CCXVIII. THE DIETARY PREVENTION OF FATTY LIVERS. TWO ANALOGUES OF CHOLINE

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CHANNON & SMITH [1936] reported that triethyl- β -hydroxyethylammonium hydroxide had choline-like action in preventing fat deposition in the livers of rats receiving diets high in fat and low in choline and protein. In that work no attempt was made accurately to determine the intensity of the action of this compound in terms of choline but the results suggested that its activity was somewhat less, perhaps of the order of 70%. In the present paper the results of experiments with two further choline analogues are recorded. The first of these compounds, tripropyl- β -hydroxyethylammonium hydroxide was investigated with a view to seeing whether further increase in the size of the alkyl radicals would cause a still greater diminution in the physiological activity than had occurred with the triethyl compound; the second, trimethyl- γ -hydroxypropylammonium hydroxide, in order to study the effect of increasing the size of the group in the other part of the molecule. For brevity of description triethyl- β -hydroxyethylammonium hydroxide, tripropyl- β -hydroxyethylammonium hydroxide and trimethyl- γ -hydroxypropylammonium hydroxide will be referred to in this paper as "triethyl-choline", "tripropyl-choline" and "homocholine" respectively.

EXPERIMENTAL

The general method of experiment was to feed groups of rats, each of 10 animals or more on diets producing either the "cholesterol" or "fat" fatty liver, and at the same time to administer to other groups receiving the same diet one of the derivatives or choline itself. The amounts of the different compounds incorporated in the diet in any given experiment were always molecularly equivalent in order to obtain figures of value in assessing the activity of any compound in terms of choline. The increasing toxicity of these compounds compared with that of choline sometimes caused a lower food consumption in certain groups and hence the amounts of the derivatives actually consumed by the animals varied somewhat from molecular proportions. These variations were, however, not sufficient to introduce uncertainty into the deductions.

In Exp. 1 the diet used to produce the "fat" fatty liver was caseinogen (alcohol-extracted) 5, fat (beef dripping) 40, glucose 44, marmite 5, salts 5, cod liver oil 1 part. In Exps. 2-4 the "cholesterol" fatty liver was studied, produced by use of a diet consisting of caseinogen (alcohol-extracted) 5, fat (beef dripping) 30, cholesterol 2, glucose 52, marmite 5, salts 5, cod liver oil 1 part. Choline and its analogues were added to the diets as aqueous solutions of the chlorides and the amount of any derivative ingested was determined by weighing the food residues daily.

The method of investigating the livers varied in the different experiments. In Exp. 1 the pooled livers of the different groups of animals were digested with 10% NaOH and, after refluxing following the addition of alcohol, the fatty acids and unsaponifiable fraction were obtained by ethereal extraction and subsequently purified by treatment with light petroleum. In Exp. 2 the pooled livers were treated by the alcohol-ether method and analysed as described by Beeston *et al.* [1935]. In order to study the variations in the individual animals of each group, in Exps. 3 and 4 the liver of each animal was ground to a fine powder with 60–70 g. anhydrous Na₂SO₄ and the mixture extracted for 4 hr. with chloroform in a Soxhlet apparatus. The material was then thoroughly reground and re-extracted for a further 4 hr. The chloroform extracts were filtered into weighed flasks and the weight of the total lipid obtained after removal of the chloroform *in vacuo*. While this chloroform method may leave traces of lipid unextracted such amounts are quantitatively insignificant from the point of view of this work.

The method used for the preparation of tripropyl- β -hydroxyethylammonium chloride was based on that described by Renshaw for choline [1910]. Freshly distilled ethylene chlorohydrin and tripropylamine in molecular proportions were heated in a sealed tube at 95° for 4 hr. with occasional shaking. The contents of the tube were then dissolved in alcohol, purified by treatment with charcoal and precipitated from the alcoholic filtrate by means of acetone and ether. The crystalline product was further purified by similar means. Its extremely hygroscopic nature rendered analysis difficult. (Found: Cl 15.3%; C₁₁H₂₆ONCl requires Cl 15.9%.) The chloroaurate contained Au 37.32% (C₁₁H₂₆ON. AuCl₄ requires Au 37.34%).

