CCCXV. THE INFLUENCE OF THE CASEINOGEN CONTENT OF DIETS ON THE NATURE OF THE "CHOLESTEROL" FATTY LIVER.

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IN continuation of investigation of factors influencing the nature of the "cholesterol" fatty liver, Channon and Wilkinson [1935] carried out experiments in which the percentage of protein (caseinogen) in the diet was varied at the expense of the carbohydrate (glucose). The results of those experiments, which were carried out with relatively small groups of animals in investigations concerned with the production of the "fat" fatty liver, indicated that the amount of glyceride in the "fat" fatty liver was governed by the amount of caseinogen in the diet. A further experiment of similar nature on the "cholesterol" fatty liver was reported. The livers of animals receiving 5% caseinogen in the diet contained 19.88% (wet liver weight) more glyceride than did those of other animals receiving 30% of the protein. Criticisms of some of the individual experiments recorded in that paper have been made by Best, Huntsman and Ridout [1935]; since these have been discussed in a joint paper from the two laboratories, they need not be referred to further [Best and Channon, 1935].

The present paper is concerned with an extension of this experiment on the "cholesterol" fatty liver, in order to confirm the lipotropic action of the protein fractions of diets containing cholesterol on the glyceride content of the liver and to study whether any effect was also exercised on the lipid constituents other than the glyceride; to attempt thereby experimentally to produce "cholesterol" fatty livers of varying glyceride content; to study the preventive action of choline on such livers in the hope of throwing light on the question of whether choline affects the cholesterol fraction directly or only indirectly by virtue of its action on the glyceride; and further to seek evidence which might differentiate the lipotropic action of dietary protein fractions from that of choline.

EXPERIMENTAL.

The first experiment was carried out by analysing the pooled livers of groups of animals by the methods previously used after 3 weeks' feeding on diets containing varying percentages of caseinogen. The materials which were in constant proportions in the diets of all the groups of animals were: beef dripping 20, cholesterol 2, salt mixture 5, marmite 5, with one drop of cod-liver oil per animal every 3 days. The four diets differed however in that the protein content (caseinogen, 5, 10, 15 and 30 parts) was varied at the expense of the carbohydrate (glucose, 63, 58, 53 and 38 parts). The caseinogen had been previously extracted industrially and was further extracted several times with hot alcohol-ether mixture before use.

Each group of animals consisted of 5 bucks and 5 does, chosen so that one animal of each sex of as nearly as possible the same weight was present in each group. The average initial weights of the animals in the groups receiving 5, 10, 15 and 30% caseinogen in the diet were 155, 158, 155 and 151 g. respectively. Two animals in group 1 and one animal in group 2 died during the experiment. At the end of the experiment there were 8, 9, 10 and 10 animals in the four groups respectively. The corresponding average changes in body weight at the end of the experiment, calculated as percentage of the initial value, were 0.0, +11.1, +18.0 and +17.0%.

The animals were allowed food *ad libitum* and at the end of the experiment were killed 24 hours after the last feeding. In order to obtain the livers in as comparable a state as possible from the point of view of their blood content, a guillotine was used for the killing and the liver dissected from each animal after as much blood as possible had been drained from the body. It was hoped that this precaution might help to avoid some of the variations which have been previously encountered in the percentages of liver fat in individual animals and this method of killing has now been adopted as routine.

The results of this experiment are recorded in Table I. Before discussing the results it is necessary to emphasise that, save for the protein and carbohydrate fractions, the diets were of constant percentage composition for all four groups. By biological assay the choline content of this constant fraction of the diet was found to be such that each animal received less than 2 mg. of choline per day, the marmite contributing practically the whole of this amount. The only difference in the intake of choline from this fraction of the diet by the different groups depended on the differences in food intake, which were small. Consequently in considering any possible effect of choline in relation to that of the protein fraction, the choline content of the latter only has to be taken into account. This was determined several times in this Department and was found to be 0.8–1.2 mg./100 g. In order to obtain confirmation of this figure a sample was submitted to Prof. Best, who very kindly carried out independent determinations at Toronto and reported a figure of less than 5.0 mg./100 g. Any possible choline content of the glucose can be neglected because, if choline were present in that material, it would diminish any effect shown by decreasing the protein content of the diet. Figures for the average daily intake of choline by the different groups are recorded in Table I.

