major conjugated acid was present in each oil; compositions of two of the oils were very similar to those recently obtained (17). The complete absence of linolenic acid from oils containing conjugated trienoic acids, which had been shown in earlier work (2), was confirmed. The oil containing the conjugated tetraenoic acid, on the other hand, did contain an appreciable proportion of linolenic acid.

ACKNOWLEDGMENTS

The author thanks M. Mazurek for ^{13}C NMR.

REFERENCES

1. Tulloch, A.P., and Bergter, L. (1979) Lipids 14, 996-1002.
2. Hopkins, C.Y. (1972) in Topics in Lipid Chemistry (Gunstone, F.D., ed.) Vol. 3, pp. 37-87, Logos Press, London.
3. McLean, J., and Clark, A.H. (1956) J. Chem. Soc. 777-778.
4. Chisholm, M.J., and Hopkins, C.Y. (1962) J. Org. Chem. 27, 3137-3139.
5. Bemis, W.P., Berry, J.W., Kennedy, M.J., Woods, D., Moran, M., and Deutschman, A.J. (1967) J. Am. Oil Chem. Soc. 44, 429-430.
6. Hopkins, C.Y., and Chisholm, M.J. (1962) Can. J. Chem. 40, 2078-2082.
7. Chisholm, M.J., and Hopkins, C.Y. (1963) Can. J. Chem. 41, 1888-1892.
8. Chisholm, M.J., and Hopkins, C.Y. (1965) Can. J. Chem. 43, 2566-2570.
9. Takagi, T. (1966) J. Am. Oil Chem. Soc. 43, 249-250.
10. Troeng, S. (1955) J. Am. Oil Chem. Soc. 32, 124-126.
11. Tulloch, A.P., and Mazurek, M. (1976) Lipids 11, 228-234.
12. Bus, J., Sies, I., and Lie Ken Jie, M.S.F. (1977) Chem. Phys. Lipids 18, 130-144.
13. Bus, J., Sies, I., and Lie Ken Jie, M.S.F. (1976) Chem. Phys. Lipids 17, 501-518.
14. Tulloch, A.P. (1977) Can. J. Chem. 55, 1135-1142.
15. Johns, S.R., Leslie, D.R., Willing, R.I., and Bishop, D.G. (1977) Aust. J. Chem. 30, 813-822.

16. Conacher, H.B.S., Gunstone, F.D., Hornby, G.M. and Padley, F.B. (1970) Lipids 5, 434-441.
17. Takagi, T., and Itabashi, Y. (1981) Lipids 16, 546-551.
18. Du Plessis, L.M., and Vladar, S. (1974) S. Afr. J. Sci. 70, 183-184.
19. Badami, R.C., and Morris, L.J. (1965) J. Am. Oil Chem. Soc. 42, 1119-1121.
20. Bagby, M.O., Smith, C.R., and Wolff, I.A. (1966) Lipids 1, 263-267.
21. Gunstone, F.D., and Subbarao, R. (1967) Chem. Phys. Lipids 1,349-359.

[Received February 11, 1982]

Occurrence of Octadecenoic Fatty Acid Isomers from Hydrogenated Fats in Human Tissue Lipid Classes

J.B. OHLROGGE, R.M. GULLEY[1] and E.A. EMKEN, *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604*

ABSTRACT

The level of *trans*-18:1 isomers in several isolated lipid classes of human liver, heart, red blood cells and plasma was determined. Phospholipids contained substantially fewer *trans*-18:1 isomers than triglycerides. The double bond distribution of the *cis* and *trans* octadecenoate fraction of triglycerides and phosphatidylcholines from human liver and heart was determined. Whereas the double bond distribution of the triglycerides correlated closely with the pattern found in dietary hydrogenated vegetable oils, the phosphatidylcholine fraction showed evidence of selective incorporation or metabolism of specific *trans* positional isomers. In general, isomers with double bonds near the methyl terminus were present at levels higher than expected from their relative abundance in the diet. Refinements in methodology needed to analyze octadecenoate double bond configuration and location in human tissues are presented.
Lipids 17:551-557, 1982.

Partial hydrogenation of vegetable oils results in rearrangement of double bonds from the *cis* to the *trans* configuration and in the movement of both *cis* and *trans* double bonds to new positions in the acyl chain (1,2).

The hydrogenated fatty acids are "unusual" in the sense that they differ structurally from the *cis*-9 and *cis*-11 octadecenoic isomers which are synthesized endogenously by animals. Over the past 60-70 years, consumption of partially hydrogenated fats has increased gradually to the level where they now constitute ca. 30-40% of total fat consumed in American diets (3). Daily per capita consumption of the 10-15 "unusual" octadecenoic *cis* and *trans* positional isomers has been estimated to be 9 g, or ca. 6-8% of total fatty acid intake (4). Dietary products and fats from ruminant animals present an additional minor source of octadecenoic isomers as a result of microbial biohydrogenations occurring in the stomachs of ruminants (5).

A number of studies have demonstrated that biological systems can respond very selectively to modifications in fatty acid double bond position or configuration (6). For example, in microbial model systems, alterations of fatty acid double bond position have led to rather striking selective consequences for the growth and viability of these cells (7,8). In vitro measurements of acyl transferase specificities have revealed the high discriminatory ability of enzymes for small changes in acyl chain structure (6).

The analysis of the double bond distribution of individual lipid classes described here provides an opportunity to determine the extent to which the selective biological responses just discussed are reflected during human metabolism of dietary *cis* and *trans* octadecenoate positional isomers.

MATERIALS AND METHODS

Human tissues were obtained during autopsies performed on male subjects of ages 17, 27, 29, 29, 57 and 64 years and on female subjects of ages 16, 26 and 62 years. In most cases, these subjects died from traumatic injuries. In addition, adipose tissue was obtained from two premature infants. Tissues were extracted using hexane/isopropanol as described previously (9). Blood samples were obtained from healthy male volunteers of ages 22-30 and were extracted and fractionated into individual lipid classes as described by Emken et al. (10).

Individual lipid classes were isolated from total lipid extracts of heart and liver by preparative thin layer chromatography (TLC) or by chromatography on a radially compressed column of silica gel as described by Patel and Sparrow (11). For preparative TLC, 2-mm silica gel TLC plates were developed in either petroleum ether/ether/acetic acid (80:20:1) for isolation of triglyceride and cholesterol esters or in chloroform/methanol/acetic acid (65:25:4) for isolation of phospholipids.

The purified lipid classes were transesterified by adding 3 ml each of benzene and 10% anhyd HCl in methanol and heating 3 hr at 65 C in a sealed tube. After adding 5 ml H_2O, the methyl esters were extracted with petroleum ether and

[1]St. Francis Medical Center, Peoria, IL 61603.

the extract was washed with ½ vol of 0.1 M $NaHCO_3$.

The *cis* and *trans* monoene fractions were isolated from methyl esters of the individual lipid classes by silver-nitrate TLC (9). Trace quantities of methyl-[1-^{14}C] oleate and methyl-[9,10-^{3}H] elaidate were added to the methyl esters prior to silver-nitrate TLC to monitor the completeness of separation and recovery of the *cis* and *trans* monoene fractions.

Prior to ozonolysis, the *cis* and *trans* monoenoic fractions that had been isolated by $AgNO_3$-TLC were further purified by preparative GLC on either 20% DEGS or 20% OV17 in ¼-in., 3-ft stainless-steel columns. This procedure removed 16:1, 20:1, or other chain length monoenes from the samples. The 18-carbon chain length monoene isomers were collected from the gas liquid chromatography (GLC) effluent after injections of 50-200 μg of each *cis* and *trans* monoenoic fraction. The GC effluent was split ca. 10:1 and the larger portion was collected (as it passed from the column oven) in a 30-cm section of 24-gauge Teflon tubing. Using radioactive methyl oleate as a standard, the collection efficiencies using this method were determined to be at least 80% if the amount injected was less than 200 μg.

The double bond distribution in the 18-carbon *cis* and *trans* monoenoic fractions was determined by the ozonolysis procedure described by Wood et al. (12) adapted as follows: 10-100 μg of the octadecenoate fractions dissolved in 20-50 μl CS_2 at -70 C was mixed with 0.2-1.0 ml ozone-saturated CS_2 at -70 C. Excess ozone was removed immediately by bubbling with N_2 and the sample was concentrated to 50-100 μl with a stream of N_2. Triphenylphosphine (100 mg/ml CS_2) was added at the level of 10 μg/μg sample and the sample was capped until it was injected into the gas chromatograph. Samples were chromatographed on 2-mm, 6-ft glass columns packed with a mixed phase of 2% OV-17 and 1% OV 210 on 100/120 mesh Supelcoport. The GC oven temperature was programmed from 50-260 C at 10 C/min (1 min initial, 5 min final). These chromatographic conditions gave resolution of all the aldehyde and aldehyde ester fragments derived from octadecenoates with double bonds between the 5 and 16 positions. Peak areas were integrated by digital computer.

The level of *trans*-18:1 in the total fatty acids of the individual lipid classes was estimated by GC on either a 1/8-in., 20-ft 15% OV-275 column or on a 0.02-in., 100-m Silar 10C capillary column.

The octadecenoate isomers used to prepare standard mixtures (as analyzed in Table 2) were gifts from R.O. Adlof, W.J. Dejarlais and H.R. Rakoff (13).

RESULTS AND DISCUSSION

Methodology for Determination of Double Bond Distributions

The analysis of both the double bond configuration and location in the octadecenoate fraction of human tissue lipids presents several problems due to the low concentration and large number of isomers present.

Although the total content of the "unusual" *cis* and *trans* isomers found in human tissues ranges from 0.5-6% (9), this value is the sum of the contributions of 15-20 individual isomers. Most of the octadecenoate isomers are present at levels below 1% and many at levels below 0.1% of the total fatty acids. Because of these low concentrations, the accurate identification of the structure of these isomers requires additional steps to ensure complete removal of other contaminating lipids that could contribute to error in the analysis.

For example, a lipid extract might typically contain 20% *cis*-9-18:1 and 1% of a mixture of *trans*-18:1 isomers. The *trans*-9-18:1 isomer usually constitutes ca. 20% of the mixture of *trans* isomers found in human tissues and thus will be present at a level of 0.2% of the total fatty acids or 1% of the *cis*-9-18:1 level.

If the silver nitrate TLC separation of *cis*- and *trans*-Δ9-18:1 is 99% rather than 100% complete, then the *trans*-9-18:1 fraction will be contaminated by an equal quantity of *cis*-9-18:1. Therefore, this 1% overlap in the TLC separation introduces a 100% error in the value determined for *trans*-9-18:1 in the sample. For this reason, we found it essential to monitor and validate separation of the *cis* and *trans* fractions using radioactive internal standards and capillary GC. In some cases, a second $AgNO_3$-TLC purification of the *trans* monoene fraction was necessary to ensure complete removal of contamination by *cis* isomers.

Although it was possible to ensure essentially complete separations of *cis*- and *trans*-Δ9-18:1 by a second $AgNO_3$-TLC step, complete *cis-trans* separations of all the isomers in these biological samples is not practicable. This is because R_f values for the octadecenoate isomers on $AgNO_3$-TLC vary with double bond position (14), causing incomplete *cis-trans* resolution of mixtures containing many isomers. The greatest overlap occurs between the *trans*-6 and *cis*-13 isomers, which had R_f values of 0.46 and 0.41, respectively, in our TLC

system, whereas elaidate and oleate had R_f values of 0.51 and 0.30, respectively. We estimate the overlap of *trans*-6 into the *cis* fraction and *cis*-13 into the *trans* fraction to be ca. 20%, but since these isomers were found to be minor constituents of the biological samples, the compositional data have not been corrected for this error.

Ozonolysis Validation

Despite improvements in separation of monoenoic positional isomers by capillary GLC (15), chromatographic conditions have not yet been established that allow complete resolution of mixtures of the 15-20 octadecenoate isomers present in samples of hydrogenated vegetable oils or their metabolic products. Thus, to obtain a complete analysis of double bond distribution in complex samples, oxidative cleavage is required.

The most frequently used oxidative technique is ozonolysis followed by triphenylphosphine reduction to yield aldehyde and aldehyde ester fragments. In our preliminary analysis of positional isomer mixtures of known composition, we observed that the peak area of the short chain (<8 carbons) aldehyde and aldehyde ester fragments was up to 60% less than expected. This phenomena was most pronounced when the quantity of sample injected onto the chromatograph column was less than 10 μg. Although this loss evidently is not observed when larger quantities (>100 μg) of isomer mixtures are analyzed (16), the tissue lipid extracts did not contain sufficient quantities of many of these isomers to allow injection of these larger amounts.

The apparent loss of short-chain fragments is not due to their volatility, because the samples were not concentrated and were injected immediately after reduction with triphenylphosphine. This suggested that a portion of the short-chain fragments was lost after injection onto the GC column. We obtained evidence that this type of loss was occurring by coinjecting various quantities of hexanal and methyl hexanoate. Methyl hexanoate was used as an internal standard to calculate the recovery of hexanal. As shown in Table 1, if 5 μg of a hexanal-hexanoate mixture is chromatographed, the recovery of hexanal is reduced 25-35% relative to its recovery when 60 μg of the mixture is injected. These results were observed with on-column injection glass columns and with several stationary phases. Evidently, a portion of the short-chain aldehyde absorbs or polymerizes during the chromatographic run.

Because of this loss of the shorter chain aldehyde and aldehyde ester fragments, it was essential for us to calculate the double bond distribution based only on the longer chain fragment of each isomer rather than using only the aldehyde ester data or an average of both fragment areas. Therefore, we used the area of the aldehyde ester fragment for double bond positions 9 and above, and the aldehyde fragment was used for double bond positions less than 9. The peak areas were corrected both for the number of ionizable carbons and also for the small difference in response of the flame ionization detector for aldehydes and aldehyde esters (16). Table 2 demonstrates the ability of this technique to give quantitatively accurate analyses of known mixtures of isomers varying in double bond location from the 6- to 13-positions.

Occurrence of Positional and Geometric Isomers in Lipid Classes of Liver, Heart, Red Blood Cells and Plasma

Although there have been several reports of the presence of *trans*-18:1 in human tissues (17-21), these studies have not determined which of the individual lipid classes contain these isomers. In Table 3, the levels of *trans*-18:1 in the triglycerides and cholesterol ester fractions and in several phospholipid classes of human heart and liver are presented. In all the subjects we have analyzed, we have consistently

TABLE 1

Influence of Sample Size on Recovery of Short Chain Aldehydes

Stationary phase	Quantity injected (μg)	Hexanal peak area/hexanoate peak area[a]
OV17/OV210	5	0.66 (7)
	60	1.03 (3)
QF1	5	0.75 (4)
	100	1.12 (2)

[a]The number of determinations is given in parentheses.

TABLE 2

Analysis by Ozonolysis of Double Bond Distribution in Two Mixtures of Known Composition

Double bond position	Mixture A composition (%)		Mixture B composition (%)	
	Real	Measured	Real	Measured
6	14.3	14.2	1.0	1.0
8	14.3	13.7	3.0	2.7
9	14.3	14.4	80.0	82.0
10	14.3	14.7	2.0	2.2
11	14.3	14.7	10.1	8.9
12	14.3	14.1	2.9	2.2
13	14.3	14.2	1.0	0.9

TABLE 3

trans-18:1 Content of Total Acyl Moieties in Lipid Classes of Human Liver and Heart

	Weight % *trans*			
	Liver		Heart	
	Avg (n)[a]	Range	Avg (n)[a]	Range
Triglyceride	1.6 (9)	1.0-2.1	2.2 (7)	0.8-3.0
Cholesterol ester	1.8 (4)	1.3-2.1	1.5 (4)	0.75-2.3
Phosphatidylcholine	0.75 (8)	0.3-1.6	0.77 (6)	0.3-1.2
Phosphatidylethanolamine	1.4 (9)	0.27-2.9	0.66 (7)	0.26-1.2
Phosphatidylinositol	0.9 (3)	0.4-1.2	0.71 (3)	0.4-1.3

[a]The number of samples analyzed is given in parentheses.

found that the highest level of *trans* isomers occurs in the neutral lipid fractions, whereas lower percentage levels occur in the phospholipids.

This observation may reflect that a greater proportion of fatty acids in phospholipids originate from de novo synthesis as opposed to dietary sources and/or that there is discrimination against incorporation of unusual isomers into the phospholipid classes. From analysis of blood samples from another set of subjects, we have determined the level of *trans*-18:1 in red blood cells and plasma lipid classes. As seen from Table 4, we again observed that triglycerides have higher levels of *trans* isomers than do the other complex lipid classes.

Our results with human tissue contrast with previous studies of animals fed diets containing hydrogenated vegetable oil. Reichwald-Hacker et al. (22), Høy and Hølmer (23) and Wood (24) fed rats diets containing *trans* isomers at levels of 12.3, 54 and 51%, respectively, of the total fatty acids. For comparison, the level of *trans* fatty acids in American diets has been estimated to be ca. 6-8% of the total fatty acids ingested, and fat consumption by Americans is roughly 35-45% of total caloric intake (3,4). After rats were fed the above hydrogenated oils at levels of 15-25% by weight (30-50% of calories) for periods of 4-12 weeks, the *trans* isomer contents in the liver and heart phospholipids were reported to have risen to levels of 10-16% of the total fatty acids (22-24).

These values are at least 10-fold higher than those we observed (Tables 3 and 4) for human phospholipids, although the dietary consumption levels of the rats and humans differed by only 2- to 6-fold. Thus, in terms of total *trans*-18:1 levels in phospholipids, the short-term rat experiments led to substantially higher incorporations than long-term human consumption. In addition, in the experiments with rats just described, the levels of *trans*-18:1 observed in phospholipids were, in general, higher than observed in tissue triglycerides. This contrasts with our consistent observation (Tables 3 and 4) that human tissues contain higher levels of *trans* isomers in triglycerides than in phospho-

TABLE 4

trans-18:1 Content of Total Acyl Moieties in Lipid Classes of Human Blood

	Weight % *trans*			
	Red blood cells		Plasma	
	Avg (n)[a]	Range	Avg (n)[a]	Range
Triglyceride	2.1 (6)	0.9-4.1	1.7 (11)	0.6-3.1
Cholesterol ester	0.92 (6)	0.1-3.2	0.25 (9)	0-0.7
Phosphatidylcholine	0.85 (6)	0.3-1.4	1.3 (12)	0.1-2.6
Phosphatidylethanolamine	0.92 (6)	0.3-2.0	0.96 (11)	0.2-1.7
Phosphatidylserine	1.3 (6)	0.4-2.2	–	–
Lysophosphatidylcholine	0.93 (3)	0.5-1.2	1.0 (12)	0.1-2.3
Sphingomyelin	0.37 (6)	0.2-0.6	1.1 (10)	0.2-2.7
Free fatty acid	1.7 (6)	0.8-2.8	2.3 (11)	1.0-3.6

[a]The number of samples analyzed is given in parentheses.

lipids.

Figure 1 presents the double bond distributions of the *cis* and *trans* octadecenoic positional isomers in the triglyceride fraction from liver and in the phosphatidylcholine fraction of liver and heart. The patterns we observe for triglyceride are very similar to those found in dietary hydrogenated vegetable oil which is probably the major source of unusual octadecenoic isomers in American diets (25). In contrast, the phosphatidylcholine double bond distribution differs from the diet by its greater proportion of isomers with double bonds near the methyl terminus of the fatty acyl chain. In particular, in phosphatidylcholine, the 11 rather than the 10 isomer is most abundant in the *trans*-18:1 fraction. In addition, an apparently highly selective metabolism of the positional isomers results in a higher level of the *trans*-14 isomer than either the adjacent 13 or 15 isomers. This selective response to individual isomers was observed previously, and in some cases to a much greater extent, by the three studies of rats just discussed. For example, Reichwald-Hacker et al. (22) reported that *trans*-14-18:1 reached a level of 27% of the liver phosphatidylcholine *trans* isomers, whereas in our results, the average level of *trans*-14-18:1 was 7%. Thus, in addition to a greater total accumulation of *trans*-18:1 in phospholipids, the rat experiments appear to result in a more specific accumulation or metabolism of individual isomers.

The pattern of *cis*-18:1 isomers shown in Figure 1 also indicates a general trend toward greater incorporation of isomers having double bonds near the methyl terminus. A quantitative comparison of the proportion in the diet is difficult because of the predominance of the endogenously synthesized $\Delta 9$ and $\Delta 11$ isomers. However, comparison of the relative dietary vs tissue abundance of the 13-15 isomers suggests that a selective accumulation of these structures occurs. In addition, the *cis*-10 isomer appears to be selectively excluded or metabolized, as evidenced by the lower ratio of its abundance relative to *cis*-8 and *cis*-12 isomers.

In Figure 2, the relative abundances of the *trans* positional isomers in the diet are plotted vs their abundance in liver lipid classes. If the concentration of an isomer falls above the straight line in Figure 2, it is an indication that its abundance in the tissue has increased relative to its abundance in the diet. This figure illustrates a consistent trend we have observed from all our analyses of human tissue double bond distributions. In all cases, the *trans* isomers with double bonds near the methyl terminus of the acyl chain fall above the line, whereas those with double bonds between the 7- and 11-positions fall on or below the line. Thus, there appears to be a small, but consistent, metabolic tendency toward either retention of the 12-14 *trans* isomers and/or exclusion of the 7-11 *trans* isomers.

Occurrence of 18:1 Isomers in Premature Infants

Using infrared (IR) spectroscopy, Johnston et al. (26) were unable to detect *trans* isomers in human fetal tissue although the maternal depot fat contained 1.5-6.8% *trans*. However, transfer of elaidic acid across the placental "barrier" of rats has been reported (27). Using capillary GLC, we have found the levels of *trans*-18:1 in samples of adipose tissue from two premature infants to be 0.1 and 0.9%. These levels would be difficult to detect by IR spectroscopy. We also observed (data not shown) that the double bond distribution in the fetal adipose tissue is similar to that of

hydrogenated vegetable oils, suggesting again a lack of selectivity by the placental barrier for or against individual isomers.

SUMMARY

To become incorporated into a tissue phospholipid, a fatty acid originating from a dietary triglyceride must serve as a substrate for a sequence of enzymes including lipases, thiokinases and acyltransferases. There have been numerous demonstrations of rather striking selective recognition of double bond position by enzymatic systems both in vitro (6,28) and in vivo (6-8, 28-32). Analysis of blood lipid changes after feeding octadecenoate isomers has provided evidence for discrimination by human metabolism based on geometry and position of double bonds (31). In the present study, the analysis of human tissue from subjects who have presumably consumed a range of fatty acid positional isomers over a 20-60 year time span provides the opportunity to examine the extent to which these specific protein-lipid interactions are reflected in the fatty acid composition of tissue lipids.

Compared to in vitro studies, the in vivo results reported here reveal somewhat less selectivity in the overall incorporation into tissue lipids of dietary octadecenoate isomers. The *cis*-10 and *trans*-14 isomers appear to be selectively metabolized or incorporated, but for all other isomers, there is a general trend toward greater accumulation of those fatty acids with double bonds farthest from the carboxyl. However, in no case did we observe large accumulation of an isomer similar to that observed with the *trans*-14 isomer in rats fed diets high in hydrogenated fats. Thus, at current levels of dietary consumption, turnover of these unusual isomers in human tissues occurs at rates sufficient to prevent any major accumulations.

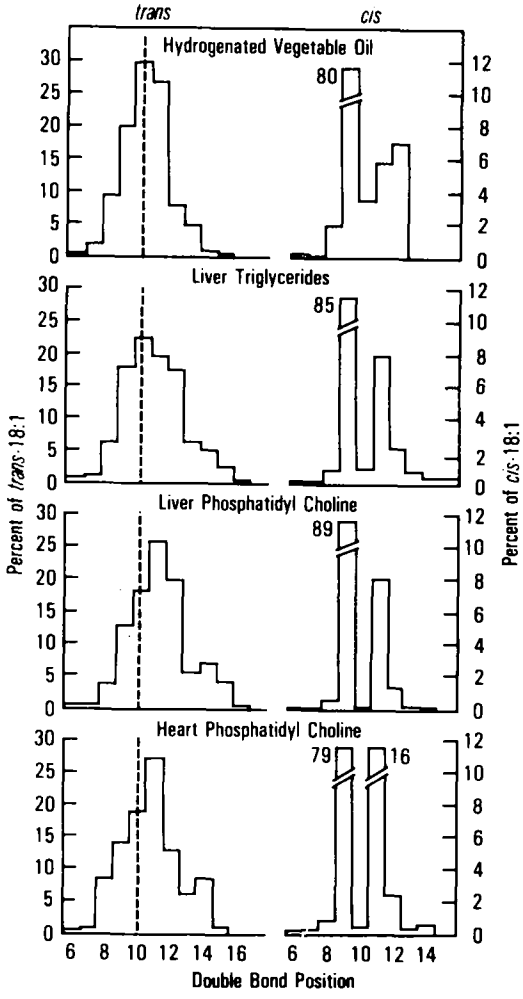


FIG. 1. Average double bond distribution in *trans* and *cis* octadecenoate fraction of hydrogenated vegetable oils and from human tissue lipid classes. The pattern shown for hydrogenated oils is calculated by averaging data published for 20 commercial margarines and cooking oils.

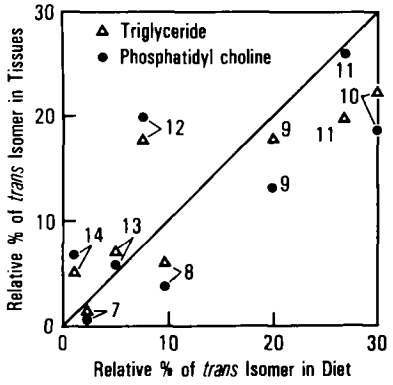


FIG. 2. Comparison of the relative distribution of *trans* octadecenoate positional isomers in diet with their distribution in liver triglyceride and phosphatidylcholine.

ACKNOWLEDGMENT

This work was supported in part by a grant from the Institute for Shortening and Edible Oils.

REFERENCES

1. Dutton, H.J. (1979) in Geometrical and Posi-

tional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 1-16, American Oil Chemists' Society, Champaign, IL.
2. Carpenter, D.L., and Slover, H.T. (1973) J. Am. Oil Chem. Soc. 50, 372-376.
3. Rizek, R.L., Friend, B., and Page, L. (1974) J. Am. Oil Chem. Soc. 51, 244-250.
4. Emken, E.A. (1981) J. Am. Oil Chem. Soc. 58, 278-283.
5. Reiser, R., and Reddy, H.G.R. (1956) J. Am. Oil Chem. Soc. 33, 155-156.
6. Lands, W.E.M. (1979) in Geometrical and Positional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 181-212, American Oil Chemists' Society, Champaign, IL.
7. Ohlrogge, J.B., Barber, E.D., Lands, W.E.M., Gunstone, F.D., and Ismail, I.A. (1976) Can. J. Biochem. 54, 736-745.
8. Vandenhoff, G., Gunstone, F.D., Brave, J., and Lands, W.E.M. (1975) J. Biol. Chem. 250, 8720-8727.
9. Ohlrogge, J.B., Emken, E.A., and Gulley, R.M. (1981) J. Lipid Res. 22, 955-960.
10. Emken, E.A., Rohwedder, W.K., Dutton, H.J., Dejarlais, W.J., Adlof, R.O., Macklin, J.F., Dougherty, R.M., and Iacono, J.M. (1979) Lipids 14, 547-554.
11. Patel, K.M., and Sparrow, J.T. (1978) J. Chromatogr. 150, 542-547.
12. Wood, R., Falch, J., and Wiegand, R.D. (1974) Lipids 9, 987-992.
13. Emken, E.A., Adlof, R.O., DeJarlais, W.J., and Rakoff, H. (1979) in Stable Isotopes (Klein, E.R., and Klein, P.D., eds.) pp. 79-87, Academic Press, New York, NY.
14. Gunstone, F.D., Ismail, I.A., and Lie Ken Jie, M. (1967) Chem. Phys. Lipids 1, 376-385.
15. Scholfield, C.R. (1979) in Geometrical and Positional Fatty Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 17-52, American Oil Chemists' Society, Champaign, IL.
16. Van der Plank, P. (1972) J. Am. Oil Chem. Soc. 49, 489-493.
17. Johnston, P.V., Johnson, O.C., and Kummerow, F.A. (1957) Science 126, 698.
18. Picciano, M.F., and Perkins, E.G. (1977) Lipids 12, 407-408.
19. Morello, A.M., and Downing, D.T. (1976) J. Invest. Dermatol. 67, 270-272.
20. Heckers, H., Körner, M., Tüschen, T.W.L., and Melcher, F.W. (1977) Atherosclerosis 28, 389-398.
21. MacBeth, L.S., and Cook, H.W. (1977) Clin. Res. 25, 709A.
22. Reichwald-Hacker, I., Grosse-Oetringhaus, S., Kiewitt, I., and Mukherjee, K.D. (1979) Biochim. Biophys. Acta 575, 327-334.
23. Høy, C.E., and Hølmer, G. (1979) Lipids 14, 727-733.
24. Wood, R. (1979) in Geometrical and Positional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 213-276, American Oil Chemists' Society, Champaign, IL.
25. Ohlrogge, J.B., in Dietary Fats and Health (Perkins, E.G., and Visek, W.J., eds.) American Oil Chemists' Society, Champaign, IL, in press.
26. Johnston, P.V., Kummerow, F.A., and Walton, C.H. (1958) Proc. Soc. Exp. Biol. Med. 99, 735-736.
27. Ono, K., and Frederickson, D.S. (1964) J. Biol. Chem. 239, 2482-2488.
28. Holman, R.T. (1979) in Geometrical and Positional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 283-300, American Oil Chemists' Society, Champaign, IL.
30. Lanser, A.C. (1979) in Geometrical and Positional Fatty Acid Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 75-97, American Oil Chemists' Society, Champaign, IL.
31. Emken, E.A. (1981) J. Am. Oil Chem. Soc. 58, 278-283.
32. Mounts, T.L. (1976) Lipids 11, 676-679.

[Received January 21, 1982]

METHODS

Direct Estimation of Dolichyl Phosphate in Rat Liver by High Pressure Liquid Chromatography

NILABH CHAUDHARY, DAVID J. FREEMAN, JACK W. RIP and
KENNETH K. CARROLL, *Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1, Canada*

ABSTRACT

A method involving reverse-phase high pressure liquid chromatography has been developed for determining the concentration of dolichyl phosphate (Dol-P) in tissues. Individual Dol-P homologs are resolved and amounts as small as 50 ng can be detected. Rat liver was found to contain 2.4 μg Dol-P/g wet weight, or ca. 4% of total liver dolichol. In contrast, rat liver microsomes contained 64 ng Dol-P/mg protein, which is about 40% of total microsomal dolichol. This enrichment in Dol-P is consistent with the role of microsomes as the major site of Dol-P-mediated glycoprotein biosynthesis in liver.
Lipids 17:558-560, 1982.

Dolichyl phosphate (Dol-P) is an essential intermediate in the biosynthesis of asparagine-linked glycoproteins (1,2), and its availability may be a rate-limiting factor in the glycosylation process (3,4). Based on indirect methods of analysis (5-7), the levels of Dol-P in tissues appear to be very low. In this paper, a method is described for the extraction of Dol-P from tissues, and for its purification and quantitation by high pressure liquid chromatography (HPLC). This method was used to analyze Dol-P in rat liver and rat liver microsomes.

MATERIALS AND METHODS

Dolichol was isolated from human liver and was chemically phosphorylated by the method of Rupar and Carroll (8). Pig liver Dol-P was obtained from Sigma Chemical Co., St. Louis, MO. [1-^{3}H]Dolichol (12.5 Ci/mmol) was purchased from New England Nuclear, Boston, MA, phosphorylated as described by Danilov and Chojnacki (9) and purified by chromatography on a silicic acid column (8). Rat liver dolichol was isolated and quantitated by the method of Rip et al. (10).

Male Wistar rats (Crl:(WI)BR strain; Canadian Breeding Farms and Laboratories Ltd., Laprairie, Que.), weighing about 200 g, were killed by decapitation. Livers were quickly removed and placed on ice. Microsomes (from 25 g liver) were prepared by the procedure of Fleischer and Kervina (11) and washed once before use. Sodium fluoride (25 mM) was added to the homogenization medium to inhibit phosphatase activity (10). Protein was determined by the method of Lowry et al. (12) using perchloric acid-precipitable material (10).

High Pressure Liquid Chromatography of Dol-P

The method was developed using authentic, chemically synthesized Dol-P. HPLC was performed on a dual-pump Hewlett-Packard 1084B liquid chromatograph with a C_{18} reverse-phase column (25 x 0.46 cm) maintained at 45 C. The mobile phase, flowing at a rate of 2 ml/min, was monitored at 210 nm. Dol-P was eluted from the column using 2 sequential gradients of HPLC-grade methanol and isopropanol (the isopropanol contained 10 mM phosphoric acid). The first gradient was from 5 to 35% isopropanol for 5 min, and the second was from 35% isopropanol to 80% isopropanol over 15 min. Retention times for homologs of Dol-P ranged from 10 to 20 min; longer homologs had longer retention times. There was a significant increase in retention time and a slight decrease in resolution when chromatography was done at ambient temperature. The absorbance of Dol-P was proportional to its concentration, as in the case of dolichol (13). Even when small amounts (e.g., 50 ng) of Dol-P were injected, the 2 major homologs could be detected and quantitated if the detector sensitivity was increased to near maximum. A blank pre-run was necessary to ensure reproducible retention times. The presence of phosphoric acid in the mobile phase was essential for elution of Dol-P, and its use over several months

did not appear to damage the column. Gradient elution gave the best resolution of Dol-P homologs but was not absolutely necessary. An isocratic run using 50:50 mix of solvents (methanol/isopropanol–10 mM phosphoric acid) could also be used, but with some loss of resolution.

Extraction of Dol-P

Liver lipids were extracted by the method of Folch et al. (14), modified by the addition of 20 mM phosphoric acid to the chloroform/methanol mixture prior to extraction. Tritiated Dol-P tracer was added at this stage to correct for losses during the isolation process. Phosphoric acid was removed later during the Folch wash. The extracted lipids were chromatographed on a silicic acid column (15) to obtain a Dol-P enriched fraction. A maximum of 100 mg lipid was loaded on a 12-g silicic acid column and eluted first with 75 ml chloroform and then with 75 ml chloroform/methanol (9:1, v/v). The chloroform fraction contained neutral lipids, including dolichol and dolichyl fatty acyl esters. The second eluate was essentially free of dolichol but was enriched in Dol-P. Lipids in this fraction were concentrated and run on preparative Silica Gel 60H thin layer chromatography plates in a solvent system of chloroform/methanol/28% aqueous ammonium hydroxide (65:35:5, v/v/v). The area corresponding to authentic Dol-P was scraped from the plates and extracted with chloroform/methanol/water (2:1:0.2, v/v/v). The organic phase was washed with water and the lipids were concentrated and analyzed by HPLC as already described. The recovery of [^{3}H] Dol-P used as an internal standard was 31 ± 4% (mean ± SEM, n=4), and ranged from 17 to 41%. Nearly all of the losses occurred at the 2 chromatographic steps preceding HPLC, and about equal amounts were lost at each step.

Microsomal Dol-P was isolated by essentially the same method but, because this extract contained much less total lipid, it was chromatographed directly on thin layer plates, followed by HPLC. The recovery of the tracer [^{3}H] Dol-P was 63 ± 4% (mean ± SEM, n=5), and ranged from 50 to 75%.

Dol-P from rat liver was characterized further by collecting HPLC fractions with retention times similar to those of standard Dol-P. These fractions were dissolved in chloroform and the solution was washed with an equal volume of water. The lipid recovered from the chloroform layer was as effective as authentic Dol-P in stimulating microsomal GDP-mannose, Dol-P mannosyl transferase activity, assayed by the method of Rupar et al. (16).

RESULTS AND DISCUSSION

HPLC chromatograms of Dol-P synthesized chemically from human and pig liver dolichol are shown in Figure 1 A and B, respectively. A chromatogram of Dol-P extracted from rat liver is shown in Figure 1C. Dol-P homologs with 18 and 19 isoprene units predominate in rat liver, as observed previously for rat liver dolichol (10).

The concentration of Dol-P in rat liver was found to be 2.4 ± 0.8 μg/g wet weight (mean ± SEM, n=4). This SEM reflects mainly the biological variation. The coefficient of variation

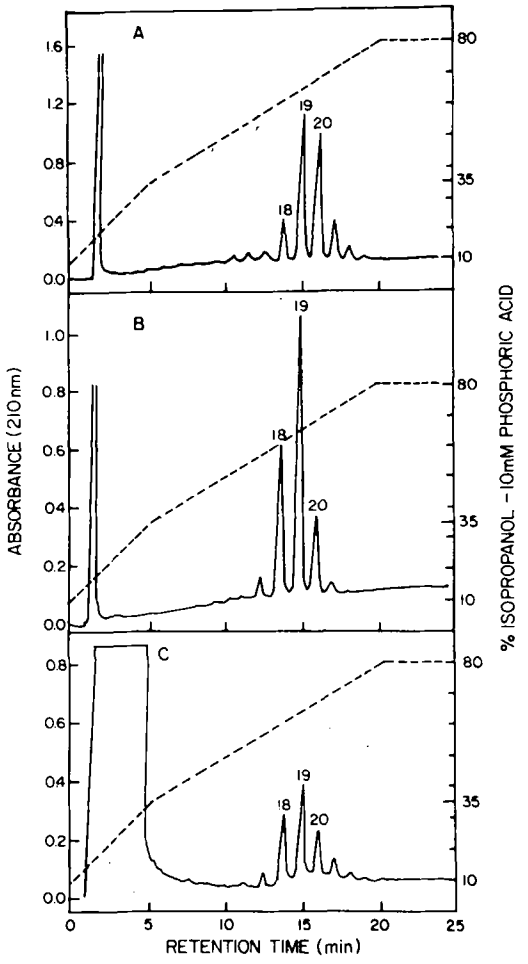


FIG. 1. HPLC chromatograms of Dol-P. (A) Chemically phosphorylated human liver dolichol (60 μg). (B) Chemically phosphorylated pig liver dolichol (40 μg). (C) Isolated directly from rat liver (20 μg). The chromatograms in B and C were run at twice the sensitivity of that in A. The number of isoprene units in major Dol-P homologs is shown above the peaks.

of the analytical method based on 6 replicate assays of one sample was 0.077. In microsomes, the Dol-P content was 64 ± 2 ng/mg protein (mean ± SEM, n=5). The concentration of dolichol (sum of free dolichol and dolichol esterified to fatty acids) in rat liver was 60.5 ± 6 μg/g wet weight (mean ± SEM, n=4) and in microsomes it was 89 ± 17 ng/mg protein (10). Therefore, Dol-P comprises only about 4% of dolichol in whole liver compared to 40% of microsomal dolichol. This is consistent with the role of microsomes as the site of asparagine-linked glycoprotein synthesis in the liver. These values do not take into account the dolichol present as dolichyl pyrophosphate (Dol-PP) or as glycosylated forms of Dol-P and Dol-PP, but these are probably present in very small amounts relative to dolichol, Dol-P and fatty acid esters of dolichol.

It is desirable to be able to measure Dol-P in tissues because of its important role in glycoprotein biosynthesis. Earlier methods for this purpose involve enzymatic assays (5,6) or the preparation of derivatives (7). Our studies have shown that HPLC offers a direct and rapid method for estimation of Dol-P, with the added advantage that homologs of Dol-P can be separated and quantitated.

ACKNOWLEDGMENT

This work was supported by the Medical Research Council of Canada.

REFERENCES

1. Struck, D.K., and Lennarz, W.J. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J., ed.) pp. 35-83, Plenum Press, New York, NY.
2. Hubbard, S.C., and Ivatt, R.J. (1981) Ann. Rev. Biochem. 50, 555-583.
3. Hemming, F.W. (1977) Biochem. Soc. Trans. 5, 1223-1231.
4. Carson, D.D., Earles, B.J., and Lennarz, W.J. (1981) J. Biol. Chem. 256, 11552-11557.
5. Dallner, G., Behrens, N.H., Parodi, A.J., and Leloir, L.F. (1972) FEBS Lett. 24, 315-317.
6. Carson, D.D., and Lennarz, W.J. (1981) J. Biol. Chem. 256, 4679-4686.
7. Keller, R.K., Tamkun, J.W., and Adair, W.L., Jr. (1981) Biochemistry 20, 5831-5836.
8. Rupar, C.A., Carroll, K.K. (1976) Chem. Phys. Lipids 17, 193-200.
9. Danilov, L.L., and Chojnacki, T. (1981) FEBS Lett. 131, 310-312.
10. Rip, J.W., Rupar, C.A., Chaudhary, N., and Carroll, K.K. (1981) J. Biol. Chem. 256, 1929-1934.
11. Fleischer, S., and Kervina, M. (1974) Methods Enzymol. 31, 6-41.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Freeman, D.J., Rupar, C.A., and Carroll, K.K. (1980) Lipids 15, 191-193.
14. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
15. Rouser, G., Kritchevsky, G., and Yamamoto, A. (1976) in Lipid Chromatographic Analysis, 2nd edn. (Marinetti, G.V., ed.), Vol. 3, pp. 713-776, Marcel Dekker Inc., New York, NY.
16. Rupar, C.A., Rip, J.W., Chaudhary, N., and Carroll, K.K. (1982) J. Biol. Chem. 257, 3090-3094.

[Received February 1, 1982]

Long-Chain Phenols: XX. Synthesis of Oxidative Degradation Products from the Methylated Component Phenols of *Anacardium occidentale* and Other Phenolic Lipids: Confirmation of the Structure of the Parent Phenols and of a Related Material[1]

AZIZ A. DURRANI, GOH CHENG SUN and **JOHN H.P. TYMAN**, *School of Chemistry, Brunel University, Uxbridge, Middlesex UB8 3PH, England*

ABSTRACT

A general procedure for the determination of the first double bond position in the side-chain of a phenolic lipid has been investigated and, in the first place, the phenols of natural cashew nut-shell liquid (*Anacardium occidentale*) have been examined. An improved oxidative degradation procedure has been applied consisting of methylation by the phase transfer procedure, hydroxylation with performic acid and oxidation of the mixture of vicinal diols with periodic acid (Malaprade reaction) followed by reduction of the aldehyde fragments with sodium borohydride. The aromatic moieties from the 15:1, 15:2 and 15:3 constituents of cardanol methyl ether, cardol dimethyl ether and dimethyl anacardate were shown to be 8-(3-methoxyphenyl)octan-1-ol, 8-(3,5-dimethoxyphenyl)octan-1-ol and methyl 6-(8-hydroxyoctyl)salicylate methyl ether, respectively. The first two octanols were identical to synthetic materials, thus confirming the 8-position for the first double bond in the side-chain of cardanol methyl ether and cardol dimethyl ether constituents. Methyl 6-(8-hydroxyoctyl)salicylate methyl ether from dimethyl anacardate was identified by a gas liquid chromatographic procedure from the relationship in a series of synthetic reference materials of log (retention time) to the methylene chain length. The synthetic acids 8-(3-methoxyphenyl)octanoic acid, 8-(3,5-dimethoxyphenyl)octanoic acid and 6-(7-carboxyheptyl)salicylic acid methyl ether have been obtained pure for the first time and correspond to the oxidation products of the aromatic aldehyde fragments from the Malaprade reaction stage. The unsaturation in pelandjauol, 17:1-bishomocardanol methyl ether, from *Pentaspadon officinalis* was confirmed to be at the 8-position by the identity of the anilides of synthetic 8-(3-methoxyphenyl)octanoic acid and of the oxidative degradation product.
Lipids 17:561-569, 1982.

INTRODUCTION

The long-chain phenols (2) of *Anacardium occidentale* in natural cashew nut-shell liquid are anacardic acid (I; R^1=CO_2H, R^2=OH, R^3=R^4=H, n=0,2,4,6), cardol (I; R^1=R^4=H, R^2=R^3=OH, n=0,2,4,6), 2-methylcardol (I; R^1=H, R^2=R^3=OH, R^4=CH_3, n=0,2,4,6) and cardanol (I; R^1=R^3=R^4=H, R^2=OH, n=0,2,4,6) whereas in the decarboxylated technical cashew nut-shell liquid only the last three components are present. They have all been described (3-5) as containing a C_{15} side-chain with unsaturation commencing at the 8-position. Early views that cardanol contained a C_{14} side-chain (6) which was dienoid (7) were discarded following oxidative (5) and ozonolytic (3,4) degradations after separation of the methylated constituents (n=0,2,4,6) by chromatography and by low-temperature crystallization.

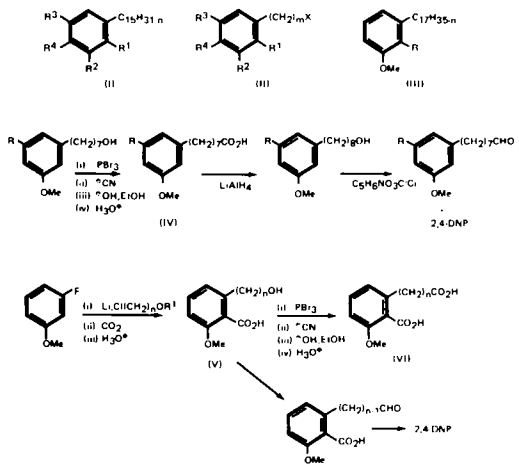
Reductive cleavage of the ozonides from the monoene, diene and triene of methylated cardanol, followed by mild oxidation, gave an acid which was considered (3) from its elementary anlysis to be "8-(3-methoxyphenyl)caprylic acid" (II; R^1=R^2=R^4=H, R^2=OCH_3, X=CO_2H, m=7). For methylated cardol, no practical results have been reported (4). The dimethyl derivative of anacardic acid, upon oxidative treatment (5) with potassium permanganate, gave an impure acid which from elementary analysis was concluded to be "8-(2-carboxy-3-methoxyphenyl)caprylic acid" (II; R^1=CO_2H, R^2=OCH_3, R^3=R^4=H, X=CO_2H, m=7). The presence of a C_{15} side-chain was based on the non-aromatic oxidative by-products isolated such as heptanal in the case of 15:1-cardanol. Our own mass spectroscopic work (1) on the constituents of these component phenols has confirmed, from accurate mass measurements, the presence of a C_{15} side-chain together with a minor proportion of C_{17}. It was desirable to synthesize the aromatic oxidation products since, in the absence of information on their molecular weights, the elementary analyses on the impure

[1] For part XIX, see ref. 1.

RRT = relative retention time; 2,4DNP = 2,4-dinitrophenylhydrazone.

materials could be interpreted in terms of closely related methylenic chain lengths (II; m=6,8).

8-(3-Methoxyphenyl)octanoic acid (IV; R=H), 8-(3,5-dimethoxyphenyl)octanoic acid (IV; R=OMe) and 6-(7-carboxyheptyl)salicylic acid methyl ether (VI; n=7) have now been synthesized in a pure state by the routes shown in Scheme 1, from 7-(3-methoxyphenyl)heptanol, 7-(3,5-dimethoxyphenyl)heptanol and 3-fluoroanisole, respectively. The first two acids (IV; R=H) and IV; R=OMe) were reduced by lithium aluminium hydride to 8-(3-methoxyphenyl)octanol and 8-(3,5-dimethoxyphenyl)octanol, respectively, which were identical to the appropriate oxidative degradation products from cardanol methyl ether and cardol dimethyl ether.



SCHEME 1

The synthesis of 6-(8-hydroxyoctyl)salicylic acid methyl ether (V; n=8) from (HO-protected) 7-chlorooctanol and 3-fluoroanisole in the presence of lithium, although similar to that used (8,9) for 6-(7-hydroxyheptyl)salicylic acid methyl ether (V; n=7), was unsuccessful. However, analogous reactions enabled 6-(3-hydroxypropyl)salicylic acid methyl ether (V; n=3) and 6-(6-hydroxyhexyl)salicylic acid methyl ether (V; n=6) to be synthesized. From the log (retention time) of the methyl esters of the C_3, C_6 and C_7 compounds, the product from the oxidative degradation treatment of dimethyl anacardate was identified by gas liquid chromatography (GLC) as 6-(8-hydroxyoctyl)salicylic acid methyl ether. The unsaturation in the sidechains of the constituents of cardanol, cardol and anacardic acid thus commences at the 8-position and this synthetic work substantiates the findings from the oxidative and ozonolytic work (3-5).

A convenient oxidative degradation of the component phenols cardanol, cardol and anacardic acid consists of phase transfer methylation, hydroxylation with performic acid, Malaprade oxidation of the mixed vicinal diols with periodic acid and sodium borohydride reduction to yield the respective methoxyarylalkanol.

The oxidative degradation of methylated pelandjauol (III; R=H, n=2,4) obtained by decarboxylation of methylated pelandjauic acid (III; R=CO_2H, n=2,4) from *Pentaspadon motleyi* or *Pentaspadon officinalis* was stated (10) to have given 8-(3-methoxyphenyl)octanoic acid which was isolated as the anilide. We are indebted to Dr. J.A. Lamberton (CSIRO, Melbourne, Australia) for a sample of this material to which the anilide of the synthetic acid was found to be identical and this confirms the commencement of unsaturation at the 8-position in the C_{17} as in the C_{15} series.

EXPERIMENTAL PROCEDURES

Melting and boiling points are uncorrected.

Thin Layer Chromatography (TLC)

TLC was performed on laboratory-coated (0.25 cm^{-1}) microscope slides, 10 × 8 cm plates on Kieselgel or Silica Gel G (Merck), type 60 as described (1). Solvents used for development were (A) chloroform, (B) chloroform/ethyl acetate (95:5, v/v), and (C) chloroform/light petroleum (40-60 C, 60:40, v/v). Direct elution with ether could sometimes be used to avoid extraction of the visualizing agent.

Gas Liquid Chromatography

Analytical GLC was carried out on a Pye 104 chromatograph and on a Pye Unicam GCD equipped with a flame ionization detector and computing integrator. Glass columns (5 ft × 3/16 in. were used with nitrogen as carrier gas (60 psi) at 18 cm^3/min and column temperatures as indicated later. The columns used (stationary phase, support) were: (A) 10% SE 30, 100-120 Diatomite M, (B) 3% SE30, 60-80 mesh Diatomite M, (C) 5% APL, 60-70 Diatomite MQ, (D) 5% APL, 100-120 celite, (E) 5% Carbowax 20M, 50-60 Diatomite MQ, and (F) 3% SE52, 60-80 Diatomite M. Relative retentions were determined four times and were found to be highly reproducible.

Column Chromatography

Column chromatography was done on silica gel (MFC) and alumina (Spence Grade H) and separations were monitored by TLC.

Spectroscopy

Infrared (IR) spectra were recorded on Perkin Elmer 700 and Unicam SP200 spectrophotometers as films (liquids) and discs (solids). ^1H nuclear magnetic resonance (NMR) spectra were determined with a Varian T60 instrument at 60 mHz with tetramethylsilane as an internal standard. Abbreviations for spectra are s=singlet, d=doublet, t=triplet, q=quartet, quin=quintet, and m=multiplet. Mass spectra were determined on an MS902 model and accurate mass determinations were carried out by the PCMU (Harwell) on Model MS50 with a computer facility.

Elemental analyses were done by G. Crouch, School of Pharmacy, University of London and BMAC Ltd., Teddington, Surrey. Reactions with lithium were carried out under nitrogen in an evacuable apparatus equipped with manometer and release valve (11).

6-Chloropropanol, 6-chlorohexanol and 8-chlorooctanol were prepared from the corresponding diols by the described procedure (1,12).

The adducts (for HO-protection) of the above three chlorohydrins with ethyl vinyl ether were prepared as described for the C_6 compound (1, 12). The respective acetaldehyde chloroalkyl ethyl acetals were obtained as fragrant-smelling oils usually in theoretical yield. IR $\nu_{(max)}$ (film) for OH nil and having characteristic ^1H NMR signals: $\delta(CCl_4)$, 4.50 (q, 1H, O<u>H</u>), 3.20-3.70 (m, 6H, 2C<u>H</u>$_2$O, C<u>H</u>$_2$Cl), 1.60-2.0 [(C<u>H</u>$_2$)$_n$], and 1.00-1.30 (s, t, 6H, Me$_2$). The chlorohydrins all possessed the expected ^1H NMR signals at (CCl_4), 3.5-4.0 (s, 1H, O<u>H</u>, D_2O exchange), 3.40-3.70 (2t, 4H, C<u>H</u>$_2$O, C<u>H</u>Cl), and 1.5-2.0 [m, (C<u>H</u>$_2$)$_n$].

8-(3-Methoxyphenyl)octanoic Acid (IV; R=H)

7-(3-Methoxyphenyl)heptanol was prepared as described (1) and the bromide was prepared in improved yield as follows. To stirred 7-(3-methoxyphenyl)heptanol (3.044 g) in dry benzene (6.1 cm^3), phosphorus tribromide (1.239 g) in dry benzene (3 cm^3) was added in one portion at 0 C. After 1.5 hr, reaction was incomplete (TLC monitoring); further phosphorus tribromide (1.239 g) in dry benzene (3 cm^3) was added, and the mixture was warmed to 60 C (1.5 hr), by which time all the starting material had reacted. The mixture was diluted with aq sodium chloride (40 cm^3), extracted with ether (3 x 50 cm^3), and the combined extracts were washed with sodium carbonate solution to remove phosphite impurities. The dried ethereal solution was recovered to give 7-(3-methoxyphenyl)heptyl bromide as a pale yellow oil (2.43 g, 66%); ^1H NMR (CCl_4)δ, 6.85 (m, 4H, <u>H</u>Ar), 3.80 (s, 3H, C<u>H</u>$_3$O), 3.29-3.49 (t, 2H, C<u>H</u>$_2$Br), 2.49-2.72 (t, 2H, C<u>H</u>$_2$Ar), and 1.43 [m, 10H, (C<u>H</u>$_2$)$_5$].

7-(3-Methoxyphenyl)heptyl bromide (1.0 g) and potassium cyanide (0.362 g) in dimethyl sulfoxide (5.6 cm^3) containing water (0.25 cm^3) were stirred at ambient temperature (16 hr). TLC (solvent B) then indicated complete reaction and the product that was isolated in the usual way by addition of water, ethereal extraction, drying ($MgSO_4 \cdot H_2O$) and recovery gave 7-(3-methoxyphenyl)heptyl cyanide as an oil which was purified by preparative TLC. (Found: C, 78.35; H, 9.35; N, 6.1. $C_{15}H_{21}OH$ requires C, 77.90; H, 9.10; N, 6.10%), IR $\nu_{(max)}$ (film) 2260 cm^{-1} (CN); ^1H NMR (CCl_4) δ, 6.85 (m, 4H, <u>H</u>Ar), 4.00 (s, 3H, C<u>H</u>$_3$O), 2.63-2.86 (t, 2H, C<u>H</u>$_2$Ar), 2.32-2.52 (t, 2H, C<u>H</u>$_2$CN) and 1.55 [m, 10H, (C<u>H</u>$_2$)$_5$].

7-(3-Methoxyphenyl)heptyl cyanide (0.728 g) in ethanol (6 cm^3) containing potassium hydroxide (0.6 g) was refluxed (20 hr), and the mixture was concentrated; water (50 cm^3) was added and the mixture was then acidified. 7-(3-Methoxyphenyl)octanamide was detected as an intermediate product in TLC monitoring of the hydrolysis. After ethereal extraction (3 x 50 cm^3), extraction with aq sodium carbonate and acidification of the alkaline solution, 8-(3-methoxyphenyl)octanoic acid was obtained (0.337 g) as prisms, mp 54-55 C (lit. [3] 52-54 C), after recrystallization (light petroleum, [bp 40-60 c]). (Found: C, 76.1; H, 10.35. $C_{15}H_{23}O_3$ requires C, 75.70; H, 9.91%), ^1H NMR (CCl_4) δ, 12.00 (s, 1H, CO_2<u>H</u>, D_2O exchange), 6.85 (m, 4H, <u>H</u>Ar), 3.38 (s, 3H, C<u>H</u>$_3$O), 2.0-2.80 (m, 4H, C<u>H</u>$_2$Ar, C<u>H</u>$_2$CO), and 1.40 [m, 10H, (C<u>H</u>$_2$)$_5$]. From the acid (0.2 g) and thionyl chloride (0.3 cm^3) warmed together followed by removal of the excess of the thionyl chloride, and refluxing with benzene (0.2 cm^3) and aniline (0.3 cm^3), the crude anilide was obtained which was purified by acidic and water washing and preparative TLC (solvent B) to give prisms (benzene/light petroleum [bp 40-60 C]), mp 67-69 C, mixed mp 67-68 C with a sample (mp 66-67 C) (10). (Found: C, 77.55; H. 8.35; N, 4.35. $C_{21}H_{27}O_2N$ requires C, 77.54; H, 8.31; N, 4.31%.)

8-(3-Methoxyphenyl)octanol

To 8-(3-methoxyphenyl)octanoic acid (0.088 g) in tetrahydrofuran (3 cm^3) was added, at 0 C, lithium aluminium hydride (0.0616 g). After stirring at 0 C (1 hr), the mixture was warmed to ambient temperature and finally refluxed (8 hr). Work-up in the usual way, following addition of ethyl acetate (1 cm^3), by acidification and ethereal extraction gave 8-(3-methoxyphenyl)octanol as an oil which was purified by

preparative TLC (solvent B). (Found: C, 76.45; H, 9.95. $C_{15}H_{24}O$ requires C, 76.27; H, 10.17%), ^{1}H NMR (CCl_4) δ, 6.97-7.32 (m, 1H, HAr), 6.50-6.77 (m, 3H, HAr), 4.27 (s, 1H, OH, D_2O exchange), 3.77 (s, 3H, CH_3O), 3.43-3.63 (t, 2H, CH_2O), 2.40-2.67 (t, 2H, CH_2Ar), and 1.33 [m, 12H, $(CH_2)_6$]. R_f 0.24 (solvent B).

8-(3-Methoxyphenyl)octanal

To stirred 8-(3-methoxyphenyl)octanol (0.025 g) in dichloromethane (3 cm^3), pyridinium chlorochromate (13) (0.157 g) was added over 2 hr. TLC (solvent B) then indicated nearly complete reaction and the mixture was kept at 0 C for 16 hr, extracted with ether, filtered, and the recovered material was extracted with light petroleum (40-60 C) to give, upon concentration, 8-(3-methoxyphenyl)octanal as an oil, (R_f 0.61, solvent B); ^{1}H NMR (CCl_4) δ, 9.70 (s, 1H, CHO), 6.93-7.37 (m, 1H, HAr), 6.50-6.93 (m, 3H, HAr), 3.72 (s, 3H, CH_3O), 2.23-2.77 (2t, 4H, CH_2Ar, CH_2CO), and 1.20-1.93 [m, 10H, $(CH_2)_5$].

The 2,4-dinitrophenylhydrazone by the addition of Brady's reagent to the aldehyde in methanol (1 cm^3) was a yellow-orange oil which semisolidified and was purified by preparative TLC (R_f 0.68, solvent B) to give pale yellow prisms (light petroleum/chloroform), mp 51-52 C (Found: C, 60.8; H, 6.35; N, 13.55. $C_{21}H_{26}O_5N_4$ requires C, 60.86; H, 6.28; N, 13.52%.)

8-(3,5-Dimethoxyphenyl)octanoic Acid (IV; R=OMe)

7-(3,5-Dimethoxyphenyl)heptyl bromide (12) (1.0 g) and potassium cyanide (0.457 g) in dimethyl sulfoxide (10 cm^3) containing water (0.25 cm^3) were stirred (20 hr) at ambient temperature; reaction was then complete (TLC monitoring, solvent B). Work-up as for the 3-methoxy compound gave 7-(3,5-dimethoxyphenyl)heptyl cyanide (0.70 g, 84%) as an oil. Preparative TLC (solvent B) of 0.48 g gave pure material with a single TLC band. (Found: C, 72.7; H, 8.8; N, 4.75. $C_{15}H_{23}O_2N$ requires C, 73.60; H, 8.80; N, 5.30%), IR $\nu_{(max)}$ film 2250 cm^{-1} (CN), ^{1}H NMR (CCl_4) δ, 6.32 (m, 3H, HAr), 3.80 (s, 6H, $2CH_3O$), 2.20-2.63 (2t, 4H, CH_2Ar, CH_2CN), 1.23 [m, 10H, $(CH_2)_5$].

A mixture of 7-(3,5-dimethoxyphenyl)heptyl cyanide (0.420 g) and 10% aq potassium hydroxide from potassium hydroxide (0.5 g) in ethanol (5 cm^3) was refluxed for 20 hr. After work-up as for the 3-methoxy compound, an oil was obtained which was crystallized (light petroleum) to give 8-(3,5-dimethoxyphenyl) octanoic acid as prisms, mp 58-59 C. (Found: C, 68.55; H, 8.85. $C_{16}H_{24}O_4$ requires C, 68.60; H, 8.60%), ^{1}H NMR (CCl_4) δ, 12.05 (s, 1H, CO_2H, D_2O exchange), 6.30-6.50 (m, 3H, HAr), 3.83 (s, 6H, $2CH_3O$), 2.26-2.72 (2t, 4H, CH_2Ar, CH_2CO), and 6.43 [m, 10H, $(CH_2)_5$]. Symes and Dawson (3) do not record a mp or description for their product of ozonolysis and oxidation.

8-(3,5-Dimethoxyphenyl)octanol

To stirred 8-(3,5-dimethoxyphenyl)octanoic acid (0.0557 g) in tetrahydrofuran (5 cm^3) at 0 C, lithium aluminium hydride (0.1089 g) was added. Reaction and work-up as for the 3-methoxy compound gave 8-(3,5-dimethoxyphenyl) octanol as an oil which was purified by preparative TLC. (Found: C, 72.65; H, 9.95. $C_{16}H_{26}O_3$ requires C, 72.18; H, 9.79%), ^{1}H NMR (CCl_4) δ, 6.20 (m, 3H, HAr), 3.73 (2s, 6H, CH_3O), 3.40-3.60 (t, 2H, CH_2O), 2.86 (s, 1H, OH D_2O exchange), 2.32-2.60 (t, 2H, CH_2Ar), and 1.3 (m, 12H, $(CH_2)_6$), R_f (solvent B) 0.25. By GLC/MS, m/e 266 M^+. $C_{16}H_{26}O_3$ requires 266.

8-(3,5-Dimethoxyphenyl)octanal

8-(3,5-Dimethoxyphenyl)octanol (.017 g) in dichloromethane (4 cm^3) was stirred and treated with pyridinium chlorochromate (0.159 g) during 2 hr. After 16 hr at 0 C, the mixture was worked-up as for the 3-methoxy compound to give 8-(3,5-dimethoxyphenyl)octanal as an oil, ^{1}H NMR (CCl_4) δ, 9.72 (s, 1H, CHO), 6.20 (m, 3H, HAr), 3.77 (s, 6H, CH_3O), 2.10-2.63 (2t, 4H, CH_2Ar, CH_2CO), and 1.07-1.67 [m, 10H, $(CH_2)_5$].

Addition of Brady's reagent to the aldehyde in methanol (1 cm^3) gave a thick yellow turbidity followed by separation of the 2,4-dinitrophenylhydrazone as a yellow-orange oil which slowly solidified. Filtration and purification by preparative TLC (solvent B) gave the pure derivative as yellow spheres (from chloroform/light petroleum, bp 40-60 C), mp 66-68 C (R_f 0.60, solvent B). Found: C, 59.55; H, 6.25; N, 12.65. $C_{22}H_{28}O_6N_4$ requires C, 59.46; H, 6.31; N, 12.61%.)

Homologous Compounds in the 6-Alkylsalicylic Acid Methyl Ether Series — 6-(7-Hydroxyheptyl)-salicylic Acid Methyl Ether (V; n=7)

Acetaldehyde 7-chloroheptyl ethyl acetal was prepared as described (8,12) and 6-(7-hydroxyheptyl)salicylic acid methyl ether was obtained in much improved yield.

Acetaldehyde 7-chloroheptyl ethyl acetal (60 g) in ether (500 cm^3) was converted into the lithium derivative by reaction with lithium (7.0 g). To the mixture, 3-fluoroanisole (6.3 g) was added during 5 min, and after an exothermic

reaction, the stirred mixture was kept at ambient temperature for 1 hr. It was then filtered to remove unreacted lithium and the filtrate was poured onto solid carbon dioxide. The organic acidic material isolated by extraction with ether of the acidified mixture and work-up as before was a viscous oil, 6-(7-hydroxyheptyl) salicylic acid methyl ether, giving a single spot (TLC), (5.43 g, 41%). (Found: C, 69.1; H, 7.7. $C_{15}H_{22}O_4$ requires C, 67.66; H, 8.27%), ^{1}H NMR ($CDCl_3$) δ, 6.60-7.43 (m, 3H, HAr), 6.50-6.60 (bs, 1H, CO_2H, D_2O exchange), 3.87 (s, 3H, CH_3O), 3.40-3.70 (t, 2H, CH_2O), 2.06-2.87 (t, 4H, CH_2Ar), 2.20 (bs, 1H, OH, D_2O exchange), and 0.97-1.86 [m, 10H, $(CH_2)_5$]. By treatment of the foregoing alcohol acid in ether solution with ethereal diazomethane, the methyl ester was formed having in its ^{1}H NMR signals similar chemical shifts with, in addition, δ(CCl_4) at 3.90 (s, 3H, CO_2Me).

In a similar way, 6-(3-hydroxypropyl)salicylic acid methyl ether (V; n=3) was prepared, mp 89-90 C. (Found: C, 62.85; H, 6.75. $C_{11}H_{14}O_4$ requires C, 62.86; h, 6.67%.) 6-(3-Hydroxyhexyl)salicylic acid methyl ether (V; n=6) was also obtained, mp 98-99 C. (Found: C, 66.3; H, 7.95. $C_{14}H_{20}O_4$ requires C, 66.66; H, 7.94%.)

6-(7-Carboxyheptyl)salicylic Acid Methyl Ether (VI; n=7)

6-(7-Hydroxyheptyl)salicylic acid methyl ether (2.5 g) in dry benzene (25 cm^3) was treated with phosphorus tribromide (6 cm^3) and the mixture was warmed (2 hr) at 60 C (TLC monitoring then indicated complete reaction). After work-up as before, the crude 6-(7-bromoheptyl)salicylic acid methyl ether in ethereal solution was methylated with ethereal diazomethane and then purified by column chromatography to give methyl 6-(7-bromoheptyl)salicylate methyl ether (1.87 g; 58%); MS, m/e 342.0835, 344.0808 (M^+). $C_{16}H_{22}O_3{}^{79}Br$ requires 342.0831. $C_{16}H_{23}O_3{}^{81}Br$ requires 344.0811.

Methyl 6-(7-bromoheptyl)salicylate methyl ether (1.3 g) in dimethyl sulfoxide (20 cm^3) containing water (4 cm^3) was stirred with potassium cyanide (3 g) for 16 hr. After work-up and column chromatographic purification, methyl 6-(7-cyanoheptyl)salicylate methyl ether (0.88 g; 80%) was obtained as a colorless, viscous oil; MS, m/e 289.1674 (M^+). $C_{17}H_{23}O_3N$ requires 289.1677. ^{1}H NMR (CCl_4) δ, 7.07-7.32 (2d, 4-H, HAr, J_O 8 Hz), 6.57-6.77 (2d, 5-H, 3-H, HAr, J_O=8Hz, J_m=2Hz), 3.80, 3.83 (2s, 6H, CH_3O), CO_2Me), 2.10-2.63 (2t, 4H, CH_2Ar, CH_2CN) and 1.12-1.73 [m, 10H, $(CH_2)_5$].

Methyl 6-(7-cyanoheptyl)salicylate methyl ether (0.5 g) was hydrolyzed by warming it in ethanol (10 cm^3) and water (20 cm^3) containing potassium hydroxide (2.5 g), for 20 hr. After work-up as before, 6-(7-carboxyheptyl)salicylic acid methyl ether was obtained as a waxy solid, showing a single spot (TLC). Crystallization (light petroleum, bp 40-60 C/ether) gave prisms, mp 72-74 C (0.18 g; 35%), lit. (5) a waxy solid (no mp given). (Found: C, 65.66; H, 7.6. $C_{16}H_{22}O_5$ requires C, 65.31; H, 7.48%.) ^{1}H NMR ($CDCl_3$) δ, 10.89 (bs, 1H, CO_2H, D_2O exchange), 6.50 (m, 3H, HAr), 3.87 (s, 3H, CH_3O), 2.30-2.73 (2t, 4H, CH_2Ar, CH_2CO_2H), and 2.52-2.87 [m, 10H, $(CH_2)_5$]. 6-(8-Hydroxyoctyl)salicylic acid methyl ether could not be prepared by reduction of the preceding acid with lithium aluminium hydride as in the 3-methoxy and 3,5-dimethoxyphenyl series because of simultaneous reduction of the aromatic carboxyl group and the lack of a selective reagent. The acetal of octamethylene chlorohydrin with ethyl vinyl ether treated with lithium failed to react with 3-fluoroanisole.

Methyl 6-(7-Formylhexyl)salicylate Methyl Ether

To methyl 6-(7-hydroxyheptyl)salicylate methyl ether (0.0143 g) in dichloromethane (3 cm^3), pyridinium chlorochromate (0.0684 g) was added. After 2 hr TLC, the oxidation appeared nearly complete and, following 16 hr at 0 C, the mixture was worked-up as in the previous oxidations to give an oil which was purified by preparative TLC (solvent: chloroform/ethyl acetate, 90:10, v/v). Several minor impurities were separated and methyl 6-(7-formylhexyl)salicylate methyl ether was obtained as an oil. Upon treatment in methanol (0.5 cm^3) with Brady's reagent, the 2,4-dinitrophenylhydrazone separated as a yellow, viscous oil which was crystallized (chloroform/light petroleum) to give orange needles, mp 116-117 C (R_f, 0.66, solvent B).

Oxidative Degradations of Methylated Component Phenols from *Anacardium occidentale*

Anacardic acid was separated from natural CNSL as described (11,14). Cardol and cardanol were obtained from technical CNSL and from the filtrate from the separation of anacardic acid (1,14). Methylation was best effected by the phase transfer method (1,14). Residual dimethyl sulfate was only removed satisfactorily by preparative TLC (solvent C). Both from the ^{1}H NMR spectrum of the recovered methyl ether and its mass spectrum, structural information (OMe to HAr ratio, and m- or o/p substitution) of value in the case of novel phenols (11, 15), can be derived. In each case, the methyl

ether contained the saturated monoene, diene and triene constituents. Although hydroxylations of the phenolic acetates could be effected, the phenolic methyl ethers were generally more suitable and more convenient for GLC purposes. With other phenolic lipids of totally unknown constitution, argentation TLC (16) separation of unsaturated constituents, before hydroxylation and oxidation of each, would be desirable since they might not possess a common first double bond position in the side-chain.

8-(3-Methoxyphenyl)octanol from Cardanol

To cardanol (1.00 g; 0.0033 mol, average molecular wt 300) in dichloromethane (16 cm^3) and water (12 cm^3) containing 3 M sodium hydroxide (3.6 cm^3) and Triton B (0.25 cm^3), dimethyl sulfate (0.5 cm^3) was added and the mixture was vibromixed. After 2 hr, the cloudy emulsion had disappeared and the clear mixture was acidified. The separated organic layer was washed repeatedly with water, dried and concentrated to give the methyl ether. TLC (solvent C, R_f 0.35) showed the absence of cardanol. Preparative TLC gave the methylated material free from dimethyl sulfate, and oxidized and polymeric material, with ^1H NMR (CCl_4) δ, 6.50-7.16 (m, 3H, HAr), 4.96-5.53 (m, CH=CH, CH=CH_2), 3.86 (s, 3H, MeO), 2.37-2.93 (m, CH_2Ar, CH_2-(CH=CH)$_2$), 1.83-2.12 (m, CH_2-CH=CH), 1.33 (m, $(CH_2)_n$), and 0.77-1.03 (t, CH_3).

Cardanol methyl ether (3.762 g; 0.0125 mol, average molecular wt 314) in 98% formic acid (23 cm^3) was stirred and treated with 30% hydrogen peroxide (1.49 g) at ambient temperature. After 20 min (TLC monitoring, solvent C), reaction was complete, the excess of formic acid was removed under reduced pressure, and the residue was diluted with water (50 cm^3). The mixture was extracted with ether and the recovered oil from the extract was stirred and warmed to hydrolyze formate esters (for small-scale work, removal of formic acid was not done and extraction with ether of the hydroxylated product diluted with water was used). The acidified mixture was extracted with ether, the extract was washed with water until it was neutral, then was dried and hydroxylated cardanol methyl ether was recovered upon concentration. ^1H NMR (CCl_4) showed absence of olefinic absorption, of methylenic absorption adjacent to unsaturation, and the presence of -CH(OH) groups (δ, CH, 3.17-3.47).

Malaprade oxidation was carried out with less than the stoichiometric proportion of potassium periodate.

To the stirred hydroxylated product in ethanol (120 cm^3), potassium periodate (3.0 g) in 1M aqueous sulfuric acid (150 cm^3) was added in one portion and the mixture was kept at 40 C (15 min). After it had been cooled, the mixture was extracted with ether; the extract was dried and concentrated. The mixture of aldehydes (odor of heptanal) showed (^1H NMR) absence of the -CH(OH) and the presence of -CHO (δ, CCl_4, 9.77). The crude oxidation product (0.142 g) in methanol (3 cm^3) was treated with sodium borohydride (0.0882 g). The mixture was stirred (3 hr), acidified and extracted with ether. The crude product possessed a major ingredient identical (R_f 0.26, solvent B) to 8-(3-methoxyphenyl)octanol. Preparative TLC (solvent B) gave three bands, the lowest (main) one of which was eluted and the product was recovered to give the pure product identical in TLC, GLC (relative retention time [RRT], column B [180 C], 7.45 min; [200 c], 3.50 min) and ^1H NMR spectrum, with synthetic 8-(3-methoxyphenyl)octanol. MS, m/e 236.1774 (M^+). $C_{15}H_{24}O_2$ requires 236.1770.

Methyl 6-(8-hydroxyoctyl)salicylate Methyl Ether from Anacardic Acid

Methylation of the carboxyl group in anacardic acid was effected at 0 C with ethereal diazomethane and the product was purified by preparative TLC (solvent, chloroform/light petroleum, 20:80, v/v).

Methyl anacardate (0.3787 g; 1.057 x 10 mol, average molecular wt 358) in dichloromethane (4 cm^3) and water (3 cm^3) was treated with 3 M sodium hydroxide solution (1 cm^3) and Triton B (0.10 cm^3). To the vibromixed mixture, dimethyl sulfate (0.5 cm^3) was added and, after 6 hr, a further 0.1 cm^3 was added. A cloudy emulsion formed which became a clear, two-phase solution. It was acidified, extracted with ether, then the ethereal extract was copiously washed with water, dried and purified by preparative TLC (solvent, chloroform/light petroleum by 40-60 C, 20:80, v/v, since some methyl anacardate and dimethyl sulfate were present. The second band from the top consisted of dimethyl anacardate which was eluted and the product was recovered (dimethyl sulfate remained near the baseline and residual methyl anacardate was the top band), ^1H NMR (CCl_4) δ, 6.54-7.30 (m, 3H, HAr), 4.93-5.50 (m, CH=CH$_2$), 3.80, 3.87 (2s, 6H, OMe, CO_2-Me), 2.27-2.87 (m, CH_2Ar, CH_2(CH=CH)$_2$), 1.70-2.20 (m, CH_2CH=CH), and 1.33 [m, $(CH_2)_n$], 1.72-1.92 (t, CH_3). The initial methyl anacardate had a similar spectrum which also contained a single OMe signal and a low-field OH signal (δ, 9.23, D_2O exchange).

Dimethyl anacardate (0.1734 g) in 98% formic acid (2 cm^3) was stirred and treated with

30% hydrogen peroxide (0.1 cm^3) during 1 hr. The mixture was worked-up as before and ^1H NMR (CCl_4) examination showed the absence of olefinic and adjacent methylene groups and th presence of CHO (δ, 8.0-8.10) as formate esters. Hydrolysis of the recovered oil at ambient temperature by stirring with 3 M sodium hydroxide solution and isolation of the product by extraction with ether gave hydroxylated dimethyl anacardate as an oil (R_f 0.15, solvent B), ^1H NMR (CCl_4) δ, 6.52-7.37 (m, 3H, HAr), 3.80, 3.85 (2s, 6H, OMe, CO_2Me), 3.53 (m, CH(OH)), 3.03-3.40 (m, OH), 2.30-2.63 (t, 2H, CH_2Ar), 1.33 (m, $(CH_2)_n$) and 1.77-1.97 (t, CH_3).

To hydroxylated dimethyl anacardate (0.1034 g) in methanol (6 cm^3), potassium periodate (0.2 g) in 1 M sulfuric acid solution (10 cm^3) was added in one portion. An immediate odor of heptanal was observed and, after 45 min, the reaction mixture was worked-up by dilution with water and extraction with ether. An aliquot of the extract was concentrated and the residual material (0.0518 g) (R_f 0.80, solvent B), in methanol (2 cm^3) was treated with sodium borohydride (0.0803 g) during 30 min. The mixture was acidified and the product was recovered by extraction with ether. It contained one prominent spot (R_f 0.20, solvent B) in agreement with the reference compound methyl (6-hydroxyhexyl)salicylate methyl ether. It possessed by GLC, RRT (column B, 200 C) 8.35 min. By comparison with the reference compounds, methyl (3-hydroxypropyl)salicylate methyl ether (log [RRT] 1.23), the 6-hydroxyhexyl compound (log [RRT] 1.63) and the 7-hydroxyheptyl compound (log [RRT] 1.78), and a plot of their log (RRT) against respective n value, the natural degradation product was clearly methyl (8-hydroxyoctyl) salicylate methyl ether with log (RRT) 1.92. The preparatively purified reduction product (TLC solvent, chloroform/ethyl acetate, 80:20, v/v) gave a single GLC peak. MS, m/e 294.1832 (M^+). $C_{17}H_{26}O_4$ requires 294.1830.

8-(3,5-Dimethoxyphenyl)octanol from Cardol

Cardol (0.4115 g, 1.302×10^{-3} mol, average molecular wt 316) in dichloromethane (13 cm^3) and water (9.6 cm^3) containing 3 M sodium hydroxide solution (2.90 cm^3) and Triton B (0.20 cm^3) was vibromixed with dimethyl sulfate (0.8 cm^3). Methylation was soon effected (2 hr), as shown by the two clear layers formed and TLC monitoring (solvent C). The mixture was acidified, extracted with ether and the recovered organic material was purified by preparative TLC (solvent C) which removed dimethyl sulfate and a small amount of polymeric material and gave cardol dimethyl ether, ^1H NMR (CCl_4) δ, 6.03 (m, 3H, HAr), 4.72-5.27 (m, CH=CH, $CH=CH_2$), 3.77 (2s, 6H, $2CH_3O$), 2.37-2.92 (m, CH_2Ar, CH_2 $(CH=CH)_2$), 1.77-2.24 (m, $CH_2CH=CH$), 1.30 [m, $(CH_2)_n$], and 0.70-1.03 (t, CH_3).

Cardol dimethyl ether (0.1925 g) in 98% formic acid (3 cm^3) was stirred and treated gradually with 30% hydrogen peroxide solution (0.25 cm^3) during 24 hr. After work-up as before, an oil (0.2318 g) was recovered. TLC indicated the presence of some unchanged cardol dimethyl ether. ^1H NMR indicated the presence of formate ester (δ CCl_4 9.07) and modified aromatic absorption consistent with the presence of a quinone resulting from nuclear hydroxylation. The material was hydrolyzed with M sodium hydroxide solution (3 cm^3) at ambient temperature under nitrogen, during which the reaction mixture became deep brown in color. The alkaline mixture after 16 hr was diluted with water, extracted with ether and recovery gave a yellow oil (0.0451 g) having ^1H NMR signals for hydroxylated cardol dimethyl ether. Acidification of the alkaline layer and extraction with ether gave, upon recovery, colored and acidic material (0.113 g) which lacked a methoxyl group and evidently comprised several substances.

The neutral product in methanol (3 cm^3) was stirred and treated with 2% periodic acid in M sulfuric acid solution (2.0 cm^3) and the mixture, after 16 hr, was diluted with water and extracted with ether. The recovered product (0.0241 g), which had a pronounced odor of *n*-heptanal, was stirred in methanol (2.5 cm^3) and the mixture was treated with sodium borohydride (0.1108 g). The solution was acidified and extracted with ether; the recovered product was purified by preparative TLC (solvent B). A band corresponding in R_f to synthetic 8-(3,5-dimethoxyphenyl)octanol was recovered. GLC examination (column B, 180 C) indicated a component with a retention of 16.9 min, (200 C, 6.65 min) identical to that of synthetic 8-(3, 5-dimethoxyphenyl)octanol. By GLC/MS, m/e 266 (M^+). $C_{16}H_{26}O_3$ requires 266.

RESULTS AND DISCUSSION

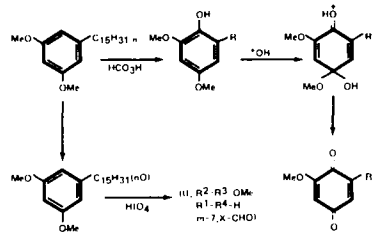
The synthesis of 8-(3-methoxyphenyl)octanoic acid and of 8-(3,5-dimethoxyphenyl)octanoic acid from the available heptanols (1,12) proceeded smoothly from the corresponding alkyl bromides to the nitriles, the hydrolysis of which afforded the required products. The acids were readily converted to the corresponding alcohols and then to the aldehydes, which were characterized as their 2,4-dinitrophenyl hydrazones for the first time. 6-(3-Hydroxy-

octyl)salicylic acid methyl ether could not be prepared from (HO-protected) 8-chlorooctanol and we were unable to find a selective reagent for the reduction for the terminal carboxyl group in 6-(7-carboxyheptyl)salicylic acid methyl ether. Because of this, identification of the natural degradation product from dimethyl anacardate was effected by a chromatographic method with the appropriate homologous reference compounds which could be synthesized.

Methylation of the component phenols, cardanol, cardol and anacardic was readily achieved by the phase transfer technique. With anacardic acid, dimethyl anacardate was formed whereas, with diazomethane, only the carboxyl group was methylated. The formation of dimethyl anacardate in this reaction has been described (17), although the product was not characterized and we believe the ensuing reactions in that report actually had been carried out unknowingly with methyl anacardate.

We have examined a number of ways of oxidatively degrading the methylated component phenols of *A. occidentale*. Neither initial hydroxylation with potassium permanganate (18), with osmic acid (19) (or in combination with potassium permanganate), acetoxylation with lead tetraacetate, ozonide formation (20), nor iodine and silver benzoate (21,22) (Prévost reagent), which led to ring iodination, was suited for our purpose. Hydroxylation with performic acid (from hydrogen peroxide and formic acid) proceeded smoothly with cardanol methyl ether and dimethyl anacardate, however, and the Malaprade reaction then led to specific cleavage of the mixture of vicinal diols. A number of volatile aldehydes, formaldehyde from trienes, butanal from dienes and heptanal from monoenes, were identified by TLC and GLC of their 2,4-dinitrophenylhydrazones and this work will be described separately. In our experience, oxidation of the aromatic aldehydic fragment by potassium permanganate in acetone solution as reported in low yield (3) did not proceed efficiently because of spectral evidence of aldehyde self-condensation and interaction with the solvent. Reduction of the reaction product with sodium borohydride in methanol occurred smoothly.

Although side-reactions involving the aromatic ring have been reported in the interaction of various peracids with alkyl benzenes and phenolic methyl ethers, such as double bond cleavage and quinone formation (23), demethoxylation accompanying quinone formation (24,25), and alkyl group migration (26), we have found that cardanol methyl ether and cardanol dimethyl anacardate reacted essentially at the side-chain and were immune to such nuclear oxidation probably due to steric hindrance and additionally some deactivation in the latter compound. With cardol dimethyl ether, side-chain hydroxylation was accompanied by nuclear oxidation, although reaction mixtures still contained some unreacted starting material. In this compound, sufficient activation evidently exists to induce some quinone formation and demethoxylation as evidenced by our spectral information. The reaction sequence may well be as shown (Scheme 2) (R = $C_{15}H_{31-n}$ or a modified grouping).



SCHEME 2

The constituents of the component phenols of *A. occidentale* and of the *Rhus* genus all have unsaturation commencing at the 8-position as confirmed in this work for the first group of compounds. Recently, with other products, unsaturation has been observed at other positions as in the phenols of *Grevillea pyramidalis* (27) (C_{15} resorcinol with unsaturation at the 10-position), *Persoona elliptica* (28) (C_{11} resorcinol with unsaturation at the 3-position), and *Cystophora torulosa* (29) (C_{14} and C_{18} resorcinols unsaturated at the 5-position). In *Pistachio vera* (C_{13}, C_{15} and C_{17} anacardic acids) monounsaturation has been found at the 8-position (J.H.P. Tyman and M. Yalpani, unpublished work).

The GLC procedure used in the present work with homologous synthetic compounds for determining the first double bond position in the side-chain of constituents of anacardic acid has, in our view, a wider use for phenolic and other lipids. Related homologous compounds have been prepared and this work will be reported subsequently.

The conversion of the methylated component phenols of *A. occidentale* into water-soluble acids also represents an alternative way of utilizing the natural products (cf. ref. 30), provided such conversions can be effected economically.

REFERENCES

1. Caplin, J., and Tyman, J.H.P. (1982) J. Chem

Res. (S), 34-35; (M) 0321-0351.
2. Tyman, J.H.P. (1979) Chem. Soc. Rev. 8, 499-537.
3. Symes, W.F., and Dawson, C.R. (1953) J. Am. Chem. Soc. 75, 4952-4957.
4. Symes, W.F., and Dawson, C.R. (1956) Nature London 171, 841.
5. Paul, V.J., and Yeddanapalli, L.M. (1956) J. Am. Chem. Soc. 78, 5675-5678.
6. Harvey, M.T., and Caplan, S. (1940) Ind. Eng. Chem. 32, 1306-1310.
7. Backer, H.J., and Haack, N.H. (1941) Rec. Trav. Chim. 60, 661-677.
8. Tyman, J.H.P. (1976) J. Org. Chem. 41, 894 (Suppl. 6 pp).
9. Durrani, A.A., and Tyman, J.H.P. (1979) J. Chem. Soc. Perkin Trans. 1, 2079-2087.
10. Lamberton, J.A. (1959) Aust. J. Chem. 12, 234-239.
11. Tyman, J.H.P. (1973) J. Chem. Soc. Perkin Trans. 1, 1639-1647.
12. Baylis, C.J., Odle, S.W.D. (in part) and Tyman, J.H.P. (1981) J. Chem. Soc. Perkin Trans. 1, 132-141.
13. Corey, E.J., and Suggs, J.W. (1975) Tetrahedron Lett. 2647-2650.
14. Lam, S.K., and Tyman, J.H.P. (1981) J. Chem. Soc. Perkin Trans. 1, 1942-1952.
15. Occolowitz, J.L. (1964) Anal. Chem., 36, 2177-2181.
16. Tyman, J.H.P. (1977) J. Chromatogr. 136, 289-300.
17. Izzo, P.T., and Dawson, C.R. (1949) J. Org. Chem. 14, 1039-1047.
18. King, G. (1938) J. Chem. Soc. 1826-1828.
19. Bader, A.R. (1948) J. Am. Chem. Soc. 70, 3938-3939.
20. Maggiolo, J. (1963) J. Am. Oil Chem. Soc. 49, 161-164.
21. Sletzinger, M., and Dawson, C.R. (1946) J. Am. Chem. Soc. 68, 345.
22. Loev, B., and Dawson, C.R. (1958) J. Am. Chem. Soc. 80, 643-645.
23. Fernholz, H. (1951) Chem. Ber. 84, 110-122.
24. Friess, S.L., Soloway, A.A., Morse, B.K., and Ingersoll, W.C. (1952) J. Am. Chem. Soc. 74, 1305-1309.
25. Davidge, H., Davis, A.G., Kenyon, J., and Mason, R.F. (1958) J. Chem. Soc. 4569-4573.
26. Chambers, R.D., Coggen, P., and Musgrave, W.K.R. (1959) J. Chem. Soc. 1804-1807.
27. Occolowitz, J.L., and Wright, A.S. (1962) Aust. J. Chem. 15, 858-861.
28. Cannon, J.R., and Metcalf, B.W. (1971) Aust. J J. Chem. 24, 1925-1931.
29. Gregson, R.O., Kazlauskas, R., Murphy, P.T., and Wells, R.J. (1977) Aust. J. Chem 30, 2577-2582.
30. Durrani, A.A., Hawkes, A.J., and Tyman, J.H.P. (1980) Eur. Patent (applied for) 15,761; Chem. Abst. (1980), 93, 240530.

[Received December 8, 1981]

COMMUNICATIONS

Stereospecificity of Premature Human Infant Lingual Lipase[1]

ROBERT G. JENSEN[a], F.A. DEJONG[a], R.M. CLARK[a], LISA G. PALMGREN[a], TERESA H. LIAO[b] and MARGIT HAMOSH[b], [a]*Department of Nutritional Sciences, University of Connecticut, Storrs, CT 06268;* and [b]*Department of Pediatrics, Georgetown University Medical School, Washington, DC 20007*

ABSTRACT

The lingual lipase in gastric aspirates from premature infants was found to be partially stereospecific for *sn*-3 esters of synthetic enantiometric triacylglycerols containing 18:1 and 16:0. The *sn*-3 ester was hydrolyzed about 4 times faster than the acid at the *sn*-1 position with no difference in rates between 18:1 and 16:0. The *sn*-2 was also hydrolyzed to some extent.
Lipids 17:570-572, 1982.

INTRODUCTION

Lingual lipase is secreted from von Ebner's glands located on the posterior surface of the tongue in humans and other species (1). The enzyme starts digestion of dietary TG in the stomach, producing DG, MG, FFA and glycerol, and contributes significantly to lipolysis of dietary TG in preterm and term infants whose pancreatic function and syntheses of bile salts have not fully developed. The delivery of the MG and FFA formed by lingual lipase into the small intestine should initiate the immediate formation of mixed micelles followed by rapid absorption. The enzyme assists in the digestion of milk fat beyond initial lipolysis. Native milk fat globules have been reported as being resistant to the action of pancreatic lipase under the conditions studied but prior exposure of the globules to lingual lipase increased the rate of lipolysis (2). The enzyme therefore enhances absorption of dietary lipids at a crucial period in the infant's life.

The identity of the digestion products passed from the stomach into the small intestine will be controlled in part by the specificity of lingual lipase. Rat lingual lipase is partially specific for primary esters of TG and for the *sn*-3 as compared to the *sn*-1 ester (3). This latter type of preferential lipolysis has been termed stereospecificity. We have investigated the stereospecificity of lingual lipase from premature infants using synthetic TG as substrates and present our findings in this paper. A preliminary report on these data has been published (4).

MATERIALS AND METHODS

Gastric aspirates containing the lingual lipase were obtained as part of routine postnatal care at the Georgetown University Hospital. The samples were taken from infants whose gestational age was 33 to 42 weeks and the sample volumes were 0.5-5.0 ml. The samples were assayed for lipolytic activity and 32 with the highest levels were frozen on dry ice and sent to the University of Connecticut in a Styrofoam shipper. All arrived and were stored in a freezer at -75 C.

The digestion mixture was 0.1% citrate-Na_2HPO_4 buffer at pH 5.4, which contained 5% bovine plasma albumin and 0.1% gum arabic with a final volume of 25-50 ml. Prior to digestion, 2% of the desired TG was added, melted by heating on a steam bath if necessary and emulsified with a Branson Sonifier. The mixture was equilibrated at 37 C in a water bath and the gastric aspirate was added. The amount of aspirate used varied depending on the volume of the sample, but usually 2 equal portions were used, one for the TG and one for a control without substrate. Two samples were divided into 3 portions and both enantiomers

[1]Scientific contribution no. 949, Storrs Agricultural Experiment Station, University of Connecticut, Storrs, CT 06268.

Abbreviations: TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acids; *sn*-18:1-16:0-16:0, 1-oleoyl-2,3-dipalmitoyl-*sn*-glycerol; *sn*-16:0-16:0-18:1, 1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol; and *sn*-16:0-18:1-18:1, 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol.

were digested separately because of the relatively large volume. This procedure was necessary, as was the addition of an internal standard later, because each aspirate contained some lipid. We wanted to avoid the risk of denaturing the lipase by extraction of the aspirates with solvent. The length of incubation was 30-60 min depending on the activity of the aspirate.

The TG used as substrates were: *sn*-18:1-16:0-16:0, *sn*-16:0-16:0-18:1, *sn*-16:0-18:1-18:1 and trioleoylglycerol. The TG were synthesized as described by Jensen and Pitas (5).

After the desired period of incubation, the samples were extracted, the digestion products were separated by thin layer chromatography and the fatty acids were identified by gas liquid chromatography (GLC) after conversion to methyl esters (6,7). A known amount of methyl heptadecanoate was added to each fraction prior to analysis by GLC. The fatty acids in the aspirate controls were subtracted from each analysis of digestion product after equalizing the internal standards and adjusting the amounts of other acids accordingly.

To check our findings on stereospecificity, we determined specific rotations of the 1,2-(2,3) DG from a digestion of trioleoylglycerol. The *sn*-1,2 dioleoylglycerol has a specific rotation of -2.8 in $CHCl_3$ (8), which we confirmed with a standard. The specific rotation of the *sn*-2,3 enantiomer is +2.8. If a lipase is stereospecific, the optical rotation of DG formed by the enzyme would approach one or the other of these figures. A nonspecific lipase would produce a racemic mixture of DG with no optical rotation.

RESULTS AND DISCUSSION

The compositions of the original TG and the MG and FFA from the digestions of the TG are presented in Table 1. The preponderance of *sn*-3 acid in the FFA is clear evidence for the partial stereospecificity of lingual lipase—about 4:1 for the *sn*-3 ester. The composition of the MG was mostly the acid originally in the *sn*-2 position. The acid in the *sn*-3 position, whether 16:0 or 18:1, was hydrolyzed at equimolar rates. We calculated these data by reference to the internal standard. There was some digestion of *sn*-2 esters in contrast to pancreatic lipase which is very specific for the *sn*-1 and -3 positions.

Further confirmation of our data was provided by our determination of the specific rotation of the DG produced by the lipase. The specific rotation of the 1,2-(2,3) DG recovered from the digestion of trioleoylglycerol was -2.8°. The configuration of the DG was therefore almost totally *sn*-1,2 as the reported rotation is -2.8° (8). This overlooked method for the determination of lipase specificity has the advantages of being nondestructive and simple. The disadvantage is lack of sensitivity. The specific rotation of either enantiomeric DG is so small that dilution with the antipode would obliterate the difference between 2.8° and zero. Preparation of a derivative would increase the specific rotation and improve the sensitivity.

Stereospecificity is a novel characteristic in glycerol ester hydrolases. The property has been observed only in various serum stimulated lipoprotein lipases (9), rat lingual lipase (3) and hepatic lipoprotein lipase (10). Interestingly, the specificity of the lipoprotein lipases is partially for the *sn*-1 position. We cannot explain the physiological significance of the *sn*-3 specificity of the lingual lipases. However, we can postulate that the *sn*-1,2 DG are better substrates for pancreatic lipase than the enantiomers, although in vitro, the rates of digestion are influenced by the fatty acid composition of the DG. Morley et al. (9) noted that the dipalmitoyl DG accumulated when either

TABLE 1

Fatty Acid Composition of the Original Triacylglycerols, and the Monoacylglycerols and Free Fatty Acids Produced by Lipolysis with Premature Human Infant Lingual Lipase

Substrate and enzyme source	N	Triacylglycerol		Monoacylglycerol		Free fatty acids	
		16:0	18:1	16:0	18:1	16:0	18:1
Human		(M%)					
sn-16:0-16:0-18:1[a]	5	64.2	35.8	84.8	15.2	24.4	75.6
SEM		0.35		7.10		5.13	
sn-18:1-16:0-16:0	5	64.4	35.2	77.8	22.2	85.8	14.2
SEM		0.56		6.28		2.20	
sn-16:0-18:1-18:1	2	34.0	66.0	27.5	72.5	12.5	87.5

[a]1,2-Dipalmitoyl-3-oleoyl-*sn*-glycerol.

sn-16:0-16:0-18:1 or *sn*-18:1-16:0-16:0 was digested with pancreatic lipase. We can also postulate that the *sn*-1,2-DG act as messengers for receptor sites in the intestine, much as FFA stimulate the release of cholecystokinin from the intestinal wall (11). Another possibility might be related to the inability of the enzyme to hydrolyze the esterified fatty acids of *sn*-3 phosphatidylcholine, although the influence is not obvious (12). To summarize, the lipase in the gastric aspirates of newborn premature infants is partially stereospecific for *sn*-3 esters of TG.

ACKNOWLEDGMENTS

This work was supported in part by federal funds made available through the provision of the Hatch Act, by the University of Connecticut Research Foundation and NIH grant HD-10823.

REFERENCES

1. Hamosh, M. (1981) in Textbook of Gastroenterology and Nutrition in Infants (Lebenthal, E., ed.), pp. 445-463, Raven Press, New York, NY.
2. Plucinski, T.M., Hamosh, H., and Hamosh, P. (1979) Am. J. Physiol. 237, E541.
3. Paltauf, F., Esfandi, F., and Holasek, A. (1974) FEBS Lett. 40, 119-124.
4. Jensen, R.G., Fey, M.B., Lambert, L.G., and Hamosh, M. (1981) J. Am. Oil Chem. Soc. 59, 133A.
5. Jensen, R.G., and Pitas, R.E. (1976) in Advances in Lipid Research (Paoletti, R., and Kritchevsky, D., eds.), Vol. 14, pp. 213-247, Academic Press, New York, NY.
6. Sampugna, J., and Jensen, R.G. (1968) Lipids 3, 519-529.
7. Pitas, R.E., and Jensen, R.G. (1974) Lipids 9, 729.
8. Buchnea, D. (1978) in Handbook of Lipid Research: Fatty Acids and Glycerides (Kuksis, A., ed.), Vol. 1, pp. 233-287, Plenum Press, New York, NY.
9. Morley, N.H., Kuksis, A., and Buchnea, D. (1974) Lipids 9, 481-488.
10. Akesson, B., Gronowitz, S., and Herslof, B. (1976) FEBS Lett. 71, 241-244.
11. Lebenthal, E., and Lee, P.C. (1981) in Textbook of Gastroenterology and Nutrition in Infancy (Lebenthal, E., ed.) pp. 1-7, Raven Press, New York, NY.
12. Liau, P.H., Hamosh, M., Scanlan, J.W., and Hamosh, P. (1981) Clin. Res. 29, 309A.

[Received April 2, 1982]

The Effects of Fasting and Streptozotocin Diabetes on the Triglyceride Lipase Activity of Rat Liver Plasma Membranes

TAKAHIDE NOMURA, AKIHISA IGUCHI, HITOSHI MATSUNAGA and **NOBUO SAKAMOTO**, *The Third Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, 466, Japan*

ABSTRACT

The activity of hepatic triglyceride lipase (H-TGL) of plasma membranes isolated from rat liver is shown to be reduced by fasting. Refeeding restores the enzyme activity. The suppressed activity of H-TGL in streptozotocin diabetic rats is restored by insulin treatment. The behavior of the enzyme activites in both situations coincides with that of plasma insulin levels. The results suggest that the H-TGL of rat liver plasma membranes is under hormonal regulation by insulin.
Lipids 17:573-575, 1982.

INTRODUCTION

The presence of triglyceride lipase in the liver is well known. However, the function of the enzyme remains obscure. Assmann et al. (1) conducted a study on subcellular distribution of the lipase in rat liver and first reported that triglyceride lipase released from liver by heparin originates primarily in the plasma membranes. Groener and Knauer (2) documented the fact that the rat liver had only one alkaline triglyceride lipase and most of it resided on the plasma membranes. It is also known that hepatocytes isolated by the collagenase method lack the enzyme activity (3), suggesting the presence of the enzyme in the outer surface of the cells. Such a localization of the lipase in the liver suggests a role of the enzyme in extracellular lipid metabolism, in the hydrolysis of serum lipid. Nevertheless, no attempt has been made to clarify the function of the lipase associated with plasma membranes.

The aim of this study was to ascertain whether hepatic triglyceride lipase (H-TGL) in plasma membranes isolated from rat liver is changed by alteration of the hormonal environment, e.g., fasting and streptozotocin diabetes. The results strongly suggest that plasma membrane-associated H-TGL is under hormonal regulation by insulin.

MATERIALS AND METHODS

Male Wistar rats (250-300 g) were used. Standard laboratory chow containing 5.1% (w/w) lipid was obtained from the Oriental Yeast Co. (Tokyo, Japan). In the fasting experiments, the animals were deprived of food for 48 hr. Some fasted animals were refed for 12 hr. Diabetes was induced by a single intravenous injection of streptozotocin (STZ) (Sigma Co., St. Louis, MO) 65 mg/kg body weight. Some diabetic rats were treated by daily subcutaneous injection of lente insulin (Novo Laboratory, Copenhagen, Denmark), 4 units per animal at 5 p.m. for 3 days. The animals were fed on the diet ad libitum. The rats were killed by decapitation in pairs at 9 a.m. The blood was collected in tubes for the determination of serum triglyceride (Glycerol and Neutralfat UV Test, Boehringer, Mannheim, West Germany) and plasma immunoreactive insulin (IRI) (IRI Radio-immunoassay Kit, Dainabot, Tokyo, Japan).

The livers were immediately excised. Partially purified plasma membranes from the rat livers were prepared by a modification of the procedure of Neville (4) as described by Ray (5). In our determination (6), the specific activity of 5′-nucleotidase in the plasma membranes was ca. 20 times higher than the homogenate. The plasma membranes' protein content was quantified by the method of Lowry et al. (7) with bovine serum albumin as the standard. Part of the obtained plasma membranes (5 mg protein/ml of 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$, pH 7.5) was incubated in the presence of 50 units of heparin (Sigma) per ml for 15 min at 37 C. After the end of incubation, plasma membranes were sedimented for 15 min (4 C) at 50,000 x g and the supernatant was obtained as heparin-released material. Preliminary data showed that 50 units per ml of heparin was sufficient to cause maximal solubilization (65%) of H-TGL from plasma membranes. The H-TGL activity of plasma membranes and heparin-released material was measured as described by Assman et al. (1) using emulsified tri-[1-^{14}C]oleate as a substrate. The fatty acids were extracted by the

method of Schotz et al. (8) and counted using the Aloka liquid scintillation system LSC-751, equipped with automatic external standardization to measure quenching (Aloka, Tokyo, Japan).

RESULTS AND DISCUSSION

H-TGL activity located on the plasma membranes was reduced by fasting, whereas the activity was partially restored by refeeding (Table 1). The H-TGL activity of heparin-released material also changed similarly under these conditions. STZ diabetes produced a marked decrease both in plasma membrane-bound and heparin-released lipase activities (Table 2). The reduced activities were partially restored by insulin treatment.

Thus, this study first made it clear that H-TGL activity located on plasma membranes changed dramatically according to the alteration in hormonal environment, e.g., fasting and STZ diabetes. The similar effects of fasting and STZ diabetes on H-TGL activity have been demonstrated by the measurement of the enzyme activity in the acetone/ether extracts of liver (9) or in the post-heparin plasma (10). However, we emphasize the importance of direct measurement of plasma membrane-bound H-TGL for the elucidation of the function of the enzyme in the liver because of the following two lines of reasoning. First, when H-TGL activity in post-heparin plasma is measured, it covers not only the enzyme activity originating in the liver, but also the activity from the ovaries and adrenal glands of rats (11). Second, recent studies (1,2) have indicated that H-TGL of the liver is mainly located on the plasma membranes and is the origin of the lipase released from liver by heparin.

The plasma IRI level of fasted rats was 21.0 ± 3.4 μU/ml (mean ± SEM) and was significantly ($p<0.05$) lower than the fed rats (60.3 ± 8.3 μU/ml). Refeeding produced a marked rise ($p<0.05$) in the IRI level (73.7 ± 11.4 μU/ml). The plasma IRI level of the diabetic rats was reduced ($p<0.0.5$) to 12.2 ± 3.2 μU/ml (the normal rats had a level of 55.0 ± 8.5 μU/ml). These results indicate that the changes of the enzyme activities coincide with those of the plasma IRI levels and strongly suggest that the H-TGL associated with plasma membranes is under hormonal regulation by insulin.

The underlying mechanism of the insulin action, however, is not yet clear. H-TGL is assumed to be synthesized and secreted by parenchymal cells and bound to cell membranes, perhaps of nonparenchymal cells (12). Insulin might regulate some of the above processes and change the amount of the enzyme associated with plasma membranes.

TABLE 1

Effect of Fasting and Refeeding on Hepatic Triglyceride Lipase Activity of Rat Liver Plasma Membranes[a]

	Hepatic triglyceride lipase activity (μmol/hr/mg protein)	
	Plasma membranes	Heparin-released
Fed	0.59 ± 0.11	1.26 ± 0.31
Fasted (48 hr)	0.15 ± 0.03[b]	0.16 ± 0.04[b]
Fasted (48 hr)-refed (12 hr)	0.38 ± 0.04[c]	0.61 ± 0.11[c]

[a]Five pairs of rats were killed. Results are expressed as means ± SEM.

p values are statistical differences from fed rats; [b] $p<0.05$, from fasted rats; [c] $p<0.05$.

TABLE 2

Effect of Streptozotocin Diabetes and Insulin Treatment on Hepatic Triglyceride Lipase Activity of Rat Liver Plasma Membranes[a]

	Hepatic triglyceride lipase activity (μmol/hr/mg protein)	
	Plasma membranes	Heparin-released
Normal	0.49 ± 0.09	1.18 ± 0.27
STZ	0.18 ± 0.04[b]	0.26 ± 0.10[b]
STZ + Insulin	0.37 ± 0.05[c]	0.74 ± 0.15[c]

[a]Six pairs of rats were killed. Results are expressed as means ± SEM.

p values are statistical differences from normal rats; [b] $p<0.05$, from STZ; [c] $p<0.05$.

H-TGL located on the plasma membranes is believed to participate in the extracellular lipid metabolism, e.g., the catabolism of very low density lipoproteins (VLDL) and chylomicron remnants (13). However, recent works have presented evidence that H-TGL activity measured in vitro against VLDL triglyceride or artificial triglyceride emulsion is inhibited in the presence of whole serum (14) or apoproteins like C (15), A-I and A-II (16). These findings make the physiological significance of the enzyme more obscure. Our serum triglyceride determination revealed a typical hypertriglyceridemia (313 ± 27 mg/dl) in diabetic rats, compared to 87 ± 14 mg/dl in normal rats. Whether the reduced H-TGL activity of plasma membranes in diabetic animals is a contributing factor to the elevation of serum triglyceride needs to be further investigated.

ACKNOWLEDGMENTS

We thank M. Ohuchi for technical assistance and M. Hayashi for her assistance in manuscript preparation. We are grateful to Prof. R.A. Harris for valuable comments on the manuscript.

REFERENCES

1. Assmann, G., Krauss, R.M., Fredrickson, D.S., and Levy, R.I. (1973) J. Biol. Chem. 248, 1992-1999.
2. Groener, J.E.M., and Knauer, T. (1981) Biochim. Biophys. Acta 665, 306-316.
3. Thomas, J., Debeer, L.J., and Mannaerts, G.P. (1978) Biochem. J. 172, 177-179.
4. Neville, D.M., Jr. (1960) J. Biophys. Biochem. Cytol. 8, 413-422.
5. Ray, T.K. (1970) Biochim. Biophys. Acta 196, 1-9.
6. Lesko, L., Donlon, M., Marinetti, G.V., and Hare, J.D. (1973) Biochim. Biophys. Acta 311, 173-179.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
8. Schotz, M.C., Garfinkel, A.S., Huebotter, R.J., and Stewart, J.E. (1970) J. Lipid Res. 11, 68-69.
9. Elkeles, R.S., and Hambley, J. (1977) Diabetes 26, 58-60.
10. Nakai, T., Yamada, S., Tamai, T., Kobayashi, T., Hayashi, T., and Takeda, R. (1979) Metabolism 28, 30-40.
11. Jansen, H., and Hülsmann, W.C. (1980) TIBS 5, 265-268.
12. Jansen, H., Kalkman, C., Zonneveld, A.J., and Hülsmann, W.C. (1979) FEBS Lett. 98, 299-302.
13. Olivecrona, T., Bengtsson, G., Marklund, S.E., Lindahl, U., and Höök, M. (1977) Fed. Proc. 36, 60-65.
14. Berry, E.M., Aldini, R., Bar-On, H., and Eisenberg, S. (1981) Eur. J. Clin. Invest. 11, 151-159.
15. Kinnunen, P.K.J., and Ehnholm, C. (1976) FEBS Lett. 65, 354-357.
16. Kubo, M., Matsuzawa, Y., Tajima, S., Ishikawa, K., Yamamoto, A., and Tarui, S. (1981) Biochem. Biophys. Res. Commun. 100, 261-266.

[Received April 13, 1982]

Increased Thromboxane B_2 Biosynthesis in Platelets

H. KAWAGUCHI*, T. ISHIBASHI and **Y. IMAI,** *Department of Biochemistry, School of Medicine, Hokkaido University, Sapporo 060, Japan*

ABSTRACT

The synthesis of thromboxane B_2 is increased in platelets from rabbits with experimental hypercholesterolemia, but the increase is not due to increased phospholipids hydrolysis. We have clarified the mechanism for the increased thromboxane synthesis. The biosyntheses of prostaglandin H_2 and thromboxane B_2 were unaffected by superoxide dismutase, xanthine oxidase, mannitol, or benzoate in other experiments designed to study the possible involvement of reactive oxygen species. These results suggest that $O_2^{\cdot -}$ and $OH^{\cdot}$ were not likely to be involved as intermediates in the synthesis of prostaglandin H_2 and thromboxane B_2 in platelets. The rate of prostaglandin H_2 biosynthesis was promoted in deuterium oxide, and this deuterium oxide enhancement effect was reversed by 2,5-diphenylfuran, suggesting that singlet oxygen may be involved in prostaglandin H_2 biosynthesis. The biosynthesis of prostaglandin H_2 was promoted by ADP-Fe^{3+} but inhibited by EDTA and EDTA-Fe^{3+}. The effect of ADP-Fe^{3+} could not be replaced by EDTA-Fe^{3+}. The effects of glutathione, glutathione peroxidase and H_2O_2 on cyclooxygenase and thromboxane synthetase were studied by using partially purified enzymes and platelet microsomes. Glutathione and glutathione peroxidase inhibited the activity of cyclooxygenase but did not inhibit that of thromboxane synthetase. H_2O_2 caused the inactivation of cyclooxygenase, but the addition of H_2O_2 did not inhibit the formation of thromboxane B_2 from prostaglandin H_2. An examination of glutathione concentration and glutathione peroxidase activity in platelets from normal and experimentally hypercholesterolemic rabbits demonstrated that both were decreased in platelets from later group. The observed alterations in glutathione levels and glutathione peroxidase activity are large enough to cause increased thromboxane B_2 synthesis in platelets but the possibility that other unidentified factors may also contribute cannot be excluded.
Lipids 17:577-584, 1982.

We have shown that the biosynthesis of thromboxane B_2 is increased in the platelets from hypercholesterolemic rabbits (1). This was shown (1) to not be due to increased phospholipids hydrolysis, suggesting that the alteration must be in the efficiency of conversion of free arachidonic acid to thromboxane B_2. It has been well established that the oxygenation of arachidonic acid by the microsomal prostaglandin synthetase complex generates prostaglandins and thromboxanes (2-4). Cyclooxygenase, the first enzyme in the complex to act on arachidonic acid, catalyzes the abstraction of hydrogen atoms from the fatty acids, and incorporates 2 molecules of oxygen into the ensuing organic radical to generate prostaglandin G_2 (5). During this process, the cyclooxygenase is irreversibly deactivated (6). A variety of studies indicate that various reactive oxygen species are involved in various prostaglandin biosynthesis systems. It has been demonstrated that methional and phenol (hydroxy radical scavengers) enhanced oxygen uptake and prostaglandin biosynthesis in microsomes (5-8), suggesting that the hydroxy radical was formed during prostaglandin biosynthesis in microsomes (7). Prostaglandin biosynthesis is stimulated by hemes, indoles (9), and reduced glutathione (10,11). These compounds act either by increasing the rate of oxygen incorporation into fatty acid or by stimulating the metabolism of the cyclic peroxides into a particular prostaglandin. Marnett et al. (8) reported that 1,3-diphenylisobenzofuran (a singlet oxygen scavenger) inhibited the conversion of fatty acids into other products, and concluded that singlet oxygen functioned in the co-oxygenating systems of sheep vesicular glands. However, it is usually difficult to ascertain the direct involvement of reactive oxygen in specific reactions because these species are interconverted rapidly under conditions suitable for prostaglandin synthesis. As previously reported (12), prostacyclin synthetase, but not thromboxane synthetase, is very sensitive to destruction by an oxidizing agent, an observation which may have important physiological implications. We have investigated the mechanism of increased thromboxane B_2 synthesis in experimental hypercholesterolemia in rabbit by studying the effect of various inhibitors and activators of thromboxane B_2 synthesis and effects of various trapping agents for specific types of reactive oxygen species. These studies indicate that decreased glutathione peroxidase activity in platelets from rabbits with experimental hypercholesterolemia

*Author to whom correspondence should be addressed.

can account for increased synthesis of thromboxane B_2, although the possibility that other factors contribute cannot be excluded.

MATERIALS AND METHODS

Chemicals

[1-^{14}C] Arachidonic acid (60.2 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. [1-^{14}C] Prostaglandin H_2 (1 Ci/mol) was prepared as described (2). Arachidonic acid, glutathione reductase, catalase, xanthine oxidase, horseradish peroxidase, superoxide dismutase, cytochrome c, mannitol, deuterium oxide (D_2O), L-tryptophane, and ADP were purchased from Sigma Chemical Co., St. Louis, MO. NADPH, reduced glutathione (GSH), oxidized glutathione (GSSG), and EDTA were purchased from the Kyowa Hakko Co., Tokyo, Japan. N-Ethylmaleimide (NEM), xanthine, and benzoate were obtained from Wako Chemicals, Tokyo, Japan. Prostaglandin (PG) A_2, E_2, $F_{2\alpha}$, H_2, and thromboxane (TX) B_2 were generous gifts from Ono Pharmaceutical Co., Ltd., Osaka, Japan.

Experimental Animals

Hypercholesterolemia was induced in rabbits weighing 3-4 kg by daily feeding with food pellets containing 1% cholesterol for 3 months. The plasma cholesterol levels in control and hypercholesterolemic rabbits were 2 mM and 19 mM, respectively, indicating that hypercholesterolemia was induced in the experimental rabbits. They were divided into four groups.

Preparation of Microsomal Fractions from the Platelets

Blood was collected into a 3.8% solution of sodium citrate (9 vol blood: 1 vol citrate). Platelet-rich plasma was prepared by centrifugation of citrated blood at 200 × g for 10 min. The resultant supernatant was again centrifuged at 2,000 × g for 20 min. The precipitate obtained was washed twice with Tyrode's solution, suspended in 0.1 M Tris-HCl buffer (pH 7.4), and disrupted by sonication (two 30-sec treatment with 2-min intervals), the microsomal preparation was prepared by differential centrifugation (2,000 × g, 15 min; 12,000 × g, 15 min; 105,000 × g, 1 hr). The pellets after the final centrifugation were suspended in the same buffer and stored in small aliquots at -70 C.

Enzyme Assays

The conversion of arachidonic acid to TXB_2 was measured by quantitative radioisotopic thin layer chromatography (TLC). A standard mixture (1.0 ml) containing [1-^{14}C] arachidonic acid (20 μM, 2.22 × 10^5 dpm), 0.1 M Tris-HCl (pH 7.4), and 0.4 mg of platelet microsomal protein was incubated at 37 C for 5 min. The mixture was acidified to pH 3.0 and extracted with ethyl acetate. TLC was performed with ethyl acetate/acetic acid (99:1, v/v) as the solvent as described previously (13). Following development, the plates were visualized with I_2 and the zones corresponding to PG standards were scraped and counted.

The conversion of arachidonic acid to prostaglandin endoperoxides was determined by modification of a previously reported method (14). The standard reaction mixture containing [1-^{14}C] arachidonic acid (10 μM), 0.1 M Tris-HCl (pH 7.4), and 0.4 mg of platelet microsomal protein was incubated at 24 C for 2 min. The extraction with ether was completed within 5 min. All procedures were done within 20 min and TLC was developed at -20 C. The solvent system was ethyl ether/petroleum ether/acetic acid (85:15:0.5, v/v/v). Cyclooxygenase and thromboxane synthetase were prepared as described (15), and the enzyme activities of these enzymes were checked according to their method (15). These enzymes were incubated with PGH_2 or arachidonic acid as follows: (a) a reaction mixture (0.2 ml) containing 10 μM [1-^{14}C] arachidonic acid, 0.5 mM L-tryptophane, 0.1 μM hemoglobin, 0.1 M Tris-HCl (pH 7.4) and cyclooxygenase (0.1 ml) was incubated at 24 C for 30 sec. (b) The reaction mixture (0.2 ml) contained 20 μM [1-^{14}C] PGH_2, 0.5 mM L-tryptophane, 0.1 μM hemoglobin, 0.1 M Tris-HCl (pH 7.4) and thromboxane synthetase (0.1 ml). The mixture was incubated for 1 min at 24 C. Extraction and TLC analysis were performed as already described.

Effect of Various Agents on Prostaglandin Biosynthesis

Compounds were added to standard assay mixture just described for PG endoperoxides and TXB_2.

Other Methods

The activities of NADPH-cytochrome c reductase (16), superoxide dismutase (17), catalase (18), and glutathione peroxidase (19) were assayed as previously described. The H_2O_2, which was generated in the incubation mixture, was determined by the established method (20, 21). The glutathione peroxidase was purified from rabbit blood according to the method of Awasthi et al. (22). The amount of GSH was measured according to the method of Tietze (23). Protein was determined by the Lowry et

al. method (24) with bovine serum albumin as standard. Triplicate samples were analyzed in all experiments.

RESULTS

In earlier work (1), we confirmed that the biosynthesis of TXB_2 by washed platelets was increased about three-fold in hypercholesterolemic rabbits compared to control. The apparent Km values for arachidonic acid were 19 μM (hypercholesterolemia) and 50 μM (control), respectively. It was also demonstrated that increased TXB_2 synthesis was not due to increased release of arachidonic acid from phospholipids, but rather to increased efficiency of conversion of released arachidonic acid to TXB_2 (1). We initiated a series of experiments in an attempt to determine the biochemical basis of this alteration.

Enzyme Activities in Microsomes from Normal and Hypercholesterolemic Rabbits

The initial approach used was to determine if there were significant differences in specific reactive oxygen species which might account for observed differences in TXB_2 synthesis either by inhibiting TXB_2 synthesis in normal platelets or by stimulating it in hypercholesterolemic platelets. The enzymes examined were: (a) NADPH-cytochrome c reductase, which can be expected to alter TXB_2 synthesis by electron transport; (b) superoxide dismutase, which can be expected to alter TXB_2 synthesis by removing superoxide anion radical ($O_2^{\cdot -}$) as has been observed in bovine seminal vesicular glands (8); (c) catalase, which can be expected to alter TXB_2 synthesis by removing H_2O_2 as has been observed in sheep seminal vesicular glands (25); and (d) glutathione peroxidase, which can be expected to alter TXB_2 synthesis because it removes GSH and H_2O_2.

NADPH-cytochrome c reductase was not significantly altered in platelet microsomes from hypercholesterolemic rabbit (HCR) (Table 1). As shown in Table 1, superoxide dismutase was not significantly different in platelet microsomes from normal and HCR, suggesting that differences in the destruction of superoxide anion radical cannot account for the observed differences in TXB_2 synthesis. Additional evidence was provided studying the effect of added superoxide dismutase on TXB_2 synthesis. As shown in Table 2, superoxide dismutase did not show any significant effect on the biosynthesis of TXB_2. Further evidence to support the exclusion of $O_2^{\cdot -}$ as a mediator of PGH_2 and TXB_2 biosyntheses is that there was no significant change in these biosyntheses when xanthine+xanthine oxidase, which generates $O_2^{\cdot -}$ (26,27), was added to the reaction mixture (Table 2). As shown in Table 1, there was a three-fold increase in catalase activity in microsomes from HCR. This increase suggests that hydrogen peroxide may influence the TXB_2 biosynthesis in the platelets from HCR. As shown in Figure 1a, the oxidation of NADPH was observed in the reaction mixture of the TXB_2 biosynthesis system. When a catalase was present in the reaction mixture, the oxidation of NADPH was inhibited about 60% (Fig. 1b). With the addition of horseradish peroxidase, the oxidation of NADPH was markedly promoted (Fig. 1c). These data indicated that H_2O_2 was generated in the TXB_2 biosynthesis system. As shown in Table 2, the addition of H_2O_2 (0.1 mM-30 mM) did not inhibit the biosynthesis of TXB_2. Van Der Quderaa et al. (25) have described that the addition of H_2O_2 or ROOH strongly enhanced the inactivation of

TABLE 1

Effect of Hypercholesterolemia on the Activities of NADPH-Cytochrome c Reductase, Superoxide Dismutase, Catalase and Glutathione Peroxidase[a]

Enzyme activities	Control	Hypercholesterolemia
NADPH-cytochrome c reductase (nmol/min/mg protein)	3.7 ± 0.5	2.8 ± 0.5
Superoxide dismutase (units/mg protein)	1.2 ± 0.2	0.9 ± 0.2
Catalase (units/mg protein)	5.7 ± 0.5	16.6 ± 0.5[b]
Glutathione peroxidase (nmol/min/mg protein)	4.9 ± 0.8	0.4 ± 0.08[b]

[a]The enzyme activities were determined by using platelet microsomes from pooled platelets of two groups. The values given represent the means of four hypercholesterolemic groups and four control groups. Student's t-test was used to analyze the results.

[b]$p<0.01$ compared to control.

TABLE 2

Effect of Various Compounds on Prostaglandin H_2 and Thromboxane B_2 Biosynthesis[a]

Additions	PGH_2 synthesis[b] pmol (% of control)	TXB_2 synthesis	
		Microsomes[c] pmol (% of control)	TX synthetase[d] nmol (% of control)
None (control)	430 ± 51(100)	397 ± 21(100)	1.43 ± 0.1(100)
Catalase, 2500 units	645 ± 45(150)	376 ± 10(95)	1.43 ± 0.05(100)
H_2O_2, 0.1 mM	131 ± 14(30)	416 ± 15(105)	1.43 ± 0.1(100)
30 mM	43 ± 2(10)	458 ± 37(115)	1.14 ± 0.1(80)
Superoxide dismutase, 100 units	430 ± 51(100)	410 ± 15(103)	1.41 ± 0.08(100)
Xanthine, 0.4 mM+xanthine oxidase, 30 μg	427 ± 52(100)	460 ± 40(115)	1.64 ± 0.03(115)
Mannitol, 10 mM	427 ± 52(100)	442 ± 5(110)	1.64 ± 0.03(115)
Benzoate, 10 mM	429 ± 50(100)	397 ± 13(100)	1.43 ± 0.2(100)
2,5-Diphenylfuran, 2 mM	252 ± 6(60)	374 ± 9(95)	1.42 ± 0.06(100)
D_2O in place of H_2O	1083 ± 20(250)	798 ± 33(200)	1.42 ± 0.06(100)
Diphenylfuran, 2 mM, in D_2O	170 ± 13(40)	257 ± 20(65)	1.43 ± 0.05(100)

[a]All conditions were the same as those described in Methods.

[b]Cyclooxygenase was assayed with arachidonic acid. The values represent the means ± SE (pmol/cyclooxygenase 0.1 ml/30 sec).

[c]Assayed with arachidonic acid. The values represent the means ± SE (pmol/mg protein/5 min).

[d]Assayed with PGH_2. The values represent the means ± SE (nmol/TX synthetase 0.1 ml/min).

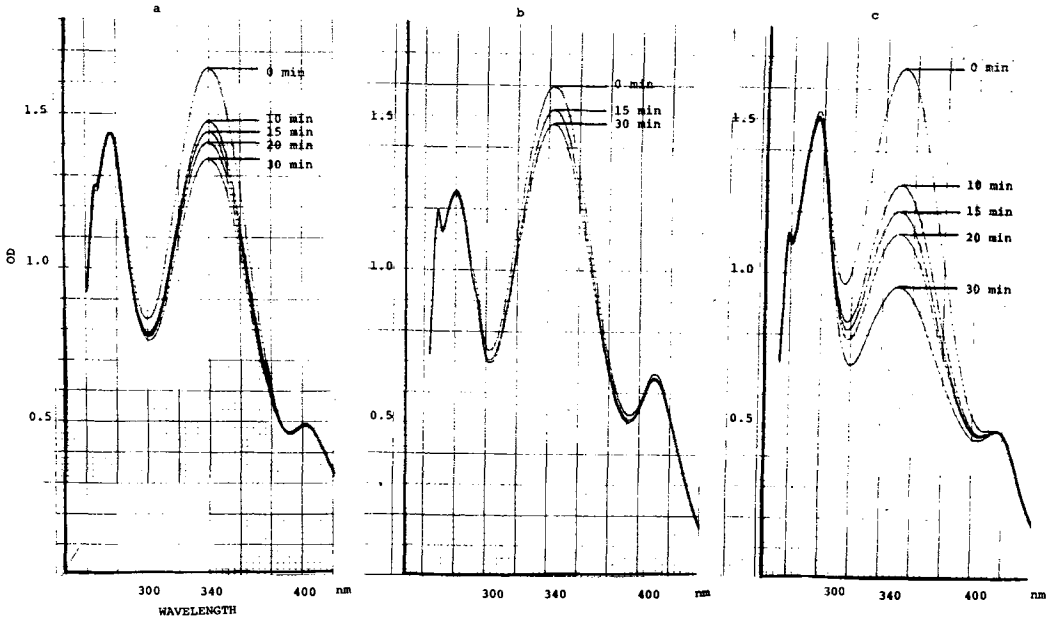


FIG. 1. H_2O_2 generation was determined by the change of NADPH concentration in reaction mixture. a: The standard reaction mixture contained platelet microsomes, 0.4 mg protein; NADPH, 0.4 mM; arachidonic acid, 20 μM; and Tris-HCl, 0.1 M, pH 7.4. b: Standard reaction mixture plus catalase, 2,500 units. c: Standard reaction mixture plus horseradish peroxidase, 600 units.

purified PG synthetase from sheep seminal vesicular glands. In our experiments, cyclooxygenase was strongly inactivated, but the biosynthesis of TXB_2 was not inhibited in any of concentration of H_2O_2 used in the platelet microsomal system and the medium containing thromboxane synthetase. The addition of catalase slightly stimulated PGH_2 biosynthesis, but there was no effect on thromboxane B_2 biosynthesis. These results suggest that the altered catalase activity is probably responsible for PGH_2 synthesis in platelets from HCR. As shown in Table 1, GSH peroxidase activity was suppressed about 92% in platelet microsomes from HCR. The GSH quantity was also decreased about 70% in platelet from HCR. These results suggest that GSH peroxidase may play a role in the increased TXB_2 synthesis in platelets from HCR. Several workers have shown that GSH stimulates the selective enzymatic isomerization of PGH_2 into PGE_2 (10,11). GSH was found to be a powerful stimulator of the formation of PGE_2 at the expense of a reduced formation of $PGF_{2\alpha}$ and other PG. In our standard reaction mixture, 0.4 nmol/5 min/mg protein and 0.6 nmol/2 min/mg protein of arachidonic acid were converted into TXB_2 and PGH_2, respectively. The conversions from arachidonic acid to TXB_2 and PGH_2 in platelet microsomes from HCR were 1.2 nmol/5 min/mg protein and 2.0 nmol/2 min/mg protein, respectively. The zones corresponding to PGE_2 and $PGF_{2\alpha}$ on the TLC were 0.07 nmol/5 min/mg protein and 0.05 nmol/5 min/mg protein, respectively. As shown in Table 3 (A and B), and Figure 2, GSH inhibited the biosynthesis of PGG_2, PGH_2, TXB_2 and oxygenation of arachidonic acid. The PGH_2/PGG_2 ratio increased when GSH peroxidase was added. However, cyclooxygenase activity was suppressed. Thromboxane synthetase activity was not affected by either of them. The levels of TXB_2 biosynthesis in platelet microsomes from normal rabbits and HCR after treatment of GSH peroxidase (20 units) were 0.18 nmol/5 min/mg protein and 0.51 nmol/5 min/mg protein, respectively. The addition of NEM reversed the inhibition of PGH_2 and TXB_2 biosynthesis. Somewhat higher amounts of TXB_2 and PGH_2 were produced by treatment of NEM without GSH.

TABLE 3

Effect of Glutathione and Glutathione Peroxidase on Prostaglandin Synthesis[a]

A

Additions	PGH_2 synthesis[b] pmol (% of control)	TXB_2 synthesis: Microsomes[c] pmol (% of control)	TXB_2 synthesis: TX synthetase[d] nmol (% of control)
None (control)	430 ± 51(100)	397 ± 21(100)	1.43 ± 0.1(100)
GSH, 2 mM	167 ± 13(40)	213 ± 34(53)	1.42 ± 0.1(100)
GSH peroxidase, 20 units	167 ± 16(40)	233 ± 48(58)	1.41 ± 0.03(100)
NEM, 2 mM	602 ± 17(140)	602 ± 36(150)	1.43 ± 0.05(100)
NEM, 2 mM, + GSH, 1 mM	602 ± 21(140)	632 ± 49(160)	1.71 ± 0.1(120)
GSSG, 2 mM	431 ± 50(100)	380 ± 16(96)	1.43 ± 0.1(100)
GSSG reductase, 1 unit	214 ± 11(50)	228 ± 27(58)	1.36 ± 0.07(95)
GSSG, 2 mM, + NADPH, 2 mM + GSSG reductase, 1 unit	86 ± 5(20)	114 ± 2(29)	1.34 ± 0.09(93)
NADPH, 2 mM	431 ± 42(100)	376 ± 17(95)	1.41 ± 0.07(100)

B[e]

Additions	PG synthesis in microsomal system: PGG_2	PGH_2	PGH_2/PGG_2	AA[f]
None (control)	100	100	100	100
GSH, 2 mM	37	47	126	120
GSH peroxidase, 20 units	14	52	371	130
NEM, 2 mM	207	211	100	80

[a]All conditions were the same as those described in Methods.

[b]Cyclooxygenase was assayed with arachidonic acid. The values represent the means ± SE (pmol/cyclooxygenase, 0.1 ml/30 sec).

[c]Assayed with arachidonic acid. The values represent the means ± SE (pmol/mg protein/5 min).

[d]Assayed with PGH_2. The values represent the means ± SE (nmol/TX synthetase, 0.1 ml/min).

[e]The control values were designated as 100.

[f]Unconverted arachidonic acid.

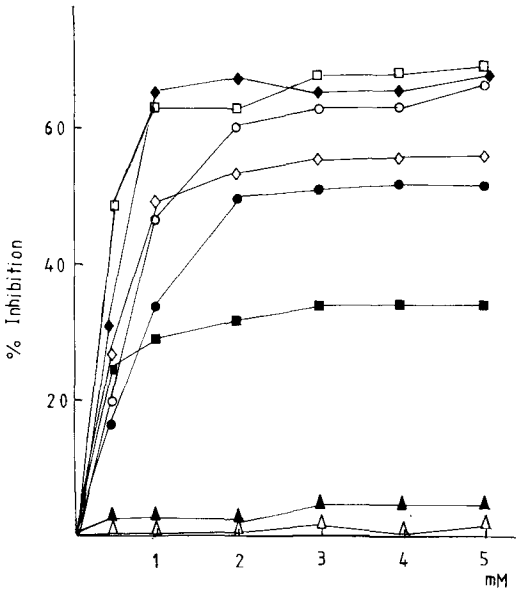


FIG. 2. Effect of GSH (○,●), GSSH (△,▲), DPF (□,■), and EDTA (◇,◆) on PGH_2 biosynthesis (○,△,□,◇) and TXB_2 biosynthesis (●,▲,■,◆). PGH_2 biosynthesis was determined by using cyclooxygenase. TXB_2 biosynthesis was determined by using platelet microsomes. DPF was added to the D_2O-containing medium.

GSSH had no effect on the biosynthesis of TXB_2 (Table 3A, Fig 2.). However, GSSH reductase and GSSH + NADPH + GSSH reductase inhibited PGH_2 biosynthesis (Table 3A). The zone corresponding to PGE_2 on the TLC was slightly increased when GSH peroxidase or GSH was added to the microsomal systems. The extent of increased PGE_2 hardly affected the conversion rate of TXB_2.

Effect of Other Oxidizing Species of TXB_2 Synthesis

The hydroxy radical ($OH^{\cdot}$) is known to be a highly oxidative species, and the generation of $OH^{\cdot}$ in PG biosynthesis has been described previously (7). Mannitol and benzoate have been widely used as scavengers of $OH^{\cdot}$ (21). In our experiments, neither mannitol nor benzoate had any effect on the biosynthesis of TXB_2 (Table 2). These results suggest that the reaction was neither mediated nor inhibited by $OH^{\cdot}$ in both of the cyclooxygenase and thromboxane synthetase systems. 2,5-Diphenylfuran (DPF) has been reported to be a singlet oxygen ($^1O_2$) trap (28). In our investigations, DPF did not affect TXB_2 biosynthesis in our standard microsomal and thromboxane synthetase systems (Table 2), whereas PGH_2 biosynthesis decreased. The rate of PGH_2 biosynthesis increased when the H_2O in the buffer was replaced with D_2O (Table 2), but thromboxane synthetase activity was unaffected. This D_2O enhancement effect is generally observed in singlet oxygen reactions (29,30); therefore, $^1O_2$ probably is a mediator in the biosynthesis of PGH_2.

Effect of Metal Ions on TXB_2 Biosynthesis

To test the possible influence of trace metal ions in the systems under examination, EDTA was added. As shown in Table 4 and Figure 2, the addition of EDTA effectively inhibited the formation of PGH_2 biosynthesis. EDTA-Fe^{3+}

TABLE 4

Effect of Metal Ions on Prostaglandin H_2 and Thromboxane B_2 Biosynthesis[a]

Additions	PGH_2 synthesis[b] pmol (% of control)	TXB_2 synthesis	
		Microsomes[c] pmol (% of control)	TX synthetase[d] nmol (% of control)
None (control)	430 ± 51(100)	397 ± 21(100)	1.43 ± 0.1(100)
Fe^{3+}, 0.1 mM	429 ± 52(100)	415 ± 15(105)	1.43 ± 0.05(100)
EDTA, 1 mM	213 ± 26(50)	143 ± 9(35)	1.19 ± 0.07(85)
EDTA, 1 mM-Fe^{3+}, 0.1 mM	213 ± 26(50)	164 ± 11(42)	1.31 ± 0.09(90)
ADP, 1.5 mM	425 ± 52(100)	417 ± 31(105)	1.43 ± 0.09(100)
ADP, 1.5 mM-Fe^{3+}, 0.1 mM	1267 ± 160(300)	1036 ± 35(250)	1.36 ± 0.05(95)
ADP, 1.5 mM-Fe^{3+}, 0.1 mM + superoxide dismutase, 100 U	427 ± 52(100)	395 ± 15(100)	1.36 ± 0.05(95)

[a]All conditions were the same as those described in Methods.

[b]Cyclooxygenase was assayed with arachidonic acid. The values represent the means ± SE (pmol/cyclooxygenase, 0.1 ml/30 sec).

[c]Assayed with arachidonic acid. The values represent the means ± SE (pmol/mg protein/5 min).

[d]Assayed with PGH_2. The values represent the means ± SE (nmol/TX synthetase, 0.1 ml/min).

also inhibited the biosynthesis of PGH_2. The addition of ADP-Fe^{3+}, however, enhanced the biosynthesis of PGH_2. This enhancement by ADP-Fe^{3+} was completely inhibited by superoxide dismutase (Table 4). ADP-Fe^{3+} was readily reduced by the reactive oxygen species or electrons from the enzyme (31). These results suggest that the reduction of ADP-Fe^{3+} may have promoted the biosynthesis of PGH_2. This conclusion is supported by the observation that superoxide dismutase had no significant effect without ADP-Fe^{3+} in PGH_2 biosynthesis (Table 2).

DISCUSSION

TXB_2 biosynthesis was increased in platelets from HCR. We investigated that the effect of reactive oxygen species, catalase, NADPH cytochrome c reductase, superoxide dismutase and GSH peroxidase on the TXB_2 biosynthesis to study the mechanism of enhanced TXB_2 biosynthesis in platelets from HCR. GSH concentration and GSH peroxidase activity were decreased under the same conditions. Therefore, cyclooxygenase, thromboxane synthetase and platelet microsomes were incubated with either GSH or GSH peroxidase to study the influence of these compounds on the increased biosynthesis of TXB_2. In earlier reports, Smith and Lands demonstrated that GSH peroxidase inhibited the oxygenation of 5,8,11,14-eicosatetraenoate by sheep vesicular gland homogenates, and catalyzed the nucleophilic cleavage of lipid hydroperoxide in the presence of GSH (6,32). Egan et al. have also reported that sulfhydryl-containing compounds such as GSH and DTT inhibited PG cyclooxygenase (5). Ohki et al. have reported that GSH peroxidase catalyzed the conversion of PGG_1 to PGH_1 (33). Our present results may support these previous data. After the addition of GSH peroxidase, the ratio of PGH_2/PGG_2 increased, but the total conversion from arachidonic acid to PG decreased (Table 3). This result shows that GSH peroxidase accelerates the conversion from PGG_2 to PGH_2, but it inhibits the activity of cyclooxygenase. When NEM was added to the reaction mixture, the inhibition by GSH was abolished (Table 3). The stimulatory effect on TXB_2 biosynthesis by GSH has been reported previously (34). However, since the microsomal fraction from the platelets used in that study contained EDTA, there may have some modification of TXB_2 biosynthesis in the assay system. We conclude from our own investigation that GSH and GSH peroxidase inhibit the activity of cyclooxygenase. These results suggest that GSH and GSH peroxidase might affect the TXB_2 biosynthesis in platelets from HCR, especially on PG endoperoxides biosynthesis.

A previous investigation has shown that the accumulation of any hydroxyl derivatives of fatty acids is harmful to the purified enzyme and leads to inactivation (25). The H_2O_2 generated in our microsomal systems, however, did not inhibit TXB_2 biosynthesis in microsomal and TX synthetase systems. To explain this phenomenon, two points must be considered. First, since there were H_2O_2 scavengers in the microsomes, the H_2O_2 could be easily and quickly trapped. Second, TX synthetase was less sensitive to inhibition by the oxidizing agent (11). These combined mechanisms may have protected the microsomal enzyme from inactivation by the hydroxide. As shown in Table 2, our results confirmed these suggestions.

In various biological reactions involving reactive oxygen species, it has frequently been difficult to identify unequivocally the specific reactions. Some workers have reported that all of reactive oxygen species are closely interrelated (27). Other investigators have reported the possible involvement of singlet oxygen in PG biosynthesis (8). In our standard incubation system, TXB_2 biosynthesis was not inhibited by the addition of DPF, but PGH_2 synthesis was slightly suppressed. Furthermore, when D_2O was substituted for H_2O in the buffer, the enhancement of PGH_2 biosynthesis was observed, and this promotion by D_2O was destroyed by the addition of DPF. DPF and D_2O did not affect TX synthetase. If $^1O_2$ is a mediator, the increase of its lifetime in D_2O increases the steady-state concentration of available $^1O_2$ needed to react with a substrate. Thus, D_2O potentiates the rate of PGH_2 biosynthesis. These results support the previous hypothesis (8), and suggest strongly that singlet oxygen was, indeed, one of the mediators in the biosynthesis of PGH_2, but not of TXB_2. Panganamala et al. has reported that PGE_1 biosynthesis by the bovine vesicular glands was not enhanced in D_2O for 30 min at 37 C (35). However, in other reports, the reaction of singlet oxygen was found to be rapid, and brief incubation times were important for detecting the involvement of singlet oxygen (21,29). It is then possible that, in D_2O, the enhancement by singlet oxygen is not detectable in long incubation times.

We have confirmed that the ADP-Fe^{3+} and D_2O enhancement effect provide the evidence that ferric ion and singlet oxygen affect PGH_2 biosynthesis. But we have not measured the concentration of these substances in platelets

from HCR and it is uncertain that these agents are involved in enhanced TXB_2 biosynthesis in platelets from HCR. Further experiments are needed to elucidate the detailed effect of these agents on TXB_2 biosynthesis in platelets from HCR.

ACKNOWLEDGMENTS

The authors are grateful to Dr. W.T. Shier, Department of Cell Biology, College of Pharmacy, University of Minnesota, Minneapolis, MN, for critical reading of this manuscript and for advice.

REFERENCES

1. Kawaguchi, H., Ishibashi, T., and Imai, Y. (1981) Lipids 16, 37-42.
2. Hamberg, M., and Samuelsson, B. (1973) Proc. Natl. Acad. Sci. USA 70, 899-903.
3. Hamberg, M., Svensson, J., Wakabayashi, T., and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 71, 345-349.
4. Hamberg, M., and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 71, 3400-3404.
5. Egan, R.W., Paxton, J., and Kuehl, F.A. (1976) J. Biol. Chem. 251, 7329-7335.
6. Smith, W.L., and Lands, W.E.M. (1972) Biochemistry 17, 3276-3285.
7. Panganamala, R.V., Gavino, V.C., and Cornwell, D.G. (1979) Prostaglandins 17, 155-162.
8. Marnett, L.J., Wlodawer, P., and Samuelsson, B. (1975) J. Biol. Chem. 250, 8510-8517.
9. Miyamoto, T., Ogino, N., Yamamoto, S., and Hayaishi, O. (1976) J. Biol. Chem. 251, 2629-2636.
10. Ogino, N., Miyamoto, T., Yamamoto, S., and Hayaishi, O. (1977) J. Biol. Chem. 252, 890-895.
11. Marnett, L.J., and Wilcox, C.L. (1977) Biochim. Biophys. Acta 487, 222-230.
12. Ham, E.A., Egan, R.W., Sonderman, D.D., Gale, P.H., and Kuehl, F.A. (1979) J. Biol. Chem. 294, 2191-2194.
13. Sun, F.F. (1977) Biochem. Biophys. Res. Commun. 74, 1432-1440.
14. Raz, A., Schwartzmen, M., Kening-Wakshal, R. (1976) Eur. J. Biochem. 70, 89-96.
15. Hammarström, S., and Falardeau, P. (1977) Proc. Natl. Acad. Sci. USA 74, 3691-3695.
16. Omura, T., and Takesue, S. (1970) J. Biochem. 67, 249-257.
17. Marklund, S., and Marklund, G. (1974) Eur. J. Biochem. 47, 469-474.
18. Choen, G., Dembec, D., and Narcus, J. (1970) Anal. Biochem. 34, 30-38.
19. Paglia, D.E., and Valentine, W.N. (1967) J. Lab. Clin. Med. 70, 158-169.
20. Yokota, K., Yamazaki, I. (1965) Biochim. Biophys. Acta 105, 301-312.
21. Bondaness, R.S., and Chan, P. (1977) J. Biol. Chem. 252, 8554-8560.
22. Awasthi, Y.C., Beutler, E., and Srivastava, S.K. (1975) J. Biol. Chem. 250, 5144-5149.
23. Tietz, F. (1969) Anal. Biochem. 27, 502-552.
24. Lowry, O.H., Rosebrough, N.S., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
25. Van Der Ouderaa, F.J., Buytenhek, M., Nugteren, D.H., and Van Dorp, D.A. (1977) Biochim. Biophys. Acta 487, 315-331.
26. Tyler, D.D. (1975) FEBS Lett. 51, 180-183.
27. Pederson, T.C., and Aust, S. (1975) Biochim. Biophys. Acta 385, 232-241.
28. Takayama, K., Noguchi, T., Nakano, M., and Migita, T. (1977) Biochem. Biophys. Res. Commun. 75, 1052-1058.
29. Merkel, P.B., Nilsson, R., and Kearns, D.R. (1973) J. Am. Chem. Soc. 94, 1030-1031.
30. Merkel, P.B., and Kearns, D.R. (1972) J. Am. Chem. Soc. 94, 7244-7253.
31. Pederson, T.C., Buege, J.A., and Aust, S.D. (1973) J. Biol. Chem. 248, 7134-7141.
32. Lands, W.E.M., Lee, R., and Smith, W.L. (1971) Ann. N.Y. Acad. Sci. 180, 107-122.
33. Ohki, S., Ogino, N., Yamamoto, S., and Hayaishi, O. (1979) J. Biol. Chem. 254, 829-836.
34. Tai, H.H., and Yuan, B. (1978) Biochim. Biophys. Acta 531, 286-294.
35. Panganamala, R.V., Brownlee, N.R., Sprecher, H., and Cornwell, D.G. (1974) Prostaglandins 7, 21-28.

[Received October 14, 1981]

Influence of Partially Hydrogenated Vegetable and Marine Oils on Lipid Metabolism in Rat Liver and Heart[1]

G. HØLMER*, C.-E. HØY and D. KIRSTEIN, *Department of Biochemistry and Nutrition, The Technical University of Denmark, Bldg. 224, DK-2800 Lyngby, Denmark*

ABSTRACT

Partially hydrogenated marine oils containing 18:1-, 20:1- and 22:1-isomers and partially hydrogenated peanut oil containing 18:1-isomers were fed as 24-28 wt % of the diet with or without supplement of linoleic acid. Reference groups were fed peanut, soybean, or rapeseed oils with low or high erucic acid content. Dietary monoene isomers reduced the conversion of linoleic acid into arachidonic acid and the deposition of the latter in liver and heart phosphatidylcholine. This effect was more pronounced for the partially hydrogenated marine oils than for the partially hydrogenated peanut oil. The content of *trans* fatty acids in liver phospholipids was similar in groups fed partially hydrogenated fats. The distribution of various phospholipids in heart and liver was unaffected by the dietary fat. The decrease in deposition of arachidonic acid in rats fed partially hydrogenated marine oils was shown in vitro to be a consequence of lower Δ6-desaturase activity rather than an increase in the peroxisomal β-oxidation of arachidonic acid. The lower amounts of arachidonic acid deposited may be a result of competition in the Δ6-desaturation not only from the C22- and C20-monoenoic fatty acids originally present in the partially hydrogenated marine oil, but also from C18- and C16-monoenes produced by peroxisomal β-oxidation of the long-chain fatty acids.
Lipids 17:585-593, 1982.

INTRODUCTION

Long-chain C22-monoenoic fatty acids, especially erucic acid, can provoke lipidosis in heart tissues of rats and monkeys after short-term feeding (3-5 days) (1). This triglyceride deposition disappears when the feeding is extended for several weeks; however, long-term degenerations have also been described in heart tissue (1).

The reversal of heart lipidosis may be caused by either a change in the fatty acids supplied by VLDL from the liver due to chain shortening (2, 3), and/or an increased peroxisomal degradation of long-chain fatty acids in the heart (4).

The symptoms just mentioned were first observed in rats fed diets containing erucic acid. Corresponding findings in animals fed partially hydrogenated marine oils, which contain a broad spectrum of C22- and C20-monoenoic fatty acids including *trans* isomers, have been reported (5-7). In other experiments (8), however, only lipidosis and no long-term lesions could be demonstrated in hearts from rats fed partially hydrogenated marine oils.

Partially hydrogenated marine oils are used in substantial amounts in northern Europe for margarine production. It is, therefore, of interest to elucidate the influence of long-term feeding of these partially hydrogenated fats on lipid metabolism.

Three different experiments with rats were performed. The first experiment showed that partially hydrogenated oils, especially partially hydrogenated marine oil, supplemented with adequate amounts of linoleic acid, influenced the fatty acid distribution in membrane phospholipids from liver mitochondria. In the second experiment, it was examined whether long-term feeding with fats containing C22-monoenoic fatty acids was followed by changes in the amount and distribution of phospholipids in heart and liver and in the fatty acid composition of the phospholipids in order to reveal possible alterations of membrane structures. In the third experiment, the influence of the dietary fat on the metabolism of linoleic acid was examined.

It will be discussed whether the changes in the distribution of membrane fatty acids observed are due to differences in the conversion of linoleic acid or rather an increased peroxisomal oxidation of the long-chain polyunsaturated fatty acids in the groups fed long-chain monoenoic fatty acids.

EXPERIMENTAL PROCEDURES

Animal Experiments

Weanling male Wistar rats (specific pathogen-free, Møllegaard Laboratory, Ll. Skensved, Denmark) were divided into groups, each containing 10 animals. The diets in the first (exp. I) and the third experiments (exp. III) contained 28% fat (by wt), 20% casein, 46% sucrose and appropriate amounts of vitamins and trace elements as described previously (9).

The fatty acid composition of the dietary fats

*Author to whom correspondence should be addressed.

[1]Part of this work was presented at the ISF-AOCS Congress, New York City, 1980.

TABLE 1

Fatty Acid Composition (%) of the Dietary Fats[a]

	Experiment I			Experiment II					Experiment III				
Dietary fat	AO	HAO	HHO	SBO	AO	HMO	RSO	LERSO	AO	HAO	HMO	RSO+ LERSO	OO
Fatty acid													
14:0	tr	tr	7.9	–	tr	6.5	–	–	tr	0.1	9.0	0.1	tr
16:0	9.9	14.9	24.2	9.8	8.7	14.0	5.3	3.5	12.5	11.1	18.0	4.6	13.8
16:1	tr	0.1	8.4	–	–	7.2	–	–	tr	0.2	8.5	0.3	1.7
18:0	4.5	14.2	7.4	3.5	2.8	5.4	1.6	1.2	3.5	14.8	6.4	1.5	2.3
18:1	36.4	57.8	23.0	23.7	52.1	14.4	21.6	58.8	38.9	63.6	16.4	41.9	65.6
18:2	39.6	4.0	1.7	54.5	28.5	2.5	26.3	21.3	38.7	4.5	2.4	19.2	15.3
18:3	–	–	–	8.1	0.5	–	7.5	10.8	0.8	–	–	11.8	0.5
20:0	2.9	2.3	1.0	0.2	1.1	4.3	0.5	0.5	1.3	1.2	2.9	0.5	0.3
20:1	1.6	1.1	8.9	0.1	1.4	16.2	9.4	2.8	1.0	1.1	13.1	4.3	0.2
20:2	–	–	2.4	–	–	4.4	0.5	–	–	–	2.0	0.2	–
22:0	3.7	3.7	0.9	0.3	2.4	3.4	0.5	0.2	3.0	2.6	3.4	tr	tr
22:1	–	–	7.8	–	1.2	16.3	25.5	1.3	–	tr	14.6	15.4	–
22:2	–	–	1.0	–	–	4.2	0.4	–	–	–	1.5	–	–
24:0	1.3	1.7	–	–	tr	–	–	–	tr	0.5	–	–	–
Total *trans*[b]	–	41.9	52.0	–	–	47.4	–	–	–	48.1	47.5	–	–

[a]Determined by GLC.

[b]Determined by IR.

AO: peanut oil; Dansk Sojakagefabrik A/S, Copenhagen, Denmark. HAO: partially hydrogenated peanut oil, mp 41 C; Aarhus Oliefabrik A/S, Aarhus, Denmark. HHO: partially hydrogenated herring oil, mp 31 C; Aarhus Oliefabrik A/S, Aarhus, Denmark. HMO: partially hydrogenated marine oil, mp 30-32 C; Jahres Fabrikker A/S, Sandefjord, Norway. SBO: soybean oil; O.J. Christensen, Copenhagen, Denmark. RSO: rapeseed oil; Karlshamns Oljefabriker A/B, Karlshamn, Sweden. LERSO: low erucic acid rapeseed oil; Karlshamns Oljefabriker A/B, Karlshamn, Sweden. RSO + LERSO: rapeseed oil + low erucic acid rapeseed oil, Karlshamns Oljebafriker A/B, Karlshamn, Sweden. OO: olive oil, cold pressed, Pharmacopea Nordica.

is given in Table 1. The partially hydrogenated marine oils, HMO and HHO, contained 22:1 and 20:1 isomers as well as 18:1 and 16:1 isomers, whereas the partially hydrogenated peanut oil, HAO, mainly contained 18:1 isomers. The total *trans* fatty acid contents of the oils were similar. Diets and water were given ad libitum for 16 weeks (exp. I) or 12-14 weeks (exp. III). The animals were killed by decapitation, and the organs were excised and cooled to about 0 C as soon as possible.

Experiment II was done in cooperation with the National Food Institute, Institute of Toxicology, Mørkhøj, Denmark. Fat comprised 20% (by wt) of the diet, but an additional 4% soybean oil was given to all groups together with the vitamins. Carbohydrates were corn starch (42%), cellulose (7%) and sucrose (2.3%); the protein source was sodium caseinate (20%). Diets were given ad libitum for 26 weeks. The fatty acid composition of the dietary fats is given in Table 1.

Isolation and Analysis of Mitochondrial Lipids

Rat liver mitochondria were prepared in ice-cold 0.25 M sucrose in 1 mM EDTA, pH 7.4, from three different pools, each containing equal weights of three livers. Isolation and preparation of mitochondrial membranes were as previously described (10). The purity of the mitochondrial fraction was checked with marker enzymes (11).

Total lipids were extracted with chloroform/methanol (2:1, v/v) and phospholipid classes were isolated and quantitated as previously described (10,11). Methyl esters of phosphatidylcholines (PC) were prepared by interesterification with dry methanol/HCl (11). Gas liquid chromatography (GLC) was performed on a Hewlett-Packard 5830A dual column instrument with flame ionization detector (FID) using 1/8 in. od × 6 ft stainless steel columns packed with 15% DEGS on Chromosorb W (AW), 80/100 Mesh (Applied Science Lab., State College, PA) at 180 C. Carrier gas was helium with a flow rate of 32 ml/min.

Analysis of Organ Lipids

Total lipids from liver and hearts were extracted with chloroform/methanol (2:1, v/v), essentially according to Folch et al. (12) with a ratio of 1:15 between organ weight and solvent volume. Phospholipid distribution and fatty acid composition were determined as already described for mitochondrial lipids.

Determination of Microsomal Δ6-Desaturase Activity

The microsomal fraction was prepared according to Larsson and Brimer (13), with the "fluffy layer" just above the microsomal pellet included. Incubations were done at 37 C for 20 min using 10 mg of microsomal protein and 1 nmol (0.05 μ Ci) of [1-^{14}C]linoleic acid from the Radiochemical Centre, Amersham, England (added in 25 μl of propylene glycol) in a total volume of 3.5 ml. The final concentrations in the incubation mixture were: 200 mM sucrose, 150 mM KCl, 4.5 mM $MgCl_2$, 35 mM KF, 0.3 mM niacinamide, 3 mM ATP, 1 mM NADH, 0.175 mM CoA, and 50 mM potassium phosphate, pH 7.0 (13).

The incubations were terminated by adding 2 ml 10% methanolic KOH. After refluxing for 2 hr at 80 C, the mixture was acidified with 1 ml 6 N HCl and the free fatty acids were extracted with 3 × 4 ml of hexane. Methyl esters were then prepared (11) and separated according to unsaturation on Silica Gel G plates impregnated with 12.5% $AgNO_3$ by development in benzene/hexane (9:1, v/v) twice. The bands were identified using appropriate standards and scraped off into scintillation vials containing 15 ml of scintillation fluid (0.4% PPO and 0.01% dimethyl-POPOP in toluene).

Desaturation activities were calculated, after quench corrections by the channels ratio method, as ^{14}C-γ-linolenic acid formed, in percentages of the total radioactivity (remaining substrate plus desaturation product).

Determination of Peroxisomal Enzyme Activities

As a measure of peroxisomal induction, catalase activity was determined in two liver fractions prepared according to Neat and Osmundsen (14), a supernatant from a centrifugation at 600 g for 10 min of a 10% homogenate, and a fraction enriched in peroxisomes (L-fraction). Furthermore, the peroxisomal β-oxidation capacity of the L-fraction was examined. Catalase was determined by following the decrease in hydrogen peroxide absorption at 240 nm (15) using a final concentration of 0.1% (w/w) BRIJ 58 (Sigma, St. Louis, MO) for membrane solubilization as suggested by Flatmark et al. (16). The rate constant K′ was calculated by linear regression of corresponding values of ln (A_{240} (sample)-A_{240} (blank)) and time.

Palmitoyl-CoA oxidation was determined as described by Lazarow and de Duve (17), except that 0.25 μg Antimycin A in 96% ethanol was used as an inhibitor of mitochondrial β-oxidation. The results are given as nmol NADH produced/min/mg protein. Protein was determined by the Lowry et al. method (18).

RESULTS AND DISCUSSION

Phospholipids in Mitochondrial Membranes (Exp. I)

The content of total phospholipid and the

TABLE 2

Phospholipids (%) in Mitochondrial Membranes from Rat Liver – Experiment I

Dietary fat	28% HHO[a]	22% HHO + 6% AO	28% AO	28% HAO	22% HAO + 6% AO
Total phospholipids (mg/mg total lipid)	0.83 ± 0.02	0.90 ± 0.02	0.94 ± 0.08	0.81 ± 0.02	0.82 ± 0.03
	%P	%P	%P	%P	%P
PC + (PS)[b]	64.4 ± 0.9[c]	61.8 ± 1.5	65.7 ± 0.8	64.8 ± 0.3	65.8 ± 0.7
PE	26.5 ± 1.0	29.8 ± 1.1	23.2 ± 0.5[d]	23.5 ± 0.2	24.6 ± 0.4
CL	4.0 ± 0.2	5.1 ± 0.8	6.6 ± 0.5	6.2 ± 0.3	6.6 ± 0.5
SPH	5.1 ± 0.3	3.3 ± 0.1	4.5 ± 0.4	5.5 ± 0.4	3.0 ± 0.5

[a]Abbreviations as in Table 1.

[b]PC, Phosphatidylcholine; PS, phosphatidylserine, PE, phosphatidylethanolamine; CL, cardiolipin; SPH, sphingomyelin.

[c]Mean ± SD; n = 3 individual pools.

[d]Including 1.8% lyso PE.

TABLE 3

Fatty Acids (%) in Phosphatidylcholines from Rat Liver Mitochondrial Membranes – Experiment I[a]

Dietary fat	22% HHO + 6% AO	28% AO	22% HAO + 6% AO
Fatty acid			
16:0	12.1 ± 0.2[b]	10.7 ± 0.1	9.5 ± 0.4
16:1(n-7)	3.6 ± 0.2[c]	0.4 ± 0.1	2.1 ± 0.2[c]
18:0	13.5 ± 0.7	20.0 ± 0.7	13.5 ± 0.3
18:1(n-9)	16.4 ± 0.2[c]	5.7 ± 0.2	17.6 ± 1.2[c]
18:2(n-6)	21.4 ± 1.5[c]	9.7 ± 0.7	14.1 ± 0.9[c]
20:3(n-6)	7.7 ± 0.2	1.1 ± 0.2	2.7 ± 0.1
20:4(n-6)	19.7 ± 1.7	38.0 ± 0.9	29.0 ± 1.8
22:4(n-6)	tr	1.0 ± 0.1	0.8 ± 0.1
22:6(n-3)	1.3 ± 0.1	6.0 ± 0.4	3.1 ± 0.2
Total *trans*[d]	18.0	0	18.1

[a]Determined by GLC.

[b]Mean ± SD; n = 3 individual pools, each of 3 livers.

[c]Includes isomers.

[d]By IR.

phospholipid distribution in liver mitochondrial membranes after 16 weeks of experiment are presented in Table 2. The groups fed partially hydrogenated herring oil (HHO) or partially hydrogenated peanut oil (HAO), both without EFA-supplement, had decreased phospholipid (PL) contents compared to the control group fed peanut oil (AO). Partially hydrogenated herring oil did not affect the total PL content, when peanut oil was supplemented (HHO +AO). However, supplementation of AO to the partially hydrogenated peanut oil (HAO+AO) did not raise the amount of total PL.

The PL distribution, which also might reflect membrane changes, did not show major differences, even for the EFA-deficient groups.

Fatty Acids in Phosphatidylcholines from Mitochondrial Membranes (Exp. I).

The fatty acid distribution in PC from liver mitochondrial membranes of rats fed partially hydrogenated oils (HHO or HAO) supplemented with peanut oil (AO) is given in Table 3. Distinct changes in the fatty acid pattern occurred with decreased amounts of arachidonic acid, but the content of docosahexaenoic acid was also lower. The decrease in arachidonic acid, which was accentuated in the group fed herring oil, was followed by a corresponding increase in 18:2 and 20:3(n-6). This might indicate a re-

duced conversion of linoleic acid to arachidonic acid, when large amounts of isomers were present in the dietary fat.

The deposition of *trans* fatty acids was equal for the two groups fed partially hydrogenated fats. Long-chain C20- and C22-monoenoic acids were only present in very small amounts in the membrane PC from rats fed (HHO+AO), 0.3% and 0.2%, respectively, although appreciable amounts were present in the diet.

Phospholipids in Total Liver and Heart (Exp. II)

In experiment II, the effect of feeding partially hydrogenated oils containing isomers of varying chain length and oils containing erucic acid on the phospholipids of total liver and total heart was examined.

The amount of total phospholipid (mg/g tissue) in liver and heart (Table 4) did not show significant differences between the experimental groups ($p<0.05$). The distribution among phospholipid classes (Table 4) was not greatly influenced by the dietary regimen, but a slight decrease in the amount of PE is observed for the groups fed rapeseed oil diets, even the one with low-erucic acid. The dietary level of erucic acid did not influence the distribution among phospholipid classes. Correspondingly, the groups fed hydrogenated marine oils with low (HMO+SBO) and high levels (HMO) of 22:1 isomers showed the same amounts of PC and phosphatidylethanolamine (PE) present, which may be interpreted as the normal amount of these phospholipids in membrane structures.

These findings are in accordance with the results found for liver mitochondrial membranes. Long-term lesions are therefore not correlated with changes in the distribution of phospholipid classes.

Fatty Acids in Phosphatidylcholines from Liver and Heart (Exp. II)

Liver. The fatty acid profile of PC (Table 5) from the group fed the highest level of 22:1, originating from partially hydrogenated marine oil (HMO), revealed changes parallel to those observed for liver mitochondrial membranes isolated from rats fed the partially hydrogenated herring oil (HHO+AO), i.e., decreased deposition of arachidonic acid and increased amount of the precursors linoleic acid and 20:3(n-6) compared to the two control groups fed either soybean oil (SBO) or peanut oil (AO).

However, such changes were not significant for the groups fed 22:1(n-9) at 1.6 or 3.3% dietary levels. This points to a specific effect of the isomers present in the partially hydrogenated marine oil.

Heart. The corresponding data for heart PC (Table 5) showed the highest values for linoleic acid when 22:1 acids were present in the diet, especially for the group fed partially hydrogenated marine oil with the lowest supplement of EFA (HMO). This increase is counterbalanced by a decrease in arachidonic acid, giving a nearly constant amount of total (n-6) fatty acids in PC for all dietary groups except the one fed low-erucic-acid rapeseed oil (LERSO), where the higher dietary 18:3(n-3)/18:2(n-6) ratio decreases the total amount of (n-6) fatty acids in the PC. Astorg and Cluzan (19) have reported a similar decrease in arachidonic acid from total heart phospholipids after feeding high amounts of 22:1 to rats for 16 weeks, and Dewailly et al. (20) have reported a decrease for rats fed LERSO.

Linoleic Acid Metabolism in Rats Fed High Amounts of Docosenoic Fatty Acids (Exp. III)

The metabolism of linoleic acid in rat liver is summarized in Figure 1. Linoleic acid may either be desaturated and chain-elongated or catabolized through β-oxidation.

Until Lazarow and de Duve (17) reported the existence of peroxisomal β-oxidation, the degradation of fatty acids was believed to occur exclusively in the mitochondria. It has since been shown that peroxisomal oxidation can be induced not only by certain drugs such as clofibrate, but also after a high dietary fat intake (21,22). This induction was most pronounced for groups fed C22 fatty acids in the diet, but also C16 and C18 fatty acids from plant oils seemed effective. The increase in peroxisomal activity was shown to correspond with chain-shortening of the long-chain fatty acids (4,23) and, therefore, may aid in the elimination of lipidosis.

However, an increased peroxisomal activity might also influence the content of arachidonic acid either by a direct oxidation of this acid, or by the degradation of the dietary C22 and C20 monoenes to the corresponding C18 and C16 monoenes which, in turn, together with the remaining long-chain monoenes, may compete with 18:2(n-6) in the Δ6-desaturation process, the rate-limiting step in the formation of arachidonic acid (24,25).

In the third experiment, a preliminary investigation of the induction of peroxisomal activity in the liver of rats fed high-fat diets with and without isomeric fatty acids of varying chain lengths and the influence of these diets on the Δ6-desaturation of linoleic acid was done.

Peroxisomal induction, measured as catalase activities and palmitoyl-CoA oxidation, is given in Table 6. The catalase activities were signifi-

TABLE 4

Major Phospholipids in Total Liver and Heart of Rats–Experiment II

Dietary fat	20% SBO[a,b]	20% AO	10% HMO + 10% SBO (HMO + SBO)	20% HMO	6.5% RSO + 13.5 % SBO (RSO + SBO, 1.6)	13% RSO + 7% SBO (RSO + SBO, 3.3)	20% LERSO
22:1 (% of diet)	0	0.2	1.6	3.3	1.6	3.3	0.3
Total phospholipid (mg/g tissue)	39.9 ± 3.2[c]	36.9 ± 2.7	41.4 ± 2.3	40.3 ± 3.3	37.3 ± 1.2	38.1 ± 0.4	36.5 ± 1.6
Liver	%P	%P	%P	%P	%P	%P	%P
PC[d]	62.4 ± 1.7	55.2 ± 2.2	57.4 ± 1.1	59.8 ± 3.0	62.2 ± 4.7	62.1 ± 2.5	65.0 ± 2.0
PE	24.9 ± 2.2	21.3 ± 2.2	25.1 ± 0.6	25.8 ± 3.0	20.5 ± 0.8	20.9 ± 1.3	19.5 ± 1.4
CL	5.4 ± 0.1	5.9 ± 0.3	4.8 ± 0.8	5.3 ± 0.3	4.8 ± 0.4	4.3 ± 0.2	4.2 ± 0.5
SPH	5.3 ± 0.2	14.8 ± 1.0	10.6 ± 0.4	6.8 ± 0.4	9.1 ± 1.9	9.2 ± 1.5	7.9 ± 0.7
Total phospholipid (mg/g tissue)	27.1 ± 1.7	27.6 ± 2.6	28.6 ± 0.4	26.2 ± 2.0	28.2 ± 0.9	26.9 ± 0.3	29.3 ± 1.8
Heart	%P	%P	%P	%P	%P	%P	%P
PC[d]	45.3 ± 0.8	46.3 ± 0.6	48.0 ± 0.4	45.4 ± 2.6	43.6 ± 0.5	46.7 ± 0.6	46.5 ± 0.2
PE	32.9 ± 0.3	32.7 ± 0.1	33.7 ± 0.8	30.9 ± 0.5	31.2 ± 0.4	31.9 ± 1.5	31.0 ± 0.5
CL	14.3 ± 1.0	13.8 ± 0.2	12.8 ± 0.3	12.4 ± 2.2	13.5 ± 0.2	12.9 ± 0.7	12.7 ± 0.8
SPH	3.5 ± 0.2	3.9 ± 0.2	3.0 ± 1.1	3.5 ± 0.3	4.3 ± 0.3	3.3 ± 0.2	3.4 ± 0.1

[a]Abbreviations as in Tables 1 and 2.

[b]All groups received additional 4% SBO containing vitamin mixture.

[c]Mean ± SD; n = 3 individual pools.

[d]Including PS.

TABLE 5

Fatty Acids (%) in Phosphatidylcholines from Rat Liver and Heart – Experiment II[a]

Dietary fat		20% SBO[b,c]	20% AO	10% HMO + 10% SBO (HMO + SBO)	20% HMO	6.5% RSO + 13.5% SBO (RSO + SBO, 1.6)	13% RSO + 7% SBO (RSO + SBO, 3.3)	20% LERSO
22:1 (% of diet)		0	0.2	1.6	3.3	1.6	3.3	0.3
	Fatty acid							
	16:0	16.2 ± 1.3[d]	14.6 ± 0.8	12.9 ± 1.0	14.1 ± 0.1	13.9 ± 1.1	14.5 ± 0.9	14.5 ± 1.4
	16:1	0.3 ± 0.0	0.3 ± 0.0	1.6 ± 0.2	2.8 ± 0.3	0.4 ± 0.0	0.5 ± 0.0	0.6 ± 0.1
	18:0	31.5 ± 0.2	33.0 ± 0.8	26.8 ± 1.4	25.4 ± 0.7	36.7 ± 2.3	28.3 ± 1.4	30.8 ± 4.4
	18:1	3.1 ± 0.6	5.3 ± 0.3	9.5 ± 0.9	12.7 ± 1.1	5.1 ± 0.4	6.7 ± 0.5	8.2 ± 0.7
Liver	18:2(n-6)	10.5 ± 0.6	5.8 ± 0.2	12.5 ± 0.6[e]	14.2 ± 2.1[e]	9.1 ± 1.4	8.6 ± 1.3	6.8 ± 0.9
	20:1	tr	0.2 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.2	1.4 ± 0.7	0.4 ± 0.1
	20:3(n-6)	0.8 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	2.3 ± 0.1	1.0 ± 0.2	1.0 ± 0.0	0.9 ± 0.2
	20:4(n-6)	30.2 ± 0.8	32.6 ± 1.8	27.5 ± 1.1	17.8 ± 1.4	25.4 ± 1.1	29.4 ± 0.8	28.3 ± 0.6
	22:1	tr	tr	0.3 ± 0.0	0.7 ± 0.2	0.1 ± 0.1	0.2 ± 0.0	0.5 ± 0.0
	22:5(n-6)[f]	0.3 ± 0.2	0.3 ± 0.2	1.3 ± 0.0	1.4 ± 0.1	1.1 ± 0.2	1.5 ± 0.2	1.2 ± 0.3
	22:6(n-3)[f]	4.4 ± 1.0	4.7 ± 0.3	3.1 ± 0.4	4.4 ± 0.2	4.1 ± 0.9	4.8 ± 0.6	5.0 ± 0.4
Σ (n-6)[f]		41.5	38.9	40.9	34.3	35.5	39.0	36.0
Σ (n-3)[f]		4.7	5.0	4.4	5.8	5.2	6.3	6.2
	Fatty acid							
	14:0	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.3	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0
	16:0	13.4 ± 0.5	13.1 ± 1.0	11.6 ± 1.1	12.1 ± 1.3	12.6 ± 1.3	13.1 ± 1.7	13.3 ± 0.9
	16:1	tr	tr	1.0 ± 0.0	2.0 ± 0.2	tr	0.3 ± 0.0	0.4 ± 0.0
	18:0	35.8 ± 0.6	37.6 ± 0.5	32.9 ± 2.2	29.7 ± 0.8	36.1 ± 1.0	35.7 ± 1.7	34.1 ± 1.4
	18:1	4.4 ± 0.3	6.2 ± 0.5	7.8 ± 0.3	11.6 ± 0.8	4.9 ± 0.4	6.0 ± 0.3	9.2 ± 0.1
Heart	18:2(n-6)	8.9 ± 0.5	6.0 ± 0.3	9.9 ± 1.0[e]	13.8 ± 2.2[e]	10.0 ± 1.2	10.9 ± 1.0	8.3 ± 0.8
	20:3(n-6)	0.2 ± 0.0	tr	0.2 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
	20:4(n-6)	30.2 ± 0.1	34.0 ± 0.3	31.0 ± 1.4	25.7 ± 2.3	30.4 ± 2.3	28.9 ± 1.1	27.1 ± 0.3
	22:5(n-3)	1.6 ± 0.2	tr	2.2 ± 0.2	tr	1.2 ± 0.0	1.2 ± 0.1	2.2 ± 0.5
	22:6(n-3)	4.6 ± 0.5	3.2 ± 0.4	4.1 ± 0.1	2.5 ± 1.0	4.0 ± 0.4	3.5 ± 0.1	4.8 ± 0.4
Σ (n-6)[f]		39.3	40.0	41.1	40.2	40.6	40.1	35.7
Σ (n-3)[f]		6.2	3.2	6.3	2.5	5.2	4.7	7.0

[a]Determined by GLC.

[b]Abbreviations as in Table 1.

[c]All groups received additional 4% SBO containing vitamin mixture.

[d]Mean ± SD; n = 3 individual pools.

[e]Includes isomers.

[f]Polyunsaturated fatty acids of (n-6) and (n-3) families.

cantly higher for both liver fractions examined when partially hydrogenated marine oil was the dietary fat (HMO+AO), as an indication of increased peroxisomal activity. In the enriched peroxisomal fraction (L-fraction), an increase in activity was also observed for the group fed erucic acid (RSO + LERSO) which indicates a correlation between peroxisomal activity and the dietary content of 22:1 fatty acids.

The peroxisomal β-oxidation of palmitoyl-CoA was not significantly different for any of the groups (Table 6). This is in contrast to the findings of Neat et al. (21), who reported a significant increase in peroxisomal activity for rats fed high amounts of 22:1 fatty acids. The peroxisomal activities observed in our experiments cannot explain the decreases in the contents of arachidonic acid, 20:4(n-6), in various tissues from rats fed partially hydrogenated marine oils. The deviation between our results and the definite changes in β-oxidation capacity reported by Neat et al. (21) may arise from differences in the animal experiments, since Neat et al. used adult rats fed experimental diets for a short period, whereas we examined rats fed diets for an extended period from weanling, which may have accustomed the rats to the dietary fatty acids. Our approach is comparable to human consumption. It remains to be clarified whether induction of peroxisomal β-oxidation of fatty acids is a mechanism which is effective only for a limited period or a permanent pathway in the catabolism of dietary substances. Furthermore, it is important to evaluate whether dietary fats induce additional peroxisomal enzyme systems and to examine the possible consequences thereof.

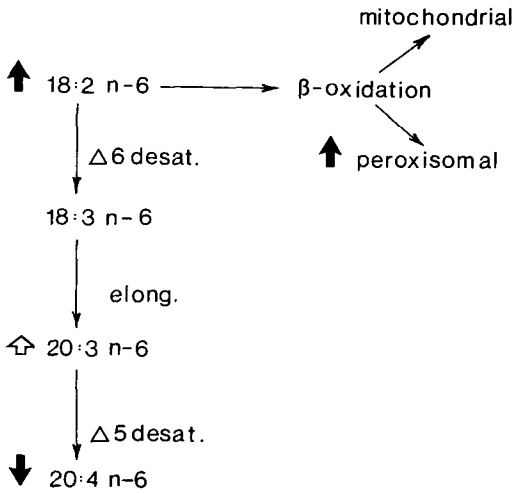


FIG. 1. Possible effects of dietary long-chain monoenoic fatty acids on linoleic acid metabolism in rats. Changes are shown by arrows. Filled arrows correspond to major changes.

The decrease in the content of arachidonic acid could also be a result of decreased conversion of linoleic acid to long-chain polyunsaturates (Fig. 1). The influence of the diet on the Δ6-desaturation of linoleic acid is shown in Table 7. The two groups that were fed partially hydrogenated fats either received a large amount of 18:1 isomers or a similar amount of monoene isomers equally distributed between 18:1, 20:1 and 22:1. These two groups have significantly lower Δ6-desaturase activities than a control group fed 28% peanut oil (AO). The decrease in the conversion of 18:2(n-6) might be related to the presence of isomers which can compete in the Δ6-desaturase reaction. For the HAO-group, the isomers are predominantly C18 monoenes. It remains to be clarified whether the HMO exerts its effect through both the C20

TABLE 6

Effect of Diet on Peroxisomal Enzyme Activities in Rat Liver – Experiment III

Dietary fat	28% AO[a]	22% HAO + 6% AO	22% HMO + 6% AO	28% RSO + LERSO	28% 00
Catalase activity K′ (min^{-1} × mg protein $^{-1}$)					
Supernatant 1	17.2 ± 2.9[b]	20.1 ± 2.0	21.9 ± 3.6[A]	18.3 ± 2.6	16.6 ± 2.3[B]
L-fraction	52.7 ± 3.8[B]	59.0 ± 4.9	64.9 ± 3.9[A]	63.8 ± 6.7[A]	50.6 ± 6.4[B]
Palmitoyl-CoA oxidation (nmol NADH × min^{-1} × mg protein $^{-1}$) L-fraction	3.9 ± 0.2	4.3 ± 0.3	5.2 ± 1.0	4.8 ± 0.7	3.8 ± 0.5

[a]Abbreviations as in Table 1.

[b]Mean ± SD; n = 4-5 individual pools. Significantly different results indicated by A and B. B<A, $p<0.05$ by Student's t-test.

TABLE 7

Effect of Diet on Δ6-Desaturase Activity in Rat Liver

Dietary fat	Δ6-Desaturase activity (% conversion)
28% AO[a]	7.9 +1.4[b,B]
22% HAO + 6% AO	5.1 ±1.2[A]
22% HMO + 6% AO	4.5 ±1.2[A]
28% RSO + LERSO	7.0 ±1.4
28% OO	6.8 ±2.2

[a]Abbreviations as in Table 1.

[b]Mean ± SD; n = 5 individual pools. A < B, $p < 0.05$ by Student's t-test.

and C22 isomers and the C18 isomers. The absence of C20 and C22 monoenes in liver lipids might be explained by a chain-shortening of these fatty acids by the peroxisomes. This is supported by the fact that the total content of 18:1 acids, as well as the amount of *trans* fatty acids, are similar in the groups fed HAO + AO and HMO + AO, respectively.

As the decrease in arachidonic acid in various tissues may be due to a competition between the substrates for Δ6-desaturation, a change in the ratio of the linoleic acid and the isomers from the partially hydrogenated oils can overcome the retardation of the 18:2(n-6) conversion.

ACKNOWLEDGMENTS

This work was supported by grants from The Danish Fat Research Foundation, MIFU Research Foundation, and The Danish Council for Technical and Scientific Research. L.K. Christensen, G. Peitersen and E. Kortegaard gave excellent technical assistance. Drs. J. Gry and P. Olsen supervised the animal experiment and delivered the organs to be analyzed in experiment II.

REFERENCES

1. Beare-Rogers, J.L. (1977) Prog. Chem. Fats Other Lipids 15, 29-56.
2. Thomassen, M.S., Strøm, E., Christiansen, E.N., and Norum, K.R. (1979) Lipids 14, 58-65.
3. Christiansen, E.N., Thomassen, M.S., Christiansen, R.Z., Osmundsen, H., and Norum, K.R. (1979) Lipids 14, 829-835.
4. Norseth, J. (1979) Biochim. Biophys. Acta 575, 1-9.
5. Beare-Rogers, J.L., Nera, E.A., and Craig, B.M. (1972) Lipids 7, 548-552.
6. Conacher, H.B.S., Page, B.D., and Beare-Rogers, J.L. (1973) Lipids 8, 256-258.
7. Ackman, R.G. (1974) Lipids 9, 1032-1035.
8. Christophersen, B.O., Svaar, H., Langmark, F.T., Gumpen, S.A., and Norum, K.R. (1976) Ambio 5, 169-174.
9. Aaes-Jørgensen, E., and Hølmer, G. (1969) Lipids 4, 501-506.
10. Høy, C.-E., and Hølmer, G. (1979) Lipids 14, 727-733.
11. Høy, C.-E., and Hølmer, G. (1981) Lipids 16, 102-108.
12. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
13. Larsson, O.M., and Brimer, L. (1979) Biochim. Biophys. Acta 572, 395-403.
14. Neat, C.E., and Osmundsen, H. (1979) Biochem. J. 180, 445-448.
15. Aebi, H. (1974) in Methods of Enzymatic Analysis, (Bergmayer, H.V., ed.) Vol. 2, pp. 673-678, Academic Press, New York/London.
16. Flatmark, T., Christiansen, E.N., and Kryvi, H. (1981) Eur. J. Cell Biol. 24, 62-69.
17. Lazarow, P.B., and de Duve, C. (1976) Proc. Natl. Acad. Sci. 73, 2043-2046.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
19. Astorg, P.-O., and Cluzan, R. (1977) Ann. Nutr. Alim. 31, 43-68.
20. Dewailly, P., Nouvelot, A., Sezille, G., Fruchart, J.C., and Jaillard, J. (1978) Lipids 13, 301-304.
21. Neat, C.E., Thomassen, M.S., and Osmundsen, H. (1980) Biochem. J. 186, 369-371.
22. Ishii, H., Fukomori, N., Horie, S., and Suga, T. (1980) Biochim. Biophys. Acta 617, 1-11.
23. Christiansen, R.Z., Christiansen, E.N., and Bremer, J. (1979) Biochim. Biophys. Acta 573, 417-429.
24. Marcel, Y.L., Christiansen, K., and Holman, R.T. (1968) Biochim. Biophys. Acta 164, 25-34.
25. Hassam, A.G., Sinclair, A.J., and Crawford, M.A. (1975) Lipids 10, 417-420.

[Received March 31, 1982]

Morphology and Fatty Acid Composition of Reticulocytes from Phenylhydrazine-Treated Rats[1]

S.C. GOHEEN*, E.C. LARKIN and **G. ANANDA RAO**, *Hematology Research Laboratory, Veterans Administration Medical Center, Martinez, CA 94553*

ABSTRACT

Reticulocytosis was induced in rats by injecting phenylhydrazine, a potent oxidizing agent. Red cell morphology was analyzed by scanning electron microscopy. The majority of red cells from rats given injections of phenylhydrazine were types 2 and 3 echinocytes. Stomatocytes were also observed, but pitted lobular reticulocytes were not detected. Echinocytes have not previously been observed in reticulocyte populations. In the reticulocytes, the relative levels of 16:1 and 18:1 were significantly greater than in erythrocytes. These differences in monoenoic acids may be due to the presence of endoplasmic reticulum, the site of desaturase activity in reticulocytes. Of all the fatty acids, the polyunsaturates are the most susceptible to attack during peroxidation. However, the polyunsaturated fatty acid composition of reticulocytes was similar both to that of erythrocytes and to reported values of young erythrocytes isolated by density. Therefore, it is unlikely that lipid peroxidation caused the formation of echinocytes.
Lipids 17:594-597, 1982.

INTRODUCTION

The fatty acid compositions of young red cells isolated according to density from humans (1,2) and rats (3) have been reported. In each case, the younger cells contained less linoleic acid (18:2) and more arachidonic acid (20:4) than the older cells. In these studies, the youngest cell fractions contained less than 50% reticulocytes. An alternate method of generating reticulocytes is by daily injections of phenylhydrazine. Using this method, 80-100% of the circulating red cells are reticuloyctes (4). We analyzed the fatty acid composition of reticulocytes collected by this method from rats to investigate whether the elevated levels of 20:4 and reduced levels of 18:2 seen previously (1-3) are characteristic of the fatty acids in reticulocytes.

It has been suggested that the morphology of red cells may be related to their level of 18:2 (5). The shape of reticulocytes has been reported (6-8). However, their morphology appears to vary with age (8). We examined the morphology of red cells by scanning electron microscopy to determine whether previously reported cell forms are adequate to describe cells from rats made severely anemic with consecutive injections of phenylhydrazine.

MATERIALS AND METHODS

Materials

Phenylhydrazine hydrochloride was purchased from Sigma (St. Louis, MO). Other reagents and solvents were of the highest purity available.

Animals

Three male Sprague-Dawley rats (Hilltop Lab Animals, Chatsworth, CA) were made anemic by injecting phenylhydrazine hydrochloride (6 mg/kg body wt) in saline intramuscularly each day for 10 days. Three control rats were given no injections. All rats were maintained on a Lab Blox diet (Allied Mills, Chicago) and weighed about 400 g.

Isolation of Cells

Rats were anesthetized 24 hr after their last phenylhydrazine injection by an intraperitoneal injection of sodium pentobarbital (170 mg/kg rat) and exanguinated using heparin-washed syringes and needles. The number of reticulocytes present in the blood was determined by counting 1,000 cells on blood smears made after staining blood with New Methylene Blue. More than 80% reticulocytes were found in red blood cells from phenylhydrazine-treated rats. The hematocrit value of rats given phenylhydrazine was 34 ± 2% whereas that of controls was 46 ± 2%. Whole blood was transferred without pressure into heparinized vacutainer tubes. Immediately after the blood was obtained, a 0.05-ml sample was fixed in 0.5% glutaraldehyde in standard incubation medium (SIM), which contained NaCl (141 mM), KCl (10 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.3 mM), NaH_2PO_4 (0.8 mM) and Na_2HPO_4 (5 mM). These samples

[1]Presented at the 72nd Annual AOCS Meeting, New Orleans, May 1981.

*Author to whom correspondence should be addressed.

were processed for scanning electron microscopy as described previously (6). Basically, samples were sedimented on glass slides, dehydrated in a graded alcohol series, and critical point-dried. After being coated with gold-palladium, they were examined and classified using an ETEC Autoscan scanning electron microscope.

Fatty Acid Analysis

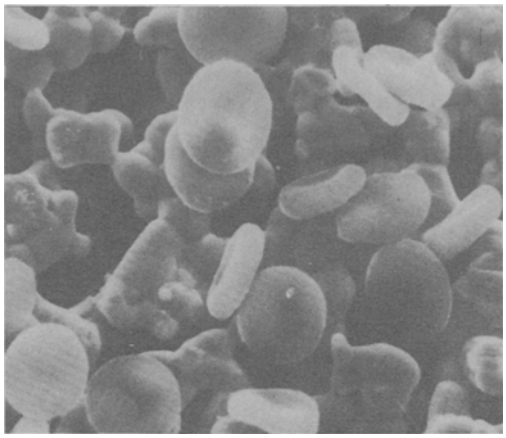
Whole blood was centrifuged at 800 × g, 22 C for 10 min (Dynac centrifuge). The plasma and buffy layer was removed and discarded. Red cells were washed three times with resuspension in SIM and centrifugation at 800 × g for 5 min.

Total lipids from erythrocytes were extracted as described by Rose and Oklander (9). Fatty acid methyl esters of erythrocyte total lipids were prepared and purified as described previously (5). Analysis of fatty acid methyl esters was done by gas liquid chromatography (GLC) at 190 C in a Varian 3700 aerograph using a flame ionization detector and a glass capillary column coated with FFAP. Areas of peaks and percentage composition of fatty acid methyl esters were computed using a Varian Chromatography Data System (CDS-111).

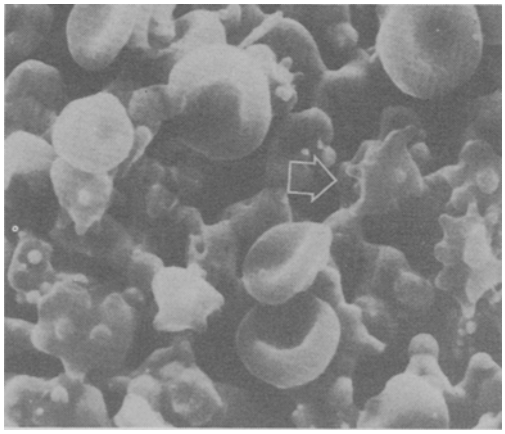
RESULTS

Morphology

The morphological differences between discocytes, knizocytes, stomatocytes and types 1, 2 and 3 echinocytes have been reported in detail (6). In control rats fed an adequate diet, more than 70% of the red cells are discocytes (Table 1) (7). Blood from rats given phenylhydrazine had relatively more knizocytes, stomatocytes and type 3 echinocytes than controls. Typical micrographs depicting red cells from control and phenylhydrazine-treated rats are shown in Figure 1, a and b, respectively.



(a)



(b)

FIG. 1. Scanning electron micrographs of red cells from (a) control and (b) phenylhydrazine-treated rats. Fresh, whole blood was fixed with 0.5% glutaraldehyde in standard incubation medium (5). A type 3 echinocyte is indicated by an arrow (× 3,650).

TABLE 1

Morphology of Red Cells from Rats Treated or Not Treated with Phenylhydrazine

Group	Disco.	Knizo.	Stomato.	Ech. 1	Ech. 2	Ech. 3
			Percent of total cells			
Control	70.8 ± 2.8	0.9 ± 0.1	1.8 ± 0.2	14.3 ± 2.1	10.3 ± 0.9	1.8 ± 0.2
Phenylhydrazine-treated	30.9 ± 3.2[a]	3.6 ± 0.8[b]	10.7 ± 3.6[c]	13.7 ± 0.6	13.3 ± 3.2	26.9 ± 3.7[c]

Abbreviations of the various cell types represent discocytes, knizocytes, stomatocytes and type 1, 2 and 3 echinocytes, respectively. Values given represent the mean of measurements of blood samples from three rats in each group ± SEM. Percentages are significantly different from controls by the two-tailed t-test when accompanied with a superscript: [a]$p<0.001$, [b]$p<0.05$ and [c]$p<0.01$.

Fatty Acid Composition

The compositions of the fatty acids from total lipids of reticulocytes and erythrocytes are shown in Table 2. Reticulocytes have a significantly higher level of 16:1, 18:1 and 20:4, and a lower level of 18:0 than erythrocytes. Previously, it was shown that young red cells have significantly more 16:0, 20:4 and 22:4, and less 18:0 and 18:2 than mature cells (3). The small differences between our results and those of Walker and Yurkowski (3) may be attributed to the differences in the cell populations. The cells used in the present study were nearly all reticulocytes, whereas the young cell fraction isolated by Walker and Yurkowski (3) may have contained only ca. 30% of these cells. Erythrocytes and reticulocytes had similar levels of other long-chain fatty acids (Table 2). The ratios of the percentages 16:0/16:1 and 18:0/18:1 were significantly smaller in reticulocytes than in erythrocytes. Both of these ratios have been used previously as a qualitative evaluation of the activity of the desaturase enzyme system.

DISCUSSION

In rats, three morphological forms of reticulocytes have been observed: pitted lobular forms (R_1), pitted discoid cups (R_2) and unpitted discoid cups (R_3) (7). These structures were observed in rats made anemic by bleeding. The ratio $R_1/(R_1 + R_2)$ increases with the severity of anemia (7). We have used the universal classification system for red cell morphology to describe the structure of reticulocytes produced in phenylhydrazine-treated rats. In the universal system, R_1 forms would be classified as reticulocytes, and both R_2 and R_3 would be classified as stomatocytes. Less than 1% reticulocytes (the R_1 form) were observed by scanning electron microscopy in this study (Table 1), whereas more than 80% of the cells were identified as reticulocytes in the same animals by New Methylene Blue staining. The higher level of stomatocytes seen in blood from phenylhydrazine-treated rats reflects an increase in reticulocytes of the R_2 and R_3 forms (Table 1) (7). However, since reticulocytes have not previously been reported to be of echinocytic shape, either the phenylhydrazine or the severity of the anemia was responsible for the effect.

Types 1, 2 and 3 echinocytes have been shown to be produced in vivo when the level of linoleate in the red cells is reduced (5). Yet, in this study, echinocytes appear to contain as much 18:2 as erythrocytes (Tables 1 and 2).

TABLE 2

Fatty Acid Composition of Red Blood Cell Total Lipids from Rats Treated or Not Treated with Phenylhydrazine

Fatty acid	Group	
	Control	Phenylhydrazine-treated
16:0	28.3 ± 2.5	27.3 ± 1.8
16:1ω7	0.7 ± 0.1	1.3 ± 0.5[a]
17:0[c]	0.7 ± 0.1	0.6 ± 0.1
17:1[d]	1.3 ± 0.2	1.2 ± 0.2
18:0	16.3 ± 0.9	11.7 ± 0.7[b]
18:1ω7 + ω9	7.2 ± 0.6	10.3 ± 1.1[a]
18:2ω6	10.4 ± 1.4	9.6 ± 1.3
20:3ω6 + ω9	1.1 ± 0.1	1.5 ± 1.2
20:4ω6	23.2 ± 0.9	24.9 ± 0.2[a]
22:4ω6	1.6 ± 0.6	1.5 ± 0.2
22:5ω6	1.5 ± 0.7	1.4 ± 0.2
22:6ω3 + 24:0	4.2 ± 1.0	4.4 ± 0.2
24:1ω9	1.2 ± 0.2	1.5 ± 0.2
16:0/16:1	40.8 ± 9.8	23.3 ± 8.9[b]
18:0/18:1	2.3 ± 0.3	1.2 ± 0.2[a]
18:2/20:4	0.45 ± 0.08	0.38 ± 0.03

Values are given as the weight percent of total fatty acids. Values are given as the mean from three rats in each group ± SD. Percentages were significantly different from control by the two-tailed t-test with:

[a] $p<0.05$.

[b] $p<0.01$.

[c] The assignment of 17:0 was based on the retention time of a standard.

[d] An alternate assignment for 17:1 is branched 18:0.

Therefore, reduced levels of 18:2 cannot alone be responsible for converting erythrocytes to echinocytes.

Phenylhydrazine injections in rats promote peroxidation of membrane phospholipids (4,10, 11). However, only minor differences exist between the fatty acid composition of reticulocytes and that of erythrocytes (Table 2). Hence, it seem unlikely that peroxidation of polyunsaturated fatty acids could have induced the generation of echinocytes. When peroxidation of membrane phospholipids was observed previously, blood samples were analyzed only 12 hr after the large doses (14 mg/kg) of phenylhydrazine (11). In other studies, peroxidation was observed either two days after very high doses of phenylhydrazine (30 mg/kg) were given (4) or immediately after similar doses (20 mg/kg) (10). In our study, 6 mg/kg phenylhydrazine was administered and blood samples were not taken until 24 hr after the last injection. If reticulocytes are harvested 24 hr after the last low dose of phenylhydrazine, lipid peroxidation may be insignificant.

Small differences have also been observed between reticulocyte plasma membrane proteins from bled animals and those treated with phenylhydrazine (11,12). In one of these studies (12), blood was collected two days after phenylhydrazine treatments (12 mg/kg). These conditions are less severe than those which induce lipid peroxidation. In sickle cell anemia, the functional groups of erythrocyte membrane proteins are abnormal (13-15). The odd morphology of sickle cells has been attributed to these differences, as well as the unusual asymmetry in their membrane phospholipids (16). Whether phenylhydrazine causes changes in membrane phospholipid asymmetry or protein functional groups is unknown.

Earlier investigations of young red cells demonstrated reduced levels of 18:2 and elevated levels of 20:4 when compared to older cells (1-3). In the present study, levels of 18:2 and 20:4 were the same in reticulocytes and erythrocytes. Therefore, as reticulocytes mature and lose membrane lipids, 18:2 is not lost preferentially as has been suggested (1). Alternatively, elevated levels of 20:4 and reduced levels of 18:2 may be characteristic of young erythrocytes as well as of reticulocytes. However, we have also observed additional and more significant differences between the fatty acid composition of reticulocytes and erythrocytes (Table 2).

The most significant differences between the fatty acids from reticulocytes and erythrocytes were in the ratios of monoenoic to saturated acids. For example, the relative level of 18:1 was greater than the level of 18:0 in reticulocytes when compared to these acids from erythrocytes. This difference may be due to the presence of endoplasmic reticulum, the site of desaturase activity. Previous studies of young erythrocytes may have failed to demonstrate this difference since erythrocytes are devoid of this membranous organelle.

In this study, the fatty acid composition and morphology of reticulocytes isolated from phenylhydrazine-treated rats is described. The fatty acid composition was similar to that described in earlier reports. Yet, the morphology of reticulocytes from phenylhydrazine-treated rats appeared unusual. These morphologies may reflect the influence of phenylhydrazine on membrane proteins. Also, if cells are harvested at least 24 hr after the last injections of relatively low doses of phenylhydrazine, membrane lipid loss from peroxidation is probably insignificant.

ACKNOWLEDGMENTS

We thank Elaine Lahue, Joseph Dardano and Dr. Carolyn Leach for their assistance. This work was supported by the National Aeronautics and Space Administration and the Veterans Administration.

REFERENCES

1. van Gastel, C., van der Berg, D., de Gier, J., and van Deenen, L.L.M. (1965) Br. J. Hematol. 11, 193-199.
2. Phillips, G.R., Dodge, J.T., and Howe, C. (1969) Lipids 4, 544-549.
3. Walker, B., and Yurkowski, M. (1967) Biochem. J. 103, 218-224.
4. Walter, H., Krob, E.J., and Ascher, G.S. (1978) Br. J. Haematol. 39, 391-398.
5. Rao, G.A., Siler, K., and Larkin, E.C. (1979) Lipids 14, 30-38.
6. Bessis, M., Weed, R.I., and Leblond, P.F. (1973) Red Cell Shape: Physiology, Pathology and Ultrastructure, Springer-Verlag, New York, NY.
7. Mel, H.C., Prenant, M., and Mohandas, N. (1977) Blood 49, 1001-1009.
8. Coulombel, L., Tchernia, G., and Mohandas, N. (1979) J. Lab. Clin. Med. 94, 467-474.
9. Rose, H.G., and Oklander, M. (1965) J. Lipid Res. 6, 428-431.
10. Goldstein, B.D., Rozen, M.G., and Amoruso, M.A. (1979) J. Lab. Clin. Med. 93, 687-694.
11. Jain, S.K., and Hochstein, P. (1980) Arch. Biochem. Biophys. 201, 683-687.
12. Tsao, D., Colton, D.G., Chang, J.S., Buck, R.L., Hudson, B.G., and Carraway, K.L. (1977) Br. J. Haematol. 11, 193-199.
13. Dzandu, J.K., and Johnson, R.M. (1980) J. Biol. Chem. 255, 6382-6386.
14. Green, G.A., and Kalra, V.K. (1981) J. Biol. Chem. 256, 10565-10571.
15. Ro, J-Y., Neilan, B., Magee, P.N., Paik, W.K., and Kim, S. (1981) J. Biol. Chem. 256, 10572-10576.
16. Lubin, B., Chiu, D., Bastacky, J., Roelfsen, B., and Van Deenen, L.L.M. (1981) J. Clin. Invest. 67, 1643-1649.

[Received April 12, 1982]

Riboflavin Deficiency and β-Oxidation Systems in Rat Liver

TAKEHIKO SAKURAI, SHOKO MIYAZAWA, SHUICHI FURUTA and **TAKASHI HASHIMOTO***, *Department of Biochemistry, Shinshu University School of Medicine, Matsumoto, Nagano, 390 Japan*

ABSTRACT

Weanling rats were fed a riboflavin-deficient diet. The mitochondrial fatty acid oxidation in liver was depressed in riboflavin deficiency but restored after supplementation of riboflavin. Among the enzymes involved in this system, only the acyl-CoA dehydrogenase (EC 1.3.99.2 and 1.3.99.3) activities varied with the change in fatty acid oxidation. An accumulation of the apoforms of acyl-CoA dehydrogenases was found in riboflavin deficiency. The levels of electron transfer flavoprotein and other enzymes involved in the β-oxidation system remained unchanged. The peroxisomal fatty acid oxidation and levels of individual enzymes of this system remained constant. No accumulation of the apoform of acyl-CoA oxidase was observed under simple, riboflavin-deficient conditions. However, accumulation of a large amount of apo-acyl-CoA oxidase was observed when the peroxisomal system was induced by administration of a peroxisome proliferator, di(2-ethylhexyl)phthalate, under riboflavin-deficient conditions.
Lipids 17:598-604, 1982.

INTRODUCTION

When rats are deprived of riboflavin (vitamin B_2), the lessening of the ability of the mitochondrial fatty acid oxidation in liver is one of the most dramatic effects (1). The dehydrogenation of acyl-CoA in the mitochondrial system is catalyzed by three flavin enzymes, which are classified by their substrate specificities into short-, medium- and long-chain fatty acyl-CoA dehydrogenases (EC 1.3.99.2 and 1.3.99.3). Hoppel et al. suggested that the most probable candidate for the decrease in the β-oxidation capacity is the acyl-CoA dehydrogenases-ETF system although they could not determine the ETF activity (1).

The other fatty acid β-oxidation system is located in peroxisomes (2). The activity of the peroxisomal system may be comparable to that of the mitochondrial system in rat liver (3,4). The initial step of the peroxisomal system, which is considered to be rate-limiting, is catalyzed by acyl-CoA oxidase, a flavoprotein (5-8). The following reactions in this system are catalyzed by the peroxisome-specific enzymes (9,10).

In this study, weanling rats were fed a riboflavin-deficient diet. The oxidation of fatty acids and activities of individual enzymes of both particles in rat liver were determined. The mitochondrial β-oxidation was depressed under the riboflavin-deficient conditions. Three acyl-CoA dehydrogenase activities were depressed but other enzymes of the mitochondrial β-oxidation system, including ETF, remained unchanged. The peroxisomal β-oxidation and activities of all peroxisomal enzymes were not altered under the riboflavin-deficient conditions.

The peroxisomal system is inducible in contrast to the mitochondrial system which is rather constitutive (2,11). When a typical peroxisomal proliferator, DEHP, was administered to riboflavin-deficient rats, a marked accumulation of the apoenzyme of acyl-CoA oxidase was found.

METHODS

Animals

Male Wistar weanling rats weighing 50-60 g were randomly distributed among the test groups. The control (standard A) and riboflavin-deficient, semi-synthetic diets were obtained from Oriental Co. (Tokyo, Japan). The control diet contained 38% corn starch, 25% vitamin-free casein, 10% α-starch, 8% cellulose, 6% safflower oil, 5% sucrose, 6% salt mixture and 2% vitamin mixture. The riboflavin-butyrate diet was supplemented with riboflavin butyrate ester at a level of 1.2 g/kg in the riboflavin-deficient diet. During the first period of feeding for 28 days, animals were maintained on either the control or the riboflavin-deficient diet. Then, animals were fed various diets in the second feeding period. DEHP was added to diets at a level of 2% (w/w) for induction of the peroxisomal enzymes. The duration of feeding of the DEHP-containing diets was 3 days, since all of the animals which had been

*Author to whom correspondence should be sent.
Abbreviations: ETF, electron transfer flavoprotein; DEHP, di(2-ethylhexyl)phthalate.

fed a riboflavin-deficient diet died 4-5 days after introduction of the reagent into the diet. The duration of feeding the diets without the reagent was 7 days.

Fatty Acid Oxidation

The mitochondrial fractions were prepared according to the method of de Duve et al. (12), except that the heavy and light mitochondrial fractions were not separated because peroxisomes were concentrated in the light fraction. The mitochondrial fractions were suspended in the basal medium that contained 300 mM mannitol, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 7 mM potassium phosphate and 0.1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,-N'-N'*-tetraacetic acid at a final pH of 7.4.

Fatty acid-dependent O_2 consumption by the mitochondrial fractions at 30 C was measured with a galvanic oxygen electrode (Bioxygraph; Kyusui-Kagaku Institute, Tokyo, Japan) (13).

The data are summarized as nmol O_2 consumed/min/g liver. Recoveries of mitochondria and peroxisomes in the subcellular fractions were based on recoveries of the marker enzymes glutamate dehydrogenase (EC 1.4.1.3) and catalase (EC 1.11.1.6), respectively.

Enzyme Assays

Activities of acyl-CoA dehydrogenases and ETF were assayed as described previously (14). Cyanide-insensitive palmitoyl-CoA-dependent NAD^+ reduction (11) and acyl-CoA oxidase activity with use of palmitoyl-CoA (15) were assayed as described previously. To distinguish other enzymes of both the peroxisomal and the mitochondrial systems, the immunoglobulins to the respective enzymes were used (16).

Glutamate dehydrogenase (17), catalase (18), and carnitine acyltransferases (EC 2.3.1.7 and 2.3.1.23) (19) were assayed according to the published procedures.

All enzyme activities were assayed at 30 C. Units of enzyme activities are expressed as μmol of substrate utilized or products formed/min. The data are summarized as units/g liver.

RESULTS

Gross Changes

The weight gains of rats in the riboflavin-deficient group were nearly zero. After 2 weeks, the epidermal change was observed in the riboflavin-deficient groups. At the end of the first feeding period (28 days), the body weights of the control animals were 250-260 g but those of the riboflavin-deficient animals were 60-65 g. However, the weight gain of the riboflavin-deficient animals was similar to that of the control (7-8 g/day) after they received the control or the riboflavin-butyrate diet in the second feeding period. The sizes of the liver (g wet weight/100 g body weight) were 4.57 ± 0.19 for the control and 5.02 ± 0.21 for the riboflavin-deficient animals. The values of the second group seemed to be reduced after they received the control (4.73 ± 0.35) or the riboflavin-butyrate diet (4.81 ± 0.29) in the second feeding period. The sizes of the liver were increased after DEHP administration in the second feeding period: 6.11 ± 0.20 for the group that had received the control diet in the first and the control diet containing DEHP in the second feeding period; 7.98 ± 0.49 for the group that had received the riboflavin-deficient diet in the first and the riboflavin-deficient diet containing DEHP in the second feeding period.

Fatty Acid Oxidation

The activity of glutamate dehydrogenase of the control group was 233 ± 44 units/g liver and that of the riboflavin-deficient group was 418 ± 66 units/g liver. Enzyme activity of the riboflavin-deficient animals was decreased to the control level after they received the control or the riboflavin-butyrate diet. Enzyme activity was unchanged after DEHP administration in the second feeding period. The recoveries of the glutamate dehydrogenase in the mitochondrial fractions were 35-40% in all groups. The activity of catalase of the control group was 135 ± 4 × 10^3 units/g liver, and that of the riboflavin-deficient group was 121 ± 8 × 10^3 units/g liver. The enzyme activity of the second group remained unchanged after the second feeding of the control and the riboflavin-butyrate diets. The catalase activity was slightly increased after administration of DEHP; increases were 1.30-fold for the control and 1.26-fold for the riboflavin-deficient group, respectively. The recoveries of the catalase activity were 50% or more for all groups.

As shown in Table 1, the mitochondrial fatty acid oxidation with palmitoyl-CoA was 344 ± 164 nmol O_2/min/g liver in the control. That of the riboflavin-deficient group was 32 ± 10 nmol O_2/min/g. The low capacity for fatty acid oxidation was overcome after the change of the diet to the control or to the riboflavin-butyrate diet. Administration of DEHP resulted in an increase in the mitochondrial β-oxidation (13). In this experiment, DEHP administration showed no effect in the control. This seems to be due to the short feeding period of 3 days.

TABLE 1

Palmitoyl-CoA Oxidation

Diet[a] I / II	Control / Control	Control / Control-DEHP	B_2-deficient / B_2-deficient	B_2-deficient / B_2-deficient-DEHP	B_2-deficient / Control	B_2-deficient / B_2-butyrate
	nmol O_2/min g liver[b]					
Mitochondria[c]	344 ± 164	350 ± 153	32 ± 10	173 ± 87	203 ± 105	312 ± 136
Peroxisomes[d]	336 ± 10	1,103 ± 256	304 ± 28	549 ± 86	352 ± 29	360 ± 33

[a]Rats were fed the control or the B_2-deficient diet in the first feeding period for 28 days (diet I), and then they were fed various diets (diet II) in the second feeding period. The durations of the second feeding period with diet II were 3 days for the groups with DEHP-containing diets and 7 days for the groups without the reagent. B_2, riboflavin; B_2-butyrate, riboflavin butyrate ester.

[b]Mean ± SD (n=4).

[c]The reaction mixture contained the mitochondrial fraction and the basal medium (compositions are described in Methods) containing 1 mg/ml of defatted bovine serum albumin (from Sigma), 1 mM ADP, 5 mM Tris-malonate, 2 mM L-carnitine, and 20 μM palmitoyl-CoA. The peroxisomal β-oxidation was inhibited by bovine serum albumin due to palmitoyl-CoA binding.

[d]The reaction mixture contained the mitochondrial fraction and the basal medium containing 0.1 mM CoA, 0.2 mM NAD^+, 50 μM palmitoyl-CoA and 0.5 ng/ml antimycin A. Antimycin A inhibited the mitochondrial β-oxidation but did not affect the peroxisomal one.

However, DEHP administration to the riboflavin-deficient group resulted in an increase in the activity.

In contrast to the mitochondrial activity, the peroxisomal activity remained unchanged by riboflavin deprivation or by its realimentation. The increase in the peroxisomal activity after DEHP administration was about three-fold in both the control and the riboflavin-deficient group.

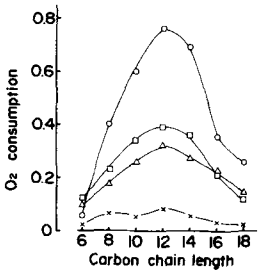


FIG. 1. Mitochondrial fatty acid oxidation with saturated fatty acids having various carbon chain lengths. Mitochondrial fractions prepared from pooled livers (n=4) were used. The reaction mixture was the same as that described in Table 1 except that 50 μM fatty acids in place of palmitoyl-CoA was used and 2 mM $MgCl_2$ and 1 mM ATP in place of ADP were added. O_2 consumption = μmol O_2/min/g liver. Nutritional conditions were as described in Table 2. ○, control–control; ×, riboflavin-deficient–riboflavin-deficient; □, riboflavin-deficient–control; △, riboflavin-deficient–riboflavin-butyrate.

Capacities for mitochondrial oxidation with various saturated fatty acids are summarized in Figure 1. Patterns of substrate specificity in all groups were the same, irrespective of the oxidation capacities. Substrate-activity patterns very similar to those in Figure 1 were found with various saturated fatty acyl-CoA (data not shown).

Enzyme Activities

Activities of carnitine acyltransferases with various acyl-CoA as substrates were unchanged by riboflavin deprivation (data not shown). Activity of acyl-CoA dehydrogenase toward palmitoyl-CoA was reduced to about one-third by riboflavin deprivation and recovered to the control level after its realimentation (Table 2).

The chain length specificity of the mitochondrial acyl-CoA dehydrogenase is shown in Figure 2, which gives sums of the three acyl-CoA dehydrogenases. In comparison to patterns of the purified enzymes (Fig. 3 in Ref. 14), the depression of the acyl-CoA dehydrogenase activity in the riboflavin-deficient group was more marked with short-chain substrates. Preincubation of the enzyme fraction with 100 μM FAD at 25 C for 5 min prior to the assays showed no effect in the control, as shown in Figure 2. However, the preincubation increased the activities toward short-, medium- and long-chain-length substrates in the riboflavin-deficient group. The data suggest that the apoenzymes of these acyl-CoA dehydrogenases accumulate under the riboflavin-deficient conditions.

The ETF activity was unchanged by ribo-

TABLE 2

Activities of Acyl-CoA Dehydrogenase and ETF

Diets[a] I / II	Control / Control	B_2-deficient / B_2-deficient	B_2-deficient / Control	B_2-deficient / B_2-butyrate
	munits/g liver[b]			
Acyl-CoA dehydrogenase	208 ± 16	65 ± 3	200 ± 23	215 ± 54
ETF	9.1 ± 0.9	8.4 ± 2.6	10.5 ± 2.8	9.8 ± 3.2

[a]Nutritional conditions were the same as described in Table 1, but the experiments were conducted with other groups of rats (n=5).

[b]Mean ± SD. The acyl-CoA dehydrogenase activities were assayed with palmitoyl-CoA as substrate.

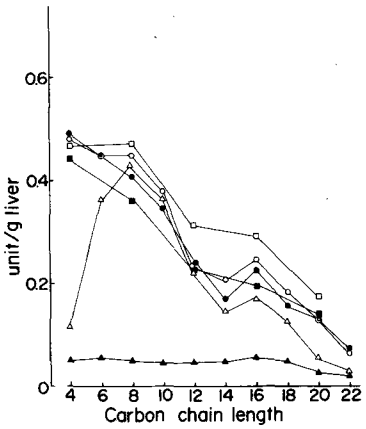


FIG. 2. Acyl-CoA dehydrogenase activities. Saturated fatty acyl-CoA were used as substrates. Samples from pooled livers from four rats were pooled and assayed with or without preincubation with FAD. For descriptions of the nutritional conditions, see Table 1. Control–control with (○) or without (●) FAD; riboflavin-deficient–riboflavin-deficient with (△) or without (▲) FAD; riboflavin-deficient–riboflavin-butyrate with (□) or without (■) FAD.

flavin deprivation (Table 2). No effect on the ETF activity of preincubation with FAD was found in any group.

The activities of other enzymes involved in the two β-oxidation systems are summarized in Table 3. Activities of mitochondrial enoyl-CoA hydratase (EC 4.2.1.17) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) were unaffected by riboflavin deprivation. Mitochondrial 3-ketoacyl-CoA thiolase (EC 2.3.1.16) seemed to be increased. Effects of administration of DEHP on the mitochondrial enzymes were not marked, since the duration was only 3 days.

It has been reported that the apoenzyme of acyl-CoA oxidase is obtained under specified purification conditions and that this form of the enzyme showed full activity in the presence of FAD (20). As shown in Table 3, FAD prompted a slight increase in the activity of the enzyme, either before or after the induction of the peroxisomal system. Riboflavin deprivation slightly increased the FAD-dependent activity. Dramatic FAD-dependent activation of the enzyme activity was found in the riboflavin-deficient DEHP group. The acyl-CoA oxidase activities of liver extracts of the control-DEHP and riboflavin-deficient DEHP were titrated with anti(acyl-CoA oxidase)-immunoglobulin (Fig. 3). The results indicated that the apoenzyme was markedly increased by the peroxisome proliferators in the riboflavin-deficient conditions.

Activities of individual enzymes of the peroxisomal system remained constant in riboflavin-deficiency. After administration of DEHP, individual enzymes of this system markedly increased irrespective of riboflavin deprivation.

DISCUSSION

Activities of individual enzymes of the mitochondrial β-oxidation systems in rat liver were determined under deprivation and realimentation of riboflavin. Hoppel et al. (1) found that the mitochondrial fatty acid oxidation of rat liver was dramatically decreased in riboflavin deficiency whereas the TCA cycle and oxidative phosphorylation were not decreased. Under their conditions, activities of acyl-CoA dehydrogenases were depressed to a great extent. Therefore, they have claimed that the site of the effect is the acyl-CoA dehydrogenation step (1).

In our study, it was found that all acyl-CoA dehydrogenase activities were depressed under riboflavin deficiency as described by Hoppel et al. (1). The activities of the riboflavin-deficient group, however, were greatly activated when the enzyme samples were assayed after preincu-

TABLE 3

Enzyme Activities of β-Oxidation Systems

Diets[a] I / II	Control / Control	Control / Control-DEHP	B_2-deficient / B_2-deficient	B_2-deficient / B_2-deficient-DEHP
		Units/g liver[b]		
Mitochondrial				
Acyl-CoA dehydrogenase[c]	160	230	61	110
Enoyl-CoA hydratase	1,063 ± 97	1,360 ± 30	1,367 ± 148	1,687 ± 362
3-Hydroxyacyl-CoA dehydrogenase	81.4 ± 8.8	62.6 ± 14.3	83.5 ± 4.5	85.3 ± 7.5
3-Ketoacyl-CoA thiolase	3.2	6.8	4.7	4.7
Peroxisomal				
Acyl-CoA oxidase[c] (+)[d]	376 ± 171	3,730 ± 900	552 ± 90	2,490 ± 620
(−)	223 ± 66	2,640 ± 640	250 ± 90	110 ± 12
Enoyl-CoA hydratase	276 ± 100	1,246 ± 391	235 ± 52	957 ± 266
3-Hydroxyacyl-CoA dehydrogenase	7.2 ± 3.0	19.7 ± 7.5	5.1 ± 1.3	20.0 ± 6.7
3-Ketoacyl-CoA thiolase	3.9	22.3	5.4	22.9

[a]Nutritional conditions of the animals were as described in Table 1.

[b]Mean ± SD (n=4), values without SD are the data of the pooled sample from 4 rats.

[c]munits/g liver; palmitoyl-CoA was used as substrate.

[d]Liver extracts were preincubated at 20 C for 10 min with (+) or without (−) 25 μM FAD.

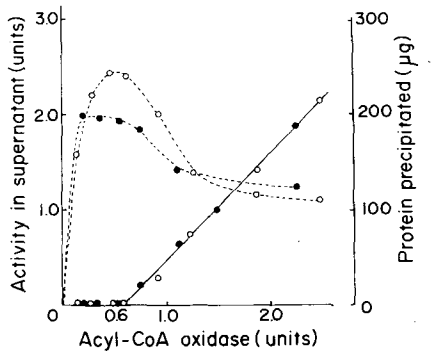


FIG. 3. Quantitative precipitin reaction of acyl-CoA oxidase. Various amounts of liver extracts were added to the fixed amount of the antibody. After incubation at 25 C for 30 min in 0.15 M NaCl and 10 mM potassium phosphate (pH 7.5), the mixture was centrifuged, an aliquot of the supernatant was used for enzyme assay and the precipitate was used for protein assay. For descriptions of the nutritional conditions, see Table 1. ○, control–control-DEHP; ●, riboflavin-deficient–riboflavin-deficient-DEHP. Solid line, enzyme activity; dotted lines, protein precipitated.

bation with FAD (Fig. 2). According to the substrate specificities of the purified preparations of rat liver acyl-CoA dehydrogenases (Fig. 3 in ref. 14), it is likely that the decrease in activity toward butyryl-CoA is due to the decrease in the quantity of short-chain acyl-CoA dehydrogenase, but the decreases in activities of medium- and long-chain acyl-CoA dehydrogenases are due to a block in the conversion of apoenzymes to holoenzymes.

The level of ETF did not change with the deficiency of riboflavin nor after it was supplied (Table 2). An accumulation of the apoform was not found. Activities of carnitine acyltransferases, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase were not decreased in riboflavin deficiency.

The dehydrogenation step of the mitochondrial system is rate-limiting when the fatty acid oxidation is assayed with the mitochondrial fraction in the presence of a sufficient amount of l-carnitine and other substrates (13). Therefore, a disturbance in the formation of the holoform of the acyl-CoA dehydrogenases seems to be the cause of the decrease of mitochondrial β-oxidation in riboflavin deficiency.

Peroxisomal β-oxidation capacity as well as activities of individual enzymes of the peroxisomal system remained constant. The initial step of the peroxisomal β-oxidation, which is supposed to be rate-limiting, is catalyzed by acyl-CoA oxidase. A decrease in the holoform and/or an increase in the apoform of this flavin enzyme were not found under the simple riboflavin-deficient conditions. However, an accumulation of a large amount of the apoform of the enzyme was found when the peroxisomal system was induced under riboflavin deficiency.

Control rats in this experiment were fed ad libitum. Simple pair-fed, riboflavin-supplemented controls were not provided since the feeding pattern of the pair-fed controls, which consumed their diet in a daily meal of a few hours, may have effects on the metabolism and the enzyme activities (21). We could not keep the rats under continuous pair-fed conditions.

Hoppel et al. (1) found that oxidation of fatty acids and the acyl-CoA dehydrogenase activities decreased in an early stage of riboflavin deficiency, and that these changes could not be reproduced by starvation of the rats. We found that the half-lives of three acyl-CoA dehydrogenases in rat liver were about one day (13). These observations suggest that turnovers of acyl-CoA dehydrogenases are at high rates, but the decreases of these enzymes in riboflavin deficiency are not due to the shortage of food intake.

ACKNOWLEDGMENTS

We thank Tokyo Tanabe Co. Ltd. (Tokyo, Japan) for supplying riboflavin butyrate ester.

REFERENCES

1. Hoppel, C., DiMarco, J.P., and Tandler, B. (1979) J. Biol. Chem. 254, 4164-4170.
2. Lazarow, P.B., and de Duve, C. (1976) Proc. Natl. Acad. Sci. USA 73, 2043-2046.
3. Shindo, Y., and Hashimoto, T. (1978) J. Biochem. 84, 1177-1181.
4. Krahling, J.B., Gee, R., Murphy, P.A., Kirk, J.R., and Tolbert, N.E. (1978) Biochem. Biophys. Res. Commun. 82, 136-141.
5. Osumi, T., and Hashimoto, T. (1978) Biochem. Biophys. Res. Commun. 83, 479-485.
6. Osumi, T., and Hashimoto, T. (1979) J. Biochem. 85, 131-139.
7. Inestrosa, N., Bronfman, M., and Leighton, F. (1979) Biochem. J. 182, 779-788.
8. Osumi, T., Hashimoto, T., and Ui, N. (1980) J. Biochem. 87, 1735-1746.
9. Osumi, T., and Hashimoto, T. (1979) Biochem. Biophys. Res. Commun. 89, 580-584.
10. Miyazawa, S., Osumi, T., and Hashimoto, T. (1980) Eur. J. Biochem. 103, 589-596.
11. Osumi, T., and Hashimoto, T. (1978) J. Biochem. 83, 1361-1365.
12. de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) Biochem. J. 60, 604-617.
13. Furuta, S., Miyazawa, S., and Hashimoto, T. (1981) J. Biochem. 90, 1751-1756.

14. Furuta, S., Miyazawa, S., and Hashimoto, T. (1981) J. Biochem. 90, 1739-1750.
15. Hashimoto, T., Miyazawa, S., Gunarso, D., and Furuta, S. (1981) J. Biochem. 90, 415-421.
16. Miyazawa, S., Furuta, S., Osumi, T., and Hashimoto, T. (1980) Biochim. Biophys. Acta 630, 367-374.
17. Schmidt, E. (1974) in Methods of Enzymatic Analysis, 2nd English edn. (Bergmeyer, H.U., ed.) Vol. 2, pp. 650-656, Verlag Chemie, Weinheim.
18. Aebi, H. (1974) in Methods of Enzymatic Analysis, 2nd English edn. (Bergmeyer, H.U., ed.) Vol. 2, pp. 673-678, Verlag Chemie, Weinheim.
19. Markwell, M.A.K., McGroarty, E.J., Bieber, L.L., and Tolbert, N.E. (1973) J. Biol. Chem. 248, 3426-3432.
20. Inestrosa, N.C., Bronfman, M., and Leighton, F. (1980) Biochem. Biophys. Res. Commun. 95, 7-12.
21. Greenfield, J., and Briggs, G.M. (1971) Ann. Rev. Biochem. 40, 549-572.

[Received January 25, 1982]

Fatty Acid Synthesis in Isolated Spermatocytes and Spermatids of Mouse Testis

W. MCLEAN GROGAN and **JAMES W. LAM**, *Department of Biochemistry, Box 614 MCV Station, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298*

ABSTRACT

In vitro incorporation of [1-^{14}C]acetate into fatty acids and lipid classes of spermatocytes, round spermatids and condensing spermatids enriched by Staput 1 × g sedimentation was measured by thin layer and gas radiochromatography. All three cell fractions showed the full range of de novo synthetase, elongation and desaturase activities necessary for biosynthesis of fatty acids characteristic of mouse testis, but synthesis of fatty acids of >16 carbons declined with progressive stages of differentiation. The magnitudes and patterns of distribution of fatty acid synthesis in the germinal cells were similar to those of whole testis incubated in vitro or injected in vivo with [^{14}C]acetate. On the other hand, complex lipid synthesis was much more variable and incorporation into triacylglycerol was generally much lower in dispersed germinal cells than in whole testis in vitro or in vivo. Cells remained viable throughout the 15-hr incubation. Thus, isolated germinal cells are fully capable of synthesizing their constituent fatty acids, including the long-chain polyenoic acids which they accumulate, but the intratubular environment or association with Sertoli cells may be necessary for maintenance of adequate complex lipid synthesis.
Lipids 17:604-611, 1982.

INTRODUCTION

The unique fatty acid metabolism and composition characteristic of the mammalian testis undergoes dramatic changes during testicular development and differentiation (1). Many lines of evidence suggest that the fatty acid composition plays an important role in normal differentiation of the germinal cells. The successive stages of spermatogenesis (spermatogonial stem cells → primary spermatocytes → round spermatids → condensing spermatids → spermatozoa) have characteristic fatty acid compositions which are different from one another and from nongerminal (Sertoli and Leydig) cells (2-4). Germinal cells are especially characterized by the accumulation of LCPA of the linoleic (n-6) and linolenic (n-3) acid families (5).

Understanding of the complex metabolic interrelationships among the various germinal and nongerminal cell types of the testis requires knowledge of the distribution of the metabolic activities involved. Fatty acid metabolism has been studied rather extensively in whole testis (1,5,6), in preparations containing highly heterogeneous mixtures of testis cell types (6,7) and in subcellular fractions representative of the entire testis cell population (8). Beckman and Coniglio (9) have examined incorporation of intratesticular injections of polyunsaturated fatty acids into lipids of Sertoli cells and mixed germinal cells in the rat, but fatty acid metabolism of individual germinal cell types in vivo or of enriched germinal cell types in vitro has not been reported. We report here the abilities of 15-hr cultures enriched in the various germinal cell types of mouse testis to incorporate [1-^{14}C]acetate into specific fatty acids and lipid classes.

Abbreviations: LCPA = long-chain polyenoic acids; 20:4(n-6) = all *cis*-5,8,11,14-eicosatetraenoic (arachidonic) acid; 22:5(n-6) = all *cis*-4,7,10,13,16-docosapentaenoic acid; 22:6(n-3) = all *cis*-4,7,10,13,16,19-docosahexaenoic acid; PS = primary spermatocytes; RS = round spermatids; CS = condensing spermatids; RB = residual bodies; WS = whole suspension; WT = whole testis; RA = radioactivity.

METHODS AND MATERIALS

Germinal Cell Enrichment

Testes from five white Swiss mice (Microbiological Associates) were decapsulated and dispersed in Eagle's Minimum Essential Media (MEM) for suspension cultures (Flow Laboratories) with hyaluronidase (Sigma, Type V) and collagenase (Sigma, Type II). Cells were sedimented at 1 g on an albumin density gradient using a Sta-Put apparatus as we have described in detail elsewhere (10). Fractions 12-17 (PS), 24-30 (RS), and 31-36 (CS or RB) from the 40 fraction gradients were pooled and found to be enriched in primary spermatocytes, round spermatids and condensing spermatids or residual bodies (cytoplasmic remnants of spermatid maturation), respectively, by histological evaluation as described previously (10). Composition of the pooled fractions used for [^{14}C]acetate incorporation is shown in Table 1. All solutions were filtered through a 0.2-μ filter, and glass-

TABLE 1

Composition of Pooled Sta-Put Fractions Incubated with [1-^{14}C] Acetate[a]

Predominant cell type	Fractions inclusive	% Composition of cells			
		PS	RS	CS	RB
Primary spermatocytes (PS)	12-17	61 ± 1	26 ± 2	6 ± 0.5	0
Round spermatids (RS)	24-30	6 ± 1	66 ± 1	18 ± 4	6 ± 1
Condensing spermatids (CS)	31-36[b]	0	0	60 ± 0	38 ± 2
Residual bodies (RB)[c]	31-36[b]	0	2 ± 2	17 ± 3	81 ± 2

[a]Forty fractions were collected from the bottom of a Sta-Put 1-g albumin density gradient. Fractions 1-11 contained most of the Leydig cells, Sertoli cells and cell aggregates.

[b]These fractions had variable amounts of RB and CS. Results of analyses are grouped according to the predominant particle present.

[c]Anucleated cytoplasmic remnants of spermatid maturation.

ware and apparatus were autoclaved prior to use. Solutions were additionally supplemented with Penicillin (25 units/ml), streptomycin (25 μg/ml), and Fungizone (2.5 μg/ml, Gibco). Concentrations of cells were determined by hemocytometer counts. Viability was checked by trypan blue exclusion, by O_2 uptake using a Clarke oxygen probe (Yellow Springs Instrument Co.), by phase contrast microscopy and by [^{14}C] acetate uptake.

[^{14}C] Acetate Incorporation

Dispersed cells (2-14 × 10^6 PS, 10-24 × 10^6 RS, 26-30 × 10^6 CS, 9-38 × 10^6 RB, and 8-22 × 10^6 WS) or single whole decapsulated testes (WT) were incubated in sealed, silanized Ehrlenmeyer flasks at 35 C for 15 hr in 3 ml of MEM supplemented with 120 μmol glucose and 10 μCi [1-^{14}C] sodium acetate (New England Nuclear, 56 mCi/mmol). Sufficient headspace was provided to prevent significant depletion of O_2 from the flasks during incubation, based on O_2 consumption of 0.5 nmol/min/10^6 cells measured by us and also reported by Romrell et al. (11). The pH of incubations was monitored by an indicator provided in the commercial MEM and did not vary significantly from 7.4 during 15 hr of incubation. For WT in vivo studies, mice were ether-anesthetized and injected intratesticularly with 10 μCi [1-^{14}C]-sodium acetate through small abdominal incisions which were then closed by surgical staples. Mice were sacrificed at the end of the incubation period and testes were removed. Incubations were terminated by extraction of the cells or tissues with $CHCl_3$/MeOH (1:1) as described below.

Radiochromatographic Analyses

Total lipids were extracted from cells and tissues by the modified Folch procedure of Bridges and Coniglio (12). Total radioactivities of the extracts were measured by liquid scintillation counting of aliquots. The extract was dried under a stream of N_2 and methyl esters of the fatty acids were formed by transesterification with sodium methoxide as we described earlier (4). Methyl esters were analyzed for mass and radioactivity by a Packard 427 gas chromatograph equipped with a sample splitter (9:1) which divided the column effluent between a Packard 497 gas proportional radiation counter (83% efficiency) and a flame ionization mass detector. Gas chromatography was otherwise carried out as we have described previously (4). Radioactivity was assigned to specific fatty acids by corresponding retention times. For WT incubations, aliquots of methyl esters were hydrogenated to confirm assignment of radioactivity by appearance of radioactivity in corresponding saturated methyl esters as described by Coniglio et al. (7).

^{14}C Incorporation into Lipid Classes

Total lipid extracts were separated into various lipid classes on Whatmak LHP-K silica gel thin layer plates developed with petroleum ether/ethyl ether/HOAc (80:20:1) as we have described previously (4). Bands of lipid visualized in an I_2 tank were scraped from the plate into counting vials and radioactivity was determined by liquid scintillation counting. Lipid remaining at the origin was classified as phospholipid.

RESULTS

Viability of Cells

Cells remained viable throughout the 15-hr incubation. Five hr after enzymatic dispersion,

93% of cells in the suspensions excluded trypan blue. The O_2 uptake of these cells was 0.5 nmol/min/10^6 cells in agreement with Romrell et al. (11). After 15 and 24 hr of incubation, respectively, 90 and 82% of the cells still excluded the trypan blue. After an initial equilibration period, [^{14}C] acetate incorporation into total lipid of cell suspensions remained approximately linear with respect to time of incubation from 10-24 hr (data not shown).

Incorporation of ^{14}C into Lipid Classes

All enriched germinal cell fractions (PS, RS, CS) and WT in vitro incorporated [^{14}C] acetate into total lipid at different levels with respect to cell number (Table 2). Although variability precluded statistical significance, in each of three experiments, values of dpm/10^6 cells were ranked in the same increasing order as the mean values, i.e., CS > WT > PS > RS. The persistence of this same pattern in values of dpm/10^6 cells for total fatty acids (Table 3) from different experiments suggests that the differences in incorporation into total lipid among the various groups are real. The same pattern also persisted among values for phospholipid incorporation (Table 2), but again, variability precluded statistical significance. Incorporation into diacylglycerol was much lower in enriched germinal cell fractions than in WT–significantly so in PS and RS. In every case, incorporations into cholesterol and cholesteryl ester were significantly lower in enriched germinal cell fractions than in WT, probably reflecting the absence of Leydig cells from the germinal cell incubations. Levels of radioactivity found in unesterified fatty acid were high and quite variable in all enriched germinal cell incubations but not significantly different from WT values. The most striking differences among groups were in incorporation into triacylglycerol which was 83-97% lower in enriched germinal cells than in WT.

The distribution of radioactivity (% RA) among lipid classes of WT injected with [^{14}C]-acetate (in vivo) was similar to that of WT incubated with [^{14}C] acetate in vitro. WT in vivo incorporated a lower percentage of radioactivity into triacylglycerol and a correspondingly higher percentage into phospholipid. Distributions of radioactivity of enriched germinal cells reflect much higher incorporations into unesterified fatty acids and lower incorporations into triacylglycerol than WT in vivo or in vitro. Total incorporation of in vivo injections (2,300 dpm/10^6 cells) was of the same order of magnitude as that of in vitro incubations, although equilibration of [^{14}C] acetate with body pools in vivo makes such comparisons questionable.

Fatty acid compositions of the enriched germinal cells (not shown) were similar to those which we have reported previously (4). The 20:4 was lower and 22:5 was higher in RS and CS, the later stages of germinal differentiation, than in PS. The 22:5/20:4 ratios for each class of cells were 0.6 (PS), 1.1 (RS), and 1.1 (CS). Composition of lipid classes in similar preparations were also reported previously (4).