Trimethyl- γ -hydroxypropylammonium chloride (homocholine) was prepared as follows. Into trimethylene chlorohydrin obtained by the method described by Adams [1928] and contained in a thick-walled tube, cooled in a freezing mixture, was passed, through a cooled delivery tube, an equimolecular quantity of trimethylamine, generated by dropping a concentrated solution of its hydrochloride on to solid NaOH. After being sealed the tube was heated at 95° for 4 hr. and the product purified as described above. (Found: Cl 23.05, N 8.9%; C₆H₁₆ONCl requires Cl 23.13, N 9.1%.) The chloroaurate contained Au 43.05% (C₆H₁₆ON. AuCl₄ requires Au 43.15%).

All the essential data are presented in a series of tables concerning which some explanatory remarks are necessary. The average weight of the rats used throughout this work was about 150 g. and the tables contain a column showing the percentage loss or gain in body weight during the period of experiment without a record of the initial weights. This column is necessary because loss of weight has to be considered in assessing the significance of the amount of fat present in the liver, for some of the choline homologues are very toxic. The tables contain also figures of the amounts of the different compounds ingested, while in addition these figures have been converted in the succeeding column into their molecular equivalents of choline, in order that accurate comparison may be made of the quantitative aspects of their actions compared with that of choline. In these two columns, the choline content of the basal diet has been ignored because, save for one or two exceptions, the food intake of all the groups was sufficiently constant to prevent the intake of choline from the basal diet varying to an appreciable extent in the different groups. The tables also contain the liver weights expressed as a percentage of the body weights, which is necessary because, with the increasing fat infiltration, the liver size increases rapidly. In addition, there are presented figures for the absolute amounts of the

different lipoids present in the livers of the 100 g. rat calculated from the percentage liver weight and the percentage fat content of the liver because, with the variation in liver size with increasing fat percentage, this absolute weight gives the truest picture of the state of affairs. It might be argued that with the considerable variations in the fat percentage and size of the liver among different individuals of any one group (many examples of which have been previously reported from this laboratory, and of which further cases appear later in this paper), it is unjustifiable to make such a calculation from analysis of the pooled livers. Investigations of this point however, which will be subsequently published, show that any error resulting is insignificant when the groups consist of 10 animals.

RESULTS

Exp. 1. In Exp. 1 the action of choline was compared with that of "triethyl-choline" and homocholine.

Table I

Exp. 1. Period of feeding 19 days

Group	No. of rats	Wt. gain or loss %	Food eaten per rat per day g.	Derivative intake		Liver % body wt.	F.A. + U.M.	
				Base per rat per day mg.	Mol. equiv. of choline mg.		% fresh liver wt.	g. per 100 g. rat (calc.)
Control	10	- 7.8	8.4	—	—	4.7	28.2	1.31
+ choline	10	+ 1.9	9.5	20.8	20.8	3.3	6.7	0.22
+ "triethyl-choline"	10	- 4.7	8.8	27.8	20.6	3.7	13.8	0.52
+ homocholine	10	- 18.8	5.5	13.5	12.1	3.1	6.4	0.20

Table I shows that the percentages of "fat" (fatty acids and unsaponifiable matter) in the choline and homocholine groups, 6.7 and 6.4 respectively, are the same within experimental limits, and that they are some 22 units % less than the control figure of 28.2 %, while "triethyl-choline" has resulted in a difference of 14 units %. The figures for the amounts of "fat" in the liver of the 100 g. rat also show that the effects of choline and homocholine in the amounts used are identical. Thus whereas the fat in the livers of the control group has risen to 1.3 g. from the normal of about 0.12 g., the livers of the choline and homocholine groups contain no more than 0.2 g. Hence these substances have prevented 1.1 g. "fat" appearing in the liver, while "triethyl-choline" has caused a lesser degree of prevention, 0.8 g. When the figures for the intakes of the different compounds are calculated in terms of choline, as is shown in the table, it is seen that while the intakes of choline and "triethyl-choline" are molecularly similar, 20.8 and 20.6 mg. per rat per day respectively, that of homocholine, 12.1 mg., is substantially less. Since the degree of prevention of fat infiltration caused by this compound is the same as that of choline, the results imply that homocholine may be considerably more active than choline itself. In this experiment, however, the food intake of the homocholine group, 5.5 g. per rat per day, was only about $\frac{2}{3}$ that of the control group, 8.4 g., and further the body weight losses, 18.8 %, were nearly two and a half times greater. While moderate weight losses have been found to play no significant part in controlling the fat content of the liver, these two factors make deduction somewhat uncertain.