Table I. *Percentage of lipoids present in the livers of rats receiving diets containing 20% fat and 2% cholesterol with varying percentages of caseinogen (g./100 g. fresh liver).*

Group no. ...	1	2	3	4
Caseinogen content of diet, %	5	10	15	30
Liver as % of final body weight	6.69	5.35	5.18	4.45
Choline intake (mg./rat/day)	1.65	1.80	1.79	1.55
Lecithin, %	1.71	2.31	2.83	3.14
Cholesterol, %	0.29	0.32	0.35	0.37
Cholesteryl oleate, %	5.89	7.26	8.30	9.72
Glyceride, %	32.75	21.42	14.06	4.23
Total, %	40.64	31.31	25.54	17.46

The figures in Table I show the striking effect of varying the caseinogen content of the diet at the expense of the carbohydrate in increasing the percentage of the total lipoids present. Inspection of the figures for the individual constituents shows that with the increase of the protein fraction of the diet

from 5 to 30 % there are consistent progressive increases in the percentages of lecithin (1.71 to 3.14 %), cholesterol (0.29 to 0.37 %), and cholesteryl ester (5.89 to 9.72 %), in a manner which might suggest a direct effect of the protein fraction in controlling these constituents in the conditions of these experiments. Further, the percentage of the glyceride increases profoundly in the reverse direction, for group 1 (5 % protein) contains 32.75 % as compared with group 4 (30 % protein) which contains 4.23 % with a regular gradation in the intermediate groups. All these findings are confirmed by groups 5*a*–8*a* in Exp. 2 to be described later.

The results appear to be dissociated from any effect of choline. In the first place the amount of caseinogen ingested by groups 1 and 2 differed by approximately 0.5 g. per animal daily and this has caused a decrease of 11.33 % in the glyceride content calculated on the fresh liver weight. On a choline content of 5 mg./100 g. this ingestion of 0.5 g. of caseinogen corresponds to an extra choline intake of 25 γ over the control group, an amount far too small on all present evidence to have so striking an effect. It is remotely possible that the glucose contains a substance which has a marked effect in causing deposition of glyceride in the liver and, if this were so, the results would be explained on that basis, but this possibility seems so remote that it is not profitable to discuss it further at the present time. On these results therefore the ingestion by each animal of an extra 0.5 g. of caseinogen daily has caused a decrease in the liver glyceride of 11.33 % between groups 1 and 2, and 7.36 % between groups 2 and 3, whilst an additional 1.5 g. daily has caused a decrease of 9.83 % between groups 3 and 4. These results therefore fully confirm the finding of Channon and Wilkinson [1935] that the amount of the protein fraction of the diet controls the percentage of glyceride in the "cholesterol" fatty liver, and they show that a similar but more limited control is exercised in the reverse sense on the percentages of lecithin and of cholesterol and its esters.

The only available figure showing the effect of dietary protein fractions in decreasing the glyceride content of the liver is that observed in the experiment of Best and Huntsman [1935], who recorded the first observation of this lipotropic effect on the liver glyceride. These authors found that transfer of animals already having fatty livers to a diet of pure sucrose caused an increase in the liver fat of some 8 %. After a similar transfer to a diet consisting of 80 % sucrose and 20 % caseinogen however this increase did not occur. In their experiment therefore a daily intake of 2 g. of caseinogen prevented an 8 % rise in the liver glyceride, although it did not cause the high initial value to fall. The very different conditions of these experiments however prevent any significant comparison of our results with those of the Toronto workers being made at the present time.

It has been pointed out that on the percentage figures in Table II the increase of the percentage of the protein fraction in the diet appears to cause significant and steady increases in the lecithin, cholesterol and cholesteryl ester fractions. These percentage figures, although instructive from one point of view, do not yield accurate information as to the extent to which the liver is acting as a reservoir, because of the great increase in the size of the livers which occurs when they become loaded with cholesteryl esters and glyceride. Thus in group 1 the liver constituted 6.69 % of the final body weight, whereas in group 4 it was but 4.45 % with intermediate figures between these values.

