CCLXV. CHOLINE AND THE "CHOLESTEROL" FATTY LIVER.

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THE large increase in the percentage of cholesteryl esters in the livers of rats which have received diets containing amounts of cholesterol of the order of 1–2 % has been demonstrated by Okey [1933, 1, 2], Chanutin and Ludewig [1933], and Best, Channon and Ridout [1934]. Best and Ridout [1933] studied the effect of choline in preventing the occurrence of this "cholesterol" fatty liver in rats. These workers estimated the amount of "fat" in the liver by saponifying the livers and weighing the combined unsaponifiable fraction and fatty acids. They demonstrated that choline would prevent cholesterol feeding from producing a fatty liver, but their results threw no light on the changes which cholesterol feeding caused in the amount and nature of the individual liver lipoids, nor on the question as to whether the lipoid material present in the livers of the animals which received choline was of normal constitution. In an attempt to obtain further evidence on the mechanism of the action of choline, Best, Channon and Ridout [1934] carried out similar experiments in which detailed analyses were made of the lipoids of the livers of groups of animals, one of which had received a diet containing cholesterol and the other the same diet with the addition of choline. The essential results of this work were to show that choline caused a substantial decrease in the glyceride content of the livers from 9·5 % to 1·5 %, while at the same time the percentage of cholesteryl ester fell from 4·35 % to 1·77 %. It is to be pointed out however that normal rat liver contains no significant amount of cholesteryl ester, and it is clear that the amounts of choline given to the animals in these experiments had not effectively prevented the appearance of cholesteryl esters in the liver. The main conclusion from the results of this and other work described in that paper was that the action of choline was primarily to prevent an increase in the glyceride fraction of the liver, with a marked but subsidiary effect on the amount of cholesteryl esters present. Best [1934] has studied the curative effect of choline on the "fat" fatty liver, produced by feeding diets of high fat content, as opposed to the preventive effect already described. The work described in the present paper is a study of the curative effect of choline on the "cholesterol" fatty liver. Its object was to study more closely the removal of the different constituents from the "cholesterol" fatty liver in the hope that this would throw further light on the mechanism by which choline exercises its effect.

EXPERIMENTAL.

The general plan of the experiments was to feed a large number of rats on a diet calculated to cause the appearance in the liver of excessive amounts of glyceride and cholesteryl esters. After this preliminary period a group of animals

was killed, their liver lipoids were completely extracted and the extracts analysed for individual constituents. These figures served as a control for the rest of the experiment. The remainder of the rats were then divided into a number of groups, one half of which received a fat-free diet, while the others received the same diet with the addition of choline. At suitable intervals groups of animals were withdrawn and their liver lipoids analysed.

The extraction of the livers and analyses of the extracts were essentially as described by Best, Channon and Ridout [1934]. The only modification made was one which provided a means whereby the water content of the livers was obtained, because it was felt that it might be important to obtain data for the dry weight of the livers in view of the fact that the water content of fatty livers is considerably below that of normal livers. The method adopted for this purpose was to add to the livers a weighed amount of silver sand and to carry on the extraction after mincing as previously described. During all the processes of transfer and extraction every care was taken to prevent loss of the protein-sand residue. At the end of each extraction this residue was transferred to a weighed vessel, dried at 105° for 3 hours and weighed. By subtracting the combined weights of the sand-protein residue and that of the total ethereal extract from the original liver weight the water content was obtained. This figure will not be more accurate than $\pm 2\%$. The results were calculated as described in the paper by Best, Channon and Ridout.

Exp. 1. Daily records of the food intake on the different diets, the compositions of which are tabulated in Table I, were made.