Exp. 2. In Exp. 2 study was made of the action of the three compounds used in Exp. 1, and also of "tripropyl-choline" in a "cholesterol" fatty liver experiment. The control, choline and "triethyl-choline" groups consisted of 19 or 20 animals

in contrast to the 10 animals of each of the "tripropyl-choline" and homocholine groups, because a larger amount of material was required in these cases for other investigations. In Exp. 2 all the animals used were does, because previous work [Channon & Smith, 1936] had shown that does reacted more readily to the triethyl compound than did bucks.

Table II

Exp. 2. Period of feeding 20 days

Group	No. of rats	Wt. gain or loss %	Food eaten per rat per day g.	Derivative intake	
				Base per rat per day mg.	Mol. equiv. of choline mg.
Control	20	- 8.3	8.5	—	—
+ choline	20	- 6.1	8.1	14.9	14.9
+ "triethyl-choline"	19	- 11.2	7.2	20.6	15.3
+ "tripropyl-choline"	10	- 7.6	8.7	3.7	2.1
+ homocholine	10	- 19.0	7.4	14.0	12.6

Table II shows that the weight losses in the homocholine group were again considerably greater than in the other groups although the food intake, 7.4 g., compared much more favourably with the control, 8.5 g., than in Exp. 1. The table shows that the molecular amounts of choline, "triethyl-choline" and homocholine ingested did not differ greatly one from the other. The amount of "tripropyl-choline", 2.1 mg. per rat per day, was deliberately kept low in the first experiment with this substance because of the probability of it being highly toxic. The detailed analyses of the liver lipoids of the groups are recorded in Table III.

Table III

Exp. 2. Analysis of the livers

Group	Liver % body wt.	Lecithin	Cholesterol	Cholesteryl oleate	Glyceride	Total
Liver lipoids (g. per 100 g. fresh liver)						
Control	4.7	2.48	0.34	5.11	21.75	29.68
+ choline	3.6	2.33	0.33	3.97	6.63	13.26
+ "triethyl-choline"	3.8	2.94	0.28	4.29	11.30	18.81
+ "tripropyl-choline"	4.5	2.44	0.32	5.29	18.00	26.05
+ homocholine	3.5	3.26	0.31	2.55	3.74	9.86
Liver lipoids (g. per liver of 100 g. rat calc.)						
Control	4.7	0.117	0.016	0.240	1.023	1.396
+ choline	3.6	0.084	0.012	0.143	0.239	0.478
+ "triethyl-choline"	3.8	0.112	0.011	0.163	0.429	0.715
+ "tripropyl-choline"	4.5	0.110	0.014	0.238	0.810	1.172
+ homocholine	3.5	0.114	0.011	0.089	0.131	0.345

Considering both the total percentage of the liver lipoids and the total absolute weight of lipoids in the liver of the 100 g. rat, it is clear that the greatest effect has again been caused by homocholine, whereas "tripropyl-choline" has had little, if any, action. Study of the figures for the individual constituents shows that the action of all these compounds consists in the main of preventing a large increase in the glyceride fraction with a lesser change in the cholesteryl ester fraction. Thus the high percentage of the control glyceride, 21.75, is to be compared with the figure of the choline group, 6.63, and the still lower one of the homocholine group, 3.74, while the latter half of the table shows corresponding differences in the absolute weights of glyceride in the livers.

Since Table II shows that the molecular equivalent of the homocholine ingested in terms of choline is 12.6 mg., an amount which has caused a greater decrease than occurred in the choline group receiving 14.9 mg. of choline daily, the results of this experiment on the "cholesterol" fatty liver confirm those of Exp. 1 on the "fat" fatty liver. Once again, however, some doubt is raised by the greater weight losses in the homocholine group. "Tripropyl-choline" has had no action at the low dosage used.

