CL. FURTHER OBSERVATIONS ON THE EFFECT OF DIETARY CASEINOGEN IN THE PREVENTION OF FATTY LIVERS.¹

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IN one experiment of a series in which study was made of the effect of choline in preventing the accumulation and accelerating the removal of fat in the liver of the rat, Best and Huntsman [1935] observed that, when rats which already had a liver fat percentage of 10–13.5 were transferred to a diet of pure sucrose, the liver fat was further increased by some 8 % during the course of 6 days. In a comparable experiment in which the transfer was made to a diet containing 80 % of sucrose with 20 % of caseinogen, this increase in liver fat did not occur. Assuming a daily food intake of 10 g. per animal, this experiment suggested that 2 g. of caseinogen had prevented an 8 % rise in the fat of a liver which was already loaded with fat. Best and Huntsman were unable to decide at that time whether this action of caseinogen was due to the fraction of a mg. of choline present in the diet and they pointed out in further possible explanation of this result that caseinogen might give rise to betaines during its metabolism, since betaine itself was shown to be lipotropically active by Best and Huntsman [1932]. At the same time Channon and Wilkinson [1935] were investigating the production of fatty livers in normal animals on synthetic diets, because they had failed to obtain such livers by the use of the diet of mixed grain and 40 % fat originally used by the Toronto workers. Various considerations discussed by them suggested that the amount of the protein present in the diet might be a governing factor in fatty liver production and they concluded from a series of experiments that the amount of fat appearing in the liver was controlled by the amount of protein in the diet, irrespective, however, of any action of choline. In a criticism of these results, Best *et al.* [1935] drew attention to their experiment mentioned above and stated that the results of their more recent unpublished experiments suggested that 1 g. of the caseinogen used by them had no greater effect on liver fat than did 0.5 mg. of choline. They further stated that, whilst the lipotropic effect of caseinogen was not to be underestimated, in their opinion the effect could be accounted for if the protein contained 0.2 or 0.3 % of choline, betaine or other substances with a similar action.

Meanwhile Beeston *et al.* [1935] had applied their previous findings on this protein effect to a further extensive study of the "cholesterol" fatty liver with results which amply confirmed their original conclusion that the caseinogen present in the diet controlled the amount of glyceride appearing in the liver, irrespective of any action of choline. As typical of this powerful action of the caseinogen may be mentioned an experiment in which the amount of glyceride in the livers of a group of animals was reduced by 18.69 % by the extra daily

¹ The substance of this paper was reported at a Meeting of the Biochemical Society, on 15th Nov. 1935 [Beeston and Channon, 1935].

intake of only 1 g. of protein per rat. Such figures as these left no doubt as to the validity of the conclusion that traces of contaminating choline could not be responsible for the effect and personal discussion of these and unpublished Toronto results led to Best and Channon [1935] stating that contaminating choline could account for only an insignificant part of the caseinogen effect.

A further point which emerged was that the lipotropic effect of the caseinogen used in Liverpool appeared to be very much greater than of that used in Toronto, for in their experiment mentioned above, Best and Huntsman found that 2 g. prevented an 8% rise in liver fat, whilst in this laboratory, in preventive experiments on normal animals both with the "fat" fatty liver and the "cholesterol" fatty liver, a very much greater effect had been repeatedly observed.

Apart from emphasising this considerable difference both in the degree of the lipotropic action of caseinogen in the results from the two laboratories and in its interpretation, Best *et al.* [1935] noted that in certain experiments the percentages of fat observed in the Toronto laboratories were higher than those obtained here. A constant amount of marmite was used in the different diets of all our groups of animals as a source of B-vitamins and Best *et al.* suggested that marmite might contain other unknown lipotropic factors which would lower the liver fat percentages. They further stated that the 2 mg. of choline received by our rats might be a significant factor in the conditions of our experiments.

The work described in this paper was therefore carried out with three objects in view. Firstly it was clearly desirable to express the preventive action of caseinogen on liver fat in terms of a standard, choline, for only by such means can the lipotropic actions of different proteins be accurately compared; secondly to investigate the effect of marmite on the degree of fat accumulation in the liver; and thirdly to determine the minimum amount of choline which exercised an effect on liver fat in the diet containing 5% caseinogen and 40% fat generally used by us. It was hoped that these experiments, the results of which have already appeared in summarised form [Beeston and Channon, 1935] would further establish or finally dispose of the apparent disparity between the results at Toronto and those at Liverpool.

