REFERENCES

- Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S. & Watson, J. G. (1957). *Brit. J. Cancer*, **11**, 212.
- Azzone, G. F. & Ernster, L. (1961). *J. biol. Chem.* **236**, 1501.
- Barnabei, O., Papa, S. & Quagliariello, E. (1963). *Arch. Biochem. Biophys.* **101**, 573.
- Borst, P. & Slater, E. C. (1961). *Biochim. biophys. Acta*, **48**, 372.
- Boyland, E. (1958). *Brit. med. Bull.* **14**, 153.
- Boyland, E. & Williams, D. C. (1956a). *Biochem. J.* **64**, 578.
- Boyland, E. & Williams, D. C. (1956b). *Rep. Brit. Emp. Cancer Campgn.* **34**, 39.
- Chance, B. & Ito, T. (1963). *J. biol. Chem.* **238**, 1509.
- Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.
- Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
- Dunning, W. F., Curtis, M. R. & Mann, M. O. (1950). *Cancer Res.* **10**, 454.
- Ehrhart, H. & Georgii, A. (1959). *Blut*, **5**, 388.
- Ivanova, V. D. & Ramonova-Tskhovrebova, O. D. (1960). *Probl. Gemat.* **10**, 34.
- Klingenberg, M. & Schollmeyer, P. (1961). *Biochem. biophys. Res. Commun.* **4**, 38.
- Lehniger, A. L. (1959). *J. biol. Chem.* **234**, 2187.
- Löw, H., Siekevitz, P., Ernster, L. & Lindberg, O. (1958). *Biochim. biophys. Acta*, **29**, 392.
- Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.
- McDonald, M. (1955). In *Methods in Enzymology*, vol. 1, p. 269. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Mentzer, C. (1948). *Bull. Soc. Chim. biol., Paris*, **30**, 872.
- Musajo, L., Benassi, C. A. & Parpajola, A. (1955). *Nature, Lond.*, **175**, 855.
- Papa, S., Budillon, G. & Quagliariello, E. (1962a). *Boll. Soc. ital. Biol. sper.* **38**, 1342.
- Papa, S., Budillon, G. & Quagliariello, E. (1962b). *Boll. Soc. ital. Biol. sper.* **38**, 1351.
- Price, J. M. & Brown, R. R. (1962). *Acta Un. int. Cancr.* **18**, 684.
- Quagliariello, E., Papa, S. & Saccone, C. (1962a). *Ital. J. Biochem.* **9**, 403.
- Quagliariello, E., Papa, S. & Saccone, C. (1962b). *Biochem. J.* **84**, 66p.
- Quagliariello, E., Saccone, C., Rinaldi, E. & Alioto, M. R. (1959). *Nature, Lond.*, **184**, 820.
- Quagliariello, E., Tancredi, F., Fedele, L. & Saccone, C. (1961). *Brit. J. Cancer*, **15**, 367.
- Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
- Tompsett, S. C. (1959). *Clin. chim. Acta*, **4**, 411.

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Composition of Tissue Lipids and Anaemia of Guinea Pigs in Response to Dietary Cholesterol

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The guinea pig is unusual among experimental animals in that it reacts to feeding with cholesterol by an accelerated destruction of erythrocytes, splenic enlargement and hyperplasia of bone marrow (Okey & Greaves, 1939; Kennedy & Okey, 1947; Okey, 1944). As a result, death usually occurs before any considerable atherosclerotic plaques are formed.

The possibility that the characteristic response of the guinea pig to cholesterol may result from changes in the lipid composition of the erythrocytes or their environment, or both, deserved investigation. The present paper reports fatty acid compositions of the lipid fractions from plasma, erythrocytes, livers and spleens of control and cholesterol-fed guinea pigs.

MATERIALS AND METHODS

Male and female guinea pigs, weighing 225–290 g. at 2–3 weeks of age, were used (Dependable Animal Supply Co., Martinez, Calif.). They were housed individually and

fed *ad libitum* on a semi-synthetic diet similar to that used by Richie (1959). For the first 2 weeks all were given the control diet, composed of the following (g./100 g. of diet): casein, 20; albumin, 10; cottonseed oil, 10; sucrose, 7; glucose, 6.8 (Cerelease from Corn Products, San Francisco, Calif.); dextrin, 20; cellophane spangles, 5 (from Rayon Processing Co., Pawtucket, R.I.); alfalfa meal, 5; agar, 5; salt mix, 4 (USP XIV; zinc carbonate was incorporated to give a concentration of 0.17% in the diet); magnesium oxide, 0.4; potassium acetate, 2; choline bitartrate, 0.36 (choline, 0.17); ascorbic acid, 0.2; inositol, 0.2; vitamin A mix, 2.0 [vitamin A mix contained (g./kg. of mix): vitamin A acetate, 0.3; viosterol, 0.2 (Nutritional Biochemical Corp.; 400 000 USP units of vitamin D/g.); α -tocopherol, 1; cottonseed oil, 998]; vitamin B mix, 2.0 [vitamin B mix contained (g./kg. of mix): thiamine hydrochloride, 0.8; ascorbic acid, 25; riboflavin, 0.8; pyridoxine hydrochloride, 0.8; calcium pantothenate, 2; nicotinic acid, 10; biotin, 0.1; folic acid, 0.5; glucose, 960]. The fatty acid composition of the diet is shown in Table 1. Analyses of the alfalfa meal and the agar showed that they contained 8.3% and 4.4% of ash respectively. These ashes were shown to contain less than 1% of magnesium (both) and 8.8% and 4.6% of

calcium respectively. Thus their contributions to the calcium content of the diet were negligible (0.03 and 0.01% respectively). Diets were prepared by combining the dry ingredients and mixing them with the oils. This mixture was then combined with water (4 l./30 kg. of diet). The diets were pelleted and dried under a fan. At the end of 2 weeks, the animals were divided into control and cholesterol-fed groups, the diet of the latter differing from the former only by the addition of 1% of cholesterol at the expense of sucrose. Acceptability of the diet proved variable. To reduce mortality, small supplements of alfalfa meal and lettuce were given occasionally to some animals. Although the diet was pelleted, food intake could not be measured with any degree of accuracy. Of 44 animals started on the experiment, 30 survived the experimental period of 50–60 days. Of the 14 animals lost (two males and three females on the control diet; four males and five females on the cholesterol-containing diet), only four showed obvious signs of illness. The others died presumably because of failure to eat. Of the remaining 30 animals, five animals of each group were chosen for tissue analyses, on the basis of uniformity of weight gain, liver and spleen enlargements, and degree of anaemia.

