The Mode of Action of Lipotropic Agents

PROOF OF THE *IN VIVO* INCORPORATION OF TRIETHYL- β -HYDROXYETHYL-AMMONIUM HYDROXIDE INTO THE PHOSPHOLIPID MOLECULE

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(Received 2 April 1947)

Several theories have been proposed to account for the lipotropic action of choline, betaine, methionine, inositol and other compounds which exert similar effects on the deposition of lipids in the liver. The discovery (Hershey, 1930; Hershey & Soskin, 1931) reported from the laboratory of one of us (C. H. B.) that lecithin prevents the accumulation of excessive amounts of fat in the liver of depancreatized dogs seemed to offer support for the hypothesis, originally advanced by Leathes, that fatty acid transport involves incorporation of these compounds into the phospholipid molecule. The demonstration by Best & Huntsman (1932) that choline is the lipotropically active constituent of the lecithin molecule did not alter the main concept. If choline cannot be synthesized by mammals, or if its rate of synthesis is not equal to all requirements, the amount available would probably be the limiting factor in the formation of new lecithin molecules. Thus the viewpoint has been adopted that ingested choline exerts its lipotropic effect, in part at least, by stimulating synthesis of lecithin. Using radioactive phosphorus as a tracer element, a more rapid turnover of liver phospholipids was shown to follow ingestion of choline (Perlman & Chaikoff, 1939) and the relatively rapid incorporation of dietary choline into the phospholipid molecule has been established (Stetten, 1941). Welch & Landau (1942) have shown that arsenocholine (an analogue of choline containing arsenic in place of nitrogen), which is lipotropic, also enters the lecithin molecule. The fact that inositol, which possesses lipotropic properties (Gavin & McHenry, 1941), is also a constituent of phospholipids (Anderson, 1930; Folch & Woolley, 1942) is of considerable interest to those who adopt the viewpoint that a lipotropic agent exerts its effect by virtue of promoting phospholipid synthesis. Methionine and betaine probably act by supplying labile methyl groups for choline synthesis.

About 10 years ago, before tracer elements were available, Channon & Smith (1936) first proposed testing the hypothesis that choline exerts its lipotropic action through promoting synthesis of lecithin. They wrote: 'Light should be thrown on this question if a base could be found which possessed an action on liver fat similar to that of choline and

which could be shown to be present in the liver... by isolation of a suitably characteristic derivative. Such a base might be found to have been incorporated in a new phosphatide molecule in which it had replaced the choline of lecithin'. Channon & Smith (1936) described the synthesis of the ethyl homologue of choline (triethyl- β -hydroxyethylammonium hydroxide) and discovered its strong lipotropic activity. The subsequent attempt by Channon, Platt, Loach & Smith (1937) to detect the compound in liver phospholipids, after feeding it to rats for 20 days, was unsuccessful. They utilized the lesser solubility of the chloreaurate of the triethyl homologue in their search for it in the hydrolyzed liver phospholipids. Channon *et al.* (1937) stated at the end of an experimental paragraph, 'The absence of triethyl choline chloroaurate was thus established'. This wording was unfortunate since all that was actually proven was that the compound sought for was either absent or present in amounts too small to be detected by the procedure used. This failure to establish the presence of the triethyl homologue in the liver phospholipids has acted as an impediment to the general acceptance of the hypothesis that the lipotropic action of choline involves reactions which utilize the intact molecule.

The writers of this communication felt, as did Channon *et al.* (1937), that '...it is a particularly attractive hypothesis... that choline should exercise its action by enabling lecithin synthesis to occur' and in view of the work of Welch & Landau (1942) it was considered desirable to reinvestigate the ingenious attempt of Channon and his colleagues to use the triethyl homologue of choline as a tracer substance. The procedure used by Channon *et al.* (1937), which would not disclose the presence of less than 20% of the homologue when mixed with choline, did not seem to us adequate for the task in hand. We were thus led to reinvestigate the problem with new analytical and fractionation procedures.