For this purpose it is therefore necessary to compare the actual weights as opposed to the percentages of the individual constituents in the liver. Since the average initial weight of the animals in the groups was approximately constant, the amounts of each lipid constituent in the livers of each group may be

Table II. *Weight of lipoids in the liver of the 100 g. rat, calculated on the final and initial body weights (g.).*

		Percentage of protein in diet			
		5	10	15	30
Lecithin	Calculated on Final body wt.	0.115	0.124	0.146	0.140
	Initial „	0.115	0.137	0.173	0.163
Cholesterol	Final „	0.020	0.017	0.018	0.016
	Initial „	0.020	0.019	0.022	0.019
Cholesteryl oleate	Final „	0.396	0.390	0.430	0.433
	Initial „	0.396	0.432	0.508	0.505
Glyceride	Final „	2.178	1.149	0.728	0.189
	Initial „	2.178	1.273	0.859	0.220
Totals	Final „	2.709	1.680	1.322	0.778
	Initial „	2.709	1.861	1.562	0.907

compared at the end of the experiment, if changes in body weight during the experiment be disregarded. This entails reference to the average animal. An alternative method is to refer all the results to the 100 g. rat and this is preferable for comparison with other results. In order to determine whether the average change in body weight, which occurred during the experiment and which varied from 0 to +18% in the different groups, plays any part in the interpretation of the results, we have calculated the amounts of the different lipoids in the liver of the 100 g. rat when referred both to the final and initial body weights respectively. This latter figure will of course correspond to that of the average animal at the end of the experiment as mentioned above, in which any body weight change occurring during the experiment is neglected. The figures so obtained will not represent quite accurately the amounts of the constituents which would have occurred in the livers if rats weighing 100 g. had been used, because liver size does not bear an entirely linear relationship to body weight. This factor however is sufficiently unimportant to be disregarded, and in any case the results from the different groups may be compared with each other.

A number of points emerge from Table II. In the first place, it is clear that whichever method of reference be adopted, the actual weight of lecithin present in the liver increases as the percentage of the dietary protein fraction is increased, at least up to 15%. This result is confirmed by the results of Exp. 2 (groups 5a-8a, Table V), where the increase continues to the 30% protein group also (group 8a). Thus the absolute weight of lecithin is increased as are the percentages recorded in Table I.

On the other hand whilst the free cholesterol is increased in all cases above normal, such slight differences as occur between the different groups are inconsistent and it is clear that the graded percentage increases recorded in Table I are merely the result of a dilution effect, the percentage falling as the liver increases in size, with decrease in the dietary protein percentage. This result also is borne out by those of Exp. 2 (Table V).

With regard to the cholesteryl ester fraction the figures are not conclusive. Whilst the lower values occur in the groups receiving less caseinogen in the diet, and the higher values with increase of dietary protein, there is no progressive change. The results in Exp. 2 (Table V) also bear this out. In two other experiments in which diets containing 5% and 30% caseinogen were used, the absolute amounts of cholesteryl ester per 100 g. rat were 0.245 and 0.246 g. for 5% protein and 0.281 and 0.308 g. for 30% protein. Yet a further experiment gave for 10% and 50% of caseinogen in the diet for 14 days 0.164 and 0.211 g.

respectively. Thus whilst there is a general tendency for the absolute amount of cholesteryl ester in the liver to be increased by increase of the amount of dietary protein consumed, no significance can at present be assigned to this finding. Thus, as in the case of the free cholesterol, the effect of lowered dietary protein in decreasing the percentage (Table I) may be a dilution effect only.

Lastly, Table II shows that virtually the whole of the difference between the amounts of total lipoids in the livers of the four groups depends on differences in the glyceride contents. The glyceride of group 1, 2.178 g., is some 60 times that of a normal liver; with increasing protein this decreases until with 30 % of caseinogen in the diet it is only 5 times that of the normal. We have no figures for the total reserve fat of animals on these particular diets, or any proof of the source of the liver glyceride. If it be assumed however that the liver glyceride has been derived from the fat depots and that the total depot fat of a 100 g. rat is 12 g., it is seen that the 5 % protein diet has caused the appearance in the liver of about one-sixth of the total reserve fat.