The animals were starved for 20–22 hr. before being killed. After the animals were anaesthetized with sodium pentobarbital, blood was collected in a heparin-treated syringe by heart puncture and placed in an ice bath. Livers and spleens were removed, weighed and immediately homogenized in 95% (v/v) ethanol. The blood was centrifuged at 3–4° within 2 hr. of removal.

Each plasma sample was extracted with 10 vol. of 95% (v/v) ethanol at room temperature (each portion of ethanol contained 1 mg. of quinol). The solution was decanted after standing at least 4 hr. in the dark. The residue was then extracted twice, each time with 10 vol. of 95% (v/v) ethanol-ether (3:1, v/v). The combined extracts were evaporated *in vacuo* in a rotary evaporator to a volume of about 1 ml. The external water-bath temperature was maintained at less than 55° during this process. The material left in the flask was extracted with light petroleum and stored at –17° until analyses could be performed.

After the plasma and the buffy coat were removed, the erythrocytes were gently washed with an equal volume of 0.85% sodium chloride and centrifuged. After a second wash with 0.85% sodium chloride, the cells were haemolysed by adding 0.25 vol. of water and stirring for 10 min.

Table 1. *Fatty acid composition of the diet*

The first figure of the fatty acid designation represents the number of carbon atoms/molecule, and the second figure the number of double bonds/molecule.

Fatty acid	Amount of fatty acid (% w/w, of total fatty acids)
12:0	Trace
14:0	1.1
16:0	19.0
16:1	1.3
18:0	1.8
18:1	16.6
18:2	60.2
20:4	0.0

Extractions of the lipids were the same as that for plasma. The liver and spleen homogenates were extracted first with ethanol, then with ether (Okey, Shannon, Tinoco, Ostwald & Miljanich, 1961).

The lipids were separated into four fractions on silicic acid columns as described in previous reports from this Laboratory (Okey *et al.* 1961; Lis, Tinoco & Okey, 1961). The fractions were: (1) cholesterol ester; (2) triglyceride; (3) unesterified cholesterol, monoglycerides and diglycerides; (4) phospholipids. The fatty acid composition of the individual fractions was determined by gas-liquid chromatography of their methyl esters (Wilkins Aerograph with thermal-conductivity detector: columns were 5 ft. coiled stainless-steel columns, packed with diethylene glycol polyester on Chromasorb W; the operating temperature was 190–200°). Methylation was accomplished by controlled heating with 1% (v/v) sulphuric acid and methanol.

The fatty acids were identified by comparing their retention times with those of known fatty acids plotted as number of carbon atoms/molecule versus the logarithm of the retention time (Woodford & van Gent, 1960). The less-common fatty acids found in these tissues were identified by rechromatography after hydrogenation of the sample (Burchfield & Storrs, 1962; Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959). This method provided a tentative identification sufficient for the present purposes.

Cholesterol was determined by the method of Sperry & Webb (1950), with the modifications described by Okey & Lyman (1957). Total lipids were determined by the method of Bloor (1928), and phosphorus was determined by the method of Sumner (1944). All analyses were performed on the tissues and blood of individual animals. Statistical comparisons of the results were made by *t* test as described by Snedecor (1957).

Erythrocytes were counted periodically on blood drawn from an ear vein and also at the time of autopsy. The blood was diluted with Hayen's solution and counted in a Bright-Line improved Neubauer counting chamber (0.1 mm.).

RESULTS

Gross effects of feeding cholesterol

Results for weight gains of the animals, size of organs, erythrocyte counts and packed-cell volumes are summarized in Table 2. Results for individual animals showed that anaemia developed first in those that ate the cholesterol diet most readily. As observed previously (Okey & Greaves, 1939; Okey, 1944), the control animals grew better than did those given cholesterol. Livers and spleens of the cholesterol-fed guinea pigs were grossly enlarged. Livers were not only fatty and friable but also occasionally showed small necrotic areas under gross examination. Microscopic examination of such livers had previously shown fat infiltration and cell destruction (Okey & Greaves, 1939). Spleens were spongy, and gall bladders were extended and frequently filled with debris. Lungs were occasionally haemorrhagic. The fall in the erythrocyte count was accompanied by an increase in the mean corpuscular volume.

Table 2. Gross effects of feeding 1% cholesterol to guinea pigs

All values are means for the groups $\pm$ S.E.M. (where given). The erythrocyte count was made at autopsy; the mean erythrocyte volume is calculated from packed-cell volume/erythrocyte count.

