CLXXVIII. THE EFFECT OF CHOLESTEROL FEEDING ON LIPOID DEPOSITION IN THE LIVER OF RATS

By PETROS ANTONIOS LOIZIDES

From the Department of Biochemistry, University of Liverpool

(Received 30 June 1938)

WHEN rats are fed on diets containing cholesterol, they develop fatty livers which are characterized by the presence in the liver of excessive amounts of cholesteryl esters, while at the same time considerable deposition of glyceride may occur. This variable increase in the glyceride content of the "cholesterol" fatty liver has been shown to depend on a number of factors, such as the dietary contents of choline [Best *et al.* 1934], of caseinogen [Channon & Wilkinson, 1935; Beeston *et al.* 1935], of methionine [Channon *et al.* 1938] and of certain choline analogues [Channon *et al.* 1937, 1, 2]. While the nature of the "cholesterol" fatty liver and the effects on its composition of a number of dietary and other factors have thus been considerably studied, the part played by the fat content of the diet has not been defined. The work described in the present paper has been designed to investigate the quantitative changes which occur in the "cholesterol" fatty liver, as a result of variations in the fat content of the diet, with particular reference to the deposition of cholesterol and of glyceride on diets of constant cholesterol content.

EXPERIMENTAL

The general method of experiment was to feed groups each of 10 rats on diets containing varying amounts of beef dripping with and without 2% cholesterol. In Exp. 1 all the animals were does and the period of feeding was 21 days. The four control groups received a diet consisting of caseinogen (alcohol-extracted) 8, marmite 5, salt mixture 5, cod liver oil 1, with 10, 20, 30 and 40 parts of beef dripping respectively, each diet being made up to 100 parts with glucose. Four other groups received similar diets with the substitution of 2 parts of cholesterol at the expense of glucose. The food intake of the animals was not measured. After 21 days the animals were guillotined and the pooled livers of each group were treated by the alcohol-ether method, and analysed for phosphatide, glyceride and free and combined cholesterol [Best *et al.* 1934]. In Exps. 2 and 3 control groups were omitted. In Exp. 2 the cholesterol-containing diets were identical with those of Exp. 1 save that the caseinogen content of the diet was decreased to 5% and the glucose correspondingly increased. The animals were bucks and the duration of the experiment 17 days. The livers of the animals were investigated individually, and the amount of the fatty acids and unsaponifiable matter determined together by the saponification method. The materials obtained from the livers of the animals of each group were then combined and analysed for their content of unsaponifiable matter, the fatty acids being obtained by difference. In group 12 (Table II) three animals in which the weight losses were greater than 20% were rejected. In Exp. 3 the

diets were the same as for Exp. 2, save that the fat contents of the four diets were 5, 15, 25 and 35 parts respectively, the glucose being correspondingly increased. The animals ($\delta\delta$) were fed for 21 days, and the pooled livers were worked up by the alcohol-ether method; the total ethereal extract was analysed for fatty acids and unsaponifiable matter only, the sterol content of the latter being determined by the digitonin method. The essential data are recorded in Tables I-III, in which the amounts of the different constituents are recorded both as percentages of the fresh liver weight and as the weight in the liver of the 100 g. rat.

RESULTS

Since the control groups, 1*a*-4*a* of Exp. 1, provide data concerning the effect of progressive increases in the fat content of the diet on the nature of the resulting fatty liver, they will be discussed together. Mention is first necessary of findings with normal animals. Work of the last few years has shown that an increasing number of dietary factors are concerned in the control of liver fat. The consequent growing realization of the complexity of liver fat control makes it uncertain as to what should be regarded as "normal" values. For comparison with groups 1*a*-4*a*, the liver of the "normal" animal on a balanced mixed diet is regarded as being 3.4% of the body weight and as containing lecithin 3.3, cholesterol 0.25-0.30, cholesteryl esters 0.03 and glyceride 1.0% of the fresh liver weight. For the 100 g. rat, these latter figures entail the presence in the liver of lecithin 111, cholesterol 8-10, cholesteryl oleate 1 and glyceride 34 mg. (total fatty acids 112 mg.).

