Effect of a Cholesterol-Biosynthesis Inhibitor on the Fatty Acid Composition of Phospholipids in the Serum and Tissues of Rats

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1. Changes produced by a cholesterol-biosynthesis inhibitor, *trans*-1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944), in the total fatty acids in the liver and brain, and in phospholipids in the serum, liver, heart, brain and lungs from male rats, have been studied. 2. Treatment with AY-9944 produced the following changes in the fatty acid composition: (a) a marked decrease in the percentage of linoleic acid and an increase in oleic acid in the total fatty acids in the liver; (b) in the serum, an overall decrease in the percentage of linoleic acid in neutral lipids and phospholipids; (c) an increased content of linoleic acid in the β -acyl chain of phosphatidylcholines in the liver and in sphingomyelins in the brain and lungs; (d) an increased content of palmitic acid and oleic acid in the β -acyl chain of phosphatidylcholine in the liver, heart and lungs; (e) an increased content of phosphatidylcholines and sphingomyelins, together with an increased percentage of saturated fatty acids in these phosphatides in the lungs. 3. Changes in the phosphatides and the production of foam cells in the lungs suggest that AY-9944 may be of use in the study of the alveolar membrane.

Stimulation of fatty acid biosynthesis and concomitant depression of cholesterol biosynthesis have been reported in rat liver (Layne, Rudolph & Bessman, 1960; Foster & Bloom, 1963). *trans*-1,4-Bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944) (Humber, 1964) given orally has been reported to inhibit the biosynthesis of cholesterol (Dvornik, Kraml, Dubuc, Givner & Gaudry, 1963) by interfering with the enzymic conversion of 7-dehydrocholesterol into cholesterol (Kraml, Bagli & Dvornik, 1964). The agent increased the incorporation of labelled acetate into fatty acids (Givner & Dvornik, 1965) and affected the incorporation of labelled acyl and nitrogen base moieties into phospholipids (Hill, 1964).

To investigate the effect of AY-9944 on phospholipids, the changes in the total fatty acid composition of the liver and brain, and of phosphatidylcholine and sphingomyelins from the liver, heart, lung and brain of rats, were determined. To determine whether the availability of cholesterol in the tissues affected the changes produced by AY-9944, rats were fed on a fat-supplemented diet known to elevate the cholesterol content in the liver (Swell, Boiter, Field & Treadwell, 1953).

EXPERIMENTAL

Male albino rats, weighing about 100g., were fed on Purina chow (reference diet) or Purina chow supplemented with 1% of cholesterol, 2.0% of sodium cholate and 7.5%

of butterfat (diet A). Treatment consisted of 4.6 mg. of AY-9944/kg. body wt. given orally per day. The duration of feeding and treatment is given in each Table.

Serum was extracted with ethanol-ether (3:1, v/v) and the extract evaporated under N_2 . The residue was taken up in light petroleum (b.p. 30–40°) and the neutral lipids and phospholipids were separated on silicic acid columns as described by Horning, Williams & Horning (1960). The phospholipids were rechromatographed on silicic acid as described by Newman, Liu & Zilvermit (1960) and the fractions containing phosphatidylserine plus phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine plus sphingomyelin were collected.

Whole brains and livers from rats fed on either diet were homogenized in ethanol-ether (3:1, v/v). The extract was evaporated under N_2 and the residue hydrolysed with methanolic KOH (1.0 ml. of 18N-KOH, 4 ml. of methanol and 5 ml. of water) at 70° for 3 hr. Phosphatidylcholines and sphingomyelins were isolated by successive silicic acid chromatography (Hill & Rochefort, 1965) from the liver, heart, brain and lungs, and the homogeneity of the isolated phosphatides was checked by thin-layer chromatography (Vogel, Doizaki & Zieve, 1962). Organs were pooled to obtain samples adequate for the fatty acid analysis of the isolated phosphatides. The β -fatty acids of phosphatidylcholines were isolated as described by Hill & Rochefort (1965).

