LXI. THE DIETARY PREVENTION OF FATTY LIVERS. COMPOUNDS RELATED TO CHOLINE

BY ALAN PERCY PLATT

From the Department of Biochemistry, University of Liverpool

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THE relative activities of various compounds related to choline in preventing the deposition of fat in the livers of rats maintained on a diet high in fat and low in other lipotropic factors have been studied as a possible source of information as to the mode of action of choline itself [Channon & Smith, 1936; Channon *et al.* 1937; Welch, 1936; Welch & Welch, 1938]. The present paper records similar investigations of a number of other compounds.

Two of the compounds used, cholamine and betaine, had been studied previously by Best & Huntsman [1932]. While cholamine had been found to be inactive, betaine had prevented the deposition of liver fat, a result confirmed by Welch & Welch [1938]. The experiments of Best & Huntsman, however, were carried out with the 40 % fat-60 % grain diet and it seemed desirable to repeat the work using a "synthetic" choline-free diet; moreover, the latter authors provided no evidence regarding the relative activity of betaine in terms of choline. Welch & Welch [1938] have since suggested that it has rather more than half the activity of choline. In view of the fact that liver tissues can oxidize choline to betaine aldehyde and probably to betaine [Mann & Quastel, 1937; Bernheim & Bernheim, 1938; Mann *et al.* 1938], the degree of activity of betaine is a point of some importance since it might possibly be the compound ultimately active when choline supplements prevent liver fat deposition.

Another compound tested was creatine, since several authors have suggested that choline is a precursor of creatine in metabolism [Riesser, 1913; 1914; Baumann & Hines, 1918; Shanks, 1921; Klee & Petropoliades, 1928]. Finally, two further compounds related to choline were investigated: choline methyl ether and tetra(β -hydroxyethyl)ammonium chloride. Since choline might act by enabling lecithin formation to take place it was decided to study the methyl ether of choline, to see whether etherification of the alcohol group robbed the compound of its lipotropic activity. The results with the tetrahydroxy derivative should show whether the alkyl groups were necessary for the activity found in choline and some of its analogues.

EXPERIMENTAL

Choline methyl ether was prepared by a method based on that described by Renshaw [1910] for choline. β -Chloroethyl methyl ether, prepared from the monomethyl ether of ethylene glycol by the action of phosphorus pentachloride, was sealed with a molecular proportion of trimethylamine in a bomb tube and heated at 95° for 6 hr. with occasional shaking. The contents of the tube were then dissolved in alcohol, purified by treatment with charcoal and precipitated by means of acetone and ether. The crystallized material was purified by similar means. Exact analysis was rendered difficult by its extremely hygroscopic

nature but the chloroaurate was a stable compound and had M.P. 155°. (Found: C, 15.90; H, 3.59; Au, 43.38 %. $C_6H_{16}ONAuCl_4$ requires C, 15.75; H, 3.51; Au, 43.15 %.)

The tetra(β -hydroxyethyl)ammonium chloride used in these experiments was a sample kindly given to us by Mr A. L. Bacharach.

Animal feeding and liver analyses

Groups of 10 rats were fed on a basal diet producing a fatty liver, one group serving as a control and the others receiving supplements of the various compounds or of choline itself, the supplements in aqueous solution being mixed in the diet. The choline content of the basal ration was negligible except in exp. 5 when marmite was included to supply vitamin B complex, and provided about 1.5 mg. choline per rat per day, the effect of which, it is considered, would be negligible. Except in exp. 5, the individual livers were analysed by digesting with 40 % NaOH and refluxing with alcohol, the fatty acids and unsaponifiable fraction then being extracted with ether and subsequently purified by treatment with light petroleum: in these cases "fat" represents fatty acids plus unsaponifiable matter. In exp. 5 the pooled livers were ground with sand and extracted with alcohol and ether and in this case therefore "fat" represents the total ether-soluble material so obtained. Details of the diets differed in the various experiments and are considered when discussing the results.

RESULTS

Betaine. The basal diet used in these experiments consisted of beef dripping 40, protein 8, glucose 46, salt mixture 5, cod-liver oil 1 part. In addition aneurin was added to the diet so that each animal received about 10 μ g. daily. The protein fraction was egg albumin in exps. 1 and 3 and caseinogen (alcohol-ether-extracted) in exp. 2. In exps. 1 and 3 the animals were all bucks while in exp. 2 there were 5 bucks and 5 does in each group. Betaine hydrochloride and choline chloride were used but the amounts are expressed in terms of the free bases.

The first two experiments were, of necessity, preliminary in nature, since none of the figures recorded in the literature was of any assistance in forming an estimate as to the lipotropic activity of betaine relative to that of choline. All the relevant data are presented in Table I together with those of exp. 3.