Animals	Sex	No. of animals	Time on diet (days)	Wt. at autopsy (g.)	Total wt. gain (g.)	10 ⁻⁶ × Erythrocyte count (per mm. ³)		Packed-cell volume %	Mean erythrocyte volume (μ ³)	Liver		Spleen	
						cyte count	volume			Wet wt. (g.)	Moisture content (%)	Percentage of body wt.	Wet wt. (g.)
Control	♂	5	57	466 ±17.0	229	5.41 ±0.14	48	88.7	15.6	67.0	3.3	0.57	0.12
Control	♀	5	54	378 ±44.7	165	5.55 ±1.11	45	81.1	13.2	67.6	3.5	0.92	0.25
Cholesterol-fed	♂	5	53	349 ±9.9	105	2.90 ±0.35	32	110.3	38.6	60.5	11.1	8.0	2.30
Cholesterol-fed	♀	5	53	322 ±10.0	98	2.13 ±0.27	26	122.2	34.8	61.2	10.8	9.9	3.10
Cholesterol-fed: con- trol ratio	♂	—	—	0.7	0.5	0.5	0.7	1.24	2.5	0.9	3.3	14	19
Cholesterol-fed: con- trol ratio	♀	—	—	0.8	0.6	0.4	0.6	1.51	2.6	0.9	3.1	11	12

Effect on lipids

Livers (Table 3). The rise of total lipid content from 10 to 20% in the cholesterol-fed animals actually represented a sixfold increase in the amount of lipid in the livers because of their larger size. The concentrations of different lipid components rose to different extents. Though feeding with cholesterol resulted in a very large rise (about 30-fold) in the concentration of liver cholesterol ester, the concentration of free cholesterol was increased only about 2.5-fold. The percentage of liver phospholipid decreased somewhat, but the actual amount was about doubled because of the enlargement of the livers. The overall effect was a large decrease in the ratio of phospholipid to cholesterol in the livers of guinea pigs as the result of feeding with cholesterol.

The triglyceride fraction of the livers represented almost half of their total lipids. Feeding with cholesterol produced a large absolute increase and a doubling of the percentage of liver triglycerides.

Plasma. The total cholesterol content increased from a mean value of about 80 mg./100 ml. for control guinea pigs to about 400 mg./100 ml. for the cholesterol-fed animals. Most of the rise in the plasma cholesterol concentration was due to an 11-fold increase in the concentration of free cholesterol, but the cholesterol ester content also increased (2.5-fold). The concentration of plasma phospholipids increased in about the same proportion as did that of cholesterol. The ratio of plasma phospholipids to cholesterol (0.6–0.7) was therefore not appreciably changed in the cholesterol-fed guinea pig. The size of samples did not permit separate determination of the total amount of lipids in triglycerides for plasma and erythrocytes.

Erythrocytes. In agreement with findings for other species (Ponder, 1947; Behrendt, 1957; Reed, Swisher, Marinetti & Eden, 1960; Burt & Rossiter, 1950), almost all the cholesterol of erythrocytes of guinea pigs was found to be unesterified. The cholesterol concentration in controls averaged 135 mg./100 ml. and that of phospholipids 250 mg./100 ml. For both components the values for females were significantly greater than those for males. The total cholesterol concentrations (mg./100 ml.) were: for controls, males 128 ± 9 , females 145 ± 6 ; for cholesterol-fed animals, males 354 ± 36 and females 404 ± 53 ($P < 0.02$); the phospholipid concentrations (mg./100 ml.) were: for controls, males 239 ± 13 , females 273 ± 11 ; for cholesterol-fed animals, males 499 ± 47 , females 636 ± 65 ($P < 0.01$). Feeding with cholesterol increased the concentration of both the cholesterol and phospholipids of erythrocytes 2.5-fold. Owing to the increased mean erythrocyte volume, these effects

of feeding with cholesterol were even greater when the results were calculated on a per cell basis.

The total erythrocyte lipid contents, as calculated from the sum of cholesterol and phospholipids, were about 400 mg. and 900 mg./100 ml. for the control and the cholesterol-fed guinea pigs respectively.

Spleens. The spleens of normal guinea pigs contained about 3 % of lipid, of which 65 % was phospholipid, 15 % was triglyceride and 15 % was cholesterol, practically all of the cholesterol being unesterified. Feeding with cholesterol led to a

moderate increase in the percentage of lipids which, however, represented a 15-fold increase in amount because of the enlarged spleens. Most of the increase of the cholesterol concentration (to 1.5 %) was due to cholesterol ester, which increased 500-fold in the males (from 0.19 to 84.3 mg.) and 200-fold in the females (from 0.50 to 94.8 mg.). The phospholipid content of guinea-pig spleen lipids decreased somewhat in the cholesterol-fed animals with the result that the ratio of phospholipid to cholesterol was decreased from about 4 to about 1.

Table 3. *Gross composition of lipids of liver, plasma, spleen and erythrocytes of guinea pigs given a diet with or without cholesterol*

The results are given as means $\pm$ S.E.M. for each of the two groups of ten animals. Means for each sex were similar in most cases and are pooled for this presentation. 'Trace' indicates that small amounts had been found in only a minority of the animals of the group. Total lipid and triglycerides of plasma and erythrocytes were not determined. The total lipid of livers was determined on the original extract, that of spleens was calculated as the sum of the total fatty acids and total cholesterol. The total cholesterol of plasma, spleen and erythrocytes was determined on the original extract, and that of liver was calculated as the sum of cholesterol ester and unesterified cholesterol. The phospholipid of plasma, spleen and erythrocytes was calculated from the phosphorus determination on the original extract, and that of liver was determined as lipid in the separated phospholipid fraction. Triglycerides of livers and spleens were calculated from the amount of total fatty acids found in fraction II of the silicic acid-column separation procedure. The values for total lipid and for triglycerides are in terms of g./100 g. fresh wt. The values for total cholesterol, cholesterol ester, free cholesterol and phospholipids are in terms of mg./100 g. fresh wt. for liver and spleen and mg./100 ml. for plasma and erythrocytes.

