CCCXIV. THE ACTION OF CHOLINE AND OTHER SUBSTANCES IN THE PREVENTION AND CURE OF FATTY LIVERS.

By CHARLES HERBERT BEST and HAROLD JOHN CHANNON.

From the Department of Physiology, the University of Toronto, and the Department of Biochemistry, the University of Liverpool.

(*Received September 26th, 1935.*)

The discovery that the choline content of the diet exercises a controlling influence on the amount of fat in the liver has provided the means for many investigations into the subject of fat metabolism, and the results already obtained have illustrated a number of aspects of the problems involved. Several new factors have come to light concerning the precautions which are necessary in order to study the action of choline and we feel that at this stage it would be helpful to other workers in the field briefly to review the conditions which must be fulfilled in studying these choline effects in various types of experiment. This seems the more important because it has come to our knowledge that certain workers have encountered difficulty in detecting the results of choline administration and this we believe to be due to a lack of appreciation of all the precautions necessary in the preparation of the experimental diets. Further, some of the results from our own two laboratories seem to be in contrast and it may be helpful to show that these results, which relate to experiments carried out under somewhat different conditions, are in fact not at qualitative variance but differ only in degree.

Choline has been shown to exercise prophylactic and curative effects on the "fat" fatty liver and the "cholesterol" fatty liver produced under a variety of experimental conditions and we propose first to discuss the "cholesterol" fatty liver. Before proceeding further it is to be emphasised that throughout this paper the discussion concerns experiments which have been carried out on rats and that it is yet to be determined to what extent these results apply to other species.

Best and Ridout [1933] investigated the action of choline in preventing the increase in the amount of the liver lipoids which occurs when a diet containing cholesterol is administered to rats. By estimation of the ether-soluble hydrolysis products of the liver (fatty acids and unsaponifiable matter) they showed that choline administration prevented the accumulation which occurred in the absence of choline. Quantitative experiments suggested that the amount of choline necessary to maintain livers at their normal lipoid content was of the order of 190 mg. per rat per day. These results were extended by Best *et al.* [1934] who analysed the individual liver lipoids of groups of animals receiving a cholesterol-containing diet, with and without the addition of 230 mg. of choline per animal per day. This amount of choline was found completely to prevent the glyceride infiltration, for the livers of the control animals contained 9·50 % of glyceride as against 1·51 % for those which received choline. Further, although it did not prevent the accumulation of cholesteryl esters completely, it was preventive to the extent of some 60 %. (Control 4·35 %; animals receiving choline 1·77 %; normal 0·0 %.)

Further results on this preventive action of choline on the "cholesterol" fatty liver have been obtained at Liverpool and will be published in detail in due course. They confirm however that choline has a very marked effect in preventing the accumulation of glyceride in the "cholesterol" fatty liver, as inspection of the figures recorded in Table II will show. In these particular experiments the animals received a constant amount of choline, about 75 mg., and the liver glyceride was prevented from rising by 23·8, 12·77, 3·85 and 0·21 % of the fresh liver weight. These experiments were carried out under conditions which caused varying degrees of glyceride infiltration in the livers of the control groups of animals. They show that, where the amount of liver glyceride is high in the absence of choline, administration of that substance causes a very striking decrease. When the liver glyceride is at a lower level, pronounced decreases also result, although the ease of removal of glyceride appears less than in those cases where the amounts of glyceride are high.

Turning now to the curative as opposed to the preventive action, Best and Huntsman [1935] showed that the "fat" fatty liver caused by feeding to rats a diet of mixed grain with 40 % of beef fat could be cured in 12 days by the administration to each animal of 5 mg. of choline daily, and Channon and Wilkinson [1934] reported curative experiments carried out on similar lines on the "cholesterol" fatty liver. The conclusion from these experiments was that in 10–12 days choline exerted no curative effect on the cholesteryl esters of the liver but did cause a slight decrease in the glyceride content. It is to be emphasised at this point that the "cholesterol" fatty livers produced in these experiments happened to be livers in which the glyceride content was low and of the order of 6 %. Best and Ridout [1935] then recorded the results of similar experiments, but in contrast showed that choline given in the amount of 100 mg. per rat per day caused a rapid fall in the glyceride in the period under discussion, although the finding that there was no effect on the cholesteryl esters in this period was confirmed.