Incorporation of ^{14}C into Fatty Acids

All enriched germinal and WT cell preparations incorporated [^{14}C] acetate into 12, 14, 16, 18, 20, 22 and 24 carbon fatty acids (Table 3). The magnitudes of incorporations (dpm/10^6 cells) into total fatty acid followed the same pattern observed for total lipid, CS > WT > PS > RS. WS, containing all of the germinal cells, was intermediate and roughly equivalent to PS, whereas RB was lowest in total incorporation, reflecting, respectively, the cellular heterogeneity of WS and the low proportion of actual cells (20%) in RB. In contrast to the similar pattern seen in total lipid incorporation, these relationships are supported by statistical analysis.

In vivo and in vitro incorporation patterns (% RA) were quite similar, although WT in vivo incorporated a much lower percentage into 14:0 than any of the in vitro incubations. All groups incorporated the highest percentages of ^{14}C (40-66%) into 16:0 among the various fatty acids.

There were numerous significant differences in incorporation into various fatty acids among the various groups. Some of these simply reflected corresponding differences in total fatty acid incorporation and were not accompanied by significant differences in % RA. For example, the values of % RA in 14:0 of PS and RS were comparable, but values of dpm/10^6 cells differed by more than 300%, roughly proportional to the respective values of dpm/10^6 cells for total fatty acid. However, due to the weighting effect of cell number, proportionality between values of dpm/10^6 cells and % RA was not observed in every case. Moreover, values for % RA and dpm/10^6 cells sometimes showed opposite trends. For example, the percentage of radioactivity incorporated into 16:0 increased with the progressive stages of germinal cell differentiation PS → RS → CS but RS actually incorporated 52% less dpm/10^6 cells than PS.

The most striking and consistent differences in incorporation among the various germinal cell groups were seen in the 20-24 carbon fatty acids. As a group, these long-chain fatty acids declined in both percentage RA and dpm/10^6

TABLE 2

Incorporation of [1-^{14}C] Acetate into Various Lipid Classes by Enriched Cell Types and Whole Testis[a]

Lipid class	Cell fraction (number of experiments)								
	PS (3)		RS (3)		CS (3)		WT in vitro (8)		WT in vivo (5)
	% RA	dpm/10^6	% RA	dpm/10^6	% RA	dpm/10^6	% RA	dpm/10^6	% RA
Total lipid	100	4443 ± 1499	100	3146 ± 1445	100	8107 ± 3539	100	6880 ± 1298	100[b]
Phospholipid	52 ± 20	2649 ± 1777	51 ± 22	1987 ± 1615	68 ± 25	6974 ± 3990	60 ± 3	3813 ± 661	83 ± 3
Diacylglycerol	0.5 ± 0.1	36 ± 9[c]	0.5 ± 0.2	20 ± 4[d]	1 ± 0.2	73 ± 20	2 ± 0.1	138 ± 28	2 ± 0.1
Cholesterol	2 ± 1	70 ± 36[e]	1 ± 0.2	38 ± 13[d]	1 ± 0.3	35 ± 6[f]	3 ± 0.2	180 ± 31	3 ± 0.2
Unesterified fatty acid	39 ± 16	1409 ± 784	31 ± 11	691 ± 218	27 ± 22	876 ± 508	6 ± 1.5	368 ± 41	3 ± 0.3
Triacylglycerol	4 ± 2	130 ± 60[c]	14 ± 11	320 ± 270[e]	1 ± 0.4	51 ± 23[c]	26 ± 4	1885 ± 525	10 ± 0.4
Cholesteryl ester	1 ± 0.6	45 ± 28[e]	1 ± 0.6	27 ± 15[g]	1 ± 0.8	46 ± 14[e]	2 ± 0.2	165 ± 40	3 ± 0.1

[a][1-^{14}C] Acetate (10 μCi) was added to media of 15-hr in vitro incubations or injected intratesticularly for in vivo studies. Total lipids were extracted and separated by thin layer chromatography. Values are reported as percentage of total radioactivity and dpm/10^6 cells ± SEM. Student's t-test was used for statistical comparison.

[b]2,300 ± 260 dpm/10^6 cells (2706 dpm/mg wet wt of decapsulated testis).

[c]Different from WT ($p < .01$).

[d]Different from WT ($p < .005$).

[e]Different from WT ($p < .05$).

[f]Different from WT ($p < .001$).

[g]Different from WT ($p < .02$).

TABLE 3

Incorporation of [1-^{14}C] Acetate into Fatty Acids of Enriched Germinal Cell Types and Whole Testis[a]

	Cell fraction incubated (number of experiments)												
	PS (5)		RS (7)		CS (2)		RB (3)		WS (5)		WT in vitro (4)		WT in vivo (5)
Fatty acid	% RA	dpm/10^6	% RA	dpm/10^6	% RA	dpm/10^6	% RA	dpm/10^6	% RA	dpm/10^6	% RA	dpm/10^6	% RA
12:0	1 ± 0.6	137 ± 47	4 ± 0.5	62 ± 11	2 ± 0.5	108 ± 62	1 ± 0.6	20 ± 8[b]	2 ± 0.5	101 ± 38	1 ± 0.5	84 ± 10	1 ± 0
14:0	16 ± 2	1162 ± 137[c,d]	18 ± 2	310 ± 57[e]	20 ± 6	788 ± 407	17 ± 1	260 ± 75[e]	18 ± 2	866 ± 203	10 ± 1	513 ± 85	3 ± 0.4
16:0	40 ± 2	1652 ± 227[f]	46 ± 4	797 ± 184[c,e]	60 ± 4	3195 ± 2305	52 ± 6	681 ± 172[e]	48 ± 4	2291 ± 521	66 ± 2	3580 ± 811	59 ± 3
16:1	9 ± 1	180 ± 84	7 ± 2	92 ± 54	4 ± 4	384 ± 384	11 ± 4	131 ± 30	5 ± 2	78 ± 33	3 ± 0	165 ± 39	2 ± 1
18:0	5 ± 0.5	135 ± 42[g]	4 ± 0.3	51 ± 7[b,h]	4 ± 0	201 ± 140	4 ± 1.5	51 ± 22[e,d]	4 ± 0.3	162 ± 30	6 ± 0.5	355 ± 78	10 ± 0.5
18:1	6 ± 1.4	89 ± 53	3 ± 1.5	39 ± 30[g]	3 ± 3.0	256 ± 256	3 ± 0.3	41 ± 8[g]	2 ± 1.3	85 ± 55	5 ± 0.8	268 ± 81	9 ± 1.0
20:1	4 ± 1.3	75 ± 53	0	0	0	0	0	0	tr	12 ± 12	0	0	tr
20:3(n-6)	2 ± 0.6	28 ± 14	1 ± 0.7	10 ± 7	tr	8 ± 8	0	0	tr	0	tr	35 ± 22	1 ± 0.4
20:4(n-6)	4 ± 1.3	85 ± 46	tr	11 ± 5	tr	8 ± 8	tr	6 ± 6	tr	24 ± 24	1 ± 0.5	83 ± 39	3 ± 0.8
22:4(n-6)	4 ± 1.0	161 ± 39[i]	4 ± 0.7	44 ± 7[j]	1 ± 1.0	15 ± 15	1 ± 1.6	11 ± 5[g]	4 ± 0.9	152 ± 61	3 ± 0.5	158 ± 48	4 ± 0.7
22:5(n-6)	2 ± 0.8	38 ± 22	2 ± 0.6	16 ± 6[k]	tr	8 ± 8	0	0	2 ± 1.6	85 ± 68	tr	24 ± 23	2 ± 0.5
22:6(n-3)	5 ± 0.5	165 ± 17[b,c,h]	3 ± 0.4	28 ± 6[h]	2 ± 0.2	79 ± 49[g]	2 ± 0.6	24 ± 8[h]	2 ± 0.4	74 ± 7	1 ± 0.4	50 ± 20	2 ± 0.5
24:4(n-6)	1 ± 0.4	145 ± 43[l]	2 ± 0.5	36 ± 10[m]	tr	64 ± 64	2 ± 0.9	27 ± 16[m]	2 ± 0.4	87 ± 11	1 ± 0.2	54 ± 22	2 ± 0.7
24:5(n-6)	1 ± 1.0	74 ± 48	2 ± 0.5	19 ± 5	2 ± 1.0	15 ± 15[e]	tr	12 ± 12[e]	4 ± 0.6	146 ± 44	3 ± 0.6	125 ± 56	2 ± 0.4
Total fatty acid	100	4328 ± 333[c]	100	1582 ± 320[d,h]	100	6200 ± 2772	100	1305 ± 332[g,h]	100	4237 ± 586	100	5515 ± 1299	

[a][1-^{14}C] Acetate (10 μCi) was added to media of 15-hr in vitro incubations or injected intratesticularly for in vivo studies. Radioactivity was measured by gas radiochromatography. Values are reported as % total radioactivity and dpm/10^6 cells ± SEM. WS is the whole cell suspension prior to separation on the Sta-Put gradient. Statistically significant differences between groups (Student's t-test) are noted by superscripts only the first time a number in a pair being compared occurs. The corresponding value being compared and the p-value of significance is given in the footnotes below. Values less than 1% are reported as trace (tr).

[b]WT (.005).

[c]RS (.001), RB (.005).

[d]WT (.02).

[e]WS (.05).

[f]RS (.001), RB (.02), WT (.01).

[g]WT (.05).

[h]WS (.005).

[i]RS (.02), CS (.02), RB (.01).

[j]RB (.005), WT (.05).

[k]RB (.05).

[l]RS (.05), RB (.05).

[m]WS (.02).

in the progressive stages of differentiation, PS → RS → CS. The corresponding values for total incorporation into LCPA are 23 → 12 → 5%, and 771 → 164 → 197 dpm/10^6 cells. Differences in values of dpm/10^6 cells between PS and RS and CS are highly significant ($p < .005$). Among the individual long-chain polyenoic acids, incorporation into 22:4 was 73 and 91% lower in RS and CS, respectively, than in PS, incorporation into 22:6 was 83% lower in RS than in PS, and incorporation into 24:4 was 75% lower in RS than in PS. Although these were the only statistically significant differences, all individual LCPA had lower levels of incorporation in RS and CS than in PS.

RB fractions, in which the major nucleated cell type is actually CS, distributed ^{14}C among the fatty acids in a pattern similar to that of CS and the level (dpm/10^6 cells) of incorporation into individual fatty acids was consistent with the number of CS present in the incubations, without regard to the RB present. WS, which is the mixture of all the cell types prior to separation on the Sta-Put gradient, had a distribution of ^{14}C consistent with this cell composition. Absolute levels of incorporation (dpm/10^6 cells) of WS into individual fatty acids were generally intermediate between PS and RS or CS, also consistent with the cell composition. Values for dpm/10^6 cells of WT, which did not go through the cell dispersal procedure, were not significantly different from those of WS for any fatty acid, although there were substantial differences in the values of percentage RA for 14:0 and 16:0.

DISCUSSION

Patterns of incorporation of [^{14}C]acetate into fatty acids in these studies suggested the presence in each of the mouse testis cell populations (PS, RS, CS, RB, WS, WT) of a full range of enzymatic activities necessary for biosynthesis of fatty acids, both de novo (12:0, 14:0, 16:0) and by elongation-desaturation reactions (1,5). The presence of ^{14}C in 16:1, 18:1, 20:3(n-6), 20:4(n-6), 22:5(n-6) and 22:6(n-3) is evidence for the presence of $\Delta 9, \Delta 8, \Delta 5$ and $\Delta 4$ desaturase activities. The patterns of incorporation of in vitro incubations are unlike those reported for rat (6,13) or human (7) testis slices, although it must be noted that incubation times differed greatly (3 hr vs 15 hr) and the present incubations took place in culture media as opposed to the phosphate buffer used in the earlier studies. On the other hand, the distribution of radioactivity (% RA) among fatty acids of mouse WT in vivo is almost identical to that of rat testis (calculated from data presented in Wharton and Coniglio, ref. 6; data not shown) injected with [^{14}C]acetate, in spite of the difference in incubation times.

The striking similarity between the fatty acid incorporation patterns seen with the various in vitro incubations and in vivo intratesticular injections is evidence that metabolic activities of germinal and nongerminal cells are unaffected by the dispersal and separation procedures. The WT and WS incubations which contained substantial numbers of Sertoli cells were not greatly different in incorporation into specific fatty acids from the PS incubations, which were practically void of Sertoli cells. Therefore, the unique fatty acid metabolism of the mouse testis does not seem to be mediated by the Sertoli cells at the level of fatty acid biosynthesis. The germinal cell preparations all had the capacity to synthesize 22:5(n-6) and 22:6(n-3), the characteristic LCPA of mouse testis. From stoichiometric considerations, the relative biosynthesis of LCPA would actually be substantially greater than that indicated by the incorporations of ^{14}C shown in Table 3, since specific activities of these fatty acids which are synthesized by elongation-desaturation reactions would be only 12-38% of the specific activity of the fatty acids synthesized de novo from the same [^{14}C]acetate pool (5).

LCPA biosynthetic activity was highest in PS among the germinal cell dispersions, declining with later stages of differentiation (RS,CS). This is consistent with previous evidence that LCPA accumulation occurs during the PS stage in the mouse (4).

On the other hand, WT distributed the newly synthesized fatty acid among the lipid classes somewhat differently from germinal cell types. The dispersed cell incubations were characterized by high levels of incorporation into unesterified fatty acid. Incorporation into phospholipid and triacylglycerol was much more variable in the germinal cell preparations and PS and CS put much less ^{14}C into triacylglycerol than did WT in vitro or WT in vivo. Diacylglycerol contained less ^{14}C in each of the germinal cell preparations than in WT. These observations suggest that the acylglycerol pathways which operate in the WT may not have remained completely intact in the germinal cell incubations. Since the cells in these incubations retained impermeable membranes and incorporated ^{14}C throughout the incubation period, it is unlikely that cell lysis would explain these results. It is possible that some substrate, cofactor or metabolic activity in these pathways may become limiting in germinal cells when they are isolated from the intratubular environment or from other testis cell

types such as Sertoli cells. It is also possible that the cells may have deteriorated in more subtle ways, causing them to lose unesterified fatty acid to the media. High levels of incorporation of [^{14}C] acetate into unesterified fatty acids have also been observed in incubations of rat testis slices (13) and human testis sections (7) with only a 3-hr incubation time, but as we have already pointed out, incubation conditions were quite different.

Beckman and Coniglio (9) have reported data suggesting that Sertoli cells are much more active in docosapentaenoic acid synthesis than germinal cells in the rat. However, in vivo studies used intratesticular injections of ^{14}C substrates prior to separation of the testis cell population into enriched Sertoli cells and germinal cells. Since their germinal cell fraction probably consisted mainly of spermatids, the metabolic activity of spermatocytes was probably not substantially reflected. Our study suggests that, in the mouse, primary spermatocytes may be highest in the elongation-desaturation activities necessary for LCPA biosynthesis. Although we have not measured [^{14}C] acetate incorporation by isolated Sertoli cells, our WT incubations, which contained Sertoli cells in intact tubules, did not reflect any localization of LCPA biosynthesis to those cells. On the other hand, Sertoli cells may play a crucial role in triacylglycerol biosynthesis which was apparently 6-37 times higher in WT than in germinal cell incubations. Since triacylglycerols are apparently the major reservoirs of 22:5 in both rat and mouse testes (2,4), triacylglycerol biosynthesis may be as important to accumulation of that LCPA as the biosynthesis of the fatty acid itself. The characteristic and highly specialized lipid composition of mouse testis germinal cells probably results from a complex metabolic interplay between the various germinal cell types and Sertoli cells, in which the germinal cells are capable of playing an important role, at least at the level of fatty acid biosynthesis.

ACKNOWLEDGMENTS

This project was supported in part by USPHS Grant HD 13019. Appreciation is expressed to Ellen Huth and W.F. Farnham for excellent technical assistance.

REFERENCES

1. Coniglio, J.G. (1977) in The Testis (Johnson, A.D., and Gomes, W.R., eds.) Vol. 4, pp. 425-449, Academic Press, New York, NY.
2. Beckman, J.K., Gray, M.E., and Coniglio, J.G. (1978) Biochim. Biophys. Acta 530, 367-374.
3. Beckman, J.K., and Coniglio, J.G. (1979) Lipids 14, 262-267.
4. Grogan, W.M., Farnham, W.F., and Szopiak, B.A. (1981) Lipids 16, 401-410.
5. Coniglio, J.G., Wharton, A.R., and Beckman, J.K. (1977) in Function and Biosynthesis of Lipids (Bazan, N.G., Brenner, R.R., and Giusto, N.M., eds.) pp. 575-589, Plenum, New York, NY.
6. Wharton, A.R., and Coniglio, J.G. (1977) J. Nutr. 107, 79-86.
7. Coniglio, J.G., Grogan, W.M., and Rhamy, R.K. (1977) J. Reprod. Fert. 51, 463-465.
8. Wharton, A.R., and Coniglio, J.G. (1975) Lipids 10, 783-789.
9. Beckman, J.K., and Coniglio, J.G. (1980) Lipids 15, 389-394.
10. Grogan, W.M., Farnham, W.F., and Sabau, J.M. (1981) J. Histochem. Cytochem. 29, 738-746.
11. Romrell, L.J., Bellve, A.R., and Fawcett, D.W. (1976) Dev. Biol. 49, 119-131.
12. Bridges, R.B., and Coniglio, J.G. (1970) Lipids 5, 628-635.
13. Evans, O.B., Jr., Zseltvay, R., Whorton, R., and Coniglio, J.G. (1971) Lipids 6, 706-711.

[Received March 8, 1982]

Fecal Steroids in Diarrhea: IV. Cholera

CHARLES T. L. HUANG[a,*], **MYRON M. LEVINE**[b], **GEORGETTE S. DAOUD**[a], **DAVID R. NALIN**[b] and **BUFORD L. NICHOLS**[a], [a]*Section of Nutrition and Gastroenterology, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030;* and [b]*Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD 21201*

ABSTRACT

Fecal bile acid and neutral sterol patterns were studied in eight healthy adult volunteers who were challenged with *Vibrio cholerae* classical Ogawa 395 strain in the course of vaccine development studies. Bacterial 7α-dehydroxylation of cholic and chenodeoxycholic acids was not altered during experimentally induced cholera diarrhea, despite the fact that fecal weight in g/day (wet wt) was increased greatly during diarrhea (1913 ± 390 vs 161 ± 11 in controls, $p < 0.005$). Consistent with the findings on bile acids, no significant changes in the production of coprostanol, epicoprostanol, or coprostanone were observed although the percentage of unmodified cholesterol was increased during the diarrheal episode (20.7 ± 3.3% vs 11.9 ± 2.3, $p < 0.02$). Total concentrations of both bile acids and cholesterol in mg/g of feces (wet wt) were decreased considerably as a result of diarrhea). However, total bile acid and neutral steroid excretions in mg/kg/day in subjects with and without diarrhea do not appear to be different. Intestinal transit times, measured in eight subjects by the use of carmine red dye, were found to be shortened in diarrhea (5.8 ± 1.1 hr vs 23.4 ± 4.1 hr in controls, $p < 0.001$). The results from this study are similar to those observed in experimentally induced travellers' diarrhea associated with toxigenic *Escherichia coli*, but they are in striking contrast to the changes in gastrointestinal steroid metabolism observed in acute shigellosis, an invasive intestinal infection.
Lipids 17:612-616, 1982.

Extensive changes in fecal bile acid (BA) and neutral steroid (NS) patterns were observed in a previous investigation conducted in normal volunteers who were infected with *Shigella flexneri* (1). Bacterial degradation of fecal steroids was found to be reduced despite the fact that total bile acid and neutral sterol concentrations were diluted. In a similar investigation (2), volunteers who had received a challenge of enterotoxigenic *Escherichia coli* did not have changes in the gastrointestinal metabolism of bile acids or neutral steroids. This contrasting pattern of response to diarrheal etiologic agents suggested specific alterations in bile acid metabolism associated with the etiology of diarrhea. To test this conclusion, the observations were extended to a study of volunteers challenged with *Vibrio cholerae*. The mechanism of diarrhea produced by both *E. coli* and *V. cholerae* is mediated through enterotoxin-induced stimulation of adenylate cyclase within mucosal enterocytes of the small bowel. The resultant intracellular accumulation of cyclic AMP results in net intestinal secretion in the absence of bacterial invasion. This stands in contrast to diarrhea mediated by *S. flexneri* which requires overt mucosal invasion, usually of the large bowel mucosa. The purpose of this investigation was to determine whether *V. cholerae*, the prototype of small bowel secretory diarrhea, would produce the same pattern of bile acid secretion as that produced in the milder disorder associated with *E. coli* (3), confirming the conclusion made in the previous publication that the mechanism of diarrhea is reflected by fecal sterol pattern.

*Author to whom correspondence should be addressed. Current address: Merichem Co. Research Center, 1503 Central, Houston, TX 77012.

Abbreviations in Fig. 1: C = cholic, CDC = chenodeoxycholic, DOC = deoxycholic, LC = lithocholic, Keto A = ketohydroxy bile acids including 7-ketodeoxycholic, 12-ketolithocholic, 7-ketolithocholic, and 3, 12-diketocholanic acids, UNIDENT = unidentified bile acids, ISODOC = isodeoxycholic, URSODOC = ursodeoxycholic. Abbreviations in Fig. 2: CH = cholesterol, CO = coprostanol, EPICO = epicoprostanol, COO = coprostanone, and UNIDENT = unidentified endogenous neutral steroids.

MATERIALS AND METHODS

Bacteriology

V. cholerae classical Ogawa 395 strain produces an enterotoxin and causes a profuse watery diarrheal syndrome in volunteers that is typical of cholera. This strain was fed to volunteers as part of a long-term program to develop vaccines against cholera (4,5).

Volunteer Studies

Volunteers were healthy adults (5 males, 3 females), ranging in age from 19 to 32 yr. Studies were carried out under quarantine in the Isolation Ward of the Center for Vaccine Development located within the University of Maryland

Hospital. The methods of medical screening, clinical surveillance and care of the volunteers, informed consent, preparation of the *V. cholerae* inoculum and bacteriologic culture techniques have been previously described (4,5). Control stool specimens were collected either prior to challenge or four weeks after recovery from diarrhea. Each subject was fasted 1-½ hr before and after oral inoculation with vibrios. In order to neutralize gastric acid, thereby ensuring occurrence of diarrhea with a smaller inoculum, 2 g $NaHCO_3$ was added to 150 ml distilled water and the volunteers drank 120 ml. The inoculum (10^6 live organisms of *V. cholerae* classical Ogawa 395 strain) was added to the remaining 30 ml and was ingested 1 min later. All subjects developed cholera diarrhea within two days postinoculation (4,5). Duplicate fecal samples collected during diarrhea, but prior to medical treatment, were studied to determine the effect of diarrhea on steroid metabolism. Methods for collection of specimens have been described previously (1). Intestinal transit times (ITT) were measured for all of the subjects prior to challenge and during diarrhea; each subject ingested 500 mg of carmine red dye, a nonabsorbable marker, that was monitored as described by Higgs et al. (6) and Dimson (7).

Steroid Analysis

Duplicate aliquots of the homogenized stools were analyzed for BA and NS. Detailed procedures of thin layer chromatography (TLC) for the separation of BA have been described elsewhere (8,9). BA were analyzed by gas liquid chromatography (GLC) according to methods described by Kuksis (10) and Yousef et al. (11). Neutral steroids were analyzed by the combined TLC and GLC method described by Miettinen et al. (12). GLC analyses were performed with a Packard Becker gas chromatograph Model 420 with dual flame ionization detectors. Chromatographic conditions and procedures have been described previously (1). 5α-Cholestane was used as an internal standard for quantitation of both BA and NS. Cholic-24-^{14}C and cholesterol-7α-^{3}H of high specific activity were added as internal recovery standards to correct for incomplete recoveries during extraction and TLC.

RESULTS

Effects of Cholera on Fecal BA

The BA profile of fecal samples collected from eight volunteers before and during infection with *V. cholerae* (but before antibiotic therapy) is shown in Figure 1. Statistical evaluation was done by Student's t-test for the paired samples. All values were expressed as (mean ± SEM)

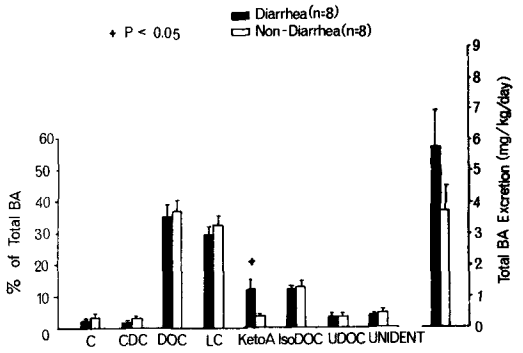


FIG. 1. Fecal bile acid profiles for eight adult volunteers challenged with *V. cholerae* classical Ogawa 395 strain. All values (mean ± SEM) are expressed as percentage of total bile acids. Total bile acid excretion is expressed as mg/kg body wt/day.

percentage of total BA in the feces so that the effect of dilution could be avoided. Fecal weights in g/day (wet wt) were increased greatly during diarrhea (1,913 ± 390 vs 161 ± 11 in controls, $p < 0.005$). Total BA concentrations, in mg/g feces (wet wt), were lower during the diarrheal episode (0.34 ± 0.15) than those in nondiarrheal controls of the same subjects (1.87 ± 0.52, $p < 0.02$). However, it can be seen from Figure 1 that there were no significant changes ($p > 0.05$) in the composition of BA for the eight *V. cholerae* challenged subjects, except that ketohydroxy BA was increased in diarrheal samples (11.9 ± 3.3% vs 3.5 ± 0.6%, $p < 0.05$). Total excretion of BA, expressed as mg/kg body wt/day, was not significantly different.

Effect of Cholera on Fecal NS

The NS profile of feces from the same eight subjects before and during cholera diarrhea, but before antibiotic treatment, is shown in Figure 2. As seen in the case of BA, total NS concentrations in mg/g feces (wet wt) were reduced greatly in diarrheal samples (0.57 ± 0.15 vs 3.97 ± 0.43, $p < 0.001$). In addition, the percentages of unmodified cholesterol (CH) were increased during the diarrheal episode (Fig. 2). However, no significant changes in NS metabolites, e.g., coprostanol (CO), epicoprostanol (EPICO), and coprostanone (COO), were observed. Total excretion of NS expressed as mg/kg body wt/day was not significantly different.

Sequence of Bile Acid Alteration

Figure 3 shows the sequence of BA alterations in the stool samples of one subject (J.P.) during cholera infection. The diarrhea started

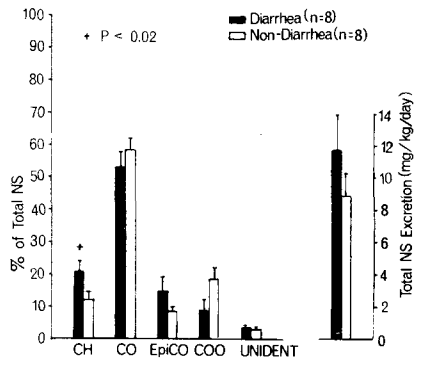


FIG. 2. Fecal neutral steroid profiles for eight adult volunteers challenged with *V. cholerae* classical Ogawa 395 strain. All values (mean ± SEM) are expressed as percentage of total cholesterol metabolites.

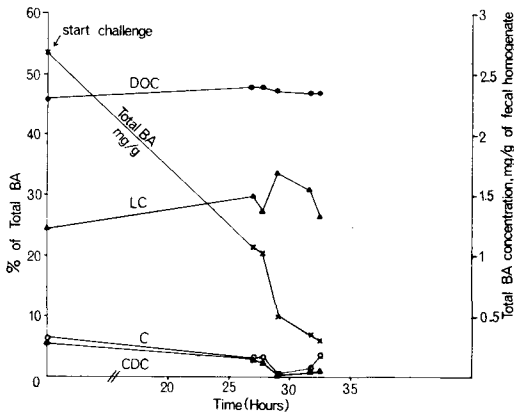


FIG. 3. Sequence of bile acid alteration in the stool samples of a subject (J.P.) with cholera. All values are expressed as percentage of total bile acid in the feces except that total bile acid concentrations are shown as mg/g fecal wet wt.

27 hr postinoculation. Four serial stools were collected within 6 hr following the initial diarrheal sample. We observed that a secondary BA, such as deoxycholic acid (DOC), remained relatively constant in all samples despite the fact that total BA concentration in mg/g feces (wet wt) decreased progressively with time elapsed after the initial episode of diarrhea. Other BA such as lithocholic (LC), cholic (C) and chenodeoxycholic (CDC) showed greater variations than DOC, but the overall pattern also appeared to be unaltered with the serial collections. This is in striking contrast with the pattern observed in acute shigellosis where a two- to five-fold reduction in DOC and LC and a concomitant increase in C and CDC were observed within 4 hr following the initial diarrheal sample (1).

Intestinal Transit Time (ITT)

ITT (or strictly speaking, the mouth-to-anus transit time) of eight subjects in this study, as measured by the use of a carmine red marker, were found to be 5.8 ± 1.1 hr during the diarrhea associated with *V. cholerae*. The corresponding values before challenge were 23.4 ± 4.1 hr for the same subjects ($p < 0.001$). No clear relationship was noted among ITT, percentage of each BA or NS, and fecal mass (wet wt) (data not shown).

Comparison of Cholera with Other Bacterial Diarrheas

Table 1 gives the mean change in fecal steroids between the paired nondiarrheal and diarrheal samples for adult volunteer subjects challenged with *V. cholerae*. For the purpose of comparison, the corresponding values from other adult subjects with travellers' diarrhea (TD) associated with toxigenic *E. coli* (2) and those with acute shigellosis (1) from previous studies are also listed. An analysis of variance comparison of these means indicates a significant difference between *V. cholerae* and shigella for both percentage C and CH. *V. cholerae* and *E. coli* are different significantly from shigella for 7α-dehydroxylase activity, defined as the ratio (× 100) of percentage DOC/(%DOC + % C), and percentage CO. There is no statistically significant difference between the three types of diarrhea for percentages of DOC, LC, CDC, or LC/(% LC + % CDC). There is no statistically significant difference between *V. cholerae* and *E. coli* for all fecal steroids listed.

DISCUSSION

We reported previously the results of a study on fecal steroid profiles of six adult volunteers who developed TD following challenge with toxigenic *E. coli* B7A and observed that bacterial 7α-dehydroxylation of cholic and chenodeoxycholic acids was not altered (2). This is consistent with the observation that the production of coprostanol from cholesterol was not changed in the same diarrheal subjects (2). These data confirm results from our earlier report (3) that bacterial modification of BA and NS was not altered in patients experiencing TD due to *E.coli* elaborating heat-stable enterotoxin. These results in *E. coli* diarrhea, however, differ markedly from the changes in gastrointestinal steroid metabolism previously observed in acute shigellosis (1). Bacterial degradation of fecal steroids was

TABLE 1

Mean (± SEM) Change in Fecal Steroid Patterns between the Paired Diarrhea and Nondiarrheal Control Samples for Adult Volunteer Subjects

%	Small bowel diarrhea *V. cholerae*	Small bowel diarrhea *E. coli*[a]	Large bowel diarrhea Shigella[b]	p Value[c]
Bile acid	(n = 8)	(n = 6)	(n = 5)	
DOC	-1.7 ± 2.6	-1.4 ± 8.9	-13.9 ± 2.5	NS
LC	-3.1 ± 3.3	-12.1 ± 3.4	-15.7 ± 4.3	NS
C	-1.1 ± 1.5	+6.2 ± 4.1	+12.6 ± 2.0<	<0.01
CDC	-1.2 ± 0.9	+2.3 ± 2.4	+5.5 ± 3.5	NS
$\frac{DOC}{DOC + C} \times 100$	+1.7 ± 4.1	-11.8 ± 8.7	-46.0 ± 7.1<	<0.001
$\frac{LC}{LC + C} \times 100$	+2.5 ± 3.1	-9.9 ± 8.2	-16.7 ± 6.2	NS
Neutral sterol				
CO	-5.6 ± 8.1	-2.2 ± 9.2	-51.7 ± 7.0<	<0.005
CH	+8.6 ± 2.8	+25.1 ± 12.5	+59.6 ± 8.0<	<0.005

[a]Taken from ref. 2. The abbreviations used are the same as those in Figs. 1 and 2.

[b]Taken from ref. 1.

[c]Differences between small bowel diarrheas (cholera and/or TD) and large bowel diarrhea (shigella) with regard to the mean changes in fecal steroids during the diarrheal episodes before the antibiotic treatment.

NS = not significant ($p > 0.05$).

found to be reduced during diarrhea associated with acute shigellosis in five volunteer subjects challenged with *S. flexneri* 2a (strain M42-43), despite the fact that total steroid (BA and NS) concentrations in mg/g feces (wet wt) were decreased in shigellosis to a magnitude comparable to that observed in TD associated with toxigenic *E. coli* (2). Specifically, the percentages of DOC and LC acids of the total BA in the feces were decreased significantly in diarrheal samples with a concomitant increase in the percentages of C and CDC acids. In addition, there was a significant reduction in coprostanol content in the feces with a concomitant increase in cholesterol in the shigella diarrhea.

In this study, we have investigated the effects of experimentally induced cholera on fecal steroid profiles of eight healthy adult volunteers. The results were found to be similar to those observed in *E. coli* diarrhea but not to those in acute shigellosis (see Table 1). Bacterial 7α-dehydroxylation of cholic and chenodeoxycholic acids was unchanged despite the fact ITT was shortened significantly during the diarrhea episode (Fig. 1). In addition, the percentage of cholesterol metabolites, such as coprostanol, epicoprostanol and coprostanone, was unchanged although the percentage of unmodified cholesterol was increased during the cholera infection (Fig. 2). Thus, there is a reflection of at least two distinct mechanisms of diarrhea production. One, typified by enterotoxin-producing *E. coli* and *V. cholerae*, is mediated through a stimulation of secretion in the small bowel in the absence of mucosal invasion. The other, exemplified in shigellosis, requires invasion of intestinal mucosa, particularly of the large bowel. In shigellosis, the increase in fecal primary BA and unmodified cholesterol, with concomitant decrease in secondary BA and coprostanol, speaks for a reduced interaction between luminal sterols and colonic bacterial flora. The absence of changes in 7α-dehydroxylation of BA and the biohydrogenation of cholesterol in TD associated with toxigenic *E. coli* and cholera indicate no appreciable alteration in interaction between intestinal sterols and bacterial flora (Table 1). This appears to be the case, even in the presence of large fecal losses and shortened ITT in cholera. Thus, it seems evident that the contrasting profiles of fecal steroids may provide a basis for biochemical differentiation between two different mechanisms of diarrhea production, i.e., stimulation of fluid secretion vs mucosal invasion. Further studies in this area are in progress.

ACKNOWLEDGMENTS

This investigation was supported by a NASA Skylab Grant #NASA-12728 from the National Aeronautics and Space Administration, Lyndon B. Johnson Space Center, Houston, TX, and by NIH 5-T22-DE-0035 and HEW 06-D-00178. The volunteer studies were supported by research contract NO1-AI-42553 from the National Institute of Allergy and Infectious Diseases, research contract DAMD 17-78-C-8011 from the U.S.

Army Research and Development Command and grants from the Middendorff Foundation and the World Health Organization. This work is a publication of the USDA/SEA, Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine and Texas Children's Hospital. We thank Ying-Ping Hsieh and Avis Hall for skilled assistance. Editorial assistance provided by Roseland Klein and Mary Boyd is gratefully acknowledged.

REFERENCES

1. Huang, C.T.L., Woodward, W.E., Hornick, R.B., Rodriguez, J.T., and Nichols, B.L. (1976) Am. J. Clin. Nutr. 29, 949-955.
2. Huang, C.T.L., Levine, M.M., Daoud, G.S., Nalin, D.R., and Nichols, B.L. (1980) Am. J. Clin. Nutr. 33, 40-44.
3. Huang, C.T.L., Udall, J.N., Merson, M., and Nichols, B.L. (1978) Am. J. Clin. Nutr. 31, 626-632.
4. Levine, M.M., Nalin, D.R., Craig, J.P., Hoover, D., Bergquist, E.J., Waterman, D., Holly, H.P., Hornick, R.B., Pierce, N.P., and Libonati, J.P. (1979) Trans. Roy. Soc. Trop. Med. Hyg. 73, 3-9.
5. Levine, M.M (1978) in Cholera and Related Diarrheas (Ouchterlony, O., and Holmgren, J., eds.), pp. 195-203, Karger, Basel.
6. Higgs, R.H., Ellis-Pegler, R.B., and Lambert, H.P. (1975) Gut 16, 458-461.
7. Dimson, S.B. (1970) Arch. Dis. Child. 45, 232-235.
8. Huang, T.L., and Nichols, B.L. (1974) J. Chromatogr. 101, 235-239.
9. Huang, C.T.L., and Nichols, B.L. (1975) J. Chromatogr. 109, 427-431.
10. Kuksis, A. (1969) in Lipid Chromatographic Analysis (Marinetti, G.V., ed.) Vol. 2., pp. 215-312. Marcel Dekker, New York, NY.
11. Yousef, I.M., Kakis, G., and Fisher, M.M. (1972) Can. J. Biochem. 50, 402-408.
12. Miettinen, T.A., Ahrens, E.H., and Grundy, S.M. (1965) J. Lipid Res. 6, 411-424.

[Received February 9, 1982]

Phospholipid Studies of Marine Organisms: III. New Phospholipid Fatty Acids from *Petrosia ficiformis*

ESER AYANOGLU[a], ROBERT D. WALKUP[a], DONATO SICA[b] and CARL DJERASSI[a],
[a]Department of Chemistry, Stanford University, Stanford, CA 94305; and [b]Istituto di Chimica Organica dell'Universita di Napoli, Naples, Italy

ABSTRACT

The fatty acyl components of the phospholipids from the sponge *Petrosia ficiformis* consisted predominantly of branched, especially iso and anteiso acids. The two major components of the complex mixture are the hitherto unknown Z,Z-25-methyl-5,9-hexacosadienoic and Z,Z-24-methyl-5,9-hexacosadienoic acids. Other unknown acids are: 7,13,16-docosatrienoic acid, 15-methyldocosanoic acid, 15-methyltricosanoic acid and 24-methyl-5,9-pentacosadienoic acid. Short branched-chain fatty acids, presumably of bacterial origin, are considered to be the possible bioprecursors of these novel phospholipid constituents. The major phospholipids were PE, PC, PG, PS and PI. The distribution of fatty acids among the phospholipid classes was also studied.
Lipids 17:614-625, 1982.

Sponges represent a low position in the evolutionary scale because of their primitive structure. Approximately 5,000 sponges are reported to occur in nature (2). Bergmann's studies (3,4) on biochemical taxonomy of sponges and recent research carried out in this and other laboratories (5-7) have shown that these primitive organisms of the metazoan phyla are rich sources of complex mixtures of sterols, generally with side-chain elongation or branching, in contrast to higher organisms which usually contain only a few major sterols.

Recently, Litchfield and colleagues (8-11) have also reported high levels of novel fatty acids in some species of demosponges. Typical fatty acids possess straight chains 14 to 22 carbon atoms long, whereas these "demospongic" acids contain 24-30 carbons. In our laboratory (1), the sponge *Aplysina fistularis* was found to contain straight-chain and methyl-branched fatty acids containing 27-30 carbon atoms. In connection with our research on membrane phospholipid structure of marine organisms and the possible interaction between their sterols and the fatty acyl chains or head groups of their phospholipids (12,13), we are concentrating on those marine animals which contain large amounts of unusual marine sterols and little or no conventional sterols such as cholesterol. We now report the presence of new branched fatty acids from the sponge *Petrosia ficiformis* (Class Demospongia, Order Haplosclerida) which contains the unusual cyclopropane-containing sterol, petrosterol, as one of the major sterols (14-16).

EXPERIMENTAL

P. ficiformis sponge colonies were collected in the Bay of Naples, Italy. The total phospholipids were extracted and separated as described earlier (1) and were kept under argon at -10 C in chloroform/methanol (1:1, v/v) containing 0.002% BHT. TLC separations of phospholipids and fatty acid derivatives were performed on 250-μ layers of Silica Gel H. Rhodamine 6G (17) was used as a nondestructive color spray reagent especially for preparative purposes. Spray reagents (18) used for the identification of phospholipid classes (see Table 1) were ninhydrin (primary and secondary amino groups, PE, PS), Drogendorff (tertiary and quaternary amino groups, PC), periodate-Schiff (vicinal

TABLE 1

The Major Phospholipids of *P. ficiformis*

Phospholipid class	Mol %
Phosphatidylethanolamine (PE)	28
Phosphatidylglycerol (PG)	14
Phosphatidylcholine (PC)	21
Phosphatidylserine (PS)	12
Phosphatidylinositol (PI)	7

[1]For preceding paper, see ref. 1.

Abbreviations: PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; DPG: diphosphatidylglycerol; BHT: butylated hydroxytoluene; GC: gas chromatography; GC/MS: gas chromatography-mass spectrometry; TLC: thin layer chromatography; HPLC: high performance liquid chromatography; ^{1}H-NMR: proton nuclear magnetic resonance.

hydroxyl groups, PG, PI), 2,4-dinitrophenylhydrazine (plasmalogens) whereas Molybdenum Blue was the general spray reagent for all phospholipids. Phospholipids were also identified by TLC comparison with known samples (Sigma Chemical Co.). For quantitative estimation of the phospholipids, TLC plates were visualized by Rhodamine 6G, each spot was scraped and was subjected to a spectrometric phosphorus assay (19, 20), and corrections were made for residual phosphate present in the adsorbent and in the eluting solvents. General developing solvent systems for the analysis of the phospholipids were chloroform/methanol/acetic acid (65:35:10, v/v/v) and chloroform/methanol/28% ammonium hydroxide (65:35:8, v/v/v). The second developing solvent was also used for the quantitative estimation of the phospholipids and for their separation for the analysis of the fatty acid contents of each class. Developing solvent systems for the fatty acid methyl esters and the synthetically intermediate fatty alcohols, tosylates and nitriles were hexane/ether (8:2 and 7:3, v/v) and for the pyrrolidides, hexane/ether (3:7, v/v).

Capillary GC was done on a Carlo Erba series 4160 Fractovap chromatograph equipped with a fused silica column (30 m x 0.32 mm) coated with SE-54 (J & W Scientific, Inc.), a Model 400 LT programmer, a cooled on-column injection system and a flame ionization detector. The initial oven temperature was 70 C or 140 C for fatty acid methyl esters and 200 C for pyrrolidides and the temperature was programmed at 3.0 C/min. Final temperature was 285 C. Isothermal temperature for the detection of methyl 14-methylhexadecanoate, methyl 15-methylheptadecanoate and methyl 16-methylheptadecanoate was 190 C. Their pyrrolidides were analyzed at 245 C. The synthetic intermediate, 14-methylhexadecanol, was analyzed at 175 C. The temperature was set at 240 C for GC analysis of methyl 24-methyl-5,9-hexacosadienoate (*19*) and methyl 25-methyl-5,9-hexacosadienoate (*18*).

HPLC separation of major fatty acid methyl esters was achieved by using a 50 cm x 9 mm Whatman ODS-2 reversed-phase column, a Waters M-6000A pump, a Valco loop injector and a Waters R401 refractometer detector. Absolute methanol was the eluting solvent at a rate of 1.5 ml/min.

GC/MS was performed either on a Ribermag GC/MS/DS system, combining a Ribermag R 10-10 quadrupole mass spectrometer with a Carlo Erba series 4160 Fractovap chromatograph equipped with a fused silica column (28 m x 0.32 mm) containing SE-54 (J & W Scientific, Inc.), or on a Varian MAT-44 GC/MS system using a spiral glass column (1.80 m x 2.0 mm), containing 3% OV-17 on GCQ. ^{1}H-NMR spectra were analyzed on a Varian Associates HA-100 NMR instrument in deuterated chloroform containing tetramethylsilane. Shift values are in ppm (δ). Infrared spectra were obtained using a Beckman Acculab 3 spectrophotometer. The total fatty acid methyl esters were prepared from the phospholipid mixture by treatment of a sample with methanolic sodium hydroxide and methanolic hydrogen chloride, respectively (21). The fatty acid content of individual phospholipid classes was estimated by separating the mixture into classes by preparative TLC and transmethylating each separated class by digestion with methanolic boron trifluoride (10 min, 100 C) while it was still on the silica gel (22). Hydrogenation of the fatty acid methyl esters was carried out by stirring (8 hr, normal pressure, room temperature) in methanol with platinum (IV) oxide under hydrogen. N-Acyl pyrrolidide derivatives were prepared by direct treatment of methyl esters with pyrrolidine/acetic acid (10:1, v/v) in a capped vial (1 hr, 100 C) followed by ether extraction from the acidified solution and purification by TLC.

Degradation studies for the location of methyl branching in the two major fatty acids were carried out by $NaIO_4/KMnO_4$ oxidation in *tert*-butanol (23) followed by methylation of the resulting carboxylic acids with methanol in the presence of 3% sulfuric acid (3 hr reflux). The resulting monofunctional esters were converted to the N-acyl pyrrolidides for mass spectral comparison with known or synthesized compounds. Methyl 16-methylheptadecanoate was obtained from Applied Science (Milton Ray Co. Laboratory Group, State College, PA). Methyl 15-methylheptadecanoate was prepared from methyl 14-methylhexadecanoate (Applied Science) by addition of one carbon atom, according to a known (24) procedure. For this purpose, the starting material was reduced with $LiAlH_4$ in tetrahydrofuran (reflux temp, 2 hr). The resulting alcohol was then tosylated (TsCl/pyr, room temp, 12 hr); the tosylate was reacted with KCN in ethanol/water (8:1, v/v) (reflux temp, 65 hr) and the resulting nitrile was saponified with KOH (10%, 7 hr) to yield 15-methylheptadecanoic acid potassium salt, which was then converted to the methyl ester. N-Acyl pyrrolidides were prepared from methyl 16-heptadecanoate and methyl 15-heptadecanoate for comparison with the naturally derived monofunctional degradation products.

RESULTS AND DISCUSSION

The capillary GC analysis of the fatty acid

methyl esters obtained from the total phospholipids of *P. ficiformis* indicated the presence of ca. 110 peaks. The identified fatty acids are listed in Table 2. A comparison of the capillary GC retention times and equivalent chain length values (ECL) of these esters with those of known compounds (25) and the interpretation of their mass spectra and those of the N-acyl pyrrolidide derivatives (26) provided information for identification. Pyrrolidides of the hydrogenated fatty acid methyl esters were also prepared and investigated by capillary GC/MS especially for the location (26) of branching.

Frequently, GC and MS data of fatty acid derivatives offer sufficient information to suggest a structure for a detected compound (25, 26). For example, GLC facilitates the recognition of saturated straight-chain, iso and anteiso acids. It is observed that, on a linear retention time scale, a C_{n+1} iso acid falls slightly more than halfway between the C_n and C_{n+1} straight-chain acids, and the corresponding anteiso acid approximately one-third of the way from iso-C_{n+1} to normal C_{n+1} (25). In the mass spectral analysis of N-acyl pyrrolidides (26) with a normal saturated acid, a regular decrease in intensity is observed in the series of these ions from low to high mass. An interruption in this trend marks the presence and location of branching. A peak of lower intensity than the analogous peak in a straight-chain fatty acid pyrrolidide is an indication of a methyl branch at that position. This is usually accompanied by an increase in the intensity of the flanking peaks (26). Mass spectra of N-acyl pyrrolidides also provide information about the position of double bonds based on the spacings between major peaks. As a general rule, the presence of 12 instead of 14 amu between the most intense peaks of fragments containing n and n-1 carbon atoms in the acid moiety points to a double bond between carbon n and n+1 in the molecule.

Thus, for example, the mass spectra of the pyrrolidide derivatives of the unsaturated acids

TABLE 2

Identified Major Fatty Acids from the Phospholipids of *P. ficiformis*[a,b]

Compound	ECL[c]	Fatty acid	Percent (by wt) in phospholipids	Distribution in phospholipid classes[d,e]			
				PI/PS	PC	PG	PE
1	14.00	Tetradecanoic (*n*-14:0; myristic)	1.5	1.1	1.7	2.3	1.3
2	14.48	4,8,12-Trimethyltridecanoic (4-Me,8-Me,12-Me-13:0)	3.5	–	8.8	–	4.6
3	14.62	13-Methyltetradecanoic (iso-15:0)	5.0	2.5	2.9	8.2	4.6
4	14.70	12-Methyltetradecanoic (anteiso-15:0)	4.7	4.8	3.2	7.9	3.1
5	15.60	14-Methylpentadecanoic (iso-16:0)	1.5	–	2.3	2.2	1.4
6	15.72	9-Hexadecenoic (Δ^9-16:1; palmitoleic)	1.3	–	2.8	2.6	1.6
7	16.00	Hexadecanoic (*n*-16:0; palmitic)	5.4	3.6	6.6	10.9	4.5
8	16.35	15-Methyl-9-hexadecenoic (Δ^9-iso-17:1)	2.8	1.7	7.1	5.1	2.4
9	16.41	10-Methylhexadecanoic (10-Me-16:0)	4.7	1.5	7.6	6.4	5.3
10	16.60	15-Methylhexadecanoic (iso-17:0)	2.0	–	3.2	2.7	1.5
11	16.69	14-Methylhexadecanoic (anteiso-17:0)	1.9	–	2.9	1.9	1.6
12	17.74	11-Octadecenoic (Δ^{11}-18:1, vaccenic)	1.2	–	2.3	2.4	1.1
13	18.00	Octadecanoic (*n*-18:0)	2.8	2.6	3.8	3.2	1.1
14	18.40	11-Methyloctadecanoic (11-Me-18:0)	5.7	9.1	5.5	4.2	2.6
*15**	21.33	7,13,16-Docosatrienoic acid ($\Delta^{7,13,16}$-22:3)*	1.5	–	4.0	–	1.9
*16**	25.08	24-Methyl-5,9-pentacosadienoic ($\Delta^{5,9}$-iso-26.2)*	3.8	4.8	1.4	1.9	4.4
17	25.42	5,9-Pentacosadienoic ($\Delta^{5,9}$-26:2)	1.4	2.1	–	1.9	2.9
*18**	26.15	25-Methyl-5,9-hexacosadienoic ($\Delta^{5,9}$-iso-27:2)*	8.4	11.8	6.6	6.2	7.9
*19**	26.37	24-Methyl-5,9-hexacosadienoic ($\Delta^{5,9}$-anteiso-27:2)*	18.4	26.2	14.8	14.2	20.0

[a]An asterisk after the compound number indicates that the acid is hitherto undescribed.

[b]Identified minor (< 1% fatty acids: *20)* iso-14:0 (ECL 13.61), *21)* *n*-15:0 (ECL 15.00), *22)* anteiso-16:0 (ECL 15.60), *23)* 17:1 (ECL 16.80), *24)* *n*-17:0 (ECL 17.00), *25)* 18:1 (ECL 17.71), *26)* 19:1 (ECL 18.79), *27)* $\Delta^{7,13}$-22:2 (ECL 21.37), *28*)* 15-Me-23:0* (ECL 22.40), *29)* Δ^{17}-24:1 (ECL 23.78), *30*)* 15-Me-24:0* (ECL 23.38)

[c]Equivalent chain length values were calculated from the methyl esters of the corresponding acids (25).

[d]Averages from these determinations; – indicates that the amount of acid is less than 1%.

[e]Full names of phospholipids are given in Table 1.

6 (M^+ 307), *8* (M^+ 321), and *12* (M^+ 335) exhibited spacings of 12 amu between C_8 (m/z 196) and C_9 (m/z 208) for the first two and between C_{10} (m/z 224) and C_{11} (m/z 236) for the third compound corresponding to double bonds at C_9 for compounds *6* and *8* and at C_{11} for compound *12*. The methyl esters and pyrrolidides of the hydrogenated derivatives showed that compounds *6* and *12* contain straight-chain 16 and 18 carbons, respectively, whereas acid *8* is an isobranched C_{17} acid (ECL: 16.60, a diminished peak at m/z 294 associated with enhanced peaks at m/z 280 and 308). Combination of these findings revealed the structures of these compounds as 9-hexadecenoic (*6*), 15-methyl-9-hexadecenoic (*8*) and 11-octadecenoic (*12*) acid. These compounds have been reported to be constituents of a marine bacterium (27). 11-Octadecenoic acid has also been found in other bacteria (28), whereas 9-hexadecenoic acid is ubiquitous among marine animals and plants (29).

As another example, a saturated component of the mixture, compound *2* (pyrrolidide M^+ 309, 16 carbon atoms), gave an unusual ECL value (14.48). The mass spectrum of the pyrrolidide derivative (Fig. 1) exhibited diminished peaks at m/z 140, 210 and 280 concurrent with enhanced flanking peaks corresponding to methyl branches at C_4, C_8 and C_{12}. Therefore, its structure was assigned to be 4,8,12-trimethyltridecanoic acid (*2*). Such an acid has already been encountered in some fish and marine mammals and is probably of zooplanktonic origin (30).

Only three straight-chain saturated fatty acids, tetradecanoic (*1*) (1.5%), hexadecanoic (*7*) (5.4%) and octadecanoic (*13*) (2.8%) acids, were encountered in the phospholipids of *P. ficiformis* as significant (>1%) acyl components. All of the other major fatty acids were either branched (predominantly iso and anteiso), unsaturated, or both.

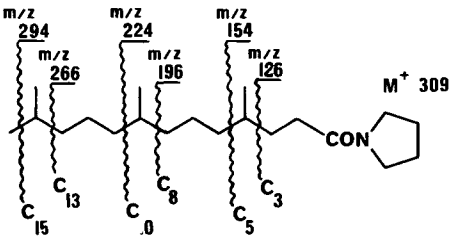


FIG. 1. The major diagnostic mass spectral fragmentations of N-(4,8,12-trimethyltridecanoyl)pyrrolidine (compound *2*, Table 2).

We now present our evidence for the structures of the important novel acids encountered in this sponge. The N-acyl pyrrolidide *15* gave a molecular ion peak at m/z 387 (Fig. 2), indicating the presence of 22 carbon atoms and three degrees of unsaturation, with no methyl branching observed. The ECL value (22.00) of the methyl ester of the saturated derivative was indicative of a straight-chain C_{22} acid. The presence of 12 amu spacings between the C_6 (m/z 168) and C_7 (m/z 180) between the C_{12} (m/z 250) and C_{13} (m/z 262) and between the C_{15} (m/z 290) and C_{16} (m/z 302) fragments of the pyrrolidide derivative clearly showed that acid *15* was 7,13,16-docosatrienoic acid. A known compound, 7,13-docosadienoic acid (31), with a slightly longer retention time was also present (0.6%) in the mixture.

Two of the minor fatty acids (*28* and *30*) remained unchanged after hydrogenation (ECL values 22.40 for *28* [0.8%] and 23.38 for *30* [0.5%]). The mass spectra of the N-pyrrolidide derivatives showed that compound *28* (M^+ 407) contained 23 carbons and compound *30* (M^+ 421) 24 carbons (Fig. 3). Peaks of relatively lower intensity at C_{15} (m/z 294) and higher intensity at C_{14} (m/z 280) and C_{16} (m/z 308) in the mass spectra of both derivatives indicated their structure to be 15-methyldocosanoic (15Me-23:0) and 15-methyltricosanoic (15Me-24:0) acids.

GC/MS analysis of acids *16-19* (Table 2) showed them to contain $\Delta^{5,9}$ unsaturation which is typical for unusually long demospongic fatty acids (1,9). The pyrrolidides of the two major acids, *18* and *19*, showed the same molecular ion at m/z 459. Both compounds exhibited a very intense peak at m/z 180 due to doubly activated allylic cleavage between C_7 and C_8 resulting from $\Delta^{5,9}$ unsaturation as well as to other related allylic cleavage peaks between C_3 and C_4 (m/z 126) and between C_{11} and C_{12} (m/z 234). Unfortunately, the low intensities of the fragments produced by other cleavages prevented any further structural assignment based on GC/MS analysis. However, MS of the hydrogenated derivatives of the pyrrolidides of *18* and *19* (M^+ 463, Fig. 4) showed diagnostic fragments for the iso and anteiso C_{27} fatty acids, respectively. The hydrogenated pyrrolidide of *18* displayed a diminished peak at m/z 434 with enhanced flanking peaks at m/z 420 and m/z 448 (Fig. 4A), whereas the hydrogenated pyrrolidide of *19* exhibited a diminished peak at m/z 420 with enhanced flanking peaks at m/z 406 and m/z 434 (Fig. 4B).

For further investigation, the methyl esters of *18* and *19* were separated from the natural

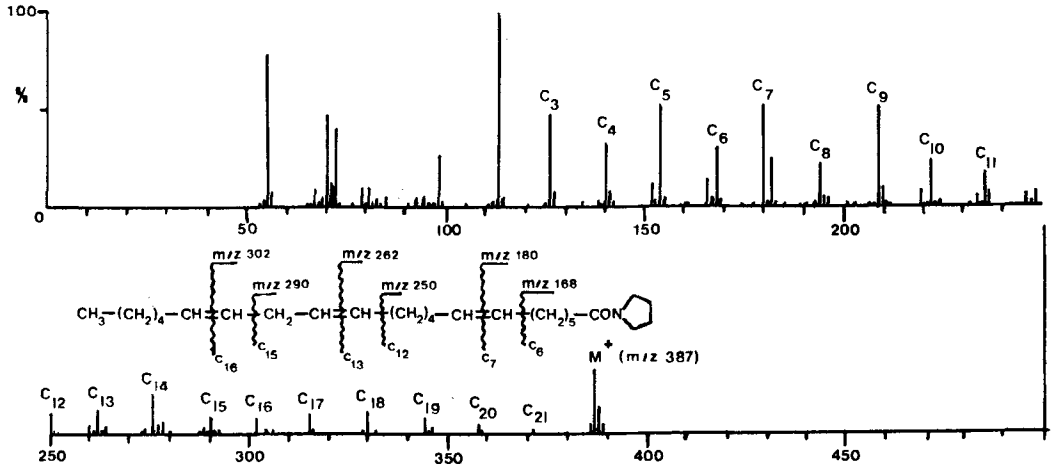


FIG. 2. Mass spectrum of N-(7,13,16-docosatrienoyl)pyrrolidine (compound *15*, Table 2).

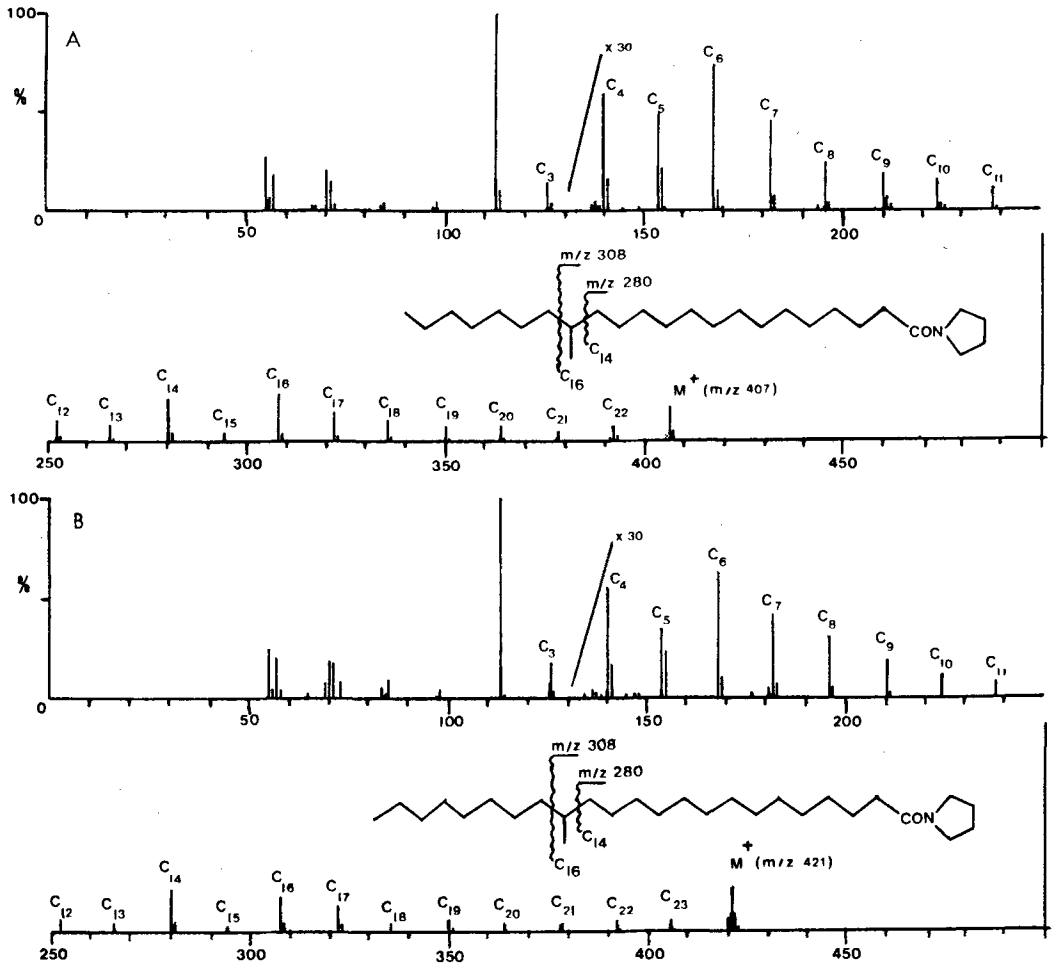


FIG. 3. (A) Mass spectrum of N-(15-methyldocosanoyl)pyrrolidine (compound *28*, Table 2). (B) Mass spectrum of N-(15-methyltricosanoyl)pyrrolidine (compound *30*, Table 2).

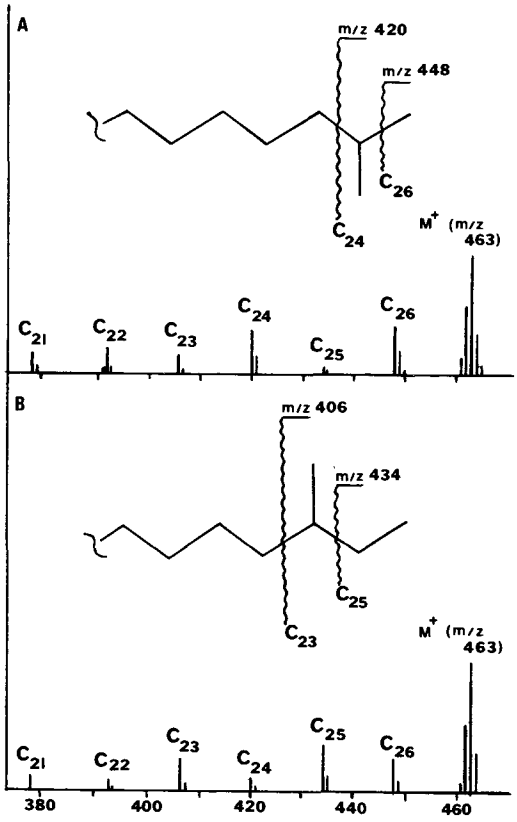


FIG. 4. (A) Partial mass spectrum of N-(25-methylhexacosanoyl)pyrrolidine (hydrogenation product of compound *18*, Table 2). (B) Partial mass spectrum of N-(24-methylhexacosanoyl)pyrrolidine (hydrogenation product of compound *19*, Table 2).

mixture by HPLC and subjected to oxidative degradation. Both short-chain fatty acid methyl esters thus obtained gave M^+ 298, when inspected by GC/MS, corresponding to 18 carbons (Δ^9 degradation). The pyrrolidides of these two products (both M^+ 337, Fig. 5) again showed the diagnostic iso and anteiso fragmentations of C_{18} acids, i.e., peaks of lower intensity at m/z 308 (Fig. 5A) and at m/z 294 (Fig. 5B). In addition, the methyl ester and pyrrolidide of the degradation product of compound *18* was compared with commercially available 16-methylheptadecanoic acid (iso-18:0) methyl ester and its pyrrolidide. The GC retention times of the two compounds were found to be identical and coinjection on capillary GC produced only one peak. Also, the pyrrolidide of the authentic 16-methylheptadecanoic acid gave an identical mass spectrum with a molecular ion peak at m/z 337 and a diminished peak at m/z 308, and enhanced peaks at m/z 294 and m/z 322.

Since an authentic sample of the degradation product of *19*, 15-methylheptadecanoic acid (anteiso-18:0), was unavailable for comparison, it was prepared from the methyl ester of 14-methylhexadecanoic acid (anteiso-17:0) (24) by one carbon homologation as outlined in the experimental section. Again, the synthetic anteiso acid was identical in all respects with the natural double bond degradation product.

^{1}H-NMR spectra of the methyl esters of the fatty acids *18* and *19* showed the expected signals: a methoxycarbonyl singlet (3H) at 3.658 ppm, from the TMS signal, a C_2 methylene triplet (2H) at 2.320 ppm, and a large singlet at 1.252 ppm corresponding to saturated methylene protons. ^{1}H-NMR and infrared spectra of these compounds also exhibited typical absorbances previously reported (1,9,32) for *cis*-5,*cis*-9-demospongic acids: a quintet (2H) at 1.680 ppm corresponding to the C_3 methylene group, and uninterpretable multiplets, centered at 2.07 ppm (6H) assignable to allylic protons on C_4, C_7 and C_8 and at 1.99 ppm arising from the protons at C_{11}. The olefinic protons were observed as a multiplet (4H) at 5.370 ppm as mentioned previously for $\Delta^{5,9}$ compounds (1). The infrared spectra had no prominent absorption at 980-968 cm^{-1}, indicating *cis* rather than *trans* unsaturation (11,32,33), as is usual for demospongic acids, whereas hydrocarbon and ester bands were present at 3050, 2820 and 1720 cm^{-1}, respectively.

Based on the GC and spectral properties just described for various derivatives of these phospholipid components and our comparison of their monofunctional degradation products with authentic samples, the structures of compounds *18* and *19* clearly corresponded to Z,Z-25-methyl-5,9-hexacosadienoic and Z,Z-24-methyl-5,9-hexacosadienoic acid.

The assignment of the 24-methyl-5,9-pentacosadienoic acid structure for acid *16* is based on the following properties of its derivatives: (a) the hydrogenation product of the methyl ester gave an ECL value at 25.60; (b) the pyrrolidide of the parent compound showed a molecular ion at m/z 445, a very strong diagnostic peak at m/z 180 with allylic fragmentations at m/z 126 and 234, typical of $\Delta^{5,9}$-unsaturation; (c) the pyrrolidide of its hydrogenation product (M^+ 449) exhibited unambiguously (Fig. 6) the presence of iso methyl branching, because a peak of diminished intensity was observed at m/z 420 accompanied by enhanced flanking peaks at m/z 406 and 434.

The relative distribution of the major phospholipids as determined by TLC (in terms of

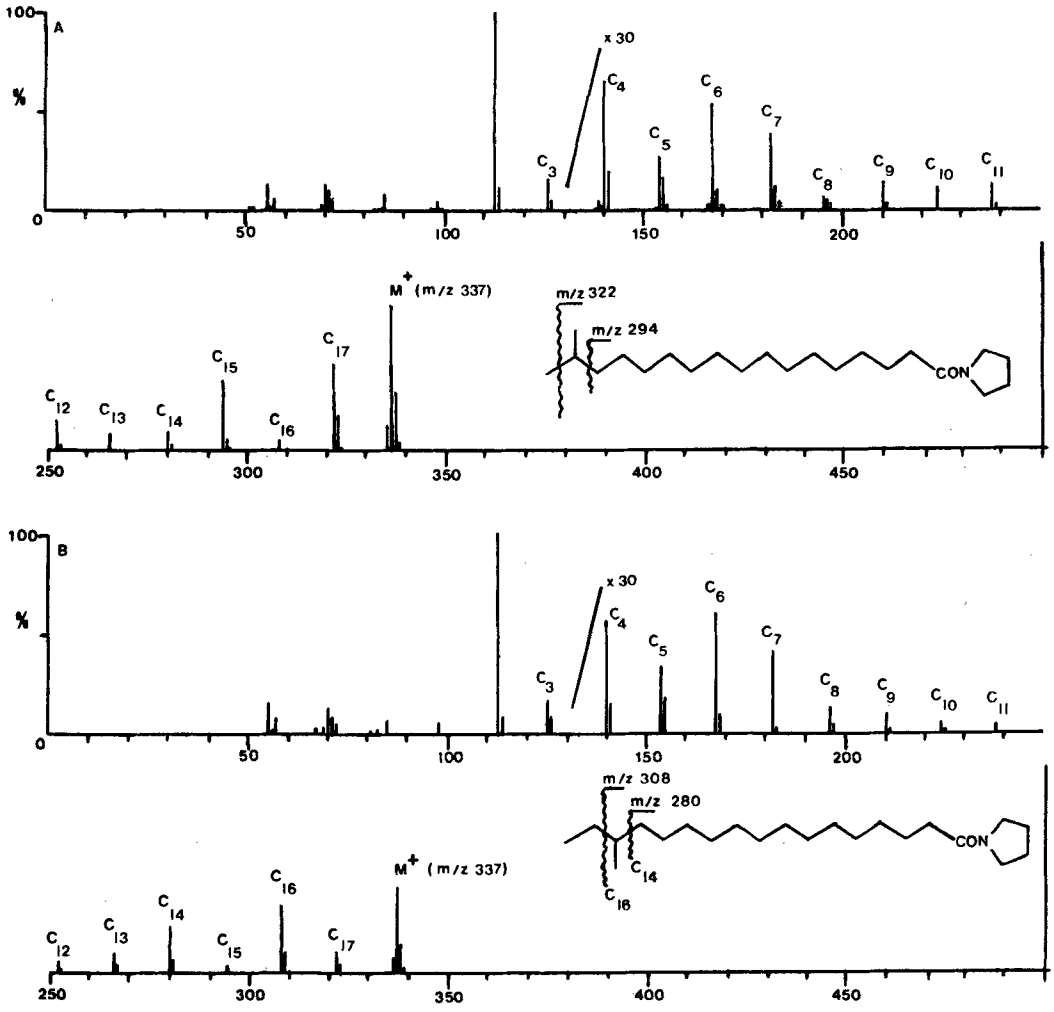


FIG. 5. (A) Mass spectrum of the pyrrolidide of the monofunctional degradation product from methyl 25-methyl-5,9-hexacosadienoate. (B) Mass spectrum of the pyrrolidide of the monofunctional degradation product from 24-methyl-5,9-hexacosadienoate.

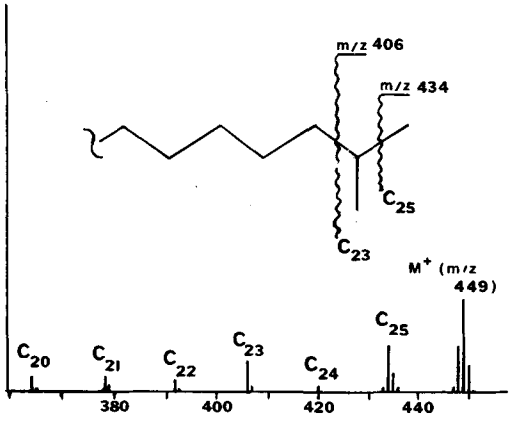


FIG. 6. Partial mass spectrum of N-(24-methyl-hexacosanoyl)pyrrolidine (hydrogenation product of compound *16*, Table 2).

head groups) is shown in Table 1. Only minor amounts of plasmalogens were detected in the phospholipid spots by two-dimensional TLC and DNP color reactions. The standard error range in the quantitative analysis of the major phospholipids was 10-15%. Some minor, unidentified spots were also detected on the chromatograms. Percentages given are averages from three determinations and are based on relative phosphate content.

Phospholipids with amino-containing head groups (PE, PC and PS) are the most common polar lipids of sponges (34). In some cases, carbohydrate-containing phospholipids (DPG, PG and PI) are also reported (1). The latter group of polar phospholipids is known to be a common major lipid class of bacteria (35,36). In *P. ficiformis*, we have detected the presence of PG (14%) and PI (7%). Amino groups containing phospholipids–PE (28%), PC (21%) and PS (12%)–were more abundant, representing about 75% of the total.

As can be observed from the data concerning the distribution of fatty acids in phospholipid classes (Table 2), the percentages of short-chain acids increase in PG, whereas demospongic acids are more abundant in PE and especially in PS/PI, in agreement with analyses of other demosponges (1,9).

About 85% by weight of the total major fatty acids found in the phospholipids of *P. ficiformis* are branched. Among them, iso and anteiso acids are predominant, comprising about 70%. Most of the branched-chain fatty acids encountered in this sponge (iso-15:0, anteiso-15:0, Δ^9-16:1, anteiso-16:0, Δ^9-iso-17:1, iso-17:0, anteiso-17:0, Δ^{11}-18:1, 11Me-19:0) are known to be typical constituents of bacteria (1) and presumably (37) arise from the large quantity of bacteria known to coexist with sponges. Our research with *A. fistularis* (1) has indicated that its long-chained, branched acids are bona fide sponge metabolites, and we believe that this is also true for the long-chained, branched acids in *P. ficiformis*.

Incorporation studies (38) have shown that demospongic acids in the sponge *Microciona prolifera* are synthesized first by chain elongation of normal chain fatty acids followed by $\Delta^{5,9}$ desaturation. Based on these results and conclusions (9,38), the following biosynthetic routes were proposed by us (1) for the new branched demospongic acids of *A. fistularis* starting with short chain length precursors present in the organism (and presumably of bacterial origin) (Scheme 1).

In a similar manner, the following plausible biosynthetic routes to the novel long-chain fatty acids in *P. ficiformis* can be proposed from (bacterial?) precursors known to be present in the sponge (numbers in parentheses correspond to numbers shown in Table 2) (Scheme 2).

P. ficiformis is the second example (cf. 1) where the existence of sterols with novel side chains suggested that unusual fatty acids should also be present. These leads have prompted additional studies in our laboratory, which will

Δ^9-16:1 → Δ^{11}-18:1 →→→→→ Δ^{21}-28:1 → $\Delta^{9,21}$-28:2 → $\Delta^{5,9,21}$-28:3
↓
Δ^{23}-30:1 → $\Delta^{9,23}$-30:2 → $\Delta^{5,9,23}$-30:3

Δ^{11}-16:1 →→→→→→ Δ^{23}-28:1 → $\Delta^{9,23}$-28:2 → $\Delta^{5,9,23}$-28:3

10Me-17:0 →→→→→ 20Me-27:0 → 20Me-Δ^9-27:1 → 20Me-$\Delta^{5,9}$-27:2
↓
22Me-29:0 → 22Me-Δ^9-29:1 → 22Me-$\Delta^{5,9}$-29:2

SCHEME 1

n-14:0 (*1*) →→ *n*-16:0 (*7*) →→→→→ *n*-26:0 →→ $\Delta^{5,9}$-26:2 (*17*)

iso-16:0 (*5*) →→→→→ iso-26:0 →→ $\Delta^{5,9}$-iso-26:2 (*16*)

iso-15:0 (*3*) → iso-17:0 (*10*) →→→→→ iso-27:0 →→ $\Delta^{5,9}$-iso-27 (*18*)

anteiso-15:0 (*4*) → anteiso-17:0 (*11*) →→→→→ anteiso-27:0
↓
↓
$\Delta^{5,9}$-anteiso-27:0 (*19*)

SCHEME 2

be reported later.

ACKNOWLEDGMENTS

We wish to thank Annemarie Wegmann for mass spectral measurements. NMR spectra were run under the supervision of Dr. Lois J. Durham. Financial support from the National Institutes of Health (Grants No. GM-06840 and GM-28352) is gratefully acknowledged.

REFERENCES

1. Walkup, R.D., Jamieson, G.C., Ratcliff, M.R., and Djerassi, C. (1981) Lipids 16, 631-646.
2. Bergquist, P.R. (1978) in Sponges, p. 9, Univ. of California Press, Berkeley, CA.
3. Bergmann, W. (1949) J. Mar. Res. 8, 137-176.
4. Bergmann, W. (1962) in Comprehensive Biochemistry (Florkin, M., and Manson, H.S., eds.) Vol. III, p. 103, Academic Press, New York, NY.
5. Djerassi, C., Theobald, N., Kokke, W.C.M.C., Pak, C.S., and Carlson, R.M.K. (1979) Pure Appl. Chem. 51, 1815-1828.
6. Djerassi, C. (1981) Pure Appl. Chem. 53, 873-890.
7. Sjöstrand, U., and Bohlin, L. (1982) in Handbook of Marine Science (Baker, J.T., and Murphy, V., eds.) Vol. III, CRC Press, Cleveland, OH, in press.
8. Litchfield, C. (1976) in Aspects of Marine Biology (Harrison, F.W., and Cowden, R.R., eds.) p. 183, Academic Press, New York, NY.
9. Litchfield, C., Tyszkiewicz, J., and Dato, V. (1980) Lipids 15, 200-202.
10. Morales, R.W., and Litchfield, C. (1976) Biochim. Biophys. Acta 431, 206-216.
11. Litchfield, C., and Marcantonio, E.E. (1978) Lipids 13, 199-202.
12. Harel, Z., and Djerassi, C. (1980) Lipids 15, 694-696.
13. Carlson, R.M.K., Tarchini, C., and Djerassi, C. (1980) in Frontiers of Bioorganic Chemistry and Molecular Biology (Ananchenko, S.N., ed.) pp. 211-224, Pergamon Press, New York, NY.
14. Sica, D., and Zoll, F. (1978) Tetrahedron Lett., 837-838.
15. Mattia, C.A., Mazzarella, L., Pulliti, R., Sica, D., and Zollo, F. (1978) Tetrahedron Lett., 3953-3954.
16. Ravi, B., Kokke, W.C.M.C., Delseth, C., and Djerassi, C. (1978) Tetrahedron Lett., 4379-4380.
17. Kates, M. (1972) in Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S., and Work, E., eds.) pp. 347-353, North Holland Pub. Co., New York, NY.
18. Skipski, V.P., and Barclay, M. (1969) Methods Enzymol. 14, 530-598.
19. Skipski, V.P., Peterson, R.F., and Barclay, M. (1964) Biochem. J. 90, 374-378.
20. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
21. Carreau, J.P., and Duback, J.P. (1978) J. Chromatogr. 151, 384-390.
22. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
23. Albro, P.W., and Dittmer, J.C. (1969) Biochemistry 8, 394-404.
24. Eglington, C., and Whiting, M.C. (1953) J. Chem. Soc., 3052-3059.
25. Ackman, R.G. (1972) Prog. Chem. Fats Other Lipids 12, 167-284.
26. Andersson, B.A. (1978) Prog. Chem. Fats Other Lipids 16, 279-308.
27. Boon, J.J., de Leeuw, J.W., Hoek, G.J.v.d., and Vosjan, J.H. (1977) J. Bacteriol. 129, 1183-1191.
28. Lennarz, W.J. (1966) Adv. Lipid Res. 4, 175-225.
29. Morris, R.J., and Culkin, F. (1976) Oceanogr. Mar. Biol. Ann. Rev. 14, 391-433.
30. Ackman, R.G., and Hooper, S.N. (1968) Comp. Biochem. Physiol. 24, 549-565 and literature cited therein.
31. Pearce, R.E., and Stillway, L.W. (1977) Lipids 12, 544-549.
32. Litchfield, C., Tyszkiewicz, J., Marcantonio, E.E., and Noto, G. (1979) Lipids 14, 619-622.
33. Wolff, I.A., and Miwa, T.K. (1965) J. Am. Chem. Soc. 42, 212-215.
34. Joseph, J.D. (1979) Prog. Lipid Res. 18, 1-30.
35. Kates, M. (1964) Adv. Lipid Res. 2, 17-90.
36. Salton, M.R.J. (1971) Biomembranes 1, 1-65.
37. Vacelet, J., and Cdonaday, C. (1977) J. Exp. Mar. Biol. Ecol. 30, 301-314.
38. Morales, R.W., and Litchfield, C. (1977) Lipids 12, 570-576.

[Received April 28, 1982]

Rat Adipocyte Utilization of Different Substrates: Effects of Cell Size and the Control of Lipogenesis

JAMES M. MAY, *Department of Medicine, Medical College of Virginia, Richmond, VA 23298*

ABSTRACT

The metabolism of labeled glucose, pyruvate and acetate was compared in adipocytes isolated from old, obese rats (>500 g) and young, lean rats (130-150 g). The larger cells from old, obese rats had markedly reduced rates of glucose, pyruvate and acetate conversion to glyceride-fatty acids, indicating that large cell fatty acid formation is reduced at some point beyond the entry of pyruvate and acetate into glucose metabolism. No evidence of a primary block in the pentose phosphate cycle of cells from old, obese rats was found. In spite of diminished glucose metabolism to several products in the large cells, both basal and insulin-stimulated rates of glyceride-glycerol synthesis from glucose and pyruvate were similar in each cell type. This indicates a relative diversion of carbon flow to α-glycerophosphate and reesterification in the large cells. Addition of low concentrations of glucose increased glyceride-fatty acid synthesis from acetate (both cell types) or pyruvate carbon (small cells), but decreased glyceride-glycerol synthesis from pyruvate carbon (both cell types). The acceleration of small cell fatty acid synthesis from pyruvate carbon by glucose and insulin was shown to be related to provision of NADPH from glucose metabolism in the pentose cycle. These studies indicate that, although the block in lipogenesis in adipocytes from old, obese rats appears to reside in the pathway of fatty acid synthesis itself, provision of additional α-glycerophosphate or NADPH from glucose metabolism may, under certain conditions, increase lipogenesis in cells from old, obese and young, lean rats.

Lipids 17:626-633, 1982.

The pentose phosphate cycle and fatty acid synthesis from glucose are known to be markedly decreased in the large adipocytes from old, spontaneously obese rats compared to the much smaller adipocytes isolated from young, lean rats (1-6). Recent studies have indicated that the primary block appears to result from diminished activity of acetyl-CoA carboxylase, the initial rate-limiting enzyme of fatty acid synthesis (4-6). Inhibition of the pentose phosphate cycle is probably a secondary event, since the cycle in large rat cells has been shown to respond normally to stimulation by the electron acceptor vitamin K_5 (4).

It is also possible to localize the block in large cell fatty acid synthesis by studying the incorporation of acetate or pyruvate into glyceride-fatty acids, since these intracellular precursors presumably bypass transport into the cell and certain intracellular enzymatic steps. Gellhorn et al. (7) have shown, for example, that fat pads from old rats incorporate [1-^{14}C] acetate into fatty acids at rates less than 10% of those found in fat pads from young rats. Richardson and Czech (4,6) reported markedly diminished rates of fatty acid synthesis and CO_2 production from [2-^{14}C] pyruvate in large cells compared to small cells, suggesting again an intracellular block. A recent study by Francendese and DiGirolamo (5) has also documented diminished acetate and pyruvate conversion to glyceride-fatty acids. The present studies were designed to extend earlier observations of glucose, pyruvate and acetate conversion in large rat cells with respect to both basal and insulin-stimulated rates as well as to investigate certain aspects of the regulation of the pentose phosphate cycle and lipogenesis in small rat cells.

Abbreviations: TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine; GSH, reduced glutathione; GSSG, oxidized gluthathione; KRB, Krebs-Ringer bicarbonate; ANOVA, analysis of variance.

MATERIALS AND METHODS

Animals

Male Wistar rats (Charles River Laboratories) were used in all experiments. Rats were fed Purina Rat Chow ad libitum until they attained body weights of either 130-150 g (at 6-8 weeks) or 500-600 g (at greater than 9 months), at which time they were sacrificed for study. Experiments were performed at similar times in the morning.

Isolation of Adipose Cells

Isolated adipocytes were prepared by the method of Rodbell (8) from the epididymal fat pads of rats killed by cervical dislocation. The pads were minced slightly and placed in 8-oz polyethylene bottles containing 5 ml of KRB buffer (pH 7.4), 4% (w/v) bovine serum albumin (Fraction V, Reheis Chemical Co., Kankakee, IN) and 2 mg/ml collagenase (Type I, Worthington Biochemicals, Freehold, NJ). Digestion was performed by placing the bottle on a slight angle from the horizontal in a shaking (60 cyc/

min) water bath at 37 C for 1 hr for the small rat cells, and slightly less for the large rat cells (in order to avoid breakage). The digested material was filtered through 250-μm nylon mesh and cells were washed 3 times with 2% albumin/KRB buffer, followed by resuspension at the proper cell concentration in 2% albumin/KRB buffer. Cell counts were performed by the hemocytometer method of Gliemann (9). There was some breakage of the larger cells, but this was minimized by a shorter digestion period. The small cells averaged 60 μm in diameter, whereas the large cells were 100 μm in diameter as measured with a micrometer attachment.

Measurements of Glucose, Pyruvate and Acetate Utilization

Cell aliquots were pipetted into polyethylene scintillation vials containing 2% albumin/KRB buffer, labeled and unlabeled substrate, and other agents as noted. Incubations were performed for 1 hr at 37 C in a total vol of 2 ml with shaking (60 cyc/min). The incubations were terminated by the addition of 0.5 ml of 8 N H_2SO_4, and CO_2 was collected by the method of Gliemann (9) as described previously (10). After CO_2 collection, 1.8 ml of the acidified incubation mixture was removed and placed in 4 ml of a 1:1 chloroform/methanol mixture (11) for measurement of lipids. This was mixed vigorously for 10 sec and allowed to stand overnight at -20 C. The upper phase was removed by suction, discarded, and 2 washes of the organic phase were performed using an upper phase wash (2 ml) consisting of 1:1 methanol/7.4 mg/ml aq KCl. Each addition of upper phase wash was followed by vigorous mixing for 10 sec, centrifugation for 5 min at 3,000 × g, and removal of the upper phase. After the last wash, the organic phase was carefully removed and evaporated to dryness at 55 C in a water bath under a stream of air. The residual lipids were saponified for 2 hr at 60 C in 2 ml of 0.5 N KOH in ethanol (saturated). The mixture was acidified to pH of less than 1 with 4 N HCl and extracted 3 times with 2 ml of heptane. The heptane extract was allowed to dry overnight and the residue was dissolved in 10 ml of toluene scintillant (10) for counting. The remaining ethanol solution was centrifuged at 1,800 × g for 5 min and the supernatant was removed and evaporated to dryness. The residue after drying was redissolved in 1 ml of water and 10 ml of ACS (Amersham) for counting. All counting was performed in a Nuclear-Chicago Mark II liquid scintillation spectrometer by single or double label techniques as required.

All incubations were done in triplicate and expressed as means with standard errors of the mean (SEM) for several experiments. Statistical calculations were performed using the paired Student's t-test and ANOVA.

Bovine insulin was purchased from Calbiochem, and purified as described previously (10). Vitamin K_5, 6-aminonicotinamide, TMPD and diamide (diazine-dicarboxylic acid-bis-[N,N-dimethylamide]) were obtained from Sigma. All radio-nuclides were purchased from Amersham.

RESULTS

Metabolism of Various Concentrations of Glucose by Large and Small Cells

Adipocytes from obese and lean rats showed similar basal rates of $^{14}CO_2$ and glyceride-glycerol production from a wide range of uniformly labeled glucose concentrations (Fig. 1A, 1D). When insulin was present, the relative responsiveness of the large cells compared to that in small cells was minimal with regard to CO_2 production (Fig. 1A), but similar with regard to glyceride-glycerol synthesis (Fig. 1D). Synthesis of glyceride-fatty acids from glucose carbon (Fig. 1B) or D-[3-^{3}H]glucose (Fig. 1C) in large cells was much lower than that of the small cells and was unresponsive to insulin. Since ^3H from glucose carbon-3 found in glyceride-fatty acid is transferred very efficiently from the pentose phosphate cycle (12), it is likely that the observed inactivity resulted from low activity of the pentose phosphate cycle, or a low rate of fatty acid synthesis, or both. Production of CO_2 in the large cells may therefore have derived largely from metabolism in the tricarboxylic acid cycle. Since basal rates of CO_2 and glyceride-glycerol production were similar in each cell type, there was a relative increase in glucose carbon flow through the glycolytic pathway in the large cells. In fact, under insulin stimulation, there was a further diversion of glucose carbon into glyceride-glycerol relative to metabolism in other pathways.

Effects of Insulin-Like Agents on Glucose Metabolism

Incubation of adipocytes with either diamide or TMPD resulted in increased CO_2 production from glucose in both cell types (Table 1), although the large cells again had diminished relative insulin responsiveness. The agents used were selected because they are known to act either by directly oxidizing reduced pyridine nucleotides (both agents) or by oxidizing GSH to GSSG (diamide) (13-15). Both a decreased [NADPH]/[$NADP^+$] ratio and GSSG have been shown to activate the pentose phosphate cycle (16). This effect may well have accounted for the stimulation of CO_2 production observed. Despite blunted insulin responsiveness in the

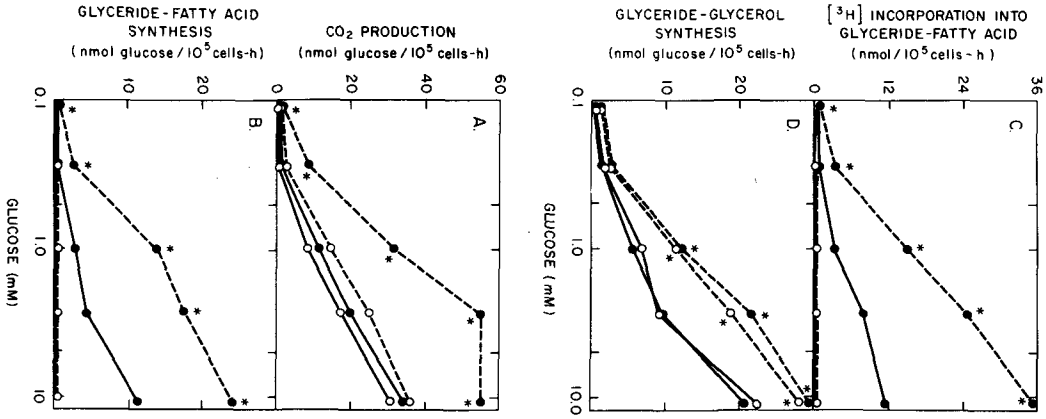


FIG. 1. Glucose metabolism at varying glucose concentrations in large and small adipocytes. The incorporation of D-[U-^{14}C]glucose (0.1 μCi/incubation) into CO_2 (A), glyceride-fatty acids (B), and glyceride-glycerol (D), as well as of D-[3-^{3}H]glucose (0.2 μCi/incubation) into glyceride-fatty acids (C) was assessed over the range of glucose concentrations shown. Cells from small (closed circles) or large (open circles) rats were incubated without (solid lines) or with (dashed lines) 5 nM insulin. Results shown are means from at least four experiments. Asterisks (*) indicate $p < .05$ comparing incubations with or without insulin.

TABLE 1

Stimulation of Glucose Metabolism by Oxidants in Large and Small Rat Adipocytes

Addition	CO_2 production	Glyceride-fatty acid synthesis	Incorporation of [^{3}H] from glucose into glyceride-fatty acid
	nmols glucose used/10^5 cells/hr		
Small cells[a]	3.2 ± 0.5	0.4 ± 0.07	0.58 ± 0.08
+ insulin	9.5 ± 0.6* (297)	1.8 ± 0.08*	2.2 ± 0.11*
+ TMPD	6.0 ± 0.8* (188)	0.24 ± 0.06	0.51 ± 0.17
+ diamide	4.5 ± 0.3* (141)	1.18 ± 1.05*	0.86 ± 0.03*
P	<.05	<.01	<.01
Large cells[a]	1.7 ± 0.4	0.11 ± 0.08	0.006 ± 0.01
+ insulin	3.5 ± 0.5* (206†)	0.18 ± 0.1	0.02 ± 0.1
+ TMPD	3.2 ± 0.4* (188)	0.08 ± 0.06	0.008 ± 0.01
+ diamide	3.0 ± 0.4* (176)	0.08 ± 0.06	0.012 ± 0.008

[a]Cells were incubated with 0.28 mM glucose, 0.2 μC of D-[U-^{14}C]glucose, 0.4 μC of D-[3-^{3}H]glucose and other agents as shown. Concentrations used were insulin, 5 nm; TMPD, 100 μM; and diamide, 100 μM. Values in parentheses indicate percentage of the respective control value. *Indicates $p < .05$ compared to rates in untreated cells of the same type. † Indicates $p < .05$ compared to small cells. The p values derived from an ANOVA comparison of all treatments in large and small cell for each column are shown in the center row.

large cells, CO_2 production responded similarly in both types of cells to agents which can directly activate the pentose phosphate cycle (most evident when expressed relative to basal values). Large cell glyceride-fatty acid synthesis and incorporation of ^{3}H from glucose into glyceride-fatty acids were very low and unresponsive to any agent. In the small cells, TMPD appeared to inhibit both basal and insulin-stimulated fatty acid synthesis from glucose carbon or hydrogen, whereas the effect of diamide was stimulatory. Although diamide was able to accelerate glucose oxidation in the large cells, fatty acid synthesis remained unresponsive. This is in contrast to the stimulation of fatty acid synthesis observed in the small cells with diamide. Incorporation of both ^{14}C and ^{3}H into fatty acid from glucose was less in the large cells than in small cells

TABLE 2

Utilization of [1-^{14}C]Acetate and [2-^{14}C]Pyruvate by Large and Small Cells

Cell type and condition	Glyceride-fatty acid synthesis from acetate	CO_2 production from pyruvate		Glyceride-glycerol synthesis from pyruvate			Glyceride-fatty acid synthesis from pyruvate	
	0.4 mM	0.15 mM	0.6 mM	2.4 mM	0.15 mM	0.6 mM	2.4 mM	0.15 mM	0.6 mM	2.4 mM
	nmols substrate used/10^5 cells/hr									
Small cells	0.08 ± 0.04	2.2 ± 0.5	5.5 ± 1.5	9.8 ± 3.3	0.7 ± 0.1	1.6 ± 0.4	2.2 ± 0.7	0.6 ± 0.1	2.2 ± 0.4	4.5 ± 0.5
+ insulin	0.14 ± 0.04	2.8 ± 0.05*	6.0 ± 1.7	9.8 ± 3.1	0.7 ± 0.3	1.6 ± 0.5	2.8 ± 0.9	0.8 ± 0.1*	2.5 ± 0.4	4.6 ± 0.4
p, (±glucose)	<.01	NS	NS	<.05	<.01	<.01	<.01	<.01	<.05	<.05
+ glucose	0.56 ± 0.1	3.2 ± 0.7	8.2 ± 1.5	16.8 ± 3.2	0.06 ± 0.04	0.1 ± 0.07	0.2 ± 0.06	1.4 ± 0.06	3.8 ± 0.7	7.8 ± 1.3
+ insulin and glucose	1.03 ± 0.2*	2.9 ± 0.7	8.6 ± 1.6	17.4 ± 1.8	0.08 ± 0.03	0.16 ± 0.07	0.2 ± 0.06	3.0 ± 0.4*	5.6 ± 0.8*	9.4 ± 1.3*
p, (small vs large)	<.01	NS	<.01	<.01	NS	<.02	<.02	<.01	<.01	<.01
Large cells	0.007 ± 0.004	1.8 ± 0.3	2.8 ± 0.3	3.8 ± 0.5	1.1 ± 0.1	2.7 ± 0.1	5.2 ± 1.4	0.1 ± 0.03	0.23 ± 0.07	0.34 ± 0.04
+ insulin	0.006 ± 0.003	2.0 ± 0.4	2.7 ± 0.4	4.1 ± 0.4	1.2 ± 0.1	2.9 ± 0.2	4.5 ± 1.4	0.08 ± 0.03	0.22 ± 0.04	0.29 ± 0.05
p, (±glucose)	<.01	NS	NS	NS	<.01	<.01	<.05	NS	NS	NS
+ glucose	0.05 ± 0.03	1.9 ± 0.6	3.6 ± 0.3	3.9 ± 0.5	0.2 ± 0.4	0.9 ± 0.1	2.0 ± 0.5	0.14 ± 0.06	0.32 ± 0.07	0.48 ± 0.06
+ insulin and glucose	0.09 ± 0.05	2.3 ± 0.3	3.2 ± 0.3	3.6 ± 0.5	0.1 ± 0.05	0.7 ± 0.1	1.4 ± 0.4	0.15 ± 0.03	0.34 ± 0.02	0.44 ± 0.06

Incubations contained as indicated [1-^{14}C]acetate, (0.2 μCi/assay), [2-^{14}C]pyruvate, (0.2 μCi/assay), glucose (0.28 mM), or insulin (5 nM). Data shown are from four experiments for each substrate. The p values listed in rows between compared groups were derived by analysis of variance. *p <.05 compared to cells of the same type not treated with insulin.

under each condition tested ($p<.05$).

Metabolism of [1-^{14}C] Acetate and [2-^{14}C] Pyruvate by Small and Large Cells

The rate of conversion of [1-^{14}C]acetate to fatty acids in the absence of glucose was lower in the large than small cells (Table 2). Large and small cells exhibited no insulin-induced increase in the incorporation of labeled acetate into fatty acids. Addition of low concentrations of glucose enhanced fatty acid synthesis from acetate carbon 5- to 10-fold in both cell types. Rates of synthesis in the large rat cells remained less than 10% of those in the small cells. In the presence of insulin and glucose, there was an additional increase over that found with glucose alone in fatty acid synthesis from acetate carbon at 0.4 mM acetate in the small cells. In the large cells, a significant insulin effect with glucose was present in only two of four experiments. Even glucose and insulin did not significantly increase fatty acid synthesis from acetate above rates found in small cells incubated without glucose or insulin. Incorporation of label from acetate to glyceride-glycerol was unmeasurable in many experiments in both cell types.

Production of CO_2 from pyruvate labeled on carbon-2 was similar in the large and small cells at 0.15 mM substrate, but diminished in the large cells at the higher concentrations (Table 2). There was a small acceleration of CO_2 production in the presence of insulin in the small cells at 0.15 mM pyruvate. Glucose (0.25 mM) increased production in the small cells at 2.4 mM pyruvate.

Synthesis of glyceride-glycerol from carbon-2 of pyruvate appeared to be greater in the large than small cells, although this difference was statistically significant only at 0.6 mM and 2.4 mM pyruvate concentrations (Table 2). There was no effect of insulin on glyceride-glycerol synthesis in either cell type. When low concentrations of glucose were included in the incubations, glyceride-glycerol synthesis from pyruvate carbon-2 virtually ceased in small cells and was markedly decreased in the large cells. This indicates preferential utilization of glucose carbon rather than that of pyruvate for glyceride-glycerol synthesis.

Synthesis of fatty acids from pyruvate carbon-2 was depressed in the large rat cells at all pyruvate concentrations tested (Table 2). The small cells had a small but significant insulin response at 0.15 mM pyruvate. Addition of glucose increased basal and insulin-stimulated rates in the small cells only. Thus, rates of fatty acid synthesis from both acetate and pyruvate were severely depressed in the large cells compared to the small cells. Although glucose did increase

rates of fatty acid synthesis from acetate in the large cells, in no instance was an effect of insulin observed. This is in marked contrast to the effects of both glucose and insulin on rates of fatty acid synthesis in small cells.

The mechanism of the glucose-enhanced rates of glyceride-fatty acid synthesis from pyruvate was evaluated. This was done only for the small cells, since rates of large cell fatty acid synthesis even with glucose and insulin were very low. The major means by which glucose could increase fatty acid synthesis from pyruvate carbon would be to provide NADPH (primarily from the pentose phosphate cycle) or to provide α-glycerophosphate for the esterification process. The latter pathway seems likely according to the data presented in Table 2 showing that glucose carbon is, in fact, preferred over pyruvate carbon for glyceride-glycerol synthesis when the two substrates are present in roughly similar amounts. However, the provision of NADPH from the pentose phosphate cycle also appears to be important. This is illustrated by the data in Table 3. The acceleration of fatty acid synthesis from pyruvate carbon by glucose and glucose and insulin was either prevented or diminished by preincubation of cells with 6-aminonicotinamide or by incubation with the electron-acceptor vitamin K_5. The pentose phosphate cycle is known to be selectively inhibited by preincubation with 6-aminonicotinamide (17, 18). From the data in Table 3, it may be seen that 6-aminonicotinamide preincubation lowered insulin-stimulated fatty acid synthesis from tritium on glucose carbon-3. As noted previously, this activity reflects pentose phosphate cycle activity (12). Although direct inhibition of fatty acid synthesis would have produced the same effect, this seems unlikely since 6-aminonicotinamide preincubation did not alter basal rates of fatty acid synthesis from pyruvate or glucose. The finding that 6-aminonicotinamide preincubation prevented the glucose and insulin effect on fatty acid synthesis from pyruvate carbon therefore suggests that the enhancing effect of glucose was due to generation of NADPH in the pentose phosphate cycle.

Incubation with vitamin K_5 also diminished glucose-enhanced synthesis of glyceride-fatty acid from pyruvate carbon but had no effect on basal rates (Table 3). The action of insulin under these conditions was also decreased by the agent. At optimal concentrations, vitamin K_5 markedly accelerates the pentose phosphate cycle by causing the transfer of electrons from NADPH directly to molecular oxygen (4). In spite of presumably increased pentose phosphate cycle activity in the presence of vitamin K_5, no more NADPH is available for reductive fatty acid synthesis, resulting in the observed effects with pyruvate. It may also be observed that incorporation of tritium from carbon-3 of glucose into

TABLE 3

Glucose- and Insulin-Enhanced Glyceride-Fatty Acid Synthesis from Pyruvate in Small Cells

Treatment	[2-^{14}C]pyruvate incorporation into glyceride-fatty acid			D-[3-^{3}H]glucose incorporation into glyceride-fatty acid
	Glucose[a]			Glucose
	p		+	+
	nmols substrate used/10^5 cells/hr			
None	0.6 ± 0.1	<.05	1.0 ± 0.1	0.14 ± 0.04
Insulin	0.9 ± 0.3	<.001	4.5 ± 0.4*	1.3 ± 0.07*
Vitamin K_5 (K_5)	0.4 ± 0.1	NS	0.4 ± 0.1	0.14 ± 0.03
6-Aminonicotinamide (6-AN)	0.6 ± 0.2	NS	0.7 ± 0.1	0.2 ± 0.06
Insulin + K_5	–		1.6 ± 0.2†	0.98 ± 0.1*†
Insulin + 6-AN	–		3.0 ± 0.6†	0.6 ± 0.2†

[a]Effects of glucose on pyruvate utilization across all treatment tested by ANOVA, $p < 01$.

Cells were preincubated 1 hr at 37 C in albumin buffer and where indicated, 17.5 μg/ml 6-aminonicotinamide. Incubations were then performed as usual for 1 hr at 37 C following addition of the remaining reagents. Each vial contained [2-^{14}C]pyruvate (0.15 mM, 0.2 μCi/assay) and where indicated D-[3-^{3}H]glucose (0.28 mM, 0.1 μCi/assay), insulin (5 nM) and vitamin K_5 (1 μg/ml). Data are from three experiments. The column headed "p" indicates th statistical significance of the effect of glucose on glyceride-fatty acid synthesis from pyruvate carbon for each treatment. *p <.05 compared to samples not treated with insulin; †p <.02 compared to samples treated only with insulin and glucose.

glyceride-fatty acids was unchanged by treatment with vitamin K_5 compared to control. This would be expected since that tritium is the one ultimately transferred to oxygen. The results with these two agents suggest that it was glucose metabolism in the pentose cycle which allowed increased fatty acid synthesis from pyruvate carbon (Table 2). Moreover, it appears that insulin-stimulated pyruvate conversion to glyceride-fatty acid (Table 3) was also a result of enhanced pentose phosphate cycle generation of NADPH.

DISCUSSION

That both basal and insulin-stimulated conversion of glucose to glyceride-fatty acids in adipocytes from old, obese rats is markedly lower than in young, lean rats has been noted previously by others (1,3,4). This has been confirmed in the present studies and extended to acetate and pyruvate metabolism. Fatty acid synthesis from acetate and pyruvate in the large cells, even with glucose and insulin present, was quite low and variable. These findings indicate that the block in fatty acid synthesis occurs beyond the locations at which pyruvate and acetate enter cellular metabolism, i.e., probably in the pathway of fatty acid synthesis itself. Similar findings and conclusions were reached in a recent study at higher substrate concentrations (5). The recent study of Jamdar and Osborne (19) showed that acetate metabolism to fatty acid was about two-fold greater on a per-cell basis in large compared to small cells. The major difference between these studies was the rather high glucose concentrations (20 mM) used by Jamdar and Osborne. In addition, the results of Francendese and DiGirolamo (5) and the present study are more consistent with the enzyme studies of Richardson and Czech (4,6) which indicate a block in fatty acid synthesis at acetyl-CoA carboxylase in cells from large rats.

One assumption made in studies of substrate utilization is that large and small rat cells have similar rates of uptake of glucose, pyruvate and acetate, as well as similar rates of acetyl-CoA formation from acetate. At least for glucose there is evidence that basal rates of transport, when expressed per cell, increase with increasing cell size (3,20-22). If this reflects increased surface area or plasma membrane (23) of the larger cells, it might be expected that cells from the large rats used in the present studies would actually take up more acetate and pyruvate as well, making the observed decrease in acetate and pyruvate utilization even more surprising.

It is evident that lower pentose phosphate cycle activity is not the primary cause of reduced fatty acid synthesis in adipocytes from large rats. It was previously shown that glucose carbon-1 oxidation stimulated by vitamin K_5 was not diminished in large rat cells (4). Large cells were also found in the present studies (Table 1) to have normal responses of glucose oxidation to the oxidants diamide and TMPD. These agents are well known to stimulate the pentose phosphate cycle by mechanisms slightly different than vitamin K_5. TMPD has been shown to accelerate the transfer of NADH into mitochondria for oxidation, causing indirectly a decrease in the [NADPH]/[$NADP^+$] ratio (24). Diamide, although it may also oxidize NADPH directly (15), has a major effect of oxidizing GSH (14). This causes a coupled decline in the [NADPH]/[$NADP^+$] ratio, as well as presumably an increase in intracellular GSSG concentrations. Both of these effects have been shown to augment pentose phosphate cycle activity (16). The pentose phosphate cycle in large rat cells is thus normally responsive to agents which directly activate the cycle by different mechanisms, but not to insulin.

In neither cell type did insulin stimulate pyruvate carbon-2 incorporation into glyceride-glycerol (Table 2). Pyruvate has been shown to be converted to glyceride-glycerol by an enzymatic pathway involving reversal of glycolytic steps by pyruvate carboxylase and phosphoenolpyruvate carboxykinase (25,26). Insulin apparently does not affect this pathway of pyruvate metabolism in large or small cells, and glucose markedly decreases it (Table 2).

The demonstration that large cell glyceride-glycerol synthesis from glucose or pyruvate carbon is not impaired in spite of markedly diminished glyceride-fatty acid synthesis might at first seem contradictory, but it is likely that the glyceride-glycerol synthesis observed resulted from reesterification of free fatty acids released during ongoing lipolysis. It has been shown that basal rates of lipolysis and intracellular free fatty acid concentrations are at least as high in large as in small cells (2). Although insulin markedly inhibits hormone-stimulated lipolysis, it has a less pronounced effect on basal lipolysis (27,28), so that intracellular free fatty acids would probably still be available for reesterification. It has further been shown that the enzymatic capacity of reesterification is actually greater in large than small cells (29). This is associated with increased rates of triglyceride synthesis from labeled free fatty acids (30,31). In both cell types, substrate availability was found to determine rates of reesterification and the latter process was reflected by glyceride-glycerol synthesis. In fact, in this and other studies (1,5), large cell glyceride-glycerol was the

major product of glucose and pyruvate metabolism. With the low rate of de novo fatty acid synthesis, maintenance of high rates of reesterification may be a major mechanism whereby large cells preserve triglyceride stores (5).

The pentose phosphate cycle may not be necessary for an insulin-induced rise in fatty acid synthesis, since the hormone caused an increase (albeit small) in glyceride-fatty acid synthesis from pyruvate carbon in the absence of glucose (Table 2). Such an effect was also observed by Halperin (32) at low pyruvate concentrations in whole fat pads. It seems unlikely that any glucose could have been available from glycogen stores in 2-hr incubated adipocytes in the presence of insulin. However, it is apparent from this and previous studies (5,32) that glucose substantially increased both basal and insulin-stimulated glyceride-fatty acid synthesis from pyruvate carbon. This effect appears for several reasons to be related to provision of NADPH from glucose metabolism in the pentose phosphate cycle. First, ^{3}H from glucose carbon-3 was found in glyceride-fatty acids (Table 3), implying that the pentose phosphate cycle was the source (12). Second, fatty acid synthesis from pyruvate carbon was actually increased in the presence of a low glucose concentration (Tables 2 and 3). Finally, prevention of NADPH generation in the pentose phosphate cycle blocked the effect of glucose, particularly with insulin present (Table 3). This occurred when NADPH generation was decreased either by inhibiting the pentose phosphate cycle (as with 6-aminonicotinamide) or by oxidizing the NADPH produced in a stimulated pentose phosphate cycle (vitamin K_5). Previous studies also using electron acceptors have provided evidence that changes in the cellular redox state per se do not regulate fatty acid synthesis (18, 33). However, by using a substrate which does not at least directly traverse the pentose phosphate cycle such as pyruvate, it has been possible to show that NADPH availability can limit rates of fatty acid synthesis. The glucose-induced increase in acetate metabolism to glyceride-fatty acids in both cell types may also be caused by increased provision of α-glycerophosphate or NADPH.

In summary, it has been shown that the low basal and insulin-stimulated fatty acid synthesis from various substrates in adipocytes from old, fat rats most likely resides in the pathway itself and is not reversible in short-term experiments. Glucose carbon flow in these cells is directed toward α-glycerophosphate synthesis and ultimate reesterification with free fatty acids which may be a major factor in the maintenance of triglyceride stores within the cells. In contrast, in small cells from young rats, triglyceride formation also results from de novo fatty acid synthesis from any of several possible precursors, including pyruvate. The pathway of fatty acid synthesis normally depends on substrate availability and enzyme activity. However, when excess substrate is present, fatty acid synthesis may be limited by the supply of NADPH.

ACKNOWLEDGMENTS

This work was supported by Clinical Investigator Award AM 00651 and a grant from the American Diabetes Association. The technical assistance of Terre Williams and manuscript typing of Jessie Reynolds and Mary Graham is appreciated. The author is also indebted to Drs. Robert G. Lamb and Donald Fletcher for review of the manuscript.

REFERENCES

1. DiGirolamo, M., and Rudman, D. (1968) Endocrinology 82, 1133-1141.
2. DiGirolamo, M., Howe, M.D., Esposito, J., Thurman, L., and Owens, J.L. (1974) J. Lipid Res. 15, 332-338.
3. Czech, M.P. (1976) J. Clin. Invest. 57, 842-851.
4. Richardson, D.K., and Czech, M.P. (1978) Am. J. Physiol. (Endocrinol. Metab. Gastrointest. Physiol. 3) 234,E182-E189.
5. Francendese, A.A., and DiGirolamo, M. (1981) Biochem. J. 194, 377-384.
6. Richardson, D.K., and Czech, M.P. (1979) Horm. Metab. Res. 11, 427-431.
7. Gellhorn, A., Benjamin, W., and Wagner, M. (1962) J. Lipid Res. 3, 314-319.
8. Rodbell, M. (1964) J. Biol. Chem. 239, 375-380.
9. Gliemann, J. (1965) Diabetes 14, 643-649.
10. May, J.M., Williams, R.H., and deHaën, C. (1978) J. Biol. Chem. 253, 686-690.
11. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
12. Katz, J., and Rognstad, R. (1966) J. Biol. Chem. 241, 3600-3610.
13. Halperin, M.L., and Robinson, B.H. (1970) Biochem. J. 116, 235-240.
14. Kosower, N.S., Kosower, E.M., Wertheim, B., and Correa, W.S. (1969) Biochem. Biophys. Res. Commun. 37, 593-596.
15. Harris, J.W., and Biaglow, J.E. (1972) Biochem. Biophys. Res. Commun. 46, 1743-1749, 1972.
16. Eggleston, L.V., and Krebs, H.A. (1974) Biochem. J. 138, 425-435.
17. Lange, K., and Proft, E.R. (1970) Naunyn-Schmiedebergs Arch. Pharmacol. 267, 177-180.
18. Kather, H., Rivera, M., and Brand, K. (1972) Biochem. J. 128, 1097-1102.
19. Jamdar, S.C., and Osborne, L.J. (1981) Lipids 16, 829-834.
20. Livingston, J.N., and Lockwood, D.H. (1974) Biochem. Biophys. Res. Commun. 61, 989-996.
21. Olefsky, J.M. (1976) J. Clin. Invest. 57, 842-851.
22. Foley, J.E., Laursen, A.L., Sonne, O., and Gliemann, J. (1980) Diabetologia 19, 234-241.
23. Lewis, D.S., Masoro, E.J., and Yu, B.P. (1981) J. Lipid Res. 22, 1094-1101.
24. Robinson, B.H., and Halperin, M.L. (1970) Biochem. J. 116, 229-233.
25. Ballard, F.J., Hanson, R.W., and Leveille, G.A. (1967) J. Biol. Chem. 242, 2746-2750.

26. Reshef, L., Hanson, R.W., and Ballard, F.J. (1969) J. Biol. Chem. 244, 1994-2001.
27. Holm, G., Jacobsson, G., Bjorntorp, P., and Smith, U. (1976) J. Lipid Res. 16, 461-464.
28. Hartman, A.D., Cohen, A.I., Richane, C.J., and Hsu, T. (1971) J. Lipid Res. 12, 498-505.
29. Jamdar, S.C., Osborne, L.J., and Zeigler, J.A. (1981) Biochem. J. 194, 293-298.
30. Jamdar, S.C. (1978) Biochem. J. 170, 153-160.
31. Zinder, O., Arad, R., and Shapiro, B. (1967) Israel J. Med. Sci. 3, 787-791.
32. Halperin, M.L. (1970) Can. J. Biochem. 48, 1228-1233.
33. Katz, J., and Wals, P.S. (1971) Arch. Biochem. Biophys. 147, 405-418, 1971.

[Received February 6, 1982]

Cholesteryl Ester Hydrolase Activity in Adrenal Homogenates from Normal and Essential Fatty Acid-Deficient Female Rats

ANGELA K. YOUNG and **BRIAN L. WALKER***, *Department of Nutrition, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

ABSTRACT

Cholesteryl ester hydrolase was assayed in adrenal homogenates from mature female rats fed a control (corn oil-containing) or essential fatty acid (EFA)-deficient diet. Cholesteryl ester of 16:0, 18:0, 18:1, 18:2(n-6), 20:4(n-6) and 22:4(n-6) were used as substrates. In control rats, the unsaturated esters were hydrolyzed more rapidly than the saturated esters and cholesteryl arachidonate was the preferred substrate of the six investigated; cholesteryl oleate elicited the highest activity in the deficient group. Polyunsaturated esters were hydrolyzed at a significantly lower rate by homogenates from EFA-deficient rats than by those from control animals. The esters of 18:1, 18:2(n-6) and 20:4(n-6) were hydrolyzed more extenstively in relation to their concentrations in adrenal tissue than were cholesteryl esters of 16:0, 18:0 and 22:4(n-6). This difference was more pronounced in control than in EFA-deficient rats. No simple relationship of adrenal cholesteryl ester hydrolase activity to ester fatty acid structure or to nutritional essentiality was evident.

Lipids 17:634-638, 1982.

INTRODUCTION

The adrenal glands of many species contain high concentrations of cholesteryl esters which appear to act as a reservoir of cholesterol for glucocorticoid biosynthesis. Administration of ACTH to animals results in a marked decrease in the cholesteryl ester content of the adrenal, concomitant with the release of glucocorticoids to the plasma (1-3). Hydrolysis of cholesteryl esters to free cholesterol occurs in response to ACTH as a result of increased cholesteryl ester hydrolase activity (4). Specific cholesteryl esters may be preferred substrates for this hydrolytic process (5-8).

EFA deficiency has been reported to impair glucocorticoid production both in vitro (9) and in vivo (5,10) and cold-stressed, EFA-deficient male rats exhibited a lower decline in adrenal cholesteryl ester concentration than did control animals. Moreover, the decrease in adrenal cholesteryl ester concentration, associated with increase in plasma corticosterone during the natural circadian cycle of this hormone in the normal female rat, was not observed in the EFA-deficient animal (8). Corticosterone elaboration appeared to occur in the latter rats without a significant net decrease in adrenal total cholesteryl esters, although there were changes in specific esters. We have observed a similar situation after administering low doses of ACTH to female rats. The current investigation was undertaken to determine activity of adrenal cholesteryl ester hydrolase in normal and EFA-deficient female rats and to investigate the specificity of this enzyme for a variety of esters.

*Author to whom correspondence should be addressed.

Abbreviations: EFA, essential fatty acid(s); x:y(n-z), fatty acid with x carbon atoms and y olefinic bonds with the terminal olefinic being z carbon atoms from the methyl group; ACTH, adrenocorticotrophic hormone.

MATERIALS AND METHODS

All solvents and inorganic constituents of buffer solutions were purchased from Fisher Scientific Co., Toronto. Cholesterol was obtained from Serdary Research Laboratories, London, Ontario, and [7-^{3}H]cholesterol from Amersham-Searle Corp., Toronto, Ontario. Fatty acyl chlorides were purchased from Nu-Chek-Prep Inc., Elysian, MN. Dietary constituents were purchased from Teklad Mills, Chargrin Falls, OH (vitamins, mineral mix), Canada Starch Co., Toronto (dextrose), Lee Chemicals, Ltd., Toronto (cellulose) and Canada Packers Ltd., Toronto (coconut oil). Corn oil was purchased at a local supermarket.

Weanling female Wistar rats were purchased from Woodlyn Farms, Guelph. They were housed individually in stainless steel cages and fed ad libitum a purified casein/dextrose-based diet (11) containing 10% by wt of fat. The control fat was corn oil and the EFA-deficient fat was hydrogenated coconut oil; the fatty acid compositions of these oils have been reported

previously (8). A 12-hr light (07:00-19:00)/12-hr darkness photoperiod was maintained in the animal room. The experiment was terminated after 20 weeks when the rats fed coconut oil exhibited the classical symptoms of EFA deficiency (scaly skin on paws and tail, low weight gain).

Cholesteryl esters of 16:0, 18:0, 18:1, 18:2, 20:4 and 22:4 acids were utilized in the assay of cholesteryl ester hydrolase activity and were synthesized as described by Deykin and Goodman (12). The [7-^{3}H] cholesterol was esterified by the appropriate acyl chloride in the presence of pyridine and the resulting esters were purified by thin layer chromatography (TLC) on Silica Gel-G with petroleum ether/diethyl ether/acetic acid (90:10:1, v/v) as the developing solvent. After detection by Rhodamine 6G and UV light, the products were recovered from the silica by extraction with diethyl ether and stored in benzene at 4 C until they were used for this assay. The specific activities of the esters (mCi/mmol) were: 16:0, 5.7; 18:0, 4.8; 18:1, 5.1; 18:2, 7.7; 20:4, 4.3; and 22:4, 3.1.

At 14:00 on each of six consecutive days, eight animals from one of the two dietary groups were killed by ether anesthesia and the adrenals were rapidly removed, trimmed of extraneous fat and weighed; tissues from eight rats were pooled so that three replicate samples were obtained for each dietary group in the experiment. Adrenals were rapidly homogenized in 10 ml of ice-cold 0.25 M sucrose and aliquots were removed, immediately frozen and stored at -10 C for protein (13), total cholesteryl ester assay and for determination of individual cholesteryl esters by TLC and gas liquid chromatography (8). Cholesteryl ester hydrolase activity was assayed in the remaining homogenate by monitoring release of labeled free cholesterol according to the procedure of Eto and Suzuki (14) with some modification to the substrate solution. Approximately 0.9 μCi of [7-^{3}H] cholesteryl ester, dissolved in 200 μl of acetone, was added to 3.8 ml phosphate buffer (0.4 M, pH 7.4) containing 22.8 μmol of sodium taurocholate. The solution was mixed thoroughly by vigorous agitation and 1-ml aliquots were used for each assay. Substrate solution (1 ml) and 0.5 ml of 0.25 M sucrose were transferred to a 25-ml Erlenmeyer flask and preincubated for 15 min at 37 C with shaking. Fresh adrenal homogenate (0.5 ml, containing ca. 4 mg protein) was added and the incubation was continued for 30 min at 37 C with shaking. Air served as the gas phase above the mixture and a blank, prepared with boiled homogenate, was assayed simultaneously with the test solutions.

After termination of the reaction by addition of 10 ml of chloroform/methanol (2:1, v/v), the contents of the flask were transferred to a conical centrifuge tube (12 x 120 mm), mixed thoroughly and centrifuged (3,000 x g, 20 min). The upper aqueous methanol phase was discarded and the lower chloroform phase was filtered through Whatman No. 41 paper; the tube and residue were washed three times with 3-ml aliquots of chloroform/methanol which were also filtered. The combined filtrates were evaporated to dryness under nitrogen and the residue was dissolved in 6 ml of chloroform/methanol (2:1, v/v). The free cholesterol in 0.5 ml of this solution was isolated by TLC on Silica Gel-G with petroleum ether/diethyl ether/acetic acid (90:10:1, by vol) as the developing solvent. Cholesterol was recovered by extraction of the gel with 2 x 5-ml aliquots of diethyl ether. After removal of the ether, the liquid was dissolved in 0.5 ml of benzene and transferred to a scintillation vial and the [^{3}H]-cholesterol was assayed in a liquid scintillation counter with external standard quench correction. Data were corrected for cholesterol released in the blank incubation. The enzyme activity was linear with time over a 60-min period and was directly proportional to protein concentration up to at least 6 mg of protein. The assay was conducted for 30 min in the presence of ca. 4 mg adrenal protein.

Data were subjected to Student's t-test to evaluate differences between normal and deficient rats (15).

RESULTS

The activity of adrenal cholesteryl ester hydrolase toward the six esters investigated is presented in Table 1. When rats were fed the control corn oil, the enzyme was more active with the unsaturated esters as substrates. Cholesteryl arachidonate (20:4(n-6)) was most rapidly hydrolyzed even though it was not the major ester in the tissue (cf. Table 2). In the homogenates from EFA-deficient rats, cholesteryl oleate (18:1) was the most abundant of the six adrenal esters investigated (Table 2) and elicited the highest hydrolase activity. The esters of 18:2(n-6), 20:4(n-6) and 22:4(n-6) were present in significantly lower concentrations (Table 2) and were hydrolyzed at significantly lower rates by adrenal homogenates from EFA-deficient rats, which had, of course, been deprived of dietary essential (n-6) acids. There were no significant differences in enzyme activity between the two groups with respect to the saturated and monoenoic esters even though cholesteryl oleate was present in significantly higher concentration in the deficient

animals.

In order to assess the differences in activity of the enzyme in terms of total utilization of esters in the adrenal, the degree of hydrolysis and the extent of hydrolysis relative to a reference compound (cholesteryl stearate) were calculated for each ester (Table 2). In homogenates from control animals, the extent of hydrolysis ranged from 6.1% for 22:4(n-6) to 15.0% for 20:4(n-6); it was independent of the concentration of ester in the tissue, as was particularly evident for 22:4(n-6). Substantially more 18:1, 18:2(n-6) and 20:4(n-6) were hydrolyzed than the other three esters. This pattern was observed to a lesser extent in the EFA-deficient group. However, significantly smaller proportions of all the esters except 18:0 were hydrolyzed by adrenal homogenates from these rats.

TABLE 1

Activity of Adrenal Cholesteryl Ester Hydrolase to Different Substrates

Cholesteryl ester substrate	Dietary group	
	Normal	EFA-deficient
	nmol/min/mg protein	
16:0	0.13 ± 0.01	0.12 ± 0.01
18:0	0.07 ± 0.01	0.05 ± 0.01
18:1(n-9)	0.26 ± 0.01	0.25 ± 0.04
18:2(n-6)	0.17 ± 0.02	0.03 ± 0.00*
20:4(n-6)	0.59 ± 0.06	0.08 ± 0.01*
22:4(n-6)	0.30 ± 0.07	0.03 ± 0.01†

,†Difference between normal and EFA-deficient group was significant ($p<0.01$; †$p<0.05$). Values are the means of assays on 3 composite samples of tissues from 8 rats ± SEM.

The relative differences in utilization among esters in the two dietary groups are evident in the final section of Table 2, in which the percentage of hydrolysis is related to a common reference, i.e., 18:0. This ester did not differ significantly in concentration between the two groups, rendering the two sets of data reasonably comparable. In both dietary groups, it is evident that esters of 16:0 and 22:4(n-6) were hydrolyzed less extensively, and those of 18:1, 18:2(n-6) and 20:4(n-6) more extensively than cholesteryl stearate, again emphasizing that the pattern of hydrolysis is not simply correlated with ester concentration in the tissue homogenate.

DISCUSSION

In this study, hydrolysis of endogenous cholesteryl esters was measured after addition of tracer amounts of the ester under investigation and the results should reflect the relative affinities of the esters for the hydrolase at the concentrations in the tissue. A basic premise of this approach is that the endogenous pool of cholesteryl esters is equally available to the added tracers. Differences in solubilities of the added esters may affect their availability to the endogenous pool and to the hydrolase. Repeated sampling of the substrate solutions revealed no evidence of inhomogeneity with any of the esters. Solubility should decline with increasing chain length but the proportion of [^{3}H] cholesteryl stearate hydrolyzed by homogenates from either group of animals was not smaller than that of [^{3}H] cholesteryl palmitate hydrolyzed. Polarity and solubility would also increase with unsaturation. In homogenates from control rats, hydrolysis of the 18:1 and 18:2 esters was more extensive than that of 18:0 but the two

TABLE 2

Relative Utilization of Adrenal Cholesteryl Esters in Normal and EFA-Deficient Rats[a]

Cholesteryl ester	Concentration		% Hydrolysis[b]		Relative % hydrolysis	
	Normal	EFA-deficient	Normal	EFA-deficient	Normal	EFA-deficient
	nmol/mg protein					
16:0	63.0 ± 4.80	90.3 ± 14.42	6.3 ± 0.5	3.9 ± 0.5†	0.77 ± 0.10	0.78 ± 0.05
18:0	25.0 ± 3.50	30.8 ± 6.51	8.6 ± 1.6	5.1 ± 0.8	1.00	1.00
18:1(n-9)	56.0 ± 4.71	116.4 ± 21.30	14.3 ± 1.4	6.7 ± 0.7†	1.75 ± 0.28	1.38 ± 0.07
18:2(n-6)	38.0 ± 1.59	13.0 ± 0.35*	14.0 ± 2.5	6.5 ± 0.4†	1.66 ± 0.19	1.35 ± 0.27
20:4(n-6)	119.3 ± 12.07	31.6 ± 1.43*	15.0 ± 1.4	7.6 ± 0.3*	1.86 ± 0.33	1.56 ± 0.24
22:4(n-6)	145.9 ± 2.31	73.0 ± 8.18*	6.1 ± 1.4	1.5 ± 0.2†	0.70 ± 0.04	0.32 ± 0.09

[a],*,†Difference between normal and EFA-deficient group were significant (*$p<0.01$; †$p<0.05$). Values are the means of assays on 3 composite samples of tissues from 8 rats ± SEM.

[b]Percentage of esters hydrolyzed in 30 min.

unsaturated esters did not differ; in homogenates from deficient animals, the proportions of saturated, monoenic and dienoic esters hydrolyzed were similar. Although the evidence is indirect, it does not appear that the results obtained may be attributed to differences in availability of the added esters to the general ester pool.

The current experiment demonstrated that adrenal cholesteryl ester hydrolase activity does exhibit specificity for the fatty acid present in the ester. No consistent relationship of fatty acid structure to hydrolase activity was apparent. In the control rats, increasing the chain length of the saturated acid by two carbon atoms (16:0 vs 18:0) made little difference to the rate of hydrolysis of the ester, whereas a similar difference in chain length in the polyunsaturated esters (20:4[n-6] vs 22:4[n-6]) substantially affected enzyme activity. Increasing unsaturation at constant chain length (18:0, 18:1, 18:2) also failed to produce a consistent trend in enzyme activity; of these three, the monoenoic ester was the preferred substrate in both dietary groups.

Cholesteryl ester hydrolase activity in liver has been investigated to a greater degree than that in the adrenal and fatty acid specificity is observed in that tissue. Deykin and Goodman (12) reported that the rat liver enzyme hydrolyzed unsaturated esters more rapidly than saturated esters. Furthermore, liver cholesteryl ester hydrolase has been reported to hydrolyze medium-chain esters more readily than long-chain esters, those with $\Delta 9$ unsaturation more readily than esters with the double bond located elsewhere in the chain, and esters with *cis* unsaturation more readily than those with *trans* unsaturation (16-18). Cholesteryl esters of odd-chain fatty acids were hydrolyzed more slowly than esters of even-chain-length acids (19). It is evident that fatty acid specificity is observed with cholesteryl ester hydrolase activity in tissues other than the adrenal although the patterns may not be the same. Studies involving the direct measurement of adrenal cholesteryl ester hydrolase activity are few in number. Beckett and Boyd (20) did observe that cholesteryl erucate (22:1) was less readily hydrolyzed than cholesteryl oleate by rat adrenal homogenates, confirming earlier in vivo observations in cold-stressed rats (7). Much of the current information concerning the specificity of the adrenal enzyme has been obtained indirectly from measurements of net changes in cholesteryl ester concentration in vivo (6-8). The present investigation is consistent with the earlier studies with esters of oleic, linoleic and arachidonic acids being preferentially hydrolyzed (in relation to tissue concentration) by rat adrenal homogenates. These same esters are preferentially depleted in stressed rats (6,7) or during the natural circadian cycle of plasma corticosterone production (8). The lack of specificity of the enzyme for 22:4(n-6) in relation to the high tissue concentration also confirms the absence of preferential utilization of this ester in vivo (6-8).

The current investigation does appear to explain the relative lack of utilization of adrenal cholesteryl esters during the circadian cycle of corticosterone production in EFA-deficient rats (8), since there was a lower hydrolytic activity to polyunsaturated esters in adrenal homogenates from such animals. However, it must be emphasized that two major esters of EFA-deficient rat adrenals, 20:3(n-9) and 22:3(n-9), were not investigated in this study since these acids are not readily available. The lower activity of the EFA-deficient adrenal homogenates toward 18:2(n-6), 20:4(n-6) and 22:4(n-6) may not apply to the 20:3(n-9) and 22:3(n-9) esters. Indeed, in vivo studies indicate that the latter esters are utilized during corticosteroidogenesis (7,8). Lack of net utilization of adrenal cholesteryl esters in EFA-deficient rats in vivo is probably due to a balance between synthesis and hydrolysis of the esters in the tissue, but further work is necessary before this can be ascribed simply to decreased hydrolysis.

The basis for the fatty acid specificity of adrenal cholesteryl ester hydrolase is not readily apparent. The high activity with the oleate (n-9) substrate, particularly in EFA-deficient rats, precludes dietary essentiality as a basis for high specificity. There is some indication that dietary induction of the enzyme may occur, since in EFA-deficient rats, the low concentrations of the (n-6) acids were accompanied by lower absolute enzyme activities toward these esters. However, 22:4(n-6), the major ester in control rats, was not as good a substrate for the enzyme as 20:4(n-6) which was less abundant. The current study, while confirming the high degree of specificity of rat adrenal cholesteryl ester hydrolase for the esters of 18:1, 18:2(n-6) and 20:4(n-6), in relation to tissue concentration, fails to indicate the basis for this specificity.

REFERENCES

1. Sayers, C.T., Sayers, M.S., Fry, E.G., White, S., and Long, C.N.H. (1944) Yale J. Biol. Med. 16, 361-390.
2. Duncommun, B., and Mach, R.S. (1949) Acta Endocrinol. 3, 17-26.
3. Glick, D., and Ochs, M.J. (1955) Endocrinology 56, 285-298.

4. Davis, W.W., and Garren, L.P. (1966) Biochem. Biophys. Res. Commun. 24, 805-810.
5. Muraoka, R. (1965) Arch. Jpn. Chir. 34, 35-41.
6. Gidez, L.I., and Feller, E. (1969) J. Lipid Res. 10, 656-659.
7. Walker, B.L., and Carney, J.A. (1971) Lipids 6, 797-804.
8. Young, A.K., and Walker, B.L. (1977) Lipids 13, 181-186.
9. Hayashida, T., and Portman, O.W. (1959) Am. J. Physiol. 197, 893-896.
10. Hikasa, Y., Nagase, M., Matsuda, S., Satomura, K., Muraoka, R., and Fukada, H. (1966) Arch. Jpn. Chir. 35, 381-394.
11. Lamptey, M.S., and Walker, B.L. (1976) J. Nutr. 106, 86-93.
12. Deykin, D., and Goodman, D.S. (1962) J. Biol. Chem. 237, 3649-3656.
13. Lowry, D.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
14. Eto, Y., and Suzuki, K. (1971) Biochim. Biophys. Acta 239, 293-311.
15. Steel, R.G.D., and Torrie, J.H. (1960) in Principles and Procedures of Statistics, pp. 43-67, 99-132, McGraw-Hill Book Co., Inc., New York, NY.
16. Sgoutas, D.S. (1968) Biochim. Biophys. Acta 164, 317-326.
17. Goller, H.J., and Sgoutas, D.S. (1970) Biochemistry 9, 4801.
18. Goller, H.J., Sgoutas, D.S., Ismail, I.A., and Gunstone, F.D. (1970) Biochemistry 9, 3072-3076.
19. Sgoutas, D.S. (1971) Biochim. Biophys. Acta 239, 469-474.
20. Beckett, G.J., and Boyd, G.S. (1975) Eur. J. Biochem. 53, 335-342.

[Received September 10, 1981]

Fatty Acid Patterns in Iron-Deficient Maternal and Neonatal Rats

ADRIA ROTHMAN SHERMAN[a,*], **SANDRA J. BARTHOLMEY**[a] and **EDWARD G. PERKINS**[b], *Department of Foods and Nutrition*[a] *and Department of Food Science*[b], *University of Illinois, Urbana, IL 61801*

ABSTRACT

To determine the effects of maternal iron deficiency on lipid composition and fatty acid patterns in offspring, rats were fed ad libitum diets containing 5 ppm iron (deficient) (n=8) or 320 ppm iron (control) (n=7) and deionized water from day-1 of gestation through day-18 of lactation. On day-2 of lactation, litters were standardized to three male and three female pups. On day-18, pups were fasted for 4 hr before tissue and blood collection. Significant changes in serum and liver lipid concentrations and fatty acid patterns were observed in deficient pups. Serum triglycerides, cholesterol and phospholipids and liver triglycerides, cholesterol, and cholesteryl esters were increased. In deficient pups, percentage total fatty acids of 14:0, 16:1, 18:1, 18:2 from serum lipids were increased; in liver, 14:0, 18:2, 18:3 were increased; 18:0 and 20:4 were decreased in both serum and liver. Dam serum lipid levels did not differ between groups. Lipid changes observed in iron-deficient pups did not consistently reflect the milk, serum or liver lipid patterns observed in dams. Altered lipid composition and fatty acid patterns of iron-deficient pups thus appear to be of endogenous origin.
Lipids 17:639-643, 1982.

Reports by this (1-5) and other investigators (6-8) have demonstrated that dietary iron restriction in rats and chicks results in elevated serum lipid levels. Sherman et al. have studied serum lipid metabolism in suckling pups of iron-deficient dams. Maternal iron deficiency during both pregnancy and lactation produces hyperlipidemia in 18-day-old offspring but not in maternal rats fed diets containing 5 ppm iron (1). Serum from pups whose mothers were deficient in iron during both pregnancy and lactation had significantly greater triglycerides, phospholipids, and total cholesterol levels than serum from pups whose dams were fed adequate iron during pregnancy or lactation. Since no significant differences were found among the serum lipid concentrations in the dams, it appears that maternal iron deficiency affects serum lipids of pups but not of dams.

Previous studies (2,3) to investigate the mechanism responsible for the hyperlipidemia in pups included measurement of milk lipids to determine if iron deficiency altered lipid content of dam's milk (2). No differences were found in triglyceride or cholesterol content of milk produced by iron-deficient and control dams. Lipid clearance from the blood was not different between iron-deficient and control pups as indicated by in vitro lipoprotein lipase activity. In vitro incorporation of [U-^{14}C]glucose into triglycerides and $^{14}CO_2$ was significantly greater in liver slices from iron-deficient pups than from control pups. Hyperlipidemia in 18-day-old, iron-deficient rat pups thus appears to be related to increased endogenous production of triglycerides. Histopathological study of tissues from iron-deficient pups has shown fatty degeneration of the liver associated with the other aberrations in lipid metabolism (3).

Post-weaning iron restriction and serum lipid metabolism have also been studied by this investigator (4). Hypertriglyceridemia develops at 60 days of age when iron-deficient diets are fed to weanling rats. Studies of lipogenesis in adult rats indicate that the in vitro incorporation of $^{3}H_2O$ into triglycerides in adipose tissue is elevated in iron deficiency (5). The iron deprivation in these adult rats results in a less severe anemia in which serum lipids are not yet elevated.

The purpose of this investigation was to further study the hyperlipidemia and triglyceride-rich livers of iron-deficient rat pups. In addition to lipid composition, the fatty acid patterns were characterized in serum, liver and milk of maternal rats and their neonates.

MATERIALS AND METHODS

Female Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 160-190 g were housed individually in stainless steel, mesh-bottomed cages and fed stock diet (Purina Rat Chow, Ralston-Purina Co., St. Louis, MO) and tap water. The estrous cycles of the rats were determined by microscopic examination of vaginal smears and rats were bred when they reached a weight of 200-225 g. When females

*Author to whom correspondence should be addressed.

were in estrous, normal Sprague-Dawley males were introduced into the cage overnight, and if either copulation plugs or sperm in the vaginal smear were detected the next morning, the time was considered day 1 of gestation. Eight dams were then randomly assigned to the iron-deficient diet (5 ppm iron) and seven to the control diet (320 ppm iron) (Table 1), fed ad libitum and given double-distilled water from the first day of pregnancy until day 18 of lactation. The control diet was designed to provide adequate levels of all known essential nutrients for the rat during reproduction (9). The diet providing 5 ppm iron was identical except that it was prepared without addition of ferrous sulfate. On the 19th day of gestation, rats were placed in a stainless steel, mesh-bottomed maternity cage with a plastic nesting box containing iron-free bedding (Sanicel, Paxton Processing, Inc., Paxton, IL).

Diet and water were available only to the dams outside the nesting boxes to insure that maternal milk was the sole nutriture of pups. On the day following parturition, litters were weighed and adjusted to contain three male and three female pups. All rats were weighed weekly and maternal food intake was determined by weight weekly throughout the study.

TABLE 1
Diet Composition

	Percent
Casein[a]	22
Sucrose	30
Cornstarch	31.5
Corn oil[b]	10
Cellulose[c]	2
Vitamin mix[d]	1
Iron-free AIN-76 mineral mix[e]	3.5

[a]Vitamin-free test casein, Teklad, Madison, WI.

[b]Mrs. Tucker's Pure Corn Oil, Anderson Clayton Foods, Dallas, TX.

[c]Alphacel, ICN Nutritional Biochemical Co., Cleveland, OH.

[d]#40060, Teklad, Madison, WI. Composition of vitamin mix (mg/kg diet): *p*-aminobenzoic acid, 110.1; ascorbic acid, coated (97.5%), 991.2; biotin, 0.441; vitamin B_{12}, 0.0297; calcium pantothenate, 66.1; choline, 0.443; folic acid, 1.98; inositol, 110.1; menadione (vitamin K_3), 49.6; niacin, 99.1; pyridoxine HCl, 22.0; thiamin HCl, 22.0. Supplies in IU/kg diet: dry retinyl palmitate, 19,824; dry ergocalciferol, 2202.5; dry tocopherol acetate, 121.15; cornstarch, 4.7 g.

[e]AIN-76 mineral (10) mixture prepared without iron. Composition of mineral mix (g/kg mineral mix): $CaHPO_4$, dibasic, 500.0; NaCl, 74.0; potassium citrate, monohydrate, 220.0; K_2SO_4, 52.0; MgO, 24.0; $ZnCO_3$, 1.6; $MnCO_3$, 3.5; cupric carbonate, 0.485; KIO_3, 0.01; $Na_2SeO_3 \cdot 5H_2O$, 0.01; $CrK(SO_4)_2 \cdot 12H_2O$, 0.55; sucrose, 124.03. $FeSO_4 \cdot 7H_2O$ is added to the supplemented diet to provide the desired iron concentration and to replace an equal amount of sucrose.

On day-18 of lactation, dams were separated from the pups and retained access to food and water. Final body weights were recorded and tail blood samples were taken from pups and from dams for hemoglobin and hematocrit determinations (11). Milk was collected from dams 5-6 hr after removal of young. The dams were given intraperitoneal injections of Nembutal (5.40 mg/100 g) and oxytocin (0.25 USP/100 g). Milk was expressed manually from each nipple, collected by vacuum into a plastic tube on ice, stored under nitrogen and frozen for analysis of lipids. After collection of milk from the dams, cardiac punctures were done, serum was removed and stored under nitrogen.

Following a 4-hr fasting period and chloroform anesthesia, blood was collected from pups by cardiac puncture, serum removed, sera from pups in each litter were pooled, stored under nitrogen and frozen for lipid analysis.

Livers of pups and dams were removed, rinsed with double-distilled water, blotted dry, weighed and ca. 2 g of liver was homogenized (Polytron PT 10-35, Brinkmann Instruments, Westbury, NY, at setting 5 for 30-60 sec) and extracted overnight in 2:1 chloroform/methanol for lipid analysis. Two pup livers from each litter were pooled for lipid extraction.

Lipids were extracted from serum, liver and milk samples by the method of Folch et al. (12). Triglycerides, cholesterol, cholesteryl ester, phospholipids and total fatty acids were determined in all tissues following separation on thin layer chromatography plates (Merck precoated Silica Gel 60) developed in a solvent system of petroleum ether/ethyl ether/acetic acid (70:30:1, v/v/v). After brief exposure to iodine vapors, lipid fractions were identified by comparison with triolein, cholesterol and cholesteryl ester standards run simultaneously. The spots were recovered and the following colorimetric assays were used to quantitate the lipid fractions: triglyceride concentration was determined by the method of Stern and Shapiro (13); nonesterified cholesterol and cholesteryl ester were measured by the method of Searcy and Bergquist (14); phospholipid concentration was determined by measuring phosphorus in the lipids remaining at the origin of the chromatograph using the method of Bartlett (15).

Fatty acid composition of tissue samples was determined by gas liquid chromatography (GLC) of methyl ester preparations. An aliquot of total lipid (1-10 mg) was converted to the corresponding methyl ester by heating with 10-20 ml of anhyd methanol containing 2%

concentrated H_2SO_4. After heating under reflux conditions for 2 hr, the reaction mixture was cooled to room temperature and an equal volume of glass-distilled hexane was added. After shaking for 1 min, the mixture was transferred to a separatory funnel and 10 ml of double-distilled water was added. The mixture was again shaken for 1 min and the water layer was drained off. A second aliquot of water was added, the mixture was shaken and the water layer was drained off. The hexane layer was dried over anhyd sodium sulfate, decanted into a storage vial and evaporated to dryness under a nitrogen stream. One ml of isooctane was added to the samples which were stored under nitrogen and frozen until analyzed, usually within two days of preparation.

Methyl esters of fatty acids were analyzed by GLC on a flame ionization detector gas chromatograph (Hewlett-Packard 5830A). Separations were achieved with a 6 ft x 2 mm id glass column packed with 10% SP-2330 Chromosorb coated on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA) at a column temperature of 175 C; the detector and injector temperatures were 250 C. The flow rate of carrier gas used was 15 ml/min. Quantitation and identification of the esters were achieved by comparison of retention times and amounts with standard mixtures of esters (Nu-Chek-Prep, Elysian, MN) on an electronic integrator (Hewlett-Packard 18850A).

The statistical significance between means of the two dietary treatments was determined by Student's t-test (16).

RESULTS

Restriction of dietary iron (5 ppm) during pregnancy and lactation resulted in anemia in both maternal and suckling rats (Table 2). In the dams, blood hemoglobin concentrations and hematocrit levels were significantly reduced in the iron-deficient group. The severity of anemia present in the suckling pups of dams fed 5 ppm iron was greater than in the maternal rats.

Pups in the 5-ppm iron group had a marked hyperlipidemia characterized by significant elevations in serum triglycerides, cholesterol and phospholipids (Table 3). Although there were elevations in serum triglycerides and cholesterol in the deficient dams, these were not significantly different from control levels. Fatty acid patterns in serum of iron-deficient pups were also quite different than those of control pups. These alterations in fatty acids from serum lipids of iron-deficient pups included increased levels of myristate (14:0), palmitoleate (16:1), oleate (18:1), and linoleate (18:2) and lower levels of stearate (18:0) and arachidonate (20:4) than in control pup serum. No significant differences in serum fatty acid patterns were found between iron-deficient and control dams.

Liver lipids of iron-deficient pups (Table 4) followed similar trends as serum lipids. Triglycerides were seven-fold higher in iron-deficient pup livers than in control pup livers, and cholesterol and cholesteryl ester concentrations were two-fold higher in iron-deficient pup livers than control levels with no change in phospholipid concentration. In iron-deficient pup livers, myristate, linoleate and linolenate increased whereas oleate and arachidonate decreased as in serum.

Iron-deficient dam livers contained 60% more cholesteryl ester than livers of control dams. Changes in fatty acid patterns were limited to an increase in linoleate and a decrease in palmitoleate.

In general, there were not significant differences between the iron-deficient and control dams with regard to milk lipid composition and

TABLE 2

Final Body Weights, Hemoglobin and Hematocrit Levels and Total Food Intake

Treatment	Body wt (g)	Hemoglobin (g/dl)	Hematocrit (%)	Total food intake (g)
Dams				
Control	281 ± 9[a]	15.9 ± 0.3	51.9 ± 0.8	949 ± 36
Deficient	276 ± 6	10.9 ± 0.2*	36.9 ± 0.9*	733 ± 32*
Pups[b]				
Control	48 ± 3	9.6 ± 0.4	36.8 ± 1.2	
Deficient	27 ± 2*	4.7 ± 0.4*	16.1 ± 1.2*	

[a]Mean ± SEM, n = 7-8/group.

[b]Group means for pups are derived from average of 4 values per litter, and 7-8 litters/group.

*$p < 0.001$ as determined by Student's t-test.

TABLE 3

Serum Lipid Concentrations (mg/dl) and % Fatty Acids

	Dams		Pups	
	Control	Deficient	Control	Deficient
Triglycerides	83.7 ± 23.8[a]	174.2 ± 36.2	68.1 ± 16.2	960.7 ± 204.6**[b]
Cholesterol	84.6 ± 19.9	115.5 ± 21.8	69.7 ± 6.1	146.2 ± 24.2**
Cholesteryl ester	116.7 ± 11.8	125.8 ± 18.3	132.5 ± 18.2	168.0 ± 13.1
Phospholipids	199.7 ± 12.5	197.9 ± 16.1	278.8 ± 18.3	506.8 ± 42.8***
Fatty acids[c] (% of total)				
14:0	0.1 ± 0.1	0.4 ± 0.2	1.9 ± 0.2	3.0 ± 0.4*
16:0	17.2 ± 1.1	17.0 ± 1.0	20.5 ± 0.6	22.1 ± 0.5
16:1	1.8 ± 0.5	1.8 ± 0.2	0.8 ± 0.2	1.5 ± 0.1**
18:0	16.2 ± 0.5	16.4 ± 0.8	11.0 ± 0.3	6.6 ± 0.5***
18:1	13.8 ± 0.8	14.6 ± 1.4	11.9 ± 0.6	22.5 ± 1.5***
18:2	17.4 ± 0.7	19.7 ± 0.8	22.7 ± 0.5	30.8 ± 0.6***
18:3	-0-[d]	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
20:4	27.7 ± 1.7	25.5 ± 2.5	28.4 ± 1.2	9.7 ± 1.6***

[a]Mean ± SEM, n = 7-8/group.

[b]*p < 0.05, **p < 0.02, ***p < 0.001, as determined by Student's t-test comparing the two dietary treatments.

[c]Minor percentages resulting from fatty acids less than 14 carbons or odd chains are not included.

[d]Not detectable.

TABLE 4

Liver Lipid Concentrations (mg/g) and % Fatty Acids

	Dams		Pups	
	Control	Deficient	Control	Deficient
Triglycerides	12.3 ± 2.2[a]	13.2 ± 1.9	18.2 ± 1.8	132.2 ± 16.8***[b]
Cholesterol	1.7 ± 0.5	4.4 ± 1.7	2.6 ± 0.3	5.7 ± 0.9**
Cholesteryl ester	0.8 ± 0.2	2.1 ± 0.3**	2.0 ± 0.2	4.1 ± 0.3***
Phospholipids	12.3 ± 0.7	12.5 ± 1.0	9.2 ± 1.3	10.5 ± 1.1
Fatty acids[c] (% of total)				
14:0	0.8 ± 0.1	0.7 ± 0.1	1.6 ± 0.2	2.9 ± 0.5*
16:0	26.1 ± 1.4	23.2 ± 1.2	21.9 ± 0.7	23.0 ± 0.8
16:1	4.2 ± 0.6	2.8 ± 0.3*	0.6 ± 0.1	0.9 ± 0.2
18:0	15.5 ± 1.3	18.4 ± 1.3	12.5 ± 1.4	6.7 ± 1.3**
18:1	25.6 ± 1.1	22.2 ± 1.6	16.8 ± 1.1	18.9 ± 0.8
18:2	10.9 ± 1.0	14.8 ± 1.0**	24.2 ± 1.2	33.0 ± 0.5***
18:3	-0-[d]	-0-	0.02 ± 0.02	0.3 ± 0.1*
20:4	14.2 ± 0.9	15.6 ± 1.8	16.5 ± 1.8	8.5 ± 0.9**

[a]Mean ± SEM, n = 7-8/group.

[b]*p < 0.05, **p < 0.02, ***p < 0.001, as determined by Student's t-test comparing the two dietary treatments.

[c]Percentages resulting from fatty acids less than 14 carbons or odd chains are not included.

[d]Not detectable.

fatty acid alterations (data not shown).

DISCUSSION

The experiments reported here confirm our previous reports that restriction of maternal dietary iron during gestation and lactation produces a severe hyperlipidemia (1,2) and fatty liver (2,3) in the offspring during suckling. In this report, we have further characterized these lipid aberrations. In addition to increases in triglycerides, cholesterol and phospholipids,

iron deficiency produces alterations in the fatty acid patterns of liver and serum.

In general, fatty acid patterns of serum from iron-deficient pups correspond with the patterns found in their livers. Both liver and serum had lipids characterized by increased percentages of fatty acids, 14:0, 18:2 and 18:3, and decreased percentages of 18:0 and 20:4. Two interpretations can be postulated to explain the altered fatty acid patterns of iron deficiency. Since 18:0 is decreased whereas 18:2 and 18:3 are increased, there may be an increased desaturation of 18-carbon fatty acids in iron deficiency. Alternatively, a blockage in desaturation and elongation of 18:2 and 18:3 to 20:4 may contribute to increased percentages of 18:2 and 18:3 while decreasing 20:4. The decrease in 20:4 may also be due to an increased utilization of this fatty acid. We cannot determine if this is the case, since further desaturation and elongation products were not measured by the GLC procedure used.

Changes in fatty acid patterns of pup tissues appear to be primarily of endogenous origin. No significant relationships were observed between the exogenous supply of maternal milk fatty acids and those of pup serum or liver. It is possible that some alterations in lipid metabolism due to iron deficiency were transmitted from the mother to offspring during fetal development or early in the postnatal period. The available evidence, however, indicates that the primary defect in lipid metabolism is in the pups. Maternal serum, liver, and milk lipids and fatty acids did not show the severe aberrations due to iron deficiency which were observed in their pups. It is unknown if this is related to the greater severity of iron deficiency in the pups or due to age-related differences in lipid metabolism.

The exact mechanism responsible for the elevations in serum and liver lipids, as well as the altered fatty acid patterns of tissues from iron-deficient neonatal rats, has not been established. The role of iron in lipids or fatty acid synthesis and/or utilization is unknown. Data presented here suggest that iron may have a role in the desaturation or elongation process involved in the metabolism of fatty acids.

Since iron deficiency is a world-wide health problem, the mechanism by which it affects lipid metabolism warrants further research.

ACKNOWLEDGMENTS

This work was supported by research funds from the University of Illinois Agricultural Experiment Station (60-337) and by a grant from the National Live Stock and Meat Board to Adria Rothman Sherman.

REFERENCES

1. Guthrie, H.A., Froozani, M., Sherman, A.R., and Barron, G.P. (1974) J. Nutr. 104, 1273-1278.
2. Sherman, A.R., Guthrie, H.A. Wolinsky, I., and Zulak, I.M. (1978) J. Nutr. 108, 152-162.
3. Rothenbacher, H., and Sherman, A.R. (1980) J. Nutr. 110, 1648-1654.
4. Sherman, A.R. (1979) Lipids 14, 888-892.
5. Sherman, A.R. (1978) Lipids 13, 473-478.
6. Lewis, M., and Iammarino, R.M. (1971) J. Lab. Clin. Med. 78, 547-554.
7. Amine, E.K., and Hegsted, D.M. (1971) J. Nutr. 101, 1575-1582.
8. Amine, E.K., Desilets, E.J., and Hegsted, D.M. (1976) J. Nutr. 106, 405-411.
9. National Research Council (1978) National Research Council Nutritional Requirements of Laboratory Animals, Washington, DC.
10. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies (1977) J. Nutr. 107, 1340-1348.
11. Richterich, R. (1969) Clinical Chemistry, Academic Press, New York, NY.
12. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
13. Stern, I., and Shapiro, B. (1953) J. Clin. Pathol. 6, 158-160.
14. Searcy, R.L., and Bergquist, L.M. (1960) Clin. Chim. Acta 5, 192-199.
15. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
16. Steel, R.G., and Torrie, J.H. (1960) Principles and Procedures of Statistics, McGraw-Hill, New York, NY.

[Received March 15, 1982]

Effect of Detergents on in vitro 7α-Hydroxycholesterol Formation by Rat Liver Microsomes

AJIT SANGHVI*, ENRICO GRASSI, CANDACE BARTMAN and WARREN F. DIVEN,
Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

ABSTRACT

Formation of 7α-hydroxycholesterol by rat liver microsomes was quantitated using a gas chromatograph-mass spectrometer (GC/MS) operated in selected ion monitoring (SIM) mode. Microsomes from normal rat livers incubated for different periods were found to yield increased 7α-hydroxycholesterol with time. This was also true when incubations contained Tween-80, but in this instance, the rate of 7α-hydroxycholesterol production was lower and dependent on the concentration of Tween used. Similarly, Triton X-100, Renex-30, Kyro EOB, Cutscum, and Emulgen 911 all lowered the formation of 7α-hydroxycholesterol by rat liver microsomes, whereas Triton WR-1339 stimulated its production. Analysis of data obtained from following the enzyme reaction over an extended period using an integrated Michaelis-Menten equation indicated the enzyme possesses a very significant affinity for the product ($K_S > K_p$). Similar analysis shows that Tween-80 is a noncompetitive inhibitor of the enzyme. *Lipids* 17:644-649, 1982.

The enzyme cholesterol-7α-hydroxylase (E.C.1.14.) is a mixed-function oxidase which uses microsomal cholesterol as its substrate. Methods which utilize incorporation of radioactivity into the product 7α-hydroxycholesterol from labeled cholesterol as an estimate of enzyme activity routinely use a nonionic detergent as the vehicle for exogenous substrate. Detergents such as Triton X-100, Lubrol (1), Cutscum (2) and Emulgen 911 (3) have been used in the study of this enzyme; however, Tween-80 is the most commonly used detergent for this purpose. Recent development of a mass spectrometric (MS) procedure in our laboratory (4) to quantify cholesterol 7α-hydroxylase activity has allowed us to directly measure the effects of Tween-80 and other detergents on the activity of this enzyme. We report here that, with the exception of Triton WR-1339, all the detergents tested lowered the production of 7α-hydroxycholesterol by rat liver microsomes, whereas Triton WR-1339 increased its formation.

MATERIALS AND METHODS

Cutscum and Tween-20 were from Fisher Scientific. Kyro EOB was a gift from Procter and Gamble. Tween-80 was purchased from J.T. Baker, Fisher Scientific and Sigma. Triton WR-1339 was from Ruger Chemical Co. (Irvington, NJ 67111). Triton X-100 was from Sigma. Renex-30 and Emulgen-911 were gifts of ICI Americas and Kao Atlas Chemicals (Tokyo, Japan), respectively.

Male Sprague-Dawley rats weighing 220-280 g were used. The animals were maintained on a 12-hr light-dark cycle (8 p.m. to 8 a.m. light cycle). Each rat was offered 30 g Purina Rat Chow per day at 8 a.m., and the remainder was withdrawn at 6 p.m.; water was allowed ad libitum. Average daily food consumption was 20 g/rat. Animals were killed at 2 p.m. by decapitation. Livers were excised, quickly chilled in 0.25 M sucrose containing 1.0 mM EDTA (pH 7.1), minced and homogenized in four volumes of the same solution. After removal of cell debris and mitochondria at 500 × g and 10,000 × g, respectively, the microsomes were obtained by centrifugation of the supernatant at 100,000 × g. The microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4) containing 10 mM β-mercaptoethylamine, recentrifuged at 100,000 × g and the resulting pellet was resuspended in the same buffer so that 4 ml of suspension contained the microsomes from 1.0 g of liver.

Each of the detergents to be tested for its effect on cholesterol 7α-hydroxylase was mixed with 0.1 M phosphate buffer first by vortex, and then by a 2-min sonication at 20 kHz. Aliquots of detergent solutions representing different amounts ranging from 0.4-3.6 mg were mixed with sufficient microsomal suspension to contain 1-2 mg microsomal protein. Incubation of microsomes was performed for 30 or 60 min at 37 C in air in 0.1 M phosphate containing 1 mM EDTA, 0.8-1.2 mg microsomal protein, 10 mM β-mercaptoethylamine, two units glucose-6-phosphate dehydrogenase, 20 mM glucose-6-

*Author to whom correspondence should be addressed: Clinical Chemistry Laboratory, University Health Center of Pittsburgh, 203 DeSoto Street, Room 8206, Pittsburgh, PA 15261.

phosphate, 2 mM NADP, and concentrations of detergents as indicated in the tables in a total vol of 1.5 ml. The amount of 7α-hydroxycholesterol formed was determined by gas chromatograph/mass spectrometer (GC/MS) operated in selected ion monitoring (SIM) mode, as described previously (4).

RESULTS

Table 1 contains results of experiments showing the effect of various nonionic detergents on the microsomal production of 7α-hydroxycholesterol after a 30-min incubation. All detergents in this group of experiments inhibited the formation of 7α-hydroxycholesterol in proportion to their concentration in the experiment. Renex-30 and Kyro EOB at 0.8 mg/mg microsomal protein virtually abolished the enzyme activity.

During the GC/MS and SIM analyses of lipid extracts obtained from microsomal incubations containing Emulgen-911 and Triton X-100, the presence of a large number of extraneous peaks was noted. None of these peaks, however, gave rise to the ions m/z 443, 456, or 546 at retention times at which cholesterol, 7α- and 7β-hydroxycholesterol appear, and which are used in the calculations of the amount of 7α-hydroxycholesterol. To ascertain if these peaks were present in these two detergents, Emulgen-911 and Triton X-100 were extracted with chloroform/methanol (2:1, v/v). The GC/MS and SIM analyses of these extracts indicated that the extraneous peaks originate either from contaminations in the detergents or from the detergents themselves. Ethyl acetate and petroleum ether extracts of the detergents, when similarly analyzed, gave identical information. Emulgen-911 extract was further analyzed by thin layer chromatography (TLC) on Silica Gel G, 250 μ-plates, using benzene/ethylacetate (40:60, v/v) as the eluting solvent system (3). Again, a large number of spots throughout the channel were observed when the plates were exposed to iodine vapors. The R_f values (0.11, 0.15, 0.21, 0.25, 0.28) of some of the unknown compounds were similar to those for the authentic 7α- and 7β-hydroxycholesterol (0.21, 0.27), respectively, suggesting a potential for inter-

TABLE 1

Effect of Several Nonionic Detergents on Microsomal Production of 7α-Hydroxycholesterol[a]

Detergent	Concentration (mg/mg protein)	pmol 7α-Hydroxycholesterol/mg protein 30 min
Control	–	917 ± 72
		% of control
Cutscum	0.2	68
	0.5	26
	1.8	23
Emulgen-911	0.2	17
	0.6	14
	1.0	11
Kyro EOB	0.3	27
	0.5	6
	0.8	4
Renex-30	0.3	9
	0.5	2
	0.8	1
Triton X-100	0.2	46
	0.5	25
	0.7	21
Tween-20	0.2	95
	0.6	61
	0.9	35
	1.6	10

[a]Effect of the indicated detergents on 7α-hydroxycholesterol formation in 30-min incubation by rat liver microsomes was studied in three experiments with combined liver microsomes from four rats in each experiment. Two detergents were included in each study. Each determination was in duplicate. Results are average of two experiments for each detergent.

ference in the estimation of cholesterol 7α-hydroxylase activity by methods which use Emulgen-911 or Triton X-100 and analyze the products of reaction by TLC (3).

Tween-20, at the lowest concentration, did not significantly affect 7α-hydroxycholesterol production. At all other concentrations, it, too, was inhibitory.

The effects of Triton WR-1339 and of glycerol on the cholesterol 7α-hydroxylase activity were studied separately and as a mixture. The results in Table 2 show that both of these agents have a stimulating effect on the formation of 7α-hydroxycholesterol. Increasing the concentration of Triton WR-1339 from 0.3 mg to 1.0 mg/mg microsomal protein did not produce an incremental change in 7α-hydroxycholesterol formation. Glycerol over a wide range of concentrations, from 5.0 to 52 mg/mg protein, increased the 7α-hydroxycholesterol production over that in controls an average of 13% after 30 min of incubation. The combined effect of these two agents is additive.

The time course of 7α-hydroxycholesterol formation was studied in the presence of several concentrations of Tween-80 and in its absence. The mean critical micelle concentration (cmc) of Tween-80 is 0.036 mM (5). In our studies, 0.04 mg Tween-80/mg microsomal protein (0.03 mM, mol wt 1310), a concentration just below the cmc of Tween-80, was inhibitory to 7α-hydroxycholesterol formation, as were all other concentrations tested (0.04 mg-1.25 mg/mg protein). When 7α-hydroxycholesterol formation at 10 min of incubation was plotted as a function of different Tween-80 concentrations (Fig. 1), it gave an apparently biphasic curve with a sharp break at a Tween-80 concentration of 75 μg/mg protein. The steep portion of the curve may represent, following membrane disruption, a rapid trapping of cholesterol into a Tween-cholesterol micelle of that fraction of endogenous cholesterol which may either be a part of "substrate pool," or may at least be easily accessible to the enzyme. Once most of this presumed "substrate pool" cholesterol had been sequestered, there is little cholesterol available to the enzyme for hydroxylation as may be evidenced by the large decrease in 7α-hydroxycholesterol production beyond the Tween-80 concentration of 75 μg/mg protein.

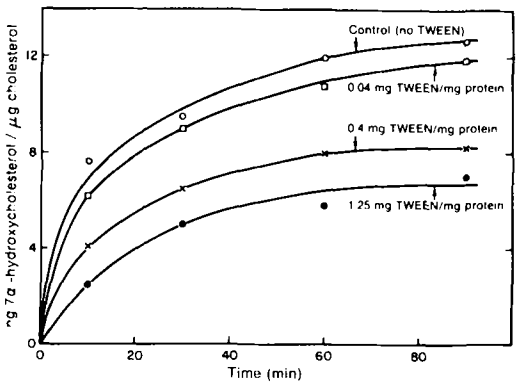


FIG. 1. Nanograms of 7α-hydroxycholesterol formed per mg protein in a 10-min incubation plotted as a function of the concentration of Tween-80. Tween-80 concentrations are expressed as μg/mg of microsomal protein in the assay. Each point is an average of duplicate measurements. Microsomes from four rat livers were used. The data have been reproducible in other experiments.

TABLE 2

Individual and Combined Effects of Triton WR-1339 and Glycerol on 7α-Hydroxycholesterol Formation[a]

	pmol 7α-Hydroxycholesterol/mg protein	
		% of control
Control	933 ± 140	100
Glycerol (53 mg/mg protein)	1058 ± 153 p<0.025	113.5 ± 4.8
Triton WR-1339 (1 mg/mg protein)	1219 ± 180 p<0.005	130.8 ± 1.6
Triton WR-1339 + glycerol	1282 ± 183 p<0.001	138.0 ± 2.2

[a]Liver microsomes from four rats were incubated separately with the indicated agent for 30 min. All assays were in duplicate. Significance values are relative to the control group.

The observed inhibition of enzyme activity could be due to the presence in Tween-80 of traces of free fatty acid, itself capable of being hydroxylated, which could function as an alternate substrate (6). To test this possibility, microsomal 7α-hydroxycholesterol formation was measured in the presence of Tween-80 obtained from different manufacturers, which contained different amounts of free oleic acid (7). As shown in Table 3, the inhibition by Tween-80 of 7α-hydroxycholesterol formation appears related to its content of free oleic acid.

TABLE 3

Effect of Different Concentrations of Free Oleic Acid in Tween-80 on 7α-Hydroxycholesterol Production[a]

% FFA[b]	pmol 7α-Hydroxycholesterol/mg protein	% of control
(Control)	1251	100
0.24% (Sigma)	813	64
0.58% (Fisher)	693	55
0.71% (Baker)	513	40

[a]The experiment was conducted using combined liver microsomes from four rats, incubated for 30 min. Results are average of two experiments with each analysis in duplicate. Sources of Tween-80 are in parentheses. Concentration of Tween-80 in each experiment was 0.9 mg/mg protein.

[b]FFA = free fatty acid (oleic).

The nature of Tween-80 inhibition was further probed by an additional kinetic experiment in which the formation of 7α-hydroxycholesterol was studied, both in the presence and absence of the detergent. This and other experiments described here use endogenous cholesterol as substrate with saturating concentrations of all required cofactors. The data obtained from this experiment were subjected to analysis by an integrated form of Michaelis-Menten equation (8) with the assumptions that (a) the reaction can be approximated as a one substrate-one product reaction; (b) the reaction has a large equilibrium constant; and (c) no interaction between enzyme and product takes place. Thus,

$$\frac{1}{t} \ln \frac{[S]_0}{[S]_0 - [P]} = -\frac{1}{K_m}\frac{[P]}{t} + \frac{V_m}{K_m}. \qquad [1]$$

In order to apply the integrated rate equation to the data from the above experiment, an estimate of $[S]_0$ was required. It was clear from these experiments that only a small fraction of the total cholesterol present in the microsomes was hydroxylated. When $[S]_0$ was estimated to be 1 μg or greater, a linear plot with a positive slope was obtained. Since Equation 1 predicts a linear relationship with a negative slope, the observed behavior can only be interpreted by a more complex equation based on the assumption of a large equilibrium constant as before, but allowing for product inhibition (8). Therefore,

$$\frac{1}{t} \ln \frac{[S]_0}{[S]_0 - [P]} = -\frac{1}{K_{ms}}\left(\frac{K_{mp} - K_{ms}}{K_{mp} + [S]_0}\right)\frac{[P]}{t} + \frac{V_m}{K_{ms}\left(1 + \frac{[S]_0}{K_{mp}}\right)}. \qquad [2]$$

The slope of this equation can be positive or negative, depending on the relative values of Michaelis constants for substrate and product. Thus, the data would appear to be consistent with the hypothesis that 7α-hydroxycholesterol has a high affinity for the enzyme and thus effectively limits the reaction.

Equation 2 can be rearranged into a form (Equation 3) where the vertical axis intercept is independent of $[S]_0$. Using the equation

$$\frac{t}{P} = \frac{K_{ms}}{V_m}\left(1 + \frac{S_0}{K_{mp}}\right)\left(\frac{\ln \frac{S_0}{S_0 - P}}{P}\right) + \frac{1}{V_m}\left(1 - \frac{K_{ms}}{K_{mp}}\right), \qquad [3]$$

a family of lines is obtained with both slopes and intercepts that vary with Tween-80 concentration, as shown in Figure 2. A negative intercept for the line resulting from the experiment in the absence of Tween-80, as seen in Figure 2, is what would be expected if $K_{ms} > K_{mp}$—again showing the marked affinity of the enzyme for the product. A kinetic pattern with both slope and intercept functions of inhibitor concentration, as seen in Figure 2, would be predicted if the detergent were acting as a noncompetitive inhibitor, thus reducing the effective enzyme concentration.

DISCUSSION

Nonionic detergents are routinely used to clarify microsomal suspensions. They serve as vehicles for lipophilic substrates and are generally used as "solubilizers" for steroids and other nonpolar substances in studies involving microsomal enzymes (9-11). For similar reasons, it has been necessary to use nonionic detergents to study the various properties of

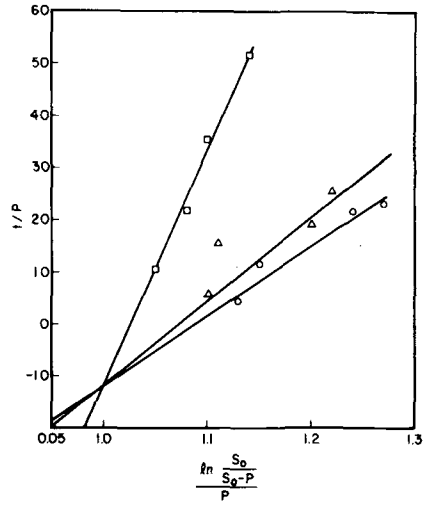


FIG. 2. Plot of time course of 7α-hydroxycholesterol formation according to equation 3 (text). For these experiments, $[S]_0$ was estimated to be 1 μg of cholesterol. Experiments were conducted in the absence of any detergent (○—○) with a Tween-80 concentration of .041 mg/mg protein (△—△) and 0.41 mg/mg protein (□—□). Each point represents duplicate determinations using the microsomes prepared from four animals.

cholesterol 7α-hydroxylase, a mixed-function oxidase enzyme, when the assay system uses conversion of labeled cholesterol to labeled 7α-hydroxycholesterol as an index of enzyme activity. We have studied the effect of several nonionic detergents themselves on the activity of this enzyme by a GC/MS procedure (4) which measures directly the mass of 7α-hydroxycholesterol formed as an index of enzyme activity. The results of our investigations reported here show that, with the sole exception of Triton WR-1339, all of the detergents exerted an inhibitory effect on the microsomal production of 7α-hydroxycholesterol. Inhibition of 7α-hydroxycholesterol formation by several detergents, including Tween-80 and Triton X-100, has been reported by Boyd and Hattersley using an isotopic technique (1); however, those studies were carried out using the acetone powder prepared from microsomes. We found Kyro EOB, Renex-30, and Emulgen-911 to be the most inhibitory of the agents tested.

Information regarding the inhibition due to Emulgen-911 may be significant in view of a recent report concerning solubilization of cholesterol 7α-hydroxylase using this detergent (3). At concentrations of Emulgen-911 similar to those used by Nimmannit and Porter (3), our studies indicate the enzyme possesses only 17-11% of activity relative to that in controls. Since the exact mechanism of inactivation of cholesterol 7α-hydroxylase activity by different detergents is unknown, the use of severely inhibitory detergents in the study of this enzyme would seem to complicate the interpretation of experimental data. A further complication peculiar to Emulgen-911 and Triton X-100 is the finding that they appear to contain or to be contaminated with compounds which are extracted by lipid solvents, and two of these have R_f values similar to those for 7α- and 7β-hydroxycholesterol upon TLC. The GC/MS procedure avoids difficulties of this nature.

Tween-80 is one of the most widely used detergents as a vehicle for lipophilic substrates, specifically in studies concerning cholesterol 7α-hydroxylase. The effects of Tween-80 on microsomal drug hydroxylations are diverse. Thus, it is found to competitively inhibit 2- and 4-hydroxylations of biphenyl, a fungistat and a lipophilic substrate, by hamster liver mixed-function oxidases (12). In the same system, Tween-80 stimulates 4-hydroxylation of aniline; on the other hand, in the rat liver microsomes, it inhibits aniline hydroxylation. In studies reported here, Tween-80 acts to reduce the formation of 7α-hydroxycholesterol by rat liver microsomes at all concentrations tested.

A possible explanation for the inhibitory action of Tween-80, and perhaps for other detergents, would be competition with substrate for the enzyme. If this were the case, kinetic studies such as those presented in Figure 2 would give a family of curves where only the slopes are functions of detergent concentration. The data presented are more consistent with the possibility that Tween-80 is interacting with the enzyme and reducing the effective enzyme concentration, while having no effect on the binding of cholesterol with the enzyme.

Tween-80 may also sequester cholesterol into Tween-cholesterol micelle, which may account for reduced 7α-hydroxycholesterol formation in the presence of Tween, as shown in Figure 1.

The inhibition by Tween-80 of 7α-hydroxycholesterol formation may be at least partly due to the presence of free oleic acid, since unsaturated fatty acids are known to be hydroxylated by microsomal enzyme systems, and they also competitively inhibit both the microsomal hydroxylations and interaction of other Type I substrates (6). The results in Table 3, showing progressive inhibition of 7α-hydroxycholesterol formation with increasing concen-

tration of free oleic acid in different Tween-80 preparations, are consistent with this reasoning.

Stimulation of cholesterol 7α-hydroxylase activity by Triton WR-1339 is in keeping with its known stimulatory effect on hydroxymethylglutaryl-CoA reductase activity, although the mechanisms involved must be different. Administration of Triton WR-1339 to animals is shown to result in a depletion of hepatic cholesterol content, which, in turn, enhances hydroxymethylglutaryl-CoA reductase activity and cholesterogenesis (13). The increase in vitro of cholesterol 7α-hydroxylase activity, on the other hand, is likely due to perturbation of microsomal membrane by Triton WR-1339, leading to the unmasking of latent enzyme activity.

REFERENCES

1. Boyd, G.S., and Hattersley, N.G. (1975) FEBS Lett. 53, 80-83.
2. Shefer, S., Hauser, S., and Mosbach, E.H. (1968) J. Lipid Res. 9, 328-333.
3. Nimmannit, S., and Porter, J.W. (1980) Arch. Biochem. Biophys. 201, 533-543.
4. Sanghvi, A., Grassi, E., Bartman, C., Lester, R., Galli-Kienle, M., and Galli, G. (1981) J. Lipid Res. 22, 720-724.
5. Malik, W.U., and Jhamb, O.P. (1972) J. Am. Oil Chem. Soc. 49, 170-172.
6. Di Augustine, R.P., and Fouts, J.R. (1969) Biochem. J. 115, 547-554.
7. Official and Tentative Methods of the Am. Oil Chem. Soc. (revised 1977) Official Method Ca5a-40, AOCS, Champaign, IL.
8. Segel, I.H. (1975) in Enzyme Kinetics, pp. 55-61, Wiley-Interscience Publication, John Wiley & Sons, New York, NY.
9. Mendelsohn, D., Mendelsohn, L., and Stapel, E. (1966) Biochemistry 5, 1286-1290.
10. Narasimhulu, S., Cooper, D.Y., and Rosenthal, O. (1965) Life Sci. 4, 2101-2107.
11. Swindel, A.C., and Gaylor, J.L. (1968) J. Biol. Chem. 243, 5546-5555.
12. Burke, D.M., Bridges, J.W., and Parke, D.V. (1975) Xenobiotica 5, 261-277.
13. Goldfarb, S. (1978) J. Lipid Res. 19, 489-494.

[Revision received April 6, 1982]

Estolide Triglycerides of *Trewia nudiflora* Seed Oil

RICHARD V. MADRIGAL* and **CECIL R. SMITH, Jr.**, *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604*

ABSTRACT

The seed oil of *Trewia nudiflora* is known to contain glycerides of α-kamlolenic (18-hydroxy-*cis*-9,*trans*-11,*trans*-13-octadecatrienoic) acid. We have shown that a large part of these glycerides contain estolides in which the hydroxyl group of α-kamlolenic acid is esterified to a molecule of another acid, either a hydroxy acid or an ordinary fatty acid. By preparative thin layer chromatography, we isolated a series of tri-, tetra-, penta- and hexaacyl glycerols. By lipolysis and gas chromatography-mass spectrometry, we isolated and characterized estolide-linked fatty acids containing two acid moieties. *Lipids* 17:650-655, 1982.

Trewia nudiflora L. (Euphorbiaceae, false white teak) is a large deciduous tree. It is found in India in the region east and south of the Jumna river and also in Burma and is characteristic of the swamp forests of the sub-Himalayan tract. It occurs in moist deciduous as well as in evergreen forests. The wood of *Trewia* is white and soft and is used in India for carving and making toys, packing cases, planking and plywood (1).

There has been interest in the biological activity of extracts of *T. nudiflora* seed. The seed has been shown to contain several novel pyridone alkaloids (2-4) and also an inhibitor of protein synthesis (5). From the ethanol extracts of these seeds, Powell et al. (6) isolated and characterized three new maytansinoid tumor inhibitors: trewiasine, dehydrotrewiasine and demethyltrewiasine. We have also shown in another publication (7) that crude *T. nudiflora* extracts and trewiasine are biologically active against various insects, suggesting possible use as a pest control agent.

Because of this interest in the biological activity of the *Trewia* seed extracts and the high oil content of these seeds, we investigated the composition of its seed oil. The oil was reported by Sarkar and Chakrabarty (8) to be similar to tung oil. They erroneously reported the major fatty acid as being α-eleostearic acid. Chisholm and Hopkins (9) isolated this conjugated acid and showed it to be α-kamlolenic (18-hydroxy-9,11,13-octadecatrienoic) acid rather than α-eleostearic acid. They observed that the infrared (IR) spectrum of the oil showed only a very weak hydroxyl band near 3620 cm^{-1}, presumably because nearly all of the hydroxy acid is combined in the form of estolides; they made no further investigation of the glyceride structure. Until its presence was established in *T. nudiflora*, α-kamlolenic acid had been found only in the seed of *Mallotus* species. Aggarwal et al. (10) reported that kamala (*Mallotus philippinensis* Muell. Arg., Euphorbiaceae) seed contained an oil with drying properties much like those of tung oil. The principal acid was identified as 18-hydroxy-9,11,13-octadecatrienoic acid by Gupta et al. (11,12) who proposed the name α-kamlolenic acid. Hopkins et al. (13) later established the complete structure and stereochemistry of α-kamlolenic acid as 18-hydroxyoctadeca-*cis*-9,*trans*-11,*trans*-13-trienoic acid.

Kapadia and Aggarwal (14) separated kamala seed oil into six fractions by low-temperature fractional crystallization. For each fraction, they determined mean molecular weight (cryoscopic), glycerol content, saponification value, iodine value, hydroxyl value and fatty acid composition by ultraviolet (UV) absorbance. From the data obtained, Achaya and Aggarwal (15) suggested that the six fractions contained four unusual triglycerides in which the hydroxyl group of α-kamlolenic acid was either free, esterified with the carboxyl group of another molecule of α-kamlolenic acid, or esterified with a normal long-chain fatty acid. The individual triglycerides suggested were: (a) a triacyl glycerol with one terminal hydroxyl group, (b) a tetraacyl glycerol with one terminal hydroxyl group, (c) a heptaacyl glycerol with one terminal hydroxyl group and (d) an octaacylglycerol with one terminal hydroxyl group. Rajiah et al. (16) confirmed the suggested structures using the procedures of Kapadia and Aggarwal along with ester group determination by a ferric hydroxamate colorimetric procedure, ester group determination by IR, glycerol determination by gas liquid chromatography (GLC), lipase hydrolysis and nuclear magnetic resonance.

In the present work, we confirmed the exist-

*Author to whom correspondence should be addressed.

ence of estolide triglycerides in *T. nudiflora* seed oil and showed that they are found as tri-, tetra-, penta- and hexaacyl glycerols with no more than two fatty acid moieties on one acyl chain attached to one position of glycerol.

MATERIALS AND METHODS

Seed

Trewia nudiflora. The seed was purchased from Pratap Nursery, Dehra Dun, India, and was supplied to us by Dr. James Duke, USDA, Beltsville, MD.

M. philippinensis. The seed was collected in Pakistan and was supplied to us by Dr. Quentin Jones, USDA, Beltsville, MD.

Chromatographic Methods

Thin layer chromatography (TLC). Analytical TLC was carried out on precoated Silica Gel 60 F-254 plates (0.25-mm thick) with the solvent system of hexane/ethyl ether (70:30, v/v) for the hexane extracts, methyl esters, and lipolysis products, and for preliminary examination of the hydrogenated oil. For preparative TLC, Silica Gel 60 F-254 plates (2 mm thick) were used with hexane/ethyl ether (60:40, v/v) except with the hydrogenated oils. For separation of the hydrogenated oils into their various components, multiple development (4x-5x) TLC was carried out with the solvent system ethyl ether/benzene (1:99, v/v) followed by ethyl ether/benzene (3:97, v/v). In analytical TLC, components were visualized by charring with sulfuric acid/dichromate solutions or by exposure to iodine vapors. In preparative TLC, bands were located by spraying with ethanolic dichlorofluorescein followed by viewing under UV light.

GLC. Fatty acid methyl esters derived from the hydrogenated glycerides were analyzed on an instrument equipped with a 1.2 m × 6 mm glass column packed with 5% Apiezon L on Chromosorb W which was operated isothermally at 185 C. Methyl esters of the hydrogenated lipolysates were analyzed with a 0.6 m × 3 mm glass column packed with 3% Dexsil 300 on Chromosorb W-AW, 100-120 mesh (Alltech Associates, Deerfield, IL). Trimethylsilyl (TMS) ethers were prepared by reacting selected samples with Hydrox-Sil (Regis Chemical Co., Morton Grove, IL) before analysis. Temperatures were programmed 100-400 C at 5 C/min. Methyl esters were identified by their equivalent chain length (ECL).

Spectrometric Methods

IR. IR spectra were determined on 1% (w/v) solutions in $CHCl_3$.

Gas chromatography-mass spectrometry (GC-MS). The mass spectra were obtained from a Kratos MS-30 mass spectrometer (Kratos Scientific Instruments, Westwood, NJ) at 70 eV with a source pressure of ca. 10^{-6} torr. Samples (0.1-5 μg) were introduced to the mass spectrometer by GC via a single-stage jet separator. A 1 m × 2 mm glass GC column packed with 5% OV-1 was programmed from 260 to 285 C at 2 C/min. The carrier gas was helium at 30 cc/min and the MS source was held at 200 C.

Seed Extraction

Seeds were cracked gently to remove the hulls. The endosperm was crushed in a mortar under the surface of hexane (Fisher Spectroanalyzed) and filtered at once. After filtration, the amount of oil recovered was estimated by evaporating a 1-ml aliquot and weighing the resulting residue. The hexane solution was either hydrolyzed or hydrogenated immediately, or stored under refrigeration until it was used.

Hydrolysis with Pancreatic Lipase

Hexane extracts were mixed with an equal volume of water, then the hexane was removed by vacuum evaporation at room temperature. The aqueous mixture was hydrolyzed with porcine pancreatic lipase (EC 3.1.1.3) (Calbiochem, San Diego, CA) by the procedure of Luddy et al. (17) as modified by Phillips and Smith (18), except that reaction times of 30 min were used. Stirring was done with a Brinkmann Polytron Homogenizer (Brinkmann Instruments, Westbury, NY). The reaction products were recovered by successive extraction of the aqueous buffered solution with hexane and chloroform.

Hydrogenation

Hydrogenation of triglycerides and derived lipolysates was done in hexane at ambient temperature and atmospheric pressure with palladium-charcoal (10%) catalyst.

Preparation of Methyl Esters

Methyl esters of the hydrogenated glycerides were prepared by dissolving 100 mg in 40 ml benzene and refluxing 6 hr with 40 ml H_2SO_4/MeOH (3:97, v/v). After diluting the reaction mixture with an equal volume of water, the esters were recovered by successive extraction with hexane and chloroform. Free acids from lipolysis products were converted to methyl esters by treatment with diazomethane.

Molecular Weights

Molecular weights were determined in $CHCl_3$ solutions by vapor-phase osmometry (VPO) with a Mechrolab Model 301-A osmometer. Tristearin (Nu-Chek-Prep, Elysian, MN) served as the calibration standard.

Melting Points

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected.

RESULTS AND DISCUSSION

Upon evaporation of the solvent from the petroleum ether extracts of *T. nudiflora* seed, a viscous oil is obtained whose rapid gelling and polymerizing properties suggest it to be a drying oil. In a typical extraction, 6.2 g of *T. nudiflora* seed containing 2.5 g endosperm furnished an estimated 0.573 g of oil. Analytical TLC gave a streak with about 15 evenly distributed spots having an R_f range of 0-0.8. The IR spectrum of the oil showed a weak hydroxyl absorbance at 3700 cm^{-1} and a sharp absorbance at 995 cm^{-1} with a shoulder at 968 cm^{-1} characteristic of *cis,trans,trans* conjugated trienes (19).

Because of the marked instability of the oil, it was advantageous to work with the hydrogenated oil and derived methyl esters wherever possible since they were stable and were amenable to TLC, GLC and other manipulations. The hydrogenated hexane extract was a white solid, mp 74-76 C. The IR spectrum was not unusual; hydroxyl absorbance was weak and no absorbance was noted near 1000 cm^{-1}. TLC analysis (one development, hexane/ether [70:30, v/v]) gave a chromatogram with spots at R_f 0.2, 0.3, 0.75, 0.8 (intense) and 0.95.

GLC of the mixed esters derived by acid-catalyzed methanolysis of the hydrogenated oil showed the following composition (relative percent in parentheses): 16:0 (6.4), 18:0 (57.3), 18:0 hydroxy (35.9), and 20:0 (0.4). Analytical TLC of the methyl esters showed two spots of R_f 0.2 and 0.75. After separation by preparative TLC, GLC of the nonpolar fraction showed 16:0, 18:0 and 20:0, whereas GLC of the polar fraction showed only methyl 18-hydroxystearate (ECL 20.4, Apiezon L column), the hydrogenation product of methyl kamlolenate.

Preparative TLC of estolide oils by multiple development with relatively nonpolar solvent systems was used by Morris and Hall (20) to separate the estolide-containing glycerides of ergot oil from *Claviceps purpurea*. Payne-Wahl et al. (21) used multiple-development with benzene in the separation of synthetic multiacyl triglycerides. By applying this technique, we were able to obtain a series of at least 12 discrete fractions (Table 1). Preparative multiple-development TLC of the hydrogenated oil with ethyl ether/benzene (1:99, v/v) gave five well defined fractions having R_f 0.5-0.95 (frac. 1-5 in Table 1), and an unresolved band at R_f 0-0.45. Rechromatography of this low R_f polar band by further multiple-development TLC in a more polar solvent system (ethyl ether/benzene [3:97, v/v]) gave eight more well defined components (frac. 6-13) and an unresolved streak (frac. 14).

Mean molecular weights of the fractions are shown in Table 1. Fraction 1, the least polar, appeared to be a hydrocarbon by IR. This material was not examined further. The chromatographic properties of fraction 2 corresponded exactly to those of tristearin, the expected triacyl glycerol having no estolide groups, and this fraction had the same molecular weight as tristearin. The progressive increase in molecular weights of fractions 2-5 indicates that, other factors being equal, the R_f of polyacyl glycerols of this series decreases with increasing numbers of acyl groups per molecule. Fraction 5 is a mixture of pentaacyl glycerol (also found in fraction 4) and hexaacyl glycerol. Fractions 8-10 were similar in molecular weight but different in polarity, indicating that these are hexaacyl glycerols with an increasing number of terminal hydroxyls in the acyl chains. Fractions 11 and 13 may be triacyl glycerols terminated with 2 and 3 hydroxyls, respectively.

The presence of estolide linkages in the original oil was confirmed by isolation after cleavage of the glyceride acyl groups with pancreatic lipase. The lipolysis could not be carried out on the hydrogenated oil because of its high melting point and the resulting difficulty of obtaining the necessary liquid emulsions (22). Lipolysis of the unhydrogenated triglycerides was more successful. Reaction conditions that were more rigorous than usual (temperature, time, stirring speed) were used because of the high molecular weights of the glycerides. Immediate hydrogenation of the lipolysates gave a product which gave the usual TLC spots corresponding to monoglycerides, fatty acids and a small amount of triglycerides. The fractions due to free fatty acids and to monoglycerides were separated by preparative TLC. GLC of the esters obtained by reaction of the acids with diazomethane showed the following composition (relative percent in parentheses): 16:0 (16.9), 18:0 (35.7), 20:0 (12.1), 22:0 (9.9), two peaks having ECL values (Dexsil 300) of 35 (1.7) and 37 (12.2). These last two ECL values are similar to those

TABLE 1

Preparative TLC Fractions from Hydrogenated *Trewia nudiflora* Seed Oil

Fraction[a]	Percent by wt	R_f	Molecular wt by VPO[b]	Calculated no. of acyl groups[c]	Free OH groups/molecule
1	4.6	0.95	–[d]	–	–
2	41.4	0.8	886	3.0	0
3	16.7	0.7	1,163	4.0	0
4	6.1	0.6	1,383	4.7	0
5	6.7	0.5,0.6[e]	1,562	5.4	0
6	2.5	0.95	376	1.2	?
7	1.8	0.85	1,007	3.4	1
8	3.8	0.8	1,870	6.5	1
9	3.3	0.75	1,900	6.6	2
10	1.8	0.7	2,000	6.9	3
11	0.7	0.6	925	3.1	2
12	0.5	0.5	–[f]	–	–
13	1.2	0.2	778	2.6	3
14	8.8	0.15[g]	1,196	4.1	3

[a]Fractions 1-5 were separated by multiple development with benzene/ethyl ether (99:1); fractions 6-14 were separated by further multiple development with benzene/ethyl ether (97:3).

[b]VPO = vapor-phase osmometry. The solvent was chloroform. Tristearin (MW 890) was used as the calibration standard.

[c]Calculated on C-18 basis.

[d]Molecular weight was not determined on this fraction, apparently hydrocarbon.

[e]This fraction contains two overlapping components.

[f]There was insufficient sample for analysis.

[g]This fraction may contain more than one component.

of diglycerides but, because of their polarity, diglycerides would not be expected in this fraction. GC-MS of the methyl esters showed the components with ECL 35 and 37 to be methyl 18-palmitoyloxystearate and methyl 18-stearoyloxystearate. GC-MS of these estolide esters (Table 2) showed molecular ions at m/z 552 and 580 accompanied by ions for ketenes arising from the loss of 32 (CH_3OH). Ions were observed for the loss of the methyl-terminated acyl groups with a McLafferty rearrangement ion at m/z 296 followed by loss of 32 or 74. Also prominent were the methyl-terminated acyl ions (fragment *a*, $R\text{-}C{=}O^+$) at m/z 239 and 267, respectively, which arise by cleavage at the estolide linkage. The ion at m/z 315 is the other ion resulting from the cleavage. The ion at m/z 356 is due to a McLafferty rearrangement and is followed by a loss of 32. Ions at 285 and 257 (fragment *b*) are formed from the molecular ion M^+ by cleavage of the $O\text{-}R'$ bond followed by a transfer of two hydrogen atoms from the R' moiety (23).

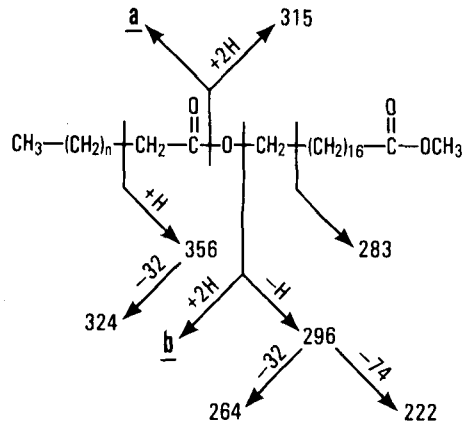
The TLC band which would normally be expected to contain monoglycerides from the lipolysis was silylated and examined by GLC and GC-MS. We identified a monostearin (24), but did not detect monoglycerides of estolide fatty acids or of hydroxyl-terminated acids.

Thus, by pancreatic lipolysis and subsequent GC-MS, we isolated and identified estolide components composed of one molecule of normal acid and one molecule of ω-hydroxy acid. The presence of estolide fatty acids in the lipolysis products demonstrates that some of the estolides are esterified in the 1- and/or 3-positions of *sn*-glycerol. However, during GLC of the methyl esters from lipolysis, we were unable to find any evidence of a product composed of three or more acyl units.

To obtain a direct TLC comparison of the two oils, we extracted *M. philippinensis* in the same manner as *T. nudiflora*. Immediate hydrogenation yielded a white solid, mp 56-58 C, not a sticky semisolid as reported by Rajiah et al. (16). It is possible that their hydrogenation product had been derived from *Mallotus* oil that had already begun to polymerize. Multiple-development TLC applied in parallel to both hydrogenated oils (Fig. 1) revealed that *Trewia* glycerides differ considerably in their make-up from those of *Mallotus*. Of the various components in hydrogenated *Trewia* oil, the only ones detected in hydrogenated *Mallotus* corresponded to frac. 2 (tristearin) and frac. 7, a tri- or tetraacyl glycerol with one terminal hy-

TABLE 2

Mass Spectra of Estolide Methyl Esters



	M^+		M^+-32		a		b								
m/z[a]	552	580	520	548	239	267	257	285	222	264	283	296	324	315	356
Methyl 18-palmitoyloxystearate, n=13 Relative intensity (%)	4		5		24		29		9	36	8	21	2	6	7
Methyl 18-stearoyloxystearate, n=15 Relative intensity (%)		8		7		24		29	11	37	8	26	2	7	9

[a]Principal ions.

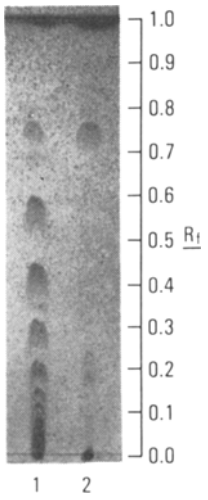


FIG. 1. Multiple-development TLC of hydrogenated triglycerides of (1) *Trewia nudiflora* and (2) *Mallotus philippinensis* on silica gel (0.25 mm). Developed 4× with ethyl ether/benzene (1:99, v/v).

droxyl group. In addition, a considerable proportion of the hydrogenated *Mallotus* oil did not move appreciably above the origin under these TLC conditions. It is possible that these are polar, high-molecular-weight (hexaacyl and higher) triglycerides. These observations are consistent with the data reported by the previous workers (15,16), although they do not necessarily substantiate their conclusions.

Estolide glycerides have been of considerable interest in recent years. In 1965, Sprecher et al. (25) showed by MS that the oil of *Sapium sebiferum* (Euphorbiaceae) contained a tetraacyl glycerol in which one of the primary hydroxyls was esterified to the allenic 8-hydroxy-5,6-octadienoic acid, and that its ω-hydroxyl group in turn is esterified to 2,4-decadienoic acid. Morris and Hall (20) showed that, in ergot oil, ricinoleic (12-hydroxy-*cis*-9-octadecenoic) acid is esterified with a normal long-chain acid. Ricinoleic acid estolides were found by Kleiman et al. (26) in *Lesquerella auriculata* (Cruciferae) along with those of densipolic (12-hydroxy-*cis*-9,*cis*-15-octadecadienoic), auricolic (14-hydroxy-*cis*-11,*cis*-17-eicosadienoic), and lesquerolic (14-hydroxy-*cis*-11-eicosenoic) acids. Lesquerolic acid estolides were also found by Plattner et al. (27) in *Heliophila amplexicaulis* (Cruciferae). Phillips and Smith (18) found estolides of *S*-coriolic (13-hydroxy-*cis*-9,*trans*-11-octadecadienoic) acid in *Monnina emarginata* (Polygalaceae) and Mikolajczak and Smith (28) reported pentaacyl triglycerides of 9,10,18-trihydroxy-*cis*-12-octadecenoic acid in *Chamaepeuce afra* (Compositae) seed oil.

ACKNOWLEDGMENTS

GC-MS analyses were performed by R.D. Plattner and R.E. England.

REFERENCES

1. Bor, N.L. (1953) in Manual of Indian Forest Botany, 1st edn., pp. 179-180, Oxford University Press, Indian Branch, Bombay.
2. Mukherjee, R., and Chatterjee, A. (1966) Tetrahedron 22, 1461-1466.
3. Ganguly, S.N. (1970) Phytochemistry 9, 1667-1668.
4. Sastry, S.D., and Waller, G.R. (1972) Phytochemistry 11, 2241-2245.
5. Gasperi-Campani, A., Barbieri, L., Lorenzoni, E., and Stirpe, F. (1977) FEBS Lett. 76, 173-176.
6. Powell, R.G., Weisleder, D., and Smith, C.R., Jr. (1981) J. Org. Chem. 46, 4398-4403.
7. Freedman, B., Reed, D.K., Powell, R.G., Madrigal, R.V., and Smith, C.R., Jr. (1982) J. Chem. Ecol. 8, 409-418.
8. Sarkar, S., and Chakrabarty, M.M. (1956) Sci. Cult. 21, 473-474.
9. Chisholm, M.J., and Hopkins, C.Y. (1966) J. Am. Oil Chem. Soc. 43, 390-392.
10. Aggarwal, J.S., Bhatnagar, S.S., Narain, P., and Karimullah (1948) J. Sci. Ind. Res. 7B, 136-138.
11. Gupta, S.C., Sharma, V.N., and Aggarwal, J.S. (1952) J. Sci. Ind. Res. 11B, 463-468.
12. Gupta, S.C., Gupta, S.S., and Aggarwal, J.S. (1953) J. Sci. Ind. Res. 12B, 240-242.
13. Hopkins, C.Y., Chisholm, M.J., and Ogrodnik, J.A. (1969) Lipids 4, 89-92.
14. Kapadia, V.H., and Aggarwal, J.S. (1958) J. Sci. Ind. Res. 17B, 117-119.
15. Achaya, K.T., and Aggarwal, J.S. (1962) Chem. Ind. 1616-1617.
16. Rajiah, A., Subbaram, M.R., and Achaya, K.T. (1976) Lipids 11, 87-92.
17. Luddy, F.E., Barford, R.A., Herb, S.F., Magidman, P., and Riemenschneider, R.W. (1964) J. Am. Oil Chem. Soc. 41, 693-696.
18. Phillips, B.E., and Smith, C.R., Jr. (1970) Biochim. Biophys. Acta 218, 71-82.
19. Hopkins, C.Y. (1972) in Topics in Lipid Chemistry (Gunstone, F.D., ed.) Vol. 3, p. 72, Elek Science, London.
20. Morris, L.J., and Hall, S.W. (1966) Lipids 1, 188-196.
21. Payne-Wahl, K., Plattner, R.D., Spencer, G.F., and Kleiman, R. (1979) Lipids 14, 601-605.
22. Desnuelle, P., and Savary, P. (1963) J. Lipid Res. 4, 369-384.
23. Aasen, A.J., Hofstetter, H.H., Iyengar, B.T.R., and Holman, R.T. (1971) Lipids 6, 502-507.
24. Johnson, C.B., and Holman, R.T. (1966) Lipids 1, 371-380.
25. Sprecher, H.W., Maier, R., Barber, M., and Holman, R.T. (1965) Biochemistry 4, 1856-1863.
26. Kleiman, R., Spencer, G.F., Earle, F.R., Nieschlag, H.J., and Barclay, A.S. (1972) Lipids 7, 660-665.
27. Plattner, R.D., Payne-Wahl, K., Tjarks, L.W., and Kleiman, R. (1979) Lipids 14, 576-579.
28. Mikolajczak, K.L., and Smith, C.R., Jr. (1968) Biochim. Biophys. Acta 152, 244-254.

[Received March 17, 1982]

METHODS

The Quantitative Determination of Plasmalogen by Its Reaction with Mercuric Chloride

ERIC M. CAREY, *Department of Biochemistry, The University of Sheffield, Sheffield S10 2TN, United Kingdom*

ABSTRACT

The alk-1-enyl group of 1-alk-1'-enyl-2-acyl-glycerophospholipids (plasmalogens) rapidly combines with mercuric chloride. At 0 C, there was a 1:1 stoichiometry for Hg binding to the reactive enol group of plasmalogens. Aldehydes were not released, indicating that the alkenyl ether bond was not cleaved. Hg binding to less reactive double bonds in unsaturated fatty acids was not significant. Quantitative estimation of bound Hg afforded a rapid and sensitive assay for alkenylacyl lipids and gave values similar to those obtained with other methods of analysis. The proportion of plasmalogens in bovine myelin glycerophosphatides and in ethanolamine glycerophosphatide was 35 and 75%, respectively. Plasmalogens account for 23.3% of the total glycerophospholipid of rat erythrocytes.
Lipids: 17:656-661, 1982.

Alk-1-enyl glyceryl ethers of neutral glycerides and phosphoglycerides occur in a wide variety of tissues and organisms (1). Phosphoglycerides that contain alk-1-enyl groups (plasmalogens) account for a substantial proportion of the phospholipids of the mammalian brain and cardiac membranes (2). Plasmalogens account for 31-36% of the total phospholipid content of CNS myelin, constituting about 70% of the EPG fraction (3). The function of plasmalogens in membranes is not understood but may relate to differences in physical properties between alkenylacyl and diacylphospholipids (4) or modulation of membrane phospholipid turnover (5).

The enhanced susceptibility of the enol ether double bond to electrophilic attack by H^+, I_2 or mercuric salts has formed the basis of several methods for the quantitative determination of alkenyl ether groups in tissue lipid extracts and in purified lipid fractions. Selective acid hydrolysis of alkenyl ethers has been used to quantitate plasmalogens (6-9). Released aldehydes are estimated as *p*-nitrophenyl hydrazones (a method which requires a correction factor to be applied because the yield is below the theoretical maximum) after Schiffs base formation or by GLC of ADMA (6,7,9). A frequently used method is the specific iodination of the enol ether double bond and quantitation of unbound I_2, a method which also gives values 7-8% below the theoretical maximum (10,11). Two-dimensional TLC has been used to separate lysophospholipids derived from plasmalogens from diacylphospholipids after exposure of the lipid on the silica gel to HCl fumes (3) or mercuric chloride (12). Wood and Snyder (13) have developed a method for the simultaneous determination of plasmalogens and *o*-alkyl lipids, which has been modified by Blank et al. (9). Each method may have some specific usefulness in, e.g., the determination of the alkenyl and acyl composition, or sensitivity. However, no method is particularly useful for the quantitation of plasmalogen in a large number of samples as is required for the study of enzyme-catalyzed plasmalogen hydrolysis.

The alkenyl double bond of plasmalogens is hydrolyzed upon prolonged exposure of lipid emulsions at room temperature to aqueous solutions of mercuric chloride at neutral (14) or acid (12) pH. However, Norton (15) showed that Hg remained bound to plasmalogen and this has been used in a histochemical method for the detection of plasmalogens in tissue slices of the brain (16) and in the detection of plasmalogens in lipid extracts (15). This paper describes a quantitative method for the determination of plasmalogen based on Hg binding. It was developed to provide a direct, one-stage method

Abbreviations: CNS, central nervous system; CPG, choline phosphoglycerides; EPG, enthanolamine phosphoglycerides; FAME, fatty acid methyl esters; ADMA, aldehyde dimethylacetals; DPCZ diphenylcarbazone; GLC, gas liquid chromatography; TLC, thin layer chromatography.

which can be used for studying the enzymic hydrolysis of the alkenyl ether group of plasmalogens.

EXPERIMENTAL PROCEDURES

Lipid Extraction and Purification

Myelin was isolated from bovine brain white matter as described previously (17). Rat erythrocytes were isolated by centrifugation and washed three times with 0.9% saline. Lipids were extracted by the method of Folch et al. (18). EPG was purified from the lipid extract of bovine brain white matter by alumina column chromatography and TLC (3). Ethanolamine plasmalogen was isolated from the EPG fraction by the method of Paltauf (19) and CPG was isolated from egg yolk by the method of Rhodes and Lea (20). FAME and ADMA were prepared from myelin lipids; EPG and ethanolamine plasmalogen were prepared by BF_3-catalyzed methanolysis (21).

Lipid Analyses

Lipid phosphorus was determined by the method of Bartlett (22). The alk-1-enyl content of lipid extracts was determined by the iodine addition method of Williams et al. (11). Aldehydes released by acid hydrolysis were quantitated by Schiffs base formation with rosaniline (7). Hydrogenation of double bonds in isolated plasmalogen was carried out with Adams catalyst as described by Snyder and Blank (23).

Mercury Binding to Plasmalogen

Samples of purified plasmalogen and lipid extracts (containing up to 80 nmol plasmalogen) were transferred to test tubes and $CHCl_3$ was removed in vacuo using a Buchler vortex evaporator. Lipid was redissolved in 0.2 ml $CHCl_3$; the test tubes were placed on ice and 1.0 ml of an ice-cold solution of 1% $HgCl_2$ in methanol/water (1:1, v/v) was added. The contents of the tubes were quickly mixed with a vortex mixer and returned to the ice bath. After 2 min, 2 ml ice-cold $CHCl_3$ was added, followed by 5 ml ice-cold $CHCl_3$-saturated water. The contents of the tube were thoroughly mixed on a vortex mixer, allowed to separate at 0 C and most of the upper layer was removed by aspiration using a water pump. The washing of the lower layer with ice-cold $CHCl_3$ saturated water was repeated a further 4 times, a procedure which took 10 min to perform. Mercury which was present in the lower $CHCl_3$ layer was determined as the colored complex with DPCZ. 2.0 ml of 0.01% DPCZ solution in $CHCl_3$ was added to the $CHCl_3$ layer, then the tube contents were mixed and centrifuged using an M.S.E. bench centrifuge. The absorbance of the lower layer was determined immediately at 546 nm using a Cecil Model 272 spectrophotometer against a reagent blank.

In some assays, plasmalogen was first hydrolyzed with 0.5 ml 90% acetic acid containing 0.5 N HCl for 45 min at 50 C (7). Tubes were cooled to 0 C before addition of the $HgCl_2$ solution. In control assays, 0.5 ml ice-cold 90% acetic acid was added to lipid samples before the addition of the $HgCl_2$ solution.

A standard curve for Hg was determined using 2 ml water-saturated $CHCl_3$ or 2 ml of an acidic synthetic $CHCl_3$ phase (made from 1 vol 90% acetic acid and 4 vol $CHCl_3$, extracted five times with 5 vol $CHCl_3$-saturated water) and 0-300 μl of 0.001%, (w/v) solution of mercuric chloride (100 mg/ℓ).

DPCZ was obtained from Aldrich Chemical Co., Milwaukee, WI. Pararosaniline-HCl and methyl arachidonate were purchased from Sigma Chemical Co., Poole, U.K. All other reagents and solvents were of A.R. purity. Bovine brains were obtained from the local abattoir and were transported on ice. Male Wistar rats were bred in the Animal House, University of Sheffield.

RESULTS

The colorimetric determination of mercury as the Hg-DPCZ complex, extracted into water-saturated $CHCl_3$, is shown in Figure 1. The color development under the simulated conditions of acid hydrolysis of plasmalogen was lower than with water-saturated $CHCl_3$. Acetic acid does not completely partition into the aqueous phase even after five washings of the lower acid-$CHCL_3$ phase. Under acid conditions, the Hg-DPCZ complex is unstable, giving a decrease of 25% in the absorbance at 546 nm over a period of 1 hr. However, under neutral conditions, the complex is stable at room temperature for at least 1 hr.

Figure 2 shows the recovery of Hg in the lower $CHCl_3$ phase with increasing amounts of ethanolamine plasmalogen, EPG and myelin lipid after 2 min incubation at 0 C. The absorbance at 546 nm is nonlinear with respect to the amount of lipid added but the amount of bound Hg (using the standard curve in Fig. 1) is proportional to the amount of plasmalogen added (up to 80 nmol). With myelin lipids, the same value for the amount of Hg bound was obtained under neutral and acid conditions, although the absorbance was lower under acid conditions. The ratio of 5 nmol plasmalogen/μg bound Hg (Fig. 2) is equivalent to the binding of 1 g atom Hg/mol of added plasmalogen. At low levels of plasmalogen in the assay, the stan-

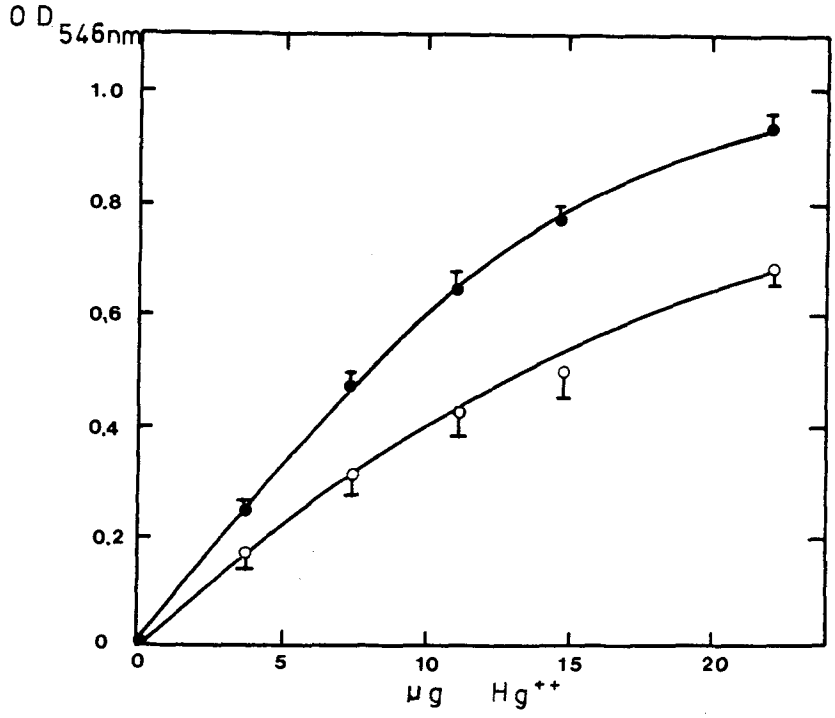


FIG. 1. Quantitative determination of mercury by complex formation with DPCZ. Assays contained 2.0 ml water-saturated $CHCl_3$ (•—•) or 2.0 ml synthetic, acidic $CHCl_3$ phase, prepared by adding 1 vol 90% acetic acid to 4 vol $CHCl_3$ and extracting 5 times with 5 vol $CHCl_3$-saturated water (○—○), and 0-300 µl 0.001% (w/v) mercuric chloride solution. The OD_{546nm} read immediately after mixing. Values are means of duplicate assays. Error bar indicates difference from mean.

dard deviation was 10% of the mean value, with an SEM for three determinations at each point of 5%. At higher concentrations, the SEM was 2.4% of the mean. The deviation of the color response from linearity for the estimation of bound Hg reduced the upper limit of the assay to 80 nmol plasmalogen.

The results for the determination of the plasmalogen content of purified lipid fractions by the Hg binding assay, aldehyde release and iodine additions are shown in Table 1. Similar values were obtained with all three methods after applying a correction factor for 92% conversion of aldehyde to the corresponding hydrazone.

Mild acid hydrolysis resulted in loss of plasmalogen as determined by the aldehyde content and iodination, with a corresponding loss of Hg binding. Mercuric salts form adducts with the double bonds of unsaturated fatty acids (24,25) but FAME and ADMA prepared from ethanolamine plasmalogen did not bind Hg at concentrations up to100 nmol under the assay conditions. Likewise methyl arachidonate (100 nmol) in the assay did not bind Hg (results not shown). Hydrogenation of plasmalogen with Adams catalyst resulted in loss of Hg binding. Hydrogenation, however, reduces double bonds in the acyl chain in addition to the vinyl ether double bond. CPG from egg yolk, which has a low plasmalogen content, did not bind Hg.

In preliminary experiments (results not presented), the binding of Hg to lipid was carried out at room temperature. The amount of Hg recovered in the lower $CHCl_3$ phase was found to be much lower than when the assay was done at 0 C, possibly as a result of Hg-catalyzed cleavage of the vinyl ether double bond. At 0 C, no aldehyde could be detected even after 30 min, suggesting that breakdown of the plasmalogen was minimal under these conditions.

The time course for Hg binding to EPG and myelin lipids at 0 C is shown in Figure 3. The binding was rapid, reaching a maximal value by 1 min. Incubating for longer than 10 min caused an increase in Hg binding to intact EPG and

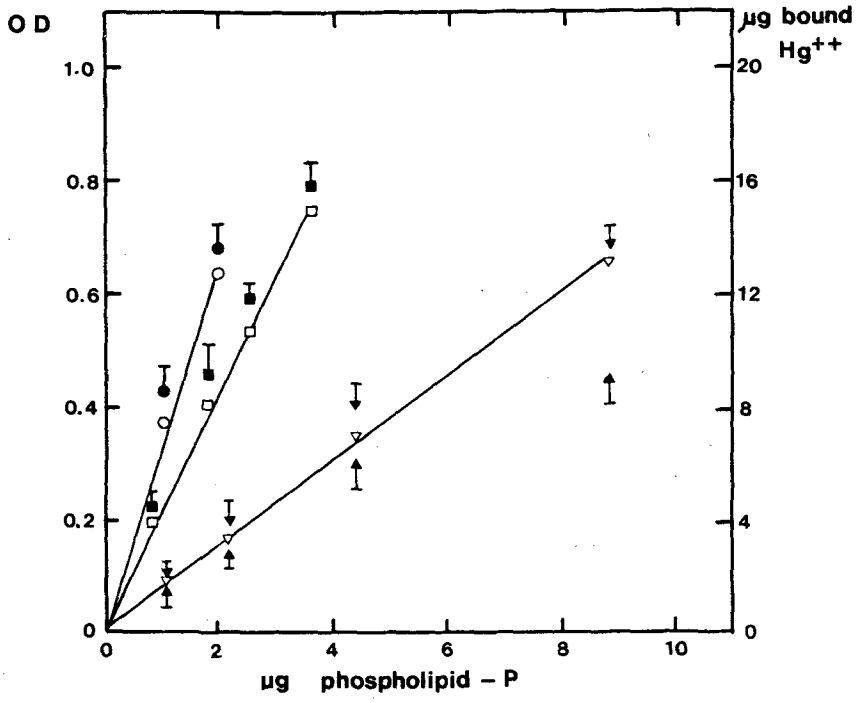


FIG. 2. The quantitative determination of mercury bound to purified ethanolamine plasmalogen, EPG and myelin lipid. Closed symbols, $OD_{546\,nm}$; open symbols, bound Hg (µg) calculated using the standard curves in Fig. 1; (●—●) ethanolamine plasmalogen, (■—■) EPG fraction. With myelin lipids, Hg binding assay was done under neutral (▼—▼) and acid (▲—▲) conditions and Hg binding was calculated using the appropriate standard curve in Fig. 1. Values are means of triplicate incubations with error bar showing standard deviation.

TABLE 1

A Comparison of the Mercury Binding Assay with Other Procedures for Determination of Plasmalogen in Purified Phospholipid Fractions

Lipid fraction	Phospholipid-P, µg (nmol)	OD 546 nm	Hg binding assay		Plasmalogen from:	
			Hg bound (µg)	Plasmalogen[a] (nmol)	Aldehyde release (nmol)	I_2 addition (nmol)
Ethanolamine phosphoglyceride	0.9 (29)	0.27	4.4	22.0 ± 0.8	22.5 ± 0.7	21.8 ± 0.4
Ethanolamine plasmalogen	1.0 (32)	0.38	5.9	29.5 ± 0.3	29.3 ± 0.5	30.1 ± 0.1
Acid-hydrolyzed ethanolamine plasmalogen	1.0 (32)	0.02	0.3	1.6 ± 0.6	0.9 ± 0.3	1.2 ± 0.6
FAME + ADMA	–	0.02	0.3	1.6 ± 0.4	1.0 ± 0.4	1.5 ± 0.3
Ethanolamine plasmalogen (hydrogenated)	1.0 (32)	0.015	0.24	1.2 ± 0.4	0.9 ± 0.2	1.0 ± 0.4
Choline phosphoglyceride	3.0 (97)	0.06	0.9	4.5 ± 1.0	3.2 ± 0.6	3.9 ± 0.5

Ethanolamine phosphoglyceride and purified ethanolamine plasmalogen were isolated from bovine brain white matter. Plasmalogen was hydrolyzed in 90% acetic acid/0.5 N HCl for 45 min at 50 C. FAME + ADMA were obtained from ethanolamine plasmalogen (1.0 µg phospholipid-P) by BF_3/methanolysis, and quantitated by GLC. Choline phosphoglyceride was purified from egg yolk lipids. Assays in triplicate ± SEM.

[a]Plasmalogen calculated using the equivalence of 1 µg Hg:5 nmol plasmalogen (see text).

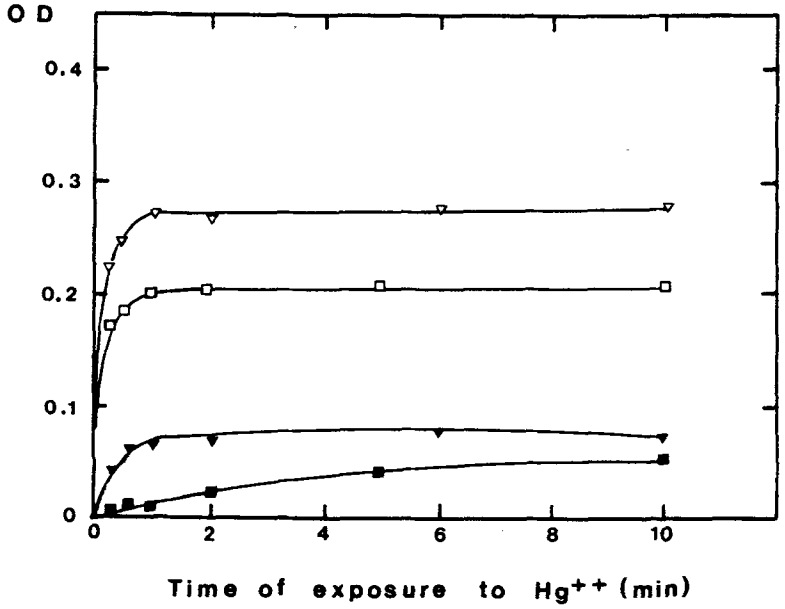


FIG. 3. Time course for mercury binding to EPG (0.6 μg pl-P) and myelin lipid (2.2 μg pl-P) from bovine white matter before (open symbols) and after (closed symbols) acid hydrolysis. Symbols are as in Fig. 2.

acid-hydrolyzed EPG, suggesting that Hg binds to double bonds other than the vinyl ether double bond. Although the amount of Hg bound to acid-hydrolyzed EPG and myelin lipids was lower than to nonhydrolyzed lipid, Hg binding followed the same time course as binding to nonhydrolyzed samples, suggesting that plasmalogen is incompletely hydrolyzed.

The values obtained by the Hg binding assay for the plasmalogen content of the total phospholipids extracted from myelin (35%), intact erythrocytes (23.3%) and erythrocyte "ghosts" (49.7%) are similar to those obtained from the aldehyde determination (Table 2). The value of the SEM was 4.2%, slightly higher than with purified plasmalogen (2.4%).

DISCUSSION

At room temperature, olefinic double bonds of unsaturated fatty acids form mercurial adducts in a reaction which takes 24 hr to reach completion (24,25). With the more reactive alkenyl ether, adduct formation occurs within 1 min at 0 C (Fig. 3 and ref. 15), with a stoichiometric amount of Hg bound. Under these conditions, mercurial addition to olefinic double bonds in phospholipids and methyl esters of polyunsaturated fatty acids is negligible (Table

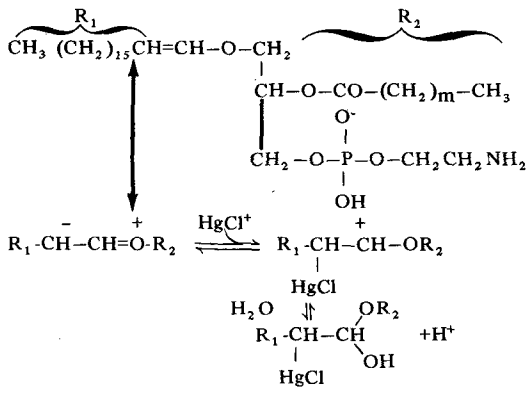
TABLE 2

Determination of the Plasmalogen Content of Membrane Lipids

Membrane	Phospholipid-P, μg (nmol)	Plasmalogen (nmol)	
		Hg binding	Aldehyde release
Bovine myelin	2.2 ± 0.1 (71)	25.0 ± 0.4	25.4 ± 0.7
—after acid hydrolysis	2.2 ± 0.1	2.6 ± 0.5	3.1 ± 0.3
Rat erythrocyte	2.3 ± 0.1 (75)	17.5 ± 1.0	18.9 ± 0.5
Rat erythrocyte "ghosts"	3.5 ± 0.1 (113)	56.2 ± 2.0	54.5 ± 1.7

1). The results indicate that the mercurial addition occurs specifically with double bonds of alkenyl ethers.

Norton et al. (16) have suggested possible reaction mechanisms involving the initial binding of mercuric chloride followed by cleavage of the vinyl ether double bond to form an aldehyde. The mechanism of Hg binding is probably:



At room temperature, the monochloromercurial adduct breaks down to yield aldehyde with or without bound Hg (16). At 0 C, we were unable to detect aldehyde products. The stoichiometric binding of mercury to plasmalogen suggests that, at 0 C, the reactions just outlined are relatively fast whereas the subsequent cleavage of the vinyl ether double bond and release of the mercury occurs slowly.

The mercury binding assay is similar in sensitivity to other methods for the determination of plasmalogen based on quantitation of released aldehydes or phospholipid phosphorus determination. The lower limit of sensitivity is 10 nmol plasmalogen, with a range of 10-80 nmol plasmalogen. In the upper part of the range, the reproducibility is better than 5% whereas, at lower plasmalogen levels, the variation is 10%. The sensitivity and reproducibility is comparable to methods based on aldehyde determination (9). The upper limit is determined by the lack of sensitivity of the Hg assay above 20 μg Hg (Fig. 1).

The Hg binding assay has been applied to the quantitation of plasmalogens in tissue lipid extracts. Similar results were obtained by quantitation of aldehyde released by acid hydrolysis. The plasmalogen content of bovine myelin phospholipid was found to be 35.2% which is similar to values reported earlier (3). The erythrocyte membrane contains a high proportion of ethanolamine plasmalogen (8). The proportion of plasmalogens in lipid extracts of intact rat erythrocytes and cell "ghosts" was found to be 24 and 49% of the total phospholipids.

The major advantage of the Hg binding assay is that, like the iodine addition assay (10,11), it does not require a two-stage analysis involving quantitation of a product derived from plasmalogen. It is more sensitive than the iodine addition method and can be done more quickly. Together with a method for definitive structural analysis of plasmalogens, the Hg binding assay is useful for sequential quantitative analysis as in a study of plasmalogen metabolism.

ACKNOWLEDGMENTS

The work was supported in part by a grant from the Multiple Sclerosis Society of Great Britian. N. Freeman and A. Morris provided excellent technical assistance.

REFERENCES

1. Horrocks, L.A. (1972) in Ether Lipids, Chemistry and Biology (Snyder, F., ed.) pp. 177-272, Academic Press, New York, NY.
2. Rapport, M.M., and Norton, W.T. (1962) Ann. Rev. Biochem. 31, 1038-138.
3. Horrocks, L.A. (1968) J. Lipid Res. 9, 469-472.
4. Shah, D.O., and Schulman, J.H. (1965) J. Lipid Res. 6, 341-349.
5. Woelk, H., and Porcellati, G. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 90-100.
6. Wittenberg, J.B., Korey, S.R., and Swenson, F.H. (1959) J. Biol. Chem. 219, 39-47.
7. Gray, G.M. (1969) in Methods in Enzymology (Colowick, S.P., and Kaplan, N.O., eds.) Vol. 14, pp. 678-684, Academic Press, New York, NY.
8. Rogiers, V. (1980) J. Chromatogr. 182, 827-833.
9. Blank, M.L., Cress, E.A., Piantadosi, C., and Snyder, F. (1975) Biochim. Biophys. Acta 380, 208-218.
10. Gottfried, E.L., and Rapport, M.M. (1962) J. Biol. Chem. 237, 329-333.
11. Williams, J.N., Anderson, C.E., and Jasik, A.D. (1962) J. Lipid Res. 3, 378-381.
12. Owens, K. (1966) Biochem. J. 100, 354-361.
13. Wood, R., and Snyder, F. (1968) Lipids 3, 129-135.
14. Schmidt, G., Ottenstein, B., Spencer, W.A., Keck, K., Blietz, R., Papas, J., Porter, D., Levin, M.L., and Thannhauser, S.L. (1959). Am. J. Dis. Child. 97, 691-708.
15. Norton, W.T. (1959) Nature London 184, 1144-1145.
16. Norton, W.T., Brotz, M., and Korey, S.R. (1965) J. Neuro Pathol. Exp. Neurol. 24, 352-360.
17. Carey, E.M., and Foster, P.C. (1977) Biochem. Soc. Trans. 5, 1412-1413.
18. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 492-509.
19. Paltauf, F. (1978) Lipids 13, 165-166.
20. Rhodes, D.N., and Lea, C.H. (1957) Biochem. J. 65, 526-533.
21. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
22. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
23. Snyder, F., and Blank, M.L. (1969) Arch. Biochem. Biophys. 130, 101-110.
24. Mangold, H.K. (1961) J. Am. Oil Chem. Soc. 39, 708-727.
25. Gunstone, F.D., and Inglish, R.P. (1973) Chem. Phys. Lipids 10, 73-88.

[Received March 9, 1982]

COMMUNICATIONS

Effect of Altered Sterol Composition on the Osmotic Behavior of Sphaeroplasts and Mitochondria of *Saccharomyces cerevisiae*

COLLEEN A. MCLEAN-BOWEN and **LEO W. PARKS***, *Department of Microbiology, Oregon State University, Corvallis, OR 97331*

ABSTRACT

The effect of sterols on the osmotic stability of mitochondrial and plasma membranes of yeast wild-types and mutants that are defective in ergosterol biosynthesis has been studied. Incorporation of the nonfungal sterol, cholesterol, into yeast membranes reduces membrane elasticity which is observed as an increased susceptibility to osmotic lysis. However, the wild-type and nystatin-resistant strains which were examined indicate that qualitative alterations in endogenously generated sterols do not affect resistance to swelling. Although these strains exhibit differences in membrane fluidity, which is influenced by the sterol accumulated by the organisms, the membrane stretching capacity shows no distinct dependence on sterol structure or bilayer fluidity.
Lipids 17:662-665, 1982.

INTRODUCTION

Saccharomyces cerevisiae has been used extensively to study the cellular role of sterols. This organism has yielded a variety of mutant strains which accumulate sterols other than ergosterol, the predominant species found in wild-type yeast. Thus, a unique system exists to examine the effects of altered lipid composition on membrane organization, membrane function and the overall physiology of the cell.

A recent report from our laboratory examined the physical state of mitochondrial membranes from wild-types and from mutants defective in ergosterol biosynthesis using a fluorescence polarization technique (1). Changes in the depolarization rate of the probe molecule as a function of temperature indicated the occurrence of a phase transition in the mitochondrial membranes isolated from mutants but not seen in mitochondria isolated from wild-types. The Arrhenius kinetics of cytochrome c oxidase (2, 3), sterol methyltransferase (2,3), and kynurenine hydroxylase (1) were also perturbed by the altered sterol composition.

Having examined the effect of sterol on bilayer fluidity, we investigated further the role of sterol in membrane elasticity. We report here the effects of qualitative sterol changes on the stretching capacity of the plasma and mitochondrial membranes.

MATERIALS AND METHODS

The strains of *S. cerevisiae* and their sterol phenotypes used in this study are summarized in Table 1. These strains and the defects in sterol biosynthesis have been characterized in previous communications (1,4).

FY3-n3 was grown on defined medium at pH 5.5 as described previously (4). Sterol was added to a final concentration of 10 μg/ml

TABLE 1

A Profile of the Organisms Used in This Study

Strain	Designation	Sterol accumulated	Comment
MCC	Wild-type, diploid	Ergosterol	
S288c	Wild-type, haploid	Ergosterol	
3701b	Wild-type, haploid	Ergosterol	
3701b-n3	$\Delta^{5(6)}$Desaturase defect	Ergosta-7,22-diene-3β-ol	nys^r clone of 3701b
Z008	Wild-type, haploid	Ergosterol ergosta-5,7,22,24(28)tetraene-3β-ol	
8R1	Steryl methyltransferase defect	Zymosterol cholesta-5,7,22,24-tetraene-3β-ol	nys^r clone of Z008
FY3-n3	*heml erg7*	Sterol auxotroph	(11)

*Author to whom correspondence should be addressed.

from a stock solution of 4 mg/ml in Brij 58: 95% ethanol (1:1, v/v). Unsaturated fatty acids were added to a final concentration of 10 μg/ml from a 10% stock solution in Brij 58:95% ethanol (1:1, v/v). All other organisms were grown in broth containing 1% tryptone, 0.5% yeast extract, 2% ethanol and 0.1% glucose.

Sphaeroplasts were prepared by treatment with snail gut enzyme (1) or an extracellular lyticase from *Oerskovia xanthineolytica* (5). The lyticase was prepared by dialysis of the medium following growth of *O. xanthineolytica* on modified Sistrom's medium (5) against buffer containing 1.1 M sorbitol, 10 mM phosphate and 1 mM EDTA, pH 7.4. Following digestion, the sphaeroplasts were washed three times and suspended in 1.1 M sorbitol, 10 mM phosphate and 1 mM EDTA, pH 7.4, in the case of FY3-n3 or in 0.7 M sorbitol, 0.3 M mannitol, 0.1 M citric acid, 0.01 M potassium phosphate and 10 mM EDTA, pH 7.4, for all other preparations. Mitochondria were isolated in buffer containing 0.9 M sorbitol, 0.5 mM EDTA and 10 mM Tris, pH 7.4, as described by McLean-Bowen and Parks (1).

Mitochondria were diluted into concentrations of sucrose or sorbitol from 0.0 to 1.0 molar. These solutions also contained 10 mM Tris and 1 mM EDTA, pH 7.4. Following incubation at room temperature for 30 min, the optical density at 520 nm (6) was determined. Sphaeroplasts were diluted into varying concentrations of sorbitol or sucrose which also contained either 10 mM Tris and 1 mM EDTA, pH 7.4, or 10 mM Tris and 10 mM succinate, pH 7.4 or 5.5, as described in the figure legends. Results are expressed as percentage lysis calculated as (7): OD in undiluted buffer–OD in diluted buffer/ OD in undiluted buffer × 100 or as percentage increase in optical density, calculated as: OD in undiluted buffer–OD in buffered H_2O/OD in undiluted buffer × 100.

Malate dehydrogenase was assayed according to the method of Vary et al. (8).

Snail gut enzyme (Glusulase) was purchased from Endo Laboratories, and was precipitated with ammonium sulfate (9) prior to use. Other reagents were from Sigma.

RESULTS AND DISCUSSION

The dependence of osmotic stability on sterol was examined using FY3-n3 sphaeroplasts. Blocked in heme synthesis, FY3-n3 requires supplementation with unsaturated fatty acids and sterol for growth, enabling dramatic manipulation of membrane composition. These experiments parallel similar experiments by Alterthum and Rose and Hassack and Rose (10,11) which used the anaerobically induced nutritional requirement of *S. cerevisiae* to manipulate the lipid composition of the organism. As shown in Figure 1, the observation of Hossack and Rose (11) that the stability of sphaeroplasts prepared from cells grown with cholesterol was diminished compared to those supplemented with ergosterol was confirmed. In view of the report of Taylor

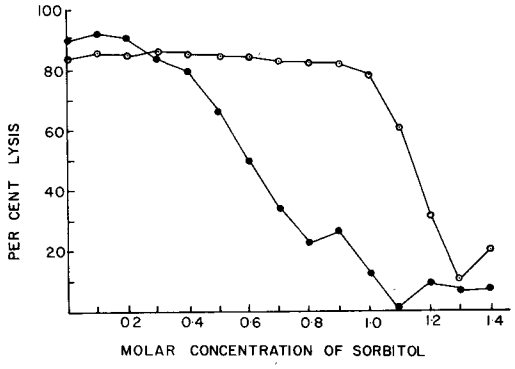


FIG. 1. Osmotic stability of FY3-n3. Sphaeroplasts from cholesterol-supplemented cultures (—o—) or ergosterol-supplemented cultures (—•—) were diluted into various concentrations of sorbitol. These solutions also contained 10 mM Tris, 10 mM succinate, 1 mM EDTA, pH 7.4.

and Parks that growth of FY3-n3 on cholesterol is pH sensitive (4), the effect of pH on the osmotic stability of sphaeroplasts from cholesterol-grown cells was examined. Optimal growth with cholesterol supplementation is at pH 5.5 whereas all previous measurements of osmotic properties were at pH 7.4. Adjustment of the pH to 5.5 resulted in a small increase in resistance to lysis, the degree of which was variable. It should be noted that the type of buffer(s) used also affected the osmotic behavior of the sphaeroplasts.

