CCLXVII. THE CONTROL OF LIVER FAT BY TRIETHYL-β-HYDROXYETHYLAMMONIUM HYDROXIDE

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WHILE the striking effect of dietary choline in preventing fat deposition in the liver or causing the removal of accumulated fat from that organ has been amply demonstrated in a variety of conditions, the mode of action of this compound has yet to be determined. The most obvious explanation is that the presence of choline in the diet enables lecithin synthesis to occur and that this phosphatide is necessary for fat transport from the liver as suggested by the desaturation hypothesis of Leathes. Since choline is a common constituent of cells, mainly in the form of lecithin, study of the method by which dietary choline exercises its effects is obviously difficult. If the lecithin hypothesis were correct, it is possible that any base having a choline-like action on liver fat would be incorporated in a new phosphatide molecule in which it had replaced choline. Such a finding would provide a ready means of investigating the control of liver fat by choline. Accordingly, we have studied a number of analogues of choline in the hope of finding one which had an action on liver fat like that of choline and which at the same time could be identified in the tissues by isolation of a suitable chemical derivative separable from that of choline. Two of these, homocholine and triethyl-β-hydroxyethylammonium hydroxide[1] were found to be active [Channon & Smith, 1936; Channon *et al.* 1937]. While the former has an action greater than that of choline, it has not been found possible to separate it from choline; the latter, however, which has about 70% of the activity of choline, can be isolated as the chloroaurate. This compound has accordingly been administered to animals and its presence sought in the liver phosphatides.

EXPERIMENTAL

As a preliminary to investigating the livers of animals which had received triethyl choline in their diet, a series of experiments was carried out to determine, first, the proportion of this base which could be detected in the presence of choline, and secondly, whether it was affected by the procedure necessary for its isolation from the mixed phosphatides.

Accordingly samples of the chloroaurates of the two bases were prepared and their melting-points and solubilities in water determined. From the data so obtained (choline chloroaurate M.P. 262–265°, solubility 0·94% at 0°, Au 44·5%; triethyl choline chloroaurate M.P. 225°, solubility 0·32% at 0°, Au 40·6%) and experiments on their separation by crystallization, it was easy to show the presence of 1 part of triethyl choline in the presence of as much as 4 parts of choline. Thus from a mixture of the chloroaurates prepared from 50 mg. of each of the bases, there was obtained a fraction M.P. 225°, Au 40·5%.

[1] For brevity this compound will be referred to as "triethyl choline".

Similarly the chloroaurates obtained from 25 mg. triethyl choline and 100 mg. choline yielded a fraction having M.P. 227° and Au 40·7 %. Attempts to apply this separation to mixtures of which triethyl choline constituted less than 20 % were, however, less successful. Typical of these experiments was one in which 10 mg. of the compound with 90 mg. choline yielded finally a chloroaurate fraction, M.P. 235°. These results indicate that while evidence of less than 20 % of triethyl choline may be obtained from the M.P. of the chloroaurates, certain proof of its presence cannot be obtained.

Having established these facts with the pure compounds, triethyl choline was added to phosphatide mixtures, and the effectiveness of the separation investigated in the more elaborate procedure necessary for isolating the bases. The phosphatide mixtures were hydrolysed with 5 % H_2SO_4 and the fatty acids extracted with ether. The aqueous mother-liquor was then neutralized with baryta and $BaSO_4$ removed by centrifuging. Cholamine was precipitated by adding slight excess of mercuric acetate in faintly alkaline 20 % solution, followed by alcohol, the mixture being then kept overnight. The precipitate was filtered, and the alcohol evaporated from the mother-liquor. After concentration and removal of the precipitated inorganic salts, excess of gold chloride was added at 0°, the low temperature being essential on account of the presence of some reducing compound, possibly a sugar residue derived from sphingomyelin or contaminating cerebroside. Two experiments were carried out in this way. In the first, 50 mg. of triethyl choline were added to 1·41 g. of crude liver phosphatide and 260 mg. of crude chloroaurate were obtained. On recrystallization, the least soluble fraction, 100 mg., had M.P. 224–7°, Au 41·3 %. This showed a 50 % recovery of the added base in the presence of three times the amount of choline. In the second experiment, 50 mg. of triethyl choline were added to 2·6 g. of phosphatide. On recrystallization of the 330 mg. of chloroaurate obtained, the final fraction had M.P. 221°. These results show that triethyl choline is unaffected by the method of hydrolysis and separation and support the previous results with the pure compounds as to the amount which can be detected if present in the phosphatides. In the animal experiments, groups each of about 20 rats were used. Such groups yield rather more than 100 g. liver containing about 3·5 % phosphatide, and 250–300 mg. choline, considerably more material than was used in the trial experiments.

Exp. 1. Three groups received a basal diet consisting of beef dripping 40, glucose 45, casein (alcohol-extracted) 5, salt mixture 5, marmite 5 parts with 1 drop of cod liver oil per animal every 3 days. One group served as the control, while the second and third groups received, in addition to the basal diet, supplements of choline and triethyl choline respectively, incorporated in the diets as aqueous solutions of the chlorides over a period of 20 days. The relevant data concerning the animal side of the experiment are recorded in Table I.