The contrast in these results is apparent rather than real, for it has become clear that both ease of removal and the degree of prevention of the liver glyceride in "cholesterol" fatty livers depend in part on the initial glyceride level. In the curative experiments of Channon and Wilkinson the initial level of liver glyceride was low and in the short term experiment little effect was seen. In the results of Best and Ridout [1935] the initial level was high and a marked effect was seen. This result, which has been confirmed in the Liverpool laboratories, is further illustrated in preventive experiments by the figures recorded in Table II.

The conclusion to be drawn therefore from the results from the two laboratories is that choline will very readily prevent the accumulation and cause the removal of the glyceride present in the "cholesterol" fatty liver, but that the degree to which these effects are obtained depends to some extent on the initial glyceride concentration.

Considering now the cholesteryl ester fraction it may be stated that in neither laboratory have experiments yet been carried out in which choline has entirely prevented the accumulation of cholesteryl esters in the liver, although, as already pointed out, a prevention of up to 60 % has been observed. The conditions of these experiments in both laboratories have however been drastic in that the diets have contained 2 % of cholesterol and 20 % of fat. In curative experiments choline has little, if any, effect on the cholesteryl esters in 10–12 days when large doses of cholesterol are used. It is to be noted however that Best and Ridout [1935] reported results which show that, whilst some mechanism exists

for the removal of cholesteryl esters from the liver in the absence of dietary choline, the presence of choline in the diet caused an accelerated rate of removal which began after some 18 days. Experiments of this duration have not been carried out in Liverpool. It is clear from all the work so far carried out that both the preventive and curative actions of choline on the cholesterol fraction of the liver are much less pronounced than those on the glyceride.

The next point to be considered is the precautions which must be taken if the results of the administration of small amounts of choline are to be observed and this leads to discussion of dietary substances, other than choline, which also exercise control on liver fat. In their original work Best and Huntsman [1932] observed that betaine also possessed a lipotropic action. More recently results from both our laboratories show that a dietary constituent, the chemical nature of which has yet to be determined, is present in the protein fraction. In this paper the term "protein fraction" is used in the general sense, without defining in any way whether the lipotropic activity associated with it is due to an integral part of the protein molecule as such or associated as a contaminant.

The first work on this subject appeared when Best and Huntsman [1935] reported some experiments concerning the effect of transferring animals which had fatty livers (fat 10 % and 13·5 %) to a diet of pure sucrose. They found that such a transfer resulted in an average increase of liver fat by some 8 %. If a diet of 80 % sucrose and 20 % caseinogen were used however this increase did not occur. Assuming daily consumption of 10 g. of the diet per animal this means that 2 g. of caseinogen had prevented an 8 % rise in liver fat. Meanwhile Channon and Wilkinson were investigating this lipotropic effect of protein from another point of view by feeding experiments on normal animals and during the course of that work became aware of the results which had been obtained at Toronto. In reporting their results, however [Channon and Wilkinson, 1935], they inadvertently failed to make reference to this Toronto experiment. Because of the low percentage of glyceride (5–7 %) which appeared in the "cholesterol" fatty livers mentioned on p. 2652, it seemed to them that some other factor besides dietary choline must be involved in controlling the glyceride infiltration and they accordingly carried out a series of experiments on the "fat" fatty liver in which the fat content of the diet was maintained at a constant level, 40 %, while the protein (caseinogen) content of the diet (0–50 %) was varied at the expense of the carbohydrate (glucose). Their conclusions from these experiments were that the amount of fat appearing in the liver was determined by the amount of protein in the diet, irrespective of any action of choline.

This paper was followed by one from Best, Huntsman, McHenry and Ridout [1935] in which it was stated that, when the fat content of the diet was 20 % (Channon and Wilkinson used 40 % fat) and the protein content as high as 15–20 %, rapid accumulation of glyceride appeared in the liver. Further, even when the fat content was as low as 3 %, glyceride still appeared in the liver, provided that the amount of lipotropic substances in the diet was very small. The results recorded in that paper were concerned with feeding experiments in which a relatively fixed percentage of protein was employed (15–21 %); the finding that such a protein percentage yet permits fatty livers on diets containing as little as 3 % of fat must not be interpreted as indicating the absence of a lipotropic effect from the protein fraction and therefore as being opposed to those of Channon and Wilkinson [1935]. Experiments have since been carried out in Toronto by methods which were the same as were used by the Liverpool workers, save that the diets were essentially choline-free and were administered to much larger groups of animals. The more complete results which these

experiments have given confirm the findings of Best and Huntsman [1935] and are in complete harmony with those of Channon and Wilkinson that in the conditions of these experiments the protein fraction of the diet exerts a lipotropic effect. These latter authors have further confirmed their previous findings and have also extended them to a study of the "cholesterol" fatty liver problem with the same results. The further data from the two laboratories, showing the lipotropic action of dietary protein, will be published in detail in due course.