The response of sphaeroplasts from the wild-type and nystatin-resistant organisms upon dilution into anisotonic sucrose and sorbitol buffers is shown in Figure 2. In all cases, the optical density of the suspension increased with increasing concentrations of either osmotic stabilizer and the sugar alcohol, sorbitol, resulted in higher optical densities than did the sucrose. The behavior of the sphaeroplasts from different strains upon dilution into varying concentrations of sorbitol was similar. Dilution into sucrose showed differences between the strains, but there was no correlation between the re-

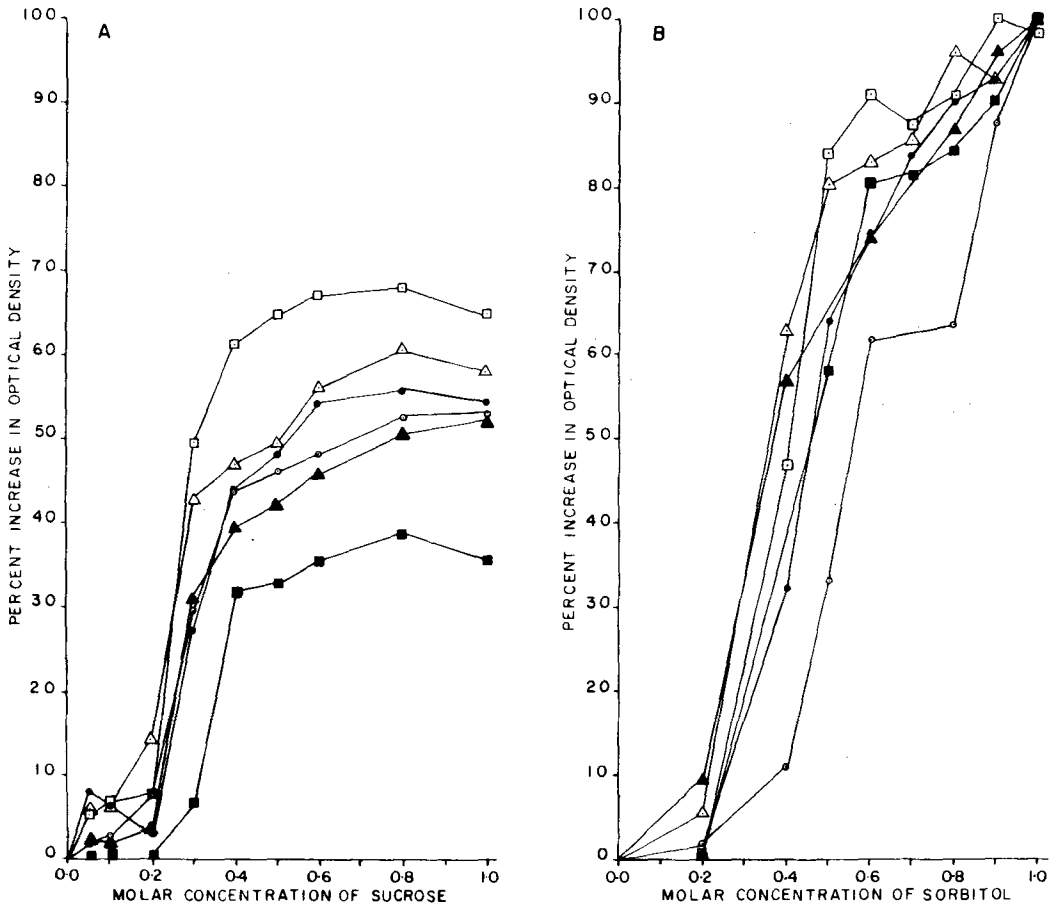


FIG. 2. Response of sphaeroplasts to anisotonic media. (A) The response of sphaeroplasts prepared from the various strains to resuspension in a range of sucrose concentration; (B) the response of the same preparation to resuspension in a range of sorbitol concentration. The source of the sphaeroplasts is as follows: ■–MCC; ●–S288c; △–3701b; □–3701b-n3; ▲–Z008; ○–8R1.

sponse and the sterol accumulated.

The osmotic stabilizer used also produced different effects when mitochondria were used. The optical density of mitochondria suspended in high concentrations of sorbitol was greater than the optical density in the same concentrations of sucrose. At levels greater than 0.3 M sucrose, there was a decrease in absorbance which was not observed with sorbitol. This behavior in sucrose was not observed in experiments with rat liver mitochondria made by Tapley (12) and Lehninger et al. (13) which showed increasing optical density with all increases in sucrose concentration.

We originally thought that the high concentration of sucrose produced a rapid efflux of water from the mitochondria which resulted in lysis and hence the low optical density. This was tested by assaying for the release of malate dehydrogenase, a soluble mitochondrial enzyme. After dilution into 1.0 M sucrose, the suspension was incubated at room temperature for 30 min, and the mitochondria were pelleted. All malate dehydrogenase activity was retained in the pellet. Further experiments showed that this decrease in optical density at high concentrations of sucrose was reversible. It therefore appears that this inconsistent optical behavior was not the result of lysis. A search of the literature indicated that the decreased optical density of the mitochondrial suspensions in concentrations of sucrose greater than 0.3 M and the variation in optical density observed between suspensions of the osmotically active particle in sucrose or sorbitol is a result of differences in the refractive indices of these solutions (6,14).

The optical density of such a suspension is affected by the wavelength of the incident light, osmotic pressure, and the refractive index of the supportive medium. In this case, the dissimilar optical density seen upon dilution with sorbitol or sucrose is the result of the different refractive indices of the suspending media, refractive index being a function of concentration and nature of the solute. Furthermore, the optical density primarily results from differences between the refractive index of the mitochondria and of the medium. Tedeschi and Harris (6) also reported a decrease in the optical density of mitochondrial suspensions at concentrations of sucrose greater than 0.3 molar, and attributed this deviation to variation of refractive index, rather than to a decrease in particle volume.

The striking similarity of the elastic behavior in sucrose buffer of mitochondria isolated from different yeast strains is shown in Figure 3. No differences were noted.

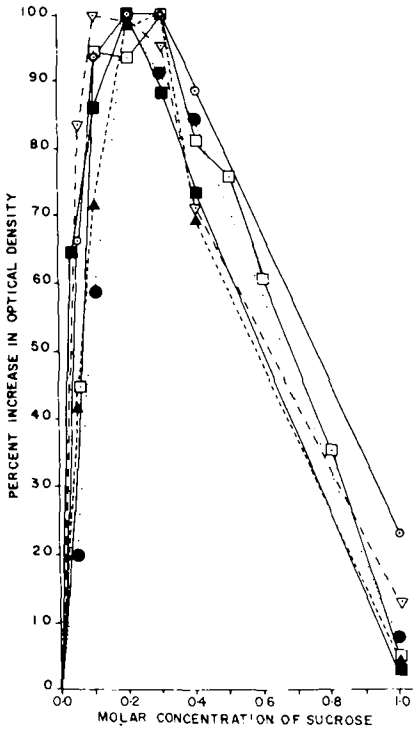


FIG. 3. Response of mitochondria to various concentrations of sucrose. Details of the experiment are described in the text. The mitochondria were isolated from: □–MCC; ▽–3701b; ■–3701b-n3; ●–Z008; ▲–8R1; ○–S288c.

The qualitative alterations in the sterol composition of nystatin-resistant yeast strains vs wild-type strains do not affect the membrane elasticity as measured by susceptibility to swelling. In contrast, cholesterol incorporation into yeast membranes increases susceptibility to osmotic lysis, as originally reported by Hossack and Rose (11). The discrepancy between these two observations may reflect differences in the means of altering the sterol composition of the organisms. With the exception of the sterol auxotrophs, these strains accumulate ergosterol or sterols which are synthesized by the organism rather than sterols which are not naturally found in yeast, e.g., cholesterol. By the criterion of membrane stretching capacity, we conclude that the structural modifications of the sterols accumulated by the mutants are not sufficiently different from ergosterol to cause a change in osmotic stability of sphaeroplasts or mitochondria, even though these sterols effect changes in bilayer organization (1) and intrinsic enzyme activities (1-3).

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation (PCM-787609) and the U.S. Public Health Service (AM-05190). C.A. McLean-Bowen was the recipient of an N.L. Tartar Fellowship during a portion of this work. This is Oregon Agricultural Experiment Station Technical paper no. 6332.

REFERENCES

1. McLean-Bowen, C.A., and Parks, L.W. (1981) J. Bacteriol. 145, 1325-1333.
2. Thompson, E.D., and Parks, L.W. (1974) Biochem. Biophys. Res. Commun. 57, 1207-1213.
3. Thompson, E.D., and Parks, L.W. (1974) J. Bacteriol. 120, 779-784.
4. Taylor, F.R., and Parks, L.W. (1980) Biochem. Biophys. Res. Commun. 95, 1437-1445.
5. Scott, J.H., and Schekman, R. (1980) J. Bacteriol. 142, 414-423.
6. Tedeschi, H., and Harris, D.L. (1958) Biochim. Biophys. Acta 28, 392-402.
7. Razin, S., Tourtellotte, M.E., McElhaney, R.N., and Pollack, J.D. (1966) J. Bacteriol. 91, 609-616.
8. Vary, M.J., Edwards, C.L., and Stewart, P.R. (1969) Arch. Biochem. Biophys. 130, 235-243.
9. Schatz, G., and Kovac, L. (1974) Methods Enzymol. 31, 627-632.
10. Alterthum, F., and Rose, A.H. (1973) J. Gen. Microbiol. 77, 371-382.
11. Hossack, J.A., and Rose, A.H. (1976) J. Bacteriol. 127, 67-75.
12. Tapley, D.F. (1956) J. Biol. Chem. 222, 325-339.
13. Lehninger, A.L., Ray, B.L., and Schneider, M. (1959) J. Biophys. Biochem. Cytol. 5, 97-108.
14. Tedeschi, H., and Harris, D.L. (1955) Arch. Biochem. Biophys. 58, 52-67.

[Received April 23, 1982]

ERRATUM

In the article "Improved Methods for the Isolation and Study of the C_{18}, C_{20} and C_{22} Monoethylenic Fatty Acid Isomers of Biological Samples: Hg Adducts, HPLC, $AgNO_3$-TLC/FID, and Ozonolysis" by J-L. Sebedio, T.E. Farquharson and R.G. Ackman (*Lipids* 17:469-475, 1982), a section was inadvertently omitted from the text. On p. 474, the second column, after line 19, the following text should be inserted:

". . . mixture of *cis* 18:1Δ9 and *trans* 18:1Δ9 was submitted to an HPLC analysis using a solvent mixture of MeOH/H_2O (90:10) at 0.7 ml/min. No difference was observed between the actual isomer weight percentage and the chart area percentage given by the HPLC analysis, indicating no difference in the detector response factor for the *cis* and *trans* 18:1Δ9 isomers. The quantitation of *trans* fatty acids by the 2 other methods ($AgNO_3$-TLC/GLC on SILAR-7CP; $AgNO_3$-Iatroscan) was not influenced by the chain lengths. These two methods can therefore be applied to any of the common chain lengths (C_{16}-C_{22}). An advantage of the $AgNO_3$-Iatroscan method over GLC methods is the small sample size (10 μg) and the short time needed for an analysis (Fig. 3A) (24). An advantage of the HPLC quantitation using a refractive index detector is the possibility of recovering the sample after analysis. The main disadvantage with HPLC is the lack of sensitivity of the refractive index detector.

The approach to the study of monoethylenic fatty acids, based on the HPLC fractionation of the monoenoic fraction recovered from the methoxy-bromomercuri-adducts, is especially useful for biological samples and/or also for partially hydrogenated oils containing significant amounts of interfering conjugated diethylenic fatty acids. Moreover, although this method will be more time-consuming than the method using preparative GLC as a technique . . . "

Immobilized Lipoxygenase in Continuous Production of Fatty Acid Hydroperoxides

SIMO LAAKSO, *The Department of Biochemistry, University of Turku, 20500 Turku 50, Finland*

ABSTRACT

Soybean lipoxygenase-1 was covalently coupled to agarose with 75% recovery of catalytic activity. Because evidence was obtained that the immobilization resulted in improved operational stability of the enzyme, a lipoxygenase-reactor and a continuous process for the synthesis of 13-hydroperoxylinoleic acid and 15-hydroperoxyarachidonic acid were developed. A procedure based on spectrophotometric hydroperoxide assay and constant oxygraphic monitoring of the effluent is presented for the calibration of the reactor to operate at the highest conversion efficiency when oxygenating quantitatively the substrate. Under these conditions, the reactor was capable of producing about 0.6 mg of hydroperoxy fatty acid/1.0 ml of wet gel/hr. The covalently coupled enzyme has been stable during six months of storage at 3 C in 0.2 M Na-borate buffer, pH 9.0, and during the same period, its operational stability in the column has been unaltered under the conditions used.

Lipids 17:667-671, 1982.

Fatty acid hydroperoxides are compounds of current interest in several areas of biological research, especially in studies concerned with membrane damage or synthesis of prostaglandins. This fact has created a growing demand for synthetic procedures improving the availability of these unstable, noncommercial compounds. Several lipoxygenase-catalyzed oxygenations are known to yield, under properly selected conditions, hydroperoxides that are homogeneous with respect to positional and enantiomorphous composition (1-3). It is not, therefore, surprising that enzymic synthesis is the preferred method of most laboratories. However, a failure either in maintaining strictly aerobic reaction conditions or using insufficiently purified enzymes may lead to unpredictable side-reactions (4,5) and, furthermore, during subsequent removal of the enzyme and unreacted substrate from the product, a time-dependent isomerization of the hydroperoxy group may become significant (6).

These problems could be largely overcome by using substrate solutions dilute enough to guarantee a molar excess of dissolved oxygen and by carrying out the synthesis as a continuous process in which the rate of supply of fatty acids is adjusted favorably for their quantitative oxygenation. However, this requires that a stable catalyst, preferably bound to an insoluble matrix, is available. In this study, such a stable enzyme was developed through immobilization of lipoxygenase-1 (linoleate:oxygen oxidoreductase, EC 1.13.11.12) to agarose. The report shows further that this immobilized enzyme can be used to synthesize mg quantities of 13-hydroperoxylinoleic acid and 15-hydroperoxyarachidonic acid, respectively. The applicability of this technique for the synthesis of other hydroperoxy fatty acids is discussed.

MATERIALS AND METHODS

Materials

Soybean lipoxygenase-1 from Sigma Chemical Company was freed from contaminating lipoxygenase-3 by DEAE-Sephadex column chromatography (7). The purified enzyme behaved as a homogeneous protein in disc SDS electrophoresis at pH 9.5 and had a specific activity of 160 U/mg protein.

CNBr-activated Sepharose 4B came from Pharmacia, Uppsala, Sweden. Linoleic acid (grade III) and arachidonic acid (>99%) were purchased from Sigma. Other reagents needed were of reagent grade and they were used without purification.

Enzyme Immobilization

Cyanogen bromide activated Sepharose 4B (1.0 g dry wt) was washed with 200 ml of 10 mM hydrochloric acid followed by 20 ml of deionized water and rapidly with 200 ml of the coupling buffer, 0.2 M Na-borate, pH 9.0. Wet gel (3.5 ml) was immediately poured into a 50-ml beaker containing 20 mg enzyme in 4.0 ml of the coupling buffer and the mixture was incubated in a rotary shaker at 8 C for 16 hr. The gel was recovered by filtration and washed under slight vacuum according to the following protocol: 40 ml of the coupling buffer, 50 ml of 1 M NaCl, 50 ml of 1 mM glycine in 10 mM Na-borate buffer, pH 9.0, and 100 ml of the coupling buffer. All the filtrates were collected separately and tested for protein content and enzyme activity.

Enzyme Assay and Protein Determination

The catalytic activity of both the soluble and immobilized enzyme was determined at 25 C as the rate of O_2 consumption (8) using the Hansatech D.W. Oxygen Electrode Unit equipped with Goerz RE 511 recorder. The enzyme unit was defined as 1 μmol of O_2 consumed/min.

Protein in the filtrates was assayed spectrophotometrically at 280 nm using a Perkin-Elmer 550S spectrophotometer and a Perkin-Elmer 561 recorder. A multiplying factor of 0.7 was used to obtain the protein concentration in mg/ml (8).

Enzyme Reactor and its Monitoring

Aliquots of the Sepharose-bound lipoxygenase-1 were applied onto a reactor column consisting of a vertically positioned 9 x 80 mm glass tube and a plunger to adjust the volume of the column to correspond to that of the settled gel. Substrate, a freshly diluted 0.1 mM solution from 10 mM stock solution in Tween 20 (9), was applied to the column using a Pharmacia P-3 peristaltic pump. The column outlet was connected by a 1-mm (id) tubing with the oxygraph reaction chamber equipped with a flow-through stopper for constant monitoring of oxygen content of the effluent. At intervals, samples were withdrawn from the outlet of the oxygraph chamber for ultraviolet (UV)-spectrophotometric quantitation of the hydroperoxy fatty acid as the content of conjugated dienes (234 nm) and the carbonyl compounds absorbing at 280 nm (4). In calculations, a molar extinction coefficient of 25,000 was used for both the conjugated dienes and trienes.

Chemiluminescence Measurements

The emission of chemiluminescent light during lipoxygenase reaction was followed luminometrically (LKB Wallac 1250) as described previously (10). The assay mixtures contained (in 1.0 ml of 0.2 M Na-borate buffer, pH 9.0) 0.4 mM linoleic acid, 0.004 mM luminol (5-amino-2,3-dihydrophtalazine-1,4-dione from Sigma) and either 0.2 U of soluble or 0.28 U of immobilized lipoxygenase-1. During the measurements, the reaction vial was continuously mixed with an LKB Wallac 1250-105 mixer. The total emitted light was calculated from the recorded reaction velocity curves by cut and weight method.

Product Analysis

The hydroperoxide-containing products were reduced by $NaBH_4$. The pH was adjusted to 3.0 with 1 M citric acid and the mixtures were extracted twice with 2 vol of diethyl ether. The extracts were dried over anhydrous Na_2SO_4 before evaporating to dryness. The residues were methylated at 25 C in the dark with ethereal diazomethane containing 10% methanol. After evaporation, the residues were dissolved in pyridine and silylated for 20 min at 80 C with bis (trimethylsilyl)trifluoro-acetamide containing 1% trimethylchlorosilane. Gas chromatographic analysis (Varian Model 3700) of both the methylated hydroxy fatty acids and their silylated derivatives was done with a column packed with 3% SE-30 and temperature-programmed from 150 to 260 C at 6 C/min.

RESULTS AND DISCUSSION

In general, immobilization of an enzyme, while improving stability, results in considerable loss of catalytic activity. Although the main goal of this work was to improve the stability of lipoxygenase-1, its immobilization to the agarose gel was routinely achieved with less than 25% reduction in total activity. The enzyme activities per ml of settled gel varied between 700 and 750 U, making possible the construction of efficient lipoxygenase-reactors to produce fatty acid hydroperoxides in a continuous process. Tests indicating that immobilization led to improved stability of the enzyme against accumulating reaction products such as peroxides also favored the development of such reactors (Fig. 1).

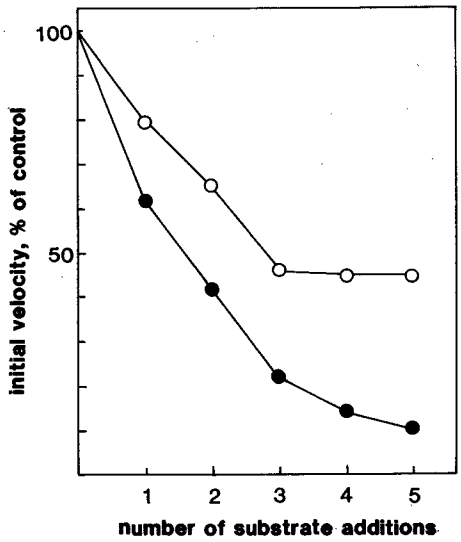


FIG. 1. The effect of repeated linoleic acid additions on the initial velocities of linoleic acid oxygenation catalyzed by soluble and immobilized lipoxygenase-1. Reaction mixtures containing either 10 μl (0.28

U) of 1:25 dilution (v/v) of lipoxygenase gel suspension (–o–) or 0.2 U of soluble enzyme (–•–) in 1.0 ml of 0.2 M Na-borate buffer, pH 9.0, were repeatedly supplemented with 0.4 μmol of substrate and the initial velocities of oxygen consumption under constant mixing were recorded and compared to that obtained after the first addition (100%). Prior to each linoleate adddition, the reaction mixture was aerated to the original concentration of dissolved oxygen at 25 C.

To take full advantage of the immobilized enzyme, the reactor to be developed should fulfill at least the following performance criteria: (a) total conversion of substrate to minimize subsequent purification of the product, (b) capability to maintain strictly aerobic reaction conditions throughout the process, and (c) from an economical viewpoint, the possibility for continuous operation at the minimal gel volume required for total substrate conversion. In practice, the requirement for oxygen in the reaction limits the applicable substrate concentration range in the solution that is passed through the reactor below the dissolved oxygen concentration (about 0.24 mM at 25 C). Therefore, a reactor operating under the above criteria can be constructed using either the flow rate of the substrate solution or the volume of the gel as a variable. The second procedure, shown in Figure 2, in which the minimal gel quantity necessary for total oxygenation of 0.1 mM linoleic acid is determined at a fixed flow rate (11 ml/hr), is the preferred choice for economical small-scale production of hydroperoxides. The volume of the packed bed can be minimized according to the lowest obtainable nonpulsating flow rate and the rate of product formation can be estimated in advance. Tests with columns of varying diameters (6, 8 and 12 mm) indicated that, when the bed volume of the gel was increased stepwise until maximal conversion at the chosen flow rate (11 ml/hr) was reached, the oxygraphic values corresponded to 95-98% conversion efficiencies. About 5% higher efficiencies were invariably obtained from spectrophotometric readings at 234 nm (ϵ_m = 25,000). No evidence of anaerobically formed carbonyl compounds was obtained by spectrophotometric measurements of the reactor products at 280 nm. The volume of the gel required for total linoleate conversion was linearly dependent on substrate concentration up to about 0.11 mM solutions. The solubility of molecular oxygen would permit the use of higher fatty acid concentrations but this was found unfavorable in the continuous hydroperoxide production because the required bed volume was increased sharply above the 0.11 mM limit. For example, if the original 0.1-mM substrate solution was changed to 0.13- or 0.15-mM solutions, the original packed bed volume of 0.8 ml had to be increased to 3.3 and 8.4 ml, respectively, suggesting that the sharp reductions in conversion efficiencies were due to limitations imposed by the availability of dissolved oxygen.

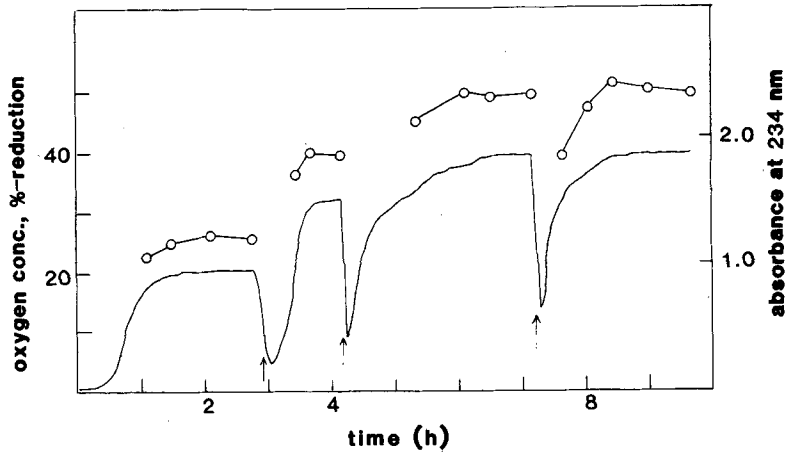


FIG. 2. Calibration of lipoxygenase-reactor for the total conversion of 0.1 mM linoleic acid. Linoleate (0.1 mM) in 0.2 M Na-borate buffer, pH 9.0, was passed through the reactor (8 mm id) at a flow rate of 11 ml/hr under constant monitoring of oxygen content of the effluent (solid line). The volume of the packed bed of immobilized enzyme was increased stepwise (arrows) by adding 200-μl aliquots of settled gel to the column. Prior to each addition, the column was allowed to reach balanced reaction conditions, evidenced as unchanged oxygraphic readings (solid line) and absorbance values at 234 nm (–o–). For the spectrophotometric assay, 1:10 dilutions of the effluent were used.

Reactors calibrated for 0.1 mM linoleic acid at a fixed flow rate are, as such, applicable to continuous hydroperoxide production. However, their conversion efficiencies (in mg product formed/hr/ml packed gel) could be further improved up to 80% by increasing the flow rate (Fig. 3). This behavior probably reflects the fact that the reaction kinetics is governed by diffusion of two different substrates and that a majority of the fatty acid is present as a complex micellar phase. When the flow rate is used as a variable, the product of the reactor should be continuously monitored by oxygraphy as shown in Figure 3 because the correlation between the flow rate and the product formed is nonlinear and independent of the reactor diameter as tested with 6, 8 and 12 mm (id) columns. The oxygraphic readings at the nonlinear region suggested that the reactors failed to exhaust the substrate. However, a 1.5- to 1.8-fold increment in the original flow rate was normally sufficient to restore the reactors' original oxygen consumption capacity, indicating that all the substrate eluted through the column was oxygenated. Reactors operating under these conditions were used throughout the stability tests.

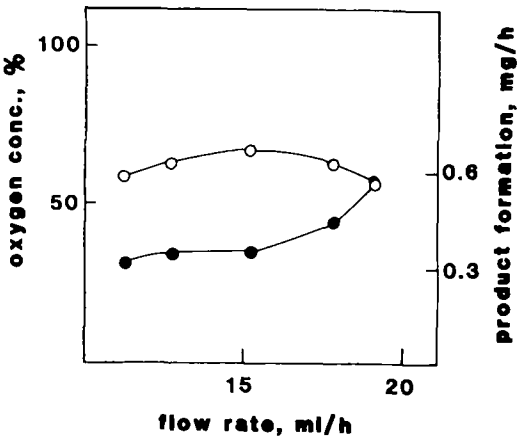


FIG. 3. The response of immobilized lipoxygeanse-reactor to increased substrate flow rates. Linoleic acid (0.1 mM) in 0.2 M Na-borate buffer, pH 9.0, was passed through the reactor column (8 mm id) filled with 0.8 ml of lipoxygenase-1 gel. The effluent corresponding to each flow rate was sampled for the spectrophotometric conjugated diene assay (–•–) after steady-state levels of oxygen were established (–o–).

Lipoxygenase-1 produces 15-hydroperoxy derivative from arachidonic acid (11). When linoleic acid in the elution buffer was replaced by arachidonic acid (0.1 mM), no change in oxygen or conjugated diene content of the effluent occurred, suggesting quantitative substrate utilization also in this case and the absence of double dioxygenation reaction.

Soybean lipoxygenase is stable (12) and the covalently coupled enzyme exhibited unchanged activity during six months of storage at 3 C in 0.2 M Na-borate buffer, pH 9.0. Therefore, the operational stability of the immobilized enzyme in the reactor remained the critical factor when the utility of the present method was evaluated, especially because the lipoxygenases are reported to undergo self-destruction when catalyzing the oxygenation reaction (13). The extent of this suicidal process was estimated by operating the reactor continuously for 125 hr at 25 C at its maximal capacity. Although any time-dependent changes in the activity should be immediately reflected as unbalanced oxygraphic and spectrophotometric readings, both of these monitoring systems indicated that the effluent remained unchanged in its composition.

It has been suggested that lipoxygenases are inactivated during the reaction either through cooxidation of the enzyme protein (14) or through the action of the hydroperoxides accumulated (13). Against this background, the observed increment in the half-life obtained by covalent coupling through amide bonds to agarose may either reflect a reduction or favorable rearrangement of targets on the enzyme, making it less susceptible to free radical-mediated cooxidation or, in the latter case, because of the flow-through type of reaction, the accumulation above inhibitory levels of hydroperoxides was eliminated due to limitations imposed by the solubility of oxygen.

Free radical species dissociated from the lipoxygenase-substrate complex are responsible for the nonspecific cooxidation of the fatty acid substrates and thereby reduce the enantiomorphous homogeneity of the hydroperoxide product (15). The formation of such radicals can be coupled to the chemiluminescent luminol oxidation (10) and, therefore, it was possible to compare the cooxidation potentials of soluble and immobilized lipoxygenases by sensitive luminometric measurements. The two enzymes gave immiscible patterns of light emission when oxygenating linoleic acid. Therefore, it is suggested that the primary mechanism of fatty acid oxygenation was not altered during enzyme immobilization. In contrast, immobilization reduced the total yield of chemiluminescence. Calculation of the integrated light yields in arbitrary units (cut and weight method) showed an average reduction of 97% in the lipoxygenase chemiluminescence. This may indicate that the

insoluble matrix absorbs the free radicals which are not actually participants in the oxygenation reaction, but rather dead end-products of limited stability. Covalent links to the matrix may also have changed the enzyme configuration unfavorably for free radical formation.

Samples for gas chromatographic product analyses were prepared either from reactor products collected during the 2- and 5.5-hr period or from the product of soluble lipoxygenase obtained from 0.1 mM linoleic acid at 25 C. After methylation, all the samples gave a single main peak appearing at 2.0 min (± 0.1 min) whereas methylated linoleic acid standard was eluted at 10.9 min (for conditions of chromatography, see Materials and Methods). The presence of unreacted linoleic acid could not be detected in any of the samples, whereas two impurities, each constituting less than 0.4% of the main peak, appeared at 13.2 and 13.8 min and were present in each case. These peaks probably represented "nonsubstrate" fatty acids which might have been present as trace impurities in the linoleic acid and were detected after accumulation in the product.

Silylation of the methylated products increased the retention time of the main peak to 3.2 min (± 0..2 min) and resulted in the appearance of an additional unidentified peak at 3.9 min. This peak constituted about 2% of the main peak in samples derived from soluble lipoxygenase reaction or from the reactor effluent after 2 hr collection whereas its proportion was increased up to 4% when the 5.5-hr collection time was used. Therefore, continuous extraction and/or derivatization of the reactor product might become necessary if purity above 96% is required.

Our preliminary experiments on immobilization of lipoxygenases from other sources suggest that the exceptionally high recovery of activity in the present study is not a general property of lipoxygenases but that values normally range from 10 to 15%. However, if they fulfill the stability requirements of continuous operation, controlled syntheses of different hydroperoxy derivatives of unsaturated fatty acids will be possible. At present, combinations of different lipoxygenase-reactors are being tested to obtain double dioxygenated arachidonic acids using a single continuous process.

ACKNOWLEDGMENTS

The skillful technical assistance of Dr. Kalevi Kurkijärvi and Dr. Timo Metsä-Ketelä with the immobilization of lipoxygenase and product analysis is greatly appreciated.

REFERENCES

1. Gardner, H.W. (1975) Lipids 10, 248-252.
2. Matthew, J., Chan, H.W.-S., and Galliard, T. (1977) Lipids 12, 324-326.
3. Yamamoto, A., Fujii, Y., Yasumoto, K., and Mitsuda, H. (1980) Lipids 15, 1-5.
4. Axelrod, B., Pistorius, E.K., and Christopher, J.P. (1972) Proc. Intern. World Congress Soc. Fat Res., 11th (abstr.), p. 117.
5. Ishimaru, A., and Yamazaki, I. (1977) J. Biol. Chem. 252, 6118-6124.
6. Chan, H.W.-S., Constaras, C.T., Prescott, F.A.A., and Swoboda, P.A.T. (1975) Biochim. Biophys. Acta 398, 347-350.
7. Axelrod, B., Cheesbrough, T.M., and Laakso, S. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) vol. 71, pp. 441-451, Academic Press, New York, NY.
8. Christopher, J.P., Pistorius, E., and Axelrod, B. (1970) Biochim. Biophys. Acta 198, 12-19.
9. Surrey, K. (1964) Plant Physiol. 39, 65-70.
10. Lilius, E.-M., and Laakso, S. (1982) Anal. Biochem. 119, 135-141.
11. Hamberg, M., and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329-5335.
12. Galpin, J.R., Tielens, L.G.M., Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1976) FEBS Lett. 69, 179-182.
13. Smith, W.L., and Lands, W.E.M. (1972) J. Biol. Chem. 247, 1038-1047.
14. Schewe, T., Wiesner, R., and Rapoport, S.M. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) vol. 71, pp. 430-441, Academic Press, New York, NY.
15. Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1977) Prog. Chem. Fats Other Lipids 15, 131-166.

[Revision received June 9, 1982]

Effect of Chlorpromazine on Rat Arterial Lipid Synthesis, in vitro

FRANK P. BELL* and **EDNA V. HUBERT**, *Diabetes and Atherosclerosis Research, The Upjohn Company, Kalamazoo, MI 49001*

ABSTRACT

The effect of chlorpromazine, a major tranquilizer, on arterial lipid metabolism was studied in vitro in rat aortas incubated with [^{14}C]acetate and [^{14}C]mevalonate as lipid precursors. Chlorpromazine at a level of 0.25 mM in the incubation medium significantly reduced the incorporation of [^{14}C]acetate into free fatty acids ($p < 0.01$) and total phospholipids ($p < 0.001$) but not triglycerides. Chlorpromazine also altered the pattern of arterial phospholipids synthesized from [^{14}C]acetate by significantly increasing the relative proportion of phosphatidylinositol plus phosphatidylserine ($p < 0.02$) and reducing the relative proportion of sphingomyelin ($p < 0.001$). [^{14}C]Acetate incorporation into the combined fractions of steryl esters plus hydrocarbons and sterols plus diglycerides was also significantly reduced ($p < 0.001$) by 0.25 mM chlorpromazine. Studies with [^{14}C]mevalonate showed that chlorpromazine is also an inhibitor of sterol biosynthesis in arterial tissues as evidenced by 35-40% reductions ($p < 0.05$) in the formation of ^{14}C-labeled squalene and C_{27} sterols.

Lipids 17:672-675, 1982.

INTRODUCTION

Chlorpromazine (CPZ), a major tranquilizer, modifies several aspects of lipid metabolism in vivo and in vitro. The drug is an inhibitor of lecithin:cholesterol acyltransferase (EC 2.3. 1.43, LCAT) in plasma from man and experimental animals (1), inhibits bile salt synthesis in the monkey (2), and alters the pattern of phospholipids synthesized by liver (3,4), brain (5) and various isolated cells (6,7). Therapeutic doses of CPZ in patients lead to hypercholesterolemia (8,9). Hypercholesterolemia also occurs in normal rabbits given CPZ (10) whereas in cholesterol-fed rabbits, CPZ exerts a hypocholesterolemic effect (10,11). Although this hypocholesterolemic effect may account for the less severe atherosclerosis observed in cholesterol-fed rabbits treated with CPZ (10,11), we have recently presented evidence that CPZ can directly modify arterial lipid synthesis as evidence by its ability to alter patterns of [^{14}C]oleate incorporation into lipids of rat and rabbit aortas in vitro (12,13). In the present studies, the effect of CPZ on lipid synthesis from [^{14}C]acetate was investigated in normal rat aorta in vitro.

MATERIALS AND METHODS

Animals

Normal male rats (Upj: TUC (SD) spf, 225-250 g) were used in the studies. All animals were individually housed with free access to food (Purina Chow) and water.

*Author to whom correspondence should be addressed.

Tissues

The rats were killed by decapitation. The aortas were quickly excised, rinsed in chilled 0.9% NaCl solution, stripped of adventitial tissue, and then opened longitudinally over their entire length.

Incubation

The aortas were incubated for 3 hr at 37 C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 5.0 μCi [1-^{14}C]acetic acid, sodium salt (SA 56.0 mCi/mmol) or 2.0 μCi DL-[2-^{14}C]mevalonic acid, dibenzylethylenediamine salt (SA 50.1 mCi/mmol) (14). All the isotopically labeled lipids were purchased from New England Nuclear Corp., Boston, MA. Chlorpromazine HCL was purchased from Sigma Chemical Co., St. Louis, MO, and added to all incubations dissolved in 25 μl saline.

Analyses

Following incubation with [^{14}C]acetate or [^{14}C]mevalonate, the aortas were rinsed in five 250-ml changes of 0.9% NaCl solution and then extracted by homogenization in $CHCl_3$/MeOH (2:1, v/v,) (14,15); 0.2 vol of 0.73% NaCl solution was added to the $CHCl_3$/MeOH extracts to cause phase separation (16). The lipid-containing lower phase was washed twice with pure upper phase as described by Folch et al. (16). A portion of the lipid extracts was fractionated by thin-layer chromatography (TLC) on glass plates coated with Silica Gel G using a developing solvent consisting of *n*-hexane/diethyl ether/acetic acid (146:50:4, v/v/v) (15,17). The

various lipid bands (fractions) were visualized under ultraviolet (UV) light after spraying the chromatoplates with rhodamine 6G (0.05% in ethanol) and were then scraped from the chromatoplates and assayed for radioactivity as previously described (17,18). In studies using [^{14}C] mevalonate as a precursor, the C_{27}-sterol and squalene fractions were scraped for radioactive assay (15,19). Additional portions of extract from three control samples and three chlorpromazine-treated samples were chromatographed as above and the squalene band was eluted from the TLC plates with $CHCl_3$. The eluate was evaporated under N_2, saponified with alcoholic-KOH (19), *n*-hexane extracted (19), and the hexane extracts were rechromatographed as above; 97-98% of the radioactivity was reisolated as squalene while 2-3% appeared exclusively in a band cochromatographing with C_{27}-sterols which indicated that 2-3% of the radioactivity originally chromatographing with squalene was steryl ester. Squalene data were not corrected for this small amount of steryl ester formed. In studies using [^{14}C]acetate, the lipid fractions corresponding to phospholipids, sterols plus diglycerides, free fatty acids, triglycerides, and steryl esters plus hydrocarbons were assayed for radioactivity. A second portion of the aortic lipid extracts from the [^{14}C]acetate experiments was fractionated by TLC to obtain the individual phospholipid classes by the method of Skipski et al. (20). The phospholipids were visualized with dichlorofluorescein, scraped from the chromatoplates and assayed for radioactivity in ACS-II counting fluid (Amersham Corp., Arlington Heights, IL).

RESULTS

The incorporation of [^{14}C]acetate into rat aortic lipids in vitro is shown in Table 1. The results indicate that 0.25 mM CPZ dramatically modifies the synthesis rat arterial lipids from [^{14}C]acetate. Incorporation of [^{14}C]acetate into phospholipids, and the combined fractions of sterols + diglycerides, and steryl esters + hydrocarbons was reduced 70-80% ($p < 0.001$) in the presence of CPZ. Synthesis of fatty acids was also significantly reduced ($p < 0.01$) by about 45% with CPZ but triglyceride synthesis was not statistically significantly affected.

Table 2 shows the results of fractionating the tissue phospholipids from the experiments of Table 1. It is clear that CPZ not only reduces the total incorporation of [^{14}C]acetate into phospholipids as given in Table 1, but also alters the pattern of acetate incorporation into the various phospholipids synthesized. The percentage of distribution of [^{14}C]acetate into phosphatidylcholine (PC) was about 50% and was not significantly affected ($p > 0.05$) by CPZ. However, the percentage distribution of [^{14}C] acetate into the combined fraction of phosphatidylserine (PS) plus phosphatidylinositol (PI) increased from 12 to 19% with CPZ treatment ($p < 0.02$). The percentage incorporation of [^{14}C]acetate into sphingomyelin was also significantly reduced from 7.9 to 3.8% ($p < 0.001$) with CPZ treatment whereas lysolecithin and phosphatidylethanolamine were not statistically affected.

The effects of CPZ on arterial lipid synthesis were not restricted to the saponifiable lipids. At a level of 0.25 mM, CPZ also exerted an inhibitory effect on the incorporation of [^{14}C] mevalonate into rat arterial nonsaponifiable lipids in vitro (Table 3). Incorporation into squalene, a C_{30} hydrocarbon intermediate in sterol synthesis, was significantly reduced ($p < 0.05$) by about 35%. This inhibition of squalene formation was reflected in a similar reduction (about 40%, $p < 0.05$) in the labeling of the C_{27}-sterol

TABLE 1

Effect of Chlorpromazine on the Incorporation of [1-^{14}C] Acetate into Various Lipids in the Normal Rat Aorta in vitro (dpm/g wet wt)[a]

	Phospholipids	Sterols + diglycerides	Free fatty acids	Triglycerides	Steryl esters + hydrocarbons
Control	20935 ± 3380[b]	7980 ± 1175	10470 ± 1315	4870 ± 820	605 ± 85
Chlorpromazine (0.25 mM)	5635[c] ± 1100	2180[c] ± 680	5855[d] ± 910	3465 ± 910	130[c] ± 25
Ratio: CPZ/control	0.27	0.27	0.56	0.71	0.27

[a]Aortas from normal male rats (225-250 g) were incubated 3 hr at 37 C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 5 μCi[1-^{14}C]acetic acid, sodium salt. Chlorpromazine (CPZ), when present, was added to the incubation medium in 25 μl saline to yield a final concentration of 0.25 mM. Lipids were extracted from the aortas with $CHCl_3$/MeOH (2:1, v/v) and the extracts were fractionated by TLC as described in Methods.

[b]Values are means ± SEM of 10 aortas per group.

[c,d]Significantly different from control values using Student's independent t-test (c: $p < 0.001$; d: $p < 0.01$).

TABLE 2

Effect of Chlorpromazine on the Percentage Distribution of [1-^{14}C]Acetate Incorporated into Phospholipids of Rat Aortas in vitro[a]

	Lyso[b]	Sph	PC	PS+PI	PE
Control	5.3 ± 1.0[c]	7.9 ± 0.5	50.0 ± 2.0	12.1 ± 2.0	24.7 ± 1.5
Chlorpromazine (0.25 mM)	3.4 ± 0.5	3.8 ± 0.4[d]	54.1 ± 1.3	19.4 ± 1.7[e]	20.3 ± 1.7

[a]See footnote to Table 1.

[b]Lyso, lysolecithin; Sph, sphingomyelin; PC, phosphatidylcholine; PS+PI, phosphatidylserine plus phosphatidylinositol; PE, phosphatidylethanolamine.

[c]Values are means ± SEM of 10 aortas per group.

[d,e]Significantly different from control values by Student's independent t-test (d: $p < 0.001$; e: $p < 0.02$).

TABLE 3

Effect of Chlorpromazine on the Incorporation of DL-[2-^{14}C]Mevalonate into Sterols and Squalene in the Normal Rat Aorta in vitro (dpm/g/wet wt)[a]

	Sterols	Squalene
Control	7215 ± 1110[b]	3370 ± 210
Chlorpromazine (0.25 mM)	4380 ± 625[c]	2260 ± 410[c]
Ratio: CPZ/Control	0.61	0.67

[a]Aortas from normal male rats (225-250 g) were incubated 3 hr at 37 C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 2.0 μCi DL-[2-^{14}C] mevalonic acid. Chlorpromazine (CPZ), when present, was added in 25 μl saline to yield a final concentration of 0.25 mM. Lipids were extracted from the aortas with $CHCl_3$/MeOH (2:1, v/v) and fractionated by TLC to separate the C_{27}-sterols and squalene as described in Methods.

[b]Values are means ± SEM of 5 control aortas and 6 chlorpromazine-treated aortas.

[c]Significantly different from control values ($p < 0.05$) using Student's independent t-test.

fraction of the arteries, as well (Table 3).

DISCUSSION

Agents that can alter lipid metabolism are of particular interest in view of the role of increased lipid synthesis and lipid accumulation during the atherogenic process (18). Our previous studies have shown that CPZ reduces the incorporation of exogenous preformed fatty acid (oleic acid) into various glycerolipids in rabbit and rat aortas in vitro (13), and inhibits arterial cholesterol esterification by ACAT (acylCoA:cholesterol acyltransferase, EC 2.3.1.26) (12). The present studies, using rat aortas, were undertaken to evaluate the effect of CPZ on the biosynthesis of fatty acids from [^{14}C]acetate and the utilization of the labeled fatty acids for neutral lipid and phospholipid synthesis. In addition, the effect of CPZ on nonsaponfiable lipid synthesis from [^{14}C]mevalonate was evaluated.

The effects of CPZ on rat arterial lipid synthesis as observed in the present studies are complex and are unlikely to be attributable to a single mechanism of action of CPZ. For instance, an inhibition of acetate activation to acetyl CoA could account for the decreased incorporation of [^{14}C]acetate into arterial free fatty acids (Table 1). Although a decrease in fatty acid synthesis could explain the decreased incorporation of [^{14}C]acetate into total arterial phospholipids, it does not explain the disproportionate labeling of the PS+PI and sphingomyelin fractions (Table 2) nor does it explain the apparent lack of effect of CPZ on [^{14}C]acetate incorporation into the triglyceride fraction of these tissues (Table 1). A possible explanation for the increase of PS+PI labeling may be a result of a partial inhibition in the activity of phosphatidate phosphohydrolase (EC 3.1.3.4) by CPZ as has been reported in liver (21,22). A decrease in the activity of this enzyme which converts phosphatidic acid into 1,2-diglyceride would result in an increased availability of phosphatidate for the formation of CDP-diacylglycerol which leads to PI. Since PI (and PS) are minor arterial phospholipids in terms of amount, a small change in activity of the enzyme could substantially affect the relative combined amount of these phospholipids without having obvious effects on the other lipids such as triglycerides and PC (Tables 1 and 2).

In this study, we did not attempt to fractionate the combined lipid fractions of sterols plus diglycerides and steryl esters plus hydrocarbons derived from [^{14}C]acetate. However, through the use of [^{14}C]mevalonate as a precursor of arterial lipids, it is clear that CPZ significantly

affects the synthesis of squalene (a C_{30} hydrocarbon) and the C_{27} sterols (Table 3). This inhibition of arterial sterol synthesis illustrates further the complex nature of CPZ action on the artery.

The effects of CPZ on arterial lipid synthesis as presented here have not been previously reported. There are, however, several reports that the administration of CPZ to rabbits fed atherogenic (cholesterol-containing) diet results in the development of less severe atherosclerosis than observed in untreated controls (10,11). The treatment of rabbits with CPZ, however, tended to reduce the level of hypercholesterolemia achieved and led to the tentative conclusion that the less severe development of atherosclerosis reflected differences in the plasma cholesterol levels, rather than a direct effect of CPZ. Our results as presented here, however, suggest the possibility that CPZ may have modified atheroma development in the rabbits by altering arterial lipid metabolism directly.

REFERENCES

1. Bell, F.P., and Hubert, E.V. (1981) Lipids 16, 815-819.
2. Ros, E., Small, D.M., and Carey, M.C. (1979) Eur. J. Clin. Invest. 9, 29-41.
3. Brindley, D.N., and Bowley, M. (1975) Biochem. J. 148, 461-469.
4. Ide, H., and Nakazawa, Y. (1980) J. Pharm. Dyn. 3, 612-619.
5. Hokin-Neaverson, M. (1980) Biochem. Pharmacol. 29, 2697-2700.
6. Allan, D., and Michell, R.H. (1975) Biochem. J. 148, 471-478.
7. Plantavid, M., Chap, H., Lloveras, J., and Douste-Blazy, L. (1981) Biochem. Pharmacol. 30, 293-297.
8. Clark, M.L., Ray, T.S., Paredes, A., Ragland, R.E., Costiloe, J.P., Smith, C.W., and Wolf, S. (1967) Psychosom. Med. 29, 634-642.
9. Clark, M., Dubowski, K., and Colmore, J. (1971) Clin. Pharmacol. Therap. 11, 883-889.
10. Wilens, S.L., McCluskey, R.T., and Somoza, C. (1956) Proc. Soc. Exp. Biol. Med. 93, 121-124.
11. LaFrance, L., and LeBlanc, J. (1967) Can. J. Physiol. Pharmacol. 45, 215-224.
12. Bell, F.P., and Hubert, E.V. (1981) Atherosclerosis 39, 517-525.
13. Bell, F.P. Exp. Mol. Pathol., in press.
14. Bell, F.P. (1981) Atherosclerosis 38, 81-88.
15. Bell, F.P. (1976) Exp. Mol. Path. 25, 279-292.
16. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
17. Bell, F.P., and Nazir, D.J. (1976) Lipids 11, 216-221.
18. Day, A.J., Bell, F.P., Moore, S., and Friedman, R. (1974) Circ. Res. 34, 467-476.
19. Bell, F.P. (1976) Lipids 11, 769-773.
20. Skipski, V.P., Peterson, R.F., and Barclay, M. (1964) Biochem. J. 90, 374-378.
21. Brindley, D.N., Allan, D., and Michell, R.H. (1975) J. Pharm. Pharmac. 27, 462-464.
22. Brindley, D.N., Bowley, M., Sturton, R.G., Pritchard, P.H., Burditt, S.L., and Cooling, J. (1977) Biochem. Soc. Trans. 5, 40-43.

[Received April 19, 1982]

Metabolism of Arachidonic Acid in Leukocytes: Isolation of a 5,15-Dihydroxy-Eicosatetraenoic Acid[1]

PIERRE BORGEAT*, SERGE PICARD, JEAN DRAPEAU and **PIERRE VALLERAND,**
Laboratoire d'Endocrinologie Moléculaire, Le Centre Hospitalier de l'Université Laval, Quebec, Canada G1V 4G2

ABSTRACT

A novel metabolite of arachidonic acid was isolated from incubations of peripheral blood leukocytes with the fatty acid and the ionophore A23187. The compound was purified by high performance liquid chromatography and identified by ultraviolet photometry and gas chromatography-mass spectrometry as a 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid. The compound was also isolated from incubations of human leukocytes with the 15S-hydroperoxy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid, suggesting a double dioxygenation mechanism in the formation of this new metabolite of arachidonic acid. A 5S,15S-dihydroxy-6,8,11,13(E,Z,Z,E)-eicosatetraenoic acid was obtained from incubations of 5S-hydroxy-6,8,11,14(E,Z,Z,Z)-eicosatetraenoic acid with the soybean lipoxygenase and reduction with stannous chloride, and was used as reference compound.
Lipids 17:676-681, 1982.

INTRODUCTION

In the last few years, it has been shown that leukocytes transform arachidonic acid into a family of bioactive compounds, the leukotrienes, in a reaction involving a lipoxygenase acting specifically at carbon-5 of the fatty acid (ref. 1 for a review). Other metabolites of arachidonic acid oxygenated at C-15 were isolated from human leukocytes, i.e., the 15S-HETE (2) and several isomeric 14,15-diHETEs and 8,15-diHETEs (3-5), suggesting the presence of a lipoxygenase specific for carbon-15 of arachidonic acid in these cells. Such an enzyme has recently been isolated from rabbit peritoneal neutrophils (6). In a previous study (7), we have reported that arachidonic acid can undergo a double dioxygenation by two leukocyte lipoxygenases with C-5 and C-12 specificities to yield the 5S,12S-diHETE. The presence of a lipoxygenase with C-15 specificity in leukocytes has led us to investigate the formation of a 5,15-diHETE in these cells.

*Author to whom correspondence should be addressed.

Abbreviations: GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; Me, methyl ester; Me_3Si, trimethylsilyl; PG, prostaglandin; LTB_4, leukotriene B_4, 5S,12R-dihydroxy-6,8,10,14(Z,E,E,Z)-eicosatetraenoic acid; 5S,12S-diHETE, 5S,12S-dihydroxy-6,8,10,14(E,Z,E,Z)-eicosatetraenoic acid; 12-HETE, 12S-hydroxy-5,8,10,14 (Z,Z,E,Z)-eicosatetraenoic acid; HHT, 12S-hydroxy-5,8,10 (Z,E,E)-heptadecatrienoic acid; 15S-HETE, 15S-hydroxy-5,8,11,13 (Z,Z,Z,E)-eicosatetraenoic acid; 15S-HPETE, 15S-hydroperoxy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid; EDTA, ethylene diamine tetraacetate.

[1]See ref. 16 for a preliminary report.

MATERIALS AND PROCEDURES

Arachidonic acid (>99% pure, from Nu-Chek-Prep, Elysian, MN), was purified by silicic acid chromatography before use. A stock solution of the ionophore A23187 (mixed sodium and potassium salts, Calbiochem, La Jolla, CA) was prepared in ethanol (3 mg/ml). The soybean lipoxygenase (Type 1 from Sigma Co., St. Louis, MO) and the methods described previously (8) were used to prepare the 15S-HPETE from arachidonic acid; the 15S-HETE was prepared by chemical reduction of the 15S-HPETE with stannous chloride; 5S-HETE was obtained from incubations of leukocytes with arachidonic acid and the ionophore (7). The compounds were purified by HPLC and analyzed by GC-MS (HETE only) for assessment of purity and identity.

Preparation and Incubation of Leukocytes

Porcine leukocyte suspensions were prepared by centrifugation of sodium EDTA-treated blood, dextran sedimentation and ammonium chloride lysis of red cells, as described before (7). Human leukocytes were prepared using similar procedures. The mononuclear and polymorphonuclear leukocytes were not separated. The cell preparations were slightly contaminated with platelets. Porcine leukocytes were suspended (250×10^6 cells/ml) in Dulbecco's phosphate buffered saline (9) (without calcium and magnesium). The cell suspensions were then warmed to 37 C and calcium and magnesium chlorides, arachidonic acid and the ionophore A23187 were added to final concentrations of 0.5 mM, 2.0 mM, 170 μM and 17 μM,

respectively, if not otherwise indicated. After 5 min, the reactions were stopped by addition of 1.5 vol of methanol. Human leukocytes were incubated as described in the legend to Figure 5 (vide infra).

Extraction and Chromatography

Ether extractions and fractionations on silicic acid columns were performed as described (7). HPLC analysis of the fraction of the ether extract containing HETEs and diHETEs was performed on a 50 x 1 cm column packed with silica gel (Li Chroprep Si60, 15- to 25-μm particles, E. Merck, Darmstadt, Germany) using a linear gradient of isopropanol in hexane (0.1% acetic acid) from 0 to 15% in 30 min at a flow of 4 ml/min. Elution of arachidonic acid metabolites was monitored at 235 and 280 nm. The materials in absorption peaks was collected; the compounds were treated with diazomethane to form the methyl esters and further purified on a silica gel column (Hibar II, 4.7 x 250 mm, Merck) using isopropanol/hexane (4:96, v/v) as solvent at 1 ml/min. The compounds obtained were finally purified by reversed-phase HPLC using a RadialPak C_{18} column (octadecyl silica, 10-μm particles, 100 x 8 mm from Waters Associates, Milford, MA) using methanol/water (80:20, v/v) at a flow of 2 ml/min and 20 C.

Gas Chromatography-Mass Spectrometry

The Me_3Si ether derivatives and the hydrogenated compounds were prepared as described (7,10). GC-MS analyses were performed as described before (7) using glass columns (6 ft long, 2 mm id) packed with SE-30 1% on Chromosorb W-HP (80/100 mesh), or OV-210 3% on Supelcoport (100/120 mesh).

RESULTS

Porcine peripheral blood leukocytes were incubated with arachidonic acid and the ionophore A23187. The fraction from the silicic acid chromatography of the ether extract containing the HETEs and diHETEs was analyzed by adsorption HPLC with detection at 235 and 280 nm. In addition to compounds previously identified, i.e., 5S-HETE, 12S-HETE, 15S-HETE, HHT, 5S,12S-diHETE, LTB_4, Δ^6-*trans*-12-epi-LTB_4 and Δ^6-*trans*-LTB_4, and absorption peak (235 nm) which did not correspond to any known metabolite of arachidonic acid was dedetected. The material was collected, esterified and further purified by adsorption and reversed-phase HPLC as described under "Extraction and Chromatography." A total of about 50 μg of the unknown product (as estimated by ultraviolet [UV] photometry using the molar absorption coefficient of 33,500 reported for the 5S,15S-diHETE; see ref. 11) were obtained from three incubations, i.e., about 0.15% of added arachidonic acid (40 x 10^9 leukocytes were incubated at a time). In the same experiments, the yield of LTB_4 was 10-20 times higher.

Ultraviolet Spectrometry

The UV spectrum of the unknown material showed a major band at λmax (MeOH) = 243 nm and a shoulder at 226 nm (5,15-diHETE in Fig. 1; the absorption coefficient was not measured). This spectrum is compatible with a conjugated diene chromophore. The UV spectra of HHT, 5S-HETE are shown in Figure 1 for comparison.

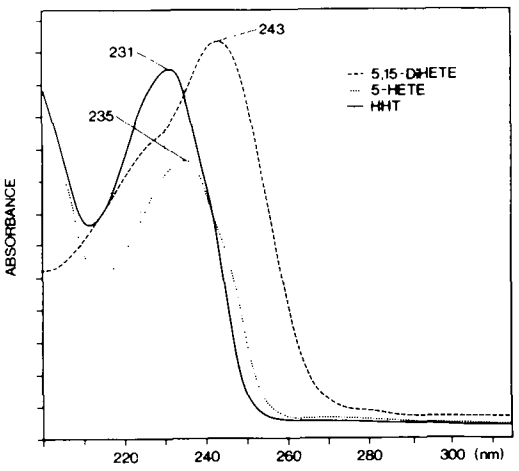


FIG. 1. Ultraviolet spectra of the 5,15-diHETE isolated from porcine leukocytes and of 5S-HETE and HHT. Spectra were recorded in methanol. The intensities of the absorption bands do not reflect the actual molar absorption coefficients of the three compounds.

Analytical HPLC

The chromatographic behavior of the new metabolite was studied on silica gel and octadecyl silica columns using isocratic elution systems (Table 1). In adsorption chromatography, the compound (5,15-diHETE in Fig. 2) showed a polarity intermediate to that of PGB_2 and 5S-HETE; in the reversed-phase HPLC system used, the compound was eluted between PGB_2 and LTB_4. The details of chromatographic conditions are given in the legend to Figure 2.

Gas Chromatography-Mass Spectrometry

GC-MS analysis of the Me_3Si ether derivative of the methyl ester of the unknown compound showed a peak with a C-value of 23.6 or 23.9 (columns SE-30 1% and OV-210 3%, re-

TABLE 1

Liquid Chromatography Data

Columns:	Silica gel[a]	C_{18}[b]	C_{18}[c]	C_{18}[d]
Compounds		k'(capacity factor)		
PGB_2	14.1*	2.4*	3.0	2.2
5,15-diHETE	5.4*	3.4*	4.3	3.2
LTB_4	–	4.2*	5.3	3.9
HHT	–	5.9*	7.2	5.3
15-HETE	–	11.6*	15.5	11.3
5-HETE	2.4*	–	–	15.7

*Values for the methyl esters of the compounds.

[a]Column: 4.7 X 250 mm packed with Nucleosil 50, 5- μm particles (Macherey Nagel, W. Germany). Solvent: isopropanol/hexane (4:96, v/v), 1 ml/min, 20 C.

[b]Column: Ultrasphere ODS, 4.7 X 250 mm, 5- μm particles (Beckman Instruments). Solvent: methanol/water (80:20, v/v), 1 ml/min, 20 C.

[c]Column: Radial Pak C_{18}, 5 X 100 mm, 10- μm particles (Waters Assoc.). Solvent: methanol/water (75:25, v/v) containing 0.01% acetic acid, 1 ml/min, 20 C.

[d]Column: see note b. Solvent: see note c.

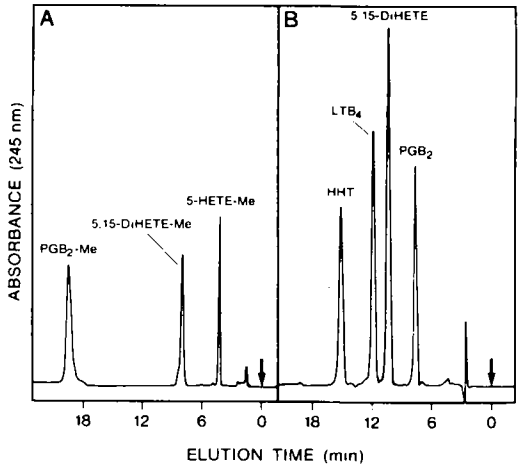


FIG. 2. (A) Adsorption HPLC chromatogram of the methyl esters of 5S-HETE, 5,15-diHETE and PGB_2. The column used (250 X 4.7 mm) was packed with silica gel (5 μm particles, Nucleosil 50-5 from Macherey-Nagel Co., W. Germany). The compounds were eluted with hexane/isopropanol (96:4, v/v) at 1 ml/min and 20 C. (B) Reversed-phase HPLC chromatogram of a mixture of PGB_2, 5,15-diHETE, LTB_4 and HHT. The column used was an Ultrasphere ODS (C_{18}) (250 X 4.7 mm, 5- μm particles, from Beckman Instruments). The compounds were eluted with methanol/water (70:30, v/v) (containing 0.01% acetic acid), at 1 ml/ min and 20 C. Arrows indicate injections.

spectively). The mass spectrum of this compound (Fig. 3) showed ions at m/e 494 (M), 479 (M-15), 463 (M-31), 423 (M-71, loss of $.CH_2\text{-}(CH_2)_3\text{-}CH_3$), 404 (M-90, loss of trimethylsilanol), 394 (probably $.CH = CH - CH = CH - CH_2\text{-}(CH = CH)_2 - CH(OSiMe_3)\text{-}(CH_2)_3\text{-}C(OCH_3) = O^+SiMe_3$, from a rearrangement; see ref. 12), 393 (M-101, loss of $.CH_2\text{-}(CH_2)_2\text{-}COOCH_3$), 333 [M-(71 + 90)], 303 [M-(101 + 90)], 255 $[(CH = CH)_2\text{-}CH(OSiMe_3)\text{-}(CH_2)_3\text{-}COOCH_3]^+$, 225 $[(CH = CH)_2\text{-}CH(OSiMe_3)\text{-}(CH_2)_4\text{-}CH_3]^+$, 203 ($Me_3SiO^+ = CH\text{-}(CH_2)_3\text{-}COOCH_3$), 173 (base peak, $Me_3SiO^+ = CH\text{-}(CH_2)_4\text{-}CH_3$), 159, 129, 113, 103, 99, 75 and 73, supporting the Me_3Si ether, methyl ester derivative of a 5,15-dihydroxy-(6,8,11,13)-eicosatetraenoic acid. The mass spectrum (not shown) of the Me_3Si ether, ethyl ester derivative (obtained by treatment of the acid with diazoethane) was in agreement with the interpretation of the ions given above for the Me_3Si ether, methyl ester derivative.

A few μg of the substance were subjected to catalytic hydrogenation over platinum oxide (10) and analyzed by GC-MS as the methyl ester, Me_3Si ether derivative: a peak with a C-value of 24.2 (column SE-30 1%) was observed. The mass spectrum (Fig. 4) showed ions at m/e 487 (M-15), 471 (M-31), 431 (M-71, loss of $.CH_2\text{-}(CH_2)_3\text{-}CH_3$), 402 (probably $.CH\text{-}(CH_2)_8\text{-}CH(OSiMe_3)\text{-}(CH_2)_3\text{-}C(OCH_3) = O^+SiMe_3$, from a rearrangement; see ref. 12), 401 (M-101, loss of $.CH_2\text{-}(CH_2)_2\text{-}COOCH_3$), 341 [M-(71 + 90)], 311 [M-(101 + 90)], 203 (base peak, $Me_3SiO^+ = CH\text{-}(CH_2)_3 - COOCH_3$) and 173 ($Me_3SiO^+ = CH\text{-}(CH_2)_4 - CH_3$). This spectrum showed conclusively that the hydrogenated substance was a 5,15-dihydroxy eicosanoic acid. Comparison of the mass spectra of the unsaturated and saturated compounds confirmed the presence of four double bonds located be-

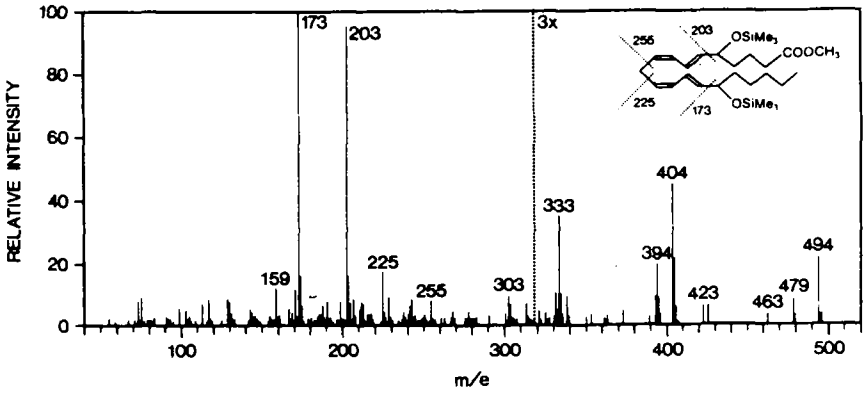


FIG. 3. Mass spectrum of the Me_3Si derivative of the methyl ester of the 5,15-diHETE isolated from porcine leukocytes (electron impact, 25 eV).

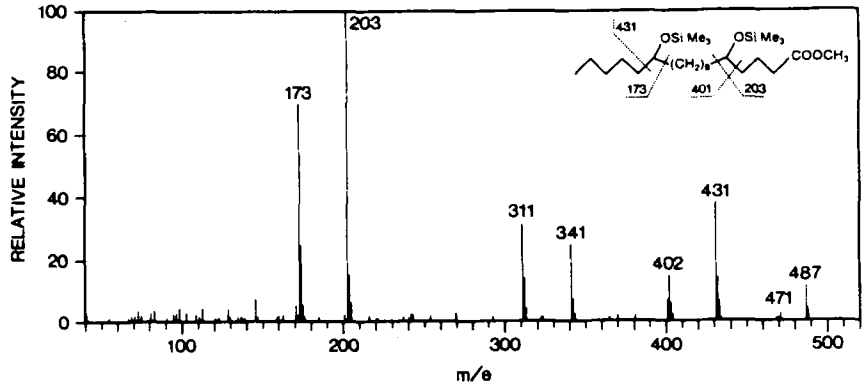


FIG. 4. Mass spectrum of the Me_3Si derivative of the methyl ester of the hydrogenated 5,15-diHETE (electron impact, 25 eV).

tween C-5 and C-15. The UV spectrometry data (supporting the presence of a conjugated diene structure) as well as the ions at m/e 203, 229 (.CH=CH-CH(OSiMe$_3$)-(CH$_2$)$_3$-COOCH$_3$)$^+$, 255 225, 199 (.CH=CH-CH(OSiMe$_3$)-(CH$_2$)$_4$-CH$_3$)$^+$ and 173 in the mass spectrum of the methyl ester, Me_3Si ether of the compound (unsaturated) supported that double bonds were located at Δ^6, Δ^8, Δ^{11} and Δ^{13}. The structure of the compound was thus established as 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid.

Biosynthesis of a 5S,15S-diHETE from 5S-HETE and Soybean Lipoxygenase

It has been shown recently that soybean lipoxygenase transforms arachidonic acid into dihydroperoxy derivatives (8,15- and 5,15-dihydroperoxy acids) with the "S" configurations in a two-step mechanism (11,13). We have used this enzyme to prepare a reference compound (a 5,15-diHETE) for the assessment of the present structural analysis of the new arachidonic acid metabolite.

Soybean lipoxygenase (Sigma Type I, 100 μg/ml in sodium borate buffer, pH 10) was incubated under an atmosphere of air with 5S-HETE at the concentration of 10 μg/ml. After 30 min at room temperature, the reaction mixture was acidified to pH 3 and extracted with ether. The extract was reduced with stannous chloride and fractionated on a silicic acid column as described (7); the fraction containing the 5,15-diHETE (as detected by UV absorbance) was purified by reversed-phase HPLC (see Materials and Procedures) using methanol/water (75:25, v/v, 0.01% acetic acid). About 100 μg (50% yield) of 5S,15S-diHETE was obtained. The material showed high degree of purity (>95%) as judged by adsorption HPLC and GC-MS analysis and was identical (in terms of UV spectrum and mass spectrum of the hydrogenated form)

to the 5S,15S-diHETE obtained from double dioxygenation of arachidonic acid by the soybean lipoxygenase (11).

Considering the stereochemistry of the substrate used (natural 5S-HETE; see ref. 14) and the stereospecificity of the soybean lipoxygenase reaction (11), we assumed that the detailed structure of the product obtained upon reaction of 5S-HETE with the enzyme was 5S,15S-dihydroxy-6,8,11,13-(E,Z,Z,E)eicosatetraenoic acid.

This compound and the leukocyte 5,15-diHETE were compared rigorously; they were found to be undistinguishable on the basis of their UV spectra, chromatographic properties (GC, adsorption and reversed-phase HPLC) and mass spectra (data not shown) suggesting that the two compounds were identical.

Formation of a 5,15-diHETE from 15S-HPETE in Human Leukocytes

Human blood leukocytes (mixed cells) were incubated with 15S-HETE or 15S-HPETE in the presence of the ionophore A23187 (see legend to Fig. 5). Both substrates gave rise to the formation of a 5,15-diHETE. Figure 5 shows a HPLC chromatogram of the products obtained upon incubation of human leukocytes with 15S-HPETE and the ionophore A23187. The metabolites of the C-5 lipoxygenase, i.e., LTB_4, Δ^6-*trans*-12-epi-LTB_4, Δ^6-*trans*-LTB_4 and 5S-HETE (derived from the endogenous arachidonic acid released by the ionophore) were detected, as well as the 5,15-diHETE. Comparisons (UV spectrometry, HPLC and GC-MS) of this product with the reference 5S,15S-diHETE indicated that both compounds were identical, in agreement with the stereochemistry of the substrates (15S-HPETE or 15S-HETE) used and the stereospecificity of the enzymatic reaction likely involved in their transformation (reaction with the C-5 lipoxygenase; ref. 14).

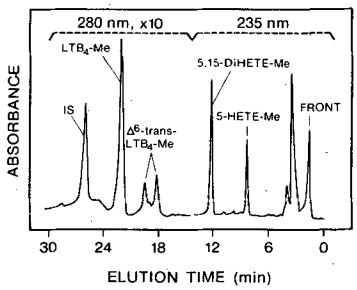


FIG. 5. Silica gel HPLC chromatogram (for column, see legend to Fig. 2A) of the products (methyl esters) contained in the diethylether/methanol (95:5, v/v) fraction of the silicic acid fractionation of the ether extract. Human blood leukocytes (60×10^6/2 ml) were incubated 5 min at 37 C with 15-HPETE (7.5 μM) and the ionophore A23187 (2 μM) before addition of 3 ml of methanol containing 500 ng of the internal standard (IS,5S-hydroxy-12-(2-hydroxy)ethoxy-6,8,10,14(E,E,E,Z)-eicosatetraenoic acid). The sample was dissolved in diethyl ether for injection. The compounds were eluted using gradients of isopropranol in hexane, at 2 ml/min and 20 C, as follows: 0-1 min, 1%; 1-7 min, 1 to 3.8%; 7-8 min, 3.8 to 4.5%; 8-19 min, 4.5 to 5.6%; 19-22 min, 5.6 to 8.7%; 22-28 min, 8.7%. The elution was monitored by ultraviolet photometry at 235 nm (0-14 min) and 280 nm (14-30 min). The Δ^6-*trans*-LTB_4 are epimeric at C-12, the first eluting compound being the 12R-hydroxy isomer (10). The amounts (approximate) of each compound formed, as measured by comparison of peak areas (corrected for differences in attenuation settings and absorption coefficients) were: LTB_4, 0.9 μg; Δ^6-*trans*-LTB_4, 0.1 μg (each); 5,15-diHETE, 4 μg; 5-HETE, 2.5 μg. The material eluting after the front peak contained some 15-HETE.

DISCUSSION

In this paper, we have reported the formation of a novel metabolite of arachidonic acid in leukocytes; GC-MS analysis, UV photometry together with HPLC data conclusively demonstrate that the compound is a 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid.

The geometry of the double bonds and the configuration of the alcohols have not been analyzed in these studies. However, biogenetic considerations as well as comparison of the compound with the 5S,15S-diHETE obtained from incubation of 5S-HETE with the soybean lipoxygenase suggest that the detailed structure of the new metabolite is the 5S,15S-dihydroxy-6,8,11,13(E,Z,Z,E)-eicosatetraenoic acid.

The presence in this compound of two conjugated double bonds α to the hydroxyl groups (a structural characteristic of several lipoxygenase products; see refs. 8,11, and 14), as well as its formation from 15S-HETE or 15S-HPETE in leukocytes, support a mechanism of synthesis involving two dioxygenations (Fig. 6), in analogy with the mechanism of formation of the 5S,12S-diHETE (15). The presence of both C-5 and C-15 lipoxygenase activities in leukocytes (2) supports the postulated mechanism of biosynthesis of the 5,15-diHETE.

The biological significance of this novel transformation of arachidonic acid in leukocytes is under investigation.

ACKNOWLEDGMENTS

This work was supported by a grant of the National Cancer Institute of Canada to P. Borgeat.

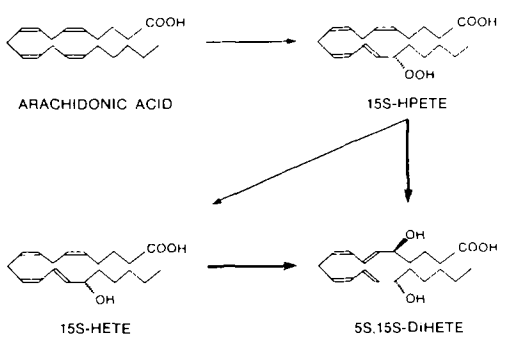


FIG. 6. Hypothetical mechanism of formation of the 5,15-diHETE in leukocytes involving the successive reactions of arachidonic acid with the C-15 and the C-5 lipoxygenases. The similar mechanism involving the reaction of arachidonic acid with the C-5 lipoxygenase first and then with the C-15 lipoxygenase, has not been excluded. The stereochemistry of the 5,15-diHETE as depicted above is based on biogenetic considerations but has not been conclusively demonstrated.

REFERENCES

1. Borgeat, P., and Sirois, P. (1981) J. Med. Chem. 24, 121-126.
2. Borgeat, P., and Samuelsson, B. (1979) Proc. Natl. Acad. Sci. USA 76, 2148-2152.
3. Jubiz, W., Radmark, O., Lindgren, J.-Å., Malmsten, C., and Samuelsson, B. (1981) Biochem. Biophys. Res. Commun. 99, 976-986.
4. Lundberg, U., Radmark, O., Malmsten, C., and Samuelsson, B. (1981) FEBS Lett. 126, 127-132.
5. Maas, R.L., Brash, A.R., and Oates, J.A. (1981) Proc. Natl. Acad. Sci. USA 78, 5523-5527.
6. Narumiya, S., Salmon, J.A., Cottee, F.H., Weatherley, B.C., and Flower, R.J. (1981) J. Biol. Chem. 256, 9583-9592.
7. Borgeat, P., Picard, S., Vallerand, P., and Sirois, P. (1981) Prostaglan. Med. 6, 557-570.
8. Gardner, H.W. (1975) Lipids 10, 248-252.
9. Dulbecco, R., and Vogt, M. (1954) J. Exp. Med. 99, 167-182.
10. Borgeat, P., and Samuelsson, B. (1979) J. Biol. Chem. 254, 7865-7869.
11. Van Os, C.P.A., Rijke-Schilder, G.P.M., Van Halbeek, H., Verhagen, J., and Vliegenthart, J.F.G. (1981) Biochim. Biophys. Acta 663, 177-193.
12. Eglington, G., Hunneman, D.H., and McCormick, A. (1968) Anal. Biochem. 22, 142-153.
13. Bild, G.S., Ramadoss, C.S., and Axelrod, B. (1977) Arch. Biochem. Biophys. 184, 36-41.
14. Borgeat, P., Hamberg, M., and Samuelsson, B. (1977) J. Biol. Chem. 251, 7816-7820; correction (1977) Ibid. 252, 8772.
15. Borgeat, P., Fruteau de Laclos, B., Picard, S., Drapeau, J., Vallerand, P., and Corey, E.J. (1982) Prostaglandins 23, 713-724.
16. Borgeat, P., Fruteau de Laclos, B., Picard, S., Vallerand, P., and Sirois, P. (1982) in Leukotrienes and Other Lipoxygenase Products, pp. 45-51, Raven Press, New York, NY.

[Received May 17, 1982]

The Effects of Phosphate on the Biosynthesis of Cholesterol in Rat Liver Homogenates

S. STEVEN HOTTA, *Department of Biochemistry, Eastern Virginia Medical School, Norfolk, VA 23501*

ABSTRACT

The biosyntheses of cholesterol from acetate and mevalonate were determined in rat liver homogenates that were prepared and incubated in buffers containing varying concentrations of phosphate. Relatively little acetate or mevalonate was incorporated into cholesterol in the absence of added phosphate. When phosphate was added, there was an increase in incorporation of both substrates. The addition of phosphate resulted in an increase in the incorporation of mevalonate to a maximum, whereas phosphate appeared to increase the incorporation of acetate at low phosphate levels and decrease the incorporation at higher phosphate levels. The results appear to be consistent with the possibility that, at low phosphate levels, the biosynthesis of cholesterol is limited by some phosphate-requiring reaction(s) in the pathway after mevalonate, and at higher phosphate levels, the biosynthesis is limited by the 3-hydroxy-3-methylglutaryl coenzyme A reductase-catalyzed step.
Lipids 17:682-685, 1982.

The hepatic biosynthesis of cholesterol from acetate has been shown to be regulated by a variety of factors (1,2). Most of the investigations on the regulation of this pathway have been focused on the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (3-7). The available evidence suggests that this regulation of cholesterol synthesis is determined by the effects of the regulatory factors on the amount and/or activity of this enzyme and that the reaction catalyzed by HMG-CoA reductase is the rate-limiting step in the biosynthetic pathway (8-10). Recent reports (11, 12) suggested that the activity of another enzyme, 5-pyrophosphomevalonate decarboxylase, may also be subject to control and may contribute to the regulation of cholesterol biosynthesis.

In the course of our studies of the hepatic synthesis of cholesterol, we found that the buffer used for the preparation and incubation of the liver homogenates affected the amounts of acetate and mevalonate incorporated into cholesterol. Further studies showed the effects appeared to depend on the presence or absence of phosphate in the buffer (13). Thus, the present investigations were undertaken to determine the effects of varying phosphate concentrations in the buffers on the incorporations of acetate and mevalonate into cholesterol by rat liver homogenates.

MATERIALS AND METHODS

Male, Wistar strain rats, weighing 200-300 g, were obtained from the Hilltop Laboratories. The rats were maintained on normal rat chow in a room with a 12-hr light/dark cycle (light on at 7 a.m. and off at 7 p.m.). Sodium [1-^{14}C]-acetate and DL-[2-^{14}C]mevalonolactone were obtained from New England Nuclear Corp. DL-Mevalonolactone, coenzyme A, NADP, ATP and glucose-6-phosphate were obtained from Sigma Chemical Co.

The rats were sacrificed by cervical fracture between 9 a.m. and 10 a.m. Their livers were removed and placed in ice-cold buffer. The buffers used for the preparation of the homogenate varied with the experiment and are given in the legends accompanying the figures and tables. Homogenates were prepared with 2.5 vol of the appropriate buffer (pH in all buffers was 7.8) by five passes of a loosely fitting Teflon pestle in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 x g for 15 min at 4 C. After aliquots of the supernate were preincubated at 37.5 C for 20 min, the substrate and cofactors were added and the incubation continued at 37.5 C for 1 hr. The final vol of the incubation media was 1.0 ml and contained the following substrate and cofactors (final concentrations): 2.0 mM [1-^{14}C]acetate (0.25 mCi/mmol) + 0.050 mM coenzyme A or 0.5 mM [2-^{14}C]-mevalonolactone (0.25 mCi/mmol), 1 mM ATP, 0.5 mM NADP, and 3.0 mM glucose-6-phosphate. The incubation was terminated by adding 2 ml 95% ethanol and 0.5 ml 60% (w/v) KOH. The mixture was heated at 80 C for 90 min to saponify the lipids. Cholesterol was extracted with hexane and isolated as the digitonide. The cholesterol digitonide was suspended in a scintillation cocktail and the amount of ^{14}C incorporated into cholesterol

was determined with the Beckman LS 7000 liquid scintillation counter. Proteins were determined by the method of Lowry et al. (14).

RESULTS

Our initial experiments were done with homogenates prepared and incubated in a buffer containing 100 mM Tris and 10 mM phosphate according to the procedures described by Goodwin and Margolis (15). The results obtained with acetate as the substrate were similar to their findings, but differed when mevalonate was used. Preincubation of our homogenates resulted in increases in incorporations of both acetate and mevalonate into cholesterol, whereas they had not observed any increase in mevalonate incorporation with preincubation. However, when more phosphate was present in the buffer, the results with acetate were relatively unchanged (Table 1), whereas that with mevalonate showed that, as the phosphate level was increased, the incorporation of mevalonate increased in the control, without a corresponding increase in the preincubated homogenates, such that preincubation no longer increased the mevalonate into cholesterol when phosphate was present in higher concentrations (Table 2).

When the phosphate contents of the buffer were varied, the incorporation of acetate into cholesterol underwent a biphasic response to the added phosphate (Fig. 1). In the absence of added phosphate, there was relatively little incorporation of acetate into cholesterol in the homogenate (control) unless the homogenate was preincubated. The presence of 2.5 mM phosphate in the buffer increased the incorporation dramatically. However, the addition of more phosphate to the buffer resulted in a progressive decrease in acetate incorporation, which approached the incorporation seen in the absence of added phosphate. Whether phosphate was absent or present in the buffer, preincubation of the homogenate always resulted in an increased incorporation of acetate into

TABLE 1

Effect of Phosphate on Cholesterol Synthesis from Acetate[a]

Buffer		[1-^{14}C]Acetate incorporation		
Tris (mM)	Phosphate (mM)	Control (pmol/hr/mg protein)	Preincubated (pmol/hr/mg protein)	Preincubated/control
100	10	61.4 ± 37.7	168.6 ± 98.5	3.25 ± 1.08
75	25	26.5 ± 16.0	166.6 ± 105.1	5.91 ± 1.53
50	50	38.1 ± 23.5	214.3 ± 134.7	6.45 ± 1.35
0	100	35.5 ± 20.8	166.4 ± 103.7	5.56 ± 1.35

[a]Portions of livers were homogenized with 2.5 vol of buffers containing varying amounts of Tris and phosphate. All buffers were pH 7.8 and contained 30 mM nicotinamide, 0.1 mM EDTA, 0.6 mM $MgCl_2$ and 5 mM glutathione. Aliquots of the 12,000 X g supernates were preincubated with shaking at 37.5 C for 20 min. Cholesterol synthesis was initiated in the unincubated control and preincubated preparations by addition of substrate and cofactors to the following concentrations: sodium [1-^{14}C]-acetate (2.0 mM, 0.25 mCi/mmol), coenzyme A (50 μM), ATP (1.0 mM), NADP (0.5 mM), and glucose-6-phosphate (3.0 mM). The data represent the incorporation of ^{14}C into cholesterol as the mean ± SEM.

TABLE 2

Effect of Phosphate on Cholesterol Synthesis from Mevalonate[a]

Buffer		[2-^{14}C]Mevalonate incorporation		
Tris (mM)	Phosphate (mM)	Control (pmol/hr/mg protein)	Preincubated (pmol/hr/mg protein)	Preincubated/control
100	10	174.4 ± 44.2	699.1 ± 230.9	4.17 ± 0.66
75	25	449.7 ± 113.2	547.8 ± 218.1	1.02 ± 0.18
50	50	532.8 ± 170.4	551.3 ± 237.8	0.99 ± 0.16
0	100	605.8 ± 244.4	546.6 ± 203.9	0.94 ± 0.10

[a]The buffers used and the procedures were as given in the legend to Table 1, except for the substitution of DL[2-^{14}C]mevalonolactone (0.5 mM, 0.25 mCi/mmol) in place of sodium [1-^{14}C]acetate and coenzyme A. The results are given as the mean ± SEM.

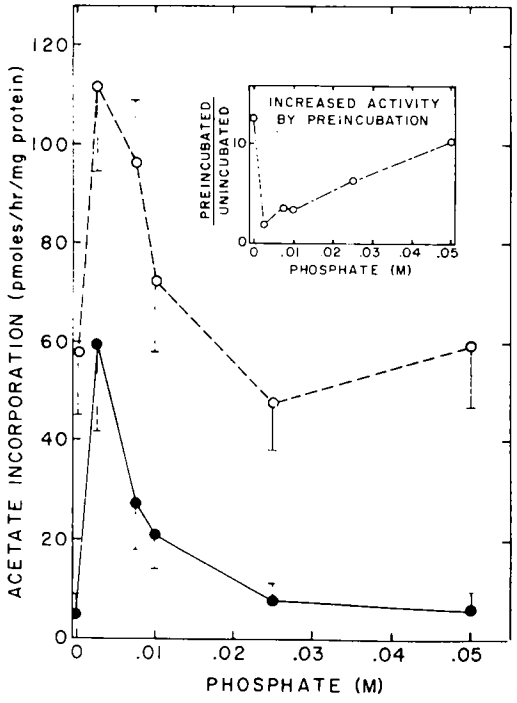


FIG. 1. The effect of phosphate on the incorporation of [1-^{14}C]acetate into cholesterol by rat liver homogenates. Livers were homogenized in 2.5 vol of 0.1 M Tris buffer, pH 7.8, containing 0.03 M nicotinamide, 0.1 mM EDTA, 0.6 mM $MgCl_2$, 5 mM glutathione, and variable amounts of phosphate as indicated on the abscissa. Aliquots of the 12,000 X g supernate kept in ice-water served as the control (•) for the preincubated samples (○). The results are means ± SEM for eight separate experiments. The inset shows the ratio of the activity in the preincubated sample to that in the control.

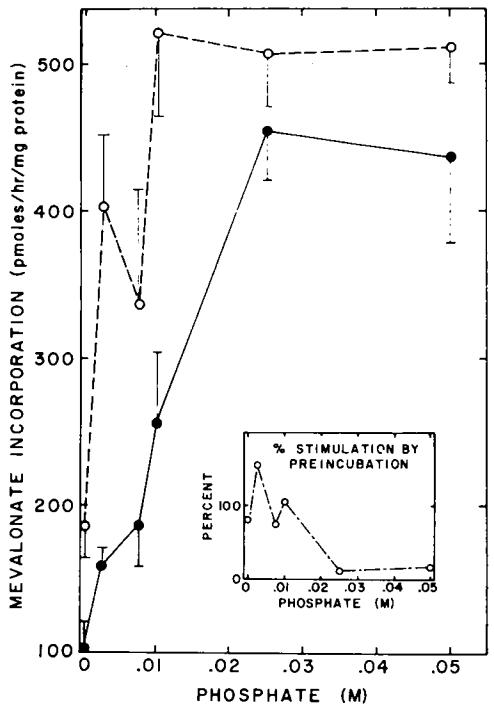


FIG. 2. The effect of phosphate on the incorporation of [2-^{14}C]mevalonolactone into cholesterol by rat liver homogenates. The buffers used were as given in Fig. 1. Results from eight experiments with the preincubated (○) and control (•) homogenates are shown as the mean ± SEM. The inset shows the effects of preincubating the preparations containing various amounts of phosphate: the points represent the percentage increase in activity as the result of preincubation.

cholesterol, the greatest incorporation being observed in the preincubated homogenate in the presence of 2.5 mM phosphate. At this phosphate concentration, the relative increase in activity by preincubation was minimal (inset, Fig. 1). The stimulatory effect of preincubation gradually increased to a maximum of about 5- to 10-fold stimulation as the phosphate content in the buffer was increased.

In contrast to the effects of phosphate on acetate incorporation, phosphate only showed a stimulatory effect on the incorporation of mevalonate into cholesterol (Fig. 2). The low incorporation of mevalonate into cholesterol in the absence of added phosphate progressively increased to a maximum as the phosphate content of the buffer was increased. The maximal rate of mevalonate incorporation, without prior preincubation, was obtained in the presence of 25 mM phosphate. Preincubation of the homogenate stimulated the incorporation of mevalonate about 2-fold in the absence and at low concentrations of added phosphate (inset, Fig. 2). Maximal incorporation in the preincubated homogenate was obtained when the buffer contained 10 mM or more phosphate. Preincubation of the homogenate in the presence of 25 mM or more phosphate had no significant stimulatory effect on the mevalonate incorporation.

Tables 1 and 2 show that the incorporations of acetate and mevalonate into cholesterol are not affected significantly when phosphate concentrations were varied from 25 to 100 mM.

DISCUSSION

In agreement with the report by Goodwin

and Margolis (15), our results showed the expected effect of preincubation on the incorporation of acetate into cholesterol. It seems reasonable to assume that this increased incorporation of acetate with preincubation of the homogenate was due to the activation of HMG-CoA reductase by dephosphorylation by endogenous phosphatases (8,10,16,17).

On the other hand, we found that the incorporation of mevalonate into cholesterol was also stimulated by preincubation. That this stimulation with preincubation occurred only when the phosphate levels in the buffer were low suggested that the incorporation of mevalonate into cholesterol seemed to require the presence of phosphate in the buffer. In accordance with this, we found that very little mevalonate was incorporated into cholesterol in the absence of added phosphate (Fig. 2). The minimal amount of phosphate that was required for the optimal incorporation of mevalonate into cholesterol was 25 mM. The difference in the concentrations of phosphate where maximal mevalonate incorporations were observed in the preincubated and control homogenates might readily be explained by the additional phosphate presumably released during the preincubation period. This released phosphate would increase the phosphate concentration of the media and result in a greater incorporation of mevalonate into cholesterol in preincubated homogenates that were prepared in the presence of suboptimal concentrations of phosphate.

A closer look at the effects of phosphate on the incorporations of acetate and mevalonate into cholesterol raises some possible explanations for our observations. First, the apparent requirement for phosphate in the biosynthesis of cholesterol from mevalonate suggests that, at very low phosphate levels, the step or steps requiring phosphate in going from mevalonate to cholesterol may be rate-limiting in the biosynthesis of cholesterol. Second, the low incorporation of acetate into cholesterol in the absence of added phosphate (Fig. 1) was probably due to the rate-limiting conversion of mevalonate to cholesterol. This and the finding that HMG-CoA reductase was most active in microsomes prepared and incubated in the absence of added phosphate (18) would eliminate the necessity to consider possible biphasic (stimulation and inhibition) effects of phosphate on the incorporation of acetate into cholesterol. The apparent inhibition of acetate incorporation at the higher phosphate levels may be the result of the known inhibitory effects of phosphate on protein phosphatases (19,20). This would prevent the activation of the normally inactive, phosphorylated form of HMG-CoA reductase during the preparation of the homogenate. The incomplete inhibition of the phosphatases, allowing the dephosphorylation and activation of HMG-CoA reductase, may account for the increased incorporation of acetate in the preincubated preparations. Third, the maximal incorporation of acetate into cholesterol occurring in the presence of 2.5 mM added phosphate and the apparent phosphate requirements for the biosynthetic pathway from mevalonate to cholesterol suggest that, at phosphate concentrations below 2.5 mM, some phosphate-requiring step beyond mevalonate limits the biosynthesis of cholesterol from acetate, and that, at phosphate concentrations above 2.5 mM, the activity of HMG-CoA reductase is rate-limiting in our preparations.

ACKNOWLEDGMENTS

This research was supported in part by a NIH Biomedical Research Development Grant and EVMS institutional research support grant.

REFERENCES

1. Dietschy, J.M., and Wilson, J.D. (1970) N. Eng. J. Med. 282, 1128-1138, 1179-1183, 1241-1249.
2. Ott, D.B., and Lachance, P.A. (1981) Am. J. Clin. Nutr. 34, 2295-2306.
3. Siperstein, M.D., and Guest, M.J. (1960) J. Clin. Invest. 39, 642-652.
4. Siperstein, M.D., and Fagan, V.M. (1966) J. Biol. Chem. 241, 602-609.
5. Linn, T.C. (1967) J. Biol. Chem. 242, 990-993.
6. Shapiro, D.J., and Rodwell, V.W. (1969) Biochem. Biophys. Res. Commun. 37, 867-871.
7. Lakshmanan, M.R., Dugan, R.E., Nepokroeff, C.M., Ness, G.C., and Porter, J.W. (1975) Arch. Biochem. Biophys. 168, 89-95.
8. Brown, M.S., Goldstein, J.L., and Dietschy, J.M. (1979) J. Biol. Chem. 254, 5144-5149.
9. Kleinsek, D.A., Jabalquinto, A.M., and Porter, J.W. (1980) J. Biol. Chem. 255, 3918-3923.
10. Arebalo, R.E., Hardgrave, J.E., and Scallen, T.J. (1981) J. Biol. Chem. 256, 571-574.
11. Ramachandran, C.K., and Shah, S.N. (1976) Biochem. Biophys. Res. Commun. 69, 42-47.
12. Jabalquinto, A.M., and Cardemil, E. (1980) Lipids 15, 196-199.
13. Hotta, S.S. (1980) Fed. Proc. 39, 1720.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Goodwin, C.D., and Margolis, S. (1973) J. Biol. Chem. 248, 7610-7613.
16. Brandt, H., Capulong, Z.L., and Lee, E.Y.C. (1975) J. Biol. Chem. 250, 8038-8044.
17. Killilea, S.D., Mellgren, R.L., Aylward, J.H., Meteieh, M.E., and Lee, E.Y.C. (1979) Arch. Biochem. Biophys. 193, 130-139.
18. Srikantaiah, M.V., Noble, N., Orenstein, L., and Morin, R.J. (1981) Lipids 16, 934-936.
19. Bingham, E.W., Farrell, H.M., Jr., and Dahl, K.J. (1976) Biochim. Biophys. Acta 71, 468-471.
20. Philipp, B.W., and Shapiro, D.J. (1979) J. Lipid Res. 20, 588-593.

[Received March 15, 1982]

Effect of Vitamin E on Pentane Exhaled by Rats Treated with Methyl Ethyl Ketone Peroxide

L.A. HERSCHBERGER and **AL L. TAPPEL***, *Department of Food Science and Technology, University of California, Davis, CA 95616*

ABSTRACT

One useful method to monitor in vivo lipid peroxidation is the measurement of volatile hydrocarbons, mainly pentane and ethane, that derive from unsaturated fatty acid hydroperoxides. Vitamin E, the biological antioxidant, inhibits lipid peroxidation and the production of pentane and ethane. The rates of pentane production by male Sprague-Dawley rats fed a diet that contained 10% vitamin E-stripped corn oil and 0, 1, 3, 5 or 10 IU dl-α-tocopherol acetate/kg were monitored over a 12-wk period. During the eleventh and twelfth weeks, the rats were injected intraperitoneally with 3.3 and 13 mg of methyl ethyl ketone peroxide (MEKP)/kg body wt, respectively. Pentane production was then measured at intervals over a 50-min period, and the total amount of pentane produced over this time interval was estimated. An asymptotic function was found to describe the relationship between exhaled pentane and the low levels of dietary vitamin E that were fed to the rats. As measured by pentane production, rats had a higher minimal vitamin E requirement after they were treated with the potent peroxidation initiator MEKP than they did prior to treatment. The level of pentane exhaled by rats injected with 13 mg MEKP/kg body wt was significantly correlated with kidney and spleen tocopherol levels.
Lipids 17:686-691, 1982.

Lipid peroxidation has been implicated as a cause of cellular damage (1) and has been associated with a variety of diseases (2). Vitamin E, an in vivo biological antioxidant, protects membranes against lipid peroxidation (3). Vitamin E-deficient experimental animals exhibit diverse symptoms, some of which are species-specific (4). Deficiencies of most vitamins result in characteristic pathological symptoms, but vitamin E deficiency symptoms are more difficult to recognize and to quantify (5). Vitamin E deficiency symptoms have been used to determine minimal vitamin E requirements (6-10) and relative potencies of tocopherol isomers and esters (11-14).

One of the newest methods to detect lipid peroxidation in vivo directly is the measurement of exhaled volatile hydrocarbons, particularly pentane and ethane. Pentane and ethane are minor products formed during decomposition of ω6- and ω3-unsaturated fatty acid hydroperoxides, respectively (15). A variety of oxidants initiate lipid peroxidation in vivo. Riely et al. (16) first demonstrated that the level of ethane increased in breath from mice injected with carbon tetrachloride. Ethane and/or pentane were elevated in expired breath of animals treated with halogenated hydrocarbons (17), ethanol (18), ozone (19), iron (20) or methyl ethyl ketone peroxide (MEKP) (21), and vitamin E inhibited the ensuing lipid peroxidation.

MEKP is used industrially to initiate polymerization reactions in the manufacture of plastics and fabrics. Unlike most other organic peroxides, MEKP decomposes to free radicals in the presence of metal ions at room temperature. Litov et al. (21) reported that injected MEKP caused severe lipid peroxidation as determined by measurement of the immediate high-level production of pentane by vitamin E-deficient rats. The measurement of expired volatile hydrocarbons to index in vivo lipid peroxidation has gained acceptance in recent years.

The possibility that the technique could be used in bioassay of antioxidant status was suggested (22). Dietary vitamin E has its major effect on basal pentane production by rats in the range of 0-10 IU dl-α-tocopherol acetate/kg diet (19,23). However, at low levels of dietary vitamin E, the relationships between expired pentane and dietary vitamin E levels and between expired pentane and tissue vitamin E levels have not been adequately investigated.

The quantitative relationships between low levels of dietary vitamin E and both basal pentane production and MEKP-induced pentane production are examined in this study. Kidney and spleen vitamin E levels were measured to correlate pentane production with tissue vitamin E status. The possible use of the technique in bioassay of antioxidant status is discussed.

*Author to whom correspondence should be addressed.

METHODS

Animals and Diets

Nineteen weanling male Sprague-Dawley rats were divided into five groups and were fed a basal 10% tocopherol-stripped corn oil diet (24) with mineral mix 4179 (Teklad Test Diets) for 12 wk and 0, 1, 3, 5 or 10 IU dl-α-tocopherol acetate/kg for 12 wk. All of the groups contained four rats except there were only three rats in the group fed 1 IU vitamin E/kg diet. The rats were housed singly or in pairs in hanging wire cages and were kept on a 14-hr light and 10-hr dark cycle at 22-24 C. The diets and water were provided ad libitum.

Pentane Measurements and Oxidant Treatments

Sample collection and measurement of pentane were done as described by Dillard et al. (22). The rats were fasted 18-24 hr before their rates of pentane production were determined. Measurements of exhaled pentane were made during weeks 3, 7, 10, 11 and 12. After a 10-min exposure to hydrocarbon-free air, a 500-ml air-breath sample was collected and analyzed by flame-ionization gas chromatography on a 10-ft alumina column at 160 C. The nitrogen carrier gas flow rate was 25 ml/min. The injector and detector temperatures were 210 and 300 C, respectively. The amount of pentane was calculated from the peak height × width of the peak at half-height. The instrument was calibrated daily with a 0.89-ppm pentane standard. The basal rates of pentane production are expressed as pmol pentane/100 g body wt/min.

Basal pentane production by the rats was measured prior to each treatment with MEKP. Each rat was injected intraperitoneally (ip) with 3.3 mg MEKP/kg body wt during week 11 and 13 mg MEKP/kg body wt during week 12. The solution injected was 6.6 mg MEKP/ml tributyrin. Tributyrin was used to maximize the absorption of MEKP. The lower level of MEKP was chosen to ensure that the rats would survive. The higher MEKP dose was used to obtain highly significant differences among the rates of pentane production by the rats in the different dietary groups. Pentane was measured in samples of breath 10, 20, 35 and 50 min following injections. The basal measurement of pentane was used as a zero time point. After MEKP was injected into a rat, the total number of pmol of pentane exhaled above the basal level of pentane was estimated by integrating the rate of pentane production over 50 min and subtracting from this the number of pmol that would have been produced during the 50 min under basal conditions. The latter was estimated by multiplying the basal rate of pentane production (pmol/100 g body wt/min) by 50 min. The integrated amount of pentane exhaled following the oxidant treatment is expressed as pmol pentane/100 g body wt/50 min.

Tissue Tocopherol Analysis

The rats were anesthetized with pentabarbital ca. 75 min after the second injection of MEKP was given. The blood was removed by heart puncture to remove as much blood from the tissues as possible. The kidney and spleen were removed, rinsed in 0.9% KCl and frozen at -10 C under nitrogen. The kidney and spleen were analyzed for total tocopherol by fluorescence assay (25).

Statistical Analysis

The curves obtained by plotting pentane production vs dietary vitamin E were modeled to the general equation $Y = a + br^x$ (26) using the Biomedical Computer Program BMD06R. This equation describes a decrease in the pentane response, y, that reaches an asymptote at high levels of x, the dietary vitamin E. In this equation, a is the minimal pentane level obtainable, b is the difference in the rate of pentane production between rats fed no vitamin E and those fed higher amounts of vitamin E and r is a dimensionless number that describes how quickly the asymptote is approached. To determine if the data fit the asymptotic model, a comparison was made between the residual sum of squares, calculated using the asymptotic model (SSE_{MODEL}), and the minimal residual sum of squares possible over all models (SSE_{MEANS}). The SSE_{MEANS} is the sum of the squares of the deviation of each observation from its group mean. The ratio $(SSE_{MODEL} - SSE_{MEANS})/SSE_{MODEL}$ was calculated for each fitted line. A Burroughs 7800 computer was used for the above computations. All p values <0.05 were considered to be significant.

RESULTS

Basal Pentane Measurements

The amounts of pentane produced by rats fed varying vitamin E levels for 3, 7, 11 and 12 wk are shown in Figure 1. The data obtained during the tenth week were similar to the eleventh week data, but they were excluded from the figure for clarity. Over the concentration range of dl-α-tocopherol acetate used in the diets, pentane production was inversely related to vitamin E intake. During the third week, the levels of exhaled pentane were higher than at other time points. The 7-wk curve and

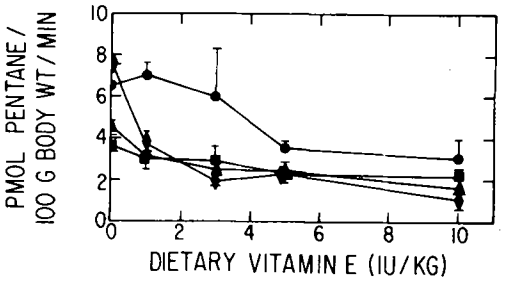


FIG. 1. Basal rates of pentane production as a function of dietary vitamin E (dl-α-tocopherol acetate). The lines represent basal rates of pentane production at (●) 3, (■) 7, (▲) 11 and (◆) 12 wk of the dietary regime. The values plotted are means ± SE. Error bars are drawn in a single direction for clarity.

subsequent curves were all similar, and by the seventh week, pentane production was stabilized.

Effect of MEKP on Pentane Production

Following each injection of MEKP, the rate of pentane production by the rats rose to a maximum by 20 min, and by 50 min, the rates had returned to near-basal levels. Pentane production by the rats following injection of the two different amounts of MEKP is shown in Figure 2. The protective effect of dietary vitamin E is demonstrated by the lower levels of pentane produced by rats fed the higher amounts of vitamin E. Twenty min after injection of 3.3 mg MEKP/kg body wt, rats fed diets with 0 and 10 IU dl-α-tocopherol acetate/kg had, on the average, 3.6- and 2-fold increases, respectively, in pentane production. Intraperitoneal injection of tributyrin or dibutyl phthalate into vitamin E-deficient rats did not increase the level of exhaled pentane over a 1-hr time period.

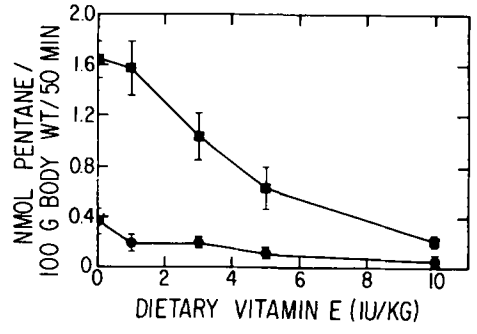


FIG. 2. Total pentane produced during 50 min following injection of MEKP as a function of dietary vitamin E (dl-α-tocopherol acetate). The two lines represent pentane production following the ip injection of (●) 3.3 mg MEKP/kg body wt and (■) 13 mg MEKP/kg body wt. The values plotted are means ± SE.

Asymptotic Models

With the exception of the 3-wk basal curve and the 13-mg MEKP/kg body wt curve, the curves of pentane production vs dietary vitamin E are adequately described using the general equation $Y = a + br^x$. The calculated estimates of the parameters a, b and r and the estimates of variance for each are shown in Table 1. The 3-wk curve for basal pentane production could not be modeled because of the large variation among the rats at this early time point. The high MEKP dose curve was approximately linear for 0-10 IU dl-α-tocopherol acetate/kg diet; therefore, it was not modeled by this equation.

Pentane vs Tissue Tocopherol

Kidney tocopherol levels ranged from 1.5-7.0 μg tocopherol/g kidney, and the spleen levels ranged from 0.44-16 μg tocopherol/g spleen. All but one spleen had less than 9 μg tocopherol/g. A plot of kidney tocopherol vs the level of pentane produced by the rats following treatment with 13 mg MEKP/kg body wt is shown in Figure 3. The correlation between pentane production and kidney tocopherol was higher ($r=0.74$, $p<0.001$) than that between pentane production and spleen tocopherol ($r=0.49$, $p<0.05$), though both were significant.

DISCUSSION

The finding that vitamin E protects against lipid peroxidation induced by the oxidant MEKP is consistent with the findings of other researchers who have measured pentane and/or ethane production by rats under basal conditions without oxidant treatment (17,22,27-29) or after treatment with various oxidants (16-19, 21,30). After 7 wk of feeding the diets to the rats, their basal pentane production stabilized, and no further change was observed by 12 wk. Bieri (31) analyzed tissue tocopherol levels in young rats fed diets that contained 0-32 mg d-α-tocopherol acetate/kg and 5.2% linoleic acid. The tissue tocopherol levels stabilized by 8 wk. Therefore, the stabilized pentane levels observed in the present study may reflect stabilization of tissue tocopherol levels in the rats.

The direct influence of the oxidant dose on the rate of pentane exhaled was expected

TABLE 1

Asymptotic Relationships between Pentane Production and Dietary Vitamin E[a]

Feeding period (wk)	MEKP dose (mg/kg body wt)	(pmol pentane/100 g body wt/min) a	b	r	$\frac{SSE_{MODEL} - SSE_{MEANS}}{SSE_{MODEL}}$
3	0	_[b]	_[b]	_[b]	
7	0	2.1	1.5	0.74	0.13
10	0	1.7	1.6	0.60	0.01
11	0	1.8	2.6	0.62	0.09
12	0	1.6	5.9	0.36	0.03
11	3.3	59[c]	280[c]	0.74	0.13
12	13	_[b]	_[b]	_[b]	

[a] $Y = a + br^x$.

[b] No fit.

[c] pmol pentane/100 g body wt/50 min.

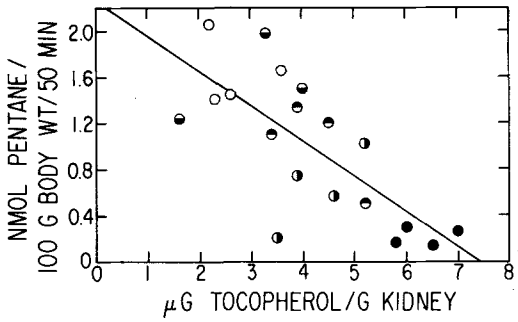


FIG. 3. Total pentane produced during 50 min following injection of 13 mg MEKP/kg body wt as a function of kidney tocopherol. Each point represents the total amount of exhaled pentane and the corresponding kidney level of tocopherol from indivdual rats fed 0 (○), 1 (◒), 3 (◓), 5 (◑) or 10 (●) IU dl-α-tocopherol acetate/kg body wt. The linear regression equation is Y = 2.3 - 0.3X and the correlation coefficient, r, is 0.74.

because higher levels of the oxidant can initiate a greater number of free-radical chain reactions. In preliminary experiments, rats fed diets that were adequate in vitamin E exhibited a linear relationship between exhaled pentane and the dose of MEKP (32).

The animals were given two oxidant treatments, and the question arises as to whether the first oxidant dose affected the animals' response to the second dose. The first treatment did not appear to substantially affect the results of the second treatment because the basal pentane levels on the week following the first injection of MEKP were the same as they were prior to the first oxidant treatment.

Comparison of the rates of pentane production vs dietary vitamin E after the two MEKP injections illustrates an important point (Fig. 2). The two curves show that, to a point of minimal production, expired pentane was inversely related to vitamin E. The curve generated from the data obtained after the animals were treated with 3.3 mg MEKP/kg body wt reaches the asymptote between 1 and 5 IU dl-α-tocopherol acetate/kg diet. However, the curve generated from data obtained after administration of 13 mg MEKP/kg body wt does not reach the asymptote even at 10 IU dl-α-tocopherol acetate/kg diet. A minimal vitamin E requirement can be estimated as the amount required to minimize the deficiency as measured by exhaled pentane. These data show that, in a range of low dietary vitamin E, rats exposed to higher levels of an oxidant require higher amounts of vitamin E for maximal protection. It is well known (33) that the vitamin E requirement increases with higher intakes of polyunsaturated fatty acids. Based on the reports of others (21,34,35), a greater vitamin E requirement is expected for rats treated with oxidants.

The data for the basal pentane production and pentane production after treatment with the lower MEKP dose were modeled to the general equation $Y = a + br^x$. The low ratios of $(SSE_{MODEL} - SSE_{MEANS})/SSE_{MODEL}$ indicate that the model describes the data well. The curve plotted from data obtained after treatment of rats with 13 mg MEKP/kg body wt could be modeled if higher dietary levels of vitamin E were included.

Animal experiments frequently involve a large number of variation among animals similarly treated. A large number of animals fre-

quently must be used to detect significant differences among groups treated differently. The experimental design of the present study circumvents the problem in two ways. First, the rates of pentane production were monitored in the same animals throughout the feeding program and also following their treatment with the oxidant MEKP. Second, a potent initiator of lipid peroxidation was administered to accentuate the differences in vitamin E status among the groups fed various levels of vitamin E. High levels of a toxicant are frequently used to minimize the number of animals required to detect significant differences.

The bioassay described in this study uses the response of in vivo pentane production to determine the antioxidant effectiveness in the whole animal. When 13 mg MEKP/kg body wt was injected into the rats, there were highly significant differences in pentane production among rats fed low levels of vitamin E. The incorporation of an oxidant in antioxidant bioassays is often used in tests of erythrocyte hemolysis. There are some distinct advantages to using exhaled pentane to assess vitamin E status in animals. One advantage is that the exhaled pentane derives from hydroperoxides, which are the initial products of peroxidation. A second advantage is that the amount of pentane exhaled by an animal is a reflection of its in vivo antioxidant status. The level of pentane produced is a function of the absorption, distribution and excretion of the vitamin E and te specific isomers ingested. The pentane level thus reflects the in situ antioxidant concentrations and effectiveness. A third advantage of the pentane methodology is that it is theoretically applicable to most animal species (16,22, 36-38). A fourth advantage of this in vivo bioassay is that incorporation of MEKP could be used to test the effectiveness of many different antioxidants.

The pentane method also has some disadvantages that may limit its use in a bioassay of vitamin E that does not include an oxidant. It was observed in some studies (21) that differences in pentane production between rats fed high and low vitamin E occurred only after an oxidant was used to initiate peroxidation. Another possible disadvantage is that a lower minimal vitamin E requirement was estimated by measurement of exhaled pentane than was estimated in studies using erythrocyte hemolysis as an index of vitamin E deficiency. Under basal conditions, this requirement was estimated to be between 1 and 5 IU dl-α-tocopherol acetate/kg diet. The National Research Council vitamin E requirement for rats (39) was based on a study by Jager and Houtsmüller (10). In their study, erythrocyte hemolysis was used to index vitamin E deficiency in rats fed diets containing 3.5% linoleic acid. The minimal vitamin E requirement was estimated to be 13 mg d-α-tocopherol acetate/kg diet (39). This is equivalent to 17.6 mg dl-α-tocopherol acetate/kg diet (39). When the higher dose of the MEKP was included, the minimal vitamin E requirement was greater than 10 IU dl-α-tocopherol acetate, which is closer to that estimated by Jager and Houtsmüller (10). Other incongruities among bioassay methods used to determine vitamin E requirements can be found in the literature (11).

Litov et al. (21) found a high correlation between pentane exhaled by rats injected with MEKP and the ratio of mg tocopherol/g lipid found in plasma. In the present study, analysis of tissue for tocopherol was done to determine whether tissue vitamin E levels were more highly correlated with pentane levels than were the dietary vitamin E levels. If this were true, some of the variation in the pentane levels could be explained by differences in vitamin E absorption, distribution or excretion. The correlations of pentane exhaled by rats injected with 13 mg MEKP/kg body wt with tocopherol levels in the kidney and spleen were low, but both were significant ($p<0.05$). The correlation coefficient between the exhaled pentane and log(dietary vitamin E + 1) was much higher. Liver may be the most important organ related to lipid peroxidation. Over 95% of conjugated dienes in organs of rats treated with CCl_4 was found in the liver (40). Another possible explanation for the low correlation of exhaled pentane with the tissue tocopherol levels may be that the MEKP oxidized the low levels of tocopherol to tocopherol quinone, which is not detected by the fluorescence assay.

The measurement of in vivo pentane production as an index of lipid peroxidation was shown in this study to be useful for studying the effects of low levels of vitamin E in rats. Under conditions of high oxidant stress, the minimal vitamin E requirement was greater than that estimated for rats under basal conditions.

ACKNOWLEDGMENT

This research was supported by funds from Best Foods—a unit of CPC North America, and by research grant AM 09933 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

REFERENCES

1. Bus, J.S., and Gibson, J.E. (1979) in Reviews in Biochemical Toxicology (Hodgson, E., Bend, J.R., and Philpot, R.M., eds.), vol. I, pp. 125-149,

Elsevier North Holland, Inc., New York, NY.
2. DelMaestro, R.F. (1980) Acta Physiol. Scand. Suppl. 492, 153-168.
3. Molennaar, I., Hulstaert, C.E., and Hardonk, M.J. (1980) in Vitamin E: A Comprehensive Treatise (Machlin, L.J., ed.), pp. 372-389, Marcel Dekker, Inc., New York, NY.
4. Desai, I.D. (1980) in Vitamin E: A Comprehensive Treatise (Machlin, L.J., ed.), pp. 67-98, Marcel Dekker, Inc., New York, NY.
5. Horwitt, M.K. (1980) in Vitamin E: A Comprehensive Treatise (Machlin, L.J., ed.), pp. 621-636, Marcel Dekker, Inc., New York, NY.
6. Ames, S.R. (1974) Am. J. Clin. Nutr. 27, 1017-1025.
7. Gabriel, E., Machlin, L.J., Filipski, R., and Nelson, J. (1980) J. Nutr. 110, 1372-1379.
8. Bieri, J.G., and Poukka, R.K.H. (1970) J. Nutr. 100, 557-564.
9. Jager, F.C. (1972) Nutr. Metabol. 14, 1-7.
10. Jager, F.C., and Houtsmüller, V.M.T. (1970) Nutr. Metabol. 12, 3-12.
11. Bieri, J.G., and Evarts, R.P. (1974) J. Nutr. 104, 850-857.
12. Ames, S.R. (1971) Lipids 6, 281-290.
13. Ames, S.R. (1979) J. Nutr. 109, 2198-2204.
14. Scott, M.L., and Desai, I.D. (1964) J. Nutr. 83, 39-43.
15. Dumelin, E.E., and Tappel, A.L. (1977) Lipids 11, 894-900.
16. Riely, C.A., Cohen, G., and Lieberman, M. (1974) Science 183, 208-210.
17. Hafeman, D.G., and Hoekstra, W.G. (1977) J. Nutr. 107, 656-665.
18. Litov, R.E., Irving, D.H., Downey, J.E., and Tappel, A.L. (1978) Lipids 13, 305-307.
19. Dumelin, E.E., Dillard, C.J., and Tappel, A.L. (1978) Arch. Environ. Health 33, 129-134.
20. Dougherty, J.J., Croft, W.A., and Hoekstra, W.G. (1981) J. Nutr. 111, 1784-1786.
21. Litov, R.E., Matthews, L.C., and Tappel, A.L. (1981) Toxicol. Appl. Pharmacol. 59, 96-106.
22. Dillard, C.J., Dumelin, E.E., and Tappel, A.L. (1977) Lipids 12, 109-114.
23. Litov, R.E., Gee, D.L., Downey, J.E., and Tappel, A.L. (1981) Lipids 16, 52-57.
24. Draper, H.H., Bergan, J.G., Chiu, M., and Csallany, A.S. (1964) J. Nutr. 84, 395-400.
25. Taylor, S.L., Lamden, M.P., and Tappel, A.L. (1976) Lipids 11, 530-538.
26. Stevens, W.L. (1951) Biometrics 7, 247-267.
27. Dillard, C.J., Litov, R.E., and Tappel, A.L. (1978) Lipids 13, 396-402.
28. Hafeman, D.G., and Hoekstra, W.G. (1977) J. Nutr. 107, 666-672.
29. Downey, J.E., Irving, D.H., and Tappel, A.L. (1978) Lipids 13, 403-407.
30. Burk, R.F., and Lane, J.M. (1979) Toxicol. Appl. Pharmacol. 50, 467-478.
31. Bieri, J.G. (1972) Ann. N.Y. Acad. Sci. 203, 181-191.
32. Herschberger, L.A. (1982) M.S. thesis, University of California, Davis.
33. Harris, P.L., and Embree, N.D. (1963) Am. J. Clin. Nutr. 13, 385-392.
34. Chen, L.H. (1981) Am. J. Clin. Nutr. 34, 1036-1041.
35. Chow, C.K., and Tappel, A.L. (1972) Lipids 7, 518-524.
36. Dillard, C.J., Litov, R.E., Savin, W.M., Dumelin, E.E., and Tappel, A.L. (1978) J. Appl. Physiol. Respirat. Environ. Exercise Physiol. 45, 927-934.
37. Young, R.G., and Tappel, A.L. (1978) Exp. Gerontol. 13, 457-459.
38. Dumelin, E.E., Dillard, C.J., and Tappel, A.L. (1978) Environ. Res. 15, 38-43.
39. National Research Council/NAS, Nutrient Requirements of Laboratory Animals, No. 10, National Academy of Sciences, Washington, DC, 1978, pp. 21-22.
40. Sagai, M., and Tappel, A.L. (1979) Toxicol. Appl. Pharmacol. 49, 283-291.

[Received March 8, 1982]

Plasma Lipid and Lipoprotein Concentrations in an Egyptian Male Sample

MALAK I. SHOUKRY* and **SAMIA FAREED**, *Department of Biochemistry, Faculty of Medicine, University of Kuwait, PO Box 24923, Kuwait, Arabia*

ABSTRACT

The distributions of plasma total cholesterol and triglyceride levels as well as plasma lipoprotein abnormalities were studied in 50 Egyptian males aged 20-69 years. Total cholesterol increased gradually with advancing age up to the seventh decade. In contrast, triglycerides peaked in the fifth decade, then declined. Type IV lipoprotein pattern was the most common abnormality (12%). Type II was less common (2.0%). Types I, III and V were not encountered. The mean plasma triglyceride and cholesterol levels were not markedly different from similar studies done on non-Arab populations. The high incidence of hyperlipidemia among this group is worth noting, especially in the search for the coronary-prone, since all of the type IV group had normal total cholesterol levels.
Lipids 17:692-695, 1982.

A knowledge of plasma concentrations of lipids and lipoproteins is an essential tool to the physician in the diagnosis and management of hyperlipoproteinemias. Clinical laboratories usually set their upper limit of normal for fasting plasma triglycerides (TG) and cholesterol levels based on the 95th-percentile determinations for a given population. However, most of these values are taken from studies mainly done on Americans or Europeans, which are not necessarily applicable to other populations. This is because lipid and lipoprotein concentrations in plasma are affected by diet, stress, degree of physical activity, and by genetic and environmental factors. This study was undertaken as part of a program to characterize the "normal values" of plasma cholesterol and triglyceride in different Arab populations, and to estimate lipoprotein concentrations as well as the prevalence of hyperlipoproteinemia, since it is a major risk factor in the development of atherosclerotic cardiovascular disease.

MATERIALS AND METHODS

Subjects

Sixty-six volunteers were recruited and from this group, 50 male participants between the ages 20 and 70 yr were selected. These subjects were in good health and had no history of diabetes. Four of the rejected volunteers were diabetics, two had high blood pressure and the rest were females (n=10), which we did not include because of their small number.

Blood Samples

Ten ml of venous blood was collected from each participant into a tube containing 10 mg EDTA after a 12- to 14-hr fast. Plasma was obtained by centrifugation at 2,000 rpm at 4 C for 20 min. Lipoprotein electrophoresis was performed on the same day whereas the major part of the plasma was kept frozen at -26 C until lipid analysis could be performed.

Cholesterol and TG Analysis

Total cholesterol was determined by the method of Allain et al. (1). TG were measured by the enzymatic method of Bucolo and David (2) after hydrolysis with alcoholic potassium hydroxide. Reproducibility of the methods used was monitored by running a standard plasma periodically during the analysis (TG = (95 ± 6.4; n = 9; cholesterol = 202 ± 7.7; n = 6).

Electrophoresis

Lipoproteins were separated by the use of commercially available equipment and supplies (Corning Medical, Palo Alto, CA); 1.0 μl of each plasma sample was applied to one of the eight wells in an agarose gel (universal gel). Electrophoresis at 90 V for 35 min separated the three classes of lipoproteins: high density (HDL), low density (LDL) and very low density (VLDL). The electrophoretograms were scanned using Beckman spectrophotometer Model 26 fitted with a gel scanning accessory. Area under the peaks was integrated by an HP45 computer fitted with a digitizer. Correction for baseline drift was made by drawing a horizontal line from each peak through and measuring half-peak area. The area of each peak is expressed as a percentage of the total areas.

Body Mass Index (BMI)

This was calculated from the formula =

*Author to whom correspondence should be addressed.

weight(kg)/height^2(m^2). This index was used as a measure of adiposity (3).

RESULTS

Population Characteristics

The ages of the group studied ranged from 26 to 68 yr, with a mean age of 42 (SD = 10). The participants were generally from middle socioeconomic group. Adiposity, as represented by BMI, varied widely, with a mean of 26 (SD = 4.0) and range of 19-43 (Fig. 1). Thirty-six percent of the subjects were above the desirable body weight, since a BMI in the range 20-25 is considered desirable for males. Fasting plasma TG concentrations were between 46 mg/dl and 348 mg/dl, with a mean of 135 mg/dl (SD = 71), and the distribution was skewed to the right (Fig. 2). Cholesterol concentrations were normally distributed with a mean of 199 mg/dl (SD = 38) and a range of 121-298 mg/dl (Fig. 3).

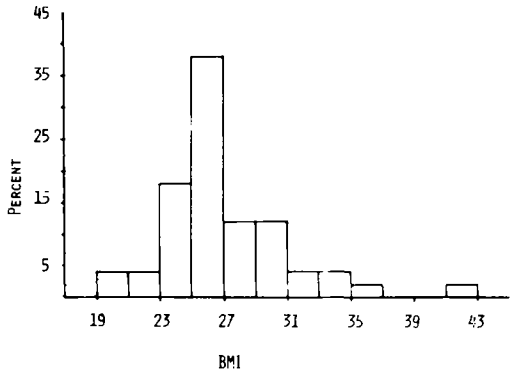


FIG. 1. Frequency distribution of BMI in normal Egyptian males.

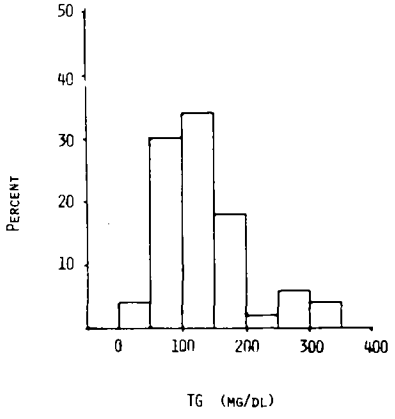


FIG. 2. Frequency distribution of plasma TG in normal Egyptian males.

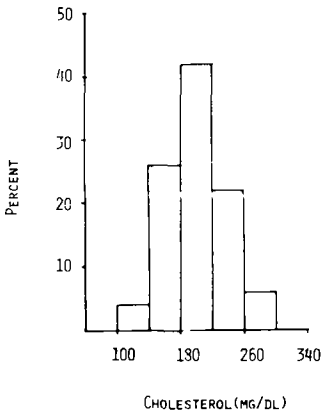


FIG. 3. Frequency distribution of plasma cholesterol in normal Egyptian males.

Lipid Concentration and Age

Cholesterol and TG concentrations by decade are shown in Table 1. Cholesterol rose gradually with advancing age and reached a peak in the seventh decade. TG concentrations peaked in the fifth decade, two decades earlier than cholesterol, then declined. The percentage of participants exceeding certain commonly used limits of normal for plasma total cholesterol and TG are shown in Table 2. A large number exceeded the lower of the two limits both for cholesterol (240 mg/dl) and for TG (150 mg/dl). Twelve percent exceeded the higher limit for TG (200 mg/dl).

Lipoprotein Abnormalities

Subjects with plasma total cholesterol level of 270 mg/dl or above, or a TG level of 200 mg/dl or above or both were considered hyperlipidemic. The lipoprotein patterns of these individuals were classified according to the Fredrickson et al. scheme (4). In Figure 4, some typical patterns encountered in this study are shown. Type IV (taken as cholesterol < 270 mg/dl and TG ≥ 200 mg/dl) was the most common among the subjects examined (12%). Only 2% (1 subject out of 50) were of type II (cholesterol ≥ 270 mg/dl and TG < 200 mg/dl). Types I, III and V were not encountered. Prevalence of hyperlipoproteinemic patterns among the participants by age is shown in Table 3. The highest incidence of hyperlipoproteinemia occurred in the fifth and sixth decades. The distribution of the three classes of lipoproteins are shown in Table 4. There was no statistically significant difference in the distribution of lipoproteins with increasing age. The expected rise in LDL and VLDL levels with increasing cho-

TABLE 1

Plasma Cholesterol and Triglycerides Levels in Normal Egyptian Males

Age range (yr)	N	Cholesterol (mg/dl) Mean ± SD	Cholesterol (mg/dl) Range	Triglyceride (mg/dl) Mean ± SD	Triglyceride (mg/dl) Range
20-29	4	193 ± 38	(158-248)	115 ± 42	(62-161)
30-39	18	193 ± 36	(121-247)	124 ± 58	(57-259)
40-49	16	199 ± 38	(145-266)	159 ± 88	(46-348)
50-59	9	208 ± 48	(151-298)	135 ± 82	(52-307)
60-69	3	210 ± 37	(168-235)	112 ± 28	(79-130)
20-69		199 ± 38	(121-298)	135 ± 71	(46-348)

TABLE 2

Percentage of Subjects Examined with Hypercholesterolemia and Hypertriglyceridemia

Age range (yr)	Cholesterol (mg/dl) ≥240	Cholesterol (mg/dl) ≥270	TG (mg/dl) ≥150	TG (mg/dl) ≥200
20-29	25	0	25	0
30-39	11	0	33	5.6
40-49	13	0	38	19
50-59	22	11	22	22
60-69	0	0	0	0
20-69	16	2.0	30	12

TABLE 3

Prevalence of Lipoprotein Abnormalities among Normal Egyptian Males

Age range (yr)	% with each type II	% with each type IV
20-29	0	0
30-39	0	5.6
40-49	0	19
50-59	11	22
60-69	0	0
20-69	2.0	12

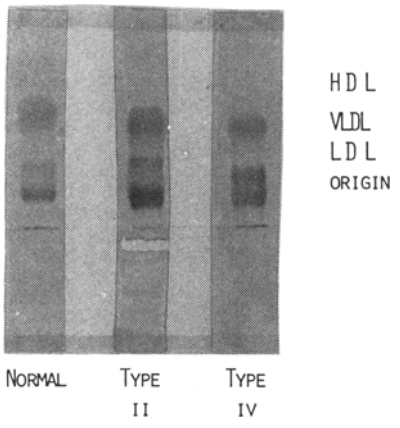


FIG. 4. A typical electrophoresis run showing the three patterns encountered: normal, Type II (elevated LDL), Type IV (elevated VLDL).

lesterol and triglyceride concentrations was confirmed (Types IV and II vs normal) (see Table 4).

DISCUSSION

This study demonstrated a gradual increase of total cholesterol concentrations with advancing age up to the seventh decade, in agreement with earlier reports (5,6). Unlike cholesterol, triglyceride concentrations increased with age, peaked in the fifth and sixth decades, then declined. It is unclear whether this decline is due to an actual decrease of TG with age, or due to natural elimination of subjects with higher TG levels. However, in view of the small number of subjects involved, especially in the third, sixth and seventh decades, it is difficult to make a conclusive statistical interpretation. A rather impressive proportion of subjects (12%) had TG concentrations above 200 mg/dl. Although this value is an arbitrary cut-off point, many physicians probably would consider it undesirably high. Elevation of plasma TG levels has been reported to be an indepen-

TABLE 4

Lipoprotein Profile of Normal Egyptian Males

Age range (yr)	% HDL	% LDL	% VLDL	α/β	Pre β/β
20-29	38 ± 14	49 ± 11	13 ± 5.0	0.87	0.34
30-39	30 ± 8.9	50 ± 7.5	20 ± 7.8	0.62	0.42
40-49	33 ± 6.4	47 ± 7.3	21 ± 8.3	0.72	0.47
50-59	35 ± 11	47 ± 9.5	17 ± 9.2	0.80	0.39
60-69	32 ± 7.6	52 ± 9.9	15 ± 2.1	0.66	0.29
Normal subjects (n=43)					
20-69	33 ± 9.1	49 ± 8.0	18 ± 6.7	0.73	0.38
Type IV (n=6)	26 ± 5.0 ($p<.05$)	45 ± 8.5	29 ± 9.7 ($p<.005$)	0.59	0.69
Type II (n=1)	32	57	11	0.56	0.18

dent risk factor in the developing ischemic heart disease (7) and, in some families with inherited hyperlipidemias, hypertriglyceridemia without hypercholesterolemia appears to be associated with premature cardiovasular disease (8,9). The high incidence of obesity encountered in this group would most likely correlate with the high percentage of individuals with elevated TG concentrations. Obesity has been associated with elevation of TG concentration (10). It has been noted that all subjects with hyperlipidemia have either elevated TG or cholesterol concentrations, which permitted the evaluation of the increase of LDL and VLDL in these cases compared to normal. None of the subjects had elevations of both TG and cholesterol. The high incidence of Type IV between the ages of 40 and 60 yr found in this group probably represents the change in eating habits and decreased physical activity. However, it is interesting to note that, in a similar study done on a larger group of American population (494 males and 503 females, of central valley, California) (5), the prevalence of Type IV between males is 13%, in close agreement with the present finding (12%). In this same group, the incidence of Type II was greater than its incidence in this group, and this might reflect the difference between diets in these two groups (high consumption of eggs and meat by the American population); or it might be due to genetic factors. The present study confirms that Type IV is relatively more common than Type II (at least in males). The distribution of the three classes of lipoproteins did not change with advancing age, which is expected, though the small increase in VLDL between the fourth and sixth decades was consistent with the triglycerides finding.

The study presented in this paper is, to the authors' knowledge, the only one that reports on lipoprotein patterns and abnormalities in Egyptians. The values reported for triglycerides and cholesterol in a group of normal Egyptians can only be used as a reference range, since a much larger sample size must be studied in order to establish normal ranges in a certain population. However, with the great difficulty encountered in recruitment of volunteers, this was not possible to achieve.

ACKNOWLEDGMENTS

This work is supported by grant no. MB009 from the Kuwait University Research Council.

REFERENCES

1. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., and Fu, C.P. (1974) Clin. Chem. 20, 470-475.
2. Bucolo, G., and David, H. (1973) Clin. Chem. 19, 476-482.
3. Thomas, A.E., McKay, D.A., and Cutlip, M.B. (1976) Am. J. Clin. Nutr. 29, 302-304.
4. Fredrickson, D.S., Levy, R.I., and Lees, R.S. (1967) New Engl. J. Med. 276, 32, 94, 148, 215, 273.
5. Wood, P.D.S., Stern, M.P., Silvers, A., Reaven, G.M., and Von Der Groeben, J. (1972) Circulation 45, 114-126.
6. Kwiterovich, P.O., Chase, G.A., and Bachorik, P.S. (1978) Johns Hopkins Med. J. 143, 32-42.
7. Carlson, L.A., and Bottiger, L.E. (1972) Lancet 1, 865-868.
8. Goldstein, J.L., Schrott, H.G., Hazzard, W.R., Bierman, E.L., and Motulsky, A.G. (1973) J. Clin. Invest. 52, 1544-1568.
9. Murphy, E.A., and Kwitervoich, P.O. (1977) in Hyperlipidemia, Diagnosis and Management (Levy, R.I., and Rifkind, B.M., eds.) Chapter 12, pp. 217-248, Grune and Stratton, New York, NY.
10. Greenfield, M.S., Kraemer, F., Tobey, T., and Reaven, G. (1980) Metabolism 29, 1095-1099.

[Received February 16, 1982]

Hydrolysis of Triglycerides in the Isolated Perfused Rat Lung[1]

SUZANNE K. COMPTON, MARGIT HAMOSH* and **PAUL HAMOSH**, *Departments of Physiology and Biophysics and Pediatrics, Georgetown University Medical Center, Washington, DC 20007*

ABSTRACT

The purpose of this study was to investigate the hydrolysis of saturated and unsaturated triglycerides by lung lipoprotein lipase and to measure the incorporation of triglyceride fatty acids into lung tissue lipids. Lipolytic activity was studied in the isolated ventilated rat lung, perfused for 100 min in a recycling system with Krebs Ringer bicarbonate containing bovine serum albumin, 5.6 mM glucose, and 1.5 or 10 mM triglyceride. Saturated triglycerides were hydrolyzed at significantly ($p<0.05$) lower rates than unsaturated triglycerides; tricaprylin, trimyristin and tripalmitin were hydrolyzed at 8.1 + 1.8, 5.4 + 1.5 and 9.5 + 1.8 μmol free fatty acids/g dry wt/100 min, respectively, whereas triolein and trilinolein were hydrolyzed at 20.2 + 1.8 and 20.6 + 0.3 μmol free fatty acids/g dry wt/100 min, respectively. The polyunsaturated triglycerides, trilinolein and triarachidonin were hydrolyzed at even higher rates (44.3 + 3.0 and 50.9 + 5.4 μmol free fatty acids/g dry wt/100 min, respectively). Intralipid infused at a concentration of 10 mM triglyceride was hydrolyzed at a significantly higher rate than at 1.5 mM triglyceride (58 + 6.3 μmol free fatty acids/g dry wt/100 min vs 16.6 + 1.7 μmol free fatty acids/g dry wt/100 min, respectively). Labeled unsaturated triglycerides were broken down at significantly higher rates than labeled saturated triglycerides. Incorporation of triglyceride-fatty acid into lung lipid was greater into neutral lipids than into phospholipids. The data suggest that (a) the factors that appear to affect lung lipoprotein lipase activity are composition and concentration of circulating triglyceride, (b) uptake of fatty acids into the tissue was proportional to the rate of hydrolysis of the emulsion, and (c) triglyceride-fatty acids could therefore be used by the lung for metabolic needs.

Lipids 17:696-702, 1982.

INTRODUCTION

Dietary fatty acids are transported in the plasma as triglycerides, which form the major constituents of chylomicrons and very low density lipoproteins (VLDL). Due to their large size, these lipoproteins cannot cross the endothelial barrier. Hydrolysis of triglyceride in lipoproteins is a necessary step for transfer of triglyceride-fatty acids to extrahepatic tissues (1). This hydrolysis is catalyzed by lipoprotein lipase, an enzyme which is functional at the capillary endothelial surface (2). Lipoprotein lipase is found in most tissues which utilize plasma triglyceride and its level of activity usually reflects the capacity of the tissue to break down blood triglyceride (3).

High intake of dietary fats and elevated serum levels of triglyceride are established risk factors for the development of atherosclerosis (4). The pulmonary vasculature is the initial capillary bed to be traversed by newly formed lipoproteins from the liver and intestine. Since the lung has high lipoprotein lipase activity (5), it may play a role in modifying or removing potentially atherogenic substances from the plasma before they reach the coronary, cerebral and other systemic circulations.

The purpose of this study was to determine the hydrolysis of different saturated and unsaturated triglycerides by lung lipoprotein lipase and to measure the incorporation of triglyceride-fatty acids into the tissue lipids of the isolated perfused rat lung.

MATERIALS AND METHODS

Lung Perfusion

Male Sprague-Dawley rats (Flow Lab, Dublin, VA), weighing 200-250 g and fed ad libitum, were anesthesized with an intraperitoneal (ip) injection of sodium pentobarbital (50 mg/kg). The trachea was cannulated and attached to a Harvard rodent respirator. Lungs were ventilated with 95% air/5% CO_2, a tidal volume of 2.0-2.5 ml at a rate of 60 cycles/min, and an end expiratory pressure of 2-3 cm H_2O. The lungs were cleared of blood by perfusing Krebs Ringer bicarbonate solution (KRB) through the hepatic portal vein. The chest was opened, the pulmonary artery was cannulated,

*Author to whom correspondence should be addressed.

[1]The data presented in part at the Annual Meetings of the American Physiological Society, Atlanta, GA, April 1981, and the American Thoracic Society, Detroit, MI, May 1981, and published in abstract form—*Fed. Proc.* 40, 621 (1981), and *Am. Rev. Respir. Dis.* 123, 219 (1981).

and the lungs were perfused with KRB. The lungs were removed from the thorax and placed in a 37 C, water-jacketed plexiglass incubation chamber. Perfusion was maintained through the pulmonary artery at a flow rate of 10 ml/min by a peristaltic pump (LKB Industries, Rockville, MD). The perfusate consisted of 40 ml of KRB containing 3% fatty acid-poor bovine serum albumin (BSA) (dialyzed fraction V; Miles Laboratories, New York, NY), 5.6 mM glucose, and the appropriate triglyceride emulsion (prepared as described below). The perfusate was maintained at a pH of 7.3-7.4 and was aerated with the same gas mixture throughout the experiment. The effluent dripped from the cut left atrium into the bottom of the incubation chamber and was then recycled. Samples of the effluent were taken at 10, 20, 30, 50, 70 and 100 min after the beginning of the experiment. Each experimental group consisted of four successful lung preparations. Lungs were discarded if visible signs of edema occurred.

Preparation of Triglyceride Emulsions

The triglycerides perfused were tricaprylin (8:0), trimyristin (14:0), tripalmitin (16:0), triolein (18:1), trilinolein (18:2), trilinolenin (18:3) and triarachidonin (20:4) at a concentration of 1.5 mM. All triglycerides were purchased from Sigma Chemicals, St. Louis, MO. Approximately 300 μmol of triglyceride, 10 mg of phosphatidylcholine (Sigma), 0.5 ml glycerol (Fisher, Silver Springs, MD), 2.5 ml 4% BSA in saline and 2 ml fresh, inactivated (heated at 60 C for 30 min to inactivate lipases) rat serum were emulsified by sonication on ice for 30 sec at maximal speed using a Polytron PCU-2-110 Sonifier C (Brinkman Instruments, Inc., Westbury, NY). The resulting emulsion was incubated at 37 C for 30 min immediately prior to perfusion. During this incubation, the emulsified lipid binds the C-II apoprotein of serum lipoproteins, the specific activator of lipoprotein lipase (6). The particle size of the different emulsions was similar as measured by electron microscopy; it ranged ca. 0.2-2.0 μm in diameter. The emulsified triglyceride (300 μmol in 5 ml) was added to 195 ml perfusion fluid to give a final concentration of 1.5 mM triglyceride. Each emulsion was circulated throughout the perfusion apparatus to evaluate breakdown of the triglyceride in the absence of a lung. Lungs were also perfused with 3% BSA in KRB alone to quantitate endogenous release of free fatty acids.

Intralipid (Vitrum, Stockholm, Sweden), a 10% emulsion of soybean oil stabilized with 1.2% egg phospholipid used extensively for parenteral nutrition (7), was also perfused. Intralipid was incubated with 2 ml of serum for 30 min before infusion at a final concentration of 1.5 or 10 mM triglyceride.

Perfusate Analysis

Total lipid was extracted from all perfusion samples by a modification (8) of the method of Dole and Meinertz (9). Free fatty acids were quantitated by microtitration; the hexane extracts of perfusion samples were removed to a separate tube and air-dried. Lipid was redissolved in 0.2% Nile Blue in ethanol and titrated with 0.02 N NaOH using a Menisco microtitrator (Metrohm, Model #E 457; Brinkman Instruments, Westbury, NY). Palmitic acid was used to prepare a standard curve for calculation of the amount of free fatty acids in each sample.

Preparation of Radioactive Liver Triglycerides

Radioactive liver triglycerides were prepared according to the method of Scow and Egelrud (10). Five mc of [9,10-^{3}H(N)]palmitic acid (New England Nuclear, Boston, MA) was complexed to 14 ml of 14% BSA and injected ip into a fed, male Sprague-Dawley rat, weighing 350 g. After 4 hr, the rat was anesthetized with sodium pentobarbital and the liver, weighing ca. 12 g, was removed and homogenized in 80 ml of ice-cold chloroform/methanol. The homogenate was extracted overnight at 4 C and filtered the following day. Lipids in the filtrate were extracted into chloroform by the method of Folch et al. (11). Lipids in the extract were separated on a silicic acid column (10 g of Unisil-activated silicic acid, 100-200 mesh, Clarkson Chemical Co., Williamsport, PA, in a plastic column; Evergreen Scientific Co., Los Angeles, CA). Neutral lipids and fatty acids were eluted from the column with 5% methanol in chloroform. Fatty acids were separated from neutral lipids by a modification of the method of Borgstrom (12). The purity of the triglyceride fraction was verified by thin layer chromatography (TLC). An aliquot of the fraction was spotted onto Silica Gel G chromatogram plates (Analabs, North Haven, CT). Neutral lipids were separated using the solvent system petroleum ether/ethyl ether/glacial acetic acid (110:60:2). Individual spots were identified by comparison with standards of neutral lipids (Applied Science Lab TLC standard #21999, St. College, PA). The amount of triglyceride present was quantitated by the method of Rapport and Alonzo (13).

Preparation of Radioactive Triglyceride Emulsions

Liver triglycerides, glycerol tri-^{3}H-oleate (New England Nuclear) or glycerol tri-1-^{14}C-palmitate (New England Nuclear) were perfused through the lung to quantitate the hydrolysis and uptake of both saturated and unsaturated fatty acids and their incorporation into lung lipids. Triglyceride emulsions were prepared as already described with a sp act of 0.5 μc/μmol triglyceride and perfused at a final concentration of 1.5 mM. After perfusion for 100 min, the lungs were postperfused for 10 min with 2% BSA (Reheis Pharmaceuticals, Phoenix, AZ) in KRB to remove radioactivity sticking to the endothelial surface or trapped in the vasculature.

Radioactive Perfusate Analysis

Total lipids were extracted from effluent samples by a modification (8) of the method of Dole and Meinertz (9). Fatty acids were separated from hexane extracts by a modification of the method of Borgstrom (12). Aliquots of the hexane, fatty acid and glyceride fractions were taken for liquid scintillation counting in 3.5% PPO-POPOP in toluene (New England Nuclear) in a Beckman LS 3150T liquid scintillation counter.

Radioactive Tissue Analysis

Lipids were extracted from ca. 300 mg wet weight of tissue, selected randomly from the lung. Column chromatography was used to separate neutral lipids and phospholipids. Neutral lipids were eluted with 100% chloroform and phospholipids were eluted with 100% methanol. Column fractions were evaporated to dryness under N_2, and then redissolved in small amounts of chloroform. An aliquot of each lipid was spotted onto Silica Gel G chromatogram plates (Analabs). Neutral lipids were separated and identified as already described. Phospholipids were separated using the solvent system chloroform/methanol/H_2O (120:80:7). Individual spots were identified by comparison with phospholipid standards (Applied Science Lab TLC standard #21994), scraped into scintillation vials, and counted as already described.

Data Analysis

Dry wt/wet wt ratios were determined for each lung. Data were expressed as μmol fatty acids released into the perfusate/g dry wt of lung. Mean and standard error of the mean were calculated for each TG emulsion and compared using the two-tailed Student's t-test at the $p<0.05$ level of significance.

RESULTS

Perfusion of Different Triglyceride Emulsions

No endogenous release of free fatty acids occurred when 3% BSA in KRB alone was perfused through the lung. In addition, none of the emulsions were broken down to free fatty acids by perfusion through the apparatus in the absence of the lungs. Dry wt/wet wt ratios for the lung tissue were ca. 16% for all lungs in these experiments, indicating that edema did not develop during the course of the perfusions. Dry wt/wet wt ratios for the unperfused lungs ranged from 15-17%.

We studied the hydrolysis of triolein as a function of triglyceride concentration previously (14) and found the rate of hydrolysis was directly proportional to the concentration of the infused triglyceride in the range of 0.38-2.2 mM. The data indicated that, in the lung, lipoprotein lipase is not saturated at physiological levels of circulating triglyceride (0.3-0.5 mM during fasting, 1.0-2.0 mM postprandially). In the present study, we decided to use the concentration usually present in the circulation of normal, fed rats, i.e., 1.5 mM. We have therefore compared the various triglyceride substrates at the concentration present in the circulation 1.5 mM to evaluate their hydrolysis by the isolated, perfused lung, under physiological conditions. The hydrolysis of different saturated and unsaturated triglyceride emulsions by the lung is shown in Figure 1. The saturated triglycerides tricaprylin, trimyristin and tripalmitin were broken down at rates of 8.1 + 1.8, 5.4 + 1.5 and 9.5 + 1.8 μmol fatty acids/g dry wt/100 min perfusion, respectively. There was no statistical difference in hydrolysis of these three saturated triglycerides in the perfused lung. The unsaturated triglycerides triolein and trilinolenin were broken down to fatty acids at a significantly higher rate than the saturated emulsions (20.0 + 1.8 and 20.6 + 0.3 μmol free fatty acids/g dry wt lung/100 min, respectively). The lung hydrolyzed the polyunsaturated triglycerides trilinolein and triarachidonin at significantly higher rates than the other unsaturated triglycerides studied (44.3 + 3.0 and 50.9 + 5.4 μmol free fatty acids/g dry wt/100 min, respectively).

Perfusion of Intralipid

Intralipid (1.5 mM) was hydrolyzed at approximately the same rate as triolein and trilinolenin (16.6 + 1.7 μmol free fatty acids/g dry wt/100 min). The results are shown in Figure 2. Intralipid at a concentration of 10 mM was broken down at a significantly higher rate than at the lower concentration (58 + 6.3

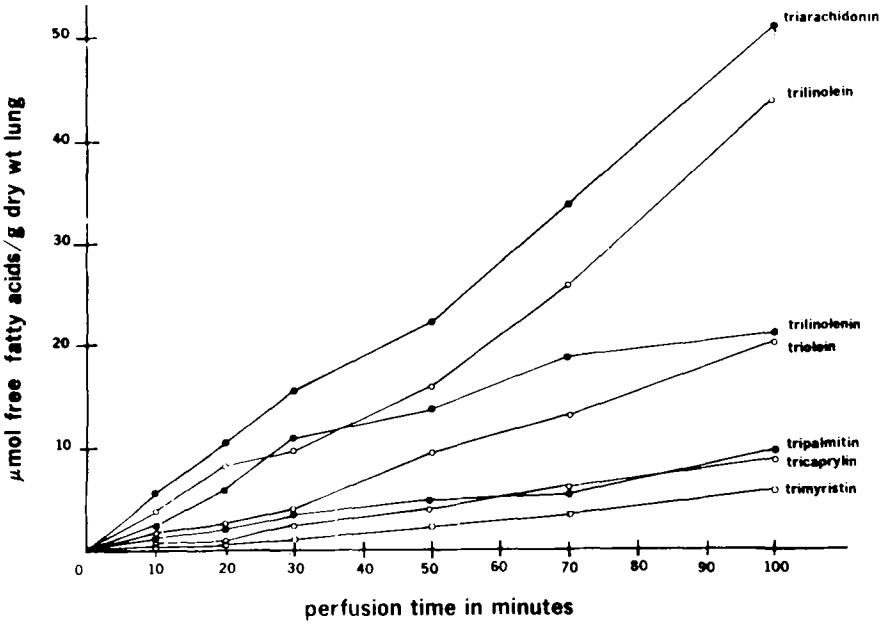


FIG. 1. Hydrolysis of different saturated and unsaturated triglyceride emulsions by lung lipoprotein lipase. Each triglyceride was perfused for 100 min at a final concentration of 1.5 mM. Each point represents the mean of four lungs. Statistical analysis of the differences between the rates of hydrolysis of various triglycerides showed: (1) no difference between the three saturated triglycerides, (2) significant differences ($p < 0.05$) between saturated and mono- or polyunsaturated species at all time periods tested and (3) significant ($p < 0.05$) differences between triolein and trilinolenin (at 20 and 30 min perfusion) triolein vs trilinolein and triolein vs triarachidonin (throughout the 100 min of perfusion).

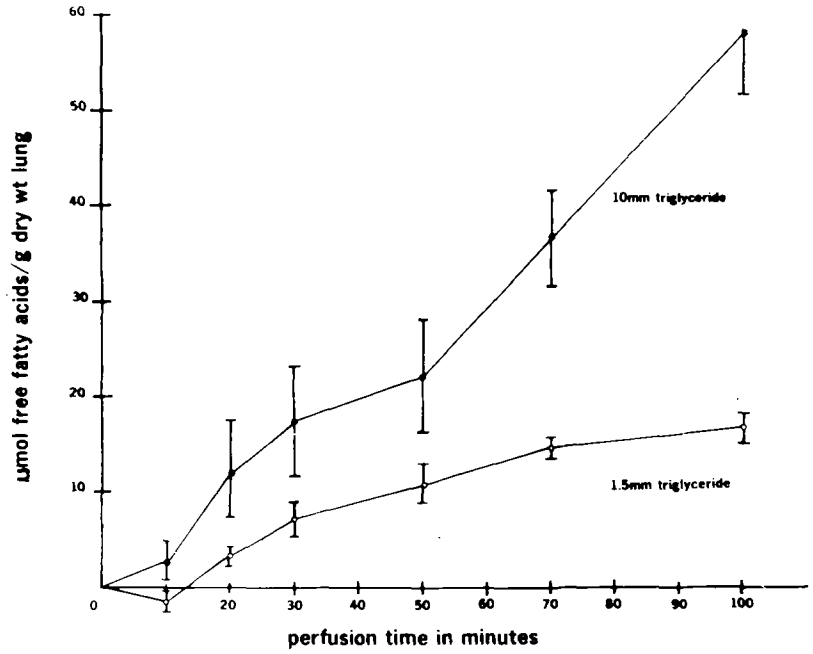


FIG. 2. Hydrolysis of Intralipid by lung lipoprotein lipase. Intralipid was perfused at a final concentration of either 1.5 or 10 mM triglyceride. Each point represents the mean ± SEM of four lungs.

μmol free fatty acids/g dry wt/100 min).

Perfusion of ^{3}H-Labeled Liver Triglycerides, Tri-^{3}H-oleate and Tri-^{14}C-palmitate

Emulsions of labeled triglycerides were perfused through the lung to measure the incorporation of triglyceride-fatty acids into lung lipids. Triolein was used as representative of the unsaturated triglycerides hydrolyzed by lung lipoprotein lipase at an intermediate rate, and tripalmitin was representative of the saturated triglycerides, which were broken down by lung lipoprotein lipase at a much lower rate. No radioactive triarachidonin or trilinolein were available at the time of this study. In vivo labeled liver triglycerides contain both saturated and unsaturated fatty acids.

Figure 3 shows the release of fatty acids into the effluent after perfusion with liver triglycerides and triolein. Free labeled fatty acids could not be detected in the effluent of lungs perfused with tripalmitin. Triolein was hydrolyzed at 2.6 + 0.2 μmol fatty acids/g dry wt/100 min. Liver triglycerides were broken down at a significantly higher rate (16.5 + 2 μmol fatty acids/g dry wt/100 min).

There was no significant difference between the amount of free fatty acid found in the effluent and the amount of fatty acid found in the lung tissue after 100 min of perfusion with either triolein or liver triglycerides.

The amount of radioactivity incorporated into lung lipids is shown in Figures 4 and 5. Incorporation of fatty acid into esterified lipids was greatest for liver triglycerides, followed by triolein, and was least for tripalmitin.

Figure 4 shows the incorporation of fatty acids into monoglycerides, diglycerides and tissue free fatty acids. Most of the label was present as free fatty acids in the tissue with the three different triglycerides infused. The amount of fatty acid in monoglycerides was significantly lower than for diglycerides for all emulsions.

Lung neutral lipids and free fatty acids incorporated a significantly larger amount of radioactivity than the lung phospholipid. Figure 5 shows the incorporation of fatty acid into phosphatidylcholine, lysophosphatidylcholine and phosphatidylethanolamine. No significant difference was found between the incorporation of ^{14}C-fatty acid from tripalmitin into any of these phospholipids. Incorporation of fatty acid into phosphatidylcholine was significantly

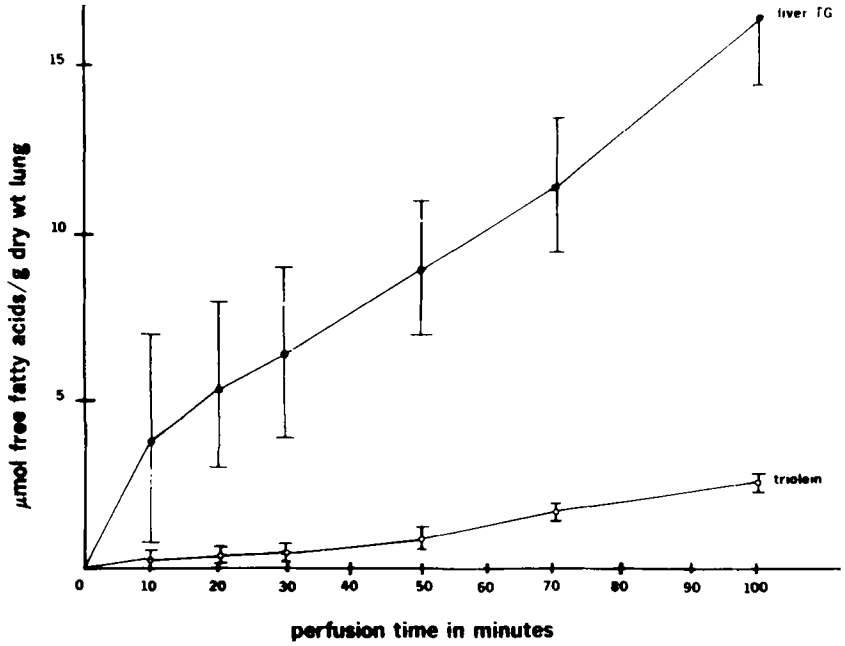


FIG. 3. Hydrolysis of different saturated and unsaturated radioactive triglyceride emulsions by lung lipoprotein lipase. Each triglyceride emulsion was perfused at a final concentration of 1.5 mM. Each point represents the mean ± SEM of four lungs. Rates of hydrolysis of triolein vs liver triglycerides, significantly different ($p < 0.05$) throughout the entire 100 min of perfusion.

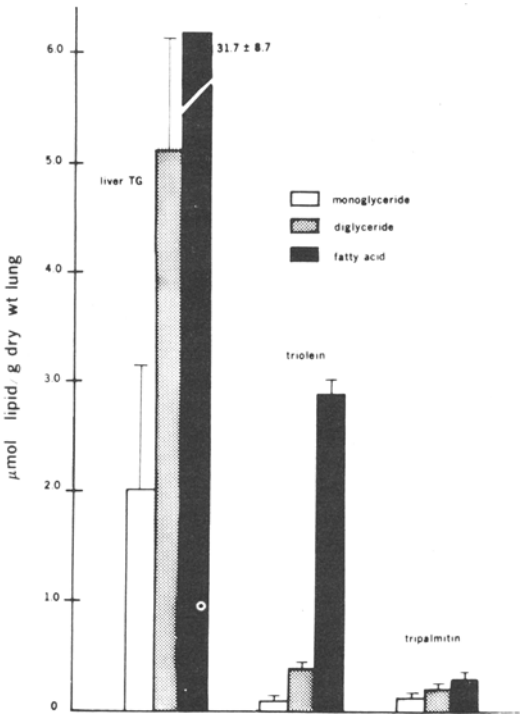


FIG. 4. Incorporation of triglyceride-fatty acids into tissue neutral lipids. Lungs were perfused with either ^{3}H-liver triglyceride (liver TG), glycerol tri-^{3}H-oleate (triolein), or glycerol tri-^{14}C-palmitate (tripalmitin) at a final concentration of 1.5 mM. Each bar represents the mean ± SEM of four lungs. Differences in the incorporation into tissue lipid, of the three triglycerides infused, statistically significant ($p < 0.05$).

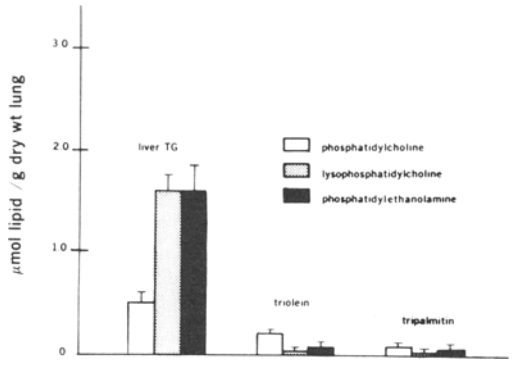


FIG. 5. Incorporation of triglyceride-fatty acids into tissue phospholipids. Lungs were perfused with ^{3}H-liver triglycerides (liver TG), glycerol tri-^{3}H-oleate (triolein), or glycerol tri-^{14}C-palmitate (tripalmitin) at a final concentration of 1.5 mM. Each bar represents the mean ± SEM of four lungs. Differences in the incorporation into tissue lipid, of the three triglycerides infused, statistically significant ($p < 0.05$).

greater for ^{3}H-oleic acid than for ^{14}C-palmitic acid.

DISCUSSION

The composition of circulating triglyceride has a marked effect on the rate of their hydrolysis by lung lipoprotein lipase. Saturated triglycerides were hydrolyzed at significantly lower rates than unsaturated triglycerides. All three saturated triglycerides were broken down at similar rates. Triolein, trilinolenin and Intralipid were hydrolyzed at an intermediate rate, and trilinolein and triarachidonin were broken down at significantly higher rates. Therefore, one factor determining the rate of removal of triglycerides from the circulation by lung lipoprotein lipase may be the composition of the triglyceride-fatty acids.

Intralipid has a metabolic fate similar to naturally occurring chylomicrons. In man, Intralipid clearing from the plasma is directly proportional to its plasma concentration (7). In the isolated rat lung, this was also the case. Hydrolysis of Intralipid was significantly greater at the higher concentration (10 mM) than at the lower concentration (1.5 mM). In a previous study, Gal et al. (14) found that breakdown of triolein to fatty acids was also directly proportional to the concentration of circulating triglyceride in the range of 0.35-2.2 mM.

When labeled triglycerides were infused, the unsaturated radioactive emulsion triolein was also broken down at a significantly higher rate than the saturated tripalmitin emulsion. Liver triglycerides containing both saturated and unsaturated fatty acids were hydrolyzed at an even faster rate. These liver triglycerides are probably the precursors for circulating VLDL-triglyceride. Lung lipoprotein lipase can hydrolyze significant amounts of this triglyceride to free fatty acids, perhaps indicating that, in vivo, this enzyme might modify VLDL before they reach the systemic circulation.

When triglycerides are hydrolyzed in peripheral tissues, some free fatty acids are released into the blood and either taken up by other tissues or by the liver for reesterification. A rapid release of fatty acid will occur as long as there are sufficient quantities of free fatty acid acceptor (albumin) present (15). These studies indicate that the lung releases significant quantities of free fatty acids during hydrolysis of triglyceride.

Newly hydrolyzed free fatty acids from plasma triglyceride are incorporated into both neutral lipids and phospholipids in the lung tissue. Although palmitic acid is a precursor of

surfactant dipalmitoylphosphatidylcholine, it was not incorporated into tissue phosphatidylcholine at a rate greater than oleic acid. In these experiments, the rate of hydrolysis by lipoprotein lipase and thus, substrate availability, not substrate specificity, appeared to determine the amount of incorporation of fatty acids into tissue lipids. The pool sizes of free palmitic and oleic acids in the adult rat lung are unknown; therefore, we cannot evaluate whether the difference in tissue level of the labeled fatty acids are, in addition, affected by different amounts of oleic and palmitic acid in the lung. Most of the fatty acids were present as free fatty acids or diglycerides, with only small amounts of monoglycerides.

It appears from our data that lung lipoprotein lipase has a low substrate affinity for circulating triglyceride. A low affinity means that the rate of triglyceride uptake is a function of its concentration (16). This is also the case for adipose tissue. On the other hand, because of high-affinity lipase sites, the rate of uptake of triglyceride by the heart remains constant under conditions of both scarcity and abundance of circulating triglyceride (17). Therefore, when plasma levels of triglyceride are low, lung lipoprotein lipase may not effectively compete with heart lipoprotein lipase for fatty acids necessary for tissue metabolism.

In conclusion, two factors which appear to affect lung lipoprotein lipase activity are (a) composition of circulating triglyceride-fatty acids, and (b) concentration of circulating triglycerides. Uptake and incorporation of fatty acids into tissue lipids was proportional to the rate of hydrolysis of the particular substrate. Fatty acids taken up by the lung are used for energy metabolism, phospholipid synthesis and de novo fatty acid synthesis (18). Free fatty acids are also released into the circulation, possibly as an energy source for other extrahepatic tissues.

Lung lipoprotein lipase may play a protective role toward the systemic circulation by changing the composition of circulating lipoproteins in the plasma. A better understanding of the mechanism of action of lung lipoprotein lipase is necessary to evaluate its role in lipoprotein metabolism.

ACKNOWLEDGMENTS

This work was supported by NIH grant HL 19056 and by a grant from the Council for Tobacco Research, USA.

REFERENCES

1. Scow, R.O., Hamosh, M., Blanchette-Mackie, E.L., and Evans, A.J. (1972) Lipids 7, 497-505.
2. Nilsson-Ehle, P., Garfinkel, A.S., and Schotz, M. (1980) Ann. Rev. Biochem. 49, 667-693.
3. Bensadoun, A., and Kompiang, I.P. (1979) Fed. Proc. 38, 2622-2626.
4. Norday, A. (1976) Thromb. Haemostas. 35, 32-38.
5. Hamosh, M., and Hamosh, P. (1975) Biochim. Biophys. Acta 380, 132-140.
6. Havel, R.J., Shore, V.G., and Bier, D.M. (1970) Circ. Res. 27, 595-600.
7. Bryan, M.H., Shennan, A.T., Griffen, E., and Angel, A. (1976) Pediatrics 58, 787-790.
8. Chernick, S.S., and Novac, M. (1970) Diabetes 19, 563-570.
9. Dole, V.P., and Meinertz, H. (1960) J. Biol. Chem. 235, 2595-2599.
10. Scow, R.O., and Egelrud, T. (1976) Biochim. Biophys. Acta 431, 538-549.
11. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-505.
12. Borgstrom, B. (1952) Acta Physiol. Scand. 25, 111-129.
13. Rapport, M.M., and Alonzo, M.J. (1959) J. Biol. Chem. 217, 193-198.
14. Gal, S., Bassett, D.J.P., Hamosh, M., and Hamosh, P. (1979) Fed. Proc. 38, 1328.
15. Smith, L.C., and Scow, R.O. (1979) Prog. Biochem. Pharmacol. 15, 109-138.
16. Fielding, C.J. (1976) Biochemistry 15, 879-884.
17. Fielding, C.J., and Havel, R.J. (1977) Arch. Pathol. Lab. Med. 101, 225-229.
18. Bassett, D.J.P., Hamosh, M., Hamosh, P., and Rabinowitz, J.L. (1981) Exp. Lung. Res. 2, 37-47.

[Received April 7, 1982]

Autoxidation of Cholesterol Fatty Acid Esters in Solid State and Aqueous Dispersion

V. KORAHANI, J. BASCOUL* and A. CRASTES de PAULET, *Unité de Recherches sur la Biochimie des Stéroïdes, INSERM U.58 - 60, Rue de Navacelles, 34100 Montpellier, France*

ABSTRACT

Cholesteryl stearate, oleate, linoleate, linolenate and arachidonate were oxidized in solid form (at 100 C) and in a water dispersion (in the presence of potassium stearate, pH 7.5, 80 C). The unsaponifiable fraction was analyzed by capillary gas liquid chromatography. In the solid state, the oxidation rates of esterified cholesterol were high for stearate and oleate, low for the polyunsaturated esters and very low for free cholesterol. In water dispersion, the rates were reversed, e.g., free cholesterol oxidized more quickly than its stearic and oleic acid esters. The fatty chains in 18:0 and 18:1 inhibited the autoxidation of cholesterol. Hydroxylation of the cholesterol side chain only occurred during solid-state autoxidation as previously observed by others. The 20- and 25-hydroxycholesterols were never detected in the products of micellar reactions, regardless of which surfactant was used for micelle formation.

Lipids 17:703-708, 1982.

INTRODUCTION

Because of the toxicity of oxidized sterols (O-sterols), particularly the hydroxycholesterols (1-5), and their role in experimental atherosclerosis (1,6), it is essential that we know the conditions under which they are formed from the free or esterified precursors which might be present in human food fats.

Oxidized sterols (hydroxy-, keto-, or epoxysterols) might be formed by the autoxidation of free or esterified sterols during the thermal processing of certain food containing fats (6,7). Autoxidation in the presence of atmospheric oxygen is enhanced by temperature, light radiation, transition metals (e.g., Fe, Cr, Co) or initiators of free radical reactions, which often occur in the processing of food.

Since the early paper of Bergström and Wintersteiner (8), Smith, Ansari and Teng et al. have done important work on the study of free cholesterol autoxidation in solid and dispersed state (9-11). The products formed by this reaction, in the presence of ground-state ($^3O_2$) atmospheric oxygen (9), are the 7α- and 7β-hydroperoxides (products of substitution), 5,6α-epoxy-5α-cholestan-3β-ol (product of addition) and to a very small extent the 20- and 25-hydroperoxides. Singlet oxygen ($^1O_2$) yields 5α-hydroperoxy derivatives and small quantities of 6α- and 6β-hydroperoxides (12). A third, minor component of the autoxidation mixture is the 3-keto-cholesta-5-ene resulting from the dehydrogenation of the 3β-hydroxy group (10).

All these products epimerize and/or decompose, resulting in a significant number of hydroxyl and carbonyl compounds. Essentially, the 7α- and 7β-hydroxycholesterols, 7-oxocholesterol, 3,5-cholestadiene-7-one and 5α-cholestan-3β,5,6β-triol are formed (13).

Cholesteryl fatty acid esters are present in large quantities in animal fats, but their autoxidation is not as well known. This is the motivation for the work described in this paper.

*Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Reagents

Solvents for extraction and chromatography were double-distilled before use. Pyridine, hexane, hexamethyldisilazane and trichloromethylsilane were Merck reagents. The methoxyamine hydrochloride was supplied by Pierce (Rockford, IL).

Steroids

Cholestane, dihydrocholesterol, 7α-, 7β-, 25-, (20 R)-20-hydroxycholesterol and 3β-acetoxy-5,7-cholestadiene were obtained from Steraloids (Wilton, NH). The (22 S)-22-, (24 R)-24- and (24 S)-24-hydroxy-; the (25 R)-26-hydroxy- and the 22ξ, 23ξ-dihydroxycholesterol were generously provided by Professor Van Lier (Sherbrooke, Quebec, Canada). 5,6α-Epoxy-5α-cholestan-3β-ol, 3β,5α,6β-triol and 3,5-cholestadien-7-one were prepared in our laboratory.

Apparatus

For gas liquid chromatography (GLC), a Carlo Erba Fractovap 2300 chromatograph equipped with a capillary column (25 m × 0.25

mm) impregnated with OV-101 was used and helium was the carrier gas at a flow rate of 1 ml/min. For injection, a 1-μl Pyrex stream splitter, type ROS (Carlo Erba, Milan, Italy) was used. Other conditions: column temperature, 285 C; injection temperature, 350 C; flame ionization detector; recorder speed, 30 cm/min.

Mass spectra were recorded on an LKB 2091 gas chromatograph-mass spectrometer at 70 eV voltage. The chromatograph was equipped with a column packed with SE-30 (25 m x 0.25 mm). The helium flow rate was 4 ml/min, and the column temperature was programmed from 250 to 310 C at 10 C/min.

Thin Layer Chromatography (TLC)

Silica Gel $60F_{254}$ plates, 20 x 20 cm, 0.2 mm thick (E. Merck, Darmstadt, G.F.R.), were developed twice with diethyl ether/cyclohexane (9:1, v/v). The sterols were revealed by their ultraviolet (UV) absorption at 254 nm or by spraying with a 50% aqueous solution of H_2SO_4, followed by heating. Preparative TLC was performed in the same manner, but with 0.25-mm-thick plates. After scraping off the zones outlined by UV spectroscopy at 254 nm, they were eluted with 50 ml of chloroform/methanol (2:1, v/v).

Derivatization of Sterols

The residue to be derivatized (generally obtained by preparative TLC) was deposited in a screw-cap culture tube by evaporating the solvents under nitrogen. Then 0.5 ml methoxyamine hydrochloride solution in pyridine (20 mg/ml) was added. The tube was plugged with a Teflon stopper and heated in a water bath for 4 hr at 60 C. After evaporation of the excess of pyridine under nitrogen, 150 μl of pyridine/hexamethyldisilazane/trichloromethylsilane (1:1:1, v/v) was added and the tube was allowed to stand at ambient temperature for 12 hr. Excess of solvent was evaporated under nitrogen. The residue was taken-up in hexane (1 ml/100 μl) and injected into the gas chromatograph.

Autoxidation of Free and Esterified Cholesterol

In solid state. A 10- to 50-mg amount of sterol (free or esterified form) was placed in a tube and heated in an oven at 100 C in the presence of trace amounts of benzoylperoxide. After heating for a period of 1 hr to 30 days, the sample was hydrolyzed in 10% KOH/MeOH (w/v) in a nitrogen atmosphere overnight at room temperature. The solvents were evaporated in a nitrogen stream. The crude products were extracted with $CHCl_3$ and the solvents removed in vacuo. A fraction of the crude products was chromatographed on silica plates using diethyl ether/cyclohexane (3:1, v/v) as solvent. The remainder was derivatized as already described for subsequent analysis by capillary columns impregnated with OV-101 (14).

In aqueous dispersion. Sodium stearate was obtained from a reaction of stearic acid and sodium in anhydrous diethyl ether at room temperature for 10 hr. Standard aqueous dispersions were prepared by dissolving 130 mg of sodium stearate in 400 ml of distilled water ($4.2\ 10^{-4}$ M). The pH was adjusted to 7.5 by acetic acid. Aqueous dispersions of Triton-X100, cetyltrimethylammoniumbromide and sodium dodecylsulfate were prepared by dissolving the detergent in distilled water without modifying the pH value. An aliquot of 50 ml of these solutions was heated at 80 C with vigorous stirring. Ten mg of ester dissolved in 5 ml of hot dioxane was added and air was bubbled through the solution with occasional addition of water to maintain the initial volume. Cooled aliquots were neutralized, extracted and analyzed as described for autoxidation of solid-state samples.

RESULTS AND DISCUSSION

The autoxidation of cholesteryl esters results in the peroxidation of the cholesteryl and fatty acid part. We describe only the modifications of the cholesteryl part (contained in the unsaponifiable fraction) during these reactions.

Kinetic studies of various solidified esters in the presence of benzoyl peroxide (Fig. 1)

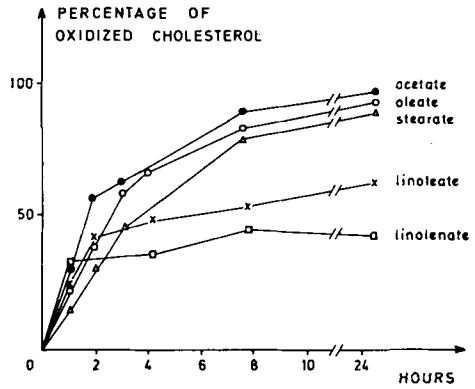


FIG. 1. Percentage of oxidized cholesterol (measured as 100 minus percentage of recovered cholesterol) obtained by autoxidation and hydrolysis (as described in the experimental procedure) of cholesteryl esters: acetate, oleate, stearate, linoleate and linolenate.

showed that the initial rate of the cholesterol oxidation reaction increased in proportion to how unsaturated the ester was. This acceleration is probably due, on one hand, to the peroxide content of the medium, which rises with unsaturation and facilitates propagation, and on the other hand, to the development of an intramolecular autoxidation reaction. The oxidized acyl part ($R_{\bar{a}}$ $OO^{\cdot}$) might react on the cholesteryl rings as in the following equation where R_S represents the steroid and R_a the fatty acid moiety:

$$\begin{array}{ccccc} R_sH & & R_s^{\cdot} & O_2 & R_sOO^{\cdot} \\ | & \rightarrow & | & \rightarrow & | \\ R_a\text{-}O\text{-}O^{\cdot} & & R_a\text{-}OOH & & R_a\text{-}OOH \end{array}$$

The initial rate of oxidation of the cholesteryl part was greater for unsaturated fatty esters than for saturated ones (Fig. 1). This rate decreased with time. After 10 hr, the percentage of oxidized cholesterol was greater for saturated than unsaturated esters.

These experiments do not resolve the mode of reaction of the acyl part. However, we observed that the R_f of autoxidized cholesteryl acetate, oleate or stearate is analogous to that of nonoxidized products. On the contrary, the R_f of autoxidized cholesteryl linoleate or linolenate is markedly diminished. This accounts for the formation of a polar site probably formed by a cleavage of the unsaturated acyl chain.

The nature and relative abundance of the principal products formed by the autoxidation of cholesterol and its esters are given in Tables 1 and 2. Identification was accomplished by TLC, GLC and GLC-mass spectrometry (GLC-MS). GLC using OV-101 did not resolve 3β-hydroxy-5-cholesten-7-one and 5α-cholestan-3β,5,6β-triol (14) (asymetric signal with the lower retention time for the trihydroxylated sterol). The 3,5-cholestadien-7-one also could have shielded compounds such as 4-cholestan-3-one and desmosterol (14). These compounds, especially the first pair, are easily resolved by TLC and GLC using another stationary phase (15) or HPLC (16).

With GLC we observed: (a) a constant chromatographic profile regardless of the autoxidation mode, whether solid-state or water-dispersion, and (b) complete absence of 20- and 25-hydroxycholesterol when the autoxidation occurred in a micellar medium. On the other hand, these products were formed when the reaction occurred on solid-state esters. This has already been observed by Teng and Smith (11).

The absence of reactivity of the cholesterol side chain in aqueous dispersion is probably due to the inclusion of the molecule in the hydrocarbon-like micellar core. In contrast, the oxidation at positions 20 and 25 in the solid state was a result of the molecular arrangement of the anhydrous or monohydrated crystals (17,18). This arrangement consists of a superposition of bilayers of cholesterol. Each bilayer is composed of two monolayers of 33.3 Å. The cholesterol molecules are juxtaposed in quasi-linear form (the C_8H_{17} chain is held quasi-linearly in the axis of the rings). The rings are contiguous, as well as the side chains. The two monolayers are joined by hydrogen bonds whose configuration varies according to whether the cholesterol is anhydrous or monohydrated. Hence, the terminal atoms 24, 25 and 26 constitute the external part of the bilayers. These atoms have a large thermal mean square amplitude of vibration. Thus, they are at the same time more reactive and more exposed to the attack of reagents than the other atoms. On the other hand, the allylic position on carbon 7 is shielded by the crystal's structure.

An analogous interpretation would also explain the formation of 20- and 25-hydroxycholesterol from polyunsaturated esters. Here again, it can be assumed that the side chains are on the surface of the bilayers where the thermal movement is greatest (19). However, a partial melting of the esters at 100 C makes this hypothesis unlikely.

None of our attempts using the protection of the allylic position against autoxidation by including it in an unreactive environment succeeded in producing 20- or 25-hydroxycholesterol. Various methods were used: absorption on silica (20), the use of monolayers (21), the inclusion of reagents in the micelles of Triton-X100, of cetyl trimethyl ammonium bromide, or of dodecyl sodium sulfate. In the micellar medium, except with sodium stearate micelles, autoxidation was partially or totally inhibited, just as it was in the presence of bovine serum albumin or human plasma (22). No selectivity could be detected.

The results in Figures 1 and 2 and Tables 1 and 2 are mean values obtained from several reactions. The proportions of various compounds were calculated by the internal normalization method. Solid state autoxidation gave more reproducible results than the micellar reactions. This is because of the difficulty of reproducing the micellar medium.

We observed a large difference between the percentage of oxidation of free cholesterol and that of its stearic and oleic esters, regardless of the autoxidation mode. After four days of reaction in the solid state, only 3% of the cholesterol was oxidized, as opposed to 95% of the two esters mentioned (Fig. 2 and Table 1). In a

TABLE 1[a]

Autoxidation in Solid State: Percentage of Recovery of Cholesterol and Oxidized Cholesterol after Autoxidation and Hydrolysis

	Time (days)	Autoxidation products[a]							
		7α-Hydroxy	Cholesterol	7-Keto $\Delta^{3,5}$[b]	7β-Hydroxy	Epoxide[c]	20-Hydroxy	3β,5α,6β-Triol + 7-keto	25-Hydroxy
Cholesterol	4		97						
	30	0.1	80	1.1	0.6	1.1	0.75	0.75	1.5
Stearate	4	3	5	26	2	12	0	25	0
	1	0.5	40	17	3	5	0	18	0
Oleate	4	2	4	28	3	9	0	28	0
Linoleate	4	3	40	18	5	5	0.5	14	0.5
Linolenate	4	1	80	5	3	1	0.2	3	0.2
Arachidonate	4	1	77	8	3	1	0.7	6	0.7

[a]Abbreviations used in Tables 1 and 2: 7α-hydroxy for 5-cholesten-3β,7α-diol; 7-keto $\Delta^{3,5}$ for 3,5-cholestadiene-7-one; 7β-hydroxy for 5-cholesten-3β,7β-diol; epoxide for 5,6β-epoxy-5β-cholestan-3β-ol; 20-hydroxy for 5-cholesten-3β,(20R)-20-diol; 3β,5α,6β-triol for 5α-cholestane-3β,5,6β-triol; 7-keto for 3β-hydroxy-5-cholestene-7-one; 25-hydroxy for 5-cholesten-3β,25-diol. The products described in the different columns are in the order of their retention times (14). No mention is made of hydroperoxysterols which are not measurable by GLC and which readily decomposed by hydrolysis or autoxidation in solid state at 100 C.

[b]The ratio of 3,5-cholestadien-7-one to the other components of the mixture was determined by GLC on the autoxidation mixture. It was slightly increased in percentage determined by GLC after scraping 3,5-cholestadien-7-one from thin-layer plates, suggesting the presence of an unresolved compound of different R_f.

[c]The epimers 5-6α-epoxy-5α-cholestan-3β-ol (predominantly formed by epoxydation of cholesterol) and 5-6β-epoxy-5β-cholestan-3β-ol (predominantly formed by autoxidation of cholesterol: ratio 8:1 [8]) are unresolved by OV-101 at 285 C.

TABLE 2[a]

Autoxidation in Aqueous Dispersion: Percentage of Recovery (Determined by Capillary GLC) of Cholesterol and Oxidized Cholesterol after Autoxidation (96 hr) and Hydrolysis

	Autoxidation products[a]							
	7α-Hydroxy	Cholesterol	7-Keto $\Delta^{3,5}$[b]	7β-Hydroxy	Epoxide[c]	20-Hydroxy	3β,5α,6β-Triol + 7-keto	25-Hydroxy
Cholesterol	8.9	50	3	17	0.5	0	19	0
Stearate	1	95	1	1	0.5	0	0	0
Oleate	3	85	3	6	1	0	1	0
Linoleate	4.5	50	14	5	3	0	8	0
Linolenate	3	75	3	4	2	0	5	0
Arachidonate	3	80	2	4	1	0	4	0

[a-c]Cf. abbreviations in Table 1.

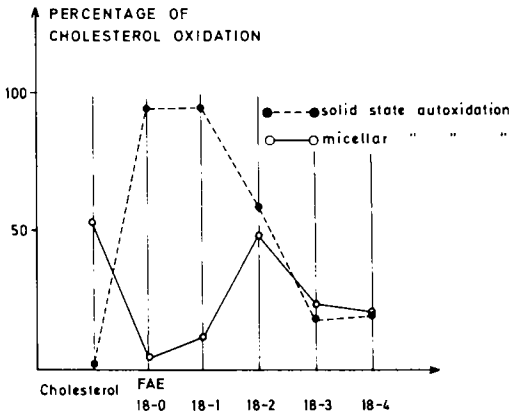


FIG. 2. Percentage of cholesterol oxidation in the autoxidation of free and fatty acid esters (FAE) of cholesterol.

micellar dispersion, the difference is less significant (Fig. 2 and Table 2). We also observe an opposite effect between the two oxidation modes, as seen in the profiles of the curves in Figure 2, with respect to cholesterol and its 18:0 and 18:1 esters. The polyunsaturated esters behaved in an analogous way in both cases. Finally, higher proportion of 7-keto-cholesterol and of triol was observed when autoxidation occurred in a solid state. The same was observed for 3,5-cholestadien-7-one. The 7-hydroxycholesterol was always present in about the same proportion.

By comparing the results of Teng et al. (11) with earlier results (8), we already know that, in micellar medium, free cholesterol autoxidizes better than its esters (22).

The weak oxidation ratio of cholesteryl stearate and oleate in a water dispersion can be assumed to be a result of the shielding of the apolar ester molecule in the micellar core, which is inaccessible to the oxidizing reagent. On the other hand, free cholesterol, with its polar hydroxyl group situated in the hydrophilic zone, is more susceptible to oxidation. This shielding has been observed with other micellar media (22). It is probably enhanced by the affinity which exists between the cholesteryl part of esterified cholesterol and the stearyl chain of the micelles. We have previously observed that mixed micelles containing Triton-X100 and (5α)cholestan-3α-yl-(methylene hydroxamic acid) specifically catalyze the hydrolysis of paranitrophenylstearate, resulting from a hydrophobic interaction between the stearyl and cholesteryl residues. The relatively easy autoxidation of cholesteryl stearate and oleate is analogous to that of cholesteryl acetate.

In conclusion, for all of the esters, solid-state autoxidation by exposure to air at 100 C was faster than autoxidation in a water dispersion at 80 C. We observed the opposite for free cholesterol. The 20- and 25-hydroxycholesterol did not form in micellar medium; they only appeared if the reaction occurred on solid products. Cholesteryl stearate and oleate were the esters which oxidized best in solid state. On the other hand, they oxidized poorly in dispersion. The stearyl and oleyl chains had a significant inhibiting or activating effect, according to the mode of reaction. The weak oxidation ratio of solid cholesterol or of esters in dispersion can be explained in terms of the shielding of the reactive and allylic sites. Theoretically, this should permit a selective autoxidation of the side chain, but until now, our attempts have not succeeded. These results demonstrate the remarkable ease with which cholesteryl esters autoxidize under conditions closely resembling those present in human food processing, especially in cooking. The correlation underscores the necessity for a systematic study of the oxidized sterols found in these food products, which in certain circumstances (1) possess angiotoxic and cytotoxic properties.

ACKNOWLEDGMENTS

We thank N. Domergue for technical assistance and R. Doucet for GLS-MS. This work was supported by the French CNRS and INSERM.

REFERENCES

1. Imai, H., Subramanyam, V., Le Quesne, P.W., Soloway, H., and Kanisawa, M. (1980) Science 207, 651-653.
2. Smith, L.L., Smart, V.B., and Ansari, G.A.S. (1979) Mutat. Res. 68, 23-30.
3. Peng, S.K., Taylor, C.B., Tham, P., Werthessen, N.T., and Mikkelson, B. (1978) Arch. Pathol. Lab. Med. 102, 57-61.
4. Kanisawa, M., Kojima, K., and Werthessen, N.T. (1977) Fed. Proc. 36, 392, abstr.
5. Kandutsch, A.A., Chen, H.W., and Heiniger, H.J. (1978) Science 201, 498-501.
6. Atherosclerosis and Autoxidation of Cholesterol (1980) Lancet 1, 964-965.
7. Kandutsch, A.A. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 589-597, Plenum Publ. Corp., New York, NY.
8. Bergström, S., and Wintersteiner, O. (1942) J. Biol. Chem. 145, 309-326.
9. Smith, L.L., Kulig, M.J., Miiller, D., and Ansari, G.A.S. (1978) J. Am. Chem. Soc. 100, 6206-6211.
10. Ansari, G.A.S., and Smith, L.L. (1978) Chem. Phys. Lipids 22, 55-60.
11. Teng, J.I., Kulig, M.J., Smith, L.L., Kan, G., and Van Lier, J.E. (1973) J. Org. Chem. 38, 119-123.

12. Teng, J.I., and Smith, L.L. (1973) J. Am. Chem. Soc. 95, 4060-4061.
13. Smith, L.L. (1980) in Autoxidation in Foods and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 119-132, Plenum Publ. Corp., New York, NY.
14. Korahani, V., Bascoul, J., and Crastes de Paulet, A. (1981) J. Chromatogr. 211, 392-397.
15. Van Lier, J.E., and Smith, L.L. (1968) Anal. Biochem. 24, 419-430.
16. Tsai, L.S., and Hudson, C.A. (1981) J. Am. Oil Chem. Soc. 58, 931-934.
17. Beckwith, A.L.J. (1958) Proc. Chem. Soc. 2, 194-195.
18. Craven, B.M. (1976) Nature 260, 727-729.
19. Duax, W.L., Griffin, J.F., Roher, D.C., and Weeks, C.M. (1980) Lipids 15, 783-787.
20. Guey-Shuang Wu, Spein, R.A., and Mead, J.F. (1978) Lipids 13, 517-524.
21. Gershfeld, N.L., and Pagano, R.E. (1972) J. Phys. Chem. 76, 1244-1249.
22. Kimura, M., Jin, Y., and Sawaya, T. (1979) Chem. Pharm. Bull. 27, 710-714.

[Revision received February 18, 1982]

Cholesterol Metabolism in Human Monocyte-Derived Macrophages: Stimulation of Cholesteryl Ester Formation and Cholesterol Excretion by Serum Lipoproteins

DANIEL H. ALBERT[1], **MARET G. TRABER** and **HERBERT J. KAYDEN***, *Department of Medicine, New York University School of Medicine, New York, NY 10016*

ABSTRACT

The role of lipoproteins and serum in the formation and accumulation of cholesteryl esters in human monocyte-derived macrophages (HMD macrophages) was investigated; studies were also carried out with IC21 cells (a cell line derived from mouse peritoneal macrophages). Following preincubation of HMD macrophages with lipoprotein-depleted serum (LPDS), both native and acetylated low density lipoprotein (LDL and AcLDL, respectively) stimulated the formation of cholesteryl esters with a resultant increase in cellular cholesteryl ester content. Cholesteryl ester formation and accumulation was also stimulated in macrophages exposed continuously to 25-hydroxycholesterol. However, the stimulation of cholesterol esterification by either lipoproteins or 25-hydroxycholesterol was not inhibited by progesterone in HMD macrophages, but was in the IC21 cells. Cholesterol efflux and the hydrolysis of cellular cholesterol ester, promoted by serum components, were studied in HMD macrophages preloaded with cholesteryl ester by incubation with 25-hydroxy cholesterol. Replacement of the medium with one devoid of 25-hydroxycholesterol resulted within 24 hr in at least a 30% decrease in the cholesteryl ester content of the HMD macrophages; replacement with a medium high in cholesterol acceptor content (LPDS or high density lipoprotein) and incubation for three days led to the most marked decreases in cellular cholesterol content. Thus, hydrolysis of the cholesteryl esters by HMD macrophages was not dependent on the presence of cholesterol acceptors in the medium, but cellular cholesterol content was.
Lipids 17:709-715, 1982.

HMD macrophages have been shown to degrade both LDL and AcLDL via separate high-affinity processes (1,2). Continuous incubation with native LDL does not lead to a striking accumulation of cholesteryl ester by these cells; uptake of LDL has been shown to be closely regulated by the HMD macrophages (1-4). However, incubation of canine or rodent peritoneal macrophages with lipoproteins that have been chemically modified to have a negative charge (such as AcLDL) has resulted in accumulation of cholesteryl ester in lipid droplets in these cells (5-8). Macrophages in human atheromatous and xanthomatous lesions in vivo also contain a high proportion of esterified cholesterol (9,10). Therefore, we have investigated various parameters which could stimulate cholesteryl ester formation and its accumulation in human macrophages in vitro.

Abbreviations: LDL, low density lipoprotein; acetylated LDL, AcLDL, HDL, high density lipoprotein; HS, human serum; LPDS, lipoprotein-depleted human serum; FCS, fetal calf serum; BSA, bovine serum albumin; HMD macrophages, human monocyte-derived macrophages.

[1]Present address: Oak Ridge Associated Universities, Medical and Health Sciences Division, PO Box 117, Oak Ridge, TN 37830.

*Author to whom correspondence should be addressed.

HMD macrophages and IC21 cells, a cell line derived from mouse peritoneal macrophages which had been transformed with Simian Virus 40 (11), were used for these studies. The cellular cholesterol content and the rate of cholesterol esterification were monitored to assess the effect of LDL, AcLDL, 25-hydroxycholesterol and progesterone on cholesteryl ester metabolism by macrophages.

MATERIALS AND METHODS

For the lipoproteins, LDL (d=1.019-1.063 g/ml), HDL (d=1.063-1.21 g/ml), and LPDS (d>1.21 g/ml) were prepared from pooled normal human plasma by differential, sequential ultracentrifugation (12). Pooled HS was obtained by combining at least three units of plasma, followed by treatment with thrombin, and stored frozen (-70 C) until it was used. Serum and lipoprotein fractions were sterilized by filtration (0.45 μ, Nalge filter unit) and refiltered immediately before addition to culture medium.

LDL was acetylated as described by Basu et al. (13) with acetic anhydride. The AcLDL migrated as one band with enhanced mobility compared to LDL in agarose gel electrophoresis, pH 8.6.

Human monocytes were isolated from a leukocyte concentrate, a by-product of the isolation of platelets for transfusion, and cultured as described previously (1). *IC21 cells were provided by Dr. Vittorio Defendi (New York University School of Medicine). They were grown in 10% FCS as described (2).

The rate of cholesterol esterification in intact cells under various conditions was determined using [9,10-^{3}H]oleic acid (25 Ci/mol, Amersham) bound to BSA (4.5:1, mol/mol) as a substrate. In some experiments, cholesterol, 25-hydroxycholesterol or progesterone, dissolved in ethanol (50 μl), was added to the incubation medium 20 hr prior to the addition of the oleic acid substrate. Following addition of the substrate to the culture medium (final concentration of oleic acid 0.2 mM), the cells were incubated for 4 hr (14). To harvest the cells, the culture flasks or dishes were chilled on ice, the medium was removed and the cells were washed extensively three times each with ice-cold buffer C (50 mM Tris, 0.15 M NaCl, 2 mg/ml BSA, pH 7.4) then with ice-cold buffer without BSA (15); finally, the cells were scraped from the flasks and centrifuged for 10 min at 600 x g. The resultant pellet was resuspended in isopropanol (1 ml) containing 10^5 dpm ^{14}C-cholesterol, sonicated for 10 sec and centrifuged to pellet the disrupted cells. The pellet was assayed for protein by the method of Lowry et al. (16). Portions of the isopropanol extract (amounts comparable to ca. 10 μg cellular protein) were used to determine the total and unesterified cholesterol by the fluorometric assay procedure described by Heider and Boyett (17). After the addition of unlabeled lipids as carriers, the remaining extract was applied to thin layer Silica Gel G plates for development in the solvent system hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Areas corresponding to cholesterol, unesterified fatty acids, triglycerides and cholesteryl ester, made visible by exposure to iodine, were scraped into scintillation vials for the determination of radioactivity (14). Calculation of the amount of cholesteryl ester formed was based on the assumption that the specific activity of the substrate was not appreciably lowered by endogenous fatty acids.

RESULTS

Rate of Cholesteryl Ester Formation

LDL and AcLDL, previously shown to be degraded via high-affinity processes by HMD macrophages (1,2), were included in the culture medium and the rate of cholesterol esterification was measured, as depicted in Figure 1. HMD macrophages, preincubated for 24 hr with LPDS, were incubated with increasing amounts of LDL or AcLDL for 20 hr, and then incubated with the addition of ^{3}H-oleic acid for 4 hr. The maximal rate of esterification achieved with LDL (1.5 nmol/mg protein/4 hr) was ca. one-third of that achieved by incubation with AcLDL (4.6 nmol/mg protein/4 hr); the rate followed the same general pattern for both lipoproteins--after an initial rapid increase, it leveled off at ca. 40-80 μg lipoprotein/ml (Fig. 1).

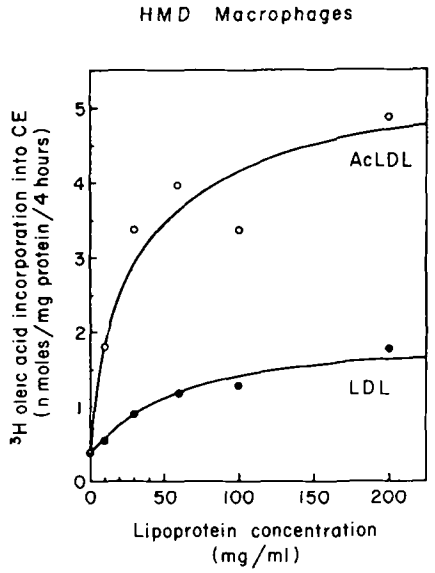


FIG. 1. Stimulation of cholesterol esterification with LDL and AcLDL in HMD macrophages. Cells cultured for 8 days in 5% HS were incubated in 10% LPDS for 24 hr and then in serum-free media (2 mg human albumin/ml) containing lipoproteins at the concentrations indicated for 20 hr. After the addition of ^{3}H-oleic acid substrate, the cells were incubated for an additional 4 hr, harvested and the rate of incorporation of oleic acid into cholesteryl esters was determined. Values are the means of 5 determinations.

AcLDL (same preparation as used in Fig. 1) stimulated cholesteryl ester formation and accumulation in IC21 cells in a concentration-dependent manner up to ca. 100 μg/ml (Fig. 2). The maximal rate of cholesterol esterification achieved in IC21 cells (12 nmol/mg protein/4 hr) was more than double that achieved in HMD macrophages (4.6 nmol/mg protein/4 hr). These data show that AcLDL used in Figure 1 stimulates the esterification of cholesterol in IC21 cells to a greater extent than it does in HMD macrophages. (The protocols for the

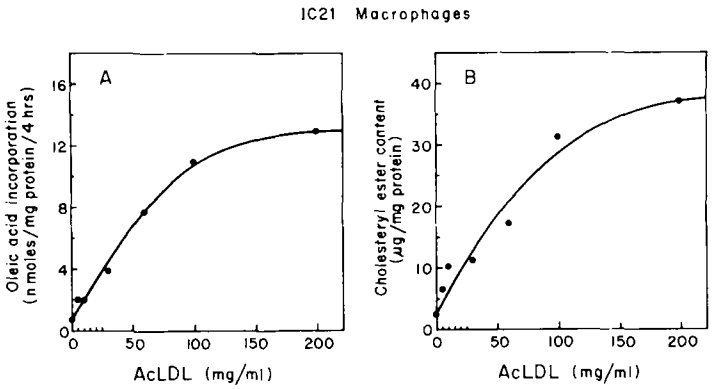


FIG. 2. AcLDL stimulation of cholesterol esterification in IC21 cells. IC21 cells grown in 10% FCS were incubated with 10% LPDS containing AcLDL for 20 hr. After the addition of ^{3}H-oleic acid, the cells were incubated for an additional 4 hr, harvested and the rate of incorporation of oleic acid into cholesteryl esters (panel A) and the mass of cellular cholesteryl ester (panel B) were determined. Values are the means of duplicate incubations.

TABLE 1

Cholesterol Content of HMD Macrophages Incubated with LDL or AcLDL[a]

Culture medium	Cellular cholesterol[b]	Addition to medium		
		None	LDL	AcLDL
10% LPDS	FC	48 ± 14(18)	61 ± 7(8)	57 ± 5(7)
	CE	1 ± 1(18)	16 ± 3(8)	14 ± 4(7)
Serum-free	FC	63 ± 16(9)	103 ± 20(9)	91 ± 16(8)
	CE	5 ± 2(8)	34 ± 12(9)	49 ± 21(8)
5% HS	FC	55 ± 7(3)	nd[c]	nd
	CE	16 ± 7(3)	nd	nd

[a]HMD macrophages cultured for 8 days with 5% HS were incubated in 10% LPDS for 24 hr and then for an additional 24 hr with either 10% LPDS or serum-free medium (2 mg human albumin/ml) containing the indicated lipoprotein (200 μg/ml). Cells continuously incubated in 5% HS are also shown.

[b]The cholesterol contents of isopropanol extracts of the cells were measured as described in the text. Shown are the means of the unesterified (FC) and esterified (CE) cholesterol contents of the cells in μg/mg protein ± SE (number of observations).

[c]nd = not determined.

experiments in Figs. 1 and 2 were slightly different—HMD macrophages were incubated in serum-free medium plus AcLDL whereas IC21 cells were incubated in 10% LPDS plus AcLDL, which should have promoted maximal rates of esterification in HMD macrophages, but not in IC21 cells.)

Cellular Cholesteryl Ester Content

The cholesterol content of HMD macrophages preincubated with 10% LPDS for 24 hr, then incubated with LDL or AcLDL, is shown in Table 1. The preincubation resulted in a decrease of both free and esterified cholesterol from the cells compared to those that were continuously maintained in 5% HS (control); the subsequent addition of either LDL or AcLDL to the LPDS medium resulted in cholesterol contents at control levels, but cells incubated in serum-free medium with lipoproteins had cholesterol contents that were twice that of controls. These data show that HMD macrophages do not accumulate large amounts of cholesteryl ester, even when incubated with modified lipoproteins under conditions in which there are few promoters of cholesterol efflux.

25-Hydroxycholesterol Promotes Cholesteryl Ester Formation and Accumulation

HMD macrophages were incubated with 25-hydroxycholesterol which stimulated the rate of incorporation of oleic acid into cholesteryl esters in a dose-dependent response up to 10 μg/ml medium (Fig. 3A). Simultaneously, there was an increase in cellular cholesteryl ester with a decrease in free cholesterol of HMD macrophages (Fig. 3B); the proportion of cholesteryl ester increased to nearly 50% of total cholesterol (Fig. 3C). Addition of cholesterol in ethanol was relatively ineffective in stimulating cholesterol esterification; cellular free cholesterol content increased by 40 μg/mg protein (Fig. 3B). These data show that stimulation of cholesterol esterification by 25-hydroxycholesterol in HMD macrophages leads to an increased proportion of cholesteryl ester; however, uptake and accumulation of free cholesterol does not result in cholesterol ester accumulation. HMD macrophages can accumulate free cholesterol without esterifying it; this phenomenon has also been observed in HMD macrophages incubated with LDL for prolonged periods (1).

The rate of cholesterol esterification in IC21 cells incubated for 24 hr with 25-hydroxycholesterol (20 μg/ml) in 10% FCS was also increased: 19.1 ± 0.9 nmol/mg protein/4 hr compared to 0.7 ± 0.3 for control cells; cholesteryl ester content also increased from 2.5 to 28.8 μg/mg protein.

Effect of Progesterone on Cholesterol Esterification

The rate of cholesterol esterification in IC21 cells incubated with both 25-hydroxycholesterol (20 μg/ml) and progesterone (10 μg/ml), a known inhibitor of acyl CoA:cholesterol acyl transferase (ACAT) (18), was reduced from 19.1 ± 0.9 to 3.8 ± 1.2 nmol/mg protein/4 hr with a cholesteryl ester content of only 5.3 μg/mg protein. In contrast, the same preparation and amount of progesterone (20 μg/ml) had little effect on the rate of cholesterol esterification or on the accumulation of cholesteryl esters by HMD macrophages incubated with 25-hydroxycholesterol (Table 2). As shown in Figure 4, progesterone had an inhibitory effect in IC21 cells exposed to both AcLDL (100 μg/ml) and progesterone, but progesterone did not inhibit cholesterol esterification when HMD macrophages were stimulated to esterify cholesterol by incubation with either AcLDL or LDL (data not shown). These experiments, using conditions for maximal cholesterol esterification (incubation with 25-hydroxycholesterol in HMD macrophages and with AcLDL in IC21 cells), show that progesterone inhibits

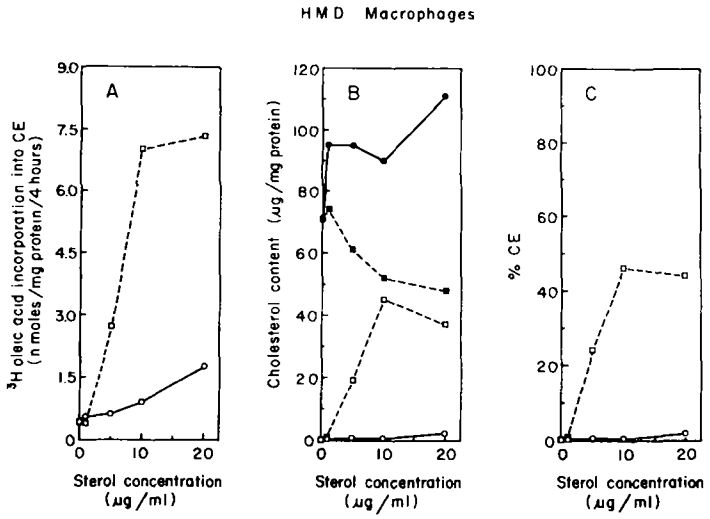


FIG. 3. Cholesterol esterification by HMD macrophages in response to 25-hydroxycholesterol. HMD macrophages cultured for 8 days with 5% HS were incubated with 10% LPDS for 24 hr and then for 20 hr with 10% LPDS containing either 25-hydroxycholesterol (boxes) or cholesterol (circles) at the indicated concentrations. After the addition of ^{3}H-oleic acid, the cells were incubated for an additional 4 hr, harvested and the rate of cholesteryl ester formation (panel A) and the cellular content (panel B) of free cholesterol (closed symbols) and cholesteryl ester (open symbols) were determined. The percentage of total cholesterol as cholesteryl ester is shown (panel C). Values are the means of 3 determinations.

TABLE 2

Failure of Progesterone to Inhibit the Stimulation of Cholesteryl Ester Formation and Accumulation by 25-Hydroxycholesterol in HMD Macrophages[a]

Progesterone concentration[b]	Cholesterol esterification[c]	Cholesteryl ester content[d]
0	7.0 ± 0.9	55 ± 7
1	7.9 ± 0.2	nd[e]
5	7.5 ± 0.2	nd
10	7.8 ± 1.2	nd
20	6.4 ± 0.3	51 ± 8

[a]HMD macrophages cultured for 8 days in 5% HS were incubated in 10% LPDS for 24 hr. The cells were then incubated for 24 hr in media containing the indicated concentration of progesterone and 5% HS with 25-hydroxycholesterol (10 μg/ml).

[b]Progesterone concentration in μg/ml medium.

[c]Shown: nmol ^{3}H-oleic acid incorporated into cholesteryl ester/mg protein/4 hr, mean ± SE (n=3).

[d]Cholesteryl ester content measured as described in Methods, μg/mg protein, mean ± SE (n=3).

[e]nd = not determined.

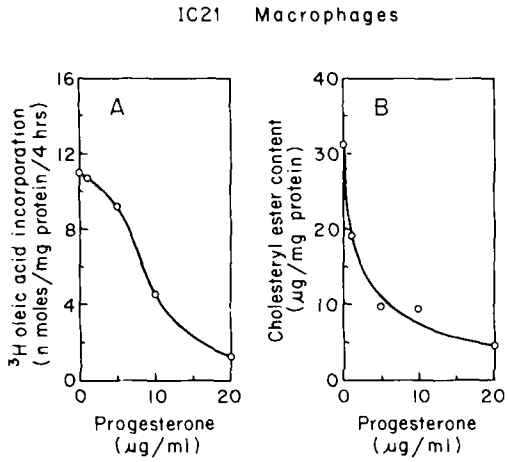


FIG. 4. Inhibition of cholesteryl ester formation and accumulation by progesterone in IC21 macrophages incubated with AcLDL. Following incubation of cells for 20 hr in media with 10% LPDS containing AcLDL (100 μg/ml) and the indicated concentration of progesterone, cholesteryl ester formation (panel A) and content (panel B) were measured as described in Fig. 2. Values are the means of duplicate determinations.

cholesterol esterification in IC21 cells, but not in HMD macrophages.

Role of Cholesterol Acceptors in the Medium in Maintaining Cholesterol Content

To determine how medium components effect cholesterol efflux from HMD macrophages, cells that had been stimulated to accumulate cholesteryl ester (60-70 μg/mg protein) by exposing them to 25-hydroxycholesterol in 5% HS for 24 hr were thoroughly washed and placed in medium containing either 5% HS, LPDS, human albumin (serum-free), or serum-free with HDL (350 μg/ml). Cellular free and esterified cholesterol were then monitored for three days. This method did not directly measure cholesterol efflux; however, it was assumed that the cholesterol homeostasis of the cells would be primarily affected by the presence or absence of cholesterol acceptors in the medium. The contribution of cellular cholesterol synthesis and uptake of lipoproteins on cholesterol homeostasis was considered to be minimal under the conditions of this experiment, as it has been previously shown that preincubation of HMD macrophages in LPDS is required to induce elevated levels of these processes (1). Figure 5 indicates that, under each of the conditions used, regardless of the composition of the medium, after one day of incubation, there was a decrease of at least 30% in the cellular cholesteryl ester content. That this decrease occurred in cells in serum-free media demonstrates that hydrolysis of cholesteryl ester by HMD macrophages does not require the presence of serum. However, the level of unesterified cholesterol in HMD macrophages after three days of incubation did depend on the composition of the medium; it increased by 20 μg/mg protein in cells in serum-free medium, but remained relatively constant in cells in 5% HS (Fig. 5). In contrast, the unesterified cholesterol content of cells incubated in LPDS or in medium containing HDL decreased by at least 15 μg/mg so that, by the third day of incubation, these cells had only about 50% as much total cholesterol as the cells maintained in serum-free medium. These results show that factors present in LPDS and HDL decrease the cholesterol content of HMD macrophages and probably increase the rate of cholesterol efflux from these cells.

DISCUSSION

The results of these studies demonstrate that cholesteryl ester accumulation in HMD macrophages is influenced by at least two processes: one which controls the rate of uptake of lipoprotein-derived cholesterol and the other that affects the rate of cholesterol efflux from the cells. From the data in Table 1 and Figure 1,

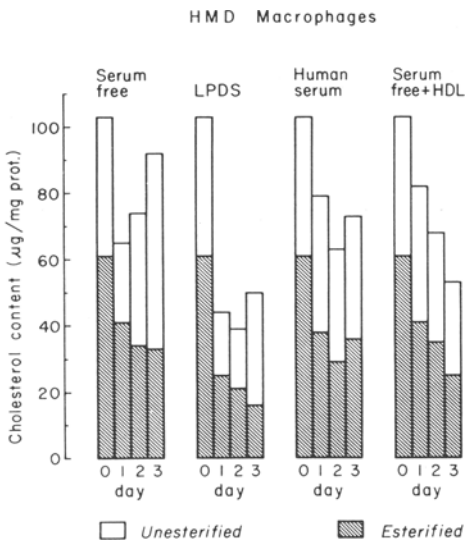


FIG. 5. Response of HMD macrophages stimulated to accumulate cholesteryl ester with 25-hydroxycholesterol to medium containing serum, LPDS and HDL. HMD macrophages cultured with 5% HS for 8 days were incubated with 25-hydroxycholesterol (10 μg/ml) in 5% HS for 24 hr, thoroughly washed and then incubated with the replacement medium for the indicated lengths of time. The replacement media used were: serum-free medium (2 mg human albumin/ml), 10% LPDS, 5% HS, or serum-free medium containing HDL (350 μg/ml). After the indicated time, cells were harvested and the cholesterol (open bar) and cholesteryl ester (filled bar) contents were determined. Shown are the averages of duplicate determinations.

it is apparent that both the rate of LDL degradation and the degree of stimulation of cholesteryl ester formation in HMD macrophages are dependent on the receptor-mediated process for the uptake of native LDL. The enhanced ability of AcLDL to stimulate cholesteryl ester formation is most likely due to the activity of the high-affinity process responsible for AcLDL uptake (1,2). However, the level of cholesteryl ester formation is not as high as that observed in mouse peritoneal macrophages as described by Goldstein et al. (5). Under our experimental conditions, even prolonged incubation (over a week) with high concentrations of AcLDL (>200 μg/ml) does not promote cholesteryl ester accumulation in excess of 100 μg/ml protein (unpublished observations), yet mouse macrophages were reported to accumulate as much as 1 mg cholesteryl ester/mg protein (5). As HMD macrophages become macrophages in vitro under quite defined conditions, and peritoneal macrophages are macrophages when they are isolated, peritoneal macrophages may have been stimulated to be more effective scavengers than HMD macrophages.

Cholesteryl ester accumulation was promoted by incubating HMD macrophages and IC21 cells with 25-hydroxycholesterol, a known stimulator of ACAT activity (19). These data are consistent with the stimulatory response to this sterol reported for cultured human fibroblasts (19).

Perhaps the most striking difference between the response of HMD macrophages and other cell types is that, under the conditions described, progesterone has virtually no effect on the rate of cholesterol esterification in HMD macrophages. This is in sharp contrast to the potent inhibition of ACAT by progesterone in fibroblasts (18), mouse peritoneal macrophages (20), and IC21 cells (present study). The failure of progesterone to inhibit cholesterol esterification suggests that the enzymes responsible for this process in HMD macrophages are different with regard to either their sensitivity or accessibility to the inhibitor.

The second process which influences the amount of cholesteryl ester accumulated by HMD macrophages is the rate of cholesterol efflux. Factors present in HDL and LPDS fractions promote efflux of cholesterol from mouse peritoneal macrophages (21) and appear to promote cholesterol efflux from HMD macrophages. Table 1 shows that cells incubated in serum-free medium supplemented with LDL or AcLDL have a greater accumulation of cholesterol and cholesteryl ester than cells in medium containing LPDS and the same lipoproteins. Furthermore, medium containing LPDS or HDL is more effective than medium that does not contain serum components in promoting efflux of free cholesterol from cells which previously have been stimulated with 25-hydroxycholesterol to accumulate ester (Fig. 5). Taken together, these data are consistent with the proposed role of apolipoproteins in promoting the excretion of cholesterol from cells (21,22). However, hydrolysis of cholesteryl ester by HMD macrophages does not seem to require the response of these apolipoproteins; only the removal of 25-hydroxycholesterol from the medium was necessary. This is in contrast to mouse peritoneal macrophages in which net cholesteryl ester hydrolysis occurs only in the presence of cholesterol acceptor (21). Preliminary evidence suggests that the HMD macrophages secrete apolipoprotein E (M.G. Traber and H.J. Kayden, unpublished observations), which has been demonstrated to act as a mechanism in the secretion of cholesterol by mouse peritoneal macrophages (23).

Perhaps the level of apolipoprotein E secretion by HMD macrophages is sufficient to allow cholesteryl ester hydrolysis and some secretion of cholesterol when the cells are incubated in serum-free medium containing albumin.

ACKNOWLEDGMENTS

Excellent technical assistance was provided by Frank Maschio. This work was supported by grant HL-06481 from the U.S. Public Health Service.

REFERENCES

1. Traber, M.G., and Kayden, H.J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5466-5470.
2. Traber, M.G., Defendi, V., and Kayden, H.J. (1981) J. Exp. Med. 154, 1852-1867.
3. Schechter, I., Fogelman, A.M. Haberland, M.E., Seager, J., Hokom, M., and Edwards, P.A. (1981) J. Lipid Res. 22, 63-71.
4. Fogelman, A.M., Haberland, M.E., Seager, J., Hokom, M., and Edwards, P.A. (1981) J. Lipid Res. 22, 1131-1141.
5. Goldstein, J.L., Ho, Y.K., Basu, S.K., and Brown, M.S. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 333-337.
6. Brown, M.S., Goldstein, J.L., Krieger, M., Ho, Y.K., and Anderson, R.G.W. (1979) J. Cell Biol. 82, 597-613.
7. Mahley, R.W., Innerarity, T.L., Weisgraber, K.H., and Oh, S.Y. (1979) J. Clin. Invest. 64, 743-750.
8. Mahley, R.W., Innerarity, T.L., Brown, M.S., Ho, Y.K., and Goldstein, J.L. (1980) J. Lipid Res. 21, 970-980.
9. Schaftner, T., Vesselinovitch, D., and Wissler, R.W. (1979) Fed. Proc. 38, 1076a.
10. Fowler, S., Shio, H., and Hayley, N.J. (1979) Lab. Invest. 41, 372-378.
11. Mauel, J., and Defendi, V. (1971) J. Exp. Med. 134, 335-350.
12. Havel, R.J., Eder, H.H., and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353.
13. Basu, S.K., Goldstein, J.L., Anderson, R.J.W., and Brown, M.S. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3178-3182.
14. Fielding, P.E., Vlodavsky, I., Gospodarowicz, D., and Fielding, C.J. (1979) J. Biol. Chem. 254, 749-755.
15. Ho, Y.K., Brown, M.S., Kayden, H.J., and Goldstein, J.L. (1976) J. Exp. Med. 144, 444-455.
16. Lowry, O.H., Rosebrough, M.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
17. Heider, J.G., and Boyett, R.L. (1978) J. Lipid Res. 19, 514-518.
18. Goldstein, J.L., Faust, J.R., Dygos, J.H., Chorvat, R.J., and Brown, M.S. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1877-1881.
19. Brown, M.S., Dana, S.E., and Goldstein, J.L. (1975) J. Biol. Chem. 250, 4025-4027.
20. Brown, M.S., Ho, Y.K., and Goldstein, J.L. (1980) J. Biol. Chem. 255, 9344-9352.
21. Ho, Y.K., Brown, M.S., and Goldstein, J.L. (1980) J. Lipid Res. 21, 391-398.
22. Stein, O., Fainaru, M., and Stein, Y. (1979) Biochim. Biophys. Acta 574, 495-504.
23. Basu, S.K., Brown, M.S., Ho, Y.K., Havel, R.J., and Goldstein, J.L. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 7545-7549.

[Received March 6, 1982]

cis-5-Olefinic Unusual Fatty Acids in Seed Lipids of Gymnospermae and Their Distribution in Triacylglycerols

TORU TAKAGI* and **YUTAKA ITABASHI**, *Department of Chemistry, Faculty of Fisheries, Hokkaido University, Hakodate, Japan*

ABSTRACT

Open-tubular gas chromatographic analysis of fatty acids in the lipids from the seeds of 20 species of Gymnospermae showed that they all contained nonmethylene-interrupted polyenoic (NMIP) acids as minor components and palmitic, oleic, linoleic and α-linolenic acids as major components. The NMIP acids have an additional 5,6-ethylenic bond in ordinary plant unsaturated fatty acids and the following C_2 elongation acids: *cis*-5, *cis*-9-octadecadienoic acid (5,9-18:2) (I); 5,9,12-18:3 (II); 5,9,12,15-18:4, 5,11-20:2, 5,11,14-20:3 (III); and 5,11,14,17-20:4 (IV). The main NMIP acids found in neutral lipids are I in two species of *Taxus*, II in seven species of Pinaceae, III in two species of Podocarpaceae, *Torreya nucifera*, *Cycas revoluta*, and *Ginkgo biloba*, and III and IV in each of three species of Taxodiaceae, and Cupressaceae. The polar lipids constitute the minor fraction of seed lipids in general. The content and composition of NMIP acids in these lipids differ considerably from those in neutral lipids. Analysis of the partial cleavage products of triacylglycerols showed that the NMIP acids distribute mainly in the 1,3-position.

Lipids 17:716-723, 1982.

INTRODUCTION

Nonmethylene-interrupted polyenoic (NMIP) fatty acids have been reported as constituents of lipids from certain classes of plants (1,2), marine invertebrates (3-6), insects (7), and others. In Gymnospermae, the occurrence of NMIP acids with an isolated 5:6 double bond has been reported in the lipids of different species, since *cis*-5,*cis*-11,*cis*-14-eicosatrienoic acid (5,11,14-20:3) was found in *Podocarpus nagi* seed oil in 1962 (8). The major NMIP acids found in the seeds of Gymnospermae are 5,11-18:2 and 5,11,14-20:3 in *Ginkgo biloba* (9,10); 5,9,12-18:3 in two species of Pinaceae: *Larix leptolepsis* (11) and *Pinus koraienesis* (12); 5,9-18:2 and 5,11,14-20:3 in *Taxus baccata* (13); 5,11,14-20:3 in *Torreya nucifera* (12,14); 5,11, 14-20:3 and 5,11,14,17-20:4 in *Ephedra campylopoda* (15); 5,11-18:2 and 5,11,14-20:3 in the lipids of roots, leaves and stems of *G. biloba* (16); 5,9,12-18:3 in the geranylgeranyl esters of Norway spruce, *Picea abies*, wood (17); plus 5,9-18:2, 5,9,12-18:3, 5,9,12,15-18:4, 5,11,14-20:3 and 5,11,14,17-20:4 in the leaf lipids of many species of conifer (18). The occurrence of *trans*-5-olefinic fatty acids in the seeds of various plants has been reported (1,2), but *trans*-5-olefinic NMIP acids have not been found in Gymnospermae.

In this study, the fatty acids of seed lipids from 20 species of Gymnospermae have been investigated with particular attention to NMIP acids, and their positional distribution in triacylglycerols.

MATERIALS AND METHODS

Materials

The species of Gymnospermae studied are listed in Table 1. All seeds were air-dried products from a nursery company (Daiichi Engei Co. Ltd., Tokyo) except those described below. Air-dried seeds of *Cycas revoluta* and dried stalks of *Ephedra sinica* were obtained from a pharmacy dealing in Chinese drugs. Seeds of *G. biloba* and *P. koraiensis* were obtained at a grocery store. The seeds of *Taxus cuspidata*, and *Chamaecyparis pisifera* were collected at Hakodate. Seeds of *P. nagi* and *Taxodium districhum* were obtained from Nara and Hiroshima, respectively. Seeds of *Podocarpus macrophylla* and *T. nucifera* were obtained from Kagoshima. Seeds of *Taxus canadensis* were obtained from Halifax, Canada.

Preparation of Methyl Esters

The seeds were ground to powder with an electric mill and extracted by the method of Bligh and Dyer (19). Total lipids were fractionated into polar and neutral lipids by liquid chromatography on a silica gel (Kieselgel 60, Merck) column using chloroform and methanol

*Author to whom correspondence should be addressed.

TABLE 1

Gymnosperm Seeds and Their Contents of Lipids and NMIP Acids

Family	Sample no.	Genus and species	Total[a] lipids (%)	Neutral[b] lipids (%)	Average[c] wt of a seed (mg)	NMIP acid[d] NL	NMIP acid[d] PL
Cycadaceae	1	*Cycas revoluta*	0.82	64.35	7140	4.20	2.61
Ginkgoceae	2	*Ginkgo biloba*	1.78	84.45	–	7.55	4.16
Taxaceae	3	*Taxus cuspidata*	15.62	90.58	60	22.42	7.85
	4	*Taxus canadensis*	30.40	93.52	–	17.08	6.04
	5	*Torreya nucifera*	49.70	99.37	1050	7.55	–
Podocarpaceae	6	*Podocarpus macrophylla*	9.24	54.66	410	11.01	–
	7	*Podocarpus nagi*	17.27	–	–	24.06	11.79
	8	*Pinaceae jezoensis*	38.68	94.76	11	29.09	8.70
	9	*Larix leptolepsis*	14.68	94.74	3	29.71	8.16
	10	*Cedrus deodra*	51.50	88.05	116	13.99	8.08
	11	*Pinus densiflora*	33.51	97.54	9	25.68	10.76
	12	*Pinus thunbergii*	26.56	93.16	–	25.13	11.41
	13	*Pinus koraiensis*	65.00[e]	–	166	17.78	12.19
	14	*Pinus pentaphylla*	16.55	96.82	167	22.97	8.42
Taxodiaceae	15	*Taxodium districhum*	2.02[e]	–	–	11.16	–
	16	*Sciadopitys verticillata*	36.80	96.65	19	17.47	14.27
	17	*Cryptomeria japonica*	9.87	94.20	18	6.73	4.04
Cupressaceae	18	*Chamaecyparis pisifera*	12.78[e]	–	3	10.68	–
	19	*Juniperus rigida*	6.52	74.29	22	23.27	9.49
	20	*Juniperus chinensis*	8.41	96.09	14	20.56	13.48
Ephedraceae	21	*Ephedra sinica*	2.76	–	–	2.27	2.12

[a]Wt %: total lipid to dried seeds.
[b]Wt %: neutral lipids to total lipids.
[c]Wt of a seed without its coat.
[d]NMIP acid % to total lipids. NL, neutral lipids. PL, Polar lipids.
[e]Extracted with ether.

as solvents. Samples 13, 15 and 18 in Table 1 were extracted with ether and the extracts were used as the total lipids. The lipid fractions were directly converted to methyl esters with 0.5 M sodium methoxide/methanol reagent. These esters were purified with thin-layer chromatography (TLC) on a 0.5-mm layer of Silica Gel G by developing with hexane/ether (8:2, v/v).

Gas-Liquid Chromatography (GLC)

Open-tubular GLC of the methyl esters was done with a Shimadzu GC-7A (Shimadzu Seisakusho Co., Kyoto) equipped with a dual FID detector on a wall-coated, open-tubular glass column coated with Silar 5CP (66 m × 0.28 mm id). The carrier gas was N_2 and had a flow rate of 0.5 ml/min and split ratio of 1/155. The column temperature was 170 C, and the injector and detector were 230 C. All carrier gas pathways were made of glass tubes. Peak area percentages were obtained with a Shimadzu integrator E1A.

Argentation TLC ($AgNO_3$-TLC)

The separation of the NMIP acids was done by preparative $AgNO_3$-TLC on silver nitrate impregnated layers of Silica Gel G by developing with ethyl acetate/hexane, 1:19 for the dienoate and 1:9 for the trienoate and tetraenoate.

Nuclear Magnetic Resonance (NMR)

A JEOL FX-200 spectrometer (Nippon Denshi Co., Tokyo) in the Fourier transform mode at 199.50 MHz (^1H) and 25.00 MHz (^{13}C) was used to obtain ^1H and ^{13}C NMR spectra of samples in DCl_3.

Mass Spectrometry (MS)

The MS of the pyrrolidides of the unsaturated fatty acids for determining the double bond positions was done with a JEOL mass spectrometer JMS-D3000 operated at an ionization potential of 70 eV and ion source temperature of 170 C. The pyroolidides of fatty acids were prepared by heating 10 mg of the methyl esters in freshly distilled pyrrolidine, 1 ml, and acetic acid, 0.1 ml, at 100 C for 30 min on a microscale (20).

RESULTS AND DISCUSSION

Unusual Fatty Acid Components

The lipid content in the seeds of Gymno-

spermae is shown in Table 1. The total lipids consisted primarily of neutral lipids. The GLC of the fatty acid methyl esters prepared from each lipid indicated some unusual peaks. The typical chromatograms are shown in Figure 1. The unusual peaks 5,7,9,12,14 and 16 appeared soon after the peaks of 9-18:1, 9,12-18:2, 9,12,15-18:3, 11-20:1, 11,14-20:2 and 11,14,17-20:3, respectively. The components of peaks 12 and 14 were identified as 5,11-20:2 and 5,11,14-20:3 on the basis of the agreement of the retention data with those of the reference specimen obtained from sea urchins (3) and *P. nagi* seeds (8).

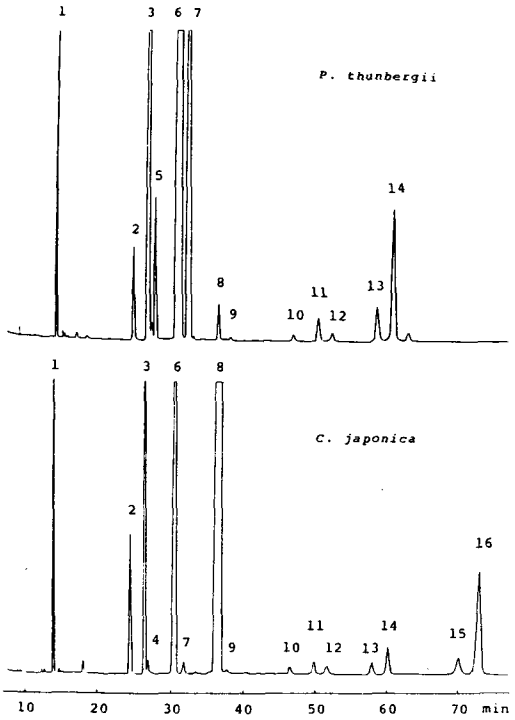


FIG. 1. GLC resolution of methyl esters of 5-olefinic NMIP acids in gymnosperm seed lipids on a WCOT Silar 5CP column. (1) 16:0; (2) 18:0; (3) 18:1(n-9); (4) 18:1(n-7); (5) 18:2(5,9); (6) 18:2(n-6); (7) 18:3(5,9,12); (8) 18:3(n-3); (9) 18:4 (5,9,12,15); (10) 20:0; (11) 20:1(n-9); (12) 20:2(5,11); (13) 20:2 (n-6); (14) 20:3(5,11,14); (15) 20:3(n-3); (16) 20:4 (5,11,14,17).

Separation of the NMIP Acids

Methyl esters of fatty acids from the seed lipids showed spots in the following order from the top to the origin in $AgNO_3$-TLC; (9-18:1), (9,12-18:2), (peak 5 and 12 components), (9,12,15-18:3), (peak 7 and 14 components), and (peak 9 and 16 components). The components of peaks 5, 7, 14 and 16 were separated by $AgNO_3$-TLC of the methyl esters from *T. cuspidata, P. koraiensis, P. nagi,* and *J. chinensis* seed lipids, respectively. The peak 7 and 16 components were concentrated by the urea adduct method before the application of $AgNO_3$-TLC. Each sample showed a purity exceeding 96% in GLC.

Mass Spectra of Pyrrolidides

The mass spectrum of pyrrolidide from the peak 5 fraction showed the following irregular intervals of m/e 12 between the maxima in the fragment ion peaks for each carbon atom: C_4 (m/e 140, intensity 0.8% to the parent peak)-C_5 (m/e 152, 0.4%), and C_8(194, 0.5%)-C_9(206, 0.3%). A molecular ion peak appeared at m/e 333. The results show 5,9-18:2 for the structure of the peak 5 component. The mass spectrum of pyrrolidide from the peak 7 fraction showed the following intervals of m/e 12 between the maxima: C_4(140, 0.8%)-C_5(152, 1.0%), C_8(194, 0.5%)-C_9(206, 0.4%), and C_{11}(234, 1.0%)-C_{12}(246, 1.0%). A molecular ion peak appeared at m/e 331. The results show 5,9,12-18:3 for the structure of the peak 7 component. The pyrrolidide of the peak 14 fraction showed the following intervals of m/e 12 between the maxima: C_4(140, 3.2%)-C_5(152, 2.1%), C_{10}(222, 1.5%)-C_{11}(234, 1.8%), and C_{13}(262, 1.8%)-C_{14}(274, 1.0%). A molecular ion peak appeared at m/e 359. The results show 5,11,14-20:3 as the structure of the peak 14 component. The pyrrolidide of the peak 16 fraction showed intervals of m/e 12 between the maxima as follows: C_4(140, 1.1%)-C_5(152, 0.7%), C_{10}(222, 0.5%)-C_{11}(234, 0.7%), C_{13}(262, 0.4%)-C_{14}(274, 0.5%), and C_{16}(302, 0.3%)-C_{17}(314, 0.3%). A molecular ion peak appeared at m/e 357. The results show 5,11,14,17-20:4 for the structure of the peak 16 component.

NMR, IR and UV Spectrometry

IR and UV spectrometric analyses of each fraction were done by the procedure Cd 14-61 and Cd 7-48 of the *AOCS Official and Tentative Methods of Analysis.* The limits of detection of isolated *trans* double bonds by IR analysis and conjugated double bonds by UV analysis were 2% as methyl elaidate and 1% as methyl α-eleostearate, respectively. The spectra did not show the presence of *trans* and conjugated unsaturated compounds in all of the samples.

The ^{13}C NMR of each fraction showed the peaks listed in Table 2. The ^{13}C NMR spectra for each NMIP acid were in fair agreement with those calculated by the set of additive substituent parameters (21-23). Coexistence of *trans* 5

TABLE 2

^{13}C Chemical Shifts of Methyl Esters of 5-Olefinic NMIP Acids from Seeds

Locant of C atom	5,9-18:2		5,9,12-18:3		5,11,14-20:3		5,11,14,17-20:4	
	Found	Calcd[a]	Found	Calcd	Found	calcd	Found	Calcd
1	174.20	174.20	174.10	174.20	174.15	174.20	174.15	174.20
2	33.55	33.55	33.49	33.55	33.54	33.55	33.55	33.55
3	24.98	25.05	24.92	25.05	25.00	25.05	25.00	25.05
4	26.68	26.65	26.65	26.65	26.68	26.65	26.64	26.65
5	128.84	128.90	129.03	129.05	128.49	128.40[b]	128.48	128.40[b]
6	130.54	130.65	130.38	130.40	130.92	131.30[b]	130.92	131.30[b]
7	27.39	27.35	27.36	27.35	27.23	27.30	29.23	29.30
8	27.39	27.35	27.36	27.35	29.43	29.40	29.39	29.40
9	128.98	129.05	129.32	129.35	29.43	29.40	29.39	29.40
10	130.48	130.55	128.70	128.70	27.23	27.30	27.23	27.30
11	27.48	27.30	25.74	25.75	130.21	130.13[c]	130.13	130.28[d]
12	29.82	29.80	127.91	127.95	128.16	128.07[c]	127.79	127.87[d]
13	29.41	29.40	130.81	130.40	25.76	25.75	25.70	25.75
14	29.62	29.65	27.27	27.25	128.16	128.07[c]	128.26	128.33[d]
15	29.41	29.40	29.39	29.45	130.21	130.13[c]	128.26	128.33[d]
16	32.00	31.90	31.59	31.55	27.23	27.25	25.70	25.75
17	22.78	22.75	22.63	22.60	29.43	29.45	127.19	127.31[d]
18	14.18	14.05	14.09	14.05	31.64	31.55	131.95	131.86[d]
19					22.68	22.60	20.66	20.55
20					14.16	14.05	14.39	14.40
OCH_3	51.46	51.45	51.45	51.45	51.43	51.45	51.42	51.45

[a]Calculated shifts for each carbon are obtained with the method reported by Bus et al. (22), except those for olefin carbons which are taken from the data of [b]5-18:1 (21); [c]11,14-20:2; [d]11,14,17-20:3 (23), without correction for the effect induced between olefinic bonds.

isomers in the 5-olefinic NMIP acids has been reported in the fatty acids of some seed oils. The δ values of these 5-isomers are ca. 0.5 higher in the 5- and 6-ethylenic carbons, and 5.4 higher in the 4- and 7-allylic carbons than those of the corresponding carbons of the *cis* 5 isomers, respectively (21-23). In this study, there were no signals for the *trans* 5-isomers in the ^{13}C NMR of the NMIP acid fractions. In Table 2, the reference values for the ethylenic carbons of 5,11,14-20:3 and 5,11,14,17-20:4 are taken from the data of 5-18:1 (21), 11,14-20:2 and 11,14,17-20:3 (23) without correction for the induced effect between 5 and other double bonds. Since it was reported that a *cis* double bond has effects on other *cis* double bond in the ϵ position (23), the 11,14-diene and 11,14,17-triene system must have some effect on the δ values of the 5- and 6-ethylenic carbons in 5,11,14-20:3 and 5,11,14,17-20:4. The appreciable deviation in the value of the 6-carbon from the reference values in Table 2 is attributable to a downfield shift resulting from the deshielding effect of the 11,14-diene and 11,14,17-triene on the 6-carbon.

