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The influence of exogenous cholesterol on hepatic lipid composition of the rat*

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SUMMARY

Rats were fed diets containing cottonseed oil and/or cholesterol, cholic acid, methyl esters of long-chain fatty acids, and tocopherol. Livers, hearts, and aortas were examined histologically; liver lipids were fractionated by silicic acid chromatography; and the fatty acid composition of the sterol esters, triglycerides, and phospholipids was determined by gas-liquid chromatography. The degree of deposition of liver sterol esters seemed to be related to the availability of dietary fatty acids for esterification with exogenous cholesterol. No differences in sterol ester deposition were noted among the groups in which the dietary cholesterol was supplemented with methyl esters of fatty acids of different degrees of unsaturation. Fatty acid analysis revealed an accentuation by cholesterol feeding of the increase in monoenoic fatty acids and the decrease of linoleic and arachidonic acids characteristic of essential fatty acid deficiency. Two eicosatrienoic acids, occurring primarily in the phospholipids, were identified. The 5,8,11-eicosatrienoic acid appears to be related to essential fatty acid deficiency, whereas the 8,11,14-isomer is probably an intermediate in the conversion of linoleic to arachidonic acid and is increased when exogenous cholesterol induces an increased arachidonic acid synthesis.

The liver has been shown to be essential in the metabolism and turnover of both endogenous and exogenous cholesterol (1). Alterations in liver fatty acid composition resulting from cholesterol feeding have been reported in rats (2, 3), rabbits (4, 5, 6), and chickens (7, 8). The primary aspect of the present study was to investigate the nature of these cholesterol-induced aberrations in fatty acid metabolism and their significance in relation to exogenous cholesterol.

A diet containing cholesterol and cholic acid has been demonstrated to produce hypercholesterolemia and atheroma formation in rats (9). Cholesterol fed either with no fat or with saturated fat to rabbits produced a greater degree of atheroma formation than cholesterol fed with unsaturated fat (10, 11). Supplementation of high cholesterol diets fed to rabbits with small amounts of essential fatty acids, however, failed to produce a noticeable difference in the development of atherosclerosis, although marked differences in tissue lipid fatty acids were noted (4).

The possibility that unprotected, oxidized, unsaturated fatty acids may inhibit or accentuate atherogenesis must also be considered. Weitzel (12) administered large oral doses of Vitamin E to cholesterol-fed chickens and reported an increase in serum cholesterol, no effect on liver cholesterol, and a slight reduction in atheromatous deposits.

Another purpose of the present study was to determine any differential effect of the administration to cholesterol-fed rats of methyl esters of different saturated and unsaturated long-chain fatty acids and excess vitamin E on liver lipid deposition and atherogenesis.

METHODS

Four-month-old male albino rats of our colony (U.S.C. strain) were divided into 9 groups of 12 each. Group I received a diet containing cottonseed oil (A), group II a diet containing cottonseed oil and cholesterol (B), group III a fat-free diet (C), and groups IV–IX

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a fat-free diet containing cholesterol (D). Groups V–IX were given force-fed oral supplements of 100 mg/animal/day of methyl palmitate (V), stearate (VI), oleate (VII), linoleate (VIII), or linoleate plus 10 mg/day of α-tocopherol (IX). All cholesterol-containing diets included 1% cholic acid.[1] The compositions of the basal diets are shown in Table 1.

After 12 weeks on the diets, the animals were killed by intraperitoneal injection of sodium pentobarbital (Nembutal). Livers were immediately excised and frozen for later chemical analysis. Hearts, aortas, and parts of livers were removed and placed in 10% formalin. Frozen sections were prepared and stained for lipids by the oil red-O method. The stained sections were also examined under the polarizing microscope for the demonstration of birefringent refractile crystals typical of cholesterol.

Livers were pooled in pairs, cut into small pieces, lyophilized, and dried under high vacuum. Samples (0.3–0.4 g) were ground to a powder with a mortar and pestle and extracted in a Servall blendor by using one 100-ml and three successive 50-ml portions of methylal–methanol 4:1. The extraction mixtures were centrifuged, the supernatant layers were evaporated to near dryness at 40° under a stream of prepurified nitrogen, and the drying was completed under high vacuum. The lipid was redissolved in 50 ml of pentane and the solutions were centrifuged, leaving a small water-soluble residue. The pentane extracts were evaporated under nitrogen, vacuum dried, and weighed. The lipid was then dissolved in 5 ml of pentane and stored under nitrogen at −20°.