EXPERIMENTAL.

The method of experiment was to feed ten groups of rats, each of 8–10 animals, on a basal diet consisting of caseinogen (alcohol- and ether-extracted) 5, fat (beef dripping) 40, glucose 45, salt mixture 5, cod-liver oil 1 part. Five of the groups received in addition 5 parts of marmite, whilst in the remaining five this was substituted by a further 5 parts of glucose. In each of the two series varying percentages of choline chloride were added to the diet. Two further groups of animals received, with and without marmite, a diet differing from the basal diet in containing 30 parts of caseinogen, 25 parts of the glucose of the basal diet being substituted by this protein. After 14 days the animals were killed by guillotine and the amount of fatty acids and unsaponifiable matter in the livers determined by the methods previously used [Channon and Wilkinson, 1935]. In Table I are recorded the essential data regarding the animal side of the experiment. Choline determinations on the food constituents were made by the method of hydrolysis followed by acetylation and biological assay on the isolated rabbit intestine. The marmite used in these experiments contained 440 mg. of choline per 100 g. and the caseinogen 0.6 mg. per 100 g. The daily food consumption was measured and in the figures in Table II the total daily choline intake includes not only the added choline in groups 1 *b*–1 *e*, and 2 *b*–2 *e*, but in series 1 that

Table I.

	Diet		1. Marmite present				2. Marmite absent			
	Caseinogen %	Choline chloride %	No. of animals	Av. final wt. g.	Av. wt. change $\pm$ % initial body wt. g.	Food consumption g./rat/day	No. of animals	Av. final wt. g.	Av. wt. change $\pm$ % initial body wt. g.	Food consumption g./rat/day
(a)	5	+0.00	9	156	-0.1	8.2	8	146	-8.7	7.8
(b)	5	+0.02	8	169	-3.0	8.7	8	156	-5.2	8.2
(c)	5	+0.05	9	167	-0.4	9.1	9	152	-7.9	7.3
(d)	5	+0.10	9	162	+3.3	9.2	7	152	-5.7	7.5
(e)	5	+0.20	9	167	+2.3	8.6	8	160	-3.2	7.7
(f)	30	+0.00	9	178	+10.0	8.6	9	164	+1.0	7.3

Table II.

	Diet		1. Marmite present			2. Marmite absent		
	Protein %	Choline chloride %	Daily intake			Daily intake		
			Total choline mg./rat	Caseinogen g./rat	Liver fat % wet wt.	Total choline mg./rat	Caseinogen g./rat	Liver fat % wet wt.
(a)	5	+0.00	1.8	0.41	20.45	0.0	0.39	23.60
(b)	5	+0.02	3.4	0.43	13.14	1.4	0.41	24.33
(c)	5	+0.05	5.9	0.45	9.62	3.2	0.36	12.44
(d)	5	+0.10	10.0	0.46	8.58	6.5	0.38	8.32
(e)	5	+0.20	16.8	0.43	6.20	13.3	0.38	6.74
(f)	30	+0.00	1.9	2.58	5.62	0.0	2.20	6.84

present also in the marmite provided in the diet. It is to be mentioned that the groups 1*f* and 2*f* in Table II in which about 2 g. more caseinogen were ingested than in groups *a-e*, received in consequence 0.01 mg. additional choline per rat per day, an amount which is insignificant and which may be disregarded in the discussion.

The lipotropic action of caseinogen in reference to choline as standard.