All the fractions of 5,9-18:2, 5,9,12-18:3, 5,11,14-20:3 and 5,11,14,17-20:4 showed quartets in the ^1H NMR spectra at δ 1.67, associated with the C_3 methylene group which is in the β position to both a double bond and a carboxylic group in the fatty acids having an isolated 5-double bond (24). ^1H NMR spectra of 5,9-18:2 and 5,9,12-18:3 fractions were identical to those reported by Madrigal et al. for 5,9-18:2 from *T. baccata* seed oil (13), and by Smith et al. for 5,9,12-18:3 from *Teucrium depressum* seed oil (25), respectively. The spectra of the 5,11,14-20:3 and 5,11,14,17-20:4 fractions include a 2-proton triplet at δ 2.30 for the CH_2 group which is in the α position to the carboxyl group, and a 3-proton singlet at δ 3.65 for the CH_3O group in common. The spectra of 5,11,14,17-20:4 fraction substained an n-3 double bond with a sharp triplet for the terminal methyl group at δ 0.95 as described in the literature (16). The other three acids showed triplets for the methyl group at δ 0.88. The spectra of the 5,11,14,17-20:4 fraction indicated 4-proton triplets for two diallyl methylene groups at δ 2.78; those of the 5,9,12-18:3 and 5,11,14-20:3 fractions indicated 2-proton triplets for one diallyl methylene group at δ 2.77. All the spectra showed 8-proton complex multiplets for the methylene groups, which is in the α position to double bonds, at δ 2.0-2.2. The spectra of the 5,11,14-20:3 and 5,11,14,17-20:4 fractions indicated 10 and 4 proton complex multiplets for the other methylene groups

at δ 1.3-1.4. These features agree with the structures for each compound deduced by MS of the pyrrolidides and ^{13}C NMR.

GLC Behavior of the Methyl Esters of NMIP Acids

The 5-olefinic acids found in this study have an additional 5:6 double bond in ordinary plant unsaturated fatty acids such as oleic, linoleic and linolenic acids, and their C_2 elongation compounds: 11-20:1, 11,14-20:2 and 11,14,17-20:3. The methyl ester peaks of the NMIP acids appear soon after the peaks of the methyl esters of the corresponding ordinary acids. The separation factors and differences in equivalent chain length (ΔECL) between these fatty acid ester pairs remained about the same. The average with standard deviation for the separation factors and ΔECL was 1.038 ± 0.02 and 0.12 ± 0.01, respectively. The relations in GLC data can be used for a tentative identification of the 5-olefinic NMIP acid esters. According to this method, the component of peak 9 was tentatively identified as 5,9,12,15-18:4 from the separation factor 1.030 and ΔECL 0.13 between peak 9 and the 9,12,15-18:3 peak. In the GLC of methyl esters of fatty acids from *Cycas, P. macrophylla*, and *Ginkgo* seed lipids, a small peak was found behind the 5,9-18:2 peak. The peak was tentatively identified as 5,11-18:2 by the separation factor 1.037 and ΔECL 0.11 between the subjected peak and the 11-18:1 peak.

Unusual Fatty Acid Composition

The fatty acid composition of neutral lipids from the seeds is shown in Table 3. The composition is usually more complicated than that for vegetable oils. The lipids of *Cycas* and *Ginkgo* seeds contain low levels of some NMIP acids such as 5,11,14-20:3, 5,11-20:2 and 5,9- and 5,11-18:2. The lipids of *T. cuspidata* seeds obtained in Japan contain a higher percentage of 5,9-18:2 compared to that of *T. canadensis* obtained in Canada and *T. baccata* obtained in the U.S. (12.2%) (13). *T. nucifera* belongs to Taxaceae with *Taxus*, but the major NMIP acid of *T. nucifera* seed lipids is 5,11,14-20:3, as in the case of Podocarpaceae. The lipids of the *P. nagi* seeds used in this study showed a somewhat higher percentage of 5,11,14-20:3 compared to that reported in the previous paper (20.5%) (8). The major component of the NMIP acid found in Pinaceae was found to be 5,9,12-18:3 (10, 11,16). All seed lipids of the nine species of Pinaceae examined in this study showed 5,9,12-18:3 as the major NMIP acid in the GLC analysis. Among these, the neutral seed lipids of *P. Jezoensis* and *L. leptolepsis* contain an especially high percentage of 5,9,12-18:3. The characteristic NMIP acids of the neutral lipids of Taxo-

TABLE 3

Fatty Acid Compositions of Neutral Lipids from Seeds of Gymnospermae

Acid	ECL[a]	Sample no. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
12:0		[b]	–	–	–	–	0.10	–	–	t	–	t	–	–	–	0.20	–	–	–	0.03	–	1.22
13:0		–	–	–	–	–	0.14	0.10	t	0.04	0.03	0.05	0.07	–	–	0.26	–	t	0.03	0.09	t	0.06
14:0		0.22	0.09	t	0.05	–	0.09	–	0.04	0.04	0.03	0.03	0.04	–	0.03	0.52	–	0.03	0.04	0.09	0.03	1.47
15:0		0.11	0.09	–	–	–	0.09	–	–	t	–	0.03	0.04	t	0.04	1.11	–	t	t	0.06	t	0.24
16:0		15.20	6.66	2.64	2.35	6.03	24.52	3.21	2.57	2.62	3.78	4.92	4.73	4.94	4.09	13.31	2.93	6.11	5.62	5.30	4.30	15.00
16:1(n-9)	16.22	0.98	0.09	t	t	t	0.47	0.09	0.06	0.04	0.03	0.08	0.09	t	0.04	1.02	0.06	0.06	0.10	0.13	0.04	0.47
(n-7)	16.30	t	3.08	0.04	t	t	0.06	0.04	0.15	0.30	0.33	0.09	0.08	0.10	0.05	0.07	t	t	0.18	0.03	t	0.22
16:2(n-6)	16.68	0.08	0.68	t	t	t	0.06	0.04	0.15	0.30	0.33	0.09	0.08	t	0.07	–	t	t	–	0.03	t	0.19
17:0		–	0.10	t	t	t	0.06	0.04	t	0.02	t	0.04	0.04	0.04	0.03	0.34	0.05	0.21	0.08	0.16	0.07	0.13
16:3(n-3)	17.27	–	0.12	t	–	0.03	t	–	t	t	t	t	t	t	t	t	0.03	–	–	0.03	–	0.10
18:0		3.77	0.86	0.80	1.85	2.51	1.27	1.05	1.46	1.26	1.50	1.82	1.61	1.98	2.13	3.52	2.13	2.62	3.14	2.03	2.62	6.18
18:1(n-13)	16.11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.27
(n-9)	18.24	34.47	13.34	36.50	46.77	30.35	8.59	17.10	15.41	17.64	45.80	19.55	17.13	26.29	20.74	11.68	22.86	9.58	11.93	10.70	12.48	10.23
(n-7)	18.32	0.30	17.30	0.51	0.17	0.57	2.65	0.54	0.35	0.59	0.47	0.19	0.12	0.17	t	0.46	0.17	0.14	0.17	0.31	t	0.58
18:2 5,9	18.37	0.54	1.04	16.02	13.65	–	–	–	3.37	2.25	3.87	2.91	2.65	1.87	3.54	t	t	t	–	–	t	t
5,11	18.42	0.28	1.76	–	–	–	0.57	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.07
(n-6)	18.69	32.67	44.14	32.88	27.93	51.26	30.56	40.23	49.54	46.02	29.35	45.12	48.27	46.70	47.75	23.56	46.01	22.43	28.39	36.77	32.95	20.84
18:3 5,9,12	18.82	0.64	0.34	3.36	1.48	0.08	0.22	–	25.01	27.00	9.47	19.06	18.15	15.04	18.10	t	0.04	0.28	0.10	0.29	0.24	–
(n-3)	19.24	3.66	1.73	1.58	1.53	0.24	16.43	0.12	0.17	0.36	2.41	0.34	0.64	0.14	0.25	16.54	1.67	49.63	35.09	12.57	24.35	5.92
18:4 5,9,12,15	19.37	t	t	0.24	–	–	0.07	–	t	0.08	–	t	0.06	t	0.03	–	–	0.08	0.32	0.05	t	–
20:0		–	0.27	t	t	–	0.74	–	0.25	0.16	0.18	0.28	0.18	0.30	0.27	0.41	0.29	0.29	0.21	0.96	t	11.97
20:1(n-9)	20.21	0.39	0.27	1.44	1.53	0.28	0.08	1.15	0.24	0.38	1.00	0.63	0.49	1.01	0.73	0.92	1.02	0.53	1.01	0.77	0.69	0.62
(n-7)	20.30	–	0.35	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20:2 5,11	20.32	0.39	0.39	t	t	0.79	0.20	0.18	t	0.08	t	0.29	0.25	0.08	0.11	0.28	0.67	0.35	0.18	0.36	0.65	–
(n-6)	20.66	0.33	0.36	0.95	0.65	0.98	0.15	12.27	0.44	0.27	0.92	0.21	0.47	0.64	2.20	4.99	0.55	1.94	2.46	1.42	1.18	1.18
20:3 5,11,14	20.97	1.75	4.02	2.80	1.95	6.68	9.67	23.88	0.71	0.30	0.65	3.42	3.94	0.79	1.11	14.95	3.81	1.21	2.96	14.08	12.31	1.89
(n-3)	22.21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.28	0.22	0.80	1.34	1.15	0.45	–
20:4 5,11,14,17	21.33	–	–	–	–	–	0.28	–	–	–	–	–	0.08	–	0.08	7.07	1.81	4.81	7.12	8.49	7.36	0.31
22:0		–	0.27	–	–	–	0.26	–	–	–	–	–	0.04	–	–	0.25	–	–	–	0.32	–	11.83
Others		–	0.12	2.65	0.24	0.09	0.20	0.38	–	0.18	0.43	0.13	0.40	0.36	0.21	0.17	11.19	0.10	0.29	0.05	2.75	0.04	8.01

[a]ECL: equivalent chain length.
[b]t: trace.

diaceae and Cupressaceae seeds are 5,11,14,17-20:4 which occur with 5,11,14-20:3. However, the content of these acids is less than 10%. In the previous paper, *Ephedra campylopoda* seed oil was found to contain the maximal amount of 5,11,14,17-20:4 (21.9%) (15). The occurrence of 5,11,14,17-20:4 was also found in the stalk lipids of *E. sinica* as the major component of the NMIP acids in this study, though the content was quite low.

Desaturation of Fatty Acids at 5:6

Recently, 5-desaturation of fatty acids from oilseeds was demonstrated for the first time by incubation of ^{14}C-labeled substrates with developing seed slices and with a cell-free homogenate of meadowfoam (*Limnanthes alba*) seeds (26). The 5-desaturase activity of the extracts of the developing seeds was characterized (27). The acyl group in meadowfoam seed oil contains about 60% of 5-20:1 with small amounts of 5-22:1, 5-18:1 and 5,13-22:2 as 5-olefinic fatty acids. The 5-olefinic NMIP acids in the gymnosperm seeds can be formed in a similar manner by 5-desaturase.

The seed lipids of certain kind of grass belong to Compositae, Raunculaceae, Labiatae, Limnanthaceae and Chemopodiaceae containing 5-olefinic unsaturated acids, including 5-monoenoic acids (1,2). However, 5-monoenoic acids were not detected in the fatty acids of lipids from the seeds of the 20 species of Gymnospermae in this study. The efficiency of the open-tubular GLC column used in this study (theoretical plates; 140,000 to 18:1) was enough to separate the peaks of 5-16:1, 5-18:1 and 5-20:1 from those of the ordinary fatty acids included in gymnosperm seed lipids. Typical ECL values and separation factors under the conditions of this study are: ECL 5-16:1 16.11, 7-16:1 16.19, 9-16:1 16.30, 5-18:1 18.12, 9-18:1 18.25, 11-18:1 18.32, 5-20:1 20.10, 11-20:1 20.22, 13-20:1 20.31, and separation factors 7-16:1/5-16:1 = 1.028, 9-18:1/5-18:1 = 1.046 (28). The absence of 5-monoenoic acids suggests one of the characteristics of the desaturase in the gymnosperm seeds.

The desaturation of acyl groups resulting in the formation of linoleate from oleate in phospholipids by cotyledons and microsomes of developing seeds has been reported (29,30). However, as was described in the previous paper, 5-desaturase does not show activity toward polar lipids as substrates in *L. alba* seeds, since endogenous phosphatidylcholine and -ethanolamine contain very low levels of 5-monoenoic acids (26,27). In this study, a considerable amount of 5-olefinic NMIP acid was found in the polar lipids taken from the gymnosperm seeds tested (Table 4). However, the content was generally

TABLE 4

Fatty Acid Compositions of Polar Lipids from Seeds of Gymnospermae

Acid	Sample no. 1	2	3	4	7	8	9	10	11	12	14	16	17	19	20	21
12:0	0.14	0.05	0.04	t	0.96	1.10	0.95	0.23	0.11	0.17	0.03	0.05	t	0.85	0.05	2.36
13:0	0.10	0.29	0.54	0.48	3.74	3.34	2.18	0.25	0.11	0.49	3.60	0.77	0.82	6.96	0.80	0.55
14:0	0.14	0.11	0.69	0.55	t	0.54	0.38	0.09	0.04	0.06	0.16	t	0.40	0.39	t	0.13
15:0	0.13	0.12	0.07	0.34	0.07	0.12	0.21	t	0.06	0.08	0.15	0.09	0.19	0.21	0.08	0.32
16:0	25.72	24.82	22.65	12.58	20.80	11.24	9.64	11.88	12.20	13.90	14.23	20.61	17.70	22.99	20.49	33.40
16:1(n-9)	0.74	0.14	0.14	0.28	0.10	0.14	0.29	0.12	0.10	0.15	0.13	0.33	0.43	0.23	0.20	0.33
(n-7)	t	0.75	0.07	0.12	0.12	t	0.19	0.13	0.05	0.09	0.09	t	0.06	–	t	0.49
16:2(n-6)	t	1.54	0.07	0.16	–	0.60	0.91	1.04	0.25	0.27	0.03	–	t	0.52	t	t
17:0	0.32	0.31	0.38	0.35	0.23	0.20	0.23	0.12	0.14	0.19	0.14	0.33	0.28	t	0.35	0.26
16:3(n-3)	0.03	0.11	1.22	0.67	–	–	t	–	–	–	–	–	t	2.87	–	0.21
18:0	4.69	1.94	3.95	3.40	3.29	4.26	3.15	3.40	3.70	3.92	5.43	4.09	5.63	–	5.62	3.19
18:1(n-13)	–	–	–	–	–	–	–	–	–	–	–	–	–	2.87	–	0.15
(n-9)	32.56	9.20	30.70	37.98	14.40	18.95	21.20	53.25	29.99	27.30	28.05	18.91	15.56	11.29	24.12	8.46
(n-7)	0.48	17.03	9.25	0.10	1.06	1.32	1.97	1.83	1.18	1.11	0.99	–	0.58	0.47	0.30	1.06
18:2 5,9	0.26	0.97	5.22	3.95	–	2.88	1.94	4.76	1.46	1.94	2.27	–	0.51	–	t	0.07
5,11	0.19	0.98	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(n-6)	26.13	31.93	10.08	15.88	31.93	42.47	41.51	15.21	37.97	37.11	33.90	33.16	20.55	28.34	25.15	13.98
18:3 5,9,12	0.29	0.49	0.30	0.13	–	4.86	5.27	2.48	3.55	3.63	5.11	0.12	0.09	t	0.08	–
(n-3)	4.55	1.30	17.30	16.51	0.83	0.62	0.63	0.25	0.55	0.71	0.29	0.69	17.82	6.50	3.92	24.47
18:4 5,9,12,15	–	–	t	t	–	–	0.05	–	–	–	–	–	–	–	–	–
20:0	0.48	0.38	0.66	0.20	–	1.16	1.02	0.98	0.29	0.40	0.65	0.86	4.20	1.40	0.56	1.19
20:1(n-9)	0.21	0.13	0.99	1.08	1.01	t	0.61	1.09	0.56	0.60	0.59	0.76	0.64	0.51	0.80	0.10
(n-7)	–	0.21	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20:2 5,11	0.89	0.35	0.38	0.37	t	–	0.16	0.16	0.42	0.48	0.20	1.12	0.45	0.23	1.23	0.10
(n-6)	0.06	0.16	0.64	0.79	6.86	0.63	0.94	0.25	0.98	1.06	0.61	3.36	1.09	1.18	1.71	0.25
20:3 5,11,14	0.98	1.47	1.42	1.39	11.79	0.96	0.74	0.68	5.33	5.36	0.84	12.70	1.86	7.48	10.82	1.62
(n-3)	–	–	0.03	–	–	–	–	–	–	–	–	0.05	0.36	–	0.33	0.10
20:4 5,11,14,17	–	–	0.53	0.20	–	–	–	–	–	–	–	0.33	1.74	1.78	1.35	0.33
22:0	0.35	2.25	0.99	0.20	–	0.98	2.07	0.58	0.10	0.23	0.63	0.73	8.17	1.39	1.31	2.50
Others	0.66	3.07	0.69	2.29	2.81	3.03	3.59	1.17	0.86	0.75	1.49	0.36	1.55	4.41	0.63	3.76

TABLE 5

Distribution of Fatty Acids in Triacylglycerols from the Seeds[a]

Acid	1. *C. revoluta*			2. *G. biloba*			3. *T. cuspidata*			5. *T. nucifera*		
	TG	2-MG	F	TG	2-MG	F	TG	2-MG	F	TG	2-MG	F
16:0	16.48	7.80	0.47	6.08	2.33	0.38	3.05	1.92	0.63	6.78	2.10	0.31
16:1(n-9)	1.26	0.85	0.67	–	–	–	–	–	–	–	–	–
(n-7)	–	–	–	4.55	2.54	0.58	–	–	–	–	–	–
18:0	4.02	0.74	0.18	–	–	–	1.51	0.24	0.16	2.80	0.36	0.13
18:1(n-9)	36.06	49.14	1.36	10.94	17.94	1.64	41.83	55.71	1.33	31.05	48.88	1.57
(n-7)	–	–	–	13.16	7.03	0.53	–	–	–	–	–	–
18:2 5,9	–	–	–	2.22	1.71	0.77	18.56	1.89	0.10	–	–	–
5,11	–	–	–	3.76	2.42	0.64	–	–	–	–	–	–
(n-6)	34.08	37.74	1.11	45.91	59.09	1.29	29.73	40.05	1.35	51.23	48.08	0.94
18:3 5,9,12	–	–	–	1.06	0.55	0.52	2.22	0.19	0.09	–	–	–
(n-3)	4.33	3.33	0.77	3.74	2.41	0.64	–	–	–	–	–	–
20:0	2.40	–	0	–	–	–	–	–	–	–	–	–
20:1(n-9)	–	–	–	–	–	–	1.42	–	0	–	–	–
20:2(n-6)	–	–	–	–	–	–	–	–	–	1.11	–	0
20:3 5,11,14	1.37	0.40	0.29	8.58	3.98	0.46	1.68	–	0	7.03	0.58	0.08
20:4 5,11,14,17	–	–	–	–	–	–	–	–	–	–	–	–

Acid	7. *P. nagi*			8. *P. jezoensis*			13. *P. koraiensis*			17. *C. japonica*		
	TG	2-MG	F	TG	2-MG	F	TG	2-MG	F	TG	2-MG	F
16:0	3.43	2.45	0.71	3.01	1.25	0.42	5.56	1.17	0.21	6.76	1.76	0.26
16:1(n-9)	–	–	–	–	–	–	–	–	–	–	–	–
(n-7)	–	–	–	–	–	–	–	–	–	–	–	–
18:0	1.10	0.38	0.35	1.70	0.34	0.20	2.21	0.26	0.12	2.88	0.36	0.13
18:1(n-9)	17.33	20.08	1.16	15.91	22.31	1.40	26.88	26.90	1.00	9.95	17.27	1.74
(n-7)	–	–	–	–	–	–	–	–	–	–	–	–
18:2 5,9	–	–	–	3.62	1.04	0.29	2.11	0.28	0.13	–	–	–
5,11	–	–	–	–	–	–	–	–	–	–	–	–
(n-6)	41.11	61.76	1.50	50.96	72.12	1.42	46.92	70.16	1.50	22.99	42.09	1.83
18:3 5,9,12	–	–	–	24.80	2.94	0.12	15.23	1.06	0.07	–	–	–
(n-3)	–	–	–	–	–	–	–	–	–	51.03	37.07	0.73
20:0	–	–	–	–	–	–	–	–	–	–	–	–
20:1(n-9)	1.11	0.34	0.31	–	–	–	1.09	0.17	0.16	–	–	–
20:2(n-6)	12.25	4.65	0.38	–	–	–	–	–	–	–	–	–
20:3 5,11,14	23.67	10.34	0.44	–	–	–	–	–	–	1.21	0.38	0.31
20:4 5,11,14,17	–	–	–	–	–	–	–	–	–	5.18	1.07	0.21

[a]TG: triacylglycerol. 2-MG: 2-monoacylglycerol. F: enrichment factor for the 2-position. Mol % of the major fatty acid components are listed.

lower than that in neutral lipids. Thus, the possibility of direct desaturation of acyl groups in polar lipids should be considered a possible pathway for the formation of 5-olefinic acid in germnosperm seeds.

In the *Ginkgo* seeds used in this study, 11-18:1 was present in a greater amount than 9-18:1, which is the main 18:1 acid in most gymnosperm seeds. On the other hand, the peculiar feature of the NMIP acid in the *Ginkgo* seed lipids is the predominant occurrence of 5,11-18:2 as 18:2 which was reported in the previous paper (9,10) and again observed in this study. The usual 18:2 acid found as a NMIP acid in gymnosperm seeds is 5,9-18:2. These facts suggest the biochemical formation of 5,11-18:2 from 11-18:1 and 5,9-18:2 from 9-18:1 by the 5-desaturase, based on the analogy to the reported pathway from 13-22:1 to 5,13-22:2 in the cotyledons of developing *L. alba* (26).

Fatty Acid Distribution in Triacylglycerols

The cleavage of triacylglycerols from neutral lipids with ethylmagnesium bromide was performed according to the Yurkowski and Brockerhoff procedure (31). The fatty acid composition of the original triacylglycerols and 2-monoacylglycerols, and the enrichment factors (content of the acids in the 2-monoacylglycerols/content of the same acid in the triacylglycerols) in mol % (32) are shown in Table 5. The results show that saturated and ordinary C_{20} acids are distributed mainly in the 1,3-position, and oleic an linoleic acids are preferentially in th 2-position. These results are in agreement with the rule presented for the ordinary seed oils (33). In this study, the 5-olefinic NMIP acids showed low enrichment factors (0-0.4) for the seven species tested except *Ginkgo* seed lipids, which showed higher enrichment factors for the NMIP acids. In stereospecific analysis of triacylglycerols of *Limnanthes douglasii* seed oil, the occurrence of 5-olefinic acids at the outer acylglycerol positions was reported (34). The distribution of the 5-olefinic NMIP acids obtained in this study showed the analogous feature as shown by low enrichment factors.

ACKNOWLEDGMENTS

We thank Dr. Tatsuichi Tsujii for his kind advice in regard to the species of Gymnospermae.

REFERENCES

1. Smith, C.R., Jr. (1970) in Progress in Chemistry of Fats and Other Lipids (Holman, R.T., ed.) Vol. XI, Part 1, pp. 137-177, Pergamon Press, London.
2. Hitchcock, C., and Nichols, B.W. (1971) in Plant Lipid Biochemistry, pp. 1-42, Academic Press, London.
3. Takagi, T., Eaton, C.A., and Ackman, R.G. (1980) Can. J. Fish. Aquat. Sci. 37, 195-202.
4. Litchfield, C., and Marcantonio, E.E. (1978) Lipids 13, 199-202.
5. Paradis, M., and Ackman, R.G. (1977) Lipids 12, 170-176.
6. Walkup, R.D., Jamieson, G.C., Ratcliff, M.R., and Djerassi, C. (1981) Lipids 16, 631-646.
7. Worthington, R.E., Brady, U.E., and Hitchcock, H.L. (1981) Lipids 16, 351-354.
8. Takagi, T. (1964) J. Am. Oil Chem. Soc. 41, 516-519.
9. Gellerman, J.L., and Schlenk, H. (1963) Experimentia 19, 522-525.
10. Schlenk, H., and Gellerman, J.L. (1965) J. Am. Oil Chem. Soc. 42, 504-511.
11. Plattner, R.D., Spencer, C.F., and Kleiman, R. (1975) Lipids 10, 413-416.
12. Seher, A., Krohn, M., and Ko, Y.S. (1977) Fette Seifen Anstrichm. 79, 203-206.
13. Madrigal, R.V., and Smith, C.R., Jr. (1975) Lipids 10, 502-504.
14. Koyama, Y., Okada, Y., and Toyama, Y. (1968) Nippon Kagaku Zasshi 89, 95-97.
15. Kleiman, R., Spencer, C.F., Earle, F.R., and Wolff, I.A. (1967) Chem. Ind., 1326-1327.
16. Gellerman, J.L., and Schlenk, H. (1969) Lipids 4, 484-487.
17. Eckman, R. (1980) Phytochemistry 19, 321-322.
18. Jamieson, G.R., and Reid, E.H. (1972) Phytochemistry 11, 269-275.
19. Bligh, E.G., andDyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
20. Anderson, B.A., Christie, W.W., and Holman, R.T. (1975) Lipids 10, 215-219.
21. Bus, J., Sies, I., and Lie Ken Jie, M.S.F. (1977) Chem Phys. Lipids 18, 130-144.
22. Bus, J., Sies, I., and Lie Ken Jie, M.S.F. (1976) Chem. Phys. Lipids 17, 501-518.
23. Gunstone, F.D., Pollard, M.R., Scrimgeour, C.M., and Vedanayagam, H.S., (1977) Chem. Phys. Lipids 18, 115-129.
24. Frost, D.J., and Gunstone, F.D. (1975) Chem. Phys. Lipids 15, 53-85.
25. Smith, C.R., Jr., Freidinger, R.M., Hagemann, J.W., and Spencer, G.F. (1969) Lipids 4, 462-465.
26. Pollard, M.R., and Stumpf, P.K. (1980) Plant Physiol. 66, 649-655.
27. Moreau, R.A., Pollard, M.R., and Stumpf, P.K. (1981) Arch. Biochem. Biophys. 209, 376-384.
28. Itabashi, Y., and Takagi, T. (1980) Yakagaku 29, 855-865.
29. Slack, C.R., Roughan, P.G., and Balasingham, N. (1978) Biochem. J. 170, 421-433.
30. Stymne, S., and Appelqvist, L. (1978) Eur. J. Biochem. 90, 223-229.
31. Yurkowski, M., and Brockerhoff, H. (1966) Biochim. Biophys. Acta 125, 55-59.
32. Conacher, H.B.S., Gunstone, F.D., Hornby, G.M., and Padley, F.B. (1970) Lipids 5, 434-441.
33. Breckenridge, W.C. (1978) in Fatty Acids and Glycerides (Kuksis, A., ed.) pp. 197-232, Plenum Press, New York, NY.
34. Phillips, B.E., Smith, Jr., C.R., and Tallent, W.H. (1971) Lipids 6, 93-99.

[Received February 9, 1982]

The Effect of Dihydroergotoxine on Lipid Peroxidation in vitro

KINGA KORÉH[a,c,*], **MYRON L. SELIGMAN**[a,b] and **HARRY B. DEMOPOULOS**[b],
Departments of Neurosurgery[a] *and Pathology*[b], *New York University School of Medicine and Department of Biochemistry*[c], *New York University College of Dentistry, New York, NY 10016*

ABSTRACT

Dihydroergotoxine mesylate (DHET), an ergot alkaloid derivative, is widely used to treat senile cerebral vascular insufficiency. Aspects of this age-related phenomenon may be due to deterioration by lipid oxidation of cellular membranes. DHET stabilizes EEG alpha frequencies, increases cerebral blood flow and oxygen uptake and accumulates in lipid-rich structures of the brain. The effect of DHET was studied on iron-catalyzed peroxidation of liposomes as measured by the thiobarbituric acid assay. It was found that DHET inhibits peroxidation in vitro in a dose-dependent manner. These results suggest that DHET acts in part as a lipid antioxidant when used to treat senile cerebral vascular insufficiency.
Lipids 17:724-726, 1982.

The ergot alkaloids and their derivatives are a group of compounds with a wide variety of chemical and pharmacologic effects, the basic mechanisms of which have not as yet been explained. Their diverse actions include alpha adrenergic receptor blockade, contraction of uterine smooth muscle, influence on body temperature, stimulation of dopaminergic and serotoninergic receptors and the inhibition of norepinephrine-stimulated cyclic AMP synthesis (1,2).

The structure of the ergot alkaloids is composed of a basic ergoline system which is N-methylated at position 6, carries a carboxyl group at position 8 and usually a double bond at the 8-9- or 9-10-position (Fig. 1A). This heterocyclic structure can be linked through the carboxyl group to a series of tricyclic tripeptides (Fig. 1B) to produce a family of peptide alkaloids with powerful pharmacologic properties. Four dihydrogenated peptide alkaloids of the ergotoxine group—dihydroergocornine, dihydroergocristine, dihydro-α- and dihydro-β-ergokryptine—in a ratio of 3:3:3:1 comprise dihydroergotoxine methylsulfonate (DHET) (3).

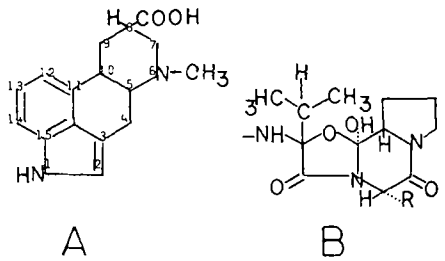


FIG. 1. Components of DHET. (A) Ergoline system; (B) tricyclic tripeptide.

DHET, in particular, is widely used to treat senile cerebral vascular insufficiency, a term which encompasses many aspects of mental and behavioral deterioration associated with the aging process. Its mechanism of action is not fully understood in humans, and although much work has been done on its pharmacologic effects, it is unknown which of these are most relevant to its clinical application (3).

DHET is therapeutically effective in ameliorating certain age-related phenomena which may be partially due to the breakdown of cell membranes. For instance, studies have shown that patients with senile cerebral insufficiency maintained on a regimen of DHET exhibited marked increases in EEG alpha frequencies (4). DHET also increased cerebral blood flow and oxygen uptake, and reduced cerebral circulation time (3). In vivo, DHET accumulates in the lipid-rich structures of the brain; notably, 60% is concentrated in the synaptosomes (5).

Due to its lipophilic nature and characteristic actions, it was postulated that DHET exerts a stabilizing or protective effect on lipid components. Therefore, DHET was tested as an antioxidant on iron-catalyzed peroxidation in liposomes as measured by the thiobarbituric acid (TBA) assay.

MATERIALS AND METHODS

Liposome Preparation and Incubation

*Author to whom correspondence should be addressed.

Liposomes were prepared by combining 80% ovolecithin and 20% dicetylphosphate in chloroform in each of two round-bottom flasks. Sufficient DHET (Hydergine®, Sandoz, Ltd.), dissolved in methanol, was added to one of the flasks to yield a final concentration of 0.01, 0.05, 0.1 or 1.0 mM. An equal amount of vehicle (methanol) was added to the other flask. The solvents were removed in vacuo (Rinco flash evaporator) and a thin film of lipid was formed. Phosphate buffer, 0.05 M, pH 7.0, was added to each flask to yield a final concentration of 17.2 mM lipids. The mixtures were agitated by pasteur pipet and sonicated for 15 min. The two suspensions were each divided in half to yield two portions containing DHET and buffer and two portions containing vehicle and buffer. Ferrous sulfate (1.0 mM final concentration) was added to one flask containing DHET and buffer and to one flask containing vehicle and buffer. The resulting liposome suspensions were incubated in loosely stoppered, 25-ml erlenmeyer flasks in a shaking water bath at 37 C.

Thiobarbituric Acid Assay

The thiobarbituric acid (TBA) reagent was prepared by making a saturated TBA solution in 10% perchloric acid and diluting it with 20% trichloroacetic acid (2:3, v/v).

Aliquots (0.1 ml) of each of the four liposome solutions were taken every 15 min up to 1 hr and every 30 min thereafter, and added to 5.0 ml of TBA reagent. The incubation was run for a total of 3 hr. The solutions were heated for 30 min in a boiling water bath and centrifuged at 2,000 rpm for 15 min. The solutions were then read in a Beckman DU spectrophotometer at 532 nm.

Calculations

Standard solutions using four different concentrations of malondialdehyde (MDA) were run in duplicate with each assay. A standard curve of absorbance at 532 nm compared to MDA concentration was constructed for each assay. MDA was added as the tetraethyl acetal. The molar extinction coefficient was found to be, on the average, 78,503. Absorbance values were converted to nmol MDA/ml liposomes. Student's t-test was performed for each time period as compared to corresponding untreated (iron-containing) liposomes. P values were determined by the use of the two-tailed Z scale of a standard t-table.

RESULTS

The effects of 1.0 mM, 0.1 mM and 0.01 mM DHET on the course of iron-catalyzed peroxidation in liposomes are presented (Fig. 2). DHET at a concentration of 1.0 mM produced virtually complete inhibition of lipid peroxidation ($p<0.001$). Reduction of the DHET concentration by a factor of 10 also yielded statistically significant decreases in peroxidation for all sample times with the exception of 90 min. With the additional 10-fold reduction (0.01 mM) in concentration, DHET ceased to be protective.

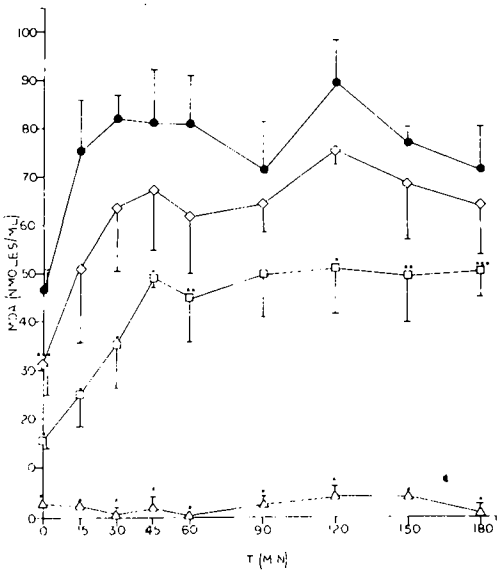


FIG. 2. The effect of DHET on iron-catalyzed peroxidation in liposomes as measured by the TBA assay. The control curve represents a pooling of 11 runs of the assay, and at least 3 runs were performed for each concentration of DHET. Vertical bars represent the standard error of the mean. Control (1 mM Fe) •; 1 mM Fe + 0.01 mM DHET ◇; 1 mM Fe + 0.1 mM DHET □; 1 mM Fe + 1 mM DHET △; *$p<0.001$; **$p<0.01$; ***$p<0.05$. Symbols without asterisks indicate nonsignificant values.

Two experiments using a concentration of 0.05 mM DHET produced values of which only 44% were significantly different from the untreated iron-containing liposomes. These experiments are not included in Figure 2.

DHET appears to exhibit a dose-dependent protective effect against peroxidation in liposomes, with the lowest effective concentration lying between 0.01 and 0.05 mM.

DISCUSSION

DHET at a concentration of 1 mM almost completely inhibits iron-catalyzed peroxidation

in liposomes. It is hypothesized that this in vitro action of DHET may be due to an antioxidant mechanism.

The structure of DHET is suited to such a mechanism, since it carries two hydrogen atoms at the 9- and 10-positions (Fig. 1A). These atoms may be extractable by free radical intermediates which are the products of lipid peroxidation. They may also serve to reduce radical oxygen species such as superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($^{\cdot}OH$) which are generated in such reactions (6).

DHET has been tested in many in vitro and animal studies, but doubt remains as to the clinical applicability of the results. Several distinct experimental effects nevertheless suggest that DHET may exert a central nervous system antioxidant or membrane-stabilizing action in vivo.

The decreased blood flow elicited by animal models of temporary ischemia leads to a disturbance of normal EEG scans and a decrease in the diameters and lengths of cerebral blood vessels. The administration of DHET prevents the reduction of EEG frequencies and promotes an improvement in cerebral vascular morphology (7).

The effect of DHET on Purkinje cell firing has been studied. Drugs such as beta blockers and barbiturates alter the firing rates of Purkinje cells. Addition of DHET to Purkinje cells in vitro acts not to increase or decrease the firing rate, but rather to regularize the interspike interval without changing the average firing rate (8). It is plausible that DHET directly stabilizes the neuronal membrane to elicit this response.

Cerebral vascular insufficiency results in a number of deleterious effects on the CNS, including impaired neurotransmitter biosynthesis, disordered neurotransmitter storage and release, altered cAMP levels and mitochondrial damage (9). Lipofuscin accumulates in neurons with advancing age and its levels can be heightened when an important endogenous antioxidant, α-tocopherol, is lacking in the diet (10).

Oxidative damage to membranes and other components of the CNS seems to be a consequence of aging, and treatment with antioxidants may ameliorate this process. If its mechanisms are further elucidated, DHET may prove to be a valuable pharmacologic agent not only in the management of the aging process, but also of other phenomena due to lipid oxidative processes.

ACKNOWLEDGMENTS

This work was supported by grants from Pioneer Systems, Inc., and Puritan Fashions, Inc. We are grateful to Sandoz, Ltd., for their generous gift of Hydergine®, and to D. Pierson and S. Shaw for some valuable concepts.

REFERENCES

1. Boissier, J.R. (1978) Pharmacology 16 (Suppl. 1), 12-26.
2. Markstein, R., and Wagner, H. (1975) FEBS Lett. 55, 275-277.
3. Berde, B., and Schild, H.O. (eds.) (1979) Pharmacology and Clinical Pharmacology of Hydergine, Springer-Verlag, Berlin/Heidelberg.
4. Gygax, P., Wiernsperger, N., Meier-Ruge, W., and Baumann, T. (1978) Gerontology 24 (Suppl. 1), 14-22.
5. Iwangoff, P., Meier-Ruge, W., Schwieweck, C., and Enz, A. (1976) Pharmacology 14, 27-38.
6. Swern, D. (1961) in Autoxidation and Antioxidants (Lundberg, W.O., ed.) vol. I, pp. 1-54, Interscience Publishers, New York, NY.
7. Gygax, P., Hunziler, O., Schulz, U., and Schweitzer, A. (1975) Triangle (Sandoz, Ltd.) 14, 80-89.
8. Gähwiler, B.H. (1978) Gerontology 24 (Suppl. 1), 71-75.
9. Davis, J.N. (1975) Neurology 25, 382.
10. Sulkin, N.M., and Srivary, P. (1960) J. Gerontol. 15, 2-9.

[Received March 22, 1982]

Inhibition of Lymphocyte Capping by Fatty Acids in Mouse and Man[1]

EDUARDO A. SANTIAGO-DELPÍN, *Surgical Service, San Juan VA Hospital,*
ANGEL A. ROMÁN-FRANCO and **JULIO I. COLÓN**, *Department of Pathology,*
University of Puerto Rico, San Juan, Puerto Rico 00936

ABSTRACT

Lipid-enriched diets have been related to a high cancer incidence in experimental animals for many years, and more recently, to assorted defects on the immune response. We investigated the effect of incubating human or murine (C3H/HEJ) lymphocytes with saturated (16:0) and unsaturated (18:1, 18:2, 18:3, 20:4) fatty acids (12 μg for each 10^7 cells), on the ability to cap with antihuman or anti-mouse anti-IgM, μ-chain specific antibody. Capping was also tested in obese (ob/ob, C57BL/6J) mice. Capping at 30 and 60 min was reduced by fatty acid incubation to 10-30% of control values in humans ($p<.001$), and to 30% of control values in mice ($p<.01$), regardless of degree of unsaturation. ob/ob capped normally. A lymphocyte membrane effect caused by fatty acids is observed in these experiments. Whether this is related to the dysimmunity caused by lipid diets cannot be assessed from our data, especially since all fatty acids, regardless of unsaturation, reduced the capping phenomenon.
Lipids 17:727-732, 1982.

Lipids have been related to an abnormally high incidence of cancer in experimental animals for almost 40 years. Tannenbaum initially observed that mice fed a diet high in lipids had a higher incidence of spontaneous tumors (1). Szepsenwol systematically studied this problem in mice and identified a carcinogenic effect of lipids (2). This effect was not limited to a particular strain or organ and it did not appear to be related to the type of lipid used (3-6). Concomitant observations in humans have suggested that diets high in fat and/or cholesterol may be related to a higher incidence of cancer of the pancreas (7). Similarly, fat is known to enhance the effect of some carcinogenic substances (8).

Several hypotheses have been advanced to explain this effect, including cocarcinogenesis (9), an alteration of the hormonal environment with changes in the prolactin and estrogen levels (10), and a direct effect on mitogenesis (11). An additional possibility has been studied by us (12) and others—an effect on the immune response. Di Luzio was the first to study the effect of lipids on the immune response in a comprehensive fashion and he detected reduced antibody production and impaired macrophage activity in animals fed fat-enriched diets (13). Subsequently, lipids were shown to prolong skin (14) and tumor allografts survival in mice across both weak and strong histocompatibility barriers (12). In vitro experiments showed that unsaturated fatty acids suppressed phytohemaglutinin (PHA) lymphocyte transformation (15). Experiments in our laboratory have shown a decreased migration inhibition factor (MIF) production (submitted for publication) and diminished delayed hypersensitivity (16) and antibody production (17). Taken together, these data suggest that lipids, specifically fatty acids, alter to some extent the immune reactivity of an otherwise healthy animal.

These observations led us to postulate that one critical effect of lipids is on the immune response and that perhaps a combination of these changes, together with the low antigenicity seen in the majority of tumors, could be factors in tumor escape and growth (18). Furthermore, since many surface and behavioral properties of normal and cancerous cells depend on the properties of its membrane, and lipids are closely involved in the control of cell membrane composition and properties, an effect on the membrane would be a possible factor in explaining the effect of lipids on the immune response. We present experiments utilizing the capping phenomenon in an attempt to identify an effect of lipids on the lymphocyte membrane.

MATERIALS AND METHODS

Experimental Design

Experiments were done using both human peripheral lymphocytes and mouse spleen cells. Lymphocytes were washed and incubated with one of five different fatty acids of varying

[1]Experiments carried out at Immunology Laboratory, Department of Pathology, University of Puerto Rico Medical School, San Juan, Puerto Rico.

*Author to whom correspondence should be addressed.

degrees of saturation: N-hexadecanoic (16:0, palmitic), *cis*-9-octadecenoic (18:1, oleic), *cis,cis*-9,12-octadecadienoic (18:2, linoleic), all-*cis*-9,12,15-octadecatrienoic (18:3, α-linolenic), and all-*cis*-5,8,11,14-eicosatetraenoic (20:4, arachidonic). After incubation, the cells were washed and incubated with either antihuman or antimouse fluoresceinated anti-IgM anti' ody, μ-chain specific. The antibody reacts with the μ-chain of the IgM on the surface of the lymphocyte membrane and a ring is rapidly formed around the cell. Small sections of the ring complex fashion themselves into patches or aggregates and these eventually coalesce to one pole of the cell to form a small cap. To induce and assay cap formation, we have modified the method described by Hudson and Hay (19) using direct immunofluorescence. The percentage of cells with the cap were counted at the end of a specified time and compared with suitable controls. Additional experiments were done using obese (ob/ob) mice.

Human Experiments

Thirty to 50 (30-50) ml of blood was drawn by venipuncture into a heparinized syringe from an anticubital vein of healthy volunteers (23-40 years old). Lymphocytes were separated by density gradient centrifugation; a triple wash was performed with minimal essential medium (MEM); cells were diluted to 10^7 cells/ml, centrifuged at 150 × g for 10 min, resuspended and incubated at 37 C for 1 hr with or without the corresponding fatty acid (2.5 μl of fatty acid solution, 12 μg each, were used per 10^7 cells). Cell incubation with lipids was done for only 1 hr because lipids are rapidly (within minutes) taken up by the cell membrane (20-23). (With time, incorporation into phospholipids may occur with different results [21]. Conversely, some types of dissociated cells may lose their capacity to rearrange surface components with the passage of time [24].) Control cells were incubated with or without 2.5 μl of 95% alcohol, the vehicle of dilution of the fatty acids (25). After 1 hr, the cells were centrifuged at 150 × g for 10 min, washed with MEM and viability tests were performed on aliquots using the Trypan Blue dye exclusion technique. The pellet was incubated with fluoresceinated anti-human-IgM, μ-chain specific antibody, and the reaction was stopped with sodium-azide at 30 or 60 min. Again the cells were centrifuged at 150 × g for 10 min, washed twice with MEM, covered with glycerol and examined under ultraviolet (UV) light. The percentage of cells with caps was noted in a total 25-100 cell count. The observer was ignorant of the origin of the sample.

Mouse Experiments

Female mice of the C3H/HEJ strain, 9-10 months of age, weighing 22-28 g, were used for these experiments. Prior to the experiments, they had been kept under temperature-controlled conditions and fed Purina Laboratory Chow and fluids ad libitum. They appeared free of disease. The animals were lightly anesthetized with ether and sacrificed by cervical dislocation. The spleen was removed and macerated. The cells were washed with sodium-chloride solution and reacted with 0.83% ammonium-chloride and Tris buffer. They were washed with either RPMI 1640 or MEM, diluted to 10^7 cells and centrifuged at 150 × g for 10 min. At this point, they were incubated at 37 C for 1 hr with or without the corresponding fatty acid, and the controls with or without 95% alcohol. Viability was tested with Trypan Blue. The cells were centrifuged at 150 × g for 10 min, washed in RPMI 1640 or MEM, and incubated with fluoresceinated antimouse-IgM, μ-chain specific antibody. The reactions were stopped with sodium-azide at 30 or 60 min; the cells were centrifuged at 150 × g for 10 min, washed twice with RPMI 1640, had glycerol added, were examined under UV light, and the percentage of cells with caps was noted in total counts of 100 cells.

Fatty Acids

Palmitic, oleic, linoleic, linolenic and arachidonic acids (see Experimental Design) obtained from Sigma Chemical (Sigmagrades 1, 3) and Baker Company (chromatography) were dissolved in 95% ethyl alcohol to a concentration of 4.8 mg/ml (4.8 μg/μl); 2.5 μl (ca. 12 μg) of each fatty acid were used for 10^7 cells. Controls were incubated with normal saline or 2.5 μl of 95% ethyl alcohol.

Capping in Obese Mice

Female obese mice of the ob/ob strain, 11-12 months of age, were used. The rationale was that the ob/ob gene might predispose to lipid alterations, and that impaired immunity has been reported (26). Also, obese C3H mice show an increased incidence of mammary tumors over controls when fed a fat-enriched diet (27). The corresponding controls were C57/BL females of identical age since ob/ob mutation originates from C57/BL. All mice were obtained from Jackson Laboratories, Bar Harbor, ME, and were kept under temperature- and humidity-controlled conditions on Purina Laboratory Chow. All animals appeared healthy. C57/BL mice weighed 25 g and ob/ob mice weighed 60 g average at the time of the experi-

ment. The animals were lightly anesthetized and sacrificed by cervical dislocation. The spleen was removed and lymphocytes were purified as already described. Spontaneous capping formation was performed with mouse anti-IgM antiserum, μ-chain specific. The percentage of cells with caps were counted as we have described.

Statistical Analysis

In human experiments, analysis was done by comparing each fatty acid with the control at both 30 and 60 min with analysis of variance (ANOVA). The controls were also compared with a t-test against all the fatty-acid-incubated cells together, at both 30 and 60 min. In the mouse experiment, all the fatty-acid-incubated cells were compared with their respective controls at 30 and 60 min using the t-test. In the obese mouse experiments, the t-test was used to compare ob/ob capping at 30 and 60 min against the C57/BL controls. A programmable desk calculator was used.

RESULTS

Viability

All cells were incubated with saline or with alcohol alone which was the vehicle for dissolving the fatty acids. Viability testing was done at the end of the incubation period of 1 hr, when aliquots were taken and stained with Trypan Blue. At least 90% viability was found with all incubations with the fatty acids, the saline controls and the alcohol controls.

Human Experiments

Table 1 illustrates our results with humans. Capping is expressed in the text and tables as a percentage of total cells. It is evident from this table that capping proceeded normally from 16.7% at 30 min to 20% at 60 min. It is also apparent that capping is markedly inhibited at both 30 and 60 min when cells are incubated with fatty acids. Controls compared to palmitic and the unsaturated fatty acids at both 30 and 60 min using ANOVA show differences significant to p values of less than .004 and .0001, respectively ($F = 6.8163$; $dfl = 2$, $df2 = 25$; $F = 20.4$; $dfl = 2$, $df2 = 24$). There is an increase in capping in all unsaturated fatty acids at 60 min from the 30-min baseline, but not on palmitic acid. In spite of this increase at 60 min, there is still a significant difference between experimentals and controls at this time. Differences appeared to be more marked with unsaturated than with palmitic acids at 30 min, and a trend toward less capping was thought to occur with increasing unsaturation, but the difference was not too pronounced and was not found to be significant. Indeed, at 60 min, this trend was lost.

Mouse Experiments

Table 2 summarizes the results of experiments with capping using mouse spleen cells. Significant inhibition of capping is observed at 30 and 60 min when cells are incubated with fatty acids. Controls show a rise in percentage capping, from 9.1 ± 1.39% at 30 min to 14.6 ± 2.09% at 60 min, as expected. However, capping after incubation with fatty acids was very low at both 30 min ($3.57 \pm .59$, $p<.01$) and 60 min ($3.13 \pm .65$, $p<.01$). No differences were apparent between the different fatty acids.

Table 2 also illustrates data concerning obese mice. Comparison of capping of spleen cells from these mice against the respective C57/BL controls were not different either numerically or statistically. The table gives the corresponding values and standard errors. Two points are appropriate. Differences between C57/BL and C3H mice are apparent: there is more capping

TABLE 1

Results of Capping in Humans

		Fatty acids[a]				
	Control	P	O	L	L′	A
Capping at 30 min[b] (%)	16.7	6.6	4.6	2.4	2	3.2
Capping at 60 min (%)	20.4	3	7.3	3	5	6.9
No. of experiments	7	7	4	5	5	5

[a]Abbreviations: P – palmitic acid; O – oleic acid; L – linoleic acid; L′ – linolenic acid; A – arachidonic acid.

[b]Statistical validity: C vs P vs OLL′A at 30 min: $p<.004$; at 60 min: $p<.0001$ (ANOVA). C vs POLL′A at 30 min: $p<.001$; at 60 min: $p<.001$ (t-test).

TABLE 2

Results of Capping in Mice[a]

	Controls	POLL'A[b]	C57BL controls	OB/OB[c]
Capping at 30 min ± SEM (%)	9.1 ± 1.39	3.57 ± .59	35 ± 6.49	34.5 ± 5.68
Capping at 60 min ± SEM (%)	14.6 ± 2.09	3.13 ± .65	40 ± 5.61	39.2 ± 6.05
No. of experiments	10	25	9	17

[a]Abbreviations: see legend for Table 1.

[b]Statistical validity: control vs POLL'A at 30 min: $p<.01$; controls vs POLL'A at 60 min: $p<.01$.

[c]C57BL vs OB/OB: not significant at both 30 and 60 min.

in the C57/BL than in the C3H mice. Also, the increase in percentage capping of 30-60 min is not marked in the C57 blacks as it is in the C3H mice and in humans.

DISCUSSION

Several experiments demonstrate an effect of lipids on immune function. Alderson and Green (28) enriched lymphocyte membranes with cholesterol. Upon stimulation with lectins, the cells showed binding into the membrane, but lymphocyte transformation was diminished. Mihas and coworkers (15) incubated cells with oleic, linoleic and arachidonic acids and found that these fatty acids suppressed PHA transformation. Tonkin and Brostoff have questioned the role of fatty acids using in vitro experiments and have suggested the possible existence of artifacts in the system. We have performed preliminary PHA transformation using lymphocytes of BALB/C mice fed coconut-oil-supplemented diets, and have shown that 25 μg of PHA diminished the stimulation index (Santiago-Delpín and Román-Franco, unpublished observations). In a separate set of experiments, we observed diminished MIF reactivity in the lymphocytes of mice fed high-lipid diet when stimulated with PHA in vitro.

Experiments by Mertin (14,30) showed that primary and secondary cell-mediated immune response is inhibited by polyunsaturated fatty acids in vitro and that mice fed polyunsaturated fatty acids have an increase in skin graft survival. Our own experiments with skin and tumor allografts also show that survival of these grafts is significantly increased in those animals fed corn-oil-supplemented diet and that this occurred both across weak and strong histocompatibility barriers (12). Experiments are in progress in our laboratory to investigate other aspects of cellular immunity in mice fed a high-lipid diet.

Di Luzio showed that diets enriched with fatty acids lead to decreased antibody production and to decreased phagocytic and chemotactic activity of macrophages. Also, mice fed methylpalmitate grew larger allotumors than controls (13). Preliminary studies in our laboratory demonstrate quantitative and qualitative changes in antibody production in mice fed diets with a high lipid content (17). Sheffy and Schultz (31) showed partial suppression of immune reactivity, most marked in dogs fed diets high in polyunsaturated fatty acids. Kollmorgen et al. (32) have shown inhibition of lymphocyte function in rats fed high-fat diets, and a transfer of this hyporeactivity with serum from fat-fed rats. However, one experiment by Moldawer et al. showed preservation of cell-mediated immunity (PPD in rats) by a lipid diet (33). More recently, Utermohlen et al. reported a decrease in direct migration inhibition of healthy human leukocytes when incubated with some unsaturated fatty acids (34). We have shown a suppressive effect of lipid diets on skin reactivity to DNCB in rats (16).

From these and other studies, it is evident that lipids affect immune reactivity both in vivo and in vitro in experimental animals. The exact mechanism of this effect is not yet clear but one possibility is an effect on the lymphocyte membrane with impairment of initial events during sensitization. The effect of lipids on biological membranes is well known and has been amply studied and documented (see ref. 16 for a comprehensive review).

Mandel and Clark used the patching time as an index of membrane fluidity (35). Anti-H2 antisera was used in a T-cell leukemia model. Incubation with 19:0 fatty acid resulted in a decrease in patching time, as did incubation

with an 18:3 fatty acid. These data are consonant with our own experiment in which capping is decreased with all the fatty acids tested, regardless of degree of unsaturation. It has also been shown that mouse lymphocytes incubated with unsaturated fatty acids have a decrease in membrane microviscosity, but that in spite of this, linoleic acid inhibits capping of immunoglobulin by more than 80% and also inhibits the capping of H2 and θ-antisera on T-cells (36).

Our experiments extend those of Mandel and Clark (35) and Bhalla et al. (36) to multiple unsaturated and one saturated fatty acids, and to the effect on the human lymphocyte. All tested fatty acids, irrespective of degree of saturation, produced a pronounced and significant decrease in cap formation in human and mouse lymphocytes. This was not due to impaired viability since the Trypan Blue test showed the cells of all groups to be alive. Neither was it due to the effect of the alcoholic solvent since capping and viability were the same in saline and alcohol controls. However, we are unable to explain the comparable effect of both saturated and unsaturated fatty acids on the capping phenomenon, although this was found by Mandel and Clark also in their patching model (35), by Di Luzio in his in vivo experiments with methylpalmitate (13) and by our group in the in vivo susceptibility of rats to intraperitoneal sepsis (37). Although evidence is presented that human and murine lymphocytes incubated with saturated and unsaturated fatty acids decrease their ability to form caps when incubated with anti-IgM antiserum, μ-chain specific, and although these data are in harmony with other immunological experiments and suggest that a membrane alteration could be present, alternatives other than changes in fluidity must be considered in order to explain the similar effect of different fatty acids (21,35,38-41). Further experiments are needed to identify the molecular basis of this action.

ACKNOWLEDGMENTS

The authors gratefully recognize the excellent assistance of Mrs. Diana Román, Mrs. Margarita Ramos, Mrs. Viola Rodríguez, and Mrs. Nancy Benitez. This work was supported in part by NCI Center Core Grant #CA-16598.

REFERENCES

1. Tannenbaum, A. (1942) Cancer Res. 2, 468-475.
2. Szepsenwol, J. (1963) Proc. Soc. Exp. Biol. Med. 112, 1073-1076.
3. Szepsenwol, J. (1966) Proc. Soc. Exp. Biol. Med. 112, 981-981.
4. Szepsenwol, J., Román, A., and Santiago, J. (1966) Proc. Soc. Exp. Biol. Med. 122, 1284-1288.
5. Szepsenwol, J., Marchosky-Kogan, J.A., and Pastrana-Iturregui, M.S. (1969) Cellule 68, 45-55.
6. Reddy, B.S., Narisawa, T., Vukusick, D., Mangat, S., Wynder, E.L., and Weisburger, J.H., (1975) Fed. Proc. 34, 900.
7. Wynder, E.L. (1975) Cancer Res. 35, 2228-2233.
8. Nigro, N.D., Singh, D.V., Campbell, R.L., and Sook Pak, M., (1975) J. Natl. Cancer Inst. 54, 439-442.
9. Nixon, J.E., Sinnhuber, R.D., Lee, D.J., Landers, M.K., and Harr, J.R., (1974) J. Natl. Cancer Inst. 53, 453-458.
10. Szepsenwol, J., and Ydrach, A.A. (1969) Cellule 68, 59-66.
11. Scarpelli, D.G. (1975) Cancer Res. 35, 2278-2283.
12. Santiago-Delpín, E.A., and Szepsenwol, J. (1977) J. Natl. Cancer Inst. 59, 459-461.
13. Di Luzio, N.R. (1972) Adv. Lipid Res. 10, 43-88.
14. Mertin, J. (1976) Transplantation 21, 1-4.
15. Mihas, A.A., Gibson, R.G., and Hirschowitz, B.I. (1975) Proc. Soc. Exp. Biol. Med. 149, 1026-1028.
16. Santiago-Delpín, E.A., and Román, D. (1981) Fed. Proc. 40, 346.
17. Llende, M., Santiago-Delpín, E.A., and Habeeb, A.F.S.A. (1981) Fed. Proc. 40, 346.
18. Román-Franco, A.A., and Santiago-Delpín, E.A. (1977) Med. Hypoth. 3, 235-240.
19. Hudson, L., and Hay, F.C. (1976) in Practical Immunology, pp. 44-45, Oxford Blackwell Scientific Publ.
20. Cyong, J., and Okada, H. (1976) Immunology 30, 763-767.
21. Karnovsky, M.J. (1979) Am. J. Pathol. 97, 212-221.
22. Shinitzky, M., and Inbar, M. (1974) J. Mol. Biol. 85, 603-615.
23. Dietschy, J.M. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., ed.) pp. 1-5, Am. Physiol. Soc. Bethesda, MD.
24. McDonough, J., and Lilien, J. (1975) Nature 256, 416-417.
25. Hatten, M.E., Horwitz, A.F., and Burger, M.M. (1977) Exp. Cell Res. 107, 31-34.
26. Sheena, J., and Meade, C.J. (1978) Int. Arch. Allergy Appl. Immunol. 57, 263-268.
27. Waxler, S.H., Brecher, G., and Beal, S.L. (1979) Proc. Soc. Exp. Biol. Med. 162, 365-368.
28. Alderson, J.C.E., and Green, C. (1975) FEBS Lett. 52, 208-215.
29. Tonkin, C.H., and Brostoff, J. (1978) Int. Arch. Allergy Appl. Immunol. 57, 171-176.
30. Meade, C.J., and Mertin, J. (1976) Int. Arch. Allergy Appl. Immunol. 51, 2-24.
31. Sheffy, B.E., and Schultz, R.D. (1979) Fed. Proc. 38, 2139.
32. Kollmorgen, G.M., Sansing, W.A., Lehman, A.A., Fischer, G., Longley, R.E., Alexander, S.S., King, M.M., and McCay, P.B., (1979) Cancer Res. 39, 3458-3462.
33. Moldawer, L.L., Nakano, Y., Trerice, M.S., Etter, G.P., Bistrian, B.R., Flatt, J-P., and Blackburn, G.L. (1978) Surg. Forum 29, 67-68.
34. Utermohlen, V., Besner, G., and Berkowitz, M.G. (1980) Clin. Immunol. Immunopathol. 16, 324-335.
35. Mandel, G., and Clark, W. (1978) J. Immunol. 120, 1637-1643.
36. Bhalla, D.K., Hoover, R.J., Inbar, M., and Karnovsky, M.J. (1978) Cell Biol. 79, 42a.

37. Llende, M., Rosario, M., Echegaray, S., and Santiago-Delpín, E.A. (1982) Fed. Proc. 41, 934.
38. Chapman, D. (1975) Quart. Rev. Biophys. 8, 185-235.
39. Fuchs, P., Parolas, A., Robbins, P.W., and Blout, E.R. (1975) Proc. Natl. Acad. Sci. USA 72, 3351-3354.
40. Tsao, Y.K., and Lands, W.E.M. (1980) Science 207, 777-779.
41. Lenaz, G. (1979) in Subcellular Biochemistry (Roodyn, D.B., ed.) Vol. 6, pp. 233, Plenum Press, New York, NY.

[Received December 22, 1981]

Preferential Oxidation of Linolenic Acid Compared to Linoleic Acid in the Liver of Catfish (*Heteropneustes fossilis* and *Clarias batrachus*)

GAUTAM K. BANDYOPADHYAY[a], JYOTIRMOY DUTTA[a] and SUDHAMOY GHOSH[b,*],
Departments of [a]Chemistry and [b]Biochemistry, Bose Institute, Calcutta 700 009, India

ABSTRACT

The fate of [1-^{14}C] linoleic acid and [1-^{14}C] linolenic acid in the liver slices and also in the liver tissues of live carnivorous catfish, *Heteropneustes fossilis* and *Clarias batrachus*, was studied. Incorporation of the fatty acids into different lipid classes in the live fish differed greatly from the tissue slices, indicating certain physiological control operative in vivo. The extent of desaturation and chain elongation of linoleic and linolenic acids into long-chain polyunsaturated fatty acids was low. Linolenic acid was oxidized (thus labeling the saturated fatty acid with liberated ^{14}C-acetyl-CoA) in preference to linoleic acid, and this oxidation also seemed to be under physiological control since both of the fatty acids were poorly oxidized in the tissue slices and in the killed fish. These fish can therefore recognize the difference in the acyl chain structures of linoleate and linolenate. The higher oxidation of linolenic acid and poor capacity for its conversion to longer chain, highly unsaturated derivatives indicates a higher demand for the dietary supply of these essential fatty acids in these two species. *Lipids* 17:733-740, 1982.

INTRODUCTION

Requirements for PUFA in mammals vary. Most mammals need linoleic acid as EFA whereas some of the carnivorous mammals such as cats and lions are dependent on higher derivatives of linoleic acid, e.g., arachidonic acid, because they cannot desaturate linoleic acid due to a lack of desaturase enzyme (1-3). Most fish studied require linolenic acid as EFA but many of them cannot desaturate linolenic acid efficiently (4). For example, rainbow trout convert a greater part of labeled 18:3(n-3) to 22:6(n-3) but red sea bream, rockfish and globefish fail to do so (4,5). The latter species would therefore be expected to be nutritionally dependent on a dietary source of higher derivatives of linolenic acid. It therefore seemed desirable to ascertain the capacity of the carnivorous catfish to metabolize ingested linolenic acid. In addition, the differences in EFA requirement by mammals and fish pose the question of whether these systems can recognize the differences in the structures of linoleic acid and linolenic acid in any of the metabolic steps.

It has already been found by the present authors that the two catfish *Heteropneustes fossilis* and *Clarias batrachus* cannot synthesize linoleic and linolenic acids (6). It has also been observed that both of these fatty acids are poorly desaturated and chain elongated.

In this communication, studies on the metabolic fate of incorporated labeled linoleic and linolenic acids in the livers of live fish and in tissue slices of the two catfish are reported. Linolenic acid is preferentially oxidized compared to linoleic acid and this oxidation is inhibited in the tissue slices.

MATERIALS AND METHODS

H. fossilis and *C. Batrachus* were collected live from the market and kept in different glass jars containing 10 ℓ of tap water (32 ± 2 C) and fed with a dough prepared from wheat powder and dry fish meal. They were kept under these conditions for two days before onset of the experiments. The Krebs-Ringer phosphate buffer, pH 7.4 (7), used for suspending tissues, contained 2.5 mM $CaCl_2$. [1-^{14}C]-Linoleic acid (sp act 60 mCi/mmol) and [1-^{14}C] linolenic acid (sp act 60 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, England. Omnifluor was purchased from New England Nuclear. Reference fatty acid methyl esters, neutral lipids and phospholipids were purchased from Nu-Chek-Prep (Elysian, MN) and the solid support and liquid phases for gas chromatography were from Applied Science Lab (State College, PA).

Incorporation of Labeled Fatty Acids into Fish Liver

[1-^{14}C] Linoleic acid and [1-^{14}C] linolenic acid were dissolved in 75% ethanol containing

*Author to whom correspondence should be addressed.

Abbreviations: PUFA, polyunsaturated fatty acid; EFA, essential fatty acid; ECL, equivalent chain length; TG, triglycerides; FFA, free fatty acid; PL, polar lipids; WE, wax ester; and TLC, thin layer chromatography.

1% NaCl; 100 μl of each of these fatty acid solutions containing 5 μCi of the labeled fatty acid was injected into the peritoneum of the live fish. Injected fish were kept at 32 ± 1 C in tap water without feeding for 4 hr, after which they were sacrificed. In the case of the killed fish experiment, fish were hit on the head with a mallet or killed by prolonged exposure to chloroform 10 min after injection of radioactive materials. The killed fish were kept in water for a total period of 4 hr after which they were sacrificed and the livers were removed. Tissues were washed first with cold Krebs-Ringer phosphate buffer (pH 7.4) and then with cold 50% ethanol.

In the case of tissue slice experiments, livers, washed free of blood with Krebs-Ringer phosphate buffer, were sliced (at 0 C) into pieces about 0.5 mm thick. These slices were placed in 10 ml of Krebs-Ringer phosphate buffer (pH 7.4) and incubated with 5 μCi of labeled fatty acids at 32 ± 1 C for 4 hr. To end the incubation, the contents of the flasks were chilled in an ice-bath and centrifuged. The resulting pellets were washed with cold 50% ethanol.

Lipid Extraction from Fish Liver

About 20 ml of water was added to 1 g of tissue in the stainless steel container (immersed in ice bath) of a Sorvall Omnimixer and homogenized three times for 45 sec with 1-min intervals. The volume of the extract was noted, a 0.2-ml aliquot was put into a vial, dried in a vacuum oven and counted for radioactivity in a scintillation counter using toluene/Triton X-100 fluor. This represents total incorporation of radioactivity into the tissues. For lipid isolation, a mixture of methanol/chloroform (2:1) was added to the tissue extract maintaining the final ratio of methanol/chloroform/water (2:1:0.8) which constituted a monophasic system. Lipids were extracted following a modification of the Bligh and Dyer procedure (8). The chloroform layer containing lipids was dried over anhydrous Na_2SO_4, concentrated under vacuum and stored at -20 C if it was not used immediately.

Separation of Lipid Classes by Thin Layer Chromatography on Silica Gel G

Separation of lipid classes by TLC was done on Silica Gel G (Kieselgel G, Type 60, E. Merck). Coated glass plates (0.5 mm thick x 20 cm x 14 cm) were activated at 110 C for 1 hr. Lipid samples were applied side by side, with the reference lipids along a line 2 cm above the bottom edge of the plates, and were then developed in solvent mixtures such as petroleum ether (bp 40-60 C)/diethyl ether (peroxide-free)/acetic acid (80:20:1.5, v/v/v) for neutral lipids and chloroform/methanol/water (65:25:4, v/v/v) for polar lipids. Spots were detected either by iodine vapor or by specific staining reagents (9).

Preparation of Fatty Acid Methyl Esters

Total lipid (50-100 mg) was saponified by refluxing with 10% methanolic KOH. The nonsaponifiable fraction was removed by diethyl ether extraction. Free fatty acids generated by acidification of K-soaps were extracted with diethyl ether (peroxide-free). These fatty acids were then subjected to either acid- (10) or BF_3- (11,12) catalyzed methanolysis. The methyl esters were then collected in hexane after addition of water and dried over anhydrous Na_2SO_4.

Argentation TLC

Fatty acid methyl esters were separated according to their number of double bonds on $AgNO_3$-treated Silica Gel G plates. The method of either Bandyopadhyay and Dutta (13) or Dudley and Anderson (14) was followed, depending on the humidity of the laboratory. The methyl esters were separated into seven different bands, starting from saturated (at the top) to hexaenoate (at the bottom) on the chromatogram. Esters were extracted from the silica gel bands by a mixture of chloroform/methanol (2:1) and were further purified by rechromatography.

Gas Chromatography of Fatty Acid Methyl Esters

A gas chromatograph with dual column and dual flame ionization detector (Model GCD, Pye Unicam Ltd., Cambridge, U.K.) was used. Chromatograms were taken at 170 C with 6 ft x 1/8 in. stainless steel columns packed with 15% DEGS on 100-120 mesh Gas-Chrom P. The carrier gas (nitrogen) flow rate was maintained at 30 ml/min. Injection port and detector temperatures were kept at 230 C. Hydrogen and air flow rates for the detectors were 150 ml/min and 300 ml/min, respectively. Reference fatty acid methyl esters were run under the same conditions and the ECL values of the peaks were compared for identification of fatty acids (15). The fatty acid composition was computed from peak areas. To avoid overlapping of peaks, fatty acid esters were first separated by argentation TLC, extracted from each TLC band, mixed with a known amount of methyl ester of either 15:0 or 19:0 as internal standard, and subjected to gas chromatography.

Total composition was obtained by normalizing against those standards.

Decarboxylation of Labeled Fatty Acids

Schmidt reaction with NaN_3 was done for the decarboxylation of labeled fatty acids (16, 17). Instead of measuring the radioactivity in the involved $^{14}CO_2$, counts remaining in the product were measured. Fatty acids (1-2 mg) were placed in Thunberg tubes kept at 0 C. Fifty mg of NaN_3 and 0.5 ml of an ice-cold mixture of fuming H_2SO_4 and conc H_2SO_4 (1:3) were added. After partial removal of the gases from inside, the tubes were heated at 70 C for 2 hr with occasional shaking. At the end of the reaction, the mixture was neutralized with 6 N NaOH and lyophilized to dryness. The dry mass was extracted with a mixture of methanol/chloroform/water (2:1:0.8). The solvent was concentrated, dried in a scintillation vial and counted for radioactivity.

Radioactive Counting

Radioactivity in a sample was counted in a liquid scintillation counter (Beckman, LS-100). Toluene containing 0.4% Omnifluor (New England Nuclear) was used as counting fluor. For aqueous samples, a mixture of toluene/Triton X-100 (2:1, v/v) was used as counting base. The efficiency of the instrument for ^{14}C-isotope was about 85%.

RESULTS

Typical fatty acid compositions of the total hepatic lipid of the two catfish *H. fossilis* and *C. batrachus* are given in Table 1. A notable point in the composition is the presence of high concentrations of 20:4(n-6) compared to the precursor 18:2(n-6). Although it has been reported that some catfish from the temperate latitudes contain very low levels of 20:4(n-6), analysis of fatty acid composition of many tropical air-breathing fish, including *H. fossilis* and *C. batrachus* collected from their natural habitats, showed deposition of these higher levels of 20:4(n-6) (18-22).

Incorporation of labeled fatty acids (as percentage of input) into the hepatic tissues of the live catfish was much less than that of the tissue slices. Incorporation was still lower in the killed fish livers (Table 2). This was expected because heartbeat and blood flow stopped soon after death (fish were killed 10 min after injection) and sufficient labeled fatty acids could not be carried to the liver.

Incorporation into lipids, expressed as percentage of tissue incorporation, was more with [1-^{14}C]linoleic acid (ca. 70%) than with [1-^{14}C]linolenic acid (ca. 55%). Incorporation of [1-^{14}C]linolenic acid was further reduced (33-34%) in the case of killed fish.

Labeling of hepatic lipid classes by the labeled fatty acids in live fish and in tissue slice experiments demonstrated that, compared to live fish, tissue slices incorporated less fatty acids into PL and WE but more into FFA and TG (Table 3). The results were similar with the two fatty acids used. There was also some significant variation in the labeling of polar lipid components. [1-^{14}C]linolenic acid was incorporated more in the cardiolipin and glycolipid fractions in the tissue slices than the other fractions, whereas in the live fish, higher incorporation took place in the lecithin, lysolecithin and phosphatidylethanolamine (Table 4).

Incorporation of radioactivity from the labeled fatty acids into the long-chain PUFA of the live fish was quite low. With [1-^{14}C]linoleic acid, some 2-3% of total radioactivity was found in each of 20:2(n-6), 20:3(n-6) and 22:6(n-3) as shown in Table 5. Of these, 6-8% radioactivity of 22:6(n-3) was retained after decarboxylation (Table 6), demonstrating that this fatty acid was terminally labeled at the carboxyl end by ^{14}C-acetyl-CoA liberated during oxidation of [1-^{14}C]linoleic acid. Incor-

TABLE 1

Fatty Acid Composition of Total Lipids of Fish Liver

Fatty acids	Distribution of fatty acids (wt %)	
	H. fossilis	*C. batrachus*
14:0	2.10	1.70
14:1	0.40	0.53
16:0	22.60	19.50
16:1(n-7)	4.25	3.20
18:0	11.50	5.32
18:1(n-9)	21.60	24.50
18:2(n-6)	4.10	7.11
20:0	0.80	0.65
18:3(n-3)	2.30	6.70
20:1(n-9)	–	0.50
18:4(n-3)	0.30	-
20:2(n-6)	1.34	0.72
20:3(n-6)	0.65	0.53
20:3(n-3)	1.72	1.15
20:4(n-6)	11.30	13.50
20:4(n-3)	0.43	0.31
20:5(n-3)	3.30	4.65
22:4(n-6)	0.90	1.05
22:5(n-6)	2.10	0.54
22:5(n-3)	1.30	1.52
22:6(n-3)	7.80	7.00

Results are mean of three sets of experiments, each set consisting of two fish for each species. (–) = Below 0.2%.

TABLE 2

Incorporation of Labeled Fatty Acids into Hepatic Tissues and Lipids of Fish

Name of fish	Total incorporation in liver (% of input)		Incorporation into lipid (% incorporated into tissue)[a]	
	[1-^{14}C] Linoleic acid	[1-^{14}C] Linolenic acid	[1-^{14}C] Linoleic acid	[1-^{14}C] Linolenic acid
			Live fish	
H. fossilis	3.25	4.00	68.80	54.50
C. batrachus	2.95	3.15	69.50	56.40
			Killed fish	
H. fossilis		1.30		33.30
C. batrachus		1.40		34.10
			Liver slice	
H. fossilis	11.80	6.00	71.20	55.42
C. batrachus	17.30	8.50	70.80	56.66

Results are the mean of three experiments. Five μCi of labeled fatty acids were either injected into the peritoneum of live fish (body wt 80-90 g) or incubated with sliced liver (700 mg). The period of incubation was 4 hr at 32 ± 1 C. Killed fish (killed 10 min after injection) were similarly incubated for 4 hr.

[a]Total incorporation in lipid X 100/total incorporation into liver.

TABLE 3

Incorporation of Radioactivity from [1-^{14}C] Linoleic Acid and [1-^{14}C] Linolenic Acid into Lipid Classes of Fish Liver

	% Distribution of radioactivity							
	After [1-^{14}C] linoleic acid incorporation				After [1-^{14}C] linolenic acid incorporation			
	H. fossilis		*C. batrachus*		*H. fossilis*		*C. batrachus*	
	Live fish	Liver slice	Live fish	Liver slice	Live fish	Liver slice	Live fish	Liver slice
Polar lipids	31.20	13.83	38.30	14.30	42.91	21.83	43.55	18.80
Diglycerides + cholesterol	7.50	7.72	2.10	10.00	6.35	7.10	11.71	9.90
Free fatty acids	26.12	27.66	19.90	24.24	15.04	25.70	14.73	12.90
Triglycerides	14.66	46.30	17.60	48.10	20.62	37.03	14.40	52.40
Wax ester	17.90	3.00	16.53	2.00	10.66	4.50	11.00	4.70
Cholesteryl ester	2.50	1.40	5.60	0.70	4.40	3.80	9.64	1.34

Results are the mean of three experiments.

TABLE 4

Incorporation of Radioactivity from [1-^{14}C] Linolenic Acid into Polar Lipid Classes of Fish Liver

Polar lipid class	% Distribution of radioactivity			
	H. fossilis		*C. batrachus*	
	Live fish	Liver slice	Live fish	Liver slice
Sphingomyelin	7.74	5.20	9.74	4.41
Lysolecithin	10.24	4.60	14.60	3.33
Lecithin	37.12	22.52	34.90	24.36
Phosphatidylethanolamine	22.82	10.11	17.44	8.20
Cardiolipin	10.70	21.43	12.42	19.60
Phosphatidic acid + glycolipid	11.40	36.24	10.30	40.30

Results are the mean of three experiments.

poration in the tissue slices gave similar results. Most of the radioactivity (75-85%) of [1-^{14}C]-linoleic acid was found in the 18:2(n-6) of the tissue. Saturated fatty acids contained very few counts. Very low conversion of labeled 18:2-(n-6) to 20:4(n-6), as shown in Table 5, indicates that the accumulation of higher percentages of 20:4(n-6) in the liver of the catfish (Table 1) is due to the dietary uptake of preformed 20:4(n-6).

TABLE 5

Incorporation of Radioactivity from [1-^{14}C] Linoleic Acid into Hepatic Fatty Acids of Fish

	% Distribution of radioactivity			
	H. fossilis		*C. batrachus*	
Fatty acids	Live fish	Liver slice	Live fish	Liver slice
14:0	0.40	–	0.53	–
16:0	1.56	0.44	1.60	0.60
18:0	1.46	0.41	1.10	0.40
20:0	–	–	–	–
14:1	–	–	–	–
16:1(n-7)	1.90	1.72	2.20	1.40
18:1(n-9)	4.92	4.45	4.00	4.41
20:1(n-9)	0.20	0.20	–	–
18:2(n-6)	79.10	82.50	75.20	85.00
20:2(n-6)	3.15	4.30	3.40	2.50
18:3(n-3)	–	–	–	–
20:3(n-6)	2.70	2.00	2.65	1.12
20:3(n-3)	–	–	1.10	–
18:4(n-3)	–	–	–	–
20:4(n-3)	–	–	–	–
20:4(n-6)	0.66	0.72	1.43	1.00
22:4(n-6)	0.50	0.54	0.70	0.50
20:5(n-3)	–	–	0.30	–
22:5(n-6)	0.50	0.42	1.10	0.50
22:5(n-3)	0.50	0.42	0.93	0.43
22:6(n-3)	2.00	2.00	3.40	1.00

Results are the mean of three experiments. (–) = Below 0.2%.

Experiments with [1-^{14}C] linolenic acid gave somewhat different results. In contrast to [1-^{14}C] linoleic acid, saturated fatty acids were very significantly labeled (39-44%) after incorporation of [1-^{14}C] linolenic acid into the hepatic tissues of live fish (Table 7). This indicates profuse oxidation of [1-^{14}C] linolenic acid and subsequent labeling of saturated fatty acids during synthesis or chain elongation by ^{14}C-acetyl-CoA liberated by β-oxidation of [1-^{14}C]-linolenic acid. Interestingly, this labeling of saturated fatty acids was drastically cut to 1-2% when incorporation was carried out in the liver slice (Table 7). If the fish were killed 10 min after injection of [1-^{14}C] linolenic acid, saturated fatty acids were labeled to an intermediate degree (8-10%). All these results relating to the labeling of saturated fatty acids are summarized in Table 8 where the ratio of radioactivity in the total saturated fatty acids x 100/18:2(n-6), after incorporation of [1-^{14}C] linoleic acid, and in the total saturated fatty acids x 100/18:3(n-3) after incorporation of [1-^{14}C]-linolenic acid, were computed from the results presented in Tables 5 and 7. This comparison (Table 8) shows that, in the live fish, linolenic acid was more readily oxidized relative to linoleic acid, and that the killing of fish destroyed this capacity for specific oxidation, as a result of which there was poor oxidation of the fatty acids in the liver slices.

Incorporation of radioactivity from [1-^{14}C]-linolenic acid into the long-chain PUFA of live fish was 3-5% or less of total radioactivity. This was observed in 18:4(n-3), 20:4(n-3), 20:5(n-3) and 22:6(n-3). In the killed fish tissues and liver

TABLE 6

Chemical Decarboxylation of Labeled Fatty Acids Obtained after Incorporation of [1-^{14}C] Linoleic Acid and [1-^{14}C] Linolenic Acid

	% Radioactivity retained in the fatty acids after decarboxylation			
	After incorporation of [1-^{14}C] linoleic acid into:		After incorporation of [1-^{14}C] linolenic acid into:	
Labeled fatty acids	*C. batrachus*	*H. fossilis*	*C. batrachus*	*H. fossilis*
18:0	84	88	87	85
18:2(n-6)	2	3	ND	ND
18:3(n-3)	ND	ND	3.50	2.5
20:4(n-6)	78	80	10	8
20:5(n-3)	13	20	88	80
22:6(n-3)	8	6	82	79

ND = experiments not done. Labeled fatty acids were isolated by argentation TLC and preparative gas chromatography. Fatty acids containing a minimum of 3×10^3 counts was used for decarboxylation.

TABLE 7

Incorporation of Radioactivity from [1-^{14}C] Linolenic Acid into Hepatic Fatty Acids of Fish

Fatty acids	% Distribution of radioactivity					
	H. fossilis			*C. batrachus*		
	Live fish	Killed fish	Liver slice	Live fish	Killed fish	Liver slice
14:0	3.02	0.70	–	2.70	0.80	–
16:0	26.62	6.00	1.03	15.74	4.53	0.70
18:0	14.60	3.30	0.60	10.05	2.90	0.43
20:0	–	–	–	0.72	0.20	–
14:1	–	–	–	0.44	0.32	–
16:1(n-7)	1.52	1.46	0.60	3.50	2.50	0.90
18:1(n-9)	1.82	1.75	0.71	6.10	4.40	1.53
20:1(n-9)	–	–	–	–	–	–
18:2(n-6)	1.70	1.10	1.20	2.15	1.50	1.30
20:2(n-6)	3.84	3.10	2.60	5.10	4.50	4.14
18:3(n-3)	20.10	33.61	66.00	30.74	38.00	56.70
20:3(n-6)	2.10	3.50	6.85	1.90	2.20	3.45
20:3(n-3)	4.52	6.22	7.16	6.25	11.80	4.22
18:4(n-3)	3.04	7.63	2.15	3.60	3.20	5.90
20:4(n-3)	4.60	10.15	3.00	2.60	3.60	8.70
20:4(n-6)	1.06	3.16	0.82	0.85	1.05	2.21
22:4(n-6)	1.80	5.26	1.35	2.06	2.55	5.36
20:5(n-3)	2.43	3.80	0.80	1.40	4.10	0.72
22:5(n-6)	0.42	1.40	–	0.26	1.13	–
22:5(n-3)	1.72	3.16	0.60	1.00	3.53	0.53
22:6(n-3)	5.05	4.62	3.55	2.80	6.80	1.16

Results are the mean of three experiments. (–) = Below 0.2%.

TABLE 8

Relative Incorporation of Radioactivity into Total Saturated Fatty Acids from Labeled Linoleic and Linolenic Acids in Fish

Radioactivity in:	*H. fossilis*			*C. batrachus*		
	Live fish	Killed fish	Liver slice	Live fish	Killed fish	Liver slice
Total saturated/18:3(n-3) X 100 after incorporation of [1-^{14}C]linolenic acid	220	29.60	2.60	95.00	22.10	2.20
Total saturated/18:2(n-6) X 100 after incorporation of [1-^{14}C]linoleic acid	4.46	ND	1.20	4.25	ND	1.35

Data were calculated on the basis of the results presented in Tables 5 and 7. ND = not done.

slices, incorporation into some of these fatty acids improved to some extent. However, from the decarboxylation studies, it may be concluded that incorporation into 20:5(n-3) and 22:6(n-3) occured mainly through metabolic conversion of intact molecules of [1-^{14}C]linolenic acid, and not by terminal labeling with ^{14}C-acetyl-CoA. In both cases, more than 80% of the counts were retained after decarboxylation (Table 6). It also showed that saturated fatty acids retained a major part of their radioactivity, whatever may be the incorporating fatty acids (Table 6).

DISCUSSION

We have observed in our laboratory that the catfish *H. fossilis* and *C. batrachus* cannot synthesize linoleic acid and linolenic acid (6). The fate of these fatty acids in the liver of these fish was therefore investigated. In the live fish, it is quite likely that related enzymes for incorpo-

rating fatty acids into different lipid classes are under strict hormonal control. This control, which channeled fatty acids more toward PL and WE in our experiments, was inoperative in the tissue slices (Table 3). As a result, the pattern of incorporation in tissue slices changed significantly from that in live fish. It should also be noted here that, among the polar lipids, incorporation of fatty acids into cardiolipin and glycolipids of tissue slices increased significantly, whereas labeling of sphingomyelin, lysolecithin, lecithin and phosphatidylethanolamine decreased (Table 4). It appears that, in these cases, slices do not resemble the in vivo metabolic situation.

One important finding of the present work is associated with the fate of the incorporated linoleic and linolenic acids in the two species of catfish. In general, [1-^{14}C]linoleic acid was poorly desaturated and elongated (Table 5); as a result, low percentages (2-3%) of counts were found in long-chain PUFA. Incorporation of radioactivity of [1-^{14}C]linolenic acid into PUFA was slightly more in the killed fish tissues or in the liver slices. Desaturation and chain elongation of linolenic acid were almost the same as in the case of linoleic acid (Table 7) and the processes were much weaker in these two species of fish in comparison to the results obtained with rainbow trout, where about 70% of the incorporated radioactivity from [1-^{14}C]-linolenic acid was channeled to 22:6(n-3) (4). It may be concluded that enzyme activities for desaturation and elongation in these species of catfish are lower than in other species, but may not be altogether missing. The lower conversion of linoleic and linolenic acids to their long-chain polyenoic derivatives, e.g., 20:4(n-6), 20:5(n-3) and 22:6(n-3), probably makes these fish dependent on the dietary supply of these C_{20} and C_{22} fatty acids for their growth and well being. Like carnivorous animals (e.g., cat and lion), many fish such as turbot, red sea bream, black sea bream, mullet, opaleye, rockfish and globefish possess very poor capacity for desaturation and chain elongation of C_{18} PUFA (2-5,23). With respect to the metabolic activities related to chain elongation and desaturation of PUFA, the two catfish investigated appear to be closer to marine fish (5,23).

Another important feature regarding the fate of the incorporated EFA is profuse oxidation of [1-^{14}C]linolenic acid in the fish, resulting in significant labeling of saturated fatty acids. About 40% of the radioactivity of [1-^{14}C]linolenic acid was present in the saturated fatty acids (Table 7). If the relative incorporation of radioactivity into total saturated fatty acids from the labeled linoleic and linolenic acids are calculated (Table 8), the significance of these differences become more evident. These results show that, in the liver of the live catfish, linolenic acid was preferentially oxidized in comparison to linoleic acid and the liberated ^{14}C-acetyl-CoA was incorporated in the saturated fatty acids. However, it was observed by Murata et al. (24) in common carp that starvation caused greater utilization of stored 18:2(n-6) as an energy source compared to 18:3(n-3), where the level remained almost unchanged. It may be said, therefore, that fish can distinguish the acyl chain structure of linoleic and linolenic acids during oxidation.

Interestingly, oxidation of both linoleic and linolenic acids was reduced more drastically in the case of linolenic acid in the liver tissue slices (Tables 5, 7 and 8), indicating that the preferential response toward the oxidation of linolenic acid in live fish could be under physiological control and this control was lost in the tissue slices. Hormone(s) can stimulate fatty acid oxidation, as was observed in the isolated rat hepatocytes in the presence of Ca^{2+} and vasopressin (25), catecholamines (26) or glucagon (27). This type of hormone-mediated control of fatty acid oxidation may also operate in live fish but with some discrimination in the structure of the fatty acids to be oxidized.

Results of decarboxylation experiments show that radioactivity in (n-6) and (n-3) polyenoates of 20- and 22-carbon chain lengths were obtained mostly by metabolic inclusion of intact [1-^{14}C]linoleic acid and [1-^{14}C]linolenic acid molecules (Table 6).

The differential oxidation of linoleate and linolenate, which occurred in the hepatic tissues of the two species of catfish when they were alive, may result from: (a) a process related to oxidation which could recognize the difference in the acyl chain structures of the two fatty acids and channel one of them preferentially toward oxidation, or (b) from the difference in K_m values between carnitine acyltransferase, glycerol-3p-acyltransferase or cholesterol acyltransferase for the two fatty acids. In the latter case, should there be a greater affinity of carnitine acyltransferase compared to the other two enzymes for linolenic acid, it would lead to increased transport of this fatty acid into mitochondria and, therefore, higher oxidation.

It may be noted that the extensive labeling of saturated fatty acids by ^{14}C-acetyl-CoA (derived from β-oxidation of incorporated [1-^{14}C]linolenic acid) occurred without much dilution by the unlabeled cytoplasmic acetyl-CoA pool. This is expected since oxidation takes place inside the mitochondria and ^{14}C-acetyl-CoA is protected from dilution (by the

cytoplasmic pool) by the mitochondrial membrane barrier which prevents free permeation of acetyl-CoA. The mitochondrial chain elongation system, on the other hand, uses this relatively less diluted ^{14}C-acetyl-CoA for chain lengthening.

The significance of the preferential oxidation of linolenic acid in these catfish is not very clear at this time. Higher oxidation of linolenic acid in the live fish will create a greater nutritional demand for this fatty acid as EFA in these fish. It is possible that these fish require long-chain PUFA as essential nutrients (which they must get from the diet) and use, whenever necessary, linolenic acid rather than linoleic acid as the preferred source of energy.

ACKNOWLEDGMENT

We thank K. Hait for typing the manuscript.

REFERENCES

1. Holman, R.T. (1977) in Polyunsaturated Fatty Acids (Kunau, W.H., and Holman, R.T., eds.) pp. 163-182, Am. Oil Chem. Soc., Champaign, IL.
2. Rivers, J.P.W., Hassam, A.G., Crawford, M.A., and Brambell, M.R. (1976) FEBS Lett. 67, 269-270.
3. Sinclair, A.J., Mclean, J.G., and Monger, E.A. (1979) Lipids 14, 932-936.
4. Owen, J.M., Adron, J.W., Middleton, C., and Cowey, C.B. (1975) Lipids 10, 528-531.
5. Kanazawa, A., Teshima, S.I., and Ono, K. (1979) Comp. Biochem. Physiol. 63B, 295-298.
6. Bandyapadhyay, G.K., Dutta, J., and Ghosh, S., Ind. J. Biochem. Biophys., in press.
7. Cohen, P.P. (1957) in Manometric Techniques (Umbreit, W.W., Burris, R.H., and Stanffer, J.F., eds.) pp. 147-150, Burgess Publishing Co., Minneapolis, MN.
8. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
9. Kates, M. (1972) in Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S., and Work, E., eds.) vol. 3, pp. 278-600, Elsevier Publishers, Amsterdam.
10. Chalverdjian, A. (1964) Biochem. J. 90, 518-521.
11. Morrison, W.K., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
12. Bazan, N.G. (1970) Biochim. Biophys. Acta 218, 1-10.
13. Bandyopadhyay, G.K., and Dutta, J. (1975) J. Chromatogr. 114, 280-282.
14. Dudley, P.A., and Anderson, R.E. (1975) Lipids 10, 113-115.
15. Ackman, R.G. (1963) J. Am. Oil Chem. Soc. 40, 558-564.
16. Goldfine, H., and Bloch, K. (1961) J. Biol. Chem. 236, 2596-2601.
17. Brady, R.O., Bradley, R.M., and Trams, E.G. (1960) J. Biol. Chem. 235, 3093-3103.
18. Murray, M.W., Andrews, J.W., and DeLoaeh, H.L. (1977) J. Nutr. 107, 272-280.
19. Worthington, R.E., Boggess, T.S., Jr., and Heaton, E.K. (1972) J. Fish. Res. Board Can. 29, 113-115.
20. Sen, P.C., Ghosh, A., and Dutta, J. (1976) J. Sci. Food Agric. 27, 811-818.
21. Sen, P.C., Ghosh, A., and Dutta, J. (1976) J. Indian Chem. Soc. LIII, 920-922.
22. Das, A.K. (1980) Ph.D. Thesis, University of Calcutta, India, pp. 81-83.
23. Kazuo, Y., Kobayashi, K., and Yone, Y. (1980) Bull. Jpn. Soc. Sci. Fish. 46, 1231-1234.
24. Murata, H., and Higashi, T. (1980) Bull. Jpn. Soc. Sci. Fish. 46, 1333-1338.
25. Sudan, M.C., Ball, A.J., Ilic, V., and Williamson, D.H. (1980) FEBS Lett. 116, 37-40.
26. Sudan, M.C., Tordoff, A.F.C., Ilic, V., and Williamson, D.H. (1980) FEBS Lett. 120, 80-84.
27. Lund, H., Borreback, B., and Bremer, J. (1980) Biochim. Biophys. Acta 620, 364-371.

[Received February 8, 1982]

A Study of the Plasma Lipoproteins and the Tissue Lipids of the Migrating Lamprey, *Mordacia mordax*

FLORENCE C.I. FELLOWS and **ROBYN M. MCLEAN***, *Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia*

ABSTRACT

Sea lampreys, *Mordacia mordax*, were collected in spring from the Yarra River, Victoria, during their upstream spawning migration, to study the lipid composition in their tissues and plasma and their lipid transport system. Plasma lipoproteins were isolated by preparative ultracentrifugation and their chemical compositions were analyzed. The major classes of lipoproteins were found to be similar to those of man and higher animals. Lipids from the muscle and liver were analyzed for fatty acids. The striking feature of the lipids in the migrating lamprey is the presence of very high levels of cholesterol in both plasma and muscle. The possible metabolic roles of cholesterol have been discussed.
Lipids 17:741-747, 1982.

INTRODUCTION

Sea lampreys, upon reaching their adult stage, migrate to freshwater rivers to spawn and, during this period, they do not feed. The energy source during this migration is mainly the lipid and protein from the muscle of the body wall (1). Lamprey muscle contains a considerable amount of lipids, 20-45% of the body dry wt (2-4). It has been shown (1) that the triacylglycerol levels in the muscle of the body wall and in the liver fall by 90% by the end of the spawning period. The body protein has also decreased to one-half.

Although the migratory animal is in a fasting state, the plasma of the short-headed lamprey, *Mordacia mordax*, contains a low concentration (0.1 mM) of nonesterified fatty acids (NEFA) (5). It has a fatty acid transport system similar to those of higher vertebrates. A protein-binding fatty acid is present in amounts comparable to those in teleost fish (6).

This paper reports the composition of plasma lipoproteins, muscle and liver lipids of the short-headed lamprey, *M. mordax*, during its spring migration.

MATERIALS

Chemicals

Triheptadecanoin, diheptadecanoyl-L-α-lecithin, cholesteryl heptadecanoate and polyunsaturated fatty acid esters (PUFA no. 1, marine source and PUFA no. 2, animal source) were obtained from Supelco, Inc., Bellefonte, PA. Butylated hydroxytoluene (BHT), cetyl alcohol, stearyl alcohol, palmitic acid stearyl ester, steric acid oleyl ester and squalene were purchased from Sigma Chemical Co., St. Louis, MO. Boron trifluoride methanol complex (14% BF_3) was obtained from The British Drug Houses, Poole, England. Silica Gel G (type 60) for thin layer chromatography (TLC) was from Merck, Darmstadt, West Germany. For gas chromatography, 10% SILAR-10C on Gas Chrom-Q (100-120 mesh) was obtained from Applied Science Laboratories Inc., State College, PA. Solvents used were redistilled from analytical-grade commercial preparations. Diacylglycerols were a gift from Dr. F.A. Shamgar, Department of Physiology, University of Melbourne. Alkyldiacylglycerols were prepared chromatographically from liver oil of *Squalus acanthias* (7).

*Author to whom correspondence should be addressed.

Animals

Short-headed lampreys (*Mordacia mordax* [Richardson]) were collected from the Yarra River (Victoria, Australia) during the upstream spawning migration (Sept.-Oct.). The weight range of the lampreys was 35-51 g with a mean weight of 40 g. The average length was 35 cm.

METHODS

Blood Sample, Muscle and Liver

Lampreys were rendered insensible by severing the spinal cord about 1 cm posterior to the eyes. This procedure maintained the function of the heart for some time, allowing blood samples to be collected. These samples were collected from the tails by cutting off 4 cm and making sure that urine was drained away before making the cut. The blood was mixed with EDTA (4 mM final concentration) and the antioxidant, BHT, was added at 50 mg/l blood. It was centrifuged and the plasma was stored at -20 C. Muscle (lateral musculature from the

middle section of the trunk) and liver were removed from the animal after taking the blood and were frozen in liquid nitrogen. They were stored at -70 C.

Isolation of Lipoprotein

Five ml of pooled plasma was used as the starting material. The procedure used for the isolation of the major classes of lipoproteins was the sequential preparative ultracentrifugation of Lindgren (8). Subfractionation was not done due to the shortage of plasma sample. The density adjustments were made with solutions containing NaCl and NaBr. The centrifugation was performed in a 50-Ti rotor in a Beckman preparative ultracentrifuge L2-65.

Lipid Extraction

Plasma and Lipoproteins. The lipids were extracted by the method of Folch et al. (9) with (and without) added triheptadecanoin, diheptadecanoyl-L-α-lecithin, cholesteryl heptadecanoate and heptadecanoic acid as internal standards.

Muscle and liver. The extraction procedure was modified from Nevenzel et al. (10). Frozen muscle (~2 g) was dissected free of the notochord and intestine and put into liquid nitrogen immediately. It was then powdered by shaking with a precooled ball-bearing, in a stainless steel centrifuge tube. The powder (1 g) was first homogenized in 4 ml of Krebs-Ringer phosphate buffer, pH 7.4, with a Dounce homogenizer, followed by the addition of 20 ml of chloroform/methanol (1:1, v/v). The homogenate was filtered through a sintered glass funnel and the residue washed with 10 ml of chloroform, followed by 10 ml of diethyl ether. The livers were thawed and cut finely with scissors and homogenized using the same procedure as for the muscle. Lipid standards were added at the homogenization stage.

Separation and Determination of Lipids

Plasma lipids were separated by TLC according to Fellows et al. (5). Muscle and liver lipids were separated by TLC with a different solvent system (petroleum ether/diethyl ether/acetic acid, 80:20:1, v/v/v) which resolved diacylglycerols from cholesterol and alkyldiacylglycerols from triacylglycerols. However, wax esters, squalene and cholesteryl esters all moved together and close to the solvent front in the latter system. This fraction was collected and eluted with diethyl ether and rechromatographed in *n*-hexane/diethyl ether (95:5, v/v) (11). Squalene was well separated from wax esters and cholesteryl esters but the latter two were not completely separated. In an attempt to determine whether wax esters were present, the following procedure was used. The ester fraction was recovered with diethyl ether and dried under N_2. It was hydrolyzed at 100 C for 1 hr with boron trifluoride methanol reagent (14% BF_3/methanol/benzene, 30:30:35, v/v/v) which methylated the fatty acids and liberated the free fatty alcohols and cholesterol. Water (1 ml) was added to the hydrolysate and it was followed by three extractions with light petroleum ether (bp 40-60 C). The combined extracts were evaporated to dryness under N_2. The extract was separated by TLC using petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v). Free fatty alcohols were recovered and esterified with acetic anhydride in pyridine (12). Both methyl esters of fatty acids and acetate esters of fatty alcohols were analyzed by gas-liquid chromatography (GLC) under the conditions described earlier (5). Standard wax esters treated as above were recovered (98-101%) as their fatty acid and fatty alcohol derivatives.

Nonesterified fatty acids, triacylglycerols, cholesteryl esters and phospholipids were separately methylated with boron trifluoride methanol reagent and the methyl esters of fatty acids liberated from these lipids were analyzed by GLC and identified by the use of standards. Squalene was separately measured by GLC under the same conditions as those for fatty acid esters.

Diacylglycerols and alkyldiacylglycerols were estimated gravimetrically after recovery from TLC plates and after drying to constant weight. The component fatty acids were methylated by trifluoride methanol reagent and analyzed by GLC. Comparable results were obtained from the above two methods. Total cholesterol and cholesteryl esters were determined by the method of Webster (13). In this method, digitonin was used to separate cholesteryl esters from free cholesterol. The total and ester cholesterol were determined by glacial acetic acid/ferric chloride reagent. Cholesteryl esters obtained using the method just described were in good agreement with those determined by GLC.

Protein Determination

The three major fractions of lipoproteins were thoroughly dialyzed against phosphate buffered saline, pH 8.0, at 2-4 C. Apolipoproteins were separated by extraction with ethanol/ether (3:1, v/v) according to Mills et al. (14). The precipitated protein fraction, after washing several times with the solvent, was dried under vacuum and dissolved in 1 M NaOH. The protein was determined by the method of Lowry et al. (15). Bovine serum albumin was used as the standard.

Bilirubin Determination

Bilirubin content in the liver lipid extract was measured spectrophotometrically in chloroform at 452 nm. A molar extinction coefficient of 62,000 cm^{-1} M^{-1} in chloroform was used for calculation. The lipid extraction procedure was done in a dark room.

RESULTS

The lipid content of the tissue and plasma are shown in Table 1. Close to 70% of the total muscle lipid was found to be neutral fats, including small amounts of diacylglycerols and alkyldiacylglycerols. Muscle also contained considerable amount of cholesterol, 380 mg (free and esterified) per 100 g. The liver was about 0.5% of the fresh body wt (40 g) and varied in color from brown to deep green, depending on the amount of bilirubin and biliverdin present (16). Bilirubin was found to be extracted along with the lipids, but the green-colored biliverdin was removed during washing procedure. The amount of bilirubin in the lipid extract ranged up to 1% of the total. The liver lipids were low in triacylglycerols, but contained an appreciable amount of diacylclycerols (~7%). Phospholipids and cholesterol were the main components in liver lipids. Squalene was present in both liver and muscle. The plasma contained very high concentrations of cholesterol and a small amount of triacylglycerols. A thorough search was made for wax ester in the tissues and plasma, but no detectable amount was found in either the tissues or plasma.

The lamprey plasma lipoproteins isolated by sequential ultracentrifugation showed three major classes, i.e., very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) as defined in Table 2. Chylomicrons were not detected in the plasma. The VLDL mainly transported triacylglycerols and cholesterol (free and esterified) whereas the LDL contained almost exclusively cholesterol and its esters. The cholesterol present in these two fractions was largely esterified (80%). However, only 45% of the cholesterol in HDL was present in an esterified form. Phospholipids and protein were the major components in HDL.

The fatty acid composition in the lamprey lipids is shown in Tables 3-5. The saturated fatty acids (11-39%) are mainly palmitic and stearic. The monoene acids (20-58%) are predominantly C_{18} accompanied by C_{16} and C_{20}. The remaining acids are mainly n-6 (18:2 to 22:4) and n-3 (18:3 to 22:6) families. The lamprey lipids are very rich in polyunsaturated acids, particularly C_{20} and C_{22} acids. The fatty acid esters of diacyl and alkyldiacylglycerols were also shown to be similar to the general pattern of tissue lipids.

TABLE 1

Lipid Content of Tissues and Plasma

Lipid content	Muscle (9)	Liver (6)	Plasma (4)
Total lipid (mg/g wet wt)	57.4 ± 3.3[a]	68.4 ± 3.5[b]	10.7 ± 0.5 mg/ml
		Wt%	
NEFAc	1.1 ± 0.3	3.0 ± 1.0	0.2 ± 0.01
Triacylglycerol	68.5 ± 2.3	8.6 ± 3.1	8.0 ± 0.3
Diacylglycerol[d]	1.0 ± 0.4	6.9 ± 2.0	0
Aliyldiacylglycerol[d]	0.5 ± 0.3	1.0 ± 0.3	0
Phospholipid	20.3 ± 2.2	54.7 ± 3.3	34.5 ± 1.3
Cholesterol	3.7 ± 0.8	10.6 ± 1.5	9.3 ± 1.0
Cholesteryl ester	4.9 ± 0.2	14.6 ± 1.2	48.0 ± 2.7
Squalene	Trace	0.6 ± 0.5	0

The number of determinations is in parentheses; liver and plasma samples were from pooled sources.

[a]Mean ± SEM.

[b]Total liver lipid includes bilirubin which varies from 0.03 to 0.12 mg/g wet wt.

[c]NEFA - nonesterified fatty acids.

[d]The results are from three determinations of pooled lipid extracts. Animals were of both sexes.

TABLE 2

Plasma Lipoproteins

Lipoprotein classes	Total[a] (mg/100 ml plasma)	Wt %				
		Protein	Triacyl-glyceride	Phospholipid	Cholesteryl esters	Cholesterol (free)
VLDL (<1.006 g/ml)	226	8.0	36.0	20.0	31.0	5.0
LDL (1.006-1.063 g/ml)	664	17.0	2.0	11.0	60.0	10.0
HDL (1.063-1.21 g/ml)	507	43.0	0.4	46.8	5.6	4.2

The results are the mean of two determinations from two pooled batches of plasma samples.

[a]The amount of lipoprotein was measured by the analysis of lipid and protein contents in each class.

TABLE 3

Fatty Acid Composition of Triacylglycerols

Fatty acids	Wt %		
	Muscle (9)	Liver (6)	Plasma (4)
Saturated	(37.6)	(39.0)	(28.0)
14:0	2.1 ± 0.3[a]	7.9 ± 2.5	3.3 ± 1.0
16:0	13.2 ± 1.1	18.2 ± 3.0	14.7 ± 1.4
18:0	20.7 ± 0.8	11.9 ± 2.5	8.4 ± 0.9
20:0	1.6 ± 0.4	1.0 ± 0.4	1.6 ± 0.6
Monoenes	(22.1)	(23.0)	(39.9)
16:1	1.0 ± 0.1	0.9 ± 0.4	11.6 ± 2.2
18:1	21.1 ± 2.2	22.1 ± 1.5	28.3 ± 3.1
20:1[b]	–	–	–
n-6 Polyenes	(5.5)	(14.8)	(9.1)
18:2	2.6 ± 0.3	8.4 ± 0.8	1.7 ± 0.7
20:4	0.9 ± 0.2	4.3 ± 0.6	5.5 ± 1.3
22:4	2.0 ± 0.3	2.1 ± 0.5	1.9 ± 0.1
n-3 Polyenes	(34.8)	(29.0)	(26.0)
18:3[b]	17.6 ± 0.4	15.5 ± 0.5	2.8 ± 0.6
20:5	2.5 ± 0.4	4.7 ± 1.4	7.8 ± 2.0
22:5	2.7 ± 0.5	1.7 ± 0.6	5.4 ± 1.1
22:6	11.8 ± 2.4	7.1 ± 2.4	10.0 ± 2.0
Unidentified	1.2 ± 0.5	4.2 ± 0.6	0

The number of determinations is in parentheses.

n-3 and n-6 refer to the first double bond in position 3 and position 6 with respect to the CH_3 group, respectively.

[a]Mean ± SEM.

[b]In the analyses, no distinction was made between 20:1 and 18:3(n-3) and the peak was assigned to 18:3 only.

TABLE 4

Fatty Acid Composition of Phospholipids

Fatty acids	Wt %		
	Muscle (9)	Liver (7)	Plasma (4)
Saturated	(38.5)	(21.4)	(14.3)
14:0	0.9 ± 0.2[a]	2.1 ± 1.0	1.1 ± 0.6
16:0	23.2 ± 1.3	9.2 ± 1.7	10.4 ± 0.9
18:0	11.3 ± 0.6	7.9 ± 1.3	2.8 ± 0.5
20:1	3.1 ± 0.3	2.2 ± 0.7	0
Monoenes	(26.9)	(19.5)	(39.5)
16:1	5.5 ± 0.5	4.2 ± 0.8	3.1 ± 0.3
18:1	21.4 ± 1.0	15.3 ± 0.9	36.4 ± 3.6
20:1[b]	–	–	–
n-6 Polyenes	(12.3)	(28.0)	(24.2)
18:2	1.8 ± 0.5	2.2 ± 0.6	2.4 ± 0.6
20:4	5.5 ± 0.9	22.7 ± 1.7	19.8 ± 2.7
22:4	5.0 ± 0.6	3.1 ± 0.2	2.0 ± 0.5
n-3 Polyenes	(20.3)	(28.8)	(21.0)
18:3[b]	2.0 ± 0.3	1.7 ± 0.2	2.6 ± 0.4
20:5	4.8 ± 0.6	10.8 ± 0.5	5.8 ± 2.0
22:5	1.5 ± 0.1	2.7 ± 0.2	4.8 ± 1.0
22:6	12.0 ± 1.4	13.6 ± 0.6	7.8 ± 0.7
Unidentified	2.0 ± 0.6	2.3 ± 0.5	1.0 ± 0.4

The number of determinations isin parentheses.

n-3 and n-6 refer to the first double bond in position 3 and position 6 with respect to the CH_3 group, respectively.

[a]Mean ± SEM.

[b]In the analyses, no distrinction was made between 20:1 and 18:3(n-3) and the peak was assigned to 18:3 only.

DISCUSSION

This study examined the plasma lipoproteins and tissue lipids in the sea lamprey *M. mordax* during its upstream spawning migration. This migration occurs in the southern hemisphere spring from early September to the end of October. It is only at this time of the year that adult lampreys may be found and caught in the Yarra River in Victoria (8 km from the sea). Attempts to obtain nonspawning lampreys from the offshore fishing grounds have been unsuccessful.

The average body wt of the lampreys in these studies was about 40 g. This is much less than that for some other sea lampreys, such as *P. marinus* (3,4). It is known that migrating lampreys lack food and water intake and that atrophy of the intestine occurs (1,16). In the specimens studied, there were no observable

TABLE 5

Fatty Acid Composition of Cholesteryl Esters

Fatty acids	Wt %		
	Muscle (3)	Liver (4)	Plasma (4)
Saturated	(13.1)	(10.6)	(16.8)
14:0	3.2 ± 0.5[a]	0.3 ± 0.1	0.3 ± 0.1
16:0	8.2 ± 1.2	3.1 ± 0.7	9.4 ± 1.3
18:0	1.0 ± 0.2	3.2 ± 0.5	3.9 ± 0.4
20:0	0.7 ± 0.1	4.0 ± 0.7	3.2 ± 0.5
Monoenes	(25.6)	(58.5)	(42.0)
16:1	7.2 ± 1.0	5.4 ± 1.6	4.0 ± 0.3
18:1	18.4 ± 2.1	53.1 ± 10.1	38.0 ± 4.6
20:1[b]	—	—	—
n-6 Polyenes	(23.2)	(5.7)	(7.3)
18:2	12.0 ± 1.5	2.6 ± 0.5	2.0 ± 0.3
20:4	7.2 ± 1.0	2.0 ± 0.3	3.6 ± 0.4
22:4	4.0 ± 0.5	1.1 ± 0.2	1.7 ± 0.3
n-3 Polyenes	(35.5)	(21.9)	(30.9)
18:3[b]	3.0 ± 0.5	7.2 ± 2.3	8.0 ± 2.3
20:5	12.4 ± 2.1	7.0 ± 0.7	14.0 ± 1.9
22:5	12.0 ± 1.4	1.1 ± 0.2	3.2 ± 0.1
22:6	8.1 ± 0.5	6.6 ± 1.2	5.7 ± 0.7
Unidentified	2.3 ± 0.8	3.4 ± 1.0	3.0 ± 0.6

The number of determinations is in parentheses. n-3 and n-6 refer to the first double bond in position 3 and position 6 with respect to the CH_3 group, respectively.

[a]Mean ± SEM.

[b]In the analysis, no distinctionw as made between 20:1 and 18:3(n-3) and the peak was assigned to 18:3 only.

traces of food or fluid in the intestines. The intestines themselves were small with thin, flattened walls which had a defalted appearance.

The relative size of the liver (0.5% of the body weight) was also considerably smaller than that of other lampreys (3,4). The liver color in nonmigrating lampreys is usually orange (16). In the animals studied, it varied from brown to deep green. This color change is caused by increased amounts of biliverdin in the liver. It is associated with a progressive degeneration of the liver tissues and a decrease in the ability to convert biliverdin to bilirubin (16). Judged by the size and amount of eggs developed in the females, the lampreys were at various stages of sexual development.

There are two findings of particular interest in relation to muscle lipids. The first is that the lamprey stores its energy source, triacylglycerols, almost entirely in the muscle. Most fish appear to use mainly the liver and/or skeletal muscle for triacylglycerol storage (17). This is in contrast with mammals which have well developed adipose tissue. It is generally considered that, in the more active fish, skeletal muscle acts as the storage function (18,19). Eels (20) and salmon (17) have large amounts of triacylglycerols stored in this manner. The second finding of interest in relation to muscle lipid is that the lamprey has a very high cholesterol content in the muscle. Our results show a level of 380 mg (free and esterified) in 100 g muscle (wet wt). Crustaceans such as shrimps and lobsters have also been shown to have high cholesterol levels (200 mg and 170 mg/100 g muscle, respectively) (21). It is known, however, that these animals are incapable of the biosynthesis of cholesterol (22-24) and store it from dietary intake. The reason for lampreys storing such an unusually high level of cholesterol in muscle is unknown. It may be related to the spawning process. During the spawning migration, liver and intestine are known to degenerate (1,16) and to lose their normal functions. At the same time, the lampreys cease feeding. It is therefore possible that the lamprey draws upon the reserve source of cholesterol in the muscle for the development of gonads and eggs.

There were two findings of particular interest in relation to plasma lipids. The first is that cholesterol and its esters accounted for 57% of the total plasma lipids in the migrating lampreys. Three-quarters of the total cholesterol was present in an esterified form. The high level of cholesterol in the plasma may be related to physiological changes during spawning. The salmonidae have been found to have an elevated level of plasma cholesterol during the upstream spawning migration (25,26). It has been observed, in general, that many teleost fish have much higher cholesterol levels than that of mammalian plasma (19,27). The high level of cholesterol in the cyclostome, *Myxine glutinosa*, is correlated with its utilization of cholesterol for the synthesis of bile salt, myxinol disulfate (28). The lamprey is also a cyclostome and may use the same pathway to produce its bile salt.

The second finding of interest in relation to plasma lipids in the lampreys is the very low level of triacylglycerols. This contrasts with findings for teleost fishes and elasmobranches (F. Fellows and R. McLean, unpublished results). The triacylglycerol levels in the plasma of teleosts are three to 17 times greater than that found in lampreys. In the elasmobranch, they are two to 14 times greater.

In evolution, the lamprey is regarded as primitive. Nevertheless, its use of lipoproteins as binding agents to transport plasma lipids is similar to higher animals. The major classes of its lipoproteins resemble those found in higher vertebrates. Their protein content is, in general, very similar. The lipid composition of lamprey lipoproteins has a lower percentage of triacylglycerols and a higher percentage of cholesterol

than those of man and other animals (29). A distinct feature is that the lamprey possesses a large amount of plasma LDL containing a very high concentration of cholesterol–70% of LDL weight. This is considerably higher than LDL in other animals (29) and contrasts with that of hagfish (14).

In higher animals, one of the important roles of LDL is carrying cholesterol to the body cells (30). The nonhepatic cells in the body obtain the cholesterol necessary for structural maintenance through LDL. It is known (31-33) that the high-affinity specific receptors on the cell surface-bind plasma LDL. In this way, the cells take-up the lipoprotein by endocytosis, use its cholesterol and suppress their own endogenous cholesterol synthesis. The cells, by regulating the number of LDL receptors on their surface, are able to control the entering amount of cholesterol (34). Thus, they are protected from excessive cholesterol accumulation which may lead to pathological conditions, such as atherosclerosis. It is unknown how the lamprey metabolizes LDL or by what mechanism the LDL delivers its cholesterol to the cells.

Great variations in the levels of nonesterified fatty acids have been observed in the plasma of fish (5,19). Most teleost species have levels exceeding 400 μM (19). In the lampreys studied, these levels were relatively low (75 μM). The circulating level of nonesterified fatty acids in fish is believed to be determined by the site of fat storage (muscle, liver, other depots) (19) and is possibly also related to the muscular activity of the species. It is possible that the migrating lampreys mobilize nonesterified fatty acids by direct lipolysis in the muscle tissue without being released into the bloodstream.

The fatty acid composition of the lipids in the lamprey is consistent with that commonly present in marine fishes (35). They are straight-chained and even-numbered acids varying in chain length from C_{14} to C_{22}. The saturated acids are mainly palmitic and stearic. The monoene acids are predominantly C_{18}. The polyunsaturated acids of the C_{18}, C_{20} and C_{22} series are important constituents of lamprey lipids. They are based on the patterns of linoleic (n-6) and linolenic (n-3) acids. They have up to five double bonds in the C_{20} series and up to six double bonds in the C_{22} series. The origin of these highly unsaturated acids in lamprey lipids is probably associated with their dietary sources. The migratory lampreys are known to be parasitic (1) and their hosts are mainly the teleost fish (4). The physiological importance of having large proportion of polyunsaturated acids in the lipids of lamprey and other fish is because the melting points of these acids are low. This facilitates the cellular exchange of lipids at the low temperature of their habitats.

ACKNOWLEDGMENTS

We thank Prof. F.J.R. Hird for useful suggestions and support, and L. Aprinis for the supply of lampreys in this study. The financial support and a Fellowship (F.C.I. Fellows) from the Australian Research Grants Committee are gratefully acknowledged.

REFERENCES

1. Larsen, L.O. (1980) Can. J. Fish. Aquat. Sci. 37, 1762-1779.
2. Bentley, P.J., and Follett, B.K. (1965) Life Sci. 4, 2003-2007.
3. Kott, E. (1971) Can. J. Zool. 49, 801-805.
4. Beamish, F.W.H., Potter, I.C., and Thomas, E. (1979) J. Anim. Ecol. 48, 1-19.
5. Fellows, F.C.I., Hird, F.J.R., McLean, R.M., and Walker, T.I. (1980) Comp. Biochem. Physiol. 67B, 593-597.
6. Fellows, F.C.I., and Hird, F.J.R. (1981) Comp. Biochem. Physiol. 68B, 83-87.
7. Malins, D.G., Wekell, J.C., and Houle, C.R. (1965) J. Lipid Res. 6, 100-105.
8. Lindgren, F.T. (1975) in Analysis of Lipids and Lipoproteins (Perkins, E.G., ed.) pp. 204-224, Am. Oil. Chem. Soc., Champaign, IL.
9. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
10. Nevenzel, J.C., Rodegker, W., and Mead, J.F. (1965) Biochemistry 4, 1589-1594.
11. Nicolaides, N., Fu, H.C., and Ansari, M.N.A. (1970) Lipids 5, 299-307.
12. Farquhar, J.W. (1962) J. Lipid Res. 3, 21-30.
13. Webster, D. (1962) Clin. Chim. Acta 7, 227-284.
14. Mills, G.L., Taylaur, C.E., Chapman, M.J., and Forster, G.R. (1977) Biochem. J. 163, 455-465.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Kott, E. (1970) Can. J. Zool. 48, 745-750.
17. Vague, J., and Fenasse, R. (1965) in Handbook of Physiology, Section 5, Adipose Tissue (Renold, A.E., and Cahill, G.F., eds.) pp. 25-36, American Physiological Society, Washington, DC.
18. Tashima, L., and Cahill, G.F. (1965) in Handbook of Physiology, Section 5, Adipose Tissue (Renold, A.E., and Cahill, G.F., eds.) pp. 55-58, American Physiological Society, Washington, DC.
19. Larsson, A., and Fägue, R. (1977) Comp. Biochem. Physiol. 57B, 191-196.
20. Lewander, K., Dave, G., Johansson, M-L., Larsson, A., and Lidman, U. (1974) Comp. Biochem. Physiol. 47B, 571-581.
21. DiTullio, N.W., and Holmes, W.L. (1967) J. Food Sci. 32, 64-66.
22. Van den Oord, A.H.A. (1964) Comp. Biochem. Physiol. 13, 461-467.
23. Zandee, D.I. (1964) Nature Lond 202, 1335-1336.
24. Zandee, D.I. (1967) Comp. Biochem. Physiol. 20, 811-822.
25. Idler, D.R., and Tsuyuki, H. (1958) Can. J. Biochem. Physiol. 36, 783-791.
26. Robertson, O.H., Krupp, M.A., Favour, C.B., Hane, S., and Thomas, S.F. (1961) Endocrinology 68, 733-745.
27. Morris, B. (1959) J. Cell. Comp. Physiol. 54, 221-230.
28. Haslewood, G.A.D. (1966) Biochem. J. 100, 233-237.

29. Chapman, M.J. (1980) J. Lipid Res. 21, 789-853.
30. Brown, M.S., and Goldstein, J.L. (1976) Science 191, 150-154.
31. Goldstein, J.L., and Brown, M.S. (1974) J. Biol. Chem. 249, 5153-5162.
32. Brown, M.S., and Goldstein, J.L. (1974) Proc. Natl. Acad. Sci. USA 71, 788-792.
33. Anderson, R.G.W., Goldstein, J.L., and Brown, M.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2434-2438.
34. Goldstein, J.L., and Brown, M.S. (1977) Ann. Rev. Biochem. 46, 897-930.
35. Gunstone, F.D., Wijesundera, R.C., and Scrimgeour, C.M. (1978) J. Sci. Food Agric. 29, 539-550.

[Received January 26, 1982]

Differential Uptake of Cholesterol and Plant Sterols by Rat Erythrocytes in vitro[1]

P. CHILD[a] and A. KUKSIS*[,b], [a]Department of Biochemistry and [b]Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada M5G 1L6

ABSTRACT

The in vitro uptake of radioactively labeled cholesterol and the plant sterol β-sitosterol has been examined in rat erythrocytes. From mixed micellar solutions containing egg yolk phospholipid and sodium taurocholate, the erythrocytes showed a nonlinear uptake of the two sterols. The uptake leveled off after about 45 min with the attainment of a 1:1 total sterol-to-phospholipid ratio within the cell membrane, as determined on a mass basis. From soltuions containing egg yolk phospholipid, or purified egg yolk phosphatidylcholine, a preference for cholesterol over the plant sterol was observed, increasing with time from a cholesterol/β-sitosterol uptake ratio of unity (the media ratio) to a maximum of 2 after a 60-min incubation. Correction of the data for nonspecifically bound sterol increased the ratio to a maximum of 5 at the 30-min time point. The increase in the cholesterol/β-sitosterol uptake ratio with time, following an initial nonspecific association, showed that penetration of the plasma membrane by the sterol was required for the selectivity to be expressed. The presence of lysophosphatidylcholine or bovine serum albumin did not exert any noticeable influence over the extent or selectivity of absorption. Replacement of the egg yolk phospholipid with synthetic dipalmitoyl-phosphatidylcholine led to a loss of the sterol selectivity. No evidence was found to support a selective extraction of sterol from the erythrocyte membrane to account for the observed effects, nor was there any sign of a mass accumulation of phospholipid during the incubation. It is suggested that the media phospholipid influences the membrane permeability toward cholesterol and β-sitosterol.

Lipids 17:748-754, 1982.

INTRODUCTION

Studies on the uptake and lymphatic transport of sterols by the intestinal mucosa in vivo have demonstrated that cholesterol is absorbed about 10 times more readily (1-4) and campesterol about four times more readily (5) than β-sitosterol. Furthermore, the plant dily (5) than β-sitosterol. Furthermore, the plant sterols have been shown to interfere with the uptake of lumenal cholesterol (6-8), which has generated much interest in the dietary control of cholesterol absorption by β-sitosterol (9). Attempts to establish the mechanism of plant sterol inhibition of cholesterol absorption, however, have been unsuccessful (2,3). These studies have been complicated by the failure of several well characterized *in vitro* preparations of intestinal tissue, including rings (10) and everted sacs (11), to mirror the in vivo findings. Thus, it has been suggested (10,12) that the intestinal absorption of sterols cannot be investigated by conventional methods in vitro. This suggestion appeared to be borne out by a similar lack of selective sterol absorption in isolated intestinal enterocytes (13), which can be prepared in a highly active metabolic state and without the mucous glycocalyx that may nonselectively retain sterol substrates.

Common to all of the above in vitro preparations, however, is a susceptibility to anoxia and mechanical damage during the isolation procedure (14), and the presence of endogenous proteolytic and lipolytic enzymes (15). These factors would all be expected to influence their behavior. To assess their influence, it was desirable to examine micellar sterol uptake in an independent in vitro system which does not suffer these drawbacks. In this study, we have examined the in vitro uptake of cholesterol and plant sterols by rat erythrocytes and have found that the relative rates of the sterol uptake depend on the amount and nature of the phosphatidylcholine present in the sodium taurocholate micelles containing the sterols.

MATERIALS AND METHODS

Sodium taurocholate, fatty acid-free bovine serum albumin, cholesterol, β-sitosterol, [4-^{14}C] cholesterol and [22,23-^{3}H]β-sitosterol were as previously described (13). *sn*-1,2-Dipalmitoyl-phosphatidylcholine (98%) and egg yolk sphingomyelin were obtained from Sigma Chemical

[1]Presented in part at the 72nd annual meeting of the American Oil Chemists' Society, New Orleans, LA, May 1981.

*Author to whom correspondence should be addressed.

Co. (St. Louis, MO). Egg phospholipid was purchased from British Drug Houses (Toronto, Canada) and contained 75% phosphatidylcholine, 13% phosphatidylethanolamine, 12% lysophosphatidylcholine and traces of sphingomyelin and free fatty acid, as estimated by thin-layer chromatography (TLC). An egg phosphatidylcholine preparation obtained from Nutritional Biochemicals (Cleveland, OH) contained 61% phosphatidylcholine and 21% lysophosphatidylcholine. Purified egg phosphatidylcholine was obtained from this preparation by TLC.

Erythrocyte Preparation

Fresh blood from 250-300- g male Wistar rats was treated with 4 mM EDTA. The cells were pelleted at 1,200 x g for 10 min and the supernatant and buffy coat were removed by aspiration. The cells were then washed several times with 5 vol of Hank's balanced salt solution containing 15 mM 2-(N-2-hydroxyethylpiperazin-N′-yl) ethanesulfonic acid (HEPES) buffer (phenol red omitted to allow detection of hemolysis) and stored at room temperature in the same buffer (30% hematocrit) until use (maximum 2 hr). In some experiments, the cells were suspended in the above buffer containing 4% fatty acid-free bovine serum albumin.

Preparation of Micellar Solutions

Micellar solutions were prepared by mixing the lipid components in amounts sufficient to give 6.6 mM sodium taurocholate, 0.5-1.5 mM phospholipid and 100-600 μM sterol (plus the appropriate tracer sterol) in the desired volume. In some cases, oleic acid (1.2 mM) and *sn*-2-monooleoylglycerol (0.6 mM) were included. Following evaporation of the solvent under nitrogen, the required volume of Hank's balanced salt solution (containing 15 mM HEPES) (pH 7.4) was added and the resulting turbid suspension was incubated at 37 C with shaking (65 cycles/min) in a Dubnoff shaker bath for 1 hr. After cooling, the suspensions were filtered through a 0.2-μm Milex filter disc (Millipore Corp., Mississauga, Ontario, Canada) attached to a disposable syringe under light plunger pressure. The mixed micellar solutions containing the crude egg phospholipid were water-clear in appearance. The proportions of the various lipids in the final filtered solutions were assessed following total lipid extraction by gas liquid chromatography (GLC) of the TMS derivatives as previously described (16). The actual composition of the solutions is indicated in the legends to the figures.

Measurement of Sterol Uptake

Incubations were initiated by the addition of 1 ml of the erythrocyte suspension (30% hematocrit) to 4 ml incubation medium, prewarmed to 37 C in a 50-ml plastic beaker, and the suspension was shaken at 100 cycles/min in a Dubnoff shaker bath. Samples were withdrawn at each time point and released into 5 ml iced saline and centrifuged at 1,200 x g for 5 min, followed by a wash with 10 ml iced saline. After careful removal of the remaining solution by Pasteur pipette, the cell pellets were frozen in dry ice/methanol for storage at -20 C, if necessary. Upon thawing, the erythrocyte pellets were slowly dispersed with stirring into 50 vol of methanol as described by Nelson (17) followed by the addition of chloroform to bring the ratio to 2:1 chloroform/methanol. The organic phase was washed with 0.9% saline using the ratios of Folch et al. (18), and following removal of the lower phase, the aqueous layer was re-extracted once more with freshly equilibrated lower phase. The lipid extracts were reduced to a small volume under N_2 and aliquots were transferred to glass scintillation vials. Following evaporation of the solvent, 10 ml of Aquasol (New England Nuclear Corp., Boston, MA) was added and the samples counted in an Isocap 300 liquid scintilation counter (Searle Corp., Oakville, Ontario, Canada), using the external standard ratio to correct for quenching. The dual label deconvolution and the dpm calculations were carried out on a desk top computer.

Calculation of Sterol Uptake Ratio

A comparison of the mean ratio of [^{14}C] cholesterol to [^{3}H]β-sitosterol at each time point (three or more replicates of cell preparations) was made with that in the incubation medium on the basis of the radioactivity (dpm). The significance of the difference between the means was assessed using Student's t-test. Following this, the ratios were normalized to a media dpm ratio of 1.00, giving the normalized cellular uptake ratios reproduced in Figures 1 and 2 and discussed in the text. Uptake values corrected for nonselectively bound sterol were obtained by a subtraction of either the 1 min (37 C) or zero degree control values from those measured at 30 min (37 C). Unless otherwise indicated, however, the data presented in the tables and figures are uncorrected.

Mass Analyses

For mass analyses of the sterol and phospholipid classes, aliquots of the total lipid extracts of the cells were applied to thin layers of magnesium acetate-treated Silica Gel H and the plates were developed with chloroform/methanol/acetic acid/water (100:45:20:6.8 by vol),

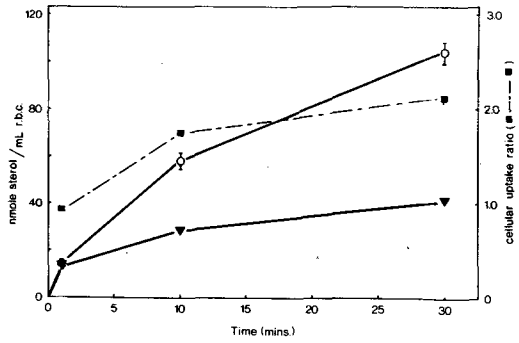


FIG. 1. Uptake of radiolabeled cholesterol and β-sitosterol by rat erythrocytes from mixed micellar solutions. Rat erythrocytes were incubated in media containing 6.6 mM Na taurocholate, 1.5 mM egg phospholipid, 280 μM cholesterol plus [4-^{14}C] cholesterol tracer and 240 μM β-sitosterol plus [22,23-^{3}H]β-sitosterol tracer. Open circles refer to cholesterol uptake, closed triangles to β-sitosterol uptake, and open squares to the cellular uptake ratio of cholesterol to β-sitosterol.

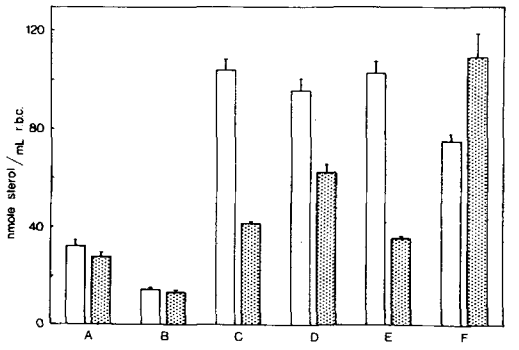


FIG. 2. Effects of variation of the incubation conditions on sterol uptake by rat erythrocytes. The cells were incubated as follows: (A) media as in Fig. 1, incubation for 30 min at 0 C. (B) As in A, incubation for 1 min at 37 C. (C) As in A, incubation for 30 min at 37 C. (D) Media containing 6.6 mM Na taurocholate, 600 μM egg phosphatidylcholine, 200 μM egg lysophosphatidylcholine, 210 μM cholesterol plus [4-^{14}C] cholesterol and 230 μM β-sitosterol plus [22,23-^{3}H]β-sitosterol, incubation for 30 min at 37 C. (E) As in A, with the inclusion of 0.8% (w/v) BSA, incubation for 30 min at 37 C. (F) Media containing 6.6 mM Na taurocholate, 1 mM dipalmitoyl phosphatidylcholine, 130 μM cholesterol plus [4-^{14}C] cholesterol and 134 μM sitosterol plus [22,23-^{3}H]-β-sitosterol, incubation for 30 min at 37 C. Clear bars refer to the uptake of cholesterol and the hatched bars to β-sitosterol. Values are presented as the means ± SD for three determinations. The cellular uptake ratios of cholesterol to β-sitosterol, normalized to that in the media, were as follows: A, 0.99 ± 0.03; B, 0.94 ± 0.03; C, 2.14 ± 0.07; D, 1.68 ± 0.03; E, 2.47 ± 0.07 and F, 0.74 ± 0.02.

as previously described (19). The sterols were scraped from the solvent front of the plate, eluted with diethyl ether/pentane (1:1), and analyzed as the TMS ethers by temperature-programmed GLC using 3% OV-1 columns (16). The remainder of the thin-layer plate was exposed to iodine vapor and the various phospholipid classes were located and scraped directly into test tubes. The scrapings were digested with H_2SO_4-$HClO_4$ and the phosphorus was determined as described by Nelson (17).

Measurement of Hemolysis

Any hemolysis of the red blood cells (RBC) was assessed spectrophotometrically according to the method of Roelofsen et al. (20).

RESULTS

Prevention of Hemolysis

The incubation of rat erythrocytes in micellar media containing sterols, oleic acid, monoacylglycerol and sodium taurocholate, as used previously with isolated intestinal villus cells (13), led to an instant hemolysis. Under these conditions, it was not possible to isolate pelletable material by centrifugation at 30,000 x g-min. A replacement of the oleic acid and monoacylglycerol with a similar amount (1.0-1.5 mM) of crude egg yolk phospholipid prevented hemolysis. Table 1 shows that comparable proportions of egg phosphatidylcholine containing 20 mol % lysophosphatidylcholine, as well as synthetic *sn*-1,2-dipalmitoyl-phosphatidylcholine, had a similar protective effect, although a complete range of concentrations was not examined. Table 1 also demonstrates that low levels (200 μM) of free fatty acid did not influence the extent of cell lysis, but the addition of larger amounts of oleic acid completely lysed the cells. Under appropriate conditions, therefore, the incubations could be carried out without detectable hemolysis for up to 60 min, allowing the sterol uptake to be assessed.

Uptake of Sterols by Erythrocytes

Figure 1 shows the cellular accumulation of radioactive cholesterol and β-sitosterol with time. The uptake is nonlinear and reaches a plateau after about 45 min. At points corresponding to the plateau region of the uptake curve, the total sterol contents of the cells had increased by about 10%, raising the cellular total sterol/total phospholipid ratio (mol/mol) from 0.92 before incubation to 1.03 after incubation, as determined on a mass basis (Table 2). From Figure 1, it is seen that the radioactive cholesterol was taken up about two times more readily than β-sitosterol. Thus, the cellular ratio

TABLE 1

Effect of Micellar Lipid Composition on the Hemolysis[a] of Rat Erythrocytes

Media lipid composition	Hemolysis 30 min	Hemolysis 60 min
6.6 mM sodium taurocholate 300-500 μM sterol +500 μM oleic acid and 400 μM monooleoylglycerol	++	
+1.5 mM egg phospholipid (contains 200 μM free fatty acid)	–	–
+1.5 mM egg phospholipid and 0.8% bovine serum albumin	–	–
+1.5 mM egg phospholipid and 1.0 mM oleic acid	+	+
+1.0 mM egg phosphatidylcholine	–	–
+0.6 mM egg phosphatidylcholine and 0.2 mM egg lysophosphatidylcholine	–	+
+1.0 mM dipalmitoyl-phosphatidylcholine	–	–
+0.8 mM dipalmitoyl-phosphatidylcholine and 0.2 mM egg lysophosphatidylcholine	–	ND
+0.5 mM dipalmitoyl-phosphatidylcholine and 0.5 mM egg phosphatidylcholine	–	ND

[a]Cells were incubated at 37 C as described in Methods; ++, cells dissolved; +, cells lysed; –, no hemolysis; and ND, not determined.

TABLE 2

Erythrocyte Sterol to Phospholipid Ratio before and after Incubation in Mixed Micellar Solution

Sample	Cholesterol / total PL	Campesterol / total PL	Sitosterol / total PL	Total sterol / total PL
	(nmol/nmol)			
Unincubated erythrocytes	0.88 ± .05	0.024 ± .008	0.017 ± .002	0.92 ± .02
Incubated[a] erythrocytes	0.95 ± .03	0.030 ± .006	0.051 ± .013	1.03 ± .06
Sterol uptake	0.07 (130)	0.006 (11)	0.034 (63)	0.11 (204)

[a]Cells were incubated for 60 min in HBSS + HEPES buffer containing 6.6 mM sodium taurocholate, 1 mM egg phospholipid, 280 μM cholesterol and 250 μM β-sitosterol. Values are presented as means ± SD for three replicate determinations. Values in parentheses are the uptake values expressed as nmol sterol/ml packed erythrocytes.

of cholesterol/β-sitosterol (normalized to that in the medium) increased with time from the media ratio (1.0) to a maximum of 2.1 at 30 min. The overall mean uptake ratio obtained over several experiments (n=12) was 1.83 ± 0.21. This uptake ratio was also found to be reflected in the mass analysis (Table 2).

The similarity of the cellular uptake ratio to that in the medium during the first minute of incubation, which corresponded to the most rapid rate of accumulation, suggested the presence of adherent fluid, perhaps leading to an overestimation of the actual sterol uptake. Several subsequent saline washes of the cell pellet, however, did not reduce the level of uptake significantly or change the ratio of the radioactivies of the sterols. Figure 2 shows that the incubation of the cells for 30 min on ice (panel A) led to a comparable nonspecific accumulation of labeled sterol. This was two times higher than that observed after 1 min of incubation at 37 C (panel B). Following incubation under any of

the above conditions, the phospholipid profile of the erythrocytes did not differ significantly from that in unincubated cells (Table 3). Thus, there was no evidence for a cellular accumulation of media phospholipid.

Role of Micellar Components

Figure 2 also shows that the replacement of the egg phospholipid with an egg phosphatidylcholine preparation containing lysophosphatidylcholine (panel D) did not lead to an overall alteration in the cellular sterol uptake. Although the sitosterol uptake is somewhat larger than that in the control sample (panel C), the normalized cellular uptake ratio of 1.68 is well within the range of experimental variation obtained in the presence of egg phospholipid. Similar results were found with purified egg phosphatidylcholine. Thus, the presence of lysophosphatidylcholine does not appear to influence the overall uptake characteristics significantly. Similarly, the inclusion of 0.8% bovine serum albumin (panel E) in the incubation medium did not perturb the overall uptake or the ratio of the two sterols. This finding, and the known ability of albumin to bind free fatty acids, demonstrate that the small amounts of free fatty acid contained in the egg phospholipid preparation do not contribute significantly to the effects noted here.

Replacement of the egg yolk phospholipid with synthetic *sn*-1,2-dipalmitoyl-phosphatidylcholine, however, led to a dramatic loss of the sterol uptake specificity (panel F). The overall shape of the uptake curve and total sterol uptake (about 200 nmol/ml RBC in 60 min) was similar to that obtained with egg phospholipid, but the cellular ratio of radioactivities decreased to a minimum of 0.74 in the presence of dipalmitoyl-phosphatidylcholine. A comparable loss of sterol uptake specificity was experienced with egg yolk sphingomyelin (results not shown).

The inclusion of 20 mol % lysophosphatidylcholine or 50 mol % purified egg phosphatidylcholine into dipalmitoylphosphatidylcholine preparations did not restore a preferential cellular accumulation of cholesterol, although the cellular ratio of cholesterol to sitosterol radioactivity in these instances did not fall below that in the medium.

DISCUSSION

This study confirms the previously reported protective effect of phosphatidylcholine upon the hemolysis of RBC by bile salt micelles. It had been shown by Coleman et al. (21) that the addition of phosphatidylcholine to incubation solutions containing bile salt prevented the hemolysis of erythrocytes when the ratio of phospholipid to bile salt was greater than 0.125. The 1.0-1.5 mM egg phospholipid used in the present experiments exceeds this ratio and resembles that found in many mammalian biles (21). In the presence of egg phospholipid, the lipolytic products normally found in the intestinal micellar millieu, such as free fatty acids and lysophosphatidylcholine, did not lead to an increased lysis of the cells at concentrations less than 200 μM and did not impair the selective accumulation of radioactive cholesterol (over β-sitosterol) by the cells.

The general agreement between the radioactive tracer data presented in Figures 1 and 2 and that obtained on a mass basis shown in

TABLE 3

Phospholipid Composition (Mole Percentage) of Incubated and Unincubated Rat Erythrocytes

Phospholipid	Control cells[a] (unincubated)	Incubated cells: Egg phospholipid[b]	Incubated cells: Dipalmitoyl-phosphatidylcholine[c]
Cardiolipin/phosphatidic acid	2.87 ± 0.28	4.28 ± 0.67	3.89 ± 1.94
Phosphatidylethanolamine	8.49 ± 0.54	8.03 ± 2.35	7.24 ± 0.58
Phosphatidylserine/ phosphatidylinositol	8.88 ± 0.22	10.61 ± 1.74	8.20 ± 2.82
Phosphatidylcholine	49.40 ± 0.91	47.15 ± 2.53	54.84 ± 1.33
Sphingomyelin	20.35 ± 0.33	17.11 ± 0.73	18.12 ± 0.79
Lysophosphatidylcholine	10.01 ± 0.19	12.82 ± 2.07	7.69 ± 0.42

[a]Cells were obtained as described in Methods.

[b]Cells were incubated as in Fig. 2D, for 60 min.

[c]Cells were incubated as in Fig. 2F, for 60 min.

Table 2, suggest that the sterols are taken up by the cells, rather than simply undergoing exchange with endogenous sterol. Thus, the net increase in the cellular cholesterol content excludes a simple exchange of labeled cholesterol as an explanation for the enhanced accumulation of radioactive cholesterol in the presence of egg phospholipid. The plateau of the sterol uptake following only a 10% increase in the total cellular sterol supports this supposition. The accumulation of labeled cholesterol would be expected to continue if exchange were the dominant mechanism.

The attainment of a sterol/phospholipid molar ratio of 1.03 within the cell at a point corresponding to the leveling off in total sterol uptake, supports the idea of a physicochemically defined endpoint for cellular sterol absorption, as was previously noted for erythrocytes (22) and isolated villus cells (13). Fifty mol % sterol is believed to represent the maximal amount that can be accommodated by a phospholipid bilayer (23) without bringing about a more extensive sterol-sterol interaction than dimer formation (24). It should be pointed out, however, that sterol/phospholipid ratios greater than unity have been reported for human RBC following incubation with sterol-rich phospholipid liposomes (25). Under these conditions, sterol uptake had also occurred.

The measured sterol accumulation is expected to contain a component derived from adherent media, which would also obscure the observed selectivity. The absence of phospholipid accumulation in the erythrocytes during the incubations indicates that the contribution of adherent fluid to the measured sterol uptake is small in this case. The presence of some adsorbed nonmicellar sterol is, however, implied by the initial, nonspecific accumulation of radioactivity at 37 C (Fig. 1 and 2). If this is considered to be indicative of sterol adsorbed to the outer membrane surface, the overestimation in the total sterol uptake would be about 15% for cholesterol and about 30% for β-sitosterol. This correction enhances the cholesterol/β-sitosterol uptake ratio significantly. If the ice blank value (Fig. 2, panel A) were used for a similar correction of the data obtained at 37 C (Fig. 2, panel C), the cellular uptake of cholesterol at 30 min would be five-fold greater than that of β-sitosterol and would presumably reflect only that sterol that was partitioned into the cell membrane. This implies that a penetration of the cell membrane by the sterol is required for the selectivity to be expressed. The adherence of some sterol to the cell surface is likely a prerequisite for absorption and would be enhanced in cases where diffusion to the membrane surface was not rate-limiting.

The ability of the plasma membrane of the RBC to absorb lower molecular weight sterols preferentially over those of higher molecular mass was previously indicated by the experiments of Edwards and Green (26), who showed that the plant sterol campesterol (C_{28}) exchanges for cholesterol (C_{27}) more readily than β-sitosterol (C_{29}) when the plant sterols, in the form of egg phosphatidylcholine liposomes, are incubated with the cells. Rat erythrocytes were found to accumulate 2.5 times more campesterol than β-sitosterol during a 16-hr period (26). This difference is somewhat greater than that seen for the small amounts of campesterol contaminating the β-sitosterol preparation tested in this study. However, the micellar properties of the sterols in the presence of sodium taurocholate and differences between the uptake and exchange processes may have contributed to the difference in the magnitude of the effect.

The ability of the plasma membrane to selectively absorb cholesterol over β-sitosterol was found to be greatly influenced by the nature of the diacyl phospholipid incorporated into the micellar incubation medium. Of particular interest is the loss of the selective sterol uptake in the presence of dipalmitoyl-phosphatidylcholine and egg yolk sphingomyelin, despite the similarity of the antihemolytic effects of these compounds to that of the unsaturated phospholipid. Since the sterol uptake ratio remained constant in the presence of these saturated phospholipids, a preferential extraction of cholesterol from the cell membrane is precluded as a rationale for the enhanced accumulation of β-sitosterol with dipalmitoyl-phosphatidylcholine. Similarly, a preferential release of β-sitosterol over cholesterol from the dipalmitoyl-phosphatidylcholine micelles, followed by a nonselective uptake by the cell membrane, is difficult to rationalize with changes in the cellular uptake ratio with time in the presence of egg phospholipid. Furthermore, the results obtained with the saturated phospholipids cannot be attributed to the absence of phosphatidylethanolamine or lysophosphatidylcholine from these preparations.

The differing effects of the saturated and unsaturated phospholipid types may arise through interactions of the micellar phospholipid with the cell membrane, leading to a modification of the membrane permeability toward the two sterols. A modification of intestinal membrane permeability by mixed micellar phospholipid has been suggested by Rampone and Machida (27) to account for the ability of dipalmitoylphosphatidylcholine to decrease the in vitro uptake of cholesterol in the absence of demonstrable micelle expansion. Similarly, egg phos-

phatidylcholine and dipalmitoyl-phosphatidylcholine have been shown to interact differently with cell membranes, as reflected in the ability of egg phospholipids to induce microvilli on the surface of mouse lymphocytes and the inability of the saturated phospholipids to do so (28). In the absence of a net mass uptake of either the saturated or unsaturated phospholipid by the erythrocyte membrane in the present experiments, an increased phospholipid content cannot be considered to be a contributing factor. However, an exchange of endogenous species for exogenous phosphatidylcholines cannot be ruled out.

Regardless of the mechanism of action of the unsaturated phosphatidylcholines, the present experiments demonstrate that a preferential uptake of cholesterol over β-sitosterol can be obtained in vitro in the presence of sodium taurocholate and other lipolytic products. Therefore, it becomes of interest to determine whether the inclusion of egg phosphatidylcholine in sodium taurocholate micelles will reestablish a preferential uptake of cholesterol over β-sitosterol in villus cells in vitro. The application of the incubation conditions used here, to isolated jejunal villus cells, forms the subject of a subsequent report.

ACKNOWLEDGMENTS

These studies were supported by funds from the Ontario Heart Foundation, Toronto, Canada, and the Medical Research Council of Canada, Ottawa, Canada.

REFERENCES

1. Hernandez, H.H., Peterson, D.W., Chaikoff, I.L., and Dauben, W.G. (1953) Proc. Soc. Exp. Biol. Med. 83, 498-499.
2. Swell, L., Trout, E.C., Field, H., and Treadwell, C.R. (1959) J. Biol. Chem. 234, 2286-2289.
3. Sylven, C., and Borgstrom, B. (1969) J. Lipid Res. 10, 179-182.
4. Shidoji, Y., Watanabe, M., Oku, T., Muto, Y., and Hosoya, N. (1980) J. Nutr. Sci. Vitaminol. 26, 183-188.
5. Kuksis, A., and Huang, T.C. (1962) Can. J. Biochem. Physiol. 40, 1493-1504.
6. Swell, L., Trout, E.C., Vahouny, G.V., Field, H., von Schuching, S., and Treadwell, C.R. (1958) Proc. Soc. Exp. Biol. Med. 97, 337-339.
7. Grundy, S.M., and Mok, H.Y.I. (1977) J. Lipid. Res. 18, 263-271.
8. Sugano, M., Morioka, H., and Ikeda, I. (1977) J. Nutr. 107, 2011-2019.
9. Subbiah, M.T.R. (1973) Am. J. Clin. Nutr. 26, 219-225.
10. Feldman, E.B., and Borgstrom, B. (1966) Biochim. Biophys. Acta 125, 148-156.
11. Sylven, C. (1970) Biochim. Biophys. Acta 203, 365-375.
12. Borgstorm, B. (1974) in Biomembranes, Intestinal Absorption, (Smyth, D.H., ed.), Vol. 4B, pp. 555-620, Plenum Press, New York, NY.
13. Child, P., and Kuksis, A. (1980) Can. J. Biochem. 58, 1215-1222.
14. Gardner, M.L.G. (1978) Q. J. Exp. Physiol. 63, 93-95.
15. Nilsson, A. (1969) Biochim. Biophys. Acta 176, 339-347.
16. Kuksis, A., Myher, J.J., Marai, L., and Geher, K. (1975) J. Chromatogr. Sci. 13, 423-430.
17. Nelson, G.J. (1972) in Blood Lipids and Lipoproteins; Quantitation, Composition and Metabolism, Wiley-Interscience, New York, NY.
18. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
19. Shaikh, N.A., and Palmer, F.B.St.C. (1976) J. Neurochem. 26, 597-603.
20. Roelofsen, B., Zwaal, R.F.A., Comfurius, P., Woodward, C.B., and vanDeenen, L.L.M. (1971) Biochim. Biophys. Acta. 241, 925-929.
21. Coleman, R., Iqbal, S., Godfrey, P.P., and Billington, D. (1979) Biochem. J. 178, 201-208.
22. Bruckdorfer, K.R., Graham, J.M., and Green, C. (1968) Eur. J. Biochem. 4, 512-518.
23. Ladbrooke, B.D., Williams, R.M., and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333-340.
24. Martin, R.B., and Yeagle, P.L (1978) Lipids 13, 594-597.
25. Cooper, R.A., Leslie, M.H., Fischkoff, S., Shinitzsky, M., and Shattil, S.J. (1978) Biochemistry 17, 327-331.
26. Edwards, P.A., and Green, C. (1972) FEBS Lett. 20, 97-99.
27. Rampone, A.J., and Machida, C.M., (1981) J. Lipid Res. 22, 744-752.
28. Margolis, L.B., and Bergelson, L.D. (1979) Exp. Cell Res. 119, 145-150.

[Revision received March 29, 1982]

COMMUNICATIONS

Synthesis of Diene Prostaglandins in Freshwater Fish

GAUTAM K. BANDYOPADHYAY[a], JYOTIRMOY DUTTA[a] and SUDHAMOY GHOSH[b,*],
Departments of [a]Chemistry and [b]Biochemistry, Bose Institute, Calcutta 700 009, India

ABSTRACT

Biosynthesis of PGD_2, PGE_2 and $PGF_{2\alpha}$ in four species of freshwater fish, *Tilapia mossambica, Cyprinus carpio, Heteropneustes fossilis* and *Clarius batrachus* was studied. Both arachidonic acid and PGH_2 were used as substrates. When PGH_2 replaced arachidonic acid in the enzymic reaction, there was a 3- to 4-fold increase in PGE_2 synthesis, but no such increase in the synthesis of $PGF_{2\alpha}$ and PGD_2 was observed.
Lipids 17:755-758, 1982.

INTRODUCTION

Occurrence and synthesis of prostaglandins in some fish tissues have already been reported (1-6). Various studies suggested that prostaglandins play important roles in fish reproduction, particularly in ovulation and spawning behavior (7-11). In most of the in vitro studies in fish, monoene prostaglandins were synthesized from dihomo-γ-linolenic acid (5,6). In this communication. studies have been extended to include a few more species of fish and the synthesis of diene prostaglandins from arachidonic acid in four species of freshwater fish, *Tilapia mossambica, Cyprinus carpio, Heteropneustes fossilis* and *Clarias batrachus*, are reported. Of these, the two non-air-breathing fish (*T. mossambica* and *C. carpio*) are omnivorous, and the two air-breathing catfish (*H. fossilis* and *C. batrachus*) are carnivorous. Using ^{3}H-20:4(n-6) and ^{3}H-PGH_2 as substrates and microsomal preparations of liver, kidney, stomach and intestine as enzyme source, it was observed that, although biosynthetic activities were low with 20:4(n-6), the use of endoperoxide intermediate (PGH_2) as substrate gave increased levels of PGE_2 synthesis.

Labeled PGH_2 was prepared from ^{3}H-20:4 (n-6) using fractionated, solubilized microsomes of goat seminal vesicles as the source of cyclooxygenase activity. The goat is a readily available animal in India and its seminal vesicles are a rich source of prostaglandin synthetase as observed in our laboratory (12).

*Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals

^{3}H-Arachidonic acid (5,6,8,9,11,12,14,15-^{3}H-20:4[n-6]; 60 Ci/mmol) and Omnifluor were purchased from New England Nuclear. Hemin, epinephrine and glutathione were purchased from Sigma Chemical Co. All reference prostaglandins were obtained as gift from Upjohn Co., Kalamazoo, MI.

Preparation of Microsomes

Fish tissues were collected in the laboratory but goat seminal vesicles were procured from a local slaughterhouse. The tissues were cleaned and freed from adhering fatty materials and then suspended by adding 2 ml of 20 mM potassium phosphate buffer (pH 7.4) per g of tissue. The starting materials were 20 g of goat vesicular tissue and about 3-5 g of fish tissues which were homogenized at 0 C in an omnimixer (Sorvall) for 2 min and then centrifuged at 10,000 x g for 10 min. The supernatants were filtered through cheese cloth and then centrifuged in a Beckman ultracentrifuge at 105,000 x g for 60 min. Microsomal pellets were resuspended in 20 mM phosphate buffer, pH 7.4, bringing the protein concentration to ca. 9-10 mg/ml. Protein concentrations were determined by the method of Lowry et al. (13). Microsomal suspensions were stored at -20 C.

Fractionation of Microsomes and Synthesis of ^{3}H-PGH_2

Microsomes of vesicular tissue were solubilized by 1% (w/v) Tween-20 in 20 mM potassium phosphate buffer, pH 7.4, and fractionated by

DEAE-cellulose column chromatography following the procedure of Miyamoto et al. (14). The DEAE-cellulose column, after loading with the solubilized microsomes, was washed with 20 mM phosphate buffer, pH 7.4, containing 0.2% Tween-20. The middle fractions of the washings were pooled and designated fraction I, which contained cyclooxygenase activity.

For preparative scale synthesis of ^{3}H-PGH$_2$ from ^{3}H-20:4(n-6), the procedure of Miyamoto et al. (14) for PGH$_1$ preparation was followed. ^{3}H-20:4(n-6) was incubated with fraction I protein in the presence of hemin and epinephrine. The products were analyzed by thin layer chromatography (TLC) at -20 C in the solvent mixture diethyl ether/hexane/acetic acid (85:15:0.1). In the chromatogram, a product carrying significant radioactivity which moved with the same R_f as PGB$_2$ was identified as PGH$_2$ since (a) it was converted to PGF$_{2\alpha}$ by the treatment of $SnCl_2$, (b) its synthesis was reduced in the absence of epinephrine, and (c) it was inhibited by indomethacin (results not shown).

Assay for Prostaglandin Synthesis

For the assay of prostaglandin synthesis in fish tissues, the following procedure was adopted. Microsomes (500 μg protein) prepared from various fish tissues was added to an assay mixture (0.5 ml) consisting of Tris-HCl, pH 8.0 (10 μmol), epinephrine (0.5 μmol), hemin (0.1 nmol), reduced glutathione (5 μmol), unlabeled 20:4(n-6) (5 nmol) and ^{3}H-20:4(n-6) (sp. act 60 Ci/mmol; 0.1 μCi). The reaction mixture was incubated at 30 C for 0 and 2 min. Whenever boiled microsomes were tested as control, levels of prostaglandins found were less than O min control. Incubation was terminated by adding 0.5 ml of precooled (-20 C) solvent mixture consisting of diethyl ether/methanol/1 (N) HCl (30:4:1). The upper solvent layer was removed, concentrated, mixed with reference prostaglandins (PGF$_{2\alpha}$, PGE$_2$, PGD$_2$, PGB$_2$) and spotted on an activated Silica Gel G plate. The chromatogram was developed up to 16 cm at room temperature using the upper layer of the solvent mixture ethyl acetate/isooctane/acetic acid/water (11:5:2:10). Spots were located by iodine vapor, scraped off and counted for radioactivity using 4% Omnifluor in toluene as counting fluor. When intermediate PGH$_2$ was used as a substrate, 5 nmol of ^{3}H-PGH$_2$ (0.1 μCi) was incubated with fish microsomes at 30 C for 2 min without epinephrine and hemin. In these cases, boiled microsomes, prepared by heaing at 100 C for 10 min, were used as a control in addition to the O-min control. The thin layer chromatogram was developed at -20 C in the solvent mixture dethyl ether/hexane/acetic acid (85:15:0.1). Recovery of radioactivity in the chromatogram was 70-75%. About 2-3 nmol of arachidonic acid and 1-2 nmol of PGH$_2$ were found as unreacted substrate.

RESULTS AND DISCUSSION

When fish tissue microsomes were incubated with ^{3}H-20:4(n-6), PGD$_2$, PGE$_2$ and PGF$_{2\alpha}$ were formed (0.2-0.4 nmol/mg protein/2 min) as shown in Table 1. The production of prostaglandins from 20:4(n-6) by the fish microsomes was sensitive to indomethacin inhibition and dependent on the presence of reduced GSH (results not shown). Incubation of ^{3}H-PGH$_2$ with microsomes resulted in increased synthesis (3- to 4-fold) or PGE$_2$ (Table 2), but there was hardly any increase in the synthesis of PGF$_{2\alpha}$ and PGD$_2$. When boiled microsomes were incubated with ^{3}H-PGH$_2$, nonenzymatic synthesis of PGD$_2$, PGE$_2$and PGF$_{2\alpha}$ occurred, the level of PGF$_{2\alpha}$ being higher than PGE$_2$ and PGD$_2$ (values shown in the footnote to Table 2). However, enzymatic synthesis of PGE$_2$ from

TABLE 1

Synthesis of Prostaglandins from ^{3}H-20:4(n-6) by Fish Tissue Microsomes[a]

Name of fish	Prostaglandin synthesized (nmol/mg protein/2 min)								
	PGE$_2$			PGF$_{2\alpha}$			PGD$_2$		
	Liver	Kidney	Stomach + intestine	Liver	Kidney	Stomach + intestine	Liver	Kidney	Stomach + intestine
T. mossambica	0.22	0.25	0.33	0.20	0.30	0.22	0.26	0.30	0.30
C. carpio	0.20	0.32	0.32	0.22	0.36	0.22	0.20	0.20	0.34
H. fossilis	0.26	0.36	0.24	0.18	0.28	0.26	0.24	0.22	0.30
C. batrachus	0.30	0.28	0.40	0.20	0.26	0.32	0.25	0.20	0.25

[a]Results are the mean of three experiments.

TABLE 2

Synthesis of Prostaglandins from ^{3}H-PGH_2 by the Microsomal Preparation of Fish Tissues

Name of fish	Prostaglandin synthesized[a] (nmol/mg protein/2 min)								
	PGE_2			$PGF_{2\alpha}$			PGD_2		
	Liver	Kidney	Stomach + intestine	Liver	Kidney	Stomach + intestine	Liver	Kidney	Stomach + intestine
T. mossambica	0.28	0.88	0.68	0.14	0.68	0.36	0.16	0.26	0.36
C. carpio	0.48	0.78	0.98	–[b]	0.12	0.06	0.15	0.16	0.18
H. fossilis	0.34	0.84	0.68	–	0.04	–	0.14	0.45	0.35
C. batrachus	0.24	0.60	0.92	–	–	0.04	0.06	0.10	0.20

Results are the mean of three experiments.

[a]Incubation of ^{3}H-PGH_2 with boiled microsomes (500 μg of protein) of the tissues produced 0.24 ± 0.04 nmol of PGD_2, 0.32 ± 0.05 nmol of PGE_2 and 0.56 ± 0.08 nmol of $PGF_{2\alpha}$ per mg protein in 2 min. Experimental values over these controls are presented.

[b]Denotes values less than 0.04.

^{3}H-PGH_2 over the nonenzymatic level was 3- to 4-fold higher. The reasons for low level of synthesis of PGE_2 from arachidonic acid could be due to (a) reduced formation of intermediate PGH_2 because of competitive inhibition of cyclooxygenase (15,16) by fatty acids like 18:3(n-3), 20:5(n-3), and 22:6(n-3) whose concentrations in fish tissues are generally higher than mammals (6,17,18); (b) dilution of labeled arachidonic acid by endogenous free arachidonic acid or (c) low levels of cyclooxygenase in these fish. In spite of the possibility of competition or dilution, the first two reasons seem to be untenable here since the free acid content of the washed microsomal preparations are negligible. On the other hand, the activity of cyclooxygenase and thereby the level of intermediate PGH_2 could be limiting, which would explain why the exogeneous addition of PGH_2 resulted in the increased synthesis of PGE_2. Here, it should be noted that increased production of PGE_2 was found with microsomes of kidney and stomach plus intestinal tissues, but not of liver (Table 2). Synthesis of diene prostaglandins in fish may be considered to be more relevent in fish physiology in comparison to monoene prostaglandins as the concentration of 20:4(n-6) in fish tissues is generally found to be higher than 20:3(n-6) (substrate of monoene prostaglandins). It may also be speculated that, in fish, the activities of synthesis of triene prostaglandins (e.g., PGH_3, PGE_3, $PGF_{3\alpha}$) from 20:5(n-3) may be more prominent than the diene prostaglandins synthesized from 20:4(n-6). The basis of this speculation is that n-3 fatty acids are generally considered to be essential fatty acids for fish (19) and the levels of $PGF_{3\alpha}$ and PGE_3 are found to be significant in some fish tissues (3,20). Moreover, a new prostaglandin, $PGF_{4\alpha}$, has been discovered from the trout gill tissue which is synthesized from 22:6(n-3), a fatty acid deposited in fish tissues in significant amounts (20). Direct comparative studies using different substrates for prostaglandin synthesis may give physiologically relevent results.

REFERENCES

1. Nomura, T., Ogata, H., and Ito, M. (1973) Tohoku J. Agric. Res. 21, 138-144.
2. Goetz, F.W. (1980) Am. Zool. 20, 730.
3. Nomura, T., and Ogata, H. (1976), Biochim. Biophys. Acta 431, 127-131.
4. Ogata, H., and Nomura, T. (1975), Biochim. Biophys. Acta 388, 84-91.
5. Ogata, H., Nomura, T., and Hata, M. (1978), Bull. Jpn. Soc. Sci. Fish. 44, 1367-1370.
6. Christ, E.J., and VanDorp, D.A. (1972) Biochim. Biophys. Acta 270, 537-545.
7. Jalabert, B. (1976) J. Fish. Res. Board Can. 33, 974-988.
8. Stacey, N.E. (1981) Am. Zool. 21, 305-316.
9. Stacey, N.E., and Peter, R.E. (1979) Physiol. Behav. 22, 1191-1196.
10. Stacey, N.E., and Pandey, S. (1975) Prostaglandins 9, 597-607.
11. Stacey, N.E., and Goetz, F.W. (1982) Can. J. Fish. Aquat. Sci. 39, 92-98.
12. Ghosh, D., and Dutta, J. (1982) J. Biosci. 4, 7-18.
13. Lowry, O.H., Rosenbrough, R.A., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
14. Miyamoto, T., Ogino, N., Yamamoto, S., and Hayaishi, O. (1976) J. Biol. Chem. 251, 2629-2636.
15. Lands, W.E.M., Hemler, M.E., and Crawford, C.G. (1977) in Polyunsaturated Fatty Acids (Kunau, W.H., and Holman, R.T., eds.), pp. 193-228, Am. Oil Chem. Soc., Champaign, IL.
16. Needleman, P., Raz, A., Minkes, M.S., Ferrendelli, J.A., and Sprecher, H. (1979) Proc. Natl. Acad. Sci. USA 76, 944-948.
17. Sinclair, A.J. (1975) Lipids 9, 809-818.
18. Ackman, R.G. (1967) Comp. Biochem. Physiol.

22, 907-922.
19. Kanazawa, A., Teshima, S.I., and Ono, K. (1979) Comp. Biochem. Physiol. 63B, 295-298.
20. Mai, J., Goswami, S.K., Buckner, G., and Kinsella, J.E. (1981) Prostaglandins 21, 691-698.

[Received March 10, 1982]

Fatty Acid Chain Length Combinations in Ascitic Fluid Triglycerides Containing Lymphatic Absorbed Medium-Chain Fatty Acids

A. CHRISTOPHE[a,*], G. VERDONK[a], M. MASHALY[b] and P. SANDRA[b], *Departments of [a]Gerontology, Dietetics and Nutrition Research and [b]Organic Chemistry, University of Gent, Gent B 9000, Belgium*

ABSTRACT

Information on the fatty acid chain length combinations in lymph triglycerides containing medium-chain fatty acids was obtained by combining the fatty acid composition and the carbon number composition of triglycerides from ascitic fluid of patients on medium-chain triglyceride-containing diets. In these triglycerides, the major part of the medium-chain fatty acids was present in combination with long-chain fatty acids. These results indicate that part of the lymphatic absorbed medium-chain fatty acids are absorbed as triglycerides which also contain long-chain fatty acids.
Lipids 17:759-761, 1982.

Lymphatic absorption of medium-chain fatty acids fed as MCT is enhanced by the presence of LCT in the diet (1). In human diets with MCT, LCT are always present (small amounts as invisible food fat and often present in the MCT preparation as natural oils rich in essential fatty acids [EFA] to insure the EFA requirements of the patients). Accordingly, medium-chain fatty acids have been demonstrated in ascitic- (2) and in pleural- (3) fluid triglycerides of patients on MCT-diets. It is unknown, however, if the medium-chain fatty acids present in lymphatic absorbed triglycerides are present as MCT, or in combination with long-chain fatty acids. The aim of this study was to obtain information on the fatty acid chain length combinations in triglycerides that are transported in man after feeding diets that contain MCT.

MATERIALS AND METHODS

Lymph-derived lipids were obtained from ascitic fluids of selected patients. Accumulation of ascitic fluid was caused by traumatic injury (patient C), congenital stenosis (patient D) or lymphoma-induced (patient A) obstruction of the ductus thoracicus or by portal hypertension due to alcoholic cirrhosis (patient V). Ascitic fluid had to be removed periodically to reduce intraabdominal pressure.

The patients were fed a diet containing a mixture of MCT with sunflower seed oil (94:6, w/w), about 0.5 g/kg body weight/day (fatty acid composition is given in Table 1, carbon number composition in Table 2). The amount of invisible food fat (LCT) (from bread, lean meat, skimmed yogurt, cheese from skimmed milk) was estimated to be about 0.2 g/kg/day, the MCT/LCT ratio of the diet being about 67:33 (w/w). The protein/fat/carbohydrate ratio in the diet was about 90:50:150 (w/w/w).

Lipids were extracted from the MCT preparation and from the ascitic fluid according to Folch et al. (4), fractionated by thin layer chromatography (5) and the triglycerides were eluted with CH_2Cl_2. Triglyceride carbon number composition was determined by capillary gas chromatography (GC). An aliquot of the triglyceride solution was introduced (6) on a 15 m/0.3 mm HTS-OV-1 capillary column (7) programmed from 230 C to 350 C (3 C/min).

The fatty acid composition of the triglycerides was determined by GC of the propyl esters as described previously (5).

Because the major long-chain fatty acids of ascitic fluid triglycerides were fatty acids with 16 or with 18 carbon atoms (respectively, palmitic + palmitoleic and stearic + oleic + linoleic acid) and the major medium-chain fatty acid had 10 carbon atoms (capric acid, Table 1), most of the triglycerides with a certain carbon number were made up mainly of those triglycerides which contained these fatty acids in such a combination that the sum of their chain lengths equaled the carbon number. For each carbon number, the most probable combination of fatty acids was calculated, based on the relative abundance of the different fatty acids shown in Table 1. There are other possible combinations for most of these, but the amounts

Abbreviations: MCT: medium-chain triglycerides; LCT: long-chain triglycerides; MML-TG: long-chain acyl-di-medium-chain-acylglycerols; MLL-TG: medium-chain-acyl-di-long-chain-acylglycerols.

*Author to whom correspondence should be addressed.

would be rather small compared to the composition suggested in Table 2.

RESULTS AND DISCUSSION

The results of the analyses of the fatty acid composition of the MCT preparation and of the ascitic fluid triglycerides after MCT/LCT feeding are reported in Table 1.

Ascitic fluid triglyceride carbon number composition was determined in several patients after feeding diets with butter, with natural fats containing only long-chain fatty acids, or with MCT/LCT. After butter feeding, the major ascitic fluid triglycerides had carbon numbers from 42 (14 + 14 + 14) to 54, whereas after feeding LCT, they had carbon numbers from 48 to 54.

Table 2 shows the carbon number compositions of the MCT-preparation and of the ascitic fluid triglycerides after MCT/LCT feeding and the major chain length combinations they represent.

A representative result of the analyses after butter feeding and after LCT feeding is also shown. After feeding, the mixture of MCT with LCT triglycerides with carbon numbers 30, 36, 38, 42 and 44 were considerably elevated, the medium-chain containing triglycerides (carbon numbers 24 to 46) making up more than half of the total triglycerides present. Most of the triglycerides which contain medium-chain fatty

TABLE 1

Fatty Acid Composition (mol %) of MCT Preparation and of Ascitic Fluid Triglycerides after Feeding MCT-Containing Diets

Fatty acid	Ascitic fluid triglycerides				MCT preparation
	A[a]	C[a]	D[a]	V[a]	
8:0	4.2	2.3	3.2	4.3	21.8
10:0	26.7	42.1	22.7	26.4	74.0
12:0	tr	2.1	5.1	1.1	0.3
14:0	4.4	2.4	2.1	3.0	
16:0	22.0	14.5	15.4	16.2	0.3
16:1	3.7	1.4	0.9	2.0	
18:0	4.7	5.6	3.3	5.5	0.2
18:1	23.4	15.3	18.5	22.1	0.7
18:2	10.8	12.9	28.0	18.8	2.8
20:4	0.2	1.4	0.8	0.6	

[a]Different patients. For details, see Materials and Methods.

TABLE 2

Carbon Number Composition (mol %) of Ascitic Fluid Triglycerides and of MCT Preparation

Carbon number	Major chain length combinations[b]	Ascitic fluid triglycerides						MCT preparation
		MCT/LCT				Butter	LCT	
		A[a]	C[a]	D[a]	V[a]	C[a]	V[a]	
24	8 + 8 + 8			1.0				6.6
26	8 + 8 +10		0.3	2.8	1.2			12.6
28	8 +10 +10	0.6	2.3	2.8	1.6			19.8
30	10 +10 +10	3.5	15.0	2.0	10.0			55.3
32	8 + 8 +16	1.3	1.8	1.9	1.0			2.4[c]
34	8 +10 +16	2.1	2.6	2.0	1.5	0.1		
36	10 +10 +16	6.6	11.4	7.8	7.3	0.3		
38	10 +10 +18	8.7	19.0	8.4	13.3	0.5		
40	10 +14 +16	3.2	2.7	3.2	1.2	1.2	0.1	
42	10 +16 +16	7.6	4.8	4.3	3.2	2.6	0.2	
44	10 +16 +18	12.6	11.0	10.4	9.1	5.1	0.6	
46	10 +18 +18	11.6	12.0	10.7	11.4	9.2	1.3	
48	16 +16 +16	6.2	2.1	3.1	4.4	15.2	3.6	
50	16 +16 +18	12.3	3.6	3.8	7.0	23.2	9.2	0.1
52	16 +18 +18	16.3	5.4	11.3	14.0	26.0	23.3	0.6
54	18 +18 +18	8.0	5.1	20.7	14.0	15.3	56.4	2.7
56	18 +18 +20			3.8		0.9	2.8	
24-30	MCT	4.1	17.6	8.6	12.8			
32-38	MML-TG	18.7	34.8	20.1	23.1			
40-46	MLL-TG	35.0	30.5	28.6	24.9			
48-56	LCT	42.8	16.2	42.7	39.4			

[a]Different patients.

[b]Based on the relative abundance of the fatty acids present in ascitic fluid triglycerides after LCT or MCT/LCT feeding (not valid after butter feeding resulting in triglycerides containing considerable amounts of myristic acid).

[c]Mainly lauryloyldicaprinoylglycerol (see footnote b).

acids were MLL-TG and MML-TG. In analogy to MCT and LCT, we suggest the name "mixed-chain triglycerides" for these components.

On a mole basis, medium-chain fatty acids make up 3/3 of the fatty acids of MCT, 2/3 of these of MML-TG and 1/3 of these of MLL-TG. Thus, most of the medium-chain fatty acids in ascitic fluid triglycerides were present as mixed-chain triglycerides (about 72 mol % as an average; about 43 mol % as MML-TG and 29 mol % as MLL-TG) and not as MCT (about 28 mol %).

The composition of the triglycerides in ascitic fluid, is not necessarily the same as that in lymph. Indeed, there could be differential influx in ascitic fluid of different lymph lipoproteins, which might or might not differ in triglyceride composition. There could be some difference in the rate of clearance between different triglycerides in ascitic fluid, although the available evidence suggests that this is not the case (2). At any rate, our results indicate that a considerable fraction of lymphatic absorbed medium-chain fatty acids were transported as mixed-chain triglycerides in our patients on MCT-containing diets. In all probability, this would also be the case in individuals on MCT diets who do not accumulate ascitic fluid.

The finding that substitution of an MCT/long chain monoglyceride mixture for a MCT/LCT mixture in the diet resulted in reduced lymphatic transport of medium-chain fatty acids (A. Christophe and G. Verdonk, unpublished data) suggests that formation of mixed-chain triglycerides after MCT/LCT feeding is probably due to acylation of medium-chain partial glycerides with long-chain acyl-CoA. This mechanism and the finding that there are MCT in ascitic fluid would imply that there is also absorption of intact MCT in the intestinal cell. These MCT might be protected against further intestinal hydrolysis (as occurs in the absence of LCT in the diet) (8) by "dissolving" in the triglyceride droplets or in the intestinal lipoproteins formed due to LCT administration. Whatever the mechanism of enhancement of lymphatic absorption of medium-chain fatty acids fed as MCT by simultaneous administration of LCT in the diet (1), it is clear from the relative proportions of capric and caprylic acid in ascitic fluid triglycerides and in the fat fed that there is a considerable difference in chyloportal repartition between these two acids.

ACKNOWLEDGMENT

Financial support of the Nationaal Fonds voor Wetenschappelijk Onderzoek is greatly appreciated.

REFERENCES

1. Bach, A., and Metais, P. (1970) Ann. Nutr. Aliment. 24, 75-144.
2. Malagelada, J.R., Iber, F.L., and Linscheer, W.G. (1974) Gastroenterology 67, 878-886.
3. Gershanik, J.J., Jonsson, H.T., Riopel, D.A., and Packer, R.M. (1974) Pediatrics 53, 400-403.
4. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biochem. 226, 497-509.
5. Matthys, F., Christophe, A., and Verdonk, G. (1972) Clin. Chim. Acta 36, 341-350.
6. Galli, M., Trestianu, S., and Grob, K., Jr. (1979) J. High Resolut. Chromatogr. Chromatogr. Commun. 42, 366-370.
7. Godefroot, M., Van Roelenbosch, M., Verstappe, M., Sandra, P., and Verzele, M. (1980) J. High Resolut. Chromatogr. Chromatogr. Commun. 3, 337-344.
8. Hashim, S.A. (1967) J. Am. Diet. Assoc. 51, 221-227.

[Received April 13, 1982]

Effect of Eicosatetraynoic Acid on Liver and Plasma Lipids

RANDALL WOOD, *Department of Biochemistry and Biophysics, Texas Agricultural Experiment Station, Texas A&M University System, College Station, TX 77843*

ABSTRACT

Groups of rats were fed a fat-free diet supplemented with 0.5% safflower oil (control) or the control diet containing 0.5% of 5,8,11,14-eicosatetraynoic acid (TYA). Blood was collected weekly and plasma lipids analyzed. After 4 weeks, the animals were killed and the liver lipids were analyzed in detail. The acetylenic fatty acid perturbed plasma neutral lipid and phospholipid class concentrations and reduced growth rates. Liver triglyceride concentrations were reduced dramatically in the TYA fed animals, suggesting interference with complex lipid synthesis. Plasma and liver triglycerides were shifted to higher molecular weight species suggesting that TYA affected fatty acid metabolism. The phospholipids showed an accumulation of 18:2 and a fall in 20:4 percentages indicating an inhibition in the conversion of linoleate to arachidonate. All major lipid classes exhibited an increase in 18:1 levels. Analysis of the octadecenoate positional isomers indicated the proportion of oleate increased substantually in all lipid classes whereas vaccenate proportions had fallen dramatically. All of the data collectively suggest that TYA inhibits the elongation of unsaturated fatty acids. A group of rats bearing hepatoma 7288CTC were also fed the TYA diet. Host liver lipids were affected by TYA similar to normal TYA fed animals, but the effects on hepatoma lipids were marginal.

Lipids 17:763-770, 1982.

INTRODUCTION

Our interest in eicosatetraynoic acid (TYA) resulted from our awareness of the possibility that it might possess antitumor activity. This conclusion was based on our observation that elevated levels of linoleic acid inhibited hepatoma cell growth in culture [unpublished data, (1)] and the observation made by Robinson et. al (2) that indicated acetylenic CoA esters inhibited fatty acid synthesis and blocked the condensation of malonyl-CoA with acetyl-CoA in rat tissues. Since the microsomal elongation system uses malonyl CoA for the biosynthesis of polyunsaturated fatty acids (3), it was reasoned that TYA might block the elongation of linoleic acid causing a build up that would inhibit hepatoma growth. It was also known in 1975, when our studies began, that TYA inhibited prostaglandin biosynthesis (4-7), which might also play a role in the inhibition of tumor growth. Since then a large number of papers have appeared that have described a variety of effects on lipid metabolism and these have been covered in a recent review by Tobias and Hamilton (8). This report describes the effect of dietary TYA on the plasma and liver lipids. A preliminary report of this work has appeared (9).

MATERIALS AND METHODS

Groups of male Buffalo strain rats weighing ca. 220 g were selected at random, marked for identification, and placed on one of the following diets: a fat-free diet supplemented with 0.5% safflower oil (control) or the control diet supplemented with 0.5% 5,8,11,14-eicosatetraynoic acid (TYA). The fat-free diet was formulated according to Wooley and Sebrell (10). A group of rats was also transplanted with hepatoma 7288CTC (11) and placed on the TYA and control diets. Blood was collected at the start of the study and weekly from the tail of individual animals. Bleeding was facilitated by using a heated rat-holding unit from Narco Biosystems Inc., Houston, TX.

After 4 weeks, the animals were sacrificed, livers removed, blotted, weighed, lyophilized and the total lipids extracted by the Bligh and Dyer procedure (12). Blood samples were centrifuged and the plasma extracted by the same procedure (12). Neutral lipids were separated from the polar lipids by silicic acid chromatography (13). The percentages of each fraction were determined gravimetrically for liver. The quantity of neutral lipids in the plasma was determined by the addition of a known amount of trimyristin before analysis by high temperature gas liquid chromatography (GLC) of the intact lipids (14,15). The distributions of the neutral lipid classes in liver were determined by this method also. The quantity of phospholipids in the plasma and liver was determined by phosphorous analysis (16). Phospholipid classes were resolved by thin layer chromatography (TLC) on adsorbent layers of Silica Gel HR, developed in a solvent system of chloroform/methanol/acetic acid/saline (50:25:8:4, v/v). Neutral lipids were resolved by TLC on adsor-

bent layers of Silica Gel G developed in a solvent system of hexane/diethyl ether/acetic acid (80:20:1, v/v). Individual neutral lipid classes and phospholipid classes were detected on TLC plates by spraying with Rhodamine 6G and 2',7'-dichlorofluorescein, respectively, and viewing under UV light.

Methyl esters were prepared from the lipid classes by sulfuric acid catalyzed transesterification (17) and analyzed quantitatively by GLC on SP 2330 packed columns (18). Peak identities were based on cochromatography with standards analyzed on polar and nonpolar liquid phases before and after hydrogenation. The *cis* octadecenoate isomers were separated by argentation TLC after the C-18 monoenes had been isolated by preparative GLC (19). The position of the double bond in the octadecenoates was determined by GLC analysis of the ozonolysis cleavage products (20).

Eicosatetraynoic acid (TYA, Ro 3-1428) was a gift from Dr. J.G. Hamilton of Hoffman-La-Roche, Nutley, NJ. The glass distilled solvents used in this study were purchased from Burdick and Jackson Laboratories, Muskegon, MI. Lipid standards and chromatographic supplies were from Nu-Chek-Prep., Inc., Elysian, MN, and Supelco, Inc., Bellefonte, PA.

RESULTS

Growth of Animals

Rats on the control diet gained 63 ± 3 g each, whereas the TYA-fed rats gained 51 ± 8 g for the 4 weeks. The difference was significant at the 90% probability level. During the first week, the control rats gained 5 ± 4 g, whereas TYA-fed rats gained 20 ± 2 g. The growth rates of the control and TYA-fed animals for the last 3 weeks were 60 ± 3 g and 33 ± 6 g, respectively, which is significantly different. These results indicate that 0.5% TYA in the diet inhibited the growth rate of normal animals.

The general appearance of the TYA-fed host animals sacrificed after 4 weeks suggested they may have had a mean survival time longer than the normal 4-5 weeks. Although tumor weights were not recorded, the size of the hepatoma was only about one-half that normally observed on other diets after 4 weeks. Instead of the normal soft diffuse nature of the hepatoma, the hepatomas from the TYA-fed animals were firm, well encapsulated, and filled with a greenish thick fluid in the hollow center.

Plasma Lipids

The concentrations of the major neutral lipid and phospholipid classes measured at weekly intervals in the plasma are shown in Table 1. The effects of the TYA diet on plasma lipid concentrations were inconsistent and variable, but fourth week levels were generally decreased. Changes in the molecular species of plasma triglycerides as affected by TYA are reflected in the carbon number distributions given in Table 2. The distribution of the plasma triglyceride

TABLE 1

Concentration of Neutral Lipid and Phospholipid Classes in the Plasma of Untreated and TYA-Fed Rats

Time (weeks)	Diet[b]	Conc (μ moles/ml plasma)[a]				
		Sterol	Sterol E.	TG	Lyso-Pc	PC
Initial	Chow	0.33 ± .02	0.76 ± .06	1.32 ± .22	0.27 ± .03	0.92 ± .12
1	Control	0.55 ± .06	1.06 ± .04	0.72 ± .25	0.37 ± .02	1.50 ± .10
	TYA-fed	0.34 ± .10[d]	0.84 ± .05[f]	0.96 ± .21	0.38 ± .02	0.92 ± .11[f]
2	Control	0.35 ± .04	0.70 ± .09	0.38 ± .16	0.24 ± .01	0.78 ± .04
	TYA-fed	0.39 ± .06	0.92 ± .12[c]	1.39 ± .43[e]	0.34 ± .06[d]	0.66 ± .26
3	Control	0.38 ± .07	0.88 ± .09	0.66 ± .31	0.25 ± .00	0.89 ± .05
	TYA-fed	0.38 ± .03	0.67 ± .06[d]	0.94 ± .20	0.36 ± .03[f]	0.90 ± .08
4	Control	0.47 ± .07	1.09 ± .08	0.89 ± .78	0.35 ± .06	1.07 ± .10
	TYA-fed	0.32 ± .07[c]	0.77 ± .17[d]	0.50 ± .24	0.21 ± .08[c]	0.75 ± .27

Abbreviations: TYA, eicosatetraynoic acid; FFA, free fatty acids; Sterol, free sterols; Sterol E., sterol esters; TG, triglycerides; Lyso-Pc, lyso phosphatidylcholine; PC, phosphatidylcholine.

[a]Concentrations represent the mean of 3 animals ± the standard deviation.

[b]Control diet = fat-free diet + 0.5% safflower oil.

[c]Significantly different ($p \leq 0.05$) from controls (student's t-distribution).

[d]Significantly different ($p \leq 0.025$) from controls.

[e]Significantly differnt ($p \leq 0.01$) from controls.

[f]Significantly different ($p \leq 0.005$) from controls.

TABLE 2

Effect of TYA on Triglyceride Carbon Number Distribution in Plasma of Untreated and TYA-fed Rats

Time (weeks)	Animals	Mole percentages[a,b] 48	50	52	54	56	58
Initial	Group 1	2.5 ± 1.4	14.2 ± 3.2	41.7 ± 1.2	21.6 ± 1.2	12.6 ± 2.2	3.3 ± 0.6
	Group 2	1.9 ± 1.0	13.1 ± 3.7	41.6 ± 1.7	25.2 ± 2.3	13.4 ± 1.9	3.6 ± 0.8
1	Control	9.1 ± 2.2	31.3 ± 0.8	46.9 ± 1.4	12.3 ± 1.9	T	
	TYA-fed	4.5 ± 1.7[d]	22.2 ± 0.6[f]	61.6 ± 1.8[f]	11.1 ± 1.4	0.6 ± 0.4	
2	Control	4.2 ± 2.6	33.9 ± 2.9	46.4 ± 4.6	12.6 ± 2.0	2.9 ± 1.3	
	TYA-fed	2.4 ± 0.7	19.4 ± 1.0[f]	65.2 ± 1.8[f]	9.5 ± 0.6[c]	0.3 ± 0.1	
3	Control	13.4 ± 4.7	33.9 ± 2.5	42.7 ± 4.1	6.1 ± 4.4	T	
	TYA-fed	3.5 ± 0.1[e]	27.7 ± 1.3[e]	59.2 ± 0.3[f]	8.7 ± 0.6	T	
4	Control	9.0 ± 0.1	31.2 ± 2.3	44.2 ± 1.3	14.4 ± 3.2	T	
	TYA-fed	T [f]	24.0 ± 2.0[e]	62.9 ± 1.8[f]	11.3 ± 0.3	T	

[a]Percentages represent the mean of 3 animals. The difference between the sum of percentages in any row and 100 represents minor amounts of shorter and longer carbon numbers.

[b]Levels of significance are the same as listed in Table 1.

carbon numbers obtained initially and for a second group analyzed 3 months later shows close agreement and is also given in Table 2. Changes in the plasma triglycerides occurred within the first week. The higher molecular weight species decreased in the groups relative to the chow-fed groups at the start of the study. TYA-fed animals exhibited significantly higher percentages of carbon number 52 and lower percentages of carbon numbers 48 and 50 than controls for all time periods.

Liver Lipids

The concentrations of the major neutral lipids and phospholipid classes in liver of control and TYA-fed animals are given in Table 3. Triglycerides and sphingomyelins were reduced significantly and sterol esters were increased in TYA-fed normal animals relative to controls. Except for sphingomyelin, the major phospholipid class concentrations were not altered significantly by the TYA.

The carbon number distributions of triglycerides obtained from liver of control and TYA-fed rats are given in Table 4. The TYA caused a shift to higher percentages of carbon number 52 and 54. Plasma triglycerides showed increased percentages of carbon number 52, but not carbon number 54.

The fatty acid compositions of the major lipid classes isolated from liver of control and TYA-fed rats are given in Table 5. Generally, TYA causes a decrease in 16:1 percentages and an increase in 18:1 percentages in all classes. The two phospholipid classes contained higher percentages of 18:2 and lower percentages of 20:4 in TYA-fed animals than controls. The decrease in phosphatidylethanolamine (PE) was much less than phosphatidylcholine (PC).

The distributions of the geometrical and positional octadecenoate isomers in the major lipid classes from liver of control and TYA-fed rats are given in Table 6. The octadecenoates from all the lipid classes of both liver and hepatoma consisted of > 98% the *cis* isomers. The *cis* octadecenoates consisted predominantly of two isomers, the Δ9 (oleate) and Δ11 (vaccenate). The neutral lipids of the TYA-fed animals consisted of > 90% oleate, while PC and PE of this group consisted of > 83% oleate. In contrast, neutral lipids from the control group consisted of 66%-75% oleate, and oleate percentages represented less than 40% of octadecenoates of PC and PE. These distributions of positional isomers for the control groups are similar to those reported earlier for animals fed chow and fat-free diets (21).

DISCUSSION

Effect of TYA on Hepatoma and Host

A subjective evaluation indicated the reduced size of the tumors may have been due to the TYA. It was reported ineffective in reducing

TABLE 3

Quantity of Major Neutral Lipid and Phospholipid Classes in Liver of Rats Fed TYA

Diet and tissue	Quantity (mg/g wet wt)[a,b]						
	Sterol	Sterol E	TG	SPH	PC	PI	PE
Control, liver	1.45 ± .71	0.78 ± .07	36.87 ± 9.71	2.42 ± 0.43	16.90 ± 1.40	3.35 ± 0.44	6.72 ± 0.70
TYA-fed, liver	1.69 ± .10	1.35 ± .15[f]	11.91 ± 2.51[e]	1.52 ± 0.02[e]	15.62 ± 0.95	2.91 ± 0.14	7.03 ± 0.39

Abbreviations: TYA, eicosatetraynoic acid; Sterol, free sterols; Sterol E, sterol esters; TG, triglycerides; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

[a]Values represent the mean of 3 animals ± the standard deviation.

[b]Levels of significance are the same as listed in Table 1.

the growth rate of hepatoma 7777 in an earlier study (8), but some reduced growth rates of mammary adenocarcinomas (22), mammary tumor Cfz No. 3 (23) and the number of skin papillomas produced by carcinogens and phorbol esters (8) have been observed. Studies conducted thus far on the inhibition of tumor growth by TYA have generally been variable and unpredictable. This may result from the virtual insolubility and presumed low absorption of TYA from the intestine.

The effect of TYA on hepatoma lipids was generally minor to insignificant from what we have reported earlier for hepatomas from host animals fed a variety of diets (11,24-27).

The effects of TYA on the host animal liver lipids were generally similar to that observed in the normal animals, except for the magnitude. In order to focus attention on the effects of TYA in normal animals, host and hepatoma data have been omitted.

Effect of TYA on Animals

The growth rates of the rats were inhibited moderately by TYA at the concentrations used. Since plasma lipid concentrations of most classes were beginning to show significant decreases by the fourth week, growth rates might have been affected even more at longer feeding times. Liver weights (4.80% ± 0.25), expressed as percent of body weight, of TYA fed animals were not statistically different from controls (4.6% ± 0.05). Increased liver weights of tumor-bearing animals given high levels of TYA by gastric intubation for 5 weeks have been reported (8). Since the experimental conditions were totally different, the different results from the two studies are not surprising.

Effect on Plasma Lipids

There have been only a few reports on the effect of TYA on plasma lipid concentrations and these have been reviewed by Tobias and Hamilton (8). Generally, TYA lowered plasma cholesterol concentrations in man and rats to a modest degree (8). Our data are in agreement: except for the second week, sterol esters were reduced significantly and free cholesterol levels were reduced the first and last week (Table 1). The inconsistent and variable hypocholestolemic effect may be related to the inert nature of TYA.

Plasma triglycerides of TYA-fed animals were elevated significantly by the second week and then declined in the third and fourth weeks (Table 1). PC concentrations were reduced dramatically the first week and then returned to normal by the third week, where they again fell. This cyclic response of the plasma lipid classes

TABLE 4

Carbon Number Distribution of Triglycerides Derived from Liver of Untreated Rats Fed TYA

Diet	Triglyceride carbon number mole percentages[a,b]				
	46	48	50	52	54
Control	1.4 ± 0.3	13.8 ± 0.6	39.6 ± 0.7	39.4 ± 1.1	6.3 ± 0.5
TYA-fed	–	1.8 ± 0.3[f]	26.6 ± 2.9[e]	57.3 ± 2.9[f]	10.5 ± 0.2[f]

[a]Percentages and standard deviations represent the mean of 3 animals.

[b]The levels of significance are the same as listed in Table 1.

to TYA is not understood, but it is interesting that the concentration of all the lipid classes in the fourth week was lower than the controls, and in many cases significantly lower. This appears to represent an alteration in a basic lipid metabolic process that takes a while to be observed.

Effect of TYA on Hepatic Lipids

Liver lipid class concentrations of TYA-fed animals show sphingomyelin, sterol ester and triglyceride levels were altered significantly after 4 weeks. Although sphingomyelin and sterol ester values were altered significantly relative to control values in this study, they fall within the extremes of values produced when chow and fat-free diets were fed (11,24). A reduction of more than 60% of the major lipid class in liver, the triglycerides, does appear to represent a major alteration produced by TYA (Table 3). This reduction in the liver triglycerdies also agrees with the lower plasma triglyceride level in the TYA-fed animals at the fourth week.

In addition to changes in triglyceride concentrations of liver, the composition of the triglycerides was also changed dramatically (Tables 4 and 5). The carbon number distributions in liver agreed with those of the plasma (Table 2), which indicates these shifts in triglyceride molecular species may have occurred within the first week. Changes in the molecular species of the triglycerides probably occurred as a result of changes in fatty acid biosynthesis (discussed later), whereas changes in liver triglyceride concentrations that reached a maximum after two weeks on the TYA diet and a minimum the fourth week, probably resulted from other perturbations in the lipid metabolism.

Effect of TYA on Fatty Acid Modification

Fatty acid analysis of total lipids from liver and other organs from animals fed TYA have revealed previously that 18:2 percentages increased, whereas 20:4 percentages decreased (22,28,29). These results led the previous investigators to conclude that TYA inhibited the conversion of linoleic to arachidonic acid. These results were examined by others (8) who concluded that TYA interfered with the desaturation of linoleate to arachidonate. Our results (Table 5) confirm that 18:2 percentages are elevated and 20:4 percentages are decreased in PC and PE of TYA-fed animals, but the present data indicate the two phospholipid classes are not affected to the same extent. Further, our data show that 18:2 percentages do not increase significantly in the two major neutral lipid classes.

A further examination of the fatty acid composition of the individual lipid classes (Table 5) revealed that 16:1 percentages were decreased and 18:1 percentages were increased significantly in all the lipid classes of TYA-fed animals. The decreases in 16:1 percentages are consistent with that reported by Rao et al. (30) for the total liver lipids. In their experiments with TYA, 18:1 percentages were not elevated, but decreased, and 20:4 percentages showed only marginal changes. These results prompted the authors (30) to suggest that dietary TYA inhibited hepatic desaturase activity. Contrary to the paper just mentioned (30), the total lipids from other studies with TYA-fed animals showed increases in 18:1 percentages (22,28,29), as our data indicate. Since most of the evidence obtained thus far indicates a single enzyme system for the desaturation of palmitate and stearate to the corresponding Δ9 monoenes (31,32), the increased percentage of 18:1 in the previous and present data do not support the notion that the Δ9 desaturase system is inhibited by TYA. The increased proportion of oleate in the octadecenoates from all the lipid classes (Table 6) likewise rules against the desaturase system

TABLE 5

Fatty Acid Composition of Individual Lipid Classes Isolated From Livers of Untreated and TYA-Fed Rats

Diet	Fatty Acid Percentages[a,b]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
				Triglycerides			
Control, liver	36.2 ± 1.3	16.8 ± 1.2	1.6 ± 0.1	42.7 ± 1.6	1.5 ± 0.1		
TYA-fed, liver	32.4 ± 0.7[e]	8.8 ± 0.7[f]	1.8 ± 0.4	55.1 ± 1.0[f]	1.6 ± 0.4		
				Sterol Esters			
Control, liver	50.2 ± 3.2	14.4 ± 0.9	4.6 ± 0.4	25.6 ± 2.4	1.6 ± 0.8	2.1 ± 0.3	
TYA-fed, liver	35.2 ± 3.5[f]	10.6 ± 1.3[e]	3.2 ± 0.2[f]	49.0 ± 4.6[f]	1.7 ± 0.3		
				Phosphatidylcholine			
Control, liver	21.2 ± 0.4	5.8 ± 1.0	20.9 ± 0.4	20.4 ± 0.2	9.8 ± 0.2	13.9 ± 1.8	3.1 ± 0.1
TYA-fed, liver	23.1 ± 0.7[e]	3.2 ± 0.5[e]	18.4 ± 1.1[d]	35.2 ± 1.2[f]	16.1 ± 0.6[f]	2.3 ± 0.3[f]	T
				Phosphatidylethanolamine			
Control, liver	24.6 ± 1.2	3.6 ± 1.1	24.5 ± 1.4	12.5 ± 0.8	2.4 ± 0.2	24.5 ± 2.1	6.7 ± 0.6
TYA-fed, liver	21.3 ± 1.6[d]	2.0 ± 0.2[c]	21.5 ± 1.0[d]	19.9 ± 1.4[f]	11.7 ± 0.8[f]	18.0 ± 2.3[d]	4.3 ± 0.8[d]

Abbreviation: TYA, eicosatetraynoic acid.

[a]Percentages represent the mean of three animals ± standard deviation. The difference between the sum of the percentages in any row and 100% represents the sum of other acids not given in the table.

[b]The levels of significance are the same as listed in Table 1.

TABLE 6

Percentage Distribution of Positional Octadecenoate Isomers in the Major Lipid Classes from Liver of Untreated and TYA-fed Rats

	Percentages[a]			
	Control diet		TYA diet	
	cis Δ9	*cis* Δ11	*cis* Δ9	*cis* Δ11
Triglycerides	75 ± 1.3	22 ± 2.0	92.8 ± 1.0	7.2 ± 1.0
Sterol esters	66	30	92.9 ± 0.9	7.1 ± 0.9
Phosphatidylcholine	39 ± 0.5	58 ± 1.1	89.1 ± 2.0	10.9 ± 2.0
Phosphatidylethanolamine	27	65	83.0 ± 3.2	17.0 ± 3.2

Abbreviation: TYA = 5,8,11,14 eicosatetraynoic acid.

[a]Mean percentages ± standard deviation are from 3 animals. Values without standard deviations represent the analysis of a pooled sample. The difference between the sum of the percentages of the two isomers and 100 represents minor amounts of other isomers not given in the table.

being inhibited. It should be noted, however, that dietary cyclopropene fatty acid, a known inhibitor of the Δ9 desaturase system (31,33), is the only other dietary compound that we have found to alter the class characteristic Δ9 to Δ11 ratios (21,25) as observed in Table 6.

The data in the present studies appear best explained if one assumes that TYA inhibits fatty acid elongation of unsaturated fatty acids. Since the conversion of linoleate to arachidonate involves an elongation step, the inhibition of this step would cause a build up of 18:2 and a decrease in the percentage of 20:4, which is consistent with the data. When TYA is added to a fat-free diet, one might expect the 20:3 percentages to be decreased if TYA inhibited elongation. Although the 0.5% safflower added to the diets in this study kept 20:3 levels too low to provide reliable values, Coniglio et al. (28) and Abraham et al. (29) have reported that TYA added to fat-free diets did lower 20:3 percentages. The increased proportion of oleate in octadecenoates from the lipid classes of the TYA-fed animals also indicates that elongation was inhibited. The inhibition of the elongation of palmitoleate, the precursor of vaccenate (34), would have reduced the Δ11 isomer levels as observed. One might argue that the decreased 16:1 percentages could have also reduced the level of the vaccenate, but that does not appear to be the case. We have shown that in chow-fed animals, where the level of 16:1 was much lower than the present study, vaccenate levels were higher (11,21,24). Finally, we have reported that 2-hexadecynoate, an acetylenic acid, inhibits fatty acid elongation in vivo and in vitro (27,35). A metabolite, the 2,3-allene that results from the isomerization of the triple bond at either the 2 or 3 position, is the proposed active inhibitor (35). After the removal of one acetate from TYA, the resulting compound with the triple bond in the 3-position may be isomerized to the active inhibitor of fatty acid elongation also. Although these data suggest that TYA inhibits the elongation of unsaturated fatty acids, it does not preclude the possibility that TYA may affect the desaturation of unsaturated fatty acids as well.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Research Grant No. CA-20136 from the National Cancer Institute.

REFERENCES

1. Wood, R. (1973) Lipids 8, 690-701.
2. Robinson, J.D., Brady, R.O., and Bradley, R.M. (1963) J. Lipid Res. 4, 144-150.
3. Wakil, S.J. (1970) in Lipid Metabolism (S.J. Wakil, ed) p. 1, Academic Press, New York, NY.
4. Ahern, D.G., and Downing, D.T. (1970) Biochim. Biophys. Acta 210, 456-461.
5. Lands, W.E.M., LeTellier, P.R., Rome, L.H., and Vanderhoek, J.Y. (1973) Adv. Biosci. 9, 15-28.
6. Willis, A.L., Davison, P., and Ramwell, P.W. (1974) Prostaglandins 5, 355-368.
7. Dalton, C., and Hope, H.R. (1973) Prostaglandins 4, 641-651.
8. Tobias, L.D., and Hamilton, J.G. (1979) Lipids 14, 181-193.
9. Wood, R., and Chumbler, F. (1978) J. Am. Oil Chem. Soc. 55, 255A.
10. Wooley, J.G., and Sebrell, W.H. (1945) J. Nutr. 29, 191-199.
11. Wood, R., Falch, J., and Wiegand, R.D. (1975) Lipids 10, 202-207.
12. Bligh, E.G., and Dyer, W.J. (1957) Can. J. Biochem. Physiol. 37, 911-917.
13. Borgstrom, B (1952) Acta Physiol. Scand. 25, 101-110.

14. Kuksis, A., Stachnyk, O., and Holub, B.J. (1969) J. Lipid Res. 10, 660-667.
15. Wood, R., and Falch, J. (1974) Lipid 9, 979-986.
16. Rouser, G., Siakotos, A.M., and Fleischer, S. (1966) Lipids 1, 85-86.
17. Wood, R. (1974) Lipids 9, 429-439.
18. Matocha, M., and Wood, R. (1980) Lipids 15, 421-427.
19. Wiegand, R.D., and Wood, R. (1974) Lipids 9, 141-148.
20. Wood, R., Falch, J., and Wiegand, R.D. (1974) Lipids 9, 987-992.
21. Wood, R., and Wiegand, R.D. (1975) Lipid 10, 746-749.
22. Rao, G.A., and Abraham, S. (1977) J. Natl. Cancer Inst. 58, 445-447.
23. Hillyard, L., Rao, G.A., and Abraham, S. (1980) Proc. Soc. Exp. Biol. Med. 163, 376-383.
24. Wood, R. (1975) Lipid 10, 736-745.
25. Wood, R., Chumbler, F., and Wiegand, R.D. (1978) Lipids 13, 232-238.
26. Wood, R., and Chumbler, F. (1978) Lipids 13, 75-84.
27. Wood, R., Lee, T., and Gershon, H. (1980) Lipids 15, 141-150.
28. Coniglio, J.G., Buch, D., and Grogan, W.M., Jr. (1976) Lipids 11, 143-147.
29. Abraham, S., McGrath, H., and Rao, G.A. (1977) Lipids 12, 446-449.
30. Rao, G.A., Siler, K., and Larkin, E.C. (1978) Lipids 13, 356-359.
31. Pande, S.V., and Mead, J.F. (1970) J. Biol. Chem. 245, 1856-1861.
32. Howling, D., Morris, L.J., and James, A.T. (1968) Biochim. Biophys. Acta 152, 224-226.
33. Raju, P.K., and Reiser, R. (1973) J. Nutr. 103, 904-907.
34. Holloway, P.W., and Wakil, S.J. (1964) J. Biol. Chem. 239, 2489-2495.
35. Wood, R., and Lee, T. (1981) J. Biol. Chem. 256, 12379-12386.

[Received July 23, 1981]

Effect of Hepatoma on Host Liver, Heart and Lung Lipids as Tumor Growth Progresses

RANDALL WOOD*, A. ZOELLER and **M. MATOCHA**, *Department of Biochemistry and Biophysics, Texas Agricultural Experiment Station, Texas A&M University System, College Station, TX 77843*

ABSTRACT

A large group of rats was transplanted with hepatoma 7288CTC and 4 animals were sacrificed at 3-day intervals for four weeks. Lipid class concentrations, fatty acid class compositions, and the distribution of *cis* octadecenoate positional isomers in the major lipid classes were determined for heart, liver and lung at each time period. The hearts of host animals decreased in dry weight as hepatoma growth progressed. At day 30, heart weights were less than two-thirds of initial weights. Lipid class concentrations changed in all three tissues: cholesterol and free fatty acids increased in liver; triglycerides and cholesterol decreased and then increased in heart; and cholesterol, triglycerides and PC decreased in lung as tumor growth progressed. Hexadecenoate percentages exhibited a progressive decrease in all the lipid classes of heart and liver. Although total octadecenoate percentages showed only minor changes, oleate concentrations generally increased and vaccenate levels decreased in heart and liver lipids as tumor growth progressed. Palmitoleate, precursor of vaccenate, exhibited decreased concentrations early that resulted in decreased vaccenate levels. Decreased palmitoleate concentrations suggest inhibition of the Δ9 desaturase system, but normal oleate concentrations complicate this interpretation. Most of the changes in the lipids were detectable 3-6 days after transplantation, indicating the hepatoma affects the lipid metabolism of the host animal early and well in advance of nutritional stresses.

Lipids 17:771-779, 1982.

INTRODUCTION

The depletion of fat stores in the advanced stages of tumor growth is common in both humans and experimental animals. Work conducted in this area prior to 1956 has been reviewed by Haven and Bloor (1). Since then, additional studies have indicated that the growth of a tumor affects the lipid metabolism of the host (2-10). Our work with minimal deviation hepatoma 7288CTC has shown that host liver phospholipids contain a higher percentage of polyunsaturated fatty acids (11) and that the octadecenoates contain a higher proportion of oleate (12). Generally, the effects on the host lipids have been observed in the advanced stages of tumor growth when nutritional stresses can also affect the host lipids.

Recently we observed changes in the lipids of the host animal well in advance of detectable tumor growth and nutritional stresses. Plasma phospholipids exhibited a detectable change in the ratio of oleate to vaccenate 3 days after hepatoma transplantation and the change was highly significant at 6 days (13). Plasma neutral lipid class concentrations of tumor-bearing animals changed dramatically over the entire growth period of the tumor, while hexadecenoate and polyunsaturated fatty acid percentages decreased in all lipid classes and plasma phospholipids, respectively (14).

The present study was carried out to determine whether lipids of host liver, heart and lung exhibit changes as hepatoma growth progresses. A preliminary report of this work has appeared (15).

MATERIALS AND METHODS

Heart, liver and lung were obtained from the same groups of animals sacrificed at 3-day intervals after hepatoma 7288CTC transplantation to study changes in plasma lipids (14). The lyophilized tissues were extracted twice by the Bligh and Dyer procedure (16) and separated into neutral lipid and phospholipids by silicic acid chromatography (17). The neutral lipid classes were quantitated by high-temperature gas liquid chromatography (GLC) (18). Phospholipid classes were analyzed quantitatively by phosphorous analysis (19) after resolution by thin layer chromatography (TLC) (14). Methyl esters were prepared by acid-catalyzed transesterification (18), separated according to configuration and degree of unsaturation by argentation TLC, hexadecenoates and octadecenoates were isolated by preparative GLC and the position of the double bonds determined

*Author to whom correspondence should be addressed.

by GLC of the ozonides (20). The percentage distribution of the fatty acids in the individual lipid classes of the various tissues was determined by GLC analysis of the methyl esters on a 10% SP-2330 column (21). Identity of the fatty acids was based upon cochromatography with authentic standards, analysis before and after hydrogenation, and analysis of methyl ester bands separated according to degree of unsaturation by silver ion TLC.

The sources of standards, solvent, reagents and other supplies were the same as given previously (14,21).

RESULTS

Liver

Neutral lipid class concentrations determined in the liver of host animals at 3-day intervals after hepatoma transplantation are given in Table 1. Free cholesterol and free fatty acid levels increased immediately after transplantation. Diglyceride concentrations, although low, increased as tumor growth progressed until the 27th day and then dropped sharply. Triglyceride levels showed little change until day 30 when concentrations plummeted. Changes in liver phospholipid class concentrations were marginal relative to day zero. Phosphatidylinositol (PI) showed the largest changes: values dropped to 50% of day zero values by the 9th day and then steadily increased to exceed initial values by 20% by day 30. Phosphatidylethanolamines (PE) exhibited a steady increase of more than 45% from day zero to the 30th day.

The fatty acid compositions of liver, triglycerides, phosphatidylcholines (PC) and PE at selected times are given in Table 2. Palmitate, octadecenoate, and octadecadienoate percentages represented between 85 and 98% of the liver triglyceride fatty acids at all times after hepatoma transplantation. Hexadecenoate percentages decreased steadily after day 6, which was offset by increased 18:2 percentages. Liver PC consisted of ca. 50% palmitate plus stearate and 50% unsaturated fatty acids. Hexadecenoate and octadecenoate percentages decreased as tumor growth progressed, while stearate and docosahexenoic acid increased. Liver PE contained the same proportion of saturated fatty acids as PC, but a higher percentage of C_{20} and C_{22} polyunsaturated fatty acids. Hexadecenoate and octadecenoate percentages decreased as tumor growth progressed while stearate percentages increased up to the 21st day. Eicosatetraenoic acid percentages decreased in liver PE after the 15th day, while docosahexenoic acid percentages increased from this time period. Free fatty acid and cholesterol ester compositions (data not shown) exhibited a dramatic decrease in hexedecenoate percentages (>65%) and a moderate to variable decrease in octadecenoate percentages as tumor growth progressed.

Percentage distributions and concentrations of the hexadecenoate and octadecenoate positional isomers isolated from host liver lipid classes as tumor growth progressed are shown in Tables 3 and 4, respectively. Palmitoleate con-

TABLE 1

Concentration of Neutral Lipids Derived from Host Liver at Various Times after Hepatoma Transplantation

Days after transplant	Neutral lipid classes (mg/g dry wt)[a]					
	CHOL	FFA	DG	TG	CE	TNL
0	7.5	1.1	0.9	25.0	1.6	36.1
3	8.7[f]	1.2	1.1[c]	25.5	1.3	37.8
9	9.5[d]	1.7[b]	1.1	28.7	1.7	42.6[c]
18	7.6	2.0[e]	1.2[c]	32.9[b]	1.4	45.1[c]
30	12.6[d]	3.4[f]	0.9	8.6[f]	1.2[b]	26.8[e]

Abbreviations: CHOL, cholesterol; FFA, free fatty acids; DG, diglycerides; TG, triglycerides; CE, cholesteryl esters; TNL, total neutral lipids.

[a]Concentrations represent the mean of 3-4 animals. Standard deviations have been omitted, but the level of significance has been indicated by a superscript.

[b]Significantly different ($p \leq 0.1$) from day zero (student's t-distribution).

[c]Significantly different ($p \leq 0.05$) from day zero.

[d]Significantly different ($p \leq 0.025$) from day zero.

[e]Significantly different ($p \leq 0.01$) from day zero.

[f]Significantly different ($p \leq 0.005$) from day zero.

TABLE 2

Fatty Acid Composition of Triglycerides, Phosphatidylcholines and Phosphatidylethanolamines Derived from Host Liver at Various Times after Hepatoma Transplantation

Days after transplant	Fatty acid percentages[a]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
				Triglycerides			
0	28.8	8.2	1.6	32.1	23.0	1.1	1.2
3	31.4	9.0	2.4	32.0	17.6	0.8	0.8
9	29.0	4.2	2.3	30.5	28.7	1.3	1.4
18	27.6	3.1	2.0	29.0	29.8	2.5	1.5
30	28.9	0.8	6.7	26.1	30.3	2.2	1.8
				Phosphatidylcholines			
0	24.4	2.2	18.9	12.0	20.3	18.1	1.8
3	24.3	2.6	20.0	10.0	19.6	15.2	2.6
9	20.8	1.1	23.5	8.4	19.1	21.3	2.7
18	24.6	1.2	25.6	9.0	15.9	18.2	3.9
30	25.5	0.6	24.6	8.6	23.4	8.7	5.6
				Phosphatidylethanolamines			
0	23.2	1.0	21.4	9.8	11.6	22.8	5.6
3	24.6	1.4	21.6	7.1	9.6	23.4	6.4
9	20.9	0.5	27.4	7.3	9.9	25.1	5.6
18	21.2	–	29.3	5.5	6.2	22.8	11.6
30	23.0	–	26.6	5.3	10.4	15.7	14.6

[a]Percentages represent the mean of 3 or 4 analyses. The differences between the sum of the percentages and 100% represent the sum of other acids present in small amounts but not shown in the table.

TABLE 3

Changes in the Distribution of Liver Hexadecenoate Positional Isomers as Hepatoma Growth Progressed

Days after transplant	Positional isomer percentages[a,b]					
	TG		PC		PE	
	Δ6 + Δ7	Δ9	Δ6 + Δ7	Δ9	Δ6 + Δ7	Δ9
0	8	90 (1.84)	10	88 (1.18)	8	90
3	8	90 (2.10)	8	88 (1.26)	8	90
9	7	91 (1.10)	18	76 (0.48)	12	84
18	11	87 (0.89)	16	79 (0.52)	24	74
30	43	54 (0.40)	36	56 (0.21)	30	65

Abbreviations: TG, triglycerides; PC, phosphatidylcholines; PE, phosphatidylethanolamines.

[a]Analyses were performed by capillary GLC (14) on a pooled sample from 3 or 4 animals, except day zero which was determined previously by ozonolysis of a pooled sample from 3 or 4 animals (20).

[b]Concentrations (μg/mg dry weight) are given in parentheses for TG and PC.

centrations decreased significantly by day 6 and continued to decrease for the rest of the growth period while the percentage of the Δ6 plus Δ7 isomers increased. The proportion of vaccenic acid decreased early after hepatoma transplantation in triglyceride and PC octadecenoates. The percentage of vaccenate also decreased in PE, but only after the 12th day of tumor

TABLE 4

Changes in the Distribution of Octadecenoate Positional Isomers of Host Liver, Heart and Lung as Hepatoma Growth Progressed

Days after transplant	Positional isomer percentage[a,b]					
	TG		PC		PE	
	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
			Liver			
0	83 (6.66)	17[c] (1.36)	[c] (2.64)	[c] (4.67)	[c] (0.76)	[c] (1.63)
3	81 (6.61)	19 (1.55)	41 (2.27)	59 (3.26)	37 (0.66)	63 (1.13)
9	89 (7.79)	11 (0.96)	50 (2.41)	50 (2.41)	36 (0.64)	64 (1.13)
18	89 (8.49)	11 (1.04)	62 (3.09)	38 (1.89)	53 (0.82)	47 (0.73)
30	87 (1.95)	13 (0.29)	71 (3.87)	29 (1.58)	67 (1.26)	33 (0.62)
			Heart			
0	90.1±1.8	9.9±1.8	31.5±1.5	68.7±1.5	39.7±2.1	60.3±2.1
9	86.7	13.3	30.9	69.1	45.4	54.6
18	88.2	11.8	36.2	63.8	51.5	48.5
30	83.5	16.5	42.4	57.6	57.6	42.4
			Lung			
0	90.5±0.5	9.5±0.5	70.0±3.9	30.0±3.9	82.5±4.4	16.3±4.4
9	90.5	9.5	77.9	22.1	90.4	9.6
18	90.3	9.7	76.5	23.5	90.3	9.7
30	87.5	12.4	75.0	25.0	87.6	12.4

Abbreviations: TG, triglycerides; PC, phosphatidylcholines; PE, phosphatidylethanolamines.

[a]Percentages were obtained from the analysis of a pooled sample from 3 or 4 animals at each time period, except day zero for heart and lung which represents the mean of 4 animals analyzed individually.

[b]Concentration (μg/mg dry weight) are given in parentheses for liver.

[c]Samples lost. Values reported previously (20) for normal animals or zero time were 81% Δ9 and 19% Δ11 for triglycerides; 31% Δ9 and 64% Δ11 for phosphatidylcholines; and 32% Δ9 and 68% Δ11 for phosphatidylethanolamines. These values were used to calculate concentrations.

growth. Oleate percentages and concentrations continued to increase in host liver PC and PE as tumor growth progressed and represented 65-75% of the octadecenoates by the 30th day. Triglyceride oleate concentrations increased to the 27th day and then dropped sharply.

Heart

The major effect of tumor growth on heart and heart lipids is shown in Table 5. Heart dry weights decreased dramatically after the 9th day and continued to decrease for the duration of the tumor growth period. Except for the 15th day, changes in the total lipid content of the heart were marginal. Free cholesterol and triglycerides, the major neutral lipids, decreased dramatically after hepatoma transplantation and remained low for the first half of the growth period and then increased. The increase in cholesterol exceeded initial concentrations, but triglyceride increases only approached one-third of the initial concentrations. The phospholipids represented 80 ± 6% of the total lipids for all time periods. The average phospholipid class composition (percentage) for all time periods was LPC, 0.5±0.5; SPH, 3.8±2.0; PC, 45.0±2.3; PI, 5.7±2.6; PS, 3.2±0.8; PE, 34.4±1.5, indicating only minor changes as hepatoma growth progressed.

Heart triglycerides were analyzed intact by high temperature GLC (18) to determine the carbon number distribution at various stages of hepatoma growth. Generally, there was a shift to higher molecular triglyceride species with time, i.e., carbon number 50 decreased from 29% to 11% while carbon number 56 increased from 1% initially to 16% at day 30. The triglyceride fatty acid compositions are compared with the fatty acid composition of the major phospholipid classes at selected time periods in Table 6. Generally, the saturated fatty acids remained constant at all time periods of all the lipid classes, whereas monoene fatty acids decreased and 22:6 increased dramatically by the 9th day in the phospholipid classes. Although PC and PE did not contain sufficient hexadece-

TABLE 5

Effect of Progressive Hepatoma Growth on Host Animal Heart Dry Weight and Lipid Content

Days after transplant	Dry wt mg/heart	Total lipid % of dry wt	NL conc (μg/mg dry wt)[a]		
			Total	CHOL	TG
0	215	17.0	71.0	7.9	61.8
3	211	17.3	15.4	4.5	10.5
9	209	15.0[b]	12.3	5.9	6.3
18	166[e]	14.4[c]	28.1	8.8	18.5
30	133[e]	13.3[c]	13.9	11.4	2.1

Abbreviations: NL, neutral lipid; CHOL, cholesterol; TG, triglycerides.

[a]Concentrations were obtained by high temperature GLC analysis of a pooled sample containing an internal standard.

[b]Significantly different ($p \leq 0.1$) from day zero (student's t-distribution).

[c]Significantly different ($p \leq 0.05$) from day zero.

[d]Significantly different ($p \leq 0.025$) from day zero.

[e]Significantly different ($p \leq 0.005$) from day zero.

TABLE 6

Fatty Acid Composition of the Major Lipid Classes from Host Animal Hearts at Selected Time Intervals after Hepatoma Transplantation

Days after transplant	Fatty acid percentages[a]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
				Triglycerides			
0	27.7±2.0	8.3±2.9	4.2±0.4	38.8±1.0	18.0±4.8		
9	24.8	5.9	5.5	33.2	21.8		
18	26.5	5.7	4.7	37.4	23.8		
30	26.0	1.5	10.2	25.5	28.6		
				Phosphatidylcholines			
0	21.5±0.4	0.6±0.2	24.2±0.6	11.1±0.6	21.0±3.0	17.4±2.9	1.7±0.4
9	21.7	0.7	24.7	7.4	11.9	24.9	5.0
18	18.0	–	31.9	5.3	9.1	28.9	4.3
30	19.0	–	28.0	6.1	18.0	20.3	6.1
				Phosphatidylethanolamines			
0	9.8±0.1	0.5±0.1	26.4±4.1	8.4±0.7	13.3±1.4	22.8±0.8	12.2±1.3
9	10.0	–	24.1	4.3	9.8	18.6	24.5
18	9.6	–	24.5	5.1	7.7	19.7	26.9
30	10.9	–	21.3	3.5	7.8	20.1	29.9
				Phosphatidylinositols and phosphatidylserines			
0	6.9	–	46.3	10.2	5.5	21.8	5.7
9	7.4	–	38.2	8.0	6.2	24.0	11.2
18	6.4	–	44.0	7.7	5.1	22.6	11.3
30	5.1	–	46.5	7.2	4.5	21.6	11.6

[a]Differences between the sum of any row and 100% represents minor amounts of other fatty acids not given in the table. Values for day zero represent the mean of individual animals, whereas the other days represent the mean of duplicate analysis of a pooled sample from 4 animals.

noate levels to permit analysis of positional isomers at each time period, a pooled sample analysis indicated substantial amounts (25-30%) of other isomers than palmitoleic. Triglyceride hexadecenoates exhibited an increase in the $\Delta 7$ isomer as tumor growth progressed at the expense of palmitoleate. Comparisons of oleate to vaccenate percentages at selected time periods

for the major lipid classes (Table 4) indicate that heart oleate percentage increased in the phospholipids as tumor growth progressed, but not in the triglycerides.

Lung

Changes in lung lipid concentrations of the host animal as hepatoma growth progressed are shown in Table 7. Total phospholipids decreased through 9 days, whereas concentrations of PE began to increase after 9 days. Total neutral lipids decreased after hepatoma transplantation and remained low through the 30th day at which time the concentration was less than 40% of the initial level. Cholesterol (free) concentrations decreased dramatically during the first 6 days of tumor growth and remained at the low level for the duration, whereas triglyceride concentrations continued to fall through the growth period (except day 18). The fatty acid compositions of host lung lipid classes at selected times are given in Table 8. Hexadecenoate percentages in triglycerides and PE showed marginal decreases as tumor growth progressed, but generally the changes in the fatty acids of the lung lipid classes were minor compared to heart and liver. Palmitoleate represented between 87 and 94% of the hexadecenoate positional isomers in lung triglycerides for the first 12 days. The $\Delta 7$ hexadecenoate isomer represented 34% of lung PC at day zero and increased to 75% by day 15, whereas palmitoleate percentages decreased. A pooled hexadecenoate fraction from lung PE showed the $\Delta 7$ isomer represented 33% and the $\Delta 9$ isomer 60%. The $\Delta 6$ isomer represented 3-7% of the hexadecenoates in all lung classes at all times. The octadecenoate positional isomers at selected time intervals for the 3 major lipid classes of host lung are given in Table 4. Unlike heart and liver, oleate was the predominant isomer in all 3 lipid classes at all times and the proportion of oleate to vaccenate did not change in lung as tumor growth progressed.

DISCUSSION

Except for the analysis of hexadecenoate and octadecenoate positional isomers in heart and lung, all the analyses in this report were made at 3-day intervals, but to conserve space, only selected time periods are given. Generally, these time periods reflect the overall changes and trends.

Effect of Hepatoma on Host Organs

The decrease in heart dry weight beginning on the 12th day after hepatoma transplantation was probably due to loss of protein since total lipid changes were marginal relative to dry weight (Table 5). Since liver and lung dry weights were not determined, it is not known if the effect is organ specific. The decreases in heart size before the 18th day after transplantation suggest that the observed decreases are not related to nutritional stresses, but probably represent an effect of the hepatoma on the protein metabolism of the host animal. Some enzyme activities have been shown to change in livers of rats bearing hepatoma 7800 (22), but it is not known whether this represented altered protein metabolism or enzyme inhibition.

Effect of Hepatoma on Host Lipid Classes

TABLE 7

Concentration of Lung Lipids Obtained from Host Animal at Various Stages of Growth

Days after transplant	μg/mg of dry wt[a]					
	Total PL	PC	PE	Total NL	CHOL	TG
0	150 ± 5	82	19	107 ± 10	47	54
3	134 ± 7[b]	67	18	81 ± 23	35	43
9	128 ± 4[c]	53	26	57 ± 9[d]	26	27
18	132 ± 6[b]	64	29	85 ± 21	29	51
30	114 ± 7[c]	ND	ND	38 ± 10[c]	27	8

Abbreviations: PL, phospholipid; PC, phosphatidylcholines; PE, phosphatidylethanolamines; NL, neutral lipids; CHOL, cholesterol; TG, triglycerides; ND, not determined.

[a]Values with standard deviations were obtained from 4 animals. Other values were obtained from the analysis of pooled samples.

[b]Significantly different ($p \leq .025$) from day zero (student's t-distribution).

[c]Significantly different ($p \leq .010$) from day zero.

[d]Significantly different ($p \leq .005$) from day zero.

TABLE 8

Fatty Acid Composition of the Major Lipid Classes Isolated from Lungs of Animals Bearing Hepatomas at Various Stages of Growth

Days after transplant	Percentages[a]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
				Triglycerides			
0	26.6±0.7	7.2±1.1	3.2±0.5	37.0±1.4	21.6±1.6	T[b]	–
9	24.8	7.8	3.9	35.2	21.1	0.8	–
18	26.6	6.4	4.5	37.1	20.4	0.7	–
30	25.8	4.9	6.8	30.6	22.5	2.0	–
				Phosphatidylcholines			
0	60.8±3.1	9.8±0.5	4.8±0.4	9.2±0.1	6.0±1.0	3.9±0.4	T
9	58.7	11.0	4.3	8.6	6.5	2.1	T
18	53.8	10.2	5.8	10.7	6.8	4.5	T
30	56.1	9.5	6.1	9.5	9.3	2.0	–
				Phosphatidylethanolamines			
0	19.8±1.5	2.9±0.6	15.9±0.8	19.0±1.1	6.0±0.7	22.6±2.7	2.1±0.3
9	11.0	1.7	15.6	17.2	5.5	29.6	2.8
18	10.1	1.4	15.2	18.0	5.2	32.7	4.3
30	12.0	1.8	19.7	19.6	7.8	21.4	6.4

[a]Zero day values represent the mean of 4 animals analyzed individually. Values for the other time periods represent the mean of duplicate determinations of a pooled sample. The difference between the sum of percentages in any row and 100 represents the amount of other fatty acids not given in the table.

[b]T denotes detectable amounts of less than 0.5%.

All 3 host tissues exhibited changes in lipid class concentrations in the first few days after hepatoma transplantation, well in advance of any nutritional stresses. Changes have been observed in the neutral lipids and phospholipids of host mice livers bearing Ehrlich ascites cells (8), but the changes bear no similarities to those reported here. In contrast to liver neutral lipids, both heart and lung exhibited large decreases in the concentrations of cholesterol and triglycerides within a few days after hepatoma transplantation (Tables 5 and 7). These results make it clear that the hepatoma affects the lipid class concentrations in heart, liver and lung of host animal soon after transplantation.

Effect of Hepatoma on Host Fatty Acids

The decrease in the percentage of hexadecenoates in all the lipid classes of all the tissues, except lung PC (Tables 2, 6, 8), was the most consistent and dramatic change in the host fatty acids. Octadecenoate percentages also decreased as tumor growth progressed in some lipid classes, but the decreases were moderate and variable. Explanation for the relatively large progressive disappearance of the hexadecenoates beginning immediately after hepatoma transplantation, while octadecenoate decreases were marginal, are difficult to imagine. Immediately one assumes that the $\Delta 9$ desaturase system is inhibited, but the small decreases in the octadecenoates and the fact that a single enzyme appears to catalyze the desaturation of palmitate and stearate (23,24) do not support this conclusion. It is possible that the $\Delta 9$ desaturase system is partially inhibited in the host animal, but that tissue levels of octadecenoates are not altered significantly because tissue levels are maintained by mobilization of adipose tissue. A lipid mobilization factor has been reported in the serum of tumor-bearing animals (25), and stearoyl CoA desaturase activities of liver from hepatoma bearing animals has been reported to be reduced relative to normal liver (26,27). The lack of a fall in the octadecenoate levels similar to that observed for the hexadecenoates may result from the biosynthesis of oleate by an alternate route when the stearoyl-CoA desaturase system is inhibited in the host animal. Oleate levels of liver phospholipids are maintained when the $\Delta 9$ desaturase system is inhibited (28) and an alternate route for oleate biosynthesis has been proposed (28,29).

Effect of Hepatoma on Host Fatty Acid Positional Isomers

The decreased percentage of hexadeceno-

ates was also accompanied by a change in the positional isomers. Decreased palmitoyl-CoA desaturase activity and an unchanged chain shortening of oleate, the precursor of the Δ7 isomer (30), could explain the apparent increase in the percentage of the Δ7 isomer.

The octadecenoate positional isomer data demonstrate that the changes in the ratio of oleate to vaccenate that we had observed in the host liver phospholipid at the 30th day (12) were detectable by the 12th day after hepatoma transplantation in liver and heart, but not lung. Changes in the proportion of oleate to vaccenate can result from an increase in the concentration of oleate or a decrease in the level of vaccenate or both. Absolute concentrations of oleate and vaccenate found in the 3 major lipid classes from liver are shown in Table 4. Except for day 30, oleate concentrations of PE remained relatively constant. Triglyceride and PC oleate levels likewise remained relatively constant until the 9th-12th day of tumor growth, but increased significantly thereafter. In contrast, vaccenate concentrations exhibited a progressive decrease as tumor growth progressed. The decrease in the vaccenate concentrations can be attributed to the decrease in palmitoleate concentrations. The correlation between palmitoleate and vaccenate concentrations is 0.81 and 0.91 for triglycerides and PC, respectively, and are highly significant. Heart lipids exhibit a similar progressive decrease in vaccenate concentrations whereas oleate changes are only marginal, especially in the phospholipids. These data indicate that the decrease in vaccenate concentrations as tumor growth progresses results from a decrease in the precursor, palmitoleate (31,32), that probably results from a decrease in the Δ9 desaturase system activity, but we are stuck with the dilemma of explaining the unchanged to higher concentrations of oleate thought to arise from the same Δ9 desaturase system.

Early Changes in Host Lipids

Comparisons of the lipids of tumor-bearing animals with normal animals have generally been made just prior to death of the host animal. At this stage of tumor growth, any differences in the lipids can be attributed to nutritional stresses, as well as the tumor. This problem can be circumvented and the real effects of the tumor on the host animal lipids can be assessed if the comparisons are made in the early stages of tumor growth, preferably in the first half of the growth period while food consumption is normal. The present study shows a number of early changes in the lipids of the host animal. All 3 tissues exhibited some dramatic changes in lipid class concentrations within 6-9 days after hepatoma transplantations and many changes occurred as early as 3 days. These early changes are consistent with those observed earlier in the plasma lipids (14). The progressive decline in hexadecenoates, predominantly palmitoleate, beginning by the 6th day in all the lipid classes of heart and liver also agrees with that observed earlier in the plasma lipid classes (14). We had reported previously from this laboratory that the proportion of oleate to vaccenate changed significantly in the plasma phospholipids by the 6th day after hepatoma transplantation (13). The present study shows the changes in the proportions of oleate and vaccenate that occur early in heart and liver phospholipids results from a decrease in vaccenate concentrations and not an increase in oleate concentrations (Table 4).

These data demonstrate quite clearly that the hepatoma affects the lipid metabolism of the host animal early, well in advance of detectable tumor growth and nutritional stresses. Studies to determine what lipid metabolic systems are affected and the mechanism by which these early effects are exerted on the host animal are planned.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Research Grant No. CA-20136 from the National Cancer Institute.

REFERENCES

1. Haven, F.L., and Bloor, W.R. (1956) Adv. Cancer Res. 4, 237-314.
2. Carruthers, C. (1969) Oncology 23, 241-256.
3. Brenneman, D.E., Mathur, S.N., and Spector, A.A. (1975) Eur. J. Cancer 11, 225-230.
4. Kralovic, R.D., Zepp, E.A., and Cenedella, R.J. (1977) Eur. J. Cancer 13, 1071-1079.
5. Ruggieri, S., and Fallani, A. (1979) Lipids 14, 781-788.
6. Edmonson, J.H. (1966) Cancer 19, 277-280.
7. Neifakh, Y.A., and Lankin, V.Z. (1967) Biofizika 12, 1085-1086.
8. Lankin, V.Z. (1971) Biokhimiya 36, 1234-1238.
9. Einisman, L.I., Gorkova, N.P., and Dyatlovitskaya, E.V. (1977) Biokhimiya 42, 320-328.
10. Kitada, S., Mwasi, L., and Mead, J.F. (1978) Lipids 13, 464-467.
11. Wood, R., and Chumbler, F. (1978) Lipids 13, 75-84.
12. Wood, R., and Wiegand, R.D. (1979) Lipids 10, 746-749.
13. Mapes, J.P., and Wood, R. (1979) Lipids 14, 70-71.
14. Matocha, M., and Wood, R. (1980) Lipids 15, 421-427.