	Liver			Plasma		
	Control	Cholesterol-fed	Cholesterol-fed: control ratio	Control	Cholesterol-fed	Cholesterol-fed: control ratio
Total lipid (g./100 g.)	9.3 $\pm$ 0.7	21.3 $\pm$ 1.2	2.3	—	—	—
(mg./organ)	1361 $\pm$ 147	7833 $\pm$ 59.0	5.8	—	—	—
Total cholesterol	405 $\pm$ 36.7	4082 $\pm$ 170	10.1	81.3 $\pm$ 6.9	431 $\pm$ 37.1	5.3
(mg./100 g. or 100 ml.)						
Cholesterol ester	111 $\pm$ 24.9	3238 $\pm$ 153	29.0	60.5 $\pm$ 4.0	151 $\pm$ 13.8	2.5
(mg./100 g. or 100 ml.)						
Free cholesterol	294 $\pm$ 12.2	857 $\pm$ 14.5	2.9	25.9 $\pm$ 2.8	270 $\pm$ 31.2	10.4
(mg./100 g. or 100 ml.)						
Phospholipid	3125 $\pm$ 47	2406 $\pm$ 52	0.8	63.5 $\pm$ 6.6	283 $\pm$ 29.8	4.5
(mg./100 g. or 100 ml.)						
Phospholipid: total cholesterol ratio	7.8	0.57	0.07	0.74	0.65	0.9
Triglycerides (g./100 g.)	5.59 $\pm$ 0.76	11.18 $\pm$ 0.98	2.0	—	—	—
	Spleen			Erythrocytes		
	Control	Cholesterol-fed	Cholesterol-fed: control ratio	Control	Cholesterol-fed	Cholesterol-fed: control ratio
Total lipid (g./100 g.)	3.2 $\pm$ 0.2	4.1 $\pm$ 0.2	1.3	—	—	—
(mg./organ)	24.4 $\pm$ 2.1	361 $\pm$ 39.9	14.8	—	—	—
Total cholesterol	427 $\pm$ 11.9	1464 $\pm$ 69	3.4	136 $\pm$ 6	376 $\pm$ 30	2.8
(mg./100 g. or 100 ml.)						
Cholesterol ester	46.0 $\pm$ 7.3	984 $\pm$ 49	21.4	Trace	Trace	—
(mg./100 g. or 100 ml.)						
Free cholesterol	381 $\pm$ 7.7	480 $\pm$ 21.7	1.3	134.6 $\pm$ 6	341 $\pm$ 15	2.5
(mg./100 g. or 100 ml.)						
Phospholipid	1842 $\pm$ 25	1456 $\pm$ 29	0.8	256 $\pm$ 3	560 $\pm$ 14	2.2
(mg./100 g. or 100 ml.)						
Phospholipid: total cholesterol ratio	4.5	1.0	0.2	1.9	1.5	0.8
Triglycerides (g./100 g.)	0.45 $\pm$ 0.05	0.29 $\pm$ 0.06	0.6	—	—	—

Spleen lipids, particularly those of anaemic guinea pigs, are mixtures originating not only from splenic tissue as such but also from plasma and from the large numbers of blood cells in all stages of maturation and destruction found in spleens. The significance of the lipid composition of this organ is therefore difficult to assess.

Fatty acid composition of lipids

Cholesterol ester fatty acids (Table 4). The cholesterol ester fatty acids of guinea-pig livers were characterized by the virtual absence of arachidonic acid. Their fatty acids were largely palmitic acid and linoleic acid. Small amounts of the following acids were also present: $C_{15:0}$, $C_{17:0}$, $C_{20:1}$, $C_{20:2}$ and $C_{20:3}$ (where, for $C_{x:y}$, x is the number of carbon atoms/molecule and y is the number of double bonds/molecule). Feeding with cholesterol increased the concentration of the unsaturated acids linoleic acid and oleic acid at the expense of the saturated acids palmitic acid and stearic acid ($P < 0.01-0.05$).

Plasma cholesterol esters contained 65% of linoleic acid and 16% of palmitic acid. Small amounts of arachidonic acid and other fatty acids were also present. Feeding with cholesterol decreased the linoleic acid content of plasma cholesterol esters by 15%, in contrast with a small increase observed in liver.

The amount of cholesterol esters in erythrocytes was too small to allow reliable determination of the fatty acid composition. The cholesterol esters of spleens of control guinea pigs contained about 25% each of palmitic acid and linoleic acid. In contrast with both livers and plasma, these cholesterol esters contained a greater variety of fatty acids, including arachidonic acid. The presence of 15% of palmitoleic acid in the males was unexpected. Feeding with cholesterol doubled the percentage of linoleic acid and of what was either a $C_{20:3}$ acid or a $C_{20:2}$ acid. Because of the increase of the cholesterol ester concentration and the enlargement of the spleens, the amount of linoleic acid in spleen cholesterol esters increased 500-fold in males and 100-fold in females. These increases were accompanied by striking decreases in the percentages of palmitoleic acid in the male and of arachidonic acid in the female guinea pig.

Triglyceride fatty acids (Table 5). Liver triglycerides contained about 60% of linoleic acid and 20% each of palmitic acid and oleic acid. Arachidonic acid was absent. Feeding with cholesterol had relatively little effect on the composition of this lipid fraction.

The plasma triglycerides were similar to those of livers in fatty acid composition, and showed similarly small responses to feeding with cholesterol.

Table 4. *Fatty acid composition of cholesterol esters of liver, plasma and spleen of guinea pigs given a diet with or without cholesterol*

The first figure of the fatty acid designation represents the number of carbon atoms/molecule, and the second figure the number of double bonds/molecule. The results are given as percentages of total methyl esters measured. They represent means $\pm$ S.E.M. for each of the two groups of ten animals. Means for each sex were similar in most cases and are pooled for this presentation. Standard errors for means less than 5 are presented only when relevant to the discussion. 'Trace' indicates that small amounts had been found in only a minority of the animals of the group. The identity of the acids listed under 'Others' has been established only tentatively. One of the isomeric $C_{20:3}$ acids could be a $C_{20:2}$ acid. The values for total fatty acids are in terms of mg./100 g. fresh wt. for liver and spleen, and mg./100 ml. for plasma.