On the animal side, both exps. 1 and 2 are obviously satisfactory, the weight losses not being such as to interfere with the interpretation of the results.

In the first experiment 0.1 % choline and betaine were given to two groups respectively and it can be seen that while the former supplement has prevented a large increase in the amount of fat deposited in the liver, the presence of 0.1 % betaine has not had a very marked effect. The livers of the control animals contained 27.01 % fat, and those of the choline group 8.18 %, values to be contrasted with the 22.14 % present in the livers of the animals receiving betaine. In our experience, the difference between the control and betaine groups is hardly significant. The figures for the amount of fat in the liver of the 100 g. rat, 1.82 and 1.28 g., show, however, a larger difference and it was accordingly decided to increase the betaine and to reduce the choline dosage in the next experiment.

Before considering the results obtained in exp. 2, it must be pointed out that the wide difference between the two control values for liver fat obtained in exps. 1 and 2 is due to the fact that in the first, egg albumin was the basal protein, while in the second it was caseinogen; this point has already been discussed by Channon *et al.* [1938]. From the results of exp. 2, it can be seen that

Table I. *Effect of betaine supplements in preventing liver fat deposition*

Group	No. of rats	Change in wt. $\pm$ % initial body wt.	Liver % final body wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.	Base intake mg./rat/day	Choline equivalent mg./rat/day
Exp. 1. Period of feeding 18 days:							
1. Control	8	+4.8	6.6	27.01	1.82	Not recorded	
2. +0.1% choline	10	+5.0	5.2	8.18	0.41		
3. +0.1% betaine	10	+8.6	5.4	22.14	1.28		
Exp. 2. Period of feeding 17 days:							
4. Control	9	-9.1	3.9	17.03	0.70	Not recorded	
5. +0.03% choline	10	-6.3	3.9	15.51	0.65		
6. +0.05% choline	10	-5.1	3.6	9.58	0.36		
7. +0.20% betaine	10	-7.9	3.3	8.50	0.28		
8. +0.50% betaine	10	-5.1	3.5	4.80	0.17		
Exp. 3. Period of feeding 18 days:							
9. Control	10	+1.3	4.8	24.10	1.24	—	—
10. +0.03% choline	10	0	4.1	15.70	0.71	2.8	2.8
11. +0.05% choline	10	+1.0	3.8	13.18	0.51	4.4	4.4
12. +0.07% choline	10	-2.2	3.7	8.79	0.32	6.5	6.5
13. +0.10% betaine	10	+1.8	4.1	16.49	0.76	9.4	8.4
14. +0.15% betaine	10	+2.2	4.1	15.23	0.68	14.1	12.7
15. +0.20% betaine	10	-1.0	3.7	12.41	0.48	17.2	15.4

the groups 6, receiving 0.05% choline, and 7, receiving 0.20% betaine, have given practically the same values for liver fat, 9.58 and 8.50%, the control figure being 17.03%; the absolute amounts support this conclusion. It is to be noted that in this experiment the inclusion of 0.50% betaine has maintained the liver fat at nearly the normal level, 4.80% and 0.17 g. in the 100 g. rat. This may be of significance in view of the fact that on several occasions even massive doses of choline have failed to show so marked an action in preventing fatty infiltration.

These results suggested that betaine had a little more activity than 25% of its weight of choline in preventing fat deposition in the liver, a conclusion which may be better expressed by saying that it is slightly more active than 32% of an equimolar amount of choline. Exp. 3 was designed to test this finding and seven groups of animals were used, six of them receiving graded quantities of choline and betaine as set out in Table I. The food intakes of the various groups were measured in order to show exactly the intake of base in each group.

In exp. 3 the weight changes and food intakes (8.6–9.4 g./rat/day) are comparable in all the groups. The figures for both the percentage of fat in the liver and the weight in the liver of the 100 g. rat show that 4.4 mg. choline and 17.2 mg. betaine have had substantially the same preventive action, the results being 13.18% and 0.51 g., 12.41% and 0.48 g. respectively, as compared with the control values of 24.10% and 1.24 g. In Table I the amounts of betaine administered are converted into their equivalents in terms of choline, and comparing the choline and betaine intakes in this form the results of groups 11 and 15 indicate a 29% activity for betaine. There is no significant difference between the groups which received 9.4 mg. and 14.1 mg. betaine, a fact which can only be referred to the individual variations among the animals. However, the values obtained for liver fat, 16.49 and 15.23% respectively, are similar to that obtained in the case of 2.8 mg. choline, 15.70%, while 100 g. rat figures for the three groups are 0.76, 0.68 and 0.71 g. respectively. The activity of betaine in these cases is 33 and 22% of that of choline. From the approximate value of 32% suggested by the results of exp. 2, and the values 29, 33 and 22% obtained

in this more accurate experiment, it is reasonable to conclude that betaine has about 30 % of the efficiency of choline in preventing fatty infiltration into the liver under these conditions.