15. Wood, R., Zoeller, A., and Matocha, M. (1981) J. Am. Oil Chem. Soc. 58, 110A.
16. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
17. Borgstrom, B. (1952) Acta Physiol. Scand. 25, 101-110.
18. Wood, R., and Falch, J. (1974) Lipids 9, 979-986.
19. Rouser, G., Siakotos, A.M., and Fleischer, S. (1966) Lipids 1, 85-86.
20. Wood, R., Chumbler, F., Matocha, M., and Zoeller, A. (1979) Lipids 14, 789-794.
21. Wood, R., Lee, T., and Gershon, H. (1980) Lipids 15, 141-150.
22. deRosa, G., and Pitot, H.C. (1978) Cancer Res. 38, 950-954.
23. Pande, S.V., and Mead, J.F. (1970) J. Biol. Chem. 245, 1856-1861.
24. Howling, D., Morris, L.J., and James, A.T. (1968) Biochim. Biophys. Acta 152, 224-226.
25. Kitada, S., Hays, E.F., and Mead, J.F. (1980) Lipids 15, 168-174.
26. Lee, T., Stephens, N., and Snyder, F. (1974) Cancer Res. 34, 3270-3273.
27. Morton, R.E., Hartz, J.W., Reitz, R.C., Waite, B.M., and Morris, H.P. (1979) Biochim. Biophys. Acta 573, 321-331.
28. Wood, R., Chumbler, F., and Wiegand, R.D. (1978) Lipids 13, 232-238.
29. Raju, P.K., and Reiser, R. (1969) Biochim. Biophys. Acta 176, 48-53.
30. Wood, R. (1979) in Geometrical and Positional Fatty Acid Isomers (Emken, E.A., ed.) Chapter 9, pp. 213-282, AOCS Press, Champaign, IL.
31. Holloway, R.W., and Wakil, S.J. (1964) J. Biol. Chem. 239, 2489-2495.
32. Wiegand, R.D., and Wood, R. (1975) Lipids 10, 194-201.

[Received July 23, 1981]

Photosensitized Oxidation of Methyl Linolenate. Secondary Products

W.E. NEFF, E.N. FRANKEL* and **D. WEISLEDER**, *Northern Regional Research Center, Agricultural Research Service, US Department of Agriculture*[1], *1815 North University Street, Peoria, IL 61604*

ABSTRACT

Previous studies of secondary oxidation products by high-pressure liquid chromatography (HPLC) of autoxidized methyl oleate, linoleate and linolenate and photosensitized-oxidized linoleate are extended to photosensitized-oxidized linolenate. Photosensitized-oxidized linolenate was fractionated by silicic acid chromatography with diethyl ether/hexane mixtures. Selected silicic acid chromatographic fractions were separated by polar phase HPLC and characterized by thin layer and gas liquid chromatography and by ultraviolet, infrared, nuclear magnetic resonance and mass spectrometry. Secondary products from the photosensitized oxidation mixtures (containing 8.2 to 29.0% monohydroperoxides) included keto- and epoxy-dienes (0.4-1.6%), hydroperoxy epidioxides (0.8-4.9%), hydroperoxy bicyclic monoenes (0.1-0.3%), dihydroperoxides (1.0-5.6%), and hydroperoxy bis-epidioxides (0.7-1.6%). Some of these secondary products are new and unique to photosensitized oxidation. Cyclization of the 10-, 12-, 13- and 15-hydroperoxides of linolenate would account for their lower relative concentration than that found for the 9- and 16-hydroperoxides. Dihydroperoxides may be derived from monohydroperoxides by singlet oxygenation or free radical oxidation. The hydroperoxy bis-epidioxides may be formed by further serial cyclization of the hydroperoxy epidioxides from 10- and 15-monohydroperoxides. Dihydroperoxides, hydroperoxy epidioxides and hydroperoxy bis-epidioxides are suggested as important flavor precursors in oxidized fats.
Lipids 17:780-790, 1982.

High-pressure liquid chromatography (HPLC) has been used extensively to isolate and identify oxidation products (1) that contribute either directly or as precursors to the flavor deterioration of unsaturated lipids (2). Reverse-phase HPLC was used to separate and characterize the secondary oxidation products of autoxidized methyl oleate, linoleate and linolenate (3,4) after reduction with $NaBH_4$ or Ph_3P. Polar phase HPLC on microporous silica was used to separate directly and identify hydroperoxy epidioxides in autoxidized methyl linolenate (4,5) and photosensitized-oxidized linoleate (6,7), and hydroperoxy bicyclic endoperoxides in autoxidized linolenate (8).

This paper extends our previous structural studies to the secondary products of photosensitized-oxidized linolenate. Unique major products included dihydroperoxides, hydroperoxy epidioxides and hydroperoxy bis-epidioxides. Minor products included hydroperoxy bicyclic compounds and epoxy- and keto-dienes. The results support 1,3-cyclization of internal monohydroperoxide isomers (10-, 12-, 13-, 15-OOH) and account for their lower concentration relative to the external monohydroperoxide isomers (9-, 16-OOH). Dihydroperoxidation apparently involves all monohydroperoxides.

EXPERIMENTAL

Materials and Methods

The methyl linolenate (100% by GLC) used was described previously (9). Before each oxidation, any traces of oxidation products were removed from linolenate by silicic acid chromatography eluting with diethyl ether/hexane (10:90, v/v). The same Ph_3P-bonded styrene-divinyl benzene copolymer was used to reduce monohydroperoxides (4). For dihydroperoxides which were more difficult to reduce, $NaBH_4$ (9) was used prior to mass spectrometry (MS). Monohydroxy compounds were silylated with bis(trimethylsilyl) trifluroacetamide. The silylating reagent was blown off with dry N_2 and the residue was dissolved in dry CS_2. The procedure for silylation of polyhydroxy compounds for gas chromatography-mass spectrometry (GC-MS) was reported previously (10). Catalytic hydrogenation was conducted by bubbling H_2 at atmospheric pressure for 15 min through an ethanol solution of sample with PtO_2 (5% by wt) in a 15 x 45 mm vial. The catalyst was removed by filtration through celite.

*Author to whom correspondence should be addressed.

[1]The mention of firm names or trade products does not imply that they are endorsed by the US Department of Agriculture over other firms or similar products not mentioned.

Oxidations

Photosensitized oxidations were carried out with 2-mg methylene blue per g of methyl linolenate in CH_2Cl_2 solution at 0 C by bubbling O_2 in an open tube exposed to a 1000-Watt air-cooled tungsten light source, shined through a 1-in. layer of water to filter infrared (IR) radiation. Oxidation was followed by TLC and peroxide value determinations (AOCS method Cd 8-53).

HPLC

Oxidized samples were first separated on a silicic acid chromatographic column eluting with diethyl ether-hexane mixtures as previously reported (4). Hydroperoxy epidioxide and bis-epidioxide fractions were further separated by HPLC using a refractive index detector and sample size of ca. 40 mg on a 100 x 0.94 cm 10 μm silica column (Magnum 9, Partisil 10, Whatman, Inc., Clifton, NJ) at 20 C. Solvent systems were hexane/CH_2Cl_2/ethyl acetate (7:4:1, v/v/v) pumped at 4 ml/min for hydroperoxy epidioxides and 5:3:1, v/v/v at 7 ml/min for hydroperoxy bis-epidioxides. The fraction containing dihydroperoxides and hydroperoxy bicyclic compounds was separated on a 25.0 x 2.12 cm 6 μm column (Zorbax Sil, Dupont, Analytical Instruments Division, Wilmington, DE) using 3% absolute ethanol in hexane, v/v at 20 ml/min. A variable wavelength ultraviolet (UV) detector was used, with range set at 0.64 ASU and wavelength at 215 nm until 10,16-dihydroperoxy octadecatrienoate was eluted. The last conjugated triene components were detected at 268 nm. Keto- and epoxy-dienes were identified by GC-MS either directly or after hydrogenations. The keto-dienes after hydrogenation were silylated and identified as the silyl ethers (9).

Characterization

The oxidation products were characterized by GC, TLC, UV, IR, nuclear magnetic resonance (NMR), MS and GC-MS as described previously (3,11). To elute the silylated pentahydroxy derivatives of the hydroperoxy bis-epidioxides, the GC temperature program required an upper temperature held at 250 C for 15 min.

RESULTS

Samples of methyl linolenate photooxidized in the presence of methylene blue to different peroxide values (PV) were fractionated by silicic acid column chromatography. Various oxidation product mixtures were thus separated and identified by TLC on the basis of functional groups by comparison with previously characterized compounds (4,6). New compounds that are identified later include hydroperoxy bis-epidioxides, hydroperoxy bicyclic endoperoxides and keto-dienes. Quantitative weight analyses show that the relative amounts of secondary products decrease in the order: dihydroperoxides, hydroperoxy epidioxides, hydroperoxy bis-epidioxides, keto-/epoxy-dienes and hydroperoxy bicyclic endoperoxides (Table 1).

Samples of photooxidized linolenate were further analyzed for isomeric hydroperoxides by GC-MS (9) at various peroxide levels. This analysis confirms previous results (12-14) in showing a consistently higher ratio of 9- (20-23%) and 16-hydroperoxides (22-29%) than of

TABLE 1

Weight-Percent Composition of Silicic Acid Fractions from Methyl Linolenate Photosensitized-Oxidized at 0 C

Compounds	Peroxide value (me/kg)[a]			
	800	1956	2720	3031
	Weight, %			
Linolenate	87.8	65.5	59.0	55.2
Keto- and epoxy-esters	0.4	1.6	1.1	1.0
Monohydroperoxides[b]	8.2	25.6	26.4	29.0
Hydroperoxy epidioxides	0.8	2.2	3.1	4.9
Hydroperoxy bicyclic endoperoxides	0.1	0.1	0.2	0.3
Dihydroperoxides	1.0	3.0	5.3	5.6
Hydroperoxy bis-epidioxides	0.7	1.0	1.6	1.2
Unidentified polar material	1.0	1.0	3.3	2.8

[a]Oxidation times: 5, 9, 10, and 10.5 hr, respectively.

[b]9 + 10 + 12 + 13 + 15 + 16-OOH (12).

the 10-, 12-, 13- and 15-hydroperoxides (ranging from 12-16% each) at all peroxide levels studied between 317 and 3031.

Hydroperoxy Epidioxides

A concentrate was first obtained by silicic acid column chromatography eluting the hydroperoxy (OOH) epidioxide mixture with a diethyl ether/hexane (40:60 to 50:50, v/v) gradient. The epidioxide mixture was then separated by HPLC on a microporous silica column (Fig. 1) into fractions containing either positional or diastereoisomers of hydroperoxy epidioxy dienes, as shown in Figure 2. The following relative weight percent composition was estimated: A-1 plus A-2, 3%; B, 34%; C, 36%; D, 14%; and E, 13%. Compounds B-E have *cis, trans*-diene conjugation. No hydroperoxy epidioxides with *trans,trans*-diene conjugation were detected.

TLC (silica, hexane/CH_2Cl_2/ethyl acetate, 7:4:1, v/v/v) Rf A: 0.36 relative to linolenate, UV inactive and B, C, D, E: 0.35, 0.32, 0.26, 0.23 relative to linolenate, UV positive, all compounds peroxide positive. GC of hydrogenated-silylated derivatives have the same respective retentions as 10,12,13-trihydroxystearate for A-1, 12,13,15-trihydroxystearate for A-2, 9,10, 12-trihydroxystearate for B, D, and 13,15,16-trihydroxystearate for C, E, relative to methyl stearate. UV (methanol) B: 233 (E_m 20100), C: 233 (E_m 24800), D: 234 (E_m 21600), E: 234 nm (E_m 26200). IR (CS_2) A-E: 3520 (free C-OOH), 3660-3150 (bonded C-OOH), 3002-3020 (olefinic-H) and A: 960 (isolated *trans* unsaturation) and B-E: 980-988, 947-952 (conjugated *cis,trans* unsaturation) cm^{-1}.

The NMR data support a 5-membered ring and the general hydroperoxy epidioxy diene structures for A-E (4,6,7) (Fig. 2). ^{1}H-NMR shows absorption at 1.00 ppm for the terminal methyl group attached to the methylene group of A-1 and 1.78 ppm for the terminal methyl

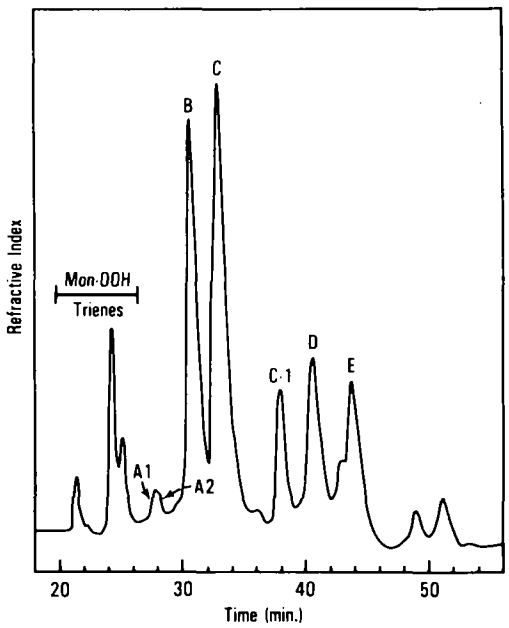


FIG. 1. 10-μm silica HPLC chromatogram of hydroperoxy epidioxide mixture from linolenate photosensitized-oxidized at 0 C in CH_2Cl_2, methylene blue, PV 3031 (flow 4.0 ml/min; mobile hexane/CH_2Cl_2/ethyl acetate, 7:4:1, v/v/v; refractive index detector X8; column temperature 20 C).

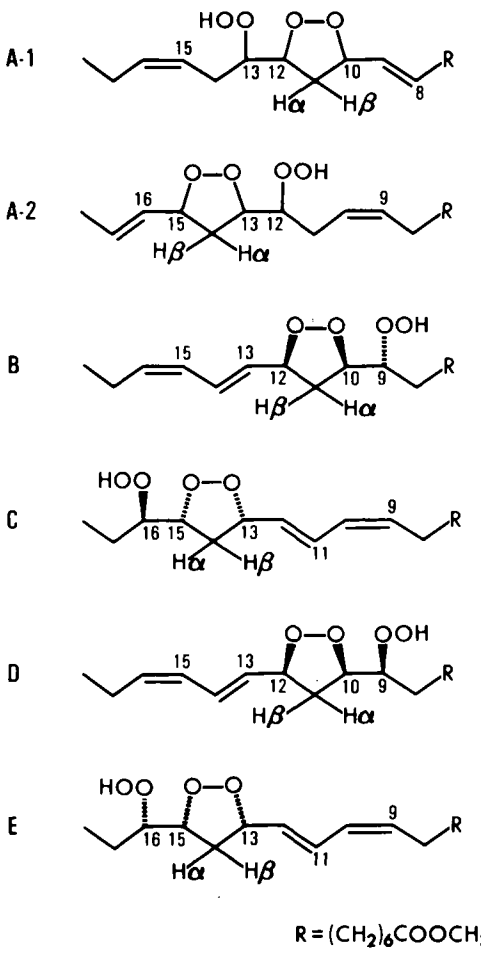
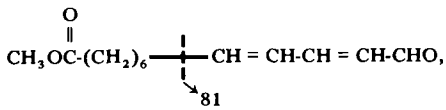


FIG. 2. Structures of hydroperoxy epidioxide fractions isolated by HPLC (see Fig. 1). Each structure shown consists of pairs of enantiomers and only one isomer is shown.

group attached to the vinyl group of A-2. For B, C, D and E, a *trans* coupling constant J = 11, 15 Hz was found due to the double bond adjacent to the ring and a *cis* coupling constant J = 8 Hz was found due to the double bond adjacent to the alkyl or ester portion of the hydroperoxy epidioxide, thus confirming the IR evidence for conjugated *cis,trans* unsaturation. The stereochemical relationship between the hydroperoxy group relative to the adjacent epidioxide (Fig. 2) is supported by the different chemical shifts of the hydroperoxy-bearing carbond methine proton for B (4.18 ppm), C (4.10 ppm), D (3.90 ppm) and E (3.85 ppm) compared to the related mono *trans* unsaturated cyclic peroxides identified in photosensitized oxidized linoleate (6,7). The chemical shift difference for the ring methylene α proton between diastereoisomers B (2.44 ppm) and D (2.13 ppm) and between C (2.47 ppm) and E (2.13 ppm) reflects the different shielding effect of the hydroperoxy group due to its rotation with respect to the peroxide ring. The ^{13}C-NMR spectra showed a chemical shift for the ring methylene carbon as the only significant difference between diastereoisomeric pairs B (41.4 ppm), D (42.4 ppm) and C (41.4 ppm), and E (43.5 ppm).

Further identification of the positional isomers was based on MS of the silylated hydrogenated and Ph_3P-reduced hydroperoxy epidioxides. GC-MS of the silylated hydrogenated mixture A-1 and A-2 produced 2 GC peaks with the same fragment ions as 10,12,13-triOTMS-stearate (7) and 12,13,15-triOTMS-stearate, m/e (rel. intensity); M-(90 + 15): 457 (0.93), C-12: 301 (41.63), C-15: 145 (100), respectively. After reduction with Ph_3P, the molecular related peaks were very weak: M-32: 380 (0.07) and M-(90 + 15): 307 (0.34). Cleavage at the carbon-bearing OTMS gave for the 13-hydroperoxy epidioxide (A-1) C-13: 171 (19.1) and ring-containing fragment 241 (0.5), and for the 12-hydroperoxy epidioxide (A-2) C-12: 299 (16.4) and ring containing fragment: 113 (3.2). For B, C, D, and E, the mass spectra were similar to those reported previously for methyl 9-hydroperoxy-10,12-epidioxy-*trans*-13,*cis*-15-octadecadienoate (B,D) and for 16-hydroperoxy-13,15-epidioxy-*cis*-9,*trans*-11-octadecadienoates (C,E) (4).

Other compounds eluting before the hydroperoxy epidioxides (Fig. 1) were identified as monohydroperoxides by ^{1}H-NMR and GC-MS of the silylated-hydrogenated derivatives (12). Fraction C-1 eluting between peaks C and D (Fig. 1) contained a mixture of aldehyde esters. IR (CS_2) 2718 (aldehyde C-H), 1735 (ester carbonyl), 1698 (aldehyde carbonyl) 970, 948 (conjugated *cis,trans* unsaturation) cm^{-1}. ^{1}H-NMR indicated at least two aldehydic compounds, 9.54 and 9.52 ppm (CHO). GC-MS, m/e (rel intensity) indicated a mixture consisting of methyl 9-oxononanoate 158 (M-28, 11.2) and 155 (M-31, 5.3) and of methyl 12-oxo-8,10-dodecadienoate, 224 (M, 6.2), 81 (100):



These aldehyde esters are apparently decomposition products from cleavage at the hydroperoxy carbon (9-oxo-nonanoate) and between carbons 12 and 13 (12-oxo-8,10-dodecadienoate) of the 9-hydroperoxy-10,12-epidioxy diene (7,15). The two fractions eluting after peak E (Fig. 2) are unidentified but are not hydroperoxy epidioxides.

Dihydroperoxides

A concentrate was first obtained by silicic acid chromatography eluting with a diethyl ether/hexane (50:50 to 60:40, v/v) gradient. The dihydroperoxide mixture was characterized by UV (methanol), 234 (E_m 13795); 256, 267, 279 nm (E_m, 267 nm, 8032) for conjugated diene and triene, respectively. GC-MS, after hydrogenation and silylation, indicated a mixture of dihydroperoxides with one OOH scattered between C-9 and C-13 on the one hand, and between C-12 and C-16 on the other hand. The dihydroperoxide mixture was then separated by HPLC on a microporous silica column (Fig. 3), and the identified dihydroperoxides are listed in Figure 4. Peaks G and I, H and J, L-1 and L-2, M, N, and P, O and Q are due to diastereoisomeric pairs, but their absolute configuration could not be assigned without suitable reference compounds. Weights of HPLC fractions in Figure 3 indicated the 9,16-dihydroperoxy conjugated triene represented 35 to 38% of the total in samples of PV 1798 to 2720 (me/kg).

TLC, GC and UV data for the dihydroperoxides are summarized in Table 2. All TLC spots were peroxide positive. TLC did not separate the two dihydroperoxide components of F. UV showed conjugated diene for F-K and conjugated triene for M-Q, with L containing two dihydroperoxides with conjugated diene and triene, respectively. IR (CS_2) F-Q: 3520-3510 (free C-OOH), 3700-3140 (bonded C-OOH), 3020-3010 (olefinic-H); F-L: 985-979, 953-935 (conjugated *cis,trans* unsaturation) 968-960

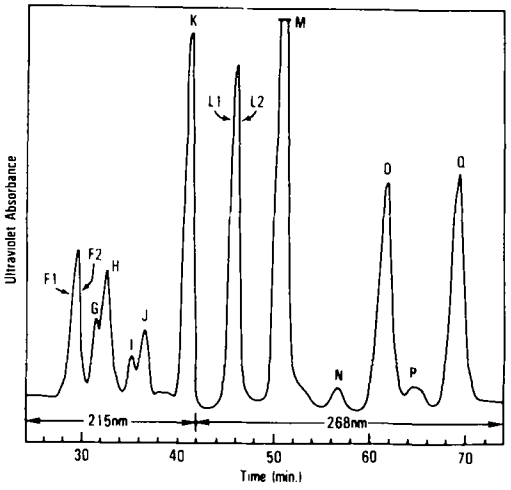


FIG. 3. 6-μm silica HPLC chromatogram of dihydroperoxides from linolenate oxidized at 0 C in CH_2Cl_2, methylene blue, PV 1798 (flow 20 ml/min; mobile 3% ethanol/hexane, v/v; UV detector 0.64 ASU with 215 nm for 42 min, then 268 nm; column temperature 20 C).

(isolated *trans* unsaturation); L, M, N, P: 960 (strong) [conjugated *trans,cis,trans* (*t,c,t*)-triene as in *t*-8,*c*-10,*t*-12-octadecatrienoic acid (16)]; O,Q: 996-991 (strong) [conjugated *trans,trans, trans* unsaturation as in β-eleostearate (*t*-9,*t*-11, *t*-13-octadecatrienoate), (17)] cm^{-1}.

^{1}H-NMR assignments in Table 3 for H,J,L-1 show the terminal methyl group bonded to a vinyl group, 1.80-1.76 ppm, and for F-1, F-2, G, I, K, L-2, M-Q, the terminal methyl group

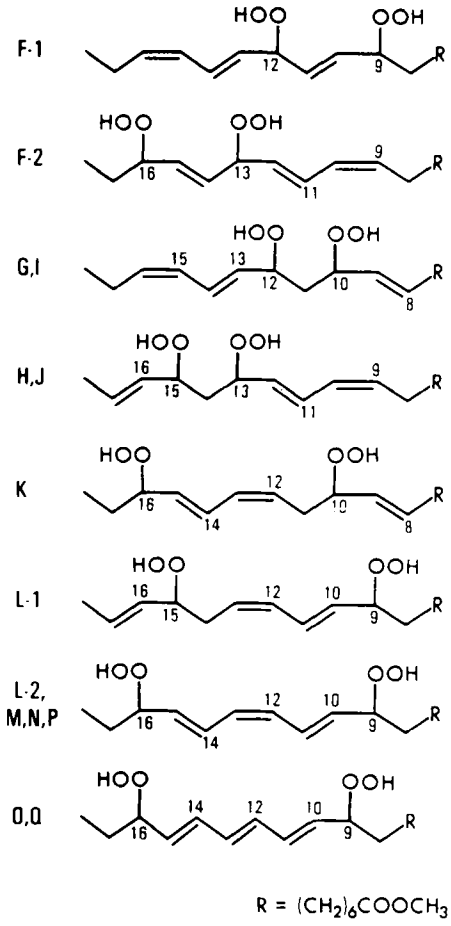


FIG. 4. Structures of dihydroperoxide fractions isolated by HPLC (see Fig. 3).

TABLE 2

Chromatographic and Ultraviolet Data of Dihydroperoxides (Figs. 3 and 4)

HPLC fractions	TLC[a] Rf	GC[b] α	UV[c] λ nm	UV[c] Em
F	0.47	1.95, 2.03	233	30,100
G	0.46	2.01	233	29,600
H	0.47	2.01	235	26,500
I	0.44	2.01	231	29,800
J	0.46	2.01	234	25,800
K	0.51	2.01	232	27,100
L	0.50, 0.46	2.12, 2.16	237, 258, 268, 279	14,300; 23,700
M	0.47	2.16	259, 268, 278	51,000
N	0.44	2.16	259, 268, 278	38,400
O	0.45	2.16	260, 268, 280	52,300
P	0.41	2.16	259, 268, 279	42,700
Q	0.39	2.16	258, 268, 279	58,000

[a]TLC: (silica, diethyl ether/hexane, 60:40, v/v), Rf relative to linolenate.

[b]GC: hydrogenated-silylated derivative; α: peak retentions relative to stearate.

[c]UV: (methanol); Fraction L: Em at 237 and 268 nm; Fractions M-Q, Em at 268 nm.

TABLE 3

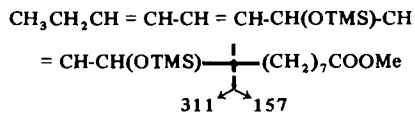
^{1}H-NMR of Dihydroperoxides (Figs. 3 and 4)

HPLC fractions	δ ppm (Multiplicity[a], proton, assignment)
F (F-1, F-2)	8.07 (br s, 2, OOH), 5.80-5.45 (m, 6, H-10, 11, 13, 15, 16, 17/H-9, 10, 11, 12, 14, 15), 4.86, 4.42 (m, 2, H-9, 12/H-13, 16), 3.66 (s, 3, CO_2CH_3), 2.30 (m, 2, H-2), 2.2-1.8 (m, 4, H-8, 17), 1.02 (t, 3, CH_3-C).
G	8.05, 8.02 (s, 2, OOH), 6.60 (m, 1, H-14), 6.15-5.25 (m, 5, H-8, 9, 13, 15, 16), 4.49 (m, 2, H-10/12), 3.66 (s, 3, CO_2CH_3); 2.31 (m, 2, H-2), 2.2-1.8 (m, 6, H-7, 11, 17), 1.01 (t, 3, H-18).
H	8.08, 7.99 (s, 2, OOH), 6.63 (m, 1, H-11), 6.20-5.25 (m, 5, H-9, 10, 12, 16, 17), 4.50 (m, 2, H-13, 15), 3.66 (s, 3, CO_2CH_3), 2.32 (m, 2, H-2), 2.1-1.9 (m, 6, H-8, 14), 1.8 (m, 3, H-18).
I	8.05 (br s, 2, OOH), 6.63 (m, 1, H-14), 6.1-5.3 (m, 5, H-8, 9, 13, 15, 16), 4.51 (m, 2, H-10, 12), 3.66 (s, 3, CO_2CH_3), 2.5-2.0 (m, 8, H-2, 7, 11, 17), 1.10 (t, 3, H-18).
J	8.04, 7.94 (s, 2, OOH), 6.63 (m, 1, H-11), 6.15-5.25 (m, 5, H-9, 10, 12, 16, 17), 4.48 (m, 2, H-13, 15), 3.66 (s, 3, CO_2CH_3), 2.31 (m, 2, H-2), 2.2-1.8 (m, 6, H-8, 14), 1.8 (m, 3, H-18).
K	8.04 (br s, 2, OOH), 6.55 (m, 1, H-14), 6.25-5.25 (m, 5, H-8, 9, 12, 13, 15), 4.34 (m, 2, H-10, 16), 3.66 (s, 3, CO_2CH_3), 2.31 (m, 2, H-2), 2.08 (m, 6, H-7, 11, 17), 0.93 (t, 3, H-18).
L (L-1, L-2)	7.88, 7.83 (s, 2, OOH), 6.90-5.25 (m, 6, H-10, 11, 12, 13, 16, 17/H-10, 11, 12, 13, 14, 15), 4.40 (m, 2, H-9, 15/H-9, 16), 3.66 (s, 3, CO_2CH_3), 2.31 (m, 2, H-2), 1.85-1.75 (m, 4, H-8, 14/H-8, 17), 1.8 (m, 3, CH_3 C = C), 0.95 (t, 3, CH_3-C).
M	7.83 (br s, 2, OOH), 6.75, 6.05, 5.68 (m, 6, H-10, 11, 12, 13, 14, 15), 4.42 (m, 2, H-9, 16), 3.66 (s, 3, CO_2CH_3), 2.34 (m, 2, H-2), 1.85-1.75 (m, 4, H-8, 17), 0.95 (t, 3, H-18).
N	7.90, 7.77 (s, OOH), 6.80-5.27 (m, H-10, 11, 12, 13, 14, 15), 4.38 (m, H-9, 16), 3.66 (s, CO_2CH_3), 2.37 (m, H-2), 1.85-1.75 (m, H-8, 17), 0.95 (t, H-18).
O	7.75 (br s, 2, OOH), 6.27, 5.75 (m, 6, H-10, 11, 12, 13, 14, 15), 4.35 (m, 2, H-9, 16), 3.66 (s, 3, CO_2CH_3), 2.37 (m, 2, H-2), 1.85-1.50 (m, 4, H-8, 17), 0.95 (t, 3, H-18).
P	7.94 (br s, OOH), 6.85-5.25 (m, H-10, 11, 12, 13, 14, 15), 4.35 (m, H-9, 16), 3.66 (s, CO_2CH_3), 2.30 (m, H-2), 1.85-1.75 (m, H-8, 17), 0.95 (t, H-18).
Q	7.75 (br s, OOH), 6.27, 5.75 (m, H-10, 11, 12, 13, 14, 15), 4.34 (m, H-9, 16), 3.66 (s, CO_2CH_3), 2.34 (m, H-2), 1.80-1.70 (m, H-4), 0.94 (t, H-18).

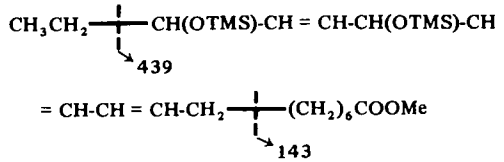
[a]Multiplicity: br = broad, s = singlet, m = multiplet, t = triplet.

bonded to methylene, 0.90-0.95 ppm. The ^{1}H-NMR could not distinguish the diastereomeric pairs G,I; H,J; L-2, M and O,Q.

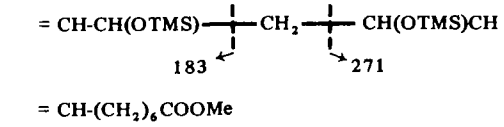
Further identification of the positional isomers was based on MS of the hydrogenated and $NaBH_4$ reduced dihydroperoxides. Hydrogenation of F-Q yielded diOH-stearates, identified by MS after silylation as 9,12-(F1); 13,16-(F2); 10,12-(G); 13,15-(H); 10,12-(I); 13,15-(J); 10,16-(K); 9,15-(L-1); and 9,16-(L-2, M, N, O, P, Q)-diOTMS-stearates. Reduction of F-M, O, Q with $NaBH_4$ yielded diOH-octadecatrienoates identified by MS, m/e (rel intensity) F-1: 453 (M-15, 0.6), 437 (M-31, 1), 378 (M-90, 2), 311 (21), 157 (7.3), 311-90 = 221 (3.5):



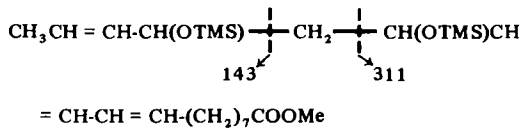
F-2: 453 (M-15, 0.1), 437 (M-31, 0.1), 378 (M-90, 0.5), 439 (13), 143 (37), 439-90 = 349 (0.2):



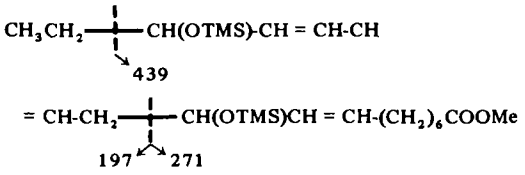
G: 453 (M-15, 0.7), (M-31, 0.7), 378 (M-90, 5.3), 183 (23), 271 (35), I: 468 (M, 0.4), 378 (M-90, 3.7), 183 (11), 271 (27):



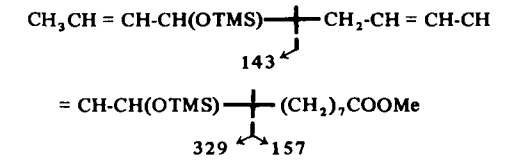
H: 498 (M, 0.3), 437 (M-31, 0.4), 387 (M-90, 0.4), 143 (100), 311 (1.8); J: 453 (M-15, 0.1), 387 (M-90, 0.7), 143 (100), 311 (3.2):



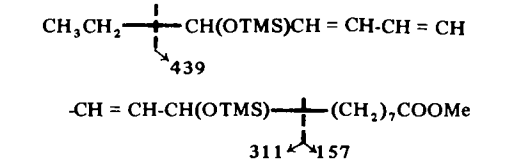
K: 468 (M, 0.2), 437 (M-31, 0.2), 378 (M-90, 8.6), 439 (0.2), 197 (2.5), 271 (26), 439-90 = 349 (4.2):



L-1: 143 (100), 329 (0.3), 157 (2.6):



L-2: 468 (M, 0.1), 453 (M-15, 0.2), 437 (M-31, 0.1), 439-90 = 349 (0.8), 311 (0.6); M: 468 (M, 1.4), 453 (M-15, 1.3), 437 (M-31, 1.9), 378 (M-90, 48), 311 (8.0), 311-90 = 221 (50), 439 (1.4), 439-90 = 349 (17), 157 (36); O: 378 (M-90, 1), 311 (0.3), 311-90 = 229 (0.3), 439-90 = 349 (0.3), 157 (7.7); Q: 468 (M, 0.1), 453 (M-15, 0.3), 437 (M-31, 0.3), 378 (M-90, 12), 311 (3), 311-90 = 221 (16), 439 (0.1), 439-90 = 349 (5), 157 (9):



Hydroperoxy bis-Epidioxides

These compounds were first concentrated by silicic acid chromatography eluting with a diethyl ether/hexane (70:30, v/v) mixture. The hydroperoxy bis-epidioxide mixture was further resolved by HPLC on microporous silica (Fig. 5) into 4 major components consisting of positional and diastereoisomeric isomers (Fig. 6) with the following composition: 9-OOH-10, 12,13,15-bis-epidioxy-*trans*-16-octadecenoate, R (32%), T (14%) and 16-hydroperoxy-10,12,13, 15-bis-epidioxy-*trans*-8-octadecenoate, S (37%), U (17%).

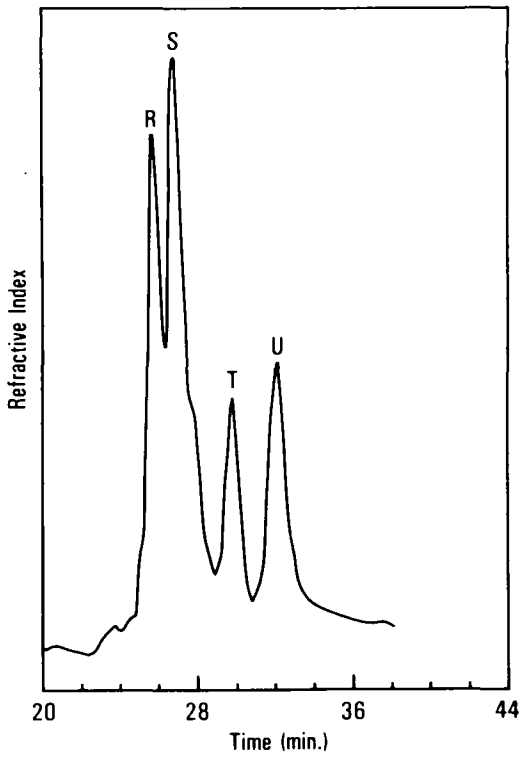


FIG. 5. 10-μm silica HPLC chromatogram of hydroperoxy bis-epidioxide mixture from linolenate photosensitized-oxidized at 0 C in CH_2Cl_2, methylene blue, PV 3031 (flow 7 ml/min; mobile hexane/CH_2Cl_2/ethyl acetate, 5:3:1, v/v/v; refractive index detector X8; column temperature 20 C).

TLC (silica, diethyl ether/hexane, 1:1, v/v) showed Rf relative to linolenate 0.26 (R, T) and 0.20 (S, U), UV negative and peroxide positive. GC, hydrogenated-silylated derivatives, peak retentions relative to stearate, gave a double peak 3.35 (R, T) and 3.58 (S, U). IR (CS_2) for all isomers: 3530 (free C-OOH), 3700-3150 (bonded C-OOH), 960 (isolated *trans* unsaturation) cm^{-1}. ^{1}H-NMR (Table 4) spectra were consistent with the assigned structures of bis-epidioxide shown in Figure 6. The double bond in R-U contains a *trans* coupling constant confirming the IR evidence for a *trans*

double bond. The positional isomers could be distinguished by the vinyl methyl absorptions (1.73 ppm) for R and T and absorptions due to terminal methyl bonded to methylene (1.05 ppm) for S and U. The diastereomers could be differentiated by the chemical shifts of protons on carbons bearing the hydroperoxy groups with 4.17 ppm for R and S and 3.88 ppm for T and U. The stereochemical relationship between the hydroperoxide group in R-U (Fig. 6) relative to the adjacent epidioxide was established by the ^{1}H-NMR data on retention volume order relative to the hydroperoxy epidioxides identified in photooxidized linoleate (7). However, the relative ring stereochemistry was deduced only on steric grounds and from examination of models.

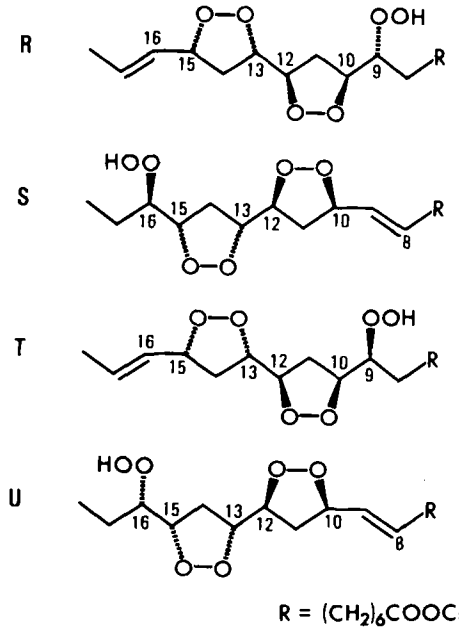
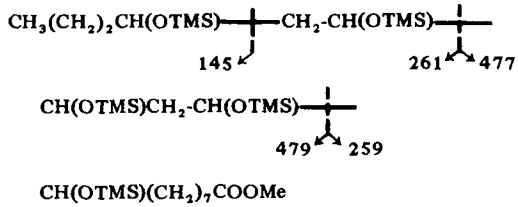


FIG. 6. Structures of hydroperoxy bis-epidioxides isolated by HPLC (see Fig. 5). Each structure consists of a pair of enantiomers and only one isomer is shown.

Further identification of the positional isomers was based on MS of the hydrogenated and Ph_3P reduced hydroperoxy bis-epidioxides. Hydrogenation of R and T produced 9,10,12, 13,15-pentaOH-stearate identified by MS (10) after silylation, m/e (rel intensity) R: 145 (100), 261 (4), 261-90 = 171 (3), 477-90 = 387 (82), 477-180 = 297 (23), 479-90 = 389 (41), 479-180 = 299 (24), 259 (43) and T: 145 (100), 261 (3), 261-90 = 171 (2), 477-90 = 387 (66), 477-180 = 297 (13), 479-90 = 389 (28), 479-180 = 299 (16), 259 (32):



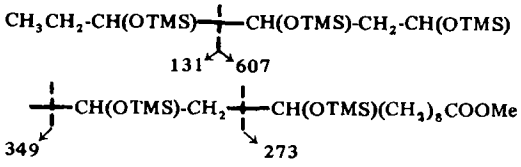
Hydrogenation of S and U produced 10,12,13, 15,16-pentaOH-stearate identified by MS (10) after silylation, m/e (rel intensity) S: 131 (40), 607-90 = 517 (4), 607-180 = 427 (13), 349 (2), 349-90 = 259 (78), 349-180 = 159 (5), 273 (100) and U: 131 (41), 607-90 = 517 (2), 607-180 = 427 (8), 349 (1), 349-90 = 259 (80), 349-180 = 169 (6), 273 (100):

TABLE 4

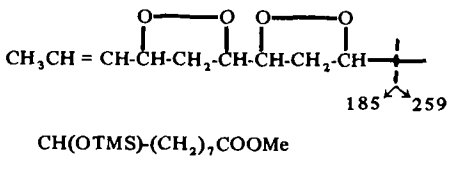
^{1}H-NMR of Hydroperoxy Bis Epidioxides (Figs. 5 and 6)

HPLC fractions	δ ppm (Multiplicity[a], proton, assignment) JHz
R	9.20 (s, 1, OOH), 5.88 (dd, 1, H-16) J: 15.1, 6.2; 5.35 (m, 1, H-17), 4.47 (m, 4, H-10, 12, 13, 15), 4.17 (m, 1, H-9), 3.66 (s, 3, CO_2CH_3), 2.1-2.8 (m, 4, H-11, 14), 2.30 (t, 2, H-2), 1.73 (dd, 3, H-18) J: 5.8, 1.5; 1.31 (m, CH_2).
S	9.20 (s, 1, OOH), 5.88 (dd, 1, H-9) J: 15.1, 6.4; 5.35 (m, 1, H-8), 4.47 (m, 4, H-10, 12, 13, 15), 4.17 (m, 1, H-16), 3.66 (s, 3, CO_2CH_3), 2.1-2.8 (m, 4, H-11, 14), 2.30 (t, 2, H-2), 1.31 (m, CH_2), 1.05 (t, 3, H-18).
T	8.73 (s, 1, OOH), 5.88 (dd, 1, H-!6) J: 15.1, 6.2; 5.35 (m, 1, H-17), 4.47 (m, 4, H-10, 12, 13, 15), 3.88 (m, 1, H-9), 3.66 (s, 3, CO_2CH_3), 2.1-2.8 (m, 4, H-11, 14), 2.30 (t, 2, H-2), 1.73 (dd, 3, H-18) J: 5.8, 1.5; 1.31 (m, CH_2).
U	8.73 (s, 1, OOH), 5.88 (dd, H-9), J: 15.1, 6.4; 5.35 (m, 1, H-8), 4.47 (m, 4, H-10, 12, 13, 15), 3.88 (m, 1, H-16), 3.66 (s, 3, CO_2CH_3), 2.1-2.8 (m, 4, H-11, 14), 2.30 (t, 2, H-2), 1.31 (m, CH_2), 1.05 (t, 3, H-18).

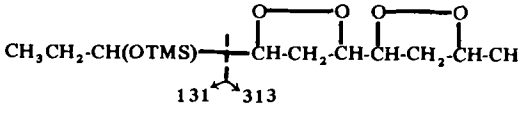
[a]Multiplicity: br = broad, s = singlet, d = doublet, m = multiplet.



Reduction of R and T with Ph_3P produced the hydroxy bis-epidioxide derivatives identified by MS after silylation R: 259 (58), 185 (11) and T: 259 (54), 185 (12):



Reduction of S and U with Ph_3P produced the hydroxy bis-epidioxide derivatives identified by MS after silylation S: 131 (96), 313 (4) and U: 131 (53), 313 (2):

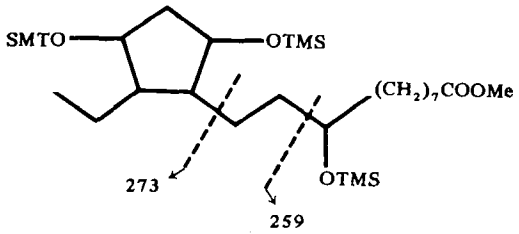


Hydroperoxy Bicyclic Endoperoxides

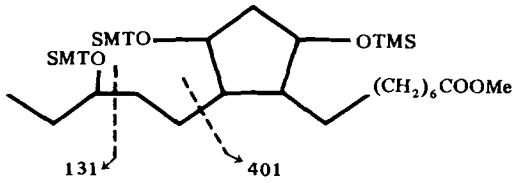
Small peaks eluting before dihydroperoxides by HPLC on microporous silica were identified as epimeric mixtures of hydroperoxy bicyclic endoperoxides similar to those reported by O'Connor et al. (18).

TLC (silica, hexane/diethyl ether, 1:1, v/v) showed one spot, UV inactive and peroxide positive, centered at Rf 0.46 relative to linolenate. GC, hydrogenated-silylated derivative, gave peak retentions for each fraction 2.80 and 2.90 relative to stearate. IR (CS_2) all HPLC fractions: 3520 (free C-OOH), 3620-3010 (bonded OOH), 960 (isolated *trans* unsaturation) cm^{-1}. The ^1H-NMR was consistent with data reported by O'Connor et al. for the linolenate 9-hydroperoxy bicyclic endoperoxide (8).

Further identification of the pair of positional isomers in the epimeric mixtures was based on MS of the hydrogenated hydroperoxy bicyclic endoperoxides. MS after silylation, m/e (rel intensity) 545 (M-15, 0.3), 455 [M-(90 + 15), 0.4], 259 (5), 273 (22):



and 545 [(M-15, (0.7)], 455 [M-(90 + 15), (0.2)], 131 (32), 401 (3):



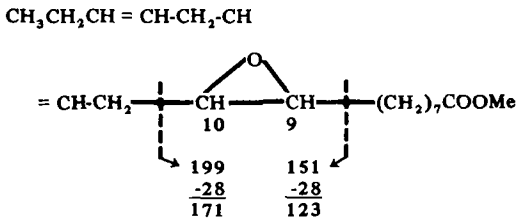
This trihydroxy ester is derived from the 16-hydroperoxy bicyclic endoperoxide found in our mixture and not reported by O'Connor et al. (8) because they started with the 13-hydroperoxide isomer of linolenate.

Keto-Dienes

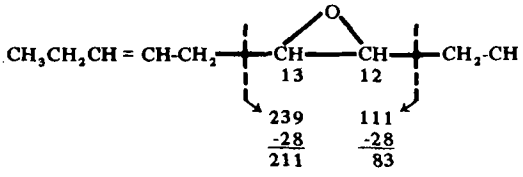
A silicic acid chromatographic fraction eluted with the first 50 ml of a diethyl ether/hexane (20:80, v/v) gradient was identified as a mixture of keto-dienes. TLC (silica, diethyl ether/hexane, 1:1, v/v) Rf relative to linolenate 0.98, UV active and peroxide negative. GC-MS indicated a mixture of 9-, 10-, 12-, 13-, 15- and 16-keto-dienes (20) m/e (rel intensity): 308 (M, 3.8), 290 (M-18, 2.6); 151 (5.1), 185 (3), (9-keto-diene); 137 (40), (10-keto-diene); 111 (14), (12-keto-diene); 259 (18), (13-keto-diene), 71 (15), (15-keto-diene); 57 (26), (16-keto-diene). Hydrogenation produced a mixture of 9-, 10-, 12-, 13-, 15- and 16-hydroxystearates identified by MS after silylation (12).

Epoxy-Dienes

A silicic acid chromatographic fraction eluted with the last 50 ml of a diethyl ether/hexane (20:80, v/v) gradient contained a mixture of epoxy-dienes. TLC (silica, diethyl ether/hexane, 1:1, v/v), Rf relative to linolenate 0.89-0.95, UV and peroxide negative. GC-MS before hydrogenation indicated the presence of epoxy-dienes (18), m/e (rel intensity) (308, 0.24), 277 (M-31, 1), 199 (2), 171 (0.9), 151 (2.2), 123 (6):

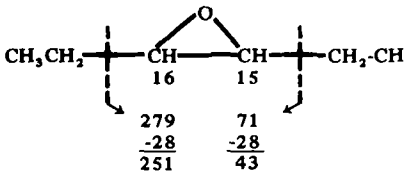


211 (10), 83 (24):



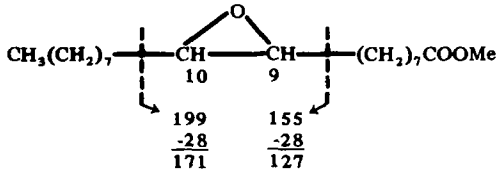
= CH-$(CH_2)_7$COOMe

71 (16), 43 (48), 279 (0.1), 251 (0.1):

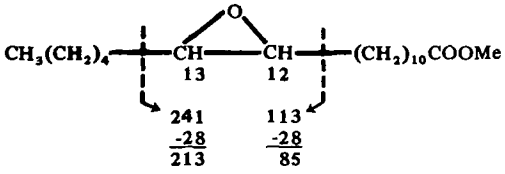


= CH-CH_2-CH = CH-$(CH_2)_7$COOMe

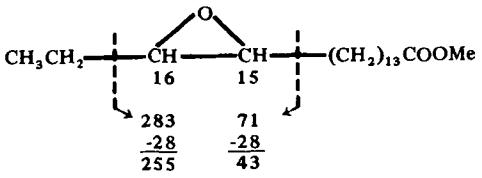
GC-MS after hydrogenation indicated the presence of epoxy stearates (18), 281 (M-31, 1.1), 199 (3), 171 (2.1), 155 (11), 127 (13):



241 (1.2), 213 (1.9), 113 (6.2), 85 (12):

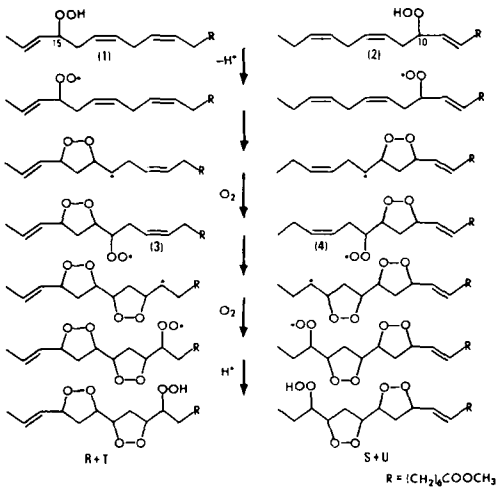


71 (52), 43 (36), 255 (0.4):



DISCUSSION

In the present study, samples of methyl linolenate photooxidized in the presence of methylene blue were analyzed for isomeric hydroperoxides at a wider range of oxidation levels than previously reported (12). The results confirm previous analyses in showing significantly higher levels of the external 9- and 16-hydroperoxides than of the internal 10-, 12-, 13- and 15-hydroperoxides. These uneven isomeric distributions can be explained in the same was as done for autoxidized methyl linolenate (4) by the tendency of the internal hydroperoxide isomers to cyclize due to their homoallylic structure. The major portion (97%) of the hydroperoxy epidioxides identified in the present work are those derived from the cyclization of the 12- and 13-hydroperoxides (B, C, D, E, Fig. 2), also identified in autoxidized methyl linolenate (4,5). The corresponding epidioxides (A-1 and A-2, Fig. 2) expected from cyclization of the 10- and 15-hydroperoxide from radicals 3 and 4 (Scheme 1) were found only in minor amounts (3%). The reason for this difference in relative concentration is



SCHEME 1. Mechanism for the serial cyclization of hydroperoxy epidioxides in photosensitized-oxidized linolenate.

that the hydroperoxy epidioxides from the 10- and 15-hydroperoxides tend to cyclize again to form the hydroperoxy bis-epidioxides R, S, T, U (19) (Scheme 1). Serial cyclization has been suggested in the autoxidation of polyunsaturated lipids on the basis of model studies with a 1,5-diene system producing 6-membered bis-cyclic peroxides (20). The present work shows, however, that the required precursors for bis-epidioxides are present only in photosensitized-oxidized methyl linolenate (10- and 15-isomers) and *not* in autoxidized methyl linolenate (12- and 13-isomers). Methyl arachidonate and other fatty esters containing *more than three* double bonds do produce internal isomeric hydroperoxides (21,22) that would be expected to undergo serial cyclization.

Dihydroperoxides were also identified in this work as a major secondary product of methyl linolenate photosensitized-oxidized with methylene blue. The 9,12-, 13,16- and 9,16-dihydroperoxides may be formed from the 9- and 16-hydroperoxides by the same mechanisms suggested for autoxidized methyl linolenate that proceeds through pentadienyl radicals (4). The remaining dihydroperoxides identified in this work are the 1,3-disubstituted (13,15 and 10,12) and 1,7-disubstituted (9,15 and 10,16). These dihydroperoxides are presumed to come from the corresponding monohydroperoxides by concerted addition of singlet oxygen:

9-OOH → 9,12- + 9,15- + 9,16-diOOH
10-OOH → 10,12- + 10,16-diOOH
12-OOH → 9,12- + 10,12-diOOH
13-OOH → 13,15- + 13,16-diOOH
15-OOH → 9,15- + 13,15-diOOH
16-OOH → 9,16- + 10,16- + 13,16-diOOH

The hydroperoxy bicyclic endoperoxides identified in this work were minor secondary products. The 9-hydroperoxy bicyclic endoperoxide isomer derived from 13-hydroperoxide is the same as that identified by O'Connor et al. (8) from the autoxidation of the corresponding enzymatically produced hydroperoxide. The 16-hydroperoxy-bicyclic endoperoxide isomer was derived from the 12-hydroperoxide, presumably by the same mechanism suggested by O'Connor et al. (8). Other identified minor secondary products include the epoxy esters that may be formed by 1,2-cyclization of the monohydroperoxides (4) and the keto esters that may be formed by dehydration of the corresponding monohydroperoxides.

The possible contribution of secondary products of oxidation to flavor deterioration of unsaturated fats has been discussed previously (1,2,7,15). Hydroperoxy cyclic peroxides from photosensitized-oxidized methyl linoleate were previously shown to contribute important volatile products similar to those formed by thermal decomposition of monohydroperoxides (7). The other secondary products identified in this work also may be important sources of volatile decomposition products from photooxidized fats.

ACKNOWLEDGMENT

We thank E. Selke for analytical mass spectrometry.

REFERENCES

1. Frankel, E.N. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 141-170, Plenum Press, New York, NY.
2. Frankel, E.N. (1980) Progr. Lipid Res. 19, 1-22.
3. Neff, W.E., Frankel, E.N., Scholfield, C.R., and Weisleder, D. (1978) Lipids 13, 415-421.
4. Neff, W.E., Frankel, E.N., and Weisleder, D. (1981) Lipids 16, 439-448.
5. Coxon, D.T., Price, K.R., and Chan, H., W.-S. (1981) Chem. Phys. Lipids 28, 365-378.
6. Mihelich, E.D. (1980) J. Am. Chem. Soc. 102, 7141-7143.
7. Frankel, E.N., Neff, W.E., Selke, E., and Weisleder, D. (1982) Lipids 17, 11-18.
8. O'Connor, D.E., Mihelich, E.D., and Coleman, M.C. (1981) J. Am. Chem. Soc. 103, 223-224.
9. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garword, R.F., and Weedon, B.C.L. (1977) Lipids 12, 1055-1061.
10. Dommes, V., Wirtz-Peitz, F., and Kunau, W-H. (1976) J. Chromatogr. Sci. 14, 360-366.
11. Frankel, E.N., Rohwedder, W.K., Neff, W.E., and Weisleder, D. (1975) J. Org. Chem. 40, 3247-3253.
12. Frankel, E.N., Neff, W.E., and Bessler, T.R. (1979) Lipids 14, 961-967.
13. Neff, W.E., and Frankel, E.N. (1980) Lipids 15, 587-590.
14. Terao, J., and Matshushita, S. (1980) J. Food Process. Preserv. 329-337.
15. Frankel, E.N., Neff, W.E., and Selke, E. (1981) Lipids 16, 279-285.
16. Mikolajczak, K.L., Bagby, M.O., Bates, R.B., and Wolff, I.A. (1965) J. Org. Chem. 30, 2983-2988.
17. Crombie, L., and Jacklin, A.G. (1957) J. Chem. Soc. 1632-1646.
18. Kleiman, R., and Spencer, G.F. (1973) J. Am. Oil Chem. Soc. 50, 31-38.
19. Frankel, E.N., Neff, W.E., and Weisleder, D. (1982) J. Chem. Soc. Chem. Commun. 599-600.
20. Porter, N.A., Roe, A.N., and McPhail, A.T. (1980) J. Am. Chem. Soc. 102, 7574-7576.
21. Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) J. Am. Chem. Soc. 103, 6447-6455.
22. Terao, J., and Matshushita, S. (1981) Agric. Biol. Chem. 45, 587-593, 595-599.

[Received May 7, 1982]

Cholesterol Metabolism in Gnotobiotic Gerbils

KENNETH F. BARTIZAL, JR.*, MARGARET H. BEAVER, and
BERNARD S. WOSTMANN[1], *Department of Microbiology, University of Notre Dame, Notre Dame, IN 46556*

ABSTRACT

Germfree gerbils were associated with a murine-derived hexaflora which produced only minor changes in the primary bile acid pattern of rats. These hexaflora-associated gerbils had relatively small ceca (4% of body weight) and reproduced well. Although serum cholesterol levels of both conventional and hexaflora-associated gerbils increased in response to dietary cholesterol, the hexaflora-associated gerbil showed a greater elevation in serum cholesterol than the conventional gerbil when maintained on a diet containing 0.1% cholesterol. This increase in serum cholesterol manifested itself almost totally in the very low density lipoprotein and low density lipoprotein fractions. The fecal bile acids of the hexaflora-associated gerbil were largely deconjugated, but very little further modification of either cholic or chenodeoxycholic acid had taken place. The data suggest that in the absence of elements of the intestinal microflora that can express a bile acid-modifying potential, and particularly a 7-α-dehydroxylating capacity, catabolism of cholesterol to bile acids is reduced, and cholesterol accumulates in the very low density and low density serum lipoprotein fractions.
Lipids 17:791-797, 1982.

Serum cholesterol levels rise in the gerbil and man when their dietary cholesterol and fat is increased (1). The rat appears to be a less satisfactory model for studies of the effects of dietary cholesterol on serum cholesterol and bile acid (BA) metabolism because its serum cholesterol concentration rises relatively little in response to dietary cholesterol (2). The rat metabolizes additional dietary cholesterol, increasing the muricholic acids and their secondary derivatives which are less well reabsorbed than cholic acid and its derived BAs (3). The gerbil, however, is quite sensitive to dietary cholesterol and early work comparing germfree (GF) and conventional (CV) gerbils has indicated a microflora can lower serum cholesterol concentration (4).

A major focus in the metabolism of cholesterol obviously is on its catabolic conversion to BAs. Gerbils and man have similar BA patterns, with cholic and chenodeoxycholic acids as their primary BAs (4). In the CV state, various components of the intestinal microflora are able to deconjugate, dehydroxylate, and oxidize the primary BAs, resulting in the production of a variety of secondary BAs. These modifications generally enhance excretion of BAs, thereby increasing cholesterol catabolism. The ensuing decrease in the concentration of various BAs in the enterohepatic circulation (5,6) will also reduce cholesterol absorption to a limited extent. While the effect of a CV microflora on intestinal BAs has proven to be substantial (7-11), there is only limited documentation on the ability of specific bacteria to metabolize BAs in vitro or in vivo. The in vitro 7-α-dehydroxylation of BAs appears to be mainly due to species of bacteriodes and lactobacilli (11), while clostridia, enterococci and various gram-negative organisms have been shown to deconjugate BAs and form keto-acids (8). However, the actual microorganisms involved in modification of bile acids in vivo have remained obscure. Because of the importance of the microflora effect, gnotobiotic (GN) gerbils with defined floras have been used to study the influence of selected microbial components on specific BA modifications.

Germfree gerbils were obtained (4) for baseline studies on the effect of intestinal bacteria on cholesterol and BA metabolism. Due to excessive cecal enlargement, they failed to reproduce. Germfree gerbils were then associated with a murine-derived "hexaflora" (12). In rats, this microflora left the primary BA pattern largely intact; only 6% cholic acid-derived secondary BAs were present in their feces. In the gerbil, this same hexaflora led to a substantial reduction in cecal size. These gerbils were able to reproduce and a colony of hexaflora-associated gerbils (hexa-gerbils) was established. Thus, the objectives of this study were to obtain functionally normal GN gerbils for studies on the microbial factors that affect cholesterol and bile acid metabolism and to

*Author to whom correspondence should be addressed.

[1]Please send reprint requests to Dr. Bernard Wostmann at address in byline.

Abbreviations: BA, bile acid; GF, germfree; CV, conventional; GN, gnotobiotic; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

compare the effect of dietary cholesterol on serum cholesterol, serum lipoproteins and fecal bile acids in these GN gerbils and in gerbils harboring a CV microflora.

MATERIALS AND METHODS

Experimental Animals

Adult male hexaflora-associated and CV Mongolian gerbils (*Meriones unguiculatus*) were used. The CV gerbils originated from Tumblebrook Farms, West Brookfield, MA. The hexaflora-associated animals were the offspring of caesarian-derived GF gerbils.

Germfree gerbils were obtained by removing the young from the uterus by sterile surgical procedures. Cesarian sections were performed after the dam had delivered one baby naturally in order to ensure being as close to normal parturition and development as possible. The newborn gerbils were transferred into an attached breeder isolator containing GF lactating female C3H/He mice and their newborn litters. Preferably, two foster mothers were made available. Part of one litter was replaced by no more than three newborn gerbils.

Hexaflora-associated gerbils were originally obtained by introducing a male and female GF gerbil into an isolator containing C3H/He mice associated with a hexaflora consisting of *Lactobacillus brevis, Streptococcus faecalis, Enterobacter aerogenes, Staphylococcus epidermidis, Bacterioides fragilis* var. *vulgatus*, and a *Fusobacterium* sp. The gerbils were housed in cages on bedding used by the mice. All six of these microbial species originally derived from rats or mice, became established in the hexaflora gerbils. These gerbils will be called "hexa-gerbils." In order to broaden this colony's genetic base, four additional caesarian operations were performed introducing additional baby gerbils when a female gerbil was nursing a new litter of her own. This microflora was monitored regularly by established microbiological procedures.

All gerbils were housed in plastic shoebox cages in Trexler-type plastic isolators, following established gnotobiotic procedures (13). The CV gerbils were housed in the same type plastic cages, under filter tops, in a temperature and humidty controlled animal room. All were maintained on autoclaved diet L477E4 or L477E5. Both diets are based on natural ingredient rat diet L485 (14). Diet L477E4 contained 60% L485, 36% Quaker Oats, 1.8% corn oil, 0.6% each of K- and Mg-acetate, and 0.09% inositol and represents a cholesterol-free, low fat diet. Diet L477E5 contained 58% L485, 35% Quaker Oats, 5.2% coconut oil, 0.1% cholesterol and K- and Mg-acetate and inositol as before and represents a moderate cholesterol, moderate saturated fat diet.

Cholesterol and Lipoproteins

A micromethod (2), based on the cholesterol assay of Caraway (15) was used to follow total serum or plasma cholesterol in CV and GN male gerbils. After overnight fasting, the gerbil was placed in a jar and exposed to halothane (Ayerst Laboratories, Chicago, IL). Blood was obtained by orbital bleeding using a micro-blood-collecting tube containing heparin (No. B3095-2; Scientific Products, McGaw Park, IL) and alternating eyes at each sampling. These and other samples were always collected between 8:00 and 10:00 A.M.

To determine very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL), blood was collected by cardiac puncture since large volumes of blood were needed for lipoprotein-cholesterol determination. The animals were starved overnight, anesthetized with ether, and exsanguinated. The blood was allowed to clot, centrifuged at 2000 x g for 20 min at 4 C, and serum was separated from cells with a Pasteur pipette. Cholesterol content of these fractions and total cholesterol content were determined as described above. The lipoprotein fractions were separated by microultracentrifugation after staining with Fat Red 7B stain (Beckman Instruments, Inc., Fullerton, CA) (16).

Bile Acids

Bile acids were determined after solvolysis and, where pertinent, were separated into the conjugated and nonconjugated BAs, via thin layer and gas liquid chromatography. Sulfated BAs were included in the total bile acid analysis.

Feces were collected from male gerbils over a 24 hr period and frozen. Free and conjugated fecal bile acids were determined by the method of Madsen et al. (5) based on the method of Grundy et al. (17) after desalting (18) and solvolysis according to Eyssen et al. (19).

Organs and Tissues

Conventional and gnotobiotic male gerbils used for lipoprotein-cholesterol determination were sacrificed as mentioned above. Whole body weights and weights of heart, liver, lungs, small intestine, large intestine, cecum, kidneys, thymus, spleen, adrenals and testes were recorded. Representative sections of small intestine from CV and GN gerbils were obtained. The contents were removed and the wet

weights of the wall determined. The samples were then dried in a vacuum oven for 48 hr at 60 C and were weighed immediately to determine the dry weights. Sections of Peyer's patches and spleen for histological evaluation were excised from the CV and GN gerbils, and immediately placed in Bouin's fixative. After overnight fixation, the tissues were dehydrated through increasing concentrations of ethyl alcohol. The tissues were imbedded in paraffin, sectioned and stained with hematoxylin and eosin.

Bacteriology

Bacteria were isolated from portions of either the stomach, mid-small intestine or cecum of male hexa-gerbils to determine the distribution of the hexaflora components. Gerbils were sacrificed by ether inhalation. The gastrointestinal tract was tied off, surgically removed, weighed and transferred to an anaerobic glove box. The samples were diluted in the proper amount of prereduced dilution fluid to make a 1:100 dilution and the wall plus contents were homogenized and serial dilutions were made. One mL of each dilution was inoculated onto the surface of selective growth media. Bacterial counts are expressed as the number of bacteria per gram homogenate wet weight. Isolates were characterized and identified on the basis of growth characteristics on the selective media, microscopic colony appearance, morphology and Gram stain reaction.

Since the *Fusobacterium* sp. failed to grow in any prereduced media using our anaerobic techniques, a direct microscopic determination of these bacteria was performed by the Breed milk count method (20).

RESULTS AND DISCUSSION

A major aspect of the metabolism of cholesterol is its catabolic conversion to BAs. The modification of primary BAs by an intestinal microflora generally enhances excretion of BAs, thereby, increasing catabolism of cholesterol (7-11). When the CV gerbil is compared to either the GF or the hexa-gerbil, the serum cholesterol-lowering effect of the CV microflora becomes obvious. This effect is much more apparent with the GF gerbil than with the GF rat (Table 1). The serum cholesterol-lowering effect of a limited microflora is also less obvious with the hexa- than with the GF gerbil, but in the latter case, becomes much more pronounced upon feeding a moderate cholesterol, moderate saturated-fat-containing diet. The data in Table 1 generally show a much greater serum cholesterol concentration in the gerbil compared to the rat when they are fed moderate cholesterol, moderate saturated-fat-containing diets. Table 2 demonstrates that the CV gerbils' microflora decreases the VLDL and LDL cholesterol fractions in serum.

The enhanced fecal BA elimination usually accompanying (and supposedly causing) the lowering of body cholesterol pools by a CV microflora (21), was not found in the present study. The hexa-gerbil excretes substantially more BA (as cholic acid) on cholesterol-free, low fat diet L477E4 than the CV gerbil which excretes BA mainly as deoxycholic acid (Table 3). This indicates that on diet L477E4 more

TABLE 1

Comparison of Serum (Plasma) Cholesterol in Male Rats and Gerbils, 3-6 Months Old

	Diet	Dietary cholesterol	mg cholesterol/dl ± SE		
			Germfree	Hexaflora	Conventional
Rat	L485[a]	–	104 ± 3	ND[b]	95 ± 4
	L488F[c]	0.05%	109 ± 4	ND	101 ± 4
	L490[d]	0.10%	ND	ND	120 ± 6
Gerbil	L485[a]	–	149 ± 3[e]	ND	74 ± 2[e]
	L477E4[f]	–	ND	102 ± 3	86 ± 3
	L477E5[f]	0.10%	ND	249 ± 8	170 ± 4
	L490[d]	0.10%	ND	ND	271 ± 31

[a]L485 Natural ingredient diet (14).

[b]ND: not done.

[c]L488F Casein-starch diet (26).

[d]L490 Casein-starch, coconut oil added (2).

[e]Wostmann et al. (12).

[f]L477:L485 and Quaker oats (see Materials and Methods).

TABLE 2

Serum Lipoproteins of Conventional and Hexaflora Male Gerbils[a]

	mg cholesterol/dl ± SE			
	VLDL	LDL	HDL	Total
Hexa[b]	77 ± 2[c]	136 ± 7[c]	26 ± 1	239 ± 8[c]
Conv.[b]	50 ± 1	120 ± 1	22 ± 1	174 ± 2

[a]Diet L-477E5 (0.1 cholesterol).

[b]Age: 6-8 months, 8 observations/group.

[c]Significantly different from conventional gerbils, $p < 0.01$.

endogenous cholesterol is catabolized to BA by the hexa-gerbil than by the CV gerbil. However, we also found much higher intestinal weights in the hexa-gerbil than in the CV gerbil (Table 4). Dry wt determination confirmed that this weight increase was due to tissue mass, not an increase in tissue hydration, while histological observation indicated normal cell size. Apparently this combination of murine-derived organisms or the absence of other intestinal bacteria results in intestinal tissue hyperplasia in the gerbil. Since the liver and the gut wall are considered the major producers of endogenous cholesterol, this increase in intestinal tissue

TABLE 3

Bile Acids (μg/day ± SE[a]) in the Feces of Hexa- and Conventional Gerbils on Cholesterol Free (L477E4) and Cholesterol-Containing (L477E5) Diets

Fecal bile acids	Hexa-gerbil		Conventional	
	L477E4(7)[c]	L477E5(6)	L477E4(8)	L477E5(8)
3-OH bile acids	85[1] ± 23	134[1] ± 88	154[2] ± 21	518 ± 86
Deoxycholic acid	111[1,3] ± 36	176[1,3] ± 15	2709[2] ± 158	6246 ± 355
Chenodeoxycholic	321[1,3] ± 31	333[1,3] ± 43	tr	0
Cholic acid	4587[1,2,3] ± 246	6710[1,3] ± 251	166 ± 48	125 ± 8
Bile acid 110/120[b]	0[1,3]	0[1,3]	106[2] ± 20	223 ± 34
Keto-acids	201[1,3] ± 38	271[1,3] ± 52	382 ± 36	354 ± 51
Total, mg/day	5.3[1,3] ± 0.2	7.7 ± 0.2	3.5[2] ± 0.2	7.5 ± 0.4

[a]Different superscripts (1, 2, and 3) in rows indicate significantly different values.
1: Significantly different than conventional gerbils on same diet.
2: Significantly different than L477E5 (0.1% cholesterol) fed gerbils.
3: Significantly different than conventional gerbils on either diet.

[b]Bile acid with GLC-RT relative to cholic acid: 110 on SE-30, 120 on QF-1.

[c]Number of observations ( ).

TABLE 4

Organ Weights of Hexaflora-Associated and Conventional Gerbils (4-7 Month Males)[a]

Organ weight (mg) per 100 g body weight	Organ weights (mg)[b]		
	Hexa-gerbil (9)[c]	Conventional (10)	P
Heart (mg)	408 ± 25	452 ± 27	0.08
Liver	3750 ± 164	3897 ± 172	0.36
Lung	476 ± 28	515 ± 37	0.21
Small intestine	2414 ± 169	1348 ± 84	< 0.01
Large intestine	789 ± 63	545 ± 47	< 0.01
Thymus	90 ± 17	142 ± 15	< 0.01
Spleen	71 ± 11	88 ± 21	0.30
Cecum (% body weight)	4.6 ± 0.4	1.9 ± 0.2	< 0.01

[a]Diet L477E4.

[b]Mean ± SE.

[c]Number of observations ( ).

mass could enhance cholesterol production to the extent that in the hexa-gerbil, both high serum cholesterol levels and high fecal acid sterol excretion are observed. The apparent cholesterol-lowering effect of the CV microflora could, therefore, be explained at least in part by the smaller amount of intestinal tissue of the CV animal.

When fed moderate cholesterol, moderate saturated-fat-containing diet L477E5, fecal BA excretion of the hexa-gerbil increases by 2.3 mg and of the CV gerbil by 4.0 mg, to become 7.7 and 7.5 mg/day, respectively. Both hexa- and CV gerbils consumed 5-6 g of diet per day, containing 5-6 mg cholesterol. The excretion data indicate that feedback mechanisms have now reduced hepatic cholesterol production, especially in the hexa-gerbil, to levels resulting in comparable amounts of cholesterol being catabolized to BA. However, even under these conditions, serum VLDL, LDL and total cholesterol values in the hexa-gerbil appear to be much higher than in the CV gerbil (Table 2), suggesting a relative reduction in cholesterol catabolism via BAs in the absence of the modifying elements of the CV intestinal microflora (Table 2).

Not much information is available on the influence of specific microbial components on BA metabolism in vivo. In vitro, 7-α-dehydroxylation of BAs appears to be carried out mainly by bacteriodes and lactobacillus species (11). A great variety of microorganisms such as clostridia, enterococci and a number of gram-negative organisms are able to deconjugate BAs and form keto-acids. But the species of microorganisms that are involved is still obscure.

The gastrointestinal and fecal bacterial flora of the CV gerbil was examined by Majumdar and Carroll (22). They found that lactobacilli were the most prominent type in the gastrointestinal tract and feces. Anaerobic clostridia, enterococci and flavobacteria were also present in large numbers. The bacteroides were not the most prevalent organisms in the CV gerbil's gastrointestinal tract. Coliforms were present in high numbers in feces and all parts of the gastrointestinal tract. Except for the prevalence of bacteroides and greater numbers of *S. epidermidis* in hexa-gerbils, the number and location of hexaflora bacteria were similar in the intestinal tracts of CV and hexa-gerbils. Bacteroides, which are the second most predominant organism present in hexa-gerbils, occupied the same niche as clostridia in CV gerbils. Large numbers of bacteroides in the gastrointestinal tract are not to be considered normal if one compares gerbils and mice. In mice, bacteroides are present predominantly in the lower intestinal tract.

The data in Table 5 indicate that, although deconjugation of BA by the hexaflora is almost complete, little further modification of BA occurs (also Table 3). Bile acids which are deconjugated but not further modified might have the effect of enlarging the gut by stimulating a large increase in mucosal cell turnover. Rankin et al. showed an enhanced mucosal cell turnover when deconjugated cholic acid was fed to mice (23). The large amount of deconjugated cholic acid present in hexa-gerbils may be responsible for the increase in hexa-gerbil intestinal weight (Table 4). On the other hand, the absence of certain beneficial bacteria from the hexaflora may also account for the increased intestinal weight. The predominant anaerobes in the CV gerbil's gastrointestinal tract play an important role in the maintenance of its physiological structure and function. In the hexa-gerbil, the absence of such a protecting micro-

TABLE 5

Primary Bile Acid Content and Percent Deconjugation of Fecal Bile Acids of Germfree, Hexaflora-Associated and Conventional Gerbils in Relation to Primary Bile Acid Production

Bile acids	% Bile acids		
	Germfree[a]	Hexa-gerbil[b]	Conventional[b]
Primary BA	100	92	5
Deconjugated BA	< 2	90	97
Deconj. Chenodeoxy	0	92	0
Deconj. Cholic	0	88	81
Cholic + deriv. mg/dl	0.57	4.90	3.26
Chenodeoxy + deriv. mg/dl	0.31	0.41	0.16

[a]Diet L485.

[b]Diet L477E4.

flora may lead to abnormal ecological niches and colonization patterns that can exert pressure on the physiological structure of the gastrointestinal tract.

Although lactobacilli and bacteroides were found to be predominant in all portions of the gastrointestinal tract of the hexa-gerbil, these organisms appear to carry out only very limited 7-α-dehydroxylation of primary BA. The present hexaflora appears remarkable in that it deconjugates primary BA almost as extensively as a CV microflora, but 7-α-dehydroxylates to only a minor extent (Table 5). In most cases, as in conventional animals, it has been found that deconjugation and subsequent dehydroxylation and ketone formation go hand in hand (7,11). In CV gerbils, 7-α-dehydroxylation of cholic acid is almost complete (Table 3). But the hexa-gerbil demonstrates that deconjugation may occur with little further modification of the bile acids. The hexaflora components (numerically mostly lactobacilli, bacteroides and enterococci) are able to deconjugate at least 90% of the bile acids but subsequent 7-α-dehydroxylation of cholic acid (3α,7α,12α-OH) and chenodeoxycholic acid (3α,7α-OH) to their secondary derivatives, deoxycholic acid (3α, 12α-OH) and lithocholic acid (3α-OH) is quite limited (Table 5). This agrees with the aforementioned in vitro data showing the ability of enterococci and some gram-negative organisms to deconjugate bile acids. However, the in vitro data do not agree with the apparent inability of bacteroides and lactobacilli, together with the other elements of this hexaflora, to effectively dehydroxylate at the 7-α position in vivo (11). The recent addition of a *Clostridium* sp., due to an accidental contamination to the hexaflora, resulted in moderate 7-α-dehydroxylation, but rather pronounced keto-acid formation (data not shown).

The hexa-gerbil's cecum (4.6% body weight) is much smaller than that of its GF counterpart (15% body weight). The smaller cecum probably allows the hexa-gerbil to reproduce. The heart of the hexa-gerbil, as in other GN rodents, tends to be smaller than that of the CV gerbil (24,25). However, when comparing CV gerbils to hexa-gerbils, both small and large intestines weighed substantially more and the thymus significantly less in hexa-gerbils (Table 4). The increased intestinal tissue weight, together with the lower weight of the thymus of the hexa-gerbil, suggests a certain incompatibility of this murine-derived microflora with the gerbil host. Histology showed that inflammatory reactions did not cause the significant increases in intestinal weight. Thus, the increase in intestinal weight observed in the hexa-gerbil did not appear to be caused by a pathological condition, especially since no infiltration of mononuclear cells into the intestinal tissue was observed, and the spleen demonstrated a decidedly "germfree" appearance. These observations suggest that the increased intestinal weight may be more the result of the absence of elements of a CV microflora, rather than the presence of specific bacterial components of this hexaflora.

In conclusion, the combination of the six microbial associates of the hexa-gerbil substantially reduced cecal enlargement. The hexa-gerbil caused almost total deconjugation of primary BA, but minimal dehydroxylation and keto-acid formation. When the gerbils were fed 0.1% cholesterol and a moderate saturated-fat diet, serum cholesterol levels remained substantially higher in the hexa-gerbil than in the CV gerbil. Obviously, other microorganisms must be added to this hexaflora to obtain "normal" serum cholesterol levels. The ensuing changes in bile acid metabolism may aid in elucidating the essential biochemical mechanisms underlying the cholesterol lowering capacity of the CV microflora.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Morris Wagner and Dr. Morris Pollard for their expert technical assistance, Dr. Edward Balish for helpful discussion and Donna Brackett for typing this manuscript. These studies were funded in part by the Max Baer Heart Fund of the Fraternal Order of the Eagles.

REFERENCES

1. Hegsted, D.M., and Gallagher, A. (1967) J. Lipid Res. 8, 210-214.
2. Wostmann, B.S., and Bruckner-Kardoss, E. (1980) J. Nutr. 110, 82-89.
3. Madsen, D., Chang, L., Beaver, M., and Wostmann, B.S. (1975) Fed. Proc. Fed. Am. Soc. Exp. Biol. 34, 3804.
4. Bartizal, K., Beaver, M., and Wostmann, B.S. (1980) in Gnotobiology for the 80s: Technology and Application, 18th Annual Meeting of the Association of Gnotobiotics, Houston, TX, Abst. 16.
5. Madsen, D., Beaver, M., Chang, L., Bruckner-Kardoss, E., and Wostmann, B.S. (1976) J. Lipid Res. 17, 107-111.
6. Wostmann, B.S. (1973) J. Nutr. 100, 928-990.
7. Midtvedt, T., and Norman, A. (1968) Acta. Path. Microbiol. Scand. 72, 334-337.
8. Lewis, R., and Gorbach, S. (1972) Arch. Intern. Med. 130, 545-549.
9. Wostmann, B.S., and Madsen, D.C. (1975) J. Germfree Life Gnotobiol. 5, 4-11.
10. Wostmann, B.S. (1973) J. Nutr. 103(7), 982-990.
11. Midtvedt, T., and Norman, A. (1968) Acta Path. Microbiol. Scand. 72, 313-329.

12. Wostmann, B.S., Beaver, M.H., Bartizal, K.F., and Madsen, D.C. (1978) 4th Intl. Symposium on Contamination Control Proceedings, Washington, DC, Vol. 4, pp. 132-135.
13. Reyniers, J.A. (1956) Germfree Life Methodology (Gnotobiotics) and Experimental Nutrition, Proc. 3rd Intl. Congress of Biochemistry, Brussels, p. 458.
14. Kellogg, T.F., and Wostmann, B.S. (1969) Lab. Animal Care 19, 812-814.
15. Caraway, W.T. (1960) in Macrochemical Methods for Blood Analysis (Thomas, C.C., ed.), Springfield, IL, p. 82.
16. Bronzert, T.J., and Brewer, H.B. (1977) Clin. Chem. 23, 2089-2098.
17. Grundy, S., Aherns, E., and Miettinen, T. (1965) J. Lipid Res. 6, 397-410.
18. VanBerge Henegouwen, G., and Hofmann, A. (1976) Clin. Chem. Acta 73, 469-474.
19. Eyssen, H., Parmentier, G.G., and Martins, J. (1976) Eur. J. Biochem. 66, 507-514.
20. Breed, R.S. (1911) Centralbe. F. Bakt. (Abst. 11) 30, 337.
21. Gustafsson, B.E., Bergstrom, S., Lindstedt, S., and Norman, A. (1957) Proc. Soc. Exp. Biol. Med. 94, 467.
22. Majumdar, S.K., and Carroll, W.J. (1973) Proc. Natl. Acad. Sci. 47, 199-201.
23. Rankin, R., Wilson, R., and Bealmear, P.M. (1971) Proc. Soc. Exp. Biol. Med. 138, 270-272.
24. Gordon, H.A., Wostmann, B.S., and Bruckner-Kardoss, E. (1963) Proc. Soc. Exp. Biol. Med. 114, 301-304.
25. Wostmann, B.S. (1975) World Review Nutr. Diet. 22, 40-92.
26. Wostmann, B.S., Bruckner-Kardoss, E., Beaver, M.H., Chang, L., and Madsen, D.C. (1976) J. Nutr. 106, 1782-1790.

[Received May 12, 1982]

Synthesis and Identification of Bis (Diacylglycero) Phosphoric Acid and Bis (Monoacylglycero) Phosphoric Acid

QUOC QUAN DANG, PIERRE ROGALLE, ROBERT SALVAYRE and LOUIS DOUSTE-BLAZY*, INSERM Unité 101, Biochimie des Lipides, Hôpital Purpan, 31059 Toulouse, France

ABSTRACT

Synthesis of bis-(diacylglycero)phosphoric acid from *sn*-1,2-dipalmitoylglycerol and phenylphosphoryl dichloride according to Baer (1) was revised. New data are reported about identification of the intermediate and final products: (a) bis-phosphatidic acid phenyl ester is very slowly visualized by the Zinzade reagent and can escape notice; (b) large amounts of phosphatidic acid chloride phenyl ester are also formed; and (c) very little transacylation from *sn*-1,2-dipalmitoylglycerol to the 1,3-isomer is observed. Hydrolysis of bis-phosphatidic acid to bis-lysophosphatidic acid is much easier using phospholipase A_2 from pig pancreas than from snake or bee venom.
Lipids 17:798-802, 1982.

INTRODUCTION

Bis-(diacylglycero)phosphoric acids, formerly called bis-(phosphatidic) acids (BPA), were first described by Baer and Kates (1,2) (see also reviews 3 and 4). Their biological interest was formerly due to their possible use as a substitute of cardiolipin in syphilis diagnosis, then, during the last decade, to their occurrence in developing plant tissues (5), and the presence of their lyso-derivatives in mammalian tissues and their lysosomal accumulation in the course of hereditary or induced phospholipidoses (6-14).

Bis-(1,2-dipalmitoyl-*sn*-glycero-3)-(phenyl) phosphate (BPA ϕ) (Fig. 1,IV) had been obtained as a by-product in the synthesis of phosphatidylcholines, in the reaction of one mole of phenylphosphoryl dichloride with one mole of 1,2-dipalmitoyl-*sn*-glycerol, the main product being 1,2-dipalmitoyl-*sn*-glycerophosphoric acid chloride phenylester or phosphatidic acid chloride phenyl ester (Fig. 1,III) (1). Later on, Baer reported an almost quantitative yield of bis-phosphatidic acids by the reaction of two moles of saturated diacylglycerols on one mole of phenylphosphoryl dichloride in anhydrous pyridine, followed by hydrogenolysis in order to remove the phenyl protecting group (Fig. 1)(2).

However, some problems still remain. Neither of the two intermediates, PAC1ϕ and BPAϕ, have been described and almost no chromatographic and spectrometric data has been reported (15). As stated by Brown in 1963 (16), the difference of reactivity between the first and second chlorine atoms of phenylphosphoryl dichloride may induce difficulties in obtaining the bis-phosphatidic acids in quantitative yield.

Baer did not give any analytical evidence for

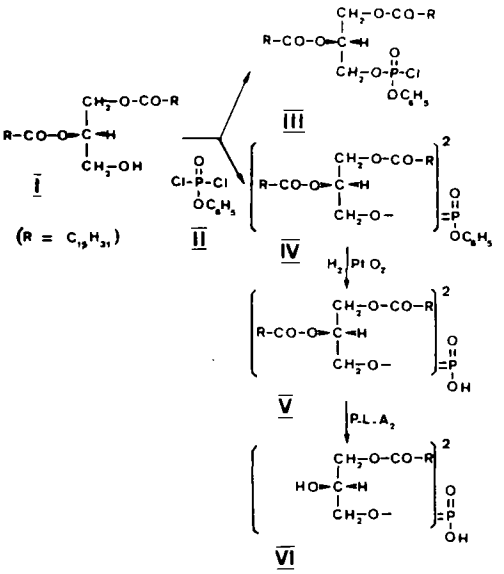


FIG. 1. Scheme of the preparation of bis-(1,2-dipalmitoyl-*sn*-glycero-3)-phosphoric acid and its lyso-derivative.

I: 1,2-Dipalmitoyl-*sn*-glycerol or D-β,γ-dipalmitine (DP).
II: Phenylphosphoryl dichloride.
III: 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoric acid chloride phenyl ester, or phosphatidic acid chloride phenyl ester (PAC1ϕ).
IV: Bis-(1,2-dipalmitoyl-*sn*-glycero-3)-(phenyl) phosphate or bis-(phosphatidic) acid phenyl ester (BPAϕ).
V: Bis-(1,2-dipalmitoyl-*sn*-glycero)-3-phosphoric acid or bis-(phosphatidic) acid (BPA).
VI: Bis-(mono-1-palmitoyl-*sn*-glycero)-phosphoric acid or bis-(lysophosphatidic) acid (BLPA).

*Author to whom correspondence should be addressed.

the structure of the bis-(1,2-diacyl-*sn*-glycero-3) phosphoric acid; perhaps isomerization such as transacylation from 1,2-diglyceride to 1,3-diglyceride (17) or some racemization could occur during the reaction. Finally, bis-(lysophosphatidic acid) (BLPA) IV prepared by Morton et al. used an enzymatic procedure which required large amounts of enzyme (1 mg of *Crotalus adamanteus* venum for 5 mg of BPA substrate) (18).

The present study was made to determine the practical prodecures, including chromatography and spectrometry, for the preparation of BPA and to ascertain its structure and also to find out a more efficient preparation of its lyso-derivative, BLPA.

MATERIALS AND METHODS

1,2-Dipalmitoyl-*sn*-glycerol puriss. was purchased from Fluka, Switzerland, and dried in vacuo over P_2O_5 for 12-24 hr. 1,3-Dipalmitoylglycerol puriss. and methyl heptadecanoate (17:0) were also supplied by Fluka. Phenylphosphoryl dichloride was synthesized as previously described (19). Anhydrous pyridine was obtained by drying the pure Merck reagent over KOH, the distillation from CaH_2. Boron trifluoride (14% in methanol) was purchased from BDH, England. Other chemicals and solvents were of analytical grade from Merck, Darmstadt. Phospholipase A_2 from pig pancreas and from bee venom were purchased from Boehringer, Mannheim, and *C. adamanteus* phospholipase A_2 was from Sigma, St. Louis, Mo.

Thin layer chromatography (TLC) was carried out on precoated Silica Gel G, 0.25 mm-thick plates from Merck, in the systems; A) $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v/v), and B) CH_2Cl-CH_2Cl/CH_3OH (98:2, v/v) and C) $CHCl_3/CH_3OH/NH_4OH$ (65/25/5, v/v/v). Visualization of all lipids was obtained first in an iodine chamber; after removal of the iodine, the phosphorus-containing lipids were visualized by spraying with Zinzade-Dittmer reagent (20).

Qualitative halogen test was performed by Beilstein flame, and also by action of metallic sodium, followed by characterization of AgCl in nitric acid medium. Melting points were determined with a Tottoli apparatus (Buchi, Switzerland). Phosphorus content was determined essentially according to Rouser et al. (21), using 1 mM KH_2PO_4 aqueous solution as a reference, and a solution of the lipid in $CHCl_3$ at about 1 mM concentration.

The acyl/phosphorus ratio was determined by alkaline hydrolysis in a large excess of 0.5 N KOH in 1:1 CH_3OH/H_2O, under gentle reflux overnight, neutralization by 6 N aq HCl, exhaustive extraction by 3:1 hexane/ether and methylation of the fatty acid with BF_3/MeOH. The methyl esters were evaporated to dryness with an approximately equivalent amount of 17:0 fatty acid methyl ester as internal standard and redissolved in hexane to about 2.5 mM final concentration for quantitative gas chromatography (IGC 120 DFL Intersmat chromatograph, capillary glass column, 31 m-long, 0.3 mm id coated with carbowax 40 M).

Microanalysis of C and H was carried out in a Perkin Elmer CHN 240 analyzer, in the Laboratory of Prof. Mila, Ecole Nationale Supérieure de Chimie de Toulouse.

Proton NMR spectra were run on a Bruker WM 250 or Bruker 90 WH using $CDCl_3$ or CCl_4, and tetramethyl silane TMS as internal reference. IR spectra were run on a Perkin-Elmer 283 spectrometer in KBr pellet.

Synthesis of Bis-(1,2-diacyl-*sn*-glycero-3)-(phenyl) Phosphate (BPA ϕ)

To a solution of 500 mg of *sn*-1,2-dipalmitoylglycerol (1) (0.88 mmol) in 4.3 ml of anhydrous pyridine was added a 200 μl solution of 92.7 mg (0.44 mmol) of phenylphosphoryl dichloride in anhydrous pyridine, in a ground-glass-stoppered flask. After a few minutes at 50 C, the reaction mixture was stirred for 24 hr at room temperature. Then, two volumes of water were gradually added, stirring continued for another half hour, and the precipitate was filtered under suction, washed thoroughly and dried in vacuo over P_2O_5 overnight, yielding 508 mg solid (91% yield).

TLC of the solid gave only one immediate phosphorus positive spot at Rf 0.52 (system A) and Rf 0 (system B), which correspond to PAC1ϕ(III). A second phosphorus positive spot appeared clearly only 24 hr after the spraying (Rf 0.88 system A, Rf 0.56 system B); this was found to be the expected BPAϕ(IV).

For separation, 494 mg of the crude solid products dissolved in a minimum of chloroform was applied to a column of 25 g of silica gel, 35-70 mesh in chloroform, and eluted successively with: (a) chloroform (containing 1% ethanol as a stabilizer), 440 ml; (b) chloroform/methanol (9:1, v/v), 200 ml; and (c) chloroform/methanol (8:2, v/v), 200 ml.

The eluates were followed by TLC (Fig. 2). Eluating solvent (a) gave IV mixed with some 1,2-dipalmitine and 1,3-dipalmitin. Identification of the dipalmitins was determined by their

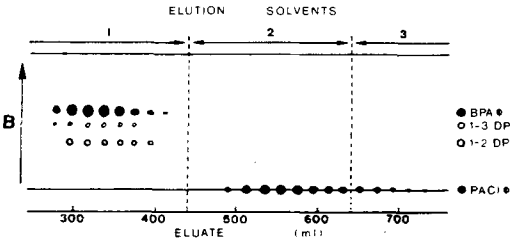


FIG. 2. Elution profile of BPAϕ and accompanying compounds from a silica gel column, TLC system B. Open spots visualized by iodine only; full spots visualized by both iodine and Zinzade reagent.

IR spectra and Rf. Preparative TLC of an aliquot of eluate (a) on Silica Gel H in system B gave 16% of 1,3-dipalmitin and 84% of 1,2-dipalmitin. Before column chromatography, only traces of 1,3-dipalmitine were observed.

The removal of dipalmitins from the evaporated eluates (a) (325 mg) by extraction with dry acetone gave 274 mg of BPA ϕ (0.215 mmol, yield based on the starting dipalmitin 49%) mp 75 C, halogen free. Elemental analysis: found C, 71.20, H 10.66, P 2.42% calcd. for BPAϕ $C_{76}H_{139}O_{12}P$(1276.7) C 71.5, H 10.98, P 2.43%, acyl/P 4.0 IR (cm^{-1}) 1740 (v.s.): C=O stretching ester, 1250 (s) P=O stretching, 3030 (w) C-H stretching aromatic, 1600 (m) 1500 (m) C=C stretching aromatic (26).

The eluate (b) yielded 77 mg PAϕCl (III) mp 205 C, yield 24% based on dipalmitin, halogen test positive; elemental analysis: P found 4.26; calcd for $C_{41}H_{72}Cl\ O_6P$ (726.5) P 4.27%. Acyl/P found 1.86, calcd 2/1. IR (cm^{-1}) 1740 (v.s.): C=O stretching ester, 1230 (s) P=O stretching, 3030 (w) C-H stretching aromatic, 1600 (m) 1500 (m) C=C stretching aromatic, 555 (m) P-Cl in $(RO_2)POCl$ (22,23).

The two phospholipids IV and III showed similar NMR spectra; they differed only in the relative intensity of the signals. IV showed δ ppm/TMS: 7.1-7.5 (multiplet C_6H_5 5H), 5.2 (multiplet 2H, HCOCOR), 3.8-4.3 (two broad signals 8H, CH_2O CO, CH_2OP), 2.3 (triplet 8H,-CH_2CO), 1.6 (shoulder 8H, CH_2-C-CO), 1.25 unresolved peak 96 H, $(CH_2)_{12}$, 0.87 triplet 12H, CH_3-C-). III showed the same chemical shifts, but based on the intensity of the signal of the phenyl as reference (5H at δ 7.1-7.5), the relative intensity of all the other signals is half the intensity of the corresponding signal in IV: δ 5.2 (1H, HCO-OR) 3.8-4.3 (4H, CH_2OCO, CH_2OP), 2.3 (4H, CH_2CO), 1.6 (4H, CH_2-C-CO), 1.25 (48H, $(CH_2)_{12}$), 0.87 (6H, CH_3-C).

Bis-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoric acid (BPA)

Dry IV (195.9 mg, 0.154 mmol) in a mixture of pure dry chloroform (2.78 ml) and absolute ethanol (1.04 ml) and 53.5 mg PtO_2 were vigorously stirred in an atmosphere of hydrogen under an initial pressure of 40 cm of water until no more hydrogen was absorbed (about 1 hr). The volume of hydrogen taken up was equal to the calculated value for the reactions $PtO_2 \rightarrow Pt$, and $-OC_6H_5 \rightarrow -OH + C_6H_{12}$. Removal of the platinum by filtration and of the solvent by evaporation under nitrogen gave 175 mg of BPA (V), yield 95%, mp 59-62 C, showing a single spot, Rf 0.60 in system A, Rf 0.00 in system B. Recrystallization from toluene-acetone gave the pure BPA (V) mp 62 C; ref. 2: 62-63 C. Analysis: found C 68.64, H 11.41, P 2.52%, calcd. for $C_{70}H_{135}O_{12}P.H_2O$ (1218) C 69.04, H 11.34, P 2.54%. Acyl/P found 3.8, calcd. 4/1, $(\alpha)D^{23} = 6.4°$, c = 10 mg/ml benzene; ref. 2: $(\alpha)\ D^{23} = 6.7°$. NMR: CCl_4, δ 5.2 (broad multiplet, 2H, HCO-COR), 3.8-4.4 (multiplet, 8 H, CH_2O-CO, CH_2OP), 2.3 (triplet, 8H, CH_2CO), 1.25 (unresolved peak, 96H, $(CH_2)_{12}$), 0.87 (triplet, 12H, CH_3-C). IR (cm^{-1}) 3450 (m, broad), O-H stretching hydrogen bonded, 1740 (v.s.) C=O stretching ester, 1250 (s) P=O stretching.

Bis-(1-palmitoyl-*sn*-glycero-3)-phosphoric acid (BLPA)

Preliminary enzymatic hydrolysis of BPA with *C. adamanteus* venom or bee venom (1 mg/5 mg substrate) in aqueous ethereal medium according to the procedure of Chadkrabarti and Khorana (24) which was a modification of (25), gave only incomplete reaction after 24 hr. Inspired by De Haas et al. (26) and Rimon and Shapiro (27), the following procedure was preferred.

BPA (V) (80 mg, 66.7 mmol) was stirred at 37 C in a well-stoppered flask with 80 ml of pH 8.3 0.1 N borate buffer containing 0.12 mM calcium chloride, 240 ml of deperoxydated ether and 200 μl of a solution containing 2 mg of phospholipase A_2 from pig pancreas. The hydrolysis was followed by TLC in the solvent A which showed that the reaction was complete after 12 hr.

After removal of the ethereal phase which contained only fatty acid, the aqueous phase and the solid phase at the interface were lyophilized, then extracted with 3:1 dry ether/acetone to remove the fatty acid, then with 2:1 dry chloroform/methanol for the separation of the lyso compound (centrifugation at room temperature was done after each extraction by vortex stirring). The $CHCl_3$/MeOH extract gave

40 mg of the lyso compound (Rf 0.42 in the system A). Recrystallization in absolute ethanol or dry chloroform/acetone gave the pure product VI, mp 152 C. Analysis: found C 59.76, H 10.71, P 4.13%; calcd. for $C_{38}H_{75}O_{10}P.2H_2O$: C 60.13, H 10.49, P 4.08%, acyl/P found 2.2, calcd. 2/1. NMR ($CDCl_3$) δ 3.8-4.3 (multiplet, broad, 10 H, CH_2-OCO, CH_2OP, CHOH), 2.3 (triplet, 4H, CH_2CO), 1.25 (unresolved peak, 48H, $(CH_2)_{12}$), 0.87 (triplet, 6H, CH_3). IR (cm^{-1}) 3450 (m, broad, O-H stretching H bonded), 1740 (C=O stretching ester), 1225 (m, P=O stretching).

In order to compare our results with data in the literature, we chromatographed our four purified products PAC1ϕ BPAϕ, BPA and BLPA, with egg phosphatidylcholine and 1,2- and 2,3-dipalmitoylglycerol in system C (28). In Figure 3, the Rf of BLPA and the Rf ratio between BLPA and PC are in reasonable agreement with those previously reported (5,28).

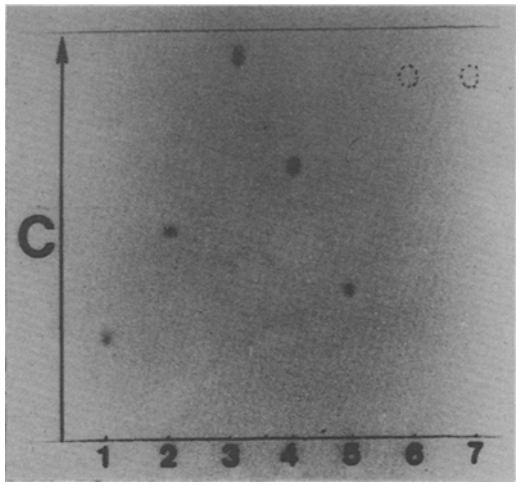


FIG. 3. TLC in system C: 1) egg phosphatidylcholine, 2) PAC1ϕ, 3) BPAϕ, 4) BPA, 5) BLPA, 6) 1,2-dipalmitoylglycerol, 7) 1,3-dipalmitoylglycerol. Visualization by Zinzade reagent, except for spots 6 and 7 with broken lines were revealed by iodine vapor only.

DISCUSSION AND CONCLUSIONS

In the synthesis of BPA (V) using Baer's procedure (1), the special TLC behavior of the intermediate BPAϕ (IV) may give problems. The latter compound is not immediately visualized by Dittmer reagent. As Baer reported, an almost quantitative yield of BPAϕ and as the by-product PAC1ϕ (III) alone is immediately visualized, this latter is easily mistaken for the desired product.

The special TLC behavior of BPAϕ might be explained by the fact that it is a phosphate triester, whereas usually phospholipids are phosphate diesters or monoesters. Phosphate triester would be less readily hydrolyzed by the sulfuric acid in the Dittmer reagent before giving the phosphomolybdate blue color.

Under the experimental conditions of Baer (1), even in rigourously anhydrous conditions, the by-product PAC1ϕ (III) was formed in about half the yield of BPAϕ. This is due to the lesser reactivity of the second chlorine atom for acyl substitution (11).

During the reaction of 1,2-dipalmitoyl-*sn*-glycerol with phenylphosphoryl dichloride in anhydrous pyridine, some isomerization into 1,3-dipalmitoylglycerol was observed by TLC on silica gel. However, one single spot has always been observed in TLC for the bis-(1,2-dipalmitoyl-*sn*-glycero-3)phosphoric acid phenyl ester obtained. Moreover, its TLC and IR spectrum showed it was not contaminated by the 1,3-dipalmitoylglycero isomer we synthesized (unpublished paper). Since TLC showed that the column chromatography eluates contain relatively larger amounts of 1,3-dipalmitoylglycerol than the product just after the reaction, the appearance of 1,3-dipalmitoylglycerol is rather due to isomerization during silica gel column chromatography than to the basicity of the pyridine reaction medium.

The BPA (V) we isolated had the same melting point as the one reported by Baer (1); however, the specific optical activity was somewhat different, although optical purity was controlled by us for the starting *sn*-1,2-dipalmitoylglycerol ($[\alpha]_D^{23} = 2.6°$ in chloroform), for the intermediate BPAϕ (IV) (see above), and although we checked by TLC and IR that our BPA (V) was not contaminated by optically inactive or less active components such as fatty acid, 1,3- and 1,2-dipalmitoylglycerols. It is noticeable that De Haas et al. reported for a mixed acid bis(1,2-diacyl-*sn*-glycero-3)-phosphoric acid, sodium salt, an $[\alpha]_D^{20} = +4.9°$ (29).

Hydrolysis of bis-(diacylglycero)-phosphoric acid (V) by phospholipase A_2 is very slow and incomplete when venom enzymes from *Crotalus* (18,24,25) or bee are used, even at high concentrations of enzyme (1/5-1/3 of the weight of substrate). In contrast, phospholipase A_2 from pig pancreas gives a quantitative yield with small amounts of enzyme. In the case of bis-(phosphatidic) acid, as well as in the case of phosphatidic acid previously reported (26,27), neither desoxycholate nor Ca^{++} are needed

when an aqueous-ethereal medium is used.

The quantitative yield obtained in phospholipase A_2-catalyzed hydrolysis leads to two conclusions: first, no racemization occurs in the preparation; this confirms the configuration of 1,2-diacyl-*sn*-glycerol or bis-(diacyl-Lα-glycero)-phosphoric acid (V) set by Baer, because the D-α form is not degraded by phospholipase A_2 (30). Secondly, it confirms that phospholipase A_2 from pig pancereas shows a predilection for anionic phospholipids, whereas the venom enzymes prefer neutral phospholipids as substrates (26,31).

ACKNOWLEDGMENTS

The authors are grateful to Professors W. Stoffel and H. Debuch (Institut für Physiologische Chemie der Universität zu Köln) and Professor H. Chap (INSERM Unité 101, Toulouse) for fruitful discussions, to Mr. C. Vieu for his contribution to gas chromatography and to Mrs. Y. Jonquière for reading the manuscript. This work was supported in part by grants from the Ministère de l'Education (Aide à la Recherche en Biologie) and from INSERM (PRC "Santé Mentale et Cerveau").

REFERENCES

1. Baer, E. (1952) J. Biol. Chem. 198, 853-859.
2. Baer, E., and Kates, M. (1950) J. Am. Chem. Soc. 72, 942.
3. Hanahan, D.J. (1960) in Lipid Chemistry, pp. 165-166, Wiley, London.
4. Van Deenen, L.L.M., and De Haas, G.H. (1964) Adv. Lipid Res. 205, 167-234.
5. Stearns, E.M., and Morton, W.T. (1977) Lipids 12, 451-453.
6. Rouser, G., Kritchevsky, G., Yamamoto, A., Knudson, A.G., Jr., and Simon, G. (1968) Lipids, 3, 287.
7. Seng, P.N., Debuch, M., Witter, B., and Wiedemann, H.R. (1971) Hoppe Seyler Z. Physiol. Chem. 352, 280-288.
8. Brotherus, J., Renkonen, O., Herrmann, J., and Fischer, W. (1974), Chem. Phys. Lipids 13, 178-182.
9. Joutti, A., Brotherus, J., Renkonen, O., Laine, E., and Fischer, W. (1976) Biochim. Biophys. Acta 450, 206-209.
10. Sommerharju, P., Brotherus, J., Kahma, K., and Renkonen, O. (1977) Biochim. Biophys. Acta 487, 154-162.
11. Joutti, A. (1979) Biochim. Biophys. Acta 575, 10-15.
12. Porthuis, B.J.H.M., and Hostetler, K.Y. (1975) J. Biol. Chem. 250, 3297-3302.
13. Porthuis, B.J.H.M., and Hostetler, K.Y. (1975) J. Biol. Chem. 251, 4596-4602.
14. Porthuis, B.J.H.M., and Hostetler, K.Y. (1978) J. Lipid Res. 19, 309-315.
15. Tremblay, P.A., and Kates, M. (1979) Can. J. Biochem. 57, 595-604.
16. Brown, D.M. (1963) in Advanced Organic Chemistry (Raphael, R.A., ed.) Vol. 3, pp. 75-157, Interscience, New York and London.
17. Hanahan, D.J. (1960) in Lipid Chemistry, pp. 194-196, Wiley, London.
18. Morton, W.T., Stearns, E.M., and Schmid, H.H.O. (1977) Lipids 12, 1083-1084.
19. Freeman, H.F., and Colver, C.W. (1938) J. Am. Chem. Soc. 60, 750-751.
20. Dittmer, J.D., and Lester, R.L. (1964) J. Lipid Res. 5, 126-127.
21. Rouser, G., Siakoutos, A.N., and Fleischer, S. (1966) Lipids 1, 85-86.
22. Bellamy, L.J. (1954) in The Infrared Spectra of Complex Molecules, pp. 54-260, Methuen, London and J. Wiley, New York.
23. Cherbuliez, E. (1973) in Organophosphorus Compounds (Kosolapoff, G.M. and Maier, L., eds.) Vol. 6, pp. 211-577, Wiley Interscience, New York.
24. Chakrabarti, P., and Khorana, G. (1975) Biochemistry 14, 5021-5033.
25. Wells, M.A., and Hanahan, D.J. (1969) Meth. Enzymol. 14, 178-184.
26. De Haas, G.H., Postema, N.M., Nieuwenhuizen, W., and Van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 159, 103-117.
27. Rimon, A., and Shapiro, B. (1959) Biochem. J. 71, 620-623.
28. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Lipids 5, 494-496.
29. De Haas, G.M., Bonsen, P.P.M., and Van Deenen, L.L.M. (1966) Biochim. Biophys. Acta 116, 114-124.
30. Van Deenen, L.L.M., and De Haas, G.H. (1964) Adv. Lipid Res. 2, 167-234.
31. Dawson, R.M.C. (1973) in Form and Function of Phospholipids (Ansell, G.B., Dawson, R.M.C., and Hawthorne, J.N., eds.) pp. 97-116, Elsevier, Amsterdam.

[Received April 26, 1982]

Sphingolipids in Immature and Mature Soybeans

MASAO OHNISHI* and **YASUHIKO FUJINO**, *Department of Agricultural Chemistry, Obihiro, University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan*

ABSTRACT

Ceramides and cerebrosides were isolated from immature and mature soybeans, and structures of the constituents were investigated. As component fatty acids, normal, 2-hydroxy and 2,3-dihydroxy acids were found in ceramides, whereas only normal and 2-hydroxy acids were identified in cerebrosides. The principal fatty acid component was 2-hydroxylignoceric acid in ceramides, and 2-hydroxypalmitic acid in cerebrosides. Sphingoids in ceramides consisted mainly of trihydroxy bases, with 4-hydroxy-*trans*-8-sphingenine being predominant. In contrast, cerebrosides contained mainly dihydroxy bases, the principal constituent being *trans*-4,*trans*-8-sphingadienine. The only sugar in cerebrosides was glucose. The constituents of the two sphingolipids were similar to each other in immature and mature seeds. Possible metabolic relations of plant sphingolipids, based on composition, are discussed.

Lipids 17:803-810, 1982.

INTRODUCTION

Sphingolipids are known to occur widely in organisms as components of the biomembranes. However, few studies have been carried out on the structure, the metabolic pathway and the physiological role of plant sphingolipids. Several analyses have been done concerning plant cerebrosides, a representative sphingolipid, but the detailed chemical composition, especially the component sphingoids, has not yet been completely elucidated. Previously, we examined cerebrosides isolated from rice, wheat grains and Azuki beans and found that typical sphingoids in seeds were 4,8-sphingadienine or 8-sphingenine, but not phytosphingosine (4-hydroxysphinganine) and dehydrophytosphingosine (4-hydroxy-*trans*-8-sphingenine) (1-3).

Free ceramide, a metabolic precursor of sphingolipids in animal tissues, was isolated for the first time in the plant kingdom from alfalfa leaves in this laboratory (4). Our recent papers reported that a comparatively large amount of ceramides was present in seeds such as rice, wheat and Azuki bean (1-3). As a preliminary survey of plant sphingolipids showed that ceramide was commonly distributed together with cerebroside in cereals, beans and leaves (3), ceramide seemed to be one of the typical sphingolipids in higher plants.

In this paper, we describe the chemical constituents and structures of ceramides and cerebrosides from immature and mature soybeans, and structural and possible metabolic relations of the two sphingolipids. Although cerebroside in immature soybeans has been isolated previously (5), individual components were not identified.

*Author to whom correspondence should be addressed.

EXPERIMENTAL METHOD

Isolation and Fractionation of Sphingolipids

Mature soybeans (Kitamusume variety, 2 kg), harvested at Hokkaido prefecture in 1978, were ground to powders, and extracted with hexane and then with chloroform/methanol (2:1, v/v) and water-saturated butanol. The latter two extracts were concentrated and washed with water to get pure lipids (1). Alternatively, soybeans of the same variety were cultured at the experimental farm in the university, and the immature plants were collected at 35 days after flowering. The beans (100 g) separated from the pods were extracted with hot isopropanol and chloroform/methanol to prepare total lipids (6). Both lipids (81 g and 40 g from mature and immature seeds, respectively) thus obtained were hydrolyzed with mild alkali to remove contaminating glycerolipids (1). Ceramides and cerebrosides were isolated from the alkali-stable lipid fractions by silicic column chromatography followed by acetylation, preparative thin layer chromatography (TLC) and subsequent deacetylation as described previously (1,2).

Purified ceramide was fractionated by silica gel TLC into three fractions according to degree of hydroxylation (7). On the other hand, cerebroside was subjected to silica gel/borax (98:2, w/w) TLC in order to separate into subfractions roughly according to the number of hydroxy groups on the ceramide moiety (8,9). These subfractions were analyzed directly by gas

chromatography-mass spectrometry (GC-MS) (7,10).

Analyses of Components

Each sphingolipid was degraded with methanolic 1 N HCl, aqueous methanolic 1 N HCl (11), aqueous dioxanic 10% $Ba(OH)_2$ (12) and methanolic 1 N KOH, respectively. Thus, methylglycosides, fatty acid methyl esters and sphingoids were obtained in every case, and analyzed as described previously (1-3).

The position of the double bonds in sphingoids was determined by periodate-permanganate oxidation (Von Rudloff degradation) and subsequent gas liquid chromatography (GLC) of the resultant monocarboxylic acid (13). The result was confirmed by GC-MS analyses of polyhydroxylated products of methyl ethers derived from sphingoids by oxidation with $NaIO_4$ followed by reduction with $NaBH_4$, methylation and subsequent oxidation with OsO_4 (1).

Each sphingolipid was dissolved in methanol and oxidized with $NaIO_4$ to determine fatty acid compositions of the two groups, which comprised the dihydroxy and trihydroxy bases (14). The products were subjected to silica gel TLC with chloroform/methanol (95:12, v/v) to be fractionated into the groups described above, and methanolyzed. 2-Hydroxy fatty acid methyl esters were isolated by silica gel TLC from the methyl ester fractions and analyzed by GLC.

For the determination of the anomeric configuration of the glycosidic linkages, acetyl cerebrosides were analyzed by a nuclear magnetic resonance spectrometer, operating in the Fourier transform mode at 200 MHz using deuterium chloroform as a solvent.

RESULTS

Confirmation of Sphingolipids

Purified ceramides were isolated in yields of 10 mg and 50 mg, and cerebrosides in yields of 33 mg and 130 mg, from immature and mature soybeans, respectively. These sphingolipids showed the same mobilities as those of authentic ones isolated from rice bran and Azuki beans on silica gel TLC (1,2). Moreover, infrared (IR) spectra of the lipids (not shown) exhibited the typical patterns of sphingolipids.

An oligoglycolipid, tentatively identified as diglycosylceramide, was also recognized in the fraction eluted from the silicic acid column by chloroform/methanol (80:20, v/v), but was not analyzed further in this work because of the insufficient amount.

Composition of Fatty Acids

The fatty acid composition, calculated from the relative ratio of each type and analyses by GLC, is shown in Table 1. The major acids in decreasing order were 2-hydroxylignoceric, 2-hydroxybehenic and palmitic acids in ceramides, whereas 2-hydroxypalmitic, 2-hydroxylignoceric and 2-hydroxybehenic acids, particularly the first one, were in cerebrosides.

Characterization of Component Sphingoids

Silica gel TLC, of the component sphingoids prepared from alkaline degradation of each sphingolipid, with chloroform/methanol/2 N ammonia (40:10:1, v/v) gave commonly three spots corresponding to *trans*-4-sphingenine (S_1), sphinganine (S_2) and 4-hydroxysphinganine (S_3). From GC-MS analyses of these spots as N-acetyl, O-trimethylsilyl ether derivatives (15), *trans*-4-sphingenine and sphingadienine were identified in S_1, sphinganine and x(not 4)-sphingenine in S_2 and 4-hydroxysphinganine and 4-hydroxysphingenine in S_3.

Von Rudloff oxidation of the acetyl sphingoids gave essentially a C_{10}-monocarboxylic acid, showing that the position of the double bond besides C-4 was exclusively C-8, which was confirmed by GC-MS analyses. The mass spectrum of the oxidized product originating from sphingadienine (Fig. 1(A)) showed ions at m/z 371 and 147 indicating the presence of a vicinal trimethylsiloxy group at C-2 and C-3 and ions at m/z 229, 257 and 289, which were the assignments for the other pair of trimethylsiloxy groups at C-6 and C-7. The mass spectrum of the polyhydroxylated compound derived from x(not 4)-sphingenine (Fig. 1(B)) exhibited ions at m/z 203 and 229, whereas that from unsaturated sphingoid S_3 (Fig. 1(C)) gave ions at m/z 189 and 229. These ions suggested that the presence of a vicinal trimethylsiloxy group were at C-6 and C-7 in the former, and at C-5 and C-6 in the latter, respectively. Thus, the positions of the double bonds in sphingoids turned out to be C-4 and/or C-8. When N-, O-acetyl derivatives of spots S_1 and S_3 in (mature) cerebroside were subjected to silica gel-$AgNO_3$ TLC (1), three spots (a, b and c) were found in both cases. Judging from their mobilities on TLC and results described above, S_1-a, -b and -c were characterized as *trans*-4-sphingenine, *trans*-4,*trans*-8-sphingadienine and *trans*-4,*cis*-8-sphingadienine, respectively. S_3-a, -b and -c were identified as 4-hydroxysphinganine, 4-hydroxy-*trans*-8-sphingenine and 4-hydroxy-*cis*-8-sphingenine, respectively (1,3, 16). Although spingoid S_2 was not analyzed in detail, 8-sphingenine was considered to be a

TABLE 1

Composition of Fatty Acids in Ceramides and Cerebrosides from Soybean Seeds (%)

Fatty acid	Immature seeds		Mature seeds	
	Ceramide	Cerebroside	Ceramide	Cerebroside
16:0	13	1	8	4
18:0	5	1	2	2
22:0	1	<1	2	<1
24:0	2	<1	1	<1
h_1 16:0[a]	3	73	2	79
h_1 22:0	14	10	26	5
h_1 23:0	4	<1	6	<1
h_1 24:0	42	13	41	9
h_1 25:0	5	<1	3	<1
h_1 26:0	2	1	<1	<1
h_2 22:0[b]	1	–	<1	–
h_2 24:0	3	–	3	–
h_2 25:0	1	–	2	–
h_2 26:0	<1	–	1	–
Others	4	1	3	1

[a] h_1 signifies 2-monohydroxy acid.

[b] h_2 signifies 2,3-dihydroxy acid.

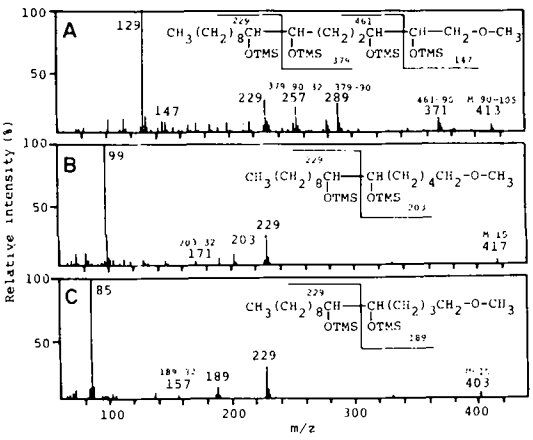


FIG. 1. Mass spectra of the trimethylsilyl ether derivatives of polyhydroxylated methyl ethers obtained from 4,8-sphingadienine (A), 8-sphingenine (B) and 4-hydroxy-8-sphingenine (C).

mixture of *cis*- and *trans*-unsaturated isomers, as in case of wheat sphingolipids (3,16).

Composition of Sphingoids

Table 2 shows the sphingoid composition analyzed and identified by GLC and GC-MS of aldehydes derived from sphingoids liberated by acid degradation (1). GLC of pentadecenals derived from the two geometric isomers of 4-hydroxy-8-sphingenine had the same retention time, so that the ratio of the isomers was determined by a combination of silica gel-$AgNO_3$ TLC and GLC of acetyl sphingoids. The principal constituents were 4-hydroxy-8-sphingenine and 4-hydroxysphinganine, the former being predominant in ceramides, whereas sphingadienines predominated in cerebrosides. Component sphingoids in soybeans generally possessed a *trans*-double bond rather than a *cis*-one at C-8, as in case of Azuki beans (2). This finding was supported by IR spectra of intact sphingolipids, in which the intensity of the absorption band at 970 cm^{-1} was significantly stronger than that of rice sphingolipid (1).

Identification of Component Sugars

Constituent sugars in cerebrosides isolated from immature and mature soybeans were found by GLC analyses to consist only of glucose. PMR spectra of the acetates (not shown) revealed the sharp doublet at 4.47 ppm ($J_{1,2}$= 7.8 Hz), indicating the β-glycosidic linkage (17,18). Therefore, the glucose linkage to the sphingoid moiety have the β-configuration.

Characterization of Molecular Species of Ceramide and Cerebroside

Table 3 shows hydroxy fatty acid composition based on sphingoid types of ceramide and cerebroside in mature soybeans. Constituent fatty acids in the dihydroxy base-containing species, which was minor in ceramide whereas major in cerebroside, were essentially only 2-hydroxypalmitic acid. On the other hand, the compositions in trihydroxy base-containing ceramide and cerebroside were similar to each other, the principal constituents being 2-hydroxylignoceric and 2-hydroxybehenic acids.

TABLE 2

Composition of Sphingoids in Ceramides and Cerebrosides from Soybean Seeds (%)

Aldehyde	Relative retention time[a]	Original base	Immature seeds		Mature seeds	
			Ceramide	Cerebroside	Ceramide	Cerebroside
15:0	0.66	4-Hydroxysphinganine	24	1	12	3
15:1 *trans*-5	0.75	4-Hydroxy-*trans*-8-sphingenine	73	16	83	10
15:1 *cis*-5	0.75	4-Hydroxy-*cis*-8-sphingenine		1	<1	7
16:0	1.00	Sphinganine	1	<1	<1	<1
16:1 *trans* & *cis*-6	1.15	*cis* & *trans*-8-Sphingenine	<1	1	<1	2
16:1 *trans*-2	1.92	*trans*-4-Sphingenine	<1	<1	–	<1
16:2 *trans*-2,*trans*-6	2.07	*trans*-4,*trans*-8-Sphingadienine	1	67	4	69
16:2 *trans*-2,*cis*-6	2.14	*trans*-4,*cis*-8-Sphingadienine	1	14	1	9
C_{15}-Aldehydes		Trihydroxy bases	97	18	95	20
C_{16}-Aldehydes		Dihydroxy bases	3	82	5	80

[a]Taking the retention time of 16:0 (10.4 min) as unity.

In order to confirm the results, subfraction I with Rf:0.4 and II with Rf:0.3 obtained by silica gel/borax TLC of immature soybeans were analyzed by GC-MS (7). The data are summarized in Table 4 (19). A single peak, comprising C_{16} hydroxy acid and sphingadienine, was found in fraction I, and five peaks in fraction II. The retention time and mass spectrum of peak 2 were identical with those of peak 1, so that peak 2 was assumed to be contaminant dihydroxy base-containing species, due to the incomplete resolution of fractions I and II. Peaks 3-6 were identified as trihydroxy base-containing species combined with hydroxy acids of C_{22-25}.

To confirm the major molecular species of ceramide, the principal ceramide components (Rf:0.6) were converted to trimethylsilyl ether derivatives and analyzed by GC-MS (Table 5) (7,19,20). Five peaks were found, the principal species being characterized as N-2′-hydroxylignoceroyl-4-hydroxysphingenine. The result was in good agreement with that deduced from the analyses of components described above. The other peaks, also identified as ceramides, were composed mainly of 4-hydroxysphingenine and 2-hydroxy acids of C_{22-26}.

DISCUSSION

In this study, the composition of ceramide and cerebroside in immature soybeans was substantially the same as that of sphingolipids in mature seeds. This suggests that the constituents in the two sphingolipids, unlike other lipid classes in soybeans (5), hardly changed during maturation.

The principal ceramide in soybean, identified as N-2′-hydroxy-lignoceroyl-4-hydroxy-*trans*-8-sphingenine, was identical with that of Azuki bean ceramide (2). Moreover, the major components in ceramide isolated from alfalfa leaves (4) and green bush bean leaves (21) were reported to be 4-hydroxysphingenine and 2-hydroxylignoceric acid. Regarding ceramide in cereals (rice and wheat) (1,3), we found that the principal fatty acid constituent was also 2-hydroxylignoceric acid, but the predominant base was the saturated homologue (4-hydroxysphinganine). Thus, in general, plant ceramide seems to consist mainly of saturated or 8-unsaturated trihydroxy bases of 18 carbons and 2-hydroxy fatty acids with longer carbon chains, particularly C_{24} acid, so that the structure of plant ceramides are not unique for plant species and organs but similar to one another. 2,3-Dihydroxy acids found in soybean ceramide have been recognized in rice, wheat and Azuki

TABLE 3

Composition of 2-Hydroxy Fatty Acids Based on Sphingoid Type in Ceramide and Cerebroside from Mature Soybean Seeds (%)

2-Hydroxy fatty acid	Ceramide		Cerebroside	
	Dihydroxy base-containing species	Trihydroxy base-containing species	Dihydroxy base-containing species	Trihydroxy base-containing species
16:0	92.4	0.4	100.0	2.9
22:0	0.3	29.9	<0.1	36.3
23:0	–	5.7	–	3.7
24:0	<0.1	58.2	<0.1	50.6
25:0	–	4.6	–	3.2
26:0	–	1.0	–	3.1
Others	7.3	0.2	–	0.2

bean seeds (1-3) and are, therefore, presumed to be widespread as the minor component of ceramide in higher plants. In any case, the stereoconfiguration of the hydroxy groups of 2,3-dihydroxy acids as well as monohydroxy ones in plant sphingolipids should be elucidated.

From the present findings, the major structure of cerebroside in soybean can be characterized as 1-O-β-glucosyl-N-2′-hydroxy-palmitoyl-*trans*-4,*trans*-8-sphingadienine, as in the case of Azuki bean (2). The main fatty acid (2-hydroxypalmitic acid) was ca. 40% in Azuki bean cerebroside, whereas ca. 70% in soybean cerebroside, though the compositions of constituent sphingoids in both cerebrosides were similar. Thus, it appears that soybean cerebroside is of the rather simple composition.

Biogenetically, ceramide has been proved to be the direct precursor of cerebroside in the animal tissues. However, overall compositions of constituents in ceramide and cerebroside isolated from soybean differed largely from each other, as in case of Azuki bean. This indicates a complex metabolic relationship between the two sphingolipids. However, the dihydroxy base- and trihydroxy base-containing ceramides were, respectively, highly similar to the ceramide moieties of dihydroxy base- and trihydroxy base-containing cerebrosides. It may suggest that the major dihydroxy base-containing cerebroside species is synthesized by the preferential glycosylation of the minor dihydroxy base-containing ceramide species. On the other hand, sphingoid and fatty acid components in the ceramide were nearly identical with those in phytoglycolipid obtained from soybeans (22). The constituents of the ceramide moiety in phytoglycolipid from green bush beans leaves were also different from those in their cerebroside, and significantly similar to those of their free ceramide (21). Thus, it seems to be general that plant ceramide is structurally related more to phytoglycolipid than cerebroside.

ACKNOWLEDGMENTS

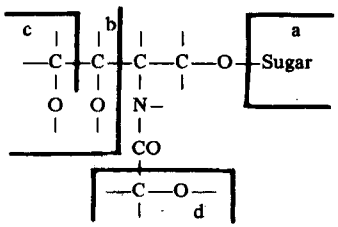
The authors wish express their thanks to Mr. M. Imai for his technical assistance and to Dr. H. Chiji, Hokkaido University, for measurement and interpretation of PMR spectra. This work was supported in part by grants for Scientific Research (1980-81) from the Ministry for Education, Science and Culture of Japan.

REFERENCES

1. Fujino, Y., and Ohnishi, M. (1976) Chem. Phys. Lipids 17, 275-289.
2. Ohnishi, M., and Fujino, Y. (1981) Agric. Biol. Chem. 45, 1283-1284.
3. Ohnishi, M., Ito, S., and Fujino, Y. (1980) Proc. Jpn. Conf. Biochem. Lipids 22, 379-382.
4. Fujino, Y., and Ito, S. (1971) Biochim. Biophys. Acta 231, 242-243.
5. Singh, H., and Privett, O.S. (1970) Lipids 5, 692-697.
6. Sastry, P.S., and Kates, M. (1964) Biochemistry 3, 1271-1280.
7. Ohnishi, M., and Fujino, Y. (1976) Agric. Biol. Chem. 40, 1419-1423.
8. Kean, E.L. (1966) J. Lipid Res. 7, 449-452.
9. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1973) Biochim. Biophys. Acta 306, 317-328.
10. Ohnishi, M., Nakano, M., and Fujino, Y. (1975) Agric. Biol. Chem. 39, 743-744.
11. Gaver, R.C., and Sweeley, C.C. (1965) J. Am. Oil Chem. Soc. 42, 294-298.
12. Morrison, W.R. (1969) Biochim. Biophys. Acta 176, 537-546.
13. Hayashi, A., and Matsubara, T. (1971) Biochim. Biophys. Acta 248, 306-314.
14. MacMurray, T.A., and Morrison, W.R. (1970) J. Sci. Food Agric. 21, 520-528.

TABLE 4

Gas-Liquid Chromatographic and Mass Spectrometric Data for Trimethylsilyl Ether Derivatives of Soybean Cerebroside



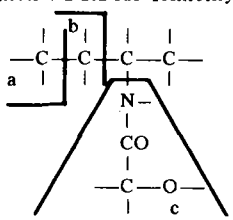
Peak number	Retention time (min)	Percentage of peak area	Component fatty acid			Component sphingoid			Molecule	
			M−a−b+73	d	Species[p]	b	c	Species[q]	M−15	Structure (sphingoid - acid - sugar)
Cerebroside I			m/z	m/z		m/z	m/z		m/z	
1	5.1	100	458	299	h_1 16:0	309	–	h_2 18:2	1130	h_2 18:2 - h_1 16:0 - Glc
Cerebroside II										
2	4.9	21	458	299	h_1 16:0	309	–	h_2 18:2	1130	h_2 18:2 - h_1 16:0 - Glc
3	16.0	25	542	383	h_1 22:0	–	297	h_3 18:1	1304	h_3 18:1 - h_1 22:0 - Glc
4	18.8	6	556	397	h_1 23:0	–	297	h_3 18:1	1318	h_3 18:1 - h_1 23:0 - Glc
5	22.5	43	570	411	h_1 24:0	–	297	h_3 18:1	1332	h_3 18:1 - h_1 24:0 - Glc
6	26.8	5	584	425	h_1 25:0	–	297	h_3 18:1	1346	h_3 18:1 - h_1 25:0 - Glc

[p] h_1 signifies monohydroxy acid.

[q] h_2 and h_3 signify dihydroxy and trihydroxy sphingoids, respectively.

TABLE 5

Gas-Liquid Chromatographic and Mass Spectrometric Data for Trimethylsilyl Ether Derivatives of Soybean Ceramide



Peak number	Retention time (min)	Percentage of peak area	Component fatty acid			Component sphingoid			Molecule	
			M−b	c+2	Species[p]	a	M−c−1−90	Species[q]	M−15	Structure (sphingoid - acid)
			m/z	m/z		m/z	m/z		m/z	
1	22.2	29	542	424	h_1 22:0	297	424	h_3 18:1	926	h_3 18:1 - h_1 22:0
2	26.9	11	556	442	h_1 23:0	297	424	h_3 18:1	940	h_3 18:1 - h_1 23:0
3	33.6	50	570	456	h_1 24:0	297	424	h_3 18:1	954	h_3 18:1 - h_1 24:0
4	39.9	8	584	470	h_1 25:0	297	424	h_3 18:1	968	h_3 18:1 - h_1 25:0
5	48.2	2	598	484	h_1 26:0	297	424	h_3 18:1	982	h_3 18:1 - h_1 26:0

[p,q]See Table 4.

15. Polito, A.J., Akita, T., and Sweeley, C.C. (1968) Biochemistry 7, 2609-2614.
16. Laine, R.A., and Renkonen, O. (1973) Biochemistry 12, 1106-1111.
17. Martin-Lomas, M., and Chapman, D. (1973) Chem. Phys. Lipids 10, 152-164.
18. Falk, K.E., Karlsson, K.-A., and Samuelsson, B.E. (1979) Arch. Biochem. Biophys. 192, 164-176.
19. Karlsson, K.-A., Pascher, I., Samuelsson, B.E., and Steen, G.O. (1972) Chem. Phys. Lipids 9, 230-246.
20. Hayashi, A., and Matsuura, F. (1973) Chem. Phys. Lipids 10, 51-65.
21. Carter, H.E., and Koob, J.L. (1969) J. Lipid Res. 10, 363-369.
22. Carter, H.E., Celmer, W.D., Galanos, D.S., Gigg, R.H., Lands, W.E.M., Law, J.H., Mueller, K.L., Nakayama, T., Tomizawa, H.H., and Weber, E. (1958) J. Am. Oil Chem. Soc. 35, 335-343.

[Revision received July 14, 1982]

Anatomical Distribution of Sterols in Oysters (*Crassostrea gigas*)

DENNIS T. GORDON*[1], and **NANCY COLLINS**, *Seafoods Laboratory, Oregon State University, 250-36th Street, Astoria, OR 97103*

ABSTRACT

Oysters (*Crassostrea gigas*) contain at least 8 predominant sterols as determined by gas liquid chromatography and a modified Liebermann-Burchard reaction. These sterols and the average amount found in mg/100 are: C_{26}-sterol (22-*trans*-24-norcholesta-5, 22-diene-3β-ol), 19.1; 22-dehydrocholesterol, 15.1; cholesterol, 46.8; brassicasterol, 27.2; $\Delta^{5,7}$-sterols (i.e., 7-dehydrocholesterol) 22.5; 24-methylenecholesterol 29.1; 24-ethylcholesta-5,22-diene-3β-ol, 1.2; and 24-ethylcholesta-5-en-3β-ol, 12.7. The distribution of these sterols appears uniform ($r^2 = 0.938$) between 5 major organs of the oyster. The percent body mass vs percent total sterols in these 5 organs are: mantle 44.1-41.4; visceral mass 30.3-36.7; gills 13.2-11.7; adductor muscle 8.3-3.7; and labial palps 4.2-6.5. The possible sources of these sterols are discussed.
Lipids 17:811-817, 1982.

Mollusks contain as broad a distribution of sterols as any found in the animal world. Early examination of oysters (*Crassostrea sp*) indicated the presence of 14 different sterols (1-3). Recently, this number has been expanded to a total of 39 (4) in the Eastern oyster (*C. virginica*) by employing gas liquid chromatography-mass spectroscopy (GLC-MS). The identity of all these sterols are progressively being confirmed (5,6). Although a uniform sterol composition in oysters has not been clearly established, sufficient information has been available to use this class of lipid in the area of phylogenetics (7). In marine crustacea (8) and vertebrates, cholesterol predominates and its function has been examined and reviewed extensively (9).

The purpose or significance of a variety of sterols in mollusks is unknown. Their diversity does appear to correspond with the low level of the mollusk in the evolutionary chain. Oysters of the Pacific Coast (*C. gigas*) are typical of mollusks containing such a mixture. The source of these sterols is also unknown. If dietary in origin, it could be speculated that greater concentrations would be found in the food trapping or digestive systems (e.g., gills and/or visceral mass) than in other organs. Alternatively, a lack of one or more sterols in other organs would suggest a nonnutrient requirement, an important observation for oyster culture operations (10). The presence of a uniform sterol distribution would indicate a functional, and possibly coordinated, role for each.

The purpose of this study was to examine the distribution of sterols in 5 major organs of the Pacific oyster (*C. gigas*).

MATERIAL AND METHODS

Samples and Lipid Extraction

Oysters were harvested from Willa Bay, WA, on March 29, 1979. Three large oysters were dissected into their 5 major organs (i.e., mantle, visceral mass, gills, adductor muscle and labial palps) as depicted in Figure 1. For comparative

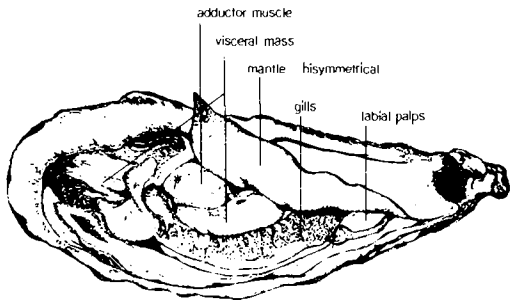


FIG. 1. Pacific oyster, *Crassostrea gigas*.

*Author to whom correspondence should be addressed.

[1]Current address: Department of Food Science and Nutrition, Eckles Hall 224, University of Missouri-Columbia, Columbia, MO 65211.

purposes, 12 oysters each (i.e., composite), of 3 smaller weight groups, were homogenized from which subsamples were withdrawn. Total lipid extractions were made of each organ and subsamples of each composite in chloroform/methanol (2:1, v/v) as described by Folch et al. (11).

Sterol Quantitation and Qualitation

Aliquots of the chloroform/methanol extracts containing an estimated 1-3 mg total sterols were removed. The extracting solvent was removed under a stream of N_2, and the residual lipids were saponified with 5 ml 1 N KOH in 80% ethanol for 30 min at 80C in a capped Teflon-lined tube. The tube was cooled and 5 ml distilled water and 10 ml heptane were added. One ml heptane was transferred to a second tube and this solvent was removed under a stream of N_2. Glacial acetic acid, 1 ml, was added and a modified Liebermann-Burchard (LB) reaction (12,13) was used to quantitate $\Delta^{5,7}$-sterols in the presence of Δ^5 sterols, as described elsewhere (14). Cholesterol and 7-dehydrocholesterol were used as standards.

For gas-liquid chromatographic analysis of sterols, isolation and identification procedures were modeled after Miettinen et al., (15). A 5 ml aliquot of heptane was removed from the saponification reaction evaporated under N_2, and the residual lipid was applied to a 20 x 20 cm Silica Gel G thin layer chromatographic (TLC) plate. Development was in hexane/ethyl ether/glacial acetic acid (90:30:1, v/v/v). Visualization of the sterol band (RF=0.3) was with a brief exposure to iodine vapor. Cholesterol was used as a standard.

Sterols were eluted from the absorbent with chloroform/methanol (2:1, v/v). This isolation step results in the loss of $\Delta^{5,7}$-sterol(s) previously quantitated by the modified LB reaction. To the sterols in the chloroform/methanol was added 250 μg 5-α cholestane in 1 ml heptane. Solvents were removed under N_2, 0.3 ml dry silylating reagent was added, and the reaction allowed to stand at room temperature (RT) for 30 min. The silylating reagent consisted of dimethylformamide/hexamethyldislazane/trimethylchlorosilane (40:40:1, v/v/v). Sterol silyl ether derivatives were separated on a 2 m x 2 mm (id) glass column containing 3% SE-30 on 100/120 mesh GAS-CHROM Q. A Hewlett-Packard Model 7610A gas liquid chromatograph (GLC) was used with the following operating parameters: column temperature 230C (isothermal), injection temperature 300C, and detector temperature 260C. Identification and quantitation of unknown sterols in oysters was made against pure silyl ether sterol standards using 5-α-cholestane (14) to obtain relative retention times (RRT).

RESULTS AND DISCUSSION

There are at least 8 major sterols in Pacific Coast oysters (*C. gigas*). Seven were indicated by GLC analysis and the presence of the eighth, or class of sterols, was strongly suggested by previous investigators (1,4) and by the modified LB reaction employed in this study. These sterols were C_{26}-sterol (22-*trans*-24-norcholesterol-5,22-diene-3β-ol), 22-dehydrocholesterol, cholesterol, brassicasterol, $\Delta^{5,7}$-sterol(s) (e.g., 7-dehydrocholesterol), 24-methylenecholesterol, 24-ethylcholesta-5, 22-diene-3β-ol and 24-ethylcholesta-5-en-3β-ol. At least 3 other sterols are believed present in oysters, desmosterol, fucosterol and isofucosterol, but they could not be separated and, thus, quantitated by the GLC system employed. Desmosterol would be included with the brassicasterol peak and fucosterol and isofucosterol, if present, partition with 24-ethyl-cholestra-5-en-3β-ol. The quantitation and identification of $\Delta^{5,7}$-sterols presents a unique problem. This class of sterols is believed to be oxidized easily (16) and subsequently lost. By employing a modified LB reaction, the presence of $\Delta^{5,7}$-sterol(s) was indicated, and as a class they were quantitated. Although 7-dehydrocholesterol is the only $\Delta^{5,7}$-sterol suggested in this investigation, it was initially reported (4) that there may be as many as 7 $\Delta^{5,7}$-sterols in Eastern oysters (*C. virginica*). The identity of these seven $\Delta^{5,7}$ sterols has subsequently been accomplished by Teshima and Patterson (5). The 7 $\Delta^{5,7}$-sterols are: 22-*trans*-24-norcholesta-5,7,22-trienol; cholesta-5,7,22-trienol; cholesta-5,7-dienol; 24-methylcholesta-5,7,22-trienol; 24-methylcholesta-5,7-dienol; 24-ethylcholesta-5,7,22-trienol and 24-ethylcholesta-5,7-dienol. Bergmann(1) lists those $\Delta^{5,7}$-sterols that have been found in a variety of mollusks.

The level of the 8 major sterols in the 5 anatomical organs of the oyster are listed in Table 1. On an individual and total sterol per gram basis, there was a wide distribution between the various organs. When comparing the coefficient of variation (CV) for levels of individual sterols in each organ as a percent, except for 24-ethylcholesta-5,22-diene-3β-ol and 24-ethylcholesta-5-en-3β-ol, the CV ranged only between 1 and 4%. This indicated that, although quantitatively the levels in organs may vary, the distribution, and thus ratio of sterols to one another, is very uniform. Lack of uniformity in 24-ethylcholesta-5,22-diene-3β-ol levels could be due to analytical variation,

TABLE 1
Sterol Levels in the Major Anatomical Components of the Oysters (*Crassostrea gigas*)

	Wt, g[1]	C_{26} sterol	22-Dehydro-cholesterol	Cholesterol	Brassicasterol	$\Delta^{5,7}$ Sterols	24-Methylene-cholesterol	24-Ethyl cholesta-5, 22-diene 3β-ol	24-Ethyl cholesta-5-en-3β-ol	Total[3]
					mg/g					
Mantle	26.75±4.81	0.158 ±0.031	0.131 ±0.030	0.422 ±0.114	0.241 ±0.084	0.27 ±0.22	0.251 ±0.098	0.014 ±0.021	0.111 ±0.050	1.61[b,c] ±0.64
Visceral mass	18.29±1.64	0.256 ±0.061	0.193 0.036	0.625 ±0.146	0.333 ±0.076	0.34 ±0.13	0.338 ±0.067	0.002[2]	0.105 ±0.098	2.19[a,b] ±0.54
Gills	8.03±1.38	0.146 ±0.018	0.127 ±0.020	0.444 ±0.094	0.250 ±0.039	0.29 ±0.13	0.266 ±0.048	0.012[2]	0.016 ±0.009	1.59[b,c] ±0.39
Adductor mass	5.06±1.46	0.064 ±0.019	0.064 ±0.018	0.278 ±0.064	0.127 ±0.023	0.08 ±0.03	0.141 ±0.030	0.008[2]	0.038 ±0.021	0.79[c] ±0.15
Labial palps	2.57±0.84	0.290 ±0.049	0.233 0.042	0.727 0.203	0.399 ±0.090	0.50 ±0.21	0.408 ±0.108	0.002[2]	0.196 ±0.025	2.76[a] ±0.69
Percent ± SD of Total		10.34± 0.39	8.40 0.13	27.77 ±0.87	15.27 ±0.18	16.49 ±0.44	15.85 ±0.27	0.57 ±0.19	5.33 ±1.07	

[1]Mean ± SD of 3 oysters.
[2]Mean of duplicate values, some samples only contained trace.
[3]Values having the same alphabetic superscript are not significantly different ($p<0.05$).

TABLE 2
Sterols in Oysters/100 g Wet Weight

	C_{26}-sterol	22-Dehydrocholesterol	Cholesterol	Brassicasterol	$\Delta^{5,7}$-Sterols	24-Methylenecholesterol	24-Ethyl cholesta-5, 22-diene 3β-ol	24-Ethylcholesta-5 en-3β-ol	Total
				mg/100g[a]					
Oyster No. 1 64.4g	18.9	14.5	50.6	24.6	25.2	29.1	1.4	5.8	170.1
Oyster No. 2 59.2g	14.3	12.1	36.7	21.1	14.2	20.7	0.2	7.6	126.9
Oyster No. 3 58.5g	22.1	17.7	59.1	34.6	47.2	32.9	1.9	16.3	231.8
Oyster composite 12 ea avg wt 15.6± 2.6g	18.7	13.7	36.7	23.7	13.5	24.7	tr	12.2	143.3
Oyster composite 12 ea avg wt 32.7± 2.0g	20.7	17.1	48.3	30.0	17.3	33.3	tr	14.3	163.6
Oyster composite 12 ea avg wt 52.9± 5.0g	19.6	15.3	49.4	29.1	17.6	33.8	tr	20.2	167.3
mg/100g Mean±SD[b]	19.1 ±2.7	15.1 ±2.1	46.8 ±8.7	27.2 ±5.0	22.5 ±12.8	29.1 ±5.4	1.2 0.9	12.7 5.4	173.7

[a]Mean values reported.
[b]n=6.

especially with the small amounts, at times only a trace, of this compound detected. As previously stated, the 24-ethylcholesta-5-en-3β-ol peak quantitated by GLC could contain other sterols such as fucosterol and isofucosterol which have identical RRT (14). One chromatographic peak consisting of 3 compounds could account for the large variation (5.33±1.07%; CV=20%).

Since the actual stereochemistry of all sterol reported in this study was not determined, unequivocal identity cannot be assigned. It can only be assumed that 24-ethylcholesta-5,22-diene-3β-ol and 24-ethylcholesta-5-en-3β-ol do have the 24β configuration, and are the compounds poriferstерol and clionsterol, respectively. Teshima et al (4) have shown these compounds predominate in *C. virginica*.

Comparing the individual and total sterols in the 3 large oysters and 3 composite samples of smaller oysters (Table 2), a wide range in total sterol levels was observed, 127 to 232 mg/100 g. Considering the previous variation in total sterols, but similarity in proportion of sterols in organs, this large variation between oysters may be due to their individual physiological state. A similar observation was made by Swift et al. (17) with regard to the neutral lipids distributed in oysters. Size was found to have no significant effect on total sterol or cholesterol content (14). Since all samples were obtained at the same time, seasonal variation can be discounted in this study. Sterol levels have been reported to change with season (18,19).

The concentration of sterols per body mass (Table 3) was highly correlated (r^2=0.938). This would indicate that all sterols found in oysters are important. It is beyond this report to speculate how these sterols function in one or more capacities as structural units in membranes as hormones, as some type of lipid solubilizing or detergent agent, or a pro-vitamin D. The two organs most involved with food intake are the gills and visceral mass (20).

It may be hypothesized that some sterols are derived from symbiotic bacteria or algae that inhabit the visceral mass and possibly the gills. The labial palps, which serve primarily for final sorting of food particles and for delivery of food to the mouth, did have a slightly higher sterol content per unit mass than the other organs. The lowest sterol content was observed in the adductor muscle, the least morphologically complex. Apparently the mantle does not store sterols, although it is the organ holding glycogen and lipid reserves. Considering the many differences in the 5 major organs of the oysters, it appears that 8, and possibly 11, major sterols are uniformly distributed and all may be vital for their proper functioning.

This study may not have totally described all sterols that could exist in oysters (*C. gigas*) nor provided direct information as to their source. A recent report (4) indicates that Eastern Coast oysters (*C. virginica*) contain 39 sterols, although many in very small amounts. It remains to be resolved which are essential and typically endogenous and those present only in low concentration because they are intermediates in biosynthetic pathways (6) or result from environment. Sterol biosynthesis is active in oysters, but to what degree remains unknown.

Recently it was indicated that 4 major sterols are synthesized in the Eastern Coast oyster. These are cholesterol, desmosterol, isofucosterol and 24-methylenecholesterol (21). In this same study, starvation of oysters resulted in lower levels of brassicasterol and 24-methylenecholesterol. Thus, although the latter compounds may be synthesized by the oyster, biosynthesis appears limited and supplementary dietary intake is necessary. Brassicasterol is abundant in marine algae (22) and East Coast oysters contain a mixture of both the 24S and 24R epimers (4).

Two sterols in oysters, and many other mollusks, are C_{26}-sterol (22-*trans*-24-norcholesta-5,22-diene-3β-ol) and 22-dehydrocholesterol. When found, these compounds generally occur together. They are also found in sponges, but in few other invertebrates. The significance and origin of these two sterols in oysters is the least known.

The presence of $\Delta^{5,7}$-sterols in bivalves has long been known and because of their presence, these animals were evaluated as a commercial source of provitamin D. Possibly the provitamin is converted to vitamin D to function in the visceral mass or mantle. However, there is no experimental evidence that the formation of vitamin D can occur without a photochemical process which is most likely unavailable to the oyster. Certain algae synthesize $\Delta^{5,7}$-sterols. In addition to 7-dehydrocholesterol, ergosterol may be a $\Delta^{5,7}$-sterol present in Pacific oysters derived from algae. Ergosterol, and its isomer epiergosterol, were found to be the most abundant $\Delta^{5,7}$-sterols in *C. virginica* (5).

The 24β sterols common in algae (22) are found in Eastern oysters (4) and are those suspected of being present in Pacific Coast oysters. However, the sterol, 22-dihydrobrassicasterol (24-methylcholesta-5-en-3β-ol) was never detected in any of the oyster sterol fractions examined nor in other mollusks of the Pacific Northwest. This may represent a major difference between *C. virginica* and *C. gigas* and/or habitat.

TABLE 3
Percent Anatomical Distribution of Sterols in Oysters (*Crassostrea gigas*)

	Body mass[a]	Total sterols
	%	
Mantle	44.1	41.4
Visceral Mass	30.2	36.7
Gills	13.2	11.7
Adductor muscle	8.3	3.7
Labial palps	4.2	6.5

[a]Mean value of 3 oysters, avg wt 66±1 g.

Fucosterol is reportedly found only in brown algae (*Phaeophyta*) which may be considered the only source of this compound (21). On very tenuous evidence, based on their presence and accumulation, poriferosterol, clionasterol and fucosterol may be required by the oyster, albeit at unknown levels.

The distribution of major sterols between Eastern Coast and Pacific Coast oysters is presented in Table 4. The analytical methodology employed by Teshima et al. (4) allowed for a more complete sterol analysis of the Eastern Coast oysters than was accomplished with Pacific Coast oysters. However, there appears to be a great deal of similarity between results of this study and those of Teshima et al. for Eastern Coast oysters examined from a variety of locations (23).

Comparing anatomical distribution of sterols in oysters with known requirements for these compounds and their presence in the ocean environment may help in meeting the nutritional needs of these biovalves under aquacultural conditions.

TABLE 4
Percent of Major Sterols in Eastern Oysters (*C. virginica*) and Pacific Coast Oysters (*C. gigas*)

Common-trivial name	Systematic nomenclature	Empirical formula	Teshima et al. (1980) % of total sterols	This study[a]
C_{26}-sterol	22-*trans*-24-Norcholesta-5,22-diene-3β-ol	$C_{26}H_{42}O$	4.0	11.0
22-dehydrocholesterol	22-*trans*-Cholesta-5,22-diene-3β-ol	$C_{27}H_{44}O$	10.2	8.7
Cholesterol	Cholesta-5-en-3β-ol	$C_{27}H_{46}O$	34.0	26.9
Brassicasterol (and epibrassicasterol)	24-Methylcholesta-5,22-diene-3β-ol	$C_{28}H_{46}O$	15.6	15.7
Desmosterol	Cholesta-5,24-diene-3β-ol	$C_{27}H_{44}O$	0.2	
Δ5,7-Sterols (e.g., 7-dehydrocholesterol)	(Cholesta-5,7-diene-3β-ol)	$C_{27}H_{44}O$	6.6[b]	13.0[c]
24-Methylenecholesterol	24-Methylenecholesta-5-en-3β-ol	$C_{28}H_{46}O$	12.6	16.8
22-Dihydrobrassicasterol	24-Methylcholesta-5-en-3β-ol	$C_{28}H_{48}O$	3.7	-----
Poriferasterol	24-Ethylcholesta-5,22-diene-3β-ol	$C_{29}H_{48}O$	2.0	0.7
Clionasterol	24-Ethylcholesta-5-en-3β-ol	$C_{29}H_{50}O$	3.7	
Fucosterol	(24E)-24-Ethylidenecholesta-5-en-3β-ol	$C_{29}H_{52}O$	1.5	7.3
Isofucosterol	(24Z)-24-Ethylidenecholesta-5-en-3β-ol	$C_{29}H_{52}O$	4.6	
		Total	98.7	100.1

[a]Percentage obtained from Table 2.
[b]Seven $\Delta^{5,7}$-sterols (5).
[c]Determined colorimetrically with modified Liebermann-Burchard reaction using cholesterol and 7-dehydrocholesterol.

ACKNOWLEDGMENT

This work was supported in part by the USDC, NOAA, Institutional Sea Grant and the National Fisheries Institute. Appreciation is extended to Connie Moorehouse, Oregon State University Sea Grant, for the free-hand illustration of the oyster in Figure 1.

REFERENCES

1. Bergmann, W. (1962) in Comparative Biochemistry (Floskin, M. and H.S. Mason, eds.) pp. 103-162. Academic Press, New York, NY.
2. Idler, D.R., and P. Wiseman (1971). Int. J. Biochem. 2, 516-528.
3. Idler, D.R., and P. Wiseman (1972). J. Fish. Res. Board Can. 29, 385-398.
4. Teshima, S., Patterson, G.W., and Dutky, S.R. (1980). Lipids 15, 1004-1011.
5. Teshima, S., and Patterson, G.W. Comp. Biochem. Physiol. 68B, 177-181.

6. Teshima, S., and Patterson, G.W. Comp Biochem. Physiol. 69B, 175-181.
7. Nes, R.W., and Nes, W.D. (1980) in Lipids in Evolution, pp. 47-54, Plenum Press, New York, NY.
8. Idler, D.R., and Wiseman, P. (1971). Int. J. Biochem. 2, 91-98.
9. Sabine, J.R. (1977) in Cholesterol (pp. 1-43). Marcel Dekker, Inc., New York, NY.
10. Parker, R.S. (1981), The Use of Liposomes as Capsulating Agents for Feeding Juvenile Pacific Oysters (*Crassostrea gigas*), Ph.D. Thesis, Oregon State University, Corvallis. pp. 15-20.
11. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957). J. Biol Chem. 226, 497-509.
12. Abell, L.L., Levy, B.B., Brodie, B.B., and Kendall, F.E. (1952) J. Biol. Chem. 195, 357-366.
13. Schoenheimer, R., and Sperry, W.M., (1934). J. Biol. Chem. 106, 745-760.
14. Gordon, D.T. (1982) J. Am. Oil Chem. Soc., scheduled for December publication.
15. Miettinen, T.A., Ahrens, E.H., Jr., and Grundy, S.M. (1965). J. Lipid Res., 6, 411-424.
16. Matsumoto, T., Tamura, T., and Ito, S. (1955). Nippon Kagaku Zasshi. 76, 951-953. Also see (1).
17. Swift, M.L., White, D., and Ghassernieh, M.B. (1980). Lipids 15, 129-132.
18. Idler, D.R., and Wiseman, P. (1972). J. Fish Res. Board Can. 29, 385-398.
19. Idler, D.R., Tamura, T., and Wainai, T. (1964). J. Fish. Res. Board Can. 21, 1035-1042.
20. Galtsoff, D.S. (1964). The American Oyster. *Crassostrea virginica* Gmelin, Fishery Bulletin, Vol. 64, U.S. Dept. of the Interior, Fish and Wildlife Service, Bureau of Commercial Fisheries.
21. Teshima, S., and Patterson, (1981). Lipids 16, 234-239.
22. Nes, W.R., and McKean, M.L. (1977), in Biochemistry of Sterols and Other Isopentenoids, pp. 458-478. University Park Press, Baltimore, MD.
23. Berenberg, C.J., and Patterson, G.W. (1981). Lipids 16, 276-278.

[Received April 12, 1982]

Composition and Biosynthesis of Sterols in Selected Marine Phytoplankton

DON S. LIN[a], AJMAL M. ILIAS[a,1], WILLIAM E. CONNOR[a,*], RICHARD S. CALDWELL[b,2], H. THERESE CORY[c], and G. DOYLE DAVES, JR.[c], [a]*Section of Clinical Nutrition-Lipid Metabolism, Department of Medicine, University of Oregon Health Sciences University, Portland, OR 97201;* [b]*Department of Fisheries and Wildlife, Oregon State University, Marine Science Center, Newport, OR 97365; and* [c]*Department of Chemistry and Biochemical Sciences, Oregon Graduate Center, Beaverton, OR 97006*

ABSTRACT

Six species of phytoplankton, *Pseudoisochrysis paradoxa, Isochrysis galbana, Monochrysis lutheri, Platymonas suecica, Thalassiosira fluviatilis* and a *Chaetoceros* species, were cultured in the laboratory and their sterol contents analyzed utilizing digitonin precipitation, thin layer and gas chromatography and gas chromatography-mass spectrometry. A total of 7 sterols were found in phytoplankton. The occurrence of these sterols, cholest-5-en-3β-ol, cholest-5,22-dien-3β-ol, 24-methylcholesta-5,24(28)-dien-3β-ol, 24-methylcholest-5-en-3β-ol, 24-methylcholesta-5,22-dien-3β-ol, 24-ethylcholest-5-en-3β-ol and 24-ethylcholest-5,22-dien-3β-ol, differed significantly among the various phytoplankton species. Cultures of *P. paradoxa* biosynthesized both of the sterols found in this species when incubated in the presence of ^{14}C- or ^{3}H-mevalonic acid for 0.5-9 days. These sterols were cholesterol and 24-methylcholesta-5,22-dien-3β-ol. Since 5 of the sterols found in the phytoplankton commonly occur in mollusks which feed on phytoplankton, it is likely that at least some of the tissue sterols in mollusks are of dietary origin.

Lipids 17:818-824, 1982.

INTRODUCTION

Many species of marine filter feeders consume phytoplankton as a major nutrient source (1). Their fatty acid composition, rich in ω-3 fatty acids, is mirrored by a similar fatty acid composition in animals higher on the food chain: marine invertebrates, fish, and sea mammals.

Certain shellfish (clams, oysters, scallops), which are filter feeders, have been found to contain many other sterols besides cholesterol, but their origin remains obscure (2-5). Because of the absence or low levels of cholesterol biosynthesis in shellfish (6-9), we have hypothesized that dietary sterols from phytoplankton must play an important role in meeting the sterol needs of these shellfish. Indeed, we recently have shown the active absorption of dietary cholesterol and plant sterols by the Florida land crab (10).

In the present study, we consider the question of whether phytoplankton could synthesize both cholesterol and the several other sterols observed in clams, oysters and scallops. Secondly, were the noncholesterol sterols of phytoplankton similar to the sterols found in these mollusks? Our findings indicate that the different species of phytoplankton studied contain many of the sterols identified in mollusks (clams, oysters and scallops) and could logically be considered their dietary sources of sterols. Active sterol synthesis was demonstrated in the phytoplankton, *Pseudoisochrysis paradoxa.*

METHODS AND MATERIALS

Phytoplankton Cultures

The following phytoplankton species were cultured: chrysophytes, *P. paradoxa, Isochrysis galbana,* and *Monochrysis lutheri;* the chlorophyte, *Platymonas suecica;* and the diatoms, *Thalassiosira fluviatilis* and *Chaetoceros* sp. Inoculates from pure cultures, maintained at the Oregon State University Marine Science Center aquaculture laboratory, were successively cultured in 250 ml, 3 and 18 liters volumes for from 10 to 15 days at each step. Cultures were maintained under constant light (300-600 foot-candles), at a temperature of 18±1.0C, and, except for the diatom cultures, were aerated with filtered oil-free compressed air through glass tubes of ca. 5 mm inside diameter.

Algae were cultured in seawater that was filtered to a final particle size of 0.3 μm, sterilized by a combination of chlorination and ultraviolet light treatment, and dechlorinated by passing through a column of activated charcoal 8 cm in diameter by 2 m in length at a rate of 1

[1]Research trainee, HL 07295-02, National Heart, Lung and Blood Institute.

[2]Current address: Northwestern Aquatic Sciences, Inc., Marine Research Laboratory, PO Box 1437, Newport, OR 97365.

liter/min. Nutrients, trace metals and vitamins were supplied to each culture at final concentrations of: $NaNO_3$, 150 mg/l; $NaH_2PO_4 \cdot H_2O$, 10 mg/l; $CuSO_4 \cdot 5H_2O$, 1.96 μg/l; $ZnSO_4 \cdot 7H_2O$, 4.40 μg/l; $CoCl_2 \cdot 6H_2O$, 2.00 μg/l; $MnCl_2 \cdot 4H_2O$, 36.0 μg/l; $Na_2MoO_4 \cdot 2H_2O$, 1.26 μg/l; ferric sodium salt of EDTA, 10 mg/l; biotin, 1 μg/l, vitamin B_{12}, 1 μg/l; and thiamine hydrochloride, 200 μg/l (11). Diatom cultures contained 30 μg/l of $Na_2SiO_3 \cdot 9H_2O$ in addition.

Chemical Analysis and Identification of Sterols

The algal cells were collected from the final 18 liters cultures by centrifugation in a Sorvall continuous flow centrifuge and dried overnight at 80C. The lipids in a weighed portion of the dried phytoplankton were extracted with chloroform/methanol (12). An aliquot of the lipid extract was saponified using alcoholic potassium hydroxide. The nonsaponifiable material was extracted with hexane and dried under nitrogen. Sterols were precipitated with digitonin (13). The precipitate was washed with diethyl ether and dried. The sterols were recovered by dissolving the digitonide precipitate in pyridine and extracting the free sterols with diethyl ether. The ether extract was dried under vacuum over concentrated H_2SO_4.

The digitonin precipitable sterols were converted to trimethylsilyl ether derivatives and subjected to gas chromatographic (GC) analysis using an instrument equipped with a hydrogen flame ionization detector (Perkin Elmer Sigma 3B Gas Chromatograph). The conditions used for these analyses were as follows: The column was a fused silica capillary coated with SE-30. The instrument was operated with a helium flow rate of 60 ml/min and 80 psi head pressure at a temperature of 280C (injection port 300C, flame detector 300C). A Hewlett-Packard model 3390A integrator was used to obtain the retention time and peak area of each compound.

For further purification and identification, the digitonin precipitable sterols were subjected to thin layer chromatography (TLC) using two systems. The first TLC system employed Florosil plates and heptane/ethyl ether (45:55) as eluting solvent. All the sterols migrate as fast as cholesterol in this system. The second TLC system used silver nitrate impregnated Florisil plates with chloroform/acetone (97:3) as solvent. 24-Methyl-cholesta-5,24(28)-dien-3β-ol (24-methylene cholesterol) migrated slower and thus was separated from the other sterols. The sterols recovered from TLC were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Varian Aerograph 2700 gas chromatograph coupled with a Dupont 21-491B mass spectrometer (14,15). The GC column (4 ft x 2 mm id) was packed with SP2250 (Supelco, 1% on 100-120 mesh Supelcoport). The operating conditions were: column temperature, 275C; injection port temperature, 290C; detector temp, 250C; injection volume, 1-2 μl; carrier gas (helium) flow rate, 40 ml/min. Sterols emerging from the GC column were monitored by flame ionization and mass spectra were recorded. Quantities of sterols in the various phytoplankton were determined from GC data using authentic standards of the sterols for calibration, as described previously (16,17). Cholesterol-4-C^{14} was used as the internal standard to monitor the recovery. Authentic samples of the sterols were obtained from the following sources: cholesterol (cholesta-5-en-3β-ol) was purchased from Applied Science Laboratories, State College, PA). Campesterol (24-methylcholesta-5-en-3β-ol), stigmasterol (24-ethylcholesta-5,22-dien-3β-ol), sitosterol (24-ethylcholesta-5-en-3β-ol), and brassicasterol (24-methylcholesta-5,22-dien-3β-ol) were purchased from Supelco, Inc., Bellefonte, PA. The 22-dehydrocholesterol (cholest-5,22-dien-3β-ol) and 24-methylene cholesterol (24-methylcholesta-5,24(28)-dien-3β-ol) were isolated from oysters in our laboratory.

Sterol Biosynthesis in Phytoplankton

The chrysophyte, *P. paradoxa*, was selected for studies of sterol biosynthesis from radiolabeled mevalonic acid. Nineteen liter cultures were started in 5-gallon glass carboys by the addition of a 3-liter inoculum from growing laboratory stocks of the algae. Culture conditions were as previously described (14). In several separate experiments, each culture was injected with up to 10 ml of the free form of mevalonic acid-2-^{14}C (6.33 MCi/MM, New England Nuclear Corp., Boston, MA) or mevalonic acid-2-^{3}H, (176 MCi/MM, Amersham Corp., Arlington Heights, IL) dissolved in sterile saline. The injected radioactivity was 10 μCi and 50 μCi, respectively, for the two isotopic forms of mevalonic acid. Individual algal cultures were collected by centrifugation at various times ranging from 12 hr to 9 days after culture initiation and dried at 80C in preparation for chemical (see above) and radiochemical analyses.

Radiochemical Analyses

There were two sterols (cholesterol and 24-methylcholesta-5,22-dien-3β-ol) found in *P. paradoxa* which will be reported subsequently. For the determination of the radioactivity in

these sterol fractions, the digitonin precipitable sterols were acetylated and separated by thin layer argentation chromatography (18). The mass of the individual sterol was determined by GLC as described previously. Its radioactivity was determined by dissolving the sample in 10 ml of scintillation mixture (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(2-[5-phenyloxazolyl])-benzene in 1 liter of toluene) and counting in a Packard Tri-Carb liquid scintillation spectrometer (model 3380, Packard Co., Downers Grove, IL) with an efficiency of 87% of ^{14}C and 41% for ^{3}H.

RESULTS

Sterol Composition in Different Species of Phytoplankton

The sterols of 6 species of cultured phytoplankton were isolated by digitonin precipitation and TLC and were analyzed by GC and by GC-MS. Relative gas chromatographic retention times for the 7 different sterols found to occur in the phytoplankton examined and structurally significant ions which occur in the sterol mass spectra are recorded in Table 1. Assignments of structures for the 7 sterols found (A-G, Table 1) were accomplished by comparison of GC and GC-MS characteristics with those obtained using authentic standards. These data serve to establish the composition of the sterols, the numbers and sites of unsaturation and the nature (methylene, methyl or ethyl) of substituents occurring at C-24. The configurations of substituents at C-24 were not assigned (19,20).

The phytoplankton species studied and the sterol composition found in each one are noted in Table 2. There were large differences in sterol composition in the different species. The sterol content varied from only 2 sterols in *P. paradoxa* to 6 different sterols in *Chaetoceros*. The sterol concentration was lowest in *Chaetoceros* (0.50 mg/g dried wt) and highest in *M. lutheri* (7.97 mg/g dried wt). 24-Methylcholesta-5,22-dien-3β-ol was the predominant sterol in *P. paradoxa* and *I. galbana*. 24-Methylcholesta-5,24(28)-dien-3β-ol (24-methylene cholesterol) was the predominant sterol in *T. fluviatilis* and *P. suecica* and the 24-ethylcholest-5-en-3β-ol was the major sterol in *M. lutheri* and *Chaetoceros*. These differ from red and brown algae which have, respectively, cholesterol and fucosterol as predominant sterols (21).

Sterol Biosynthesis in Phytoplankton

Three separate incubation experiments were conducted employing mevalonic-2-H^3 acid (experiments 1 and 2) and mevalonate-2-C^{14} acid (experiment 3) with pure cultures of *P. paradoxa*. High concentrations of radioactivity (as high as 32,350 dpm/g dried wt or 19,606 dpm/mg sterol) were found in the digitonin precipitable sterol fraction in experiment 3. This indicated biosynthesis of sterol was occurring in these actively growing phytoplankton cultures (Table 3a). Lesser incorporation was observed in experiment 1. This may be due to the natural variation of the cultures. Nevertheless, significant radioactivity levels (1925 dpm/mg dried wt or 1119 dpm/mg sterol) were detected in the sterol fraction of this experiment as well. In these multiple experiments, the specific radioactivity of 24-methylcholesta-5,22-dien-3β-ol peaked between 2 and 6 days of incubation. The averaged sterol content was 1.51 mg/g dried algae in experiment 1, 2.36 mg/g dried wt in experiment 2, and 1.16 mg/g dried algae in experiment 3. Since a minute amount of cholesterol was present in this species of phytoplankton in addition to the major component of 24-methylcholesta-5,22-dien-3β-ol, we have separated these 2 components in the 5 samples of experiment 2 and determined their mass and radioactivity (Table 3b). A preponderance of the radioactivity resided in the molecule of 24-methylcholesta-5,22-dien-3β-ol. Radioactivity was also found in cholesterol in low quantity so that the accuracy about its absolute radioactivity is less certain.

DISCUSSION

Despite the importance of phytoplankton as the cornerstone of the ocean food chain, relatively little information about their sterol content is available (7). In the present study, we cultured 6 species of phytoplankton and analyzed their sterol composition. Seven sterols were identified (Tables 1 and 2). These included cholesterol, 22-dehydrocholesterol and 24-methylene cholesterol, the major sterols present in shellfish, for example the oyster, clam and scallops (2-5). Cholesterol was also found in diatoms *Nitzschia longissima, Nitzschia frusticulum, Daganmena pamila* and *Dhaetoceros simplex calcitrans* (7). Orcutt and Patterson reported the presence of significant amounts of 22-dehydrocholesterol (33% of total sterol) in diatoms *N. ovalis* (7). 24-Methylene cholesterol was detected in diatoms *Chaetoceros simplex calcitrans* (7), and in nonphotosynthetic diatom *Nitzschia alba* (22). The other 4 sterols that we found in the phytoplankton were C_{28} and C_{29} sterols with methyl or ethyl groups at the 24 position. These sterols have structures similar to campesterol, brassicasterol,

TABLE 1

Gas Chromatographic (GC) and Gas Chromatography-Mass Spectrometric (GC-MS) Data for Phytoplankton Sterols

Sterol	Assignment	GC Rention time (cholesta-5-en-3β-ol=1.00) System 1[a]	System 2[a]	Significant ions in mass spectra m/z (% rel abundance)
A	Cholesta-5-en-3β-ol (cholesterol)	1.00	1.00	386(M^+, 100), 371 (45), 368 (55), 353 (48), 301 (45), 275 (88), 273 (29), 255 (37), 213 (50)
B	Cholesta-5,22-dien-3β-ol (22-dehydrocholesterol)	0.93	0.80	384($M^{+\cdot}$, 97), 369 (18), 366 (17), 351 (16), 300 (73), 285 (20), 273 (43), 255 (100), 213 (37)
C	24-Methylcholesta-5,24(28)-dien-3β-ol (24-methylene cholesterol)	1.20	1.26	398($M^{+\cdot}$, 100), 383 (16), 380 (18), 365 (18), 314 (10), 299 (50), 281 (34), 271 (52), 213 (28)
D	24-Methylcholesta-5-en-3β-ol (campesterol/24 epicampesterol)	1.21	1.20	400($M^{+\cdot}$, 100), 385 (32), 382 (51), 367 (27), 315 (51), 289 (43), 273 (24), 255 (27), 213 (32)
E	24-Methylcholesta-5,22-dien-3β-ol (brassicasterol/24 epibrassicasterol)	1.08	1.12	398($M^{+\cdot}$, 100), 383 (12), 380 (12), 365 (10), 355 (10), 337 (17), 314 (10), 313 (8), 300 (45), 285 (7), 271 (32), 255 (61), 213 (19)
F	24-Ethylcholesta-5-en-3β-ol (sitosterol/clionasterol)	1.44	1.40	414($M^{+\cdot}$, 100), 399 (13), 396 (20), 381 (12), 329 (15), 303 (17), 273 (9), 255 (10), 231 (8), 213 (12)
G	24-Ethylcholesta-5,22-dien-3β-ol (stigmasterol/poriferasterol)	1.28	1.37	412($M^{+\cdot}$, 100) 397 (17), 394 (14), 379 (14), 351 (41), 300 (55), 271 (60), 255 (79), 213 (18)

[a]Chromatographic conditions are described in Methods and Materials; system 1, capillary SE-30 column; system 2, packed SP-2250 column.

TABLE 2

Sterol Composition of Six Phytoplankton Species and One Clam Species (mg/g dry wt)

Species of phytoplankton	5-ene[a] (A)	5,22-Diene (B)	5,24-Diene (C)	24-Me-5-ene (D)	24-Me-5, 22-diene (E)	24-Ethyl -5-ene (F)	24-Ethyl 5,22-diene (G)	Total
Pseudoisochrysis paradoxa	0.05				2.27			2.32
Isochrysis galbana	0.02		0.01	0.01	2.35	0.03		2.42
Thalassiosira fluviatilus	0.25		0.58	0.06		0.11		1.00
Monochrysis lutheri	0.01			1.31		5.84	0.81	7.97
Platymonas suecica	0.01		0.54	0.51				1.06
Chaetoceros	0.02	0.02	0.01	0.19	0.06	0.20		0.50
Species of mollusk								
Clams[b]	2.18	0.71	1.08		0.84	0.22		5.03

[a]Sterols as listed in Table 1. A=cholest-5-en-3β-ol. B=cholest-5,22-dien-3β-ol. C=ergosta-5,24(28)-dien-3β-ol. D=24-methyl-cholest-5-en-3β-ol. E=24-methyl-cholesta-5,22-dien-3β-ol. F=24-ethyl-cholest-5-en-3β-ol. G=24-ethyl-cholest-5,22-dien-3β-ol.
[b]Cockle clam, *Clinocardium nuttalli* (ref. 3).

TABLE 3a

Biosynthesis of 24-Methylcholesta-5,22-dien-3β-ol[a] From Radioactive Mevalonic Acid in the Phytoplankton *P. paradoxa*

Days of incubation	Experiment 1			Experiment 2			Experiment 3		
	Sterol content	Sterol[b] radio-activity	Sterol specific activity	Sterol content	Sterol radio-activity	Sterol specific activity	Sterol content	Sterol radio-activity	Sterol specific activity
	(mg/g dried wt)	(dpm/g dried wt)	(dpm/mg sterol)	(mg/g dried wt)	(dpm/g dried wt)	(dpm/mg sterol)	(mg/g dried wt)	(dpm/g dried wt)	(dpm/mg sterol)
0.5	0.74	296	400						
1	1.60	734	459	2.16	7,066	3,271			
1.5	2.00	883	441						
2	1.72	1925	1119	2.38	21,029	8,835			
3				1.97	15,041	7,635	1.06	9,892	9,332
4				2.43	15,792	6,499			
5				2.87	7,740	2,697			
6							1.65	32,350	19,606
9							0.76	12,388	16,300

[a]The major sterol identified, in *P. paradoxa*.
[b]Total radioactivity of 24-methylcholesta-5,22-dien-3β-ol (major) and cholesterol (minor). See text and Table 3b.

TABLE 3b

Sterol Biosynthesis from Radioactive Mevalonic Acid in the Phytoplankton *P. paradoxa.*

Days of incubation	Sterol content (mg/g dried wt)		Sterol radioactivities (dpm/ g dried wt)		Sterol specific activities (dpm/mg sterol)	
	Cholesterol	24-Methyl-cholesta-5, 22-dien-3β-ol	Cholesterol	24-Methyl-cholesta-5, 22-dien-3β-ol	Cholesterol	24-Methyl-cholesta-5, 22-dien-3β-ol
1	0.05	2.12	281	6,698	5,620	3,159
2	0.03	2.41	240	21,089	8,000	8,751
3	0.03	2.03	283	14,893	9,433	7,336
4	0.02	2.40	204	14,695	10,200	6,122
5	0.02	2.88	157	7,525	7,850	2,612

stigmasterol and β-sitosterol, except that the configuration of substituents on C-24 was not assigned. Although campesterol, brassicasterol, stigmasterol and β-sitosterol have been reported in shellfish (3-5), in these studies also, the absolute configuration of the C-24 substituents was not definitely established. Nevertheless, in view of the low sterol synthetic rate in shellfish (6-9), and the finding of these major marine sterols and the other structurally similar sterols, further support is gained for the suggestion that phytoplankton may play in important role in supplying the sterol requirements for marine invertebrates. A possible dietary contribution to provide for the sterol needs of mollusks has also been suggested by others (7,8).

It is interesting to note the variety of sterols, both in quantity and quality, found in the different species of phytoplankton studied (Table 2). *P. paradoxa* only has 2 sterols, whereas *Chaetoceros* contain 6 different sterols. The sterol concentration of *Chaetoceros* was 0.50 mg/g dried wt and that of *M. lutheri* was 18 times higher, 7.97 mg/g dried weight. 24-Methylcholesta-5,22-dien-3β-ol accounted for 98% and 97% of the sterols in *P. paradoxa* and *I. galbana,* respectively. In *T. fluviatilis* and *P. suecica,* 24-methylcholesta-5,24(28)-dien-3β-ol (24-methylene cholesterol) was the major sterol, contributing 58% and 51% of total sterols, respectively. The major sterol in *M. lutheri* and *Chaetoceros* was 24-ethylcholesta-5-en-3β-ol (73% and 40% of total sterols). Orcutt and Patterson found that in the diatoms *Nitzschia frustulum,* 24α-methylcholesta-5,22-dien-3β-ol was the major sterol at 54% (7). 24-Methylcholesta-5,24(28)-dien-3β-ol (24-methylene cholesterol) was reported to contribute 40% of the sterols in *C. simplex calcitrans* (7) and is the only sterol in the nonphotosynthetic diatom *N. alba* (22). Boutry and Jacques observed that the sterols from a natural sample of phytoplankton consisted of about 75% cholesterol (7). These data clearly demonstrate that there are wide variations in sterol pattern in the phytoplankton. The factors governing the sterol content and composition in different species of phytoplankton generally remain obscure. However, Boutry et al. have shown that both sterol composition and content vary with different light conditions (23).

While the pathway of sterol biosynthesis has been studied extensively in vertebrates and to a lesser extent in invertebrates, there is no information concerning the biosynthesis of sterols in marine phytoplankton. Incorporation of C^{14}-labeled bicarbonate into lipids by phytoplankton has been previously reported (24). However, individual lipid classes were not identified. In the present study, we incubated isotopic mevalonic acid with cultured phytoplankton *P. paradoxa* for 0.5-9 days. Active sterol synthesis was demonstrated by the presence of large amounts of radioactivity in the digitonin precipitable sterol fraction.

Our data indicated the biosynthesis of the two sterols found in this species: cholesterol, a minor component, and 24-methylcholesta-5,22-dien-3β-ol, which was the major sterol component. Perhaps these 2 sterols are synthesized via 2 slightly different pathways (22) or there may be interconversion of these two sterols.

ACKNOWLEDGMENT

This work was supported in part by a research grant from the National Institutes of Health (AM 21478-03).

REFERENCES

1. Newell, G.E., and Newell, R.C. (1963). Marine Plankton, pp. 32-40. Hutchinson, London.
2. Austin, J. (1970) in The Sterols of Marine Invertebrates and Plants, (Briggs, M.D., eds.) pp. 73-96, Academic Press, New York, NY.
3. Connor, William E., and Lin, D.S. (1981). Gastroenterology 81, 276-284.
4. Kritchevsky, D., Tepper, S.A., Ditullio, N., and Holmes, W.L. (1967). J. Food Sci. 32, 64-66.
5. Patterson, G.W., Khalil, M.W., and Zoller, D. (1975). J. Chromatogr. 115, 153-159.

6 Pennock, J.F. (1977) in Biochemistry of Lipids II, International Review of Biochemistry, (Goodwin, T.W., ed.), Vol. 14, pp. 153, University Park Press, Baltimore, MD.
7. Goad, L.J. (1978) in Marine Natural Products: Chemical and Biological Perspectives, (Schener, P.J., ed.) Vol. II, pp. 82-84 and 124-128, Academic Press, New York.
8. Nes, W.R., and McKean, M.L. (1977) in Biochemistry of Steroids and Other Isopentenoids, pp. 433-439, University Park Press, Baltimore, MD.
9. Nes, W.R., and Nes, W.D. (1980) in Lipids in Evolution, pp. 69-71, Plenum Press, New York, NY.
10. Douglass, S.T., Connor, W.E., and Lin, D.S. (1981). J. Lipid Res. 22, 961-970.
11. Breese, W.P., and Malouz, R.E. (1975) in Hatching Manual for the Pacific Oyster, p. 22, Publication No. ORESHU-H-75-002, Oregon State University Sea Grant College Program, Corvallis, OR.
12. Folch, J., Lees, M., and Sloan-Stanley, G.H. (1957). J. Biol. Chem. 226, 497-509.
13. Sperry, W.M. (1963). J. Lipid Res. 4, 221-225.
14. Smith, R.G., and Daves, D., Jr., Biomed. Mass Spectr. 4, 146-151 (1977).
15. Frick, W., Cbang, D., Folkders, K., and Daves, D., Jr. (1977). Anal. Chem. 49, 1241-1245.
16. Lin, D.S., Connor, William E., Napton, L.K., and Geizer, R.F. (1978). J. Lipid Res. 19, 215-221.
17. Ilias, A.M., Connor, W.E., Cory, H.T., Lin, D.S., Daves, G.D., Jr., and Krippaehne, W.E. (1980). Gastroenterology 79, 539-544.
18. Vroman, H.E., and Cohen, C.F. (1967). J. Lipid Res. 8, 150-152.
19. Rubinstein, I., Goad, L.J., Clagne, A.D.H., and Mulheirn, L.J. (1976). Phytochemistry 15, 195-200.
20. Thompson, M.J., Dutky, S.R., Patterson, G.W., and Gooden, E.L. (1972). Phytochemistry 11, 1781-1790.
21. Patterson, G.W., (1971). Lipids 6, 120-127.
22. Goad, L.J. (1976) in Biochemical and Biophysical Perspectives in Marine Biology, (Malins, D.C., and Sargent, J.R., eds.), Vol. 3, Academic Press, New York, pp. 213-218.
23. Boutry, J.L., Barbier, M., Ricard, M., (1976). J. Exp. Mar. Biol. Ecol. 21, 69-74.
24. Smith, A.E., and Morris, I. (1980). Science 207, 197-198.

[Revision received July 26, 1982]

METHODS

Quantitative Measurement of Prostaglandins E_2 and E_3 by Selected Ion Monitoring

ALDO FERRETTI,* VINCENT P. FLANAGAN, and **JOHN M. ROMAN,** *Agricultural Research Service, Beltsville Human Nutrition Research Center, Lipid Nutrition Laboratory, Beltsville, MD 20705*

ABSTRACT

A method for the simultaneous quantitative analysis of prostaglandin E_2 (PGE_2) and PGE_3 is described. The PG were analyzed by selected ion monitoring as the methyl ester-TMS ether derivatives of PGB_2 and PGB_3, respectively. The internal standard for the quantification of both species was [3,3,4,4-$^2H_4$]PGE_2. A linear response over the range 0.6-50 ng (1.7-143 pmoles) was demonstrated for PGE_3. The chromatographic conditions used (2% SP-2330 column) afforded nearly baseline separation of the prostaglandins. New standard curves for PGE_3 must be developed each time the ion source parameters are changed. In a typical calibration run, the instrumental precision, expressed as coefficient of variation, ranged from 1.1 to 7.2% for PGE_2 (3 to 100 ng injected) and from 1.6 to 11.1% for PGE_3 (1.5-50 ng injected). The method was applied to the PG analysis of rat renomedullary tissues. The recovery of synthetic PGE_2 added to medullary homogenates was 100.5±1.7% (mean± SEM, n = 9), and the recovery of PGE_3 was 91.3±1.4% (n = 9).
Lipids 17:825-830, 1982.

Thrombotic tendencies and incidence of myocardial infarction are lower in Greenland Eskimos than in people subsisting on a Western-type diet (1,2). These effects were associated with dietary 5,8,11,14,17-eicosapentaenoic acid (20:5ω3, EPA) which is present in large amounts in the Eskimos' diet. Although the detailed biochemical mechanism (or mechanisms) producing those effects are still a matter of controversy, EPA – which replaces the ω6 fatty acids (primarily arachidonic acid, 20:4ω6) in tissue lipids – apparently does affect the qualitative and quantitative production of prostaglandins (PG), including a shift from the 2- to the 3-series PG. The physiological effects of the PG metabolism thus altered would be reduced platelet aggregability and a less thrombogenic state (3,4). Results of a recent animal study conducted in our laboratories (5) confirmed previous results (6,7) as to the ability of dietary ω3 fatty acids to depress the biosynthesis of PGE_2 and $PGF_{2\alpha}$ in vitro. Furthermore, endogenous eicosapentaenoic acid of dietary origin is converted in vitro to prostaglandin E_3 (PGE_3) (8). Thus, studies of essential fatty acid metabolism in animals and humans often require the measurement, in selected biological systems, of PG metabolites of arachidonic acid and EPA, e.g. prostaglandin E_2 (PGE_2) and PGE_3, respectively. To our knowledge, a method for the quantification of PGE_3, alone or together with PGE_2, by selected ion monitoring (SIM) has not been reported. We now report the successful use of [3,3,4,4-$^2H_4$]PGE_2 as internal standard for the simultaneous measurement of PGE_2 and PGE_3. The method was validated and applied to the analysis of renomedullary homogenates obtained from rats which had been fed a fish oil diet.

EXPERIMENTAL

Glassware and Materials

All analytical operations were done in silanized glassware. PGE_2, PGE_3, and [3,3,4,4-$^2H_4$]PGE_2 were provided by Dr. J. Pike and Dr. U. Axen of the Upjohn Co. All solvents were analytical grade and redistilled before use. Diazomethane was prepared from N-methyl-N-nitroso-p-toluenesulfonamide (Aldrich Chemical Co., Milwaukee, WI) and co-distilled with diethyl ether. Trimethylsilyl-

*Author to whom correspondence should be addressed.

Abbreviations: PG, prostaglandin(s); ME, methyl ester; TSI, trimethylsilylimidazole; SIM, selected ion monitoring.

imidazole (TSI) was purchased from Pierce Chemical Co., Rockford, IL, and Lipidex-5000 from Packard Instrument Co., Downers Grove, IL. Kidney medullae were recovered as described (5) from rats fed a fish oil-containing diet. The tissues were incubated and supplied frozen by Dr. N.W. Schoene of our laboratory.

Derivatization of Prostaglandins

Evaporations prior to derivatizations were conducted under a stream of dry N_2 at room temperature. Methylations were done with excess CH_2N_2 in diethyl ether/MeOH. The methyl esters (ME) of PGE were converted to the ME-TMS ether derivatives of their PGB counterparts by action of TSI in piperidine (1:1) according to a published procedure (9), and analyzed by GC-MS.

Lipidex-5000 Column Chromatography

Lipidex-5000 liquid-gel chromatography was carried out in glass columns (300 x 10 mm id) equipped with a solvent reservoir and a Teflon valve. Lipidex-5000 was sonicated and equilibrated for 1 hr in heptane/chloroform (7:3, v/v), which was also used as the eluent. The columns were packed under gravity flow to a height of 200 mm; flow rate was 0.25 ml/min. at 25±1 C. The columns, which were never allowed to dry out, were rinsed after use with heptane/chloroform (7:3, v/v) until pure solvent emerged and were used repeatedly.

Preparation and Analysis of Rat Kidney Medullae

Details of rat feeding protocol, tissue handling, and sample preparation for GC-MS analysis were reported previously (8). The wet weight of each medulla ranged from 20 to 30 mg. The concentrations of PGE_2 and PGE_3 in the kidney homogenates were calculated by linear regression analysis using the parameters of calibration curves developed on the same day under identical instrumental conditions (*vide infra*).

Instrumental Conditions

The GC-MS system we used was a Finnigan 3200F with an all-glass jet molecular separator. The glass columns, 1.50-m long x 2 mm id, were treated with dimethyldichlorosilane and packed with 2% SP-2330 on 100-120 mesh Gas Chrom Q. Temperatures were: injector, 235C; column, 225C; separator, 230C. The carrier gas (He) was supplied at a head pressure of 1.26 kg/cm^2, and the flow rate was about 20 ml/min. The mass spectrometer was operated at 70 and 40 eV, and the electron multiplier was set at 1.7 kV. We used a PROMIM (Finnigan) modular electronic programmer for selected ion monitoring, and the signals were fed to a Rikadenki 4-channel pen recorder. The ions selected were: m/z 325 and m/z 353 for monitoring $[^2H_4]PGE_2$; m/z 321 for PGE_2; and m/z 349 for PGE_3. Peak heights were used for quantitative measurements.

RESULTS

Mass Spectra

The quadrupole mass spectra of the ME-TMS ether derivatives of PGB_2, PGB_3, and $[3,3,4,4\text{-}^2H_4]PGB_2$ obtained by derivatization of their PGE counterparts were published previously (8,10).

Determination of Standard Curves

When a single standard curve for PGE_3 was determined at 70 eV, the plot response ratio (349/325) vs ng PGE_3 injected followed the equation $Y = 0.193\ X - 0.004$, with $R = 0.9958$. Figures 1 and 2 show simultaneous calibration curves for PGE_2 and PGE_3 developed by use of physically different ions sources and different ion source parameters. In all cases, 20 ng constant amount of $[3,3,4,4\text{-}^2H_4]PGE_2$ internal standard was used in each injection. The linearity ranges extend, on both upper and lower end, well beyond the levels of prostaglandins which are likely to be injected during the analysis of biological samples.

Instrumental Precision

Properly conditioned SP-2330 GC columns at 225C afforded nearly baseline separation of derivatized PGE_2 and PGE_3. This situation existed throughout the acquisition of calibration, recovery, and accuracy data reported here. Figure 3 shows a typical selected ion chromatogram of derivatized renal medullary PGE fraction from a Lipidex-5000 column. The data of Table 1, from one of the simultaneous calibrations (see Fig. 2), are typical, and indicate the instrumental precision at different levels of injected prostaglandins.

Recovery, Accuracy, and Precision

The overall accuracy of the method for analysis of rat kidney medullae was assessed by plotting ng PG measured vs ng PG added before extraction from three separate experiments. Thus, in a typical experiment, 4 nonspiked medullary homogenates (83.4 mg total wet tissue), after thawing, were pooled, acidified, and spiked with 2 μg of $[^2H_4]PGE_2$. The mixture was then brought to 14 ml with water and carefully divided into four 3.5-ml portions. Exact and increasing amounts of PGE_2 (100, 200, and 350 ng) and PGE_3 (50, 150, and 250

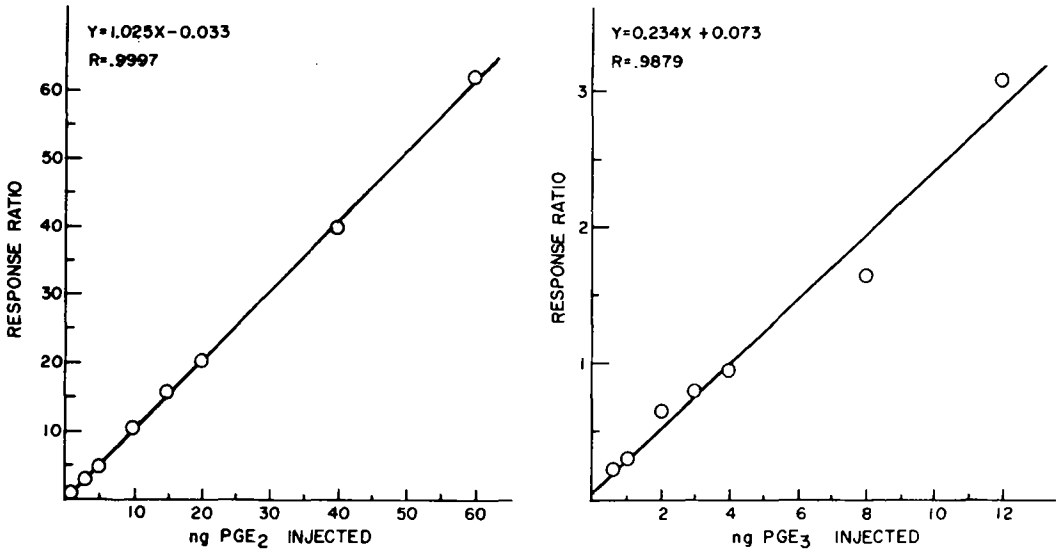


FIG. 1. Simultaneous calibration curves for PGE_2 and PGE_3 at 40 eV. The response ratios were 321 $PGB_2/325$ d_4-PGB_2 and 349 $PGB_3/325$ d_4-PGB_2.

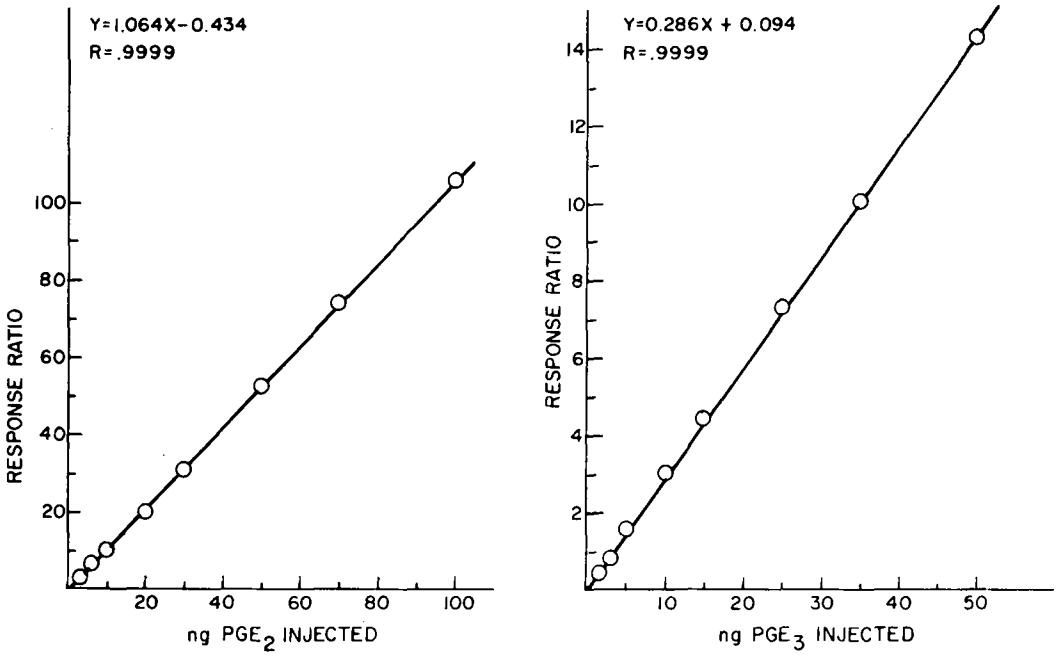


FIG. 2. Extended simultaneous calibration curves for PGE_2 and PGE_3 at 40 eV after ion source conditioning. Response ratios were as in Figure 1.

ng) were added to 3 of the 4 aliquots, and all 4 were analyzed as described in Experimental. The results are shown in Figures 4 (PGE_2) and 5 (PGE_3) where the endogenous PG levels (measured in the nonspiked aliquot) were subtracted. Please note that the abscissa values in Figures 4 and 5 are slightly offset to avoid excessive crowding. The mean amounts of PGE_2 and PGE_3 recovered from 9 samples were 100.5±1.7% and 91.3±1.4%, respectively. The data of Table 2 indicate the instrumental precision for one of the three recovery experiments described above.

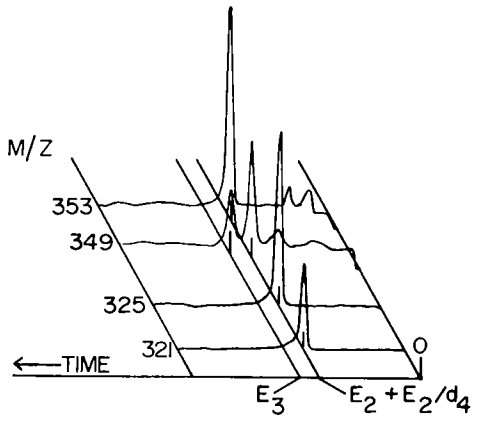


FIG. 3. Selected ion profiles of a derivatized renomedullary PGE fraction (plotted at different sensitivities). The major peaks of the m/z 325 and m/z 353 lines represent the internal standard.

DISCUSSION

This study was conducted over a period of several months during which the ion source had to be reconditioned a few times. Source parameters were adjusted to optimize sensitivity, resolution and peak shape. The choice of the ionizing energy (40 eV or 70 eV) depended on which value produced the highest intensity of the ions being monitored. The ions at m/z 321 and m/z 325 (8,10) correspond to the $[M-(C_5H_{11}+CO)]^+$ fragment of the ME-TMS derivatives of PGB_2 and $[^2H_4]PGB_2$, respectively. The m/z 349 ion in the spectrum of PGB_3-ME-TMS (8) arises from allylic cleavage and loss of the $[CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH_3]\cdot$ radical at the ω-side of the molecule.

The effects of ion source conditioning and of changes of instrumental parameters on the slope of PGE_3 standard curves are clearly demonstrated by this study (see Results and Figs. 1 and 2). Analogous observation was made in a previous study (10) in which PGE_1 calibration curves were based on a constant amount of $[^2H_4]PGE_2$. This is to say that $[^2H_4]PGE_2$ is not a perfect internal standard for quantification of PGE_1 and PGE_3. A practical corollary of the above is that PGE_3 in biological samples must be measured under instrumental conditions that are identical to those used in determining the calibration curves. As expected, the slopes of PGE_2 standard curves are not affected by changes of instrumental parameters.

The m/z 353 ion can be used in place of the m/z 325 ion to monitor $[^2H_4]PGE_2$. In that case, however, the instrumental precision was adversely affected, possibly because the m/z 353 ion represents a smaller – therefore a rela-

TABLE 1
Simultaneous Calibrations (see Figure 2)
Response Ratios and Instrumental Precision

$[^1H]PGE_2$			PGE_3		
ng Injected	$\bar{y}$ ± SEM[a]	CV[b]	ng Injected	$\bar{y}$ ± SEM[a]	CV[b]
100	106.1 ± 0.8	1.5	50	14.34 ± 0.24	3.3
70	74.4 ± 2.7	7.2	35	10.09 ± 0.56	11.1
50	52.4 ± 0.3	1.1	25	7.32 ± 0.06	1.6
30	31.0 ± 0.3	1.9	15	4.44 ± 0.07	3.1
20	20.0 ± 0.6	6.0	10	3.04 ± 0.10	6.6
10	10.30 ± 0.10	1.9	5	1.59 ± 0.02	2.5
6	6.69 ± 0.10	3.0	3	0.83 ± 0.01	2.4
3	3.05 ± 0.06	3.9	1.5	0.44 ± 0.01	4.5

[a]n = 4.
[b]Coefficient of variation (%).

tively more variable – percentage of the total ionization current (see spectrum of $[^2H_4]PGE_2$, ref. 10).

The instrumental precision in determining standard curves as well as in PG quantification in the biological matrix tends to be uniformly higher with PGE_2 than with PGE_3 (see Tables 1 and 2). We attribute that difference, at least in part, to a difference in elution time between PGE_3 and $[^2H_4]PGE_2$ in the GC column whereby minor changes in time-dependent variations in ionization conditions or ion detection sensitivity are not compensated for by a co-eluting internal standard. This compensatory effect is more important with prostaglandins than with less labile species. Notwithstanding this limitation and the fact that $[^2H_4]PGE_2$ is obviously somewhat less than a perfect carrier/internal standard for PGE_3, the recovery curves of Figures 4 and 5 and the statistics in Tables 1 and 2 indicate that our method is precise, reproducible, and accurate.

Our present study demonstrates that prostaglandins E_2 and E_3 can be quantified simultaneously by use of $[3,3,4,4\text{-}^2H_4]PGE_2$ as the carrier/internal standard without mutual interference with a GC system that affords a nearly baseline separation (Fig. 3). Furthermore, we demonstrated that Lipidex-5000 columns, which do not separate PGE_2 and PGE_3 under the conditions used, are useful for cleanup of extracts of kidney medullae. The method de-

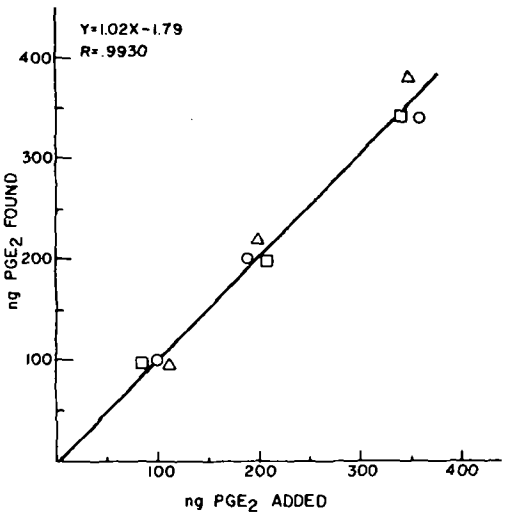


FIG. 4. Accuracy and precision: PGE_2 recovered vs synthetic PGE_2 which was added to 3 different pools of kidney medullae in 3 different amounts (100, 200, and 350 ng). Endogenous PGE_2 levels were subtracted. The abscissa values are slightly offset for clarity.

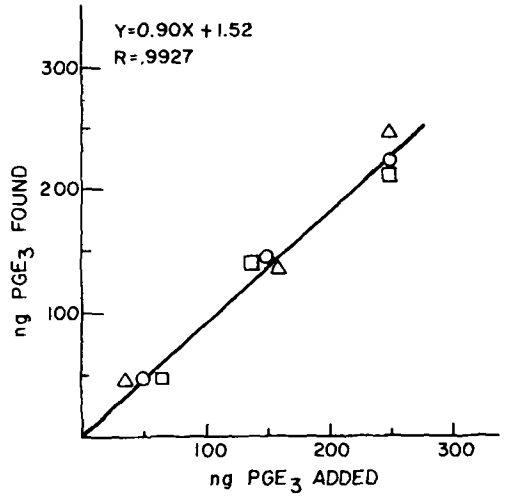


FIG. 5. Accuracy and precision: PGE_3 recovered vs synthetic PGE_3 which was added to 3 different pools of kidney medullae in 3 different amounts (50, 150, and 250 ng). Endogenous PGE_3 levels were subtracted. The abscissa values are slightly offset for clarity.

TABLE 2

Recovery, Accuracy and Precision: PG Measured vs Synthetic PG Added

$[^1H]PGE_2$			PGE_3		
ng Added	ng Found[a]	CV[b]	ng Added	ng Found[a]	CV[b]
0	191.8 ± 0.8	0.8	0	72.3 ± 1.3	3.6
100	287.0 ± 0.9	0.6	50	118.9 ± 2.8	4.7
200	411.4 ± 7.1	3.4	150	205.2 ± 5.2	5.1
350	571.1 ± 4.5	1.6	250	317.6 ± 10.7	6.7

[a]Mean ± SEM; n = 4.
[b]Coefficient of variation (%).

scribed was applied to a nutritional study with rats. The levels of prostaglandins E_2 and E_3 in medullary homogenates were reported in previous articles (5,8).

ACKNOWLEDGMENTS

We thank Dr. J. Pike and Dr. U. Axen of the Upjohn Co., Kalamazoo, MI, for generous supplies of PGE_2, PGE_3, and $[3,3,4,4\text{-}^2H_4]PGE_2$; Dr. N.W. Schoene of our laboratory for the rat kidney medullae.

REFERENCES

1. Dyerberg, J., Bang, H.O., Stoffersen, E., Moncada, S., and Vane, J.R. (1978) Lancet II, 117-119.
2. Dyerberg, J., and Bang, H.O. (1979) Lancet II, 433-435.
3. Needleman, P., Raz, A., Minkes, M.S., Ferrendelli, J.A., and Sprecher, H. (1979) Proc. Natl. Acad. Sci. USA 76, 944-948.
4. Whitaker, M.O., Wyche, A., Fitzpatrick, F., Sprecher, H., and Needleman, P. (1979) Proc. Natl. Acad. Sci. USA 76, 5919-5923.
5. Schoene, N.W., Ferretti, A., and Fiore, D. (1981) Lipids 16, 866-869.
6. Hwang, D.H., and Carroll, A. (1980) Am. J. Clin. Nutr. 33, 590-597.
7. Siess, W., Roth, P., Scherer, B., Kurzmann, I., Böhlig, B., and Weber, P.C. (1980) Lancet I, 441-444.
8. Ferretti, A., Schoene, N.W., and Flanagan, V.P. (1981) Lipids 16, 800-804.
9. Nicosia, S., and Galli, G. (1974) Anal. Biochem. 61, 192-199.
10. Ferretti, A., and Flanagan, V.P. (1979) Lipids 14, 483-491.

[Received May 3, 1982]

Glass Capillary Gas Chromatography-Mass Spectrometry of Wax Esters, Steryl Esters and Triacylglycerols

STUART G. WAKEHAM* and **NELSON M. FREW**, *Department of Chemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543*

ABSTRACT

Complex mixtures of wax esters, steryl esters and triacylglycerols isolated from representative biological and geochemical samples have been analyzed using combined high resolution gas chromatography and electron impact and chemical ionization quadrupole mass spectrometry. These low volatility neutral lipids containing up to 65 carbons were chromatographed intact on 15-20 m high-temperature (upper limit: 370 C) persilylated SE-52 and SE-30 glass capillary columns. Discrimination effects due to adsorptive losses and degradation were minimized using a nonvaporizing on-column injector and a direct all-glass capillary connection (370 C) to the quadrupole mass spectrometer. Structural information regarding the fatty acid and alcohol moieties was found to be maximal for methane-CI spectra in the case of wax and steryl esters, whereas EI spectra were most useful in interpreting triacylglycerol structures. Principal features of the EI and CI fragmentation patterns are discussed. The molecular composition of complex mixtures of these lipids is reconstructed for selected samples.
Lipids 17:831-843, 1982.

Complex mixtures of neutral lipids are present in biological systems and environmental samples. Long chain alkyl esters of long chain fatty acids (wax esters) are major components of the natural waxes of microorganisms, plants, and animals (1), being, for example, the principal metabolic energy reserve in many marine zooplankton (2). However, in other organisms, including mammals, triacylglycerols are the major energy storage form (1) and triacylglycerols are present in many natural oils and fats. Steryl esters function as intercellular transport forms of sterols (3), which themselves act as structural components and are responsible for many growth, respiratory, and reproductive processes through hormone regulation. Wax esters, steryl esters, and triacylglycerols are also important in the geosphere. They are associated with marine (4) and atmospheric (5) particulate matter and are present in aquatic sediments (6-8), making them important to geochemists studying the cycling of biologically produced organic matter in the environment.

Detailed compositional analyses of mixtures of wax esters, steryl esters, and traicylglycerols, whether for biological or geochemical interests, have generally been difficult to obtain for the intact compounds. Hydrolysis of mixtures followed by derivatization and gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) provides detailed information on the acid and alkyl moieties of the total mixture (e.g. 9-11), but structural data for individual components are lost. Limited structural information may be derived from fragment ions produced by direct probe MS of mixtures (8,11,12). GC of intact wax esters, steryl esters, and triacylglycerols using packed columns provides useful qualitative and quantitative information about carbon chain length distributions in a variety of sample types (13-29). Application of glass capillary columns to analysis of high molecular weight lipids can result in, but does not guarantee, improved resolution of complex mixtures and allows analysis of smaller samples (29-33). Structural information of intact wax esters, steryl esters, and triacylglycerols has been obtained by GC-MS (22-29,32,34), although several attempts have been hampered by low or moderate resolution and difficulties of transferring material from the gas chromatograph to the mass spectrometer. Recent advances in high-temperature glass capillary column technology (35-37), injection techniques (38-40), and GC-MS interfaces (41-45), as well as the use of short (5-10 m) capillary columns (6,46,47) have greatly improved the ability to obtain some direct structural information for small samples of complex mixtures of these high molecular weight, low volatility lipids.

Our investigations of organic matter in the marine environment depend on glass capillary GC-quadrupole MS data for structural elucidations, and limited sample availability often precludes detailed analyses by a variety of complementary techniques. In this paper, we describe application of high-temperature glass capillary GC-MS procedures for the analysis of intact

*Author to whom correspondence should be addressed.

wax esters, steryl esters, and triacylglycerols. The methods are applicable to a wide range of biological and geochemical samples as demonstrated by representative examples.

EXPERIMENTAL

Gas Chromatography

A Carlo Erba 4160 gas chromatograph equipped with a flame ionization detector and a nonvaporizing on-column injector (40) was used for analysis of intact wax esters, steryl esters, and triacylglycerols. Separations were made by a 26 m x 0.3 mm id glass capillary column coated in our laboratory with a 0.15 μm film of SE-52 according to the high-temperature silylation procedure of Grob (35). Hydrogen was the carrier gas: 1.5 kg/cm^2 (1.4 m/sec linear velocity at 180 C) for wax and steryl esters; 2.0 kg/cm^2 (1.9 m/sec) for triacylglycerols. On-column injections of hexane solutions (1-6 μl) were made at 100 C with auxiliary cooling (40), followed by temperature programming from 180-370 C at 2 C/min for wax and steryl esters and 200-370 C at 3.5 C/min for triacylglycerols. The FID temperature was set at 350 C; increasing the detector temperature is not recommended by the manufacturer, and in any event, we did not observe a significant increase in chromatographic efficiency during tests at elevated temperatures.

Gas Chromatography-Mass Spectrometry

A Finnigan Model 3200 quadrupole mass spectrometer was used to obtain electron impact and methane-chemical ionization spectra of wax esters and steryl esters and electron impact spectra of triacylglycerols. We modified the Finnigan 9500 gas chromatograph for high temperature operation by installing a water-cooled on-column injector (40) and by overriding the 300 C upper temperature safety devices on the column oven and interface. The glass capillary columns were directly coupled to the mass spectrometer by means of a silylated glass capillary (0.2 mm id) mounted in an interface after the design of Blum and Richter (43,44). The interface temperature was maintained at 370 C. At lower temperatures, significant discrimination effects were observed. All couplings were stainless steel or Vespel (E.I. duPont trademark) containing 15% graphite.

For GC-MS, the wax esters and steryl esters were separated on a 20 m x 0.3 mm id silylated SE-52 column (0.12 μm film thickness) with 1.3 kg/m^2 of helium as carrier gas. Following on-column injection at 100 C, the column temperature was programmed from 180-360 C at 3 C/min. Mass spectra were acquired beginning at 200 C. Chemical ionization mass spectra were obtained with methane as reagent gas at 950 μm ion source pressure. The ion source was operated at 130 eV and with 400 μA ionization current. The ion source temperature was about 160 C. At this temperature, there may be some cold-trapping in the ion source which appears to result in slight increases in peak tailing for higher molecular weight components compared to GC runs. However, attempts to increase source temperature resulted in unacceptably high fragmentation and loss of diagnostic ions, similar to that previously reported by Hennenberg et al. (42).

Electron impact spectra of triacylglycerols were obtained after GC on a 15 m x 0.3 mm id silylated SE-30 (0.12 μm film) column using helium at 0.75 kg/m^2 and a temperature program of 250-370 C at a rate of 4 C/min. Acquisition was begun at 250 or 275 C. The ion source was operated at 70 eV with an ionization current of 400 μA. The off-axis continuous dynode electron multiplier was equipped with an auxiliary dynode biased at -3 kV to minimize discrimination of high mass fragments (48).

Initially, mass spectral data were acquired and processed using a Finnigan Model 6000 data system. More recently an Incos Model 2300 data system was used. Scans over the range 60-700 amu were made in 3 sec with the 6000 data system and 1.5 sec with the Incos system. Data files collected on the 6000 system were subsequently reprocessed on the Incos system.

Samples

Three samples from our laboratory illustrate the techniques described. Wax and steryl esters were isolated from a sample of marine particulate matter (~ 0.25 g) collected in a floating sediment trap (FST) deployed at 36 m water depth in the highly productive Peru upwelling area (FST 20 of Staresinic (49); 15°27'S, 75 51'W). Lipids were extracted from the particulate matter with toluene/methanol (1:1, v/v) and ~ 1 mg of lipid was fractionated into constituent lipid classes by column chromatography using silica gel and eluting with a series of solvents of increasing polarity, ranging from hexane, toluene in hexane, increasing proportions of ethyl acetate in hexane, ethyl acetate, to methanol (4). Wax esters and steryl esters (~ 12 μg of each) were collected in a fraction eluted with 25% toluene in hexane. GC and GC-MS analyses were made by injection of 2.5 μl of a 0.01% solution in hexane, yielding an effective injection of about 0.2 μg total wax esters and total steryl esters.

We routinely use butter triacylglycerols as a rapid check of GC column performance. The sample of butter triacylglycerols was obtained by dissolving about 2 mg of whole butter in 5 ml of hexane, and 0.3-1 μl of the resulting 0.07% solution was injected into the GC for this investigation. A second triacylglycerol mixture was isolated from lipids extracted from a sample of mixed marine zooplankton, primarily calanoid copepods, collected at the PARFLUX E sediment trap location in the equatorial North Atlantic Ocean (13°30'N, 54°00'W (50)). Toluene/methanol-extracted lipids (~ 4 mg) were fractionated on silica gel, with triacylglycerols (~ 0.4 mg) eluted with 10% ethyl acetate in hexane. Injections of 0.25-1 μl of a 0.05% hexane solution resulted in analysis of about 0.3 μg of the triacylglycerol mixture.

Wax ester spectra were compared with spectra obtained for commercially available saturated and monounsaturated reference compounds over the range C_{26}-C_{44}. Steryl esters were prepared by reacting individual sterols (cholest-5-en-3β-ol, 5α-cholestan-3β-ol, 24-methylcholesta-5,22E-dien-3β-ol, 24-methylcholest-5-en-3β-ol, 24-ethylcholesta-5,22E-dien-3β-ol, and 24-ethylcholest-5-en-3β-ol) with 16:0 and 18:0 acyl chlorides and were purified by procedures used for the samples. Triacylglycerols (glyceryl-trimyristate, glyceryl-tripalmitate, glyceryl-tripalmitoleate, glyceryl-1,3-palmitate-2-stearate, glyceryl-1-palmitate-2-stearate-3-oleate, glyceryl-trilinolenate, glyceryl-trilinoleate, glyceryl-trioleate, glyceryl-tristearate, glyceryl-triarachidate, and glyceryl-tridocosahexaenoate) were used as received from the suppliers. Other triacylglycerols were prepared by reacting glyceryl monodocosahexaenoate and glyceryl didocosahexaenoate with the 16:0 and 18:0 acyl chlorides, followed by silica gel chromatography. Standards were analyzed by both direct insertion probe-MS and GC-MS.

RESULTS AND DISCUSSION

GC-MS of low volatility/high molecular weight lipids requires that the high temperature and high resolution capabilities of glass capillary gas chromatography be retained in the GC-MS system. Three features are critical and have resulted in limited success of past GC-MS attempts. They are the basis of our modified GC-MS system. (a) The GC-MS injector must minimize discrimination against high boiling components. The nonvaporizing on-column injector has been shown to eliminate many of the discrimination and degradation effects which are unavoidable using flash-vaporization injectors (39,40). (b) The capillary column of the GC-MS must deliver the desired resolution, and be of a length and liquid phase film thickness to elute the compounds of interest over the available temperature range (and the GC-MS oven must perform over this temperature range as well; our GC-MS oven would not without modification). Our experience has been that over 1000 on-column injections over a period of two years have not resulted in significant deterioration of the capillary columns for this particular type of analysis. However, we have observed some degradation of column quality for some types of compounds (e.g., alkanes, aromatic hydrocarbons, fatty acid methyl esters) which are due to increased surface activity of the column and to the formation of carbon particles in the injector-end of the column due to pyrolysis of nonvolatile materials in the samples. (c) The GC-MS interface must remain inert at high temperature so as to prevent deterioration of resolution and loss of compounds. Use of a silylated direct interface connection minimizes sample decomposition and reduces the pressure at the GC-MS column outlet, resulting in a lower elution temperature (or time) when comparing the same carrier gas flow (or pressure) with the GC. For example, we used 2.0 kg/cm^2 of hydrogen carrier gas in our GC analyses with a 26-m column having a 0.15-μm coating, but 0.75 kg/cm^2 of helium (a 5.3-fold lower mass flow) with a 15-m column, 0.12-μm film, in the GC-MS gave comparable elution temperatures and resolution.

Wax Esters

The Peru upwelling floating sediment trap sample (FST 20) contains a mixture of saturated and unsaturated wax esters over the range C_{28}-C_{42} (Fig. 1). Each peak is composed of a number of wax esters differing in alkyl and acyl moieties but having the same total carbon number. Resolution of the reconstructed ion current (RIC) is somewhat less than that obtained on the GC, but this is attributed primarily to the relatively long scan time (3 sec) used with the 6000 data system.

Aasen et al. (51) and Vajdi et al. (27) have shown that electron impact (EI) mass spectra of wax esters obtained by direct insertion probe and a magnetic sector mass spectrometer contain characteristic ions representing the molecular ion $(RCO\text{-}OR')^+$, $(RCO_2H_2)^+$, $(RCO)^+$, $(R'\text{-}H)^+$, and $(CO_2R')^+$. Our GC/quadrupole spectra were similar, but extensive fragmentation of $(RCO\text{-}OR')^+$, $(RCO_2H_2)^+$ and $(R'\text{-}H)^+$ ions resulted in loss of most structural information. The CI-CH_4 spectra we obtained differ from their EI counterparts in two important

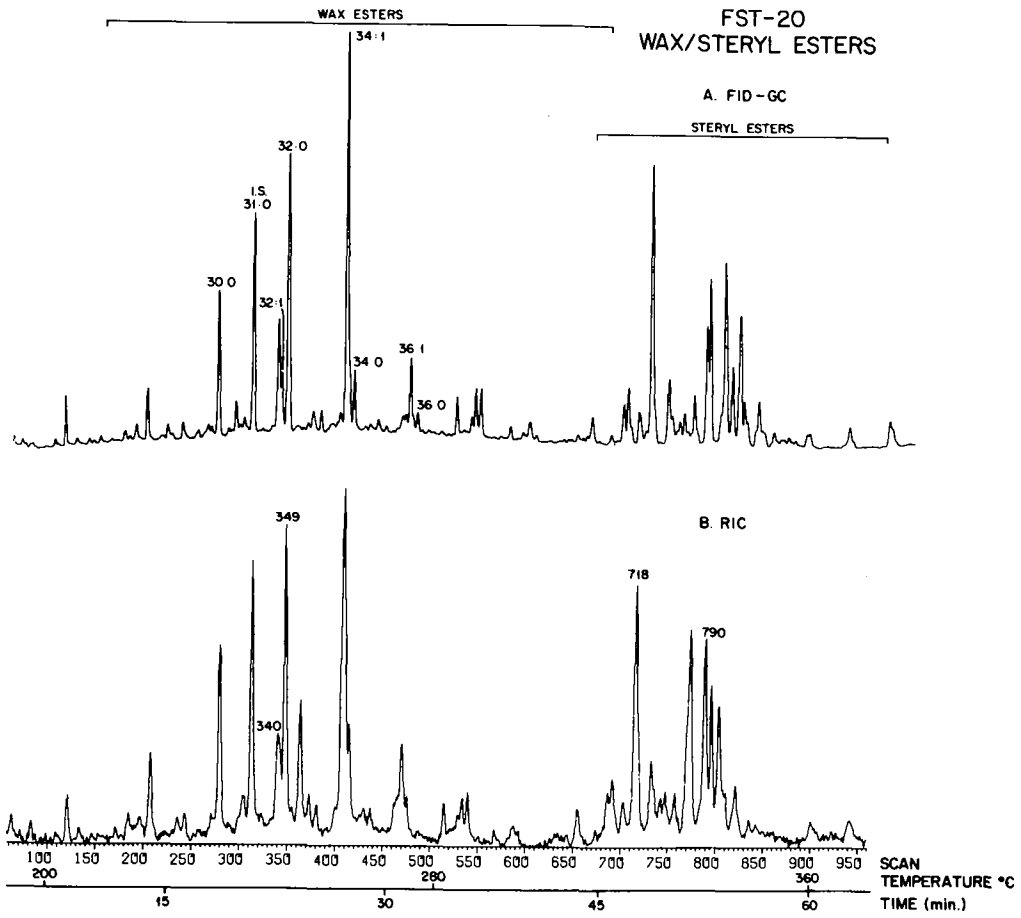


FIG. 1. (A) Flame ionization detector-glass capillary gas chromatogram (FID-GC) and (B) reconstructed ion chromatogram (RIC) of wax esters and steryl esters in Peru floating sediment trap sample FST 20. Numbers above peaks refer to carbon number:number of double bonds. Scan numbers refer to Figure 2. IS = internal standard.

aspects. Both saturated and unsaturated wax ester spectra exhibit the $(M+1)^+$, $(M+29)^+$ and $(M+41)^+$ ions typical of CI addition reactions as well as $(M-1)^+$ ions resulting from hydride ion abstraction. For saturated wax esters, the $(M-1)^+$ ion predominates, whereas unsaturated esters show $(M-1)^+$ and $(M+1)^+$ ions in approximately equal abundance (scans 340 and 349 in Fig. 2). Also, the alcohol moiety is represented by an $(R'-2H)^+$ ion. This alkenyl ion may arise from CH_5^+ attack on the alkyl chain followed by hydride abstraction and a hydrogen rearrangement (52,53).

Scans 340 and 349 for FST 20 wax esters shown in Figure 1 are the monounsaturated and saturated wax esters containing 32 carbon atoms (32:1 and 32:0, respectively). By examining the $(RCO_2H_2)^+$ and $(R'-2H)^+$ fragments, it is possible to reconstruct the several wax esters coeluting in a given GC peak. An estimate of the relative abundances of the individual esters may be made from the $(RCO_2H_2)^+$, $(RCO_2H)^+$, and $(R'-2H)^+$ ions (31). Thus, the 32:1 peak consists of a mixture of alcohol/acid combinations 16:0/16:1 (52%), 14:0/18:1 (32%), and 18:1/14:0 (16%), and the 32:0 wax ester is composed of 16:0/16:0 (81%), 18:0/14:0 (6%), and 14:0/18:0 (13%). However, caution is required in comparing intensities of the $(RCO_2H_2)^+$ ions of unsaturated and saturated fatty acid moieties because the ions of the unsaturated acids may be more readily fragmented and hence, less intense. This is illustrated by $(RCO_2H_2)^+/(RCO)^+$ for 16:1 (m/z 255/237) and 16:0

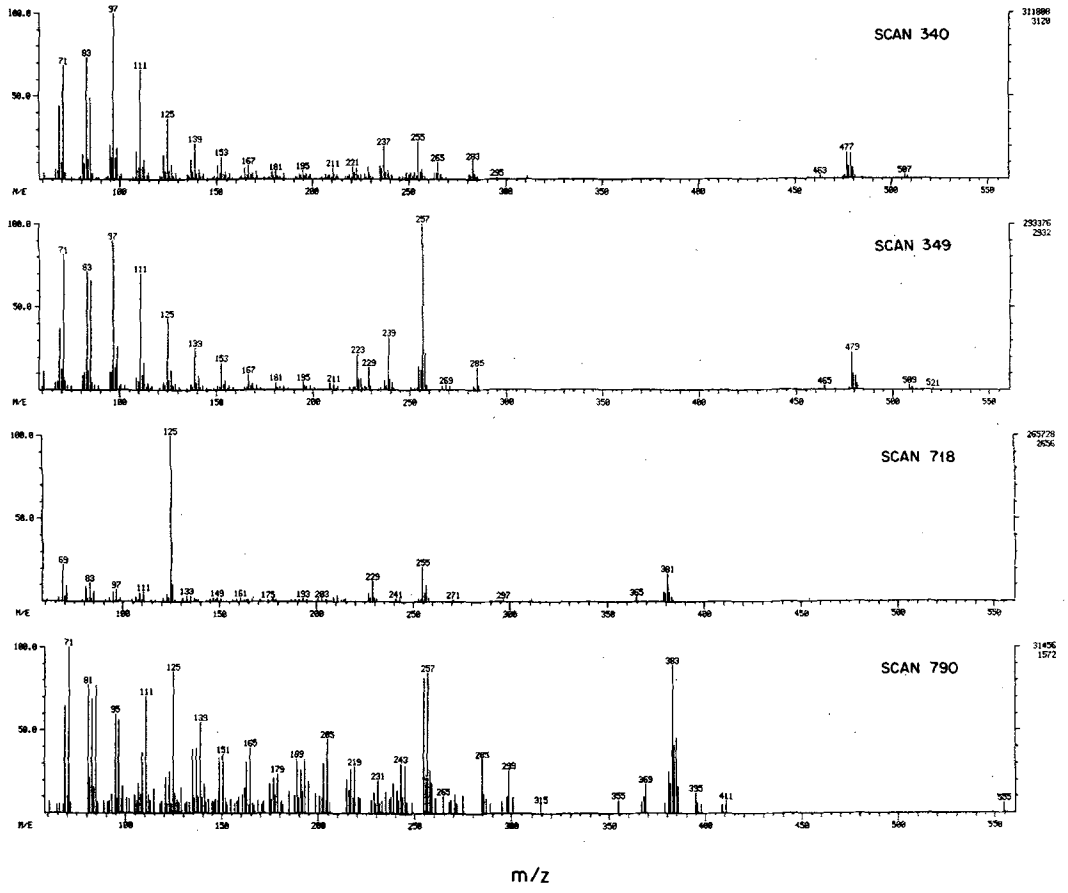


FIG. 2. Methane-chemical ionization (CI-CH_4) mass spectra of wax esters (scans 340 and 349) and steryl esters (scans 718 and 790) in the floating sediment trap sample. Scan numbers refer to Figure 1.

(m/z 257/239) in Figure 2. The GC-MS-derived molecular composition of the complete FST 20 wax ester mixture thus obtained is given in Table 1.

Steryl Esters

Steryl esters are about as abundant in the sediment trap sample as wax esters (Fig. 1), but with a carbon range of C_{40}-C_{48}. CI-CH_4 spectra of components occurring at scans 718 and 790 are given in Figure 2.

Murata (13) compared EI and CI spectra of cholest-5-en-3β-yl (cholesteryl) esters. EI spectra contain little information to aid in identifying the fatty acid group present and no molecular ions (as we have also observed). In CI-CH_4, Murata showed that the base peak was consistent with dehydrated cholesterol $((M_{sterol}+1)\text{-}H_2O)^+$ (m/z 369), while the acid moiety was represented by a strong $(RCO_2H_2)^+$ ion. Molecuions were not observed.

However, the steryl ester mixture shown in the FST 20 sample (Fig. 1) contains a variety of esters of C_{27}-C_{29} sterols and only minor amounts of esters of cholesterol (although many other biochemical samples may have predominantly cholesteryl esters). We find that CI-CH_4 fragmentation of steryl esters is more dependent on the structure of the side chain of the sterol moiety than is EI fragmentation. The critical factor is whether the 22-position is saturated or unsaturated. Table 2 illustrates the major fragment ions for a series of C_{28} sterols; exact fragment intensities vary with mass spectrometer operating parameters. If the 22-position is saturated, as is the case of 24-methylcholest-5-en-3β-ol, then the major ion (often the base peak) is the dehydrated sterol $((M_{sterol}+1)\text{-}$

TABLE 1

Reconstructed Composition of FST 20 Wax Esters Obtained by GC-MS

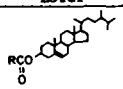
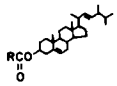
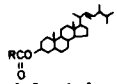
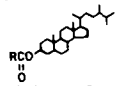
Carbon number	Alkyl/acyl combinations	Percentage of this chain length	Percentage of total wax ester
28:0	10:0/18:0	3	0.1
	12:0/16:0	75	1.8
	14:0/14:0	22	0.5
29:0	13:0/16:0	13	0.1
	14:0/15:0	11	0.1
	15:0/14:0	76	0.8
30:1	14:0/16:1	100	0.05
30:0	12:0/18:0	3	0.2
	14:0/16:0	32	2.4
	16:0/14:0	65	4.8
31:0	–	– internal standard –	–
32:1	14:0/18:1	32	1.2
	16:0/16:1	52	2.0
	18:1/14:0	16	0.6
32:0	14:0/18:0	13	3.1
	16:0/16:0	81	19.3
	18:0/14:0	6	1.4
33:1	15:0/18:1	100	1.0
33:0	17:0/16:0	100	1.1
34:2	16:0/18:2	100	1.2
34:1	14:0/20:1	19	7.2
	16:0/18:1	81	30.7
34:0	14:0/20:0	9	0.2
	16:0/18:0	46	1.2
	18:0/16:0	45	1.2
36:2	16:1/20:1	35	1.3
	18:1/18:1	27	1.0
	18:0/18:2	34	1.3
36:1	16:1/20:0	14	0.9
	16:0/20:1	39	2.6
	18:0/18:1	36	2.4
	18:1/18:0	11	0.6
36:0	16:0/20:0	21	0.4
	18:0/18:0	35	0.7
	20:0/16:0	44	0.8
38:2	18:0/20:2	5	0.2
	20:1/18:1	33	1.0
	22:1/16:1	25	0.8
	22:0/16:2	37	1.1
			97.3%

$H_2O)^+$. A less intense ion from the acyl moiety is observed, as well as a weak ion resulting from further fragmentation of the sterol itself. However, an unsaturated side chain, such as in 24-methylcholesta-5,22-dien-3β-ol, can be readily cleaved, forming a stable fragment and base peak. Ions of moderate intensity are observed for the dehydrated sterol, the (dehydrated sterol-side chain), and the acid group. As shown in Table 2, fragment ions for the (dehydrated sterol minus side chain) occur at m/z 255 and 257, depending on the nature of the sterol skeleton, and may complicate the determination of 16:1 and 16:0 (m/z 255 and 257, respectively) acyl moieties. It is necessary to estimate the relative contributions to ions m/z 255 and 257 from sterol and acyl groups by comparison of mass spectra and retention times with authentic steryl ester standards.

Table 3 gives the molecular composition of steryl esters for FST 20. For example, the peak centered at scan 718 (Figs. 1 and 2, and Table 3) is 24-methylcholesta-5,22-dien-3β-yl tetradecanoate (24-methylcholesta-5,22-dien-3β-ol m/z 125, 255, 381; 14:0 m/z 229) and has been confirmed by coinjection. Scan 790, on the other hand, represents a complex mixture of steryl esters, each with sterol and acyl moieties totaling 44 carbon atoms. The fatty acids present are 15:0 at m/z 243, 16:1 at m/z 255, 16:0 at m/z 257, 17:0 at m/z 271, and 18:0 at m/z 285. The sterols are 26:1 (24-norcholest-5-en-3β-ol, m/z 355), 27:1 (cholest-5-en-3β-ol, m/z 369), 28:1 (24-methylcholest-5-en-3β-ol, m/z

TABLE 2

CH_4-CI Fragmentation of Fatty Acid Esters of C_{28}-Sterols

Steryl Ester	Acyl Moiety	Sterol Moiety Fragments (m/z) $(M_{sterol}+1)-H_2O$	$(M_{sterol}-H_2O$-Side Chain)	Side Chain
 24-Methylcholest-5-en-3β-yl ester	$RCO_2H_2^+$	383 (s)[a]	255 (w)	125 (w)
 24-Methylcholesta-5,22-dien-3β-yl ester	$RCO_2H_2^+$	381 (m)	255 (m)	125 (s)
 24-Methyl-5α-cholest-22-en-3β-yl ester	$RCO_2H_2^+$	383 (m)	257 (m)	125 (s)
 24-Methyl-5α-cholestan-3β-yl ester	$RCO_2H_2^+$	385 (s)	257 (w)	125 (w)

[a] Relative intensity of fragment: s = strong, m = moderate, w = weak

TABLE 3

Reconstructed Composition of the Major FST 20 Steryl Esters Obtained by GC-MS

Scan number[a]	Major steryl ester assignment	Percentage of total steryl esters
657	24-Methylcholesta-5,22-dien-3β-yl dodecanoate	2.0
674	Cholest-5-en-3β-yl tridecanoate	0.7
684	24-Methylcholesta-5,22-dien-3β-yl tridecanoate	3.1
691	Cholesta-5,22-dien-3β-yl tetradecanoate	3.8
702	Cholest-5-en-3β-yl tetradecanoate	2.8
718	24-Methylcholesta-5,22-dien-3β-yl tetradecanoate	17.4
733	Cholest-5-en-3β-yl pentadecanoate	4.7
742	24-Methylcholesta-5,22-dien-3β-yl pentadecanoate	2.2
748	Cholesta-5-22-dien-3β-yl hexadecanoate/hexadecenoate	2.6
757	Cholest-5-en-3β-yl hexadecanoate/hexadecenoate	3.9
771	24-Methylcholesta-5,22-dien-3β-yl hexadecanoate/ hexadecenoate	8.0
775	24-Methylcholest-22-en-3β-yl hexadecanoate/ hexadecenoate	7.7
790	24-Methylcholest-5-en-3β-yl hexadecanoate/ hexadecenoate	14.1
795	24-Ethylcholest-22-en-3β-yl hexadecanoate/ hexadecenoate	4.9
802	Cholest-5-en-3β-yl octadecanoate/octadecenoate	12.0
808	Unidentified	0.6
821	24-Methylcholesta-5,22-dien-3β-yl octadecanoate/ octadecenoate	5.3
836	Unidentified	1.5
842	Cholest-5-en-3β-yl eicosanoate	1.5
		99.0%

[a]Scan numbers refer to Figure 1B.

383), and 29:2 (24-ethylcholesta-5,22-dien-3β-ol, m/z 395 and 139). The predominance of the m/z 383 sterol fragment and the 255/257 ions indicates that the major components are 24-methylcholest-5-en-3β-yl hexadecenoate and 24-methylcholest-5-en-3β-yl hexadecanoate.