Fatty acid	Liver		Plasma		Spleen	
	Control	Cholesterol-fed	Control	Cholesterol-fed	Control	Cholesterol-fed
<16:0	6.0 $\pm$ 0.6	5.7 $\pm$ 0.4	2.0	3.0	5.6 $\pm$ 0.8	2.0
16:0	25.2 $\pm$ 0.8	19.8 $\pm$ 0.9	16.6 $\pm$ 1.5	20.9 $\pm$ 0.7	21.7 $\pm$ 0.6	13.9 $\pm$ 1.2
16:1	4.9 $\pm$ 0.7	2.8	2.2	2.6	14.9 $\pm$ 2.2*	1.5*
18:0	10.6 $\pm$ 0.4	6.1 $\pm$ 0.4	2.4	3.6	9.7 $\pm$ 0.8	3.1
18:1	14.8 $\pm$ 0.8	19.2 $\pm$ 0.3	8.4 $\pm$ 0.8	11.5 $\pm$ 0.3	12.8 $\pm$ 0.8	16.1 $\pm$ 0.3
18:2	34.6 $\pm$ 0.6	39.2 $\pm$ 1.4	66.3 $\pm$ 3.1	53.3 $\pm$ 0.9	24.0 $\pm$ 0.8	41.0 $\pm$ 0.7
20:4	Trace	Trace	1.5 $\pm$ 0.4	1.7 $\pm$ 0.2	4.4 $\pm$ 1.9*	5.0 $\pm$ 0.4*
Others						
15:0	1.0	0.9	—	—	—	—
17:0	0.9	0.9	—	—	—	—
20:0	—	—	—	—	2.3	0.3
20:1	—	—	—	—	0.7	1.0
20:3	Trace	3.0 $\pm$ 0.3	—	—	3.9 $\pm$ 0.6	8.6 $\pm$ 0.2
20:3	—	—	—	—	2.8	3.5
Total fatty acids (mg./100 g. or 100 ml.)	81.3 $\pm$ 18.2	240.1 $\pm$ 98.1	44.1 $\pm$ 2.9	110 $\pm$ 10.6	73.8 $\pm$ 14.3	71.6 $\pm$ 35.6

* Results are for the groups of males. Females had the following values: $C_{16:1}$ acid, 4.7 $\pm$ 0.7 and 1.5 $\pm$ 0.6 for the control and cholesterol-fed groups respectively; $C_{20:4}$ acid, 10.6 $\pm$ 1.0 and 5.9 $\pm$ 0.4 for the two groups respectively.

Table 5. *Fatty acid composition of triglycerides of liver, plasma and spleen of guinea pigs given a diet with or without cholesterol*

The first figure of the fatty acid designation represents the number of carbon atoms/molecule, and the second figure the number of double bonds/molecule. The results are given as percentages of total methyl esters measured. They represent means $\pm$ S.E.M. for each of the two groups of ten animals. Means for each sex were similar in most cases and are pooled for this presentation. Standard errors for means less than 5 are presented only where relevant to the discussion. 'Trace' indicates that small amounts of the compound had been found in only a minority of the animals of the group. The identity of the acids listed under 'Others' has been established only tentatively. One of the isomeric $C_{20:3}$ acids could be a $C_{20:1}$ acid. The total amount of plasma triglyceride fatty acids was not determined. The values for total fatty acids are in terms of mg./100 g. fresh wt. for liver and spleen, and mg./100 ml. for plasma.

Fatty acid	Liver		Plasma		Spleen	
	Control	Cholesterol-fed	Control	Cholesterol-fed	Control	Cholesterol-fed
16:0	20.8 $\pm$ 0.5	16.2 $\pm$ 0.5	19.4 $\pm$ 1.0	16.4 $\pm$ 0.8	23.8 $\pm$ 0.3	21.7 $\pm$ 0.3
18:0	2.4	5.5 $\pm$ 0.2	3.3	6.9 $\pm$ 0.7	8.2 $\pm$ 0.5	9.5 $\pm$ 0.6
18:1	14.9 $\pm$ 0.2	16.0 $\pm$ 0.3	14.3 $\pm$ 1.5	17.4 $\pm$ 0.6	19.0 $\pm$ 0.5	15.4 $\pm$ 0.7
18:2	58.8 $\pm$ 7.8	60.0 $\pm$ 0.7	57.5 $\pm$ 1.6	57.0 $\pm$ 1.8	41.5 $\pm$ 0.7	36.0 $\pm$ 1.9
20:4	—	Trace	1.7	Trace	1.2	3.0
Others	—	—	—	—	—	—
20:1	—	—	—	—	0.9	1.1
20:3	—	—	—	—	1.2	4.2
20:3	—	—	—	—	0.2	2.0
22:4	—	—	—	—	—	2.0
Total fatty acids (mg./100 g. or 100 ml.)	5232 $\pm$ 723	10994 $\pm$ 896	—	—	432 $\pm$ 52.8	276 $\pm$ 18.8

The triglyceride fraction of erythrocytes was too small to allow reliable analyses. The major constituents of spleen triglycerides were 40 % of linoleic acid and 20 % of palmitic acid. Feeding with cholesterol produced a notable increase in the concentration of the unusual long-chain unsaturated pair of isomeric $C_{20:3}$ acids (one of those may have been a $C_{20:2}$ acid) and a $C_{22:4}$ acid.

A mixture of unesterified cholesterol, unesterified fatty acids, monoglycerides and diglycerides, which was isolated as 'fraction III' by our analytical procedure, represented a small proportion of the lipids in the tissues investigated.

Phospholipid fatty acids (Table 6). The main components of liver phospholipids were 45 % of linoleic acid and 30 % of stearic acid. About 8 % of arachidonic acid was also present. Feeding with cholesterol did not appreciably alter the composition. Normal guinea-pig-plasma phospholipids had a fatty acid composition similar to that of liver phospholipid. Feeding with cholesterol did not change this appreciably. In spite of the proportionately small decrease in the concentration of linoleic acid, the increased amount of this fraction represents a large rise in the absolute amounts of linoleic acid in the blood of the cholesterol-fed guinea pig.