When it was shown (McArthur, Lang & Lucas, 1945) that the *cholineless* mutant (34486) of the bread mould *Neurospora crassa* was unable to utilize the triethyl homologue of choline (cf. Horowitz, Bonner & Houlahan, 1945), and that there was a significant difference between the enneaiodide assay

for choline (Erickson, Avrin, Teague & Williams, 1940) and the value obtained by microbiological assay (Horowitz & Beadle, 1943), we were encouraged to search for an improved fractionation procedure which would clearly differentiate the trimethyl and triethyl homologues. After many preliminary trials it was found that the tertiary amines obtained by oxidative degradation of the 'choline fraction' (Lintzel & Fomin, 1931; Lintzel & Monasterio, 1931) could be separated out by fractional 'micro-distillation'. The trimethylamine and triethylamine were identified as chloroaurates.

Since the correct chemical name of the compound (triethyl- β -hydroxyethylammonium hydroxide) is a lengthy one and since there are serious objections to the use of the chemically incorrect name 'triethyl choline' we propose, for the sake of brevity, to refer to the free base as TE (i.e. the triethyl homologue) and to its chloride as TE.Cl.

It has been possible to establish unequivocally that the triethyl homologue of choline is incorporated in appreciable amount in the liver phospholipids of rats ingesting this substance (McArthur, 1946). The fact that this unnatural quaternary ammonium base, which possesses lipotropic activity, is utilized in the formation of liver 'lecithin', may be taken as support for the view that the lipotropic action of choline is dependent upon the intact choline molecule and that the natural substance acts in part at least by favouring lecithin synthesis.

sucrose of such a composition that at a food intake of 8 g./day the rats would receive 30 μ g. aneurin hydrochloride, 25 μ g. riboflavin, 20 μ g. pyridoxin hydrochloride, 100 μ g. calcium pantothenate and 100 μ g. nicotinic acid. The diets, freshly prepared about every 5 days, were stored in tightly covered tin canisters in the refrigerator. The rats, housed in individual cages with false bottoms of coarse wire screen, were supplied daily with fresh water (*ad lib.*) and food (8.0 g.). Left-over food averaged 1.7–2.0 g. daily. From the individual food consumption the average daily intake of TE.Cl per rat for each group was calculated to be: 10-day group, 71 mg.; 20-day group, 68 mg.; 30-day group, 69 mg.; 40-day group, 69 mg. Thus the average daily intake of the substance, calculated as the base (69 mg. $\times$ 163.3/181.7 = 62.1 mg.) was nearly six times that of the rats in the experiment of Channon *et al.* (1937) (11.5 mg.). The average loss of weight of rats fed this diet for 10 days was 11.2%, for 20 days 9.2%, for 30 days 14.5% and for 40 days was only 9.1%.

The animals were stunned, the livers were removed, weighed and disintegrated immediately in a Waring blender, under acetone, and extracted repeatedly with boiling absolute ethanol. The combined extracts were taken to dryness *in vacuo*, under N_2 , at a bath temperature below 45°. The oily residue was dehydrated by repeated evaporation *in vacuo* of 50 ml. portions of benzene-absolute ethanol (4:1 v/v) which were added to the flask. The dry lipid material was taken up in light petroleum (b.p. 30–60°) and made up to volume (500 ml.) at about 3–4°. The solutions were kept in a refrigerated room and all samples were removed there. The analytical procedures applied to these lipids have been described recently (Best *et al.* 1946). Certain of the results are presented in Table 1.

Table 1. *Effect of feeding triethyl- β -hydroxyethylammonium chloride*

(20 rats (10 ♂, 10 ♀, average weight 138 g./group) fed test diets for periods shown. Average daily intake of TE.Cl 69 mg. Average weight loss of 9% after 40 days on diet.)