Triacylglycerols

High temperature GC-MS of intact triacylglycerols offers a rapid means of obtaining compositional information for a mixture of these compounds. As an example, whole butter contains triacylglycerols rangning from C_{28}-C_{54} as shown in Figure 3. In the RIC, the relative intensities of the peaks are similar to those obtained in the FID-GC, although there is a moderate loss of resolution attributable to ion source cold-trapping and/or to the relatively long 3-sec scan time. Peak widths in both the FID-GC and RIC are noticeably broadened compared to individual triacylglycerol standards. GC-mass fragmentography indicated the peaks are mixtures of compounds with varying degrees of unsaturation but the same carbon chain lengths. A major advantage of MS detection is the ability to apply commercially available enhancement software which utilizes coincidence of ion current maxima to enhance GC resolution (Fig. 3). However, caution must be observed since relative intensities of enhanced spectra may be distorted.

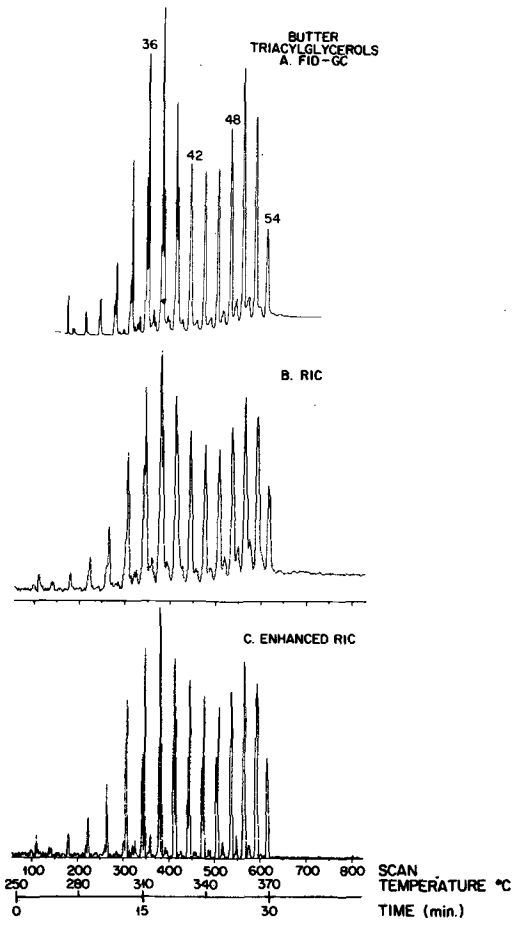


FIG. 3. Capillary gas chromatograms of triacylglycerols in whole butter: (A) FID, (B) RIC; (C) computer-processed ("enhanced") RIC.

Grob has reported losses of the higher molecular weight triacylglycerols (e.g. triolein) due to thermal degradation on persilylated glass capillary columns (54). In our GC and GC-MS analyses, some discrimination against higher boiling triacylglycerols was observed, particularly for those triacylglycerols containing unsaturated acids. Caution is, therefore, advised when making quantitative interpretations. Using either GC or GC-MS, the degree of success in eluting unsaturated triacylglycerols decreases considerably as unsaturation increases. Whereas triolein, for example, elutes with a symmetrical peak shape, trilinolein and trilinolenin show increasing tailing. Triacylglycerols containing polyunsaturated fatty acids exhibit peak shape distortion and tailing in the extreme. At present, we have no evidence relating to the question of whether poor chromatography of polyunsaturated triacylglycerols is due to decomposition during injection or to adsorption on the column.

In addition to chromatographic difficulties, triacylglycerols containing unsaturated acids showed considerably enhanced fragmentation (for both probe MS and GC-MS) when compared with saturated triacylglycerols. The relative abundance of the $(RCO)^+$ fragment corresponding to the unsaturated acyl moiety was found to decrease in the series triolein (base peak) to trilinolein (20%) and trilinolenin (10%). The $(RCO)^+$ and $(M\text{-}RCO_2)^+$ fragments are very weak or nonexistent in the spectra of triacylglycerols containing polyunsaturated acids (e.g. 22:6). Using the methods described here, useful GC-MS analyses of triacylglycerols containing polyunsaturated acyl groups do not appear feasible at present.

Triacylglycerols isolated from the PARFLUX E zooplankton lipids range from C_{42}-C_{60} (Fig. 4). EI spectra of selected GC peaks are given in Figure 5. Ions representing $(RCO)^+$ (the acyl moiety), $(RCO+74)^+$, $(RCO+115)^+$, and $(RCO+128+14n)^+$ (the glycerol moiety with one acyl group), and $(M\text{-}RCO_2)^+$ (the glycerol moiety with two acyl groups) are present, as described previously for triacylglycerols analyzed by

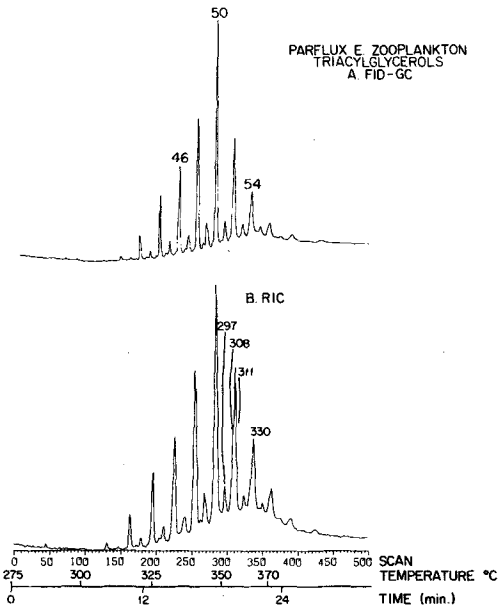


FIG. 4. FID-GC and RIC of triacylglycerols from PARFLUX E mixed zooplankton sample. Numbers above peaks refer to acyl carbon number. Scan numbers refer to Figure 5.

probe MS (8,55,56). In addition, unsaturation in the acyl moiety results in formation of $(RCO-1)^+$ (55) (e.g. m/z 264/265 for 18:1 in Fig. 5). Since we were using a quadrupole MS with a practical upper mass limit of 750 daltons, we did not expect to observe molecular or $(M-18)^+$ ions.

A great deal of compositional information may be derived from the triacylglycerol mass spectra obtained with a quadrupole mass spectrometer, even without molecular ions, and in conjunction with the carbon chain length data from the GC. For example, the GC peak centered at scan 297 in Figure 4 is the C_{51} triacylglycerol. In the mass spectrum (Fig. 5), the constituent acyl moieties $(RCO)^+$ are 14:0 (m/z 211), 15:0 (m/z 225), 16:1 (m/z 236/237), 16:0 (m/z 239), 17:0 (m/z 253), 18:1 (m/z 264/265), 18:0 (m/z 267), 19:0 (m/z 281), and 20:0 (m/z 295). The strong $(M-RCO_2)^+$ ions at m/z 563 and 565 are due to $C_{36}H_{67}O_4$ and $C_{36}H_{69}O_4$ fragments, respectively, meaning that they represent 33:1 and 33:0 acyl groups. Other $(M-RCO_2)^+$ ions at m/z 537, 551, 577, 591, 593, and 605 correspond to combinations of shorter and longer acyl chains on the glycerol moiety. Thus, a number of triacylglycerols having combinations of acyl moieties totaling 51 carbons are present in the peak. By comparing the intensities of RCO^+ and $(RCO+74)^+$ ions for each acyl chain length and correcting for variations in ion intensity due to increasing chain length and unsaturation as determined for standard compounds, it is possible to deduce the probable composition of the triacylglycerols (Table 4). The dominant C_{51} triacylglycerol is 16:0/17:0/18:1 or 18:0, although several other acyl combinations are apparently present to give the observed fragments. Likewise, the C_{54} triacylglycerol (scan 330 in Figs. 4 and 5) contains a mixture of 14:0, 16:1, 16:0, 18:1, 18:0, 20:1, and 22:1 fatty acids, and probable fatty acid combinations are (Table 4): $C_{14}/C_{20}/C_{20}$ (16% of the C_{54} compounds), $C_{16}/C_{16}/C_{22}$ (31%), $C_{16}/C_{18}/C_{20}$ (44%) and $C_{18}/C_{18}/C_{18}$ (9%). From the mass spectra obtained, it is difficult to assign unambiguously the saturated/unsaturated acyl moieties in a given combination (molecular ions would help) and impossible to assign positions of the acyl chains on the glycerol backbone.

However, it is possible to use the mass spectral data to help "resolve" triacylglycerols which differ in their degree of unsaturation but which are not effectively separated by the GC column. This is demonstrated for the C_{52} triacylglycerols of the plankton sample. The GC trace (Fig. 4) shows a barely discernible shoulder in the C_{52} peak, although the shoulder is less evident in the RIC (Fig. 5). (By enhancing the RIC or by using a shorter scan time, the presence of two partially resolved components becomes clearer.) The mass spectra of the shoulder (scans centered at 308) and the major component (scans around 311) are given in Figure 5. From the major fragment ions, we deduce that scan 308 represents primarily a 16:0/18:1/18:1 triacylglycerol (52:2) while scan 311 is 16:0/18:1/18:0 (a 52:1 triacylglycerol).

The proportion of each acyl combination in the total triacylglycerol mixture may be estimated from the percentage each combination represents for a given chain length and from the relative abundance of each chain length in the sample. Relative abundances of chain length groups are obtained from the GC or reconstructed ion current (RIC) trace, applying appropriate detector response factors from standard analyses (relative responses for standard triacylglycerols C_{42}:C_{48}:C_{54} were 1.0:0.95:0.80). The composition thus obtained, given in Table 4, shows that the dominant triacylglycerols in the total sample were $C_{14}/C_{14}/C_{16}$ (6.2%), $C_{14}/C_{16}/C_{16}$ (7.7%), $C_{14}/C_{16}/C_{18}$ (10.2%), $C_{16}/C_{16}/C_{18}$ (22.6%), $C_{16}/C_{18}/C_{18}$ (12.2%), and $C_{16}/C_{18}/C_{20}$ (2.5%). A comparison of the overall fatty acid composition resulting from the GC-MS deter-

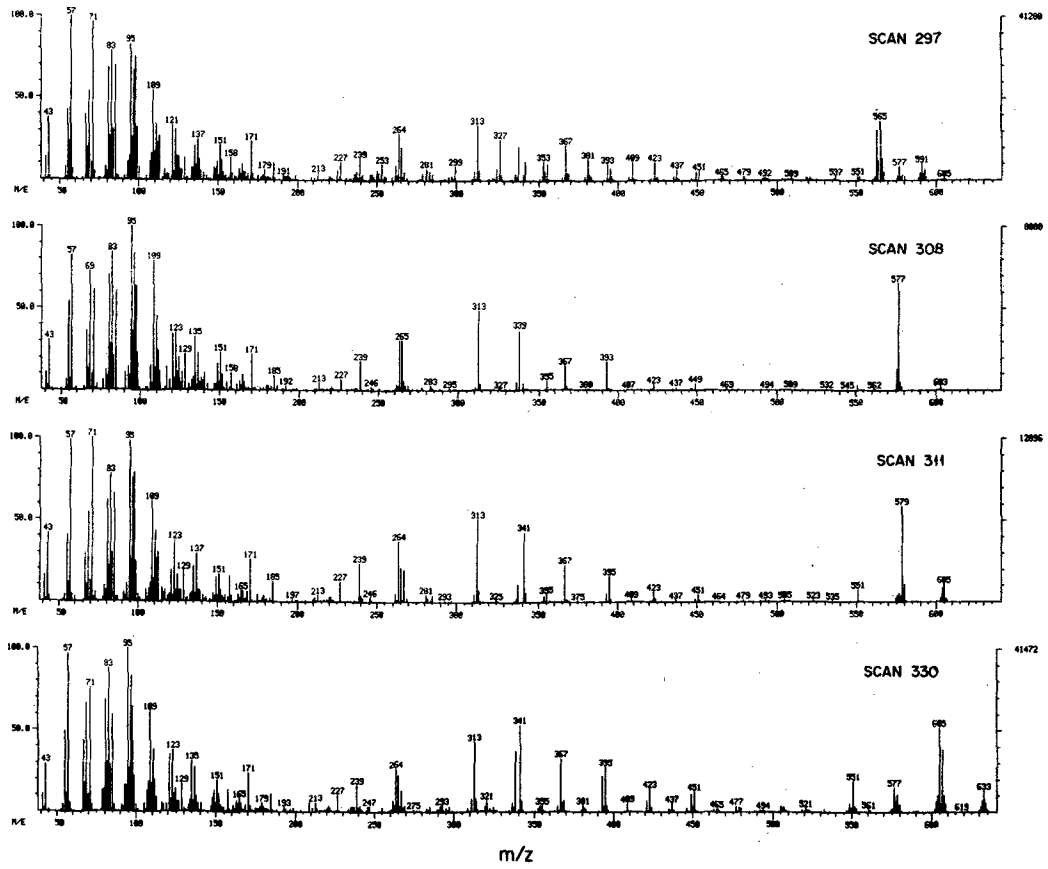


FIG. 5. Electron impact mass spectra of triacylglycerols from the zooplankton sample. Scan numbers refer to Figure 4.

mination with that obtained by saponification of the sample and subsequent analysis of fatty acid methyl esters is shown in Table 5. In general, the agreement is fairly good, although discrepancies exist in the proportions of unsaturated and saturated species of the same carbon number. In particular, the GC-MS method overestimated the contribution of 16:1 but underestimated the abundance of 18:0. Since the GC-MS results were based on ion intensities determined by analyses of standards, the reasons for the discrepancy remain unresolved; nevertheless, the results are encouraging.

In conclusion, detailed compositional analyses of small samples of mixtures of naturally occurring wax esters, steryl esters, and triacylglycerols can be carried out with high temperature glass capillary gas chromatography/quadrupole mass spectrometry with only minor modifications of instrumentation. High resolution capabilities of the high temperature glass capillary columns were retained in the GC-MS system. With a quadrupole mass spectrometer, CI-CH_4 provided more structural information for wax esters and steryl esters than EI spectra. For triacylglycerols, EI spectra were useful. Analysis of the GC-MS data thus obtained made possible determination of the molecular composition of individual wax and steryl esters in a complex mixture and provided similar, but less precise, structural information for triacylglycerols.

ACKNOWLEDGMENTS

This work was supported by Contract N00014-79-C0071 from the Office of Naval Research. We thank J.W. Farrington and R.B. Gagosian for assistance and advice on the manuscript. Contribution No. 4963 of the Woods Hole Oceanographic Institution.

REFERENCES

1. Kolattukudy, P.E. (1976) Chemistry and Biochemistry of Natural Waxes, Elsevier, New York, NY.

TABLE 4

Fatty Acid Composition of the PARFLUX E Zooplankton Triacylglycerols Obtained by GC-MS

Carbon number	Acyl combinations	Percentage of this chain length	Percentage of total	Approximate acyl unsaturated/saturated ratio for each chain length		
				16:1/16:0	18:1/18:0	20:1/20:0
42	10/16/16	13	0.4	–	–	–
	12/14/16	8	0.2	–	–	–
	14/14/14	79	2.3	–	–	–
43	12/14/17	12	0.08	–	–	–
	13/13/17	9	0.06	–	–	–
	13/14/16	18	0.1	–	–	–
	14/14/15	61	0.4	–	–	–
44	12/14/18	19	1.4	0.16	nd[a]	–
	14/14/16	81	6.2	–	–	–
45	13/14/18	10	0.2	0.19	0.5	–
	13/16/16	3	0.05	–	–	–
	14/14/17	16	0.3	–	–	–
	14/15/16	71	1.3	–	–	–
46	14/14/18	29	3.2	0.27	0.7	–
	14/16/16	71	7.7	–	–	–
47	13/16/18	9	0.2	0.42	nd[b]	–
	14/15/18	28	0.5	–	–	–
	14/16/17	19	0.3	–	–	–
	15/16/16	44	0.8	–	–	–
48	14/16/18	64	10.2	0.32	3.3	nd[a]
	16/16/16	36	5.7	–	–	–
49	14/16/19	23	0.6	0.37	2.3	–
	14/17/18	20	0.5	–	–	–
	15/17/18	40	1.0	–	–	–
	16/16/17	17	0.4	–	–	–
50	14/16/20	12	3.3	0.18	3.1	nd[a]
	14/18/18	6	1.7	–	–	–
	16/16/18	82	22.6	–	–	–
51	14/18/19	17	0.4	0.35	5.0	nd[a]
	15/16/20	6	0.1	–	–	–
	15/18/18	15	0.3	–	–	–
	16/16/19	12	0.3	–	–	–
	16/17/18	50	1.2	–	–	–
52	14/18/20	9	1.2	0.22	2.3	2.0
	16/18/18	91	12.2	–	–	–
53	14/19/20	6	0.1	0.47	1.6	nd[a]
	15/18/20	6	0.1	–	–	–
	16/16/21	22	0.4	–	–	–
	15/16/22	10	0.2	–	–	–
	16/17/20	3	0.05	–	–	–
	16/18/19	10	0.2	–	–	–
	17/18/18	42	0.7	–	–	–
54	14/20/20	16	0.9	0.26	1.6	2.8
	16/16/22	31	1.8	–	–	–
	16/18/20	44	2.5	–	–	–
	18/18/18	9	0.5	–	–	–
56	14/20/22	13	0.3	0.27	3.1	2.7
	16/16/24	12	0.3	–	–	–
	16/18/22	42	1.1	–	–	–
	16/20/20	21	0.6	–	–	–
	18/18/20	12	0.3	–	–	–
			97.9%			

[a]Ratio not determined since little of the unsaturated component was present.

[b]Ratio not determined since little of the saturated component was present.

TABLE 5

PARFLUX E Plankton Triacylglycerol Fatty Acids Determined by GC-MS and Saponification Followed by Methylation and GC

Acid carbon number	GC-MS Rel wt %	Saponification rel wt %
10	0.7	–
11	–	–
12	0.7	1.1
13	0.3	2.0
14	19.6	15.0
15	1.8	3.1
16:1	8.5	1.6
16:0	34.1	29.3
17	1.1	2.9
18:1	20.4	19.6
18:0	7.9	15.6
19	0.4	0.9
20:1	2.4	2.0
20:0	0.9	1.7
21	0.01	0.7
22:1	1.1	2.0
22:0	0.1	0.9
23	–	0.2
24:1	–	1.0
24:0	–	0.5

2. Sargent, J.R., Gatten, R.R., and Henderson, R.J. (1981) Pure Appl. Chem. 53, 867-871.
3. Goad, L.J. (1978) in Marine Natural Products (Scheuer, P.J., ed.) pp. 75-172, Academic Press, New York, NY.
4. Wakeham, S.G., Farrington, J.W., Gagosian, R.B., Lee, C., DeBaar, H., Nigrelli, G.E., Tripp, B.W., Smith, S.O., and Frew, N.M. (1980) Nature 286, 798-800.
5. Peltzer, E.T., Wakeham, S.G., and Gagosian, R.B. (1981) SEAREX Newsletter 4, 15-19.
6. Cranwell, P.A., and Volkman, J.K. (1981) Chem. Geol. 32, 29-43.
7. Boon, J.J., and de Leeuw, J.W. (1979) Mar. Chem. 7, 117-132.
8. Boon, J.J., Rijpstra, W.I.C., de Leeuw, J.W., and Burlingame, A.L. (1980) Geochim. Cosmochim. Acta 44, 131-134.
9. Nevenzel, J.C. (1969) Lipids 5, 308-319.
10. Ackman, R.G., Eaton, C.A., and Litchfield, C. (1971) Lipids 6, 69-77.
11. Volkman, J.K., Gatten, R.R., and Sargent, J.R. (1980) J. Mar. Biol. Assoc. U.K. 60, 759-768.
12. Hites, R.A. (1970) Anal. Chem. 42, 1736-1740.
13. Murata, T. (1977) Anal. Chem. 49, 2209-2213.
14. Kuksis, A., and MacCarthy, M.J. (1962) Can. J. Biochem. Biophys. 40, 679-687.
15. Kuksis, A. (1964) Can. J. Biochem. Biophys. 42, 419-429.
16. Kuksis, A., Marai, L., and Gornall, D.A. (1967) J. Lipid Res. 8, 352-358.
17. Litchfield, C. (1965) J. Am. Oil Chem. Soc. 42, 849-857.
18. Breckenridge, W.C., and Kuksis, A. (1968) Lipids 3, 291-300.
19. Tulloch, A.P. (1972) J. Am. Oil. Chem. Soc. 49, 609-610.
20. Tulloch, A.P. (1975) J. Chromatogr. Sci. 13, 403-407.
21. Takagi, T., Itabashi, Y., Ota, T., and Hayashi, K. (1976) Lipids 11, 354-356.
22. Takagi, T., Sakai, A., Itabashi, Y., Hayashi, K. (1977) Lipids 12, 228-232.
23. Bezard, J., Bugaut, M., and Clement, G. (1971) J. Am. Oil Chem. Soc. 48, 134-139.
24. Spencer, G.F., and Tallent, W.H. (1973) J. Am. Oil Chem. Soc. 50, 202-206.
25. Spencer, G.F., Plattner, R.D., and Mina, T. (1977) J. Am. Oil Chem. Soc. 54, 187-189.
26. Spencer, G.F. (1979) J. Am. Oil Chem. Soc. 56, 642-646.
27. Vajdi, M., Nawar, W.W., and Merritt, C., Jr. (1981) J. Am. Oil Chem. Soc. 58, 106-110.
28. Murata, S., Takahashi, S., and Takeda, T. (1975) Anal. Chem. 47, 577-580.
29. Murata, T., and Takahashi, S. (1973) Anal. Chem. 45, 1816-1823.
30. Dielmann, G., Meier, S., and Rapp, U. (1979) J. High Resolut. Chromatogr. Chromatogr. Commun. 2, 343-350.
31. Geigert, J., Dalietos, D., and Heidleman, S.L. (1980) J. High Resolut. Chromatogr. Chromatogr. Commun. 3, 473-474.
32. Dewitt, S., Ervin, J.L., Howes-Orchison, D., Dalietos, D., Neidleman, S.L., and Giegert, J. (1982) J. Am. Oil Chem. Soc. 59, 69-74.
33. Grob, K., Jr., Neukom, H.P., and Battaglia, R. (1980) J. Am. Oil Chem. Soc. 57, 282-286.
34. Schmid, P.P., Müller, M.D., and Simon, W. (1979) J. High Resolut. Chromatogr. Chromatogr. Commun. 2, 675-676.
35. Grob, K., (1980) J. High Resolution Chromatogr. Chromatogr. Commun. 3, 493 and references given therein.
36. Schomburg, G., Dielmann, R., Borwitzky, H., and Hussmann, H. (1979) J. Chromatogr. 167, 337.
37. Godefroot, M., Van Roelenbosch, M. Van, Verstappe, M., Sandra, P., and Verzele, M. (1980) J. High Resolut. Chromatogr. Chromatogr. Commun. 3, 338.
38. Schomburg, G., Behlau, H. Dielmann, R., Weeke, F., and Hussmann, H. (1977) J. Chromatogr. 142, 87-102.
39. Grob, K., and Grob, K., Jr. (1978) J. Chromatogr. 151, 311-320.
40. Galli, M., and Trestianu, S. (1981) J. Chromatogr. 203, 193-205.
41. Hennenberg, D., Henrichs, U., and Schomburg, G. (1978) J. Chromatogr. 112, 343-352.
42. Hennenberg, D., Henrichs, U., Hussman, H., and Schomburg, G. (1978) U. Chromatogr. 167, 139-147.
43. Blum, W., and Richter, W.J. (1977) J. Chromatogr. 132, 249-259.
44. Frew, N.M., and Sawdo, R.M. (1980) Woods Hole Oceanographic Institution Technical Report WHOI-79-26, 85 pp.
45. Schmid, P.P., Müller, M.D., and Simon, W. (1979) J. High Resolut. Chromatogr. Chromatogr. Commun. 2, 225-228.
46. Eglinton, G., Hajlbrahim, S.K., Maxwell, J.R., Quirke, M.M.E., Shaw, G.J., Volkman, J.K., and Wardroper, A.M.K. (1979) Phil. Trans. R. Soc. Lond. 293, 69-91.
47. Traitler, H., and Prevot, A. (1981) J. High Resolut. Chromatogr. Chromatogr. Commun. 4, 109-114.
48. Stafford, G. (1979) American Society for Mass Spectrometry, 27th Annual Conference, Seattle, WA.
49. Staresinic, N. (1978) The Vertical Flux of Particulate Organic Matter in the Peru Coastal Upwel-

ling as Measured with a Free-drifting Sediment Trap. Ph.D. Thesis, Woods Hole Oceanographic Institution/Massachusetts Institute of Technology, Woods Hole, 255 pp.
50. Honjo, S. (1980) J. Mar. Res. 38, 53-97.
51. Aasen, A.J., Hofstetter, H.H., Iyengar, B.T.R., and Holman, R.T. (1971) Lipids 6, 502-507.
52. Munson, M.S.B., and Field, R.H. (1976) J. Am. Chem. Soc. 88, 4337-4345.
53. Munson, M.S.B., and Field, F.H. (1966) J. Am. Chem. Soc. 88, 2621-2630.
54. Grob, K., Jr. (1981) J. Chromatogr. 205, 289-296.
55. Lauer, W.M., Aasen, A.J., Graff, G., and Holman, R.T. (1970) Lipids 5, 259-265.
56. Aasen, A.J., Lauer, W.M., and Holman, R.T. (1970) Lipids 5, 267-275.

[Received January 18, 1982]

Characteristics of the Formation of the Platelet Lipoxygenase Product from Endogenous Arachidonic Acid

DANIEL H. HWANG, *Louisiana Agricultural Experiment Station, Human Nutrition, Home Economics Building, Louisiana State University, Baton Rouge, LA 70803-4300*

ABSTRACT

The concentration of 12-hydroxyeicosatetraenoic acid (12-HETE) formed in rat platelets aggregated by collagen suspension increased continuously during a 115-min incubation period, whereas the concentration of TXB_2 or $PGF_{2\alpha}$ reached the maximum within 3 min and stayed at the plateau for the remaining incubation period. These data indicate that platelet lipoxygenase is not completely inactivated as is cyclooxygenase by the oxidizing agent. Platelets of essential fatty acid deficient (EFAD) rats resuspended in plasma of control rats produced more 12-HETE than platelet-rich plasma (PRP) of EFAD rats, whereas platelets of control rats resuspended in plasma of EFAD rats formed less 12-HETE than PRP of control rats. However, the concentration of TXB_2 or $PGF_{2\alpha}$ produced was not changed in both cases implying that platelet cyclooxygenase preferentially utilizes arachidonic acid (AA) derived from platelet lipids. Radioactivity of phosphatidylcholine (2-arachidonyl-1-^{14}C) suspended in the plasma of PRP was incorporated into 12-HETE but not to TXB_2, indicating again that only lipoxygenase can utilize AA derived from plasma phospholipids. The significance of this observation is that the effects of platelet lipoxygenase products, although their physiological roles are not known, would be much more persistent than cyclooxygenase products after platelets are stimulated or aggregated in vivo.

Lipids 17:845-847, 1982.

INTRODUCTION

In platelets of most species of animals, thromboxane A_2 (TXA_2) is the major cyclooxygenase derived product of arachidonic acid (AA); minor components being prostaglandins E_2 (PGE_2) and $PGF_{2\alpha}$. The lipoxygenase derived product of arachidonic acid is 12-hydroxyeicosatetraenoic acid (12-HETE) in platelets (1,2). In studies so far reported on the formation of 12-HETE in platelets, exogenous arachidonic acid or prelabeled platelets with radioactive arachidonic acid have been used. In addition, most studies have used washed platelet suspensions; thus, effects of plasma lipids on the formation of lipoxygenase and cyclooxygenase derived products were eliminated. Exogenous arachidonic acid may not represent the physiological substrate pool for platelet lipoxygenase and cyclooxygenase. In this study, characteristics of the formation of 12-HETE from endogenous arachidonic acid, were evaluated in comparison with TXB_2, in rat platelet-rich plasma.

MATERIALS AND METHODS

Platelet Aggregation and Sampling Procedure

Weanling male Sprague-Dawley rats (Gibco Animal Research Lab., Madison, WI) fed a commercial stock diet for 12 weeks were used. In the experiment described in Table 1, two groups of weanling rats ($n = 3$) were fed semipurified diets for 20 weeks. The essential fatty acid deficient group (EFAD) received the diet containing 5% hydrogenated coconut oil, and the control group received the diet containing safflower oil.

Preparation of platelet-rich plasma (PRP), platelet-poor plasma (PPP), and platelet aggregation were carried out as described in a previous report (3). At appropriate time intervals after preincubation of inhibitors or vehicle, and addition of collagen suspension, 5% formic acid was added to PRP to adjust the pH to 3.5. Plasma samples were prepared after centifugation and stored at -15 C until they were assayed.

For the experiment described in Table 1, PRP of both EFAD rats and control rats

TABLE 1

Exchanging Plasma between EFAD and SO Platelet-Rich Plasma Affected the Formation of 12-HETE without Changing the Concentration of TXB_2[a,b]

Platelet-rich plasma		12-HETE	TXB_2
Platelets	Plasma	(ng/ml)	ng/ml
EFAD	EFAD	574 ± 153	322 ± 40
EFAD	SO	806 ± 160	365 ± 77
SO	SO	1990 ± 220	552 ± 53
SO	EFAD	1270 ± 120	601 ± 10

[a]EFAD, essential fatty acid deficient rat; SO, safflower oil fed rat.

[b]Values are mean ± SEM of 3 observations.

were centrifuged (1000 X g for 5 min), and 0.35 ml of EFAD rat PRP was replaced by PPP of a control rat, or vice versa. Paired platelets (EFAD vs control) were resuspended in Chronolog aggregometer for 10 min before aggregation was induced by collagen suspension. The sampling procedure was the same as described above.

For the experiment described in Figure 1, 0.5 ml of PPP was incubated for 5 min with 5 μCi of phosphatidylcholine (L-α-1-palmitoyl-2-arachidonyl, arachidonyl-1-^{14}C, 40-60 mCi/mmol, New England Nuclear, Boston, MA). Three ml of PRP were then added to the PPP, and immediately aggregated by collagen suspension. After 115 min of incubation, 9 ml of cold acetone were added, and the mixture was shaken vigorously for 1 min. This mixture was extracted twice with 9 ml of petroleum ether to remove neutral lipids. Radioactivity lost in petroleum ether layer was primarily cholesterol ester as determined by thin layer chromatography (TLC) described below. The pH of the aqueous phase was adjusted to 3.5 with 5% formic acid, and the mixture was extracted twice with 5-ml diethyl ether. The trace formic acid was eliminated by adding additional amounts of diethyl ether and repeating evaporation. The residue was dissolved in a mixture of chloroform/methanol (2:1, v/v) for TLC.

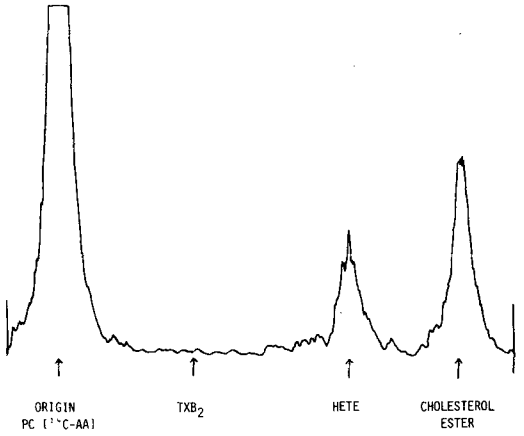


FIG. 1. Thin layer chromatogram radioactivity scan of the reaction products of phosphatidylcholine, L-α-1-palmitoyl-2-arachidonyl (arachidonyl-1-^{14}C) with platelet rich plasma. No detectable radioactivity was found in TXB_2 band.

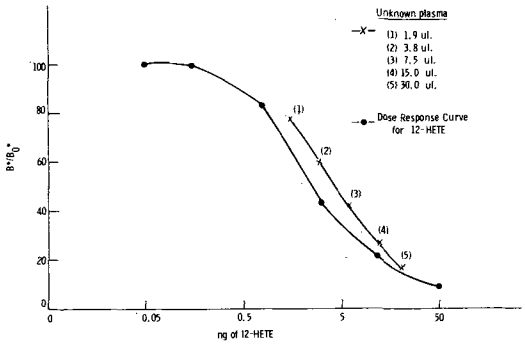


FIG. 2. Demonstration of parallelism between the dose response curve for 12-HETE and serial dilutions of an unknown plasma sample. (B* denotes labeled 12-HETE bound to antibody; B_0^* denotes labeled 12-HETE bound to antibody in absence of unlabeled 12-HETE.)

Analyses

Thin layer chromatography. The sample dissolved in chloroform/methanol was applied to TLC plates (Kontes 5F, Vineland, NJ) and developed in a solvent containing chloroform/methanol/water/acetic acid (90:8:1:0.3, v/v/v/v). Bands of TXB_2 and 12-HETE were identified by comparison to tritiated TXB_2 and 12-HETE. The cholesterol ester band was identified by cholesterol oleate using 2 different solvent systems, the solvent mentioned above and diethyl ether/ petrolem ether/acetic acid (30:70:1, v/v/v). Radioactivity in TLC plate was scanned by radiochromatogram scanner (Packard Model 7230, Downers Grove, IL).

Radioimmunoassay. Preparation and characterization of $PGF_{2\alpha}$ and TXB_2 antibodies, and assessment of the validity of the assay systems were reported in previous reports (4,5). Antiplasma for 12-HETE was a gift from Dr. Laurence Levine, Brandeis University, Waltham, MA. The highest cross-reactivity (0.3%) for the antiplasma was shown by 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) among compounds tested (arachidonic acid, 15-HETE, HHT, TXB_2, $PGF_{2\alpha}$, PGE_2, 6-keto-$PGF_{1\alpha}$, 5-HETE). Recoveries of 12-HETE standard added into an unknown plasma sample at multiple dose (2, 4, 8, 16 and 32 ng/ml) were 99.5, 104.5, 113.7, 111.1 and 90%, respectively. Parallelism between the dose response curve and serial dilutions of an unknown sample is demonstrated in Figure 2.

RESULTS AND DISCUSSION

The concentration of 12-HETE in platelets aggregated by collagen suspension increased continuously during the 115-min incubation period, whereas the concentration of TXB_2 or $PGF_{2\alpha}$ reached the maximum within 3 min

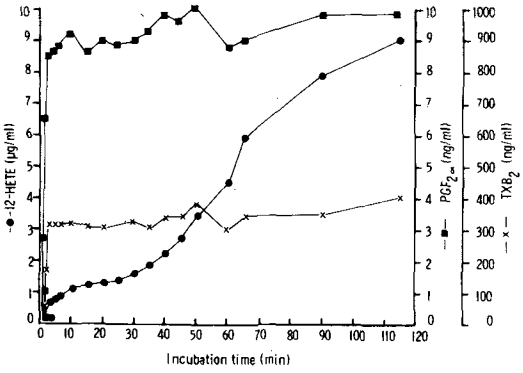


FIG. 3. Time courses for the formation of 12-HETE, TXB_2, or $PGF_{2\alpha}$ from endogenous arachidonic acid in platelet rich plasma.

and stayed at the plateau for the remaining 115 min (Fig. 3). This indicates that platelet lipoxygenase was not completely inactivated by the oxidizing agent, postulated to be derived from the peroxidation of PGG_2 to PGH_2 (6). The significance of this result is that effects of platelet lipoxygenase products are much more persistent than cyclooxygenase products after platelets are aggregated. It has been shown that hydroperoxides derived from arachidonic acid inhibit the biosynthesis of prostacyclin which possesses potent vasodilating effect and inhibits platelet aggregation (7). It is an intriguing question whether lipoxygenase-derived hydroperoxide (12-HPETE) would be released continuously following aggregation of platelets in vivo. If so, 12-HPETE could inhibit the formation of prostacyclin in arterial walls. This, in turn, may increase the tendency of vasoconstriction and further aggregation of platelets.

Since free arachidonic acid can be derived from both platelet and plasma lipids in PRP, it was important to determine whether platelet lipoxygenase and cyclooxygenase can utilize arachidonic acid derived from plasma lipids. During collagen-induced aggregation, platelets of EFAD rats resuspended in plasma of control rats formed more 12-HETE than PRP of EFAD rats, whereas platelets of control rats suspended in plasma of EFAD rats synthesized less 12-HETE than PRP of control rats (Table 1).

Platelet lipoxygenase does not seem to have specificity for degree of unsaturation or position of double bonds of polyunsaturated fatty acids (8). Plasma phospholipids of EFAD rats contained 13.9% of 20:3(n-9) and 3.6% of 20:4(n-6), whereas those of control rats contained trace amounts and 27.5% of 20:3(n-9) and 20:4(n-6), respectively; total amounts of 20 carbon polyunsaturated fatty acids in EFAD plasma phospholipids were much less than those of the control plasma phospholipids. Consequently, the amounts of hydroxy fatty acids derived from EFAD plasma phospholipids would be much less than those from control plasma phospholipids. Therefore, these data can best be interpreted as an indication that platelet lipoxygenase can utilize arachidonic acid derived from plasma phospholipids. However, amounts of TXB_2 synthesized were not changed in both cases (Table 1), implying that platelet cyclooxygenase preferentially utilizes arachidonic acid derived from platelet lipids.

In order to substantiate this, platelets were aggregated in the presence of phosphatidylcholine (L-α-1-palmitoyl-2-arachidonyl, arachidonyl-1-^{14}C) in plasma. Radioactivity was incorporated to 12-HETE and cholesterol esters; however, no detectable radioactivity was found in TXB_2 (Fig. 1), indicating again that only lipoxygenase can utilize arachidonic acid derived from plasma phospholipids.

It has been shown that platelet cyclooxygenase was found in the microsomal fraction, whereas lipoxygenase was found in the soluble cytoplasmic fraction (2). It is not known whether different localization of these enzymes is responsible for utilization of preferential substrate pool by each enzyme. Ability of platelet lipoxygenase to utilize arachidonic acid derived from plasma phospholipids, and its resistance to inactivation imply that aggregation of platelets in vivo produce more lipoxygenase derived products than cyclooxygenase-derived products.

ACKNOWLEDGMENT

This work was supported by USDA competitive human nutrition grant (5901-0410-0311-0), Institutional Biomedical Research Grant, Office of Research (LSU), and Louisiana Agricultural Experiment Station.

REFERENCES

1. Hamberg, M., and Samuelsson, B. (1974) Proc. Nat. Acad. Sci. USA 71, 3400-3404.
2. Nugteren, D.H. (1975) Biochim. Biophys. Acta 299-307.
3. Hwang, D.H. (1980) Prostaglandins Med. 5, 163-173.
4. Hwang, D.H., and Carroll, A. (1980) Am. J. Clin. Nutr. 33, 590-597.
5. Hwang, D.H., Godke, R.A., and Rings, R.A. (1980) Lipids 15, 597-600.
6. Kuehl, F.A., Jr., Humes, J.L., Ham., E.A., Egan, R.W., and Dougherty, H.W. (1980) in Advances in PGs and TXB Research (Samuelsson, B. and Rammwell, P.W., eds.) Vol. 6, pp. 77-86, Raven Press, New York, NY.
7. Gryglewski, R.J., Bunting, S., Moncada, S., Flower, R.J., and Vane, J.R. (1976) Prostaglandins 12, 715-737.
8. Needleman, P., Wyche, A., Sankarappe, S.K., Jakschik, B.A., and Sprecher, H. (1981) Prog. Lipid Res. 20, 415-422.

[Received May 3, 1982]

Tissue Culture of Cocoa Bean (*Theobroma cacao* L.): Incorporation of Fatty Acids into Lipids of Cultured Cells

C.H. TSAI and **J.E. KINSELLA***, *Institute of Food Science, Cornell University, Ithaca, NY 14853*

ABSTRACT

Suspension cell cultures of cocoa bean rapidly incorporated palmitic, stearic, oleic and linoleic acids into cellular lipids. Thus, 75 and 20% of [1-^{14}C]palmitic acid was incorporated into polar lipids and triglycerides, respectively, after 48 hr. When [1-^{14}C]oleic and [1-^{14}C]linoleic acid were added separately, polar lipids consistently contained most of the radioactive fatty acids. Ca. 60% of the stearic acid accumulated as unesterified fatty acid in the cells. Palmitic and stearic acid were not desaturated, but oleic acid and linoleic acid were further desaturated. The kinetics of conversion of oleic acid and linoleic acid suggested a sequential desaturation pathway of 18:1 → 18:2 → 18:3 in cocoa bean cell suspensions.

Lipids 17:848-852, 1982.

INTRODUCTION

Plant cell cultures are increasingly used for the study of plant lipid metabolism (1-6). Suspensions of soybean cells absorb and acylate exogenous fatty acids into cellular glycerolipids (3). The lipid composition of cultured cocoa bean cells has been characterized, and these cells synthesize a variety of fatty acids from exogenous acetate (7,8). Significantly, the patterns of fatty acids synthesized were similar to those of immature cocoa beans. To determine if cultured cocoa bean cells could synthesize cocoabutter triglycerides, we have studied the capacity of cultured cells to incorporate exogenous fatty acids into glycerides.

Mature cocoa beans contain ca. 60% fat which is predominantly triglycerides (TG). These contain mostly palmitic (16:0), stearic (18:0), and oleic (18:1) acids (*sn*-palmito-oleo-stearin is the predominant triglyceride in cocoabutter). Trace amounts of linoleic acid (18:2) occur in cocoabutter (9,10).

The present study was conducted to determine if cultured cells of cocoa bean can utilize exogenous fatty acids typical of those found in cocoa bean, and if these are incorporated into cellular TG.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]Palmitic acid (57.0 mCi/mmol, [1-^{14}C]stearic acid (50.0 mCi/mmol), [1-^{14}C]-oleic acid (57.0 mCi/mmol and [1-^{14}C]linoleic acid (57.0 mCi/mmol) were purchased from New England Nuclear (Boston, MA). PCS Liquid scintillant was obtained from Amersham Corporation (Arlington Heights, IL). Precoated thin layer chromatography (TLC) plates (silica gel 60H) were purchased from Applied Science Lab (State College, PA).

Cell Culture

Suspension cultures of cocoa beans were initiated from calli of cocoa bean cotyledons as described previously (7,11). Suspension cultures were grown in 40 ml of MS basal medium containing 0.5 ppm 2,4-D and 0.1 ppm kinetin in 125-ml Erlenmeyer flasks on a Lab-Line Orbin Eviron Shaker (Melrose, IL) rotating at 120 rpm and at 28 C in the dark (7,11). The cells were subcultured every 12-14 days. Cells were harvested by filtration with miracloth (Calbiochem) and weighed (11).

Incorporation of [1-^{14}C] Fatty Acids

Cells were grown for 10 days in the culture medium (40 ml) to a concentration of 100 mg cells per ml. Labeled [1-^{14}C] fatty acid (20 μM; 3-4 × 10^6 cpm) in 20 μl of ethanol was then added to the culture medium and cells were incubated at 28 C for a specific period of time. At the appropriate time, 5-ml aliquots were removed. The test tube containing the cells was immersed in boiling water to stop all metabolic reactions. The cells were then centrifuged in a Precision clinical centrifuge (Chicago, IL). The clear supernatant was removed with a disposable Pasteur pipette. The solvent (5 ml), chloroform/methanol (C/M; 2:1, v/v), was added to the pellet and the mixture was shaken for 30 min to extract lipids (8,12). Distilled

*Author to whom correspondence should be addressed.

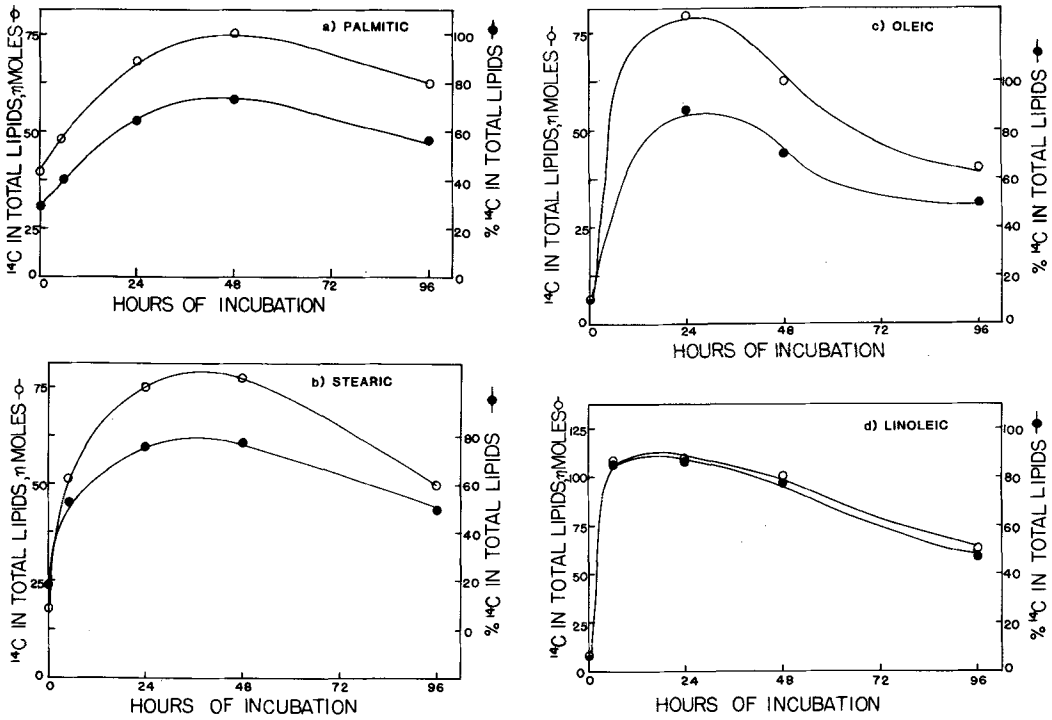


FIG. 1. Incorporation of [1-^{14}C] fatty acids into lipids of cocoa bean suspensions following incubation with (a) [1-^{14}C] palmitic; (b) [1-^{14}C] stearic; (c) [1-^{14}C] oleic, 20 μM each; and (d) [1-^{14}C] linoleic acid (30 μM), respectively. Results are given as nmoles of fatty acid incorporated per g of fresh cells and as % of total radioactive substrate available. Lipids were extracted and analyzed as described in Experimental Procedures. For the "zero time" sample, ca. 1.5 min elapsed between the addition of the fatty acid and addition of extracting solvent.

water (1 ml) was added, followed by mixing on a vortex and centrifugation for 1 min to separate the two phases. The lower chloroform layer was carefully recovered with a Pasteur pipette and evaporated to dryness under N_2 gas. Lipid extraction of cells was done twice. The extracted lipids were dissolved in 1 ml of C/M (2:1, v/v). Aliquots (0.1 ml) of this solution were placed in vials and the radioactivity was determined using a Packard Tri-Carb (Downers Grove, IL) liquid scintillation counter using the external standard ratio method to correct for quenching. The remaining lipid solution was used to determine the distribution of the labeled fatty acids in lipid classes.

The distribution of the labeled fatty acids in the lipid classes was determined by TLC. An aliquot (0.5 ml) of the lipid extract was spotted on silica gel 60H thin-layer plates. TLC plates were developed with the solvent system petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v). The spots corresponding to each lipid class (free fatty acids, (FFA), triglycerides and phospholipids) were located by 2',7'-dichlorofluorescenin spray. These zones were scraped into counting vials, liquid scintillation fluid was added, and the radioactivity determined in the liquid scintillation counter.

An aliquot (0.3 ml) of the lipid solution was used for preparation of fatty acid methyl esters using boron trifluoride as described previously (7,8). The methyl esters of saturated, mono-, di- and trienoic fatty acids were cochromatographed with authentic standards of fatty acid methyl esters on TLC plates impregnated with 5% $AgNo_3$, as described (8). The $AgNO_3$ TLC plates were developed with a solvent system of chloroform/ethanol/acetic acid (11:1:9, v/v/v). Spots containing methyl esters of saturated, mono-, di- and trienoic acids were localized with 2',7'-dichlorofluorescein spray reagent, scraped into counting vials and the radioactivity determined. The data presented are means from triplicate studies.

RESULTS

The incorporation of various [1-^{14}C] fatty acids into total lipids of cell suspensions of cocoa bean is shown in Figure 1a-1d. [1-^{14}C]-

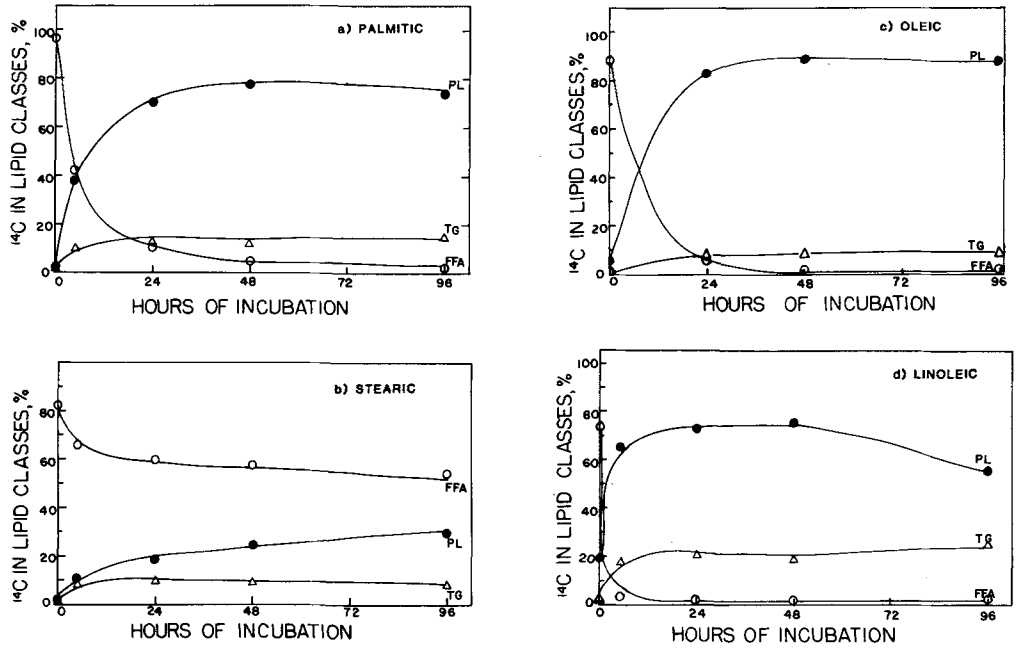


FIG. 2. Distribution of [1-^{14}C] fatty acids in lipid classes of cocoa bean cell suspension following incubation with (a) [1-^{14}C] palmitic acid, (b) [1-^{14}C] stearic acid, (c) [1-^{14}C] oleic acid, and (d) [1-^{14}C] linoleic acid. Lipid classes were analyzed as described in Experimental Procedures. PL = polar lipids; TG = triglycerides; FFA = free fatty acids.

Palmitic, [1-^{14}C] stearic, [1-^{14}C] oleic and [1-^{14}C] linoleic acids were rapidly taken up and incporporated into lipids of cocoa bean cell suspensions, indicating that the cocoa bean suspension cultures readily utilized exogenous FFA. To ensure that exogenous FFA were not only nonenzymatically associated with cell wall components as indicated by Stumpf and Weber (3) but further acylated to form complex lipids, the total lipids were separated into various lipid classes by TLC.

The patterns of incorporation of exogenous [1-^{14}C] fatty acids into various lipid classes of cocoa bean cell suspensions are summarized in Figure 2a,b. Except for the stearic acid, the exogenous fatty acids were readily incorporated into complex lipids. About 60% of the [1-^{14}C]-stearic acid associated with the cellular lipids remained as FFA and only 30% and 10% was associated with polar lipids and triglycerides after 24 hr of incubation. In contrast, when [1-^{14}C] palmitic, [1-^{14}C] oleic or [1-^{14}C] linoleic acid were added, most of the fatty acid appeared in polar lipids and triglycerides; very little remained in the FFA pool after 24 hr. Polar lipids consistently contained most of the incorporated fatty acids (Fig. 2b,c,d).

The conversion of exogenous FFA into other fatty acids as a function of time was determined (Fig. 3). Palmitic and stearic acid were not desaturated (data not shown). In contrast, [1-^{14}C] oleic acid was converted to linoleic and [1-^{14}C] linolenic acids; [1-^{14}C]-linoleic acid was desaturated to [1-^{14}C] linolenic acid. Precursor-product relationships between oleic, linoleic, and linolenic acid were apparent (Fig. 3,a,b). These results suggested the presence of a sequential desaturation pathway 18:1 → 18:2 → 18:3 in cocoa bean suspension cultures.

DISCUSSION

As reported for other plant cell cultures (2), cocoa bean cell suspensions easily incorporated exogenous fatty acids into cellular lipids. Linoleic acid was most rapidly incorporated, followed by oleic, stearic and palmitic acid.

Following incubation of the cells with 16:0, 18:1, and 18:2, the polar lipids contained 60–80% of radioactivity and triglycerides contained 10–20%. These data concerning the distribution of exogenous fatty acids in lipid classes were in general agreement with those reported for soybean cell suspension (3,4). However, in the case of stearic acid, almost 60% remained in the

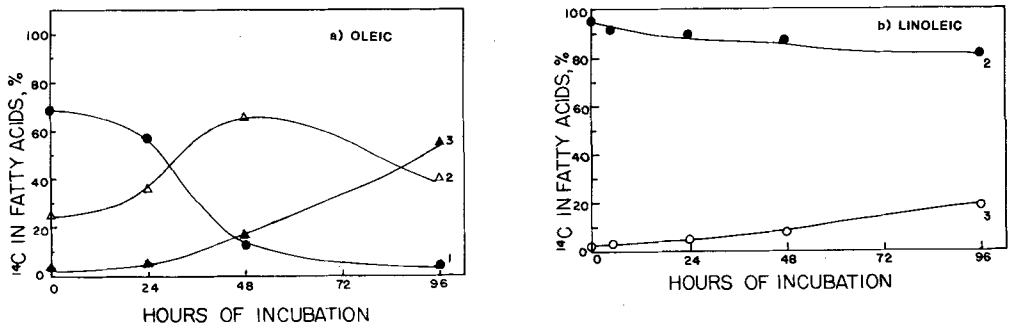


FIG. 3. Distribution of [1-^{14}C] fatty acids in fatty acids of cocoa bean cell suspensions following the incubation with (a) [1-^{14}C] oleic acid; and (b) [1-^{14}C] linoleic acid. Fatty acids were separated as methyl esters by argentation TLC as described in Experimental Procedures. 0 = saturated; 1 = monoene; 2 = diene; 3 = triene.

unesterified state. This observation is inconsistent with the low levels (2–4%) of free stearic acid normally observed in cultured cocoa bean cells (7). Conceivably, in the present experiments, the accumulation of free stearic acid reflected an impaired capacity of these cells to activate exogenous stearic acid to stearyl-ACP and this limited further metabolism via acylation or desaturation.

However, neither the palmitate nor stearate taken up by the cells were converted into other fatty acids, whereas [1-^{14}C] oleate was rapidly desaturated to [1-^{14}C] linoleate and exogenous [1-^{14}C] linoleate was desaturated to linolenate to a limited extent. These results were consistent with those reported for soybean slices (13) and for soybean cell suspensions (3). Stumpf and Porra (13) observed that acetate was converted rapidly to C16 and C18 fatty acids but palmitate and stearate were ineffective substrates for elongation and desaturation, whereas oleate was readily converted to linoleate in slices of developing soybean cotyledons. Stumpf and Weber (3) observed the same results and also the conversion of linoleate to linolenate linolenate in soybean suspension cultures. These results were readily explained in terms of an ACP-dependent de novo sequence which converts acetate to palmityl-ACP which is elongated to stearyl-ACP, and stearyl-ACP desaturase which converts stearyl-ACP to oleyl-ACP (3,14,15). Since free palmitic and stearic acid cannot be activated to acyl-ACP derivatives, they do not enter the acyl-ACP pool, and hence are not further metabolized. However, 18:1 can be activated via thiokinase to oleyl-CoA which is subsequently desaturated to linoleyl-CoA which may be further desaturated to linolenyl-CoA (14,15). The cocoa bean cells apparently contain the enzymes (thiokinases and desaturases) required for these reactions but lack the acyl-ACP synthetase. Significantly, much more oleic acid than linoleic acid was ultimately converted to linolenic acid by the cocoa bean cells. This may reflect the fact that the oleic acid was more facilely incorporated into the phosphoglycerides where it is the preferred substrate for the desaturase (16).

The kinetics of conversion of fatty acids in cocoa cell suspensions were clearly suggestive of the sequential desaturation of 18:1 → 18:2 → 18:3. Stearns and Morton (1) and Wilson et al. (4) using ^{14}C-acetate reported the same sequential desaturation in soybean suspension cultures. This pathway was also observed in cocoabean suspension culture using [1-^{14}C] acetate as substrate (8).

The tendency of the cells to desaturate oleic acid and to incorporate exogenous fatty acids, mostly into phospholipids, indicated that these cocoa bean cell suspensions may not be suitable for the production of a cocoa butter like fat. This is consistent with previous analyses of endogenous lipids of cultured cocoa bean cells (7). Current research is concerned with enhancing the capacity of cultured cells to synthesize mostly triglycerides from endogenous and exogenous fatty acids. Initial studies indicated that increasing the concentration of 18:0 in the medium and the addition of coconut water to the culture medium significantly increased the triglyceride content of cultured cells (9).

REFERENCES

1. Stearns, E.M., and Morton, W.T. (1975) Lipids 10, 597–601.
2. Radwan, S.S., and Mangold, H.K. (1980) Adv. Biochem. Eng. 16, 109–133.
3. Stumpf, P.K., and Weber, N. (1977) Lipids 12, 120–124.
4. Wilson, A.C., Kates, M., and de la Roche, A.I. (1978) Lipids 13, 504–510.

5. MacCarthy, J.J., and Stumpf, P.K. (1980) Planta 147, 384–388.
6. MacCarthy, J.J., and Stumpf, P.K. (1980) Planta 147, 389–395.
7. Tsai, C.H., and Kinsella, J.E. (1981) Lipids 16, 577–582.
8. Tsai, C.H., and Kinsella, J.E. (1982) Lipids 17 (press).
9. Tsai, C.H., and Kinsella, J.E. (1982) J. Food Sci. 47, 768–773.
10. Leherian, D.W., and Keeney, P.G. (1980) J. Am. Oil Chem. Soc. 57, 61–65.
11. Tsai, C.H., and Kinsella, J.E. (1980) Ann. Bot. 48, 549–556.
12. Folch, P.J., Lees, M., and Stanley, G.M. (1957) J. Bill. Chem. 226, 497–506.
13. Stumpf, P.K., and Porra, R.J. (1976) Arch. Biochem. Biophys. 176, 63–70.
14. Jaworski, J.G., Goldschmidt, E.E., and Stumpf, P.K. (1974) Arch. Biochem. Biophys. 163, 769–776.
15. Jaworski, J.G., and Stumpf, P.K. (1974) Arch. Biochem. Biophys. 162, 158–165.
16. Stymme, S., and Glad, G. (1981) Lipids 16, 298–305.

[Received May 18, 1982]

Identification of 27-Nor-(24*R*)-24-methylcholesta-5,22-dien-3 β -ol and Brassicasterol as the Major Sterols of the Marine Dinoflagellate *Gymnodinium simplex*[1]

L. JOHN GOAD* and **NANCY WITHERS**[2], *Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool, L69 3BX, United Kingdom*

ABSTRACT

The major 4α-monomethyl sterol of the dinoflagellate *Gymnodinium simplex* was identified as (24*S*)-4α,24- dimethylcholestan-3β-ol. The major 4-demethyl sterols were characterized as (24*R*)-24-methylcholesta-5,22-dien-3β-ol (brassicasterol) and 27-nor-(24*R*)-24-methylcholesta-5,22-dien-3β-ol. The latter sterol has the opposite configuration at C-24 to that assigned to occelasterol, which has the same basic structure and has previously been reported as a constituent of the sterols of a marine worm. 24-Nor-cholesta-5,22-dien-3β-ol was also identified along with several other trace sterols. The co-occurrence of 27-nor -(24*R*)-cholesta-5,22-dien-3β-ol together with 24-nor-cholesta-5,22-dien-3β-ol and brassicasterol provides new evidence for the biosynthetic origins of the two former nor-sterols. It is suggested that they may be produced de novo by a route involving nor-isoprenoid pyrophosphates and nor-squalene as intermediates, rather than as bacterial degradation products of brassicasterol (or related sterols) as previously suggested in the literature.
Lipids 17:853-858, 1982.

INTRODUCTION

Many unusual sterols have now been reported as constituents of marine invertebrate animals (1-3) and the list is being increased at a steady rate as more refined analytical techniques allow trace sterols to be purified and characterized. The origin of these sterols has aroused great interest since they present challenging biosynthetic problems. Many of the sterols possess hitherto unimagined side chain alkylation patterns and this indicates that they may be of algal origin and are incorporated into invertebrate tissue via their passage through the complex marine food chain (2-4).

In the search for the algal producers of these sterols, a number of dinoflagellate species have been examined and these have yielded a rewarding array of new sterols (5-18). As a continuation of our own studies on marine invertebrate and dinoflagellate sterols, we now report the identification of (24*S*)-4α,24-dimethylcholestan-3β-ol *(1)*, 24-norcholesta-5,22-dien-3β-ol *(2)*, 27-nor-(24*R*)-24-methylcholesta-5,22-dien-3β-ol *(3)* and (24*R*)-24-methylcholesta-5,22-dien-3β-ol *(4)* in the dinoflagellate *Gymnodinium simplex*.

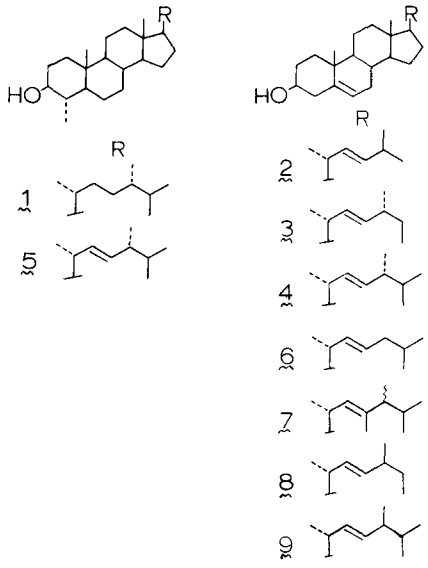
[1]Presented at the "Sterol Symposium" of the American Oil Chemists' Society Annual International Congress, New Orleans, LA, May 1981.

[2]Current address: Hawaii Institute of Marine Biology, University of Hawaii, P.O. Box 1346, Kaneohe, HI 96744.

EXPERIMENTAL

G. simplex (Loh.) Kof. & Sw. was cultured in a 200 ℓ vat of GPM medium (19) at 18 C with 1000 μ W/cm^2 continuous illumination. The cells were harvested after 5 weeks culture and lyophilized to give 14.9 g dry wt of cells. Extraction by reflux with 200 ml of $CHCl_3$/MeOH (2:1) gave 3.34 g of the total lipid. This was fractionated by chromatography on a 50 g column of alumina, Brockmann grade III, eluted with diethyl ether-petrol mixtures to give fraction 3 (30.3 mg), fraction 4 (20.6 mg) and fraction 5 (40.2) which were shown by thin layer chromatography (TLC) and gas liquid chromatography (GLC) to contain mixtures of 4α-methylsterols and 4-demethylsterols. Fractions 3-5 were submitted to preparative TLC on silica gel developed with $CHCl_3$/EtOH (98:2). Bands with R_f values corresponding to the 4α-methyl sterols and 4-demethyl sterols were eluted with diethyl ether and submitted to GLC analysis on a 3% OV-17 column (260C).

The 4-monomethyl sterol fraction (11 mg) contained one major component (95%) which was identified as (24*S*)-4α,24-dimethylcholestan-3β-ol, *(1)*. GLC, 3% OV-17, RR_t = 1.45 (cholesterol RR_t = 1.00). Electron impact mass spectrometry (EIMS) *m/z* (rel int): 416(48), 401(20), 383(17), 369(4), 289(5), 276(13), 262(11), 247(48), 229(61), 179(44), 43(100). ^{1}H NMR ($CDCl_3$): δ 0.642 (*s*, H-18), 0.769 (*d*, H-28), 0.778 (*d*, H-27), 0.822 (*s*, H-19), 0.850 (*d*, H-26), 0.896 (*d*, H-21), 0.940



(*d*, H-30), 3.1 (ddd). The minor 4α-methyl sterol component had a short retention time (RR_t = 1.25) and it was tentatively identified as 4α,24-dimethylcholest-22-en-3β-ol *(5)*. EIMS *m/z* (rel int): 414(32), 316(29), 301(11), 287(34), 271(40), 229(7), 69(100).

The 4-demethyl sterol fraction (35 mg) was shown by GC-MS to have 4 components. Peak 1 (0.7%), RR_t = 0.67 (cholesterol RR_t = 1.00); EIMS *m/z* (rel int): 370(14), 352(4), 337(3), 300(14), 285(7), 271(15), 255(36), 213(15), 97(84), 55(100) corresponding to 24-norcholesta-5,22-dien-3β-ol *(2)*. Peak 2(32%), RR_t = 0.89, EIMS *m/z* (rel int): 384(39), 369(8), 366(12), 351(10), 300(32), 285(8), 271(29), 255(68), 213(22), 55(100), corresponding to 27-nor-24-methylcholesta-5,22-dien-3β-ol *(3)* or cholesta-5,22-dien-3β-ol *(6)*. Peak 3 (53%), RR_t = 1.13, EIMS *m/z* (rel int): 398(36), 383(6), 380(8), 365(6), 337(11), 300(23), 285(6), 271(24), 255(47), 213(14), 55(100) corresponding to (24*R*)-24-methylcholesta-5,22-dien-3β-ol *(4)*. Peak 4 (14%), RR_t = 1.34, EIMS *m/z* (rel int): 412(2), 397(0.5), 369(2), 351(2), 323(1), 314(11), 300(9), 271(22), 255(13), 253(4), 69(100) corresponding to 23,24ξ-dimethylcholesta-5,22-dien-3β-ol *(7)*.

The 4-demethylsterols were acetylated (pyridine-acetic anhydride) and the steryl acetates submitted to preparative TLC on 10% $AgNO_3$-silica gel developed with ethanol-free $CHCl_3$. After spraying with 0.005% berberine and viewing under UV light, one major band and several very minor bands were observed. These were eluted and examined by GC-MS. Band 1, R_f 0.75; MS *m/z* (rel int): 444(5), 369(4), 276(6), 275(4), 215(16); identified as 24ξ-methylcholestan-3β-yl acetate. Band 2, R_f 0.68; MS (*m/z* (rel int): 456(1), 353(2), 344(4), 329(3), 315(7), 271(2), 257(10), 255(2), 215(3), 69(100); tentatively identified as 23,24ξ-dimethylcholest-22-en-3β-yl acetate. Band 3, R_f 0.53; 4 components by GLC; peak 1, MS *m/z* (rel int): 368(100), 353(19), 260(16), 255(16), 247(22), 213(14), identified as cholesteryl acetate; peak 2, MS *m/z* (rel int): 442(22), 344(26), 339(14), 315(25), 257(63), 215(10), 69(100), identified as 24ξ-methylcholest-22-en-3β-yl acetate; peak 3, MS *m/z* (rel int): 382(100), 367(18), 274(14), 255(14), 213(14), identified as 24ξ-methylcholest-5-en-3β-yl acetate; peak 4, MS *m/z* (rel int): 396(29), 381(4), 358(5), 329(9), 271(15), 255(4), 213(2), 69(100), tentatively identified as 23,24ξ-dimethylcholest-5-en-3β-yl acetate. Band 4, R_f 0.43, MS *m/z* (rel int): 394(18), 379(2), 351(4), 323(2), 296(1), 282(6), 267(2), 255(14), 253(11), 213(12), 69(100), tentatively identified as 23,24ξ-dimethylcholesta-5,22-dien-3β-yl acetate. Band 5, R_f 0.36, MS *m/z* (rel int): 380(58), 255(37), 213(7), 69(100). Band 6, R_f 0.21, was the major fraction and it was shown by GLC to contain two major components. This material was carefully chromatographed on silver nitrate-silica gel TLC to give two bands which were eluted. The less polar compound (5.6 mg) was identified as (24*R*)-24-methylcholesta-5,22-dien-3β-yl *(4)* acetate (brassicasteryl acetate) mp 153-156.5C, literature (20) 158C; EIMS *m/z* (rel int): 380(96), 365(6), 337(7), 282(6), 255(50), 228(9), 213(11), 69(100). ^1H NMR ($CDCl_3$, 220 MHz): δ 0.690 (*s*, H-18), 0.815 (*d*, H-26), 0.832 (*d*, H-27), 0.906 (*d*, H-28), 1.007 (*d*, H-21), 1.016 (*s*, H-19).

The more polar material (4 mg) was identified as 27-nor-(24*R*)-24-methylcholesta-5,22-dien-3β-yl *(3)* acetate, mp 137-140C. EIMS *m/z* (rel int): 366(100), 351(8), 282(6), 255(55), 69(98). ^1H NMR ($CDCl_3$, 400 MHz): δ 0.690 (*s*, H-18), 0.827 (*t*, H-26), 0.925 (*d*, H-28), 1.010 (*d*, H-21), 1.020 (*s*, H-19), 2.025 (*s*, 3β-acetate), 4.58-4.68 (*m*, H-3α), 5.17 (*m*, H-22, H-23), 5.39 (*m*, H-6). A sample of synthetic 27-nor-(24*S*)-24-methylcholesta-5,22-dien-3β-yl *(8)* acetate (occelasteryl acetate, mp 142-144C (21), generously provided by Dr. M. Kobayashi) had an identical mass spectrum; ^1H NMR ($CDCl_3$, 400 MHz): δ 0.690 (*s*, H-18), 0.830 (*t*, H-26), 0.926 (*d*, H-28), 1.003 (*d*, H-21), 1.020 (*s*, H-19), 2.025 (*s*, 3β-acetate), 4.58-4.68 (*m*, H-3α), 5.16 (*m*, H-22, H-23), 5.40 (*m*, H-6).

RESULTS AND DISCUSSION

The total lipids were extracted from the

lyophylized cells of *G. simplex* and separated by alumina column chromatography followed by preparative TLC on silica gel to yield the 4α-methylsterol and 4-demethylsterol fractions. GLC analysis showed the 4α-methyl sterol fraction to contain one major component. This had a molecular ion in the mass spectrum at *m/z* 416, revealing it to be a saturated 4α-methyl compound with an extra methyl group which was shown to be located in the side chain by examination of the mass spectral fragmentation ions (*m/z* 289 [M-side chain]$^+$, *m/z* 247[M-C-15,C-16,C-17 and side chain]$^+$, *m/z* 229 [247-H_2O]$^+$). This suggested that the sterol was 4α,24-dimethyl-5α-cholestan-3β-ol which has previously been recognized as a constituent of the zooxanthellae of *Briareum asbestinum* (16). This assignment was confirmed by the ^{1}H NMR spectrum which exhibited the appropriate signals for all the methyl groups (see the Experimental). In particular, the chemical shifts of the doublets for the C-21, C-26, C-27 and C-28 methyl group protons, when compared with the corresponding values for known sterols (22), permitted the configuration at C-24 to be assigned as (24*S*). Thus, the major 4α-methyl sterol was identified as (24*S*)-4α,24-dimethyl-5α-cholestan-3β-ol *(1)*. A very minor component of the 4α-methyl sterol fraction had a shorter retention time than *1* which was indicative that it was the Δ^{22}-analogue of *1*. The molecular ion (*m/z* 414) and fragmentation ions substantiated this view and the compound was tentatively identified as 4α,24-dimethyl-5α-cholest-22-en-3β-ol *(5)*, which has also been indicated previously as a minor component of the sterols of the zooxanthellae of *B. asbestinum* (16).

The 4-demethyl sterol fraction was found by GC-MS analysis to consist of two major components and two more minor constituents. The most minor component, peak 1 (0.7%), had a short retention time, suggesting it to be a C_{26}-sterol. This was confirmed by the mass spectrum (M$^+$ at *m/z* 370) which showed the compound to be most probably 24-norcholesta-5,22-dien-3β-ol *(2)* although the alternative structure, 27-nor-cholesta-5,22-dien-3β-ol, could not be eliminated due to the lack of material for a more definitive identification. Sterol *2* was first isolated from a clam (23) and it is now recognized to be ubiquitous in its occurrence in animals of all marine invertebrate phylla (1,2).

Peaks 2 (32%) and 3 (53%) had mass spectra identical to those of cholesta-5,22-dien-3β-ol *(6)* and (24*R*)-24-methylcholesta-5,22-dien-3β-ol *(4*, brassicasterol), respectively. However, a careful comparison of the GLC retention time of peak 2 with that of a synthetic sample of cholesta-5,22-dien-3β-ol *(6)* showed a small discrepancy with peak 2 eluting slightly the faster of the two. Co-injection of cholesta-5,22-dien-3β-ol with the dinoflagellate sterol sample onto two different GLC columns (3% OV-17 and 1% HiEFF 8) gave a noticeable broadening of peak 2 which was sufficient to indicate the nonidentity of the peak 2 sterol with cholesta-5,22-dien-3β-ol *(6)*.

Peak 4 (14%) had a retention time and mass spectrum (M$^+$ at *m/z* 412) which indicated it to be a C_{29}-$\Delta^{5,22}$ sterol. However, the intensities of the molecular ion and other fragment ions were not consistent with its identification as (24*R*)-24-ethylcholest-5,22-dien-3β-ol (poriferasterol) which is a commonly occurring algal sterol. The base peak at *m/z* 69 suggested this compound to be 23,24ξ-dimethylcholesta-5,22-dien-3β-ol *(7)* which has previously been reported as a constituent of several coelenterates (24) and the alga, *Hymenomonas carterae* (25).

In order to characterize these compounds, the 4-demethyl sterols were acetylated and separated by TLC on silver nitrate-silica gel. One major band and several minor bands were observed. Mass spectrometry of the steryl acetates recovered by elution of the bands allowed the acetates of cholesterol, 24ξ-methylcholestan-3β-ol, 24ξ-methylcholest-5-en-3β-ol and 24ξ-methylcholest-22-en-3β-ol, to be identified by comparison of their mass spectra with those of authentic steryl acetates.

Three of the other trace components were tentatively identified as the acetates of 23,24ξ-dimethylcholesta-5,22-dien-3β-ol *(7)*, 23,24ξ-dimethylcholest-5-en-3β-ol and 23,24ξ-dimethylcholest-22-en-3β-ol. The mass spectrum of compound *7* displayed all the fragmentation ions reported to be characteristic of this sterol (24) but all the higher mass fragments were of very much lower intensity than reported (24) and the base peak was at *m/z* 69 as indicated by Volkman et al. (25). All of these compounds were present in such small amounts that they could not be further characterized.

Rechromatography of the major steryl acetate fraction on silver nitrate-silica gel TLC resolved it into two bands each of which was shown by GLC to contain only one component. The less polar material was shown by its ^{1}H NMR and mass spectra to be the acetate of (24*R*)-24-methylcholesta-5,22-dien-3β-ol *(4)*. The C-24 configuration was confirmed to be (24*R*) by comparison of the ^{1}H NMR spectrum of the isolated material with the ^{1}H NMR spectra of authentic samples of (24*R*)-24-methyl-

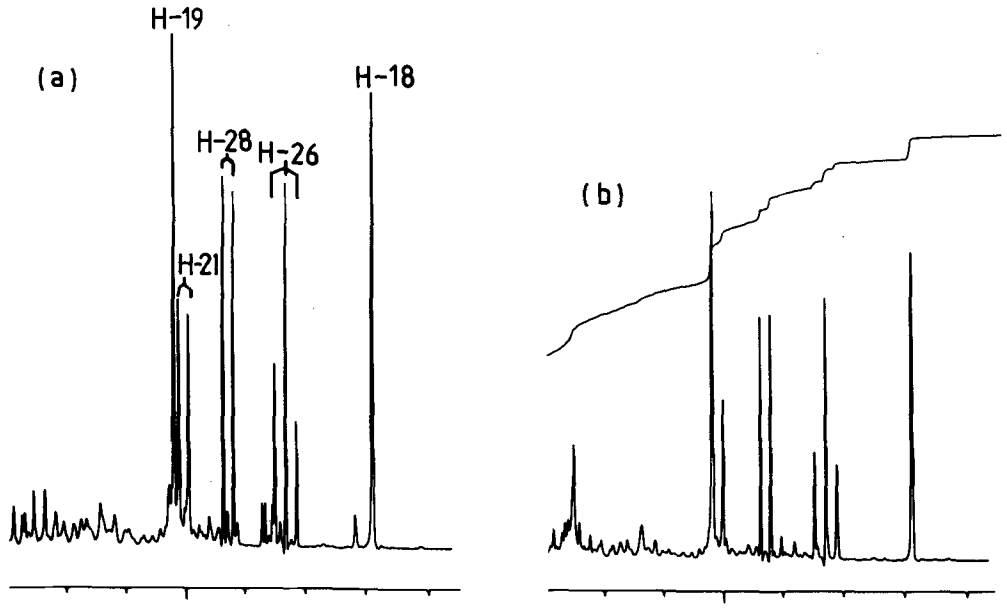


FIG. 1. The 400 MHz NMR spectra of (a) synthetic 27-nor-(24*S*)-24-methylcholesta-5,22-dien-3β-yl acetate and (b) the 27-nor-(24*R*)-24-methylcholesta-5,22-dien-3β-yl acetate isolated from *G. simplex*.

cholesta-5,22-dien-3β-yl *(4)* acetate (brassicasteryl acetate) and (24*S*)-24-methylcholesta-5,22-dien-3β-yl *(9)* acetate (epibrassicasteryl acetate). The natural product and brassicasteryl acetate had superimposable spectra and the H-21 doublet (δ 1.007) in the spectra of these compounds was significantly downfield compared to the corresponding signal for the H-21 protons (δ 1.003) of epibrassicasteryl acetate, thus establishing (22) the (24*R*)-configuration of the dinoflagellate sterol.

The steryl acetate of higher polarity from the TLC separation had a retention time and mass spectrum very similar to those of cholesta-5,22-dien-3β-yl *(6)* acetate. However, its ^{1}H NMR spectrum confirmed the earlier GLC retention time information, that it could not be this compound. The 220 MHz ^{1}H NMR spectrum displayed a triplet at δ 0.827 for a terminal ethyl group and two doublets for methyl groups at δ 0.925 and 1.009. These features, together with the mass spectrum, indicated that the sterol was 27-nor-24ξ-methylcholesta-5,22-dien-3β-yl acetate. This sterol has previously been isolated from a marine worm and given the trivial name occelasterol (21). Further examination of the ^{1}H NMR spectrum of the dinoflagellate steryl acetate showed that the signal for the H-21 doublet (δ 1.009) had a similar chemical shift to that of brassicasteryl acetate, thus strongly suggesting that the configurations of the two compounds at C-24 must be the same, that is (24*R*). However, occelasterol was concluded by Kobayashi and Mitsuhashi (21) to have the (24*S*)-configuration by comparison of its mp and ^{1}H NMR spectrum with the corresponding data of a synthetic sample of 27-nor-(24*S*)-24-methyl-cholesta-5,22-dien-3β-ol *(8)*. Dr. Kobayashi kindly provided us with a sample of the synthetic occelasteryl *(8)* acetate and we were, therefore, able to make a direct comparison of this compound with the dinoflagellate steryl acetate to resolve the C-24 configuration of the latter. The two compounds had very similar mp but their 400 MHz ^{1}H NMR spectra (Fig. 1) allowed them to be differentiated. The H-21 doublet (δ 1.010) of the dinoflagellate sterol was clearly downfield compared to the corresponding H-21 signal (δ 1.003) for the synthetic 27-nor-(24*S*)-24-methylcholesta-5, 22-dien-3β-yl *(8)* acetate, thus showing the two compounds to be epimeric at C-24 and establishing that the dinoflagellate sterol had the (24*R*)-configuration. The *G. simplex* sterol is, therefore, identified as 27-nor-(24*R*)-24-methylcholesta-5,22*E*-dien-3β-ol *(3)*. This assignment is now confirmed by the recent chemical synthesis of *3* by Hirano and Djerassi (26); synthetic *3* had the same ^{1}HNMR spectrum as our dinoflagellate sterol.

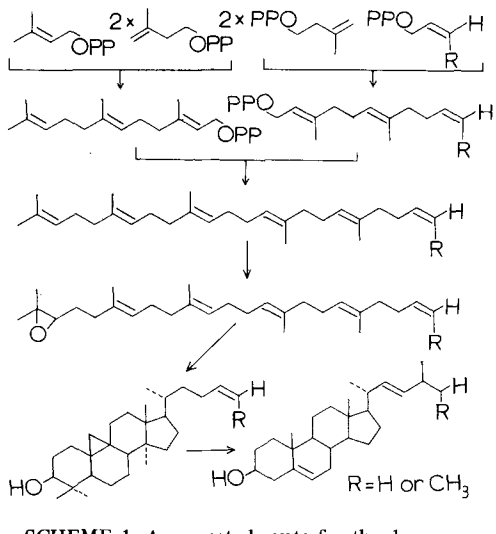
The identification of 27-nor-(24*R*)-24-methylcholesta-5,22-dien-3β-ol *(3)*, together with a small amount of 24-nor-cholesta-5,22-

dien-3β-ol *(2)*, in a dinoflagellate poses an interesting question regarding their mode of biosynthesis. Sterol *2*, or its Δ^7-analogue, occurs in trace amounts in many of the marine invertebrates examined (1,2,27) and the 24-epimer of sterol *3* (occelasterol), which was first reported in a marine worm (21), now appears also to occur more widely in small amounts, often accompanying cholesta-5,22-dien-3β-ol *(6)* which may perhaps have masked its presence in some earlier analyses. Since the *G. simplex* sterol is the epimer of occelasterol, it will be interesting to pay more careful attention to the analysis of the C_{27}-$\Delta^{5,22}$ sterols of marine invertebrates to establish if, in fact, it contains both the epimers *3* and *8*. Dr. Kobayashi (personal communication) has now informed us that reexamination of the H-21 doublet in the ^1H NMR spectrum of the occelasterol isolated from a scallop showed that, although the major component is the (24*S*)-epimer *(8)*, it does appear to contain a small amount of the (24*R*)-sterol *(3)*.

Regarding the origin of the nor-sterols *2* and *8*, it has been suggested that they may arise by degradation of the C_{28} sterol (24*S*)-24-methylcholesta-5,22-dien-3β-ol *(9)* by sequential removal of the terminal C-27 and C-26 methyl groups to give, in turn, *8* and then *2* (1,2,21,28). The organisms thought most likely to be responsible for these demethylations are bacteria which act upon sterol *9* (28) which, on the basis of present evidence, seems to be the main source of this sterol (1,2). Sterols *2* and *8* will then be incorporated into the sterol mixtures of invertebrate animals via the food chain. Support for this proposal has been provided by Boutry and Barbier (28-31) who have indicated that incubation of C_{27}, C_{28} and C_{29} sterols with a mixed culture of marine bacteria results in the appearance of short side chain sterols.

Teshima et al. (12) in their analysis of the sterols of the dinoflagellate, *Noctiluca milialis*, observed small amounts of *2* (0.7%) and *8* (or *3)* (0.5%) in a mixture which contained 24ξ-methyl-cholesta-5,22-dien-3β-ol *(4* or *9*, 72.5%) as the major component. However, it is not clear if *2* and *8* were true constituents of the dinoflagellate, in this case, as the material for analysis was not cultured axenically. The organism is halozoic and was collected from the sea near the coast of Japan and, therefore, may have included other plankton flora and fauna which would make their contribution to the sterol mixture extracted and analyzed. With regard to this point, Boutry et al. (32) reported that plankton contained a small amount of 24-nor-cholesta-5,22-dien-3β-ol *(2)* in addition to the larger amounts of C_{27}, C_{28} and C_{29} sterols and they suggested that a plankton organism may be an important primary source of the C_{26}-sterol.

Our observation of a small amount of the C_{26} sterol *(2)* together with a much larger amount of 27-nor-(24*R*)-24-methylcholesta-5,22-dien-3β-ol *(3)* in a culture of *G. simplex* now reveals the possibility that this alga, and perhaps other related dinoflagellates, are important primary producers of the 24-nor- and 27-nor-sterols, *2* and *3*, respectively, found in the marine environment. Since *G. simplex* also contains (24*R*)-24-methylcholesta-5,22-dien-3β-ol *(4)*, it is conceivable that sterols *2* and *3* were derived in this organism by demethylation of *4* either by the dinoflagellate itself or perhaps by unsuspected bacterial contamination during culture or subsequent storage (at -20C) of the cells prior to extraction. However, we have previously speculated in our laboratory that nor-sterols could be of algal origin and result from de novo synthesis rather than sterol degradation (27,33). We would like to suggest that they may be produced by a biosynthetic route leading from nor-isoprenoid pyro-



SCHEME 1. A suggested route for the de novo synthesis of nor-sterols commencing from dimethylallyl pyrophosphate, isopentenyl pyrophosphate and either propenyl pyrophosphate or butenyl pyrophosphate and passing via farnesyl pyrophosphate and norfarnesyl pyrophosphate to nor-squalene and nor-squalene-2,3-oxide. The first cyclized sterol precursor is shown here as a 9β,19-cyclopropane sterol. However, it has not yet been established if sterol biosynthesis in dinoflagellates involves 9β,19-cyclopropane sterols, as found in other algae, or if it proceeds via the Δ^8 sterol, lanosterol, as occurs in nonphotosynthetic organisms.

phosphate precursors through nor-farnesyl pyrophosphate and nor-squalene to cyclized products lacking one or two carbons in the side chain (Scheme 1). These latter compounds then undergo the appropriate modifications to the ring system and C-24 alkylation occurs to produce *2* or *3*.

Evidence has been presented from studies employing nor-squalene-2,3-oxide analogues that the trisubstituted epoxide moiety is critical for cyclization to occur when catalysed by squalene-2,3-oxide-lanosterol cyclase of rat liver (34,35). A similar requirement for a trisubstituted epoxide grouping may be exhibited by the dinoflagellate cyclase. In this case, only the mono- or dinor-squalene-2,3-oxide analogues with methyl groups lacking from the putative sterol sidechain portion of the molecule (Scheme 1) would serve as a substrate for the cyclase. The (24*R*)-24-methylcholesta-5,22-dien-3β-ol (*4*) of *G. simplex* would, of course, be produced concomitantly by the conventional route involving squalene. An examination of the hydrocarbon fraction of this alga for mono- or dinor-squalene analogues, or the presence of other nor-polyisoprenoids such as carotenoids in the dinoflagellate, would perhaps provide substantiating evidence for the operation of such a pathway as that shown in Scheme 1.

ACKNOWLEDGMENTS

We thank the Science Research Council for financial support during this work, Dr. M. Rose and Mr. M. Prescott for determining mass spectra, the Department of Chemistry, University of Liverpool, for 220 MHz NMR spectra and the Department of Chemistry, Sheffield University, for the 400 MHz ^{1}H NMR spectra.

REFERENCES

1. Schmitz, F.J. (1978) in Marine Natural Products (Scheuer, P.J., ed.), Vol. 1, pp. 241-297, Academic Press, New York, N.Y.
2. Goad, L.J. (1978) in Marine Natural Products (Scheuer, P.J., ed.), Vol. 2, pp. 76-172, Academic Press, New York, N.Y.
3. Djerassi, C. (1981) Pure Appl. Chem. 53, 873-890.
4. Goad, L.J. (1981) Pure Appl. Chem. 53, 837-852.
5. Shimizu, Y., Alam, M., and Kobayashi, A. (1976) J. Am. Chem. Soc. 98, 1059-1060.
6. Withers, N.W., Tuttle, R.C., Holz, G.G., Beach, D.H., Goad, L.J., and Goodwin, T.W. (1978) Phytochemistry, 17, 1987-1989.
7. Withers, N.W., Goad, L.J., and Goodwin, T.W. (1979) Phytochemistry, 18, 899-901.
8. Alam, M., Schram, K., and Ray, S.M. (1978) Tetrahedron Lett. 3517-3518.
9. Alam, M., Sansing, T.B., Busby, E.L., Martinez, D.R., and Ray, S.M. (1979) Steroids 33, 197-203.
10. Alam, M., Martin, G.E., and Ray, S.M. (1979) J. Org. Chem. 44, 4466-4467.
11. Alam, M., Sansing, T.B., Guerra, J.R., and Harmon, A.D. (1981) Steroids 38, 375-382.
12. Teshima, S.I., Kanazawa, A., and Tago, A. (1980) Mem.Fac. Fish Kagoshima Univ. 29, 319-326.
13. Kokke, W.C.M.C., Withers, N.W., Massey, I.J., Fenical, W., and Djerassi, C. (1979) Tetrahedron Lett. 3601-3604.
14. Withers, N.W., Kokke, W.C.M.C., Rohmer, M., Fenical, W., and Djerassi, C. (1979) Tetrahedron Lett. 3605-3608.
15. Kokke, W.C.M.C., Fenical, W., and Djerassi, C. (1981) Phytochemistry 20, 127-134.
16. Bohlin, L., Kokke, W.C.M.C., Fenical, W., and Djerassi, C. (1980) Phytochemistry 20, 2397-2401.
17. Swenson, W., Tagle, B., Clardy, J., Withers, N.W., Kokke, W.C.M.C., Fenical, W., and Djerassi, C.J. (1980) Tetrahedron Lett. 21, 4663-4666.
18. Kokke, W.C.M.C., Fenical, W., Bohlin, L., and Djerassi, C. (1981) Comp. Biochem. Physiol. 68B, 281-290.
19. Tuttle, R.C., and Loeblich, A.R. (1975) Phycologia 14, 1-6.
20. Fernholtz, E., and Stavely, H.E. (1940) J. Am. Chem. Soc. 62, 1875-1881.
21. Kobayashi, M., and Mitsuhashi, H. (1974) Steroids 24, 399-410.
22. Rubinstein, I., Goad, L.J., Clague, A.D.H., and Mulheirn, L.J. (1976) Phytochemistry 15, 195-200.
23. Idler, D.R., Wiseman, P.M., and Safe, L.M. (1970) Steroids 16, 451-461.
24. Kanazawa, A., Teshima, S-I., and Ando, T. (1977) Comp. Biochem. Physiol. 57B, 317-323.
25. Volkman, J.K., Smith, D.J., Eglington, G., Forsberg, T.E.V., and Corner, E.D.S. (1981) J. Mar. Biol. Assoc. 61, 509-527.
26. Hirano, Y., and Djerassi, C. (1982) J. Org. Chem., in press.
27. Goad, L.J. (1976) in Biochemical and Biophysical Perspectives in Marine Biology (Malins, D.C., and Sargent, J.R., eds.) Vol. 3, pp. 213-318, Academic Press, London.
28. Boutry, J.-L., and Barbier, M. (1980) Bull. Soc. Chim. Belg. 89, 1075-1079.
29. Boutry, J.-L., and Barbier, M. (1980) Biochem. Syst. Ecol. 8, 455-456.
30. Boutry, J.-L., and Barbier, M. (1980) Presented at the IUPAC Third International Symposium on Marine Natural Products, Brussels, Belgium.
31. Boutry, J.-L., and Barbier, M. (1981) Abstracts of the 72nd Annual Meeting of the American Oil Chemists' Society, New Orleans, LA.
32. Boutry, J.-L., Alcaide, A., and Barbier, M. (1971) C.R. Acad. Sci. Paris 272, 1022-1024.
33. Rubinstein, I. (1973) Ph.D. Thesis, University of Liverpool.
34. Clayton, R.B., Van Tamelen, E.E., and Nadeau, R.G. (1968) J. Am. Chem. Soc. 90, 820-823.
35. Van Tamelen, E.E., and Freed, J.H. (1970) J. Am. Chem. Soc. 92, 7206-7207.

[Received March 1, 1982]

Phosphatidate Phosphatase of Dermatophytes

C. KASINATHAN, A. CHOPRA and **G.K. KHULLER***, *Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh-160 012, India*

ABSTRACT

Phosphatidate phosphatase (EC 3.1.3.4) was detected in filamentous pathogenic fungi. In both dermatophytes, *Microsporum gypseum* and *Epidermophyton floccosum*, the enzyme was located in the mitochondrial and microsomal subcellular fractions with a pH optimum of 6.0. The *E. floccosum* enzyme was more active than that of *M. gypseum*. Although the enzyme in both fractions of the dermatophytes was susceptible to inhibition by Fe^{2+}, Mn^{2+}, Cu^{2+}, Ba^{2+} and Hg^{2+}, the *E. floccosum* microsomal enzyme was completely inhibited by Mn^{2+} and Fe^{2+}. The enzyme of *M. gypseum* as well as of *E. floccosum* exhibited a Mg^{2+} dependency in presence of EDTA. Sulphydryl reagents did not inhibit either the mitochondrial or the microsomal enzyme of these fungi. Phosphorylcholine and lysophosphatidylcholine markedly inhibited the enzyme in the two dermatophytes; however, the inhibition was less pronounced with phosphatidylcholine.
Lipids 17:859-863, 1982.

INTRODUCTION

Phosphatidic acid occupies the branch point between neutral and polar lipid synthesis in animals (1), plants (2) and yeasts (3) and phosphatidate phosphatase (EC 3.1.3.4) has been suggested to be rate-limiting for acylglycerol synthesis in the rat liver (4). Although this enzyme has been extensively studied in the rat liver (4) and lungs (5), it has not been demonstrated in filamentous fungi (6) and has been rather poorly investigated in other microorganisms (7). Earlier, it has been shown from this laboratory that the acylglycerol content of *Microsporum gypseum* (8) and *Epidermophyton floccosum* (9) is much higher than the phosphatides. Moreover, during labeled acetate incorporation studies with *E. floccosum* (9), it was observed that labeling of triacylglycerols was much higher than other neutral lipids, suggesting an extremely active phosphatidate phosphatase in this dermatophyte. The present study was, therefore, initiated to detect, localize and investigate the phosphatidate phosphatase of these pathogenic fungi.

MATERIALS AND METHODS

The source of *E. floccosum* was as given earlier (10), whereas *M. gypseum* was obtained from the Public Health Laboratory Service, London School of Hygiene and Tropical Medicine, London. These fungi were grown in Sabouraud's medium containing 4% glucose and 1% peptone, pH 5.4-5.6, at 27 C. Logarithmic phase growing cells were harvested by filtration and washed with chilled normal saline, to remove any adhering medium. Washed cells were homogenized in a mortar in 0.25 M sucrose, 10 mM Tris-HCl buffer, pH 8.0. The homogenate was sonicated for 5 min and then centrifuged at 5000 x g for 20 min to remove cell debris. All these procedures were carried out at 4 C. Supernatant, thus obtained after the above centrifugation, was used for crude characterization of the enzyme.

Subcellular Fractionation

For subcellular fractionation, a slightly modified procedure of Chavant et al. (11) was used. The 5,000 x g supernatant, obtained above, was spun at 15,000 x g for 30 min. The pellet was suspended in the above mentioned buffer and sonicated, whereas the supernatant was centrifuged at 105,000 x g for 90 min. The pellet so obtained was suspended in the same buffer and sonicated. To characterize the various fractions, cytochrome C-oxidase was assayed for mitochondria and glucose-6-phosphatase for microsomes. Pellet obtained from the 15,000 x g centrifugation was the mitochondrial fraction, whereas pellet from the 105,000 x g operation was the microsomes. Both fractions were found to be relatively free of contamination from other fractions. Freshly prepared fractions were used for enzyme assay either immediately or stored at -70 C till required.

Protein Estimation

Proteins were estimated by Lowry's method (12), using bovine serum albumin as standard.

Assay of Phosphatidate Phosphatase

Phosphatidic acid, prepared as an aqueous suspension, was used for this study. A total

*Author to whom correspondence should be addressed.

TABLE 1

Localization of Phosphatidate Phosphatase in Dermatophyte Subcellular Fractions

Fraction	Specific activity (μmol/mg protein/hr)	
	M. gypseum	*E. floccosum*
Mitochondrial	0.876 ± 0.06	1.129 ± 0.00
Microsomal	1.008 ± 0.03	1.496 ± 0.08
Cytosol	0.172 ± 0.01	0.457 ± 0.00

Values are mean ± SD of 4 independent determinations.

volume of 250 μl contained 3.25 mM phosphatidic acid, 0.1 M Tris-Maleate, pH 6.0, and 200-250 μg of enzyme protein. The reaction was carried out at 37 C for 60 min with continuous shaking. The reaction was terminated with 0.5 ml chloroform/methanol (2:1, v/v) and the two layers separated by centrifugation. Liberated phosphorus in the aqueous layer was estimated by Buell's method (13). In controls, enzyme was added after termination of the reaction.

Visualization of DG

In order to confirm hydrolysis of phosphatidic acid, the reaction was allowed to continue for 3 hr and all the enzyme assay constituents were increased to 4 times the quantities given above. The reaction was terminated as above and the chloroform layer was dried under a stream of nitrogen. The dried mass was dissolved in a known volume of chloroform, and a suitable aliquot was spotted on preactivated Silica Gel G plates. These plates were developed in petroleum ether/solvent ether/acetic acid (90:10:1, v/v/v) and exposed to iodine vapor for visualization. Dipalmitin was used as a marker.

Chemicals and Solvents

Phosphatidic acid, as a sodium salt, was purchased from CSIR Centre for Biochemicals, New Delhi; dipalmitin was obtained from Sigma and Silica Gel G from E. Merck. All other solvents and chemicals were of the highest purity available.

RESULTS AND DISCUSSION

To optimize the assay system of phosphatidate phosphatase (EC 3.1.3.4), homogenates from log phase cultures of *M. gypseum* and *E. floccosum* were used as the enzyme source. Visualization of diacylglycerol (DG), obtained after phosphatidic acid hydrolysis, was carried

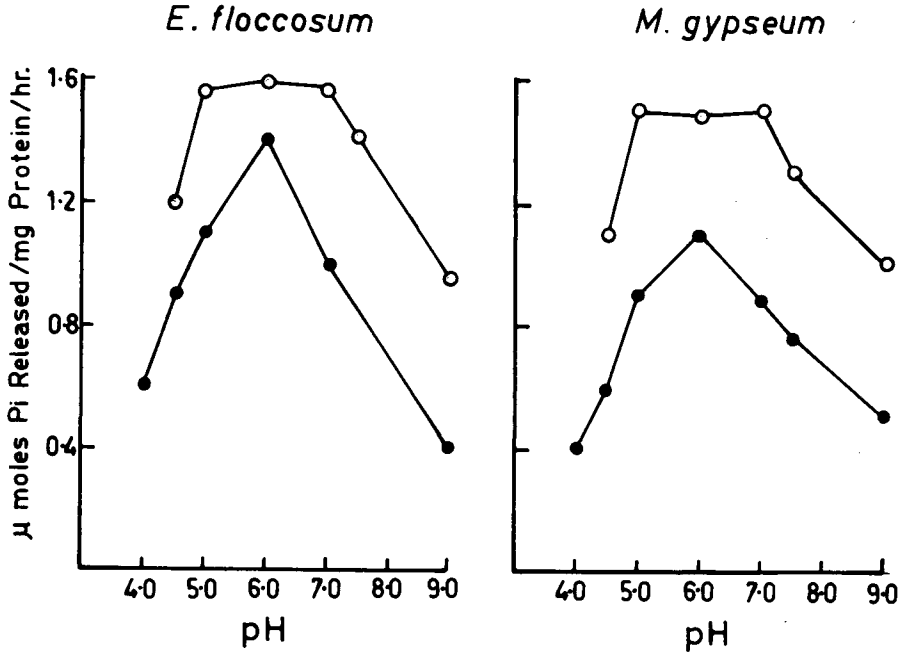


FIG. 1. Effect of pH on phosphatidate phosphatase activity of dermatophytes. •—• mitochondrial; ○—○ microsomal.

TABLE 2

Effect of Metal Ions on Mitochondrial and Microsomal Phosphatidate Phosphatase of *M. gypseum* and *E. floccosum*

Divalent metal ion (10 mM)	Activity (% control)			
	Mitochondrial		Microsomal	
	M. gypseum	*E. floccosum*	*M. gypseum*	*E. floccosum*
None	100	100	100	100
Mn	19	35	29	–
Fe	21	21	18	–
Cu	64	53	98	41
Ba	61	50	89	22
Hg	42	51	53	22

Values are mean of 4 independent determinations.

out using these homogenates. The controls showed faint spots of DG on the TLC plate, which were obviously due to endogenous content. Diacylglycerol spots from the experimental assay tubes were more prominent and had similar Rf to that of marker dipalmitin. This observation confirms our earlier observation, indicating the existence of a phosphatidate phosphatase in *E. floccosum* (9) and *M. gypseum*. To the best of our knowledge, this is the first report indicating the presence of phosphatidate phosphatase in filamentous fungi although phosphatidic acid has always been assumed to be a precursor for DG synthesis in fungi, mainly yeasts (6).

Subcellular Localization of Phosphatidate Phosphatase

In both dermatophytes, the enzyme was found to be localized in the mitochondrial and the microsomal fractions (Table 1). Enzyme activity was higher in the microsomal fraction, compared to mitochondria in *M. gypseum*, as well as in *E. floccosum*. Activity of the enzyme in *E. floccosum* mitochondrial and microsomal fractions was higher than that in the corresponding fractions of *M. gypseum*. Association of phosphatidate phosphatase activity with membranous fractions of the cell has been earlier reported in several mammalian tissues (14-16) and plants (6). Occurrence of phosphatidate phosphatase in the mitochondrial and microsomal fractions is not unexpected since most of the lipid synthesis takes place in these two cellular fractions although it is not known, of the two fractions, which one contributes more to the cellular lipid pool (6).

Since maximum activity of the enzyme was associated with mitochondrial and microsomal fractions of *M. gypseum* and *E. floccosum*, further work was carried out in these two cellular fractions.

Properties of Mitochondrial and Microsomal Phosphatidate Phosphatase

The microsomal phosphatidate phosphatase of both fungi had a broad pH optima, from pH 5.0-7.0, while the mitochondrial enzyme of these fungi was most active only at pH 6.0 (Fig. 1). Difference in pH optima of phosphatidate phosphatase, has been earlier reported in rat liver also (17). Since the microsomal fraction phosphatidate phosphatase was equally active between pH 5.0 and 7.0, all further studies in this fraction were carried out at pH 6.0, similar to the mitochondrial fraction.

Different Km values were obtained for the enzyme localized in the two subcellular fractions of the dermatophytes. The mitochondrial enzymes had Km (mM) of 0.26 and 2.20 for *E. floccosum* and *M. gypseum*, respectively. Similarly, these values were 0.25 and 1.42 for the respective microsomal enzymes. The Km values for mitochondrial and microsomal phosphatidate phosphatase of *E. floccosum* were lower than those of *M. gypseum* indicating the *E. floccosum* enzyme to be more active than that of *M. gypseum*, as suggested earlier.

Effects of metal ions on mitochondrial and microsomal phosphatidate phosphatase of *M. gypseum* and *E. floccosum* were compared (Table 2). In both subcellular fractions of the dermatophytes, the enzyme activities were more susceptible to inhibition by Fe^{2+}, Hg^{2+}, Ba^{2+}, Cu^{2+} or Mn^{2+}. In *E. floccosum*, however, the microsomal enzyme was completely inhibited by Mn^{2+} and Fe^{2+}, and was more susceptible to Ba^{2+} and Hg^{2+}, as compared to the *M. gypseum* microsomal phosphatidate phosphatase.

Although variable effects of Mg^{2+} have been reported, even for the same tissue (18,19), except for the mitochondrial phosphatidate phosphatase of *E. floccosum*, the enzyme was

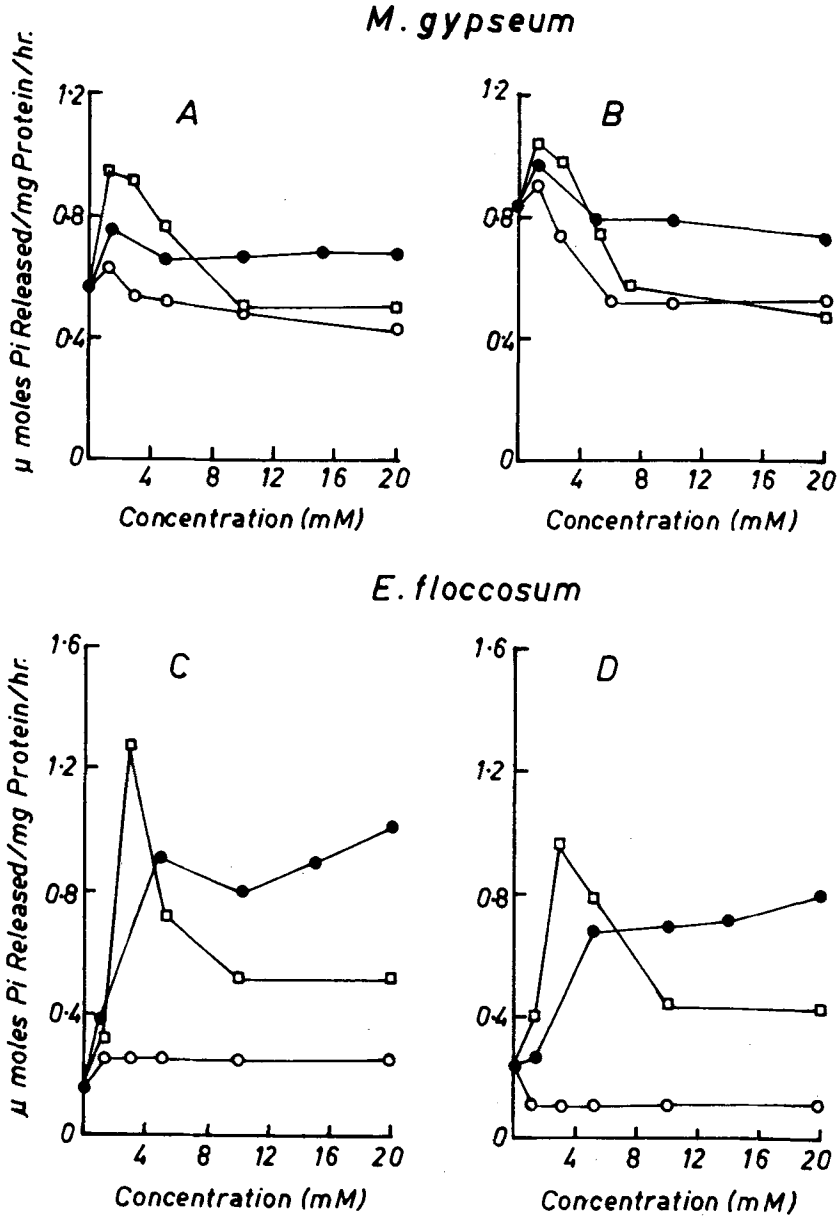


FIG. 2. Effect of Mg^{2+} (o—o), EDTA (•—•) and EDTA + Mg^{2+} complex (□—□) on mitochondrial (A,C) and microsomal (B,D) phosphatidate phosphatase of *M. gypseum* and *E. floccosum*.

activated by this metal in both dermatophytes (Fig. 2). Addition of EDTA alone stimulated the enzyme in both subcellular fractions of the dermatophytes and increasing concentrations of Mg^{2+}, in presence of 5 mM EDTA, considerably stimulated the enzyme activity (Fig. 2). Although a Mg^{2+} ion requirement was not evident under standard incubation conditions, the enzyme exhibited Mg^{2+}-dependent activation in presence of EDTA (5 mM) in *M. gypseum* as well as *E. floccosum*. This phenomenon of Mg^{2+} activation has earlier been demonstrated in rat liver microsomes also (4).

Several studies (20-22) have demonstrated that phosphatidate phosphatase is sensitive to inhibition by sulfhydryl reagents. In contrast to these reports, in both dermatophytes, β-mercaptoethanol or iodoacetate had no effect on the

TABLE 3

Effect of Phospholipid Intermediates on Mitochondrial and Microsomal Phosphatidate Phosphatase of *M. gypseum* and *E. floccosum*

Phospholipid intermediates	Activity (% control)			
	Mitochondrial		Microsomal	
	M. gypseum	*E. floccosum*	*M. gypseum*	*E. floccosum*
α-Glycerol-3-phosphate (10 mM)	99	103	92	95
Choline phosphate (10 mM)	23	29	31	44
Lysophosphatidylcholine (3.25 mM)	26	34	18	39
Phosphatidylcholine (3.25 mM)	86	91	77	93

Values are mean of 4 independent determinations.

enzyme activity. These observations indicate that phosphatidate phosphatase catalysis is not thiol group dependent in these pathogenic fungi.

Effects of phospholipid intermediates on mitochondrial and microsomal phosphatidate phosphatase of *M. gypseum* and *E. floccosum* were studied (Table 3). Glycerol-3-phosphate did not inhibit the enzyme, but a considerable decrease in the activity was observed in presence of phosphorylcholine. Similar observations have been reported with the rat lung phosphatidate phosphatase (23). Phosphatidylcholine as well as its hydrolysis product, lysophosphatidylcholine, inhibited phosphatidate phosphatase in both subcellular fractions of *M. gypseum* and *E. floccosum*.

From the above study, it can be concluded that phosphatidate phosphatase activity in *M. gypseum* and *E. floccosum* is associated with their mitochondrial and microsomal fractions. However, further studies at the enzymatic level need to be carried out to understand the exact mechanisms involved in regulation of neutral and polar lipids in these pathogenic fungi.

ACKNOWLEDGMENTS

This study was supported by grants from the Council of Scientific and Industrial Research and the Indian Council of Medical Research, New Delhi.

REFERENCES

1. van den Bosch, H., Van Golde, L.M.G., and Van Deenen, L.L.M. (1972) Rev. Physiol. Biochem. Pharmacol. 66, 13-145.
2. Galliard, T. (1973) in Form and Function of Phospholipids (Ansell, G.B., Dawson, R.M.C., and Hawthorne, J.N., eds.) 2nd edn., pp. 253-288, Elsevier Scientific Publishing Company, Amsterdam.
3. Wassef, M.K. (1977) Adv. Lipid Res. 15, 159-232.
4. Lamb, R.G., and Fallon, H.J. (1974) Biochim. Biophys. Acta 348, 166-178.
5. Casola, P.G., and Possmayer, F. (1981) Can. J. Biochem. 59, 500-510.
6. Weete, J.D. (1980) in Lipid Biochemistry of Fungi and Other Organisms, 2nd edn., pp. 130-156, Plenum, New York, NY.
7. Finnerty, W.R., and Makula, R.A. (1975) CRC Crit. Rev. Microbiol. 41, 1-40.
8. Khuller, G.K. (1978) Experientia 34, 342.
9. Chopra, A., and Khuller, G.K. (1981) Ind. J. Med. Res. 73, 325-330.
10. Khuller, G.K., Chopra, A., Bansal, V.S., and Masih, R. (1981) Lipids 16, 20-22.
11. Chavant, L., Dargent, R., Rami, J., and Motant, C. (1981) Biol. Cell. 37, 299-303.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Buell, M.V., Lowry, O.H., Roberts, N.R., Chang, M.W., and Kapphahn, J.I. (1958) J. Biol. Chem. 232, 979-993.
14. Schultz, F.M., Jimenez, J.M., MacDonald, P.C., and Johnston, J.M. (1974) Gynecol. Invest. 5, 222-229.
15. Ravinuthala, H.R., Miller, J.C., and Weinhold, P.A. (1978) Biochim. Biophys. Acta 530, 347-356.
16. Mavis, R.D., Finkelstein, J.N., and Hall, B.P. (1978) J. Lipid Res. 19, 467-477.
17. Sedgwick, B., and Hubscher, G. (1965) Biochim. Biophys. Acta 106, 63-77.
18. Mitchell, M.P., Brindley, D.N., and Hubscher, G. (1971) Eur. J. Biochem. 18, 214-220.
19. Jamdar, S.C., and Fallon, H.J. (1973) J. Lipid Res. 14, 517-524.
20. Coleman, R., and Hubscher, G. (1962) Biochim. Biophys. Acta 56, 479-490.
21. Johnston, J.M., Reynolds, G., Wylie, M.B., and MacDonald, P.C. (1978) Biochim. Biophys. Acta 531, 65-71.
22. Johnston, J.M., Rao, G.A., Lowe, P.A., and Schwart, G.E. (1967) Lipids 2, 14-20.
23. Yeung, A., Casola, P., Wong, C., Fellows, C.F., and Possmayer, F. (1979) Biochim. Biophys. Acta 574, 226-239.

[Received June 21, 1982]

Effect of Inhibitors on ω - and (ω-1)-Hydroxylation of Lauric Acid by Frog Liver Microsomes

YOSHIRO MIURA, *Department of Biochemistry, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan*

ABSTRACT

To investigate the involvement of different cytochrome P-450 monooxygenases in fatty acid hydroxylation in frog liver microsomes, the effect of various inhibitors of cytochrome P-450 monooxygenases on the ω- and (ω-1)-hydroxylation of laurate was examined. The ω/ω-1-hydroxylation ratios were changed significantly by various levels of carbon monooxide (CO) inhibition; the formation of ω-hydroxylaurate was more sharply inhibited by various levels of CO than was the formation of (ω-1)-hydroxylaurate. On the contrary, metyrapone inhibited only the formation of (ω-1)-hydroxylaurate and stimulated the formation of ω-hydroxylaurate. 7,8-Benzoflavone as well as CO was more inhibitory to the ω-hydroxylation of laurate. At low concentrations of KCN (0.2 and 0.1 mM), the (ω-1)-hydroxylase activity was stimulated, but both the ω- and (ω-1)-hydroxylase activities were inhibited at the higher concentrations (5-10 mM). The effect of drugs and hydroxylaurate isomers on the ω- and (ω-1)-hydroxylation was also examined. Aminopyrine showed a stimulative effect on ω-hydroxylase activity and no effect on the (ω-1)-hydroxylase activity, while *p*-nitroanisole inhibited the (ω-1)-hydroxylase activity and showed almost no effect on the ω-hydroxylase activity. 12-Hydroxylaurate inhibited both the ω- and (ω-1)-hydroxylase activities, but the ω-hydroxylase activity was inhibited to a much greater extent. 11-Hydroxylaurate had no effect on either hydroxylation. These findings strongly support the hypothesis that different cytochrome P-450 species are involved in the hepatic microsomal hydroxylation of laurate at ω- and (ω-1)-positions in the frog.

Lipids 17:864-869, 1982.

INTRODUCTION

A number of the so-called ω-hydroxylating systems have been found in a variety of organisms (1-10). All the hydroxylating systems except for a bacterial ω-hydroxylating system (2) involve cytochrome P-450, which plays a role in the hydroxylation of fatty acids. Most of the hydroxylating systems catalyze the hydroxylation of fatty acids at the ω- and (ω-1)-positions. However, a hydroxylating system obtained from *Bacillus megaterium* is unique in that it carried out the hydroxylation of fatty acids, alcohols, and amides at ω-1, ω-2, and ω-3 positions (11-12). Salaün et al. also reported the hydroxylation of lauric acid at ω-2, ω-3, and ω-4 positions by the microsomal fractions from Jerusalem-artichoke-tuber tissues (13). In previous papers (14-15), we reported that frog liver microsomes catalyzed the conversion of free fatty acids and alcohol to an isomeric mixture of ω- and (ω-1)-monohydroxy derivatives. It was also shown that the ratio of ω- and (ω-1)-hydroxy fatty acids varied with the chain length of fatty acids (14). Moreover, the formation of ω-hydroxydodecanol was more sharply inhibited by carbon monoxide (CO) than was the formation of (ω-1)-hydroxydodecanol (15). These findings have led to the suggestion that two or more cytochrome P-450 species are involved in hydroxylation at ω- and (ω-1)-positions by frog liver microsomes. Recently, the problem of determining whether one or several monooxygenase species are involved in ω- and (ω-1)-hydroxylation of fatty acids and prostaglandins has attracted special interest (16-22). In order to investigate the possible involvement of different cytochrome P-450 monooxygenases in fatty acid hydroxylation in frog liver, we decided to subject frog liver microsomes to various inhibitors (CO, metyrapone, and 7,8-benzoflavone) of cytochrome P-450 monooxygenases and to examine the alteration of the ratios of ω- and (ω-1)-hydroxy isomers of laurate. The effect of KCN and drugs (aminopyrine and *p*-nitroanisole) on the ω- and (ω-1)-hydroxylations of laurate by frog liver microsomes was also examined. From our previous observation and the results obtained in this study, we concluded that more than one cytochrome P-450 is involved in the ω- and (ω-1)-hydroxylation of laurate by frog liver microsomes.

MATERIALS AND METHODS

Substrate and Standards

1-[1-^{14}C]lauric acid (32 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, England). The ω- and (ω-1)-hydroxylauric acids were synthesized chemically (16). 7,8-Benzoflavone, KCN, and *p*-nitroani-

TABLE 1

Distribution of Hydroxylaurate Isomers Formed by Frog Liver Microsomes Partially Inhibited by CO[a]

$CO-O_2$ (v/v)	Hydroxylation activity [A]	Distribution of hydroxylaurate isomers(%)[B]		ω/ω-1 hydroxylation	Absolute amount ([A] X [B])	
		ω	ω-1		ω	ω-1
1:9	74	36	64	0.56	27(68)	47(78)
3:7	66	24	76	0.32	16(40)	50(83)
6:4	49	23	77	0.30	11(28)	38(63)
8:2	39	0	100	0	0(0)	39(65)
control	100[b]	40	60	0.67	40(100)	60(100)

[a]Each tube contained 20 ml of a $CO:O_2$ mixture for this inhibitor study. The amount of microsomal protein was 2.06 mg.

[b]Actual conversion: 7.2 nmol/mg/15 min.

sole were purchased from Tokyo Kasei Che. Co. (Tokyo, Japan). Aminopyrine was a gift of Dr. K. Yamaoka, Pharmaceutical Division of Teikyo University School of Medicine. NADPH and metyrapone were obtained from Sigma Chem. Co. (St. Louis, MO) and Aldrich Chem. Co. (Milwaukee, WI), respectively. All other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan) and were of analytical grade.

Preparation of Frog Liver Microsomes

Japanese bullfrogs (*Rana catesbeiana*) weighing 200-250 g were obtained from Tokyo Experimental Animal Laboratories (Tokyo, Japan). The preparation of frog liver microsomes was done as previously described (23). The protein concentration of the microsomal suspension was determined by the method of Lowry et al. (24).

Assay for Hydroxylation of Fatty Acids

The method described by Miura et al. (14) was used with minor modification. The incubations contained microsomes (2.1-3.4 mg), 1 mmol potassium phosphate (pH 7.5), 2 μmol NADPH, and 0.3 μmol of potassium salt of [1-^{14}C]lauric acid (1.8×10^6 cpm). The final volume was 5 ml; incubation time was 15 min at 37 C. Metyrapone was dissolved in methanol and 7,8-benzoflavone in acetone. Control experiments containing an equal volume of methanol or acetone were performed when inhibitors were used. After treatment of reaction products with diazomethane, they were chromatographed on a column of 2 g of silicic acid (Wako Pure Chemical Industries Ltd). The polar esters (ω- and (ω-1)-hydroxy laurate) were eluted with 100% ether. Recovery of radioactivity was 81-95% in all experiments. The several procedures used for identification of methyl ω- and (ω-1)-hydroxylaurates have been described in detail previously (14).

Radio-Gas Chromatographic Analyses

The general procedures used for radio-gas chromatographic analyses of the O-acetyl derivatives of hydroxylauric acid have already been described (14), except that an Aloka model PDC-R8376 proportional counter (Aloka Instrument Inc., Tokyo, Japan) was used for radioactive determinations.

RESULTS

Hydroxylation of Laurate by Frog Liver Microsomes at Various Levels of CO

Since it is well known that CO binds specifically to the cytochrome P-450 from various organisms, the effect of CO are various levels on laurate hydroxylation was examined (Table 1). It was observed that the ω/ω-1-hydroxylation ratio changed significantly by various levels of CO inhibition. It was also observed that the formation of ω-hydroxylaurate was more sharply inhibited than that of (ω-1)-hydroxylaurate by various levels of CO. At a $CO:O_2$ ratio of 4.0, no ω-hydroxylaurate was formed in the incubation of [1-^{14}C]laurate with frog liver microsomes.

Effect of Metyrapone and 7,8-Benzoflavone on ω- and (ω-1)-Hydroxylation of Laurate by Frog Liver Microsomes

Since inhibitors of cytochrome P-450 monooxygenases have often been used to distinguish the characteristics of the P-450 species involved in metabolism of many compounds and xenobiotics, metyrapone and 7,8-benzoflavone were selected and the effect of these inhibitors on frog liver laurate hydroxylase activity was examined (Table 2). The total hydroxylation ac-

TABLE 2

Effect of Metyrapone and 7,8-Benzoflavone on ω- and (ω-1)-Hydroxylation of Laurate by Frog Liver Microsomes[a]

Inhibitor	Hydroxylation activity (% of control)			ω/ω-1 Hydroxylation
	ω	ω-1	ω+(ω-1)	
Metyrapone 0.5 mM	152	61	102	2.03
Metyrapone 1.0 mM	141	47	90	2.45
Metyrapone 2.0 mM	145	37	86	3.20
7,8-Benzoflavone 0.03 mM	90	109	104	0.33
7,8-Benzoflavone 0.06 mM	38	89	74	0.18
7,8-Benzoflavone 0.16 mM	6	48	36	0.05
7,8-Benzoflavone 0.24 mM	5	51	38	0.04

[a]The specific activities of ω-hydroxylaurate and (ω-1)-hydroxylaurate formations for control experiment for metyrapone inhibition were, 3.40 and 4.15 nmol/mg/15 min, respectively. The rates of ω-hydroxylaurate and (ω-1)-hydroxylaurate formations for control experiment for 7,8-benzoflavone inhibition, were 1.65 and 4.04 nmol/mg/15 min, respectively.

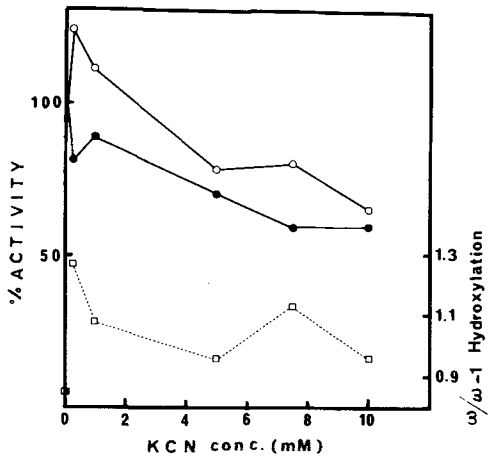


FIG. 1. Effect of KCN on ω- and (ω-1)-hydroxylation of laurate by frog liver microsomes. The amount of microsomal protein was 2.57 mg. The specific activities of ω- and (ω-1)-hydroxylaurate formations for control experiment were 4.65 and 5.46 nmol/mg/15 min, respectively. ○, ω-hydroxylation; ●, (ω-1)-hydroxylation; □, ω/ω-1-hydroxylation ratio.

tivity (the sum of ω- and (ω-1)-hydroxylase activity) decreased with an increase in the concentration of metyrapone, but, rather, the ω-hydroxylase activity was stimulated and only the (ω-1) hydroxylase activity was significantly inhibited by metyrapone. The ω/ω-1-hydroxylation ratio changed at various inhibition degrees. On the other hand, 7,8-benzoflavone as well as CO was more inhibitory to the ω-hydroxylation of laurate than to the (ω-1)-hydroxylation of laurate; the ratio of ω/ω-1-hydroxylation decreased with increasing concentration of 7,8-benzoflavone.

Effect of KCN on ω- and (ω-1)-hydroxylation of Laurate by Frog Liver Microsomes

Although it is widely accepted that most drug oxidations by liver microsomes are insensitive to cyanide (25-27), it has been observed that microsomal oxidations of several drugs, including aniline hydroxylation, are sensitive to cyanide (28-29). Since we already reported that KCN was inhibitory to laurate hydroxylase activity in frog liver microsomes (14), the effect of KCN on the ω- and (ω-1)-hydroxylation of laurate was examined (Fig. 1). At low concentrations of KCN (0.2 and 1.0 mM), the ω-hydroxylase activity was somewhat stimulated, but both the ω- and (ω-1)-hydroxylase activities were inhibited at the higher concentrations (5-10 mM); the formation of (ω-1)-hydroxylaurate was more significantly inhibited by KCN than was the formation of ω-hydroxylaurate.

Effect of Aminopyrine and *p*-Nitroanisole on ω- and (ω-1)-Hydroxylation of Laurate by Frog Liver Microsomes

Ellin and Orrenius (18) reported the relationship between the ω- and (ω-1)-hydroxylase of fatty acids and drug hydroxylase; they suggested that (ω-1)-hydroxylating activity is similar or identical to the "nonspecific drug hydroxylase" and, by contrast, the ω-hydroxylase is most probably highly specific for fatty acids. On the basis of this suggestion, we decided to examine the effect of several drugs on the ω- and (ω-1)-hydroxylation of laurate. Since Moldeus et al. (17) reported the effect of aminopyrine on the ω- and (ω-1)-hydroxylation of laurate by liver

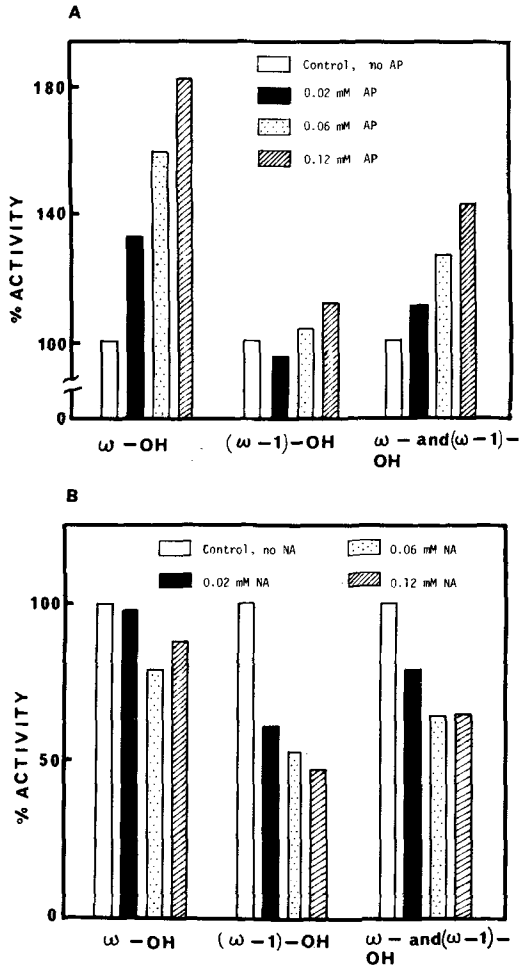


FIG. 2. Effect of aminopyrine (A) and *p*-nitroanisole (B) on ω- and (ω-1)-hydroxylation of laurate by frog liver microsomes. The amount of microsomal protein was 2.57 mg. The specific activities of ω- and (ω-1)-hydroxylaurate formations for control experiment were 3.76 and 5.00 nmol/mg/15 min, respectively. AP, aminopyrine; NA, *p*-nitroanisole.

and kidney cortex microsomes in the rat, we examined the effect of two different drugs, aminopyrine and *p*-nitroanisole, on the ω- and (ω-1)-hydroxylation of laurate by frog liver microsomes (Figs. 2A and 2B). Surprisingly, increasing concentrations of aminopyrine enhanced the ω-hydroxylase activity of laurate, while aminopyrine had almost no effect on the (ω-1)-hydroxylase activity. On the other hand, *p*-nitroanisole showed an inhibitory effect on the (ω-1)-hydroxylase activity at all concentrations, but almost no inhibitory effect on ω-hydroxylase activity at 0.02 and 0.12 mM.

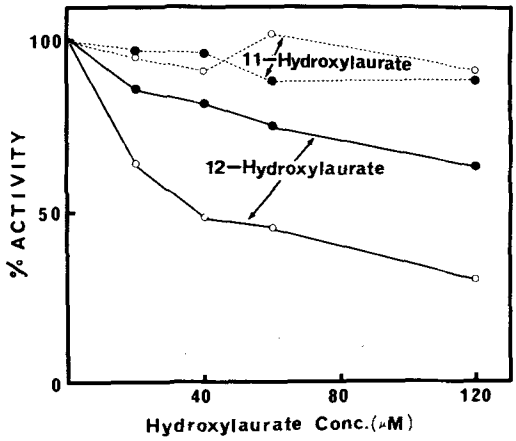


FIG. 3. Effect of 12- and 11-hydroxylaurate on ω- and (ω-1)-hydroxylation of laurate by frog liver microsomes. The amount of microsomal protein was 2.57 mg. The specific activities of ω- and (ω-1)-hydroxylaurate formations for control experiment were 4.44 and 5.66 nmol/mg/15 min, respectively. ○, ω-hydroxylation; ●, (ω-1)-hydroxylation.

Effect of 12- and 11-Hydroxylaurate on ω- and (ω-1)-Hydroxylation of Laurate by Frog Liver Microsomes

Finally, the effect of ω- and (ω-1)-hydroxylated products, 12- and 11-hydroxylaurate on the hydroxylation of laurate was also examined (Fig. 3). 12-Hydroxylaurate inhibited both the ω- and (ω-1)-hydroxylation, but it was more inhibitory to the ω-hydroxylase activity than to the (ω-1)-hydroxylase activity. On the other hand, 11-hydroxylaurate had no inhibitory effect on either hydroxylase activity.

DISCUSSION

The experiments reported in this paper were designed to determine whether one or several cytochrome P-450 species are involved in ω- and (ω-1)-hydroxylation of fatty acids by frog liver microsomes. The observation that CO, which is known to bind specifically to cytochrome P-450, changed the ω/ω-1-hydroxylation ratio dramatically (Table 1), strongly supports the concept that ω- and (ω-1)-hydroxylations of laurate are catalyzed by more than one cytochrome P-450. Moreover, it was suggested that CO has a higher affinity for the cytochrome P-450 catalyzing the ω-hydroxylation because the formation of ω-hydroxylaurate was more sharply inhibited by CO than was the formation of (ω-1)-hydroxylaurate.

Interesting differences in the action of inhibitors on the ω- and (ω-1)-hydroxylation of laurate by frog liver microsomes were observed

when metyrapone and 7,8-benzoflavone were used as an inhibitor (Table 2). Metyrapone specifically inhibited the (ω-1)-hydroxylase activity. Okita et al. (8) also reported that metyrapone inhibited human liver laurate (ω-1)-hydroxylase activity but had no inhibitory effect on the laurate ω-hydroxylase activity. On the other hand, 7,8-benzoflavone inhibited both the ω- and (ω-1)-hydroxylase activities in frog liver microsomes, but the ω-hydroxylase activity was more sensitive to the inhibitory action of 7,8-benzoflavone than was the (ω-1)-hydroxylase activity. These results indicate that different cytochrome P-450 species are involved in laurate hydroxylation at ω- and (ω-1)-positions by frog liver microsomes. Moldeus et al. (17) and Okita et al. (20) also reached a similar conclusion based on their results using metyrapone, 7,8-benzoflavone, and SKF 525-A on control rat liver microsomes. Similarly, Kupfer et al. (19,22) showed the clear evidence for several cytochrome P-450 species catalyzing ω- and (ω-1)-hydroxylation of prostaglandins in guinea pig using metyrapone and 7,8-benzoflavone.

The inhibitory study using KCN also suggests the involvement of different cytochrome P-450 species in the ω- and (ω-1)-hydroxylation of laurate in frog liver microsomes because ω/ω-1-hydroxylation ratios were changed at various concentrations of KCN (Fig. 1). It seems that KCN is more inhibitory to the cytochrome P-450 catalyzing (ω-1)-hydroxylation of laurate than to that catalyzing ω-hydroxylation. A similar finding was reported by Kamataki et al. (29) for the effect of KCN on aniline hydroxylation in rat liver microsomes. They indicated that the degree of KCN inhibition for aniline hydroxylation was dependent on the species of cytochrome P-450 used in reconstituted systems.

The different effects of aminopyrine and *p*-nitroanisole on the ω- and (ω-1)-hydroxylase activity of laurate (Figs. 2A and 2B) suggest also that frog liver microsomes contain different cytochrome P-450 species catalyzing ω- and (ω-1)-hydroxylation of laurate. The fact that *p*-nitroanisole showed almost no effect on ω-hydroxylase activity, while it showed an inhibitory effect only on the (ω-1)-hydroxylase activity in frog liver microsomes (Fig. 2B), suggests that the O-demethylase activity of *p*-nitroanisole is similar to the (ω-1)-hydroxylase of laurate. The observation that aminopyrine showed almost no effect on the (ω-1)-hydroxylase activity (Fig. 2A) suggests that the N-demethylase activity of aminopyrine is different from the (ω-1)-hydroxylase of laurate.

Ellin et al. (16) reported 12-hydroxylaurate inhibited the ω- and (ω-1)-hydroxylation of laurate in rat kidney cortex microsomes to a similar extent and 11-hydroxylaurate did not inhibit either hydroxylation. They conlcuded that this observation is strong evidence that only one hydroxylase is involved in fatty acid hydroxylation by kidney microsomes. In frog liver microsomes as well as in rat kidney microsomes, 11-hydroxylaurate had no effect on either the ω- or (ω-1)-hydroxylation of laurate, but 12-hydroxylaurate inhibited ω- and (ω-1)-hydroxylation of laurate to different extents (Fig. 3). This observation suggests also that the ω- and (ω-1)-hydroxylations are catalyzed by different cytochrome P-450 species.

From our previous results and the observations in this study, we concluded that two or more cytochrome P-450 species are involved in the microsomal hydroxylation of laurate at ω- and (ω-1)-positions in frog liver.

ACKNOWLEDGMENTS

The author is grateful to Dr. N. Ueta (Department of Biochemistry, Teikyo University School of Medicine) for his encouragement in performing this work, to Dr. A.J. Fulco (Department of Biological Chemistry, UCLA School of Medicine, University of California, Los Angeles) for his critical reading of this manuscript, and Dr. K. Yamaoka (Pharmaceutical Division, Teikyo University School of Medicine) for providing aminopyrine. The author would also like to thank H. Hisaki for the preparation of the microsomes.

REFERENCES

1. Preiss, B., and Bloch, K. (1964) J. Biol. Chem. 239, 85-88.
2. Kusunose, M., Kusunose, E., and Coon, M.J. (1964) J. Biol. Chem. 239, 1374-1380.
3. Lu, A.Y.H., and Coon, M.J. (1968) J. Biol. Chem. 243, 1331-1332.
4. Ichihara, K., Kusunose, E., and Kusunose, M. (1969) Biochim. Biophys. Acta 176, 704-712.
5. Björkhem, I., and Danielsson, H. (1970) Eur. J. Biochem. 17, 450-459.
6. Heinz, E., Tulloch, A.P., and Spencer, J.F.T. (1970) Biochim. Biophys. Acta 202, 49-55.
7. Ellin, Å., Jakobsson, S.V., Schenkman, J.B., and Orrenius, S. (1972) Arch. Biochem. Biophys. 150, 64-71.
8. Okita, R.T., Jakobsson, S.V., Prough, R.A., and Masters, B.S.S. (1979) Biochem. Pharmacol. 28, 3385-3390.
9. Ichihara, K., Yamakawa, I., Kusunose, E., and Kusunose, M. (1979) J. Biochem. 86, 139-146.
10. Kusunose, E., Ogita, K., Ichihara, K., and Kusunose, M. (1981) J. Biochem. 90, 1069-1076.
11. Miura, Y., and Fulco, A.J. (1974) J. Biol. Chem. 249, 1880-1888.
12. Miura, Y., and Fulco, A.J. (1975) Biochim. Biophys. Acta 388, 305-317.
13. Salaün, J.-P., Benveniste, I., Reichhart, D., and Durst, F. (1978) Eur. J. Biochem. 90, 155-159.
14. Miura, Y., Hisaki, H., and Ueta, N. (1978) Biochim. Biophys. Acta 531, 149-157.
15. Miura, Y. (1981) Lipids, 16, 721-725.
16. Ellin, Å., Orrenius, S., Pilotti, Å., and Swahn,

C.-G. (1973) Arch. Biochem. Biophys. 158, 597-604.
17. Moldeus, P., Ellin, Å., Thor, H., and Orrenius, S. (1974) in Regulation of Hepatic Metabolism (Lundquist, F. and Tygstrup, N., eds.) pp. 659-675, Munksgaard, Copenhagen.
18. Ellin, Å., and Orrenius, S. (1975) FEBS Lett. 50, 378-381.
19. Kupfer, D., Miranda, G.K., Navarro, J., Piccolo, D.E., and Theoharides, A.D. (1979) J. Biol. Chem. 254, 10405-10414.
20. Okita, R.T., Prough, R.A. and Masters, B.S.S. (1980) in Microsomes, Drug Oxidations and Chemical Carcinogenesis (Coon, M.J., Conney, A.H., Estabrook, R.W., Gelboin, H.V., Gillette, J.R., and O'Brien, P.J., eds.) Vol. II, pp. 741-744, Academic Press, New York, NY.
21. Kupfer, D. (1980) Pharmac. Ther. 11, 469-496.
22. Theoharides, A.D., and Kupfer, D. (1981) J. Biol. Chem. 256, 2168-2175.
23. Miura, Y., Hisaki, H., and Ueta, N. (1980) Comp. Biochem. Physiol. 67B, 541-547.
24. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
25. La Du, B.N., Gaudette, L., Trousof, N., and Brodie, B.B. (1955) J. Biol. Chem. 214, 741-752.
26. Gillette, J.R., Brodie, B.B., and La Du, B.N. (1957) J. Pharmac. Exp. Ther. 119, 532-540.
27. Oshino, N., Imai, Y., and Sato, R. (1966) Biochim. Biophys. Acta 128, 13-28.
28. Matsubara, T., and Tochino, Y. (1976) Arch. Biochem. Biophys. 172, 696-705.
29. Kamataki, T., Kitada, M., Chiba, K., Kitagawa, H., Imai, Y., and Sato, R. (1980) Biochem. Pharmac. 29, 1141-1147.

[Received July 6, 1982]

Oxidation of Lipids: III. Oxidation of Methyl Linoleate in Solution[1]

YORIHIRO YAMAMOTO, ETSUO NIKI* and **YOSHIO KAMIYA**, *Department of Reaction Chemistry, Faculty of Engineering, University of Tokyo, Hongo, Tokyo 113, Japan*

ABSTRACT

The effects of oxygen pressure, substrate concentration and solvent on the rate and products of oxidation of methyl linoleate were studied at 50 C with azobisisobutyronitrile as a radical initiator. The absolute and quantitative numbers for oxygen uptake, substrate disappearance, and formation of conjugated diene and hydroperoxides were measured. Under the present conditions, 4 conjugated diene hydroperoxides, 13-hydroperoxy-9-*cis*,11-*trans*-(**2a**), 13-hydroperoxy-9-*trans*,11-*trans*-(**3a**), 9-hydroperoxy-10-*trans*,12-*cis*-(**4a**), and 9-hydroperoxy-10-*trans*,12-*trans*-(**5a**) octadecadienoic acid methyl esters, were formed almost quantitatively. The rate of oxidation decreased with decreasing oxygen pressure. However, the ratio of *cis,trans* to *trans,trans* hydroperoxides, (**2a** + **4a**)/(**3a** + **5a**), was independent of oxygen pressure, and this ratio increased with increasing methyl linoleate concentration, as found recently by Porter. Further, the rate of oxidation and the ratio of *cis,trans/trans,trans* hydroperoxides were dependent on solvent and increased with an increase in dielectric constant of solvent. A mechanism of methyl linoleate oxidation consistent with these results is discussed.
Lipids 17:870-877, 1982.

Oxidations of unsaturated fatty acids have been the subject of investigations since the onset of the study on oxidation in the 1940s (1). Recently, the nonenzymatic oxidation of polyunsaturated fatty acids and esters (PUFA) by molecular oxygen has received renewed attention in connection with formation and the behavior of lipid peroxides in biological systems (2) as well as with the oxidative deterioration of foods, fats, and oils (3).

Among the PUFA, oxidation of methyl linoleate was studied most extensively. Howard and Ingold (4) observed that the hydroperoxides were formed quantitatively and measured the absolute rate constants for propagation k_p and termination k_t in the autoxidation of methyl linoleate in chlorobenzene at 30 C as 62 $M^{-1}s^{-1}$ and 4.4 x 10^6 $M^{-1}s^{-1}$, respectively. Chan and Levett (5) reported that the hydroperoxides consisted of 4 conjugated diene hydroperoxides, 13-hydroperoxy-9-*cis*,11-*trans*-(**2a**), 13-hydroperoxy-9-*trans*,11-*trans*-(**3a**), 9-hydroperoxy-10-*trans*,12-*cis*-(**4a**), and 9-hydroperoxy-10-*trans*,12-*trans*-(**5a**) octadecadienoic acid methyl esters. We observed that oxygen uptake, substrate disappearance, peroxide, and conjugated diene formation all agreed well with each other in the early stage of oxidation of methyl linoleate (6). Porter et al. (7) studied the factors which determined the distribution of 4 conjugated diene hydroperoxides and observed that the proportion of hydroperoxides was dependent on the concentration of substrate and inhibitor but independent of oxygen pressure. After we first submitted this manuscript, a more detailed study on the oxidation of PUFA was published by Porter et al. (8). They proposed a mechanism for linoleic acid oxidation by which they interpreted the above results (Scheme 1).

Several papers have been published in addition to those referred above on the oxidation of linoleic acid and its esters (9-15).

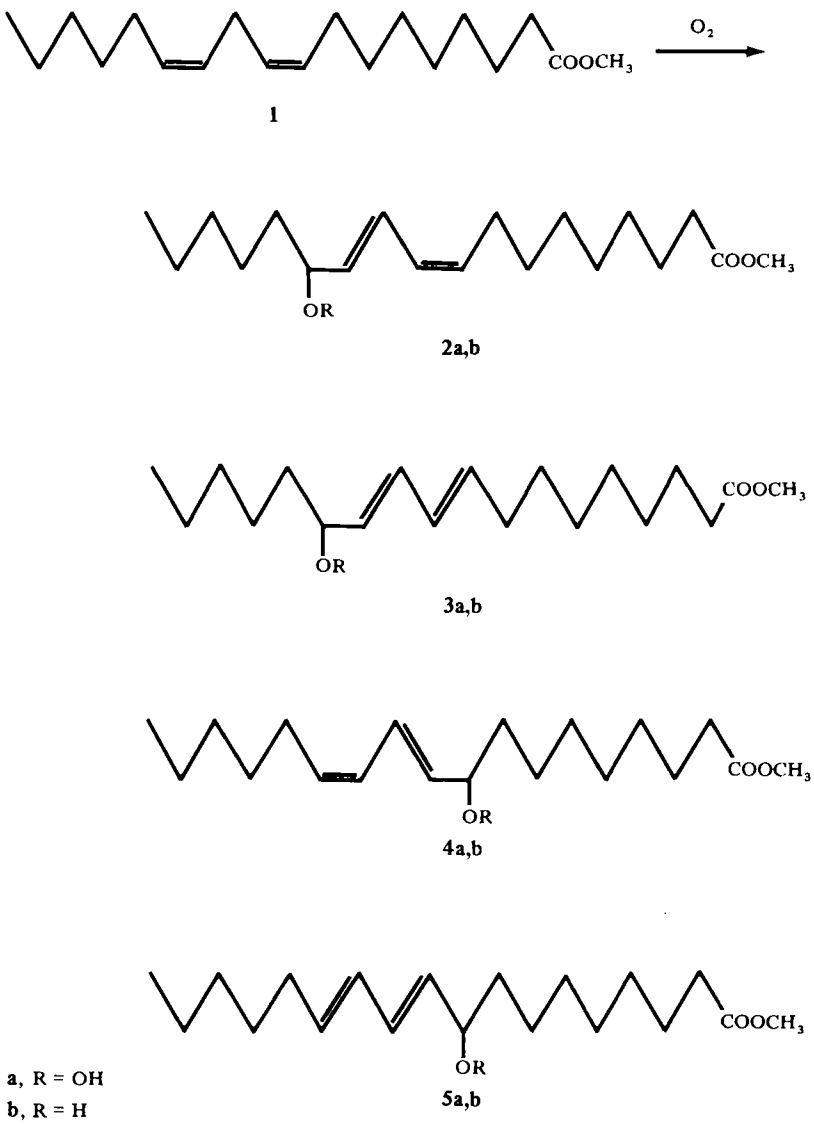
Considering that the concentration of oxygen is low in biological systems, we intended to reinvestigate the oxygen pressure effect on the rate and products of the oxidation of methyl linoleate in solution. We found that the rate of oxidation decreases with decreasing oxygen pressure but the distribution of 4 hydroperoxides is independent of oxygen pressure. We also measured the effect of substrate concentration and solvents on the rate and products of oxidation. Although this paper may appear now to be a rather straightforward extension of the work of Porter and his colleagues (7), our data confirm their observation and add some new data. We believe these studies are fundamental and essential to our understanding of the peroxidation of lipids in biological systems.

EXPERIMENTAL SECTION

Methyl linoleate was obtained from Sigma Chemical Co., and used as received. Prior to the

[1]Presented at the 15th Symposium on Oxidation Reactions, Nagoya, October 1981.

*Author to whom correspondence should be addressed.



SCHEME 1. Oxidation of methyl linoleate.

oxidation, neither conjugated diene nor peroxides were detected by ultraviolet (UV) spectrometry and iodometric titration, respectively (see later text). Organic solvents such as acetonitrile were those of the highest grade available. Azobisisobutyronitrile (AIBN) was recrystallized from methanol. Appropriate amounts of methyl linoleate, AIBN, and solvent were taken into ca. 30 ml pyrex ampoule. It was degassed by freeze and thaw cycle and then oxygen was introduced into the vessel. Initial and final amounts of oxygen were measured by a Toepler pump. The oxidation was carried out at 50 C for the desired time. The rate of oxygen uptake was followed by pressure decrease using a pressure transducer connected to the vessel. After the oxidation, the gases in the vessel were collected and measured by Toepler pump and then analyzed by a gas chromatography with Molecular Sieve 13X column. The amount of nitrogen evolved from AIBN was quite small and it was calculated from the measured rate constant for its unimolecular decomposition, $k_d = 1.25 \times 10^{-6}\ s^{-1}$ at 50 C.

The amount of methyl linoleate consumed was measured by gas liquid chromatography

TABLE 1

Effect of Oxygen Pressure on the Oxidation of Methyl Linoleate (LH) in Acetonitrile at 50 C, 60 min

Run no.	1	2	3	4
LH, μmol	871	2103	2053	2104
LH, M	0.380	0.370	0.362	0.370
AIBN, mM	22.6	22.0	22.4	22.7
Initial O_2, μmol	125.7	368.7	952.1	1175.3
Initial O_2, torr	90.1	316	776	959
Final O_2, μmol	86.4	283.6	830.4	1041.3
Final O_2, torr	62.0	231	677	850
$-dO_2/dt \times 10^6$, M/s	4.91	5.21	6.50	6.71
$R_i \times 10^8$, M/s	5.03	5.23	5.32	5.39
Kinetic chain length	91.0	99.2	122	124
$k_p/(2k_t)^{1/2}$, $(M \cdot s)^{-1/2}$	0.056	0.062	0.078	0.078
ΔO_2, μmol	39.3	103.1	121.7	134.0
ΔLH, μmol	40	95	127	143
$(C=C)_2$, μmol	34	93	99	110
Peroxides[a], μmol	38	108	125	137
Hydroperoxides analyzed for HPLC after reduction with triphenylphosphine, μmol (relative):				
13-*c,t*-LOOH (2a)	6.1(1)	16.9(1)	19.5(1)	21.9(1)
13-*t,t*-LOOH (3a)	12.8(2.1)	35.6(2.1)	41.8(2.1)	45.8(2.1)
9-*t,c*-LOOH (4a)	6.2(1.0)	16.7(0.99)	17.3(0.89)	21.2(0.97)
9-*t,t*-LOOH (5a)	12.7(2.1)	35.2(2.1)	39.4(2.0)	44.5(2.0)
Total	38	104	118	133
c,t/t,t[b]	0.48	0.48	0.45	0.48
$\Delta O_2/(LH)_0$	0.045	0.049	0.059	0.064

[a]By iodometric titration.

[b]*c,t/t,t* = (2a + 4a)/(3a + 5a).

(GLC) with an FON (Wako Pure Chemical Industries; PEG 20M modified by nitroterephthalic acid) column. The total amount of hydroperoxides formed was measured by iodometric titration. Conjugated diene was measured spectrometrically, λ_{max} = 233-236 nm and ϵ = 28,000 $M^{-1}cm^{-1}$ (5). Four hydroperoxides, 2a, 3a, 4a, and 5a, were analyzed as their corresponding alcohol, 2b, 3b, 4b, and 5b, after reduction of the product solution with triphenylphosphine, by high pressure liquid chromatography (HPLC), using a silica gel column and hexane/isopropyl alcohol/acetic acid (1000:10:1, v/v/v) as the eluent. The extinction coefficient for the alcohol was taken from the literature (5).

The rate of initiation was determined from the induction period produced in the presence of radical inhibitor (16,17).

RESULTS AND DISCUSSION

Oxidation of methyl linoleate proceeded smoothly at 50 C and constant rate of oxygen uptake was observed. Under mild conditions and at the initial stage of oxidation, it gave 4 different hydroperoxides, 2a, 3a, 4a, and 5a, quantitatively as observed previously (5-7).

The oxidation of methyl linoleate (LH) initiated by azo compounds proceeds by the mechanism shown below.

Initiation:

$$IN_2I \xrightarrow{k_d, O_2} 2eIO_2\cdot + N_2 \quad [1]$$

$$IO_2\cdot + LH \xrightarrow{O_2} IOOH + LO_2\cdot \quad [2]$$

Propagation:

$$LO_2\cdot + LH \xrightarrow{k_p} LOOH + L\cdot \quad [3]$$

$$L\cdot + O_2 \underset{k_\beta}{\overset{k_\alpha}{\rightleftharpoons}} LO_2\cdot \quad [4]$$

Termination:

$$LO_2\cdot + LO_2\cdot \xrightarrow{k_t} \quad [5]$$

$$LO_2\cdot + L\cdot \xrightarrow{k_t'} \text{nonradical products} \quad [6]$$

$$L\cdot + L\cdot \xrightarrow{k_t''} \quad [7]$$

The rate of initiation is given by

$$R_i = 2ek_d[IN_2I] \quad [8]$$

where e and k_d are the efficiency of radical

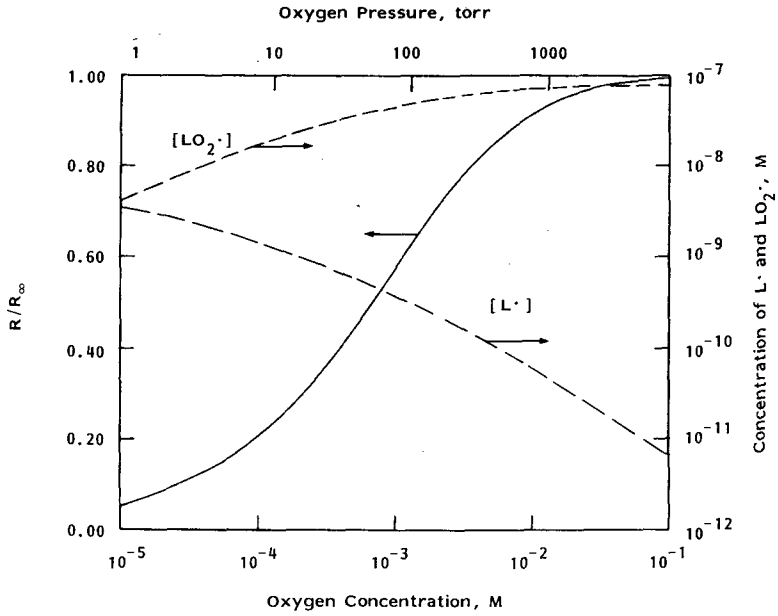


FIG. 1. Calculated ratio of rate of oxidation to limited rate of oxidation (R/R_∞) and calculated concentration of L· and LO_2· at various oxygen concentrations in the oxidation of 0.37 M methyl linoleate with 0.022 M AIBN in acetonitrile at 50 C. $k_0 = 10^8\ M^{-1}s^{-1}$, $k_p = 230\ M^{-1}s^{-1}$, $k_\beta = 800\ s^{-1}$, $k_t = 4.4 \times 10^6\ M^{-1}s^{-1}$, $k_t' = k_t'' = 10^9\ M^{-1}s^{-1}$, $R_i = 5.2 \times 10^{-8}$ M/s.

production and rate constant for decomposition of azo compound, respectively. The equilibrium constant for reaction 4 is given by

$$K = [LO_2\cdot]/[L\cdot]\,[O_2] = k_0/(k_\beta + k_p\,[LH]) \quad [9]$$

and the rate of oxygen uptake (R) in the propagation step is given by

$$R = -dO_2/dt = \frac{k_p(R_i/2k_t)^{1/2}[LH]}{\left(1 + \frac{k_t'}{k_t}\frac{1}{K[O_2]} + \frac{k_t''}{k_t}\frac{1}{K^2[O_2]^2}\right)^{1/2}} \quad [10]$$

When the oxygen pressure is high and $[LO_2\cdot] \gg [L\cdot]$, the rate of oxidation (R_∞) is expressed by a simple equation:

$$R_\infty = k_p\,(R_i/2k_t)^{1/2}[LH] \quad [11]$$

Effect of Oxygen Pressure

Table 1 shows the effect of oxygen pressure on the rate and products of the oxidation of methyl linoleate in acetonitrile at 50 C. Initial oxygen pressure was varied from 90 to 959 torr. The conversion (percent molar ratio of oxygen uptake to initial substrate concentration) was between 4.5 and 6.4%. The kinetic chain length (rate of propagation/rate of initiation) was long. Under these conditions, the oxygen uptake, substrate disappearance, and the amounts of peroxides and conjugated diene formed all agreed satisfactorily well.

The amount of peroxides measured by iodometric titration and that of total peroxides, **2a** + **3a** + **4a** + **5a**, measured by HPLC agreed well. The ratio of 4 hydroperoxides remained constant; **2a**:**3a**:**4a**:**5a** = 1:2:1:2, and *cis,trans*-LOOH/*trans,trans*-LOOH ratio was 0.48.

The rate of oxidation decreased with decreasing oxygen pressure as predicted by equation 10. Figure 1 shows the calculated ratio of rate of oxidation to limited rate of oxidation (R/R_∞) and calculated concentration of L· and LO_2· at various oxygen concentrations. In this calculation, we assume (18) $k_p = 230\ M^{-1}s^{-1}$, $k_0 = 10^8\ M^{-1}s^{-1}$, $k_\beta = 800\ s^{-1}$, $k_t = 4.4 \times 10^6\ M^{-1}s^{-1}$, $k_t' = k_t'' = 10^9\ M^{-1}s^{-1}$, [LH] = 0.37 M, $R_i = 5.2 \times 10^{-8}$ M/s, and $[O_2] = 10^{-2}$ M at atmospheric oxygen pressure. The temperature effect on k_t was assumed to be small (19) and k_t was taken as $4.4 \times 10^6\ M^{-1}s^{-1}$ obtained by Howard and Ingold (4) at 30 C. The k_p was calculated as $230\ M^{-1}s^{-1}$ from $k_p/(2k_t)^{1/2} = 0.078$ (Table 1). The rate constant k_β was calculated as $800\ s^{-1}$ from $k_\beta = 144\ s^{-1}$ (9) at 30 C and $\Delta H^{\neq}_\beta = 17$ kcal/mol (7). Although these rate constants may admittedly involve some

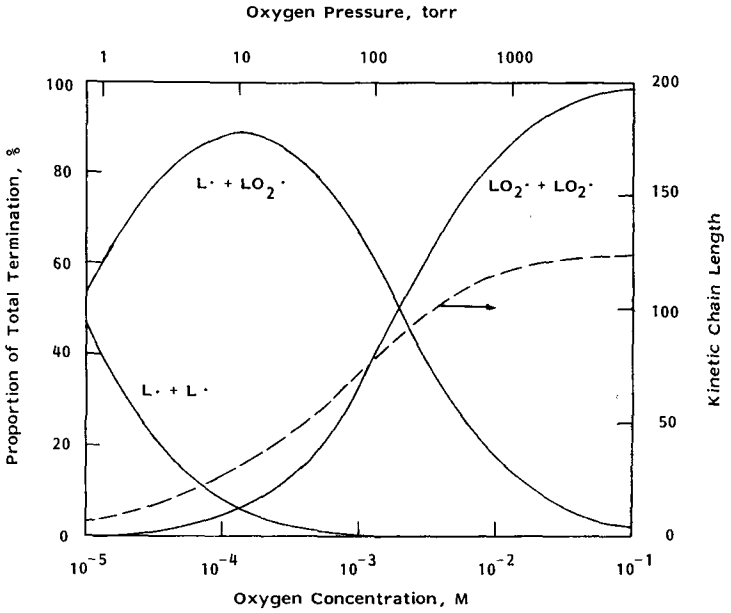


FIG. 2. Calculated relative importance of three termination reactions and kinetic chain length at various oxygen concentrations in the oxidation of 0.37 M methyl linoleate with 0.022 M AIBN in acetonitrile at 50 C. $k_0 = 10^8\ M^{-1}s^{-1}$, $k_p = 230\ M^{-1}s^{-1}$, $k_\beta = 800\ s^{-1}$, $k_t = 4.4 \times 10^6\ M^{-1}s^{-1}$, $k_{t'} = k_{t''} = 10^9\ M^{-1}s^{-1}$, $R_i = 5.2 \times 10^{-8}$ M/s.

uncertainty, Figure 1 clearly shows the general trend of the effect of oxygen concentration on the rate of oxidation.

From equation 9, we obtain $K = 1.1 \times 10^5\ M^{-1}$ and $[LO_2\cdot]/[L\cdot] = 1.5 \times P_{O_2}$, where P_{O_2} is the oxygen partial pressure in torr. The ratio of the rates of reactions 3, 4, and -4 in the propagation step is also calculated as $R_3:R_4:R_{-4} = 1:10.4:9.4$, independent of oxygen pressure. This ratio shows that these 3 reactions have comparable rates. This indicates that the carbon radical L·, formed by the abstraction of doubly allylic hydrogen at the 11 position followed by rearrangement, are stabilized by conjugated double bonds and the concentration of carbon radicals L· is high enough for this radical to participate in termination steps especially at low oxygen pressure. Thus, the rate of oxidation decreases with decreasing oxygen pressure. Figure 2 shows that the relative importance of 3 termination reactions depends on the oxygen pressure and cross-termination (reaction 6) plays an important role under a wide range of oxygen pressure.

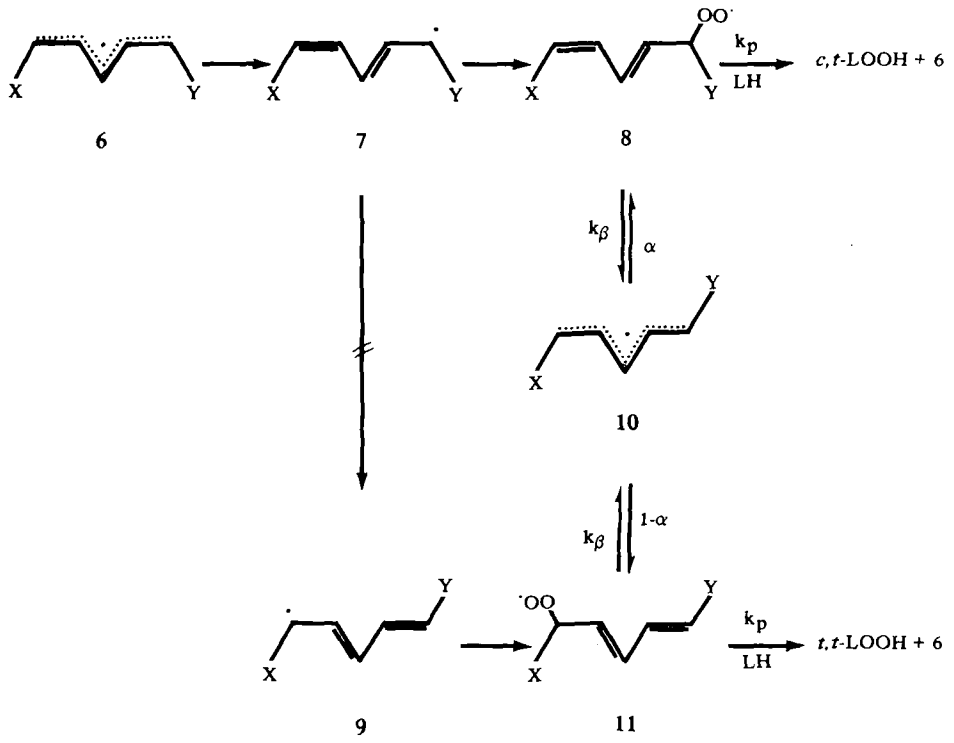
On the other hand, the oxygen pressure had little effect on the products over the oxygen pressure range from 90 to 959 torr. Porter and his coworkers (7) have also found that the product distribution was identical when the oxidation was carried out under 80 or 1000 torr oxygen. The absence of the effect of oxygen pressure on the *cis,trans* to *trans,trans* hydroperoxide ratio suggests that the isomerization of carbon radical (7→9) does not take place, since in such a case, more *trans,trans* product should be formed with decreasing oxygen pressure.

The hydroperoxides, **2a**, **3a**, **4a** and **5a**, were found to be thermally stable at 50 C (Yamamoto, Y., Saeki, N., Niki, E., and Kamiya, Y., unpublished data), and the most plausible precursor of *trans,trans* hydroperoxides is carbon radical **10** formed by the β-scission of peroxy radical 8 (the reverse reaction 4), as proposed originally by Porter et al. (7). They derived equation 12 from the following scheme, where α and 1-α are the ratio of carbon radicals **10** that gives *cis,trans* peroxy radical 8 and *trans, trans* peroxy radical **11**, respectively (Scheme 2). Equation 12 indicates that the ratio of *cis, trans* to *trans,trans* LOOH is independent of oxygen pressure.

$$\frac{cis,trans\ \text{LOOH}}{trans,trans\ \text{LOOH}} = \frac{k_p[LH]}{k_\beta(1-\alpha)} + \frac{\alpha}{(1-\alpha)} \qquad [12]$$

Effect of Substrate Concentration

The results of oxidation at different methyl



X = C_5H_{11}, Y = $C_7H_{14}COOCH_3$ (or X = $C_7H_{14}COOCH_3$, Y = C_5H_{11})

SCHEME 2. Formation of *cis,trans* and *trans,trans* hydroperoxides in the oxidation of methyl linoleate.

linoleate concentrations in acetonitrile are summarized in Table 2. The conversions ranged from 5 to 12%. The oxygen uptake, substrate disappearance, and the amounts of peroxides and conjugated diene formed were in fair agreement. The amounts of peroxides measured by iodometric titration and those of total peroxides measured by HPLC agreed well.

Table 2 shows that the ratio of *cis,trans* to *trans,trans* hydroperoxides increased with increasing concentration of methyl linoleate as expected by equation 12. Porter et al. have also found the same dependence and observed a linear correlation between the ratio of *cis,trans/trans,trans* hydroperoxides and substrate concentration and total hydrogen atom donating ability to the medium (7,8). In contrast, in the present study, the plot of this ratio as a function of methyl linoleate concentration in acetonitrile and benzene shows upward and downward convex curvatures rather than a straight line. The intercept at the y-axis is the same in

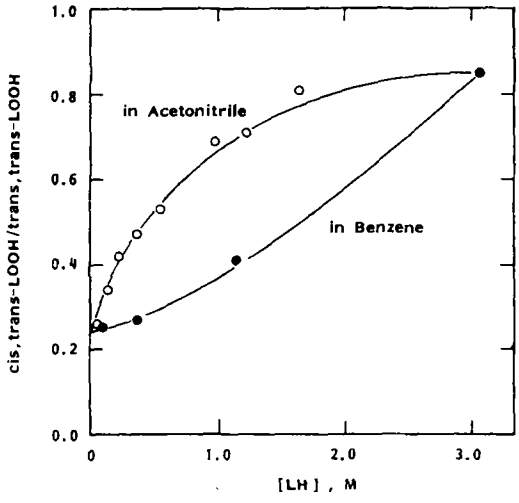


FIG. 3. Ratio of *cis,trans*-LOOH to *trans,trans*-LOOH in the oxidation of methyl linoleate (LH) in acetonitrile and benzene at 50 C.

TABLE 2

Effect of Substrate Concentration in the Oxidation of Methyl Linoleate (LH) in Acetonitrile at 50 C

Run no.	5	6	4	7	8	9
LH, μmol	705	1223	2104	1701	2308	2306
LH, M	0.141	0.226	0.370	0.664	1.22	3.06
AIBN, mM	27.6	22.4	22.7	47.7	52.2	135
Time, min	75	65	60	55	60	75
Initial O_2, μmol	1003.8	1272.5	1175.3	1018.0	1398.8	
Initial O_2, torr	797	1001	959	737	981	∿800
Final O_2, μmol	921.1	1173.3	1041.3	895.8	1236.8	
Final O_2, torr	731	923	850	649	868	
$-dO_2/dt \times 10^6$, M/s	3.25	4.83	6.71	14.3	25.6	35.5
$R_i \times 10^8$, M/s	6.69	5.38	5.39	11.1	11.9	
Kinetic chain length	48.0	89.4	124	128	214	
$k_p/(2k_t)^{1/2}$, $(M \cdot s)^{-1/2}$	0.089	0.092	0.078	0.065	0.061	
ΔO_2, μmol	82.7	99.2	134.0	122.2	162.0	107
ΔLH, μmol	77	86	143	82	170	
$(C=C)_2$, μmol	53	66	110	106	114	
Peroxides[a], μmol	82	75	137	150	124	
Hydroperoxides analyzed by HPLC after reduction with triphenylphosphine, μmol:						
13-*c,t*-LOOH (**2a**)	9.2	12.4	21.9	24.3	29.5	24.6
13-*t,t*-LOOH (**3a**)	27.9	29.4	45.8	38.5	41.0	30.2
9-*t,c*-LOOH (**4a**)	9.9	12.2	21.2	24.7	29.7	24.4
9-*t,t*-LOOH (**5a**)	28.0	28.9	44.5	38.5	42.9	27.3
Total	75	83	133	126	143	107
c,t/t,t[b]	0.34	0.42	0.48	0.64	0.71	0.85
$\Delta O_2/(LH)_0$	0.12	0.081	0.064	0.072	0.070	0.053

[a]By iodometric titration.

[b]*c,t/t,t* = (**2a** + **4a**)/(**3a** + **5a**).

both solvents. These results suggest that the rate constant k_p or $k\beta$ varies with solvent.

Effect of Solvents

Table 3 summarizes the results of oxidation of 0.37 M methyl linoleate in various solvents. The initial oxygen pressure was higher than 850 torr to minimize the effect of oxygen pressure. Again, the oxygen uptake, substrate disappearance, and the amounts of peroxides (measured by both iodometric titration and HPLC) and conjugated diene formed agreed well.

Table 3 shows that the rate of oxidation and the ratio of *cis,trans* to *trans,trans* hydroperoxides increased with an increase in the dielectric constant of the solvent. These results and equations 11 and 12 suggest an increase of the rate constant for propagation, k_p, and/or the decrease of the rate constant for termination, k_t, and/or the decrease of the rate constant for β-scission of peroxy radical, $k\beta$, with an increase of solvent polarity.

It has been observed that the rate of oxidation increases with increasing dielectric constant of the solvent (20-27), and this has been explained by the contribution of dipolar structure at the transition state for the hydrogen atom abstraction reaction as illustrated below.

Thus, the larger the rate constant k_p,

$$LO_2\cdot + LH \rightarrow [LOO{\cdot}H{:}L \longleftrightarrow LOO{\cdot}H{\cdot}L \longleftrightarrow \overset{\delta-}{LOO}{:}\overset{\delta+}{H}{\cdot}L] \rightarrow LOOH + L\cdot$$

the faster the oxidation rate and the more *cis, trans* hydroperoxides should be formed. It is unlikely that k_t decreases with increasing polarity of the medium. It is not clearly known whether or not the solvent polarity has any effect on $k\beta$. If the conjugative delocalization of the unpaired electron of the peroxy radicals occurs and the canonical structure **12** and **13** contributes to the stabilization of peroxy radicals as discussed by Barclay (28), $k\beta$ may decrease with increasing polarity of the medium. We, however, believe that solvents

$$\underset{\mathbf{12}}{R\text{-}\ddot{O}\text{-}\dot{O}} \longleftrightarrow \underset{\mathbf{13}}{R\text{-}\overset{+}{\dot{O}}\text{-}\ddot{O}^{-}}$$

have greater effect on k_p than on k_t and $k\beta$.

The results and discussion given above show that the effects of oxygen pressure, substrate concentration, and solvents on the rate and products of the oxidation of methyl linoleate are interpreted consistently by the mechanism presented originally by Porter and his colleagues (7) where the competition between reactions 3,

TABLE 3

Solvent Effect in the Oxidation of Methyl Linoleate (LH) at 50 C

Run no.	10	11	12	13	14	4
LH, μmol	2127	2078	2051	2077	2142	2104
LH, M	0.373	0.365	0.362	0.366	0.376	0.370
AIBN, mM	25.3	23.6	25.0	24.8	23.3	22.7
Solvent	CCl_4	PhH	PhCl	[c]	tBuOH	CH_3CN
Dielectric constant	2.24	2.28	5.62	9.93	12.47	37.5
Time, min	120	120	120	120	120	60
Initial O_2, μmol	1091.4	1052.4	1187.8	1249.1	1227.1	1175.3
Initial O_2, torr	891	859	968	1019	1002	959
Final O_2, μmol	1004.1	953.9	1054.8	1101.9	1113.1	1041.3
Final O_2, torr	820	778	860	899	906	850
$-dO_2/dt \times 10^6$, M/s	2.03	2.67	3.21	3.66	2.78	6.71
ΔO_2, μmol	87.3	98.5	133.0	147.2	114.0	134.0
ΔLH, μmol						143
$(C{=}C)_2$, μmol	69	81			88	110
Peroxides[a], μmol	86	109	117		107	137
Hydroperoxides analyzed by HPLC after reduction with triphenylphosphine, μmol:						
13-*c,t*-LOOH (**2a**)	9.5	10.7	13.6	16.5	15.1	21.9
13-*t,t*-LOOH (**3a**)	33.9	40.0	49.2	54.3	43.6	45.8
9-*t,c*-LOOH (**4a**)	9.3	10.6	14.0	16.1	13.9	21.2
9-*t,t*-LOOH (**5a**)	34.7	39.9	48.7	53.6	38.1	44.5
Total	87	101	125	140	110	133
c,t/t,t[b]	0.27	0.27	0.28	0.30	0.35	0.48
$\Delta O_2/(LH)_0$	0.041	0.047	0.065	0.071	0.053	0.064

[a]By iodometric titration.

[b]*c,t/t,t* = (**2a** + **4a**)/(**3a** + **5a**).

[c]*o*-Dichlorobenzene.

4, and -4 plays an important role.

ACKNOWLEDGMENT

This work was in part supported by The Toyota Foundation.

REFERENCES

1. Lundberg, W.O. (1962) Autoxidation and Antioxidants, Interscience, New York, NY.
2. Pryor, W.A., ed. (1976) Free Radicals in Biology, Vol. I, Academic Press, New York, NY.
3. Simic, H.G., and Karel, M., eds. (1980) Autoxidation in Food and Biological Systems, Plenum Press, New York, NY.
4. Howard, J.A., and Ingold, K.U. (1967) Can. J. Chem. 45, 793-802.
5. Chan, H.W.S., and Levett, G. (1977) Lipids 12, 99-104.
6. Yamamoto, Y., Niki, E., and Kamiya, Y. (1982) Bull. Chem. Soc. Jpn. 55, 1548-1550.
7. Porter, N.A., Weber, B.A., Weenen, H., and Khan, J.A. (1980) J. Am. Chem. Soc. 102, 5597-5601.
8. Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) J. Am. Chem. Soc. 103, 6447-6455.
9. Pryor, W.A., Stanley, J.P., Blair, E., and Cullen, G.B. (1976) Arch. Environ. Health 31, 201-210.
10. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedom, B.C.L. (1977) Lipids 12, 908-913.
11. Chan, H.W.S., Levett, G., and Matthew, J.A. (1978) J. Chem. Soc. Chem. Commun., 756-757.
12. Chan, H.W.S., Levett, G., and Matthew, J.A. (1979) Chem. Phys. Lipids 24, 245-256.
13. Dumelin, E.E., and Tappel, A.L. (1977) Lipids 12, 894-900.
14. Porter, N.A., Wolf, R.A., and Weenan, H. (1980) Lipids 15, 163-167.
15. Fatemi, S.H., and Hammond, E.G. (1980) Lipids 15, 379-385.
16. Boozer, C.E., Hammond, G.S., and Sen, J.N. (1955) J. Am. Chem. Soc. 77, 3233-3237.
17. Burton, G.W., and Ingold, K.U. (1981) J. Am. Chem. Soc. 103, 6472-6477.
18. Ingold, K.U. (1973) in Free Radicals I (Kochi, J.K., ed.) pp. 37-112, John Wiley & Sons, New York, NY.
19. Howard, J.A. (1972) Adv. Free Radical Chem. 4, 49-173.
20. Zaikov, G.E. (1963) Neftekhimiya 3, 381-389.
21. Zaikov, G.E., and Maizus, Z.K. (1963) Dokl. Akad. Nauk SSSR 150, 116-119.
22. Zaikov, G.E. (1968) Kinet. Katal. 9, 511-515.
23. Hendry, D.G., and Russel, G.A. (1964) J. Am. Chem. Soc. 86, 2368-2371.
24. Howard, J.A., and Ingold, K.U. (1964) Can. J. Chem. 42, 1044-1056.
25. Howard, J.A., and Ingold, K.U. (1964) Can. J. Chem. 42, 1250-1253.
26. Kamiya, Y. (1965) Bull. Chem. Soc. Jpn. 38, 2156-2159.
27. Niki, E., Kamiya, Y., and Ohta, N. (1969) Bull. Chem. Soc. Jpn. 42, 3224-3229.
28. Barclay, L.R.C., and Ingold, K.U. (1981) J. Am. Chem. Soc. 103, 6478-6485.

[Revision received September 9, 1982]

Covalent Binding of Peroxidized Linoleic Acid to Protein and Amino Acids as Models for Lipofuscin Formation

HIROYUKI SHIMASAKI*,[a], NOBUO UETA[a] and O.S. PRIVETT[b], [a]*Department of Biochemistry, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi, Tokyo 173, Japan;* and [b]*The Hormel Institute, University of Minnesota, Austin, MN 55912*

ABSTRACT

The fluorescent substances produced by the reaction of linoleic acid hydroperoxides (LOOH) with ca. 20 different amino acids and bovine serum albumin (BSA) were studied. Only the amino acids, lysine, glycine, arginine, histidine and phenylalanine, gave products with strong fluorescent properties. Products of lysine had a fluorescence intensity of ca. 10 times those of glycine and 100 times those of phenylalanine. The N-acylation of amino acids greatly reduced the fluorescence of the products of the reaction except lysine and arginine. The fluorescence of the products of the reaction of LOOH with N-acetyl BSA was only ca. 25% of the control BSA under the same conditions. It appeared that the substances formed from the reaction of LOOH with BSA were crosslinked polymers as evidenced by column chromatography and polyacrylamide gel electrophoresis. These products were insoluble in common organic solvents and their fluorescent intensities correlated well with the thiobarbituric acid (TBA) test. These observations appear to be highly important in the formation of lipofuscin substances, particularly those associated with the aging pigments which accumulate during aging in mammalian tissues.

Lipids 17:878-883, 1982.

INTRODUCTION

Generally, lipofuscin substances associated with aging in mammalian tissues are extracted with organic solvents and analyzed via the fluorescent properties directly (1,2), after fractionation by column (3,4) or thin layer chromatography (TLC) (5-7). Although the fluorescent substances correlated generally with histologic analysis of the aging pigment, it became apparent from our TLC studies (5-7) and recent studies by high performance liquid chromatography (HPLC) (8) that the organic soluble fluorescent substances extracted from mammalian tissues make up only a small fraction of the aging pigment. It has been well demonstrated that lipofuscin and ceroid pigments consist of a heterogeneous polymeric lipid-protein complex which can be isolated by density gradient ultracentrifugation (9,10). Studies reported here indicate that most of the lipofuscin substances are oxidized fat bound tightly to protein and are insoluble in organic solvents.

MATERIALS AND METHODS

Materials

Linoleic acid was purchased from Nu-Chek Prep, Inc. (Elysian, MN) and checked for purity by TLC and gas liquid chromatography (GLC). LOOH was obtained by the method described by Privett et al. (11) using sodium salt of the linoleic acid with lipoxidase (Sigma Chemical Co., St. Louis, MO). The purified hydroperoxides had a single spot on silica gel TLC and the peroxide value of the preparation was 6200-6150 me/kg, indicating a highly pure product.

N-acetyl-α-lysine, N-acetyl-α-arginine, N-acetyl-α-phenylalanine, N-acetyl-α-histidine, N-acetyl-α-glycine, and BSA were purchased from Sigma Chemical Co. The other L-α-amino acids were obtained from Nippon Rikagaku Co. (Tokyo, Japan). N-acetylimidazole was purchased from Nakarai Co. (Kyoto, Japan), and G-10 and G-200 Sephadex gel were obtained from Pharmacia Fine Chemicals (Sweden). Thiobarbituric acid (TBA) and silica gel thin-layer plates were purchased from Merck (Darmstadt, Germany).

Reaction of LOOH with bovine serum albumin (BSA) and N-acetyl BSA

LOOH in chloroform solution was pipetted into a test tube (1 × 14 cm) and the solvent evaporated in a stream of nitrogen. A 0.5 ml solution of BSA (0.1 M borate buffer pH 7.2) was added to the dry lipid and emulsified by simultaneous vigorous shaking on a vortex mixer for 1 min. The final concentration of LOOH was 2 mM and that of the BSA was 10 mg/ml. The emulsion in the test tube was loosely capped and incubated at 45 ± 2C for periods up to 24 hr.

Acetylation of BSA with N-acetylimidazole was carried out as described by Roosdorp et al.

*Author to whom correspondence should be addressed.

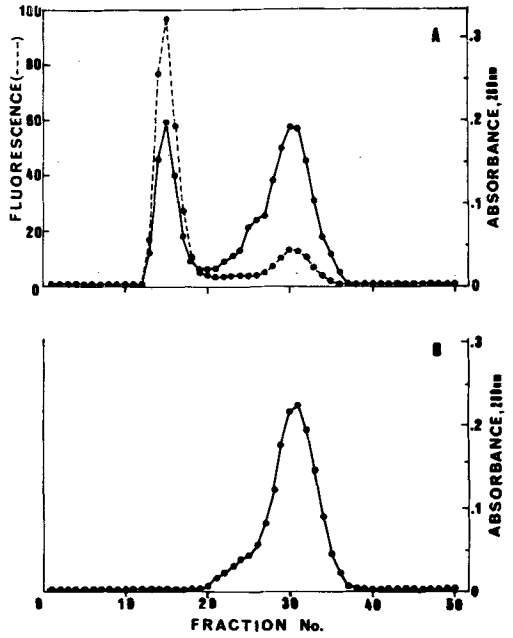


FIG. 1. Sephadex G-200 Gel filtration of (A) BSA-LOOH incubated for 6 hr at 45 C; and (B) BSA as control. The solid line shows relative protein concentration measured by absorbance at 280 nm, and the dotted line shows relative fluorescence intensity of products at 427 nm when excited at 360 nm.

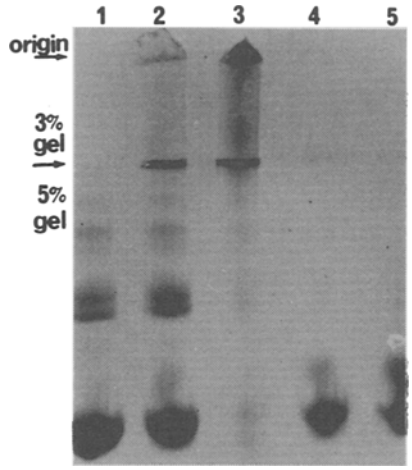


FIG. 2. SDS-polyacrylamide slab gel electrophoresis of BSA-LOOH reaction products. Slots 1 and 5 control BSA before and after Sephadex G-200 gel filtration, respectively; slot 2, BSA-LOOH incubated for 6 hr at 45 C; slot 3, fractions corresponding to the first peak; and slot 4, fractions corresponding to the second peak.

(12); about 95% of the free amino groups were acetylated in the procedure. The emulsion of the LOOH and the acetylated BSA was prepared in the same manner as described for BSA.

Fractionation of Fluorescent Products Binding to BSA by Gel Filtration on Sephadex G-2000

After 6 hr incubation, 0.5 ml of the sample was chromatographed on a 1 x 45 cm column of Sephadex G-200 in a borate buffer, 0.1 M, pH 7.2 containing 0.9% NaCl. Flow rate was 0.09 ml/min and fractions of 0.9 ml were collected for the measurement of fluorescent intensity at 427 nm with excitation at 360 nm. Protein concentration was determined on the eluent by absorption at 280 nm.

Agarose and SDS-polyacrylamide Gel Electrophoresis

The electrophoresis was performed with 1.0% agarose gel in barbiturate buffer at pH 8.6 (μ= 0.05) for 1 hr at 2 mA/cm and stained with 0.2% Ponceau 3R (Atomic Chemicals Corp., Plainview, NY). Sodium dodecyl sulphate (SDS) polyacrylamide slab gel electrophoresis was performed by a slight modification of the method of Maizel (13) as follows. Samples were dialyzed at 4 C against 25 mM Tris-HCl buffer, pH 8.5, containing 0.5% SDS and 0.19 M glycine for 40 hr prior to use, and then the electrophoresis was performed using 5% running gel with the same buffer. The gel was stained with 0.025% Coomassie Brilliant Blue (CBB).

Reaction of LOOH with Amino Acids and N-Acetyl Amino Acids

A mixture of 2 mM LOOH and 0.1 M amino acids in 0.1 M borate buffer, pH 7.2, containing 2 mM SDS was emulsified by mixing on a vortex mixer and incubated at 45 ± 2C for up to 24 hr. The fluorescent products of the emulsion were extracted with 2.0 ml of ethanol/ether (3:1, v/v), by vigorous mixing on a vortex mixer followed by centrifugation for 10 min at 3000 rpm. The fluorescent intensity of the solution was measured at 420 nm with excitation at 360 nm. The emulsion of the LOOH and N-acetyl amino acids was prepared by the same procedure.

Extraction and Analysis of Flurescent Products

Initially, the procedure described previously (14) was used to quantify the fluorescent products which are soluble in the organic solvents in the emulsions of LOOH with BSA or amino acids. The emulsions were extracted with 2.0 ml of ethanol/ether (3:1, v/v) by vigorous mixing on a vortex mixer followed by centrifugation. The fluorescent intensities of 2 ml aqueous solution of the particulate material sedimented

TABLE 1

Extraction and Relative Fluorescent Intensity of Products Formed in the Reaction of LOOH and BSA

Extraction methods[a]	Solvents	Fluorescence maxima		Fluorescent intensity	
		Ex(nm)	Em(nm)	6 hr	24 hr
H_2O	H_2O	360	430	100	154
CM	CM	–	–	0	0
	H_2O	–	–	0	0
CM (ppt)	H_2O	360	430	52	83
EE	EE	–	–	0	0
	H_2O	–	–	108	152
DMP	DMP	–	–	0	0
	H_2O	–	–	0	0
DMP (ppt)	H_2O	360	430	83	132

[a]CM = chloroform/methanol (2:1, v/v); EE = ethanol/ether (3:1, v/v); DMP = dimethoxypropane; ppt = sediment obtained by ultracentrifugation.

in the bottom of the tube were measured with a Hitachi 204 fluorospectrophotometer. In addition to this method, the procedures of Fletcher et al. (2) and Shimasaki et al. (7) were used for measuring the fluorescence of organic and aqueous solutions prepared in the same manner. The fluorescence measurements were determined relative to a quinine sulfate standard (1 μg/ml of 0.1 N H_2SO_4) with a relative fluorescence intensity of 24.

TBA-test of Substances Binding to BSA

TBA-reactive substances in the emulsion were extracted with ethanol/ether (3:1, v/v) solution by mixing on a vortex mixer followed by centrifugation, and dissolved in 2.0 ml of distilled water after removal of the solvents. The protein moiety which sedimented by the centrifugation was also dissolved in 2.0 ml of water. These solutions were added to 1.0 ml of 0.67% TBA solution containing 50% acetic acid in water and heated for 20 min in a boiling water bath. TBA values were determined by the absorbance at 535 nm (14) and the fluorescence intensity of 550 nm with excitation at 515 nm (15).

RESULTS

The chromatography and elution pattern of the albumin treated with LOOH and untreated albumin in Sephadex G-200 are shown in Figures 1A and 1B, respectively. Although there were some reaction products corresponding to the albumin peak, the major products of the reaction of BSA with LOOH were fluorescent and the fluorescence corresponded to the protein peak separated from albumin as shown in Figure 1A. The fluorescent maxima in the separated fraction had an emission maximum at 427 nm and an excitation maximum at 360 nm. Separate gel filtration of albumin on Sephadex G-200 showed that it was eluted in a single fraction that had no fluorescence. Thus, it appeared that fluorescent products were formed in the reaction of LOOH with albumin.

The reaction of LOOH with albumin appeared to increase the size of the protein as indicated by the elution pattern on the Sephadex chromatography (Fig. 1A) and by SDS-polyacrylamide gel electrophoresis shown as in Figure 2. In the latter analysis, fractions corresponding to the first peak did not move into the 5% gel, which indicated that its molecular size was greater than that of unreacted albumin, fractions 30 and 31. No fluorescent products were extractable from LOOH treated albumin with organic solvents, chloroform/methanol (2:1, v/v), ether/ethanol (1:3, v/v), absolute ethanol or dimethoxypropane (Table 1). On the other hand, the aqueous solution of the sediments obtained by centrifugation were highly fluorescent exhibiting emission and excitation maxima at 430 and 360 nm, respectively. A comparison of the fluorescent intensities of aqueous and ethanol extracts is shown in Figure 3. These experiments indicate that the fluorescent substances were covalently bound in the reaction of LOOH with albumin. Some water soluble fluorescent material with an emission maxima of ca. 300 nm was also present in the LOOH treated albumin as shown in Figure 3. This material was unaffected by acetylation (Fig. 4) and because its fluorescence maximum is not in the range of that generally considered characteristic of lipofuscin substances, it does not appear to be a primary product of the reaction.

In order to obtain a further insight into the

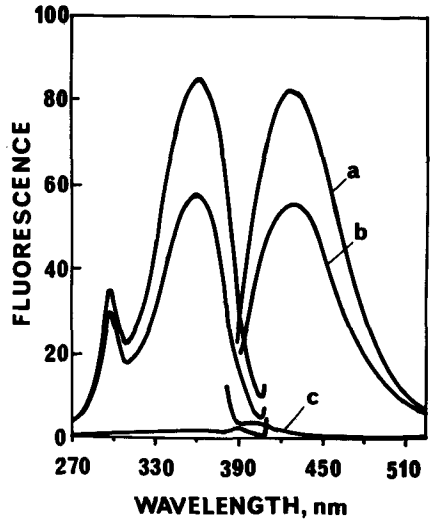


FIG. 3. Fluorescence spectra of the water soluble fluorescent products formed during reaction of BSA and LOOH. (a) 24 hr incubation; (b) 6 hr incubation; (c) absolute ehtanol extracts from 6 and 24 hr BSA-LOOH reaction products.

reaction, the fluorescent products of the reaction of LOOH with various amino acids were studied. LOOH (1 mM) was incubated with 100 mM of a number of amino acids at 45 ± 2 C for periods of 6-24 hr, the reaction mixture was extracted with ethanol and the relative fluorescence of the extracts determined (Table 2). The reaction of LOOH with lysine gave the strongest fluorescence. The fluorescence given by the other amino acids was relatively weak, and varied in the order histidine > glycine > arginine > phenylalanine. No fluorescent products were produced with the N-α-acetyl amino acids except lysine, which has a free amino group in the ϵ-position. Reactions of LOOH with N-α-acetyl-L-arginine, which has a guanido group, gave a weakly fluorescent product.

When BSA was acetylated, the fluorescent intensity of the products was reduced to less than 25% of the control (Fig. 4). These observations indicated that the fluorescent products of the reaction of LOOH with albumin were mainly with the ϵ-amino group of the lysine moieties.

Yagi (15) observed that, in general, TBA color pigments are also fluorescent with an emission maximum at 550 nm and an excitation maximum at 515 nm. However, the total incubation mixture of LOOH with BSA gave only a weak positive TBA color reaction. When the reaction mixture was separated into ethanol/ether (3:1, v/v) soluble and sedimented protein, the soluble fraction gave a positive TBA color reaction, but this fraction did not give any fluorescence characteristic of lipofuscin substances.

TABLE 2

Relative Fluorescent Intensity of LOOH-Amino Acid Reaction Products

	Incubation time (45 C)					
	6 hr			24 hr		
Amino acids	Ex	Em	Fluorescent intensity	Ex	Em	Fluorescent intensity
Glycine	360	435	100	360	435	240
Alanine	335	430	24	355	430	35
Arginine	335	415	72	330	410	104
Asparagine	355	435	15	360	430	54
Aspartic acid	350	(430)	2	360	(430)	3
Cysteine	350	(430)	12	350	(430)	18
Glutamine	355	430	17	355	430	36
Glutamic acid	350	(430)	4	350	(430)	6
Histidine	338	410	228	338	420	324
Isoleucine	345	420	13	345	425	20
Leucine	355	430	16	355	430	25
Lysine	360	430	1008	360	430	1184
Methionine	350	420	9	350	420	15
Phenylalanine	365	430	78	365	335	91
Proline	350	450	5	350	450	6
Serine	340	410	35	335	405	95
Threonine	340	410	37	335	405	93
Tyrosine	330	420	13	330	410	21
Valine	340	420	13	340	420	18

( ) = Fluorescene maximum not well defined.

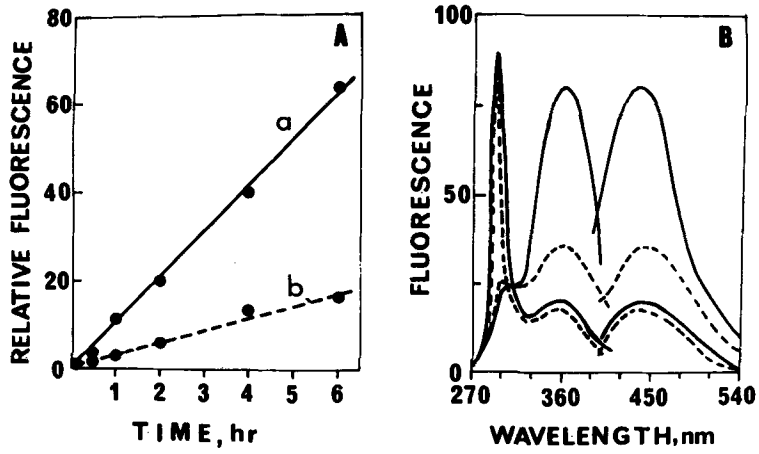


FIG. 4. A. Formation of fluorescent products as a function of time. (a) LOOH with BSA; (b) LOOH with acetylated-BSA. B. Fluorescence spectra of aqueous extracts from LOOH-BSA (——), and LOOH-acetylated BSA (-----) reaction mixture at 0 and 6 hr.

On the other hand, not only was the sedimented fraction fluorescent, but the products formed with TBA also were fluorescent with an excitation maximum of 515 nm and an emission maximum at 550 nm. These observations indicated that TBA reacted with products in both the ethanol/ether extract and the sedimented protein, giving fluorescent substances with the latter. A plot of the fluorescence of the protein sedimented fraction and that of the products of the TBA reaction of this fraction gave a linear relationship (Fig. 5) indicating that the same groups were responsible for the fluorescence in both reactions.

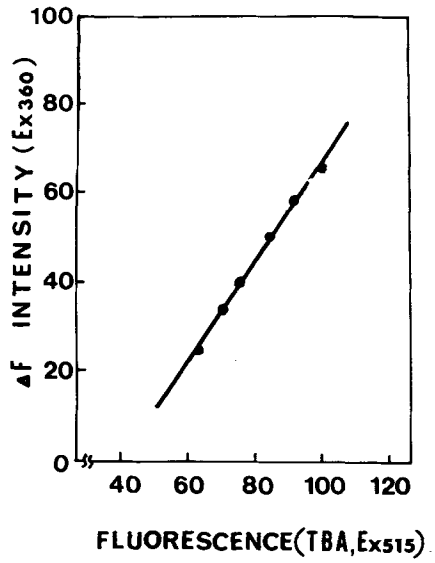


FIG. 5. Relationship between fluorescent products and TBA-reactive fluorescent substances binding to BSA.

DISCUSSION

Most methods for the determination of lipofuscin substances in mammalian tissues are based on the extraction of the fluorescent compounds in organic solvents and the measurement of their fluorescence directly or after separation by various types of chromatography (3). The fluorescent material in the aqueous phase of the lipid extracts is generally disregarded. In this study, we rationalize that if the lipofuscin substances are lipid-protein complexes, as generally suggested, they might be water soluble. This view was further strengthened by the fact that in recent work (5) we observed that the mass of the fluorescent material recovered in organic extracts was very small, relative to that which might be expected from histologic analysis. The present study shows, indeed, that fluorescent compounds are formed by the reaction of LOOH and protein (albumin) and that these substances are insoluble in organic solvents but soluble in water. Studies on the Sephadex chromatography of albumin after reaction with LOOH showed that it contained a component of high molecular mass and that the water soluble fluorescent substances were concentrated in it. Polyacrylamide electrophoresis of the products of the reaction of albumin with LOOH also showed that unaltered albumin was gradually

replaced by substances with a higher molecular mass. Thus, it appeared that LOOH reacted with albumin to give a covalently bound product with strong fluorescent properties. Studies on the reaction of LOOH with amino acids and albumin before and after acetylation indicated that LOOH reacted with the free amino groups in the protein. Moreover, studies of the fluorescent properties of the products of the reaction with individual amino acids indicated that the reaction with albumin occurred primarily with the lysine moieties.

The experiments with TBA showed that, although this reagent gave a positive fluorescent test with the protein fraction which correlated well with the fluorescence of this fraction, it was not specific for lipofuscin substances in as much as it also gave a positive absorption test at 550 nm on the alcohol/ether extracts which exhibited no fluorescence characteristics of lipofuscin substances.

Although the present study was carried out on model compounds, the reaction appears to be relevant to the formation of lipofuscin substances and the aging pigment in mammalian tissues.

ACKNOWLEDGMENTS

This work was supported in part by US Public Health Service Grants AG-00174 from the National Institute on Aging; HL-08214 from the Program Project Branch, Extramural Programs, National Heart, Lung and Blood Institute, and by The Hormel Foundation.

REFERENCES

1. Tappel, A.L. (1973) Fed. Proc. 32, 1870-1874.
2. Fletcher, B.L., Dillard, C.J., and Tappel, A.L. (1973) Anal. Biochem. 52, 1-9.
3. Csallany, A.S., and Ayaz, K.L. (1976) Lipids 11, 412-417.
4. Taubold, R.D., Siakotos, A.N., and Perkins, E.G. (1975) Lipids 10, 383-390.
5. Shimasaki, H., Nozawa, T., Privett, O.S., and Anderson, W.R. (1977) Arch. Biochem. Biophys. 183, 443-451.
6. Shimasaki, H., Privett, O.S., and Keane, W.F. (1978) Radiat. Res. 74, 393-404.
7. Shimasaki, H., Ueta, N., and Privett, O.S. (1980) Lipids 15, 236-241.
8. Phillips, F.C., and Privett, O.S. (1981) J. Am. Oil Chem. Soc. 58, 590-594.
9. Siakotos, A.N., Watanabe, I., Saito, A., and Fleisher, S. (1970) Biochem. Med. 4, 361-375.
10. Siakotos, A.N., Goebel, H.H., Patel, V., Watanabe, I., and Zeman, W. (1972) in Advances in Experimental Medicine and Biology (Volk, B.W., and Aronson, S.M., eds.) Vol. 19, pp. 53-61, Pergamon Press, New York, NY.
11. Privett, O.S., Lundberg, W.O., and Nickell, E.C. (1953) J. Am. Oil Chem. Soc. 30, 17-21.
12. Roosdorp, N., Wann, B., and Sjoholm, I. (1977) J. Biol. Chem. 252, 3876-3880.
13. Maizel, J.V. (1971) in Methods in Virology (Maremorosch, K., and Koprowsky, H., eds.) Vol. 5, p. 188, Academic Press, New York, NY.
14. Shimasaki, H., Privett, O.S., and Hara, I. (1977) J. Am. Oil Chem. Soc. 54, 119-123.
15. Yagi, K. (1976) Biochem. Med. 15, 212-216.

[Received February 12, 1982]

Source of Lung Surfactant Phospholipids: Comparison of Palmitate and Acetate as Precursors

TADAHIRO SATO and **TOYOAKI AKINO***, *Department of Biochemistry, Sapporo Medical College, Sapporo 060, Japan*

ABSTRACT

The phospholipids and the fatty acid compositions of major phospholipids in rat lung parenchyma, microsomes, lamellar bodies and alveolar wash were quantified. Adult rats were injected simultaneously with [^{3}H]palmitate and [^{14}C]acetate into the femoral vein. The appearance of labeled phosphatidylcholine (PC), disaturated phosphatidylcholine (DSPC) and phosphatidylglycerol (PG) in each lung fraction was measured during short periods of time (5 min to 2 hr) after isotope administration. Relatively more PC, DSPC and PG labeled with acetate radioactivity in lung microsomes entered lamellar body and alveolar wash fractions than those labeled with palmitate radioactivity. However, there was no difference between palmitate and acetate labeled phospholipids in the transport from microsomes to lamellar bodies by phospholipid exchange proteins. On the other hand, prior injection of colchicine resulted in decrease in the transport of PC from microsomes to alveolar space to a relatively greater extent in the acetate radioactivity than in the palmitate radioactivity.

Lipids 17:884-892, 1982.

INTRODUCTION

1,2-Dipalmitoyl species of phosphatidylcholine (PC) and phosphatidylglycerol (PG) are the main constituents of lung surfactant (1), which has been implicated in the maintenance of lung compliance (2). In order to supply sufficient amounts of palmitate for the synthesis of these dipalmitoyl species, the lung tissue actively takes up palmitate from the circulation and synthesizes palmitate endogenously de novo. Labeled palmitate administrated intravenously is rapidly taken up by the lung and incorporated preferentially into phospholipids, particularly into PC (3-5). On the other hand, lung slice experiments with labeled acetate have shown that palmitate accounts for greater than 80% of the radioactivity incorporated into phospholipids (6). Namely, the major product of the de novo fatty acid synthesis from acetate appears to be palmitate (7,8). This notable characteristic in the de novo fatty acid synthesis in the lung permits a comparison of the endogenously synthesized palmitate as the acetate radioactivity and the exogenously supplied palmitate as palmitate radioactivity in the metabolism of lung phospholipids. Studies on their relative differences revealed that they were metabolized differently in the transfer into alveolar space (9,10), as membrane bound substrate for microsomal phospholipase A_2 (11), or in the synthesis of dipalmitoyl PC by the effect of essential fatty acid deficiency (12). Jobe (9) demonstrated that the relative specific activities of surfactant PC and DSPC labeled with acetate radioactivity were ca. twice those measured using the palmitate radioactivity.

The present study was made to confirm the preferential appearance of palmitate synthesized de novo in the surfactant phospholipids, and further to study possible differences between the endogenously synthesized and exogenously supplied palmitate in the transfer mechanisms of phospholipids from microsomes to the surfactant related fractions.

MATERIALS AND METHODS

Materials

[9,10-^{3}H]Palmitic acid (sp act, 500 mCi/mmol), [1-^{14}C]palmitic acid (sp act, 50 mCi/mmol), [1-^{14}C]acetic acid (sp act, 60 mCi/mmol) and [^{3}H]acetic acid (sp act, 300 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, England. The labeled palmitic acids were complexed with fatty acid-free bovine serum albumin (Sigma Co.) according to the method described by Åkesson et al. (13).

In vivo Experiments

Male Wistar rats weighing ca. 250 g were fasted for 16 hr before isotope injection. 0.25 ml of 4% albumin-0.9% saline solution containing 50 μCi (26 μg) of [9,10-^{3}H]palmitic acid and 200 μCi (50 μg) of [1-^{14}C]acetic acid were injected within 5 sec into the femoral vein of the rats under slight ether anesthesia. Some rats were pretreated by the intraperitoneal injection of colchicine (10 mg) in 0.9% saline (0.5 ml)

*Author to whom correspondence should be sent.

TABLE 1

Phospholipid Composition of Rat Lung Fractions

	Parenchyma (n=5)	Microsomes (n=5)	Lamellar bodies (n=4)	Alveolar wash (n=4)
Total phospholipid content (μmol/g wet tissue)	18.1 ± 2.5	1.93 ± 0.6	0.23 ± 0.04	1.16 ± 0.3
Phosphatidylcholine	45.5 ± 4.4	41.7 ± 5.2	74.2 ± 4.5	81.7 ± 2.0
Phosphatidylethanolamine	25.6 ± 2.8	23.1 ± 2.1	11.5 ± 1.7	4.4 ± 1.0
Phosphatidylglycerol	2.0 ± 0.3	4.6 ± 4.7	8.5 ± 0.2	9.4 ± 0.9
Phosphatidylinositol	9.0 ± 1.9	9.6 ± 1.6	0.6 ± 0.8	1.6 ± 0.7
Phosphatidylserine	2.6 ± 1.5	3.6 ± 2.4	1.5 ± 1.7	
Sphingomyelin	13.1 ± 3.4	12.3 ± 2.1	3.0 ± 0.6	1.9 ± 0.9
Lysophosphatidylcholine	1.6 ± 1.3	3.2 ± 0.1	0.6 ± 1.0	0.2 ± 0.3
Lysophosphatidylethanolamine	0.2 ± 0.2	1.8 ± 1.5	tr	tr
Lyso-*bis*-phosphatidic acid	0.4 ± 0.2	0.1 ± 0.1	0.1 ± 0.6	0.8 ± 0.8

The results are presented as mean percent composition ± standard deviation (n).

according to the description by Delhunty and Johnston (14). After 3 hr, the isotopes were injected into the animals in the same manner as above. After different time intervals, the rats were killed by bleeding through the abdominal aorta. The trachea was opened in the neck and cannulated with a polyethylene tube connected to a syringe. The lungs were washed 4 times with the same 5 ml of 0.9% saline. The effluent volume recovered was about 14 ml. The alveolar wash was not centrifuged to remove cellular materials, as in the method for rabbit lung alveolar wash described by Jobe (15).

Isolation of Rat Lung Fractions

The isolation procedure for rat lung fractions was carried out generally according to the method described for rabbit lung fractions by Jobe (15). The recovery of phospholipid in the lamellar body fraction and the microsomes isolated from rat lung by this procedure was 1.3% and 10.7% of the total lung phospholipid. The ratio of phospholipid (μmol) to protein (mg) was 0.33 ± 0.6 for the microsomes and 2.15 ± 0.52 for the lamellar body fraction.

Phospholipid-transfer Experiments

Labeled microsomes from rat lung were prepared by lung slice experiments with [1-^{14}C] palmitic acid and [^{3}H]acetic acid; tissue slices from two rat lungs (2.4 g) were incubated at 37 C in 20 ml of Krebs-Ringer medium containing [1-^{14}C]palmitic acid (500 μ Ci, 2.6 mg) complexed with bovine serum albumin or [^{3}H] acetic acid (5 mCi, 1.0 mg). The lung slices were removed after 1.5 hr incubation, rinsed in cold 0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl (pH 7.4) (buffer A), and the microsomes were isolated as mentioned above except for the use of buffer A. The isolation procedures for unlabeled mitochondria and lamellar bodies from rat lung, and the assay procedures for phospholipid-transfer activity were essentially the same as described by Engle et al. (16). The 100,000 × g supernatant fractions, isolated as described by Vereyken et al. (17), were adjusted to pH 5.1 with 3 M HCl. After standing in ice for 30 min with occasional stirring, the suspensions were centrifuged at 15,000 × g for 15 min. The supernatants were then adjusted to pH 7.4 with solid Tris. In this study, they were designated as pH 5.1 supernatant, which were used as phospholipid exchange proteins in phospholipid-transfer experiments.

Lipid Analysis

Analytical procedures for lipids were generally the same as described in our previous paper (12,18,19). Lipids of the lung parenchyma, microsomes, lamellar bodies and alveolar wash were extracted by the method of Bligh and Dyer (20), after which they were subjected to two-dimensional thin layer chromatography (TLC) to separate individual lipid classes (21). For analysis of phospholipid composition, the spots on the plates were detected by charring and analyzed for phosphorus. The spots were also detected by fluorescein spray and each phospholipid was recovered from the gel by the method of Arvidson (22). PC isolated was converted to 1,2-diacyl-3-acetylglycerol and separated into molecular classes using the procedures described by Okano et al. (19). The PC was also subjected to permanganate/periodate oxidation to isolate desaturated phosphatidylcholine species according to the method of Shimojo et al.

TABLE 2

Fatty Acid Composition of Phosphatidylcholine and Phosphatidylglycerol from Rat Lung Fractions

	Phosphatidylcholine				Phosphatidylglycerol		
	Parenchyma (n=5)	Microsomes (n=5)	Lamellar bodies	Alveolar wash (n=5)	Parenchyma	Microsomes	Alveolar wash
Total							
14:0	1.3 ± 0.3	1.1 ± 0.2	2.2	2.3 ± 0.2	0.6	0.4	2.4
16:0	51.8 ± 2.1	49.5 ± 1.2	65.4	66.1 ± 2.2	46.5	38.7	57.2
16:1	3.7 ± 1.5	4.0 ± 1.7	6.5	10.1 ± 0.6	2.3	2.8	8.9
18:0	9.5 ± 0.4	10.2 ± 0.7	4.0	3.2 ± 0.5	7.0	8.4	4.0
19:1	13.4 ± 1.6	13.1 ± 0.5	7.4	6.9 ± 1.0	18.4	20.1	12.3
18:2	8.8 ± 0.8	9.1 ± 0.5	8.5	7.4 ± 1.2	15.3	17.8	7.2
20:3	0.3 ± 0.1	0.4 ± 0.1	0.5	tr	tr	tr	tr
20:4	7.1 ± 1.0	8.3 ± 0.9	4.7	3.1 ± 0.8	5.1	6.0	3.3
20:5	0.5 ± 0.1	0.6 ± 0.05	0.3	0.4 ± 0.2	0.3	tr	tr
22:5	0.8 ± 0.1	0.9 ± 0.1	tr	tr	0.7	0.8	tr
22:6	1.0 ± 0.2	1.0 ± 0.2	0.4	tr	2.2	2.8	1.1
Others	1.8 ± 0.5	1.8 ± 0.5	0.1	0.5 ± 0.2	1.6	2.2	3.6
2-Position							
14:0	1.2 ± 0.4	0.9 ± 0.3	2.9	3.1 ± 0.6	tr	–	–
16:0	34.8 ± 4.1	32.3 ± 2.3	45.5	49.0 ± 4.3	24.5	–	–
16:1	6.3 ± 2.9	9.0 ± 0.7	12.2	18.7 ± 0.9	4.1	–	–
18:0	2.0 ± 0.5	1.7 ± 0.2	1.5	0.3 ± 0.2	tr	–	–
18:1	17.2 ± 6.7	18.0 ± 1.4	11.4	8.5 ± 1.1	22.6	–	–
18:2	15.0 ± 1.2	15.0 ± 0.01	13.6	12.9 ± 2.4	29.0	–	–
20:3	0.6 ± 0.3	0.6 ± 0.4	1.0	tr	0.4	–	–
20:4	14.3 ± 2.0	15.8 ± 1.1	9.3	6.2 ± 1.7	10.2	–	–
20:5	0.9 ± 0.3	1.2 ± 0.1	0.6	0.8 ± 0.8	1.4	–	–
22:5	1.5 ± 0.3	1.8 ± 0.2	tr	tr	0.6	–	–
22:6	1.9 ± 0.3	1.9 ± 0.5	0.7	0.1 ± 0.1	4.4	–	–
Others	3.1 ± 0.8	2.8 ± 0.9	1.3	1.3 ± 0.8	2.8	–	–

The results are presented as mean percent composition ± standard deviation (n). Where no standard error is given, the values were obtained from single analyses of samples pooled from 8 rats. tr, trace amounts; –, not determined.

(23). The positional distribution of the fatty acids of PC and PG was analyzed by hydrolysis with phospholipase A_2 (*Crotalus adamanteus* and *Crotalus atrox*). The free fatty acids and lysophospholipids prepared were separated by TLC. The fatty acid methyl esters prepared by BF_3/CH_3OH (24) were analyzed by gas liquid chromatography (GLC).

A part of the labeled phospholipids from lung parenchyma was transmethylated by BF_3/CH_3-OH (24). The fatty acid methylesters prepared were separated into saturates, monoenes, dienes and polyenes by argentation TLC (25). The saturates were further separated according to the carbon numbers by reverse-phase TLC (25).

Protein was determined by the method of Lowry et al. (26). Phosphorus was determined by the method of Bartlett (27). The amount of 1,2-diacyl-3-acetylglycerol was estimated by glycerol determination according to the method of Van Handel and Zilversmit (28). Radioactivity counting was carried out with a Packard Liquid scintillation spectrometer using a toluene based scintillator as described by Snyder (29), and Aquasol (New England Nuclear) when determined with silica gel.

RESULTS

The phospholipid composition of four lung fractions, lung parenchyma, microsomes, lamellar bodies and alveolar wash, are presented in Table 1. As noted earlier (30,31), the phospholipid profiles of the lung parenchyma and microsomes were quite similar, but significantly different from those of the lamellar bodies and alveolar wash. Compared to the phospholipid profiles in microsomes, PC and PG increased approximately 2-fold in the surfactant related fractions, e.g., lamellar bodies and alveolar wash. In contrast, sphingomyelin and phosphatidylethanolamine (PE) decreased significantly in the surfactant related fractions. The fatty acid compositions of the major phospholipids, i.e., PC and PG, in the surfactant related fractions are given in Table 2. In these 2 phospholipids, palmitic acid was the major fatty acid, not only in the total but also in the 2-position. Its relative concentration increased in the order of microsomes, lamellar bodies and alveolar wash. Concomitant decreases in the relative concentration of oleic and arachidonic acids were also found in the supernatant related fractions.

Table 3 provides a comparison of the proportion of the molecular classes of PC from the 4 lung fractions. Consistent with the findings on their fatty acid patterns, significant increases were found in the disaturated classes in the surfactant related fractions and, conversely, de-

TABLE 3

Composition of Molecular Species of Phosphatidylcholine and Phosphatidylglycerol from Rat Lung Fractions

	Phosphatidylcholine				Phosphatidylglycerol	
	Parenchyma (n=5)	Microsomes (n=5)	Lamellar bodies (n=4)	Alveolar wash (n=4)	Parenchyma	Alveolar wash
Pool size (μmol/g wet tissue)	8.25 ± 1.2	0.78 ± 0.2	0.17 ± 0.03	0.95 ± 0.2	0.36 ± 0.05	0.11 ± 0.03
Saturated	34.3 ± 4.7	29.9 ± 0.8	52.8 ± 0.9	54.2 ± 5.5	22.3	36.4
Monoenoic	21.5 ± 3.8	26.1 ± 5.6	27.1 ± 0.2	26.3 ± 3.6	28.5	35.6
Dienoic	20.6 ± 3.9	19.7 ± 4.2	11.2 ± 0.6	11.8 ± 1.2	21.2	18.5
Trienoic	3.8 ± 0.7	3.9 ± 1.0	2.4 ± 0.6	2.0 ± 0.3	7.7	2.5
Tetraenoic	16.5 ± 1.8	17.1 ± 4.8	4.3 ± 1.8	4.0 ± 1.1	11.1	5.3
Polyenoic	3.2 ± 1.4	3.3 ± 1.3	2.2 ± 1.6	1.6 ± 0.4	7.3	1.7

The results are presented as mean percent composition ± standard deviation (n). Where no standard error is given, the values were obtained from duplicate analyses of samples pooled from 8 rats.

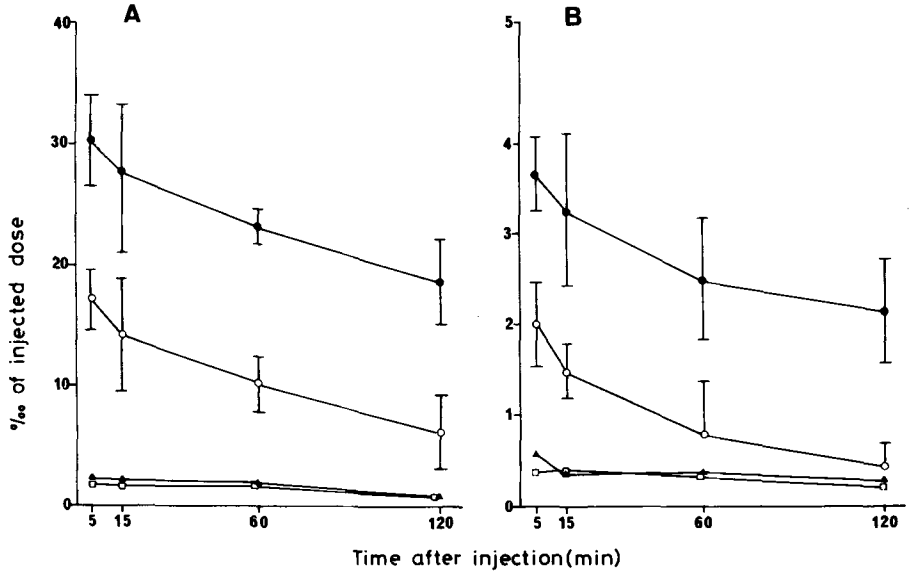


FIG. 1. Incorporation of [9,10-^{3}H]palmitic acid (A) and [1-^{14}C]acetic acid (B) into each lipid clss of rat lung parenchyma after intrafemoral injection. For phosphatidylcholine and triacylglycerol, the data are given as the average values ± SD for 3 independent experiments. The range of deviation was omitted in other fractions. •, phosphatidylcholine; ○, triacylglycerol; ▲, phosphatidylglycerol; □, phosphatidylethanolamine.

creases were observed in the dienoic and tetraenoic classes. Our analytical results were essentially in agreement with those reported in rabbits (31) and rats (30).

In order to elucidate the source of palmitate acylating the main phospholipids in the surfactant related fractions, a comparison of the labeling profiles was made using [9,10-^{3}H]palmitate and [1-^{14}C]acetate as the sources of surfactant phospholipids. Following the injection of the above-mentioned isotopes, the radioactivity distribution among fatty acids acylating in the total phospholipids from lung parenchyma was determined. Ten min after injection, ^{14}C-activity was 85.4% for palmitate and 6.0% for unsaturated acids. There was little change in these percentages during the experimental time periods, which seems to indicate that palmitate was primarily synthesized from the acetate radioactivity in the lung tissue. On the other hand, ca. 95% of the ^{3}H-activity was recovered as palmitate.

Figure 1 shows the appearance of [^{3}H]palmitate and [^{14}C]acetate in various lipid classes of rat lung parenchyma during short periods of time after the simultaneous injection of both radioactivities. The labeling profiles of lipid classes were similar for both radioactivities. The ^{3}H and ^{14}C activities in lipid classes rapidly decreased from 5 min to 120 min after the injection. Among lipid classes, PC was predominantly labeled with both precursors. These results indicate that both radioactivities incorporated into lung lipids with similar kinetics.

Figure 2 shows the time-dependent changes in specific radioactivities of PC, DSPC and PG in 3 lung fractions after injection of [^{3}H]palmitate and [^{14}C]acetate. In order to compare labeling profiles of both precursors, the specific radioactivities of these phospholipids are shown as specific activities relative to the unity of the specific radioactivities of microsomal PC at 60 min after injection of each isotope. The specific activities in lung microsomes were highest at the initial time point of 5 min after the injection, after which they rapidly decreased. The appearance in the lamellar body fraction of the labeled phospholipids was also rapid. Maximal specific activities were achieved within 1 hr after injection. The specific activities of these phospholipids in lamellar bodies exceeded those in microsomes 2 hr after injection. This seems to indicate that the microsomal subpool of phospholipids destined for the lamellar bodies may exist in the lung. The labeled phospholipids appeared in the alveolar space in a linear fashion for a period of 2 hr, although their specific activities were still lower compared to those in lamellar bodies. These labeling profiles were similar in both precursors. It should, however,

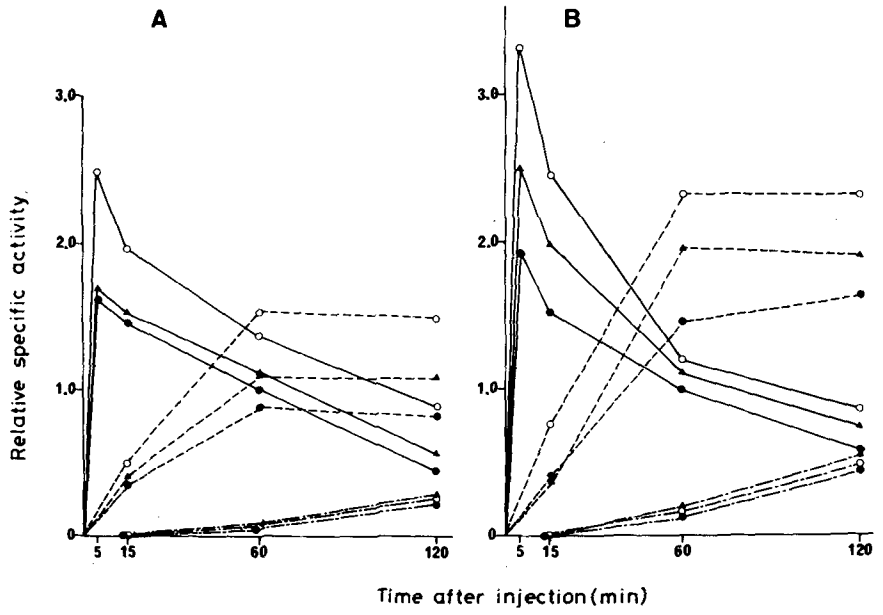


FIG. 2. Changes of specific radioactivity of phosphatidylcholine, disaturated phosphatidylcholine and phosphatidylglycerol in microsomes, lamellar bodies and alveolar wash of rat lung after intrafemoral injection of [9,10-^{3}H]palmitic acid (A) and [1-^{14}C]acetic acid (B). The data are given as relative specific activity calculated as unity of the specific radioactivity of microsomal phosphatidylcholine at 60 min after injection of each isotope, which was 21.6 ± 1.7 dpm/nmol for ^{3}H-activity and 9.3 ± 0.8 dpm/nmol for ^{14}C-activity. Each mark is the average value from 2 to 3 animals. The range of deviation was omitted for technical reasons. —— line, microsomes; ------ line, lamellar bodies; - - - -line, alveolar wash. •, phosphatidylcholine; ○, disaturated phosphatidylcholine; ▲, phosphatidylglycerol.

be noted that the relative specific activities of these phospholipids in the surfactant related fractions were significantly higher in the acetate radioactivity than those in the palmitate radioactivity.

In order to compare the rate of appearance of PC, DSPC and PG labeled with both [^{3}H] palmitate and [^{14}C]acetate from microsomes to the surfactant related fractions, the ratios of ^{14}C-activity to ^{3}H-activity of these phospholipids in the 3 lung fractions were calculated (Table 4). The results showed that, when the ^{14}C/^{3}H ratios of these phospholipids in lamellar body and alveolar wash fractions were calculated as the unity of those in microsomal phospholipids, ^{14}C-activity was ca. from 1.5 to 1.8 times of ^{3}H-activity in the lamellar body fractions and ca. from 1.6 to 2.7 times in alveolar wash in the 3 phospholipids. These data indicate that relatively more PC, DSPC and PG labeled with [^{14}C] acetate in lung microsomes entered the surfactant related fractions than those labeled with [^{3}H] palmitate. These metabolic findings strongly suggest that PC and PG utilize both palmitates synthesized de novo and supplied exogenously for their synthesis in lung microsomes with similar kinetics, but that phospholipids acylating palmitate synthesized de novo appear preferentially in the surfactant related fractions.

Possible differences in metabolic fate between palmitate and acetate radioactivities were further studied with regard to the transport of surfactant phospholipids from microsomes to the surfactant related fractions. A fraction containing phospholipid exchange proteins catalyzed the transfer of various phospholipids from microsomes labeled with palmitate and acetate to unlabeled mitochondria or lamellar bodies. However, there was no difference between palmitate and acetate radioactivities in the transport from microsomes to mitochondria or lamellar bodies (data not shown). It is, therefore, likely that the phospholipid exchange proteins do not distinguish between the palmitate and acetate labeled phospholipids in their transport from microsomes. Another transport system, the microtubular system, was tested using colchicine-treated rats. The results are shown in Table 5. The prior injection of colchicine resulted in marked decreases in the secretion of PC and DSPC labeled with both precursors into the alveolar space. This decrease was more signifi-

TABLE 4

Comparison of Labelings with [^{3}H] Palmitate and [^{14}C] Acetate in Phosphatidylcholine, Disaturated Phosphatidylcholine and Phosphatidylglycerol of Rat Lung Fractions

Time after injection (min)	Lung fraction	$^{14}C/^3H$ ratio of relative specific activity[a]		
		Phosphatidyl-choline	Disaturated phos-phatidylcholine	Phosphatidyl-glycerol
60	Microsomes	1.00	0.87 (1.00)	1.02 (1.00)
	Lamellar bodies	1.65	1.62 (1.86)	1.77 (1.74)
	Alveolar wash	2.23	2.07 (2.38)	2.81 (2.75)
120	Microsomes	1.34	0.98 (1.00)	1.23 (1.00)
	Lamellar bodies	2.02	1.54 (1.57)	1.77 (1.44)
	Alveolar wash	1.93	1.87 (1.91)	2.00 (1.63)

Values in parenthesis are the values calculated as unity of the $^{14}C/^3H$ ratio in microsomes. The results are means from three independent experiments.

[a]Relative specific activities were calculated as unity of specific activity of phosphatidylcholine in microsomes at 60 min after the injection of [^{3}H] palmitate and [^{14}C] acetate.

cant in the acetate labeled phospholipids than in the palmitate labeled phospholipids. These findings suggest that the lung microtubular system may participate in the secretion of surfactant phospholipids into alveolar space. They also suggest that phospholipids that acylate palmitate de novo synthesized may be transfered preferentially to the alveolar space by the microtubular system.

DISCUSSION

Jobe (9) demonstrated that in in-vivo experiments to determine the specific activities of PC and DSPC, more palmitate synthesized from labeled acetate than that supplied from circulation was preferentially incorporated into lung PC and DSPC destined to become surfactant. The present study confirmed the results of earlier reports by Jobe (9) and Jobe et al. (10) in experiments carried out during relatively short periods of time (from 5 min to 2 hr) after simultaneous administration of labeled palmitate and acetate. In those experiments and ours, relatively more PC, DSPC and PG acylating de novo synthesized palmitate than that acylating exogenously supplied palmitate entered the surfactant related fractions. There, however, has been no conclusive evidence presented to explain this preferential appearance of phospholipids labeled with acetate in the surfactant fractions.

There are several possible explanations for this preferential appearance: (a) whole lung microsomes are derived from many different cell types. Compared to other lung cell types, alveolar type II cells may utilize more acetate for the surfactant phospholipid synthesis than plasma-born plamitate. It has been reported, however, that a relatively greater amount of exogenous palmitate is utilized by alveolar type II cells for the synthesis of dipalmitoyl PC and PG than is utilized by other lung cell types (32). In addition, the metabolic profiles in PC, such as that for positional distribution, are almost the same for palmitate synthesized de novo from acetate as for that supplied exogenously (33). (b) There may be metabolically different palmitate pools in the lung microsomes. De novo synthesized palmitate may enter preferentially a pool destined for the surfactant phospholipids. In contrast, exogenously administrated palmitate may be primarily acylated to membrane phospholipids. The existence of different palmitate pools in lung microsomes was suggested by Longmore et al. (11), who reported that microsomal PC labeled with [1-^{14}C] acetate were utilized as substrate by microsomal phospholipase A_2, while those labeled with [9,10-^{3}H] palmitate were not degraded by this enzyme. Groener and van Golde (34) also showed in studies with isolated rat hepatocytes that palmitate synthesized endogenously from acetate did not mix completely with exogenously supplied palmitate. (c) There may be a preferential transport of phospholipids acylating de novo synthesized palmitate from the microsomes to the surfactant fractions. If this is the case, there may be a specific transfer system available for the specific phospholipid components including preferentially de novo synthesized palmitate in the surfactant fractions. This possibility has not been tested previously.

The mechanisms by which surfactant phospholipids are transported from their synthetic site in alveolar type II cells to the lamellar bodies are unknown. The phospholipid exchange proteins, which are present in cytosol and cata-

lyzed the intracellular transfer of phospholipids, have been demonstrated in several mammalian tissues (35-39). In the lung, the occurrences of such soluble proteins have been described for PC (40,41) and PG (42,43). However, the present study showed that there was no difference between palmitate and acetate labeled phospholipids in the transport by phospholipid exchange proteins from labeled microsomes to mitochondria or lamellar bodies. This would seem to indicate that phospholipid exchange proteins do not distinguish between microsomal phospholipids having endogenously synthesized palmitate and palmitate exogenously supplied during intracellular transport. On the other hand, the results of our experiments with colchicine-treated rats showed that the microtubular system in the lung might participate in translocation of PC from microsomes to surfactant fractions, and that the phospholipids acylating palmitate which was synthesized de novo from acetate might be preferentially transferred from microsomes to surfactant fractions by the microtubular system. Namely, the present observation strongly suggests that, when certain microsomal phospholipids are directed toward the surfactant fractions, the microtubular system may have a relative specificity for phospholipids acylating palmitate synthesized endogenously compared to phospholipids acylating palmitate supplied exogenously.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid for scientific research from the Ministry of Education, Science and Culture, Japan, and a grant from Hokkaido Geriatric Institute.

REFERENCES

1. King, R.J., and Clements, J.A. (1972) Am. J. Physiol. 223, 715-726.
2. Tierney, D.F. (1974) Annu. Rev. Physiol. 36, 209-231.
3. Toshima, N., Akino, T., and Ohno, K. (1972) Tohoku J. Exp. Med. 108, 265-277.
4. Moriya, K., and Kanoh, H. (1974) Tohoku J. Exp. Med. 111, 241-256.
5. Young, S.L., and Tiereney, D.F. (1972) Am. J. Physiol. 222, 1539-1544.
6. Chida, N., and Adams, F.H. (1967) J. Lipid Res. 8, 335-341.
7. Wang, M.C., and Meng, H.C. (1972) Lipids 7, 207-211.
8. Wang, M.C., and Meng, H.C. (1974) Lipids 9, 63-67.
9. Jobe, A. (1979) Biochim. Biophys. Acta 572, 404-412.
10. Jobe, A., Ikegami, M., and Sarton-Miller, I. (1980) Biochim. Biophys. Acta 617, 65-75.
11. Longmore, W.J., Oldenborg, V., and van Golde, L.M.G. (1979) Biochim. Biophys. Acta 572, 452-460.

TABLE 5

Effects of Colchicine on the in-vivo Transport of Phospholipids Labeled with [9.10^{-3}H]Palmitate and [1-^{14}C]Acetate in Rat Lung

		Specific radioactivity (dpm/nmol-lipid)					
		Palmitate label-^{3}H			Acetate label-^{14}C		
		Control	Treated	Treated/control	Control	Treated	Treated/control
Phosphatidylcholine	Microsomes	13.7	48.5	3.54	12.0	45.2	3.78
	Lamellar bodies	34.4	55.6	1.62	36.1	45.6	1.26
	Alveolar wash	2.0	0.9	0.45	3.5	0.7	0.20
Saturated phosphatidylcholine	Microsomes	31.7	68.8	2.17	28.0	84.2	3.01
	Lamellar bodies	32.4	94.2	2.90	33.6	82.6	2.45
	Alveolar wash	2.6	1.7	0.65	4.1	1.2	0.29

The rats were pretreated by the intraperitoneal injection of colchicine (10 mg) per rat in saline (0.5 ml). After 3 hr, the animals were injected simultaneously with [9.10^{-3}H]palmitate (50 μCi) and [1-^{14}]acetate (200 μCi) complexed with 4% bovine serum albumin into the femoral vein. 2 hr after the isotope administration, the animals were killed and the specific radioactivities of phospholipids in rat lung fractions were determined. Data are averages of 2 experiments.

12. Nakamura, M., Kawamoto, T., and Akino, T. (1980) Biochim. Biophys. Acta 620, 24-36.
13. Åkesson, B., Elovson, J., and Arvidson, G.A. (1970) Biochim. Biophys. Acta 218, 44-56.
14. Delahunty, T.J., and Johnston, J.M. (1976) J. Lipid Res. 17, 112-116.
15. Jobe, A. (1977) Biochim. Biophys. Acta 489, 440-453.
16. Engele, M.J., van Golde, L.M.G., and Wirtz, K.W.A. (1978) FEBS Lett. 86, 277-281.
17. Vereyken, J.M., Montfoort, A., and van Golde, L.M.G. (1972) Biochim. Biophys. Acta 260, 70-81.
18. Itoh, K., Nakamura, M., and Akino, T. (1981) Lipids 16, 876-880.
19. Okano, G., Kawamoto, T., and Akino, T. (1978) Biochim. Biophys. Acta 528, 385-393.
20. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
21. Poorthuis, J.H.M., Yasaki, P.J., and Hostetler, K.Y. (1976) J. Lipid Res. 17, 433-437.
22. Arvidson, G.A.E. (1968) Europ. J. Biochem. 4, 478-486.
23. Shimojo, T., Abe, M., and Ohta, M. (1974) J. Lipid Res. 15, 525-527.
24. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
25. Mangold, H. (1969) in Thin-layer Chromatography Stahl, E. ed. pp. 363-421, Spring-Verlag, New York, NY.
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
27. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
28. Van Handel, E., and Zilversmit, D.B. (1957) J. Lab. Clin. Med. 50, 152-157.
29. Snyder, F. (1964) Anal. Biochem. 9, 183-196.
30. Hallman, M., and Gluck, L. (1975) Biochim. Biophys. Acta 409, 172-191.
31. Jobe, A., Kirkpatrick, E., and Gluck, L. (1978) J. Biol. Chem. 253, 3810-3816.
32. Darrah, H.K., and Hedley-Shyte, J. (1973) J. Appl. Physiol. 43, 205-213.
33. Batenburg, J.J., Post, M., and van Golde, L.M.G. (1981) Prog. Respir. Res. 15, 1-19.
34. Groener, J.E.M., and van Golde, L.M.G. (1978) Biochim. Biophys. Acta 529, 88-95.
35. Kamp, H.H., Wirtz, K.W.A., Baer, P.R., Slotboom, A.J., Rosenthal, A.F., Paltauf, F., and van Deenen, L.L. M. (1977) Biochemistry 16, 1310-1316.
36. Johnson, L.W., and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 165-175.
37. Helmkamp, G.M., Harvey, M.S., Wirtz, K.W.A., and van Deenen, L.L.M. (1974) J. Biol. Chem. 249, 6382-6389.
38. Lumb, R.H., Kloosterman, A.D., Wirtz, K.W.A., and van Deenen, L.L.M. (1976) Eur. J. Biochem. 69, 15-22.
39. Lutton, C., and Zilversmit, D.B. (1976) Biochim. Biophys. Acta 441, 370-379.
40. Robinson, M.E., Wu, L.N.Y., Brumley, G.W., and Lumb, R.H. (1978) FEBS Lett. 87, 41-44.
41. Spalding, J.W., and Hook, G.E.R. (1979) Lipids 14, 606-613.
42. van Golde, L.M.G., Oldenborg, V., Post, M., Batenburg, J.J., Poorthuis, B.J.M., and Wirtz, K.W.A. (1980) J. Biol. Chem. 255, 6011-6013.
43. Whitlow, C.D., Pool, G.L., Brumley, G.W., and Lunb, R.H. (1980) FEBS Lett. 113, 221-224.