The total lipid was fractionated by silicic acid chromatography using a modification of the method of Mead and Gouze (13). Silicic acid (7.5 g of Baker 100 mesh), prewashed six times with methanol and oven-dried, was added dry to 10-mm i.d. columns, tamped down, and then washed with successive 50-ml portions of methanol, acetone, ethyl ether, and pentane. The lipid sample in pentane was then added to the column, and fractions were collected as follows: hydrocarbons, 50 ml pentane; sterol esters, 100 ml 3% ethyl ether in pentane; triglycerides, 150 ml 15% ethyl ether in pentane; free sterol, 150 ml 30% ethyl ether in pentane; mono- and diglycerides, 100 ml ethyl ether; phospholipids, 100 ml methanol. The solvents were evaporated under a nitrogen stream and the fractions dried under high vacuum and weighed. Thin-layer chromatography and cholesterol analyses using the Liebermann-Burchardt method were performed on all fractions initially to establish the efficiency of separation. Overlapping of the fractions was estimated at between 1 and 3%. Average recovery from the columns was 98%.

[1] Cholic acid was added to facilitate intestinal absorption of cholesterol. It has been shown to have no effect on liver lipid composition (R. B. Alfin-Slater, unpublished data, 1953).

TABLE 1. DIETS

Component	A	B	C	D
	%	%	%	%
Casein	8.1	8.1	8.1	8.1
Salt mixture*	5.0	5.0	5.0	5.0
Cellulose†	5.0	5.0	5.0	5.0
Choline chloride	0.3	0.3	0.3	0.3
Sucrose	59.5	55.5	79.5	75.5
Cottonseed oil	20.0	20.0	..	..
Cholic acid	..	1.0	..	1.0
Cholesterol	..	3.0	..	3.0
Vitamin mix‡	2.2	2.2	2.2	2.2

* Wesson modification of Osborne-Mendel Formula (*Science* **75**: 339, 1932).

† Solka-Floc.

‡ The vitamin mixture had the following composition: Vitamin Test Casein, 61.35 g; p-aminobenzoic acid, 2.42 g; inositol, 2.0 g; α-tocopherol acetate, 1.3865 g; ascorbic acid, 0.8 g; thiamine, 0.288 g; Ca-pantothenate, 0.24 g; niacin, 0.24 g; vitamin B_{12} triturate, 0.24 g; riboflavin, 0.11 g; pyridoxine, 0.108 g; crystalets (500,000 U.S.P./g vitamin A, 50,000 U.S.P./g vitamin D), 0.052 g; folic acid, 0.046 g; menadione, 0.022 g; biotin, 0.016 g.

The fatty acids of the sterol ester, triglyceride, and phospholipid fractions were converted to methyl esters by refluxing for 8 hr with 6 ml of a solution prepared by mixing 1 ml of concentrated sulfuric acid, 61.5 ml of methanol, and 123 ml of benzene. After appropriate extraction and purification, the methyl esters were applied to the gas-liquid chromatograph (Barber-Coleman Model 20, 15% diethylene glycol succinate polyester on 80–100 mesh Gaschrom P, with a column temperature of 180° and an argon gas pressure of 26 lb). Chromatographic peaks were identified by comparison of retention times with those of standards (available for all fatty acids except 14:1 and 22:4), graphic representation of retention times, and catalytic hydrogenation.

The probability that apparent differences in the data were due to chance was calculated, and only those results considered statistically significant have been commented upon.

RESULTS

Morphological Studies. Liver sections from animals fed a diet containing only cottonseed oil exhibited no discrete lipid droplets or globules. Only very fine, barely visible, lipid droplets were demonstrable in a few cells of the lobule (Fig. 1). Fig. 2a and b illustrates a liver section from an animal maintained on the choles-

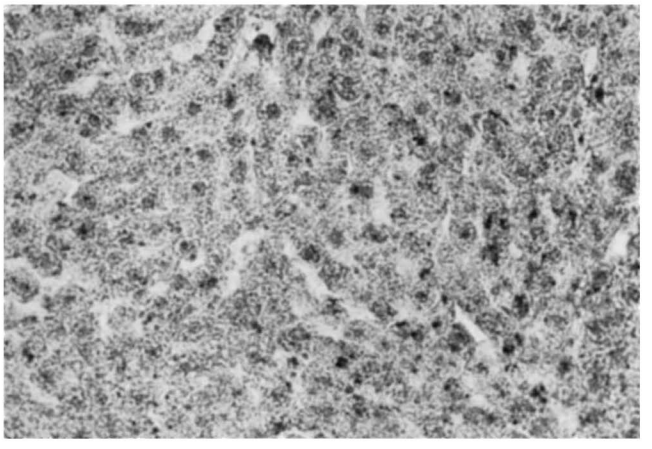

FIG. 1. Frozen section of liver from a rat fed a cottonseed oil-containing diet (I). Only very few small lipid droplets are visible.