Duration of feeding TE.Cl (days)	No. of survivors	Wet wt. of livers (g.)	Total lipids (g.)	Total lipid P (mg.)	Total* choline (enneaiodide) (m.equiv.)	True* choline (<i>N. crassa</i>) (m.equiv.)	TE*
							(mg.)
10	18	117.5	7.135	153.1	2.45	2.11	55.5
20	20	118.9	11.66	187.5	2.62	2.37	40.9
30	18	112.9	9.38	173.5	2.60	2.08	84.9
40	19	115.6	13.36	154.2	2.54	1.91	103.0

* 'Total choline' was determined by precipitation with $KI-I_2$ and titration of the enneaiodide (i.e. true choline plus any other quaternary bases precipitated by this reagent), and expressed as milliequivalents. True choline, estimated by microbiological assay with the *cholineless* mutant (34486) of *Neurospora crassa*, was similarly expressed. The difference (assumed to be due to TE) was multiplied by the m.equiv. weight of TE (163.3) to give the figures in the last column. The liver fat of animals receiving no TE (i.e. basal group or groups receiving choline supplements) showed no appreciable difference ($\pm 2\%$) between choline assays determined chemically (enneaiodide) and microbiologically.

EXPERIMENTAL

Four groups of rats were fed a diet containing 1.125% of TE.Cl for periods of 10, 20, 30 and 40 days, respectively. Each group consisted of 10 males and 10 females (100–170 g.). The diet had the following composition: beef dripping 25; corn oil (Mazola) 5; casein 8; gelatin 12; salts (McCormick 185) 5; agar 2; sucrose 39.9; 'vitamin powder' 2; cod-liver oil concentrate (Ayerst, McKenna & Harrison) 0.010. The 'vitamin powder' was a mixture in powdered

Isolation of liver phospholipids

The solvent was evaporated from the main portion of the light petroleum solution of the total lipid and the residue was redissolved in a small volume (10 ml.) of ether. To this solution 20 vol. of anhydrous acetone were added. The precipitation of the phospholipids was enhanced by a small addition (1.2 ml.) of saturated ethanolic $MgCl_2$ solution. The mixture, after standing overnight, was centrifuged and the supernatant solution was set aside. The phospholipid,

reprecipitated from ethereal solution with acetone and MgCl_2 , was dissolved in moist ether and a small amount of ether-insoluble matter was removed. Both the ethereal solution of the phospholipids and the pooled acetone mother liquors were analyzed for lipid P and choline. About 3–5% of the P and about 10% of the choline (enneaiodide determination) escaped precipitation.

The remainder of the phospholipid solution was evaporated to dryness and hydrolyzed as described below (p. 615). Choline was determined in the hydrolysate by the enneaiodide procedure and also by microbiological assay. One minor modification was found advisable in the enneaiodide procedure of Erickson *et al.* (1940), viz. to use a more concentrated oxidizing reagent: 1 ml. bromine in 100 ml. glacial acetic acid containing 10% potassium acetate. On known solutions and hydrolysates of normal liver phospholipids the results obtained by the two methods were in excellent agreement.

Preliminary chemical investigation of choline homologues

A search of the literature and some preliminary investigations in this laboratory failed to reveal a suitable method of separating choline from its mono-, di- or triethyl homologues. Finally, resort was made to permanganate oxidation of these compounds to form tertiary amines which were found to be separable by distillation and then identifiable as their chloroaurates. All boiling-points and melting-points reported in this paper have been corrected.

Source of compounds required

(a) Trimethylamine hydrochloride (Hoffmann-La Roche) was used without further purification.

(b) Triethylamine (Eastman Kodak) was dried over solid KOH and fractionally distilled to yield a product boiling at 88–89°.

(c) Dimethylethylamine and methyldiethylamine were prepared from ethylamine and diethylamine (Eastman Kodak), respectively, by methylation with paraformaldehyde and formic acid according to the procedure of Clarke, Gillespie & Weisshaus (1933) (cf. Sommelet & Ferrand, 1924). The products thus obtained were dried over solid KOH and fractionated in a Widmer column. The dimethylethylamine (b.p. 36–37°) yielded a chloroaurate m.p. 212°. The methyldiethylamine (b.p. 65.5°) formed a chloroaurate melting at 151–152°.

(d) Ethylene chlorohydrin (British Drug Houses) was fractionally distilled to give a product boiling at 127–128°.

(e) Triethyl- β -hydroxyethylammonium chloride was prepared by heating equimolecular proportions of ethylene chlorohydrin and triethylamine in sealed tubes at 120° for 24 hr. The reaction mixture was dissolved in warm absolute ethanol (0.5 vol.) and the crystalline quaternary ammonium salt was precipitated by the addition of 1 vol. of acetone and 5 vol. of dry ether. After several recrystallizations from mixtures of absolute ethanol, acetone and ether a small portion of the product was converted to the chloroaurate, which had a satisfactory m.p. (230°).