[Received March 15, 1982]

Fatty Acid[1] Specificity in the Inhibition of Cell Proliferation and Its Relationship to Lipid Peroxidation and Prostaglandin Biosynthesis[2]

N. MORISAKI, H. SPRECHER, G.E. MILO and **D.G. CORNWELL**, *Department of Physiological Chemistry, The Ohio State University, Columbus, OH 43210*

ABSTRACT

Primary cultures of smooth muscle cells were established from the medial layer of guinea pig aorta. Cells at passage level 4 were treated with different series of fatty acids belonging to the n-9, n-6 and n-3 families. Lipid peroxidation was measured by the thiobarbituric acid assay and prostaglandin biosynthesis was measured by the radioimmunoassay of PGE and 6-keto-$PGF_{1\alpha}$. Cell proliferation was estimated from the total cell number of cultures seeded at low density. 18:1(n-9) did not form lipid peroxides and this fatty acid stimulated cell proliferation. All fatty acids which generated lipid peroxides inhibited cell proliferation, but inhibition was correlated with the degree of lipid peroxidation only in the n-9 fatty acid family. 22:4(n-6) and 22:6(n-3) inhibited prostaglandin biosynthesis. 18:2(n-6), 18:2(n-9), 18:3(n-3), 20:2(n-9), 20:3(n-3) and 20:5(n-3) had no effect on prostaglandin biosynthesis. 18:3(n-6), 20:3(n-6) and 20:4(n-6) generated prostaglandins. 20:3(n-9) generated metabolites with prostaglandin immunoreactivity. The inhibition of cell proliferation did not correlate with enhanced or inhibited prostaglandin synthesis. The inhibition of cell proliferation was related to the structures of the different polyunsaturated fatty acid families decreasing in the order n-9 > n-6 > n-3. Eicosatrienoic acids were the most effective inhibitors of cell proliferation in each fatty acid family and 20:3(n-9) was the most potent eicosatrienoic acid. These data show that specific as yet unrecognized products of fatty acid metabolism are responsible for the inhibition of cell proliferation.
Lipids 17:893-899, 1982.

INTRODUCTION

Polyunsaturated fatty acids decrease cell proliferation (cloning potential) in cultures of smooth muscle cells (1-6), fibroblasts (2,6), neuronal cells (7) and a glioma cell tumor clone (7). Studies with several antioxidants show that the inhibitory effect on cell proliferation is related to lipid peroxidation. Polyunsaturated fatty acids generate lipid peroxides in tissue culture and agents which block lipid peroxidation restore cell proliferation (3-5,7,8).

Lipid peroxidation may represent only one of several pathways in fatty acid metabolism that alter cell proliferation. Some polyunsaturated fatty acids function as substrates both for lipid peroxidation and for prostaglandin biosynthesis. Several prostaglandins such as PGE_1 and PGE_2 stimulate cell proliferation at low or physiological concentrations and inhibit cell proliferation at high or pharmacological concentrations (3,). Other prostaglandins such as $PGF_{1\alpha}$ and $PGF_{2\alpha}$ have no effect on cell proliferation at low concentrations and stimulate cell proliferation at high concentrations (3,8). It has been suggested that fatty acid metabolism, antioxidant status and prostaglandin biosynthesis all contribute to the regulation of cell multiplication (8).

Polyunsaturated fatty acids appear to vary in their inhibitory effects on cell proliferation (2,6). However, fatty acid specificity has not been examined in a systematic manner. In the present investigation, we have measured lipid peroxidation, prostaglandin biosynthesis and the inhibition of cell proliferation with fatty acids derived from the desaturation-chain elongation of oleic acid (the n-9 family), linoleic acid (the n-6 family), and linolenic acid (the n-3 family).

MATERIALS AND METHODS

Materials

20:5(n-3) and 22:6(n-3) were kindly sup-

[1] Fatty acids are designated by the number of carbon atoms: number of double bonds and the position of the first double bond from the methyl terminus of the acyl chain is noted in parenthesis: 18:1(n-9), 9-octadecenoic acid; 18:2(n-9), 6,9-octadecadienoic acid; 18:2(n-6), 9,12-octadecadienoic acid; 18:3(n-6), 6,9,12-octadecatrienoic acid, 18:3(n-3), 9,12,15-octadecatrienoic acid; 20:2(n-9), 8,11-eicosadienoic acid; 20:3(n-9), 5,8,11-eicosatrienoic acid; 20:3(n-6), 8,11,-14-eicosatrienoic acid; 20:3(n-3), 11,14,17-eicosatrienoic acid, 20:4(n-6), 5,8,11,14-eicosatetraenoic acid; 20:5(n-3), 5,8,11,14,17-eicosapentaenoic acid; 22:4-(n-6), 7,10,13,16-docosatetraenoic acid, 22:6(n-3), 4,7,10,13,16,19-docosahexaenoic acid.

[2] Presented at the 73rd AOCS annual meeting, Toronto, Canada, May 1982.

plied by the Nippon Oil and Fat Company (Tokyo). 18:2(n-9), 20:2(n-9) and 20:3(n-9) were synthesized in this laboratory (9). Other fatty acids were purchased from NuChek (Elysian, MN). All fatty acids were tested for lipid peroxides (1) since these compounds inhibit cell proliferation. Antisera to 6-keto-$PGF_{1\alpha}$ and PGE (cross-reactivity: PGE_1, 70%; PGE_2, 100%; PGE_3, 94%) were kindly supplied by Dr. L. Levine. Antimyosin (chicken gizzard) was kindly supplied by Dr. Ute G. Stewart.

Tissue Culture

Primary cultures of smooth muscle cells were established from the dissected medial layer of guinea pig aorta from prepubertal males (1-7,10). Smooth muscle cells were identified by their reactivity to antibodies prepared from smooth gizzard muscle (11). The medium for growing cells to confluency (Growth Medium) was prepared from 1X Eagle's minimum essential medium containing Hank's salts and 25 mM HEPES buffer (GIBCO, Grand Island, NY) supplemented with 50 μg/ml of gentamycin sulfate (Schering, Kenilworth, NJ), 2 mM glutamine, 1X nonessential amino acids (Microbiological Associates, Walkersville, MD), 1 mM sodium pyruvate, and 1.3 mg/ml of sodium bicarbonate. This medium was supplemented with 5% fetal bovine serum (Sterile Systems, Logan, UT: Hyclone, Lot 100331). The medium in cell proliferation, lipid peroxidation, PGE and PGI_2 experiments (Experimental Medium) consisted of Growth Medium supplemented with 20% fetal bovine serum, 1X essential amino acids, and 1X essential vitamins. Cells were used at passage level 4.

Fatty acids were dissolved in 95% ethanol and diluted 1:500 with Experimental Medium. Control cultures were treated with Experimental Medium containing the same amount of ethanol.

Lipid Peroxidation

Nonenzymatic lipid peroxidation was initiated by incubating fatty acids with cumene hydroperoxide. Lipid peroxides were assayed with the thiobarbituric acid (TBA) reagent. The fatty acid concentration was 1 mM. The incubation conditions and TBA assay are described elsewhere (5). Lipid peroxides in this system are reported as the absorbance at 532 nm (A_{532}).

Lipid peroxidation in tissue cultures was measured with cells seeded at 2.5×10^5 cells/flask containing 4 ml of Experimental Medium. Lipid peroxides were measured with TBA and were reported as nmol malondialdehyde (MDA)/culture. The details of our assay for lipid peroxides in tissue cultures are described elsewhere (5).

Prostaglandin biosynthesis

PGE was estimated in media from Corning T-25 flasks by a standard RIA procedure. The cross-reactivity of the PGE antibody was: 6-keto-$PGF_{1\alpha}$, 0.4%; PGF_1, 0.76%; PGF_2, 0.31%; PGD_2, 0.051%; arachidonic acid, 0.00045%. Data for immunoreactive PGE metabolites are expressed as nmol/culture.

Prostacyclin (PGI_2) was estimated as 6-keto-$PGF_{1\alpha}$ in media from Corning T-25 flasks by a standard RIA procedure. The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ antibody was: PGE_2, 0.15%; PGD_2, 0.02%; $PGF_{2\alpha}$, 0.10%; arachidonic acid, 0.005%. Data for immunoreactive PGI_2 metabolites are expressed as nmol/culture.

Cell Proliferation

Smooth muscle cells, 3–5 days postconfluent, were seeded at low densities (40 cells/cm^2) in Falcon single-well (60 by 15 mm) plates. Cells were allowed to attach to the plastic petri plates for 1 day before initial treatment. Cells were retreated with a media change at day 5 of the incubation period. After an 8–10 day incubation period, cells were fixed in 2.5% phospate-buffered glutaraldehyde or 3% phospate-buffered formalin and stained with filtered Giemsa.

A relative cell count was obtained from the total cell area on the Falcon plate. Total cell area was measured by image analysis using an Optomax Visual Analysis System (Optomax, Inc., Wallis, NH). The relationship between cell area and cell number was validated both with a microscope (12) and with a Coulter Counter.

Statistics

Data are reported as mean ± SEM. The significance of differences in a treatment series was determined by a one-way analysis of variance (F-statistic). Individual fatty acids were compared with the control by Dunnett's test. The 95% confidence interval for each fatty acid was used in comparisons involving groups containing several fatty acids. The Student t-test was used when only two means were compared.

RESULTS

Lipid Peroxidation

Lipid peroxides were formed (TBA assay) when polyunsaturated fatty acids were incubated with cumene hydroperoxide in an aqueous system. Peroxidation data are summarized in

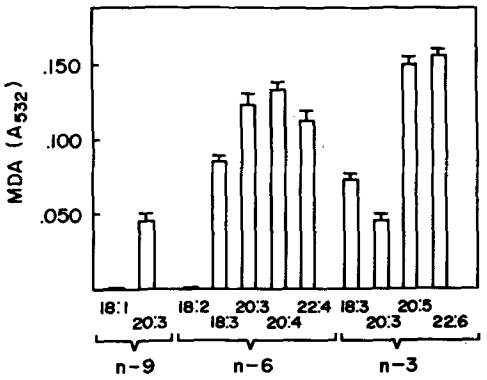


FIG. 1. Formation of lipid peroxides (MDA) from fatty acids incubated with cumene hydroperoxide. Lipid peroxides were estimated in absorbance at 532 nm (A_{532}) in the TBA assay.

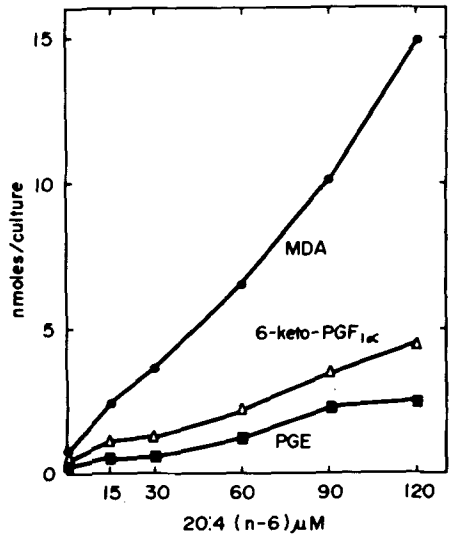


FIG. 2. Formation of lipid peroxides (MDA), immunoreactive PGE and immunoreactive 6-keto-$PGF_{1\alpha}$ when confluent smooth muscle cells were incubated for 24 hr with different concentrations of 20:4(n-6).

Figure 1. A one-way analysis of variance showed that significant differences existed between fatty acids in their ability to form lipid peroxides (F ratio 57.970 and F probability 0). Two fatty acids, 18:1(n-9) and 18:2(n-6), did not form lipid peroxides under the conditions used in this study. All fatty acids with 3 or more double bonds showed significant lipid peroxidation with cumene hydroperoxide.

Lipid peroxides were also formed when polyunsaturated fatty acids were incubated with confluent smooth muscle cells in tissue culture. The amount of lipid peroxide varied directly with fatty acid concentration increasing throughout a 15-120 μM concentration range (Fig. 2). We used 120 μM fatty acid in subsequent experiments to insure that sufficient amounts of lipid peroxides were available for analysis. Peroxidation data are summarized in Figure 3. A one-way analysis of variance showed that significant differences existed between fatty acids and their ability to form lipid peroxides (F ratio 234.062 and F probability 0). Biological peroxidation (Fig. 3) and chemical peroxidation (Fig. 1) were correlated in that only fatty acids with 3 or more double bonds formed large amounts of lipid peroxides. No peroxidation was found in cultures incubated with the fatty acid containing one double bond, 18:1(n-9). Fatty acids containing two double bonds, 18:2(n-6) and 20:2 (n-9), generated small but significant ($p < 0.05$, Dunnett's test) amounts of lipid peroxides in tissue culture. When all fatty acids containing 3 double bonds were considered as one group and all fatty acids containing 4, 5 or 6 double bonds were considered as a second group, the first group generated less MDA (9.34 ± 1.98 nmol/culture) than the second group (14.03 ± 0.98 nmol/culture). The difference between the 2 groups was highly significant ($p < 0.005$, Student-t-test).

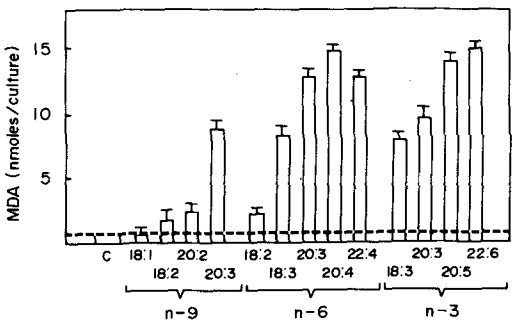


FIG. 3. Formation of lipid peroxides from 120 μM fatty acid incubated for 24 hr with confluent smooth muscle cells. MDA was calculated from absorbance at 532 nm in the TBA assay. Dotted line is the mean for control cultures.

Prostaglandin Biosynthesis

The amounts of immunoreactive PGE and 6-keto-$PGF_{1\alpha}$, like lipid peroxides, increased with increasing fatty acid concentration (Fig. 2). We again used 120 μM fatty acid to insure that sufficient amounts of immunoreactive products were available for RIA analyses.

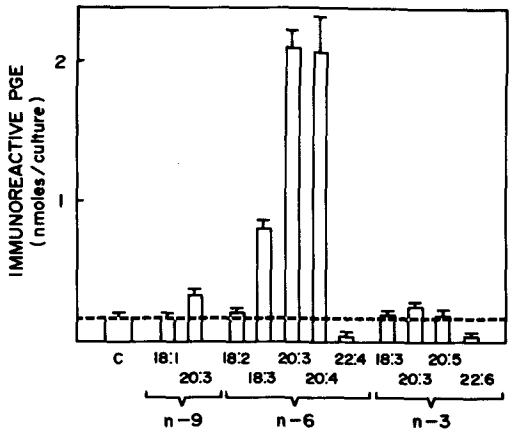


FIG. 4. Biosynthesis of immunoreactive PGE from 120 μM fatty acid incubated for 24 hr with confluent smooth muscle cells. PGE was estimated by RIA with antisera to PGE_2. Dotted line is the mean for control cultures.

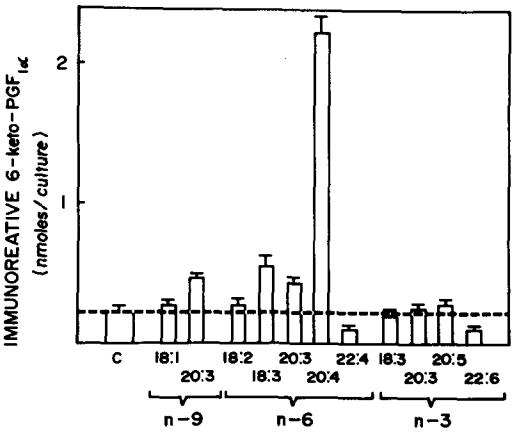


FIG. 5. Biosynthesis of immunoreactive 6-keto-$PGF_{1\alpha}$ from 120 μM fatty acid incubated for 24 hr with confluent smooth muscle cells. 6-Keto-$PGF_{1\alpha}$ was estimated by RIA with antisera to 6-keto-$PGF_{1\alpha}$. Dotted line is the mean for control cultures.

Specific polyunsaturated fatty acids varied widely in their ability to promote or to inhibit the biosynthesis of prostaglandins in the E series. These data are summarized in Figure 4 (F ratio 68.548 and F probability 0). Several desaturation-chain elongation derivatives of 18:2(n-6) including 18:3(n-6), 20:3(n-6) and 20:4(n-6) synthesized large amounts of PGE ($P < 0.01$, Dunnett's test). Although 18:2(n-6) generated lipid peroxides (Fig. 3), this fatty acid did not enhance PGE synthesis with cells in culture. Several fatty acids including 18:1 (n-9), 18:3(n-3), 20:3(n-3) and 20:5(n-3) had no effect on PGE biosynthesis. Two fatty acids, 22:4(n-6) and 22:6(n-3), inhibited PGE biosynthesis (the upper limit of the 95% confidence interval for these fatty acids was 0.051 nmol/culture, while the lower limit of the 95% confidence interval for other fatty acids was 0.067 nmol/culture). One fatty acid, 20:3(n-9), unexpectedly appeared to promote the synthesis of compounds with immunoreactivity to PGE (the lower limit of the 95% confidence interval for 20:3(n-9) was 0.269 nmol/culture while the upper limit of the 95% confidence interval for fatty acids which did not synthesize PGE was 0.277 nmol/culture). Two additional n-9 fatty acids, 18:2(n-9) and 20:2(n-9), did not promote the synthesis of significant amounts of PGE when they were incubated with cells in a new batch of media (Hyclone 100348).

Specific fatty acids had similar effects on the biosynthesis of compounds with 6-keto-$PGF_{1\alpha}$ immunoreactivity and compounds with prostaglandin E immunoreactivity. 6-Keto-$PGF_{1\alpha}$ data are summarized in Figure 5 (F ratio 137.265 and F probability 0). Desaturation-chain elongation products of 18:2(n-6) synthesized large amounts of 6-keto-$PGF_{1\alpha}$ ($P < 0.01$, Dunnett's test) although 18:3(n-6) and 20:3(n-6), as expected, synthesized much less 6-keto-$PGF_{1\alpha}$ than its immediate precursor, 20:4(n-6). 18:1(n-9), 18:2(n-6), 18:3(n-3), 20:3(n-3) and 20:5(n-3) again failed to enhance prostanoid biosynthesis. Two fatty acids, 22:4 (n-6) and 22:6(n-3), again inhibited prostanoid biosynthesis (the upper limit of the 95% confidence interval for these fatty acids was 0.145, while the lower limit of the 95% confidence interval for other fatty acids was 0.147). 20:3 (n-9) promoted the synthesis of compounds with immunoreactivity to 6-keto-$PGF_{1\alpha}$ ($P < 0.05$, Dunnett's test). Neither 18:2(n-9) nor 20:2(n-9) promoted the synthesis of significant amounts of compounds with immunoreactivity to 6-keto-$PGF_{1\alpha}$ (fatty acids incubated in Hyclone 100348).

Inhibition of Prostaglandin Biosynthesis

Two fatty acids, 22:4(n-6) and 22:6(n-3), inhibited prostanoid biosynthesis when they were added to Experimental Media at a 120 μM concentration (Figs. 4 and 5). These naturally occurring fatty acids also inhibited prostaglandin biosynthesis in concentrations as low as 30 μM (Fig. 6). Furthermore, these fatty acids in the 30-120 μM concentration range inhibited prostaglandin biosynthesis when they were added to confluent cultures treated with Experimental Media enriched with 120 μM

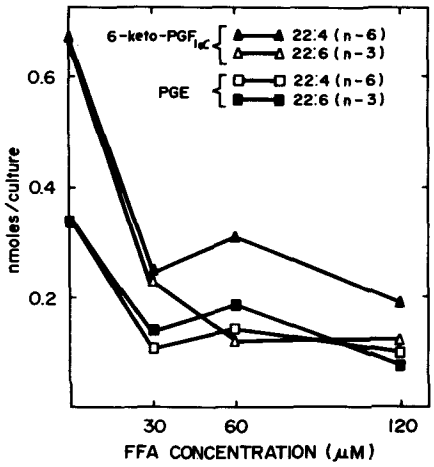


FIG. 6. Biosynthesis of immunoreactive PGE and 6-keto-$PGF_{1\alpha}$ (RIA assay) when confluent smooth muscle cells were incubated for 24 hr with Experimental Media containing different concentrations of 22:4(n-6) or 22:6(n-3).

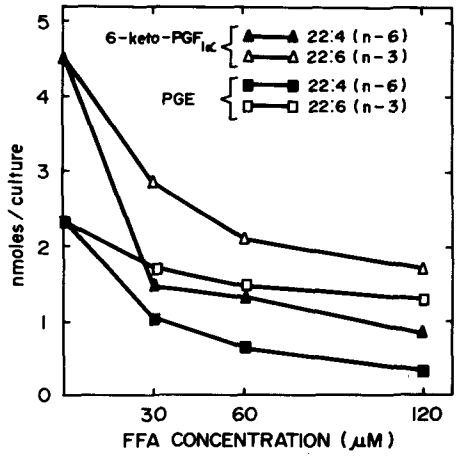


FIG. 7. Biosynthesis of immunoreactive PGE and 6-keto-$PGF_{1\alpha}$ (RIA assay) when confluent smooth muscle cells were incubated for 24 hr with Experimental Media containing 120 μM 20:4(n-6) and different concentrations of 22:4(n-6) or 22:6(n-3).

20:4(n-6) (Fig. 7). In contrast to prostanoid biosynthesis, 22:4(n-6) and 22:6(n-3) had an additive effect on lipid peroxidation when they were supplied to confluent cultures treated with Experimental Media enriched with 120 μM 20:4(n-6) (Fig. 8).

Inhibition of Cell Proliferation

Fatty acids belonging to the n-9, n-6 and n-3 families were examined at three concentrations

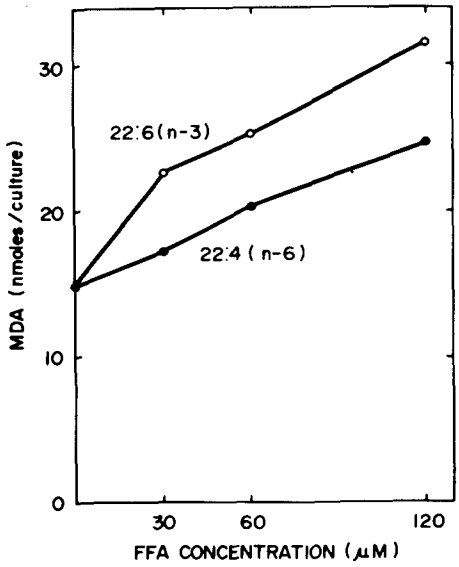


FIG. 8. Formation of lipid peroxides (MDA) when confluent smooth muscle cells were incubated for 24 hr with 120 μM 20:4(n-6) and different concentrations of 22.4(n-6) and 22:6(n-3).

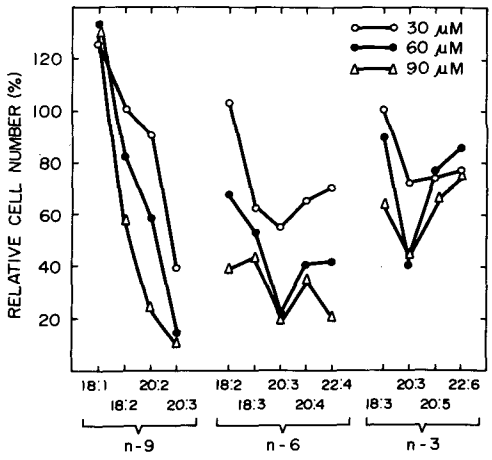


FIG. 9. Proliferation of smooth muscle cells seeded at 40 cells/cm^2 and incubated for 8 days with different concentrations of a specific fatty acid. Relative cell number was calculated from mean of total cells in treatment cultures divided by mean of total cells in control cultures. Each mean was obtained from a minimum of 7 Falcon plates.

for their effects on the proliferation of smooth muscle cells. These data are summarized in Figure 9.

The first member of the n-9 family, 18:1(n-9), stimulated cell proliferation at all concentrations. Other members of the n-9 family showed

concentration-dependent inhibition of cell proliferation. Inhibition with these fatty acids increased with chain elongation and desaturation in the sequence 18:2(n-9) < 20:2(n-9) < 20:3(n-9).

The first members of both the n-6 and n-3 fatty acid families, 18:2(n-6) and 18:3(n-3), were the least inhibitory fatty acids in these families only inhibiting cell proliferation at higher concentrations. The inhibitory effects of fatty acids in the n-6 and n-3 families increased with chain elongation and desaturation to a maximum with the eicosatrienoic acid members of each family. Further chain elongation and desaturation diminished the inhibitory effects of n-6 and n-3 fatty acids.

Eicosatrienoic acids were the most potent inhibitors of cell proliferation in the 3 fatty acid families. At all concentrations, inhibition with these fatty acids increased in the sequence 20:3(n-3) < 20:3(n-6) < 20:3(n-9). 20:3(n-9) was the most potent inhibitor of cell proliferation among all fatty acids examined in this study.

DISCUSSION

A number of studies have shown that polyunsaturated fatty acids inhibit the proliferation of cells in tissue culture (1–8). Polyunsaturated fatty acids generate lipid peroxides (5,7). Antioxidants which block lipid peroxidation restore cell proliferation (3–5,7,8). A fatty acid which did not generate lipid peroxides, 18:1 (n-9), enhanced cell proliferation. Fatty acids which formed lipid peroxides inhibited cell proliferation even though they formed widely different amounts of TBA reactive materials.

Individual fatty acids showed large differences in their ability to form TBA reactive products in nonbiological and biological systems. Fatty acids with 2 double bonds formed, as expected (13), less TBA reactive material than more unsaturated fatty acids. However, these fatty acids showed concentration-dependent inhibition of cell proliferation. A diene fatty acid is the preferred substrate for some lipoxygenases (14). Specific oxidation products of the diene fatty acids should be explored.

In the present study, fatty acids with 3 double bonds formed less TBA reactive material than fatty acids with 4, 5 or 6 double bonds. However, triene fatty acids were the most effective inhibitors of cell proliferation in the 3 fatty acid families. The TBA reaction is a nonspecific test for the breakdown of lipid hydroperoxides. The TBA reaction would be expected to correlate with the inhibition of cell proliferation if proliferation was inhibited by nonspecific lipid peroxidation and cooxidation. Fatty acid specificity unrelated to the TBA reaction shows that specific lipid peroxidation products may be involved in the inhibition of cell proliferation.

Polyunsaturated fatty acids affect prostaglandin biosynthesis in a number of different ways. Two n-6 fatty acids, 20:3(n-6) and 20:4 (n-6), are prostaglandin precursors. 20:4(n-6) yields large amounts of both PGE and PGI_2 when it is incubated with smooth muscle cells. 20:3(n-6) yields a large amount of PGE and a small amount of PGI_2 when it is incubated with smooth muscle cells. 20:3(n-6) is not desaturated to 20:4(n-6) by smooth muscle cells in culture (15). 20:3(n-6) replaces 20:4(n-6) in phospholipids when it is incubated with cells in culture and the 20:4(n-6) released through this process is now available for PGI_2 synthesis. 20:3(n-9) apparently functions in the same way to release 20:4(n-6) for PGE and PGI_2 synthesis. Significant amounts of chain elongation metabolites are formed when polyunsaturated fatty acids are incubated with smooth muscle cells in culture (15). The chain elongation of 18:3(n-6) to 20:3(n-6) explains prostaglandin synthesis when this fatty acid is added to cells in culture. The absence of desaturase activity (15) explains why 18:2(n-6) did not generate prostaglandins in these cultures.

A number of fatty acids including 18:1 (n-9), 18:2(n-6), 20:3(n-9) and all members of the n-3 family were shown in early studies either to inhibit microsomal prostaglandin biosynthesis (16,17,22) or vesicular gland oxygenase activity (18,19). High concentrations of inhibitor fatty acids were necessary. 22:6(n-3) was the strongest inhibitor in these studies. The inhibitory effect of 20:5(n-3) was not confirmed in a recent study (20). We found that only two fatty acids, 22:4(n-6) and 22:6(n-3), inhibited prostaglandin biosynthesis in cultures of smooth muscle cells. These fatty acids were strong inhibitors even in the presence of a 4-fold substrate excess. Their effect on prostaglandin biosynthesis in other cells and tissues should be explored.

Prostaglandins show concentration-dependent stimulation and inhibition of cell proliferation (3,8). Fatty acid precursors of the prostaglandins stimulate cell proliferation in the 2–20 μM concentration range and inhibit cell proliferation at higher concentrations (1,2,21,22). In our studies, the inhibitory effect of a specific polyunsaturated fatty acid was unrelated to its effect on prostaglandin biosynthesis. 20:4(n-6) synthesized large amounts of both PGE and PGI_2 but the inhibitory effect of 20:4(n-6) did not differ significantly from 22:4(n-6), a

fatty acid that inhibited prostaglandin biosynthesis. 22:4(n-6) and 22:6(n-3) both inhibited prostaglandin biosynthesis yet differed significantly in their effects on cell proliferation. Only small amounts of PGE and PGI_2 were generated when cells were incubated with 20:3 (n-9), yet this fatty acid was the most potent inhibitor of cell proliferation. As the fatty acid concentration increased, other inhibitory pathways of polyunsaturated fatty acid metabolism overrode any stimulatory or inhibitory effect that prostaglandins had on cell proliferation.

Cell proliferation is stimulated by a growth factor released from aggregating platelets (23). This growth factor may be important in the proliferation of smooth muscle cells that is characteristic of atherosclerosis. Fatty acids in the n-3 family and 20:3(n-9) both diminish platelet aggregation (24-26). However, we found that the n-3 fatty acids were less effective than 20:3(n-9) in the inhibition of cell proliferation. Since 20:3(n-9) suppresses both platelet aggregation and smooth muscle cell proliferation, the effect of synthetic 20:3 (n-9) as a dietary supplement should be explored in experimental atherosclerosis.

ACKNOWLEDGMENTS

This study was supported in part by Research Grant HL-11897 from the National Institutes of Health. We appreciate the technical assistance of Judith Stitts, Louise Tomei and David A. Cornwell. Statistical analyses were kindly supplied by Dr. Larry Sachs.

REFERENCES

1. Huttner, J.J., Gwebu, E.T., Panganamala, R.V., Milo, G,E., Cornwell, D.G., Sharma, H.M., and Geer, J.C. (1977) Science 197, 289-291.
2. Huttner, J.J., Milo, G.E., Panganamala, R.V., and Cornwell, D.G. (1978) In Vitro 14, 854-859.
3. Cornwell, D.G., Huttner, J.J., Milo, G.E., Panganamala, R.V., Sharma, H.M., and Geer, J.C. (1979) Lipids 14, 194-207.
4. Miller, J.S., Gavino, V.C., Ackerman, G.A., Sharma, H.M., Milo, G.E., Geer, J.C., and Cornwell, D.G. (1980) Lab. Invest. 42, 495-506.
5. Gavino, V.C., Miller, J.S., Ikharebha, S.O., Milo, G.E., and Cornwell, D.G. (1981) J. Lipid Res. 22, 763-769.
6. Gavino, V.C., Miller, J.S., Dillman, J.M., Milo, G.E., and Cornwell, D.G. (1981) Prog. Lipid Res. 20, 323-325.
7. Liepkalns, V.C., Icard-Liepkalns, C., and Cornwell, D.G. (1982) Cancer Lett. 15, 173-178.
8. Bettger, W.J., and Ham, R.G. (1981) Prog. Lipid Res. 20, 265-268.
9. Sprecher, H. (1978) Prog. Chem. Fats Other Lipids 15, 219-254.
10. Huttner, J.J., Cornwell, D.G., and Milo, G.E. (1977) T.C.A. Manual 3, 633-639.
11. Groschal-Stewart, U., Chamley, J.H., McConnell, J.D., and Burnstock, G. (1975) Histochemistry 43, 215-224.
12. Gavino, V.C., Milo, G.E., and Cornwell, D.G. (1982) Cell Tissue Kinet. 15, 225-231.
13. Ohkawa, H., Ohishi, N., and Yagi, K. (1978) J. Lipid Res. 19, 1053-1057.
14. Gardner, H.W. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 447-504, Plenum Press, New York, NY.
15. Gavino, V.C., Miller, J.S., Dillman, J.M., Milo, G.E., and Cornwell, D.G. (1981) J. Lipid Res. 22, 57-62.
16. Pace-Asciak, C., and Wolfe, L.S. (1968) Biochim. Biophys. Acta 152, 784-787.
17. Ziboh, V.A. (1973) J. Lipid Res. 14, 377-384.
18. Lands, W.E.M., LeTellier, P.R., Rome, L.H., and Vanderhoek, J.Y. (1973) Adv. Biosci. 9, 15-28.
19. Ziboh, V.A., Vanderhoek, J.Y., and Lands, W.E.M. (1974) Prostaglandins 5, 233-240.
20. Dyerberg, J., and Jorgensen, K.A. (1980) Artery 8: 12-17.
21. Kidwell, W.R., Monaco, M.E., Wicha, M.S., and Smith, G.S. (1978) Cancer Res. 38, 4091-4100.
22. Wicha, M.S., Liotta, L.A., and Kidwell, W.R. (1979) Cancer Res. 39, 426-435.
23. Ross, R. (1981) Arteriosclerosis 1, 293-311.
24. Friedman, Z. (1981) Prog. Lipid Res. 20, 59-66.
25. Harris, W.S., Connor, W.E. and Goodnight, Jr., S.H. (1981) Prog. Lipis Res. 20, 75-79.
26. Hornstra, G. (1981) Prog. Lipid Res. 20, 407-413.

[Received May 18, 1982]

Stimulation of Hepatic Squalene and Triglyceride Synthesis by Dimethylsulfoxide, in vitro

FRANK P. BELL* and **EDNA V. HUBERT**, *Diabetes-Atherosclerosis Research, The Upjohn Company, Kalamazoo, MI 49001*

ABSTRACT

The incorporation of [^{14}C] mevalonate and [^{14}C] acetate into squalene by rat liver slices was increased over 7-fold by the presence of 5% dimethylsulfoxide (DMSO) in the incubation medium. The stimulation of squalene synthesis was dose-related over the concentration range of 1–5% DMSO and did not affect the incorporation of [^{14}C] mevalonate into the C_{27}-sterol fraction (cholesterol) but did increase (about 50%) incorporation into C_{30}-sterol (lanosterol) at a level of 5% DMSO. The stimulation of squalene synthesis was observed under both anaerobic (N_2 atmosphere) and aerobic (ambient air or 95% O_2/5% CO_2) conditions and may represent a direct effect of DMSO on squalene synthetase. At a level of 5%, DMSO also stimulated 7-fold the incorporation of [^{14}C] acetate into triglycerides by liver slices; this occurred without changes in incorporation into the phospholipid or free fatty acid fractions. The disproportionate increase in lipid labeling from [^{14}C] acetate suggests that the effects of DMSO are not simply a matter of increasing [^{14}C] acetate entry into the tissue. *Lipids* 17:900-904, 1982.

INTRODUCTION

Dimethylsulfoxide (DMSO) has diverse biological and pharmacological effects and has been the subject of a number of symposia (1,2). DMSO is miscible in water and readily penetrates tissues and cells (3). Its list of biological activities includes bacteriostatic and anti-inflammatory activity (4), inhibition of cyclic AMP phosphodiesterase (5), a reversible inhibition of protein synthesis in cultured cells (6), cytoprotection (7) and enhancement of cell-mediated immunity (8). DMSO also appears to be capable of modifying some aspects of lipid metabolism such as increasing the rate of exchange of cholesterol between plasma lipoproteins and erythrocytes and tissues (9), inhibiting phospholipid synthesis in Friend erythroleukemia (FL) cells (10) and decreasing the uptake of low-density lipoproteins (LDL) by cultured fibroblasts (11). In the studies reported here, the effect of DMSO on lipid synthesis in rat liver slices was investigated with [1-^{14}C] acetate and [2-^{14}C] mevalonate. DMSO was found to modify the pattern of saponifiable and nonsaponifiable lipids synthesized in that it stimulated triglyceride and squalene biosynthesis.

MATERIALS AND METHODS

Animals and Tissues

Male Spraque-Dawley rats of the Upjohn strain (Upj: TUC (SD) Spf, 225–250g) were maintained on Purina Chow ad libitum. The rats were decapitated between 9 AM and 10 AM and the livers were excised, rinsed in 0.9% NaCl solution, and used to prepare liver slices (12,13).

Incubation Procedures

Liver slices (500 mg) were incubated at 37 C for 90 min to 3 hr in 3.5-ml Krebs-Ringer-bicarbonate buffer, pH 7.4, or the buffer which was made up with dimethylsulfoxide (Matheson Coleman and Bell, Norwood, OH) substituting for 1–5% of the aqueous volume. Each flask contained either 3.0 μCi [1-^{14}C]-acetic acid, sodium salt (sp act 56.0 Ci/mol) or 2.0 μCi of DL-[2-^{14}C] mevalonic acid, DBED salt (sp act 47.0 Ci/mol) (New England Nuclear Corp., Boston, MA). All incubations were performed in 25-ml Erlenmeyer flasks that were stoppered under air (ambient) or stoppered after flushing the flasks with N_2 or 95% O_2/5% CO_2.

Analyses

After incubation, all tissues were extracted with chloroform/methanol (2:1, v/v) (14). Lipid extracts of tissues incubated with [^{14}C]-acetate were divided. One portion was fractionated by thin layer chromatography (TLC) in order to isolate the phospholipids, free fatty acids, and triglycerides (15) which were scraped from the chromatoplates into counting vials containing 15 ml of Liquifluor (New England Nuclear Corp., Boston, MA) and assayed for radioactivity by liquid scintillation spectrom-

*Author to whom correspondence should be addressed.

TABLE 1

Effect of Dimethylsulfoxide on the Incorporation of [1-^{14}C]Acetate into Lipids in Rat Liver Slices (dpm g wet wt × 10^{-1})[a]

	Free fatty acids	Phospholipids	Triglycerides	Digitonin-precipitable sterols	Squalene
Control	418[b] ±58	1140 ±139	256 ±40	170 ±20	77 ±10
5% DMSO	384 ±75	1230 ±130	1705 ±140	240 ±40	860 ±185
	NS[c]	NS	$p < 0.001$	$p < 0.05$	$p < 0.001$

[a]Liver slices (500 mg) from each of 8 normal rats were incubated for 3 hr at 37 C under air with 3 μCi [1-^{14}C]acetic acid, sodium salt (SA 56.0 Ci/mol) in 3.5-ml Krebs-Ringer bicarbonate buffer, pH 7.4, and in 3.5 ml of the buffer which was made with dimethylsulfoxide (DMSO) substituting for 5% of the aqueous volume.

[b]Values are means ± SEM of data obtained from 8 animals.

[c]Statistical analysis was performed using Student's paired t-test. NS denotes values not differing significantly from control values.

etry (Packard Tricarb Mod. 3375, Packard Instruments, Downers Grove, IL). A second portion of the lipid extracts was evaporated and resuspended in alcoholic KOH, and saponified (16) and the nonsaponifiable lipids were extracted with *n*-hexane (13). The nonsaponifiable lipids were fractionated into digitonin-precipitable sterols (12) and squalene (17) as previously described and assayed for radioactivity (17).

Lipid extracts of tissues incubated with [^{14}C]mevalonate were fractionated by TLC to yield squalene, and the C_{27}-sterols and C_{30}-sterols which were identified by cochromatography with authentic cholesterol and lanosterol, respectively (13). The various fractions were then scraped from the chromatoplates and assayed for radioactivity as above. In the studies reported in Table 2, the squalene fraction taken from the TLC plates was eluted with chloroform and the identity of the squalene confirmed by formation and recrystallization of the hexahydrochloride derivative (17,18). From 95–97% of the radioactivity in the squalene fraction which was isolated from the TLC plates was recoverable as squalene hexahydrochloride.

RESULTS

Rat liver slices were incubated with [^{14}C]-acetate in the presence and absence of 5% dimethylsulfoxide (DMSO) (Table 1). In the presence of DMSO, [^{14}C]acetate incorporation into triglycerides was increased about 7-fold ($p < 0.001$) relative to paired control values; incorporation into phospholipids and free fatty acids, however, was not affected. The presence of DMSO also resulted in an 11-fold increase in [^{14}C]acetate incorporation into squalene ($p < 0.001$) with a smaller, but significant increase (40%, $p < 0.05$) in [^{14}C]acetate incorporation into digitonin-precipitable sterols (sterols having a 3 β-OH group, principally cholesterol).

The results of Table 2 show the effect of DMSO on the incorporation of [^{14}C]mevalonate into the nonsaponifiable lipids (sterols and squalene) of rat liver slices. At a level of 5% in the incubation medium, DMSO resulted in a 7-fold increase in [^{14}C]mevalonate incorporation into squalene ($p < 0.001$) and increased incorporation into C_{30}-sterols by 50% ($p < 0.01$); incorporation into the C_{27}-sterols, however, was not affected. Confirmation that the increased incorporation of [^{14}C]mevalonate into the squalene fraction of the livers was indeed squalene was obtained by forming the hexahydrochloride derivative; 95–97% of the radioactivity in the squalene fraction was recoverable as the hexahydrocloride derivative.

The effect of DMSO on the incorporation of [^{14}C]mevalonate into nonsaponifiable lipids was examined in more detail in experiments presented in Figure 1. The stimulation of labeled squalene production from [^{14}C]mevalonate was concentration-dependent over the range 1–5% DMSO. As was observed in Table 2, incorporation of [^{14}C]mevalonate into the C_{27}-sterols was essentially unchanged in the presence of DMSO. Incorporation into the C_{30}-sterols was also unchanged but only up to a level of 4% DMSO. At 5% DMSO, incorporation of [^{14}C]mevalonate into C_{30}-sterols was

TABLE 2

Effect of Dimethylsulfoxide on the Incorporation of [2-^{14}C]Mevalonate into C_{27}-Sterols, C_{30}-Sterols, and Squalene in Rat Liver Slices (dpm g wet wt × 10^{-2})[a]

	C_{27}-Sterol	C_{30}-Sterol	Squalene
Control	487[b] ±48	183 ±14	497 ±47
5% DMSO	485 ±47	287 ±23	3440 ±349
	NS[c]	$p < 0.01$	$p < 0.001$

[a]Liver slices (500 mg) from each of 8 normal male rats were incubated for 3 hr at 37 C under air with 2 μCi [2-^{14}C]mevalonic acid, DBED salt (SA 47.0 Ci/mol) in 3.5-ml Krebs-Ringer bicarbonate buffer, pH 7.4, and in 3.5 ml of buffer which was made with dimethylsulfoxide (DMSO) substituting for 5% of the aqueous volume.

[b]Values are means ± SEM of data obtained from 8 animals.

[c]Statistical analysis was performed using Student's paired t-test. NS denotes values not differing significantly from control values.

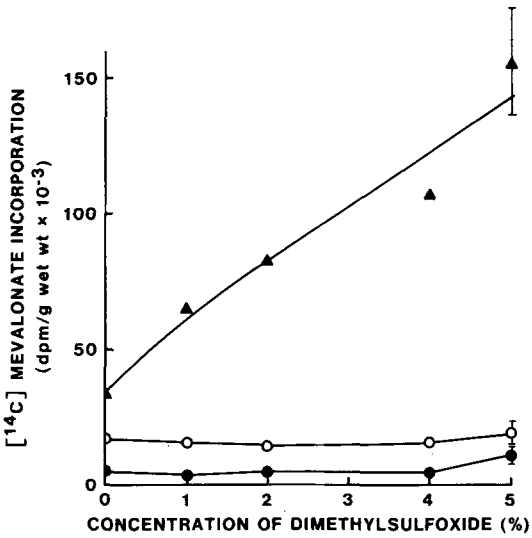


FIG. 1. Effect of various concentrations of dimethylsulfoxide on the incorporation of [2-^{14}C]-mevalonate into C_{27} sterols (-o-o), C_{30}-sterols (-●-●-), and squalene (-▲-▲-) in rat liver slices. Liver slices (500 mg) from normal male rats were incubated for 3 hr at 37 C with 2 μCi [2-^{14}C]mevalonic acid, DBED salt (47.0 Ci/mol) in 3.5-ml Krebs-Ringer bicarbonate buffer, pH 7.4, that was made to contain from 0 to 5% dimethylsulfoxide (DMSO) by substituting DMSO for an equivalent volume of water. Data points at 1, 2, and 4% DMSO are mean values obtained from 2 animals; data points at 5% DMSO are means of data from 9 animals ± SEM.

increased significantly ($p < 0.001$) from a control value of 5055 ± 760 to 11425 ± 910 dpm/g wet weight ($n = 9$), thus confirming the results of Table 2.

The effect of pure N_2 atmospheres or 95% O_2/5% CO_2 atmospheres on the stimulation of squalene synthesis by DMSO in rat liver was also investigated and the results are shown in Table 3. These studies were undertaken because the conversion of squalene into sterols requires molecular oxygen (19) which, if displaced by DMSO, could account for squalene accumulation in incubations of liver with [^{14}C]mevalonate. The results show that an increased incorporation of [^{14}C]mevalonate into squalene in the presence of DMSO per-

TABLE 3

Effect of Dimethylsulfoxide and Nitrogen on the Incorporation of [2-^{14}C]Mevalonate into Squalene in Rat Liver Slices (dpm g wet wt × 10^{-2})[a]

	n	Additions	Atmosphere	Squalene
Exp. 1	12	0	95% O_2/5% CO_2	804 ± 93[b] $p < 0.001$[c]
	10	5% DMSO	95% O_2/5% CO_2	2033 ± 87
Exp. 2	9	0	N_2	400 ± 29 $p < 0.001$
	6	5% DMSO	N_2	2779 ± 143

[a]Liver slices (500 mg) from male rats were incubated for 90 min at 37 C with 2 μCi [2-^{14}C]mevalonic acid, DBED salt (SA 47.0 Ci/mol) in 3.5-ml Krebs-Ringer-bicarbonate buffer, pH 7.4, or 3.5 ml of the buffer which was made with dimethylsulfoxide (DMSO) substituting for 5% of the aqueous volume. The gas phase over the samples during incubation was either 95% O_2/5% CO_2 or pure N_2.

[b]Values are means ± SEM of the number of animals given by n in the table.

[c]Statistical analysis was performed using Student's independent t-test.

DISCUSSION

The studies presented here demonstrate that DMSO affects the pattern of lipids synthesized from [^{14}C] acetate and [^{14}C] mevalonate in rat liver slices. The effect of DMSO is seen most dramatically with squalene biosynthesis and triglyceride biosynthesis. The incorporation of [^{14}C] acetate and [^{14}C] mevalonate into squalene increased 11-fold and 7-fold, respectively, in the presence of 5% DMSO. Since the cyclization of squalene to lanosterol requires molecular oxygen (19), and squalene is known to accumulate in liver tissue under anaerobic conditions (20), the possibility that DMSO limited oxygen availability to the tissue was explored by comparing squalene synthesis from [^{14}C] mevalonate under the extremes of a 95% O_2 atmosphere and an N_2 atmosphere (Table 3). Irrespective of the extremes of atmosphere, the incorporation of [^{14}C] mevalonate into squalene was always significantly greater in the presence of DMSO as was the case under ambient conditions as well (Table 2, Fig. 1). The results suggest that DMSO stimulates squalene synthesis per se. Whereas it could be argued that an increased synthesis of [^{14}C] squalene from [^{14}C] acetate could occur through a stimulation of 3-hydroxy-3-methylglutaryl CoA reductase (hydroxy methyl sisted under conditions of an oxygen-rich atmosphere (Table 3, exp. 1) as well as under an N_2 atmosphere (Table 3, exp. 2).
glutaryl CoA reductase, EC 1.1.1.34), the rate-limiting step in squalene and sterol synthesis, this would not explain the increased [^{14}C]-squalene synthesis from [^{14}C] mevalonate, since mevalonate is a post-hydroxy methyl glutaryl CoA reductase substrate. Instead, the increased squalene synthesis from [^{14}C] mevalonate suggests the possibility that DMSO affects the activity of squalene synthetase, a microsomal complex that catalyzes the formation of squalene from farnesyl pyrophosphate units (21). However, the greater increase in [^{14}C]-acetate vs [^{14}C] mevalonate incorporation into squalene in the presence of DMSO (11-fold vs 7-fold) does suggest the possibility that DMSO can also affect the activity of HMG CoA reductase.

The increased synthesis of [^{14}C] squalene from [^{14}C] acetate and [^{14}C] mevalonate resulted in a comparatively small (40–50%) increase in the formation of digitonin-precipitable sterols and lanosterol, respectively (Tables 1 and 2, Fig. 1), suggesting that DMSO has a relatively minor effect on the post-squalene portion of the sterol synthetic pathway. The [^{14}C] mevalonate studies offered no evidence for an effect of DMSO on cholesterol formation (Table 2, Fig. 1).

To what extent DMSO can affect squalene synthesis in tissues other than liver is unknown. The addition of 2% DMSO to cultured skin fibroblasts that were actively synthesizing cholesterol (grown with lipoprotein-free serum) did not affect the incorporation of [^{14}C]-acetate into squalene, lanosterol, or cholesterol (11). The addition of exogenous cholesterol to the cells as LDL, however, inhibited sterol and squalene synthesis as would be predicted (22), but the inhibition was partly alleviated if 2% DMSO was also added. Since DMSO also inhibited the uptake of LDL-cholesterol by the cells (11), this apparent stimulation of synthesis by DMSO is likely a consequence of the lower cellular sterol levels in the LDL/DMSO-treated cells (11), rather than a direct effect of DMSO as seen in the liver preparations discussed here.

The effects of DMSO on liver lipid synthesis from [^{14}C] acetate and [^{14}C] mevalonate are unlikely to be a result of an increased uptake of labeled precursors in the presence of DMSO; this is perhaps best illustrated with the [^{14}C]-acetate studies. All else being equal, an increased availability of [^{14}C] acetate to tissues would not be expected to result in a disproportionate labeling of the various lipids relative to control values, as was the case (Table 1). The increase in the labeling of [^{14}C] digitonin-precipitable sterols may simply be related to the increased formation of [^{14}C] squalene. Although the explanation of the increased (7-fold) labeling of the triglyceride fraction without an effect on the labeling of the phospholipids is unknown, it is possible that the acylation of 1,2-diglyceride is enhanced with DMSO.

The data presented here indicate that DMSO may be a useful tool for examining the regulation of squalene synthesis and the limitations on its flow-through to the sterols.

ACKNOWLEDGMENTS

The secretarial assistance of Mrs. B. Laurian is greatly appreciated.

REFERENCES

1. Jacob, S.W., and Herschler, R. (eds.) (1975) Biological Actions of Dimethylsulfoxide, p. 243, Ann. N.Y. Acad. Sci.
2. Wyer, E.M. (ed.) (1967) Biological actions of Dimethylsulfoxide, p. 141, Ann. N.Y. Acad. Sci.
3. Szmant, H.C. (1975) Ann. N.Y. Acad. Sci. 243, 20–23.
4. Wood, D.C., and Wood, J. (1975) Ann. N.Y. Acad. Sci. 243, 7–19.

5. Wieser, P.B., Zeiger, M.A., and Fain, J.N. (1977) Biochem. Pharmacol. 26, 775-778.
6. Saborio, J.L., and Koch, G. (1973) J. Biol. Chem. 248, 8343-8347.
7. Ashwood-Smith, M.J., (1967) Ann. N.Y. Acad. Sci. 141, 45-62.
8. Bartfeld, H., and Goldstein, A. (1975) Ann. N.Y. Acad. Sci. 243, 81-90.
9. Bell, F.P. (1973) Exp. Mol. Pathol. 19, 293-303.
10. Harel, L., Lacour, F., Friend, C., Durbin, P., and Semmel, M. (1979) J. Cell. Physiol. 101, 25-32.
11. Alam, S.S., and Layman, D.L. (1982) Biochim. Biophys. Acta 710, 306-313.
12. Bell, F.P., and Hubert, E.V. (1980) Biochim. Biophys. Acta 619, 302-307.
13. Bell, F.P. (1976) Lipids 11, 769-773.
14. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
15. Bell, F.P., and Nazir, D.J. (1976) Lipids 11, 216-221.
16. Daly, M.M. (1971) J. Lipid Res. 12, 367-375.
17. Bell, F.P. (1976) Exp. Mol. Pathol. 25, 279-292.
18. Loud, A.V., and Bucher, N.L.R. (1958) J. Biol. Chem. 233, 37-41.
19. Tchen, T.T., and Bloch, K. (1957) J. Biol. Chem. 226, 921-930.
20. Bucher, N.L.R., and McGarrahan, K. (1956) J. Biol. Chem. 222, 1-15.
21. Sabine, J.R. (1977) in Cholesterol, pp. 94-95, Marcel Dekker, Inc., New York, N.Y.
22. Brown, M.S., Kovanen, P.T., and Goldstein, J.L. (1980) Ann. N.Y. Acad. Sci. 348, 48-66.

[Received June 14, 1982]

Modulation of Tissue Prostaglandin Synthesizing Capacity by Increased Ratios of Dietary Alpha-Linolenic Acid to Linoleic Acid[1,2]

LISA A. MARSHALL and **PATRICIA V. JOHNSTON***, *Department of Food Science and Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, 1208 West Pennsylvania Avenue, Urbana, IL 61801*

ABSTRACT

Semipurified diets containing ratios of α-linolenic acid (18:3ω3) to linoleic acid (18:2ω6) of 1/32, 1/7, 1/1, and 3.5/1 in the form of corn oil, soybean oil, soybean/linseed oil mix and linseed oil were fed to rats for 2 months. The first 3 diets were fed to another group of rats for 4 months and to a group through the second generation. Fatty acid analysis of liver and spleen ethanolamine glycerophosphatide revealed that, as the level of 18:3ω3 in the diet increased, the elongated, desaturated metabolites of the ω6 series decreased and the ω3 series increased. Noteworthy was the depression in the amount of the precursor of the 2-series prostaglandins (PG) as the ω3 levels increased. Synthesis of PG by liver of rats fed 2 or 4 months markedly decreased, but at 2 months in thymus and spleen, it showed a trend toward decreasing only. Brain slices showed no decrease in $PGF_{2\alpha}$ synthesis after 4 months, but did decrease significantly after feeding the diets to the second generation. Synthesis of PGE_2 by spleen homogenate from the second generation also significantly decreased. The replacement of ω6 series fatty acids by ω3 series is explained by the effective competition of 18:3ω3 over 18:2ω6 for the Δ6 desaturase. Depressions in PG synthesis by high dietary 18:3ω3 is explained by the competitive inhibition of the PG synthetase complex by 20:5ω3 as well as by the decreased levels of 20:4ω6. *Lipids* 17:905-913, 1982.

INTRODUCTION

Unequivocal evidence that α-linolenic acid (18:3ω3) is essential in the diet of mammals has been lacking (1,2). Tinoco et al. (2) were unable to demonstrate classical clinical deficiency symptoms of 18:3ω3, i.e., those affecting appearance, growth or reproduction. Perhaps this type of evaluation is an unsuitable criterion for the essentiality of 18:3ω3, the biochemical function of which may not readily be assessed clinically. Thus far, only one case of 18:3ω3 deficiency has been reported and this was in a patient on intravenous hyperalimentation with a very low 18:3ω3 component.

Circumstantial evidence that 18:3ω3 is an essential fatty acid (EFA) does exist. Omega-3 fatty acids are important components of membrane-based lipids in many tissues, notably in brain. Moreover, during an 18:3ω3 deficiency, the ω3 fatty acids tend to be sequestered (1). There have also been some reports that ω3 deficiency leads to physiological changes which can be reversed on feeding 18:3ω3 (3,4). The essentiality of 18:3ω3 is also implicit in the hypothesis of Crawford and coworkers (5,6) that the conversion of the parent EFA, both linoleic (18:2ω6) and 18:3ω3, to the derived long chain EFA, may not always proceed fast enough to meet the physiological demand for the derived ω3 series. This hypothesis is supported by the fact that the first step in this pathway, the Δ6-desaturation, is rate-limiting in most animals while in others it does not occur at all. This results in an absolute dietary requirement for both parent and derived EFA in some animals (7). Two other facts of interest are first, the Δ6 desaturase is easily inhibited (8,9); second, the enzyme prefers the ω3 substrate before the ω6 and the ω6 before the ω9 (8).

The properties of the desaturase and its preference for the ω3 series led a number of investigators to examine the effect of dietary ω3 fatty acids on the capacity of tissues to synthesize prostaglandins (PG). Galli et al (10) found that increasing the amount of 18:3ω3 in the diet of rats led to decreased levels of free arachidonic acid (20:4ω6) and a decreased capacity for $PGF_{2\alpha}$ synthesis in the cerebral cortex. Hwang and Carroll (11) fed purified methyl linolenate to EFA-deficient rats and found that, as the amount of dietary linolenate increased, there was a decreased synthesis of PG by platelets as measured by serum PG concentration. In this laboratory (12), we found that after a 2-month feeding period with 18:

[1] Part of a dissertation submitted by Lisa A. Marshall in partial fulfillment of requirements for the Ph.D. degree in Nutritional Sciences.

[2] Presented in part at the 72nd AOCS annual meeting, New Orleans, May 1981.

*Author to whom correspondence should be addressed.

TABLE 1

Composition of Semipurified Diets

	CO	SO	SL[d]	LO
		(g/100 g)		
Corn oil[a]	10	–	–	–
Soybean oil[b]	–	10	2.3	–
Linseed oil[c]	–	–	7.7	10
Casein[e]	20	20	20	20
Dextrose[f]	18	18	18	18
Cornstarch[g]	30.5	30.5	30.5	30.5
Cellulose[h]	5	5	5	5
Salt mix[i]	4	4	4	4
Vitamin mix[j]	12.5	12.5	12.5	12.5

[a]Fatty acid analysis: 16:0, 8.7%; 18:0, 1.4%; 18:1ω9, 16.5%; 18:2ω6, 71.0%; 18:3ω3, 2.2%. Courtesy of Best Foods, CPC International, Inc., Englewood Cliffs, NJ.

[b]Fatty acid analysis: 16:0, 11.8%; 18:0, 3.7%; 18:1ω9, 20.3%; 18:2ω6, 56.3%; 18:3ω3, 7.7%. Courtesy of Kraft, Inc., Memphis, TN.

[c]Fatty acid analysis: 16:0, 5.5%; 18:0, 2.6%; 18:1, 12.5%; 18:2, 17.5%; 18:3ω3, 61.8%. Courtesy of Cargill, Inc., Minneapolis, MN.

[d]Fatty acid analysis: 16:0, 1.0%; 18:0, 3.1%; 18:1ω9, 20.0%; 18:2ω6, 38.2%; 18:3ω3, 37.7%.

[e]Shamrock Brand, Erie Casein Company, Erie, IL. Supplemented with .3 g DL methionine/100 g diet.

[f]Staleydex 333, A.E. Staley Company, Decature, IL.

[g]Cornstarch, A.E. Staley Company, Decatur, IL.

[h]Solka-Floc, Brown Company, Berlin, NH.

[i]Jones-Foster Salt Mix (modified by addition of $ZnCO_3$), Nutritional Biochemicals Corporation, Cleveland, OH.

[j]Vitamins (mg/kg diet): thiamin.HCl, 30; riboflavin, 30; pyridoxine.HCl, 8; calcium pantothenate, 100; nicotinamide, 100; inositol, 220; folic acid, 2; biotin, 0.2; cyanocobalamin, 0.05; vitamin K_5, 2; retinyl acetate, 10; vitamin D_2, 4; dl-α-tocopherol, 738; choline chloride, 1000; brought up to 12.5 g with Dextrose.

3ω3, in the form of a soybean/linseed oil mix, organs of the immune system reflected the high 18:3ω3 intake, but their PG synthesizing capacity was not altered unless the animals were immunochallenged. We have now examined the effects of feeding higher levels of 18:3ω3 on the PG synthesizing capacity of several tissues.

MATERIALS AND METHODS

Dietary Treatment

Female and male weanling Lewis/Mai F rats (Microbiological Associates, Walkersville, MD) were maintained on one of 3 or 4 semipurified diets, each adequate in all nutrients (Table 1). Ten percent of the diet, by weight, was composed of either corn oil (CO) containing less than 1% 18:3ω3, nonhydrogenated soybean oil (SO) containing 7–8% 18:3ω3, soybean/linseed oil mixture (SL) containing 37.7% 18:3ω3 and 38.2% 18:2ω6 or linseed oil (LO) containing 61.8% 18:3ω3. All animals were housed in polypropylene cages with Sanicel^R bedding (Paxton Processing Company, Inc., Paxton, IL). A diurnal light cycle of 12 hr was maintained and food and water were available ad libitum. Body weight was monitored once a week throughout the feeding trials. The experimental diets were prepared fresh every 2 days using preweighed aliquots of oil stored at -20 C. The diets and oils were periodically checked for deterioration by determination of peroxide number (13).

Three feeding trials were conducted. The first consisted of male weanling rats divided into 4 dietary groups (CO, SO, SL, LO) for a 2-month period. The second consisted of male weanling rats fed 3 diets (CO, SO, SL) for 4 months. In the third, the diet was fed through the second generation. Weanling female rats were maintained on either CO, SO or SL throughout growth, pregnancy, delivery and lactation. When weaned at 21 days of age, both male and female offspring were placed on the same diets as the mothers for another 50–70 days. Upon completion of the feeding trials, the animals were sacrificed and brain, liver, spleen, and thymus quickly excised. The tissue was weighed and used for determination of PGE_2 or $PGF_{2\alpha}$ synthesizing capacity. Half of the liver and spleen were frozen in dry ice/acetone and stored at -20 C for lipid analysis.

Lipid Analysis

Although it is well established that the feeding of 18:3ω3 is reflected in the fatty acid composition of tissue lipids (1,2), samples of liver and spleen were examined for fatty acid changes. The tissues were extracted with (2:1 v/v chloroform/methanol) (14). The ethanolamine phosphoglycerides (EPG) were collected after separation of the lipids using 2-dimensional thin layer chromatography (TLC) (15). This phospholipid was examined because it is known that higher levels of 18:2ω6 and 18:3ω3 elongated and desaturated products reside in this lipid fraction relative to the other major phospholipids. Transmethylation was carried out in 4% sulfuric acid in methanol by heating for 1 hr on a steam bath. The dimethylacetals were removed after separation by TLC using toluene as the solvent. The methyl esters were analyzed by gas liquid chromatography (GLC) using a Hewlett-Packard chromatograph model 7610A equipped with a 180 cm X 0.4 cm glass column packed with 10% SP-2340 on 100/120 Chromo-

sorb W AW (Supelco, Inc., Bellefonte, PA) and operated isothermally at 185 C. The methyl esters were identified by means of equivalent chain lengths based on the retention times of standards (NuChek Prep, Elysian, MN, and Supelco, Inc., Bellefonte, PA). Percent of fatty acids was obtained by triangulation.

The fat was extracted from the diets in a Soxhlet apparatus with diethyl ether as the solvent. The methyl esters were prepared and the fatty acid composition was determined to check that it was the same as the fresh oil.

Determination of PG

Spleen and thymus PGE_2 and liver and brain $PGF_{2\alpha}$ were determined by radioimmunoassay (RIA) using Miles-Yeda antisera (Kiryat, Weizmann, Rehovot, Israel) as described in detail by Weston and Johnston (16). As determined in this laboratory, the Miles-Yeda PGE_2 antiserum had a cross-reactivity of 10% with both PGE_1 and PGE_3. The $PGF_{2\alpha}$ antiserum (Miles–Yeda) was specific for $PGF_{1\alpha}$ and $PGF_{2\alpha}$ and had only 3% and 5% cross-reactivity with PGE_1 and PGE_2. No correction for cross-reactivities was made. Standard PG were provided by Dr. John E. Pike of the Upjohn Company, Kalamazoo, MI.

Tritiated $PGF_{2\alpha}$ (178.0 Ci/mmol) and PGE_2 (165.0 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Standard curves were constructed by plotting the percentages of the ^{3}H-labeled PG bound against the concentration of standard PG added. Samples for assay were diluted so that PG extrapolations would be made from the most linear portion of the standard cruve to provide good precision and reliability. Three serial dilutions made of selected samples demonstrated parallel curves to that of the PG standard curve. Recovery of various PG concentrations added to selected samples always approached 100%. Assay results were determined using logit transformation and linear regression of the standard PG curve.

The liver, spleen and thymus samples were prepared in a similar manner. The only difference was the time of incubation used, which was based on preliminary investigations constructing plots of time vs. PG production. The times selected were those at which the PG in the homogenate were still increasing linearly, and where we could qualitatively evaluate PG synthesizing capacity. A portion of the tissue was weighed and homogenized at 4 C with 10 vol of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) One ml of homogenate was directly added to 0.5 ml of a 42 mM aspirin-potassium phosphate buffer solution, resulting in a final concentration of 14 mM aspirin (17). Preliminary investigations showed no significant difference in PG production between tissue homogenized in the presence of the PG inhibitor, aspirin, and tissue homogenization followed by prompt addition to the aspirin solution. The latter method was chosen as being the most efficient and acted as a zero control representing the PG formed primarily during excision and weighing, as well as homogenization. One ml of the homogenate was also added to a tube without aspirin and both the zero control tube and the tube containing homogenate alone were incubated in a water bath shaker at 37 C; the spleen and thymus for 30 min and liver for 15 min. At the end of the incubation period, 0.5 ml of the aspirin solution was added to the tubes containing only homogenate. Selected liver homogenate samples from rats fed 2 months were also incubated for 15 min in the presence of either 99% arachidonic acid or 99% 5,8,11,14,17-eicosapentaenoic acid (EPA) (NuChek Prep., Inc., Elysian, MN), at a final concentration of 50 μM. All tubes were stored at −20 C until analyzed. Prostaglandin E production by the 2 immune organs was analyzed because this is the main PG of interest in cellular immune interactions (18). Preliminary investigations showed that $PGF_{2\alpha}$ is produced in greater concentrations in the liver than either PGE_2 or PGE_1 and therefore it was determined. The data were expressed as PG produced/mg of wet tissue weight corrected for the production of PG in the zero control. The brain was analyzed for $PGF_{2\alpha}$ production using 0.5 mm thick brain slices cut from each hemisphere with a Stadie-Riggs microtome at room temperature in a humid chamber (19). One-half of each slice was weighed and placed in 3 ml of Krebs-Ringer phosphate medium, pH 7.4. The other half slice was weighed and incubated in 3 ml of the same medium, acidified to pH 1–2 with 1 N HCl. The slices were gently shaken at a constant speed in a 37 C water bath in air for 30 min (16). At the end of the incubation, the tissue slices were removed by filtration over glass wool and the incubation medium stored at −20 C until analyzed. The determination of the incubation time point as well as the validation of sample preparation and RIA analysis of the incubation medium are described in detail by Weston and Johnston (16). Several investigators including Wolfe (20) have shown that $PGF_{2\alpha}$ may be the main PG of significance in vivo, and, therefore, $PGF_{2\alpha}$ was analyzed by RIA. The values were corrected for production during excision and slice preparation by subtracting the production in the other half slice after stopping synthesis by bringing the medium to pH 1–2.

TABLE 2

Effect of α-Linolenate Feeding on Lewis Rat Body Weights

	Dietary treatment[f]			
	CO	SO	SL	LO
Two-month study[b]	396 ± 6[a] (11)	388 ± 10 (11)	384 ± 7 (11)	383 ± 9 (11)
Four-month study[c]	478 ± 20 (10)	461 ± 55 (9)	476 ± 21 (8)	
Second-generation study[d,e]	307 ± 24 (6)	228 ± 13 (8)	268 ± 20 (12)	

[a]Mean ± SEM ( ) = number of animals.

[b]No significant difference.

[c]No significant difference.

[d]Significant difference due to dietary treatment as determined by ANOV ($P<0.10$). See text for explanation.

[e]CO significantly different from SO, Duncan's Multiple Range Test ($P<0.01$). See text for explanation.

[f]CO = 10% corn oil, SO = 10% soybean oil, SL = soybean/linseed oil mixture, LO = 10% linseed oil.

TABLE 3

Effect of Different Ratios of Dietary α-Linolenic Acid to Linoleic Acid on Liver Ethanolamine Phosphoglyceride Fatty Acid Composition[a]

Diet[f]	CO (n=3)	SO (n=3)	SL (n=3)	LO (n=3)	P Value
Fatty Acid					
16:0	20.1 ± 1.3[b]	19.6 ± 0.5	17.2 ± 1.1	19.7 ± 1.0	NS[c]
18:0	14.8 ± 0.8	16.1 ± 1.0	18.1 ± 0.5	19.2 ± 0.6	P<0.025[d]
18:1ω9	6.1 ± 1.0	4.9 ± 0.4	5.7 ± 0.8	5.4 ± 0.1	NS
18:2ω6	11.4 ± 1.4	11.1 ± 0.8	9.4 ± 1.4	11.2 ± 1.2	NS
18:3ω3	1.0 ± 1.0	0.2 ± 0.2	1.2 ± 0.2	2.2 ± 0.2	NS
20:4ω6	30.7 ± 2.5	31.7 ± 1.5	20.4 ± 1.5	13.7 ± 0.8	P<0.005
20:5ω3	ND[e]	2.1 ± 0.1	6.1 ± 0.6	11.9 ± 0.9	P<0.005
22:3ω6	3.0 ± 0.2	0.0 ± 0.2	ND	ND	P<0.005
22:4ω6	5.4 ± 0.3	0.1 ± 0.1	ND	ND	P<0.005
22:5ω3	2.2 ± 1.6	1.4 ± 0.7	3.6 ± 0.6	4.4 ± 0.5	NS
22:6ω3	5.1 ± 1.2	12.6 ± 1.6	14.8 ± 1.5	12.0 ± 1.7	P<0.010

[a]Results of a 2-month feeding study.

[b]Mean ± SEM, precent of total.

[c]NS = not significant.

[d]P value is the result of ANOV.

[e]ND = not detectable.

[f]CO = 10% corn oil, SO = 10% soybean oil, SL = soybean/linseed oil mixture, LO - 10% linseed oil.

Statistical Analysis

All data were analyzed using ANOV and, where appropriate, Duncan's Multiple Range Test. Data are expressed as mean ± standard error of the mean (SEM).

RESULTS

The means of the body weights of the Lewis rats at the end of each trial are shown in Table 2. There was no significant dietary effect of α-linolenate on body weight. The variation in body weights of rats fed throughout the 2 generations is due to differences in age. The animals fed the soybean oil diet were approximately 2 weeks younger than the animals on the other dietary treatments when the study was terminated.

Tissue Fatty Acid Composition

The fatty acid composition of liver EPG

TABLE 4

Effect of Different Ratios of Dietary α-Linolenic Acid to Linoleic Acid on Liver Ethanolamine Phosphoglyceride Fatty Acid Composition[a]

Diet[f]	CO (n=3)	SO (n=3)	SL (n=3)	P Value
Fatty acid				
16:0	30.0 ± 4.9[b]	21.6 ± 1.9	28.1 ± 1.1	P<0.05[d]
18:0	18.3 ± 1.8	17.8 ± 2.1	18.1 ± 1.1	NS[c]
18:1ω9	4.8 ± 0.6	8.7 ± 3.0	5.0 ± 0.4	NS
18:2ω6	6.0 ± 0.9	6.3 ± 1.2	12.8 ± 1.5	P<0.025
18:3ω3	0.5 ± 0.2	0.3 ± 0.3	1.1 ± 0.2	P<0.01
20:4ω6	28.4 ± 0.6	26.5 ± 1.6	22.1 ± 1.3	P<0.05
20:5ω3	0.2 ± 0.2	0.4 ± 0.2	5.4 ± 0.4	P<0.005
22:3ω6	2.0 ± 0.2	0.4 ± 0.2	ND[e]	P<0.005
22:4ω6	4.0 ± 0.8	0.4 ± 0.3	ND	P<0.005
22:5ω3	0.3 ± 0.3	2.3 ± 0.5	3.5 ± 0.4	P<0.005
22:6ω3	4.9 ± 0.8	14.2 ± 1.4	12.3 ± 1.3	P<0.005

[a]Results obtained from a 4-month feeding study.
[b]Mean ± SEM, percent of total.
[c]NS = not significant.
[d]P value is a result of ANOV.
[e]ND = not detectable.
[f]CO = 10% corn oil, SO = 10% soybean oil, SL = soybean/linseed oil mixture.

from animals fed for 2 months (Table 3) shows a significant increase of 20:5ω3, from nondectable levels in the CO group to 6.1 ± 0.6% in the SL group and 11.9 ± 0.9% in the LO group. Docosahexaenoic acid (22:6ω3) increased from 5.1 ± 1.2% (CO) to ca. 13% in both SL and LO groups. Arachidonic acid, on the other hand, was depressed from the control levels of 30.7 ± 2.5% to 13.7 ± 0.8% in the LO group. The other ω6 metabolites were depressed to nondetectable levels in the high α-linolenate group. The saturated fatty acids were essentially unaffected except in the case of 18:0, in which a slight increase was observed in the LO group (19.2 ± 0.6%) when compared to the CO group (14.8 ± 0.8%).

After 4 months of high α-linolenate relative to linoleate feeding the liver EPG fatty acid composition (Table 4) showed the same changes as in the 2-month trial. In the SL group, ω3 fatty acids all increased to levels equal to those observed in the 2-month study. Arachidonate, and the other ω6 fatty acids decreased with the high α-linolenate diet, again to levels observed in the 2-month feeding trial.

The effect of feeding dietary 18:3ω3 through the second generation can be seen in the spleen EPG fatty acid composition (Table 5). Arachidonic acid was significantly depressed from 26.9 ± 1.6% in the CO group to 21.3 ± 2.4% in the SL group (P<0.005). The same trend was shown in 22:4ω6 levels. The ω3 fatty acids, 22:5ω3 and 22:6ω3, increased from barely detectable levels in the CO group to 5.6 ± 0.6% (P<0.05) and 2.4 ± 0.3% in the SL group (P<0.01). Eicosapentaenoic acid (20:5ω3) was present only in small amounts in the spleen.

Synthesis of PG by Tissues

Consistent with the change in fatty acid composition in only 2 months, the liver $PGF_{2\alpha}$ synthesis (Table 6) was significantly depressed by high dietary levels of 18:3ω3 when compared to synthesis by the CO group. The LO group $PGF_{2\alpha}$ synthesis (195.6 ± 20.7 pg/mg tissue) was reduced to less than one-half the values of the CO group (470.1 ± 56.6 pg/mg tissue). The $PGF_{2\alpha}$ synthesis by liver of rats fed for 4 months showed a statistically significant depression even though the highest 18:3ω3 level fed was the SL diet. When arachidonate was added to the liver homogenate samples from animals fed the CO diet and the LO diet, there was no statistically significant difference between the elevated levels of $PGF_{2\alpha}$ produced (pg $PGF_{2\alpha}$/mg tissue) (CO, 1124.8 ± 148.7; LO, 803.9 ± 86.4). Eicosapentaenoic acid addition to samples from the two dietary groups resulted in PG production by the CO group (605.5 ± 55.1 pg/mg tissue) which was still statistically (P<0.0005) higher than the LO group (168.8 ± 31.1 pg/mg tissue). However, when the CO liver homogenate was incubated with EPA and compared to the values obtained from CO samples incubated for 15 min with no additions, the PG production was significantly reduced by one-third on EPA addition (P<0.05).

TABLE 5

Effect of Different Ratios of Dietary α-Linolenic Acid to Linoleic Acid on Spleen Ethanolamine Phosphoglyceride Fatty Acid Composition[a]

Diet[e]	CO (n=3)	SO (n=3)	SL (n=3)	P Value
Fatty Acid				
16:0	19.9 ± 2.7[b]	13.7 ± 1.9	16.7 ± 2.5	NS[c]
18:0	17.9 ± 0.8	18.9 ± 2.1	18.3 ± 2.2	NS
18:1ω9	7.9 ± 0.8	10.5 ± 0.6	9.6 ± 0.2	NS
18:2ω6	9.3 ± 0.3	10.4 ± 1.6	10.1 ± 1.5	NS
18:3ω3	0.9 ± 0.4	1.7 ± 0.2	3.0 ± 1.8	NS
20:2ω6	1.4 ± 0.1	1.4 ± 0.4	2.1 ± 0.1	NS
20:3ω6	3.5 ± 0.7	2.2 ± 0.5	4.5 ± 0.3	NS
20:4ω6	26.9 ± 1.6	27.2 ± 1.7	21.3 ± 2.4	P<0.005[d]
20:5ω3	1.1 ± 0.4	1.2 ± 1.1	1.7 ± 0.1	NS
22:4ω6	7.4 ± 0.9	6.4 ± 0.5	3.6 ± 0.6	P<0.01
22:5ω3	0.4 ± 0.3	2.2 ± 0.6	5.6 ± 0.6	P<0.05
22:6ω3	0.7 ± 0.4	2.8 ± 0.4	2.4 ± 0.3	P<0.01

[a]Results obtained from the second generation dietary study.

[b]Mean ± SEM, percent of total.

[c]NS = not significant.

[d]P value is a result of ANOV.

[e]CO = 10% corn oil, SO = 10% soybean oil, SL = soybean-linseed oil mixture.

No difference was noticed between the LO sample incubated with EPA for 15 min and the LO liver homogenate incubated with no additions for 15 min.

The values presented in Table 6 are corrected for liver PG produced during excision, weighing, and homogenization. These aspirin control values were consistently 10-fold lower than the values obtained after 15 min of incubation at 37 C (2-month study (CO) 35.7 ± 5.5, (SL) 28.8 ± 31.1, (LO) 25.9 ± 4.6; 4-month study (CO) 51.1 ± 8.4, (SO) 35.0 ± 5.0, (SL) 30.6 ± 3.3 pg $PGF_{2\alpha}$/mg tissue). In view of this rapid response of the liver to the diets, it was not examined in the second-generation rats.

Because of the known resistance of neural lipids to short-term diet-induced change, brain $PGF_{2\alpha}$ was not determined after the 2-month trial. After 4 months on the diets, the brain $PGF_{2\alpha}$ still showed no response. However, when the diets were fed to rats through the second generation, brain slice $PGF_{2\alpha}$ production was depressed when fed a high dietary ratio of α-linolenate to linoleate ($P<0.05$) (Table 6). The PG produced by the slice in the acidified control incubation medium was 4- to 10-fold lower than the amount produced in ng $PGF_{2\alpha}$/100 mg of wet tissue weight by the incubated slice (4-month study (CO) 0.4 ± 0.1, (SO) 0.5 ± 0.1, (SL) 0.4 ± 0.1; second-generation study (CO) 1.4 ± 0.3, (SO) 1.7 ± 0.8, (SL) 1.6 ± 0.3).

The PGE_2 of both the spleen and thymus tissue homogenate represent production by nonchallenged immune tissue. Therefore, the values obtained after 30 min of incubation were only 2- to 3-fold higher than the aspirin control. Control values for both thymus and spleen after 2 months of feeding in pg PGE_2/mg tissue were (CO) 65.3 ± 8.1, (SL) 64.1 ± 8.2, (LO) 73.2 ± 9.8 and (CO) 35.1 ± 4.7, (SO) 27.4 ± 3.8, (SL) 26.7 ± 2.9, (LO) 20.8 ± 2.0, respectively. The second-generation values for rat aspirin-control spleen homogenate PG production were (CO) 11.7 ± 4.6, (SO) 9.1 ± 1.4 and (SL) 5.7 ± 1.2 pg PGE_2/mg tissue. Spleen PGE_2 synthesis showed a trend toward being depressed as more 18:3ω3 was added to the diet (Table 6). Synthesis of PGE_2 by the thymus also showed this trend but was not statistically significant (Table 6). Neither tissue was, therefore, examined until the second-generation feeding trial. The PGE_2 production by spleens from the SL group was then found to be reduced to approximately one-half (59.3 ± 8.1 pg/mg tissue) that of the CO group (106.5 ± 18.9 pg/mg tissue).

DISCUSSION

High dietary ratios of 18:3ω3 to 18:2ω6 effectively decrease the incorporation of ω6, and especially 20:4ω6, into tissue lipids. This finding is consistent with the preference of the Δ6 desaturase for the ω3 series. As the precursor of the 2-series PG is decreased, the 3-series precursor, 20:5ω3, is reciprocally increased. Moreover, the PG synthetase system

TABLE 6

Prostaglandin Synthesis by Liver, Spleen and Thymus Homogenates and Brain Slices of Rats Fed 10% Corn Oil (CO), 10% Soybean Oil (SO), 10% Soybean/Linseed Mixture (SL) or 10% Linseed Oil (LO) Diets for 2 Months, 4 Months, or Through the Second Generation

Diet (2 months)	CO	SO	SL	LO
		$PGF_{2\alpha}$ pg/mg tissue[b]		
Tissue				
Liver[c]	470.1 ± 56.6[a] (10)	441.4 ± 32.2 (7)	298.6 ± 28.9 (10)	195.6 ± 20.7 (9)
		PGE_2 pg/mg tissue[d]		
Thymus[e]	122.2 ± 25.8 (9)	103.7 ± 11.4 (8)	100.8 ± 20.6 (9)	86.6 ± 10.6 (9)
Spleen[e]	83.0 ± 26.2 (4)	–	63.3 ± 8.7 (7)	45.9 ± 11.8 (8)

Diet (4 months)	CO	SO	SL
	$PGF_{2\alpha}$ pg/mg tissues[b]		
Tissue			
Liver[f]	368.9 ± 46.5 (9)	248.6 ± 34.6 (9)	153.6 ± 24.1 (9)
	ng $PGF_{2\alpha}$/100 mg of wet tissue weight[d]		
Brain slices[g]	3.2 ± 0.7 (9)	3.6 ± 0.5 (9)	4.7 ± 0.9 (9)

Diet (2nd generation)	CO	SO	SL
	PGE_2 pg/mg tissue[d]		
Tissue			
Spleen[h]	106.5 ± 18.9 (6)	103.4 ± 18.5 (9)	59.3 ± 8.1 (11)
	ng $PGF_{2\alpha}$/100 mg of wet tissue weight[d]		
Brain slices[i]	3.4 ± 0.7 (4)	2.5 ± 0.4 (4)	1.5 ± 0.2 (10)

[a]Mean ± SEM. ( ) = Number of animals.

[b]Synthesis after 15-min incubation and corrected for zero-time synthesis.

[c]Significant difference ($P<0.005$), CO significantly different from SL and LO, $P<0.01$, SO significantly different from SL and LO, $P<0.01$, Duncan's Multiple Range Test.

[d]Synthesis after 30-min incubation and corrected for zero-time synthesis.

[e]No significant difference as analyzed by ANOV.

[f]Significantly different, $P<0.01$, (ANOV); CO significantly different from SO and SL ($P<0.05$), Duncan's Multiple Range Test.

[g]No significant difference as analyzed by ANOV.

[h]Significant difference, $P<0.025$, (ANOV); CO and SO significantly different from SL ($P<0.01$), Duncan's Multiple Range Test.

[i]Significant difference, $P<0.05$, (ANOV); CO and SO significantly different from SL ($P<0.01$), Duncan's Multiple Range Test.

has been shown to prefer 20:5ω3 over 20:4ω6, particularly when peroxide tone is high (21,22). Effective competion of the 20:5ω3 for the PG synthetase may further reduce the PG_2 series synthesizing ability of the tissues. It may be that 3-series PG and lipoxygenase products of 20:5ω3 are produced at the expense of 1- and 2-series PG and other 20:3ω6 and 20:4ω6

products. Unfortunately, we do not presently have the means of measuring 3-series PG, nor is there any strong evidence that they are produced or possess a function in vivo. At this time, investigative efforts (12) indicate that the presence of the $\omega 3$ fatty acids may primarily act as a physiological mode for regulating PG biosynthesis.

The results of this study are essentially in agreement with the findings of Galli et al. (10) and Hwang and Carroll (11). Galli et al. fed a diet in which linolenic acid (linseed oil) made up 10.2% of the caloric intake. They induced in vivo brain $PGF_{2\alpha}$ production by the administration of a convulsive drug and measured the concentration after killing the animals by microwave radiation. They found that the 20:$4\omega 6$ released in the brain after ischemia was reduced in the rats fed linseed oil and suggested a preference of the phospholipase for 20:$5\omega 3$. Hwang and Carroll (11) fed rats an EFA-deficient diet for 15 weeks and then fed graded amounts of methyl linolenate daily for 6 weeks. They then measured the serum concentration of PG after incubation at 37 C. Although these studies differ from ours in a number of respects, they all demonstrate that the higher ratio of α-linolenate to linoleate inhibits the synthesis of PG and thromboxane of the 2-series. We have demonstrated lowered PG synthesizing capacity of several tissues by feeding a higher α-linolenate to linoleate ratio. The length of feeding trial and amount of α-linolenate required to demonstrate lowered PG synthesis has been shown to depend on the tissue of interest. This is primarily due to the variation in the rate of dietary lipid uptake by the tissue. Once the dietary fatty acid is incorporated into the phospholipids, the tissue quickly elongates and desaturates to the more unsaturated metabolites. When the tissue is stimulated to produce PG, both the presence of available substrate and the relative concentration of competitive inhibitors influence the quantity, and perhaps the type, of PG produced. When arachidonic acid was added to both CO and LO homogenates, no difference in PG production was observed. This indicates that the α-linolenate effect is not directly on the enzyme complex, but is primarily through arachidonate availability. This was further strengthened by the observations made when EPA addition to CO group liver homogenates reduced the PG production to two-thirds that of the CO liver homogenate incubated alone for 15 min.

Prostaglandins are known to play a role in events of the immune responses (23). The changes induced in the immune tissues by feeding the high α-linolenate to linoleate diets were not observed until extensive feeding trials were completed. The reduction in PG synthesis by the LO groups was one-half that of the CO groups, but this is not surprising in view of their naive or nonimmunochallenged state. Osheroff et al. (24) demonstrated greatly enhanced $PGF_{2\alpha}$ production by whole spleen homogenate when they challenged mice in vivo with sheep erythrocytes as compared to mice injected with saline solution. Since altered PG synthesis can be accomplished by feeding the $\omega 3$ fatty acids, we may be able to modify specific functions of immune cell populations in which PG play a regulatory role. We are presently investigating the effects of both in vivo and in vitro stimulation of immunocompetent cell populations, which are purified from animals fed diets with high ratios of α-linolenate to linoleate.

ACKNOWLEDGMENT

This research was supported by the Science and Education Administration of the US Department of Agriculture under Grant Number 5901-0410-8-0061-0 from the Competitive Research Grants Office.

REFERENCES

1. Tinoco, J., Babcock, R., Hincenbergs, J., Nedwadowski, I., and Miljomuch, P. (1978) Lipids 13, 6-17.
2. Tinoco, J., Babcock, R., Hincenbergs, I., Medwadowski, B., Miljomuch, P., and Williams, M.A. (1979) Lipids 14, 166-173.
3. Fiennes, R.N., Sinclair, A.J., and Crawford, M.A. (1973) Med. Primatol. 2, 155-169.
4. Lamptey, M.S., and Walker, B.L. (1976) J. Nutr. 102, 86-93.
5. Crawford, M.A., and Sinclair, A.J. (1972) in Lipids, Malnutrition and the Developing Brain, (A Ciba Foundation Symposium jointly with the Nestle Foundation), pp. 267-292. Elsevier, Amersterdam.
6. Crawford, M.A., Msuya, P.M., and Munhambro, A. (1973) in Dietary Lipids and Postnatal Development (Galli, C., Jacini, and Pecile, A., eds.), pp. 41-56, Raven Press, New York.
7. Rivers, J.P.W., and Frankel, T.L. (1980) in Nutrition of the Dog and Cat (Anderson, R.S., ed.), pp. 67-99, Pergamon Press, Oxford.
8. Mead, J.F. (1971) Prog. Chem. Fats Other Lipids 9, 159-192.
9. Crawford, M.A., Hassan, A.G., and Rivers, J.P.W. (1978) Am. J. Clin. Nutr. 31, 2181-2185.
10. Galli, G., Spagnuolo, C., Bosisio, E., Tosi, L., Folco, G.C., and Galli, G. (1978) in Advances in Prostaglandin and Thromboxane Research (Coceani, E., and Olley, P.M., eds.), Vol. 4, pp. 181-189, Raven Press, New York, NY.
11. Hwang, D.H., and Carroll, A.E. (1980) Am. J. Clin. Nutr. 33, 590-597.
12. Marshall, L.A., and Johnston, P.V. (1982) Prog. Lipid Res. 20, 731-733.
13. American Oil Chemists' Society Official Method Cu-8-53 (1977 Peroxide Value), Tentative Methods of the American Oil Chemist Society Sampling and Analysis of Vegetable Oil Source

Material, Section A, American Oil Chemists' Society, Champaign IL.
14. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–502.
15. Rouser, G., Kritchevksy, G., Galli, C., and Heller, D. (1965) Am. Oil Chem. Soc. 42: 215–227.
16. Weston, P.G., and Johnston, P.V. (1978) Lipids 13, 408–414.
17. Mathias, M.M., and Dupont, J. (1979) Lipids 14, 247–252.
18. Morley, J., Bray, M.A., Jones, R.W., Nugteren, D.H., and van Dorp, D.A. (1979) Prostaglandins 730–736.
19. Elliot, K.A.C. (1969) in Handbook of Neurochemistry (Laj A., ed.) Vol. 2, pages xx-xx, Plenum Press, New York, NY.
20. Wolfe, L.S. (1978) in Advances in Prostaglandins and Thromboxane Research (Coceani, F., and Olley, P.M., eds.) Vol. 4, pp. 215–220, Raven Press, New York, NY.
21. Culp, B.R., Titus, B.G., and Lands, W.E.M. (1979) Prostaglandin Med. 3, 269–278.
22. Lands, W.E.M. (1981) in Nutritional Factors: Modulating Effects on Metabolic Processes (Beers, R.F., and Bassett, E., eds.) pp. 489–493, Raven Press, New York, NY.
23. Stenson, W.F., and Parker, C.W. (1980) J. Immun. 125, 1–5.
24. Osherhoff, P.L., Webb, D.R., and Paulsrud, J. (1975) Biochem. Biophys. Res. Commun. 66, 425–429.

[Received March 8, 1982]

Fatty Acid and Phospholipid Composition of *Bacillus megaterium* Spores with Altered Germination Properties

J.F. SKOMURSKI and **J.C. VARY***, *Department of Biochemistry, University of Illinois Medical Center, Chicago, IL 60612*

ABSTRACT

The ability of spores to trigger germination was altered by growing spores at either a suboptimal temperature or in a rich medium modified by substituting L-isoleucine for D-glucose. Compared to the control, spores grown in the presence of isoleucine germinated more readily between 20 and 28 C, while spores grown at 20 C germinated slower than the control at any temperature tested. Analysis of the composition of these spores indicated that spores grown in the presence of isoleucine had much higher levels of anteiso-C15 fatty acids than the control, while the phospholipid composition and the phospholipid to protein ratio were unchanged. The fatty acid composition for spores grown at 20 C was comparable to that of the control spores, but the levels of diphosphatidylglycerol and phosphatidylglycerol were altered as well as the ratio of phospholipid to protein. Steady-state fluorescent anisotropy measurements were made with 1,6-diphenyl-1,3,5-hexatriene incorporated into membrane isolated from these spores. The membranes from spores grown in the presence of isoleucine were more "fluid" between 10 and 20 C than membranes from the control spores. Membranes from 20 C grown spores were less "fluid" between 10 and 38 C than membranes from the control spores. These results show that triggering of spore germination was altered by growing spores under conditions that altered the composition of spore membranes.

Lipids 17:914-923, 1982.

INTRODUCTION

Bacterial spore germination is triggered by a few specific amino acids or carbohydrates (germinants). Triggering has been hypothesized to entail a stereospecific interaction of the trigger compound with a receptor located in the spore inner membrane and a subsequent alteration of the permeability properties of the membrane (1,2). Support for this model has been furnished by the following observations. First, a membrane protein(s) that may be involved in the trigger reaction was found to be differentially labeled by ^{3}[H]-proline chloromethyl ketone, an affinity analog of proline, when spores capable of being triggered for germination on L-proline were compared to mutant spores unable to trigger on L-proline (3). A similar membrane protein(s) was reported to be differentially labeled by acetylation of spores with ^{3}[H]acetic anhydride (4). Second, inhibition of spore germination by lipophilic compounds, medium chain lengths alcohols (5) and inert gases (6) led to the conclusion that triggering involved a membrane-associated enzyme(s) or protein(s). Third, spectroscopic studies using both ESR (2) and fluorescence depolarization (4) showed that purified spore membranes reacted specifically with known germinants in vitro. Finally, the earliest events during triggering are the extrusion of ions, apparently resulting from permeability changes in the spore inner membranes (7).

Changes in membrane "fluidity" have been reported to influence membrane-associated events such as transport (8,9), enzyme activity (10) and receptor mediated responses to external stimuli (11). Therefore, the chemical composition of spore membranes was altered in vivo to test the effect of altered membrane properties on spore germination. The composition of membranes was changed by growing spores under different growth conditions that are known to change the composition of bacterial cell membranes.

*Author to whom correspondence should be addressed.

Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; HEPES, N-2-hydroxyethylpiperazine-N'-2'-ethane sulfonic acid; Tris, tris (hydroxymethyl) amino methane: n-C14, tetradecanoic acid; i-C14, 12-methyltridecanoic acid; n-C15, pentadecanoic acid; i-C15, 13-methyltetradecanoic acid; a-C15, 12-methyltetradecanoic acid; n-C16, hexadecanoic acid; i-C16, 14-methylpentadecanoic acid; i-C17, 15-methylhexadecanoic acid; a-C17, 14-methylhexadecanoic acid; t½, time required during germination for 50% change of the total adsorbance at 660 nm in 30 min; SNB, supplemented nutrient broth medium; SNI, supplemented broth medium with isoleucine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; and Glc-NH_2-PG, glucosaminylphosphatidylglycerol.

Bacillus megaterium QM B1551 can be enriched for branched-chain fatty acids by supplementing the growth medium with branched-chain amino acids (12). Since anteiso branched-chain fatty acids have been suggested to play a comparable role to unsaturated fatty acids in modulating membrane fluidity in other organisms (13–16), we attempted to enrich the spore membrane for the lower melting fatty acids (a-C15 and a-C17) by adding L-isoleucine to the growth medium. Supplementation of the growth medium with valine also alters the fatty acid composition of vegatative cells and spores by increasing the amounts of branched fatty acids (12). We chose to test the effect of isoleucine supplementation because it is the precursor to anteiso fatty acids which should have the largest effects on fatty acid packing density in the membranes. Isoleucine supplementation also caused the largest changes in branched-chain fatty acid composition of sporulating cells when compared to growth in either valine or glucose (12). Additionally, the effect of growth at different temperatures on the composition of the spore membranes was examined, similar to experiments reported for other bacteria (17,18) but never tested in *B. megaterium*. The results of these studies suggest that some component(s) of the trigger reaction for spore germination is sensitive to changes in the composition of the spore inner membranes.

METHODS AND MATERIALS

Organisms, Growth Conditions and Germination Assay

B. megaterium QM B1551 spores were grown in SNB medium containing D-glucose, salts and nutrient broth at 30 C in Erlenmeyer flasks in a constant temperature shaker as previously described (19). To alter the fatty acid composition of spores, spores were grown at 30 C in the above medium modified by substituting L-isoleucine at 1 mg/ml (SNI medium) for D-glucose (12). Spores were also grown in SNB medium at 20, 25, 30, 33.5 and 37 C and they were harvested and washed after 72, 48, 36, 33 and 24 hr of growth, respectively. Spores were harvested, washed and lyophilized as previously described (19) and stored dry in vacuo. All references to spore weights are on a dry weight basis.

Vegetative cells were grown at 30 C in SNB or in SNI media to an adsorbance of about 1 at 660 nm, harvested by centrifugation at 5,000 X g for 5 min, washed once with a minimal salts medium (20) and used immediately for fatty acid analysis or membrane isolation.

Triggering of spore germination was measured spectrophotometrically as previously described (19) with spores that were heat-activated for 10 min at 60 C. To determine the effect of altered sporulation conditions on the temperature requirements for triggering germination, spores heat-activated for 10 min at 60 C were added to a germination solution preequilibrated to the desired temperature and triggering of germination followed spectrophotometrically as above.

Fatty Acid Analysis

Spore fatty acids were saponified by refluxing 100 mg of spores for 4 hr in 150 ml of a 10% (v/v) KOH, 50% (v/v) CH_3OH solution (21), containing 200 μg of octadecanoic acid as an internal standard. Octadecanoic acid was used because *B. megaterium* QM B1551 contains only trace amounts of this fatty acid (12). Similarly, vegetative cell fatty acids from 200 ml of cell culture were saponified in 50 ml of methanolic KOH. The fatty acids were extracted and converted to the corresponding methyl ester with diazomethane as previously described (22).

Fatty acid methyl esters were analyzed on a Hewlett-Packard gas chromatograph (Model 5730A) equipped with a flame ionization detector and Model 7123A recorder. Separation of fatty acid methyl esters was performed isothermally at 185 C on a glass column (6 ft X 1/8 in.) packed with 10% diethylene glycol succinate on 80/100 Chromosorb W AW DMCS (Supelco Inc.). The injection port and detector temperature were operated at 250 and 300 C, respectively. Nitrogen at 18 ml/min was the carrier gas.

Spore fatty acid methyl esters were identified by comparing retention times to known standards and the percent composition of individual fatty acids was determined by calculating the peak area of each fatty acid by triangulation (21). Total fatty acids were quantitated by comparison with a known amount of octadecanoic acid added prior to saponification. The reliability of octadecanoic as a standard was confirmed by calculating the recovery of [1-^{14}C] octadecanoic acid (60,000 CPM) added prior to saponification and the recovery was greater than 90%.

Membrane Isolation and Labeling with DPH

Membranes were isolated from heat-activated spores by lysis with lysozyme and sonication as previously described (23,24) except that 2 mM phenylmethylsulfonyl fluoride was added during lysis (4). These procedures yield mainly spore inner membranes that are biologically active as judged by marker enzyme assays (24) and amino acid transport (Skomurski et al., submitted).

TABLE 1

Fatty Acid Composition of *B. megaterium*

Fatty acid	Percentage of total fatty acids[a] Vegetative cells[b] SNB	SNI	Spores[c] SNB	SNI
i-C14	9.0	–[d]	16.6	1.6
n-C14	10.8	14.9	34.0	37.5
i-C15	20.5	1.7	14.4	1.8
a-C15	36.2	64.0	17.8	42.6
n-C15	1.0	–	–	4.0
i-C16	3.7	–	5.9	–
n-C16	13.9	10.4	7.1	7.1
i-C17	1.5	–	2.0	1.1
a-C17	3.3	9.6	2.2	4.5
Grouped Components				
Branched				
Iso	34.7	1.7	38.9	4.5
Anteiso	39.5	71.6	19.9	47.1
Unbranched	25.7	25.3	41.1	48.6

[a]Values are the mean of at least 2 determinations and the range was within ± 5% of the mean. Recovery was greater than 90% as measured with octadecanoic acid added before saponification.

[b]Vegetative cells were grown in SNB or SNI media at 30 C and harvested during exponential growth as described in Methods.

[c]Spores were grown in SNB or SNI media at 30 C as described in Methods.

[d]Constitutes trace amounts.

Membranes from vegetative cells were isolated from 1 ℓ of cells grown in SNB or SNI medium at 30 C to an adsorbance of 1 at 660 nm. The cells were centrifuged at 5,000 X g for 5 min, washed once with a minimal salts medium (20) and the membranes were isolated in the same way as for spore membranes.

The membranes, after purification on a sucrose gradient, were suspended in about 1 ml of 10 mM HEPES (pH 7.5), incubated with DPH for 15 min at 25 C in a Ultramet III bath sonicator (Buehler Ltd.), and centrifuged at 304,000 X g for 2 hr to remove unincorporated DPH as previously described (4). The membrane pellets were resuspended in 10 mM HEPES, pH 7.5, at a protein concentration of 15–20 mg/ml determined by the method of Lowry et al. (25) and used within 2–3 days.

Fluorescence Depolarization Studies

Steady-state fluorescence anisotropy of DPH incorporated into spore and vegetative membranes was determined as previously described (4, Skomurski et al., submitted). Briefly, DPH labeled membranes were diluted with 10 mM HEPES, pH 7.5, to 2 ml to yield a final protein concentration of 0.5 mg/ml, placed in a 1 cm cuvette and anisotropy values calculated as a function of temperature between 10 and 38 C. All measurements were made on a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with polarization accessories and a thermostated 4-cell cuvette holder.

Phospholipid Analysis

Lipids from isolated spore membranes or vegetative cell membranes were extracted as previously described (26). Lipids were separated, identified and quantitated as previously described (24,26,27).

Materials

L-proline and D-glucose were from Calbiochem. The fatty acids, i-C15 and i-C17, were from Regis Chemical, [1-^{14}C] octadecanoic acid was from ICN, and all other fatty acid standards were from Supelco.

Spectrophotometric grade gold label *n*-hexane was from Aldrich. Phenylmethylsulfonyl fluoride, lysozyme, Tris, HEPES, DPH, and L-isoleucine were from Sigma. All other materials are reagent grade.

RESULTS AND DISCUSSION

Fatty Acid Composition of Vegetative Cells and Spores Grown with Isoleucine

The effect of supplementing the growth medium with isoleucine on the total fatty acid composition of *B. megaterium* vegetative cells and spores was examined. *B. megaterium* vegetative cells grown in SNB medium at 30 C (control) and harvested during midexponential growth were found to contain 9 different fatty acids with the branched-chain fatty acids comprising about 75% of the total fatty acids (Table 1). Fatty acid analysis of vegetative cells grown in SNI medium showed that these cells contained the same complement of fatty acids as found in the control cells, but there was a marked increase in the anteiso-fatty acids and a concomitant decrease in the iso-fatty acids (see column 2). Also observed were some minor changes in the relative proportions of the straight-chain fatty acids. These results show for the first time that the anteiso-fatty acids can be enriched in this strain by growing vegetative cells in the presence of L-isoleucine. A previous study with this organism (12) did not identify the individual fatty acids nor the specific effect of supplementing the growth medium with branched-chain fatty acid precursors.

Total fatty acids from spores grown in SNB medium (Table 1, see column 3) were compared

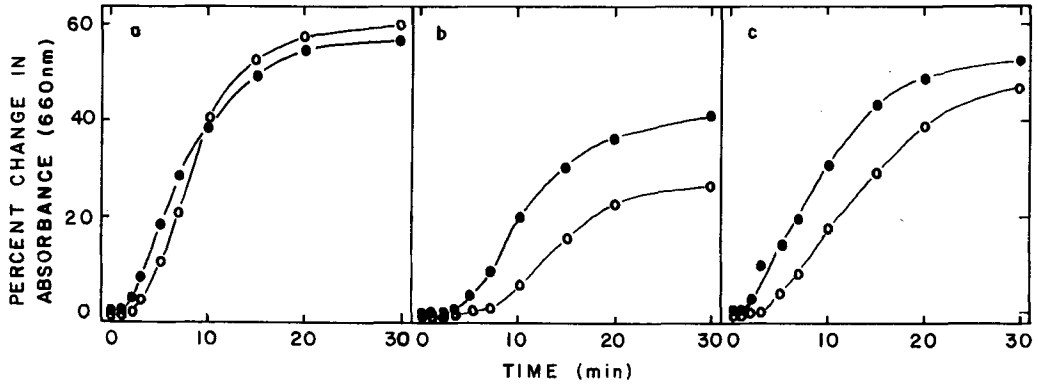


FIG. 1. Germination of spores grown in SNB and SNI. Spores (3 mg/ml) were heat-activated for 10 min at 60 C and germination was triggered by adding 0.2 ml of spores to 2.8 ml of 5 mM Tris (pH 8) containing 10 mM D-glucose (a), 10 mM L-leucine (b) or 10 mM L-proline (c) at 25 C. The absorbance loss for spores grown in SNB (○—○) or SNI media (●—●) was recorded and plotted as percent decrease in the initial absorbance.

with those from vegetative cells grown in SNB medium. While spores were observed to contain the same fatty acid species as vegetative cells, there was a marked increase in n-C14 and a decrease in a-C15 fatty acids relative to vegetative cells, plus other minor changes. Although the significance of the difference in the fatty acid composition between vegetative cells and spores is unknown, one possibility is that it provides a unique method of increasing the rigidity of spore membrane lipids by decreasing the low melting point (23 C) a-C15 fatty acid and increasing the high melting point (54 C) n-C14 fatty acid (28). Studies on other *Bacilli* (29–31) have also reported variations in the fatty acid composition between spores and vegetative cells, but the direction of this change has not been consistently towards increasing the high melting point fatty acids in membrane lipids.

Analysis of the fatty acid composition of spores grown in SNI medium (see column 4) showed an increase in the a-C15 and a decrease in iso-fatty acids compared to the control spores. These results show that the amount of anteiso-fatty acids can be increased by growing spores in the presence of L-isoleucine. Changes in spore fatty acid composition have also been reported for *B. subtilis* (32) and *B. megaterium* (12) when spores were grown on either α-keto acid or amino acid precursors of fatty acids. In both cases, the investigators observed that the ability of vegetative cells to form spores was unaffected by the added fatty acid precursors. However, in no case have any of the properties of these modified spores been examined to test the effect of the fatty acid alterations.

Germination of Spores Grown with L-Isoleucine

The ability of spores grown in SNB (control) or SNI media to germinate was examined by adding an aliquot of heat-activated spores to a solution containing 5 mM Tris (pH 8.0) and a trigger compound, and the decrease in adsorbance at 660 nm was measured. Decrease in adsorbance is the conventional method to follow spore germination, and a 60% decrease in adsorbance indicates greater than 95% germination of spores (19). Shown in Figure 1a are the losses in adsorbance triggered by D-glucose at 25 C for the control and spores grown on isoleucine. Spores grown on isoleucine were triggered slightly faster than the control spores if measured by the time required to reach 50% of the total change in adsorbance within 30 min ($t_{1/2}$). Moreover, the total change in adsorbance in 30 min was somewhat less than the control. Although the difference in $t_{1/2}$ values between the two types of spores was small, it was reproducible and was even more obvious when germination was triggered by either L-leucine or L-proline (see Fig. 1b and 1c). Here there is a clear difference in both rate and extent of adsorbance loss. These results show that the germination properties of spores were altered in vivo by growth on isoleucine.

Since the effect of increasing the a-C15 fatty acid in spores may have its greatest influence on spore germination at temperatures closer to its melting point, germination was examined in the temperature range of 20–45 C, and the results of these experiments are shown (see Fig. 2) plotted as $t_{1/2}$ vs the temperature of germination. Above 28 C, the two types

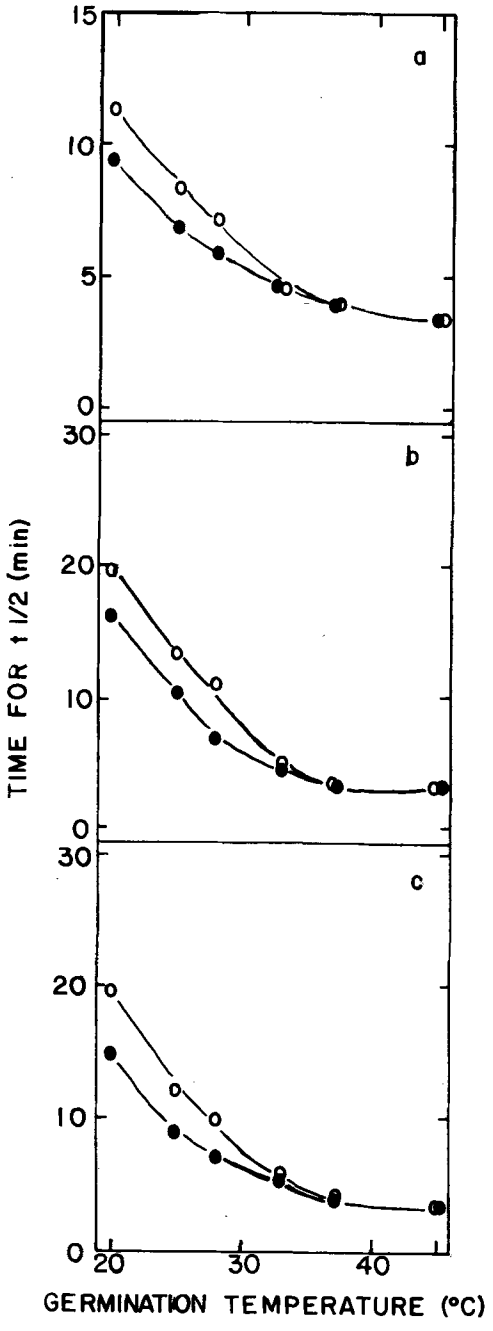


FIG. 2. Temperature dependence for triggering germination. Triggering of germination was measured as described in the legend of Figure 1, except that the temperature of germination was varied from 20 to 45 C for SNB (o–o) and SNI (•–•) grown spores; (a) 10 mM D-glucose, (b) 10 mM L-leucine or (c) 10 mM L-proline. The percent decrease in absorbance was measured and plotted as t½.

of spores germinated at about the same rate, as measured by t½. Also, the rates of germination of the two types of spores were similar when assayed with L-proline or L-leucine. The extent of adsorbance losses was also about the same. Thus, for one particular germinant, spores grown with isoleucine were similar to the control above 28 C. But, as the temperature of germination was decreased to 28 C and below, spores grown on isoleucine triggered germination more readily than the control. At 20 C, spores grown on isoleucine were triggered by D-glucose, L-leucine and L-proline faster than the control by 2, 4, and 5 min, respectively. These results show that growing spores on isoleucine decreased the temperature requirement for triggering germination. As anticipated, the greatest difference between the control and spores grown on isoleucine was observed at temperatures where the low melting a-C15 fatty acids (23 C) would be expected to have their greatest effect on the physical state of membrane lipids.

Effect of Sporulation Temperature on the Triggering of Germination

It has been shown that the fatty acid composition of bacteria can also be modified by varying the growth temperature (9,17,18). Therefore, spores were grown between 20 and 37 C to determine if we could employ a second method to alter the fatty acid composition and if this method would also alter the germination properties of the spores. The results for germination triggered at one temperature (25 C) are shown in Figure 3. It can be seen in Figure 3a that spores grown at 25, 30 and 33.5 C triggered germination similarly on D-glucose, as judged by losses in absorbances. This was also found to be generally the case when germination was triggered by L-leucine or L-proline although the results with leucine are not as obvious as with other germinants (see Fig. 3b and 3c). However, spores grown at 20 C were not triggered for germination as readily as spores grown at 30 C (control), by D-glucose L-leucine or L-proline (Fig. 3). When other germination temperatures (20 to 45 C) were examined, the same general pattern of results shown in Figure 3 was found for spores grown at 25, 30 and 33.5 C, while spores grown at 20 C always germinated poorly (data not shown). In addition, longer times of heat-activation or different temperatures of heat-activation did not change the general pattern of poor germination with spores grown at 20 C compared to spores grown between 25 and 33.5 C. These results showed that, compared to control spores, varying the sporulation temperature

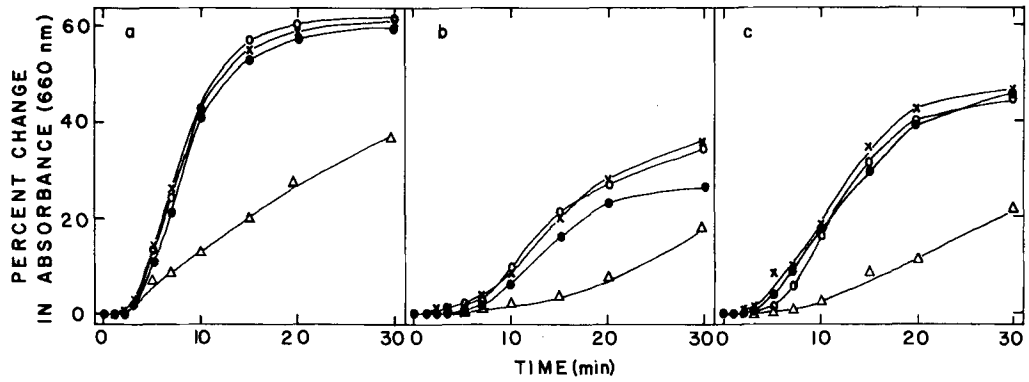


FIG. 3. Germination of spores grown at different temperatures. Triggering of germination was measured as described in the legend of Figure 1, at 25 C in: (a) 10 mM D-glucose, (b) 10 mM L-leucine, or (c) 10 mM L-proline. The absorbance loss for spores grown in SNB medium at 20 C (△—△), 25 C (X—X), 30 C (●—●) and 33.5 C (○—○) was measured and plotted as percent change of initial absorbance.

from 25 C to 33.5 C had little effect on spore germination in vivo. However, by growing spores at 20 C, the ability to germinate was markedly reduced.

Fatty Acid Analysis of Spores Grown at Different Temperatures

Since varying the sporulation temperatures altered the triggering of spore germination in spores grown at 20 C, the effect of sporulation temperature on the fatty acid composition of spores was examined. As shown in Table 2, spores grown between 20 and 33.5 C contained the same complement of fatty acids with some small differences. A general trend can be seen in that decreasing the growth temperature increased the a-C15 fatty acids slightly and decreased the i-C14 and i-C16 fatty acids, in addition to other minor changes. These changes are in agreement with findings in other organisms that decreasing the growth temperature increases the proportion of low melting point fatty acids and decreases the high melting fatty acids (15,17).

Vegetative cells of some *Bacilli*, when grown at 20 C, replace up to 5% of their total fatty acids with unsaturated iso-fatty acids (33). The presence of unsaturated fatty acids in our spores grown at 20 C was examined by argentation chromatography (34). We detected no unsaturated fatty acids with a limit of detection in our assays at less than 3% of the total fatty acid present (data not shown). Therefore, we cannot rule out a potential of role of unsaturated fatty acids in accounting for the poor germination properties of spores grown at 20 C. However, as we show below, several other factors may also account for the poor germination.

TABLE 2

Fatty Acid Composition of *B. megaterium* Spores

Fatty acid	Percentage of total fatty acid[a] Growth temperature[b] 20 C	25 C	30 C	33.5 C	37 C
i-C14	12.8	13.4	16.6	18.6	28.1
n-C14	34.7	36.6	34.0	32.8	7.4
i-C15	16.2	14.0	14.4	13.0	19.2
a-C15	21.4	18.0	17.8	15.2	21.6
n-C15	–[c]	1.0	–	–	1.1
i-C16	3.2	5.9	5.9	8.8	15.5
n-C16	6.3	6.2	7.1	6.8	3.6
i-C17	3.3	2.8	2.0	1.6	1.2
a-C17	1.4	1.9	2.2	2.2	2.2
Grouped Components					
Branched					
Iso	35.5	36.1	38.9	42.0	64.0
Anteiso	22.8	19.9	20.0	17.4	23.8
Unbranched	41.0	43.8	41.1	39.6	12.1

[a]Values are the mean of at least 2 determinations and the range was within ± 5% of the mean. Recovery was greater than 90% as measured with octadecanoic acid added during saponification.
[b]Spores were grown in SNB as described in Methods.
[c]Constitutes trace amounts.

Finally, in Table 2, the fatty acid composition of spores grown at 37 C is also shown. This was done to compare our findings with those of Scandella and Kornberg (12) and to identify the individual fatty acids they had not reported. The fatty acid composition of spores grown at 37 C was similar to previously reported

TABLE 3

Fatty Acid Composition of Spore Membranes

Fatty acid	Percentage of total fatty acid[a] Growth medium and temperature SNB (30 C)	SNI (30 C)	SNB (20 C)
i-C14	15.9	–	12.8
n-C14	32.9	36.7	35.3
i-C15	14.8	1.3	15.0
a-C15	18.9	42.3	22.0
n-C15	–[b]	3.8	–
i-C16	6.1	–	3.0
n-C16	6.4	8.3	6.1
i-C17	2.3	1.3	3.5
a-C17	2.1	5.1	1.5
Grouped Components			
Branched			
Iso	39.1	2.6	34.3
Anteiso	21.0	47.4	23.5
Unbranched	39.3	48.4	41.4

[a]Spores were grown in SNB at 30 C or 20 C or in SNI at 30 C and membranes were isolated as described in Methods. Fatty acid analyses were done as described in Methods.

[b]Constitutes trace amounts.

values (12) but was quite different from spores grown between 20 and 33.5 C. First, there was a large increase in the proportion of branched chain fatty acids and a decrease in straight chain fatty acids. Second, the amount of n-C14 was reduced from about 34% to 7.4%. These differences have not been previously reported because spores grown at different temperatures were never compared.

It may be noted that we have not reported the germination properties of spores grown at 37 C. This is because these spores are very unstable and spontaneously germinate (spores used for our fatty acid analyses were washed about 5 times and immediately saponified before any germination occurred). We do not know if the spontaneous germination is due to the increased ratio of branched- to straight-chain fatty acids or other changes in membrane composition, and such a question will be difficult to answer because of the problem of getting enough clean spores to analyze.

The above results demonstrated that altering the sporulation temperature from 20 to 33.5 C did not change the fatty acid composition of spores as dramatically as when spores were grown in the presence of isoleucine. Also, the ability of 3 different germinants to trigger germination was not very different for spores grown at 25, 30 or 33.5 C. Only for spores grown at 20 C was germination inhibited, but these changes are not reflected by dramatic changes in the fatty acid composition. As will be discussed below, the observed changes in germination could be attributed to other changes in spore composition. The important point in this study is that changing the growth temperature did not provide a simple alternative method for changing the fatty acid composition of spores.

The Composition of Isolated Membranes

Since changes in the fatty acid composition of spores would most likely influence the fatty acid composition of spore membranes, the effect of varied growth conditions on spore membrane fatty acids was examined. Membranes isolated from spores grown in SNB medium at 30 C contained the same fatty acids and in the same proportions as whole spores (compare Table 3, column 1 and Table 2, column 3). The analysis of membranes isolated from spores grown in SNI medium at 30 C and spores grown in SNB medium at 20 C also indicated that the fatty acid composition of isolated membranes was similar to that of whole spores (compare Table 3, column 2 and Table 1, column 4, and Table 3, column 3 and Table 2, column 1, respectively). These results show that alterations in the fatty acid of whole spores reflect changes in the fatty acid composition of the spore membrane.

The effect of varied growth conditions on membrane components in addition to fatty acids was also determined. The phospholipid composition of membranes isolated from vegetative cells grown on SNB medium was analyzed (see Table 4) and four different phospholipids were present. The 2 predominant phospholipids were PG and PE which together comprised greater than 75% of the total phospholipids present, and the remaining phospholipids were accounted for by DPG and Glc-NH_2-PG. These results are in agreement with previously published results for vegetative cells of this strain (26). The same phospholipid species were present in membranes isolated from spores grown on SNB medium at 30 C (control), but the relative proportion of the individual phospholipids was different. Most dramatic was the increase in DPG and the decrease in PE, and these results agree with previously published values for whole spores (24,26) and membranes isolated from spores (24). The phospholipid composition of membranes from spores grown in SNI medium was also analyzed (Table 4) and found to contain the same complement of phospholipids and in the same proportions as the control. This result suggests

that the observed changes in the germination properties of spores grown in an isoleucine medium were not due to changes in phospholipid head group ratios.

Also, membranes from spores grown at different temperatures were analyzed to determine if the phospholipid composition changed with growth temperature as was observed for fatty acids. Membranes from spores grown at 30 C and 25 C contained the same phospholipids and in the same proportions as the control. However, membranes isolated from spores grown at 20 C on SNB medium contained the same phospholipid species as the control, but the amount of PG was increased while DPG decreased (see Table 4, column 5). This suggests that the changes in germination properties observed with spores grown at 20 C could be in part attributed to the altered phospholipid composition.

Finally, the phospholipid to protein ratio (see Table 4) showed little or no difference between membranes isolated from vegetative cells, spores grown in isoleucine, spores grown at 25 C in SNB medium, or spores grown at 30 C (control). However, in spores grown at 20 C, the phospholipid to protein ratio was decreased relative to the control. These results show that with spores grown at 20 C there was little change in fatty acid composition, but both phospholipid head group ratios and phospholipid to protein ratio were altered. While the latter changes in membrane composition might account for the reduced germination, we cannot rule out other factors. However, these results do suggest a possible relationship between the triggering of germination and the spore membrane composition. This is supported by the observation that when spores were grown in the presence of isoleucine, the property of the spore membrane that was altered was the fatty acid composition. Furthermore, when spores were grown at 20 C, both the phospholipid composition as well as the phospholipid to protein ratio was altered. In both cases, altered growth conditions changed the composition of spore membranes and changed the germination of these spores in vivo.

TABLE 4

Phospholipid Composition of Isolated Membranes

	Percentage of total phospholipid[a]				
	Vegetative[b] cells	Spores[c]			
	SNB	SNI	SNB		
Phospholipid	(30C)	(30C)	(30C)	(25C)	(20C)
DPG	14	29	30	29	19
PG	47	44	41	46	57
PE	31	16	16	16	12
Glc-NH_2-PG	8	12	13	15	13
Ratio of phospholipid to protein[d]	0.23	0.26	0.26	0.24	0.16

[a]Values are the mean of 2 determinations and the range was within ± 5% of the mean.

[b]Membranes were isolated from vegetative cells grown in SNB medium at 30 C and harvested during exponential growth.

[c]Membranes were isolated from spores grown in SNB media at 30 C, 25 C and 20 C as well as spores grown in SNI media at 30 C as described in Methods.

[d]The ratio is based on mg of phospholipid/mg of protein. The average mw of phospholipid used for the conversion were: 779 mg/mmol for vegetative cells, 846 mg/mmol for spores grown in SNB medium at 30 C, 869 mg/mmol for spores grown in SNI medium at 30 C, 864 mg/mmol for spores grown in SNB medium at 25 C, and 882 mg/mmol for spores grown in SNB medium at 20 C. These values were calculated by determining the average mw of fatty acid for each membrane from the fatty acid analysis and using this value for the acyl moiety of the phospholipid. Then the relative contribution of each phospholipid was used to determine the average mw of the phospholipid.

Fluorescence Depolarization Studies of Isolated Membranes

Previous studies with other organisms have shown that alterations of the lipid and/or protein composition of membranes could be correlated with changes in membrane "fluidity" as determined by a number of different techniques (35–39). Since membranes isolated from vegetative cells and spores exhibited different membrane compositions, these membranes were examined to see what effect these changes might have on the rotational mobility of the rod-shaped lipophilic fluorescent probe, DPH, as determined by steady-state fluorescence anisotropy measurements. Membranes isolated from spores grown in SNB at 30 C (control) exhibited a decrease in the anisotropy of DPH, when the temperature was increased from 10 to 38 C (Fig. 4). These results agree with other studies on changes in anisotropy of DPH as a function of temperature (40–42). No attempts have been made to draw lines through these points since it is doubtful whether discontinuities in Arrhenius curves reported by DPH have simple physical interpretations in complex biological membranes (Michael Glaser, University of Illinois, Urbana, personal communication). The anisotropy of DPH in membranes isolated from vegetative cells was less than in spore membranes. The increased mobility of DPH in membranes isolated from vegetative cells agrees with the fatty acid analysis (see Table 1) which showed these membranes to

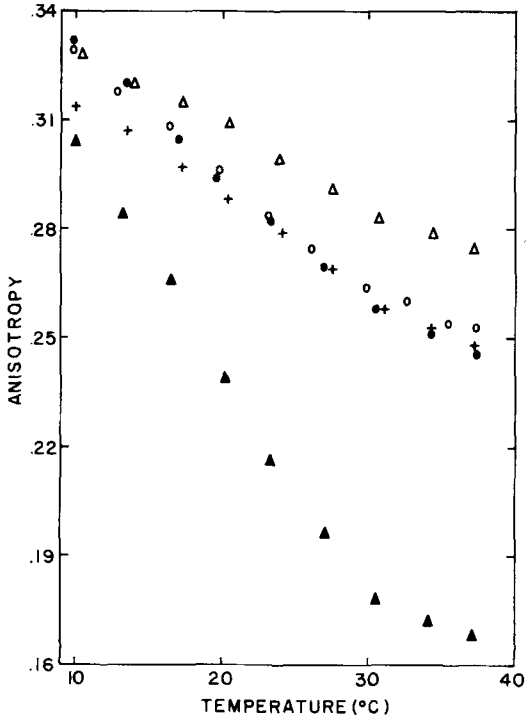


FIG. 4. Isolated membranes studied by fluorescence depolarization of DPH. Isolated membranes labeled with DPH were diluted with 10 mM HEPES (pH 7.5) to a final protein concentration of 0.5 mg/ml and 2 ml transferred to cuvettes preequilibrated to 10 C. The dependence of anisotropy on temperature was measured in the range of 10–38 C. All points represent the mean calculated from a minimum of 2 experiments. Membranes were isolated from vegetative cells grown in SNB medium at 30 C (▲–▲), or from spores grown in SNB medium at 30 C (●–●), 25 C (o–o) and 20 C (Δ–Δ) and from spores grown in SNI medium at 30 C (+–+) as described in Methods.

have a higher proportion of low melting fatty acids. In addition, these membranes have a greater percentage of branched-chain fatty acids, which have been shown not to pack as tightly as straight-chain fatty acids (14,43, 44), and therefore should permit increased DPH mobility.

Membranes isolated from spores grown on isoleucine showed no difference in the anisotropy of DPH from the control at temperatures greater than 20 C but, in the range of 10-20 C, the anisotropy of DPH in these membranes was less than the control. These results agree with the following: First, in vivo studies on the triggering of germination showed that spores grown on isoleucine germinated more readily at temperatures below 28 C (see Fig. 2). Second, the increased mobility of DPH at the lower temperature agrees with the results of fatty acid analysis (see Table 1) which showed that spores grown on isoleucine had increased levels of the low melting fatty acids. It, therefore, suggests a possible correlation between the physical properties of spore membrane and its role in the triggering of germination. This finding should not be confused with another study that reported an increase in the anistropy of DPH when labeled membranes were exposed to trigger compounds (Skomurski et al., submitted). Although both studies are focusing on the role of spore membranes during the triggering of germination, the above study examined the effect of trigger compounds on the physical state of membranes while the present study investigated the effect of altering the physical state of membranes on the ability of spores to trigger germination.

As anticipated from the analysis of membrane composition, the anisotropy of DPH in membranes isolated from spores grown at 25 C was similar to membranes from spores grown at 30 C (Fig. 4). But the anisotropy of DPH in membranes isolated from spores grown at 20 C appeared greater than the control. The interpretation of this result is complicated by the finding that in these membranes the relative proportion of phospholipids was changed (PG increased while DPG decreased), while the ratio of phospholipid to protein decreased. Since increasing PG while decreasing DPG would be expected to lower the melting point of phospholipids (45), these membranes might be expected to allow less anisotropy of DPH than the control. However, the anisotropy of DPH in these membranes was greater than the control, in agreement with the effect of decreasing phospholipid to protein ratio (42,46,47). This would suggest that decreasing the ratio of phospholipid to protein had a greater effect on DPH anisotropy in these membranes than did changes in the proportions of PG to DPG. These results further support the hypothesis that some component of the trigger reaction may be associated with the membrane, since altering the physical property of the spore membrane changed the spore's ability to trigger germination. However, we cannot exclude the possibility that some other components of the spores may also have been altered.

In summary, the results of this study show that altering the fatty acid composition of spores and spore membranes influences the ability of spores to germinate in vivo. Changes in the fatty acid composition of spore membranes correlated with changes in the physical properties of isolated membranes as determined

by steady-state anisotropy measurements of DPH. These findings suggest that the germination of spores can be altered by changing their fatty acid and/or phospholipid composition as well as the phospholipid to protein ratio.

ACKNOWLEDGMENTS

The authors thank T.O. Henderson and J.A.F. Op den Kamp for their advice and K. Joseph for technical assistance. This work was supported by a Public Health Service grant AI 12678.

REFERENCES

1. Vary, J.C. (1978) in Spores VII, (Chambliss, G., and Vary, J.C., eds.) pp. 104–108, American Society for Microbiology, Washington, D.C.
2. Janoff, A.S., Coughlin, R.T., Racine, F.M., McGroarty, E.J., and Vary, J.C. (1979) Biochem. Biophys. Res. Comm. 89, 565–570.
3. Rossignol, D.P., and Vary, J.C. (1979) Biochem. Biophys. Res. Commun. 89, 547–551.
4. Racine, F.M., Skomurski, J.F., and Vary, J.C. (1981) in Sporulation and Germination, (Levinson, H., Sonenshein, A.L., and Tipper, D.J., eds.) pp. 224–227, American Society for Microbiology, Washington, D.C.
5. Yasuda-Yasaki, Y., Namiki-Kanie, S., and Hachisuka, Y. (1978) J. Bacteriol. 136, 484–490.
6. Enfors, S.O., and Molin, G. (1978) in Spores VII, (Chambliss, G., and Vary, J.C., eds.) pp. 80–84, American Society for Microbiology, Washington, D.C.
7. Swerdlow, B.M., Setlow, B., and Setlow, P. (1981) J. Bacteriol. 148, 20-29.
8. Im, W.B., Deutchler, J.T., and Spector, A.A. (1979) Lipids 14, 1003–1008.
9. Melchior, D.L., and Czech, M.P. (1979) J. Biol. Chem. 254, 8744–8747.
10. McMurchie, E.J., and Raison, J.K. (1979) Biochim. Biophys. Acta 554, 364–374.
11. Klausner, R.D., Bhalla, D.K., Dragsten, P., Hoover, R.L., and Karnovsky, M.J. (1980) Proc. Natl. Acad. Sci. USA 77, 437–441.
12. Scandella, C.J., and Kornberg, A. (1969) J. Bacteriol. 98, 82–86.
13. Esser, A.F., and Souza, K.A. (1974) Proc. Natl. Acad. Sci. USA 71, 4111–4115.
14. Haest, C.W.M., Verkleij, A.J., De Gier, J., Scheek, R., Ververgaret, P.H.J., and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 17–26.
15. McElhaney, R.N., and Souza, K.A. (1976) Biochim. Biophys. Acta 443, 348–359.
16. Rilfors, L., Wieslander, A., and Stahl, S. (1978) J. Bacteriol. 135, 1043–1052.
17. Bhakoo, M., and Herbert, R.A. (1979) Arch. Microbiol. 121, 121–127.
18. Ray, P.H., White, D.C., and Brock, T.D. (1971) J. Bacteriol. 106, 25–30.
19. Shay, L.K., and Vary, J.C. (1978) Biochim. Biophys. Acta 538, 294–292.
20. Lammi, C.J., and Vary, J.C. (1972) in Spores, V. (Halvorson, H.O., Hanson, R., and Campbell, L.L., eds.) pp. 277–283, American Society of Microbiology, Washington, D.C.
21. Ackman, R.G. (1979) in Methods in Enzymology, (Lowenstein, J.M., ed.) Vol. 14, pp. 329–381, Academic Press, New York, NY.
22. Schlenk, H., and Gellerman, J.L. (1960) Anal. Chem. 32, 1412–1414.
23. Vary, J.C. (1973) J. Bacteriol. 116, 797–802.
24. Racine, F.M., and Vary, J.C. (1980) J. Bacteriol. 143, 1208–1214.
25. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
26. Bertsch, L.L., Bonsen, P.P.M., and Kornberg, A. (1969) J. Bacteriol. 98, 75–81.
27. Chen, P.S., Toribara, T.Y., and Warner, H. (1965) Anal. Chem. 28, 1756–1758.
28. Cronan, J.E., and Gelmann, E.P. (1975) Bacteriol. Rev. 39, 232–256.
29. Nickerson, K.W., Bulla, L.A., and Mounts, T.L. (1975) J. Bacteriol. 124, 1256–1262.
30. Kaneda, T. (1967) J. Bacteriol. 93, 894–903.
31. Yao, M., Walker, H.W., and Dillard, D.A. (1970) J. Bacteriol. 102, 877–878.
32. Boudreaux, D.P., and Freese, E. (1981) J. Bacteriol. 148, 480–486.
33. Quint, J.F., and Fulco, A.J. (1973) J. Biol. Chem. 248, 6885–6895.
34. Wood, R., and Snyder, F. (1966) J. Amer. Oil Chem. Soc. 43, 53–54.
35. Eisenberg, A.D., and Corner, T.R. (1978) Can. J. Microbiol. 24, 386–396.
36. Blume, A., Dreker, R., and Poralla, K. (1978) Biochim. Biophys. Acta 512, 489–494.
37. Dickens, B.F., Martin, C.E., Kig, G.P., Turner, J.S., and Thompson, G.A. (1980) Biochim. Biophys. Acta 598, 217–236.
38. Furtado, D., Williams, W.P., Brain, A.P.R., and Quinn, P.J. (1979) Biochim. Biophys. Acta 598, 352–357.
39. King, M.E., and Spector, A.A. (1978) J. Biol. Chem. 253, 6493–6501.
40. Andrich, M.P., and Vanderkooi, J.M. (1976) Biochemistry 15, 1257–1261.
41. Brasitus, T.A., Tall, A.R., and Schacter, D. (1980) Biochemistry 19, 1256–1261.
42. Stubbs, G.W., Litman, B.J., and Barenholz, Y. (1976) Biochemistry 15, 2766–2772.
43. Silvius, J.R., and McElhaney, R.N. (1979) Chem. Phys. Lipids 24, 287–296.
44. Silvius, J.R., and McElhaney, R.N. (1980) Chem. Phys. Lipids 24, 67–77.
45. Pluschke, G., and Overath, P. (1981) J. Biol. Chem. 256, 3207–3212.
46. Chapman, D., Gomez-Fernandez, J.C., and Goni, F.M. (1979) Fed. Eur. Biochem. Soc. 98, 211–223.
47. Fraley, R.T., Yen, G.S.L., Leuking, D.R., and Kaplan, S. (1979) J. Biol. Chem. 254, 1987–1991.

[Received January 28, 1982]

Effects of Cereals and Culture Filtrate of *Trichoderma viride* on Lipid Metabolism of Swine[1,2]

ASAF A. QURESHI[a,*], WARREN C. BURGER[a], CHARLES E. ELSON[b], and NORLIN J. BENEVENGA[b], *[a]USDA, SEA-AR, Barley and Malt Laboratory, 501 N. Walnut Street, Madison, WI 53705;* and *[a]Department of Agronomy, and [b]Department of Nutritional Sciences, University of Wisconsin, Madison, WI 53706*

ABSTRACT

Swine were fed corn- or barley-based diets with, or without, culture filtrate (CF) of *Trichoderma viride* for 21 days. Weight gains were nonsignificantly but slightly increased by CF. The activities of β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase, acetyl-CoA carboxylase (ACX), fatty acid synthetase (FAS) and other lipogenic enzymes in several tissues were determined. Significant decreases in the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase in all tissues of swine fed the CF-diets were observed. The major site for the regulation of cholesterol biosynthesis was adipose tissue followed by the intestine, liver, lung and muscle in order of activity. The concentrations of cholesterol in serum and muscle were decreased 27% and 23%, respectively, by CF. ACX and FAS activities increased ca. 2-fold when CF was fed with either of the cereal-based diets. The major sites for fatty acid synthesis was the adipose tissue and, to a lesser extent, the liver. Very low rates of synthesis were detected in intestine, lung and muscle. Similar distributions of activities were found for related lipogenic enzymes.
Lipids 17:924-934, 1982.

INTRODUCTION

We recently reported that dietary cereals and material in the culture filtrate (CF) of *Trichoderma viride* influence the rate of cholesterol biosynthesis and that the lowest rate of fatty acid biosynthesis were present in livers of chickens fed a corn-based diet. A barley-based diet produced the greatest fatty acid synthetase (FAS) activity and lowest β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase (ED 1.1.1.-34) activity. CF added to each cereal-based diet further increased fatty acid synthesis and reduced cholesterol biosynthesis (1,2).

In a number of studies, it has been established that the relative contributions of tissues sites to overall fatty acid and cholesterol synthesis vary appreciably depending on the animal species studied. Essentially all fatty acid synthesis takes place in the liver of chicken (3-8), whereas in the rat the adipose tissue assumes this major role (5). Cholesterol biosynthesis in both species occurs primarily in the liver (5-9). Tissues of the gastrointestinal tract also contribute to overall cholesterol synthesis (9). In swine, virtually all fatty acid synthesis occurs in the adipose tissue (10-14). Cholesterol biosynthesis, supported by acetate or glucose, is shared by the liver and adipose tissue (14,15).

The present investigation was conducted to determine whether corn- and barley-based diets with and without CF exert the same influence on the mammalian lipid metabolism as was previously reported in the avian system (1,2). In addition to liver and adipose tissue lipogenic activities, the enzymic activities were also examined in swine lung, muscle and intestinal tissue. The list of cholesterogenic and lipogenic enzyme activities examined included: HMG-CoA reductase, cholesterol 7α-hydroxylase (EC 1.14), FAS, acetyl-CoA carboxylase (ACX, EC 6.4.1.2), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), malic enzyme (ME, EC 1.1.1.40) and citrate-cleavage enzyme (CCE, EC 4.1.3.8). Cholesterol concentrations in serum and muscles were also determined. The addition of CF to either diet caused a marked decrease in cholesterol synthesis and concentration, whereas fatty acid synthesis was stimulated. Results of this study are discussed in relation to the dietary modification of cholesterol and fatty acid metabolism, assuming swine are representative of other mammalian models.

MATERIALS AND METHODS

Experimental materials were purchased from the following sources: acetyl-CoA, malonyl-

[1]Cooperative investigation between the Science and Education Administration, US Department of Agriculture, and College of Agricultural and Life Sciences, University of Wisconsin, Madison.

[2]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

CoA, RS-mevalonic acid, glucose-6-phosphate, dithiothreitol, DL-isocitrate, glutathione, $NADP^+$, NADPH, NADH, ATP, glucose-6-phosphate dehydrogenase, cysteamine, Tween-80, triethanolamine hydrochloride, sodium malate, coenzyme A, malate dehydrogenase, nicotinamide and 6-phosphogluconate from Sigma Chemical Co. (St. Louis, MO); cholesterol, Aldrich Chemical Co. (Milwaukee, WI), was recrystallized twice in glacial acetic acid; 7α-hydroxycholesterol (5-cholesten-3β,7α-diol) and 7-ketocholesterol (5-cholesten-3β-ol-7-one) from Steraliods, Inc. (Wilton, NH); EDTA from Fisher Scientific Co. (Itasca, IL); bovine serum albumin from Nutritional Biochemicals Corporation, (Cleveland, OH); and DL-3-hydroxy-3-methyl-[3-^{14}C] glutaryl-CoA (sp act, 26.3 mCi/mmole), [4-^{14}C] cholesterol (sp act 50-60 mCi/mmole), [^{14}C] sodium bicarbonate (sp act, 48 mCi/mmole) and Aquasol (scintillation solution) from New England Nuclear (Boston, MA). Barley, var. "Larker," was purchased from the Ladish Malting Company (Jefferson, WI). Other diet components were obtained locally. All other chemicals were of analytical grade. The lyophilized *T. viride* CF was the commercial enzyme product, "Cellulase Onazuka" (Kinki Yakult Co., Ltd., Tokyo).

Animals and Diets

Twelve Yorkshire and 10 Hampshire gilts (young female swine that have not produced a litter) 5 months of age, 100 kg, were obtained locally. Prior to this study, the gilts were fed a diet providing 14.3% protein and 3% fat supplied by ground yellow corn plus soybean meal and supplemented with a commercial vitamin-mineral premix.

The gilts were distributed into 4 groups, 2 of which included 2 Hampshire and 3 Yorkshire gilts, the remaining 2 groups each included 3 Hampshire and 3 Yorkshire gilts. A corn-based diet (control group) was fed to each group of 5 gilts and barley-based diet was fed to each group of 6 gilts. Culture filtrate (0.008%) was added to the diets of one group of gilts fed each diet (Table 1). The proximate analysis of each diet is given in Table 2 (16). Amino acid composition of the protein fraction was determined (17). Diets and water were provided ad libitum; the gilts were fed in confinement with 12-hr light and dark periods. The gilts were weighed and blood samples were drawn from the jugular vein at 1000 hr at 5-day intervals. After 21 days, the gilts were slaughtered (at the Muscle Biology Laboratory, UW) and samples of liver, lung, intestine (duodenum), adipose tissue (inner, perineal; and outer at the 8th (rib) and muscle (semimembranous) tissues were taken for analyses.

TABLE 1

Percent Composition of the Swine Diets

Ingredients	Diets	
	Corn	Barley
Corn[a]	82.0	–
Barley	–	82.0
Soybean meal (44% protein)	17.0	17.0
Iodized salt	0.5	0.5
Vitamin mixture[b]	0.5	0.5

[a]Analyses of corn and barley were 9.1 and 12.0% protein, respectively.

[b]The vitamin premix supplies per kg of complete diet: lysine, 250 mg; calcium as calcium carbonate and dicalcium phosphate, 1 g; calcium iodate, 200 μg; zinc, 900 μg; iron, 9 mg; manganese, 7 mg; sodium and potassium chlorides, 750 mg; riboflavin, 100 mg; pantothenic acid, 200 mg; niacin, 660 mg; choline, 4.4 g; vitamin B_{12}, .77 μg; vitamin A palmitate, 132,000 U; vitamin D_3, 132,000 U; and vitamin E, 363 U.

TABLE 2

Proximate Analysis of Corn- and Barley-based Diets ("as is" percentage basis)

Component	Corn diet	Barley diet
Nitrogen	2.31	2.56
Protein (N X 6.25)	14.4	16.0
Ether extractables	4.5	3.2
Moisture	9.9	9.9
Ash	5.3	6.4
Carbohydrate	72.0	60.5
beta-glucan	0.6	7.8
arabinose	0.2	0.9
xylose	7.0	8.4
Acid det. fiber	3.0	6.2
Dietary fiber[a]	3.8	13.9
Kcal/g diet	3.4	3.3

[a]Dietary fiber = 100 - (protein + ether extractables + ash + carbonhydrate).

Preparation of Tissues for Analyses

Tissue homogenates were prepared in 0.1 M potassium phosphate buffer, pH 7.4 containing 4 mM $MgCl_2$, 1 mM EDTA and 2 mM dithiothreitol. The tissues were chopped and suspended in the buffer (1:2, w/v except muscle, 1:1, w/v) and homogenized with a Polytron homogenizer. These procedures were done at 4 C for all tissues except the adipose tissues which were prepared at 10 C. The 100,000 × g supernatant (cytosol) and microsomal fractions were stored at -20 C until assayed for enzymatic activities (18). Enzymatic assays of adipose tissue were

conducted with fresh preparation specifically for lipogenic enzymes, which confirmed earlier observations (19). Protein concentrations were estimated by a modification of the Biuret method using the bovine serum albumin as a standard (20).

Assays for the Cholesterol Biosynthesis, Oxidation and Concentration

β-Hydroxy-β-methylglutaryl Coenzyme A Reductase- Ten μl microsome suspensions (300 μg protein) were incubated at 37 C with 0.3 unit glucose-6-phosphate dehydrogenase in a vol of 70 μl. After 10 min, 80 μl of a coefactor-substrate solution containing 4.5 μmol glucose-6-phosphate, 5 μmol potassium phosphate/buffer, pH 7.4, 0.3 μmol dithiothreitol, 25 nmol DL-3-hydroxymethyl-[3-^{14}C]glutaryl CoA (2×10^5 dpm) and 450 nmol $NADP^+$ were added. After 15 min, 25 μl of 10 N HCl was added to end the reaction. After waiting for 30 min to permit mevalonic acid to lactonize, the denatured protein was sedimented by centrifugation. Aliquots of the protein-free solution was applied to activated Silica Gel G thin layer plates.

DL-mevalonolactone standard was applied to the end channel plate. The plate was developed in 1:1 benzene/acetone, the end channels and regions from the plate corresponding to the light brown standard were scraped into counting vials. Aquasol was added and radioactivity measured. Enzyme activities reflect nmoles mevalonic acid synthesized/min/mg microsomal protein (21).

Cholesterol 7α-Hydroxylase

Fifty μg microsomal protein in 145 μl homogenizing buffer were incubated for 10 min at 4 C with 5 μl buffer containing 0.1 unit glucose-6-phosphate dehydrogenase. Then 800 μl cofactor mixture containing 50 μmol potassium phosphate buffer, pH 7.4, 5 μmol cysteamine, 5 μmol $MgCl_2$, 2 μmol glucose-6-phosphate, 100 μmol cholesterol (0.1 Ci [4-^{14}C]cholesterol), 1 mg Tween 80 and 50 μmol $NADP^+$ were added. The incubation for 30 min was at 37 C. The reaction was terminated by the addition of 1 ml ethanol. This mixture was extracted twice with light petroleum ether. The extract was taken to dryness, dissolved in benzene/methanol, 4:1, and applied with reference standards of cholesterol and 7α-hydroxycholesterol under nitrogen to an activated Silica Gel G plate. The plate was developed with benezene/ethyl acetate, 2:3. The cholesterol and 7α-hydroxycholesterol bands were identified under ultraviolet light and the bands removed and assayed for radioactivity as described (22).

Estimation of Cholesterol in Serum and Muscle

The blood (3 ml) was collected from the jugular vein, centrifuged at 10,000 × g for 10 min and the serum was stored at -20 C until the analyses were performed. Cholesterol concentrations in serum and muscle samples were estimated using Worthington "Cholesterol Reagent" obtained from Worthington Diagnostics Division of Millipore Corporation, Freehold, NJ.

Assay for the Lipogenic Enzymes

Acetyl-CoA carboxylase. Cytosolic protein (200-400 μg) was incubated at 37 C for 10 min in a vol of 175 μl containing 15 μmol Tris HCl, pH 7.0, 0.5 μmol glutathione, 2 μmol $MgCl_2$, 25nmol EDTA, 0.15 mg bovine serum albumin and 5 μmol potassium citrate. The reaction was initiated by the additions of 50 μl solution containing 0.5 μmol ATP, 50 nmol acetyl-CoA and 25 μl of [^{14}C]$NaHCO_3$ (2.5 nmol and 1×10^6 dpm). Following incubation for 10 min at 37 C, the reaction was stopped by the addition of 50 μl 6 N HCl. Following centrifugation at 5000 × g for 10 min, 200 μl of the supernatant solution were placed in a counting vial and taken to dryness, the precipitate dissolved in 200 μl water, 6 ml Aquasol added and the samples counted for radioactivity. The enzyme activity reflects nmol malonyl-CoA formed/min/mg cytosolic protein (23).

Spectrophotometric assays. These assays were monitored at 340 nm with recorder set for 0.1 full scale. The reaction in 0.5 ml was run at room temperature; the reaction initiated by the addition of 25-250 μg cytosolic protein to the reaction mixture. The absorbance of 1 mM NADPH or NADH is 6.22. Enzyme activities reflect nmol NADPH or NADH (or $NADP^+$) oxidized (or reduced)/min/mg cytosolic protein.

Fatty Acid Synthetase

The reaction mixture contained 250 μmol potassium phosphate, pH 7.0, 16 nmol acetyl-CoA, 50 nmol malonyl-CoA, 0.5 μmol EDTA, 0.5 μmol β-mercaptoethanol, and 50 nmol NADPH (24).

Glucose-6-phosphate Dehydrogenase

The reaction mixture (0.5 ml) contained 50 μmol Tris-HCl, pH 8.0, 15 μmol KCl, 5 μmol nicotinamide, 1 μmol glucose-6-phosphate and 0.125 μmol $NADP^+$ (25).

6-Phosphogluconate Dehydrogenase

The reaction mixture contained 50 μmol Tris-HCl, pH 8, 15 μmol KCl, 5 μmol $MgCl_2$, 5 μmol nicotinamide, 1 μmol 6-phosphogluconate and 0.5 μmol $NADP^+$ (25).

Malic Enzyme

The reaction mixture contained 35 μmol triethanolamine hydrochloride, pH 7.4, 0.25 μmol sodium L-malate, 2.5 μmol $MnCl_2$ and 0.125 μmol $NADP^+$ (26).

Citrate-cleavage Enzyme

The reaction mixture contained 100 μmol Tris-HCl, pH 8.4, 10 μmol potassium citrate, 2.5 μmol ATP, 0.1 μmol coenzyme A, 5 μmol β-mercaptoethanol, 5 μmol $MgCl_2$, 0.1 unit malate dehydrogenase and 0.1 μmol NADH (27).

Following differential centrifugation of adipose tissue and liver homoegenates, the protein concentrations of the cytosolic and microsomal fractions were determined. Separable fat from pigs of comparable back ground ranges from 15 to 20% of live weights (personal communication, R.G. Kaufman, University of Wisconsin). We estimated that the liver and adipose tissue contained 58 and 200 g cytosolic protein and 50 and 140 g microsomal protein, respectively. Total enzyme activities, based on these estimates, of the two tissues are presented for discussion purposes: The average value for the inner and outer adipose tissue specific activities were used in our calculations.

Expression of Data and Statistical Methods

Enzyme data were expressed as specific activities (units/mg cytosolic or microsomal protein/min). Statistical comparisons of these results were performed by two-way analysis of variance. When the F test indicated significance, the differences between the means analyzed by protected LSD test using the correction for samples of unequal size (28).

RESULTS

The corn- and barley-based diets provided about 3.4 and 3.3 kcal/g, respectively. During the experiment, energy consumption calculated per individual animal fell within the range 178,000 and 190,000 kcal (183,000 ± 4,900) (Table 3). Weight gains of the groups ranged from an average of 18.3 ± 3.0 to 21.6 ± 1.8 kg (Table 3). The average gain in weight by gilts fed diets with 0.008% CF was about 8% (NS) greater. Diet intake was not influenced by the addition of CF.

The specific activity of HMG-CoA reductase in tissues from gilts fed the corn diet varied from 55.2 (muscle) to 485 (inner adipose) (Table 4). The activity in tissues of gilts fed the barley diet varied from 42.3 (muscle) to 395.0 (inner adipose); the activity in each tissue of the latter dietary group was only 75-87% ($p<$

TABLE 3

Effect of Cereals and Culture Filtrate of *T. viride* on Weight Gain, Feed Consumption and Feed Conversion of Swine

Nutritional state	Days on feed				Δ	Feed Total kg	kcal[a] pig	Conversion ratio[b]
	0	5	10	21				
	kg body wt							
Corn	98.5 ± 7.1[c]	103.3 ± 6.8	109.2 ± 7.9	116.8 ± 8.1	18.3 ± 3.0	279.5	1.90	0.327
Corn + culture filtrate	99.0 ± 9.8	104.4 ± 5.4	110.4 ± 5.2	119.1 ± 4.7	20.1 ± 2.5	262.7	1.78	0.382
Barley	100.3 ± 8.4	104.8 ± 8.2	112.3 ± 8.0	120.5 ± 9.4	20.1 ± 3.2	330.0	1.81	0.366
Barley + culture filtrate	100.8 ± 7.2	106.3 ± 7.1	113.4 ± 5.9	122.4 ± 5.6	21.6 ± 1.8	331.8	1.82	0.390

[a]kcal ($\times 10^{-5}$) consumed per pig during the experiment.
[b]Feed conversion, unit weight gain/unit feed consumed.
[c]Data expressed as means ± SD; N=5 for corn groups and 6 for barley groups.

TABLE 4

Effect of Cereals and Culture Filtrate of *T. viride* on the Activities of β-Hydroxy-β-methylglutaryl-CoA Reductase and Cholesterol 7α-Hydroxylase in Certain Swine Tissues[1]

Nutritional state		Tissue					
		Liver	Adipose (inside)	Adipose (outside)	Intestine	Lung	Muscle[4]
Corn	HMG[2]	163.0 ± 9.0[a]	485.0 ± 10[a]	478.2 ± 15.0[a]	212.3 ± 9.0[a]	78.3 ± 5.0[a]	55.2 ± 2.0[a]
	7α OH[2]	4.6 ± 0.2[a]	19.7 ± 1.0[a]	11.8 ± 1.5[a]	5.4 ± 0.5[a]	1.8 ± 0.6[a]	1.1 ± 0.1[a]
Corn + culture filtrate	HMG	115.0 ± 4.0[c](71)[3]	307.0 ± 8.0[c](63)	274.2 ± 8.0[c](57)	101.3 ± 7.0[c](48)	52.2 ± 4.0[c](67)	30.2 ± 3.0[c](55)
	7α OH	3.0 ± 0.1[b](65)	8.8 ± 0.6[c](45)	4.6 ± 0.2[c](39)	3.3 ± 0.3[c](61)	1.1 ± 0.1[b](61)	0.6 ± 0.1[bc](55)
Barley	HMG	142.0 ± 3.0[b](87)	395.0 ± 10[b](81)	381.2 ± 9.0[b](80)	158.2 ± 8.0[b](75)	62.1 ± 5.0[b](79)	42.3 ± 2.0[b](77)
	7α OH	3.3 ± 0.2[b](72)	16.8 ± 1.0[b](85)	8.4 ± 0.4[b](71)	4.1 ± 0.3[b](76)	1.4 ± 0.2[b](78)	0.8 ± 0.1[b](73)
Barley + culture filtrate	HMG	95.0 ± 3.0[d](58)	207.0 ± 7.0[d](43)	198.2 ± 6.0[d](41)	85.0 ± 5.0[d](40)	30.3 ± 4.0[d](39)	22.2 ± 2.0[d](40)
	7α OH	2.5 ± 0.2[c](54)	8.6 ± 0.4[c](44)	3.4 ± 0.3[d](29)	1.9 ± 0.2[d](35)	0.7 ± 0.1[c](39)	0.4 ± 0.1[c](36)

[1] Feeding period was 3 weeks. Time of killing was 0800 hr. Data expressed as means ± SD; N=5 swine per group.

[2] HMG = β-hydroxy-β-methyl-glutaryl-CoA reductase and 7α OH = cholesterol 7α-hydroxylase as *p*-mol of mevalonic acid synthesized/min/mg microsomal protein (HMG) or *p*-mol of [^{14}C]cholesterol into [^{14}C]7α-OH-cholesterol/min/mg of microsomal protein (7αOH).

[3] Percentage of respective control (corn-diet) activity data are in parentheses.

[a-d] Means for a given enzyme within a column and without a common superscript letter are significantly different, $p<0.01$.

[4] Enzyme activities recorded for the muscle tissue may actually represent contribution of the intramuscular fat cells which account for about 5% of the weight of the semimembranous muscle (35).

0.01) of that present in the respective tissue of gilts fed the corn diet. The pattern of cholesterol 7α-hydroxylase activity varied in a similar manner. Although inner and outer adipose tissues exhibited similar HMG-CoA reductase specific activities, cholesterol 7α-hydroxylase activity was significantly higher ($p<0.01$) in the inner adipose tissue (Table 4). The addition of CF to either diet produced a significant ($p<0.01$) decreased in HMG-CoA reductase (57 ± 8%) and cholesterol 7α-hydroxylase (55 ± 9%) activities.

The values in parentheses represent percent of mean activity of the respective diet compared to corn control diet. The CF-mediated inhibitions were least effective in the liver (Table 4). Ratios of the activities of biosynthetic and oxidative enzymes (Tables 5 and 6) were calculated for each tissue. In outer adipose tissue, lung and muscle, biosynthetic activity was 49-fold the oxidative activity; in liver and intestine, 36-fold, and in the inner adipose tissue, biosynthetic activity was only 26-fold the oxidative activity. These calculations failed to reveal individual responses to either the CF or the dietary cereal. The inner adipose tissue, which had the highest biosynthetic activity, exhibited several-fold higher oxidative activity.

Inititally, the gilts had an average serum cholesterol concentration of 95.2 mg/100 ml (Table 7). During the feeding trial, the cholesterol concentration in the serum of gilts fed the corn diet remained at this level. The cholesterol concentration decreased progressively in the serum of the gilts fed the barley diet. After 21 days, their cholesterol concentration was 18% ($p<0.01$) lower. Addition of the CF to either diet reduced the cholesterol concentration by 11-12% compared to the control diet (Table 7). The cholesterol concentration in muscle of gilts fed the barley diet was 17% lower than that in muscle of corn-fed gilts ($p<0.01$). A minimum cholesterol concentration, 64 mg/100 g tissue, was found in the muscle of gilts fed diets which contained CF ($p<0.01$, Table 7).

Specific activities of key enzymes in the pathway leading to fatty acid synthesis are shown on Tables 6-8. Those activities providing reducing equivalents, G6PD, 6PGD and ME, (Tables 5 and 6) were greater in each tissue (inner adipose, 29%; muscle, 91%) taken from gilts fed the barley diet compared to corn-based diet. In most tissues, ME activity was most elevated by the barley diet. The activities of CCE, ACX and FAS were also increased, generally in proportion to the increased $NADPH_2$ flow, when barley diet was fed. The influence of the dietary cereal was greatest in the muscle tissue and least in the inner adipose tis-

TABLE 5

Effect of Cereals and Culture Filtrate on *T. viride* on the Activities of Pentose Pathway Dehydrogenases in Certain Swine Tissues[1]

Nutritional state		Tissue					
		Liver	Adipose (inside)	Adipose (outside)	Intestine	Lung	Muscle
Corn	G6PD[2]	54.4 ± 5.5^{b}	320.0 ± 14.9^{d}	259.8 ± 23.0^{d}	111.3 ± 7.3^{d}	174.0 ± 14^{d}	3.7 ± 0.2^{d}
	6PGD[2]	76.0 ± 4.2^{d}	167.3 ± 11.4^{d}	123.7 ± 14.6^{d}	67.4 ± 3.0^{c}	76.7 ± 5.0^{d}	21.4 ± 3.0^{d}
Corn + culture filtrate	G6PD	81.3 ± 6.8^{a}(149)[3]	801.2 ± 41.3^{a}(250)	387.0 ± 24.0^{c}(149)	174.0 ± 5.0^{b}(156)	290.0 ± 12^{b}(167)	7.1 ± 0.3^{c}(192)
	6PGD	121.5 ± 13.4^{b}(160)	304.4 ± 23.9^{b}(182)	223.1 ± 8.4^{b}(180)	100.7 ± 2.0^{b}(149)	113.0 ± 8.0^{b}(147)	33.4 ± 3^{c} (156)
Barley	G6PD	54.9 ± 3.2±(101)	360.9 ± 19.0^{c}(113)	451.0 ± 16.0^{b}(175)	158.3 ± 5.0^{c}(142)	191.0 ± 7.0^{c}(110)	13.4 ± 0.5^{b}(362)
	6PGD	85.4 ± 8.0^{c}(112)	219.5 ± 9.8^{c}(131)	200.0 ± 8.4^{c}(162)	100.4 ± 4.0^{b}(149)	103.2 ± 4.0^{c}(135)	52.2 ± 4.0^{b}(244)
Barley +culture filtrate	G6PD	78.3 ± 4.8^{a}(144)	695.3 ± 102^{b}(217)	657.0 ± 17.0^{a}(253)	219.0 ± 2.0^{a}(197)	317.5 ± 17^{a}(182)	28.9 ± 0.7^{a}(781)
	6PGD	141.1 ± 3.8^{a}(186)	395.1 ± 24.7^{a}(236)	282.0 ± 8.0^{a}(228)	156.4 ± 4.0^{a}(232)	169.2 ± 7.0^{a}(221)	80.4 ± 3.0^{a}(376)

[1]Feeding period was 3 weeks. Time of killing was 0800 hr. Data expressed as means ± SD; N=5 swine per group.

[2]G6PD = glucose-6-phosphate dehydrogenase and 6PGD = 6-phosphogluconate dehydrogenase as nmoles of $NADP^{+}$ reduced/min/mg cytosolic protein.

[3]Percentage of control (corn-diet) activity data are in parentheses.

a-dMeans within a column and without a common superscript letter are significantly different, $p<0.01$.

TABLE 6

Effect of Cereals and Culture Filtrate of *T. viride* on the Activities of Malic and Citrate Cleavage Enzymes in Certain Swine Tissues[1]

Nutritional state		Tissue					
		Liver	Adipose (inside)	Adipose (outside)	Intestine	Lung	Muscle
Corn	ME[2]	204.3 ± 25.0^{d}	879.0 ± 42.0^{d}	1104.0 ± 43.0^{d}	596.0 ± 77.0^{d}	340.3 ± 13.0^{d}	32.3 ± 8.0^{d}
	CCE[2]	3.5 ± 0.4^{c}	8.3 ± 0.7^{d}	17.0 ± 1.2^{d}	4.8 ± 0.4^{d}	8.4 ± 0.2^{d}	1.6 ± 0.1^{c}
Corn + culture filtrate	ME	354.0 ± 28.0^{b}(173)[3]	1499.0 ± 63.0^{b}(171)	1507.0 ± 50.0^{c}(137)	951.6 ± 25.0^{c}(160)	558.0 ± 78.0^{c}(164)	51.0 ± 5.0^{b}(158)
	CCE	5.7 ± 0.3^{b}(163)	19.2 ± 1.4^{b}(231)	34.2 ± 2.0^{c}(201)	6.9 ± 0.3^{c}(144)	14.1 ± 0.7^{b}(168)	3.4 ± 0.1^{b}(213)
Barley	ME	319.0 ± 11.0^{c}(156)	969.0 ± 73.0^{c}(110)	1667.0 ± 150^{b}(151)	1121.0 ± 32.0^{b}(188)	655.0 ± 17.0^{b}(192)	39.2 ± 2.0^{c}(121)
	CCE	6.1 ± 0.6^{b}(174)	13.6 ± 1.8^{c}(164)	43.0 ± 2.0^{b}(253)	7.6 ± 0.2^{b}(158)	10.2 ± 1.0^{c}(121)	3.1 ± 0.2^{b}(194)
Barley + culture filtrate	ME	401.0 ± 45.0^{a}(196)	1616.0 ± 86.0^{a}(184)	2442.0 ± 72.0^{a}(221)	1574.0 ± 17.0^{a}(264)	870.0 ± 36.0^{a}(256)	91.0 ± 7.0^{a}(282)
	CCE	9.9 ± 0.2^{a}(283)	23.8 ± 2.7 (287)	80.0 ± 3.8^{a}(471)	10.5 ± 0.8^{a}(219)	16.6 ± 0.6^{a}(198)	6.8 ± 0.2^{a}(425)

[1]Feeding period was 3 weeks. Time of killing was 0800 hr. Data expressed as means ± SD; N=5 swine per group.

[2]ME = malic enzyme and CCE = citrate cleavage enzyme as nmol of $NADP^{+}$ reduced/min/mg cytosolic protein (ME) or nmol of product formed/min/mg cytosolic protein (CCE).

[3]Percentage of control (corn-diet) activity data are in parentheses.

[a-d]Means within a column and without a column superscript letter are significantly different, $p<0.01$.

sue; a reflection most likely of the very active biosynthetic process in the latter tissue.

Whereas the addition of CF to the diets effected a halving of HMG-CoA reductase activity in each tissue, CF addition to the diet significantly increased fatty acid biosynthesis in each tissue (Tables 5,6 and 8) compared to the dietary control, CF appears to elevate the activities of ACX and FAS disproportionately higher than it elevates the activity of CCE and the production of reducing equivalents. Ratios of NADPred/CCE, NADPred/ACX and NADPred/FAS were calculated for each tissue and treatment. The first ratio was not greatly influenced by the CF; the latter two activity ratios were considerably lower in all tissues except the inner adipose tissue of gilts fed diets containing CF.

DISCUSSION

Although these studies were carried out to examine the influence of dietary cereal and CF on certain lipogenic activities in swine, the data also provide information regarding the relative lipogenic capacities of liver and adipose tissue. Our estimate is that the liver possesses 8% of the FAS activity which is present in adipose tissue. The ratio of the FAS activity in adipose tissue to that present in liver is 12.6 ± 1.5. The ratios for ME (16.0 ± 1.8), CCE (15.8 ± 2.3) and ACX (13.3 ± 2.0) approach the FAS activity ratio. The tissue activity ratios for the $NADP^+$-dependent pentose phosphate/shunt enzymes, G6PD and 6PGD, were 24.7 ± 4.7 and 7.7 ± 0.8, respectively. These estimates are consistent with earlier reports (10-14) that adipose tissue serves as the major site of porcine lipogenesis.

In regard to cholesterol metabolism, the adipose biosynthetic and oxidative capacities were 7.2 ± 1.0-fold and 8.2 ± 2.2-fold for those of the liver, respectively. These calculations suggest that liver has only 14% of the biosynthetic capacity and 12% of the oxidative capacity present in adipose tissue. These estimates and the order of specific activities shown on Table 7 are in agreement with the observations of Huang and Kummerow (15).

The cereal base of the CF-free diet influenced weight gain (Table 3), serum and muscle cholesterol concentrations (Table 7), and the specific activities of all enzymes (Tables 4-6,8)) except hepatic G6PD (Table 6) and inner adipose tissue FAS (Table 8). Weight gains by barley-fed gilts were 12% (diet consumed/kg) or 15% (diet consumed/kcal) more efficient than that produced by the corn-based diet (Table 3). The proximate analyses of the diets (Table 2) show that the major differences in the diets are the

TABLE 7

Effect of Cereals and Culture Filtrate of *T. viride* Supplemented Diets on the Level of Cholesterol in Serum and Muscle of Swine[1]

Nutritional state	Days on feed							
	(Serum[2])						(Muscle[3])	
	0		5		10	15	21	21
Corn	92.5 ± 2.0		98.3 ± 3.0[a]		96.7 ± 3.0[a]	97.3 ± 4.0[a]	95.3 ± 3.0[a]	83.0 ± 4.6[a]
Corn + culture filtrate	98.6 ± 3.0		93.6 ± 3.0[b](95)[4]		77.6 ± 3.0[b](92)	85.2 ± 3.0[b](88)	83.6 ± 2.0[b](88)	65.0 ± 3.0[c](78)
Barley	98.6 ± 2.0		94.2 ± 3.0[b](96)		87.6 ± 3.0[b](89)	81.7 ± 2.0[c](84)	78.6 ± 2.0[c](82)	69.2 ± 2.0[b](83)
Barley + culture filtrate	98.3 ± 2.0		94.6 ± 2.0[b](96)		83.2 ± 2.0[c](86)	75.4 ± 3.0[d](77)	70.1 ± 2.0[d](73)	64.3 ± 1.5[c](77)

[1]Feeding period was 3 weeks. Time of killing was 0800 hr. Data expressed as means ± SD; N=5 swine per group.
[2]The cholesterol concentration is expressed as mg/100 ml of serum.
[3]The cholesterol concentration is expressed as mg/100 g of muscle.
[4]Percentage of respective control (corn-diet) concentration data are in parentheses.
[a-d]Means within a column and without a common small superscript letter are significantly different, $p<0.01$.

TABLE 8

Effect of Cereals and Culture Filtrate of *T. viride* on the Activities of Acetyl-CoA Carboxylase and Fatty Acid Synthetase in Certain Swine Tissues[1]

Nutritional state		Tissue					
		Liver	Adipose (inside)	Adipose (outside)	Intestine	Lung	Muscle
Corn	ACX[2]	9.2 ± 0.6[d]	43.8 ± 3.8[d]	42.7 ± 2.0[d]	2.3 ± 0.2[d]	1.3 ± 0.2[d]	0.6 ± 0.1[c]
	FAS[2]	13.2 ± 0.8[d]	55.5 ± 2.8[c]	52.0 ± 1.2[d]	2.7 ± 0.1[d]	1.6 ± 0.1[d]	0.8 ± 0.1[c]
Corn + culture filtrate	ACX	21.2 ± 2.2[b](230)[3]	72.4 ± 4.8[b](165)	74.7 ± 7.0[b](175)	5.5 ± 0.3[b](204)	3.9 ± 0.3[b](300)	1.4 ± 0.3[b](233)
	FAS	24.6 ± 1.0[b](186)	85.4 ± 1.9[b](154)	92.6 ± 6.6[b](178)	6.6 ± 0.2[b](244)	4.6 ± 0.4[b](288)	1.7 ± 0.2[b](213)
Barley	ACX	14.7 ± 0.6[c](160)	49.8 ± 5.4[c](114)	55.5 ± 5.0[c](130)	4.0 ± 0.2[c](174)	2.7 ± 0.3[c](208)	1.2 ± 0.1[b](200)
	FAS	19.1 ± 0.7[c](145)	58.3 ± 6.2[c](105)	60.4 ± 6.0[c](116)	4.6 ± 0.2[c](170)	2.8 ± 0.3[c](175)	2.0 ± 0.2[b](333)
Barley + culture	ACX	27.5 ± 1.8[a](299)	106.8 ± 10.5[a](244)	95.8 ± 7.8[a](224)	7.1 ± 0.3[a](309)	5.8 ± 0.3[a](446)	3.4 ± 0.2[a](567)
	FAS	30.4 ± 0.8[a](230)	123.7 ± 9.0[a](223)	115.4 ± 13.7[a](222)	8.9 ± 0.2[a](330)	6.6 ± 0.2[a](413)	5.3 ± 0.3[a](663)

[1]Feeding period was 3 weeks. Time of killing was 0800 hr. Data expressed as means ± SD; N=5 swine per group.

[2]ACX = acetyl-CoA carboxylase and FAS = fatty acid synthetase as nmol of product formed/min/mg cytosolic protein (ACX) or nmol of NADPH oxidized/min/mg cytosolic protein (FAS).

[3]Percentage of control (corn-diet) activity data are in parentheses.

[a-d]Means within a column and without a common superscript letter are significantly different, $p<0.01$.

concentrations of β-glucan and total dietary fiber. These materials increase cholesterol excretion and hence decrease plasma cholesterol (29-32). The lower FAS activity generally present in the tissues of the corn-fed gilts may represent a response to the greater quantity of linoleic acid present in this feed stuff. The addition of linoleic acid to fat-free diets inhibits hepatic lipogenesis (33). Conversely, the slightly greater quantity of fat in the corn-based diet (Table 2) may underlie the elevation of both the synthetic and oxidative activities in cholesterol metabolism. The factors underlying the increased feed efficiency of the gilts fed the barley-based diet are not defined. This diet provided 6.4% ash and 16.0% protein, whereas the corn diet provided 5.3% and 14.4% of the respective fractions. Our analysis of the amino acid contents of the two grains showed that lysine, threonine, valine, and isoleucine concentrations were 48, 12, 23 and 16%, respectively, higher in barley, whereas leucine was 64% higher in corn. These differences were diluted by the addition of the soybean meal.

The addition of CF to chicken diets increased weight gains, inhibited hepatic cholesterol biosynthesis and oxidation, lowered plasma cholesterol levels and stimulated hepatic fatty acid synthesis (1,2). We have now observed similar responses in the pig; additionally, we found that the CF produced similar enzymatic responses in adipose tissue, lung, intestine and muscle. The coordinate responses of HMG-CoA reductase and cholesterol 7α-hydroxylase have been observed in liver of rats (22) and chickens (1,2, 34). A review of our data reveals that the inhibitory factor in CF was equally effective towards adipose tissue and hepatic HMG-CoA reductase but, in regard to cholesterol 7α-hydroxylase, it was less inhibitory of the hepatic enzyme than of the adipose tissue enzyme.

The tissue of gilts fed diets containing CF exhibited greater lipogenic activity than the tissues of gilts fed the non-CF diets. This was evident by the increase in activity of the two pentose phosphate/shunt enzymes by 167.2 ± 25.3%, malic enzyme 158.8 ± 26.8%, CCE 177.6% ± 28.1%, ACX 215.2 ± 44.1% and FAS 210.5 ± 43.5%. These studies support previous research which indicates the majority of de novo fatty acid synthesis occurs at adipose tissue site in swine and to some extent in the liver (10,11). The effectiveness of CF to inhibit cholesterol biosynthesis was evident at the cellular level in the relatively short time-frame of 20 days in adult swine. However, these studies failed to identify a specific site of action of the CF factor. Consistent with the data would be the interpretation that a factor in the CF carries the properties of an anabolic hormone. A problem now under study is whether or not the factor in the CF that inhibits cholesterol biosynthesis and oxidation is the factor that increases feed efficiency and stimulates lipogenic activity.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Bray for his valuable suggestions and Dr. Burt Olson for the use of the Isocap/300 Nuclear Liquid Scintillation counter. We also thank Steven W. Faber and Margaret Dietrich for their excellent technical assistance and Faye Roed for her excellent editorial assistance.

This investigation was supported in part by Hatchfunds No. 1718 of the Research Division, College of Agricultural and Life Sciences, University of Wisconsin-Madison and by Research Grants HL25591 from National Heart and Lung Institute, National Institutes of Health, United States Public Health Service, and from USDA-SEA/AR Competitive Research Grants, Human Nutrition Program, grant number 8000597.

REFERENCES

1. Qureshi, A.A., Burger, W.C., Prentice, N., Bird, H.R., and Sunde, M.L. (1980) J. Nutr. 110, 1014-1022.
2. Qureshi, A.A., Burger, W.C., Prentice, N., and Elson, C.E. (1980) J. Nutr. 110, 1473-1478.
3. Leveille, G.A., Romsos, D.R., Yeh, Y.Y., and O'Hea, E.K. (1975) Poultry Sci. 54, 1075-1093.
4. Waterman, R.A., Romsos, D.R., Tsai, A.C., Miller, E.R., and Leveille, G.A. (1975) Proc. Soc. Exp. Biol. Med. 156, 220-225.
5. Leveille, G.A., O'Hea, E.K., and Chakabarty, K. (1968) Proc. Soc. Exp. Biol. Med. 128, 398-401.
6. Yeh, S.J.C., and Leveille, G.A. (1973) Proc. Soc. Exp. Biol. Med. 142 115-119.
7. Goodridge, A.G. (1968) Biochem. J. 108, 655-661.
8. O'Hea, E.K., and Leveille, G.A. (1968) Comp. Biochem. Physiol. 26, 111-120.
9. Dietschy, J.M., and Siperstein, M.D. (1967) J. Lipid Res. 8, 97-104.
10. Mersmann, H.J., Allen, C.D., Steffen, D.G., Brown, L.J., and Danielson, D.M. (1976) J. Anim. Sci. 43, 140-150.
11. O'Hea, E.K., and Leveille, G.A. (1969) J. Nutr. 99, 338-344.
12. Mersmann, H.J., Houk, J.M., Phinney, G., Underwood, M.C., and Brown, L.J. (1973) Am. J. Physiol. 224, 1123-1129.
13. Allee, G.L., Romsos, D.R., Leveille, G.A., and Baker, D.H. (1972) J. Anim. Sci. 35, 41-47.
14. Romsos, D.R., Allee, G.L., and Leveille, G.A. (1971) Proc. Soc. Exp. Biol. Med. 137, 570-573.
15. Huang, W.Y., and Kummerow, F.A. (1976) Lipids 11, 34-41.
16. Association of Official Analytical Chemists (1975) Official Methods of Analysis (12th Ed.) p. 926, Association of Official Agricultural Chemists, Washington, DC.
17. Pomeranz, Y., and Robbins, G.S. (1972) Am. Assoc. Cereal Chem. 49, 560-565.
18. Qureshi, A.A., Burger, W.C., Prentice, N., Bird, H.R., and Sunde, M.L. (1980) J. Nutr. 110, 388-393.
19. Anderson, D.B., and Kauffman, R.G. (1973) J. Lipid Res. 14, 160-168.
20. Gornall, A.G., Bardawill, C.J., and David, M.M.

(1949) J. Biol. Chem. 177, 751-766.
21. Shapiro, D.J., Norstrom, J.L., Mitchelen, J.J., Rodwell, V.W., and Shimke, R.T. (1974) Biochim. Biophys. Acta 370, 369-377.
22. Carlson, S.E., Mitchell, A.D., and Goldfarb, S. (1978) Biochim. Biophys. Acta 531, 115-124.
23. Craig, M.C., Nepkroeff, C.M., Lakshmanan, M.R., and Porter, J.W. (1972) Arch. Biochem. Biophys. 152, 619-630.
24. Nepokroeff, C.M., Lakshmanan, M.R., and Porter, J.W. (1975) in Methods in Enzymology (Lowensein, J.M., ed.) pp. 45-49, Academic Press, New York, NY.
25. Bottomley, R.H., Pitot, H.C., Potter, V.R., and Morris, H.P. (1963) Cancer Res. 23, 400-409.
26. Hsu, R.H., and Lardy, H.A. (1967) J. Biol. Chem. 245, 520-526.
27. Inoue, H., Suzuke, F., Fukunishi, K., Adachi, K., and Takeda, Y. (1966) J. Biochem. 60, 543-553.
28. Snedecor, G.W., and Cochran, W.G. (1971) Statistical Methods, 6th edn. pp. 258-298, The Iowa State University Press, Ames, IA.
29. Anderson, J.W., and Chen, W.J.L. (1979) Am. J. Clin. Nutr. 32, 346-363.
30. Reddy, B.S., Watanabe, K., and Sheinfil, A. (1980) J. Nutr. 110, 1247-1253.
31. McNaughton, J.L. (1978) J. Nutr. 108, 1842-1848.
32. Jenkins, D.J.A., Leeds, A.R., Newton, C., and Cummings, J.H. (1975) Lancet 1, 1116-1117.
33. Muto, Y., and Gibson, D.M. (1970) Biochem. Biophys. Res. Commun. 38, 9-15.
34. Mitchell, A.D., Carlson, S.E., McGibbon, W.H., and Goldfarb, S. (1979) Atherosclerosis 32, 11-21.
35. Elson, C.E., Fuller, W.A., Kline, E.A., and Hazel, L.N. (1963) J. Anim. Sci. 22, 946-952.

[Received April 14, 1982]

Nonspecific Lipid Transfer Proteins as Probes of Membrane Structure and Function[1]

RICHARD C. CRAIN, *The Biological Sciences Group, U-125, The University of Connecticut, Storrs, CT 06268*

ABSTRACT

A protein that accelerates transfer of phospholipids of varying head group and fatty acid composition has been purified from bovine liver. As previously found for other phospholipid transfer proteins, "nonspecific lipid transfer protein" stimulates a kinetically biphasic transfer of radioactively labeled phospholipid from small unilamellar vesicles to unlabeled multilamellar vesicles. The kinetics are consistent with rapid transfer of phospholipid from the outer monlayer and slow transfer of that localized in the inner monolayer (half-times greater than 3 days for phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol). Protein catalyzed transfer is inhibited by high ionic strength and has an activation energy of 35 kJ/mol. The broad lipid specificity and ease of large-scale purification make these proteins candidates for membrane phospholipid compositional modification. The compositions of rat liver mitochondrial and microsomal membranes and Morris hepatoma 7288c mitochondrial membranes were altered by incubation with lipid vesicles and nonspecific lipid transfer protein. Incubation with phosphatidylcholine vesicles led to increased levels of phosphatidylcholine and decreased levels of other transferrable lipids (phosphatidylethanolamine, phosphatidylinositol, and cholesterol) unless the latter were included in the vesicles. When vesicles containing dipalmitoylphosphatidylcholine were incubated with microsomal membranes, a large increase in disaturated phosphatidylcholine was also observed. These changes in composition were correlated with activities of membrane enzymes. It appears that microsomal glucose-6-phosphatase is inhibited by increased phosphatidylcholine saturation. Moreover, this enzyme is also inhibited by decreases in the phosphatidylethanolamine/phosphatidylcholine ratio whereas NADPH cytochrome c reductase is not. Likewise, decreased cholesterol to phospholipid ratios did not greatly affect the abnormally low levels of hepatoma succinate cytochrome c reductase activity.
Lipids 17:935-943, 1982.

The spontaneous transfer of phospholipid between single-walled phospholipid vesicles is slow (1,2); it exhibits half-times of the order of 13-24 hr (1). However, it can be accelerated by addition of post-microsomal supernatant fractions from a number of tissues (3). Phospholipid transfer activities have now been purified and characterized from a variety of sources including plants (4) and animals (5-9). The presence of these proteins in the cytosol has been suggested to explain the similarity of rates of phospholipid turnover found for the different subcellular organelle membranes (10,11), as well as the relatively rapid labeling of mitochondrial PtdCho and PtdEtn that occurs after injection of radioactive precursor, despite the fact that biosynthesis of these lipids occurs only in the endoplasmic reticulum (12,13). The important role of phospholipid transfer activities in membrane biogenesis has also been suggested by the observations that these activities increase during development, such as at the onset of myelogenesis (14) or of synthesis of PtdCho surfactant (15). Further evidence is found in hepatomas having aberrant phospholipid transfer protein where abnormal phospholipid compositions for mitochondrial, microsomal, and nuclear membranes are observed (16).

In addition to their putative role in membrane turnover and biogenesis, phospholipid transfer proteins have been applied as probes of membrane structure and function (17). PtdCho-transfer protein from beef liver and PtdIns/PtdCho-transfer protein from beef heart have been used to examine PtdCho asymmetry and transbilayer movement in artificial and biological membranes (18-21). The recent large-scale purification of a nonspecific lipid transfer protein from beef liver (9) has allowed these studies to be extended to include other classes of phospholipid (22). In addition, the availability of pure nonspecific lipid transfer protein permits the manipulation of phospholipid head group as well as fatty acid composition (23) and serves as a tool for examining the lipid dependence of membrane enzyme activities

[1]This paper was presented at the 73rd AOCS annual meeting, Toronto, Canada, May 1982.

Abbreviations: PdtCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; SphMye, sphingomyelin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; NS-TP, nonspecific lipid transfer protein; diPtdGro, diphosphatidylglycerol; diPal, dipalmitoyl.

TABLE 1

Relative Specificity of Purified Phospholipid Transfer Proteins

Source (ref)	Phospholipids transferred	Relative specificity	MW	Isoelectric point
Beef liver (29)	PtdCho	1	24,681	5.8
Beef liver (9)	PtdCho/PtdIns/PtdEtn/SphMye	1.6:2.2:1.0:0.5	13,900	9.5-9.7
Rat liver (7)	PtdCho/PtdIns/PtdEtn/SphMye	1.0:2.3:1.0:0.16	12,400	8.8
Beef heart (8)	PtdCho/PtdIns/PtdEtn/SphMye	3.5:10:0.01:0.5	33,400[a]	5.3-5.6
Beef brain (30)	PtdCho/PtdIns	1.0:8.0	32,000[a]	5.2-5.5
Rat hepatoma (31)	PtdCho/PtdIns/PtdEtn/SphMye	1:1:1:1	11,200	5.2

[a]Value obtained by SDS polyacrylamide gel electrophoresis.

(24). In the present report, the properties of nonspecific lipid transfer proteins are described that are important for their use in membrane studies, and their application to studies on artificial and biological membranes.

EXPERIMENTAL

^{32}P-labeled phospholipid was purified as previously described (7) from the liver of a rat injected intraperitoneally with ^{32}P-inorganic phosphate. Small unilamellar vesicles (25) and multilamellar vesicles (26) were prepared by standard procedures. Beef liver nonspecific lipid transfer protein (9) and PtdCho-transfer protein (5) were purified by published methodology. Phospholipid transfer was determined by measuring the movement of radioactively labeled phospholipid from donor to acceptor membrane. Labeled triolein was included as a nonexchangeable marker in order to correct for "sticking" or fusion of membranes. One unit of transfer activity is defined as the transfer of 1 nmol phospholipid/min at 37 C from PtdCho small unilamellar vesciles (64 nmol) to heat treated mitochondria (3). Mitochondrial and microsomal membranes were isolated from rat liver as previously described (24,27). Glucose-6-phosphatase (24) and NADPH cytochrome c reductase (28) were assayed in the absence of detergent as previously described. Details of experimental procedures are included in the legends to figures and tables.

RESULTS

Purification and Characterization

Nonspecific lipid transfer protein, which was measured by its catalytic rate of transfer of PtdEtn from PtdCho/PtdEtn (1:1, mol/mol) small unilamellar vesicles to heat treated mitochondria (7), was purified in large quantities from the post microsomal supernatant (pH 5.1 supernatant) of beef liver (9). Transfer activity in the crude supernatant is quite stable at -20 C, though less stable in purified fractions. Purification involves concentration by ammonium sulfate precipitation, ion exchange chromatography on CM cellulose, heat treatment, and hydrophobic chromatography on an octyl agarose column (9). Two fractions were eluted from the octyl agarose column. The first had a specific activity of 66 nmol/min mg and was purified 2,160-fold compared to the pH 5.1 supernatant. The second had a specific activity of 137 nmol/min mg and was greater than 90% pure as judged by SDS-polyacrylamide gel electrophoresis. Both fractions were free of phospholipase A_2 activity found as a contaminant at earlier stages of purification. The activity of the final fractions has a half-life of one month when stored at 4 C, though greater stability can be achieved by storage at -20 C, with or without 50% glycerol (half-life about 6 mo).

Specificity can be measured from the relative rates of transfer of various lipids from small unilamellar vesicles to multilamellar vesicles (9). As seen in Table 1, nonspecific lipid transfer protein from beef liver has a broad specificity, which is similar to that observed for the basic protein isolated from rat liver but contrasts to the high specificity of the acidic proteins from beef heart, liver, and brain. In agreement with this, antibody prepared against the beef liver nonspecific lipid transfer protein inhibits transfer catalyzed by the rat liver basic protein, but not the more specific transfer activities of the acidic proteins. It is interesting to note that the basic proteins appear to be identical (as determined by amino acid composition) to the sterol carrier protein$_2$ purified by Scallen and coworkers (9,32). In addition to their broad specificity for phospholipid class composition, these proteins have little specificity for fatty acid composition. DipalmitoylPtdCho is transferred nearly as well as rat liver PtdCho from small unilamellar vesicles containing both in varying proportions to multilamellar vesicles (Table 2). At very high dipalmitoyl PtdCho concentrations, transfer is inhibited, presumably because

TABLE 2

Transfer of Rat Liver PtdCho and diPalPtdCho from Small Unilamellar Vesicles to Multilamellar Vesicles by Nonspecific Lipid Transfer Protein[a]

Vesicles (% diPalPtdCho)	Phospholipid transferred (% transferred)	
	Rat liver PtdCho	diPalPtdCho
5%	14.3	10.3
10%	14.1	10.6
25%	12.9	8.6
50%	11.2	7.9

[a]Small unilamellar vesicles were prepared from ^{14}C-dipalmitoyl PtdCho and ^{32}P-rat liver PtdCho in the ratios indicated above. ^{3}H-Triolein was included as nonexchangeable marker and butylated hydroxytoluene as antioxidant (9). Small unilamellar vesicles (50 nmol) were incubated with 2 μmol multilamellar vesicles of the same composition (with 10 mol% diPtdGro) for 30 min at 37 C in the presence of 0.5 units transfer protein. Vesicles were separated by centrifugation and transfer calculated as described previously (3), subtracting blank transfer in the absence of protein.

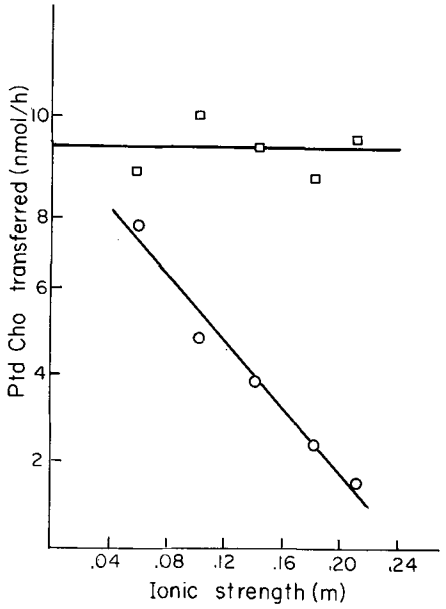


FIG. 1. Effect of ionic strength on protein catalyzed transfer of PtdCho. 50 mM PtdCho small unilamellar vesicles were incubated for 60 min at 37 C with 1.2 μmol PtdCho/diPtdGro (9:1, mol/mol) multilamellar vesicles and 0.06 units either nonspecific lipid transfer protein (○) or PtdIns/PtdCho beef heart transfer protein (□) in 0.5 ml of 1 mM EDTA, 50 mM Tris, pH 7.4, containing varying concentrations of NaCl to adjust the ionic strength (molal). Vesicles were separated and transfer calculated as described previously (9).

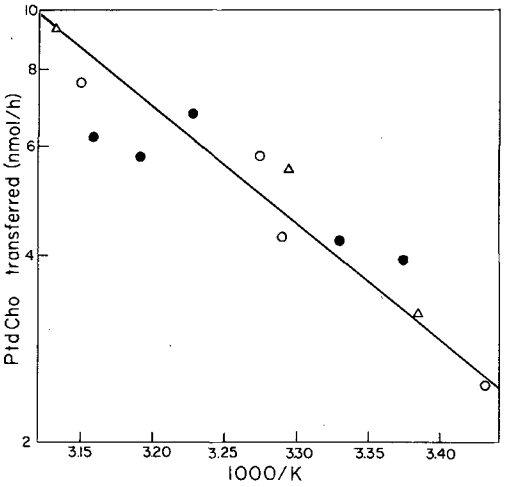


FIG. 2. Temperature dependence of nonspecific lipid transfer protein catalyzed transfer of PtdCho from small unilamellar vesicles to multilamellar vesicles. In a total volume of 1 ml of 1 mM EDTA, 50 mM Tris (pH 7.4) were suspended 50 nmol PtdCho small unilamellar vesicles, 2 μmol PtdCho/diPtdGro (9:1, mol/mol) multilamellar vesicles, and 0.1 units nonspecific lipid transfer protein. Transfer was measured after 30 min incubation and calculated as described in Figure 1. Different symbols represent different experiments.

the bilayer is below the gel-liquid crystalline phase transition (data not shown).

Finally, two additional properties of the beef liver nonspecific lipid transfer protein must be considered before they are used as membrane probes. First, in contrast to transfer catalyzed by PtdIns/PtdCho transfer proteins from beef heart, transfer stimulated by nonspecific lipid transfer proteins is markedly inhibited at elevated ionic strength (Fig. 1). Therefore, care should be taken to use incubation conditions of low ionic strength or to compensate by using increased protein concentration. Second, the temperature dependence of transfer of phospholipid (Fig. 2) is similar to other lipid transfer proteins investigated, the energy of activation of 35 kJ/mol is identical to values of 34 kJ/mol for bovine brain and bovine liver PtdCho transfer proteins (33). As can be seen in Fig. 2, transfer can be effected over a broad range of temperatures. However, as mentioned above, at temperatures below the phase transition of the bilayer, the rate of lipid transfer is greatly reduced.

Phospholipid Transfer Between Lipid Bilayers

It has previously been established that, in incubations of PtdCho small unilamellar vesicles

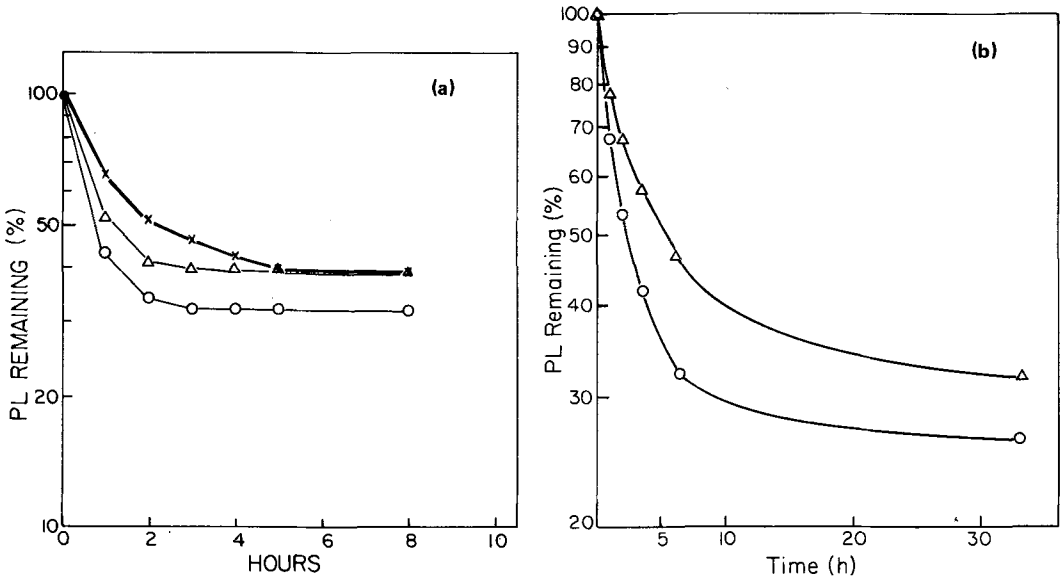


FIG. 3. Time course of nonspecific lipid transfer protein catalyzed transfer of PtdCho, PtdEtn, PtdIns, and SphMye. (a) 250 nmol small unilamellar vesicles composed of radioactively labeled PtdCho/PtdEtn/PtdIns (45:45:10, mol/mol) were incubated at 37 C with 10 μM multilamellar vesicles and 12 units of non-specific lipid transfer protein in 5 ml of 1 mM EDTA, 50 mM Tris, pH 7.4. At time points, multilamellar vesicles were sedimented by centrifugation for 15 min at 25,000 × g. Transfer of phospholipid was quantitated as previously described (3). The decanted small unilamellar vesicles were incubated with an additional 10 μmol multilamellar vesicles. The logarithm of PtdCho (○), PtdEtn (×), and PtdIns (△) remaining are plotted as a function of time of incubation. (b) 125 nmol small unilamellar vesicles composed of PtdCho/SphMye (50:50 mol/mol) were incubated at 37 C with 5 μmol multilamellar vesicles of the same composition containing 10 mol% diPtdGro and 8 units nonspecific lipid transfer protein in 2.5 ml buffer as described above. Log PtdCho (○) and SphMye (△) remaining are plotted as a function of time.

and PtdCho or PtdIns/PtdCho transfer protein, only phospholipid from the outer monolayer is utilized (19,20). In agreement with this conclusion, it was found that incubation of nonspecific lipid transfer protein with radioactively labeled PtdCho/PtdEtn/PtdIns (45:45:10 mol/mol) small unilamellar vesicles and unlabeled multilamellar vesicles of the same composition resulted in a biphasic kinetic plot of phospholipid transfer (Fig. 3a). The initial transfer was rapid and dependent on the concentration of transfer protein, whereas the latter, slow phase was independent of the protein concentration (data not shown). Nonspecific lipid transfer protein also catalyzed movement of phospholipid from radioactively labeled PtdCho/SphMye (50:50 mol/mol) small unilamellar vesicles to unlabled multilamellar vesicles (Fig. 3b). The initial phases of PtdCho transfer was more rapid than that for sphingomyelin transfer, which is consistent with the protein's known specificity (Table 1). However, both lipids exhibited similar slow phases of transfer.

These results are consistent with the notion that rapid transfer of phospholipid occurs from the external monolayer and that this is followed by a slow transfer, which is dependent on the transbilayer movement (flip-flop) of phospholipid. Although this second phase of transfer is very slow for synthetic vesicles (Fig. 3), more rapid transbilayer movement of phospholipid has been found by these techniques for red cell (22) and microsomal membranes (34) labeled in vivo with $^{32}P_i$.

The broad specificity of the nonspecific lipid transfer proteins for phospholipid head group (Table 1) and fatty acid (Table 2) makes them ideal for the modification of membrane phospholipid composition to relate membrane lipid composition to membrane function. To determine the applicability of this technique to the alteration of biological membranes, rat liver mitochondrial and microsomal membranes were incubated with PtdCho small unilamellar vesicles and nonspecific lipid transfer protein. After separation from vesicles, the phospholipid composition of the altered membranes was determined (after correcting for cosedimentation of vesicles by quantitation of the nonexchangeable marker ^{3}H-triolein). As shown in

TABLE 3

Incubation of PtdCho Vesicles with Nonspecific Lipid Transfer Protein and Rat Liver Mitochondrial or Microsomal Membranes[a]

Membrane	PtdCho-Vesicles (μmol)	NS-TP[b] (units)	Phospholipid composition (% total) PtdCho	PtdEtn	PtdIns	DiPtdGro	Total phospholipid (nmol/mg)
Mitochondria	–	–	39	40	4.2	17	200
Mitochondria	1.2	–	41	39	4.3	16	190
Mitochondria	1.2	1	61	23	2.2	14	290
Microsomes	–	–	63	22	14	–	580
Microsomes	1.2	–	64	22	14	–	560
Microsomes	1.2	1	77	14	9.4	–	630

[a]Inner mitochondrial membranes (8 mg) or microsomal membranes (3 mg) were incubated at 37 C with 1.2 μmol egg PtdCho small unilamellar vesicles and 1 unit nonspecific lipid transfer protein in 70 mM sucrose, 200 mM mannitol, 2 mM HEPES, pH 7.4. Controls were performed without transfer protein and without vesicles or transfer protein. After 60 min, membrane was sedimented by centrifugation, homogenized in 1 ml buffer, extracted in chloroform/methanol, and analyzed by thin layer chromatography (35). Phospholipid compositions are corrected for cosedimentation of vesicles by quantitation of ^{3}H-triolein and expressed as percent of total phospholipid (27).

[b]NS-TP, nonspecific lipid transfer protein.

Table 3, very little change in lipid composition resulted from coincubation of PtdCho vesicles alone with mitochondrial or microsomal membranes. However, inclusion of nonspecific lipid transfer protein resulted in a significant change in the composition. In addition to an increase in the proportion of PtdCho and a decrease in the proportion of PtdEtn and PtdIns in total membrane lipid, an increase was found in the total phospholipid content of microsomal and particularly mitochondrial membranes (Table 3). This finding, which indicates the occurrence of a net transfer of phospholipid from vesicle to membrane, is in agreement with a previous finding that these transfer proteins are capable of net transfer of lipid (27).

The capability of nonspecific lipid transfer proteins to carry out net phospholipid transfer should be considered when making conclusions about membrane structure and function using them as probes. The extent of net transfer depends on the nature of the donor and acceptor membranes and the conditions of incubation (27).

Effects of Altered Lipid Composition on Membrane Enzyme Activity

The effect of induced alterations of membrane phospholipid concentration on enzyme activity has also been examined. Initial studies were carried out using rat liver microsomes for the following reasons: these membranes can be prepared relatively easily, they contain a number of well characterized membrane bound enzymes that appear to be lipid dependent, and finally, they exhibit a rapid transbilayer movement (flip-flop) of phospholipid (34,36,37), thus allowing a maximum change in phospholipid composition to be completed within relatively short periods of time. In order to study the dependence of microsomal function on phospholipid composition, microsomal membranes were incubated with nonspecific lipid transfer protein and vesicles containing phospholipids of varied classes and fatty acid composition. As shown in Table 4, these incubations resulted in a substantial inhibition of glucose-6-phosphatase activity when vesicles were prepared from PtdCho (either primarily unsaturated or saturated). However, when vesicles containing PtdEtn were incubated with nonspecific lipid transfer protein and microsomes, little or no inhibition resulted. On the other hand, when PtdCho transfer protein, which generally has been found to catalyze a one-for-one exchange of PtdCho (27,38,39), though under certain conditions it has been found capable of net transfer (40), was incubated with vesicles and microsomes, no inhibition of glucose-6-phosphatase resulted, except in the presence of dipalmitoylPtdCho (Table 4). From the total phospholipid compositions of the membranes after incubation (Table 4), it can be concluded that saturated PtdCho and PtdEtn content each have an independent, or at least additive, effect on enzyme activity. In contrast to this effect of membrane modification on glucose-6-phosphatase activity, changes in PtdEtn content have no effect on NADPH cytochrome c reductase activity (data not shown) as one would expect from the known exposed location of the active site of this

TABLE 4

Glucose-6-Phosphatase and Phospholipid Compositions of Microsomal Membranes Incubated with Lipid Vesicles and Phospholipid Transfer Protein[a]

Transfer protein[b]	Vesicle composition (mol/mol)	Phospholipid composition (% total)				Total phospholipid (nmol/mg)	Glc-6-pase activity (% control)
		PtdCho	diPalPtdCho	PtdEtn	PtdIns		
–	–	55	–	21	15	607	–
–	diPalPtdCho:RL PtdCho(3/1)	54	1.6	21	15	641	100
NS-TP	diPalPtdCho:RL PtdCho(3/1)	63	22	16	11	618	63
PtdCho-TP	diPalPtdCho:RL PtdCho(3/1)	54	20	23	16	633	85
–	RL PtdCho	54	–	23	15	626	100
NS-TP	RL PtdCho	65	–	14	8	645	76
PtdCho-TP	RL PtdCho	52	–	22	16	647	103
–	RL PtdCho:egg PtdEtn(3/1)	54	–	23	16	600	100
NS-TP	RL PtdCho:egg PtdEtn(3/1)	56	–	24	10	603	99
PtdCho-TP	RL PtdCho:egg PtdEtn(3/1)	54	–	21	15	603	98

[a]Microsomes (5 mg) were incubated for 60 min at 37 C in 250 mM sucrose, 5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA with 2 units nonspecific lipid transfer protein or PtdCho-transfer protein and 6 μmol small unilamellar vesicles of the following compositions: (1) diPalPtdCho/rat liver PtdCho/diPtdGro/cholesterol (7.5:2.5:1:1.5 mol/mol); (2) rat liver PtdCho/diPtdGro/cholesterol (10:1:1 mol/mol); and (3) rat liver PtdCho/egg PtdEtn/diPtdGro/cholesterol (7.5:2.5:1.0:1.0 mol/mol). Vesicles and membranes were separated by centrifugation. Membranes were suspended in buffer and analyzed for protein and glucose-6-phosphatase activity (expressed as percent of control incubated with vesicles but no transfer protein). Lipids were extracted and analyzed by thin layer chromatography (35).

[b]NS-TP, nonspecific lipid transfer protein; PtdCho-TP, phosphatidylcholine-transfer protein; RL, rat liver.

TABLE 5

Incubation of PtdCho Vesicles with Rat Hepatoma Mitochondria and Nonspecific Lipid Transfer Protein[a]

Source	NS-TP (units)	PtdCho vesicles (μmol)	PL[b] nmol/mg	Chol[b] nmol/mg	Chol/PL	Succinate cytochrome c reductase (nmol/mg min)
A. Unincubated						
liver	–	–	211 ± 20	13.7 ± 6	0.064 ± 0.023	153 ± 48
hepatoma	–	–	204 ± 50	46.6 ± 16.0	0.24 ± 0.09	16 ± 4
B. Incubated						
hepatoma	–	–	242	67	0.28	16
hepatoma	–	4	260	67.5	0.26	15
hepatoma	1	4	435	55	0.13	17

[a]Mitochondria were isolated from Morris hepatoma 7288C (41) and host liver (42). Phospholipid and cholesterol were analyzed as described (24). Succinate cytochrome c reductase was assayed by standard methodology (43). Hepatoma mitochondria (3.6 mg) were incubated for 60 min at 30 C in 250 mM sucrose, 1 mM EDTA, 50 mM Tris, pH 7.4 with 4 μmol PtdCho/diPtdGro (10:1, mol/mol) small unilamellar vesicles and 1 unit nonspecific lipid transfer protein. Controls were performed without transfer protein and without vesicles or protein. Mitochondria were sedimented by centrifugation, homogenized in 2 ml buffer and the cholesterol, phospholipid, and succinate cytochrome c reductase activity determined. Values for unincubated mitochondria represent the mean ± standard deviation of mitochondrial membranes isolated from 8 separate animals. Values for hepatoma mitochondria incubated with PtdCho vesicles were average of duplicate determinations from one experiment which is representative of three performed. Duplicates agreed within 5%.

[b]PL, phospholipid; Chol, cholesterol.

enzyme on the cytoplasmic surface of the membrane.

Nonspecific lipid transfer proteins have also been used to examine the lipid dependence of enzyme activities in hepatoma membranes. Mitochondria isolated from the rapidly growing Morris hepatoma 7288c are characterized by a high cholesterol to phospholipid ratio (Table 5) and low rate of succinate oxidation (as measured by succinate cytochrome c reductase activity). It has previously been postulated that the altered metabolic properties of hepatoma mitochondria may result in part from the abnormally high levels of cholesterol (44,45). This speculation is supported by our finding that slowly growing hepatomas, which have lower levels of cholesterol, have increased activities of succinate cytochrome c reductase (Crain et al., manuscript in preparation). In an attempt to restore enzyme activities to hepatoma mitochondria, nonspecific lipid transfer protein and PtdCho vesicles were incubated with the mitochondria. The cholesterol transfer activity of nonspecific lipid transfer protein (9) resulted in a net transfer of cholesterol to the vesicles, and this was accompanied by a net transfer of PtdCho to mitochondria (Table 5). The resultant 2-fold diminution in cholesterol to phospholipid ratio produced no change in succinate cytochrome c reductase activity. However, this decrease in the cholesterol to phospholipid ratio is caused primarily by increased mitochondrial phospholipid rather than a large decrease in the total amount of cholesterol. The latter could be the critical factor in regulating the activity of the enzyme and, therefore, the absence of an effect on enzyme activity should be interpreted with caution.

DISCUSSION

The intimate relationship of integral membrane proteins with the lipid bilayer of biological membranes suggests that the lipid environment may play an important role in the function of integral membrane systems. This possible relationship has been examined by a number of techniques. For many membrane enzymes (46-49), the importance of bilayer integrity has been established by lipid depletion using either phospholipases (50) or organic extraction (51) in order to remove phospholipid from the bilayer. Unfortunately, phospholipases produce products such as lyso PtdCho and free fatty acids which themselves may inhibit enzyme activity, whereas organic solvents are known to denature proteins in a manner that is independent of their effect on membrane disruption. Chemical modifications, which also have been used to study the dependence of enzyme activities on membrane lipid, may alter proteins either directly or by production of inhibitory byproducts.

Alterations of membrane lipid compositions have also been induced by dietary manipulations and, although this method avoids many of the complications mentioned above, its use is largely limited to bacterial auxotrophs and

certain cell types in culture. With most eukaryotic systems, these changes would be accompanied by other metabolic changes and it may be difficult to distinguish changes in function caused by membrane lipid alterations from those resulting from these metabolic changes. Reconstitution of purified enzyme with lipid has also been useful in some cases, but this technique is limited in its usefulness for examining specific lipid-protein interactions because purified enzyme is required and because difficulties often occur in distinguishing a lipid requirement from a lipid stabilization effect (52).

Nonspecific lipid transfer proteins provide another technique to induce changes in biological membranes in order to study the dependence of membrane function on lipid composition. This technique permits large changes in both fatty acid composition and phospholipid head group composition to be achieved by choice of appropriate lipid vesicles. It does not require purification of functional membrane proteins, nor is it likely to cause denaturation of these proteins. Depending on the relative amounts of transfer protein and vesicle lipid added, transfer can be achieved over a large range of temperature and within a short time span. Unfortunately, there are also some difficulties involved in the use of nonspecific lipid transfer proteins for these types of studies. First, the membrane should be readily separable from lipid vesicles. Second, because transfer proteins interact only with phospholipid at the exterior surface, unless appreciable rates of phospholipid transbilayer movement exist, only the external monolayer lipid composition can be rapidly modified. Third, because a net transfer of phospholipid mass may occur, alterations in composition may be accompanied by changes in total phospholipid content and it may, therefore, be difficult to distinguish which of these alterations is most critical in regulating the function of intrinsic membrane proteins. In spite of these problems, nonspecific lipid transfer proteins may prove to be extremely useful as a vehicle for the introduction of foreign lipid into biological membranes and as a tool for the study of the structure as well as the structure-function relationships of biological membranes.

ACKNOWLEDGMENT

This investigation was supported in part by research grants 35-252 and 35-337 awarded by the University of Connecticut Research Foundation and a Grant-in-Aid of Research from Sigma Xi, the Scientific Research Society to R.C. Crain and Public Health Service Research Grant HL 10940 to Donald Zilversmit, Cornell University. A portion of this work was completed while RCC was a United States Public Health Service Postdoctoral fellow (HL 05730). The author acknowledges the invaluable advice, criticism, and support of D. Zilversmit during that time.

REFERENCES

1. Roseman, M.A., and Thompson, T.E. (1980) Biochemistry 19, 439-444.
2. Papahadjopoulos, D., Hui, S., Vail, W.J., and Poste, G. (1976) Biochim. Biophys. Acta 448, 245-264.
3. Zilversmit, D.B., and Hughes, M.E. (1976) Methods Membr. Biol. 7, 211-259.
4. Douady, D., Grosbois, M., Guerbette, F., and Kader, J.-C. (1982) Biochim. Biophys. Acta 710, 143-153.
5. Kamp, H.H., Wirtz, K.W.A., and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 318, 313-325.
6. Helmkamp, G.M., Jr., Harvey, M.S., Wirtz, K.W.A., and Van Deenen, L.L.M. (1974) J. Biol. Chem. 249, 6382-6389.
7. Bloj, B., and Zilversmit, D.B. (1977) J. Biol. Chem. 252, 1613-1619.
8. DiCorleto, P.E., Warach, J.B., and Zilversmit, D.B. (1979) J. Biol. Chem. 254, 7795-7802.
9. Crain, R.C., and Zilversmit, D.B. (1980) Biochemistry 19, 1433-1439.
10. McMurray, W.C., and Dawson, R.M.C. (1969) Biochem. J. 112, 91-108.
11. Lee, T.C., Stephens, N., Moehl, A., and Synder, F. (1973) Biochim. Biophys. Acta 291, 86-92.
12. Ada, G.L. (1949) Biochem. J. 45, 422-428.
13. Gurr, M.I., Prottey, C., and Hawthorne, J.N. (1965) Biochim. Biophys. Acta 106, 357-370.
14. Brophy, P.J., and Aitken, J.W. (1979) J. Neurochem. 33, 355-356.
15. Engle, M.J., Van Golde, L.M.G., and Wirtz, K.W.A. (1978) FEBS Lett. 86, 277-281.
16. Bergelson, L.D., Dyatlovitskaya, E.V., Sorokina, I.B., and Gorkova, N.P. (1974) Biochim. Biophys. Acta 360, 361-365.
17. Bloj, B., and Zilversmit, D.B. (1981) Mol. Cell. Biochem. 40, 163-172.
18. DiCorleto, P.E., and Zilversmit, D.B. (1979) Biochim. Biophys. Acta 552, 114-119.
19. Johnson, L.W., Hughes, M.E., and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176-185.
20. Rothman, J.E., and Dawidowicz, E.A. (1975) Biochemistry 14, 2809-2816.
21. Bloj, B., and Zilversmit, D.B. (1976) Biochemistry 15, 1277-1283.
22. Crain, R.C., and Zilversmit, D.B. (1980) Biochemistry 19, 1440-1447.
23. Muczynski, K.A., Harris, W.E., and Stahl, W.L. (1981) Int. J. Biochem. 13, 959-962.
24. Crain, R.C., and Zilversmit, D.B. (1981) Biochemistry 20, 5320-5326.
25. Johnson, L.W., and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176-185.
26. Dicorleto, P.E., and Zilversmit, D.B. (1977) Biochemistry 16, 2145-2150.
27. Crain, R.C., and Zilversmit, D.B. (1980) Biochim. Biophys. Acta 620, 37-48.
28. Phillips, A.H., and Langdon, R.G. (1962) J. Biol. Chem. 237, 2652-2660.
29. Kamp, H.H., and Wirtz, K.W.A. (1974) in Methods in Enzymology (Fleischer, S., and Packer, L., eds.) Vol. XXXII, part B, pp. 140-146, Academic Press, New York, NY.

30. Demel, R.A., Kalsbeek, R., Wirtz, K.W.A., and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 466, 10-22.
31. Dyatlovitskaya, E.V., Timofeeva, N.G., and Bergelson, L.D. (1978) Eur. J. Biochem. 82, 463-471.
32. Noland, B.J., Arebalo, R.E., Hansbury, E., and Scallen, T.J. (1980) J. Biol. Chem. 255, 4282-4289.
33. Helmkamp, G.M., Jr. (1982) Biophys. J. 37, 112-113.
34. Zilversmit, D.B., and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99-110.
35. Crain, R.C., Marinetti, G.V., and O'Brien, D.F. (1978) Biochemistry 17, 4186-4192.
36. Brophy, P.J., Burbach, P., Nelemans, S.A., and Westerman, J., Wirtz, K.W.A., and Van Deenen, L.L.M. (1978) Biochem. J. 174, 413-420.
37. Van den Besselaar, A.M.H.P., de Kruijff, B., Van den Bosch, H., and Van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 510, 242-255.
38. DiCorleto, P.D. (1978) Ph.D. thesis, Cornell University, Ithaca, NY.
39. Helmkamp, G.M., Jr. (1980) Biochem. Biophys. Res. Commun. 97, 1091-1096.
40. Wirtz, K.W.A., Devaux, P.F., and Bienvenue, A. (1980) Biochemistry 19, 3395-3399.
41. Kaschnitz, R.M., Hatefi, Y., Pedersen, P.L., and Morris, H.P. (1979) in Methods in Enzymology (Fleischer, S., and Packer, L., eds.) Vol. 55, pp. 79-88, Academic Press, New York, NY.
42. Schnaitman, C., and Greenawalt, J.W. (1968) J. Cell Biol. 38, 158-175.
43. Sottocasa, G.L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967) J. Cell Biol. 32, 415-438.
44. Coleman, P.S., Lavietes, B.B., Born, R., and Weg, A. (1978) Biochem. Biophys. Res. Commun. 84, 202-207.
45. Coleman, P.S., and Lavietes, B.B. (1981) CRC Critical Rev. Biochem. 11, 341-383.
46. Wallach, D.F.H. (1975) Membrane Molecular Biology of Neoplastic Cells, Elsevier Scientific Publishing Company, New York, NY.
47. Coleman, R. (1973) Biochim. Biophys. Acta 300, 1-30.
48. Foucans, B., and Jain, M.K. (1974) Adv. Lipid Res. 12, 147-226.
49. Sandermann, H., Jr. (1978) Biochim. Biophys. Acta 545, 209-237.
50. Fleischer, S., Brierley, G., Klouwen, H., and Slautterback, D.B. (1962) J. Biol. Chem. 237, 3264-3272.
51. Martonosi, A., Donley, J., and Halpin, R.A. (1968) J. Biol. Chem. 243, 61-70.
52. Racker, E. (1979) in Methods in Enzymology (Fleischer, S., and Packer, L., eds.) Vol. LV, part F, pp. 699-711, Academic Press, New York, NY.

[Received May 17, 1982]

Changes in the Activities of Lipoprotein Lipase and the Lipogenic Enzymes in Tumor-Bearing Rats

SUSAN LANZA-JACOBY*, ELIZABETH E. MILLER[1] and FRANCIS E. ROSATO,
Department of Surgery, Thomas Jefferson University, Philadelphia, PA 19107

ABSTRACT

The effects of tumor growth on lipid metabolism were investigated by evaluating serum lipids, lipoprotein lipase activity (LPLA), the lipogenic enzymes, urinary catecholamines along with serum insulin and glucagon levels. We injected 1.5×10^6 cells of rat mammary tumor, AC33, and killed the rats on the 18th day. Serum triglycerides and free fatty acids of the tumor-bearing (TB) rats increased 4 and 5 times, respectively, more than the control (C) rats. Total liver lipids were not significantly different between the two groups. Tumor growth produced a 70% decrease in total epididymal fat pad LPLA; there were no changes in soleus muscle LPLA. Serum insulin levels of the TB rats were 49% less than the C rats. The TB rats had significantly lighter epididymal fat pads and lower activities of adipose fatty acid synthetase and citrate cleavage enzyme. Urinary catecholamines of the TB rats were reduced over 30% compared with the C rats. These results show that the hypertriglyceridemia of the TB rats may be due, in part, to a deficiency of adipose tissue LPLA. The data also suggest that the effects of the tumor on lipid metabolism may be mediated through insulin.
Lipids 17:944-949, 1982.

Hypertriglyceridemia and the depletion of carcass fat stores have been observed in human cancer patients (1) and tumor-bearing rodent models (2-4). Barclay et al. (5) suggested that the elevation of serum triglycerides may result from a deficiency of LPL, the key enzyme responsible for triglyceride clearance from the blood. Recently, Thompson et al. (6) reported that LPLA was reduced in adipose tissue of mice bearing large preputial gland tumors. To our knowledge, there are no studies that determine the effects of tumor growth on the hormones regulating lipid metabolism as well as the changes in serum lipids and LPL activities in the extrahepatic tissues.

This investigation was initiated to extend previous findings concerning the observed hypertriglyceridemia and tissue activities of LPL in tumor-bearing animals. Activities of LPL were measured in the adipose tissue, soleus muscle, and tumor tissue in a tumor-bearing rat model to determine whether the elevation of serum triglycerides was due to a defect in the clearing mechanism. Since hormones have been shown to regulate the LPLA (7-11), we have measured levels of serum insulin and glucagon, and the urinary catecholamines. We also assessed the ability of the tumor to alter liver and adipose tissue lipogenesis by assaying the activities of FAS, CCE, and G6PDH. These parameters are evaluated as to their relative importance in the development of hypertriglyceridemia found in tumor-bearing rodents.

METHODS

Animals

Male Lewis rats (Microbiological Associates, Walkersville, MD), weighing between 230 g and 265 g, were used in this study. On the first day of the experiment, all rats were assigned to two groups. One group of rats, tumor-bearing (TB) was inoculated subcutaneously in the dorsal area with 1.5×10^6 cells/rat from a rat mammary adenocarcinoma (AC33). This dose produced a palpable tumor on day 6. The control (C) rats in the second group were injected in a similar manner with saline. The AC33 tumor was initially induced in this strain after treatment with dimethyl-β-aziridinopropionamide (12). All rats were fed a powdered stock diet (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) and water ad libitum. Food intake was measured daily. Animals were housed individually in metabolic cages at 23 ± 1 C on a 12-hr light-dark cycle. Body weights and the dimensions of the tumor were recorded periodically. Tumor volume was calculated by using the formula for a prolate spheroid ($V = 1/6\pi AB^2$ where A = long diameter, B = short diameter) (13). On day 16, a 24-hr urine collection was made

*Author to whom correspondence should be addressed.
[1]Present address: Harrison Department of Surgery, University of Pennsylvania, Philadelphia, PA 19104. Abbreviations: LPL, lipoprotein lipase; FAS, fatty acid synthetase; CCE, citrate cleavage enzyme; G6PDH, glucose-6-phosphate dehydrogenase; TB, tumor-bearing; C, control.

TABLE 1

Effect of Tumor Growth on the Concentration of Serum Lipids

Group	Triglycerides (mg/dl)	Cholesterol (mg/dl)	Free fatty acids (FA=SA molar ratio)	β-hydroxybutyrate (μmol/ml)
Control	63 ± 2[a]	123 ± 1	1.5 ± 0.3	1.46 ± 0.11
Tumor-bearing	232 ± 22[b]	140 ± 16	8.3 ± 0.9[b]	1.04 ± 0.09[b]

[a]Means ± SEM, N = 6-8.
[b]$p < 0.05$ compared with control.

for determination of urinary catecholamines. Prior to sacrifice by decapitation, all rats were fasted for 16 hr to equalize any differences in food consumption. Final carcass weights of TB rats were calculated by subtracting the tumor weights from the total body weights. A 1-ml aliquot of blood was collected in heparinized tubes to which 0.05 ml of Trasylol (Mobay Chemical Corp., New York, NY) and 1.2 mg of EDTA were added for insulin and glucagon determination. The remaining blood was collected in glass tubes. All blood samples were centrifuged at 4 C and stored at −20 C.

Liver, epididymal fat pads, soleus muscles, and tumors were removed, weighed, and stored on ice until processed.

Assay Methods

Serum and fluid from the tumor were analyzed for triglycerides (14), free fatty acids (15), and cholesterol (16). In addition, serum-β-hydroxybutyrate (17), glucose (18), insulin and glucagon (19) were also measured. Parts of the liver were retained for determination of fat (20), triglyceride (21), and cholesterol (22). Urine was analyzed for catecholamines (23). Soleus, liver, tumor, and epididymal fat tissue homogenates (1:5, w/v) were prepared in ice-cold, 0.25 M sucrose plus 1 mM EDTA buffer, pH 7.4, and centrifuged at 15,000 × g for 15 min at 4 C. The liver supernatant solution was centrifuged at 100,000 × g for 45 min at 4 C for assay of G6PDH, FAS, and CCE activities. Adipose and liver tissue homogenates were used for assay of FAS activity according to the procedure of Hsu et al. (24) and CCE activity (EC 4.1.3.8) by method of Takeda et al. (25). Activity of G6PDH (EC 1.1.1.49) was measured on adipose and liver tissue using the method described by Kaplan and Fried (26). Data for the activities of FAS and CCE are reported as units ± SEM; 1 unit representing 1 nmole of NADPH oxidized per minute. Activity of G6PDH is expressed as units ± SEM; 1 unit represents 1 nmol of NADPH formed per minute. Units are given as per mg protein and per whole tissue. Lipoprotein lipase activity (EC 3.1.1.3) was assayed in the postmitochrondrial supernatant solution of adipose tissue, soleus muscle, and tumor tissue using the method of Schotz et al. (27) with minor modifications (28). Previous reports have shown that muscle and adipose tissue activities of LPL were similar (29) or higher (28,30,31) in the postmitochrondial supernatant solution than in acetone-ether extracts. Based upon these reports and our preliminary findings that the activities of the lipogenic enzymes were significantly reduced in adipose tissue prepared with acetone-ether, we used the postmitochrondial supernatants for all enzyme determinations. Since the other fat pads were so small in the TB rats, the epididymal fat pads were selected for assay of LPL and the lipogenic enzymes. Several investigators (29,32,33) have shown that the LPL response in the epididymal fat pad was similar to that observed in other sites such as the retroperitoneal and the inguinal depots. The enzyme activity is expressed as units ± SEM; 1 unit representing 1 μmol free fatty acids released per hr incubation. Units are given as per mg protein and per whole tissue. The activity measured in the supernatants is attributed to LPL because it had a pH optimum at 7.5 for adipose tissue and a pH at 7.0 for soleus muscle and was inhibited maximally by 0.5 m NaCl and 100 μg of protamine sulfate. Protein concentration was measured on the supernatants by the method of Bradford (34) using bovine serum albumin as the standard.

Statistical Analysis

All data were subjected to analysis of variance and group means were tested at the 5% level of significance (35).

RESULTS

Food Intake and Carcass Weight

Total food consumption of the C rats (404 ± 15 g, mean ± SEM) and the TB rats

TABLE 2

Tumor Fluid Lipid Levels

Parameter	Mean ± SEM
Tumor weight (g)	55.2 ± 4.6
Tumor volume (cm^3)	36.4 ± 3.8
Free fatty acids (FA=SA molar ratio)	76.8 ± 5.9
Triglycerides (mg/dl)	72 ± 3
Cholesterol (mg/dl)	101 ± 8

(400 ± 9 g) was similar for the 18-day period. There were no significant differences in weight gain between the C (40 ± 6 g) and the TB (25 ± 4 g) rats. These findings indicated that this tumor did not cause anorexia in our rat model.

Serum Lipids

Hyperlipidemia was present in the TB rats. Serum triglycerides and free fatty acid levels increased in the TB rats 4- and 5-fold, respectively, compared with the C rats (Table 1). There were no significant differences in serum cholesterol levels between the two groups. In contrast, β-hydroxybutyrate levels of the TB rats decreased by 29% compared with the C rats. The lipid composition of the tumor fluid is presented in Table 2. The concentration of free fatty acids in the tumor fluid was 9 times the serum concentration of the TB rats.

Lipoprotein Lipase Activity

The activities of LPL in the adipose tissue, soleus muscle and tumor tissue are given in Table 3. When LPLA is expressed as per epididymal fat pad, there was a 70% decrease in LPL activity of the TB rats compared to the C rats. A 38% decrease in LPLA was observed during tumor growth when epididymal activity was expressed as per mg of protein. There was no significant differences in soleus muscle LPLA between the two groups.

Liver Lipids

As shown in Table 4, the TB rats had larger livers than the C rats. The amounts of total lipids, triglyceride and cholesterol were not significantly different between the two groups. The concentration in mg/g of wet weight of liver was 42.4 ± 1.1 for the C rats and 39.5 ± 1.2 for the TB rats; the concentration of triglycerides was 22.7 ± 1.2 for the C rats and 17.3 ± 1.7 for the TB rats; and the concentration of cholesterol for the C rats was 4.1 ± 0.1 and 3.8 ± 0.1 for the TB rats. Only the triglyceride concentration of the TB rats was significantly ($p < 0.05$) less than the C rats.

TABLE 3

Tissue Lipoprotein Lipase Activity of Control and Tumor-Bearing Rats

Groups	Adipose tissues (U[a]/mg protein) (U/whole tissue)	Soleus muscles (U/mg protein) (U/whole tissue)	Tumor tissue (U/mg protein) (U/whole tissue)
Control	1.014 ± 0.100[b]	0.145 ± 0.017	
	15.246 ± 2.050	0.847 ± 0.186	
Tumor-bearing	0.627 ± 0.323[c]	0.162 ± 0.011	0.051 ± 0.008
	4.533 ± 0.571[c]	0.792 ± 0.053	76.133 ± 9.763

[a]U = units which are μmol of free fatty acids released/hr.
[b]Means ± SEM; N = 6–8.
[c]$p < 0.05$ compared with control.

TABLE 4

Effect of Tumor Growth on the Liver Lipids

Groups	Total liver weight (g)	Total lipid (g)	Total triglycerides (g)	Total cholesterol (g)
Control	8.7 ± 0.1[a]	370.0 ± 11.1	197.7 ± 13.1	36.2 ± 1.7
Tumor-bearing	10.3 ± 0.2[b]	408.1 ± 13.4	181.0 ± 20.3	39.1 ± 0.7

[a]Means ± SEM, N = 6–8.
[b]$p < 0.05$, compared with control.

TABLE 5

Activities of Lipogenic Enzymes in Tumor-bearing and Control Rats

Groups	Fatty acid synthetase (U^a/mg protein) (U^a/whole tissue)	Citrate cleavage enzyme (U^a/mg protein) (U^a/whole tissue)	Glucose-6-phosphate dehydrogenase (U^b/mg protein) (U^b/whole tissue)
		Adipose tissue	
Control	25.9 ± 4.0[c]	36.3 ± 7.6	100.3 ± 11.2
	321.9 ± 54.4	461.0 ± 102.2	1254.9 ± 159.0
Tumor-bearing	8.5 ± 0.9[d]	7.6 ± 1.6[d]	67.0 ± 11.9[d]
	57.1 ± 11.4[d]	38.0 ± 11.5[d]	452.4 ± 79.0[d]
		Liver tissue	
Control	2.9 ± 0.3	3.1 ± 0.3	45.1 ± 2.5
	73.3 ± 9.2	88.9 ± 8.7	1166.5 ± 74.5
Tumor-bearing	2.9 ± 0.3	2.9 ± 0.2	42.3 ± 1.1
	89.5 ± 10.2	88.4 ± 7.7	1283.8 ± 45.4

[a]U = units which are nmol NADPH oxidized/min.
[b]U = units which are nmol NADPH formed/min.
[c]Means ± SEM, N = 6–8.
[d]$p < 0.05$ compared with control.

Lipogenic Enzyme Activities

The epididymal fat pads of the TB rats were 2.46 ± 0.14 g (mean ± SEM) which was significantly less than 1.51 ± 0.21 g for the C rats (Table 5). The activities of FAS and CCE in the adipose tissue of the TB rats were also significantly less than the C rats. G6PDH activity in the adipose tissue of the TB rats was significantly less than the C rats when the activity was expressed as per whole fat pad. Moreover, the tumor did not appear to alter lipogenesis in the liver as there was no changes in the activities of FAS, CCE, or G6PDH between the two groups.

Serum Glucose, Insulin and Glucagon

The level of serum glucose was slightly higher in the TB rats compared to the C rats (Table 6). Serum insulin levels of the TB rats were half the values of the C rats, while glucagon levels of both groups did not vary significantly.

Urinary Catecholamines

The excretion values of urinary epinephrine and norepinephrine are presented in Table 7. The urinary levels of nonrepinephrine and epinephrine were significantly less in the TB rats compared to the C rats. Changes in urinary norepinephrine, but not urinary epinephrine, were shown to correlate with plasma levels in human subjects (36). Data on the correlation of the urinary catecholamine levels to plasma levels in normal and tumor-bearing rodents have not been reported.

DISCUSSION

The hypertriglyceridemia observed during tumor growth may result from a decrease in the removal of triglycerides from the blood, an increase in liver synthesis of lipids, and/or

TABLE 6

Serum Levels of Glucose, Insulin, and Glucagon in Tumor-bearing and Control Rats

Group	Glucose (ml/dl)	Insulin (U/ml)	Glucagon (pg/ml)
Control	64 ± 1[a]	34 ± 2	232 ± 10
Tumor-bearing	71 ± 2[b]	17 ± 9[b]	190 ± 23

[a]Means ± SEM, N = 6–8.
[b]$p < 0.05$ compared with control.

TABLE 7

Urinary Catecholamine Excretion

Group	Epinephrine (μg/24 hr)	Nonrepinephrine (μg/24 hr)
Control	72 ± 2[a]	90 ± 9
Tumor-bearing	44 ± 8[b]	60 ± 7[b]

[a]Means ± SEM, N = 6–8.
[b]$p < 0.05$ compared with control.

an increase in triglyceride secretion rates. The results of this study suggest that the elevation of serum triglycerides in the TB rats compared with the C rats may be due to a decrease in the removal of triglycerides as indicated by the 70% reduction in LPLA in the adipose tissue. Thompson et al. (6) reported a similar decrease in adipose tissue LPLA in mice bearing preputial gland tumors. However, our findings on LPL, along with the data on serum insulin and the lipogenic enzymes, have not been presented for a rat tumor model. In this study, 18 days of tumor growth altered the activity of LPL in the adipose tissue but not in the soleus muscle. The presence of LPLA in the tumor indicates that it is capable of competing with other tissues for uptake of triglycerides. The nutritional state such as fasting or starvation are also known to affect LPLA (37,38). However, in this study, it was not likely that the overnight fast caused a greater decrease in LPLA in the TB rats compared to the C rats as both groups were treated in a similar manner.

Changes in the activity of LPL in the adipose tissue are related to insulin levels (7,8, 11). Ashby and Robinson (8) have found that insulin increased the activity of LPL in the adipose tissue by stimulating protein synthesis of the enzyme. Our results showed that the reduction of LPLA in the adipose tissue was accompanied by a decline in insulin levels of the TB rats compared with the C rats. From this experiment, it was not possible to ascertain the order of these changes. Further studies are required to find out which event occurred first: the decrease in insulin levels or LPLA.

Previous investigators have suggested that the activities of LPL in the heart and skeletal muscle are regulated by glucagon (7,9,10) and the catecholamines (8,10). The fact that there were no changes in glucagon levels or LPL activities in the soleus muscle were consistent with the above findings.

In this study, we found no changes in liver lipids or liver lipogenic enzymes between C and TB rats. Kitada et al. (39) also reported that liver fat content of mice with advanced stage lymphoma was similar to control mice. Our finding that liver lipogenic enzymes were not altered during tumor growth would suggest that liver synthesis of triglycerides is not changed. Since synthesis of lipids or total liver lipids did not appear to be affected by the tumor growth, it is also likely that secretion rates of liver triglycerides are not altered in these rats. Recently, Kannon et al. (40) and Lyon et al. (41) have shown that the secretion rate of triglycerides from the liver decreased in tumorous mice compared to control mice. These reports did not include any data on liver lipid composition or liver lipogenic activity. However, the data on triglyceride turnover reported by Lyon et al. (41) indicated that the decrease in the removal of triglycerides from the blood contributed to the hypertriglyceridemia of tumor-bearing rodents.

Fat mobilization was increased in the TB rats as evidenced by the elevation in serum free fatty acids, decrease in adipose tissue lipogenic enzymes, and the decrease in epididymal fat pad weights compared with the C rats. Since insulin is known to regulate lipid metabolism, it is possible that the increase in free fatty acid mobilization observed in the TB rats is related to the decrease in serum insulin. The tumor may have a primary effect on insulin synthesis or secretion which, in turn, would alter lipid metabolism. Other investigators have suggested that the tumor may produce lipid mobilizing factors which would stimulate lipolysis and/or affect the hormones regulating lipolysis and lipogenesis (1,42). Whether the tumor has a direct effect on lipid metabolism or whether the effects are mediated by changes in hormones or other regulatory compounds remains to be determined.

The data presented here suggest that the deficiency of LPL activity in the adipose tissue of TB rats compared to the C rats may account, in part, for the accumulation of triglycerides in the blood. Moreover, these experiments demonstrated that insulin is affected by the tumor and is associated, either directly or indirectly, with the alterations of lipid metabolism accompanied tumor growth.

ACKNOWLEDGMENTS

Insulin and glucagon assays were performed at the Diabetes Center, University of Pennsylvania, under NIH Grant AM 19525. The excellent technical assistance of Alma Tabares and Stephan Lansey is acknowledged.