Table I

Group	No. of rats	Final body wt. g.	% loss in body wt.	Food intake g. per rat per day	Base intake mg. per rat per day	Liver % body wt.
Control	20	172	6·0	7·8	—	3·88
Control + choline	17	164	5·7	8·0	12·1	4·01
Control + triethyl choline	18	150	12·3	6·4	11·5	4·25

In the light of our previous experience, the comparable weight losses and food intakes of the different groups are regarded as satisfactory. The pooled

livers were ground with sand, the lipoids extracted by alcohol and ether and analysed as previously described [Best *et al.* 1934] with the results recorded in Table II. In this table, the glyceride and phosphatide contents of the livers

Table II. *The liver lipoids of rats receiving choline and triethyl choline*

	% fresh liver wt.			g. per liver of 100 g. rat		
Group	Glyceride	Lecithin	Total	Glyceride	Lecithin	Total
Control	12·3	2·84	15·14	0·477	0·111	0·588
Control + choline	4·7	3·11	7·81	0·190	0·124	0·314
Control + triethyl choline	4·8	2·79	7·59	0·204	0·119	0·323

only are recorded, since these alone are relevant to the discussion. While the glyceride in the control group, 12·3 %, is some 8 units lower than is usually encountered with this particular diet, the effects of choline and triethyl choline are clearly seen, for each has prevented an increase of some 8 units % and reduced the weight of glyceride in the liver by 60 % of the control value. It is to be noted that in this experiment triethyl choline has been as effective as choline, although Channon & Smith [1936] found its activity in most of their experiments to be rather less. After completion of the analyses recorded in Table II, the phosphatide fractions were precipitated from the remainder of the ethereal solutions of the total lipoid by acetone (4 vol.), redissolved in ether and reprecipitated.

The phosphatides so obtained, 4·09, 4·07 and 3·10 g. in the control, choline and triethyl choline groups respectively, were then hydrolysed and the cholamine separated. The crude chloroaurates were precipitated as previously described, the yields being 0·434, 0·492 and 0·415 g. These crude chloroaurates from the three groups were recrystallized and their gold contents determined. All of them had M.P. 262° and the gold content was the same 44·3–44·4 % Au (choline chloroaurate has M.P. 262–265°, Au 44·5 %). The compounds from the control and choline groups were next recrystallized three times more and that from the triethyl group four times more. There was no significant change in any of the final fractions (control group M.P. 264°, Au 44·2 %: choline group M.P. 264°, Au 44·2 %; triethyl group M.P. 264°, Au 44·3 %). The absence of triethyl choline chloroaurate was thus established.

Exp. 2. This was a repetition of the first experiment except that the basal diet was one designed to produce "cholesterol" fatty livers. It consisted of beef dripping 30, glucose 52, casein (alcohol-extracted) 5, salt mixture 5, marmite 5, cholesterol 2, cod liver oil 1. All the data and the complete analyses of the livers of these three groups of animals have already been presented in another connexion [Channon *et al.* 1937] and will not be discussed here. The only relevant facts which need mention are that the weight losses of animals were satisfactory, and that the percentages of glyceride in the livers of the control, choline and triethyl choline groups were 21·7, 6·6 and 11·3 % respectively, while the actual weights of glyceride in the liver of the 100 g. rat were 1·02, 0·24 and 0·43 g. Further, the average daily intakes of choline and triethyl choline in the two latter groups were 14·9 and 20·6 mg. per rat per day respectively. After completion of the routine analysis, the remainder of the lipoid material was precipitated from ether by acetone and treated as in Exp. 1 for the preparation of the chloroaurates of the phosphatide bases. The full details regarding the yields of these compounds in the different groups and their subsequent recrystallization are recorded in Table III.

Table III. *Data concerning the chloroaurates of the bases obtained from the liver phosphatides*

Group	Wt. of phosphatide hydrolysed g.	Wt. of crude chloro-aurates mg.	M.P. after one crystallization °C.	After two further recrystallizations: Wt. taken mg.	Wt. of final products mg.	M.P. °C.	Au content %
Control	4·38	475	255	300	60	263	44·30
Control + choline	3·48	410	253	285	35	262	44·39
Control + triethyl choline	3·70	400	261	200	62	262	44·46

The possibility that a phosphatide containing triethyl choline might be soluble in acetone and so escape precipitation with the other phosphatides was then considered. The material obtained by evaporation of the ether-acetone mother-liquors to dryness was therefore hydrolysed by boiling with 5% H_2SO_4 for 5–6 hr. The mixture was extracted exhaustively with ether to remove fatty acids, unsaponifiable matter and unhydrolysed glycerides, and N was then determined in the aqueous mother-liquor. The similar and small amounts of N found, 15·1, 12·6 and 14·7 mg. in the control, choline and triethyl choline groups respectively, made it appear unlikely that any triethyl choline was present in the mother-liquors from the livers of the animals which received this base. In confirmation, however, the three aqueous residues were treated exactly as described already, but in no case was a precipitate obtained with gold chloride. These small amounts of N must therefore have been derived from nitrogenous substances contaminating the phosphatides and extracted with them from the livers, an occurrence frequently reported with crude phosphatide preparations. As a further precaution, N determinations were carried out on the ether-soluble fractions obtained after the acid hydrolysis, but even when quantities as large as 0·5 g. were used, the results were negative. The acetone-ether-soluble fraction of the lipoids of the triethyl groups was thus shown to contain no triethyl choline.