terol-cholic acid diet with no other lipid added (IV). Here the lipid droplets were uniformly distributed throughout the liver and were deposited in both the Kupffer and hepatic cells. When the section was examined under the polarizing microscope, refractile granules and small needle-like crystals characteristic of cholesterol were visible mainly in the Kupffer cells, with an occasional granule seen in the hepatic cells (Fig. 2b). The addition of cottonseed oil to the cholesterol diet (II) produced a marked increase in the amount of demonstrable lipid and cholesterol in the liver (Fig. 3a, b); the lipid droplets and globules as well as the refractile (cholesterol) crystals were massed in the Kupffer and hepatic cells. In addition, free lipid droplets and refractile granules and crystals were observed in the sinusoids and central veins.

When the various fatty acid esters were added to the cholesterol diet, there were differences in the appearance

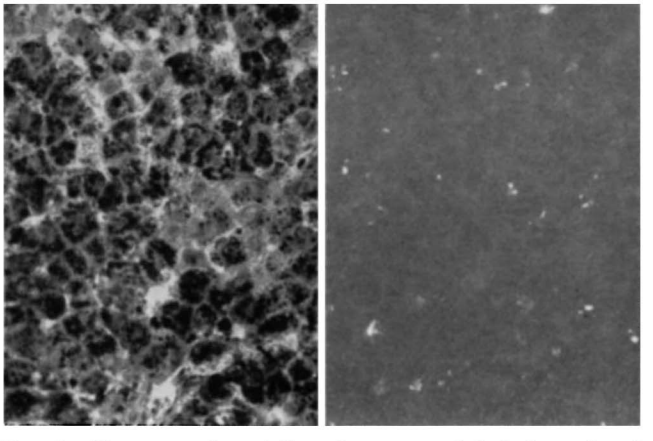

FIG. 2. Frozen section of liver from a rat fed cholesterol and 1% cholic acid with no other fat (IV): *a*, note uniform distribution of lipid droplets; *b*, under polarized light, refractile granules of cholesterol are visible in the Kupffer cells.

of the liver in relation to lipid and cholesterol. Fig. 4a and b illustrate the marked differences in the distribution of both lipid and cholesterol in the liver of an animal fed cholesterol supplemented with methyl oleate. There is no uniform distribution to the demonstrable lipid; rather, it is in the form of irregular masses found in both the Kupffer and hepatic cells or lying free in the sinusoids. This section, when observed under the polarizing microscope, exhibits only very fine refractile granules of cholesterol located mainly in the hepatic cells.

No lesions were observed in the coronary arteries of animals on the cottonseed oil and fat-free diets (I and III). Marked atherosclerotic changes were observed in the group fed cholesterol plus cottonseed oil (II), as illustrated in Fig. 5a. Similar but less extensive lesions were noted in the animals of all the groups fed

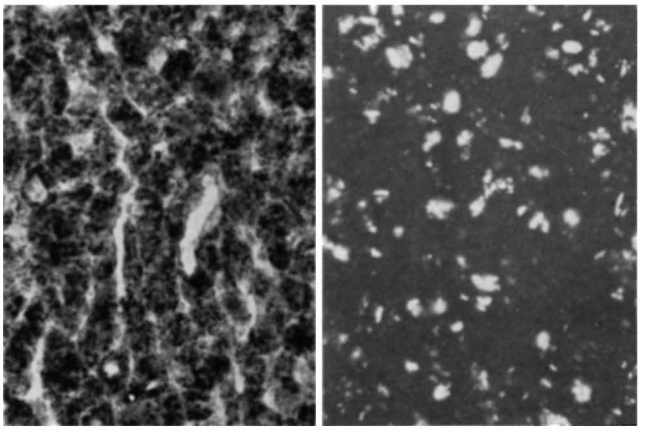

FIG. 3. Frozen section of liver from a rat fed cottonseed oil plus cholesterol (II): *a*, increased amounts of lipid droplets and globules are present; *b*, under polarized light, refractile crystals of cholesterol are seen massed in the Kupffer and parenchymal cells.

cholesterol with or without the various methyl esters of fatty acids (IV–IX), as illustrated in Fig. 5b by a typical lesion from an animal fed the oleate-supplemented diet. Both figures show an intimal thickening resulting from an increased proliferation of both cellular and fibrillar elements. In addition, lipid droplets are visible in the ground substance and macrophages of the intima.