Exp. 3. As a result of Exps. 1 and 2 it was decided to attempt to avoid the weight losses by decreasing both the homocholine intake and the length of the period of the experiment. The choline content of the diet of the choline group was correspondingly reduced whilst the amount of "tripropyl-choline" was increased in order to attempt to demonstrate a possible action of this compound in a greater dosage than had been used in Exp. 2. In this experiment the individual livers were studied and the total lipid represents the chloroform extract of the livers dried with anhydrous Na_2SO_4 ; the results are presented in Table IV. In this table the mean values for the livers as % of the body weight, for the lipids as % of the fresh liver and for the weight of lipids in the liver of the 100 g. rat are presented as before. In addition, there are recorded, following each mean value the extremes of the individual variations. These individual variations, which so often occur and which were extensively investigated and discussed in a previous paper [Channon & Smith, 1936], will not be further analysed, because experience in that work in this direction was similar to that under discussion. Thus successive experiments with "triethyl-choline" in which this type of variation was encountered, resulted in identical findings. This fact, together with the consistency of the results of all the experiments recorded in the present paper, make it appear reasonable to consider the mean values only.

Table IV

Exp. 3. Period of feeding 11 days

Group	No. of rats	Wt. gain or loss %	Food eaten per rat per day g.	Derivative intake		Liver % body wt.	Total lipid % fresh liver wt.	Total lipid g. per liver of 100 g. rat (calc.)
				Base per rat per day mg.	Mol. equiv. of choline mg.			
Control	9	- 6.8	8.0	—	—	4.6 (3.6-5.3)	27.18 (11-46)	1.304 (0.39-2.43)
+ choline	10	- 1.8	8.0	9.0	9.0	3.9 (3.6-4.4)	12.20 (5-19)	0.486 (0.18-0.83)
+ "tripropyl-choline"	9	- 10.0	7.8	16.6	9.8	4.5 (3.7-5.6)	26.69 (20-34)	1.208 (0.85-1.95)
+ homocholine	10	- 6.5	7.0	8.7	7.8	3.6 (3.2-4.6)	10.54 (7-14)	0.384 (0.28-0.63)

In this experiment the weight losses in the homocholine group were rather less than in the control group and even with the "tripropyl-choline" group, in which slightly greater weight losses occurred, no significance is to be attached to them. This is thus the first experiment in which the possible interference of this factor has been overcome. The results of Exp. 3 bear out those of Exps. 1 and 2. The lowest level both in percentage and the absolute weight of the liver lipids is caused by an amount of homocholine, 8.7 mg. per rat per day, which corresponds to 1.2 mg. less choline than was received by the choline group, 9 mg. per rat per day. Further, increasing the "tripropyl-choline" intake from 3.7 mg. in

Exp. 2 to 16.6 mg. in Exp. 3 has had no effect in preventing the liver fat deposition, for the small changes in the percentage and amount of liver lipoids shown in Table IV are considered to be insignificant.

Exp. 4. The finding that homocholine was more active in fatty liver prevention made it possible to carry out Exp. 4 in which the intake of the substances was reduced still further. Table V records the animal side of this experiment.

Table V

Exp. 4. Period of feeding 14 days

Group	No. of rats	Wt. gain or loss %	Food eaten per rat per day g.	Derivative intake	
				Base per rat per day mg.	Mol. equiv. of choline mg.
Control	9	-4.5	8.2	—	—
+ choline	10	-2.0	9.0	4.7	4.7
+ "tripropyl-choline"	10	-6.8	7.9	8.7	5.1
+ homocholine	10	-7.7	7.0	4.5	3.9

The data in Table V show that this is in every way a satisfactory experiment from the point of view of the weight losses and food intakes. The detailed analyses of the liver lipoids are presented in Table VI.