As regards the quantitative aspect of the caseinogen effect, Table II shows that when referred to group 1 the ingestion of an extra 0.5, 1.0 and 2.5 g. of caseinogen in groups 2, 3 and 4 has prevented the appearance in the liver of 1.029, 1.450 and 1.989 g. of glyceride respectively. The preventive effect of increasing the dietary protein fraction is thus the more strikingly shown at the lower levels.

One further point worthy of notice is that, whether the results for the different constituents be compared on the basis of the initial or final body weights, the general trend of each series of figures remains the same, even though the figures individually are somewhat changed.

Further work is now in progress in order to equate the lipotropic action of the protein fraction of the diet with that of choline; whether this action is due to an integral part of the caseinogen molecule or to a contaminant is at present unknown [Best and Channon, 1935].

These results show therefore that the intake of dietary protein fractions governs the amount of glyceride in the "cholesterol" fatty liver. This glyceride control, first demonstrated by Best and Huntsman [1935] in a transfer experiment on animals already having "fat" fatty livers, has thus now been extended to normal animals receiving diets which cause both "fat" and "cholesterol" fatty livers [Channon and Wilkinson, 1935]. The results show further that dietary protein also controls the absolute amount of liver lecithin and possibly that of cholesteryl esters, while they provide the means whereby "cholesterol" fatty livers of different types may be readily produced.

We have therefore used this finding for further study of the action of choline in preventing the "cholesterol" fatty liver. The one recorded experiment in which the preventive action of choline on the individual liver lipoids was studied was that of Best, Channon and Ridout [1934]. These authors found that 230 mg. of choline per rat per day entirely prevented the deposition of glyceride in the livers of animals receiving a diet containing 20 % of fat and 2 % of cholesterol. From the fact that this choline administration prevented not more than 60 % of the cholesteryl ester deposition, it was concluded that the primary effect of choline was on the glyceride fraction.

The method of experiment has been essentially the same as was used in Exp. 1, save that in addition to groups of animals receiving 5, 10, 15 and 30 % of caseinogen, further groups have received similar diets with the addition of choline chloride (1 %). On the food intakes, which were measured daily, the amount of choline ingested per rat per day varied from 80 to 74 mg. in groups

5*b*–8*b* respectively, whilst the corresponding control groups (5*a*–8*a*) received approximately 2 mg. daily in the basal diet.

The percentage composition of the liver lipoids of the two series of groups of animals are set out in Table III. If the effect of the dietary protein fraction be first considered (control groups 5*a*–8*a*) it is seen that the various liver lipoid

Table III. *Percentages of lipoids present in the livers of rats receiving diets containing 20% fat, 2% cholesterol, with varying protein percentages with and without added choline.*

Group no.	...	5 <i>a</i>	5 <i>b</i> Control + choline	6 <i>a</i>	6 <i>b</i> Control + choline	7 <i>a</i>	7 <i>b</i> Control + choline	8 <i>a</i>	8 <i>b</i> Control + choline
Protein content of diet, %		5	5	10	10	15	15	30	30
No. of animals in group		10	10	10	10	10	10	10	10
Average wt. change, % initial body wt.		-1.2	-1.2	+6.0	+3.0	+7.0	+3.1	+4.1	+1.1
Choline intake, mg.		2.0	80	1.9	77	1.8	77	1.7	74
Liver as % of final body wt.		5.36	3.70	4.82	3.86	4.02	3.83	4.17	3.99
Lecithin, %		1.88	2.95	2.82	3.04	3.46	3.62	3.76	3.71
Cholesterol, %		0.304	0.239	0.296	0.338	0.338	0.374	0.403	0.387
Cholesteryl oleate, %		5.16	3.42	7.15	4.66	7.43	4.57	7.72	6.45
Glyceride, %		24.78	0.98	16.17	3.40	6.11	2.26	2.92	2.71
Total, %		32.12	7.59	26.44	11.44	17.34	10.82	14.80	13.26