(f) The other homologues of choline were prepared from the corresponding tertiary amines in a similar manner.

Oxidative degradation of choline homologues

Preliminary quantitative experiments, one of which is reported here, on the alkaline permanganate oxidation of triethyl- β -hydroxyethylammonium hydroxide and collection of the resulting triethylamine showed that the procedure is applicable to TE.

To 50 ml. of an aqueous solution containing 62.08 mg. TE.Cl in a 250 ml. Lintzel flask, 20 g. of solid NaOH were added. A slow stream of air, washed by dilute H_2SO_4 , was drawn through the apparatus into a tube containing 10.26 ml. of 0.100N- H_2SO_4 and two drops of 0.02% methyl red in 60% ethanol. The strongly alkaline solution was heated to boiling and aqueous KMnO_4 (0.5%) was added carefully from the thistle-tube at such a rate that no green colour, due to excess manganate, persisted in the reaction mixture until the oxidation had been completed. The heating and aeration were continued for a further 45 min. At the end of this period the flask and its contents were allowed to cool while being very slowly aerated. The contents of the receiving tube required 6.84 ml. 0.100N-NaOH for back titration. The recovery of volatile base, subsequently shown to be triethylamine, was 100.1% of theory.

Since it had been established that the trimethyl and triethyl compounds behave identically as far as the oxidative degradation is concerned, it was assumed that the mono- and diethyl homologues would behave similarly.

Separation of tertiary amines by 'distillation'

In view of the great difference in the boiling-points of trimethylamine and triethylamine, 3.5° and 89°, respectively, it seemed feasible to separate small amounts of these by fractional distillation. Several small stills of various designs were made and tested. The most suitable of these had the form and specifications shown in Fig. 1.

The amine or mixture of amines, converted to the hydrogen sulphates or hydrochlorides, is transferred to the distilling flask (A) in methanolic solution and the solvent is driven off by warming the flask while a slow stream of nitrogen is passed in through tube (E). The receiver (C) is charged with 10 ml. of neutral methanol containing two drops of 0.02% methyl red indicator. The distilling flask (A), the U-tube (B) and the delivery tube (H) are joined through the ground-glass joints (F) and (G). The U-tube is cooled in a bath of ethanol and solid carbon dioxide and a boiling water-bath is placed around (A) so that not only the flask but also the side arm is kept at 100°. Through the stopcock (K), 2 ml. of aqueous NaOH (saturated at 35°) are run into (A) from the cup (D). The stopcock is now turned to close the opening from (D) and to connect (E) with (A) so that a slow stream of nitrogen may be passed through (A) to carry the amine into the U-tube condenser. After sweeping for 10 min. the boiling water-bath is removed from (A) and the ethanol-carbon dioxide bath around (B) is replaced by a cold water-bath which is maintained at a uniform temperature of 4° by mechanical stirring and occasional

additions of small pieces of ice. As the flask (A) cools, it is necessary to allow nitrogen to flow through the stopcock to prevent the methanol from being drawn over into (B). When (A) has reached room temperature, (E) is connected to a gasometer which is arranged to deliver a slow, constant flow of nitrogen (about 3 ml./min.). Sufficient standard acid is added from the burette (I) to keep the solution in (C) distinctly acidic and (K) is turned to admit nitrogen from the gasometer. When the portion of acid added initially to (C) has been neutralized, further known increments of the standard acid are added from the burette and the time intervals required for the neutralization of these by the amine carried over are recorded.

Fig. 1. Diagram of apparatus used for the separation of the tertiary amines.