The next point for consideration is the means whereby the protein fraction of the diet exercises this lipotropic effect. Various suggestions have been made as to the mechanism of this action [Best and Huntsman, 1935; Channon and Wilkinson, 1935; Best, Huntsman and Ridout, 1935] and in order to dispel any possible confusion as to our views it is to be stated that in neither laboratory has evidence yet been obtained as to whether the protein is exercising its effect through an amino-acid or some other integral part of the protein molecule, or whether there is some contaminating lipotropic substance. It can now definitely be stated however that contaminating choline can account for only an insignificant part of the caseinogen effect. Should some aspect of protein metabolism be involved in the lipotropic effect, the experiments at Liverpool, in which the action of pure amino-acids, and at Toronto where the lipotropic effects of the fractions of protein hydrolysates, are being investigated, should provide further information.

It is not possible on the basis of the figures available adequately to compare the lipotropic effects of the protein fractions used in our two laboratories. Such figures as are available have been obtained in experiments of different types. In the original experiments of Best and Huntsman [1935] 2 g. of protein caused a decrease of 8% in the liver fat, and Best, Huntsman and Ridout [1935] expressed the view that 2 g. of their caseinogen had the equivalent effect of 1 mg. of choline. In preventive experiments in Liverpool 2·5 g. of protein have generally lowered the liver fat by 20%. Accurate comparisons cannot be made however until the lipotropic effect of the proteins is assessed in terms of choline and this is at present under investigation. Meanwhile we have the general impression that the protein fractions used in Liverpool may possess lipotropic actions greater than those used in Toronto.

For the benefit of other workers it may be useful to discuss the question of the percentage of liver fat which may result from diets of varying fat and protein contents and the conditions necessary for obtaining high liver fat levels. Here difficulties arise because of complicating factors concerned with the composition of the diets, the chief of which is the provision of vitamin B_1. Dried yeast and certain yeast preparations which are commonly used as sources of vitamin B_1 may contain considerable amounts of choline. In Toronto this difficulty has been overcome by the use of crystalline vitamin B_1, which has been made available through the generosity of Messrs Merck. In Liverpool, because it has not been found possible to obtain samples of crystalline vitamin B_1, it has been the practice to use 5% marmite in the diet in order to supply this vitamin. Biological assays on this material, carried out both at Toronto and repeatedly in Liverpool, show that marmite contains about 3 mg. of choline per g., so that on a daily intake of 10 g. a rat receives 1·5 mg. of choline from this source. It is possible also that there may be present in marmite some other substance which exerts a lipotropic action, but such evidence as is available at the present time does not appear to support that possibility.

Since Best and Huntsman [1935] amply demonstrated the preventive effect of 5 mg. of choline in "fat" fatty liver production, it is clear that the daily intake

of the 1·5–2 mg. present in 0·5 g. marmite must render any comparison between the liver fat levels obtained in the two laboratories very uncertain. In experiments in which the diets contained the same amount of fat but considerably more protein, Best, Huntsman and Ridout [1935] obtained considerably higher liver fat levels than those obtained in Liverpool and it was on this finding that they based the statement that comparison of the results from the two laboratories did not appear to show that a lipotropic effect of protein *per se* was apparent. These authors did not intend to convey the impression that they believed that the protein fraction was not exerting a lipotropic effect; in fact, they had observed this effect repeatedly. Further, it may be stated here that it is very difficult to compare the results from the two laboratories when findings which may be affected by such factors as the type of protein fraction, the exact nature of the carbohydrate *etc.* are under discussion. In diets used in the two laboratories, where the only apparent substantial difference is the presence of vitamin B_1 or marmite, the general tendency must clearly be for higher levels of liver fat to appear in the Toronto results than in the Liverpool ones, although the general effects will not be obscured by the marmite, unless the particular diets are such as to cause a relatively low degree of fat infiltration. The Toronto workers [1935] quoted an average fat content of the livers of 50 animals receiving a diet of 40 % fat and 21 % protein for 3 weeks of 17 % and contrasted this with the 12 % found originally by Channon and Wilkinson in animals receiving the same amount of fat and one-quarter the amount of protein. The figures recorded by Channon and Wilkinson happened in that particular experiment to be at an unusually low level, possible reasons for which are discussed later. The mean value for all the 87 animals which have received this 5 % protein diet with 40 % of fat since those original results is however 24·6 %. Bearing in mind that in every case the diet has contained some 2 mg. of choline present in the marmite, it is yet clear from these subsequent results that the lipotropic effect of the protein fraction is demonstrated, even by comparison with the 17 % figure recorded by the Toronto workers for a diet containing 21 % protein. Thus on these figures it is possible that the protein *per se* is exerting an effect, but it is obvious from what we have already stated that the lipotropic action of the protein fraction may prove to be due in part or completely to a contaminating non-choline lipotropic factor. Such comparison also seems to indicate that lipotropic substances other than choline or protein are not present in the marmite to any significant extent.