percentages bear out the findings of Exp. 1 in every respect and they need therefore no further discussion. The only further point worthy of notice in this connection is that whereas in Exp. 1 the total lipid percentages in the groups 1–4 (5–30% caseinogen) varied from 40.64 to 17.46%, in the present experiment they range from 32.12 to 14.80%. Two other experiments carried out under identical conditions with 5 and 30% of caseinogen gave 31.92–16.44% and 38.54–16.41% respectively. The difference in the results from series of groups of animals which have received the same diet for the same period but at different times has no explanation at present. Some possible reasons for this phenomenon, which frequently occurs, have been discussed by Best and Channon [1935].

Further discussion of the figures in Table III will be confined therefore to mention of the preventive effect of choline on the different lipid percentages, because the action of this substance on liver "fat" has been usually discussed on this basis. The addition of choline has significantly increased the percentage of lecithin in all the groups where the glyceride percentage was high (*cf.* 5*b*–7*b* with 5*a*–7*a*). It has had little effect on the percentages of free cholesterol, but has caused a marked and regular decrease in those of the cholesteryl ester fractions of all the groups. Its chief effect however is on the glyceride fractions where the striking preventive action of the 80–74 mg. given per rat per day is shown in all the groups. Thus in group 5*b* the glyceride is at the normal level, whereas the control group, 5*a*, contains 24.78% glyceride. Similarly group 6*b* contains 3.4% as compared with the 16.17% of its control, 6*a*. The percentage of glyceride in groups 6*b*–8*b* varies from 3.40 to 2.7%, figures which are about 2% above normal. It appears therefore that the deposition of cholesteryl esters causes the accumulation of some 2% glyceride in spite of the presence of a considerable amount (1%) of choline in the diet.

The figures in Table IV, in which the absolute weights of the liver constituents are calculated on the basis of the 100 g. rat on the final body weight, do not differ significantly from those calculated on the average animal (initial

Table IV. *Weight (g.) of individual lipoids present in the liver of the 100 g. rat (calc.).*

Group no. ...	5a	5b	6a	6b	7a	7b	8a	8b
Protein content of diet, %	5	5	10	10	15	15	30	30
Choline intake, mg.	2	80	1.9	77	1.8	77	1.7	74
Lecithin, %	0.101	0.109	0.136	0.117	0.139	0.139	0.157	0.148
Cholesterol, %	0.016	0.009	0.014	0.013	0.014	0.014	0.017	0.015
Cholesteryl oleate, %	0.276	0.127	0.345	0.180	0.299	0.175	0.322	0.257
Glyceride, %	1.327	0.036	0.779	0.131	0.246	0.087	0.122	0.108
Total, %	1.720	0.281	1.274	0.441	0.698	0.515	0.618	0.528

body weight) and the latter are therefore not recorded. The findings of the effect of increasing the dietary protein fraction discussed under Exp. 1 appear confirmed, and the effect of choline may now be considered in relation to the latter.

It is of interest to discuss whether any conclusion may be drawn from the figures in Table IV, as to whether lecithin is functionally active in controlling the

Table V. *Degree of prevention of glyceride and cholesteryl ester deposition (g./liver of 100 g. rat).*

Group	Fatty acid	Cholesteryl oleate
5a-5b	1.291	0.149
6a-6b	0.648	0.165
7a-7b	0.159	0.124
8a-8b	0.014	0.065

degree of fat infiltration and whether choline exercises its effect through this substance. The first point for observation is that the addition of choline has not caused any increase in the amount of lecithin in any of the four groups, even though in groups 5b and 6b it has caused a profound decrease in the total lipid constituents. This might be used as an argument that the choline effect is not exercised through lecithin, an argument which consideration of the lecithin figures in the control groups of animals might appear to confirm. In these the amount of lecithin actually increases with increasing caseinogen intake, although the total lipoids of the liver are also rapidly falling (*cf.* 5a and 6a). Both these lines of argument are however open to possible objections; thus although choline has caused no increase in the amount of liver lecithin, it might yet be exercising its effect by enabling lecithin synthesis to occur, the newly synthesised lecithin being used for the transport of fatty acids to the tissues and being metabolised in the process, whilst the fact that there is an increase in lecithin with increasing dietary protein might be connected with an entirely different phenomenon, possibly the phosphorus content of the caseinogen. However on their face value these results suggest that the lipotropic effect of the dietary protein fraction is to be differentiated from that of choline, in the sense that whilst it prevents fatty livers and causes an increase in the amount of lecithin, choline prevents fatty livers but does not cause such an increase. We feel it wise to regard these results as being no more than suggestive.