No aortic atherosclerosis was observed in any of the groups, although occasional fatty streaks were noted in the aortae.

Liver Lipid Fractionation. Total lipid analyses and silicic acid chromatography data are summarized in Table 2. Liver total lipids were markedly increased in the group fed cottonseed oil plus cholesterol (II). There were no differences in amounts of total lipid when cholesterol was fed alone or in combination with any of the methyl esters.

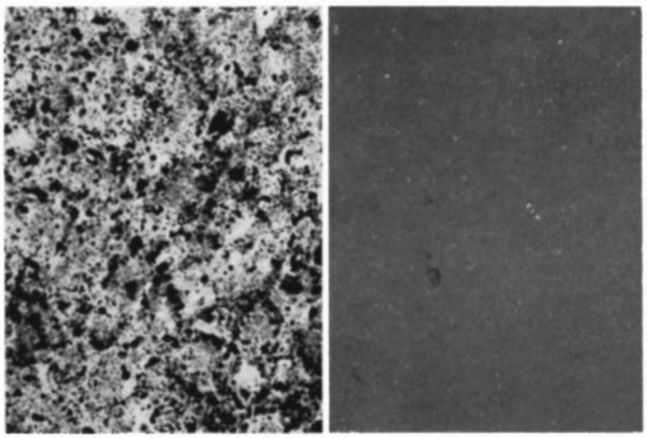

FIG. 4. Frozen section of liver from a rat fed cholesterol plus oleate: *a*, irregularly distributed masses of lipid are present; *b*, polarized light reveals very fine, uniformly distributed refractile granules of cholesterol.

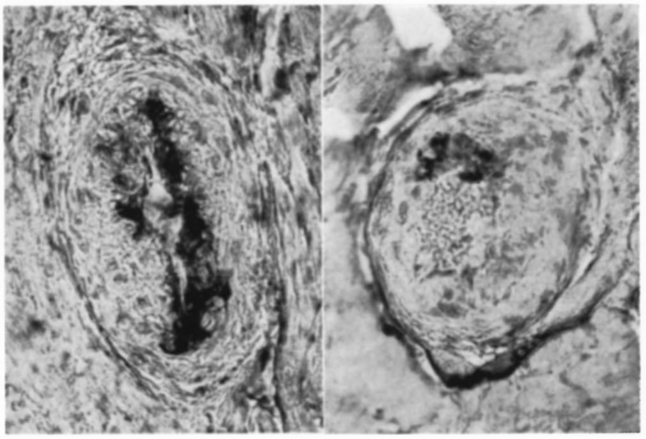

FIG. 5. Frozen sections of coronary arteries from *a*, a rat fed cholesterol plus cottonseed oil (II) and from *b*, a rat fed cholesterol plus oleate (VII). Both figures show intimal thickening and lipid deposition in the ground substance and macrophages of the intima.

The percentages of sterol esters of the cottonseed oil (I) and fat-free (III) groups were low, rising approximately 8-fold in the group in which cholesterol was added to the cottonseed oil (II) and 3–4-fold in the groups fed cholesterol plus the various methyl esters, there being no significant difference between Groups IV, V, VI, VII, VIII, and IX.

Percentages of triglycerides and free sterols of the various groups were not different, but there were increases in the absolute amounts of both of these lipid fractions in the group fed cottonseed oil plus cholesterol (II). In this latter group, the phospholipids showed a decrease in both percentage and total amount. All the other groups receiving cholesterol showed smaller decreases in percentages and similar decreases in amounts of phospholipids.

The fatty acid composition of the sterol esters, triglycerides, and phospholipids are shown in Tables 3, 4, and 5, respectively.