It is probably the presence of marmite in the diet which explains the lack of consistency compared with other results in some of the experiments reported by Channon and Wilkinson, a point to which the Toronto workers have drawn attention.

The next point to be discussed is the effect of weight losses by the animals on their content of liver fat. Channon and Wilkinson [1935] neglected the result of one of their experiments in which every animal lost weight. Best, Huntsman and Ridout [1935] however recorded a large series of figures showing that there was no correlation between weight losses in animals on a given diet and their liver fat content. Although similar results had been obtained in Liverpool, showing that on a diet which caused slight gains in weight in some animals and slight losses in others the weight change was not related to the liver fat, Channon and Wilkinson thought it unwise to base deductions on the results of the one group of animals in which weight losses had been experienced by every animal. In all the other groups the average weight change had been from −0·6 % to +31·6 %, whereas in this particular group the average weight change was −12·6 %.

As pointed out in a preceding paragraph, the mean value for the liver fat of 87 animals receiving the 5 % protein diet has been 24·6 %, as against that of 12 % originally reported, and a brief discussion is necessary on this very considerable increase. The limited groups of 6 animals which were previously used must necessarily be a factor, but cannot account for more than a small part of the difference. The Toronto workers in their earlier work pointed out that at times they failed to obtain fatty livers in groups of animals on diets which produced fatty livers in other groups of animals and no explanation of this finding is yet forthcoming. Further, they have emphasised that it is necessary in every experiment to use control groups, because even animals from the same stock do not consistently give the same figure for liver fat content when put on the same diet at different times. In Liverpool this latter observation has been confirmed many times. There appear therefore to be other factors yet to be determined which cause this variation in liver fat level. One of these clearly may be temperature, and in this connection it may be stated that when the experiments of Channon and Wilkinson were carried out the animal house was not thermostatically controlled. Further, the extreme susceptibility of the liver fat to change is illustrated by an experiment of Best and his colleagues, who observed that frequent handling and subcutaneous injection of saline had an interfering effect. The reasons for these varying results from groups of animals on the same diet clearly need further investigation.

The preceding discussion will, it is hoped, clarify the conditions necessary for the study of the effects of choline in the fatty liver problem and we think that lack of appreciation of some of these conditions has been responsible for difficulties which may have been encountered by other workers.

Obviously the ideal diet would contain no substance having any lipotropic effect. This means that in the first place the diet must be choline-free, as can be demonstrated by hydrolysis of the constituents and biological assay of the acetylated products on the isolated rabbit intestine. Secondly some of the food constituents of the diet may contain betaine or substances of the betaine class, or other unidentified lipotropic factors. No method of estimating these is available. Lastly, even in the absence of any of these substances, dietary protein itself may exercise a lipotropic effect. As already pointed out, one of the main difficulties is the provision of vitamin B_1, without which the diets on the whole are poorly consumed. Crystalline vitamin B_1 is obviously the most satisfactory material but, if it is not available, care must be taken to choose a material containing as little choline as possible. Unless the diet is extremely low in lipotropic substances (*i.e.* equivalent to less than 1 mg. of choline per day), the effects of choline administration will not be observed in experiments in which the dietary conditions are less drastic. The main difference which exists between the Toronto and Liverpool results under discussion is connected with this factor and may be summarised by saying that on diets of similar composition, differing only in the provision of vitamin B_1 in crystalline form or as marmite, livers reach a higher level of fat content in Toronto than in Liverpool. It may be stated that with diets of constant protein content, but of variable fat content, the degree of fat infiltration in the liver increases with the fat content, and further that with diets of constant fat content the liver fat increases with decreasing protein content. The most intense degree of fat infiltration will be produced when the diet is high in fat and when the protein fraction is kept at as low a level as is desirable, with the diet free also from other lipotropic substances.