The sphingomyelins were hydrolysed with methanolic HCl (1.0 ml. of 6N-HCl, 4 ml. of methanol and 5 ml. of water) at 70° for 3 hr. (Sweeley & Moscatelli, 1959–60). The fatty acids were extracted twice with ether, the solvent was evaporated under N_2 and the residue was taken up in pentane. Methylation was accomplished with diazomethane (Schlenk & Gellerman, 1960). The methyl esters were

Table 1. *Percentage composition of the fatty acids of serum neutral lipids and phospholipids in rats given AY-9944*

Serum (12 ml.) was obtained from four male rats fed on the reference diet and given 4.6 mg. of AY-9944/kg./day orally for 28 days. Neutral lipids and phospholipids were separated as described in the text. The fatty acids were identified by their retention times relative to palmitic acid on butane-1,4-diol succinate polyester at 210°. Fatty acid relative retention times for 18:2-20:4 acids have been combined. In all Tables (and in the text) the first figure of the fatty acid designation represents the no. of carbon atoms/mol. and the second figure the no. of double bonds/mol. The percentage composition is calculated as: $100 \times \text{peak area of fatty acid} / \text{total peak area}$. — indicates that the amount of the fatty acid present was less than 0.5%.

Percentage composition of fatty acids									
Relative retention time	Fatty acid	Neutral lipids		Phosphatidylserines and phosphatidylethanolamines		Phosphatidylcholines		Lysophosphatidylcholines and sphingomyelins	
		Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944
< 0.86	12:0 to 16:0	4.2	7.0	4.3	12.9	0.7	2.6	5.0	0.8
1.00	16:0	21.4	33.9	25.2	22.1	25.2	27.7	27.0	83.9
1.10	16:1	1.9	2.9	2.0	2.0	1.6	1.6	1.4	0.5
1.31	17:0	0.8	1.7	10.2	9.9	1.1	1.9	3.2	0.6
1.70	18:0	14.0	8.2	10.3	6.4	14.7	14.9	19.2	3.4
1.86	18:1	21.9	19.1	23.1	22.4	23.7	25.1	23.9	5.1
2.21	18:2	21.6	10.2	17.5	10.5	22.8	19.0	13.1	2.6
2.60-4.49	18:2 to 20:4	4.4	2.0	3.4	2.0	—	0.8	3.1	2.5
4.94	20:4	4.9	10.6	3.2	2.6	3.9	5.9	—	—
5.40-11.94	above 20:4	2.7	0.8	—	6.4	0.9	—	—	1.4

separated on butane-1,4-diol succinate polyester coated on Chromosorb W at 210° (Perkin-Elmer gas chromatograph model 800) or at 185° (Pye Argon chromatograph). Standard mixtures of methyl esters were kindly supplied by the National Institutes of Health, Bethesda, Md., U.S.A., and pure standards were purchased from the Hormel Institute, Austin, Minn., U.S.A.

RESULTS

Serum lipids. In the neutral lipids, treatment with AY-9944 increased the percentage of short-chain fatty acids (between 12:0 and 16:0) and of palmitic acid and arachidonic acid, and decreased that of stearic acid and linoleic acid (Table 1).

In the serum of rats treated with AY-9944, in the phosphatidylserine and phosphatidylethanolamine fraction the percentage of short-chain fatty acids was increased from 4 to 13%, and those of stearic acid and linoleic acid were decreased from 10 to 6% and from 17.5 to 10.5% respectively. In the fraction containing lysophosphatidylcholines and sphingomyelins, treatment increased the palmitic acid content from 27 to 84%. Little change occurred in the fatty acid composition of serum phosphatidylcholines.

Tissue lipids. The fatty acid composition of the liver and brain lipids of rats fed on either reference diet or diet A as determined by gas-liquid

chromatography is shown in Tables 2 and 3. In the liver of rats given diet A, the oleic acid content increased from 7.0 to 33%, whereas stearic acid decreased from 31 to 12% and arachidonic acid from 20.5 to 3%. Little change in the fatty acid composition of the brain lipids occurred in rats fed on diet A.

On treatment with AY-9944, the content of palmitic acid and oleic acid increased, whereas the content of stearic acid, linoleic acid and arachidonic acid decreased to different extents in the liver of rats fed on the reference diet. In rats fed on diet A, AY-9944 decreased the content of linoleic acid and increased the content of oleic acid in the liver. The content of palmitic acid and stearic acid decreased on treatment in the brain.

Phosphatidylcholines. The fatty acid composition of the β -acyl chain of phosphatidylcholines in the brain, heart, liver and lungs is shown in Table 4. AY-9944 increased the percentage of palmitic acid plus stearic acid in the lungs from 37 to 50% and decreased the percentage in the brain from 43.6 to 35%. On treatment with AY-9944, the content of palmitic acid and oleic acid increased slightly in the liver and markedly in the heart and lungs. Treatment decreased the content of the short-chain fatty acids in the heart and produced an overall decrease in the fatty acid content in the brain.