The outstanding difference between the fatty acid composition of the erythrocyte phospholipids of the guinea pig, as compared with that of their livers and plasma, was the high arachidonic acid content of about 25 %. Relatively high concentrations of higher unsaturated fatty acids were found in the erythrocyte phospholipids, especially of a

$C_{22:4}$ acid that occurred at a concentration of 5 % in females and 10 % in males. Feeding with cholesterol produced a rise in the proportion of arachidonic acid and of one of the $C_{20:3}$ acids ($P < 0.01$), which was compensated by a decrease in the concentration of the saturated fatty acids and the $C_{22:4}$ acid ($P < 0.01$). The spleen phospholipids of normal guinea pigs contained smaller proportions of arachidonic acid and greater proportions of linoleic acid than did those of the erythrocytes. Appreciable amounts of $C_{20:3}$ acid and $C_{22:4}$ acid were also present. A possibly significant difference between the fatty acid composition of phospholipids of livers, plasma and erythrocytes, on the one hand, and of spleens on the other, was the difference in the ratio of stearic acid to palmitic acid. This ratio was 1 or less in spleen phospholipids, but 2 or more in those of the other three tissues. This might indicate a difference in the composition with respect to phospholipid classes. In contrast with the response of erythrocytes, the composition of spleen phospholipids was not changed by feeding with cholesterol. The tenfold increase of spleen weights, however, resulted in a large accumulation of both linoleic acid and arachidonic acid.

DISCUSSION

Literature reports on the lipid composition of guinea-pig tissues

Liver. The total lipid and cholesterol contents found in both the control and the cholesterol-fed guinea pigs agree closely with those reported in the

Table 6. *Fatty acid composition of phospholipids of liver, plasma, spleen and erythrocytes of guinea pigs given a diet with or without cholesterol*

Fatty acid	Liver			Plasma			Spleen			Erythrocytes		
	Control		Cholesterol-fed	Control		Cholesterol-fed	Control		Cholesterol-fed	Control		Cholesterol-fed
	12.3±0.3	12.4±0.5	12.4±0.5	14.2±0.6	19.6±0.2	17.1±0.3	17.1±0.3	15.3±0.7	10.3±0.4	11.1±0.2	11.1±0.2	10.3±0.4
16:0	30.2±0.5	28.5±1.1	28.5±1.1	28.0±1.4	21.5±1.1	15.6±0.3	15.6±0.3	17.4±0.4	22.7±1.5	27.4±1.1	27.4±1.1	22.7±1.5
18:0	5.4±0.5	6.2±0.5	6.2±0.5	5.8±0.3	6.4±0.6	8.5±0.2	8.5±0.2	8.6±0.2	9.3±0.4	7.0±1.3	7.0±1.3	9.3±0.4
18:1	43.1±0.4	46.5±0.8	46.5±0.8	42.9±0.6	40.2±1.0	28.2±0.6	28.2±0.6	26.1±0.6	18.9±0.6	19.5±0.7	19.5±0.7	18.9±0.6
20:4	8.6±0.6	6.0±1.6	6.0±1.6	7.9±0.6	11.0±1.1	18.4±0.9	18.4±0.9	18.8±0.6	23.2±1.1	23.2±1.1	23.2±1.1	23.2±1.1
Others	—	—	—	—	—	3.4	3.4	4.7	4.2±0.3	1.9±0.9	1.9±0.9	4.2±0.3
20:3	—	—	—	—	—	1.6	1.6	1.5	0.1	0.9	0.9	0.1
20:3	—	—	—	0.7	0.4	4.3	4.3	3.1	6.8±0.8*	10.2±1.1*	10.2±1.1*	6.8±0.8*
22:4	—	—	—	0.3	0.2	—	—	—	—	—	—	—
24:0	—	—	—	—	—	—	—	—	—	—	—	—
Total fatty acids (mg./100 g. or 100 ml.)	2206±33.6	1680±36.0	1680±36.0	44.8±2.8	199±21.0	1300±17.2	1300±17.2	1028±64.7	396±30.7	181±7.7	181±7.7	396±30.7

* Results are for the groups of males. Females had 5.8±0.6 and traces for the control and cholesterol-fed groups respectively.

The first figure of the fatty acid designation represents the number of carbon atoms/molecule, and the second figure the number of double bonds/molecule. The results are given as percentages of total methyl esters measured. They represent means $\pm$ S.E.M. for each of the two groups of ten animals. Means for each sex were similar in most cases and are pooled for this presentation. Standard errors for means less than 5 are presented only where relevant to the discussion. The identity of the acids listed under 'Others' has been established only tentatively. One of the isomeric $C_{20:3}$ acids could be a $C_{20:3}$ acid. The values for total fatty acids are in terms of mg./100 g. fresh wt. for liver and spleen, and mg./100 ml. for plasma and erythrocytes.

literature (Kennedy & Okey, 1947; Okey, 1944.) Some authors (Marble, Grafflin & Smith, 1940; Handler, 1949) reported lower values although the ratio of phospholipids to total cholesterol was similar to that obtained by us. The discrepancies are very likely the results of differences in the diets used. The one paper on fatty acid composition of guinea-pig livers (Bieri, Pollard & Reid, 1957) reported, as we do, a very high content of dienoic acids and a low content of tetraenoic acids.

Plasma. Our values are similar to those reported in the literature (Okey, 1944; Morris & Courtice, 1955; Swell, Field & Treadwell, 1960). The high content of dienoic acids and low content of tetraenoic acids of the plasma cholesterol ester have also been found and commented on previously (Swell *et al.* 1960; Bieri *et al.* 1957).