The figures in Table II show that in all the groups *a-e* in series 1 and 2 the caseinogen intake per rat per day varied between 0.36 and 0.46 g., a figure which may be taken as sufficiently constant to permit a satisfactory comparison of the different results. In group 1*a* the caseinogen intake of 0.41 g. per rat per day has yielded a liver fat percentage of 20.45. In the same series in group 1*f* a caseinogen intake of 2.58 g. per rat per day has yielded a liver fat percentage of 5.62. Thus an extra intake of 2.17 g. of caseinogen per rat per day has lowered the liver fat percentage by 14.83. Further, in series 2, in which marmite was absent, an extra intake of 1.81 g. of protein has lowered the liver fat from 23.6 to 6.84 %, a reduction of 16.76 %. These figures correspond to reductions of 7 and 9 % in the liver fat respectively per g. of protein ingested. The lowest figures for the liver fat in the two series caused by the greatest amounts of choline given, 16.8 and 13.3 mg. per rat per day respectively, are 6.20 and 6.74 % (groups 1*e* and 2*e*). If these be taken as the same as those resulting from the 30 % protein diets in groups 1*f* and 2*f*, 5.62 and 6.84 % respectively, then in series 1, 2.17 g. of protein have had an effect equivalent to 16.8 mg. of choline, while in series 2, 1.81 g. of protein have had an effect equivalent to 13.3 mg. of choline. Hence in series 1, 1 g. of protein has yielded an effect on the percentage of liver fat equivalent to 7.7 mg. of choline, while in series 2 it is equivalent to 7.3 mg. of choline.

With these figures is to be contrasted the statement of Best *et al.* [1935] that in unpublished results following their experiment mentioned above 1 g. of caseinogen is equivalent in action to not more than 0.5 mg. of choline, which is about one-fifteenth of the value recorded here.

In experiments which have since been carried out on normal animals by the preventive technique originally used by us in investigating this problem, Best, Grant and Ridout [1936] have now fully substantiated our findings [Channon and Wilkinson, 1935; Beeston *et al.*, 1935] that caseinogen exercises a very considerable action on liver fat in this type of experiment. They now emphasise the importance of limiting the caseinogen content of the diet, if the rapid production of large fatty livers is desired, and it is for this reason that we have adopted during the last two years the use of a diet containing 5 % of caseinogen and 40 % of fat [Channon and Wilkinson, 1935; Beeston *et al.*, 1935; Channon and Smith, 1936]. It is of considerable interest also that in this type of experiment Best, Grant and Ridout [1936] find that 1 g. of their caseinogen exercises an effect comparable with "perhaps 5 or 6 mg. of choline". This confirmation of our results [Beeston and Channon, 1935] by the Toronto workers makes it very unlikely that there is any difference in the lipotropic values of the caseinogen preparations used in the two laboratories, for the difference between their figure of 5–6 mg. and ours of 7–8 mg. is, in our opinion, well within the range of experimental error. As these authors point out, it thus appears that the lipotropic action of caseinogen in the transfer experiment on which they originally based their criticism of the results of Channon and Wilkinson [1935; Best, Huntsman and Ridout, 1935], is far less than in the preventive type of experiment carried out on normal animals. If the active substance present in caseinogen is a contaminant, the possibility exists that its amount may vary in different preparations of the protein. It is therefore of interest that the Toronto workers have now obtained a result similar to our own, when employing the same experimental method, even though feeding caseinogen from a different source.

Best, Grant and Ridout [1936] also confirm that the fat content of the liver decreases to approximately normal value as the protein content of the diet is increased from 5 to 30 % and make the additional observation that further increase in the dietary protein to 50 % causes no further lowering of the liver fat percentage. With their figure for a liver fat percentage of 5.04 on the 30 % protein diet, which they mention as being only slightly above normal, may be compared the corresponding figures in Table II, which are 5.62 and 6.84 % respectively. These figures also are definitely above the normal value, which is usually taken as 3–4 %. In all our experiments of this type we have never obtained a figure as low as 4 %, although, as is discussed later, neither has choline in the amounts used by us caused a reduction to the normal figure.