Sterol Esters. The cottonseed oil group (I) showed low percentages of palmitoleic and stearic acids and rather high percentages of palmitic, oleic, linoleic, and arachidonic acids in the sterol ester fraction (Table 3). Feeding cholesterol with cottonseed oil (II) resulted in a decrease in the percentage of palmitic acid and a 7-fold decrease in the percentage of arachidonic acid. Considering that the amount of liver sterol ester of Group II was more than 16 times that of Group I, it can be concluded that there was actually an increase in the absolute amount of arachidonic acid. Two eicosatrienoic acids were identified in small amounts, the 8,11,14-isomer appearing in the group fed cottonseed oil plus cholesterol (II) and the 5,8,11-isomer appearing in the fat-free group (III). The identities of these two C_{20} fatty acids were established by catalytic hydrogenation, graphic representation of retention times, and comparison of the retention times with known standards. The

TABLE 2. LIVER LIPID COMPOSITION

Diet	CSO	CSO +Chol.	Fat-Free	Cholesterol –	+Palmitate	+Stearate	+Oleate	+Linoleate	+Linoleate+E
Group No.	I	II	III	IV	V	VI	VII	VIII	IX
Total lipid mg/g*	216.9 ± 15.1‡	418.4 ± 24.0	210.8 ± 18.8	228.7 ± 15.9	226.7 ± 17.1	223.2 ± 32.9	231.3 ± 8.4	224.0 ± 17.6	232.3 ± 15.4
Sterol ester, %†	4.6 ± 1.4	38.8 ± 3.2	5.3 ± 0.7	16.7 ± 5.6	16.7 ± 3.0	19.6 ± 3.2	15.9 ± 3.8	18.2 ± 5.0	16.6 ± 5.7
mg/g	10.0 ± 3.0	162.3 ± 13.4	11.2 ± 1.5	38.2 ± 12.8	37.9 ± 6.8	43.8 ± 7.1	36.8 ± 8.8	40.8 ± 11.2	38.6 ± 13.2
Triglyceride, %	23.5 ± 3.7	27.2 ± 2.2	21.9 ± 3.0	25.5 ± 1.1	26.0 ± 4.1	26.1 ± 3.3	26.8 ± 3.1	27.9 ± 4.6	25.5 ± 2.3
mg/g	51.0 ± 8.0	113.8 ± 9.2	46.2 ± 6.3	58.3 ± 2.5	58.9 ± 9.3	58.3 ± 7.4	62.0 ± 7.2	62.5 ± 10.3	59.2 ± 5.3
Free sterol, %	5.2 ± 0.5	5.0 ± 0.6	4.9 ± 0.7	5.1 ± 0.7	5.3 ± 0.8	5.2 ± 0.9	5.6 ± 0.9	6.0 ± 0.7	5.8 ± 1.2
mg/g	11.3 ± 1.1	20.9 ± 2.5	10.3 ± 1.5	11.7 ± 1.6	11.2 ± 1.8	11.6 ± 2.0	13.0 ± 2.1	13.4 ± 1.6	13.5 ± 2.8
Phospholipid, %	62.9 + 5.0	24.7 + 2.4	63.8 + 3.1	47.4 + 6.5	46.1 + 7.4	44.9 + 5.9	44.7 + 5.7	43.8 + 7.4	45.8 + 5.3
mg/g	136.4 ± 10.9	103.3 ± 10.0	134.5 ± 6.5	108.4 ± 14.9	104.5 ± 16.8	100.2 ± 13.2	103.4 ± 13.2	98.1 ± 16.6	106.4 ± 12.3

* Mg/g dry weight of liver.
† % of total lipid.
‡ Standard deviation of the mean $= \sqrt{\frac{\Sigma d^2}{n-1}}$.

TABLE 3. FATTY ACID COMPOSITION OF LIVER STEROL ESTERS*

Diet	CSO	CSO +Chol.	Fat-Free	Cholesterol —	+Palmitate	+Stearate	+Oleate	+Linoleate	+Linoleate+E
Group No.	I	II	III	IV	V	VI	VII	VIII	IX
Fatty Acid									
14:0	0.9 ± 0.2	0.3 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	0.5 ± 0.0	0.7 ± 0.1	0.8 ± 0.2
16:0	29.3 ± 2.9	11.2 ± 1.8	29.3 ± 2.8	23.4 ± 8.7	21.1 ± 2.3	27.6 ± 3.5	19.2 ± 2.5	21.3 ± 5.0	26.9 ± 2.9
16:1	4.5 ± 0.4	3.4 ± 0.7	10.3 ± 2.1	17.9 ± 4.6	19.0 ± 2.5	15.6 ± 2.7	18.0 ± 1.9	20.1 ± 2.5	18.6 ± 1.9
18:0	5.1 ± 0.2	1.7 ± 0.3	3.0 ± 0.3	1.9 ± 0.5	1.6 ± 0.1	2.3 ± 0.4	1.6 ± 0.3	1.8 ± 0.7	2.0 ± 0.1
18:1	18.8 ± 1.6	37.4 ± 1.5	48.7 ± 4.1	52.6 ± 2.0	54.1 ± 3.2	51.6 ± 1.2	54.5 ± 1.5	50.2 ± 3.4	46.9 ± 2.1
18:2	30.8 ± 0.9	43.5 ± 1.7	4.3 ± 2.1	2.5 ± 1.7	2.4 ± 0.3	1.8 ± 1.1	3.6 ± 1.0	4.2 ± 0.5	3.5 ± 0.8
20:3†	....	....	1.2 ± 0.2	....	....	....	....	....	....
20:3‡	....	0.9 ± 0.3	....	....	....	....	....	....	....
20:4	10.5 ± 2.0	1.5 ± 0.3	3.0 ± 0.7	1.1 ± 0.5	1.2 ± 0.8	Tr.	1.9 ± 0.8	1.7 ± 0.2	1.7 ± 0.9