Erythrocytes. The one paper that dealt with the fatty acid composition of guinea-pig erythrocytes (Bieri *et al.* 1957) reported 18–23% of dienoic acids and 24–27% of tetraenoic acids, a finding similar to ours.

Relationship of cholesterol intake, composition of tissue lipids and anaemia

As shown previously (Okey & Greaves, 1939; Okey, 1944), the anaemia produced by feeding cholesterol to guinea pigs was of the macrocytic or normocytic, normochromic, haemolytic type, with increased numbers of reticulocytes in the blood, a decreased half-life, increased fragility of erythrocytes, and much accelerated activity of the hypertrophic bone marrow and enlarged spleen. The hyperplastic spleen has been shown not to be directly responsible for the anaemia (Kennedy & Okey, 1947).

It is not known to what extent the erythrocytes of the cholesterol-fed guinea pigs were formed with defective membranes and to what extent they became defective as a result of changes in the plasma. A recent report on a cross-transfusion experiment with anaemic-rabbit erythrocytes (Pinter & Bailey, 1961) indicated that, in this case, the erythrocytes had a low resistance to haemolysis even in normal plasma. The 'environment' of the erythrocytes in our experiment, on the other hand, was different for the cholesterol-fed than for the normal animals. In addition to the changes in lipid composition of the plasma, hormonal changes might be expected to have taken place, particularly in view of the fact that adrenal hypertrophy and changes in the adrenal-lipid composition have been observed by us (Ostwald, Shannon, Miljanich & Lyman, 1964).

Apparently the composition of lipids in the blood must remain reasonably constant to ensure their normal transport and metabolism. It has been shown, for instance, that in essential-fatty acid-

deficient rats cholesterol esters accumulate in the liver (Alfin-Slater, Aftergood, Wells & Deuel, 1954) and that abnormal cholesterol esters are formed and deposited in tissues (Sinclair, 1956). Conversely, the need to transport additional cholesterol has been shown to increase the essential-fatty acid requirement of cholesterol-fed rats (Peifer & Holman, 1955). Also, the rate of arachidonic acid synthesis in the livers of such rats appears to be insufficient to maintain its normal concentration in liver and in cholesterol esters in the blood even though its total amount is greater than in rats given a diet without cholesterol (Okey *et al.* 1961; Morin, Bernick, Mead & Alfin-Slater, 1962). The hypothesis that polyunsaturated fatty acids facilitate cholesterol transport and consequently prevent its pathological accumulation in tissue has been advanced (Sinclair, 1956; Holman, 1958). In addition to these, Swell *et al.* (1960) have pointed out the inverse relationship between the amount of arachidonic acid in the blood of a species of animal and its susceptibility to atherosclerosis. The guinea pig, with very low arachidonic acid concentrations in liver and plasma lipids, would seem to be particularly vulnerable to the stress of feeding with cholesterol. The relatively large rise in the concentration of unesterified cholesterol in these lipids may, in addition, indicate a limited ability to esterify cholesterol, and suggests the possibility of marked changes in the composition of the serum lipoproteins. The fact that these animals had very fatty livers with signs of cellular damage may be a factor in the changes occurring in their lipid metabolism.

The concentrations of arachidonic acid in the tissues investigated indicate that most of the available arachidonic acid accumulated in the phospholipids of the erythrocytes. Considerable amounts of other long-chain polyunsaturated fatty acids were also present. Absolute amounts of these fatty acids increased by more than 100% in the cholesterol-fed groups. Polyunsaturated fatty acids are subject to peroxidation and other oxidative changes unless they are adequately protected. A report that vitamin E deficiency caused anaemia in monkeys (Dinning, 1962) may be evidence that the integrity of the erythrocyte wall depends in some way on the presence of unaltered polyunsaturated fatty acids. The observation that hydroperoxides of methyl linoleate facilitate the breakdown of β -lipoprotein *in vitro* (Nishida & Kummerow, 1960) may also be relevant in this connexion.

That phospholipids constitute an integral part of cell membranes seems well established, and that their qualitative and quantitative make-up have some influence on the permeability of the cells has been suggested (Rapoport, Gerischer-Mothes &

Nieradt, 1955; Turner, 1957; Turner, Anderson & Gondal, 1958; Dawson, Hemington & Lindsay, 1960). Kögl, de Gier, Mulder & Van Deenen (1960) found that, in a number of animal species, the ratio of palmitic acid to oleic acid in the erythrocyte membranes decreased in the same order as did their permeability as measured by haemolysis times. In our guinea pigs this ratio was somewhat smaller (1.1) in the erythrocytes of the cholesterol-fed animals than in those of the controls (1.6). It is known that cholesterol forms associations with phospholipids (Vandenheuvel, 1962). It is conceivable that the structure of the erythrocyte membrane is changed by increased amounts of such molecules due to the large increase of cholesterol in the erythrocytes of the cholesterol-fed guinea pigs.

One of the important functions of essential fatty acids appears to be in the construction of phospholipids of membranes, and essential-fatty acid deficiency has been shown to lead to erythrocyte fragility (King, 1960). By analogy with reports on the fatty acid composition of erythrocytes of fat-deficient monkeys (Fitch, Dinning, Witting & Horwitt, 1961) and rats (Witting, Harvey, Century & Horwitt, 1961), the appearance of two isomeric $C_{20:3}$ acids might be expected. The increase of at least one of these isomers in the lipids of the erythrocytes and spleens from the cholesterol-fed guinea pigs ($P < 0.01-0.05$) might be interpreted as a sign of a relative or marginal essential-fatty acid deficiency in these tissues in response to the need to cope with additional cholesterol (Holman, 1958; Witting *et al.* 1961). The present analyses were performed on the portion of the erythrocytes that was tough enough to survive; the composition of the more-damaged and consequently lysed cells is unknown. Also, because of the anaemia, the cells we examined probably represented a younger-than-normal population with the possibility of a lipid composition different from the normal.