Table 2. *Percentage composition of the fatty acids of liver lipids of rats given AY-9944*

Male hooded rats were fed on the reference diet for 38 days and treated rats were given 4.6 mg. of AY-9944/kg./day from day 11 to day 38. The fatty acid composition was determined on the total lipids from eight pooled livers. Samples were analysed on butane-1,4-diol succinate polyester (10% on Chromosorb W) at 185° (Pye Argon chromatograph). The percentage composition is calculated as indicated in Table 1. — indicates that the amount of the fatty acid present was less than 0.5%.

Fatty acid	Percentage composition of fatty acids			
	Reference diet	Reference diet + AY-9944	Diet A	Diet A + AY-9944
16:0	17.9	24.1	21.0	24.8
16:1	—	1.4	—	3.0
18:0	30.8	24.4	12.1	10.4
18:1	6.6	16.7	33.0	51.5
18:2	21.1	10.7	16.5	6.1
20:4	20.5	16.1	3.1	2.8

Table 3. *Percentage composition of the fatty acids of brain lipids in rats given AY-9944*

Experimental details are as given in Table 2. The percentage composition is calculated as indicated in Table 1. — indicates that the amount of the fatty acid present was less than 0.5%.

Fatty acid	Percentage composition of fatty acids			
	Reference diet	Reference diet + AY-9944	Diet A	Diet A + AY-9944
15:0	3.1	—	0.6	—
16:0	33.3	22.5	31.1	24.7
16:1	—	0.7	0.6	1.0
18:0	19.3	15.1	21.0	15.8
18:1	28.1	32.4	31.5	31.6
18:2	2.5	—	0.7	—
20:0	—	4.1	—	4.1
20:4	8.7	8.2	5.4	10.3

The relative proportions of the fatty acids between 18:2 and 20:4 (relative retention 2.38 to 4.38) varied with the tissue. On treatment, the composition and percentage of these fatty acids were altered in the brain and lungs. The content of long-chain fatty acids (above 20:4) decreased on treatment in the lungs.

Sphingomyelins. The amount of saturated fatty acids (16:0 and 18:0) in the liver, the brain and the heart varied from 43.5 to 49%, but comprised only 21% of the lung sphingomyelin fatty acids. The total content of sphingomyelin fatty acids was increased in the brain and lung on treatment (Table 5).

In the brain, the content of palmitic acid, palmitoleic acid and oleic acid increased, whereas that of linoleic acid decreased on treatment. Similar changes occurred in the lungs, except that the percentage of oleic acid decreased. In both the lungs and brain, the percentage of long-chain fatty

acids (above 20:4), including the 22:1, 24:0, 24:1 and 22:6 acids, decreased markedly on treatment.

In the heart, the change on treatment in the composition of the fatty acids appeared as a decrease in linoleic acid and an increase in arachidonic acid.

DISCUSSION

The physiological relationship between cholesterol and fatty acid biosynthesis is little understood. Wood & Migicovsky (1957) have suggested a correlation between inhibition of cholesterol biosynthesis and elongation of the carbon chain in saturated fatty acids. Citrate has been reported to stimulate fatty acid biosynthesis (Srere, 1965), while depressing cholesterol biosynthesis (Foster & Bloom, 1963). Catravas (1963) has isolated a protein-carbohydrate complex (lipogenin) that stimulated biosynthesis of both fatty acids and cholesterol.

Table 4. *Percentage composition of the β -acyl fatty acids of tissue phosphatidylcholines of rats given AY-9944*

The β -fatty acids of phosphatidylcholines were isolated by successive silicic acid chromatography, followed by hydrolysis with *Crotalus adamanteus* venom and subsequent isolation of the β -fatty acids as described by Hill & Rochefort (1965). The fatty acids were identified by their retention times relative to palmitic acid on butane-1,4-diol succinate polyester at 210°. The percentage composition is calculated as indicated in Table 1. — indicates that the amount of the fatty acid present was less than 0.5%.