Typical curves obtained with the four possible tertiary amines, viz. trimethylamine, dimethylethylamine, methyldiethylamine and triethylamine, and a known mixture of the first compound with the last, are shown in Fig. 2. Curve 1 shows that trimethylamine (80.0 mg.) 'distills' at 4° with great rapidity, the bulk of it going over in about 3 min. The relatively slow evolution of triethylamine (20.0 mg.) is apparent in curve 5, while curves 3 and 4 indicate the rates of evaporation of dimethylethylamine (101.5 mg.) and methyldiethylamine (118.4 mg.) respectively, under the same conditions. If the rate of sweeping is kept constant from one experiment to another, the amount of triethylamine present does not affect the rate of evolution, as is shown in curve 6, which represents the rate of evolution of 40 and 80 mg. of triethylamine with slower aeration (1.8 ml. N_2 /min.) than that used in obtaining curve 5. Curve 2 represents the behaviour of a mixture of 80 mg. of trimethylamine and 20 mg. of triethylamine under the standard conditions (3.1 ml. N_2 /min.) used to obtain curves 1 and 5 and in all subsequent work. Since the slope of the curve for

evaporation of triethylamine is constant with a constant sweeping rate, projection of the upper linear part of curve 2 should cut the vertical axis at a point representing the volume of standard acid required to neutralize the trimethylamine. The difference between this value and that of the total titration should, therefore, represent the amount of acid consumed by triethylamine. The agreement between theory and practice is satisfactory (see also Fig. 4)

Fig. 2. Time-distillation curves for known substances at 4°. Sweeping rate 3.1 ml./min. for curves 1-5, 1.8 ml./min. for curve 6. Curve 1, 80 mg. trimethylamine; curve 2, a mixture of 80 mg. of trimethylamine and 20 mg. triethylamine; curve 3, 101.5 mg. dimethylethylamine; curve 4, 118.4 mg. methyldiethylamine; curve 5, 20 mg. triethylamine; curve 6, $\triangle$ — $\triangle$ 40 mg. triethylamine, $\blacktriangle$ — $\blacktriangle$ 80 mg. triethylamine. Lines A, B and C cut the vertical axis at points representing the calculated titration values for the mixture of 80 mg. trimethyl- and 20 mg. of triethylamine, 80 mg. of trimethylamine alone and 20 mg. triethylamine alone, respectively.

Examination of the rat liver phospholipids

Hydrolysis. The phospholipid fractions from each of the four groups of animals were treated separately. Each was dissolved in 10 ml. of warm ethanol and emulsified by forcing the solution in a thin stream into 100 ml. of 5% (v/v) aqueous H_2SO_4 . The mixture was refluxed for 8 hr. After removal of the fatty acids by shaking several times with ether and precipitation of the bulk of the sulphuric acid with baryta the hydrolysate was neutralized to phenol red by the addition of 0.1N-NaOH and made up to a volume of 500 ml.

Choline determinations in the hydrolysates. Portions of each of the four hydrolysates obtained from the acetone-insoluble phospholipids were analyzed in replicate for choline by the chemical (enneiodide) and microbiological (*Neurospora crassa*, cholineless) methods. The results are tabulated in Table 2.

Table 2. *Choline content of hydrolysates of isolated liver phospholipids*

(Results expressed as mg./500 ml. of hydrolysate.)

Days of feeding	Chemical procedure (calculated as choline)	Microbiological procedure (calculated as choline)	Difference (calculated as TE)	TE (as % 'total choline')
10	249	216	44.4	17.1
20	263	242	28.3	10.5
30	246	202	59.3	22.7
40	254	194	80.8	29.4

Since the chemical procedure estimates total quaternary ammonium base while the microbiological method responds only to choline (and to a very much smaller extent to the methyldiethyl and dimethylethyl homologues of choline), any difference in assay was tentatively attributed to the presence of the triethyl homologue.

Oxidative degradation of phospholipid hydrolysates

40-day group. The portion (350 ml.) of the hydrolysate which remained after removal of samples for analytical purposes was oxidized directly with alkaline permanganate in the Lintzel apparatus as described above except that just sufficient standard H_2SO_4 was added to the receiver during the oxidation to keep the distillate acidic to methyl red. Because of the limited capacity of the reaction flask, the operation was performed on three separate portions. To the combined distillates containing the tertiary amines another volume of standard H_2SO_4 , equal to that required for neutralization, was added and the acidic solution was set aside for further investigation.