Table I shows that increase in the fat content of the diet causes in general increases in the size of the liver, although these increases do not run strictly parallel with the increasing fat content. Similarly the phosphatide figures show decreases in the percentages of lecithin with increasing fat infiltration, although group 3*a* contains less lecithin, 2.30%, than group 4*a*, 2.63%. On the basis of the 100 g. rat, however, the livers contain similar amounts of phosphatide 112, 114, 103 and 105 mg. respectively, and these do not differ sufficiently from the normal 111 mg. to be of significance. The percentage of free cholesterol, 0.21, is constant throughout, while the increase in liver size is not sufficiently great significantly to increase its absolute amount. More pronounced changes occur in cholesteryl ester fractions which increase from the normal 0.03% and 1 mg. to a maximum of 0.33% and 14 mg. The most striking changes occur, however, in the glyceride fractions which show successive increases from 4.10 to 17.54% (normal 1%) as the fat content of the diet is progressively increased. The absolute weight in the liver of the 100 g. rat thus progressively rises to a maximum of 0.722 g., the amounts present in the 11, 21, 31 and 41% fat groups respectively being about 4, 9, 15 and 21 times greater than the normal. Noteworthy also is the fact that the glyceride fraction whether expressed as a percentage of the liver or in terms of absolute weight, shows a linear increase with the amount of fat in the diet.

The results of the cholesterol groups 1*b*-4*b* will now be compared with those of the control groups just discussed. In all cases the liver weight is increased considerably in the cholesterol groups compared with the corresponding control groups. The greatest increase is shown in group 4*b* in which the liver constitutes 6.88% of the body weight, a 70% increase over the control group 4*a*, 4.12%. While the inclusion in the diet of 2% cholesterol thus has a profound effect on the liver size at all levels of dietary fat, among themselves the cholesterol

groups do not increase regularly in this respect, for group 3*b* at 4.97 % is lower than group 2*b*, 5.24 %. In the control groups 3*a* and 4*a* a similar inconsistency in this matter of liver size is shown. In every case also the percentages of lecithin are lower than those of the control groups, the extremely low figures of 1.62 and 1.14 % occurring in groups 3*b* and 4*b*. While the actual weights of lecithin in groups 1*b* and 2*b*, 118 and 115 mg. respectively, are normal, they are significantly decreased in the former groups to 81 and 77 mg. It has usually been found in this laboratory in experiments on the "cholesterol" fatty liver, that the absolute amounts of lecithin are unchanged with progressive infiltration. Thus Beeston *et al.* [1935] carried out four experiments, in the control groups of which the total weight of lipoids in the livers varied from 1.720 to 0.618 g. Reduction of these amounts to 0.281 and 0.528 g. respectively by large doses of choline (about 75 mg. per rat per day) had no effect in increasing the absolute weight of lecithin in the liver although the percentages were of course increased. Similarly in other experiments which provided considerable data on this point [Channon *et al.* 1937, 1, 2] the same result held. The results with groups 3*b* and 4*b* are thus contrary to the general experience in this laboratory.

The effect of the inclusion of cholesterol in the diets is most marked on the cholesterol and glyceride fractions. The free cholesterol shows increases to percentage figures which are 25-50 greater than those of the control groups, while the actual weight in the livers is approximately doubled. These changes are, however, small compared with those in the cholesteryl ester fractions, which increase from the control figures of 0.14, 0.32, 0.31 and 0.33 % in groups 1*a*-4*a* to 1.88, 2.44, 3.14 and 3.86 % in the corresponding cholesterol groups. The actual weights of cholesteryl ester in the liver of the 100 g. rat thus pass from 0.071 to 0.265 g., amounts which are 14, 11, 11 and 18 times greater than those of the corresponding control groups. The amounts of glyceride in groups 1*b*-4*b* also provide clear evidence of the influence of cholesterol in increasing the glyceride fraction of the liver. In all cases, the percentages of glyceride present in the livers of the cholesterol-fed animals are much greater than in the control groups, the minimum increase being in group 1*b* in which it is increased by 10.33 units over group 1*a*, and the maximum being in group 2*b* in which it is 20.3 units % greater than in its control group 2*a*. It might have been anticipated that the amounts of glyceride in the cholesterol-fed animals would progressively increase as the fat content of the diet increased. This, however, is not the case, for although the percentages and amounts of liver glyceride in groups 2*b* and 3*b* lie between those of groups 1*b* and 4*b*, they are not in ascending order. Since in the control groups the amounts of liver glyceride show a linear relationship to the fat content of the diet and in the cholesterol groups the cholesterol contents of the livers do likewise, it seems improbable that group variations are responsible for these irregularities. Accordingly two further experiments were carried out with the object of obtaining further evidence concerning the extent of the deposition of glyceride caused by cholesterol feeding in diets of increasing fat content, and secondly to confirm the progressive increases in cholesterol deposition in the liver observed in Exp. 1. Since both these objects appeared to be obtainable without the use of control groups no such groups were used in Exps. 2 and 3. Before presenting the results of Exps. 2 and 3 in Table II, one point needs mention. In Exp. 2 the amount of cholesterol present in the unsaponifiable fraction of the livers was not determined. Since in cholesterol-fed animals cholesterol constitutes some 90 % of the liver unsaponifiable fraction, the extreme limits in Exp. 3 being 88-92 %, for the purpose of this discussion the unsaponifiable fraction will be regarded as cholesterol. The amount of free cholesterol in the