REFERENCES

1. Mays, E.T. (1969) J. Surg. Res. 9, 273-277.
2. Frederick, G.L., and Begg, R.W. (1956) Cancer Res. 16, 548-551.
3. Brenneman, D.E., Mathur, S.M., and Spector, A.S. (1975) Eur. J. Cancer 11, 225-230.
4. Kremmer, T., and Holczinger, L. (1976) Acta Morphol. Acad. Sci. Hung. 24, 369-379.
5. Barclay, M., Calathes, D.N., Garfinkel, E., Terebuskekish, R.K., Barclay, R.K., and Skipski, V.P. (1962) Arch. Biochem. Biophys. 98, 391-396.
6. Thompson, M.P., Koons, J.E., Tan, E.T.H., and Grigor, M.R. (1981) Cancer Res. 41, 3228-3232.
7. Borensztajn, J., Samols, D.R., and Rubenstein,

A.H. (1972) Am. J. Physiol. 233, 1271-1275.
8. Ashby, P., and Robinson, D.S. (1980) Biochem. J. 188, 185-192.
9. Borensztajn, J., Keig, P., and Rubenstein, A.H. (1973) Biochem. Biophys. Res. Comm. 53, 603-613.
10. Palmer, W.K., Caruso, R.A., and Oscai, L.B. (1981) Biochem. J. 198, 159-166.
11. Garfinkel, A.S., Nilsson-Ehle, P., and Schotz, M.C. (1976) Biochim. Biophys. Acta 424, 264-273.
12. Miller, E.E., Czernobilsky, B., and Tsou, K.C. (1967) Proc. Am. Assoc. Cancer Res. 8, 182-187.
13. Reilly, J.J., Goodgame, T., Jones, D.A., and Brennan, M.F. (1977) J. Surg. Res. 22, 281-286.
14. Bucolo, G., and David, H. (1973) Clin. Chem. 19, 467-482.
15. Berde, C.B., Kerner, J.A., and Johnson, J.D. (1980) Clin. Chem. 26, 1173-1177.
16. Chianori, N., and Henry, R.J. (1959) Am. J. Clin. Path. 31, 305-309.
17. Williamson, D., Mellanby, J., and Krebs, H.A. (1962) Biochem. J. 82, 90-96.
18. Trinder, P. (1969) J. Clin. Path. 22, 246-249.
19. Herbert, V., Lau, K.S., Gottlieb, C.W., and Bleicher, S.J. (1965) J. Clin. Endocr. Metab. 25, 1375-1384.
20. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
21. Levy, A.L., and Keyloun, C. (1970) in Advances in Automated Analysis, Vol. 1, pp. 497-502, Futura, Mount Kisco, NY.
22. DeHoff, J.L., Davidson, L.M., and Kirtchevsky, D. (1978) Clin. Chem. 24, 433-435.
23. Passon, P.G., and Peuler, J.D. (1973) Anal. Biochem. 51, 618-631.
24. Hsu, R.Y., Butterworth, R.H.W., and Porter, S.W. (1969) in Methods Enzymology (Lowenstein, J.M., ed.) Vol. 14, pp. 33-34, Academic Press, New York, NY.
25. Takeda, R., Suzuki, F., and Inoue, H. (1969) in Methods Enzymology (Lowenstein, J.M., ed.) Vol. 13, pp. 153-160, Academic Press, New York, NY.
26. Kaplan, M.L., and Fried, G.H. (1973) Arch. Biochem. Biophys. 158, 711-719.
27. Schotz, M., Garfinkel, A., Huebolter, R.J., and Stewart, J.L. (1970) J. Lipid Res. 11, 68-69.
28. Hietenan, E., and Greenwood, M.R.C. (1977) J. Lipid Res. 18, 480-489.
29. Hartman, A.D. (1981) Am. J. Physiol 241, (Endocrinol & Metab 4) E108-E115.
30. Salaman, M.R., and Robinson, D.S. (1961) in Enzymes of Lipid Metabolism, pp. 218-230, Pergamon Press, New York, NY.
31. Hellman, B., Thelander, L., and Taljedal, I.B. (1963) Acta Anat. 55, 286-294.
32. Gruen, R.K., and Greenwood, M.R.C. (1981) Am. J. Physiol. 241, (Endocrinol & Metab 4) E76-E83.
33. Boulange, A., Planche, R., and deGasquet, P. (1981) Metabolism 30, 1045.
34. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
35. Ott, L. (1977) in An Introduction to Statistical Methods and Data Analysis, pp. 354-384, Duxbury Press, North Scituate, MA.
36. Schiffl, H., Weidmann, P., Meir, A., and Ziegler, W.H. (1981) Klin. Wochenschr. 59, 837-844.
37. Taskinen, M.R., and Nikkila, E.A. (1979) Atherosclerosis 32, 289-299.
38. Delorme, C.L.W., and Harris, K.L. (1975) J. Nutr. 105, 447-451.
39. Kitada, S., Mwasi, L., and Mead, J.F. (1978) Lipids 13, 464-476.
40. Kannan, R., Wilson, L., and Baker, N. (1978) Lipids 13, 887-891.
41. Lyon, I., Kannon, R., Ookhtens, M., and Baker, N. (1982) Cancer Res. 42, 132-138.
42. Kitada, S., Hays, E.F., and Mead, J.F. (1980) Lipids 15, 168-174.

[Received April 26, 1982]

Lipid Profiles of Taste and Non-Taste Epithelial Tissues from Steer Tongues

JOSEPH L. RABINOWITZ[a,c], JOSEPH G. BRAND[b,c], and DOUGLAS L. BAYLEY[b],
[a]Veterans Administration Medical Center, University of Pennsylvania; [b]Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104; and *[c]Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104*

ABSTRACT

Some hypotheses on taste reception have implicated lipids of taste cells as major receptor constituents. This study reports detailed lipid profiles of the taste bud-containing epidermis from circumvallate papillae and fungiform papillae as well as profiles from two non-taste bud tissues: circumvallate papillae dermis and epidermis from the lateral posterior of the tongue. Differences in levels of triglycerides and phosphatidylcholines were observed but these were not directly related to the presence of taste buds. At this level of analysis, it is evident that there are no unusual distributions of phospholipid classes in the taste bud epidermis when compared with the non-taste bud lingual epidermis.
Lipids 17:950-955, 1982.

Current evidence suggests that the initial site of interaction of taste stimuli is on the plasma membrane of taste receptor cells (1–4). Although the biochemical character of the recognition molecules is not firmly established, it has been postulated that lipids of the taste cell plasma membrane function at least as one major constituent in the reception of the modalities salty and sour (5–7). The question of whether or not taste cell plasma membrane contains lipids unique to this receptor cell has never been experimentally addressed. Taste buds are neuroepithelial structures located in the epidermis of specialized papillae of the tongue. The sensory nerves that innervate the papillae are necessary for differentiation of basal cells into taste receptor cells (8) and this process may initiate synthesis of unique receptor macromolecules or unique membrane lipids. An investigation of the lipid profile of various lingual regions may reveal differences among the taste and non-taste areas and could then lead to hypotheses about the role of specific lipids in taste reception.

A previous study (9) reported the lipid profiles of extracts of entire circumvallate papillae. However, the circumvallate papilla is primarily structural dermis and non-taste epidermis. Taste buds are located only within the lateral epidermal surface of steer circumvallate papillae. Rather than extract the entire papilla (as has been done before [9]), we have surgically divided the steer circumvallate papillae into two portions: the taste bud-containing lateral epidermis, and the non-taste bud-containing dermal core of the papilla. While an earlier report (10) used the entire papilla for biochemical studies, more recent ones have used the taste bud rich lateral epidermis (11–13).

In a preliminary analysis of lingual lipids (14), we observed a high percentage of free fatty acids (FFA). Since it is known that lipolytic activity is present in the posterior portion of the tongues of many species (for reviews, see 15,16), we assumed that much of this FFA could be the result of endogenous lipase. During preliminary experiments to monitor this lipase activity, we observed that lidocaine, a known inhibitor of phospholipase (17), inhibited the hydrolysis of tripalmitin, and when tissue that had been held in lidocaine was analyzed for total lipids, only 3–6% FFA were found (Rabinowitz and Brand, unpublished observation). Consequently, we determined lipid profiles of both taste and non-taste lingual epidermal samples keeping the tissue wet with lidocaine buffer for the time required for dissection. The tissue was then placed immediately in the extracting medium. We report here the results of these analyses.

MATERIALS AND METHODS

Glucose-free Tyrode's Buffer

Tyrode's solution was made to the following component concentration: NaCl, 137 mM; KCl, 2.7 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 0.5 mM; NaH_2PO_4, 0.36 mM; pH 5.2. All Tyrode's solution was autoclaved for 1 hr. Lidocaine was a product of ICN Pharmaceuticals and was dissolved to 0.75 mM in Tyrode's buffer.

Tissue

Tongues from seven steer (each animal was 18-24 months old, ca. 1100 lb) were obtained as soon as possible after slaughter (15-30 min). It was noted on purchase that in the throat portions attached to several of the tongues, the carotid bodies were beating. Tongues were immediately rinsed with Tyrode's lidocaine, then placed on ice and the throat tissue cut away. All procedures were conducted on ice (4 C). The 7 tongues were draped with towels soaked in the glucose-free Tyrode's buffer plus 0.75 mM lidocaine. Although no gross microbiological contamination was apparent and no attempt was made to sterilize the materials, tongues were periodically rinsed with copious quantities of the Tyrode s lidocaine buffer.

The following 4 types of tissues were obtained from each of the seven tongues.

Circumvallate papillae (CVP) lateral epidermis. The 15-20 circumvallate papillae of a single steer tongue (Fig. 1) contain about 90% of the total number of taste buds on the tongue (18). Only the lateral epidermis of this structure is invested with taste buds. Therefore, we dissected the CVP into a taste bud epidermal fraction and a non-taste bud-containing dermal portion. The dorsal (top) surface epidermis of the CVP was removed by a single scalpel cut. This surface tissue contains no taste buds. The remaining papilla was then excised from the tongue by a second scalpel cut. The lateral epidermis was teased away from the supporting dermal core using a microforceps. The lateral wall contains taste bud cells and normal epithelial cells. The CVP lateral epidermal pieces were placed in a glass vial with a Teflon-lined screw cap containing 8 ml of Folch (chloroform/methanol) extracting medium. Ca. 200 mg wet wt of tissue was obtained.

CVP dermis. The dermal cores from the dissection above in (a) were also placed in a separate vial with 8 ml Folch. The dermal core of the CVP contains connective tissue, dermal cells, blood vessels, secretory glands and the sensory nerve processes which innervate the taste buds. Ca. 600 mg wet wt of tissue was obtained.

Posterior epidermis. Unpapillated posterior epidermis was collected from all tongues from a region below the CVP and posterior to the intermolar eminence (Fig. 1). The epidermis at this portion of the tongue can be stripped free of the dermis (13). These epidermal strips were rapidly cut into small pieces, 3–5 mm^2, then transferred to a vial containing 8 ml Folch. Approximately 900 mg wet wt of tissue was obtained.

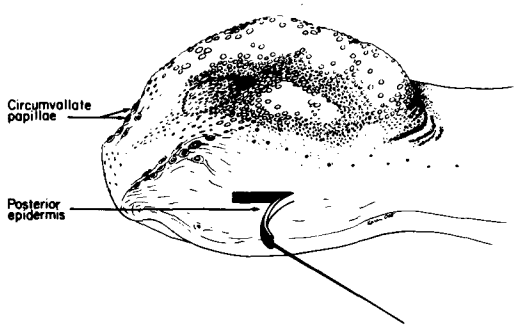


FIG. 1. Sketch of the posterior portion of a steer tongue (throat viscera removed, tip of tongue to the right) showing the circumvallate papillae, whose lateral epidermal surface contains taste buds, and the lateral posterior region from which epidermal strips were collected. The circumvallate papillae were removed and subjected to additional dissection leading to taste bud-containing "CVP lateral epidermis" and the supportive non-taste "CVP dermis."

Fungiform papillae. The anterior portion of the steer tongue contains small taste papillae whose dorsal surface is invested with from 1 to 5 taste buds. The dorsal surface of these papillae was recovered by a single scapel cut and these surfaces placed in a glass vial with 8 ml Folch. Although material was removed from each of the 7 tongues, not all available fungiform papillae were removed. Each steer tongue contains ca. 200 fungiform papillae (18). Ca. 50 mg wet wt of tissue was obtained.

During these procedures, the vials were kept on ice and periodically agitated. After all tissue was collected, the caps were screwed on tightly and the vials vigorously agitated. Each vial was then placed at –15 C for 24 hr until the analytical procedures were begun.

Analytical Procedures

All lipids or solutions containing lipids were stored in the presence of ca. 5 μg of butylated hydroxytoluene (BHT)/mg of lipid. Lipid standards were purchased from Applied Sciences, State College, PA, and were tested for purity by chromatography (several aliquots were tested), as described below. In no instance did the level of impurities exceed 1% and in most instances no detectable impurities were present. The tissue specimens were chopped finely and homogenized in all-glass piston homogenizers; they were extracted with 100 ml of chloroform/methanol (2:1, v/v) (19). Two extractions were required. To insure that no glycerolipid was left unextracted, residues were transmethylated (20) and a subsequent lipid extraction per-

formed. No additional lipid material was recovered. Repeated extractions did not yield additional lipid material. One aliquot of the extract was subjected to silicic acid (Bio-Sil A, 100–200 mesh) column chromatography to separate neutral lipids (by chloroform elution) from phospholipids (by methanol elution). Individual lipid families were obtained from the separated neutral lipids and phospholipids by chromatography on thin layer plates. Silica Gel G TLC plates were developed with petroleum ether/ethyl ether/glacial acetic acid (90:10:1, v/v/v) for separation of neutral lipids. Phospholipids were separated by two-dimensional TLC using Silica Gel H on glass plates and chloroform/methanol/acetic acid/water (200:120:25:15 by vol) in the first dimension (21) and chloroform/methanol/acetone/acetic acid/water (100:20:40.20:10 by vol) in the second dimension (21–23). Known amounts of authentic standards were "spotted" alongside each experimental sample. Iodine vapor was used to pinpoint lipid areas. The appropriate areas of each lipid class were then scraped off and placed in individual test tubes containing 2 ml of concentrated sulphuric acid. The tubes were heated at 200 ± 2 C for exactly 15 min. The test tubes were then cooled by immersion in ice and 3 ml of water was then added to each tube. Following centrifugation for a few minutes at 3000 rpm to remove the silica gel, each solution was decanted into a separate quartz cuvette. The charred lipids were quantitatively assayed by use of a Beckman DB spectrometer set at 375 nm and compared with known standards obtained in the same manner (19,24,25). Slight differences may exist between the known standards (obtained from Applied Sciences) and the unknowns from lingual tissues due to possible differences in fatty acid distribution. The sum of the charring data of each fraction was considered equal to 100% (21,26,27). The cholesterol and cholesteryl ester spots were also determined by Abell's method (28) for maximum accuracy.

To determine if the analytical procedures were breaking down phospholipid or triglyceride, separate vials were run through all procedures as described above in "Tissue", except that no tissue was added. Instead, either ^{14}C-tripalmitin (^{14}C-carboxyl) (New England Nuclear, 5 mCi/mmol) or [U-^{14}C] phosphatidylcholine (New England Nuclear, 5 mCi/mmol) was added to 1.5 ml buffer. These were carried through all procedures as though they contained tissue.

In a separate experiment, 2 vials each containing 10 intact fresh circumvallate papillae were incubated for 40 min at 30 C in 1.5 ml of Tyrode's buffer containing 50 μg of tripalmitin (^{14}C-carboxyl). One vial also contained 0.75 mM lidocaine.

RESULTS

Detailed analysis of the isotope blanks that were run separately through the extraction, elution and identification procedures (no tissue in the vials) as described in the section on analytical procedures in Methods (above) revealed the following chemical breakdowns. For ^{14}C-tripalmitin, less than 0.01% was converted to methyl ^{14}C-palimitate. For the [U^{14}C] lecithin, less than 0.01% was converted to ^{14}C-palmitic acid, and less than 0.001% was converted to methyl ^{14}C-palmitate and ^{14}C-phosphatidic acid. Thus, the lipid profiles in Table 1 represent endogenous lipids and are not a result of lipid decomposition or recombination during the analytical procedures for identification of individual lipid species.

In the experiment where 2 vials of 10 intact CVP samples were incubated with ^{14}C-tripalmitin with and without lidocaine, 48% hydrolysis to ^{14}C-palmitic acid was observed in the vial without lidocaine, 1.4% hydrolysis to ^{14}C-palmitic acid was observed in the vial containing lidocaine.

Table 1 displays the results of analyses of various lipid classes from each tissue area. For most classes of lipids in the 4 tissue regions sampled, little difference existed. The circumvallate epidermal fraction was similar to that of the CVP dermis. The dermis forms the supportive tissue for the epidermal taste-bud-containing fraction. Profiles of the epidermis from the posterior portion and of the dorsal surface of fungiform (taste) papillae showed striking similarity. Compared with the circumvallate tissues, both had higher levels of triglycerides and lower levels of phosphatidyl cholines. Percentages of all other lipid classes were similar among all four regions. The ratio of neutral to polar lipids was higher for the posterior epidermis and the fungiform epidermis compared with the epidermis and dermis of the circumvallate papillae.

DISCUSSION

This study presents a complete lipid profile of both taste bud-containing CVP epidermis and a non-taste bud-containing epidermis (as control tissue). In addition, the profile of the supportive dermis of the CVP is also reported. We also used the known phospholipase inhibitor lidocaine while taking tissue samples since earlier work suggested that lidocaine is not only a phospholipase inhibitor but may also be a lipase (glycerol ester hydrolase) inhibitor in

TABLE 1

Lipid Composition (%) of Tongue Tissues from 4 Different Regions[a]

Constituent	Region of tongue sampled			
	CVP lateral epidermis	CVP dermis	Posterior epidermis	Fungiform dorsal surface
Neutral lipids				
Free fatty acids	5.05	5.88	6.01	6.09
Mono- and diglycerides	2.53	2.73	3.40	4.92
Triglycerides	6.24	4.79	14.37	15.12
Methyl esters	1.54	1.13	1.79	2.00
Cholesterol	18.09	18.00	19.85	17.53
Cholesteryl esters	4.51	6.63	7.15	7.76
Undetermined	1.00	1.58	0.92	1.13
Total neutral lipids	38.96	40.74	53.49	54.55
Polar Lipids				
Sphingomyelins	4.60	4.98	2.59	2.26
Phosphatidylcholines	15.32	13.81	8.43	8.53
Lysophosphatidylcholines	3.58	2.69	2.11	2.24
Phosphatidylserines	6.90	6.33	4.33	4.00
Phosphatidylinositols	5.62	5.75	3.09	4.40
Phosphatidylethanolamines	13.66	14.58	14.10	12.12
Cardiolipins	9.19	7.98	9.86	10.55
Phosphatidic acids	1.72	1.88	1.03	0.85
Undetermined	0.45	1.26	0.97	0.50
Total polar lipids	61.04	59.26	46.51	45.45

[a]Values are percent of total lipid and represent the average of three parallel analyses with standard error of the mean less than 0.05%.

these tissues. The mono- and diglyceride levels were low in all tissues sampled (Table 1), in spite of the triglyceride levels being different among the fractions. This suggests that the presence of lidocaine may maintain the integrity of the triglyceride levels by inhibiting the potent lingual lipases (15,16,29).

We have taken advantage of this inhibitory ability of lidocaine in order to determine the endogenous levels of various lipid species in taste and non-taste lingual tissue. Differences in lipid profile reflect apparent epithelial type and regional position of the tissue and not the direct presence of taste buds. It is notable that the epidermis from the lateral borders of the tongue has a profile similar to the fungiform dorsal surface. The chorda tympani branch of the VIIth nerve innervates the fungiform papillae while the glossopharyngeal nerve innervates the circumvallate. Some of these differences in lipid profile may be due to differences in innervation.

The profiles we present here are not completely consistent with preliminary work we have performed on tissue that was incubated for 40 min in the presence of lidocaine before being extracted in Folch's mixture. In these preliminary experiments, we incubated CVP epidermis in Tyrode's buffer with and without lidocaine in order to determine the extent of endogenous lipase. We attribute these discrepancies (mostly differences in tryglyceride and phospholipid levels) to syntheses and degradations that may have occurred during the 40-min incubations and to possible differences in strain, age, health or prior drug treatment of steers from which the tissue was derived. The distributions reported in Table 1 are probably nearer the actual distributions in the animal since tissues now were placed as soon as possible after slaughter into Folch's extracting medium.

The report of Kurihara et al. (9) states that 41% of the total lipids extracted from entire circumvallate papillae were phospholipids, although the individual lipid classes quantitated were not given. Our value for CVP lateral epidermis is higher than this (Table 1), although it is not clear as to how Kurihara et al. defined their phospholipid classes. They also found much higher values (16%) for sphinogomyelins and lower values (4%) for phosphatidylethanolamines (reported as weight % of total lipids) than we report here. Their report of cholesterol content is lower than ours. The state of the tissue (fresh or frozen, strain, age and health of animal, etc.) is not known in the previous report (9), and the investigators did not use lidocaine during dissection. Without lidocaine, the diacylglycerophospholipids may have been

partially hydrolyzed allowing the relative amount of sphingomyelin to increase. Additionally, the analytical procedures were different.

Our study supports the efficacy of using the CVP epidermis as a tissue for biochemical studies of reception and the appropriateness of using posterior epidermis as a control tissue. It is also apparent from our data that at the levels of tissue concentration employed here, only the difference in phosphatidylcholines and triglycerides is obvious between epidermal tissues that contain taste buds and those that do not. This fact suggests that if taste reception is initiated through an interaction of cations with the lipid plasma membrane of taste cells, then that plasma membrane is probably of a composition similar to non-taste bud epithelial cells. The higher content of phosphatidylcholines in CVP taste epidermis compared with fungiform taste epidermis may have bearing on the regional specificity often considered of interest in taste. This perceptual difference is not, however, as potent as is generally assumed (30). Since the CVP lateral epidermis contains both taste bud cells and non-taste bud normal epithelial cells, the tissue sampled here may not be concentrated enough in taste bud cells to observe what may be subtle differences in lipid profile. CVP lateral epidermis is sufficiently enriched in taste bud material to show a difference in the binding of taste stimuli when compared to posterior lateral epidermis (13,31). While further purification of the taste bud cells from CVP lateral epidermis has been achieved (32), the method is very aggressive and lengthy (about 5 hr), requiring collagenase digestion and several steps of tissue disruption. All of these steps alter the lipid profile of the tissues under study.

We have determined the lipid composition of epidermis of steer tongue from both the taste bud-rich circumvallate papillae, the taste bud-containing fungiform papillae and the non-taste bud lateral posterior region. The lipid composition was similar in all regions. The considerable lipase activity known to be present in lingual tissues can be inhibited with lidocaine. We conclude that if taste reception is mediated in part or in whole by plasma membrane lipids, then, at the levels of our analyses, no unique distribution of membrane lipids is present in taste bud-containing epidermis.

ACKNOWLEDGMENT

We thank Dr. Robert H. Cagan for critical review of the manuscript. This work was supported in part by National Institutes of Health Research Grant No. NS-15565 to J.G. Brand from the National Institute of Neurological and Communicative Disorders and Stroke.

REFERENCES

1. Renqvist, Y. (1919) Skand. Arch. Physiol. 38, 97-201.
2. Cagan, R.H. (1981) in Biochemistry of Taste and Olfaction (Cagan, R.H. and Kare, M.R., eds.) pp. 175-203, Academic Press, New York, NY.
3. Price, S. and De Simone, J.A. (1977) Chem. Sens. Flav. 2, 427-456.
4. Cagan, R.H. and Rhein, L. (1980) in Olfaction and Taste VII (van der Starre, H., ed.) pp. 35-44, IRL Press, London.
5. Kamo, N., Miyake, M., Kurihara, K., and Kobatake, Y. (1974) Biochim. Biophys. Acta 367, 1-10.
6. DeSimone, J.A., and Price, S. (1976) Biophys. J. 16, 869-881.
7. Brand, J.G., and Bayley, D.L. (1980) in Biological and Behavioral Aspects of Salt Intake (Kare, M.R., Fregly, M.J., and Bernard, R.A., eds.) pp. 289-298, Academic Press, New York, NY.
8. Farbman, A.I. (1980) Cell Tiss. Kinet. 13, 349-357.
9. Kurihara, K., Koyama, N., and Kurihara, Y. (1972) in Olfaction and Taste IV (Schneider, D., ed.) pp. 234-240, Wissenschaftliche Verlagsgesllschaft, Stuttgart.
10. Cagan, R.H. (1971) Biochim. Biophys. Acta 252, 199-206.
11. Brand, J.G., Zeeberg, B.R., and Cagan, R.H. (1976) Intern. J. Neurosci. 7, 37-43.
12. Lum, C.K.L., and Henkin, R.I. (1976) Biochim. Biophys. Acta 421, 380-394.
13. Torii, K., and Cagan, R.H. (1980) Biochim. Biophys. Acta 627, 313-323.
14. Brand, J.G., and Rabinowitz, J.L. (1981) Abstract, Third Annual Meeting, Association for Chemoreception Sciences.
15. Nelson, J.H., Jensen, R.G., and Pitas, R.E. (1977) J. Dairy Sci. 60, 327-362.
16. Hamosh, M. (1981) in Textbook of Gastroenterology and Nutrition in Infancy (Lebenthal, E., ed.) pp. 445-463, Raven Press, New York, NY.
17. Scherphof, G., Scarpa, A., and van Toorenenbergen, A. (1972) Biochim. Biophys. Acta 270, 226-240.
18. Davies, R.O., Kare, M.R., and Cagan, R.H. (1979) Anat. Rec. 195, 443-446.
19. Blank, M.L., Schmit, J.A., and Privett, O.S. (1964) J. Am. Oil Chem. Soc. 41, 371-376.
20. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-609.
21. Rabinowitz, J.L., Bailey, T.A., and Marsh, J.B. (1971) Arch. Oral Biol. 16, 1195-1205.
22. Rabinowitz, J.L., Luddy, F.E., Barford, R.A., Herb, S.F., Orlean, S.L., and Cohen, D.W. (1967) J. Dent. Res. 46, 1086-1089.
23. Parsons, J.G., and Patton, S. (1967) J. Lipid Res. 8, 696-698.
24. Marsh, J.B., and Weinstein, D.B. (1966) J. Lipid Res. 7, 574-576.
25. Weinstein, D.B., Marsh, J.B., Glick, M.C., and Warren, L. (1969) J. Biol. Chem. 244, 4103-4111.
26. Rabinowitz, J.L., and Hercker, E.S. (1974) Arch. Biochem. Biophys. 161, 621-627.
27. Rabinowitz, J.L., and Hercker, E.S. (1976) Biochem. J. 160, 463-466.
28. Tietz, N. (1976) in Fundamentals of Clinical

Chemistry, pp. 510–512, W.B. Saunders, Philadelphia, PA.
29. Jensen, R.G., DeJong, F.A., Clark, R.M., Palmgren, L.G., Liao, T.H., and Hamosh, M. (1982) Lipids 17, 570–572.
30. McBurney, D.H. (1974) Chem. Senses Flav. 1, 17–28.
31. Cagan, R.H., and Morris, R.W. (1979) Proc. Natl. Acad. Sci. USA 76, 1692–1696.
32. Brand, J.G., and Cagan, R.H. (1976) J. Neurobiol. 7, 205–220.

[Revision received September 13, 1982]

Effects of Different Fractions of the Barley Kernel on the Hepatic Lipid Metabolism of Chickens[1,2]

WARREN C. BURGER[a], ASAF A. QURESHI[a,*], NEVILLE PRENTICE[a] and CHARLES E. ELSON[b], [a]*USDA, SEA-AR, Barley and Malt Laboratory, 501 N. Walnut Street, Madison, WI 53705,* and [a]*Department of Agronomy and* [b]*Department of Nutritional Sciences, University of Wisconsin, Madison, WI 53706*

ABSTRACT

Various physical fractions of the barley kernel were fed to one-day-old female and male chickens to determine their effect on hepatic β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase and the lipogenic enzymes, acetyl-CoA carboxylase (ACX), malic enzyme (ME), citrate-cleavage enzyme (CCE) and fatty acid synthetase (FAS) at the subcellular level. Significant inhibition ($p<0.01$) of cholesterol biosynthesis accompanied by significant decreases in plasma cholesterol concentrations and induction of fatty acid synthesis were found in diets based on pearled barley, barley pearlings and a high-protein barley flour (HPBF: aleurone and subaleurone layers of barley endosperm) separated from the pearlings when compared to corn. Lower weight gains in 1- to 4-week-old birds fed the high-protein barley flour were found to be the result of lower feed consumption; pair feeding of 12-week-old birds with diets based on corn and high-protein barley flour produced equal weight gains in both treatments and significant reductions in hepatic HMG-CoA reductase, plasma cholesterol and induction in several lipogenic enzymes in birds fed the high-protein barley flour. Substitutions of 5-20% high-protein barley flour for corn in a corn-based diet produced significant weight gains ($p<0.01$) of 10 to 20% in 2-week-old chickens, inhibited cholesterol biosynthesis by 45-65% and produced a 3-fold increase in a fatty acid synthetase. The results indicate that HPBF contains an inhibitor(s) of cholesterol biosynthesis and a growth factor(s) when compared to a corn-based diet.

Lipids 17:956-963, 1982.

INTRODUCTION

The role of nutritional factors such as the type of carbohydrate and dietary fiber in affecting plasma cholesterol concentration has been reported by a number of investigators (1-7). Numerous observations in man and animals have indicated that hepatic and plasma lipid concentrations are influenced by the amount and type of dietary carbohydrate (8,9). Diets rich in sugars, most notably fructose and sucrose, have been shown to induce high levels of plasma and hepatic lipids in laboratory animals compared to diets containing complex carbohydrates (8). More recently, the source of dietary protein has been recognized for its ability to change plasma cholesterol (1). Animal protein has been associated with high, and vegetable protein with low, plasma cholesterol (2). This has been observed in chickens (2,3) and rats (5,6) fed cholesterolemic diets and in rabbits fed diets low in fat without adding cholesterol (7).

Recently, we have described the effect of feeding barley as compared to corn on the lipid metabolism in the liver of chickens (10). A dramatic decrease in cholesterol biosynthesis was caused by the barley diet. This effect was accompanied by large increases in fatty acid biosynthesis.

The decrease of cholesterol biosynthesis caused by feeding a barley-based diet compared to corn in chickens (10) prompted us to determine whether a particular part of the barley kernel is more effective in lowering plasma cholesterol levels and hepatic cholesterol biosynthesis. Fractions obtained from commercial barley pearling operations were used. These were (a) the whole kernel; (b) barley pearlings which consist of hull, testa, pericarp, aleurone, germ and some starchy endosperm; (c) a high-protein barley flour (HPBF), which has the finer particles separated by sieving the pearlings, and consists of aleurone, germ and subaleurone portions of the starchy endosperm; and (d) pearled barley, a food product, which

[1]Cooperative investigation between the Science and Education Administration, US Department of Agriculture, and the College of Agricultural and Life Sciences, University of Wisconsin-Madison.

[2]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

*Author to whom correspondence should be addressed.

TABLE 1

Percent Composition of the Chicken Diets

Ingredients	Diets[1]				
	A	B	C	D	E
Corn[2]	61.5	–	–	–	–
Barley	–	68.0	–	–	–
Barley pearlings	–	–	76.5	–	–
High-protein barley flour	–	–	–	79.5	–
Pearled barley	–	–	–	–	65.0
Soybean meal (44%)	30.0	23.5	15.0	12.0	26.5
Meat scrap	5.0	5.0	5.0	5.0	5.0
Alfalfa meal (17%)	1.0	1.0	1.0	1.0	1.0
Dicalcium phosphate	1.0	1.0	1.0	1.0	1.0
Calcium carbonate	0.5	0.5	0.5	0.5	0.5
Mineral mixture[3]	0.5	0.5	0.5	0.5	0.5
Vitamin mixture[4]	0.5	0.5	0.5	0.5	0.5
Crude protein % (calculated)[5]	21.73	21.73	21.83	22.1	21.73
Calcium % (calculated)	1	1	1	1	1

[1]5% grit was also incorporated at the expense of each diet.

[2]Protein (N X 6.25) concentrations on "as is" basis of corn, barley, barley pearlings, high-protein barley flour and pearled barley were 9.5, 12.8, 16.4, 18.1 and 11.1%, respectively.

[3]Contains/kg sodium chloride (NaCl) 2 mg, zinc sulphate ($ZnSQ_4$) 50 mg and manganese dioxide (MnO_2) 50 mg.

[4]Contains/kg vitamin A 2000 IU, vitamin D_3 200 ICU, vitamin E 10 IU, vitamin K_1 5 mg, choline 1.3 g, thiamin 1.8 mg, niacin 27 mg, riboflavin 3.6 mg, pyridoxine 3 mg, calcium-pantothenate 10.0 mg, vitamin B_{12} 10 μg, lysine-HCl 1 g, methionine 0.72 g.

[5]Calculated from analyzed values for corn, barley and NRC (1977) values for other ingredients.

accounts for the remainder of the kernel and consists primarily of starchy endosperm. Identification of a particularly active fraction from barley might also serve as a preliminary step in the isolation and identification of the active principles.

The interrelationship between dietary carbohydrate and lipid metabolism is complex and, in addition, is influenced by sex (11,12) and genetic background (13,14). The present study was carried out to check the effect of each of the three physical fractions of barley on lipid metabolism of female and male chickens individually for any differences among these products in both sexes.

The activities of β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34) cholesterol 7α-hydroxylase (EC 1.14) and the lipogenic enzymes, fatty acid synthetase (FAS), acetyl coenzyme A carboxylase (ACX EC 6.4.1.2), malic enzyme (ME EC 1.1.1.40) and citrate-cleavage enzyme (CCE EC 4.1.3.8) were determined. The response of plasma cholesterol and triglycerides was also measured.

MATERIALS AND METHODS

Experimental materials were obtained commercially as reported previously (15). Barley, var. 'Larker,' was a gift from the Ladish Malting Company, Jefferson, WI; yellow dent corn, used as a control cereal, was obtained from a local merchant. The different fractions of barley used in the present studies were a gift from the Minnesota Grain Pearling Company, East Grand Forks, MN. One-day-old and 2-week-old female and male chickens, White Leghorn, weighing ca. 36 g and 106 g, respectively, were obtained from commercial sources.

Animals and Diets

Effect of dietary barley fractions compared to corn on body and liver weights and hepatic enzyme activities in 4-week-old female and male chickens. Forty one-day-old White Leghorn female chickens, weighing 36 g each, were divided into 5 groups of 8 birds or thirty 2-week-old female chicks, weighing 105 g were divided into 5 groups of 6 birds for each treatment. The birds for each treatment were housed in 4 cages containing 2 birds per cage. The first groups were fed isonitrogenous diets containing corn or different fractions of the barley kernel for 4 weeks and the latter groups were fed for 2 weeks. The compositions of the diets are shown in Table 1. Male chickens were also fed the same diets. The birds were exposed

TABLE 2

Effect of HPBF on Body Weight, Hepatic Enzyme Activities and Plasma Cholesterol in Pair-Fed 15-week-old Female Chickens

Parameters	Corn (control)	HPBF	
Corn-based diet[1]	69.4%	–	
High-protein barley flour (HPBF)	–	88.4%	
Soybean meal	25.0%	6.0%	
Initial body weight (g) (12 wk old)	912 ± 64	837 ± 78	
Final body weight (g) (15 wk old)	1078 ± 102	1000 ± 84	
Percentage gain[2]	18	19	
Liver weight (g)	30.5 ± 3.0	29.7 ± 3.0	
β-Hydroxy-β-methylglutaryl-CoA reductase[3]	372 ± 26[a]	269 ± 15[b]	(72)[7]
Cholesterol 7α-hydroxylase[4]	1.6 ± 0.19[a]	1.2 ± 0.16[b]	(75)
Plasma cholesterol (mg/100 ml)	139 ± 10[a]	117 ± 10[b]	(84)
Fatty acid synthetase[5]	64.6 ± 7.0[a]	85.1 ± 8.0[b]	(132)
Malic enzyme[6]	286 ± 12.0[a]	323.0 ± 16.4[b]	(112)

[1]Each diet also contains meat scrap 50% (1.1%); alfalfa meal 17% (2.0%); dicalcium phosphate (1%); calcium carbonate (0.5%); vitamin and mineral mixture (1%); grit (5%) was incorporated at the expense of each diet. Vitamin and mineral mixture contains/kg; vitamin A 3000 IU, vitamin D_3 500 IU, vitamin E 10 IU; vitamin K_1 5 mg; choline 1.3 g; thiamine 1.8 mg; niacin 27 mg; riboflavin 2.5 mg; pyridoxine 3 mg; calcium pantothenate 3.0 mg; vitamin B_{12} 5 mg; lysine-HCL 1 g; methionine 0.72 g; sodium chloride (NaCl) 2 mg; zinc sulphate ($ZnSO_4$) 50 mg and manganese dioxide (MnO_2) 50 mg. Analysis of corn, high-protein barley flour (9.1 and 18.2% protein on "as is" basis, respectively).

[2]Feeding period was 3 weeks; time of killing was 0800 hr; data expressed as mean ± SD; N = 10 chickens per group.

[3]pmoles of mevalonic acid synthesized/min/mg of microsomal fractions.

[4]pmoles of [^{14}C]cholesterol into [^{14}C]7α-OH-cholesterol/min/mg of microsomal fraction.

[5]nmoles of NADPH oxidized/min/mg of cytosolic fraction.

[6]nmoles of $NADP^+$ reduced/min/mg of cytosolic fraction.

[7]Percentage of respective control activity data are in parentheses.

[a-b]Means on a line and without a common small superscript letter are different, $p<0.01$.

to continuous artificial illumination and had free access to feed and water. Feed consumption and body weights were checked once a week. At the end of the feeding period, birds were weighed individually, blood samples (3 ml) were collected from the neck vessels in a tube containing 500 units of heparin in 0.3-ml saline solution (0.9%) immediately after sacrifice. Samples were stored at -20 C until used for analysis. The birds were sacrificed between 8 and 9 A.M.; livers were removed, immediately put on ice and then worked up for cytosol and microsomal fractions ro carry out studies for lipid metabolism as described earlier (10).

Effect of HPBF on body and liver weights and hepatic enzyme activities in pair-fed 15-week-old female chickens. Twenty 12-week-old White Leghorn female chickens, weighing 760-970 g, were divided into 2 groups of 10 birds (5 birds per pen) for each treatment. The birds of this age fed the corn-based diet typically consume 100 g diet per day and those fed the HPBF diet, 80 g per day. To test whether or not this difference in diet intake influenced the differences in metabolic activities being studied, each group of birds was fed 80 g/bird/day of the isonitrogenous corn- or HPBF-based diets (Table 2) for 3 weeks. The diets were fed in small batches as a wet mash (4:1 diet/water) with the diet freshly mixed and fed at 3 intervals per day to avoid microbial growth. Under these conditions, the corn-fed birds, which normally consumed 100 g per bird per day, were limited to 80 g per day, the amount normally consumed by the HPBF-fed birds. At the end of the feeding period, cytosolic and microsomal fractions were prepared as described in experiment 1.

Effects of corn and HPBF on chicken body and liver weights and on the level of enzymes regulating the synthesis of cholesterol and fatty acid in 6-week-old chicken livers. In this experiment, thirty six 2-week-old female chickens weighing ca. 95-100 g were divided into 6 groups of 6 birds for each treatment. Two groups were fed the isonitrogenous diets (Table 1) for corn- and barley-based diets as controls, the remaining 4 groups were fed the corn-based diet in which 5, 10, 15 and 20% of the corn was replaced with HPBF for 4 weeks. The rest

of the conditions were identical to those described in experiment 1.

Preparation of Chicken Liver Homogenates

The liver homogenates were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.004 M $MgCl_2$, 0.001 M EDTA and 0.002 M dithiothreitol as described earlier (10). Protein concentration was estimated by a modification of the Biuret method using bovine serum albumin as a standard (16).

Enzyme Assays and Cholesterol Estimation

Assays for HMG-CoA reductase, cholesterol 7α-hydroxylase, acetyl-CoA carboxylase, fatty acid synthetase and cholesterol in liver and blood plasma were carried out as reported previously (10,15).

The activities of malic enzyme (17), citrate-cleavage enzyme (18) and fatty acid synthetase (19) in the 100,000 g supernatant fraction were assayed spectrophotometrically at 25 C.

Estimation of Cholesterol and Triglyceride in Plasma

Cholesterol and triglyceride concentrations in plasma samples were estimated by using Worthington "Cholesterol Reagent" and "Triglycerides Reagent" obtained from Worthington Diagnostics Division of Millipore Corporation, Freehold, NJ.

Expression of Data and Statistical Methods

Statistical comparison of results was performed by one- or two-way analysis of variance. When the F test indicated a significant treatment effect differences between the means were analyzed by the protected LSD test using the correction for samples of unequal size (20).

RESULTS AND DISCUSSION

The results of feeding diets consisting primarily of the various barley fractions to one-day-old female and male chickens for 4 weeks are summarized in Table 3. Compared to the corn control diet, barley was a better feed for the females than the male. Pearled barley, HPBF and pearlings produced progressively less growth in that order. The high level of dietary fiber in pearlings suggested that this was the primary reason for the poor growth on this diet. Growth on the HPBF diet was significantly better in both sexes than that observed with the pearlings, but was not commensurate with that obtained with intact barley containing a similar concentration of starch.

A determination of the gross energy values of the corn and HPBF yielded values of 4340

TABLE 3

Effects of Different Fractions of Barley Kernels on Body and Liver Weights, Hepatic Enzyme Activities and Plasma Lipids in 4-week-old Chickens[1]

Nutritional state	Body weight (g)	Liver weight (g)	HMG-CoA reductase[2]	Cholesterol 7α-hydroxylase[3]	Plasma cholesterol[4]	Plasma triglycerides[4]	ME[5]	CCE[6]	ACX[7]	FAS[8]
Females										
Corn	263 ± 3.0[a]	8.7 ± 0.4[a]	6.0 ± 1.0[a]	27.7 ± 3.0[a]	165 ± 17[a]	117 ± 7.0[a]	10.8 ± 0.4[a]	1.0 ± 0.1[a]	41.0 ± 4.0[a]	4.8 ± 0.4[a]
Barley	258 ± 4.0[a] (98)[9]	7.6 ± 0.3[b] (87)	2.6 ± 0.8[b] (43)	17.8 ± 4.0[b] (64)	120 ± 14[b] (73)	236 ± 15[bc] (202)	16.3 ± 0.4[b] (151)	1.5 ± 0.2[b] (150)	65.2 ± 5.0[bc] (159)	18.0 ± 0.3[b] (375)
Barley pearlings	116 ± 14[b] (44)	2.8 ± 0.1[c] (32)	3.5 ± 0.8[b] (58)	18.2 ± 4.0[b] (66)	126 ± 12[b] (76)	229 ± 12[b] (195)	15.1 ± 0.5[b] (140)	1.9 ± 1.0[c] (190)	70.5 ± 4.0[c] (172)	17.6 ± 0.4[d] (367)
High-protein barley flour	186 ± 4.0[c] (71)	4.8 ± 0.1[d] (55)	2.4 ± 0.6[b] (40)	16.0 ± 2.0[b] (58)	120 ± 11[b] (73)	256 ± 11[c] (219)	40.8 ± 1.0[c] (378)	4.4 ± 0.2[d] (440)	75.8 ± 3.0[c] (190)	17.5 ± 0.2[d] (364)
Pearled barley	217 ± 6.0[d] (83)	6.3 ± 0.2[e] (72)	3.4 ± 0.4[b] (51)	23.4 ± 5.0[a] (84)	124 ± 15[b] (75)	230 ± 19[b] (197)	16.1 ± 0.5[b] (149)	1.6 ± 0.3[b] (160)	61.3 ± 3.0[b] (150)	16.2 ± 0.3[b] (338)
Males										
Corn	284 ± 11[a]	8.5 ± 1.0[a]	4.8 ± 0.2[a]	19.7 ± 3.0[a]	143 ± 12[a]	64 ± 5.0[a]	7.6 ± 0.9[a]	0.3 ± 0.2[a]	35.2 ± 3.0[a]	2.4 ± 0.2[a]
Barley	233 ± 17[b] (82)	7.2 ± 1.0[b] (85)	2.4 ± 0.8[b] (50)	13.7 ± 4.0[b] (70)	110 ± 11[b] (77)	98 ± 5.0[bc] (153)	12.0 ± 0.7[b] (158)	1.2 ± 0.1[b] (400)	58.4 ± 3.0[b] (166)	4.9 ± 0.3[b] (204)
Barley pearlings	109 ± 6.0[c] (38)	3.1 ± 0.3[c] (32)	3.3 ± 0.7[b] (69)	12.5 ± 0.9[b] (63)	114 ± 10[b] (80)	94 ± 2.0[b] (147)	9.2 ± 0.4[dc] (121)	2.0 ± 0.1[c] (667)	62.2 ± 3.0[b] (178)	5.2 ± 0.2[c] (217)
High-protein barley flour	177 ± 4.0[d] (62)	4.3 ± 0.3[d] (51)	2.4 ± 0.4[b] (50)	11.5 ± 0.7[b] (58)	109 ± 9[b] (76)	104 ± 3.0[c] (162)	16.2 ± 1.2[e] (213)	2.2 ± 0.2[c] (733)	66.7 ± 3.0[c] (191)	5.9 ± 1.2[d] (246)
Pearled barley	198 ± 20[e] (70)	6.5 ± 0.5[b] (76)	2.5 ± 0.2[b] (52)	15.7 ± 4.0[ab] (80)	118 ± 12[b] (83)	95 ± 4.0[b] (148)	10.0 ± 0.5[c] (132)	1.2 ± 0.6[b] (400)	59.5 ± 2.0[b] (169)	4.9 ± 0.4[b] (204)

[1]Feeding period was 4 weeks; time of killing was 0800 hr; data expressed as mean ± SD; N=8 chickens per group. ME = malic enzyme, CCE = citrate cleavage enzyme; ACX = acetyl coenzyme-A carboxylase; FAS = fatty acid synthetase.
[2]nmoles of mevalonic acid synthesized per min/g of liver.
[3]pmoles of [^{14}C]cholesterol converted into [^{14}C]7α-OH-cholesterol/min/g of liver.
[4]mg/100 ml of plasma.
[5]μmoles of NADP$^+$ reduced/min/g of liver.
[6]μmoles of product formed/min/g of liver.
[7]nmoles of product formed/min/g of liver.
[8]μmoles of NADPH oxidized/min/g of liver.
[9]Percentage of respective control activity data are in parentheses.
[a-e]Means within a column and without a common superscript letter are significantly different, $p<0.01$.

and 4590 kcal/kg, respectively. The daily average feed consumption for a pen of 5 birds for the 4-week experiment was 50 g of corn, barley or pearled barley, 40 g of HPBF and about 25 g of pearlings. Calculations show that the gross energy intake and weight gain of chicks fed the HPBF were 80% and 60%, respectively, those of the corn-fed group. These observations suggest that the gross energy of HPBF overestimates the metabolizable energy. Thus, it appeared that either HPBF was not being utilized for reasons not apparent from its compositional data or its extremely fine texture was preventing it being consumed in adequate quantity by the young chicks. In subsequent experiments, the latter reason was shown to explain the decreased consumption.

The HMG-CoA reductase values indicated suppression by all the various physical fractions of the barley kernel (Table 3). Except for the pearled barley diets, the barley fractions caused significant ($p<0.01$) reductions in cholesterol 7α-hydroxylase as well (Table 3). These altered enzymic activities were reflected in lowered plasma cholesterol concentrations ranging from 17 to 27% below those of the birds that received the corn-based diet. Plasma triglyceride concentrations were elevated significantly over the controls; female birds increased by about 100% and male birds by ca. 50%. The activities of HMG-CoA reductase and cholesterol 7α-hydroxylase were determined to establish which activity changed in response to the active principles in barley which effected a lowering of plasma cholesterol.

The data in Table 3 indicate that there was somewhat less overall cholesterol biosynthesis in male as compared to female chickens, which is consistent with published results (12,14). The differences in the lipid metabolism of male and female chickens (12,14), rats (14,21) and monkeys (22) have been reported by a number of investigators. Mitchell et al. (21) recently demonstrated that female rats have consistently higher activities of HMG-CoA reductase and cholesterol 7α-hydroxylase than male rats and these sex-related differences were present throughout the diurnal cycle and also on standard diets. It was suggested that these differences might be mediated by estrogen (21).

It has been established by a number of investigators that nutritional manipulations affect the activities of several lipogenic enzymes in rat liver (23). Fasting results in a decrease in the activities of fatty acid synthetase and acetyl-CoA carboxylase in rat liver (24-27). The nearly 2-fold increase of triglycerides in the female birds under these paradigms (Table 3) was also reflected in the activities of lipogenic enzymes system, which synthesize fatty acids for triglyceride formation. These are the fatty acid synthetase complex, acetyl-CoA carboxylase, citrate-cleavage enzyme, and malic enzyme.

The effect of the different fractions of the barley kernel showed a maximum induction by feeding HPBF as compared to corn or barley (Table 3). The maximum effect occurred with citrate-cleavage enzyme: up to 7-fold increases in male chickens and 4-fold in females. The increase in activity of acetyl-CoA carboxylase was the lowest and was independent of sex with any of the fractions. The increase in activity of fatty acid synthetase was 3-fold in females compared to 2-fold in males. These data clearly indicate that all of these hepatic lipogenic enzymes are coordinately regulated under the conditions employed.

The pronounced inhibition of body and liver weight gain that was observed in chicks which were started on the barley pearlings (Table 3) was not particularly surprising in view of the relatively low starch concentration and high dietary fiber of this material; however, the similarities in composition of HPBF and barley suggested the presence in HPBF of materials which one-day-old chicks were unable to utilize. As a result, a similar experiment using 2-week-old female chicks which had been previously reared on a commercial corn diet was carried out. The results (Table 4) closely paralleled in all respects the data shown in Table 3. Thus, if the earlier results were indeed caused by some inability of the one- to 2-week-old chicks to assimilate HPBF components, this condition was still operative during the third and fourth weeks of development.

The lower gain in body weight was only observed when HPBF was fed as the major source of protein in the diets of one-day-old, or 2-week-old female chickens. This was supported by pair-feeding HPBF and corn-based diet to 12-week-old female chickens for 3 weeks. The gain in weight was comparable to corn fed chickens with HPBF as shown in Table 2. The inhibition or induction of cholesterogenesis and lipogenesis persisted even under these conditions (Table 2). The quantity of feed was determined on the basis of the consumption of HPBF-based diet, which was 20% less than the corn diet. Two other control groups fed ad libitum without water treatment showed higher consumption of corn-based diet and a more pronounced effect on the inhibition of cholesterogenesis compared to pair-fed HPBF-based diet or the same period of feeding. These results clearly indicate that the poor gain in body weight with HPBF may be attributed to difficulty in eating this fine powder, which

TABLE 4

Effect of Different Fractions of Barley Kernels on Body and Liver Weights, Hepatic Enzyme Activities and Plasma Lipids in 4-week-old Female Chickens[1]

Parameters	Corn	Barley	Barley pearlings	High-protein barley flour	Pearled barley
Body weight (g)	260 ± 5.0^{a}	249 ± 6.0^{a} (96)[8]	112 ± 10^{c} (43)[8]	205 ± 14^{b} (79)[8]	210 ± 13^{b} (81)[8]
Liver weight (g)	8.6 ± 0.3^{a}	6.6 ± 0.2^{b} (77)	5.7 ± 0.2^{c} (66)	6.4 ± 0.2^{b} (74)	6.4 ± 0.3^{b} (74)
β-Hydroxy-β-methylglutaryl-CoA reductase[2]	5.5 ± 0.4^{a}	2.3 ± 0.9^{b} (42)	3.4 ± 0.8^{c} (62)	2.1 ± 0.2^{b} (38)	3.5 ± 0.3^{c} (64)
Cholesterol 7α-hydroxylase[3]	28.0 ± 3.0^{a}	20.9 ± 0.9^{b} (75)	16.7 ± 3.0^{c} (60)	17.9 ± 4.0^{c} (64)	21.7 ± 3.0^{b} (78)
Plasma cholesterol (mg/100 ml)	165 ± 18^{a}	128 ± 12^{bd} (78)	121 ± 10^{bc} (73)	115 ± 12^{c} (70)	130 ± 11^{d} (79)
Plasma triglycerides (mg/100 ml)	144 ± 15^{a}	284 ± 19^{b} (197)	271 ± 16^{b} (188)	318 ± 22^{c} (221)	265 ± 14^{b} (184)
Malic enzyme[4]	5.6 ± 0.1^{a}	11.7 ± 1.0^{b} (209)	9.4 ± 0.1^{c} (168)	18.1 ± 1.1^{d} (323)	12.0 ± 1.0^{b} (214)
Citrate-cleavage enzyme[5]	1.7 ± 0.1^{a}	3.0 ± 0.3^{b} (176)	2.9 ± 0.2^{b} (170)	4.1 ± 0.2^{c} (241)	2.8 ± 0.2^{b} (165)
Acetyl-CoA carboxylase[6]	38.1 ± 2.0^{a}	63.5 ± 3.0^{b} (167)	69.2 ± 3.0^{c} (182)	81.3 ± 4.0^{d} (214)	62.7 ± 4.0^{b} (165)
Fatty acid synthetase[7]	3.4 ± 0.1^{a}	12.4 ± 0.4^{b} (365)	12.3 ± 0.7^{b} (362)	15.7 ± 0.6^{c} (462)	12.3 ± 0.3^{b} (362)

[1] Feeding period was 2 weeks; time of killing was 0800 hr; data expressed as mean ± SD; N = 6 chickens per group.
[2] nmoles of mevalonic acid synthesized/min/g of liver.
[3] pmoles of [^{14}C] cholesterol into [^{14}C] 7α-OH-cholesterol/min/g of liver.
[4] μmoles of $NADP^{+}$ reduced/min/g of liver.
[5] μmoles of product formed/min/g of liver.
[6] nmoles of product formed/min/g of liver.
[7] μmoles of NADPH oxidized/min/g of liver.
[8] Percentage of respective control activity data are in parentheses.
$^{a-d}$ Means within a column and without a common small superscript letter are significantly different, $p<0.01$.

TABLE 5

Effect of Dietary HPBF on Body and Liver Weight and Levels of Hepatic HMG-CoA Reductase and FAS in the Chicken

Nutritional state	Body weight (g)	Liver weight (g)	HMG-CoA reductase[3]		Fatty acid synthetase	
			Enzyme activity[4]	% of control activity	Enzyme activity[5]	% of control activity
Corn	491 ± 15[ac]	12.4 ± 0.7[a]	3.8 ± 0.3[a]		1.5 ± 0.3[a]	
Barley	463 ± 12[a]	11.7 ± 0.4[c]	1.9 ± 0.2[b]	50	3.9 ± 0.4[b]	267
Corn + 5% HPBF[2]	508 ± 15[ac]	12.2 ± 1.6[c]	3.3 ± 0.3[a]	87	2.2 ± 0.1[c]	147
Corn + 10% HPBF	538 ± 20[c]	14.2 ± 1.0[b]	2.1 ± 0.2[b]	55	4.2 ± 0.2[bd]	280
Corn + 15% HPBF	573 ± 26[b]	14.3 ± 0.7[b]	1.7 ± 0.2[bc]	45	4.6 ± 0.1[d]	307
Corn + 20% HPBF	600 ± 23[b]	14.7 ± 1.3[b]	1.4 ± 0.3[c]	37	5.3 ± 0.2[e]	353

[1] Feeding period was 4 weeks; time of killing was 0800 hr; data expressed as mean ± SD; N=6 male chickens per group.
[2] HPBF = high-protein barley flour.
[3] HMG-CoA reductase = β-hydroxy-β-methylglutaryl-CoA reductase.
[4] nmoles of mevalonic acid synthesized/min/g of liver.
[5] μmoles of NADPH oxidized/min/g of liver.
[a-e] Means within a column and without a common small superscript letter are significantly different, $p<0.01$.

was accumulated on the beaks of young chickens.

A second possible explanation for the observed low weight gain was the presence in HPBF of a growth retardant which becomes evident when HPBF is fed at concentrations substantially higher than that existing in the whole kernel, i.e., 5-10%. To check this hypothesis, 2-week-old female chicks, previously reared on commercial chick starter diet with corn as the only cereal grain, were fed the corn-based diet (Table 1) for 4 weeks with substitutions of different concentrations (5, 10, 15 and 20%) of HPBF for corn. These levels of HPBF caused significant increases in body and liver weights (Table 5) over that produced by the corn-based diet. The maximum difference in protein in the diets by these additions of HPBF was only 1.4%. Significant inhibition of HMG-CoA reductase and induction of fatty acid synthetase persisted at 10% or higher concentration of HPBF. There was a curvilinear response of these two enzymes to the different concentrations of HPBF in the diets. Verification of these data were obtained in 2 repeats of the experiment. Such drastic inhibition of cholesterogenesis cannot be attributed to slight changes in the concentration of protein in the diets.

These results indicate the presence of some factor(s) responsible for the inhibition of cholesterol biosynthesis in HPBF and also the presence of some growth factor(s) not evident in the intact barley. The relatively high differences in concentrations of ether extractables (4.7% vs 2.2%), pentosans (15.5% vs 9.8%) and acid detergent fibers (8.1% vs 6.2%) in HPBF compared to the barley kernel suggest several avenues of approach for further study of the active principles involved. Work on the isolation and identification of these substances is in progress.

ACKNOWLEDGMENTS

This investigation was supported in part by USDA-SEA/AR Competitive Research Grants Office Human Nutrition Program, Proposal Number 8000597, and from Research Grants HL25591 from National Heart, Lung Institute, National Institutes of Health, United States Public Health Service and Hatch Funds No. 1718, of the Research Division, College of Agricultural and Life Sciences, University of Wisconsin-Madison.

We thank Dr. Burt Olson for the use of the Isocap/300 Nuclear Liquid Scintillation counter. We also thank Steven W. Faber, Margaret Dietrich and John Carden for their excellent technical assistance and Faye Roed for excellent editorial assistance.

REFERENCES

1. Carroll, K.K., and Hamilton, R.M.G. (1975) J.

Food Sci. 40, 18-23.
2. Kritchevsky, D. (1978) Cereal Food World (Am. Assoc. Cereal Chem.) 23, 245-247.
3. Kritchevsky, D., Kolman, R.R., Guttmacher, R.M., and Forbes, M. (1959) Arch. Biochem. Biophys. 85, 444-451.
4. Kritchevsky, D., Tepper, S.A., and Story, J.A. (1975) J. Food Sci. 40, 8-11.
5. Stamler, J. (1978) Arch. Surg. 113, 21-25.
6. Yadav, N.R., and Liener, I.E. (1977) Nutr. Rept. Int. 16, 385-389.
7. Hamilton, R.M.G., and Carroll, K.K. (1976) Atherosclerosis 24, 47-62.
8. Bender, A.E., and Damji, K.B. (1972) World Rev. Nutr. Diet 15, 104-155.
9. Winitz, M., Graff, J., and Seedman, D.A. (1964) Arch. Biochem. Biophys. 108, 576-579.
10. Qureshi, A.A., Burger, W.C., Prentice, N., Bird, H.R., and Sunde, M.L. (1980) J. Nutr. 110, 388-393.
11. Macdonald, I. (1976) Proc. Nutr. Soc. 35, 155-175.
12. Teekell, R.A., Breidenstein, C.P., and Watts, A.B. (1975) Poultry Sci. 54, 1036-1042.
13. Berdanier, C.D. (1974) J. Nutr. 104, 1246-1256.
14. Innis, S.M., and Clandinin, M.T. (1980) J. Nutr. 110, 1006-1013.
15. Qureshi, A.A., Burger, W.C., Elson, C.E., and Benevenga, N.J. (1982) Lipids 17, 924-934.
16. Gornall, A.G., Bardawill, C.J., and David, M.M. (1949) J. Biol. Chem. 177, 751-766.
17. Hsu, R.H., and Lardy, H.A. (1967) J. Biol. Chem. 245, 520-526.
18. Inoue, H., Suzuke, F., Fukunishi, K., Adachi, K., and Takeda, Y. (1966) J. Biochem. 60, 543-553.
19. Nepokroeff, C.M., Lakshmanan, M.R., and Porter, J.W. (1975) in Methods in Enzymology (Lowenstein, J.M., ed.) pp. 45-49, Academic Press, New York, NY.
20. Snedecor, G.W., and Cochran, W.G. (1971) in Statistical Methods, 6th edn., pp. 258-298, The Iowa State University Press, Ames, IA.
21. Mitchell, A.D., Carlson, S.E., McGibbon, W.H., and Goldfarb, S. (1979) Atherosclerosis 32, 11-21.
22. Rudel, L.L., and Pitts, L.L. (1978) J. Lipid Res. 19, 992-1003.
23. Carrozza, G., Livered, G., Caponetti, R., and Manasseri, L. (1979) J. Nutr. 109, 162-170.
24. Korchak, H.M., and Masoro, E.J. (1962) Biochim. Biophys. Acta 58, 354-356.
25. Allmann, D.W., Hubbard, D.D., and Gibson, D.M. (1965) J. Lipid Res. 6, 63-74.
26. Burton, D.N., Collins, J.M., Kennan, A.L., and Porter, J.W. (1969) J. Biol. Chem. 244, 4510-4516.
27. Craig, M.C., Dugan, R.E., Muesing, R.A., Slakey, L.L., and Porter, J.W. (1972) Arch. Biochem. Biophys. 151, 128-136.

[Received April 14, 1982]

Partial Purification and Characterization of a Ca^{2+}-Stimulated Lipoxygenase from Soybean Seeds[1]

THOMAS D. DREESEN[2], MARTHA DICKENS[2], and ROBERT B. KOCH*, *Department of Biochemistry, Drawer BB, Mississippi State, MS 39762*

ABSTRACT

A highly purified Ca^{2+}-stimulated lipoxygenase was isolated from the Hill variety of soybean seeds. Separation of Ca^{2+}-stimulated lipoxygenase from lipoxygenase active in the absence of Ca^{2+} (lipoxygenase-1) was readily obtained using a DEAE-cellulose column. Sample size applied to the ion exchange column was found to be critical. Both enzymes were bound to the column, although some highly active Ca^{2+}-stimulated lipoxygenase eluted with buffer in the presence of bound lipoxygenase-1. Ca^{2+}-stimulated lipoxygenase bound to DEAE-cellulose required the use of a NaCl gradient for elution. Ca^{2+}-stimulated lipoxygenase showed an apparent isoelectric point at pH 5.90 and optimum activity at pH 7.5 and at 1.1 mM calcium. Lipoxygenase-1 was inhibited over 95% in the presence of 60 μM methyl mercuric chloride, while Ca^{2+}-stimulated lipoxygenase showed a maximum of only 20% inhibition under the same conditions.
Lipids 17:964-969, 1982.

Lipoxygenase (EC 1.13.11.12) catalyzes the hydroperoxidation of *cis,cis*-pentadiene systems to the corresponding isomeric dienoic hydroperoxides (1). There are reports of at least 2 isozymes of lipoxygenase in soybean seeds (2-6) and possibly 3 (7) or 4 (8). Lipoxygenase-2 (5,9,10) has been shown to be activated by Ca^{2+}, although whether the effect was due to a Ca^{2+}-enzyme complex or a Ca^{2+}-linoleate has not been conclusively determined. Ca^{2+} stimulations of from 2- to 6-fold of purified lipoxygenase-2 have been reported (9,10). Also, Ca^{2+} stimulation of lipoxygenase activity(s) in crude water extracts of soybean seeds of from 6- to > 10-fold have been reported (3-5). We have obtained a highly purified lipoxygenase isozyme preparation from soybean seeds. The activity of the Ca^{2+}-stimulated enzyme was reduced by 97% when EGTA (ethylene glycol-*bis*- (β-amino ethyl ether) N,N' tetraacetic acid) was added to chelate Ca^{2+} in the reaction mixture. The Ca^{2+}-stimulated lipoxygenase possesses some of the previously published characteristics of lipoxygenase-2(2,5,10).

MATERIALS AND METHODS

Materials

Soybeans of the Hill variety (1978 crop) were obtained from the Seed Technology Laboratory, Mississippi State University. Methyl mercuric chloride was obtained from Alfa Products, Danvers, MA. All other chemicals were reagent grade from commercial sources.

Enzyme Purification

Soybeans were ground in a Wiley mill to pass a #20 standard sieve and extracted with 10 volumes of deionized distilled water at room temperature for 30 min. The resulting suspension was centrifuged at 16,000 × g for 10 min at 4 C. The supernatant was decanted and the material that precipitated between 30 and 50% $(NH_4)_2SO_4$ was dissolved in 10 mM Tris HCl, pH 7.0, at 2-5 mg/ml protein. One ml of the 30-50% $(NH_4)_2SO_4$ fraction was applied to a 2.4 cm × 40 cm DEAE cellulose column (Selectacel, DEAE HI-FLO, James Rimver Corp., Berlin, NH) equilibrated with 10 mM Tris HCl, pH 7.0. Proteins that bound were eluted with 400 ml of a 0–1.0 M NaCl buffered linear gradient in the above buffer. Active peaks were collected and concentrated using an Amicon ultrafiltration system with a YM10 membrane. The concentrated fractions were applied to a second DEAE-cellulose column (1.5 cm × 2.5 cm, DEAE - Sephacel, Sigma Chemical Co., St. Louis, MO) to which the lipoxygenases bound. The lipoxygenases were eluted with 200 ml of a 0–0.5 M NaCl buffered linear gradient. Active fractions containing Ca^{2+}-stimulated lipoxygenase activity or lipoxygenase-1 activity were combined and concentrated, respectively, and then applied to a Sephacryl S-200 column equilibrated with 10 mM Tris HCl, pH 7.0, to remove contaminating proteins.

Enzyme Assays

Lipoxygenase activities were measured polar-

[1] Paper No. 4993 Mississippi Agricultural and Forestry Experiment Station.

[2] Present address: University of Mississippi Medical School, Jackson, MS 39216.

*Author to whom correspondence is to be addressed.

graphically at 25 C using a Yellow Springs oxygen monitor with a Clark electrode. The reaction mixture routinely contained 3 ml buffer (0.2 M borate, pH 9.0, or 0.2 M imidazole, pH 7.5) containing 0.39 mM linoleic acid with 0.68 mM Ca^{2+} or 0.68 mM EGTA. Ca^{2+}-stimulated activity is defined as activity in the presence of Ca^{2+} minus activity in presence of EGTA.

Electrophoresis

Tube gel electrophoresis was carried out in 7.5% acrylamide, pH 8.3, by the method of Ornstein (11) at 4 mA per tube. After electrophoresis, gels were stained for activity by the methods of Guss et al. (8) or DeLumen and Kazeniac (12) and then stained for protein with Coomassie blue.

Electrofocusing was done on flat beds of Sephadex G-75 (13) with ampholytes (pH 3–11). After focusing was complete, a paper print was made, the gel bed divided into lanes, gel lanes removed, and proteins eluted with 10 mM Tris HCl, pH 7.0.

Methyl Mercury Treatment

Highly purified preparations of lipoxygenase-1 and Ca^{2+}-stimulated lipoxygenase were incubated with varied concentrations of methyl mercuric chloride for varied time periods according to Spaapen et al. (14) except the reaction was carried out in 0.2 M imidazole, pH 7.5, at 25 C. The extent of inhibition was monitored enzymatically.

Protein Determination

Protein concentrations were measured by the method of Lowry et al. (15) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Enzyme Purification

Since earlier reports (3,4) have shown a 10-fold Ca^{2+}-stimulation of lipoxygenase(s) activity in crude soybean extracts, further studies were conducted to isolate and characterize the isozyme(s) responsible. The basic purification method used was similar to methods employed for the isolation of lipoxygenase-1 and lipoxygenase-2 (2,7). The elution profile of a typical chromatographic separation of an aliquot of the 30–50% $(NH_4)_2SO_4$ fraction of soybean seed extract on DEAE-Selectacel is shown in Figure 1. A major portion of the Ca^{2+}-stimulated lipoxygenase (as indicated by activity measurements) did not bind to the column. Also, a variable amount of lipoxygenase-1 did not bind, depending on the amount of protein applied. There was a large amount of inactive protein bound to the column that required high salt concentrations for removal. Fractions from the DEAE-Selectacel column that had Ca^{2+}-stimulated activity (some of which contained

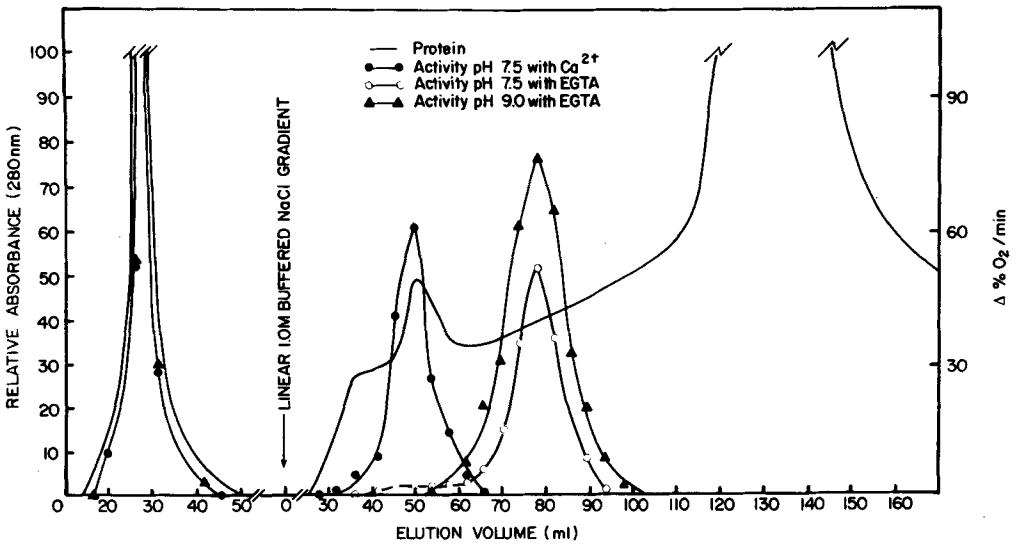


FIG. 1. DEAE-Selectacel chromatography of 30–50% $(NH_4)_2SO_4$ fraction from crude extract of soybean seeds. A_{280} was measured continuously and fractions of 3 ml collected. Enzyme activities were measured as described in Experimental Procedures.

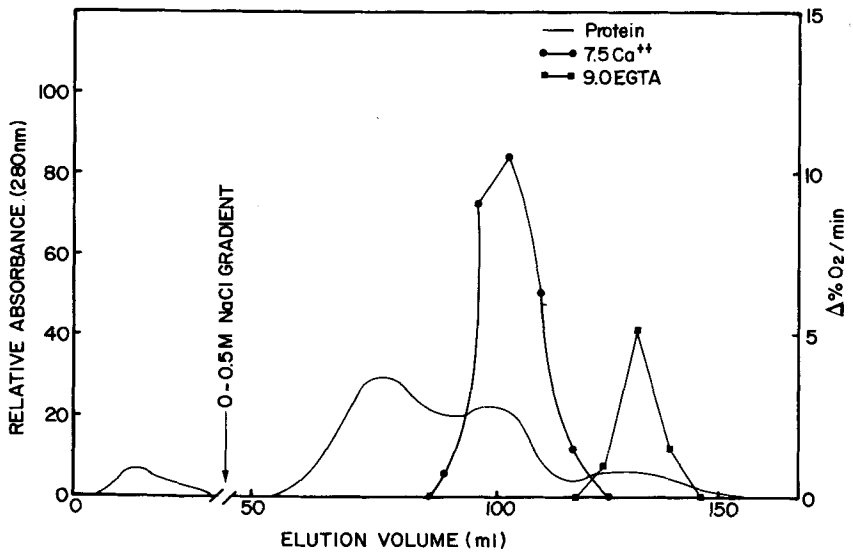


FIG. 2. DEAE-Sephacel chromatography of active fractions from DEAE-Selectacel column. See Figure 1 for other parameters.

lipoxygenase-1 activity) were combined, concentrated, and applied to a DEAE-Sephacel column which bound both lipoxygenase enzymes present in the concentrated sample. The enzymes were eluted with a buffered 0–0.5 M NaCl linear gradient. The Ca^{2+}-stimulated enzyme eluted before the lipoxygenase-1 enzyme (Figure 2). In neither chromatographic separation was any activity observed that corresponded to lipoxygenase-3 (7). To remove remaining contaminating proteins, each activity peak (lipoxygenase-1 and Ca^{2+}-stimulated) was concentrated and applied to a Sephacryl S-200 column. Ca^{2+}-stimulated lipoxygenase was the first protein to be eluted. However, the yield of total active enzyme from the Sephacryl S-200 column was only 10–20% of the activity of the DEAE-Sephacel-separated sample. The specific activity of the Ca^{2+}-stimulated lipoxygenase from the Sephacryl S-200 column was 42.7 μmol O_2/min/mg protein, which represents a 3-fold increase in specific activity compared to the 30–50% ammonium sulfate insoluble fraction.

The characteristics of the enzyme were not altered by the Sephacryl S-200 step so that the partially purified preparations from the DEAE-cellulose column were used in the studies discussed below, except that electrophoresis and methyl mercury treatment were conducted with the S-200 column preparation.

Electrophoresis

The protein and activity stained profiles of lipoxygenases (obtained by electrophoresis under nondenaturing conditions at pH 8.3) from the various fractions are depicted in Figure 3. Gel 1 (Fig. 3) contained both L-1 and Ca^{2+}-stimulated LOX activities. The activity peak of the Ca^{2+}-stimulated enzyme from Sephacryl S-200 column (Fig. 3, Gel 2) exhibited 2 bands, neither of which was activity stainable with either of the activity stains tested.

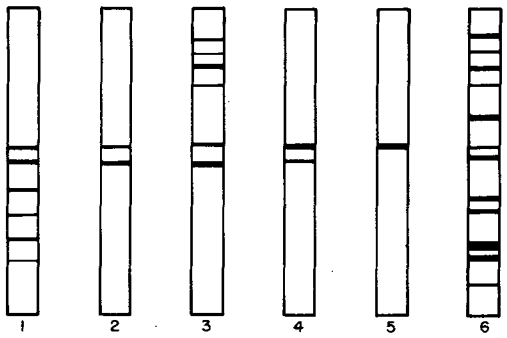


FIG. 3. Polyacrylamide gel electrophoresis of various preparations of lipoxygenases. Gels 1, 2, 3, and 6 were stained for protein, gels 4 and 5 were stained for lipoxygenase activity with starch-KI and *o*-dianisidine, respectively. (1) Ca^{2+}-stimulated, pH 7.5 activity peak from DEAE column (Fig. 2); (2) Same fraction after Sephacryl S-200 chromatography; (3) Lipoxygenase-1 activity peak from DEAE column (Fig. 2); (4) Starch KI, and (5) *o*-dianisidine activity stains of 3); (6) 30–50% $(NH_4)_2SO_4$ fraction.

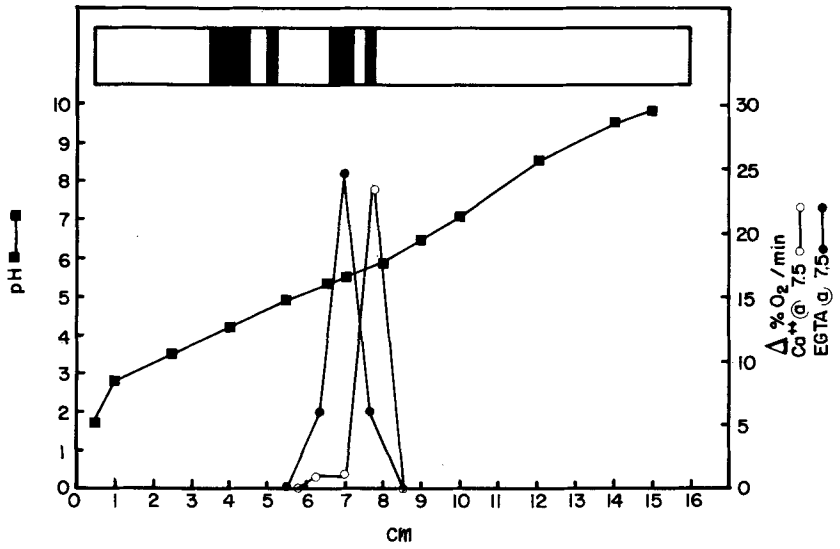


FIG. 4. Isoelectric focusing. Isoelectric focusing was performed on Sephadex G-75 beds at 4 C for 4 hr in 1% pH 3-11 ampholytes. See Experimental Procedures for conditions. Coomassie blue stained paper print at top of figure.

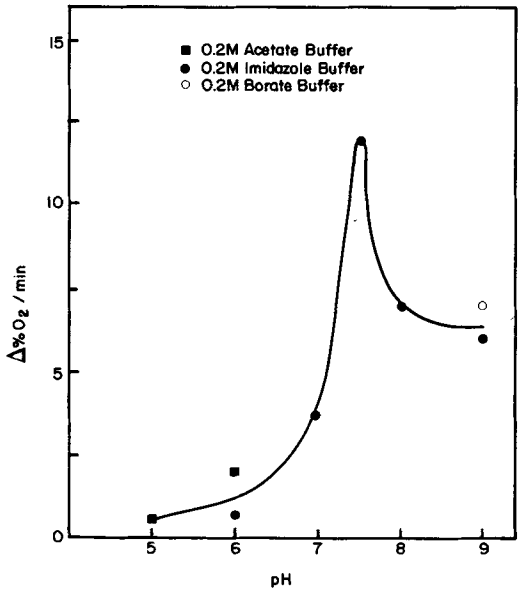


FIG. 5. Effect of pH on the Ca^{2+}-stimulated lipoxygenase. Assays were performed as in Experimental Procedures, except with the noted buffer changes.

The reason why the Ca^{2+}-stimulated enzyme does not activity stain is not known. The lipoxygenase-1 preparations, on the other hand, showed 2 activity bands with starch-KI (Fig. 3, Gel 4) but only one with *o*-dianisidine (Fig. 3, Gel 5). Whether the second activity stainable band on gel 4 is a separate lipoxygenase is not known at this time.

Isoelectric Focusing

Results from isoelectric focusing of the sample containing both enzyme activities (Gel 1, Fig. 3) are shown in Figure 4. After electrofocusing, the Sephadex G-75 bed was divided into lanes and pH measured. Each lane was removed from the plate and extracted with 10 mM Tris HCl buffer, pH 7.0. Each extract was tested for lipoxygenase activity at pH 7.5 and 9.0, with and without Ca^{2+}. The apparent isoelectric points were 5.90 and 5.53 for the Ca^{2+}-stimulated lipoxygenase and lipoxygenase-1, respectively. These values were in reasonably close agreement with published isoelectric points for lipoxygenase-1 which are 5.68 (7) and 5.65 (16). The isoelectric points for lipoxygenase-2 (6.25) and -3 (6.15) as reported by Christopher et al. (7) were only higher than the Ca^{2+}-lipoxygenase (5.90) isolated in this study.

Effect of pH and Ca^{2+} Concentration

Figure 5 shows the Ca^{2+}-stimulated enzyme activity as a function of pH with linoleic acid as substrate. Maximal activity was found at pH 7.5 in agreement with Koch and coworkers (3, 4), and Yamamoto et al. (5). Christopher et al. (2) reported a pH optimum of 6.6 for Ca^{2+}-stimulated lipoxygenase-2, but show in

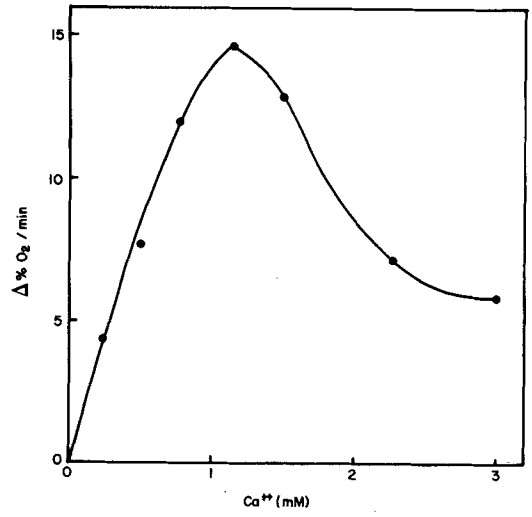


FIG. 6. Effect of Ca^{2+} on the Ca^{2+}-stimulated lipoxygenase. Assays were performed at pH 7.5 with indicated final concentrations of Ca^{2+}. The small amount of activity (<3%) in the presence of EGTA has been subtracted.

Figure 3 of reference (2) a pH optimum of ca. 7.2 for lipoxygenase-2 with linoleic acid as substrate in the presence of Ca^{2+}.

The effect of Ca^{2+} on a highly purified Ca^{2+}-stimulated lipoxygenase is shown in Figure 6. A definite optimum Ca^{2+} concentration was found at 1.1 mM Ca^{2+}. This stimulation was 50-fold over the activity in the presence of EGTA (0.68 mM).

Methyl Mercury Treatment

Spaapen et al. (14), by use of organic mercurials, have reported that lipoxygenase-1 contains 5 free sulfhydryls and no disulfide bridges. They also reported that lipoxygenase-1 was inhibited 80% with 0.8 mM methyl mercuric chloride after 1-hr preincubation of enzyme with the mercurial. We found that the inhibition of lipoxygenase-1 by methyl mercury was both time- and concentration-dependent. Thus, 95% of lipoxygenase-1 activity was lost after a 10-min preincubation with 60 μM methyl mercuric chloride (Fig. 7). In contrast, the Ca^{2+}-stimulated lipoxygenase was inhibited only 20% at 60 μM methyl mercuric chloride at all preincubation times (Fig. 7). The presence or absence of Ca^{2+} with lipoxygenase-1 or Ca^{2+}-stimulated lipoxygenase during preincubation with methyl mercuric chloride had no effect on the inhibition patterns (data not shown). These results suggest that free sulfhydryls are not important in the catalytic function of Ca^{2+}-stimulated lipoxygenase. Whether sulfhydryls are present but protected from the action of methyl mercury is yet to be determined. Comparison of our Ca^{2+}-stimulated lipoxygenase with isozymes isolated by others was hampered by the variety of assay conditions found in the literature. For example, Tween 20 has been used to solubilize linoleic acid in the assay mixture (2,7), but has been shown to inhibit Ca^{2+}-stimulation of lipoxygenase (4,5). Also, the spectrophotometric assay used by Axelrod and coworkers (2,17) was hampered by turbidity upon addition of Ca^{2+} and protein.

The Ca^{2+}-stimulated lipoxygenase reported in this work was similar to lipoxygenase-2 (2, 5,10) with respect to elution pattern from DEAE cellulose, ability of Ca^{2+} to stimulate

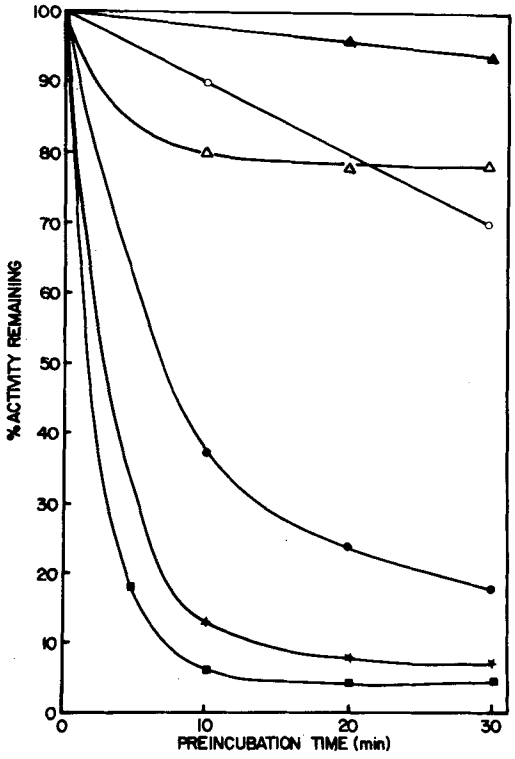


FIG. 7. Effect of methyl mercury chloride on lipoxygenase activities. Highly purified lipoxygenase-1 and Ca^{2+}-stimulated lipoxygenase were incubated with the noted concentrations of methyl mercury chloride in 0.2 M imidazole buffer, pH 7.5. ▲, Ca^{2+}-stimulated enzyme control; △, Ca^{2+}-stimulated enzyme plus 60 μM methyl mercury; ○, lipoxygenase-1 control; ●, lipoxygenase-1 plus 20 μM methyl mercury; ★; lipoxygenase-1 plus 40 μM methyl mercury; ■, lipoxygenase-1 plus 60 μM methyl mercury. Lipoxygenases were assayed at the appropriate pH as in Experimental Procedures.

activity, and specific activity. However, the Ca^{2+}-stimulated lipoxygenase in our study differs from lipoxygenase-2 reported by others (2,5,7,17) in the amount of activity present in the absence of Ca^{2+}, pH optimum, and Ca^{2+} optimum. It may be that the differences observed were due to soybean variety differences used in the different laboratories. It has been observed (unpublished data, RBK) that extracts from different varieties of soybeans show different patterns of LOX and Ca^{2+}-stimulated LOX activities.

REFERENCES

1. Holman, R.T., and Bergström, S. (1951) in The Enzymes (Sumner, J.B. and Myrback, K., eds.) Vol. II, Part 1, pp. 559–580, Academic Press, New York, NY.
2. Christopher, J.P., Pestorius, E.K., andAxelrod, B. (1970) Biochim. Biophys. Acta 198, 12–19.
3. Koch, R.B. (1968) Arch. Biochem. Biophys. 125, 303–307.
4. Koch, R.B., Brumfiel, B.L., and Brumfeil, M.N. (1971) J. Am. Oil Chem. Soc. 48, 532–538.
5. Yamamoto, A., Yasumoto, K., and Mitsuda, H. (1970) Agr. Biol. Chem. 34, 1169–1177.
6. Verhue, W.M., and Franke, A. (1972) Biochim. Biophys. Acta 284, 43–53.
7. Christopher, J.P., Pestorius, E.K., and Axelrod, B. (1972) Biochim. Biophys. Acta 284, 54–62.
8. Guss, P.O., Richardson, T. and Stahman, M.A. (1967) Cer. Chem. 44, 607–610.
9. Restrepo, F., Snyder, H.E., and Zimmerman, J. (1973) Food Sci. 38, 779–782.
10. Zimmerman, G.L., and Snyder, H.E. (1974) Agr. Food Chem. 22, 802–805.
11. Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321–349.
12. Delumen, B.O., and Kazeniac, S.J. (1976) Anal. Biochem. 72, 428–432.
13. Salin, M.L., and Bridges, S.M. (1980) Arch. Biochem. Biophys. 201, 369–374.
14. Spaapen, L.J.M., Verhagen, J., Veldink, G.A., and Vliegenthart, J.F.G. (1980) Biochim. Biophys. Acta 618, 153–162.
15. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
16. Catsimpoolas, N. (1969) Arch. Biochem. Biophys. 131, 185–190.
17. Axelrod, B., Cheesbrough, T.M., and Laakso, S. (1981) Methods Enzymol. 71, 441–451.

[Received June 7, 1982]

Temperature Sensitivity of Cholesteryl Ester Hydrolases in the Rat Testis

LUCIAN A. DURHAM, III* and **W. McLEAN GROGAN**, *Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298*

ABSTRACT

Cholesteryl ester hydrolase (CEH) (EC 3.1.1.13) activity was assayed in the 104,000 × g supernatant (S104) of rat and mouse testes and livers at various temperatures between 27 C and 44 C. The CEH activity in the testis dropped from 44 pmol [4-^{14}C] cholesteryl oleate hydrolyzed/hr/mg protein to 14 pmol hydrolyzed/hr/mg protein (a 68% decrease) between testicular and abdominal temperatures (32 C and 37 C, respectively) in the rat. This decrease in activity is essentially a reversible phenomenon. CEH from the testis S104 was stabilized in 10 mM EDTA and was purified by HPLC size exclusion. These steps did not alter the temperature effect previously noted. The temperature effect on the testicular CEH was demonstrated in vivo by assaying the enzyme following unilateral cryptorchidism. The HPLC purification yielded 3 peaks of CEH activity from the testicular S104. The 28,000 MW peak was found to be temperature insensitive while the 70,000 and 420,000 MW peaks were temperature labile. The liver CEH of both species remained relatively constant over the range 32–37 C. CEH is a potential regulator of both steroidogenesis and membrane composition in the testis and its temperature lability may suggest a unique regulatory mechanism responsible for impaired spermatogenesis seen with elevated testicular temperatures.
Lipids 17:970-975, 1982.

INTRODUCTION

Cholesteryl ester hydrolase (CEH) plays an important role in regulating the equilibrium between free and esterified cholesterol in the testis and other tissues. Cholesteryl ester (CE) is a storage form of androgen precursor cholesterol (1–3), and mobilization of CE is apparently necessary for androgen synthesis (2) which is required for maintenance of spermatogenesis. Bartke et al. have reported that a specific inhibitor of CEH, phenylmethylsulfonylfluoride (PMSF), causes a decrease in the number of Type A spermatogonia in the testis subsequent to increases in the CE pool (2). A number of other conditions resulting in impaired spermatogenesis are accompanied by shifts in the free cholesterol/CE ratio (4). One of these conditions is cryptorchidism, which is generally thought to produce its degenerative effects by elevation of the testicular temperature. An elevation in the scrotal temperature (32 C) to abdominal temperature (37 C) has significant effects on metabolism of rat testis including pronounced inhibition of protein synthesis (5) and modification of lipid metabolism (6). Specific changes in the membrane FSH receptor of Sertoli cells have also been seen with temperature increases (7). Since CEH is a potential regulator of both steroidogenesis and membrane composition in the testis, we examined the effects of temperature on this enzyme as a possible mediator of the temperature induced impairment of spermatogenesis.

*Author to whom correspondence should be addressed.

EXPERIMENTAL PROCEDURE

Preparation of Rat Testis for CEH Assay

Adult male Sprague-Dawley rats (Flow Laboratories) were used for the experiments. They had free access to food and water and were kept at 24 C on a cycle of 12 hr light/12 hr darkness. Rats weighing 100–200 g were sacrificed by cervical dislocation and their testes were removed and decapsulated. The decapsulated testes were weighed and rinsed in ice cold 0.1 M potassium-phosphate buffer, pH 7.4. The tissue was then homogenized in a tissue grinder, with a loose teflon pestle, in 2.5 ml of 0.1 m P_i buffer, pH 7.4, per gram of tissue. This preparation was centrifuged at 2,000 × g for 30 min; the supernatant was decanted and centrifuged at 10,000 × g for 30 min to remove the mitochondrial fragments, with the supernatant being decanted and centrifuged at 104,000 × g for 90 min to yield the high-speed supernatant desired (S104). The high-speed supernatant (S104) was dispersed in hexane with a loose teflon pestle in order to remove any endogenous substrate (cholesteryl ester). The phases were allowed to separate and the hexane layer containing the cholesteryl ester was discarded. Protein content in the aqueous layer was deter-

mined by the method of Lowry et al. (8). This is essentially the method of Deykin and Goodman (9) for the isolation of liver CEH, with modifications by McGovern and Quackenbush (10). The mouse testis supernatant was obtained in the same manner as above.

Preparation of Liver for CEH Assay

The livers of the same Sprague-Dawley rats and Swiss mice used in the procedures above were also excised. The liver supernatant (S104) was prepared in the same manner as described above.

Cholesteryl Ester Hydrolase Assay

Cholesteryl ester hydrolase activity was assayed in 2.0-ml final volume containing an aliquot of the high-speed supernatant (100 mg of protein) to which 200 nmol [4-^{14}C] cholesteryl oleate was added in 50 μl of acetone. This was the method of choice for the presentation of substrate (8). This mixture was then incubated at the various temperatures for 60 min. The reaction was stopped by the addition of 50-ml chloroform/methanol (2:1, v/v). Isolation by thin layer chromatography of the radioactive cholesterol and cholesteryl ester for scintillation counting has been described elsewhere (10).

Preincubation Procedure

The high-speed supernatant was incubated at 32, 37 or 40 C for 60 min; it was then allowed to equilibrate to incubation temperatures of 32 C and 37 C (ca. 5 min). Aliquots of the preincubated S104 fraction (100 mg of protein) were subsequently assayed for CEH activity as described above.

Procedure for Unilateral Cryptorchidism

The Sprague-Dawley rats were made unilaterally cryptorchid by making a 1-cm midline abdominal incision under ether anesthesia. The supporting fatty tissue was withdrawn until the testis was drawn into the abdominal cavity, at which time the gubernaculum was severed and the testis was secured to the lateral abdominal wall with a suture through the tunica albuginea. The incision was then closed with surgical staples. This is a modification of a procedure described elsewhere (11). The rats were sacrificed after 24 hr and the S104 fraction was assayed at 32 C and 37 C for CEH activity.

Stabilization of Testicular CEH

It was found that the testicular CEH was both a delicate and short-lived enzyme. CEH activity was lost after 12 hr regardless of whether the S104 was kept at room temperature, refrigerated, or frozen. Therefore, attempts were made to stabilize the enzyme in the S104 fraction using 10 mM 2-mercaptoethanol, 10 mM EDTA, 15% sucrose, and 15% glycerol in 0.1 M potassium phosphate buffer, pH 7.4.

Purification of CEH in the Rat Testis by HPLC

Three "I-series" HPLC protein columns (Waters Associates, Milford, MA) were connected to a Waters HPLC system consisting of two model 6000 pumps, UK-6 injector, and model 440 Absorbance detector. The "I-series" column setup consisted of one each I-250, I-125 and I-60 protein columns, connected in order of decreasing pore size. The elution profile was monitored at 254 nm and 313 nm. The mobile phase consisted of 0.1 M potassium-phosphate buffer containing 10 mM EDTA and 0.05% sodium cholate, pH 7.4. Aliquots of the S104 fraction were filtered through a 0.4 μ filter, injected, and run at a flow rate of 1.0 ml/min (1200 psi). Fractions were collected and assayed for protein content and CEH activity.

Chemicals

[4-^{14}C] Cholesteryl oleate (52.5 mCi/mmol) was obtained from New England Nuclear, Boston, MA. It was adjusted to give 200 nmol/50 μl acetone. The purity and specific radioactivity were determined by thin layer chromatography in hexane/ether/acetic acid (90:20:1, v/v/v).

RESULTS

Assay Parameters

Velocity (pmol [4-^{14}C] cholesteryl oleate hydrolyzed/hr) vs protein concentration (mg/ml assay volume) curves were run at 32 C and 37 C and it was determined that 50 mg protein/ml assay volume yielded values well above control while remaining in the linear range of the reaction. Therefore, this concentration was chosen as the standard protein concentration for the CEH assay. V vs [S] plots at 32 C indicated that optimum cholesteryl ester hydrolysis, with 50 mg protein/ml assay volume, was achieved with 100 μM substrate (200 nmol [4-^{14}C] cholesteryl oleate/2.0 ml assay volume).

Temperature Sensitivity of Testicular CEH

After establishing the optimum parameters for the CEH assay, we performed 60 min incubations at various temperatures to ascertain the

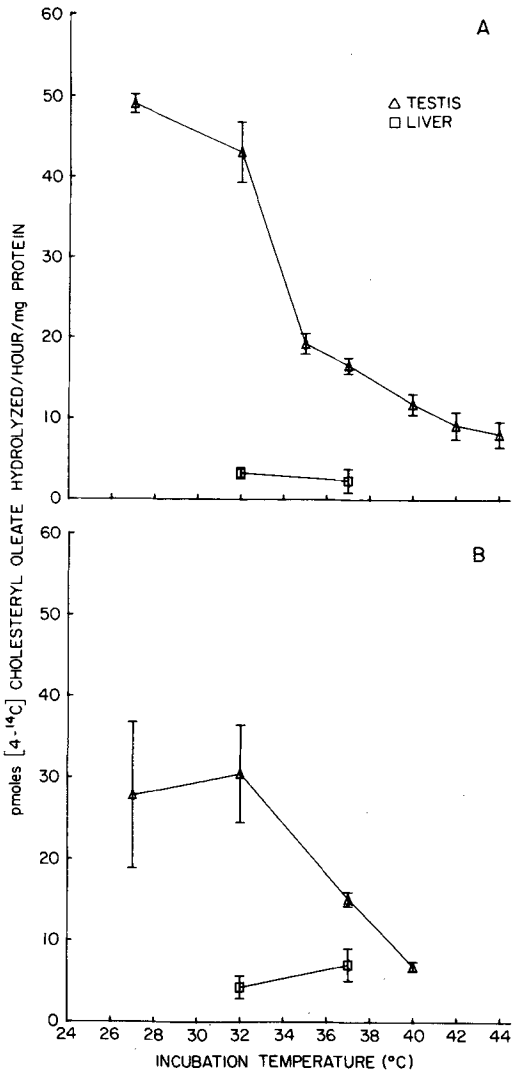


FIG. 1. Velocity vs incubation temperature for rat and mouse testicular and hepatic cholesteryl ester hydrolase. Samples were incubated for 1 hr in 2.0-ml assay volume containing 100 mg protein and 200 nmol (0.025 μCi) [4-^{14}C] cholesteryl oleate. (A) Rat tissues. (B) Mouse tissues.

temperature sensitivity of the enzyme. An overall decrease in activity was noted between 27 C and 44 C, but the extreme drop in activity (68%) between 32 C and 37 C was of particular interest as it represented a significant decrease in activity between testicular and abdominal temperatures (Fig. 1A). The experiments were repeated using mouse testis to look at possible species specificity of the testicular CEH temperature sensitivity observed in the rat. These experiments yielded similar results (51% decrease in enzyme activity between 32 C and 37 C), thus indicating that the temperature sensitivity is not unique to rat testis CEH (Fig. 1B).

TABLE 1

Summary of Preincubation Data[a]

Preincubation temperature (C)	Incubation temperature (C)	
32	43.3 ± 2.9	29.1 ± 1.5[e]
37	35.3 ± 2.2[b]	22.2 ± 1.5[c]
40	25.8 ± 1.1[c]	11.8 ± 1.9[c]

[a]Units are pmol [4-^{14}C] cholesteryl oleate hydrolyzed/hr/mg protein ± SEM (N = 12). Samples were preincubated for 1 hr prior to a 1 hr incubation in 100 μM [4-^{14}C] cholesteryl oleate (0.025 μCi).

[b]Significantly different from value immediately above at $p < 0.05$.

[c]Significantly different from value immediately above at $p < 0.001$.

[d]All values for the 37 C incubation were significantly different from value for 32 C incubation at $p < 0.001$.

[e]Different from the 37 C preincubation–32 C incubation at $p < 0.05$.

TABLE 2

Effect of Cryptorchidism on CEH Activity in the Rat[a]

Tissue type	Incubation temperature 32 C	37 C
Normal	36.1 ± 0.6[b]	24.5 ± 0.9[c]
Cryptorchid	21.3 ± 1.9	21.7 ± 0.7

[a]Activity expressed in pmol [4-^{14}C] cholesteryl oleate hydrolyzed/hr/mg protein ± SEM.

[b]Different from 32 C incubated cryptorchid CEH activity, $p < 0.01$.

[c]Different from 32 C incubated normal CEH activity, $p < 0.001$.

Temperature Sensitivity of Liver CEH

Organ specificity of the temperature sensitivity of CEH was demonstrated by assaying both rat and mouse livers for CEH activity under the same temperature conditions as for the testicular enzyme and comparing the results obtained for the livers with those of the testis. There was no significant temperature sensitivity observed for the liver CEH, which suggests that the temperature lability seen in the testicular CEH is specific for the testis (Fig. 1).

Effect of Preincubation on CEH Activity

Sample groups that were preincubated at

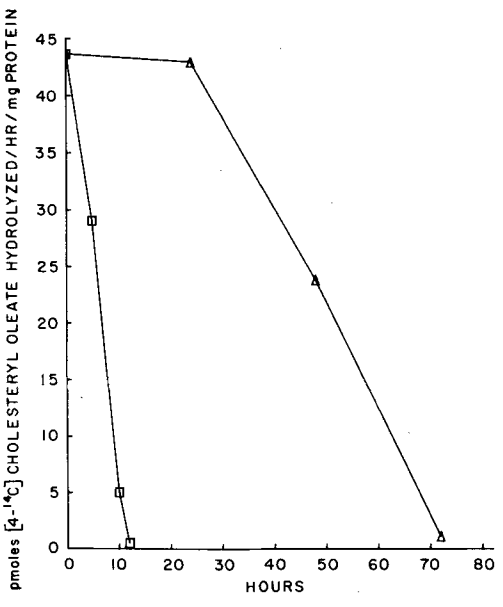


FIG. 2. Velocity (pmol [4-^{14}C] cholesteryl oleate hydrolyzed/hr/mg protein) vs time (hr). CEH prep was kept at 4 C until designated time. Aliquots (100 mg protein) were then incubated for 1 hr at 32 C with 200 nmol label (0.025 μCi). □–10 mM 2-mercaptoethanol, control, 15% glycerol, 15% sucrose. Δ–10 mM EDTA.

32 C (Table 1) retained normal CEH activity when incubated at 32 C (32 pmol [4-^{14}C] cholesteryl oleate hydrolyzed/hr/mg protein). Preincubation at 37 C or 40 C resulted in progressive decreases in activity at both incubation temperatures (32 C and 37 C). However, incubation at 32 C subsequent to preincubation at 37 C resulted in greater activity (35 pmol hydrolyzed/hr/mg protein) than in the 37 C incubation without any preincubation (15 pmol hydrolyzed/hr/mg protein) or with 32 C preincubation (29 pmol hydrolyzed/hr/mg protein). In each case, incubation at 37 C resulted in a loss of activity of ca. 14 pmol hydrolyzed/hr/mg protein, regardless of the preincubation temperature when compared to 32 C incubation. However, preincubation at 32 C resulted in a significantly lower loss of activity (8 pmol hydrolyzed/hr/mg protein) than preincubation at 37 C, regardless of incubation temperature. This indicates that the temperature lability is at least partially reversible.

Effect of Cryptorchidism on CEH Activity

The CEH activity in the cryptorchid testis remained constant, regardless of incubation temperature, while the normal testis showed a 33% decrease in activity between 32 C and 37 C (Table 2). This demonstrates that the temperature effect on the hydrolysis of cholesteryl ester in the testis occurs both in vitro and in vivo.

Effect of Stabilizing Agents on the Longevity of CEH Activity

The effect of the various stabilizing agents on CEH activity is shown in Figure 2. The 15% glycerol, sucrose, and 10 mM 2-mercaptoethanol had no effect in increasing the hydrolytic lifetime of the testicular CEH. However, 10 mM EDTA increased the lifetime of the enzyme significantly allowing further purification attempts.

Purification of Testicular CEH by HPLC

A 75-fold purification of the CEH was

TABLE 3

Purification of CEH in the Rat Testis

Method	Protein (g)	Activity (units)[a]	Specific activity (u/g)[b]	% Yield[c]
Homogenate	3.12	789	253	100
Centrifugation 2,000 X g–30 min	2.18	739	339	94
Centrifugation 10,000 X g–30 min	1.11	507	458	64
Centrifugation 104,000 X g–90 min	0.62	333	537	42
HPLC "I-series"	0.02	380	19,000[d]	48

[a]Units = nmol [4-^{14}C] cholesteryl oleate hydrolyzed/hr.
[b]Specific activity = pmol hydrolyzed/hr/mg protein.
[c]% Yield based on activity recovered.
[d]Value given is the pool of all fractions with CEH activity.

achieved using a combination of successive centrifugations and HPLC (Table 3). The pellets of the centrifugation steps were assayed and found to contain minimal amounts of CEH activity. Other purification procedures, such as $(NH_4)_2SO_4$ fractionation, PEG precipitation, and hydrophobic column chromatography using agarose-C_{0-10}-NH_2 series columns (Miles-Yeda, Ltd., Kryat-Weizmann, Rehovot, Israel) were unsuccessful, due to loss of CEH activity. The "I-series" HPLC columns allowed rapid separation of the protein components of S104 and recovery of more than 90% of the CEH activity.

Figure 3 shows the protein elution profile (254 nm absorbance) and CEH activity of fractions collected as they eluted from the columns. Three major peaks of CEH activity were noted which corresponded to molecular weights of 420,000 (420K), 70K and 28K. The 70K and 28K activities were completely resolved by recycling that portion of the effluent twice through the columns, prior to collection of the fractions with which they were associated. All 3 activities were assayed at 32 C and 37 C. Both 420K and 70K activities were completely abolished at 37 C, while the 28K activity was unaffected by the higher temperature. Recoveries were 91% of the protein and 94% of the CEH activity. Purity of the CEH was determined by electrophoresis on a 5–20% SDS-polyacrylamide slab gel. Only slight impurities persisted.

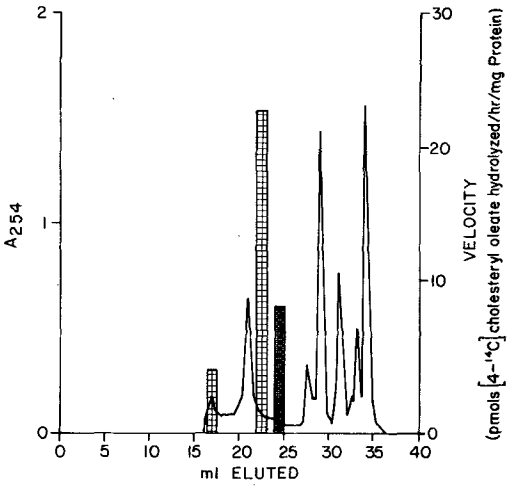


FIG. 3. Velocity (pmol [4-^{14}C] cholesteryl oleate hydrolyzed/hr/mg protein) and A_{254} vs elution volume (ml). Aliquots (100 μg protein) were passed through a 0.2 μm filter and were then injected into the HPLC running at 1.0 ml/min (1,200 psi). The samples were monitored at 254 nm from 0–2.0 absorbance units full scale (AUFS). Fractions were collected and assayed for CEH activity at 32 C and 37 C for 1 hr. Samples were incubated with 200 nmol label (0.025 μCi). — indicates elution profile at 254 nm. Large checked histogram indicates temperature sensitive CEH activity. Small checked histogram indicates temperature insensitive CEH activity.

DISCUSSION

These experiments demonstrate a direct temperature effect on testicular CEH activity which is organ specific and at least partially reversible. The effect was seen both in vivo and in vitro. Inhibition of CEH by elevation of temperature to 37 C persists in the partially purified temperature sensitive enzyme which can be separated from the temperature insensitive CEH by molecular sizing HPLC columns at a high percentage of recovery of both protein and enzyme activity. The presence of a temperature insensitive CEH explains the residual CEH activity seen in the CEH temperature assays of the testicular S104 (Fig. 1).

The instability of the CEH hindered further attempts at purification, but the purity was sufficient to suggest that temperature sensitivity is an inherent property of the major testicular CEH, independent of cofactors, proteases, etc. It is possible that the 420,000 MW protein is an aggregate of 70,000 MW subunits as both are temperature labile and this type of association in a cholesteryl ester hydrolase has been previously documented (12).

We know of no other enzyme which is so exquisitely sensitive to temperature in the immediate vicinity of its physiological temperature. A temperature-sensitive modulation of CEH could assume an important role during gestation and postnatal development when testicular temperatures are subject to dramatic changes.

ACKNOWLEDGMENT

This work was supported by a grant from the National Institute of Health, HD 13019.

REFERENCES

1. Moyle, W.R., Jungas, R.L., and Greep, R.O. (1973) Biochem. J. 134, 415–424.
2. Bartke, A., Musto, N., Caldwell, B.V., and Behrman, H.R. (1973) Prostaglandins 3, 97–104.
3. Saksena, S.K., Lau, I.F., and Bartke, A. (1974) Endocrinology 95, 311–341.
4. Johnson, A.D. (1970) in The Testis, Johnson, A.D., Gomez, W.R., and Vandermark, H.L., eds. Vol. II, pp. 233–234, Academic Press, New York, NY.
5. Nakamura, M., and Hall, P. (1980) J. Biol. Chem. 255, 2907–2913.

6. Johnson, A.D. (1970), in The Testis, Johnson, A.D., Gomez, W.R., and Vandermark, H.L., eds. Vol. II, pp. 193–xxx, Academic Press, New York, NY.
7. Hagenas, L., Ritzen, E.J., Svensson, J., Hansson, V., and Purvis, K. (1978) Int. J. Androl. Suppl. 2, 449–458.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
9. Deykin, D., and Goodman, D.S. (1962) J. Biol. Chem. 237, 3649–3656.
10. McGovern, R.F., and Quackenbush, F.W. (1973) Lipids 8, 473–478.
11. Hagenas, L., Ritzen, E.M., and Suginami, H. (1978) Int. J. Androl. 1, 477–484.
12. Gallo, L.L. (1981) Methods Enzymol. 71, 665–674.
13. Perlman, P.L. (1950) Endocrinology 46, 341–348.

[Received March 29, 1982]

Distribution of Methylene and Nonmethylene-Interrupted Dienoic Fatty Acids in Polar Lipids and Triacylglycerols of Selected Tissues of the Hardshell Clam (*Mercenaria mercenaria*)

J. SCOTT KLINGENSMITH, *Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS 39216*

ABSTRACT

Fatty acid profiles of polar lipids and triacylglycerols were determined for 6 tissues of the hardshell clam *(Mercenaria mercenaria)*, namely, mantle, gill, mouth, foot, digestive tract/gonadal tissue and adductor muscle. The largest concentrations of nonmethylene-interrupted dienoic (NMID) fatty acids were found in the gill, mantle, and foot. Structural analyses were undertaken to determine the double bond configurations of the various NMID isomers. The major 22C NMID species were Δ7,13- and Δ7,15-docosadienoic acid. The major 20C NMID species were Δ7,11- and Δ7,13-eicosadienoic acid and Δ5,11-eicosadienoic acid.

Lipids 17:976-981, 1982.

INTRODUCTION

Nonmethylene-interrupted dienoic (NMID) fatty acids have been described in a variety of plant and animal species (1-12). The reports concerning the presence of NMID fatty acids in marine organisms have primarily been derived from analyses of lipids either extracted from whole organisms or from a single tissue. Due to an interest in the functional significance of the NMID fatty acids in marine invertebrate lipids, the distribution of NMID fatty acids among tissues of the hardshell clam *(Mercenaria mercenaria)*, and between the polar lipids and triacylglycerols of those tissues, were determined. In addition, structural analyses were undertaken in an effort to determine the homology of structure existing between the two chain lengths of NMID fatty acids previously reported for the hardshell clam *(M. mercenaria)*, the 20C and 22C isomeric species (13).

EXPERIMENTAL PROCEDURES

Isolation of Lipid Classes

Mercenaria mercenaria of commercial chowder size (n = 44) were collected from the local estuarine area of Charleston, South Carolina on April 11, 1977, and transported immediately to the laboratory. The tissues of 40 clams were excised, pooled, weighed, lyophilized, and extracted with chloroform-methanol (14). The lipids of tissue from 4 whole clams were also extracted at this time and pooled. Lipids were separated into individual classes using thin layer chromatography (TLC) and a solvent system containing petroleum ether/diethyl ether/acetic acid (50:30:05). Following development, the TLC plates were sprayed with a solution of dichlorofluorescein (Eastman Kodak, Rochester, NY) in ethanol (0.1% w/v) in order to visualize the lipid classes with ultraviolet (UV) light. The areas of silica gel containing the lipid classes of interest, namely, polar lipids and triacylglycerols, were scraped from the plate into glass tubes and extracted 3 times with 4 ml of chloroform/methanol (1:1) to which one drop of water was added. The silica gel was removed by centrifugation. The combined extracts were evaporated to dryness under a fine stream of nitrogen, weighed, redissolved in a minimal amount of chloroform, and stored at −20C.

Fatty Acid Methyl Esters

Fatty acid methyl esters were prepared by placing an aliquot of the sample in a Teflon-lined screw-cap vial, adding a suitable amount of 3% methanolic H_2SO_4 and heating for 2 hr at 60C. The methyl esters were recovered from the reaction mixture by the addition of one drop of water and extraction with petroleum ether. Fatty acid methyl esters from the polar lipids and triacylglycerols of each of the tissues

as well as from the whole organism were analyzed on a Hewlett-Packard 5711A gas chromatograph (Hewlett-Packard, Lexington, MA) fitted with a 50 m X 0.24 mm id wall-coated open tubular stainless steel column coated with SILAR-5CP (Perkin-Elmer Corp., Norwalk, CT). The gas chromatograph was equipped with a flame ionization detector. Relative peak areas were determined with a CRC-110 digital integrator (Columbia Scientific Corp., Austin, TX). Tentative identification of component fatty acids was achieved by plotting the log of the relative retention times against carbon number with the aid of authentic standard mixtures containing the following fatty acid methyl esters: iso-14:0, 14:0, anteiso-15:0, 15:0, iso-16:0, 16:0, anteiso-17:0, 18:0, 18:1ω9, 18:2ω6 and 18:3ω3 (Supelco Inc., Bellefonte, PA).

NMID Isolation

Preliminary isolation of the NMID fatty acids was achieved by silver-ion thin layer chromatography using TLC plates coated with 0.26 mm of Supelcosil 12A (Supelco) containing 5% $AgNO_3$. After coating, plates were heated at 100C for 30 min. Relative humidity was an important variable in these silver-ion chromatographic analyses, and it was found necessary to expose the plates to an atmosphere containing a high relative humidity prior to their use. This was accomplished by placing the plates overnight in a large, covered aquarium in which a tray of water was placed. The samples were then applied and the plates were developed in chloroform at 5C until the solvent front was within 1 cm of the upper edge of the TLC plates. The chloroform was evaporated from the plates, the plates were sprayed with 0.1% dichlorofluorescein solution and the silica gel containing the NMID fatty acids was scraped from the plates and extracted 3 times with 4-ml chloroform/methanol (1:1) to which one drop of water was added. The silica gel was removed by centrifugation and the combined extracts were evaporated to dryness under a fine stream of nitrogen, taken up in petroleum ether and quantitatively transferred to a screw-cap vial. Isolation of individual NMID chain lengths was accomplished by preparative gas chromatography on a Perkin-Elmer 801 gas chromatograph, equipped with a flame-ionization detector and fitted with a 2 m X 3 mm id glass column packed with 10% Apiezon L on 80/100 mesh Gas Chrom P. Confirmatory chromatograms were obtained for each chain length on a 50 m X 0.24 mm id SCOT stainless steel column coated with SILAR 5CP (Perkin-Elmer Corp., Norwalk, CT) to ensure that only a single chain length was present in each fraction. Each chain length was subjected to reductive ozonolysis according to the method of Stein and Nicolaides (15). The fatty acids were dissolved in methylene chloride (Fisher Scientific Co.). Ozone, generated by an ozone generator (Supelco), was bubbled through the solution for 2 min at room temperature. The ozonides were reduced by adding a few crystals of triphenylphosphine (Supelco). The ozonolysis products were analyzed on a Beckman GC-45 gas chromatograph (Beckman Instruments, Inc., Atlanta, GA) equipped with a flame ionization detector and fitted with a 2 m X 2 mm glass column packed with 8% OV101 on 80/100 mesh Gas Chrom P. The identity of the ozonolysis products was determined by subjecting individual authentic standard fatty acids to the reductive ozonolysis procedure, followed by gas chromatograph analysis as described above. All solvents utilized were ACS grade (Fisher Scientific Co.) and redistilled in glass.

RESULTS

Fatty Acid Profiles

The fatty acid profiles of the hardshell clam polar lipids are shown in Table 1. The profile derived from the digestive tract/gonadal tissue bears a resemblance to that derived from the polar lipids of the whole organism, with high levels of 16:0, 18:0, 20:5ω3 and 22:6ω3, and a modest amount (5%) of 22C NMID. The profiles for the remaining tissues vary considerably in terms of levels of various fatty acids and degree of unsaturation. However, the most important characteristic of these fatty acid profiles is the wide range of 22C NMID levels present among the various tissues. Of particular interest are the high levels of 22C NMID in the mantle (15.1%), foot (13.1%) and gill (19.1%). The percentage composition of 22C NMID present in these profiles is the highest reported in the literature, with the exception of the marine sponge, *Microciona prolifera* (7).

In contrast, the fatty acid profiles of the triacylglycerol fraction from various tissues are quite different from that of the polar lipids (Table 2). Triacylglycerol lipids generally possessed greater levels of 14:0 and 16:0 fatty acids and lower levels of 22C polyunsaturated fatty acids. Particularly striking are the variations in the gill fatty acids. Among the polar lipid fatty acids of the gill, 16:0 and 18:0 account for 42% of the total fatty acids, while polyunsaturated fatty acids account for 27% of the total. In the triacylglycerol lipids of the gill,

TABLE 1

Principal Fatty Acids of Polar Lipids Isolated from the Whole Animal and Specific Clam Tissues

Fatty acids[a]	Whole animal tissue[b]	Digestive tract gonads	Mouth	Adductor	Mantle	Foot	Gill
14:0	3.1 ± 0.4	0.6 ± 0.0	7.9 ± 0.9	0.4 ± 0.0	0.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
16:0	33.1 ± 0.5	26.8 ± 1.6	10.6 ± 0.3	32.4 ± 4.7	18.9 ± 0.2	22.0 ± 0.7	26.3 ± 1.4
16.25[c]	T[d]	3.1 ± 1.1	6.3 ± 0.6	1.1 ± 0.5	2.9 ± 0.5	1.5 ± 0.1	1.0 ± 0.2
16:1ω9	5.6 ± 0.1	1.8 ± 0.3	1.2 ± 0.1	2.2 ± 0.5	1.7 ± 0.8	3.5 ± 0.1	3.1 ± 0.3
16:1ω7	1.7 ± 0.0	1.6 ± 0.1	0.6 ± 0.1	2.4 ± 0.4	2.5 ± 0.5	1.9 ± 0.5	1.8 ± 0.4
17:0	2.5 ± 0.9	1.1 ± 0.4	0.7 ± 0.1	2.4 ± 0.9	4.2 ± 0.4	3.8 ± 0.2	5.0 ± 0.2
17.71[c]	2.1 ± 0.7	4.5 ± 2.1	3.3 ± 0.3	7.4 ± 1.5	10.6 ± 1.5	7.3 ± 0.8	9.6 ± 0.4
18:0	7.7 ± 0.1	8.4 ± 0.2	2.3 ± 0.6	11.5 ± 0.5	13.6 ± 0.1	13.0 ± 0.2	15.2 ± 0.8
18:1ω9	2.1 ± 0.0	1.3 ± 0.1	5.3 ± 0.1	5.5 ± 0.7	1.5 ± 0.3	2.9 ± 0.9	0.6 ± 0.1
18:1ω7	4.9 ± 0.1	2.5 ± 0.1	1.6 ± 0.1	1.1 ± 0.3	0.7 ± 0.2	1.4 ± 0.1	0.9 ± 0.1
18:4ω3	1.6 ± 0.0	0.7 ± 0.1	1.1 ± 0.1	0.7 ± 0.3	0.8 ± 0.6	0.3 ± 0.0	T[d]
20:1ω13	1.9 ± 0.0	2.0 ± 0.0	0.3 ± 0.1	1.4 ± 0.2	4.4 ± 0.1	3.0 ± 0.1	7.8 ± 1.8
20:1ω9	1.1 ± 0.0	1.1 ± 0.1	2.8 ± 0.3	0.8 ± 1.0	1.3 ± 0.1	1.1 ± 0.1	2.0 ± 0.4
20:1ω7	2.1 ± 0.5	2.1 ± 0.0	0.8 ± 0.1	1.7 ± 0.2	2.6 ± 0.1	2.6 ± 0.0	3.6 ± 0.8
20C NMID[e]	T[d]	T[d]	T[d]	T[d]	T[d]	T[d]	T[d]
20:2ω6	1.1 ± 0.0	1.1 ± 0.0	1.7 ± 0.1	3.0 ± 2.3	1.0 ± 0.0	0.8 ± 0.0	1.1 ± 0.3
20:4ω6	2.3 ± 0.0	2.6 ± 0.0	1.2 ± 0.0	1.9 ± 0.2	2.9 ± 0.3	2.7 ± 0.1	0.4 ± 0.1
20:5ω3	12.0 ± 0.2	12.2 ± 0.3	4.4 ± 0.5	4.0 ± 0.5	2.4 ± 0.3	3.4 ± 0.1	0.3 ± 0.0
22C NMID[f]	2.9 ± 0.2	5.0 ± 0.3	6.9 ± 0.1	5.2 ± 0.9	15.1 ± 0.3	13.1 ± 0.1	19.1 ± 0.6
23.25[c]	0.4 ± 0.1	1.1 ± 0.2	10.7 ± 0.7	0.7 ± 0.2	0.7 ± 0.3	1.0 ± 0.0	0.5 ± 0.3
22:4ω6	0.7 ± 0.1	1.3 ± 0.1	4.8 ± 1.4	2.5 ± 0.3	3.4 ± 0.1	3.0 ± 0.1	0.6 ± 0.2
22:5ω6	0.2 ± 0.1	1.2 ± 0.1	2.8 ± 0.2	1.2 ± 0.2	1.8 ± 0.1	1.7 ± 0.0	0.2 ± 0.1
22:5ω3	0.9 ± 0.2	2.2 ± 0.1	3.0 ± 0.4	2.1 ± 0.3	1.3 ± 0.1	1.5 ± 0.1	0.3 ± 0.1
22:6ω3	9.6 ± 0.3	16.3 ± 0.1	18.7 ± 0.7	9.0 ± 0.9	6.9 ± 0.3	8.7 ± 0.2	0.7 ± 0.3

[a]All analyses were performed in triplicate on a pooled sample of 40 tissues. Values cited are relative percentage weight compositions.
[b]Klingensmith (16).
[c]ECL = equivalent chain length.
[d]Trace.
[e]Represents total of all 20C NMID isomers present.
[f]Represents total of all 22C NMID isomers present.
Other fatty acids identified include 12:0, iso-14:0, ante-iso 15:0, 15:0, iso-16:0, ante-iso 17:0, 18:2ω6, 18:3ω3, 19:0, 20:0, 20:3ω3, and 20:2ω6. All of these components were present in only trace amounts (> 0.2%).

16:0 and 18:0 comprise 61% of the total fatty acids present, while polyunsaturates account for only 2.8% of the total. The NMID fatty acid levels are uniformly low and similar to those of the whole organism.

Structural Determinations

Structural determinations made by reductive ozonolysis show that the predominant isomer for the 22C NMID fatty acid is Δ7,15-22:2, with minor amounts of Δ7,13-22:2 (Table 3). This is consistent with previous identifications made for 22C NMID fatty acids from marine species (13,17,18). The Δ7,15-22:2 NMID is the major 22C isomer in all of the tissues, in both the polar lipid and triacylglycerol fractions. However, the ratio of the Δ7,15 and Δ7,13 isomers is different between the two fractions. In the polar lipids, the Δ7,15-22:2 NMID fatty acid ranges anywhere from 4–20 times the level of the Δ7,13-22:2 isomer. In the triacylglycerol fraction, this ratio is lower and shows much less variation, with the Δ7,15 isomer averaging about 5 times that of the Δ7,13 isomer (data not shown).

The 20C NMID fatty acids were not reported in the fatty acid methyl ester profiles of each tissue because they were always present in only trace amounts. Nevertheless, in hopes of demonstrating a degree of homology between other NMID fatty acids found in the tissues, an attempt to determine their structure was made using the polar lipids of the gill as the source of the 20C NMID fatty acids.

Gas chromatographic data revealed that there are at least 3 isomers comprising the 20C NMID fatty acids of the hardshell clam gill. The information derived from reductive ozonoloysis indicates that the major isomer is Δ7,13-20:2, with smaller amounts of Δ7,11-20:2 and Δ5,11-20:2 (Table 3).

DISCUSSION

The discovery of the NMID fatty acids in

TABLE 2

Fatty Acids of Triacylglycerol Lipids Isolated from the Whole Animal and Specific Clam Tissues

Fatty acids[a]	Whole animal tissue[b]	Digestive tract gonads	Mouth	Adductor	Mantle	Foot	Gill
14:0	3.3 ± 0.3	3.2 ± 0.2	5.9 ± 0.1	2.5 ± 0.0	2.8 ± 0.1	2.7 ± 0.0	1.5 ± 0.0
16:0	29.3 ± 0.5	32.3 ± 5.9	32.9 ± 0.1	35.9 ± 0.3	32.3 ± 0.5	30.6 ± 0.4	44.9 ± 0.2
16:1ω9	6.5 ± 0.2	7.9 ± 1.7	3.1 ± 0.0	2.1 ± 0.0	1.6 ± 0.0	3.9 ± 0.0	4.2 ± 0.1
16:1ω7	1.9 ± 0.1	1.8 ± 0.2	1.7 ± 0.0	1.6 ± 0.0	1.6 ± 0.6	1.7 ± 0.0	2.4 ± 0.0
17:0[c]	1.1 ± 0.1	2.2 ± 0.6	T[d]	T[d]	T[d]	T[d]	T[d]
18:0	7.8 ± 0.2	6.6 ± 1.1	14.1 ± 0.2	13.9 ± 0.9	9.1 ± 0.1	7.7 ± 0.1	16.3 ± 0.1
18:1ω9	1.4 ± 0.0	2.1 ± 0.1	10.8 ± 0.0	2.2 ± 0.0	1.9 ± 0.1	1.5 ± 0.1	1.7 ± 0.0
18:1ω7	5.2 ± 0.3	5.3 ± 0.4	1.5 ± 0.2	5.4 ± 6.0	8.3 ± 0.1	8.2 ± 0.3	6.3 ± 0.1
18:4ω3	1.7 ± 0.1	1.8 ± 0.2	T[d]	0.7 ± 0.1	0.4 ± 0.0	0.7 ± 0.1	T[d]
20:1ω13	1.8 ± 0.0	1.9 ± 0.1	2.5 ± 0.1	2.8 ± 0.1	3.4 ± 0.1	2.6 ± 0.1	2.0 ± 0.0
20:1ω9	1.8 ± 0.0	1.0 ± 0.1	1.3 ± 0.1	2.8 ± 0.1	3.1 ± 0.1	2.6 ± 0.2	5.5 ± 0.0
20:1ω7	2.9 ± 0.6	2.2 ± 0.2	2.1 ± 0.0	4.3 ± 0.1	5.0 ± 0.1	4.3 ± 0.1	7.3 ± 0.0
20C NMID[e]	T[d]	T[d]	T[d]	T[d]	T[d]	T[d]	T[d]
20:2ω6	1.2 ± 0.4	1.1 ± 0.1	0.6 ± 0.0	1.2 ± 0.0	1.7 ± 0.1	1.4 ± 0.1	T[d]
20:4ω6	3.5 ± 0.2	1.4 ± 0.2	5.4 ± 0.1	4.2 ± 0.1	4.2 ± 0.3	5.2 ± 0.1	T[d]
20:5ω3	15.6 ± 0.3	12.4 ± 0.1	5.1 ± 0.1	3.3 ± 0.0	5.9 ± 0.2	9.5 ± 0.2	T[d]
22C NMID[f]	2.1 ± 0.1	1.8 ± 0.4	1.9 ± 0.1	4.5 ± 0.1	5.4 ± 0.4	2.7 ± 0.1	2.8 ± 0.2
23.25[c]	0.4 ± 0.1	0.8 ± 0.1	T[d]	T[d]	T[d]	T[d]	T[d]
22:4ω6	0.7 ± 0.2	0.3 ± 0.1	0.8 ± 0.0	1.3 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	T[d]
22:5ω6	0.4 ± 0.6	0.3 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.7 ± 0.0	T[d]
22:5ω3	0.6 ± 0.1	0.8 ± 0.2	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	T[d]
22:6ω3	8.9 ± 0.4	9.5 ± 0.8	5.4 ± 0.1	4.7 ± 0.2	4.4 ± 0.3	9.4 ± 0.5	T[d]

[a]All analyses were performed in triplicate on a pooled sample of 40 tissues. Values cited are relative percentage weight compositions.
[b]Klingensmith (16).
[c]ECL = equivalent chain length.
[d]Trace.
[e]Represents total of all 20C NMID isomers present.
[f]Represents total of all 22C NMID isomers present.
Other fatty acids identified include 12:0, iso-14:0, ante-iso 15:0, 15:0, iso-16:0, ante-iso 17:0, 18:2ω6, 18:3ω3, 19:0, 20:0, 20:3ω3, and 20:2ω6. All of these components were present in only trace amounts (> 0.2%).

the tissues of various organisms has naturally led to speculation on their origin and their physiological function. It has been suggested that perhaps the NMID fatty acids in higher marine organisms are essentially biochemically inert, and that the levels reported thus far represent primarily food web effects (13). This hypothesis is based on the occurrence of NMID in depot fats of the turtle, starfish and sturgeon and the lack of such NMID in the oils of most pelagic species of fish (13). In addition, the presence of NMID fatty acids in the lipids of most molluscs (13), which are primarily filter feeders, and the absence of suitable precursors for chain elongation to the observed chain lengths, are also cited (13), suggesting that NMID fatty acids in molluscs are derived almost exclusively from food sources. Furthermore, studies by Johns et al. (17) demonstrated that levels of NMID fatty acids in several Australian species of molluscs, especially the limpet, *Cellana tramoserica*, show a correlation with the levels of NMID fatty acids present in the particular algal diet of a given site.

However, the present study indicates a highly variable level of the 22C NMID fatty acids in specific tissues of the hardshell clam, as well as in specific lipid classes. Similarly, Johns et al. (17) reported an uneven tissue distribution of the 22C NMID in the sponge, *Ponerplex costata*, with the highest level in the gill. These findings imply that the 22C NMID fatty acids are not simply accumulating in the organism.

Conversely, in the common sea urchin, *Strongylocentrotus droebachiensis*, it has been shown by Takagi et al. (19) that, while individuals displayed considerable variation in total lipid and lipid classes, there was nonetheless a consistent distribution of 20C NMID fatty acids with 5,6-ethylenic unsaturation, regardless of the nutritional status of the animal. Takagi et al. pointed out that a likely precursor to the 20C NMID, Δ5-20:1 is reported to be absent from local marine seaweeds and marine microorganisms (19), which suggests an endogenous origin of the 20C NMID fatty acids in *S. droebachiensis*.

TABLE 3

Percent Composition, ECL Values, and Ozonolysis Products of Nonmethylene-Interrupted Dienoic Fatty Acids From the Polar Lipid Fraction of Clam Gill Lipids

Structure	ECL[a]	Percent composition[a]	Ozonolysis products[b]	
			Aldehydes	Aldesters
		20:2C NMID		
Δ7,11-20:2	20.13	9.8	C_7 72.6	C_5 12.0
Δ7,13-20:2	20.17	78.6	C_9 27.4	C_7 88.0
Δ5,13-20:2	20.21	13.3		
		22:2C NMID		
Δ7,13-22:2	22.47	4.8	C_7 95.2	C_5 1.7
Δ7,15-22:2	22.50	95.2	C_9 4.8	C_7 98.3

[a]ECL values and percent composition determined on SILAR 5CP as described in Methods.
[b]Mole percent.

Indeed, direct evidence for the endogenous origin of an NMID fatty acid has been reported. Morales and Litchfield (20) have shown that the marine sponge, *Microciona prolifera*, actively synthesized the two 26C NMID fatty acids reported to be present in large amounts (45-52%) in this sponge (7). It will be interesting to see if similar results can be obtained from other marine organisms, especially molluscs.

If the 22C NMID fatty acids are synthesized in the clam by a process of chain elongation, the origin of the major 22C NMID isomer, Δ7,15-22:2 is unclear since the only homologous precursor of the 22C NMID fatty acid isomers, namely, Δ5,11-20:2, was present in only trace amounts. Pearce and Stillway (12) have suggested that biosynthesis of the 22C NMID might occur via a Δ7 desaturase acting upon the corresponding monoenoate species of the same chain length which already possess a double bond distal to the Δ7 position. Similarly, Takagi et al. (19) postulate that 20C NMID fatty acids which possess a 5,6-ethylenic bond might arise from a Δ5 desaturase. Further work is needed to confirm these hypotheses.

If a marine invertebrate such as the hardshell clam can regulate tissue levels of NMID by such factors as rate of synthesis, rate of incorporation, or intrinsic resistance of the NMID fatty acid to degradation, the question arises as to what is the purpose of this manipulation. It has been suggested that, since such NMID fatty acids occur in the outer membrane of molluscs, their presence confers a measure of resistance against attack by microbial lipases (9). It is certainly possible that the anomalous NMID unsaturation pattern might prevent or at least impede the hydrolysis of phospholipids in the membrance. Lipases from a variety of sources have been shown to be highly specific relative to their substrate requirements (21). In addition, it has been noted that the NMID components of a fatty acid profile increase under nonfeeding conditions, or in diets lacking in polyunsaturates (13). This response could be an obligatory one, metabolically, or it could be that there are actually positive advantages for the clam in pursuing this metabolic scheme.

However, with such a narrow data base, one must be cautious in assigning broadly generalized roles for nonmethylene-interrupted unsaturation in marine organisms. It has been reported by Morales and Litchfield (20) that in sponges phosphatidylcholine is predominantly located in the surface of the outer cell membrane. However, the 26C NMID and NMIT are almost completely absent in the phosphatidylcholine fraction, which led the authors to suggest metabolic roles for these particular nonmethylene-interrupted unsaturation in the marine sponge. On the other hand, in the hardshell clam, high amounts of NMID were found in the polar lipids, implying a structural role for the NMID in the membrane. The consistency of the 20C NMID distribution in *S. droebachiensis*, irrespective of nutritional status, lead Takagi to suggest a basic role for these components in membrane function. Such a role could be extremely important, for the degree of unsaturation, geometric configuration, and chain length of fatty acids have profound effects on membrane properties such as transition temperature, molecular mobility and activity of membrane bound proteins.

The inverse relationship between the level of 22C NMID and the level of polyunsaturates in the polar lipid fraction, as shown in Figure 1, is particularly interesting, because it suggests that a competitive incorporation rate may exist between the 22C NMID and the two major polyunsaturates generally found in marine organisms, 20:5ω3 and 22:6ω3. However,

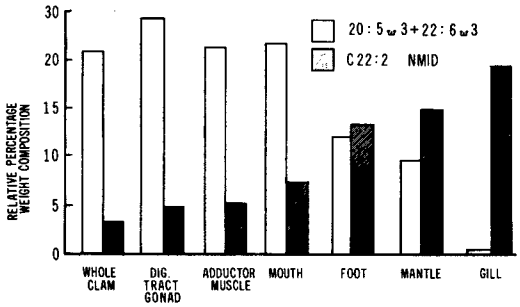


FIG. 1. Inverse relationship between the amount of eicosapentaenoic and docosahexaenoic acids and the 22C NMID fatty acids in the polar lipids of the hardshell clam. Levels are expressed in terms of relative percentage by weight of total fatty acids.

since this relationship is based on relative levels of the components, one must be careful in assigning any significance to it. Unfortunately, the physiochemical properties of NMID fatty acids and their effects on membranes are not known. It was noted in the course of this study that the NMID fatty acids appeared to be more resistant to oxidation than other polyunsaturates, particularly eicospentaenoic and docosahexaenoic acids. This was only a qualitative observation, but it suggests the possibility that such resistance may be important in mulluscan tissues exposed most often to the external environment and may supply a partial explanation for the large amounts of NMID found in the lipids of the gill, mantle, and foot of the clam.

In conclusion, in this report, a dramatic variation in levels of a 22C NMID fatty acid among tissues and within lipid classes of the hardshell clam is demonstrated. The gill, mantle, and foot in particular possess extremely high levels of this fatty acid in the polar lipid fraction. Of interest is the apparent inverse relationship between degree of normal, methylene-interrupted polyunsaturation and the presence of 22C NMID. The findings of this study, although suggestive of an endogenous origin and perhaps an important role in clam polar lipids for NMID fatty acids, emphasize the need for extensive investigation into the biosynthesis and physiochemical properties of the NMID fatty acids before their role in the lipid metabolism of marine invertebrates can be accurately assessed.

ACKNOWLEDGMENTS

This work was supported in part by research funds from the Medical University of South Carolina. The author wishes to thank Dr. Lewis Stillway for his many helpful suggestions and comments and Ms. Jewel Harper for her help in the preparation of this manuscript.

REFERENCES

1. Hoffman, G., and Meijboom, P.W. (1969) J. Am. Oil Chem. Soc. 46, 620-626.
2. Murawski, U., Egge, H., Gyorgy, P., and Zilliken, F. (1971) FEBS Lett. 18, 290-292.
3. Fore, S.P., Dollear, F.G., and Sumrel, G. (1966) Lipids 1, 73-75.
4. Smith, C.R., Freidinger, R.M., Hagemann, J.W., Spencer, G.F., and Wolff, I.A. (1969) Lipids 4, 462-465.
5. Plattner, R.D., Spencer, G.E., and Kleiman, R. (1975) Lipids 10, 413-416.
6. Madrigal, R.W., and Smith, C.R. (1975) Lipids 10, 502-504.
7. Jefferts, E., Morales, R.W., and Litchfield, C. (1974) Lipids 9, 244-247.
8. Jamieson, G.R., and Reid, E.H. (1972) Phytochemistry 11, 269-275.
9. Ackman, R.G., and Hooper, S.N. (1973) Comp. Biochem. Physiol, 46B, 153-165.
10. Paradis, M., and Ackman, R.G. (1975) Lipids 10, 12-16.
11. Murawshi, U., and Egge, H. (1975) J. Chromatogr. Sci. 13, 497-505.
12. Pearce, R.E., and Stillway, L.W. (1977) Lipids 12, 544-549.
13. Paradis, M., and Ackman, R.G. (1976) Lipids 12, 170-176.
14. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
15. Stein, R.A., and Nicolaides, N. (1952) J. Lipid Res. 3, 476-478.
16. Klingensmith, J.S. (1979) M.S. Thesis, Medical University of South Carolina, Charleston, pp. 9-12.
17. Johns, R.B., Nicholas, P.D., and Perry, G.J. (1980) Comp. Biochem. Physiol. 65B, 207-214.
18. Ackman, R.G., and McLachlan, J. (1977) Proc. N.S. Inst. Sci. 28, 47-64.
19. Takagi, T., Eaton, C.A., and Ackman, R.G. (1980) Can. J. Fish. Aq. Sci. 37, 195-202.
20. Morales, R.W., and Litchfield, C. (1977) Lipids 12, 570-576.
21. Patton, J.S., and Quinn, J.G. (1973) Mar. Biol. 21, 59-69.

[Revision received August 24, 1982]

METHODS

Synthesis of Deuterium Labeled Cholesterol and Steroids and Their Use for Metabolic Studies[1]

L.J. GOAD[a], M.A. BREEN[a], N.B. RENDELL[a], M.E. ROSE[a], J.N. DUNCAN[b] and A.P. WADE[b], [a]*Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool, L69 3BX, United Kingdom, and* [b]*Department of Endocrine Pathology, Alder Hey Childrens' Hospital, Liverpool, United Kingdom*

ABSTRACT

A simple method is described for the preparation of [6,7,7-$^2H_3$]sterols and steroids. The synthesis starts with a Δ^5-sterol or steroid and involves preparation of the 6-oxo-3α,5α-cyclosteroid, base exchange in the presence of deuterium oxide to introduce two deuteriums at the C-7 position and sodium borodeuteride reduction of the 6-oxo group to introduce the third deuterium atom at C-6. Rearrangement of the [6,7,7-$^2H_3$]6α-hydroxy-3α,5α-cyclosteroid then gives the desired [6,7,7-$^2H_3$]-Δ^5 sterol or steroid. [6,7,7-$^2H_3$]Cholesterol, [6,7,7-$^2H_3$]pregnenolone and [6,7,7-$^2H_3$]3β-hydroxyandrost-5-en-17-one were synthesized in this fashion and [6,7,7-$^2H_3$]progesterone was prepared from the [6,7,7-$^2H_3$]pregnenolone. Three examples of the use of these deuterium labeled compounds for metabolic studies are described. The metabolites were identified by gas chromatography-mass spectrometry. The chrysophyte alga, *Ochromonas malhamensis,* was shown to be capable of introducing an extra methyl or ethyl group at C-24 of the side chain of [6,7,7-$^2H_3$]cholesterol to yield brassicasterol and poriferasterol, respectively. The ovary of the echinoderm, *Asterias rubens,* was demonstrated to metabolize [6,7,7-$^2H_3$]progesterone to yield mainly the 5α-isomers of pregnane-3,20-dione and 3β-hydroxypregnan-20-one. However, the 5β-isomers of these compounds were also detected as minor products for the first time as progesterone metabolites in this animal. Isolated oocytes of the frog, *Xenopus laevis,* produced a number of metabolites of [6,7,7-$^2H_3$]progesterone. In this report, two of them were shown to be 17α-hydroxy-pregn-4-en-3,20-dione and 20α-hydroxypregn-4-en-3-one.
Lipids 17:982-991, 1982.

Investigations on sterol and steroid hormone metabolism have conventionally used radioactively labeled precursors. Metabolites, or suitable derivatives, are then identified on the basis of their chromatographic properties and their cocrystallization with added authentic carrier compound to constant specific activity. These are well established and are generally reliable criteria for product identification. However, in steroid research, the identification of a radioactive metabolite by these methods is not always certain because of problems such as cochromatography and cocrystallization (1). Another factor which may, in some circumstances, hinder steroid metabolite identification is the unavailability or high cost of authentic compounds in the amounts required for addition to labeled metabolites prior to derivative formation and crystallization to constant specific activity. Also, some studies on human steroid metabolism may be prevented due to ethical objections to the use of carbon-14 or tritium labeled steroids.

For the above reasons, we decided to investigate the use of deuterium labeled sterols and steroids for metabolic studies with identification of products by gas chromatography-mass spectrometry (GC-MS). This technique has previously been adopted to study, for example, prostaglandin and drug metabolism (2) and its application to steroid metabolic studies has been described (3-9). Numerous methods (7,10,11) have been developed to introduce deuterium atoms into specific positions in the steroid molecule and such compounds have been employed to investigate mass spectral fragmentation processes and for steroid quantification and metabolic studies (7).

The methods described in the literature for the deuterium labeling of steroids are often multistep processes with relatively poor overall yields and sometimes employing expensive starting materials or reagents. Our approach has been to develop a facile method for deuterium labeling using readily available starting compounds and reactions which give a reasonable yield of product.

MATERIALS AND METHODS

Methods were generally as described previously (12,13). Mass spectra were determined

[1]**Presented at the "Sterol Symposium" of the American Oil Chemists' Annual International Conference, New Orleans, LA, May 1981.**

on a VG70-70F instrument coupled to a Finnigan Incos data system. ^1H NMR spectra were determined at 220MHz in $CDCl_3$ solution with TMS as internal standard.

Synthesis of [6,7,7-$^2H_3$] Cholesterol

Using the methods described previously (12,13), cholesterol *(1,* 5.0 g) was converted into 3α,5α-cyclocholestan-6β-ol *(2,* 4.22 g), pure by thin layer chromatography (TLC) and gas liquid chromatography (GLC) and with ^1H NMR and mass spectra in agreement with the literature data (13). Oxidation of *2* (2.46 g) with Jones reagent (13) followed by column chromatography on alumina, Brockman grade 3, eluted with diethyl ether-petrol mixtures gave 3α,5α-cyclocholestan-6-one *(3,* 1.48 g), pure by TLC and GLC, ^1H NMR and mass spectra in accord with previous data (13).

3α,5α-Cyclocholestan-6-one (*3,* 150 mg) was dissolved in 40 ml 1,4-dioxan and 400 mg sodium methoxide in 2 ml deuterium oxide was added. After refluxing for 1.5 hr, water (40 ml) was added and the mixture was extracted with diethyl ether (3 × 50 ml). The ether extract was washed with water, dried over anhydrous sodium sulphate, and evaporated to leave [7,7-$^2H_2$] 3α,5α-cyclocholestan-6-one *(4,* 150 mg). Electron impact mass spectrometry (EIMS) *m/z* (rel int): 386[M]$^+$(100), 371[M-CH_3]$^+$(20), 358(15), 273(5), 245(6), 232(6), 231(10), 217(4), 138(96), 231(10), 217(4), 138(96), 123(45), 121(60).

Sodium borodeuteride (152 mg) was added to a solution of *4* (150mg) dissolved in 15 ml tetrahydrofuran-ethanol (1:1). After stirring for 4 hr at room temperature, water was added and the product extracted into diethyl ether. Usual work up gave [6,7,7-$^2H_3$] 3α,5α-cyclocholestan-6α-ol *(5,* 141 mg). R_f 0.45; RR_t 0.71; EIMS *m/z* (rel int): 389[M]$^+$ (12), 374[M-CH_3]$^+$ (55), 371[M-H_2O]$^+$ (28), 356[M-CH_3-H_2O]$^+$ (14), 334(36), 258(9), 247(11), 234(12), 216(12).

[6,7,7-$^2H_3$] 3α,5α-Cyclocholestan-6α-ol (*5,* 140 mg) was dissolved in 25 mL 1,4-dioxan containing 25 μl conc H_2SO_4 and the mixture held at 60C for 1 hr. Water (25 ml) was then added and the product extracted with petrol (3 x 50 ml). Usual workup and purification by chromatography on alumina, Brockman grade 3, eluted with diethyl ether-petrol mixtures, gave [6,7,7-$^2H_3$] cholest-5-en-3β-ol *(6,* 115mg). R_f 0.20; RR_t 1.00; EIMS *m/z* (rel int): 389[M]$^+$ (41), 374[M-CH_3]$^+$ (18), 371[M-H_2O]$^+$ (24), 356[M-H_2O-CH_3]$^+$ (20), 303(14), 275(59), 258(13), 234(10), 233(10), 216(18), 215(17); ^1H NMR($CDCl_3$): δ 0.67 (*s,* H-18), 0.87 (*d,* H-26 and H-27), 0.90 (*d,* H-21), 1.00 (*s,* H-19), 3.5 (*brm* H-3α).

Synthesis of [6,7,7-$^2H_3$] Pregnenolone

Pregnenolone (3β-hydroxypregn-5-en-20-one, *7)* was first converted by treatment with ethylene glycol (14) into 3β-hydroxypregn-5-en-20-ethylene ketal *(8);* EIMS *m/z* (rel int): 360[M]$^+$ not detected, 345 [M-CH_3]$^+$ (2), 316(6), 298(7), 283(5), 213(3), 87(100).

3β-Hydroxypregn-5-en-20-ethylene ketal *(8,* 0.58 g) was dissolved in pyridine (10 ml) containing *p*-toluenesulphonyl chloride (1.25 g) and the mixture left at room temp for 18 hr. Water was added and, after diethyl ether extraction and the usual workup procedure, 3β-tosyl-pregn-5-en-20-ethylene ketal (0.7 g) was obtained. R_f 0.77; EIMS *m/z* (rel int): 342 (1), 327(1), 298(1), 87(100). The 3β-tosyl-pregn-5-en-20-ethylene ketal was rearranged without further purification by refluxing for 6 hr in 100 ml acetone to which was added 5 g potassium acetate in 50 ml water. Diethyl ether extraction and the usual workup followed by chromatography on alumina, Brockmann grade 3, eluted with diethyl ether-petrol mixtures gave 6β-hydroxy-3α,5α-cyclopregnan-20-ethylene ketal (*9,* 0.35 g). R_f, 0.75; EIMS *m/z* (rel int) 345(7), 298(2), 213(1.5), 87(100); ^1H NMR ($CDCl_3$): δ 0.25 and 0.50 (*m*'s, cyclopropane), 0.79 (*s,* H-18), 1.03 (*s,* H-19), 1.28 (*s,* H-21), 3.25 (*m,* H-6α), 3.8-4.0 (*m,* ketal).

The 6β-hydroxy-3α,5α-cyclopregnan-20-ethylene ketal (*9,* 0.34 g) in pyridine (3.4 ml) was added to Sarrett reagent (0.34 g chromic trioxide in 3.4 ml pyridine) and the mixture left for 18 hr at room temperature. The reaction was quenched with water (30 ml) and extracted with diethyl ether (3 x 25 ml) followed by normal workup to yield 6-oxo-3α,5α-cyclopregnan-20-ethylene ketal (*10,* 0.3 g). R_f 0.87; EIMS *m/z* (rel int): 358[M]$^+$ (1) 343(5), 314(0.5), 87(100); ^1H NMR ($CDCl_3$): δ 0.70 (*m,* cyclopropane), 0.81 (*s,* H-18), 1.01 (*s,* H-19), 1.30 (*s,* H-21).

The 6-oxo-3α,5α-cyclopregnan-20-ethylene ketal (*10,* 50 mg) was refluxed for 1.5 hr in 10 ml 1,4-dioxan containing sodium methoxide (100 mg) and deuterium oxide (0.5 mL). Diethyl ether extraction and usual workup yielded [7,7-$^2H_2$] 6-oxo-3α,5α-cyclopregnan-20-ethylene ketal (*11,* 49 mg). EIMS *m/z* (rel int): 345(7), 316(3) 87(100). The [7,7-$^2H_2$] 6-oxo-3α,5α-cyclopregnan-20-ethylene ketal was reduced by stirring for 18 hr with 50 mg sodium borodeuteride in 5 ml tetrahydrofuran-ethanol (1:1). Workup yielded [6,7,7-$^2H_3$] 6α-hydroxy-3α,5α-cyclopregnan-20-ethylene ketal *(12,* 48 mg). R_f, 0.55; EIMS *m/z* (rel int): 348(3), 317(0.5), 87(100);

CIMS *m/z* (rel int): 364 $[M+1]^+$ (10) 346(4) 343(4), 87(100); ^1H NMR ($CDCl_3$): δ 0.60 (*m*, cyclopropane), 0.76 (*s*, C-18), 0-90(*s*, C-19), 1.29 (s, C-21), 3.8-4.0(*m*, ketal).

The [6,7,7-$^2H_3$]6α-hydroxy-3α,5α-cyclopregnan-20-ethylene ketal (48 mg) was warmed at 60C for 1 hr with 5 ml 1,4-dioxane containing 25 μl H_2SO_4. Addition of water and diethyl ether extraction followed by preparative TLC gave [6,7,7-$^2H_3$]3β-hydroxypregn-5-en-20-one (*13*, 32 mg). R_f 0.55; EIMS *m/z* (rel int): 319$[M]^+$ (100), 318(34), 316(5) 301(56), 300(25), 286(55), 285(32), 258(24), 233(39), 215(33), 205(54), 108(95); ^1H NMR ($CDCl_3$): δ 0.63 (*s*, H-18), 1.00 (*s*, H-19), 2.13 (*s*, H-21), 3.55 (*brm*, H-3α), no olefinic proton.

Conversion of [6,7,7-$^2H_3$]Pregnenolone to [6,7,7-$^2H_3$] Progesterone

[6,7,7-$^2H_3$]Pregnenolone (*13*, 1 g) was dissolved in 125 ml acetone and 0.9 ml chromium troxide reagent added (15). The mixture was stirred for 5 min, excess water added and the mixture extracted with diethyl ether to yield [6,7,7-$^2H_3$]pregn-5-en-3,20-dione (0.85 g) which was dissolved in 5.0 ml methanol and 0.1 ml of 10% hydrochloric acid added. After warming at 30-40C, for 10 min, water was added and the mixture extracted with diethyl ether to yield [6,7,7-$^2H_3$]pregn-4-en-3,20-one (progesterone, *14*) which was purified by chromatography on silica gel eluted with diethyl-ether-petrol mixtures, fractions were monitored by TLC and GLC; yield of pure material 200 mg; R_f 0.67; UV λ_{max} 235; EIMS *m/z* (rel int): 317$[M]^+$ (68), 316(24), 315(1), 314(4), 275(67), 274(29), 273(7), 272(4), 247(34), 232(55), 193(24), 125(100); ^1H NMR ($CDCl_3$): δ 0.64 (*s*, H-18), 1.17 (*s*, H-19), 2.12 (*s*, H-21), 5.70 (*s*, H-4).

Synthesis of [6,7,7-$^2H_3$]3β-Hydroxyandrost-5-en-17-one

3β-Hydroxyandrost-5-en-17-one (*15*, 2.50 g) was converted using the literature methods (12,13) into 6β-hydroxy-3α,5α-cycloandrostan-17-one (*16*, 2.40 g) mp 125-130C: TLC (silica gel, $CHCl_3$-acetone, 185:15) R_f 0.5; EIMS *m/z* (rel int): 288 $[M]^+$ (25), 273 (80), 270(49), 255(19), 233(100), 213(11), 123(47), 95(57). Oxidation of *16* (2.30 g) with Jones reagent (13) yielded 3α,5α-cycloandrostane-6,17-dione (*17*, 2.27 g); EIMS *m/z* (rel int); 285$[M]^+$ (100), 271(39), 270(27), 258(24) 243(7), 229(11), 215(9), 149(23), 136(84), 121(59); ^1H NMR ($CDCl_3$): δ 0.90 (*s*, H-18), 1.02 (*s*, H-19). The 3α,5α-cycloandrostane-6,17-dione (*17*, 2.2 g) was dissolved in 200 ml 1,4-dioxan containing 25 ml deuterium oxide and 2.5 g sodium methoxide and the mixture refluxed for 2.5 hr. Extraction with petrol and usual workup gave [7,7,16,16-$^2H_4$]3α,5α-cycloandrostane-6,17-dione (*18*, 1.5 g). EIMS *m/z* (rel int): 290$[M]^+$(100), 289(29), 275(35), 272(18), 262(24), 261(27), 231(8), 217(6), 138(89), 121(55).

Reduction of the [7,7,16,16-$^2H_4$]3α,5α-cycloandrostane-6,17-dione (*18*, 1.23 g) in 200 ml tetrahydrofuran-ethanol(1:1) by addition of sodium borodeuteride (0.5 g) with stirring for 4 hr at room temp followed by extraction into methylene chloride and usual workup gave [6,7,7,16,16,17-$^2H_6$]3α,5α-cycloandrostane-6α,17β-diol (*19*, 1.1 g): EIMS *m/z* (rel int): 296 $[M]^+$(7), 281(100), 280(40), 278(32), 277(16), 263(15), 241(76), 216(8).

The [6,7,7,16,16,17-$^2H_6$] 3α,5α-cycloandrostane-6β,17β-diol (*19*, 0.67 g) was rearranged by refluxing for 2 hr with 5 g zinc acetate in 200 ml glacial acetic acid. The mixture was then diluted with water (1ℓ) and extracted with hexane-diethyl ether (3 x 200 ml). The combined extracts were washed with saturated sodium bicarbonate solution, followed by water, dried and evaporated to yield 0.62 g of product. This was chromatographed on an alumina, Brockmann grade 3, column eluted with mixtures of diethyl ether in petrol to yield 233 mg [6,7,7,16,16,17-$^2H_6$]3β-acetoxy-androst-5-en-17β-ol (*20*) and 229 mg [6,7,7,16,16,17-$^2H_6$]androst-5-ene-3β,17β-diol diacetate (*21*). [6,7,7,16,16,17-$^2H_6$]3β-Acetoxy-androst-5-en-17β-ol, TLC (silica gel, $CHCl_3$-EtOH, 98:2) R_f 0.1, GLC (3% OV-17) RR_t 1.33 (3β-hydroxyandrost-5-en-17-one, RR_t = 1.00); EIMS *m/z* (rel int): 278$[M\text{-}CH_3COOH]^+$ (100), 263(25), 245(16). [6,7,7,16,16,17-$^2H_6$] Androst-5-ene-3β,17β-diol diacetate, TLC R_f 0.4, GLC (3% OV-17) RR_t 1.73; EIMS *m/z* (rel int): 320 $[M\text{-}CH_3\ COOH]^+$ (100), 305(7), 260-(5), 245 (11).

Jones reagent (10 ml) was added to 41 mg [6,7,7,16,16,17-$^2H_6$] 3β-acetoxy-androst-5-en-17β-ol (*20*) in 3 ml benzene-acetone (1:2) and the mixture left at 4C for 20 min. *Iso*propanol (20 ml) was then added followed by diethyl ether extraction and the usual workup to yield 37 mg [6,7,7,16,16-$^2H_5$]3β-acetoxy-androst-5-en-17-one (*22*) EIMS *m/z* (rel int): 335$[M]^+$ (15), 334(14), 275(17), 274(10), 43(100).

Finally, the [6,7,7,16,16-$^2H_5$]3β-acetoxy-androst-5-en-17-one (*22*, 35 mg) was dissolved in 8 ml methanol, 2 ml 10% aq potassium hydroxide solution added and the mixture refluxed for 2 hr. Extraction with methylene chloride and usual workup gave 21 mg [6,7,7,-$^2H_3$]3β-hydroxyandrost-5-en-17-one

(*24*); TLC (silica gel, $CHCl_3$-EtOH, 98:2) R_f 0.18; EIMS *m/z* (rel int) 291[M]$^+$ (85), 290(31), 288(8), 273(65), 258(68), 234(12), 215(20), 205(32), 177(60).

Incorporation of [6,7,7-$^2H_3$] Cholesterol into Poriferasterol by *Ochromonas malhamensis*

O. malhamensis was obtained from the Cambridge Culture collection and grown as described previously (12,17) with [6,7,7-$^2H_3$] cholesterol (2 mg/100 ml) added to the growth medium. The cells were harvested after 3-5 days and the sterols were isolated (12) and analyzed by GC-MS on a 3% OV-17 column at 260C.

Incubation of [6,7,7-$^2H_3$] Progesterone with Ovaries of *Asterias rubens*

The ovaries of a specimen of *A. rubens*, collected at Millport, Scotland in May, were removed, minced, and incubated with a mixture of [4-^{14}C] progesterone (1 μCi) and [6,7,7-$^2H_3$] progesterone (800 μg) plus 50 μl propyleneglycol in 1.0 ml of sterile sea water. After 5 hr, the total lipids were extracted by homogenization with $CHCl_3$-MeOH (2:1). The $CHCl_3$ extract was subjected to TLC (silica gel, $CHCl_3$-acetone, 95:10) and the plate scanned for radioactivity. The radioactive bands with R_f values corresponding to marker spots of 5α-pregnane-3,20-dione (band 1), progesterone (band 2) and 3β-hydroxy-5α-pregnan-20-one (band 3) were eluted and subjected to GC-MS analysis on a 1% SE-30 column. MS of band 1, peak with R_t 8.5 min, *m/z* (rel int): 319(36), 301(2), 261(14), 249(12), 234(41), 71(100); peak with R_t 9.5 min; *m/z* (rel int): 319(28) 301(19), 275(9), 261(14), 249(8), 234(30), 84(100). MS of band 3, peak with R_t 7.5 min, *m/z* (rel int): 321(8), 260(13), 236(4), 71(74), 43(100); peak with R_t 9.1 min, *m/z* (rel int): 321(17), 303(7), 291(4), 260(5), 236(7), 218(13), 84(65), 43(100).

Incubation of [6,7,7-$^2H_3$] Progesterone with Oocytes of *Xenopus laevis*

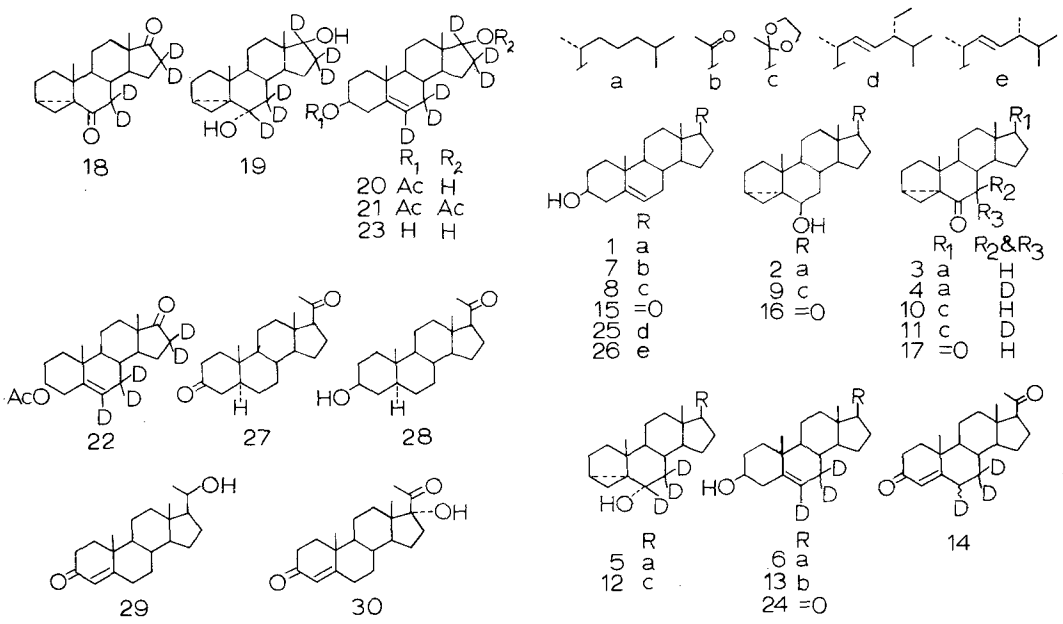
The ovary (24 g) was removed from a specimen of *X. laevis* (192 g), washed, and cut into small sections (150-200 mg each). Ovary pieces, consisting mainly of oocytes and their follicle cells (total wt 6.0 g), were incubated in 100 ml Merriams saline medium containing [4-^{14}C] progesterone (0.5 μCi) and [6,7,7-$^2H_3$] progesterone (200 μg). After 2 hr incubation at 20C, the total lipid was extracted by homogenization with $CHCl_3$-MeOH (2:1). The total lipid was chromatographed on a silica gel column (20 g) and several radioactive fractions eluted with petrol, Et_2O, $CHCl_3$ and acetone mixtures. These were then subjected to TLC on silica gel ($CHCl_3$-acetone, 90:10) and radioactive bands detected by radioscanning and eluted for GC-MS analysis on a 3% OV-17 column. Full details of the analysis of all the metabolites will be presented elsewhere.

RESULTS AND DISCUSSION

In order to aid recognition of deuterium labeled steroid metabolites by GC-MS, it is desirable to label the steroid substrates with an odd number of deuterium atoms. Thus, unless the metabolic reactions result in loss of a deuterium atom, the metabolites will have odd-numbered molecular weights and be easily distinguished by mass spectrometry from endogenous steroids. Ideally, 3 or 5 deuterium atoms should be introduced into the steroid molecule. With only one deuterium atom present, it may be difficult to recognize and quantify metabolites in the presence of the unlabeled endogenous steroids, particularly if they are produced in low yield, because of the natural abundance of the ^{13}C-containing steroid molecules (i.e. [M+1]$^+$) present in the endogenous steroid pool (6). More than 4 or 5 deuterium atoms in the steroid molecule may result in some significant separation of the deuterium labeled species from the endogenous steroid during GLC or TLC and this may be undesirable or misleading in some investigations (6) (Scheme 1).

We have previously described methods for the synthesis of sterols labeled with tritium at either the C-6 or the C-7 positions (12,13). By a combination of these two procedures, we have now developed a simple method for the introduction of three deuterium atoms into Δ^5-sterols and some steroid hormones. The starting material is a sterol or steroid with a Δ^5-bond and the method can be exemplified by the synthesis of [6,7,7-$^2H_3$] cholesterol.

The first step involved the conversion of cholesterol (*1*) into the *i*-sterol, 3α,5α-cyclocholestan-6β-ol (*2*). Oxidation of the *i*-sterol (*2*) with Jones reagent produced the 6-oxo derivative (*3*) into which two deuterium atoms were efficiently introduced at C-7 by base-catalysed enolization in the presence of deuterium oxide. The mass spectrum of the labeled compound (*4*) showed that the exchange of the two C-7 hydrogens of *3* for deuterium was virtually complete (M$^+$ at *m/z* 386) under the conditions employed. Reduction of the bideuterated 6-oxo-compound (*4*) using sodium borodeuteride restored the C-6 hydroxyl group and also introduced a third deuterium atom at C-6 to yield



SCHEME 1

the [6,7,7,-$^2H_3$]*i*-sterol (*5*). In our previous synthesis of tritiated sterols (12,13), the *i*-sterol was rearranged by reflux with acetic acid-zinc acetate to yield the Δ^5-3β-steryl acetate which then required alkaline hydrolysis to give the desired 3β-hydroxy-Δ^5-sterol. For the present work, the rearrangement of the labeled *i*-sterol to the 3β-hydroxy-Δ^5-sterol (*6*) was achieved efficiently in one step by warming the [6,7,7-$^2H_3$]*i*-sterol (*5*) in 1,4-dioxan containing sulphuric acid. The ^{1}H NMR spectrum of the recovered [6,7,7-$^2H_3$]cholesterol (*6*) showed the expected methyl proton signals, but there was no signal for a C-6 olefinic proton, thus demonstrating that the C-6 deuterium atom of *5* was retained at this position during the rearrangement reaction. The [6,7,7-$^2H_3$]cholesterol showed one peak on GLC with a retention time the same as unlabeled cholesterol. The mass spectrum of *6* showed a molecular ion at *m/z* 389 and other significant peaks were displaced by three mass units as expected. The ratio of the molecular ion peaks for the trideuterated, bideuterated and nondeuterated species, at *m/z* 389, 388 and 386, respectively, was 16:4:1 showing that trideuterated cholesterol was the major product of the synthesis.

To synthesize deuterium labeled C_{21}-steroids, we used pregnenolone (*7*) as the starting material. The first requirement was to protect the C-20 carbonyl group by formation of the C-20 ethylene ketal (*8*). This was necessary to prevent deuterium being introduced at C-17 and C-21 during the base exchange step and reduction of the C-20 carbonyl by treatment with sodium borodeuteride. The introduction of deuterium at C-7 and C-6 then proceeded from *8* by the same sequence of reactions as outlined above to yield [6,7,7-$^2H_3$]6α-hydroxy-3α,5α-cyclopregnan-20-ethylene ketal (*12*). Treatment of *12* with sulphuric acid-dioxan not only effected the restoration of the 3β-hydroxy-Δ^5-system but also removed the protective ethylene ketal to re-establish the C-20 carbonyl group thus producing [6,7,7-$^2H_3$] pregnenolone (*13*). The ^{1}H NMR spectrum and the mass spectrum again showed that three deuterium atoms had been introduced into the desired positions.

The conversion of [6,7,7-$^2H_3$]pregnenolone (*13*) into [6,7,7-$^2H_3$]progesterone (*14*) was achieved by acidic oxidation of *13* to yield [6,7,7-$^2H_3$] pregn-5-en-3,20-dione followed by acidic (HCl) isomerization (15) of the Δ^5-bond to the conjugated Δ^4-position to produce [6,7,7-$^2H_3$]progesterone (*14*). Oppenauer oxidation of *13* to *14* was avoided since the C-6 deuterium atom of *14* is labile under alkaline conditions. The ^{1}H NMR spectrum of *14* was in accord with that of progesterone and the mass spectrum had a molecular ion at *m/z* 317, as expected for trideuterated progesterone. The ratio of the molecular ion abundance of the trideuterated, bideuterated and nondeuterated

species (*m/z* 319, 318 and 316, respectively) of *13* was 18.6:6.3:1, whereas the ratio for the corresponding molecular ions of *14* (*m/z* 317, 316 and 314, respectively) was 17.7:6.2:1. This showed that there had been no appreciable exchange of the enolizable C-6 deuterium in this conversion under the acidic oxidation and isomerization conditions employed.

The synthesis of C_{19}-steroids commenced with 3β-hydroxy-androst-5-en-17-one (*15*) which was converted to the *i*-steroid (*16*) without prior protection of the 17-oxo group. This allowed two deuterium atoms to be introduced at C-16 in addition to the two at C-7 during base exchange of 3α,5α-cycloandrostane-6,17-dione (*17*) with deuterium oxide to give the tetradeuterated compound (*18*). Reduction of *18* with sodium borodeuteride introduced a further two deuterium atoms to produce [6,7,7,16,16,17-$^2H_6$]3α-cycloandrostane-6α,17β-diol (*19*). Although this could be rearranged with sulphuric acid-dioxan, as employed in the two previous sequences described above, to yield [6,7,7,16,16,17-$^2H_6$]androst-5-en-3β,17β-diol (*23*), we chose to use the alternative method of refluxing with acetic acid-zinc acetate (12,13). In this case, a mixture of the 3β-monoacetate (*20*) and the 3β,17β-diacetate (*21*) was produced and these acetates were readily separated by chromatography on an alumina column. [6,7,7,7,16,16,17-$^2H_6$] Androst-5-en-3β,17β-diol (*23*) can then be obtained from the diacetate (*21*) by alkaline hydrolysis.

The labeled 3β-monoacetate (*20*) was oxidized to give [6,7,7,16,16-$^2H_5$]3β-acetoxy-androst-5-en-17-one (*22*) which was then subjected to alkaline hydrolysis which removed the 3β-acetate group and the two enolizable deuterium atoms at C-16 to yield [6,7,7-$^2H_3$] 3β-hydroxy-androst-5-en-17-one (*24*). Again, the mass spectrum showed that the trideuterated species was the major product. The [6,7,7-$^2H_3$] 3β-hydroxy-androst-5-en-17-one could be used for the preparation of [6,7,7-$^2H_3$]androst-4-ene-3,17-dione in a manner similar to that described above for the conversion of [6,7,7-$^2H_3$]pregnenolone into [6,7,7-$^2H_3$]progesterone while [6,7,7,16,16,17-$^2H_6$]androst-5-en-3β,17β-diol (*23*) could be used as a precursor for the synthesis of deuterium labeled testosterone (16).

We are now using these labeled steroids for studies on sterol and steroid metabolism in algae, marine invertebrates and amphibian and mammalian tissues. Their use is illustrated here by examples of our studies with the chrysophyte alga *O. malhamensis*, the echinoderm *A. rubens* and the amphibian *X. laevis*.

O. malhamensis has been used extensively for our studies (12,17,18) on phytosterol biosynthesis and contains poriferasterol (*25*) as the major sterol together with about 5% of brassicasterol (*26*). The additional C-24 ethyl or methyl groups of *25* and *26*, respectively, are introduced by methyl group transfer from S-adenosylmethionine to a precursor Δ^{24}-sterol (18). During investigations (19), on the utilization of radioactively labeled 24,25-dihydrolanosterol and cholesterol (*1*) by *O. malhamensis*, evidence was obtained that these compounds were converted into poriferasterol (*25*). This was an unexpected result since it implies that the alga is capable of introducing a Δ^{24}-bond into the saturated iso-octane side chain of cholesterol. To confirm this result, we cultured *O. malhamensis* in medium containing [6,7,7-$^2H_3$]cholesterol. After 5 days, the cells were harvested and the sterols isolated and analyzed by GC-MS. Figure 1 shows the molecular ion regions of the mass spectra of the brassicasterol (*26*) and poriferasterol (*25*) isolated from control cells and those grown in the presence of [6,7,7-$^2H_3$]cholesterol. In the mass spectrum of the control poriferasterol (M^+ at *m/z* 412), no peak was observed at *m/z* 415 whereas, in the poriferasterol isolated from the culture grown with added deuterium labeled cholesterol, a peak at *m/z* 415 was clearly distinguished. This must represent a trideuterated species of poriferasterol and it thus confirms the previous evidence (19) that this alga is capable of introducing extra carbon atoms into the cholesterol side chain at C-24 to produce poriferasterol. In the previous study (19), no evidence was obtained regarding the possible conversion of cholesterol into brassicasterol (*26*). However, comparison of the brassicasterol mass spectra obtained in the present work showed that, while the control brassicasterol (M^+ at *m/z* 398) showed no peak at *m/z* 401, there was a very pronounced peak for trideuterated brassicasterol in the sample obtained from the [6,7,7-$^2H_3$]cholesterol culture. The incorporation of [6,7,7-$^2H_3$]cholesterol into brassicasterol, as well as poriferasterol, is expected on the basis of the C-24 alkylation mechanisms which operate in *O. malhamensis* (18). The first transmethylation reaction transfers the methyl group of S-adenosyl methionine to a Δ^{24}-sterol precursor to produce a 24-methylene sterol intermediate. This latter compound can then act as the substrate for a second transmethylation reaction to lead ultimately to poriferasterol (*25*) or alternatively the 24-methylene group is reduced to yield the 24β-methyl group of brassicasterol (*26*).

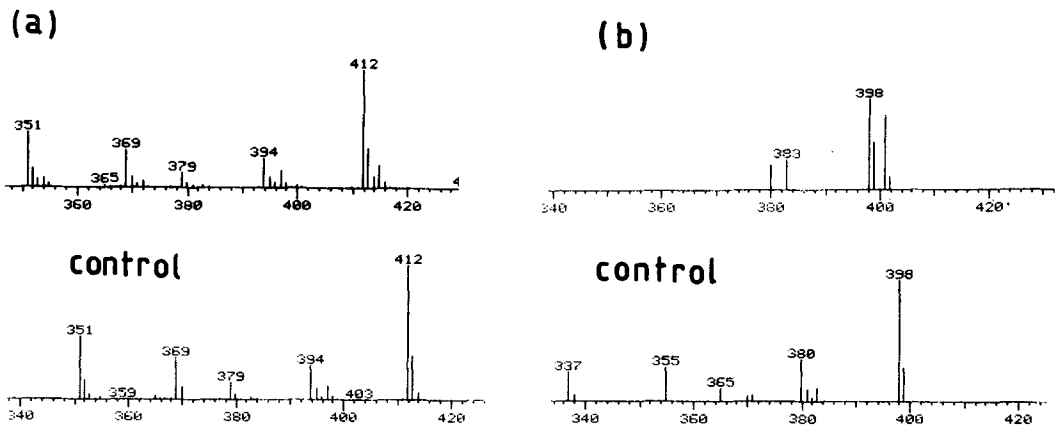


FIG. 1. Molecular ion regions of the mass spectra of (a) poriferasterol (*25*) and (b) brassicasterol (*26*) produced by *O. malhamensis* grown in the presence of [6,7,7-$^2H_3$]cholesterol. Also shown are the mass spectra of the sterols isolated from a control culture.

Although the mass spectral evidence demonstrates that cholesterol was incorporated into both brassicasterol and poriferasterol, we observed two unexpected results. First, the presence of cholesterol in the growth medium reduced the amount of brassicasterol relative to poriferasterol present in the cells. In control cells, brassicasterol constituted about 6% of the total sterol and the ratio of poriferasterol to brassicasterol was about 16:1. However, in the cholesterol grown cells, the sterol composition was brassicasterol 0.5%, cholesterol 19.4% and poriferasterol 80.1%, while the ratio of poriferasterol to brassicasterol had risen to about 160:1. Secondly, while the [6,7,7-$^2H_3$]-species of poriferasterol was only about 16% of the total poriferasterol isolated, in the case of brassicasterol, the trideuterated form was about 45% of the total sterol. Similar results were observed in several other incubations of *O. malhamensis* with [6,7,7-$^2H_3$]cholesterol. A satisfactory explanation for these observations is not apparent at the present but it seems that, under the conditions of these experiments, the pool of brassicasterol is turning over more rapidly than the poriferasterol pool and further investigation is warranted.

In order to identify deuterium labeled metabolites by GC-MS, it is often first necessary to locate and to achieve at least partial purification of the metabolite-containing fractions from tissue extracts prior to the GC-MS analysis. The location of steroid metabolites can be facilitated by admixture of radioactively labeled precursor of high specific activity to the deuterium labeled steroid prior to tissue incubation (2). Partial purification of the metabolites from the lipid extract by TLC can then be monitored by radioscanning to locate labeled metabolites. The R_f value of the radioactive band will give some indication of the possible identity of the metabolite and appropriate GC-MS conditions can then be chosen for subsequent identification of the deuterium labeled species present in the fraction. The final identification of a deuterated metabolite will, therefore, be based upon (a) the R_f value on TLC, (b) the retention time on GLC, and (c) the mass spectrum, all of which can then be compared to the corresponding data of reference compounds.

We have already briefly described the application of this technique to identify the metabolites of progesterone produced by the ovaries of the echinoderm, *A. rubens* (20). We had previously shown (21-23) using [4-^{14}C]progesterone that the main metabolites were 5α-pregnane-3,20-dione (*27*) and 3β-hydroxy-5α-pregnan-20-one (*28*). To confirm this observation, we mixed [4-^{14}C]progesterone with [6,7,7-$^2H_3$]progesterone (*14*) and, after incubation with minced ovaries of *A. rubens*, the lipid extract was submitted to TLC separation. The radioactive bands with R_f values corresponding to *27* and *28* were eluted and subjected to GC-MS analysis. Figure 2 shows the results obtained for the analysis of the band corresponding to *27*. The main GC peak had a retention time corresponding to *27* and its mass spectrum had a molecular ion at *m/z* 319 and fragment ions revealing it to be [6,7,7-$^2H_3$]5α-pregnane-3,20-dione. A minor component of shorter retention time was also apparent on the total ion GC trace and examination of its mass spectrum revealed that this compound also had a molecular ion at *m/z* 319.

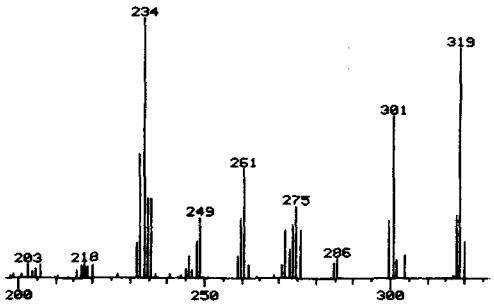


FIG. 2. Mass spectrum obtained by GC-MS analysis of one of the metabolites of [6,7,7-$^2H_3$]progesterone produced by incubation with ovary tissue of *A. rubens*. The metabolites had an R_f value corresponding to pregnane-3,20-dione in TLC. This material separated by GLC into two components. The mass spectrum of the major component is shown and it was identified as 5α-pregnane-3,20-dione. The minor component had a shorter retention time but the mass spectrum was similar (See the Experimental).

From its mass spectral and chromatographic properties, this compound appears to be [6,7,7-$^2H_3$] 5β-pregnane-3,20-dione, which had hitherto gone undetected during metabolite identification by radioisotope methods, presumably because of the relatively small amount of this metabolite produced. Similarly, GC-MS analysis of the radioactive TLC band corresponding in R_f value to *28* showed (20) a major component with a retention time and mass spectrum (M^+ at *m/z* 321) corresponding to [6,7,7-$^2H_3$] 3β-hydroxy-5α-pregnan-20-one. There was also a minor component of shorter retention time with a molecular ion at *m/z* 321 indicating it to be [6,7,7,7-$^2H_3$] 3β-hydroxy-5β-pregnan-20-one which had also been unrecognized previously (21, 22).

The power of this technique to detect minor metabolites which may conceivably be overlooked using radiochemical methods is also illustrated by an example from our studies on steroid metabolism by frog ovaries. In frogs, progesterone produced in the ovary follicle cells initiates oocyte maturation by interaction with an oocyte outer membrane receptor (24). The progesterone also enters the oocyte and is metabolized to give several products, some of which have only been tentatively identified (24,25). In order to examine further these steroid metabolites, we have incubated oocytes with a mixture of [4-^{14}C]progesterone and [6,7,7-$^2H_3$]progesterone. Partial separation of the isolated total lipid into fractions and radioscanning of the thin layer chromatograms of

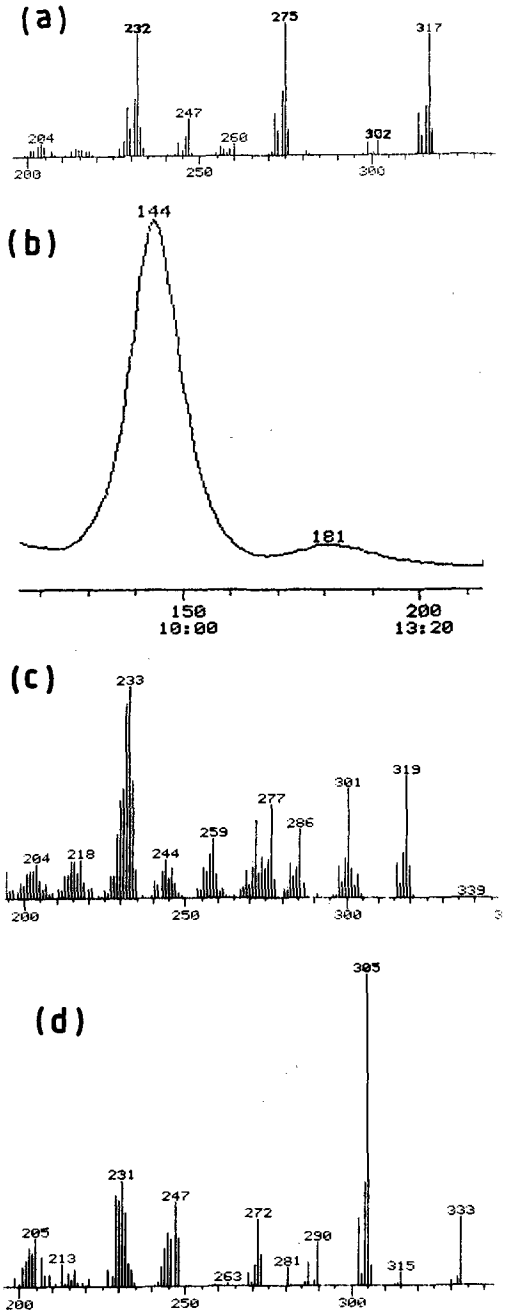


FIG. 3. Mass spectra of [6,7,7-$^2H_3$]progesterone (*14*) and two metabolites produced from this steroid by oocytes of *X. laevis*. The metabolites had an R_f value on TLC corresponding to 17α-hydroxyprogesterone (*30*). (a) Mass spectrum of [6,7,7-$^2H_3$]progesterone; (b) GLC trace of the metabolites; (c) mass spectrum of peak 144 corresponding to trideuterated 20α-hydroxypregn-4-en-3-one (*29*); (d) mass spectrum of peak 181 corresponding to trideuterated 17α-hydroxyprogesterone (*30*).

these fractions revealed several metabolite bands. These were eluted and subjected to GC-MS examination. Figure 3 shows the results obtained for a radioactive band which had an R_f indicating it to be 17α-hydroxyprogesterone.

In this experiment, a small amount of unlabeled progesterone was included with the [6,7,7-$^2H_3$]progesterone prior to incubation. This resulted in the molecular ion region of the mass spectrum showing a recognizable "fingerprint" pattern (Fig. 3a) due to the relative proportions of nondeuterated (*m/z* 314), bideuterated (*m/z* 316) and trideuterated (*m/z* 317) progesterone species present. This molecular ion pattern was observable in the mass spectrum of the major metabolite (Fig. 3c). By contrast, the mass spectrum of the minor component was weak and the molecular ion region did not display this pattern. However, it was clearly discernable in the fragment ion isotope cluster around *m/z* 302-305 showing that this compound had arisen from the precursor species of deuterium labeled progesterone. This technique has been described previously for studies on drug metabolism (26,27) and it serves as a valuable aid in the recognition of metabolites when reviewing the many mass spectra accumulated by the data system during GC-MS analysis. However, it should be noted that endogenous pools of the precursor and/or metabolites, if present, could significantly alter the isotope patterns in the mass spectra.

The major component of the oocyte metabolite fraction shown in Figure 3 had a molecular ion at *m/z* 319 and its fragmentation pattern, together with the GLC retention time and R_f value, identified it as 20α-hydroxypregn-4-en-3-one (*29*). The minor component had a molecular ion at *m/z* 333 and its chromatographic properties indicated it to be 17α-hydroxypregn-4-en-3,20-dione (*30*). Steroid *29* has previously been identified as a progesterone metabolite in *Pleurodeles waltlii* oocytes and *X. laevis* oocyte homogenate (29) and *30* is a product in oocytes of *X. laevis* (28-30) and *Trituris alpestris* (31).

The results described above show that the readily synthesized [6,7,7-$^2H_3$]sterols and steroids can be used for metabolic studies and they could also be of value as internal standards for steroid quantification work by GC-MS. Although the deuterium at C-6 in [6,7,7-$^2H_3$]progesterone (*14*) is at a potentially labile position the above studies with *A. rubens* and *X. laevis* show that this steroid can be utilized successfully for some studies. However, some incubation, extraction and analytical procedures may cause exchange of this deuterium and this must be considered when using [6,7,7-$^2H_3$]steroids. In particular, an active 3-keto-Δ^5-Δ^4 steroid isomerase in some tissues may cause loss of the C-6 deuterium atom prior to metabolism of the [6,7,7-$^2H_3$]steroids.

ACKNOWLEDGMENTS

We thank the SERC and North West Cancer Fund for financial support, Mr. M. Prescott for determining mass spectra, Miss A. Howes for technical assistance and the Department of Organic Chemistry, University of Liverpool for measuring the ^1H NMR spectra.

REFERENCES

1. Albertson, B.D., Schiebinger, R.J., Cutler, G.B., Davies, S.E., and Loriaux, D.L. (1980) Steroids 35, 351-360.
2. Baillie, T.A. (1978) Stable Isotopes: Applications in Pharmacology. Toxicology and Clinical Research, MacMillan, London.
3. Bjorkhem, I. (1975) Eur. J. Biochem. 51, 137-143.
4. Baillie, T.A., Sjövall, J., and Herz, J.E. (1975) Steroids 26, 438-457.
5. Baillie, T.A., Sjövall, J., and Sjövall, K. (1975) FEBS Lett. 60, 145-148.
6. Seamark, R.F., Phillipou, G., and McIntosh, J.E.A. (1977) J. Steroid Biochem. 8, 885-891.
7. Johnson, D.W., Phillipou, G., and Seamark, R.F. (1981) J. Steroid Biochem. 14, 793-800.
8. Pinkus, J.L., Charles, D., and Chattoraj, S.C. (1971) J. Biol. Chem. 246, 633-636.
9. Pinkus, J.L., Charles, D., and Chattoraj, S.C. (1979) Horm. Res. 10, 44-56.
10. Fetizon, M., and Gramain, J-C. (1969) Bull. Soc. Chim. 651-672.
11. Tores, L. and Throop, J.L. (1972) in Organic Reactions in Steroid Chemistry, (Freid, J., and Edwards, J.A., eds.) Vol. 1, pp. 145-221, Van Nostrand Reinhold, New York, NY.
12. Knapp, F.F., Goad, L.J., and Goodwin, T.W. (1977) Phytochemistry 16, 1683-1688.
13. Palmer, M.A., Goad, L.J., and Goodwin, T.W. (1979) Nouveau J. Chim. 2, 401-404.
14. Fonken, G.J. (1961) J. Org. Chem. 26, 2549-2550.
15. Djerassi, C., Engle, R.R., and Blowers, A. (1956) J. Org. Chem. 21, 1547-1550.
16. Gaskell, S.J., and Finlay, E.M.H. (1980) J. Lab. Comp. Radiopharm. 17, 861-869.
17. Lenton, J.R., Hall, J., Smith, A.R.H., Ghisalberti, E.L., Rees, H.H., Goad, L.J., and Goodwin, T.W. (1971) Arch. Biochem. Biophys. 143, 664-674.
18. Goad, L.J. (1977) in Lipids and Lipid Polymers in Higher Plants (Tevini, M., and Lichtenthaler, H.K., eds.) pp. 146-168, Springer-Verlag, Berlin.
19. Beastall, G., Rees, H.H., and Goodwin, T.W. (1972) Biochem. J. 128, 179-181.
20. Goad, L.J. (1981) Pure Appl. Chem. 51, 837-852.
21. Goad, L.J. (1978) in Marine Natural Products (Scheuer, P.J., ed.) Vol. 2, pp. 75-172, Academic Press, New York, NY.
22. Teshima, S., Fleming, R., Gaffney, J., and Goad, L.J. (1977) in Marine Natural Products (Falkner, D. and Fenical, W.H., eds.) pp. 136-146, Plenum Press, London.
23. Gaffney, J., and Goad, L.J. (1973) Biochem. J.

138, 309-311.
24. Masui, Y., and Clarke, H.J. (1979) Int. Rev. Cytol. 57, 185-282.
25. Schatz, F., and Morrill, G.A. (1975) Biol. Reprod. 13, 408-414.
26. Watson, J.T. (1978) in Stable Isotopes: Applications in Pharmacology, Toxicology and Clinical Research (Baillie, T.A., ed.) pp. 15-26, MacMillan, London.
27. Vanden-Heuvel, W.J.A. (1978) in Stable Isotopes: Applications in Pharmacology, Toxicology and Clinical Research (Baillie, T.A., ed.) pp. 65-84, MacMillan, London.
28. Ozon, R., Belle, R., Serres, C., and Fouchet, C. (1975) Mol. Cell. Endocrinol. 3, 221-231.
29. Thibier-Fouchet, C., Mulner, O., and Ozon, R. (1976) Biol. Reprod. 14, 317-326.
30. Fouchet, C., Serres, C., Belle, R., and Ozon, R. (1975) Comp. Biochem. Physiol. B 52, 205-210.
31. Antila, E. (1977) Differentiation 8, 71-77.

[Received March 1, 1982]

Quantitative Analysis of Lipid Classes by Liquid Chromatography Via a Flame Ionization Detector

F.C. PHILLIPS, W.L. ERDAHL and O.S. PRIVETT*, *The Hormel Institute, University of Minnesota, Austin, MN 55912*

ABSTRACT

A method is described for the direct quantitative analysis of the lipid classes of mammalian tissue lipids using high performance liquid chromatography (HPLC) with a flame ionization detector (FID). The lipid is extracted from the tissue with chloroform/methanol after deactivation of hydrolytic enzymes and removal of nonlipid substances by extraction with hot dilute acetic acid (0.05N). Separation of the lipid classes is performed with a column (45 cm × 0.2 cm id) of 8 μm silica (Spherisorb, Phase Sep, Hauppague, NY) treated with concentrated ammonium hydroxide at a solvent flow rate of 0.5 ml/min, which requires a pressure of ca. 900 psi. Cholesteryl esters (CE) and triglycerides (TG) are eluted first with Skellysolve B/methylene chloride (1:1, v/v); cholesterol (CH) is eluted with chloroform/methylene chloride (1:2, v/v) and the phospholipids with methanol containing 6% ammonium hydroxide added to the latter solvent in a linear gradient. The neutral lipids are eluted in ca. 12 min and the phospholipids in an additional 30 min. The relative amount of each lipid class was determined from standard curves of the peak areas obtained according to response factors using erucyl alcohol as an internal standard. The method was applied to samples of kidney, liver and serum of rats. Duplicate analyses were generally within ca. 1.0% and good agreement was obtained in the analysis of the lipid classes of Azolectin and liver mitochondria lipid compared to thin layer chromatography (TLC) via photodensitometry of charred spots or phosphorus analysis of recovered phospholipids.

Lipids 17:992-997, 1982.

INTRODUCTION

High performance liquid chromatography (HPLC) has had only limited application to the analysis of the lipid classes for lack of a sensitive universal detector (1-5). Photometric detectors are not ideal for the analysis of the lipid classes because they operate on the principle of the detection of specific structures that absorb light of particular wavelengths. Most lipids do not have structures that exhibit strong specific absorption bands in the visible, ultraviolet (UV) or infrared (IR) regions of the spectrum (1-5). UV light detectors have found some application with lipids by use of wavelengths at the lower end of the spectrum (190-210 μm) where unsaturated groups exhibit absorptivity (1-4,6,7). IR detectors have also had some use with lipids (8), but they severely limit the solvent systems that can be employed for the separation of lipids. Direct quantitative analysis by UV and IR detectors is complicated by the lack of standards which have specific absorption bands without interference from the absorptivity of other groups (9). These detectors also impose limitations on the solvent systems that can be used for the chromatography, because lipids being highly heterogeneous are separated best by a combination of isocratic and gradient solvent systems (5,10). Detectors based on the principle of refractive index are relatively insensitive and not ideal for use with gradient solvent systems employed for the fractionation of lipids (1). Thus, quantitative analysis of the lipid classes by photometric detectors is generally performed in conjunction with secondary analysis of recovered fractions such as phosphorus for the phospholipids (2).

Methods employing thin layer chromatography (TLC) also provide fine separation of the lipid classes (11-16). Quantitative analysis can be made by photodensitometry of charred spots (11-15), but these methods are highly empirical and depend on the cochromatography of corresponding standards. The same difficulties prevail in the use of the Iatroscan technique for lipid analysis because response factors are not reproducible (17). Thus, quantitative analysis by TLC is also performed best in conjunction with secondary methods of analyses (1,16).

Early models of flame ionization detectors (FID), which in theory should be ideal for lipids inasmuch as they are universal for carbon compounds, were beset with many mechanical problems which made them unsatisfactory. Most of these difficulties have been eliminated in a model developed in this laboratory and it has enabled the fractionation of essentially the complete profile of the lipid classes of plant and animal tissue lipids by HPLC (5,10,18-21).

*Author to whom correspondence should be addressed.

Its application to the quantitative analysis of these compounds is reported here.

EXPERIMENTAL

Standards

Highly purified erucyl alcohol and the lipid class standards were available from the Lipids Preparation Project at The Hormel Institute. The identity and purity of each preparation was monitored by TLC. Neutral lipids used as standards were cholesterol (CH), cholesteryl oleate (CE), triolein (TG) and oleic acid (FFA). Phospholipid standards included rat liver phosphatidylcholine (PC) and lysophosphatidylcholine (LPC); beef brain phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE) and phosphatidylserine (PS); egg sphingomyelin (SPH); soybean phosphatidylinositol (PI); and beef kidney diphosphatidylglycerol (DPG).

Solvents

Solvents used in the extraction of the tissues as well as for HPLC, including regeneration of the column, must be rigorously purified. Skellysolve B, methylene chloride and chloroform were shaken with concentrated sulfuric acid in a separatory funnel several times until both upper and lower phases were clear. The solvents were then washed with water and distilled in an all-glass still. Methanol was distilled in an all-glass still over potassium hydroxide pellets. Analytical grade solvents are used; there are no advantages in the use of solvents purified for photometric detectors because the same purification procedure described above must be performed.

Tissues

Blood serum, liver and kidney tissue, used as specimens in this work, were obtained from Sprague-Dawley rats fed a Lab Chow diet (Purina, St. Louis, MO), ad libitum. The animals were sacrificed by withdrawal of blood from the retroocular plexes; the livers and kidneys were excised and used immediately.

Tissue Extraction

Tissues were extracted first with hot dilute acetic acid (0.05 N) to destroy hydrolytic enzymes and to remove nonlipid substances that contaminate chloroform/methanol extracts. Details of the procedure have been described in previous work (18,22,23).

HPLC

HPLC was carried out with a Spectra Physics Model 3500B liquid chormatograph. The lipid classes were separated with a 45 cm x 0.2 cm id column of 8 μm diameter silica (Spherisorb, Phase Sep, Hauppague, NY) treated with concentrated ammonium hydroxide as previously described (10,18). The liquid chromatograph was operated at a flow rate of 0.5 ml/min, which required a pressure of ca. 900 psi.

Cholesteryl esters and triglycerides were eluted first with Skellysolve B/methylene chloride (1:1, v/v). This solvent is passed through the column for exactly 2 min. Then chloroform/methylene chloride (1:2, v/v) is passed through the column. At the end of 10 min, in which time this solvent clears the system of the first solvent, methanol containing 6% concentrated ammonium hydroxide is added in a linear gradient. Cholesterol is eluted first, followed by the phospholipids which require ca. 30 min. The column is regenerated by passing chloroform/methylene chloride (1:2, v/v), followed by Skellysolve B/methylene chloride (1:1, v/v) through it for 20 min and 10 min, respectively.

Flame Ionization Detector

The detector employed in this work has been previously described (19), a detailed schematic diagram is shown in Figure 1. The belt has a perforated structure (19,24,25) which has a high capacity to retain solvents—up to 1 ml/min with some solvents. Belts are available from Buckbee, Mears & Company, St. Paul, MN. Under normal operating conditions these belts have a lifetime of over a year of continuous operation. The evaporator is operated at 180 C with a nitrogen flow rate of 1.5 ℓ/min; the speed of the belt is 1.5 cm/sec. The reactor is maintained at 600 C; nitrogen and hydrogen, used as carrier gases, are passed through at 160 and 40 ml/min, respectively. Nitrogen is also used as the blocking gas at the external end of the reactor at a flow rate of 80 ml/min. Compressed air is passed through the chamber around the flame at 250–300 ml/min. The signal from the flame detector is amplified and passed into an IMSAI microprocessor which contains hardware and software for integration and subsequent printout of percent composition.

RESULTS

Separation of a profile of the lipid classes from the neutral lipids to LPC of the phospholipids for rat kidney, serum and liver is shown in Figure 2. It was found that erucyl alcohol served as a good internal standard because it eluted in the gap in the elution pattern between

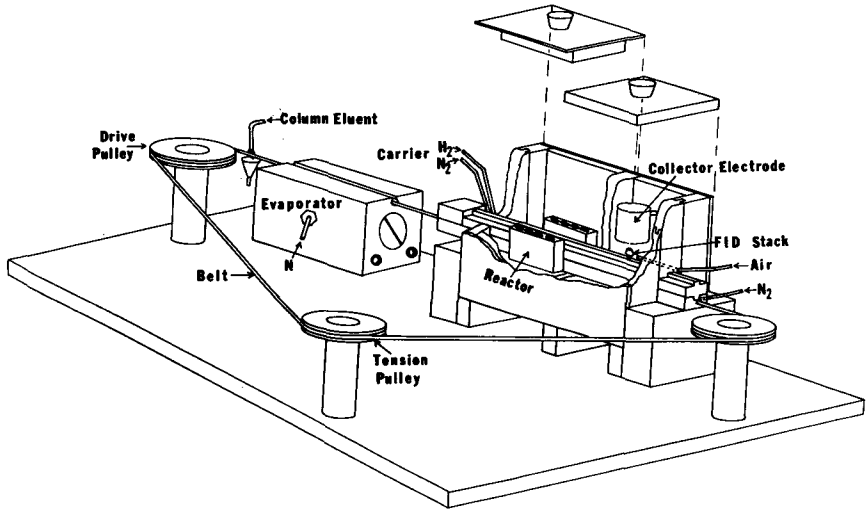


FIG. 1. Schematic diagram of the flame ionization detector.

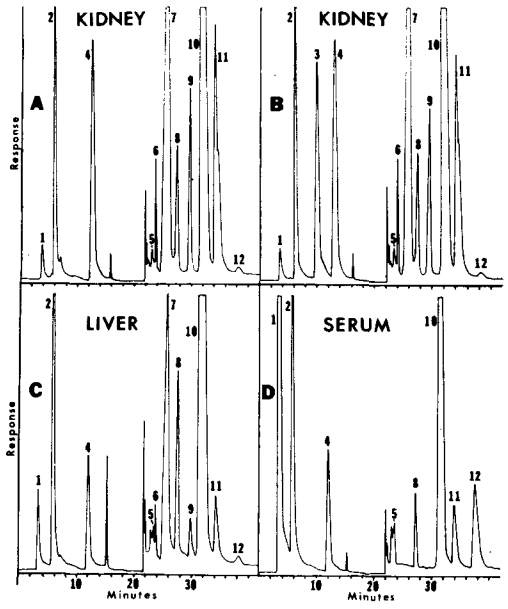


FIG. 2. 1-Cholesteryl esters; 2-triglycerides; 3-erucyl alcohol (internal standard); 4-cholesterol; 5-free fatty acids; 6-diphosphatidylglycerol; 7-phosphatidylethanolamine; 8-phosphatidylinositol; 9-phosphatidylserine; 10-phosphatidylcholine; 11-sphingomyelin; 12-lysophosphatidylcholine. (A) rat kidney total lipid; (B) rat kidney total lipid plus erucyl alcohol; (C) rat liver total lipid; (D) rat serum total lipid. Column and gradient conditions are described in text.

triglycerides and cholesterol (Fig. 2B). This compound is generally absent in mammalian tissues and serves as a good compound to standardize the conditions of the analysis and operation of the system. Each lipid class gave a curvilinear relationship between the amount of sample and response as illustrated for PC in Figure 3. While the relationship is very close to linearity over limited ranges, it becomes curvilinear over an extended range required for the analysis of the large range of lipid class concentrations in natural lipids. Otherwise, the best fit linear relationship does not pass through zero. That the relationship curves to zero is illustrated in the insert in Figure 3B of the analysis of small amounts of PC. Accordingly, response factors were calculated for each lipid class according to the following equation, $Y = AX^B$, where Y = the response in peak area, X = the amount of sample in μg, A = a constant, and B = the response index as defined by Fowlis and Scott (26) and employed by Scott and Lawrence (27). The response index takes into account the deviation from direct proportionality. The mass of each component and percent composition is determined via a microprocessor which prints out the data in percentage composition based on the response factors relative to the response of the erucyl alcohol. The response factors were highly reproducible and gave recoveries of 100 ± 3% with standard mixtures of phospholipids; hence, it was not necessary to add the internal standard to every sample.

The reproducibility of the method was illustrated by the analysis of several samples of rat serum in Table 1 taken from a study of the influence of sucrose and dextrose diets on fatty acid composition and acyl desaturase activities of the liver microsomes of Sprague-Dawley rats

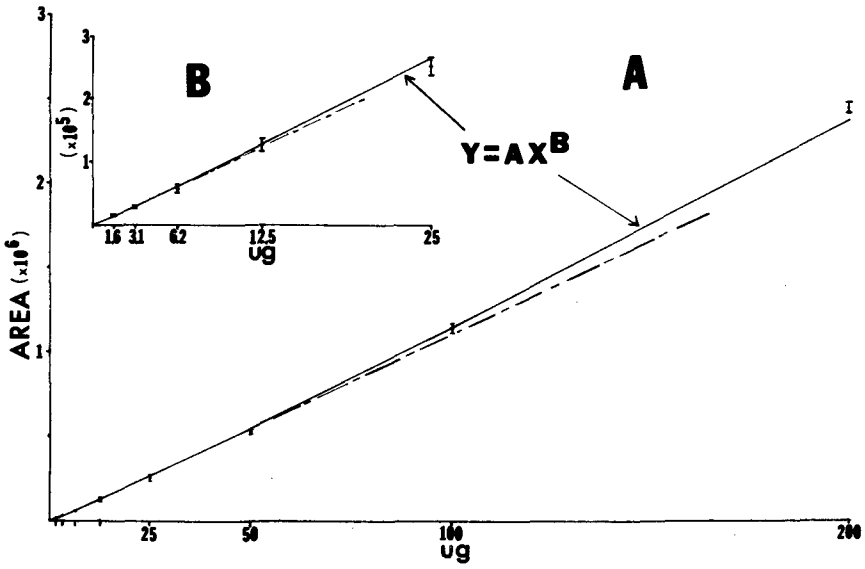


FIG. 3. (A) response curve of phosphatidylcholine. (B) enlargement of low concentration responses of phosphatidylcholine. Dashed lines are indicative of linear response. All points are the mean ± SD of 3 determinations.

TABLE 1

HPLC Analysis of Serum Lipids of Rats Fed Diets Differing in Fat or Carbohydrate (28)

Lipid class	Sucrose + 10% HCO	Dextrose + 10% HCO	Sucrose + 10% SAFF	Dextrose + 10% SAFF	Fat-free sucrose	Fat-free dextrose
CE	17.9 ± 1.3[a]	17.8 ± 1.4	16.3 ± 1.5	17.1 ± 0.6	18.3 ± 3.3	25.9 ± 1.1
TG	36.6 ± 0.2	35.7 ± 1.1	50.2 ± 1.1	46.7 ± 2.1	40.0 ± 0.8	33.1 ± 0.3
DPG	1.5 ± 0.1	2.3 ± 0.1	1.9 ± 0.2	2.8 ± 0.2	1.4 ± 0.2	2.9 ± 0.2
FFA	5.4 ± 0.6	6.0 ± 1.0	3.4 ± 0.4	3.4 ± 0.2	2.6 ± 0.1	4.1 ± 0.1
PI	3.8 ± 0.3	3.0 ± 0.5	2.5 ± 0.2	3.0 ± 0.3	3.2 ± 0.2	3.9 ± 0.3
PC	25.7 ± 0.5	26.7 ± 0.6	17.7 ± 0.3	17.6 ± 0.8	24.4 ± 1.3	20.4 ± 0.1
SpH	2.2 ± 1.1	2.0 ± 0.6	1.0 ± 0.7	2.0 ± 0.5	2.0 ± 0.4	1.9 ± 0.2
LPC	6.9 ± 0.3	6.3 ± 0.3	6.3 ± 0.2	7.1 ± 0.6	7.6 ± 0.7	7.8 ± 0.6

[a]Range of values of duplicate determinations. CE = cholesteryl esters, TG = triglycerides, DPG = diphosphatidylglycerol, FFA = free fatty acids, PI = phosphatidylinositol, PC = phosphatidylcholine, SpH = sphingomyelin, LPC = lysophosphatidylcholine.

(28). These results show that the variation between duplicate analyses was generally less than 1.0%.

Table 2 shows the comparison of the analysis of the lipid classes of Azolectin by our method and by TLC using charring-densitometry which was performed on the same sample earlier in our laboratory (20). These analyses agreed reasonably well considering they were performed many months apart and by completely different techniques.

Table 3 shows a comparison with our method of the analysis of the phospholipids of three samples of rat liver mitochondria lipid by TLC via the method of Rouser et al. (16) and subsequent phosphorus analysis by the method of Bartlett (29), of recovered spots for quantification. The agreement between the two methods likewise was very good.

DISCUSSION

The use of HPLC in conjunction with a flame ionization detector for the quantitative analysis of the lipid classes is well demonstrated in the present work. Although individual

TABLE 2

Analyses of Azolectin

Lipid class	% Composition		
	HPLC	HPLC[a]	TLC (20)
TG	2.10 ± 0.29	1.78 ± 0.14	2.26 ± 0.33
ST	0.62 ± 0.32	0.12 ± 0.02	0.25 ± 0.08
FFA	0.27 ± 0.27	0.20 ± 0.01	0.13 ± 0.02
ESG	8.20 ± 0.40	9.01 ± 0.21	7.00 ± 0.33
DGDG	2.13 ± 0.03	2.00 ± 0.18	1.92 ± 0.33
SG	4.50 ± 0.02	4.98 ± 0.48	4.18 ± 0.77
PE	27.62 ± 0.26	27.34 ± 0.27	26.54 ± 0.77
PI	16.94 ± 0.24	16.54 ± 0.23	17.05 ± 0.88
PA	6.82 ± 0.10	6.28 ± 0.44	7.91 ± 0.88
PC	30.85 ± 0.55	31.77 ± 0.69	32.75 ± 2.31

TG = triglyceride, ST = sterols, FFA = free fatty acids, ESG = esterified sterol glucoside, DGDG = digalactosyldiglyceride, SG = sterol glucoside, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PA = phosphatidic acid, PC = phosphatidylcholine.

[a]Analysis of same Azolectin sample 5 months later.

TABLE 3

Analyses of Three Different Samples of Rat Liver Mitochondria Phospholipid Classes by HPLC and TLC

Phospholipid class	% Composition					
	Sample 1		Sample 2		Sample 3	
	HPLC	TLC[a]	HPLC	TLC	HPLC	TLC
DPG	14.4	15.4	13.4	12.5	4.5	4.9
PE	37.5	35.4	36.1	35.7	37.9	41.0
LPE	Tr	0.5	1.2	2.0	1.4	2.1
PI	2.8	2.6	5.4	7.1	1.3	3.0
PC	43.8	43.4	44.0	41.7	54.9	48.2
SpH	1.5	0.8	Tr	0.5	Tr	0.6

[a]TLC: 2-dimensional TLC, followed by phosphorus analyses (16,29). DPG = diphosphatidylglycerol, PE = phosphatidylethanolamine, LPE = lysophosphatidylethanolamine, PI = phosphatidylinositol, PC = phosphatidylcholine, SpH = sphingomyelin.

molecular species of the lipid classes give different responses in the flame detector, the differences are relatively small in most cases, probably because all compounds are converted to hydrocarbons in the reactor (19). Hence, lipid classes isolated from natural lipids or esters of oleate serve very well as standards for the analysis of natural occurring mixtures of these compounds. One column can be regenerated while another is in use, thus, the method can be operated virtually continuously (18). Because of the large difference in polarity, and accordingly, difference in composition of the eluting solvents, the neutral lipids can be analyzed separately from the phospholipids. Likewise, the method can be used for the independent analysis of the phospholipids because the gradient system used to separate these compounds elutes the neutral lipids with the solvent front.

Relatively large samples were used in the present work, from a practical standpoint, to permit the analysis of minor (<1.0%) as well as major components of natural lipids. However, because virtually baseline separations are achieved with relatively large sample loads, it is evident that by taking advantage of modern column technology, the speed of the fractionation can be increased without loss of resolution and with much smaller samples in many cases. Thus, it should be possible to increase the sensitivity of the method by modification of the column parameters inasmuch as the detector is capable of the detection of nanogram amounts of the lipid classes (19). Work is currently in

progress on this aspect of the method.

ACKNOWLEDGMENTS

This work was supported in part by US Public Health Service Grants AG 00174 from the National Institute on Aging, HL 08214 from the Program Project Branch, Extramural Programs, National Heart, Lung and Blood Institute and the Hormel Foundation. The authors thank K. Broekemeier, P. Schmid and D. Pfeiffer for the TLC-phosphorus analyses of the samples of mitochondria lipid.

REFERENCES

1. Aitzetmuller, K. (1976) J. Chromatogr. 113, 231–266.
2. Patton, G.M., Fasulo, J.M., and Robins, S.J. (1982) J. Lipid Res. 23, 190–196.
3. Plattner, R.D. (1981) in Methods in Enzymology (Lowenstein, John M., ed.) Vol. 72, pp. 21-34, Academic Press, New York, NY.
4. Porter, N.A., and Weenen, H. (1981) in Methods in Enzymology (Lowenstein, John M., ed.) Vol. 72, pp. 34-40, Academic Press, New York, NY.
5. Privett, O.S., and Erdahl, W.L. (1975) in Analysis of Lipids and Lipoproteins (Perkins, E.G., ed.) pp. 123-137, Am. Oil Chem. Soc., Champaign, IL.
6. Jungalwala, F.B., Evans, J.E., and M'Cluer, R.H. (1976) Biochem. J. 155, 55–60.
7. Geurto Van Kessel, W.S.M., Hax, W.M.A., Demel, R.A., and DeGier, J. (1977) Biochim. Biophys. Acta 486, 524–530.
8. Freeman, N.K., Alpham, F.T., and Windsor, A.F. (1973) Anal. Lett. 6, 943–950.
9. Herslof, B.G., and Peluna, T.J. (1982) Abstract #295, 73rd Annual Meeting of the Am. Oil Chem. Soc., Toronto, Canada.
10. Stolyhwo, A., and Privett, O.S. (1973) J. Chromatogr. Sci. 11, 20–25.
11. Privett, O.S., Dougherty, K.A., and Erdahl, W.L. (1973) in Quantitative Thin Layer Chromatography (Touchstone, J., Waley, J., and Sons, eds.) pp. 57–78, New York, NY.
12. Nutter, L.J., and Privett, O.S. (1968) J. Chromatogr. 35, 519–525.
13. Blank, M.L., Schmit, J.A., and Privett, O.S. (1964) J. Am. Oil Chem. Soc. 41, 371–376.
14. Privett, O.S., Blank, M.L., Codding, D.W., and Nickell, E.C. (1965) J. Am. Oil Chem. Soc. 42, 381-393.
15. Rouser, G.O., Galli, E., Blank, M.L., and Privett, O.S. (1964) J. Am. Oil Chem. Soc. 41, 836–840.
16. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Lipids 5, 494–496.
17. Crane, R.T., Goheen, S.C., Larkin, E.C., and Rao, G.A. (1982) Poster Session, p. 15, 73rd Annual Meeting of the Am. Oil Chem. Soc., Toronto, Canada.
18. Phillips, F.C., and Privett, O.S. (1981) J. Am. Oil Chem. Soc. 58, 590–594.
19. Privett, O.S., and Erdahl, W.L. (1978) Anal. Biochem. 84, 449–461.
20. Erdahl, W.L., Stolyhwo, A., and Privett, O.S. (1973) J. Am. Oil Chem. Soc. 50, 513–515.
21. Privett, O.S., Dougherty, K.A., Erdahl, W.L., and Stolyhwo, A. (1973) J. Am. Oil Chem. Soc. 50, 516-520.
22. Phillips, F.C., and Privett, O.S. (1979) Lipids 14, 590–595.
23. Phillips, F.C., and Privett, O.S. (1979) Lipids 14, 949–952.
24. Erdahl, W.L., and Privett, O.S. (1977) Lipids 12, 797–803.
25. Privett, O.S., and Erdahl, W.L. (1978) Chem. Phys. Lipids 21, 361–378.
26. Fowlis, I.A., and Scott, R.P.W. (1963) J. Chromatogr. 11, 1–10.
27. Scott, R.P.W., and Lawrence, J.G. (1970) J. Chromatogr. Sci. 8, 65–71.
28. DeSchrijver, R., and Privett, O.S. (1982) Abstract #14, 73rd Annual Meeting of the Am. Oil Chem. Soc., Toronto, Canada.
29. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.

[Received July 14, 1982]

A High-Yield Saponification of Galactosylceramide I^3-Sulfate

K.M. KOSHY and **J.M. BOGGS***, *Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8*

ABSTRACT

A method for the deacylation of galactosylceramide I^3-sulphate using aqueous methanolic KOH is described. The combination of a relatively low concentration of the alkali (0.3 M) and a moderate reaction temperature (reflux point of 90% methanol) results in the formation of galactosylsphingosine I^3-sulphate in consistently high yields (61%) with a minimum of side reactions. The product was purified by column chromatography and its identity established by thin layer chromatography, infrared spectroscopy, determination of galactose content and organic sulphate assay using established methods or their modifications.
Lipids 17:998-1000, 1982.

Although galactosylceramides (cerebrosides) can be hydrolyzed with *n*-butanolic KOH to galactosylsphingosine in high yield (1), the applications of similar methods for the deacylation of galactosylceramide I^3-sulphate (I) (cerebroside sulphate, also called sulphatide)—another important lipid in myelin—has proved to be unsatisfactory (2).

The saponification of I with *n*-butanolic KOH results in side reactions other than the cleavage of the amide linkage and the yield of galactosylsphingosine I^3-sulphate (II) is very low (2). We describe here a procedure for the deacylation of I which minimizes the loss of the material to other side reactions.

EXPERIMENTAL METHOD AND RESULTS

'Bovine brain sulphatides', cerebrosides, and ceramides, purchased from Supelco Inc., Bellefonte, PA, were used without further purification. All solvents used were reagent grade. Silica gel 100 (70-230 mesh, E. Merck) was used for column chromatography and silica gel 60 (E. Merck) precoated plates were used for thin layer chromatography (TLC). Infrared (IR) spectra were recorded on a Beckman IR spectrophotometer and colorimetric measurements were made on a Beckman Acta MVI spectrophotometer.

One hundred mg of bovine brain sulphatides were placed in a 200-ml round bottomed flask, together with a magnetic stirring bar. One hundred ml of 0.3 M KOH in methanol/water (90:10, v/v) were added. The flask was fitted with a reflux water condenser provided with a guard tube containing KOH pellets. The flask was heated in an oil bath maintained at 80 C and the contents were kept gently refluxing for a period of 6 days. Most of the methanol was then distilled off under reduced pressure using a rotary evaporator (50 C). After cooling to room temperature, 1 N HCl was added carefully with stirring until the apparent pH of the solution was between 7 and 8. After removing any methanol remaining using the rotary evaporator, the residual material was lyophilized. The solid left behind was suspended in 10 ml of water and extracted with three 10-ml portions of tetrahydrofuran (THF). The combined THF extracts were evaporated to dryness, the residue resuspended in 4 ml of chloroform/methanol (2:1, v/v) and centrifuged. The supernatant solution was applied to a chromatographic column prepared with 20 g of silica gel 100 and washed with 75 ml of chloroform/methanol/concentrated ammonium hydroxide (65:25:4, v/v/v). The column was eluted with 350 ml of the same solvent mixture. The fractions of 5 ml each were monitored by TLC on silica gel 60 plates using the solvent system mentioned above. The last component eluted was identified as II as described below. The fractions containing this component were pooled and evaporated to dryness; the product weighed 36 mg.

The component identified as II was the only ninhydrin positive spot observed by TLC of the hydrolysis products in chloroform/methanol/ammonium hydroxide (65:25:4, v/v/v), and had an Rf ≈ 0.24 in agreement with the value reported in the literature (2). The IR spectrum of this product (Fig. 1) showed bands at 810 and 1240 cm^{-1} characteristic of the sulphate group and is identical in every other respect with that obtained for lysosulphatide by Nonaka and coworkers. Determinations of the galactose (3) and organic sulphate content (4)

*Author to whom correspondence should be addressed.

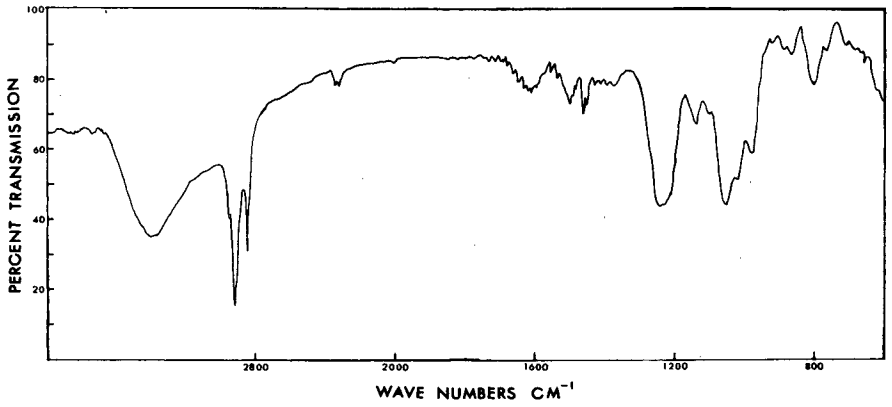


FIG. 1. Infrared spectrum of galactosylsphingosine I^3-sulphate (KBr pellet).

were in agreement with the corresponding values calculated for galactosylsphingosine I^3-sulphate: galactose found 33.2%, calculated 33.3%; sulphate found 17.4%, calculated 17.74%. Taking into account the presence of the free amino group in II, the procedure of Kean for sulphate analysis (4) was modified as follows: after evaporating to dryness, an aliquot (containing .0065 μmol) of a solution of II in chloroform/methanol (2:1, v/v), 200 μl of pyridine and 10 μl of acetic anhydride were added and the mixture was heated at 50 C for 30 min. The pyridine was then evaporated and the residue dried under vacuum. The rest of the procedure was unchanged.

Thin layer chromatographic examination (chloroform/methanol/water, 70:30:5, v/v/v) of the remaining fractions after removal of the lysosulphatide present in the hydrolysate, showed three major components besides the fatty acids. Two of these comigrated with the major component of natural ceramides and the nonhydroxy fatty acid containing portion of bovine brain sulphatides, respectively. The behavior of the third component on TLC was different from that of natural cerebrosides, ceramides, or sulphatides; therefore, it was inferred to be a product formed by degradation or rearrangement of I, other than a straight loss of sulphate group by SN_2 displacement. This observation is in perfect agreement with the remarkable stability to bases of the sulphate group in sulphatides as reported in the literature (5). Attempts to hydrolyse the recovered sulphatides under the same conditions described above gave only a further 1 mg of II, indicating that this is a component of the original sulphatides which are not deacylated readily. The absence of any α-hydroxy fatty acid containing I in this fraction as indicated above can be understood in the light of the expected faster rate of hydrolysis of the amides of α-hydroxy fatty acids.

The well known sluggish rate of hydrolysis of fatty acid amides (6) together with the tendency of I to form breakdown products under severe reaction conditions warrants careful selection of conditions to optimize the formation of II. We have found that the combination of a relatively low concentration of alkali (0.3 M) and the reflux temperature of 90% methanol (68.5 C) favor high yields of II. With various batches of bovine brain sulphatides, we have been able to obtain consistently, yields not less than 61% (based on an average MW of 894 for I) in comparison with a yield of 10% reported by Nonaka and coworkers (2).

Attempts to shorten the reaction time by raising the temperature to 100 C (sealed ampule) or employing polar aprotic solvents such as acetone resulted in the formation of significant amounts of breakdown products.

To keep the lipid completely in solution in the hydrolysis medium, it was necessary to use a ratio of 1 ml of the alkali solution for every milligram of the lipid. Extraction of the hydrolysis product with tetrahydrofuran was found to be much more convenient than using chloroform/methanol, especially in view of the 'soapy' nature of the aqueous suspensions of sulphatides and their hydrolysis products. Tetrahydrofuran is miscible with pure water, but the presence of dissolved salts in water causes separation into two distinct layers, THF forming the top layer. The solubility of the lipid products in dry THF is limited, but the small amounts of water extracted into it from the aqueous layer considerably increases its solvent power. Three extractions with the solvent almost completely removes all the lipid

products.

In our laboratory, we have applied this method to the hydrolysis of cerebroside sulphate ranging in quantity from 20 mg to 500 mg with reproducible results and we consider it as a definite improvement over the existing methods.

ACKNOWLEDGMENTS

This study was supported by a grant from the Multiple Sclerosis Society of Canada and a MS Society Career Development Award to J.M.B.

REFERENCES

1. Radin, N.S. (1974) Lipids 9, 358-360.
2. Nonaka, G., Kishimoto, Y., Seyama, Y., and Yamakawa, T. (1979) J. Biochem. 85, 511-518.
3. Kushwaha, S.C., and Kates, M. (1981) Lipids 16, 372-373.
4. Kean, E.L. (1968) J. Lipid Res. 9, 319-327.
5. Stoffyn, P., and Stoffyn, A. (1963) Biochim. Biophys. Acta 70, 218-220.
6. Klenk, E., and Harle, R. (1928) Z. Physiol. Chem. 178, 221.

[Received August 2, 1982]

COMMUNICATIONS

Anesthetics May Restructure the Hydrogen Belts of Membranes

H. BROCKERHOFF, *Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314*

ABSTRACT

Anesthetic molecules can form hydrogen bonds or organize hydrogen-bond networks. It is argued that they affect the neuronal cell membrane not by an amorphous fluidization of the hyprophobic core of the lipid bilayer but by a restructuring of its hydrogen belts, i.e., the regions occupied by the CO and OH groups of the membrane lipids. The structured disturbance of the hydrogen-bond network is translated latitudinally to hydrogen-bonding sites of the proteins of the membrane, causing allosteric changes of ion channels that result in neuronal blocking.
Lipids 17:1001-1003, 1982.

Numerous chemicals, with varied and unrelated structures, can serve as general anesthetics (and many as hypnotics, or anticonvulsants) (1-3). Because of this lack of specificity, and because the anesthetic potency of a drug correlates with its solubility in lipid, anesthetics are considered to act in an unspecific manner in and on the lipid bilayer of the neuronal membrane, by creating a disturbance which is then transferred to the proteins of the membrane. Consequently, the sodium channel proteins in particular lose their proper conformations, and neuronal conductance stops. In recent theories, anesthetics have been though to expand the lipid bilayer (1) cause it to thicken (4,5), increase the "molten" portion of the bilayer (6,7), or melt a lipid annulus that surrounds the protein (8). These effects are taken to be consequences of the "fluidization" of the membrane by anesthetics (1-3). Explanations invoking hydrogen bonding as mechanism of anesthesia (9-11) are not popular at present.

Fluidization looms large in all recent discussions of membranes, but I believe that too much is made of it: that, in the case of anesthesia, if it occurs at all, it is an accidental event without much consequence; a similar view is held by others (12). Fluidization, i.e., the increase of molecular (or segmental molecular) movement in the bilayer, can be assessed by various methods, such as fluorescence (6, 13), electron spin resonance (7,14,15), or nuclear magnetic resonance (16). Applied to anesthetics in membranes, such methods variously show that fluidization only occurs at drug concentrations higher than those clinically required (1,3) or barely appears at such drug levels (3). Some anesthetics even suppress membrane fluidity (12,15,17). But even if we accept fluidization as real, we still find that the effect at clinical drug concentrations would be very small, with membrane order parameters changing by a few percent only (3,14).

Why should a small increase in fluidity, i.e. a small decrease in the viscosity of the solvent-like lipid core, have any effect on the conformation of membrane proteins? A raise in temperature also reduces solvent viscosity, but a fever does not cause anesthesia; on the contrary, cooling does (12). Membrane fluidization is an unstructured phenomenon, a weakening of intermolecular hydrophobic (van der Waals) forces, without direction. It is an amorphous event that offers no explanation for a phenomenon that is the skeleton in the closet of all "unified" theories of anesthesia, namely, the great diversity of clinical as well as in vitro effects of different anesthetics (1-3,17).

Lipid bilayers are usually treated as consisting of a hydrophobic core sandwiched by two hydrophilic polar zones. In Figure I, attention is also given to the region which I call hydrogen belt because it is neither polar nor hydrophobic but contains hydrogen bond acceptors (CO of phospho- and sphingolipids) and hydrogen-bond donors (OH of cholesterol, sphingosine, water (18,19)). The hydrogen belt is structured because hydrogen bonds are vectorial, in contrast to the nondirected van der Waals forces of the hydrophobic core. Hydrogen bonding is also highly cooperative, i.e., it is translated intermolecularly, as exemplified by the "iceberg" formation in fluid water. Manipulation of the composition of the hydrogen belt changes the structure of the hydrogen-bond network. This is demonstrated in experiments (20,21) measuring the permeation of the same molecule, glycerol, through various bilayers differing in

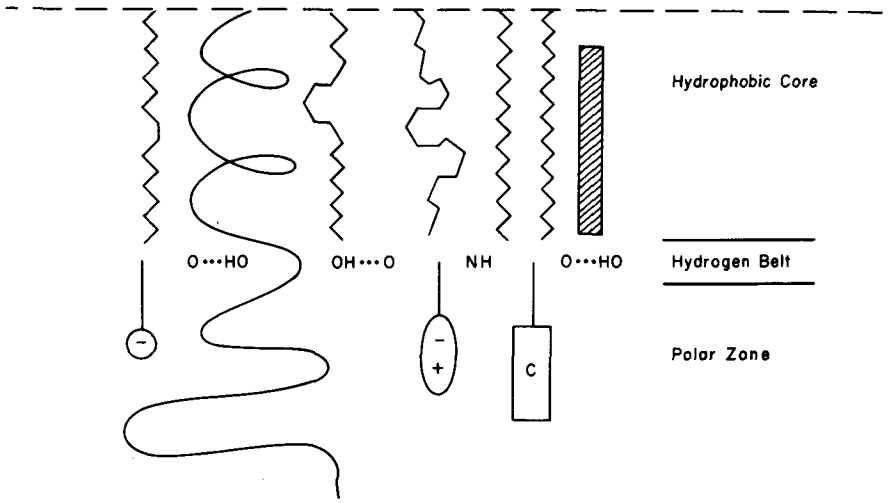


FIG. 1. Stratification of a biological membrane. One half of the membrane shown. Zigzag lines represent aliphatic chains; the shaded rectangle is cholesterol; the coil, a membrane protein; + and - are charged phospholipid heads; C is carbohydrate; and O atoms are supplied by the carbonyl groups of phospho- and sphingolipids; NH from sphingolipids and proteins; OH from cholesterol, sphingosin, water, and protein.

their hydrogen belt composition. The entropy of activation for the process is twice as large for a phosphatidylcholine-cholesterol bilayer as for a sphingomyelin-cholesterol bilayer, and twice as large again in a lysophosphatidylcholine-cholesterol bilayer. As argued elsewhere (20, 21), these results show that the hydrogen belts of different bilayers have different degrees of order. In Figure 1, it is suggested that the proteins of the membrane (represented by the helix) participate in the hydrogen-bond network. This participation is restructured when the hydrogen belt is modified by the incorporation of anesthetic molecules. Anesthetics, being amphiphatic molecules, are all likely to seek residence in the hydrogen belt, if transiently, with at least part of their molecule. Those which cannot form hydrogen bonds, e.g. rare gases and small alkanes, can organize the hydrogen bonds around them (as clathrates) (9,10). Hydrogen bonds are formed, or broken, or redirected, and the conformation of the protein is thereby changed. The proteins of the membrane must be regarded as allosteric structures in which hydrogen-bonding sites are the allosteric sites and lipids, water, or drugs are the effectors.

Evidence of lipid-protein bonding via hydrogen bonds has been found for phospholipid transfer proteins (22), but interaction of a membrane protein proper with the hydrogen belt has not yet been demonstrated. The argument for such interaction is nevertheless compelling: given the fact that membrane proteins are encircled by hydrogen belts, it would be unreasonable to assume that readjustment of such a girdle would *not* have an effect on protein conformation.

It is now easy to see how different anesthetics can have different effects on a neuron. As an example, in the membrane, the aliphatic isopentyl group of pentobarbital (a hypnotic) will be more strongly drawn toward the core than the phenyl group of phenobarbital (an anticonvulsant), conferring a different location and orientation on the barbituric ring. Similarly, the "anesthetic cut-off", i.e., the disappearance of potency when the chain of an aliphatic alcohol is lengthened to 14 carbons (23), may be explained: the O-H vector changes as the molecular chain is drawn more deeply into the hydrophobic membrane core. Even the possibility of stereospecific anesthetic action is opened: it is conceivable that the D and L species of a lipophilic drug will align differently between the stereospecific phospholipid and cholesterol molecules of the bilayer and form differently oriented hydrogen bonds in the hydrogen belt. The hypothesis also offers an explanation why the cationic local anesthetics are active only at concentrations 10 or 20 times higher than those required of general anesthetics (1). These drugs may bind to the negative head groups of phospholipids, so remote (several bond lengths) from the hydrogen belt that a massive dose is needed before any restructuring of the belt is

induced. That high pressure influences anesthesia (1–3) (it is generally antagonistic) becomes understandable if it is realized that hydrogen bonding (and not fluidity only) responds to pressure (24).

The hypothesis of hydrogen-belt restructuring allows the possibility that a drug may hydrogen-bond immediately to a protein, without intercalation of lipid or water, in the hydrogen-belt region. It does not collide with the Meyer-Overton rule which states that anesthesia commences when any chemical substance has reached a certain concentration in the lipid bilayer (1), and it does not demand that every hydrogen-bonding chemical must upset the hydrogen-belt architecture in such manner as to cause anesthesia. Since no two chemicals will have exactly the same effect on the hydrogen belt, no simple rule linking the structure of a chemical with its anesthetic activity can be expected. Still, the hypothesis may be useful as a guiding principle in the search, not only for new anesthetics, but for membrane-anchored drugs in general. Such drugs might have the general structure of hydrogen-bonding molecules provided with anchoring, lipophilic tails.

REFERENCES

1. Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
2. Roth, S.H. (1979) Ann. Rev. Pharmacol. Toxicol. 19, 159–178.
3. Miller, K.W. (1981) in Burger's Medical Chemistry, (M.E. Wolf, ed.) Part III, 4th edn, pp. 623–644, John Wiley & Sons, New York, NY.
4. Haydon, D.A., Hendry, B.M., Levinson, S.R., and Requena, J. (1977) Nature 268, 356–358.
5. Ashcroft, R.G., Coster, H.G.L., and Smith, J.R. (1977) Nature 269, 819–820.
6. Vanderkooi, J.M., Landesberg, R., Selick, II, H., and McDonald, G.G. (1977) Biochim. Biophys. Acta 464, 1–16.
7. Trudell, J.R. (1977) Anesthesiology 46, 5–10.
8. Lee, A.G. (1976) Biochim. Biophys. Acta 455, 102–108.
9. Miller, S.L. (1961) Proc. Natl. Acad. Sci. USA 47, 1515–1524
10. Pauling, L. (1961) Science 134, 15–21.
11. DiPaolo, P., and Sandorfy, C. (1974) J. Med. Chem. 17, 809–814.
12. Richards, C.D., Martin, K., Gregory, S., Keightley, C.A., Hesketh, T.R., Smith, G.A., Warren, G.B., and Metcalfe, J.C. (1978) Nature 276, 775–779.
13. Feinstein, M.B., Fernandez, S.M., and Sha'afi, R.I. (1975) Biochim. Biophys. Acta 413, 354–370.
14. Miller, K.W., and Pang, K-Y. Y. (1976) Nature 263, 253–255.
15. Pringle, M.J., and Miller, K.W. (1978). Biochem. Biophys. Res. Commun. 85, 1192–1198.
16. Shieh, D.D., Ueda, I., and Eyring, H. (1975) in Molecular Mechanisms of Anesthesia—Progress in Anesthesiology, (Fink, B.R., ed.) Vol. 1, pp. 307–312, Raven Press, New York, NY.
17. Roth, S.H. (1980). Fed. Proc. 39, 1595–1599.
18. Brockerhoff, H. (1974). Lipids 9, 645–650.
19. Brockerhoff, H. (1977) in Biorganic Chemistry, (van Tamelen, E.E., ed.) Vol. III, pp. 1–20, Academic Press, New York, NY.
20. Tirri, L.J., Ayengar, N.K.N., Lipton, L.C, Chatterjie, N., and Brockerhoff, H. (1978) Lipids 13, 267–269.
21. Ramsammy, L.S., and Brockerhoff, H. (1982). J. Biol. Chem. 257, 3570–3574.
22. Somerharju, P., Brockerhoff, H., and Wirtz, K.W.A. (1981) Biochim. Biophys. Acta 649, 521–528.
23. Pringle, M.J., Brown, K.B., and Miller, K.W. (1981) Mol. Pharmacol. 19, 49–55.
24. Vinogradow, S.N., and Linnell, R.H. (1970) in Hydrogen Bonding, p. 37, Van Nostrand Reinhold Co., New York,, NY.

[Received July 23, 1982]