It is not known at the present time whether caseinogen effects this lipotropic action through some integral part of its molecule or by virtue of some contaminating substance. Various possibilities have been discussed [Best and Huntsman, 1935; Channon and Wilkinson, 1935; Best and Channon, 1935; Beeston, Channon and Wilkinson, 1935; Best, Mawson, McHenry and Ridout, 1936]. We would like briefly to draw attention to one further possibility. The extensive experiments of Best and Huntsman [1935] showed that when animals, whether with normal livers or livers already loaded with fat, were transferred to a diet of pure sucrose only, the amount of fat in the liver was markedly increased and the authors commented on the possibility of a "specific" effect of sucrose. These results, together with the fact that the mixed grain diet with 40 % of fat used by the Toronto workers in their original work, although providing some 10 mg. of

choline daily for each animal, yet caused fatty livers, led us to enquire whether this effect of increasing protein intake might be due merely to the corresponding decrease in carbohydrate intake. We endeavoured to investigate this possibility by feeding three groups of animals on diets containing 20 % of fat, 2 % of cholesterol and 5 % of caseinogen; one group served as control, whilst in the second and third groups 5 % and 25 % of glycine were added at the expense of the corresponding amounts of glucose of the basal diet. The object of this experiment on the cholesterol fatty liver was to cut down the carbohydrate intake by substitution of a pure amino-acid which might be expected to have no lipotropic effect. This experiment was carried on for 21 days. The percentages of total ether-soluble material in the pooled livers of these three groups were: control 39.4 %; 5 % glycine 42.4 %; 25 % glycine 34.4 %. We feel that the somewhat lower figure of 34.4 % on the diet with 25 % glycine is of doubtful significance, since the food consumption on this particular diet was not good and the weight losses considerable (21.7 % of the initial body weight). Hence these results appear to dispose of the idea that "dilution" of the carbohydrate is the cause of the protein effect. Some figures recorded by Best, Grant and Ridout [1936] in an experiment designed for another purpose appear to provide confirmation of this. They fed three groups of animals on a diet containing 40 % of fat with (a) 10 % caseinogen, (b) 30 % caseinogen and (c) 10 % caseinogen with 20 % gelatin. While the increase of the caseinogen content from 10 to 30 % reduced the fat content of the liver from 24.7 to 6.5 %, the addition of 20 % of gelatin to the diet containing 10 % caseinogen had no effect, the recorded figure, 22.4 %, not being significantly different.

Apart from this interesting observation that gelatin appears to possess no lipotropic activity, Best, Grant and Ridout [1936] report that dried egg white possesses an activity approximately equal to that of caseinogen, whilst minced ox muscle proteins have a smaller activity than that of caseinogen. In this laboratory studies of the lipotropic actions of different proteins are also in progress. For this purpose we have prepared a number of plant and animal proteins of varying biological value and these are now being tested against caseinogen, with and without the addition of certain essential amino-acids. It will not be possible to express their activities in terms of caseinogen with accuracy, because of the difficulty of preparing pure proteins in sufficient amount, since 1–2 kg. of each protein are required for a single experiment on two groups of animals. At the present stage we can only report that edestin appears to exercise a considerable lipotropic action, since groups of animals receiving a diet containing 40 % of fat and 5 % of edestin yielded a liver fat of 23.7 %, while those receiving 25 % of edestin and 40 % of fat gave a figure of 9.0 %. The further results will be reported in due course.

The effect of marmite on the liver fat percentage.

The effect of marmite on the liver fat percentages can be seen from the figures in Table II, in which are recorded the choline intakes of the two series of animals, together with the percentages of liver fat resulting. Considering first series 2, in which marmite was absent, it is seen that in group 2a, with a choline intake of zero, a liver fat percentage of 23.6 % resulted. The intake of 1.43 mg., group 2b, has had no effect on the liver fat percentage, the difference between the resulting figure, 24.33 %, and that of group 2a, 23.6 %, being of no significance in view of the normal group variations. Increase of the choline intake to 3.2 mg., group 2c, has caused a marked effect in lowering the liver fat to 12 % and the percentage progressively decreases through groups 2d and 2e to 6.74 %

with increasing choline intake. In the series in which marmite was present, group 1 *a*, in which the choline intake was 1.8 mg., yielded a liver fat of 20.45 %, a figure which is little different from that obtained in group 2 *b* (marmite absent, choline intake 1.4 mg.). The increase of the choline intake to 3.4 mg., group 1 *b*, has also caused a sharp fall in the liver fat percentage to 13.14 %, a figure again of a very similar order to that occurring in group 2 *b*, 12.44 %, in which the choline intake was of the same order, 3.2 mg. A further increase to 5.9 mg. causes a further fall to 9.62 %, group 1 *c*, which again is quite similar to the figure in group 2 *d* at a similar choline intake. Similar remarks apply to group 1 *d* compared with group 2 *e*.