Percentage composition of fatty acids									
Relative retention time	Fatty acid	Liver		Heart		Brain		Lung	
		Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944
	below 16:0	2.8	2.2	35.8	11.8	3.3	3.2	7.6	7.9
1.00	16:0	17.2	26.4	10.2	14.3	11.4	10.3	14.4	20.9
1.11	16:1	1.9	1.9	2.1	1.8	—	—	—	—
1.26-1.59	16:1 to 18:0	1.7	0.7	1.8	1.0	0.6	2.5	1.9	2.4
1.70	18:0	12.1	10.8	32.1	27.6	32.2	24.4	20.3	28.7
1.86	18:1	26.0	28.9	13.2	14.2	37.3	34.1	11.8	19.4
2.10	—	—	—	—	—	1.3	1.5	—	3.4
2.20	18:2	17.0	18.0	5.0	—	—	—	1.7	1.1
2.38-2.88*	—	1.9	1.2	—	—	—	1.8	—	—
3.11	—	0.7	1.0	—	—	0.6	4.8	1.3	0.8
3.39	—	—	—	—	—	7.3	6.7	1.2	1.2
3.69-3.96†	—	0.6	—	—	29.5	0.6	0.9	1.3	3.0
4.20	—	7.7	1.3	—	—	0.8	1.2	19.2	—
4.38	—	—	—	—	—	—	4.4	—	5.8
4.85	20:4	7.5	5.1	—	—	2.0	2.8	—	—
5.10-5.63	above 20:4	2.0	2.0	—	—	—	—	—	—
5.10-6.80	—	—	—	—	—	1.6	1.0	—	—
5.10-10.80	—	—	—	—	—	—	—	12.4	4.3
Wt. of β -fatty acids (mg.)...202		233	6	12	43	28	16	27	
Wet wt. of tissue (g.).....44.1		45.1	3.8	3.2	6.0	4.9	5.5	5.1	
Wt. of β -fatty acids/g.									
wet wt. of tissue.....4.58		5.17	1.58	3.75	7.17	5.71	2.91	5.29	

* Contains three fatty acid peaks.

† Contains two fatty acid peaks.

Alternatively, it has been suggested that biosynthesis of fatty acids (Bortz & Lynen, 1963) and cholesterol (Siperstein & Bhattathirly, 1963) may be controlled by an end-product feedback mechanism, although the interdependence of the feedback is unknown.

In the present study, the addition of cholesterol together with cholate and butterfat to the diet after 28 days caused a decrease in the percentage of linoleic acid and arachidonic acid in the liver lipids, but caused little change in the brain lipids, whose fatty acid composition was comparable with that reported by Biran & Bartley (1961). The addition of cholesterol to the diet has been reported to alter phospholipid polyunsaturated fatty acids in the liver (Morton & Horner, 1961).

In the liver of rats fed on the reference diet, treatment with AY-9944 produced a marked

decrease in the percentage of linoleic acid and an increase in oleic acid, whereas in the brain the percentage of palmitic acid and stearic acid decreased. In rats fed on the supplemented diet, the changes in the fatty acid composition of the liver on treatment were less marked, except that the oleic acid increased to approx. 50% of the total fatty acids.

It has been postulated that the rate of turnover of phospholipids may be related to their role in metabolism (Collins, 1963; Redman & Hokin, 1959), which may vary with their fatty acid composition (Wurster & Copenhaver, 1965). Stoffel & Ach (1964) have reported that fatty acids biosynthesized in different tissues are characteristic of the tissue phosphatides, and Lands & Hart (1965) found different patterns of acyltransferases in different tissues.

Table 5. *Percentage composition of the fatty acids of tissue sphingomyelins of rats given AY-9944*

Experimental conditions are given in the text. Fatty acids with relative retentions 2.80–4.38 and 5.34–11.49 have been combined for clarity. The percentage composition is calculated as indicated in Table 1. — indicates that the amount of the fatty acid present was less than 0.5%.