Table VI

Exp. 4. Analysis of the livers

Group	Liver % body wt.	Lecithin	Cholesterol	Cholesteryl oleate	Glyceride	Total
Liver lipoids (g. per 100 g. fresh liver)						
Control	5.0 (3.8-7.0)	2.44	0.272	3.40	23.53	29.64
+ choline	3.9 (2.9-5.9)	3.21	0.260	2.39	13.78	19.64
+ "tripropyl-choline"	4.6 (3.5-6.6)	2.15	0.310	3.50	21.87	27.83
+ homocholine	3.6 (3.2-4.0)	3.11	0.310	2.99	9.97	16.38
Liver lipoids (g. per liver of 100 g. rat calc.)						
Control	5.0 (3.8-7.0)	0.122	0.014	0.170	1.279	1.585
+ choline	3.9 (2.9-5.9)	0.125	0.010	0.093	0.538	0.766
+ "tripropyl-choline"	4.6 (3.5-6.6)	0.099	0.015	0.161	1.006	1.281
+ homocholine	3.6 (3.2-4.0)	0.112	0.012	0.108	0.359	0.591

The lowest level in the total percentages and the absolute amounts of lipoids in the livers has again been caused by homocholine, which was given in an amount molecularly less than the choline received by the choline group. "Tripropyl-choline" has again had little, if any, action. Study of the individual constituents shows that the main effect of homocholine is like that of choline on the glyceride fraction, for 4.5 mg. of homocholine per rat per day have reduced the glyceride figure from 23.53 to 9.97 % while 4.7 mg. of choline have reduced it to 13.78 %. Similarly while the weight of glyceride in the liver of the 100 g. rat is as high as 1.279 g. in the control group, it is considerably less, 0.359 and 0.538 g., in the homocholine and choline groups respectively. It is to be observed also that homocholine prevents a partial increase in the percentage of cholesterol esters in the same way as has already been established for choline itself [Best *et al.* 1934]. The figures in Table VI suggest that the action of homocholine on the cholesteryl ester fraction is perhaps rather less than that of choline, but these figures probably lie within experimental variation since a reverse result was obtained in Exp. 2 (Table III).

DISCUSSION

Results of the four experiments just described show consistently that homocholine is more effective in preventing fat infiltration both in the "fat" and "cholesterol" fatty livers than is choline itself. This is an interesting example of a synthetic substance having a greater action than the natural one, for so far as we are aware the presence of homocholine in animal tissues has not so far been reported. Whilst it would have been satisfactory to have determined by a further experiment the minimum amount of homocholine having a demonstrable action on liver fat, this particular point was not considered worthy of further pursuit. It is clear from the results of Exps. 2 and 4 that the action of homocholine on the "cholesterol" fatty liver is of the same type as that of choline.

Whilst there are slight indications in Exps. 2 and 4 that "tripropyl-choline" may have a very slight effect in decreasing fat deposition, it is clear that any possible action it has is quantitatively negligible compared with those of choline or homocholine in the dosage used. This observation is of interest because of our experiments with "triethyl-choline" [Channon & Smith, 1936]. Whilst the exact choline equivalent of this latter compound was not accurately determined, it probably has at least $\frac{2}{3}$ of the activity of choline itself. This loss of $\frac{1}{3}$ of the activity when the three methyl groups of choline are replaced by three ethyl groups is thus greatly accentuated when three propyl groups are substituted, for the compound then becomes inactive. The toxicity of "tripropyl-choline" made it appear useless to pursue the enquiry further by increasing the dosage.

SUMMARY

1. The preventive effect of homocholine (trimethyl- γ -hydroxypropyl-ammonium hydroxide) in controlling the percentage of fat in the livers of rats receiving diets which cause either the "fat" or "cholesterol" fatty liver, has been studied in four experiments.

2. All the experiments show that homocholine is more effective than is choline.

3. The nature of the action of homocholine is similar to that of choline in that on the "cholesterol" fatty liver the main effect is to decrease substantially the glyceride content of the livers and to a lesser extent the cholesteryl ester fraction.

4. Similar studies were made in three experiments with tripropyl- β -hydroxyethylammonium hydroxide in experiments in which homocholine and choline had a profound effect in preventing fat deposition. This compound was without action.

5. This latter result is contrasted with previous findings with triethyl- β -hydroxyethylammonium hydroxide which has an action similar to and not greatly less than that of choline.

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