Table I. *Description of the diets used in Exp. 1.*

		Diet after transfer			
		For 0–5 days		For 6–7 days	
	Preparatory cholesterol diet	Choline-free	With choline	Choline-free	With choline
Group No.		1 A	1 B	1 C	1 D
Caseinogen	20	—	—	—	—
Starch	50	—	—	65	65
Marmite	5	—	—	—	—
Salt mixture	5	—	—	5	5
Beef dripping	20	—	—	—	—
Cholesterol	2	—	—	—	—
Choline	—	—	1	—	1
Caseinogen (fat-free)	—	30	30	30	30
Sugar	—	70	69	—	—
Cod-liver oil	1 drop per 3 days per animal	—	—	—	—

The preparatory cholesterol diet was administered to the 70 rats for a period of 3 weeks. In the first 5 days after transfer the food intake of the animals on the diets began to decrease and therefore after 5 days the sugar of the diet was replaced by starch and for days 6–7 the animals received a diet as set out in Table I, under 1 C and 1 D. No attempt was made to make the preparatory cholesterol diet choline-free, because the fatty liver caused by cholesterol feeding is so intense that it did not seem important to consider this question. The diet used for days 0–5 was to all intent choline-free, for the B.D.H. fat-free caseinogen which was used contains amounts of choline of less than 5 mg. per 100 g. Similar diets have been used by Best [1934] in his studies of the cure of the "fat" fatty liver by choline.

Table II. *Weight records of the animals and the choline intake.*

Exp. 1.

Diets	Pre-paratory	Without choline						With choline					
Group No.		1A	2A	3A	4A	5A	6A	1B	2B	3B	4B	5B	6B
No. of animals	10	5	5	5	5	5	5	5	5	5	5	5	5
Days on experiment	—	1	3	4	5	6	7	1	3	4	5	6	7
Average wt. at beginning of experiment (g.)	174	167	173	168	176	169	173	166	175	168	166	170	174
Average wt. at transfer (g.)	199	186	210	178	211	195	204	175	209	192	196	192	208
Gain or loss (g.)	+25	+21	+37	+10	+35	+26	+31	+9	+34	+24	+30	+22	+34
Average wt. at end of experiment (g.)	—	182	198	169	193	176	180	190	195	174	184	177	184
Gain or loss since beginning (g.)	—	+15	+25	+1	+17	+7	+7	+24	+20	+6	+18	+7	+10
Choline intake per rat per day (mg.)	0	0	0	0	0	0	0	80	60	67	68	85	53
Average loss or gain per rat from time of transfer (g.)	—	−1	−2	−2	−4	−4	−5	+3	−3	−4	−2	−3	−5

Table III. *The percentages of the liver lipoids.*

Exp. 1.

Diets	Pre-paratory	Without choline						With choline					
Group No.		1A	2A	3A	4A	5A	6A	1B	2B	3B	4B	5B	6B
Days on diet		1	3	4	5	6	7	1	3	4	5	6	7
(*a*) Total fatty acids g./100 g. liver	9·75	9·54	8·57	7·62	7·59	6·35	5·98	7·39	5·44	7·64	4·76	4·99	5·80
(*b*) Unsaponifiable matter g./100 g. liver	4·42	4·41	3·07	4·04	3·15	3·93	2·92	3·73	2·79	4·79	2·53	3·20	3·07
Total (*a* and *b*)	14·17	13·95	11·64	11·66	10·74	10·28	8·90	11·12	8·23	12·43	7·29	8·19	8·87
Iodine value of fatty acids	—	80	87·8	104·2	85·7	92·9	86·6	84·2	86·8	105·1	97·2	98·3	94·6
Lecithin	3·05	3·02	3·05	2·90	2·83	3·10	3·48	2·50	3·23	3·03	3·18	3·11	3·27
Cholesterol	0·351	0·309	0·280	0·302	0·298	0·322	0·34	0·288	0·293	0·338	0·285	0·293	0·306
Cholesteryl oleate	6·08	6·55	4·55	5·69	4·26	5·33	3·81	5·46	3·97	7·07	3·41	4·60	4·30
Glyceride	5·18	4·78	4·64	3·25	3·91	1·94	1·96	3·40	1·51	2·43	1·08	0·83	1·70
Total	14·661	14·659	12·520	12·142	11·298	10·692	9·59	11·648	9·003	12·868	7·955	8·833	9·576

Results. In Table II are recorded data concerning the number of animals used, their changes in weight during the experiment and the average choline intake per rat per day in the different series.