Table I. *Exp. 1. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

Group	Fat in diet %	Chole-sterol in diet	Liver (% body wt.)	Liver lipoids (g. per 100 g. fresh liver)				Total	Liver lipoids (g. per liver of 100 g. rat)				Total
				Lecithin	Chole-sterol	Chole-steryl oleate	Glyceride		Lecithin	Chole-sterol	Chole-steryl oleate	Glyceride	
1a	11	—	3.62	3.11	0.21	0.14	4.10	7.56	0.112	0.007	0.005	0.148	0.272
1b	11	2	4.00	2.95	0.32	1.88	14.43	19.58	0.118	0.014	0.071	0.577	0.780
2a	21	—	3.84	3.05	0.21	0.32	8.25	11.83	0.114	0.008	0.012	0.317	0.451
2b	21	2	5.24	2.19	0.27	2.44	28.55	33.45	0.115	0.014	0.128	1.496	1.753
3a	31	—	4.49	2.30	0.21	0.31	11.22	14.04	0.103	0.010	0.014	0.505	0.632
3b	31	2	4.97	1.62	0.28	3.14	24.14	29.18	0.081	0.014	0.156	1.191	1.442
4a	41	—	4.12	2.63	0.21	0.33	17.54	20.71	0.105	0.009	0.014	0.722	0.850
4b	41	2	6.88	1.14	0.28	3.86	34.40	39.68	0.077	0.019	0.265	2.367	2.728

Table III. *Exp. 3. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

Group	Fat in diet %	No. of rats	Wt. gain or loss %	Liver % body wt.	g. per 100 g. fresh liver				g. per liver of 100 g. rat			
					Fatty acids (a)	Unsap. matter (b)	Chole-sterol	Total (a+b)	Fatty acids (c)	Unsap. matter (d)	Chole-sterol	Total (c+d)
13	6	10	-4.7	4.00	15.94	2.49	2.21	18.43	0.638	0.096	0.085	0.734
14	16	10	-4.7	4.00	17.70	3.01	2.59	20.71	0.709	0.120	0.103	0.829
15	26	10	-8.7	4.37	24.70	3.26	2.83	27.96	1.078	0.140	0.122	1.218
16	36	10	-5.7	4.96	25.37	3.78	3.29	29.15	1.284	0.192	0.167	1.476

"cholesterol" fatty liver being small and subject to relatively little change, changes in total cholesterol represent substantially changes in cholesteryl esters. The results are presented in Tables II and III.

Table II. *Exp. 2. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

Group	Fat in diet %	No. of rats	Wt. gain or loss %	Liver % body wt.	Liver lipoids					
					g. per 100 g. fresh liver			g. per liver of 100 g. rat		
					Fatty acid	Unsap. matter	Total	Fatty acid	Unsap. matter	Total
9	11	10	- 7.9	4.20	14.04	1.61	15.65	0.588	0.072	0.660
10	21	10	-10.4	3.94	14.23	2.75	16.98	0.543	0.106	0.649
11	31	10	- 9.7	5.88	29.76	2.35	32.11	1.748	0.135	1.883
12	41	7	-11.1	5.81	29.63	2.85	32.48	1.732	0.156	1.888