Discussion

In both experiments, the recrystallized chloroaurates from the control, choline and triethyl choline groups had the melting-point of the choline compound and gave no depression when mixed with the pure compound. It was shown earlier that it was possible to identify triethyl choline with certainty if it constituted 20% of a mixture with choline. Further, when smaller proportions were present, the melting-points of the least soluble fractions of the chloroaurates were always depressed well below that of the choline compound. The fact that in both experiments the compounds obtained from the triethyl and choline groups had the melting-point of choline chloroaurate shows clearly that the triethyl compound, though ingested in considerable amounts (total intake 4·35 and 7·83 g. in Exps. 1 and 2 respectively), does not constitute any notable proportion of the phosphatide bases, being present in traces, if at all.

An ingenious attempt to throw light on the same problem has been made by Welch [1936] who supplemented a high fat diet with the arsenic analogue of choline which he found "approximately as effective as choline chloride" in preventing the development of fatty livers in rats. On spectrographic examination the purified lecithins prepared from the livers and brains of the animals which had received arsenocholine showed strong arsenic lines while those from "control" animals gave no such lines. This result indicates that the

preventive action of arsenocholine on liver fat deposition may well be by "lecithin" formation. Confirmation by the isolation and identification of the arsenocholine as a constituent of a phosphatide molecule is however desirable because of the extreme delicacy of the spectrographic method and of the difficulty of controlling such an experiment. It does not appear for instance from the preliminary report available that the control groups received any arsenic compounds and hence these interesting results must be accepted with reserve until a more complete account is available.

Our failure to detect the presence of triethyl choline in the liver phosphatide is not surprising perhaps in view of results obtained by us on many occasions when choline feeding has been found to have no effect on the amount of the liver phosphatide. In the present work the weights of phosphatide in the livers are, in Exp. 1, 109, 117 and 104 mg. and in Exp. 2, 117, 84 and 112 mg. per 100 g. rat respectively for the control, choline and triethyl choline groups. Save for the choline group in Exp. 2 none of these variations can be considered significant, and with this at present unexplained exception and a few other isolated examples among a very large number of observations accumulated in this laboratory, it has been found that in general the absolute weight of liver phosphatide (calculated as lecithin from the ether-soluble P) is independent of the dietary choline and the degree of fatty infiltration, though naturally its percentage falls with increasing fat deposition. However, while the weight of phosphatide thus shows no change the possibility remains that the proportion of lecithin present in it is greater in animals receiving choline. So far as we are aware the only recorded results on this aspect of the problem are those of Best *et al.* [1934] and the further results of work in this laboratory are too few to merit discussion of the subject at the present time.

In view of the relative constancy of the amount of the liver phosphatide and the apparent inability of its contained choline to exert any lipotropic action, however intense the degree of fat infiltration, it is important to consider quantitatively the efficiency of dietary supplements of choline in this respect. This matter will be discussed in detail later; meanwhile it is worthy of mention here that, even at the lowest dosages when the effect is greatest, 1 mg. of choline per day can only prevent the deposition of about 30 mg. of fat per day in the liver of the 100 g. rat. This relatively low degree of activity appears to imply a chemical utilization. A considerable volume of recent research, particularly the work of Sinclair and of Artom, seems definitely to establish the active role of phosphatides in fat metabolism. (The most recent papers of many published on this subject by these authors are Sinclair [1936] and Artom *et al.* [1937], from which references to the earlier work may be obtained.) It is a particularly attractive hypothesis, therefore, that choline should exercise its action by enabling lecithin synthesis to occur. Save for the results of Welch mentioned earlier, however, there seems to us to be little evidence in support of this hypothesis: yet it must be emphasized that our failure in the present work to find triethyl choline in the liver phosphatides does not disprove the lecithin hypothesis. Any lecithin formed might pass into the blood without accumulation in the liver.

Summary

1. Evidence has been sought on the problem as to whether the preventive action of choline on liver fat deposition is exercised through lecithin formation.

2. Triethyl-β-hydroxyethylammonium hydroxide which has an effect on liver fat similar to that of choline was used for this purpose. This compound can be separated from choline because of the lower solubility of its chloroaurate.

3. A control group of animals received a "fat" fatty liver-producing diet while other groups received the same diet with the addition of either choline or triethyl-β-hydroxyethylammonium hydroxide.

4. The phosphatides were prepared from the livers and the bases obtained by hydrolysis. After removal of cholamine, the chloroaurates were prepared and fractionally crystallized.

5. No evidence was obtained of the presence of triethyl-β-hydroxyethylammonium hydroxide in the livers of the animals receiving this base, even though the liver fat had been markedly decreased.

6. A second experiment was carried out on the "cholesterol" fatty liver with the same result.

7. The lecithin hypothesis is discussed.

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