30-day group. Because oxidation of the unfractionated hydrolysate (above) gave rise to ammonia (from hydrolytic products of cephalins) as well as tertiary amines and further because the volume of permanganate solution required was so great, it was deemed advisable to isolate the quaternary bases for oxidation. The remainder of this hydrolysate (470 ml.), made alkaline to phenol red, was concentrated *in vacuo* to about 140 ml. (bath temperature 60–70°). No volatile base was detectable in the distillate proving that the isolated phospholipid did not contain any free tertiary amine. From the concentrated hydrolysate, again made slightly acidic to phenol red, the quaternary ammonium bases were precipitated in centrifuge pots at 0° as enneaiodides. After centrifuging, the precipitate was washed once in the pots with cold potassium triiodide reagent diluted to the same concentration as was used for precipitation. The precipitate was transferred to a distillation flask with 100 ml. of water and 5 ml. $\text{N-H}_2\text{SO}_4$. The elemental iodine was removed by distillation under reduced pressure. Iodic iodine was precipitated with an excess of Ag_2SO_4 and the excess silver ions were removed by H_2S . The filtrate from the silver sulphide, concentrated to a volume of 30 ml., was oxidized in the Lintzel apparatus. After the addition of a second equivalent of standard H_2SO_4 to the distillate, it was set aside.

Combined 10- and 20-day groups. The remaining portions of the phospholipid hydrolysates from the 10- and 20-day groups of animals, each 450 ml. in volume, were combined and concentrated to a volume of 200 ml. The quaternary ammonium bases were isolated as described above, except

that the enneaiodide was washed twice by dispersing the precipitate in diluted potassium triiodide reagent. The isolated base in aqueous solution was made up to a volume of 100 ml. and 98 ml. of this was converted to tertiary amines.

Separation of the tertiary amines

40-day group. The aqueous solution obtained by titration of the volatile base formed by oxidation of the hydrolyzed phospholipids was concentrated *in vacuo*. The residue was dehydrated by repeated distillation of 5 ml. portions of absolute ethanol which were added to the distillation flask. Two 5 ml. portions of absolute ethanol were used to extract the amine salts from the contaminating $(\text{NH}_4)_2\text{SO}_4$. The ethanolic extracts were taken to dryness in the micro-distillation flask.

The amines, liberated and 'redistilled' as previously described to obtain the time-'distillation' curve, required a total of 12.95 ml. of 0.1006 $\text{N-H}_2\text{SO}_4$. The slope of the upper linear part of the curve indicated the presence of triethylamine. The acid required by this compound corresponded to 29.0 mg. of the base. (This value agrees moderately well with that calculated from the difference between the two choline analyses, 26.6 mg.) In order to obtain a sample of this material contaminated with as little trimethylamine as possible, the contents of the receiver were fractionally 'distilled' under the same conditions. The point at which to change the receiver was determined by inspection of the time-'distillation' curve. The cut was not made until the slow constant evolution of the higher boiling amine was evident. Momentarily, the flow of nitrogen was stopped while the receiver was replaced by another containing 15 ml. of 0.1 N-methanolic HCl . Hot water-baths were placed around the distillation flask and the U-tube and sweeping was resumed at a gradually increasing rate (4–8 ml./min.). Complete transfer required about 2 hr.

The mean molecular weight of the amine mixture was determined upon the crystalline residue obtained by evaporating the acidic absorbing liquid to dryness. The residue, washed with absolute ether and desiccated (21.5 mg.) was returned to the micro-still. The volatile amines required 16.00 ml. of 0.0100 $\text{N-H}_2\text{SO}_4$ (methyl red indicator). The mean molecular weight (134.4) suggested that the mixture consisted of 92.4% triethylammonium chloride and 7.6% trimethylammonium chloride.