The results presented in Table II call for comment on two aspects only, for in general they confirm those of Exp. 1. First, with regard to the cholesterol content of the liver, the percentage in Exp. 2 rises from 1.61 on the diet containing 11 % fat to 2.85 on that with 41 % fat. The percentages in groups 10 and 11, while lying between those of groups 9 and 12, are not however in ascending order. In Exp. 3 the cholesterol increases regularly from 2.21 % in group 13 (6 % fat) to 3.29 in group 16 (36 % fat). In spite of the irregularity of the percentage figures in groups 10 and 11 in Exp. 2, the actual weights of cholesterol in the livers show again progressive increases in both experiments, from 0.072 to 0.156 g. in Exp. 2 and from 0.085 to 0.167 g. in Exp. 3. These amounts include of course the free cholesterol present, which, as shown in Exp. 1, is about 10 mg. The results of Exps. 2 and 3, therefore, confirm the finding of Exp. 1 that on diets of constant cholesterol but of increasing fat content the amount of cholesterol appearing in the liver is directly related to the fat content of the diet.

Considering now the fatty acid content, Table II shows that in Exp. 2 the four groups fall into pairs, i.e. groups 9 and 10 with 11 and 21 % fat in the diet contain 14.04 and 14.23 % fatty acids while groups 11 and 12 with 31 and 41 % fat in the diet contain 29.76 and 29.63 % fatty acid in the liver. The actual weights of fatty acids in the livers behave similarly. In Table III, Exp. 3 shows a similar phenomenon as regards the percentage figures, for groups 13 and 14 have 15.94 and 17.70 % fatty acid in the liver, while groups 15 and 16 contain 24.70 and 25.37 % fatty acid. The actual weights of fatty acid in this experiment, however, show a progressive increase with increasing dietary fat, although this is not as regular as in the control groups of Exp. 1.

DISCUSSION

The results of the control groups in Exp. 1 show that increasing the fat content of the diet causes linear increases in the percentage and amount of glyceride, a decrease in the percentage but no change in the amount of phosphatide and a small but definite accumulation of cholesteryl esters. The question arises whether the changes in cholesteryl esters are metabolically significant. Previous work has shown that the inclusion of even small amounts of cholesterol in diets results in an increase in the amount of this substance in the liver. Thus Channon & Tristram [1937] found that the daily intake by the 100 g. rat of only 2.82 mg. cholesterol for 21 days caused about a 30 % increase in the total cholesterol of the liver, while 9.46 mg. caused a 70 % increase. Further, this

deposition of cholesterol as ester, may be detected within a few hours of feeding and is progressive with time, while little change takes place in the free sterol [Aylward *et al.* 1935]. For these reasons it seemed possible that the increases of cholesteryl ester caused by the increasing fat content of the control diets 1a-4a might be related to the cholesterol content of the beef dripping used in the diets rather than to the changes consequent on the accumulation of glyceride in the liver. While the cholesterol content of the actual fat used in these experiments was not determined, another sample of beef dripping was found to contain 0.2% cholesterol. Every increase of 10% in the fat content of the diet would thus result in the cholesterol intake being increased about 1 mg. per 100 g. rat per day, an amount which appears too small to account for the differences observed between group 1a and the other control groups. Work by Minovici [1935] and Eckstein & Treadwell [1935-6] indicates that diets containing unsaturated fatty acids promote sterol synthesis. This finding, if confirmed, may explain the deposition in the liver of small quantities of cholesteryl esters caused by high fat diets, low in lipotropic factors.

With regard to the results of the inclusion of 2% cholesterol in the diet all three experiments show consistently that the amount of cholesteryl ester appearing in the liver is progressively increased by the fat content of the diet. The simplest explanation of this finding would be that the amount of cholesterol absorbed depends on the amount of fat being absorbed at the same time. Evidence on this question is, however, somewhat scanty and inconclusive. That cholesterol is not significantly absorbed unless fat is present in the diet is shown by the results of Thannhauser [1923] with human beings, Sano [1924] with dogs and Cook [1936] with rats. Some evidence exists also that absorption does not depend on the fat content of the diet. Chanutin & Ludewig [1933] fed two groups of rats on diets both of which contained 2.5% cholesterol and 4% cod liver oil, one diet containing 75% carbohydrate and the other 75% fat. Surprisingly, the amount of cholesterol in the livers of the animals on the high carbohydrate diet was 6%, some 4 times greater than that in the livers of the animals receiving 75% fat in the diet. Cook [1937] found no significant difference in the cholesterol contents of the livers of three groups of rats receiving 2% cholesterol with 15, 20 and 30% arachis oil in their diets respectively. The clear contrast between the present results and those of the latter author may perhaps be connected with the chemical nature of the dietary fat used.