* Percentage of total fatty acids including standard deviation of the mean.
† 5,8,11-eicosatrienoic acid.
‡ 8,11,14-eicosatrienoic acid.

retention time of a known, synthetic 8,11,14-eicosatrienoic acid was 15.1 min, as compared to 15.1 min for the eicosatrienoic acid in the sample of Group II. A known, isolated 5,8,11-eicosatrienoic acid standard had a retention time of 13.9 min, as compared to 13.9 min for the eicosatrienoic acid in the sample of Group III.

The groups fed cholesterol alone or with the methyl esters of the various fatty acids showed higher percentages of the monoenoic acids, palmitoleic and oleic, and lower percentages of linoleic and arachidonic acids than the fat-free group. There were no significant differences among the groups fed the various methyl esters.

Triglycerides. The fatty acid patterns were essentially similar to those of the sterol esters with a few exceptions (Table 4). Small amounts of an octadecatrienoic acid appeared in both groups receiving cottonseed oil. In the group given cottonseed oil plus cholesterol, there was no decrease in palmitic or increase in oleic acids as was seen in the sterol esters. Small amounts of a tetradecenoic acid occurred in the groups fed the fat-free diet supplemented with cholesterol alone or with cholesterol plus the methyl esters of fatty acids.

Phospholipids. The fatty acid pattern of the phospholipid fraction differed from that of the other fractions in that longer chain, highly unsaturated fatty acids appeared. Group I (CSO) contains 1.3, 4.2, and 2.1% of C_{22} acids with 4, 5, and 6 double bonds, respectively.

The group fed cottonseed oil plus cholesterol (II) showed decreases in absolute amounts as well as in

TABLE 4. FATTY ACID COMPOSITION OF LIVER TRIGLYCERIDES*

Diet	CSO	CSO +Chol.	Fat-Free	Cholesterol —	+Palmitate	+Stearate	+Oleate	+Linoleate	+Linoleate+E
Group No.	I	II	III	IV	V	VI	VII	VIII	IX
Fatty Acid									
14:0	1.1 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	1.3 ± 0.2	1.2 ± 0.1	1.4 ± 0.4	0.9 ± 0.3	1.4 ± 0.1	1.3 ± 0.1
14:1	Tr.§	Tr.	Tr.	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	0.8 ± 0.1
16:0	29.4 ± 3.3	23.3 ± 2.4	27.2 ± 2.3	26.6 ± 2.4	27.4 ± 1.9	29.6 ± 2.1	25.6 ± 1.3	28.5 ± 4.0	28.8 ± 1.6
16:1	2.8 ± 0.7	1.8 ± 0.4	11.4 ± 1.0	18.6 ± 1.1	20.2 ± 2.2	17.4 ± 2.0	16.7 ± 1.7	18.0 ± 1.9	20.8 ± 2.0
18:0	4.1 ± 0.5	2.6 ± 0.3	2.9 ± 1.1	1.7 ± 0.3	1.8 ± 0.2	2.3 ± 0.4	1.9 ± 0.5	2.0 ± 0.4	2.1 ± 0.5
18:1	20.3 ± 1.8	21.7 ± 1.7	46.4 ± 2.1	47.2 ± 1.1	44.8 ± 1.9	44.6 ± 2.1	48.2 ± 1.5	43.8 ± 3.1	40.8 ± 1.7
18:2	35.3 ± 0.5	45.8 ± 4.2	7.1 ± 3.6	3.3 ± 0.9	2.8 ± 0.2	2.5 ± 0.8	4.2 ± 0.2	4.4 ± 0.3	4.5 ± 1.0
18:3	2.0 ± 1.0	1.2 ± 0.4	....	....	....	....	....	....	....
20:3†	....	....	1.2 ± 0.7	....	....	....	....	....	....
20:3‡	....	1.4 ± 0.2	....	....	....	....	....	....	....
20:4	5.4 ± 1.4	1.5 ± 0.4	2.8 ± 0.8	1.3 ± 0.4	1.5 ± 0.4	1.6 ± 0.2	1.8 ± 0.4	1.3 ± 0.2	1.3 ± 0.6

* Percentage of total fatty acids including standard deviation of the mean.
† 5,8,11-eicosatrienoic acid.
‡ 8,11,14-eicosatrienoic acid.
§ Present, but not measurable.