It will be observed that, although the animals were all heavier at the end of the experiment than they were at the beginning, they decreased very slightly in weight after transfer from the preparatory cholesterol diet. The maximum loss was 5 g. per animal of initial weight 170 g. in the one week period, and usually the losses were less than this. These weight changes are to be regarded as being of little importance in interpreting the results, for apart from their insignificance they occur to a similar extent in the corresponding groups of animals.

In Table III the composition of the liver lipoids in the successive groups is set out in detail.

The results of Exp. 1 will be considered with those of the further experiment now to be described. It may be mentioned here however that choline has not exercised any significant curative effect on the "cholesterol" fatty liver. Since Best and Ridout [1933] have shown that a considerably greater quantity of choline is necessary to prevent the production of the "cholesterol" fatty liver than the "fat" fatty liver, it was decided to carry out a more prolonged experiment in which the percentage of choline in the diet was increased.

Exp. 2. The diets used are described in Table IV.

Table IV. *Description of the diets used in Exp. 2.*

	Preparatory cholesterol diet	Diets after transfer	
		Choline-free	With choline
Fat-free caseinogen	20	30	30
Starch	39	55	53
Beef dripping	30	10	10
Salt mixture	5	5	5
Cholesterol	2	—	—
Choline (as chloride)	—	—	2
Marmite	5	—	—
Cod-liver oil	1	—	—

In the preparatory cholesterol diet which was administered for 21 days to 74 animals the percentage of beef dripping was increased to 30 % and fat-free caseinogen was employed. Both these changes were made in the hope of increasing the neutral fat fraction of the liver lipoids to a higher figure. Beef dripping (10 %) was added to the diets after transfer to render them more palatable and thereby further to increase the choline intake.

Table V. *Weight records of the animals and the choline intake.*

Diets	Preparatory	Without choline				With choline			
Group No.		1A	2A	3A	4A	1B	2B	3B	4B
No. of animals	11	8	8	7	8	8	8	8	8
Days on experiment	—	2	5	9	12	2	5	9	12
Average wt. at beginning of experiment (g.)	241	238	246	250	245	245	239	242	243
Average wt. at transfer (g.)	258	246	259	262	260	258	244	256	254
Gain or loss (g.)	+15	+8	+13	+12	+15	+13	+5	+14	+11
Average wt. at end of experiment (g.)	—	251	252	234	227	262	239	234	213
Gain or loss since beginning (g.)	—	+13	+6	−16	−18	+17	0	−8	−30
Choline intake per rat per day (mg.)	—	0	0	0	0	170	170	148	143
Average loss or gain per rat from time of transfer (g.)	—	+1	−1	−6	−7	+1	−1	−4	−8

Results. Table V contains the essential data relative to the animal side of the experiment, while the detailed composition of the liver lipoids is recorded in Table VI.

Table VI. *The percentage of the liver lipoids.*

Diets	Pre-paratory	Without choline				With choline			
Group No.		1A	2A	3A	4A	1B	2B	3B	4B
Days on diet ...		2	5	9	12	2	5	9	12
(*a*) Total fatty acid g./100 g. liver	12·24	9·35	9·53	7·50	9·54	12·46	8·40	6·25	6·43
(*b*) Unsaponifiable matter g./100 g. liver	4·75	3·72	4·79	3·31	3·85	4·75	4·70	2·92	3·00
Total (*a* and *b*)	16·99	13·07	14·32	10·81	13·39	17·21	13·10	9·17	9·43
Water content	61·8	65·4	64·5	66·9	64·4	63·2	64·9	67·8	66·3
Lecithin	3·43	3·22	3·09	3·42	3·26	3·26	3·42	3·49	3·53
Cholesterol	0·390	0·332	0·346	0·327	0·367	0·376	0·395	0·331	0·354
Cholesteryl oleate	7·06	5·48	7·02	4·93	5·79	7·26	6·70	4·30	4·33
Glyceride	7·14	4·97	4·33	3·26	4·83	7·53	3·41	2·11	2·20
Total	18·020	14·002	14·786	11·937	14·247	18·426	13·925	10·231	10·414

Discussion.

The nature of the cholesterol fatty liver.