Lastly, because of the difficulty which some other workers appear to be experiencing in demonstrating the effect of choline, we consider it of value to

Table I. *Preventive action of choline on the "fat" fatty liver.*

Exp. No.	Duration of exp. (days)	Control: No. of animals in group	Control: Liver fat %	Control + choline: No. of animals in group	Control + choline: Liver fat %	Control + choline: Choline/ rat/ day (mg.)	Fall in glyceride caused by choline administration %
B 1	21	9	20·8	10	5·65	79·0	14·1
S 1	17	6	24·1	6	5·2	80·0	18·9
S 2	20	9	24·0	10	8·5	79·0	15·5
S 3	18	9	15·75	10	5·25	61·0	10·5
S 4	18	12	26·1	12	7·2	31·0	18·9
S 5	19	10	28·2	10	6·7	20·0	21·5
S 6	13	16	25·5	16	8·2	8·7	17·3

In Exps. S 3 and S 4 the amount of choline administered varied somewhat during the periods of the experiments and the figures recorded are for the average daily intake.

Table II. *Preventive action of choline on the glyceride fraction of the "cholesterol" fatty liver.*

Exp. No.	Duration of exp. (days)	Control: No. of animals in group	Control: Liver glyceride %	Control + choline: No. of animals in group	Control + choline: Liver glyceride %	Control + choline: Choline/ rat/ day (mg.)	Fall in glyceride caused by choline administration %
W	21	10	24·78	10	0·98	80·0	23·8
W*b*	21	10	16·17	10	3·40	77·0	12·77
W*c*	21	10	6·11	10	2·26	77·0	3·85
W*d*	21	10	2·92	10	2·71	74·0	0·21

refer to results which have been secured independently in Liverpool. These results have been obtained, not with the specific purpose of demonstrating the already well established effect of choline, but merely as a routine in order to assay the lipotropic effect of other substances in terms of choline. In Table I therefore is recorded a series of preventive experiments on the "fat" fatty liver. The figures in Table I show the striking effect of choline in preventing the accumulation of fat in the liver. No figures are available concerning the minimum amount of choline which will result in a preventive, if not a completely preventive, action. On diets containing 40% of fat the above figures show however a decrease of 21·5% caused by 20 mg. of choline and one of 17·3% caused by 8·7 mg. of choline, although in neither case has the liver fat reached the normal level of 4%, results which amply confirm the Toronto finding as to the effective action of small amounts of choline.

In another series of Liverpool experiments the preventive action of choline on the "cholesterol" fatty liver has been studied and Table II records some of the results concerning the effect on the glyceride fraction. Here again no attempt has been made to reach the minimum figure showing a preventive action.

Summary.

1. Present knowledge regarding the action of choline and other substances in the prevention and cure of fatty livers induced by dietary means is discussed and the results obtained from the laboratories at Liverpool and Toronto are shown to be not at qualitative variance but to differ only in degree, governed by the somewhat different conditions under which experiments have been carried out.

2. The protein fraction of the diet exercises a controlling influence on the amount of the liver lipoids. Possible means whereby this lipotropic action of the protein fraction exerts its effect are discussed.

3. The precautions necessary for planning diets adequate for observing the effects of choline are considered in detail.

4. Figures which have been obtained independently in Liverpool on the preventive action of choline on the "fat" and "cholesterol" fatty livers are recorded. They confirm the findings obtained at Toronto.

The expenses of the work carried out at Liverpool have been borne from an expenses grant from the Medical Research Council, for which grateful acknowledgment is made.

REFERENCES.

Best, Channon and Ridout (1934). *J. Physiol.* **81**, 409.
—— and Huntsman (1932). *J. Physiol.* **75**, 405.
—— —— (1935). *J. Physiol.* **83**, 255.
—— —— McHenry and Ridout (1935). *J. Physiol.* **84**, 38 *P.*
—— —— and Ridout (1935). *Nature*, **135**, 821.
—— and Ridout (1933). *J. Physiol.* **78**, 415.
—— —— (1935). *J. Physiol.* **84**, 7 *P.*
Channon and Wilkinson (1934). *Biochem. J.* **28**, 2026.
—— —— (1935). *Biochem. J.* **29**, 350.