Cholamine and creatine. The groups of animals used in this experiment consisted entirely of bucks and the same control group served for this experiment as for exp. 1. Two groups received, in addition to the basal 8 % egg albumin diet, 1 % cholamine and 0.5 % creatine respectively.

Table II. *The effect of cholamine and creatine in fatty liver prevention*

Group	No. of rats	Change in wt. $\pm$ % initial body wt.	Liver % final body wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.
Exp. 4. Period of feeding 18 days:					
1. Control	8	+4.8	6.6	27.01	1.82
16. +1 % cholamine	9	+4.1	6.0	25.64	1.67
17. +0.5 % creatine	10	+1.1	6.4	27.96	1.71

Clearly neither compound has exerted any lipotropic action, for the groups of animals receiving them have livers as fatty as those of the control group, and in view of the relatively large quantities administered, it was not considered profitable to pursue the investigations any further.

Choline methyl ether and tetra(β -hydroxyethyl)ammonium chloride. In view of the considerable toxicity which the choline analogues previously investigated had been found to possess [Channon & Smith, 1936; Channon, Platt & Smith, 1937], it was decided to use relatively small quantities of these two compounds in preliminary experiments. The tetrahydroxyethyl derivative was tested first and two groups of does received a basal diet calculated to produce the "cholesterol" fatty liver: caseinogen (alcohol-ether-extracted) 5, beef dripping 30, glucose 52, marmite 5, salt mixture 5, cholesterol 2, cod liver oil 1 part. One group served as control and the other received a supplement of 0.14 % of the tetrahydroxyethyl derivative. The experimental data for this and the subsequent exps. 6, 7 and 8 are presented in Table III.

Table III. *Effect of choline methyl ether and tetra(β -hydroxyethyl)ammonium chloride in fatty liver prevention*

Group	No. of rats	Change in wt. $\pm$ % initial body wt.	Liver % final wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.
Exp. 5. Period of feeding 14 days:					
18. Control	10	-2.3	5.5	24.3	1.34
19. +0.14 % tetrahydroxyethyl derivative	9	+1.0	5.2	29.4	1.52
Exp. 6. Period of feeding 12 days:					
20. Control	10	-11.3	4.1	16.36	0.68
21. +0.10 % choline	10	-9.6	3.5	7.74	0.30
22. +0.11 % choline methyl ether	9	-13.6	4.1	17.09	0.74
23. +0.25 % tetrahydroxyethyl derivative	10	-10.7	4.3	15.76	0.67
Exp. 7. Period of feeding 18 days:					
1. Control	8	+4.8	6.6	27.01	1.82
2. +0.10 % choline	10	+5.0	5.2	8.18	0.41
24. +0.28 % choline methyl ether	10	+1.7	5.6	24.46	1.41
Exp. 8. Period of feeding 17 days:					
4. Control	9	-9.1	3.9	17.03	0.70
6. +0.05 % choline	10	-5.1	3.6	9.58	0.36
25. +0.50 % choline methyl ether	9	-14.3	3.6	16.32	0.62

The figures for weight change show that the compound is in no way toxic, while from the analytical figures it appears to have had little action on liver fat deposition. Actually the percentage of fat in the livers of the group receiving the tetrahydroxyethyl derivative, 29.4, is higher than in the control group, 24.3, but the difference in the absolute weights, which are 1.52 and 1.34 g., is not so great. While these groups do not differ significantly, further evidence as to the effect of a larger dose of the tetrahydroxyethyl derivative was clearly desirable, and accordingly this compound was again fed in exp. 6 in which choline methyl ether was also studied.

Four groups of does were maintained on a basal diet consisting of caseinogen (alcohol-ether-extracted) 5 parts, beef dripping 40, glucose 49, salts 5, cod liver oil 1 part, each animal receiving in addition 10 μ g. aneurin daily. Two groups received 0.11% choline methyl ether and 0.25% tetra(β -hydroxyethyl)-ammonium chloride respectively, while a third group received 0.10% choline to facilitate the assessment of lipotropic action.

The weight losses in exp. 6, although rather large, are not such as to interfere with any conclusions which may be drawn, and from the point of view of weight maintenance, the superiority of egg albumin as a basal protein in these low-protein diets is clearly demonstrated (compare groups 1 and 9 with 4 and 20). From study of these results it is seen that the tetrahydroxyethyl derivative has no choline-like action on liver fat, nor has the methyl ether in the amount employed. The percentages of liver fat in these two groups, 15.76 and 17.09 respectively, are substantially the same as the control group's 16.36%, a conclusion borne out by the values obtained for the weights in the 100 g. rat, 0.68, 0.74 and 0.67 g., for the control, choline methyl ether and tetrahydroxyethyl compound groups respectively. In contrast with these, group 21, which received 0.10% choline, had only 7.74% liver fat or 0.30 g. in the 100 g. rat, figures which demonstrate very clearly the powerful action of choline in preventing the deposition of liver fat under these conditions.