The figures recorded in Tables III and VI for the lipoids of the livers of the animals receiving the preparatory cholesterol diets illustrate the nature of the "cholesterol" fatty liver. They are in agreement with those of Okey [1933, 1, 2], Chanutin and Ludewig [1933] and Best, Channon and Ridout [1934] in that the amounts of cholesteryl ester and glyceride are markedly increased. The degrees to which these increases occur differ however in the results of the different workers. Thus Best, Channon and Ridout obtained in rats fed for 26 days on a diet containing 2 % cholesterol, 9·50 % glyceride and 4·35 % cholesteryl esters. The present results show the presence of 5·18 % and 7·14 % of glyceride and 6·08 % and 7·06 % of cholesteryl esters in Exps. 1 and 2 respectively. Calculation from the figures of Chanutin and Ludewig [1933] shows that the livers of rats fed by them on a cholesterol diet for 57 days contained 3·40 % glyceride and 5·69 % cholesteryl esters, while after 269 days these percentages had increased to 6·11 % and 7·76 % respectively. The wide variation in these results in the relative increases in these two constituents must be significant in the understanding of the part played by the liver in cholesterol metabolism, for calculation shows that the amount of glyceride in all the results mentioned above varies from 0·60 to 2·18 times that of the cholesteryl ester present. Variation in the proportions of the different dietary constituents and the duration of feeding must clearly be factors involved, although these results and other work which we have in progress suggest that the amounts in which cholesteryl esters and glyceride will appear in the liver depend on factors other than the percentage of fat and cholesterol present in the diet. The effect of increasing the fat content of the diet is shown in our Exp. 2, where the fat content was raised from 20 % to 30 % at the expense of 10 % starch. This resulted in an increase of 1·96 % in the glyceride and 0·98 % in cholesteryl esters for the same duration of feeding (21 days), which indicates the importance of the fat content of the diet in influencing these relative amounts. Yet in other investigations we have observed livers containing 16 % of cholesteryl esters and only 1 % of glyceride on a diet containing 40 %

fat. Further investigations into the significance of this finding will be reported later.

The results in Tables III and VI also confirm previous observations that the increase in free cholesterol, while positive, is not profound. The livers of our normal animals maintained on the stock diet contain 260 mg. per 100 g., while in Exps. 1 and 2 the figures are 351 and 390 mg. per 100 g. respectively.

On the other hand no decrease has occurred in "lecithin" content as a result of cholesterol feeding, for many determinations on our stock animals show a "lecithin" content of 3·3 to 3·5 %. It is usual for the phosphatide content of fatty livers to be depressed and marked decreases were observed in the "cholesterol" fatty liver by Best, Channon and Ridout [1934]. On the other hand, the livers of the rats used by Chanutin and Ludewig [1933] contained 3·12 % of "lecithin" 2 days from the beginning of the experiment and 2·91 % 267 days later, when they had become intensely fatty—an inconsiderable change. We are investigating further the factors concerned in affecting the amounts of the various lipoid constituents present in fatty livers of different types, because it seems to us that interpretation of the mechanism of the preventive and curative actions of any given agent cannot be made until these basal facts are acquired.

The effect of choline.

Before discussing any possible effect of choline in curing the "cholesterol" fatty liver, one point needs emphasis. Inspection of the amounts both of the individual constituents and of the total lipoids in Tables III and VI shows that there is no constant change in any series of groups with time. Thus in Exp. 2, group 1 A contained 14·002 % of total lipoid, which increased to 14·786 % 3 days later, fell to 11·937 % after a further 4 days and by the twelfth day had risen again to 14·247 %. These variations are probably to be taken as of no significance in considering the effect of choline.

Inspection of the many figures recorded in papers published from the Toronto laboratories on fatty liver production show the enormous variations which are encountered in the fat content of the livers of rats on a fatty-liver-producing diet and many observations in this laboratory confirm this finding. Each of the results included in Table VI represents the results obtained on the pooled tissues of eight animals. In Exp. 1 groups of five animals were used because it was thought that this would be sufficiently large with a diet causing a "cholesterol" fatty liver to yield a uniform result. The increase in the size of the groups to eight in Exp. 2 has in fact yielded results which show less rather than more consistency than those of Exp. 1.