One experiment was carried out in which the degree of absorption of cholesterol on a diet containing 40% fat and 2% cholesterol was measured. The cholesterol intake was calculated from the daily food intake and the analysis of the faeces of two groups, each consisting of eight animals kept in individual cages, and fed on the same diet with and without 2% cholesterol. The average cholesterol intake was 132 mg. and the average excretion was 63 mg. per 100 g. rat per day. This shows a daily absorption of about 70 mg. per 100 g. rat which is nearly twice as great as the constant absorption of 0.3-0.4 g. per kg. observed by Cook [1937] in his three groups of animals referred to above. Whilst the accuracy of estimations of the degree of absorption of a substance such as cholesterol by the intake and excretion method is limited, it seems certain that greater absorption occurred in this experiment with 40% fat, than in that of Cook [1937] with 15, 20 and 30% arachis oil. It would have been desirable to measure the cholesterol absorption at a series of levels of fat in the diet, but it was not possible to pursue this study further, and until further evidence is available, the progressive increase in the liver cholesterol with increasing fat in the diet must be regarded as the result of increased cholesterol absorption.

It is possible by the addition of supplements of either choline [Best *et al.* 1934] or some of its analogues [Channon *et al.* 1937, 1, 2] or by increasing the protein content of the diet [Beeston *et al.* 1935] to produce "cholesterol" fatty livers containing amounts of glyceride only a few times greater than the normal. It appears reasonable, therefore, to believe that the amount of glyceride present in the liver does not control the deposition of cholesteryl esters. The converse of this does not, however, hold, for the results reported in this paper show that increase in the amount of cholesteryl ester in the liver consequent upon the inclusion of 2% cholesterol in appropriate diets, is the cause of the marked increase in the amount of the liver glyceride. This increase is apparent at all dietary fat levels, even when the glyceride content of the liver is already high. Thus in the control group, 4a, the amount of glyceride, 0.722 g., is some 21 times the normal, yet this is further increased to 70 times normal in group 4b, and similar results occur in the other groups.

SUMMARY

1. The effect of varying the amount of dietary fat on the lipoids of the "fat" and "cholesterol" fatty livers has been investigated.
2. On a diet causing "fat" fatty livers in rats, the degree of glyceride infiltration is proportional to the amount of fat in the diet. There is a small increase in the amount of cholesteryl esters which does not appear to be related to the percentage of fat in the diet.
3. On a diet causing the "cholesterol" fatty liver, successive increases in the percentage of fat in the diet cause progressive and large increases in the cholesteryl ester content of the liver. Very considerable increases in the amounts of glyceride also occur, and the "cholesterol" fatty liver always contains much more glyceride than the "fat" fatty liver at any given level of dietary fat.
4. Small increases in the amounts of free cholesterol also occur with increases in the amount of dietary fats, and these become greater when cholesterol is present in the diets.

The expenses of this research were defrayed in part by a grant from the Medical Research Council to Prof. H. J. Channon.

REFERENCES

- Aylward, Channon & Wilkinson (1935). *Biochem. J.* **29**, 169.
 Beeston, Channon & Wilkinson (1935). *Biochem. J.* **29**, 2659.
 Best, Channon & Ridout (1934). *J. Physiol.* **81**, 409.
 Channon, Manifold & Platt (1938). *Biochem. J.* **32**, 969.
 — Platt & Smith (1937, 1). *Biochem. J.* **31**, 1736.
 — — Loach & Smith (1937, 2). *Biochem. J.* **31**, 2186.
 — & Tristram (1937). *Biochem. J.* **31**, 738.
 — & Wilkinson (1935). *Biochem. J.* **29**, 350.
 Chanutin & Ludewig (1933). *J. biol. Chem.* **102**, 57.
 Cook (1936). *Biochem. J.* **30**, 1630.
 — (1937). *Biochem. J.* **31**, 410.
 Eckstein & Treadwell (1935-6). *J. biol. Chem.* **112**, 373.
 Minovici (1935). *Bull. Soc. chim. biol.* **17**, 369.
 Sano (1924). *Tohoku J. exp. Med.* **4**, 417.
 Thannhauser (1923). *Dtsch. Arch. Klin. Med.* **141**, 290.