Clearly tetra(β -hydroxyethyl)ammonium chloride had no lipotropic action but the lack of toxicity of the choline methyl ether made it desirable to study its action in large dosages, and two further experiments were therefore carried out using this derivative with the results quoted in Table III. In exp. 7 the animals used were bucks and the basal diet consisted of egg albumin 8, beef dripping 40, glucose 46, salt mixture 5, cod liver oil 1 part and 10 μ g. aneurin per rat per day. The same diet was used in exp. 8 with the substitution of caseinogen for egg albumin as the protein while in this case the animals consisted of equal numbers of both sexes.

Considering the results of exp. 7 first, it would appear just possible that the choline ether possesses some activity, for although the results obtained for this group, 24.46% and 1.41 g., are not markedly different from the control values, 27.01% and 1.82 g., nevertheless they are somewhat lower. In view of the marked effect of 0.1% choline, which has prevented an increase in fat content of nearly 19% of the wet liver weight, any action of the ether must be slight and it was therefore decided to use a massive dosage, 0.5%, in the next experiment.

In exp. 8, the choline methyl ether has clearly exercised no lipotropic action whatsoever, the values both for the percentage of liver fat and for the weight in the 100 g. rat not differing significantly from those of the control group. On the other hand, only 0.05% choline has had a marked preventive action. The negative result obtained with this large dose provides confirmation of the conclusion from exps. 6 and 7 that choline methyl ether has no activity in preventing this fatty infiltration into the liver.

DISCUSSION

In the present work betaine has been found to possess about 30% of the activity of choline in preventing fatty infiltration in the livers of rats maintained on choline-free diets of high fat and low protein contents. This finding that betaine is lipotropically active confirms previous studies by Best & Huntsman [1932] and by Welch & Welch [1938], the latter authors also finding betaine aldehyde to be active. Neither paper, however, offers evidence as to the relative efficiencies of the two compounds although Welch & Welch [1938] suggest that betaine may have more than 50% of the activity of choline, but defer a definite statement until a later date. Quastel and his associates [1937; 1938] and Bernheim & Bernheim [1938] having shown that choline is oxidized to betaine aldehyde and then probably to betaine by surviving rat liver tissue and by enzyme systems extracted from rat liver, the present finding that betaine is less active than choline in fatty liver prevention suggests that, among other possibilities, it may be first converted into choline in the liver, this choline then being available for use: if this should be so, it would provide an interesting example of a readily reversible reaction taking place in animal tissues. Another explanation may be that while betaine itself is the active material, its activity is fully manifested only when it is produced in the liver by the oxidation of choline *in situ*, a part only of any dietary betaine being available and the rest oxidized before it is able to exercise its lipotropic action.

The very definitely negative result obtained with cholamine in exp. 4 confirms the work of Best & Huntsman [1932] and shows that if, in fatty liver prevention, lecithin formation resulting from choline feeding is the means whereby choline acts, the analogous synthesis of kephalin under similar conditions either plays no part, or cannot be influenced by cholamine feeding. This conclusion may indicate that kephalins have little or no importance in the metabolism of dietary fatty acids.

The results of the experiment with creatine indicate that this compound cannot serve as a source of choline in the body.

The results obtained with choline methyl ether show that if the primary alcohol group is etherified, choline becomes inactive. Apparently the body cannot remove a methyl ether group of this type although it appears to be able to demethylate methionine quite readily. Here again no light is thrown on the way in which choline may act, because, while the inactivation may be due to the fact that the choline ether can no longer be linked up in a phosphatide molecule, etherification may easily have inactivated the compound, whatever its mode of action.

Finally, study of tetra(β -hydroxyethyl)ammonium chloride has revealed the necessity that the alkyl radicles of the choline molecule be intact for the possession of lipotropic properties, a finding in agreement with the complete lack of activity of cholamine.

SUMMARY

1. Studies have been made of five compounds—betaine, cholamine, creatine, choline methyl ether and tetra(β -hydroxyethyl)ammonium chloride—in order to discover whether they possess choline-like properties in preventing the deposition of liver fat under certain dietary conditions.

2. Of these compounds, only betaine was found to possess lipotropic properties. Its activity in fatty liver prevention was estimated to be approximately 30% of that of choline.

3. The relationship of these results to the theory that choline acts by enabling lecithin formation to take place is discussed.

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