TABLE 5. FATTY ACID COMPOSITION OF LIVER PHOSPHOLIPIDS*

Diet	CSO	CSO +Chol.	Fat-Free	Cholesterol –	+Palmitate	+Stearate	+Oleate	+Linoleate	+Linoleate+E
Group No.	I	II	III	IV	V	VI	VII	VIII	IX
Fatty Acid									
14:0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.2
16:0	16.0 ± 1.9	16.8 ± 0.3	18.3 ± 1.1	19.4 ± 2.2	19.2 ± 0.4	19.6 ± 0.9	17.8 ± 0.7	21.1 ± 1.6	19.8 ± 0.9
16:1	0.5 ± 0.1	0.7 ± 0.1	3 8 ± 0.3	7.5 ± 0.8	7.4 ± 0.2	7.0 ± 0.7	5.8 ± 0.9	6.2 ± 1.4	6.9 ± 1.5
18:0	26.4 ± 1.3	23 3 ± 0.7	21.2 ± 0.4	16.1 ± 2.0	16.8 ± 1.8	18.9 ± 0.4	16.2 ± 2.1	16.4 ± 1.3	16.3 ± 1.1
18:1	4.3 ± 0.6	6.7 ± 0.6	15.5 ± 0.8	21.4 ± 0.5	20.2 ± 0.9	18.4 ± 1.9	19.9 ± 1.1	16.8 ± 1.1	16.5 ± 0.8
18:2	15.1 ± 0.4	27.4 ± 2.4	6.6 ± 1 4	7.4 ± 2.0	7.1 ± 0 6	6.7 ± 1.2	7.9 ± 1.3	9.3 ± 0.8	10.1 ± 1.1
20:3†	...	...	4.5 ± 1.6	4.8 ± 1.7	3.7 ± 1.5	4.7 ± 2.5	3.2 ± 0.9	1.9 ± 0.4	1.2 ± 0.6
20:3‡	0.8 ± 0.3	3 7 ± 0.5	1.4 ± 0.2	2.3 ± 1.0	2.8 ± 0.7	2.3 ± 2.3	2.2 ± 0.3	2.3 ± 0.5	1.9 ± 0.4
20:4	29.0 ± 1.8	20.3 ± 1.6	22.8 ± 3.2	17.1 ± 3.3	19.0 ± 1.7	18.7 ± 3.2	22 1 ± 2.0	23.2 ± 1.9	23.3 ± 0.9
22:4	1.3 ± 0.5	...	Tr.§	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.
22:5	4.2 ± 0.9	...	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.
22:6	2.1 ± 0.7	1.0 ± 0.3	5.5 ± 0.9	3.6 ± 1.2	3.4 ± 1.5	3.2 ± 0.6	4.7 ± 0.6	2.5 ± 0.5	3.8 ± 0.6

* Percentage of total fatty acids including standard deviation of the mean.
† 5,8,11-eicosatrienoic acid.
‡ 8,11,14-eicosatrienoic acid.
§ Present, but not measurable.

percentages of arachidonic, docosatetranoic, docosapentanoic, and docosahexanoic acids; and a 4.5-fold increase in 8,11,14-eicosatrienoic acid. The latter fatty acid was also slightly increased in the other groups receiving cholesterol. The 5,8,11-eicosatrienoic acid isomer appeared in the groups lacking essential fatty acids (Groups III through VII) but occurred in smaller amounts in both groups receiving methyl linoleate (VIII and IX). The latter groups also showed a lower percentage of oleate and higher percentages of linoleic and arachidonic acids than Groups V, VI, and VII.

Docosahexanoic acid was higher in the Groups III through IX than in either group receiving cottonseed oil (I and II).

As in the sterol esters, the phospholipids of the groups fed cholesterol with and without the methyl esters showed lower percentages of the dienoic and tetraenoic acids and higher monoenoic acids than the fat-free group.

No alteration in any of the lipid fractions or in their fatty acid composition was observed after supplementation with excess amounts of α-tocopherol.