The degree of prevention caused by the provision of choline can be more readily studied from the figures in Table V.

These figures show in relation to those in Table IV that in the four experiments choline has prevented the appearance of 97, 83, 65 and 11% of the glyceride which appeared in its absence, and that of 54, 48, 41 and 20% respectively of the cholesteryl ester. It is not possible from such figures definitely to decide whether choline is exercising a direct preventive effect on the cholesteryl

ester fraction. If its effect on the latter were dependent on a primary action in preventing glyceride infiltration, then it would have been anticipated that the figures in the two columns would have run parallel. Such however is not the case. This might suggest therefore that the action of choline in preventing cholesteryl ester deposition was independent of glyceride. In this connection it is to be observed that in groups 7*a* and 8*a* the amount of glyceride which appeared in the liver in the absence of added choline was relatively quite small compared with those of 5*a* and 6*a*. Since the amounts of choline were approximately constant for all the groups, it would have been expected, were there a direct action on the cholesteryl ester fraction, that there would have been a considerably increased degree of cholesteryl ester prevention in groups 7*a* and 8*a*, for in these two groups the choline was almost entirely free to act in this direction. The figures however do not bear this out. We feel therefore that no conclusion can be drawn as to whether there is a direct effect of choline on the cholesteryl ester fraction. It will be remembered that Best and Ridout [1935] observed that there was a process for the removal of cholesteryl esters from the liver in the absence of dietary choline, but that the rate of removal was increased after some 18 days by choline administration.

Another point which needs mention is that in these experiments the lipotropic effect of protein is confined to the glyceride fraction only, whereas that of choline affects the cholesteryl ester fraction also. This might appear at first sight to differentiate the protein fraction effect from that of choline in the way that the lecithin figures have indicated. Prof. Best has kindly pointed out to us however that, if 1 g. of protein had a lipotropic action equivalent to 3 mg. of choline, as suggested by Best, Huntsman and Ridout [1935], then this lack of effect of the dietary protein fraction on cholesteryl esters would not indicate a qualitative difference in the lipotropic action, because, say, 9 mg. of choline would have presumably little effect, if any, on the cholesteryl ester fraction.

It must be clearly realised that the amounts of choline used do not represent the minimum necessary to give a preventive effect. The object of the experiments was to choose a daily choline intake which would lie between the 230 mg. found to be completely preventive as regards the glyceride fraction by Best, Channon and Ridout [1934], and that which would have a very definite, although not completely preventive effect.

SUMMARY.

The effect of quantitative variations in the protein fraction of the diet on the amount and composition of the lipoids of the livers of rats receiving a diet of low choline content and containing 20 % of fat and 2 % of cholesterol has been studied in two series of experiments. Profound changes are caused in the amounts of the different lipoids in the resulting "cholesterol" fatty livers by such variation. The results are discussed on both a percentage basis and on that of the absolute weights. In confirmation of the results of Channon and Wilkinson, they show that in the "cholesterol" fatty liver the dietary protein fraction exercises a profound lipotropic effect. By the use of graded protein percentages in the diet it is thus possible to produce "cholesterol" fatty livers of widely varying composition and these findings have been used in further experiments for studying the preventive effects of choline. These experiments have shown that the addition of approximately 80 mg. of choline per rat per day very largely prevents the deposition of glyceride, but only partially prevents that of cholesteryl esters. No deductions could be made from the experiments as to whether choline is exerting its effect through lecithin synthesis, or whether

choline has a direct action in preventing cholesteryl ester deposition. The lipotropic effect of the protein fraction differs superficially at least from that of choline, in that the absolute amount of liver phosphatide is increased.

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