Percentage composition of fatty acids									
Relative retention time	Fatty acid	Liver		Heart		Brain		Lung	
		Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944	Reference diet	Reference diet + AY-9944
0.37–0.74	below 16:0	—	—	—	—	1.4	—	1.7	8.6
1.00	16:0	19.9	14.3	22.3	22.3	15.2	33.3	13.4	62.9
1.13	16:1	0.5	0.7	—	—	0.6	3.1	24.7	7.1
1.74	18:0	23.6	23.8	26.3	26.0	32.2	16.0	6.5	4.1
1.92	18:1	10.0	12.3	13.5	13.3	18.6	27.0	16.7	7.8
2.28	18:2	25.5	25.8	31.1	22.4	1.6	—	9.3	5.4
2.80–4.38	18:2 to 20:4	1.7	1.8	0.8	0.5	1.5	1.3	1.9	—
4.80	20:4	14.2	16.6	1.7	12.7	15.1	9.9	18.4	2.9
5.34–11.49	above 20:4	3.4	3.4	3.3	2.0	13.9	7.7	7.1	—
Wt. of fatty acids in sphingomyelin (mg.).....36									
Wet wt. of tissue (g.).....24.1									
Wt. of sphingomyelin fatty acids/g. wet wt. of tissue (mg.) 0.82									
		0.82	0.80	5.53	5.00	0.33	0.82	0.55	4.12

Further, Lands & Hart (1965) have indicated that certain fatty acids in phosphatidylcholines may affect the rate and specificity of the acyltransferases, and Leat (1964) suggested that the function of aminophosphatides, e.g. phosphatidylethanolamines, was dependent on their arachidonic acid content. Treatment with AY-9944 produced a marked increase in the content of phosphatidylcholines in the heart and lungs and a slight increase in the liver. This increase may reflect an increased synthesis of phosphatidylcholines in these tissues.

Though phosphatidylcholines may be synthesized via a similar pathway in the liver and brain (Strickland, Subrahmanyam, Pritchard, Thompson & Rossiter, 1963), the treatment produced opposite effects on the β -fatty acid content of phosphatidylcholines in the brain and liver. Specific changes in different tissues, such as the decrease in content of short-chain fatty acids (between 12:0 and 16:0) in the heart and in the long-chain fatty acids (above 20:4) in the lungs, further suggested that AY-9944 either affected the fatty acid biosynthesis or the acyltransferases or both in the tissues differently. However, the increased content of palmitic acid and oleic acid in sphingomyelin in the brain and lungs suggested a similar effect on the fatty acid metabolism in these tissues.

Allman (1964) has reported that the amount of fatty acids synthesized in a tissue, measured either by incorporation of [2-¹⁴C]acetate or oxidation of

NADPH, was proportional to the linoleic acid content in the tissues. The increased content of linoleic acid in brain and lung sphingomyelin and in the β -acyl chain of phosphatidylcholines in the liver together with the increase in the fatty acid content in these tissues suggest that AY-9944 may have increased fatty acid biosynthesis.

It is noteworthy that the percentage of linoleic acid decreased in the neutral lipids and phosphatides and the percentage of palmitic acid increased in neutral lipids, lysophosphatidylcholines and sphingomyelins in the serum, since similar changes in linoleic acid and palmitic acid were found in the fatty acid composition of the liver on treatment with AY-9944.

Rat lungs have been reported to incorporate long-chain fatty acids into phospholipids (Felts, 1962) and [¹⁴C]acetate into mitochondrial lipids (Tombropoulos, 1964). Klaus, Reiss, Tooley, Piel & Clements (1962) have shown that mitochondria in alveolar epithelial cells secrete a surface-active lipid-protein complex, probably dipalmitoylphosphatidylcholine (Brown, 1964; Klaus, Clements & Havel, 1961), which stabilizes the alveolar radius (Clements, 1962). Further, Schaefer, Jordan & Thelin (1965) correlated changes in the surface-active complex and lamellar bodies in granular pneumocytes with disruption of the hyaline membrane.

Treatment with AY-9944 has been reported to

increase the incorporation of labelled palmitic acid and choline into lung sphingomyelins (Hill & Rochefort, 1965). In rat lungs phosphatidylcholines and sphingomyelins comprise 34 and 26% respectively of the total phospholipids (Marinetti, Erbland & Stotz, 1958).

The decreased percentage of long-chain fatty acids in lung phosphatidylcholines and sphingomyelins and the marked increase in saturated fatty acids, mainly palmitic acid in the latter, on treatment may affect the function of the phosphatides (Farquhar, 1965). The increased content of lung phosphatidylcholines and sphingomyelins is of added interest since AY-9944 has been reported to produce foam cells in rat lungs (Greselin, 1965).

Evidence indicates that AY-9944, an inhibitor of cholesterol biosynthesis, affected the fatty acid composition of the β -acyl chain of phosphatidylcholines and sphingomyelins. Further, the agent may be of use in the investigation of the phospholipid metabolism of the alveolar membrane.

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