It appears therefore from these figures that the presence or absence of marmite in the diets has had no effect on the liver fat except by virtue of the choline which it contains and that no other lipotropic substances are present in sufficient amount to cause any obvious effect. These results therefore appear to discount the suggestion made by Best, Huntsman and Ridout [1935] that the lack of greater deposition of liver fat in our experimental animals could be attributed to lipotropic substances other than choline contained in the marmite. On the other hand, although marmite has exercised no lipotropic effect other than that due to its contained choline, it is not unlikely that this lack of effect is merely the balance between the opposing factors of substances tending to increase liver fat and those tending to decrease it. Thus McHenry [1936] has shown that vitamin B₁ increases the amount of fat in the liver. Whilst the results of series 1 do not show any increase over those of series 2, in spite of the vitamin B₁ provided as marmite, this is probably explained by the fact that only on diets of extremely low choline content was the effect of vitamin B₁ observed [McHenry, 1936]. Further it is probable that a yeast extract such as marmite will contain cystine, of which amounts as low as 7.5 mg. per rat per day have been found to be markedly effective in increasing the percentage of liver fat on this particular diet [Beeston and Channon, 1936]. Any effect of these two factors tending to increase liver fat may be offset by betaines, the presence of which is probable in such an extract.

The minimum effective dose of choline.

The figures in Table II show very clearly that about 3 mg. of choline have caused a marked effect in decreasing the liver fat percentage. Thus in groups 1 *a* and 2 *b*, in which the choline intake was 1–2 mg., the percentages do not differ from those of 2 *a*, in which no choline was ingested. On the other hand in groups 1 *b* and 2 *c*, in which the choline intake has been raised to 3.4 and 3.2 mg. respectively, a sharp fall in the liver fat percentages to 13.4 and 12.44 % respectively has resulted. These figures thus show that on the diet most frequently used in this laboratory, containing 40 % of fat and 5 % of protein, 3 mg. of choline have a marked preventive effect. It is interesting to note that Best, Mawson, McHenry and Ridout [1936] have obtained a similar figure on a diet containing 15 % of protein and 40 % of fat. On the other hand it is seen in groups 1 *e* and 2 *e*, in which 16.8 and 13.3 mg. of choline were administered per rat per day, that the liver fat percentages are 6.2 and 6.74 % respectively, figures which are definitely above normal. We have repeatedly encountered this finding that choline in the amounts used by us has not reduced the liver fat to normal on this particular diet. Thus in the experiments of Channon and Smith [1936] in which the effect of triethyl- β -hydroxyethylammonium hydroxide was compared with that of choline, a large number of groups of animals was used. All these groups received the 40 % fat and 5 % protein diet for periods varying from 13 to

20 days and in addition received choline in amounts varying from 8.8 to 79.8 mg. per rat per day. If all the results be pooled, the mean value for the fat content of the livers of these 64 animals was 7.75 %, a figure approaching twice the normal, while the lowest figure obtained for any group was 5.2 % and in 5 out of 11 groups a figure of 8 % or over was obtained. It thus appears that, whilst small amounts of choline have a very marked effect in preventing a rise in the liver fat percentage, a considerably greater quantity is necessary to maintain it at a normal level.

SUMMARY.

1. In continuance of previous studies of the effect of dietary protein in preventing the occurrence of fatty livers in rats receiving diets containing 40 % of fat, the action of caseinogen has been equated against that of choline. 1 g. of caseinogen is equivalent in its preventive action on liver fat deposition to 7–8 mg. of choline.

2. The provision of marmite, or its absence from the diet, had no further effect on liver fat accumulation than that anticipated from its choline content.

3. In preventive experiments on diets containing 5 % of caseinogen and 40 % of fat, 3 mg. of choline per rat per day caused the liver fat percentage to be reduced from 20 to 10 %. On the other hand it is pointed out that amounts varying from 8.8 to 79.8 mg. a day did not prevent some fat accumulation occurring in the liver.

4. Edestin is shown to have a lipotropic action.

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