DISCUSSION

An abnormally large amount of hepatic lipid, consisting primarily of sterol esters, was demonstrated both histologically and chemically in groups II and IV–IX. The excess sterol ester was most marked in the group receiving cottonseed oil with cholesterol (II). This probably was due to the greater availability of fatty acids for esterification to cholesterol in this group, resulting from the larger amounts of fatty acids in the cottonseed oil diet (20% oil by weight) as compared to the 100 mg/day of methyl esters of fatty acids received by the animals in groups V–IX, the different form in which the fatty acids were fed (triglycerides in cottonseed oil *vs.* methyl esters), or the different means of intake (cottonseed oil incorporated in the diet *vs.* methyl esters force-fed daily). Comparison of the liver sterol esters in groups IV–IX seems to indicate no differences in deposition produced by supplementation with the C_{16} methyl ester, with the C_{18} methyl esters of fatty acids of different degrees of unsaturation, or with excess α-tocopherol.

Investigations by Alfin-Slater *et al.* (14) have suggested a relationship between essential fatty acids and the transport of cholesterol from the liver to the blood plasma. The increase in serum cholesterol esters when essential fatty acids were fed to rats (15, 16) may represent an increased transport of cholesterol caused by essential fatty acids. The accentuation of symptoms of essential fatty acid deficiency when diets containing 1% cholesterol were fed to rats (17, 18) provides additional evidence for the utilization of essential fatty acids in cholesterol transport.

In the present study, a more pronounced essential fatty acid deficiency pattern in the liver (high monoenoic acids and low linoleic and arachidonic acids) was produced in the groups fed either cholesterol with no other fat or supplemented with methyl palmitate, stearate, or oleate than was observed in the completely fat-free group.

The appearance of an eicosatrienoic acid in the livers of fat-deficient rats was first noted by Nunn and Smed-

ley-MacLean (19). Mead and Slaton (20) have reported the isolation from fat-deficient rats of 5,8,11-eicosatrienoic acid. Subsequent studies (21) have indicated that oleic acid is the precursor of this acid and that palmitoleic acid is the precursor of 7,10,13-eicosatrienoic acid formed in smaller amounts under the same conditions. In the present study, the 5,8,11-eicosatrienoic acid observed in the phospholipids and in smaller amounts in the sterol esters and triglycerides appeared to be related to essential fatty acid deficiency, thus confirming the previous reports. Although the triene/tetraene ratio has been used in some investigations (22, 23) as a measure of linoleate deficiency or requirement, a C_{20} triene, different from that observed in essential fatty acid deficiency, has been identified in groups of animals receiving adequate linoleate. This 8,11,14-eicosatrienoic acid appeared in highest amounts in the phospholipids of the group receiving cottonseed oil with cholesterol.

The formation of arachidonic from linoleic acid by chain extension and dehydrogenation has been demonstrated by Mead *et al.* (24) with one of the postulated, but not identified, intermediates being 8,11,14-eicosatrienoic acid. If it is assumed that arachidonic acid is involved in cholesterol transport, it seems probable that in the present study the increased cholesterol load caused an increased transport of arachidonate into the blood, resulting in an increased arachidonate biosynthesis and an accumulation of the immediate precursor in the liver possibly because of a limitation in the rate of the final enzymatic dehydrogenation.

The increased formation of cholesterol arachidonate resulting from excess dietary cholesterol may account for the decreased arachidonic and increased 8,11,14-eicosatrienoic acids of the liver phospholipids. The mechanism may be either a direct selective esterification of arachidonic acid to cholesterol or a transfer of arachidonate from the phospholipids to cholesterol. The decrease in the amount of liver phospholipids in the animals receiving cholesterol in this study is consistent with the demonstration of a depression of liver phospholipid formation by exogenous cholesterol in an investigation by Perlman and Chaikoff (25) and may be caused by the decreased availability of arachidonate, which is probably a structural requirement of the phospholipid molecule.

Kinsell (26) has reported that arachidonate seemed to be more effective than linoleate in lowering serum cholesterol in humans. Investigation of the serum cholesterol esters of several species of animals has shown a correlation between cholesteryl arachidonate levels and resistance to spontaneous atherosclerosis (27). In the study reported here, the increased hepatic cholesteryl arachidonate in the group given cottonseed oil plus cholesterol and the increase in the 8,11,14-eicosatrienoic acid as discussed above provide evidence of increased arachidonic acid synthesis induced by exogenous cholesterol and further emphasize the vital role of arachidonic acid in cholesterol metabolism.

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