SUMMARY

1. Groups of male and female guinea pigs were given a semi-synthetic diet, with or without 1% of cholesterol, for 50-60 days. The cholesterol-fed animals developed enlarged and fatty livers, very large spleens and haemolytic anaemia.

2. Feeding with cholesterol produced not only a large increase in the cholesterol ester fraction of livers, plasma and spleens but also an increase in unesterified cholesterol. The erythrocytes responded to feeding with cholesterol by a threefold increase in their cholesterol and phospholipid contents.

3. The phospholipids of the spleens and particularly of the erythrocytes had a high arachidonic acid content which further increased in the chole-

sterol-fed animals. The cholesterol esters and phospholipids of livers and plasma and the cholesterol esters of spleens had, on the other hand, a characteristically low arachidonic acid content but a high linoleic acid content.

4. The results suggested that, in the guinea pig, the rate of arachidonic acid formation, or the rate of cholesterol esterification, or both, was not sufficient to maintain a normal tissue-lipid composition when cholesterol was added in the diet.

5. Some possibilities for the mechanism of the development of the anaemia observed in the cholesterol-fed guinea pigs are discussed.

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REFERENCES

- Alfin-Slater, R. B., Aftergood, L., Wells, A. & Deuel, H. (1954). *Arch. Biochem. Biophys.* **52**, 180.
- Behrendt, H. (1957). *Chemistry of Erythrocytes: Clinical Aspects*. New York: Charles C. Thomas.
- Bieri, J., Pollard, C. & Reid, M. (1957). *Biochim. biophys. Acta*, **23**, 650.
- Bloor, W. R. (1928). *J. biol. Chem.* **77**, 53.
- Burchfield, H. P. & Storrs, E. E. (1962). *Biochemical Applications of Gas Chromatography*, p. 548. New York: Academic Press Inc.
- Burt, N. & Rossiter, R. (1950). *Biochem. J.* **46**, 569.
- Dawson, R., Hemington, N. & Lindsay, D. (1960). *Biochem. J.* **77**, 226.
- Dinning, J. (1962). *Physiol. Rev.* **42**, 169.
- Farquhar, J. W., Insull, W., Rosen, P., Stoffel, W. & Ahrens, E. (1959). *Nutr. Rev.* **17**, suppl. 8.
- Fitch, C., Dinning, J., Witting, L. A. & Horwitt, M. K. (1961). *J. Nutr.* **75**, 409.
- Handler, P. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 70.
- Holman, R. T. (1958). *Nutr. Rev.* **16**, 33.
- Kennedy, B. M. & Okey, R. (1947). *Amer. J. Physiol.* **149**, 1.
- King, H. K. (1960). *Chemistry of Lipids in Health and Disease*, p. 74. New York: Charles C. Thomas.
- Kögl, F., de Gier, J., Mulder, I. & Van Deenen, L. (1960). *Biochim. biophys. Acta*, **43**, 95.
- Lis, E. W., Tinoco, J. & Okey, R. (1961). *Analyt. Biochem.* **2**, 100.
- Marble, A., Grafflin, A. & Smith, R. (1940). *J. biol. Chem.* **134**, 253.
- Morin, B. J., Bernick, S., Mead, J. F. & Alfin-Slater, R. B. (1962). *J. Lipid Res.* **3**, 432.
- Morris, B. & Courtice, F. (1955). *Quart. J. exp. Physiol.* **40**, 127.
- Nishida, T. & Kummerow, F. A. (1960). *J. Lipid Res.* **1**, 450.
- Okey, R. (1944). *J. biol. Chem.* **156**, 179.
- Okey, R. & Greaves, V. (1939). *J. biol. Chem.* **129**, 111.
- Okey, R. & Lyman, M. M. (1957). *J. Nutr.* **61**, 523.
- Okey, R., Shannon, A., Tinoco, J., Ostwald, R. & Miljanich, P. (1961). *J. Nutr.* **75**, 51.
- Ostwald, R., Shannon, A., Miljanich, P. & Lyman, R. L. (1964). *J. Nutr.* (in the Press).
- Peifer, J. J. & Holman, R. T. (1955). *Arch. Biochem. Biophys.* **57**, 520.
- Pinter, G. & Bailey, R. (1961). *Amer. J. Physiol.* **200**, 292.
- Ponder, E. (1947). *Ann. N.Y. Acad. Sci.* **48**, art. 7, p. 579.
- Rapoport, S., Gerischer-Mothes, W. & Nieradt, C. (1955). *Hoppe-Seyl. Z.* **300**, 174.
- Reed, C., Swisher, S., Marinetti, G. & Eden, E. (1960). *J. Lab. clin. Med.* **56**, 281.
- Richie, D. J. (1959). M.Sc. Thesis: University of California, Berkeley.
- Sinclair, H. M. (1956). *Lancet*, i, 381.
- Snedecor, G. W. (1957). *Statistical Methods*, 5th ed., p. 140. Ames: Iowa State College Press.
- Sperry, W. M. & Webb, M. (1950). *J. biol. Chem.* **187**, 97.
- Sumner, J. B. (1944). *Science*, **100**, 413.
- Swell, L., Field, H. & Treadwell, C. R. (1960). *Proc. Soc. exp. Biol., N.Y.*, **104**, 325.
- Turner, J. (1957). *J. exp. Med.* **105**, 189.
- Turner, J., Anderson, H. & Gondal, C. (1958). *Biochim. biophys. Acta*, **30**, 130.
- Vandenheuvell, F. (1962). *Canad. J. Biochem. Physiol.* **40**, 1299.
- Witting, L. A., Harvey, C. C., Century, B. & Horwitt, M. K. (1961). *J. Lipid Res.* **2**, 412.
- Woodford, F. P. & van Gent, C. M. (1960). *J. Lipid Res.* **1**, 188.