Bearing in mind these variations in the individual groups, the effect of choline feeding may now be considered. Both in Exps. 1 and 2 choline administration has had no effect in noticeably decreasing the amount of any of the constituents. Such changes as occur in the choline-fed groups are apparent in the groups which did not receive choline. Because of the individual variations, it is perhaps unwise to consider the average figures for the animals receiving diets with and without added choline. These figures have however been collected in Table VII.

In both these experiments it is seen that the fall in the amounts of the different lipoids from the control figures differs little whether the animals received choline or not. In both experiments there is a slight fall, which is probably not significant, in the percentage of cholesteryl esters, with a more marked decrease in the amount of the glyceride fraction. This latter decrease is 1·59 % in Exp. 1

Table VII. *Mean values for percentages of liver lipoids of rats with "cholesterol" fatty livers, receiving diets with and without added choline.*

	Exp. 1			Exp. 2		
	Preparatory diet	Without choline	With choline	Preparatory diet	Without choline	With choline
"Lecithin"	3·05	3·06	3·05	3·43	3·25	3·40
Cholesterol	0·351	0·30	0·300	0·390	0·343	0·360
Cholesteryl ester	6·08	5·03	4·80	7·06	5·80	5·65
Glyceride	5·18	3·41	1·92	7·14	4·35	3·81

and 0·54 % in Exp. 2, decreases which may suggest that the administration of choline has effected a diminution in the amount of the glyceride fraction. In this connection two points need mention. In Exp. 1 the average choline intake per rat per day was 65 mg. and in Exp. 2 158 mg. This would suggest that, if choline were effective in removing glyceride, a greater decrease should have occurred in Exp. 2. On the other hand, the diet used in the latter experiment contained 10 % of fat, which may have been a factor counteracting the increased choline intake, although in view of the results of Best [1934] this effect should have been relatively small.

Best, Channon and Ridout [1934] found that when 230 mg. of choline per day per rat were given to rats on a diet containing 20 % of fat and 2 % of cholesterol, the deposition of glyceride which resulted when no choline was given was entirely prevented. On the other hand, the amount of cholesteryl ester was only reduced 60 % by choline administration and it is clear therefore that even such large amounts of choline cannot prevent entirely the deposition of cholesteryl esters. In Exp. 2 158 mg. of choline per day were given to rats maintained on a normal diet in an attempt to cure the "cholesterol" fatty liver already produced. The failure to achieve this end in the conditions used in our experiments, is perhaps not very surprising in view of the results from preventive experiments just mentioned. On the other hand, it is in striking contrast to the results of Best [1934] who found that amounts as low as 10 mg. of choline per day would cure the fatty liver caused by diets of high fat content. It is to be noticed however that this type of fatty liver is characterised by a large increase in the amount of glyceride only. Such positive evidence as our results may afford seems to confirm the view previously expressed by Best, Channon and Ridout [1934] that the effect of choline is on the glyceride fraction and that its action in preventing the deposition of cholesteryl esters is secondary to this effect on the neutral fat.

Summary.

1. Large numbers of rats have been fed on a diet containing fat and 2 % cholesterol in order to produce "cholesterol" fatty livers.

2. From analyses of the total ether-soluble materials the nature of the "cholesterol" fatty liver so produced is discussed in relation to the observations of other workers.

3. Further groups of animals after receiving this preparatory diet have been transferred to various diets with and without added choline.

4. At successive periods up to twelve days pooled samples of the livers of different groups receiving diets with and without added choline have been analysed in an attempt to study whether choline had a curative effect on the "cholesterol" fatty liver.

5. Choline had no obvious effect in causing a decrease in any of the lipoid constituents save perhaps in the case of the glyceride fraction.

6. These results are discussed in relation to those of Best, Channon and Ridout [1934], concerning the preventive action of choline on "cholesterol" fatty liver production, and those of Best [1934] on the effect of choline in curing the fatty liver characterised by the presence of excessive amounts of glyceride.

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