The crude triethylamine, 'redistilled' into an excess of HCl to free it from indicator, was converted to the chloroaurate in 0.5 ml. of N-HCl . The precipitate, filtered and washed twice with 0.12 ml. portions of cold dilute HCl , when dried *in vacuo* over P_2O_5 weighed 16.8 mg., m.p. 86–87°. Analysis: C, 16.5; H, 3.68; Au, 44.2%. The com-

positions and m.p. of the possible trialkylammonium chloroaurates are tabulated below:

	m.p.	C (%)	H (%)	Au (%)
$(C_2H_5)_3N \cdot HCl \cdot AuCl_3$	89°	16.35	3.66	44.7
$(C_2H_5)_2CH_2N \cdot HCl \cdot AuCl_3$	151–152°	14.06	3.30	46.2
$C_2H_5(CH_2)_3N \cdot HCl \cdot AuCl_3$	212°	11.63	2.93	47.7
$(CH_3)_3N \cdot HCl \cdot AuCl_3$	234–235°	9.03	2.53	49.4

30-day group. The acidified distillate obtained by Lintzel oxidation of the isolated choline was evaporated to dryness and the residue was transferred to the still. The time-‘distillation’ curve shown in Fig. 3 indicated the presence of

Fig. 3. Time-‘distillation’ curve for tertiary amine mixture derived from liver phospholipids of 30-day group. Aeration rate 3.1 ml./min. The line *A* cuts the vertical axis at a point representing the total titration.

triethylamine. From the amine mixture, fractionated as described in the previous section, the higher boiling fraction was separated. It was converted to the chloroaurate (81 mg.) m.p. 87°. Mixed with triethylammonium chloroaurate, m.p. 89°.

Combined 10- and 20-day groups. All operations upon the combined material from these two groups were conducted with quantitative care in order to compare the recoveries obtained by oxidative degradation and fractional ‘distillation’ with the results of the choline analyses on the bases isolated as enneiodides. The tertiary ammonium salts were transferred to the micro-distillation apparatus. Since the amount of trimethylamine derivable from the choline in the mixture was large (30.2 ml. 0.1006N-acid, calculated from the results of the microbiological choline assay), the U-tube was kept at a temperature of 4° for 1 hr. without aeration to avoid too rapid transfer and possible incomplete absorption of trimethylamine. During this time amine equivalent to 13.35 ml. of the standard acid was collected in the receiver, which then was replaced by a second receiver. ‘Distillation’ and titration of the remaining part of the amine mixture were carried out in the manner previously described. Fig. 4 shows the ‘distillation’ curve obtained. The total titration amounted to 32.85 ml. of the standard acid (calculated requirement from the

results of the chemical analyses, 32.30 ml.). The curve (Fig. 4) indicates that the mixture consisted of 3.05 m.equiv. of trimethylamine and 0.25 m.equiv. of triethylamine; by calculation (from the results of the microbiological and chemical choline assays), the values were 3.03 m.equiv. and 0.19 m.equiv. respectively.

Fig. 4. Time-‘distillation’ curve of tertiary amines derived from liver phospholipids (10- and 20-day groups). The choline fractions from the two groups were combined for oxidative degradation and fractionation of the tertiary amines. Intercept on the vertical axis of line *A* represents the total titration of the amine mixture, of line *B* (a projection of the upper linear portion of the curve) represents the titration value for trimethylamine; the difference represents the titration value for triethylamine.

After drying, the residue from the second receiver was transferred to the micro-apparatus for fractionation. The ‘distillation’ was carried out with sweeping until 17.0 ml. of 0.1006N-acid had been consumed. At this point the receiver was exchanged for one containing 20 ml. of methanol and 0.2 ml. of conc. HCl, into which the remainder of the amine was swept. The residue remaining after the removal of the methanol was converted to the chloroaurate in dilute HCl (1.9 ml.). The yellow crystalline precipitate, washed with three 1 ml. portions of cold dilute HCl and dried (33.4 mg.) melted at 89°, mixed with methyl-diethyl-ammonium chloroaurate, m.p. 124°; mixed with authentic triethylammonium chloroaurate, m.p. 89°. A further quantity (23.5 mg., m.p. 87–89°) was obtained by concentration of the mother liquors.

DISCUSSION

Although the lipotropic effect of choline is well established and generally accepted, its mode of action has not yet been completely elucidated. Some

authorities believe that the lipotropic effect may be explained by the observation that choline accelerates the turnover of the phosphorus in liver phospholipids. The assumption has been made that choline similarly promotes an accelerated turnover of all constituents of the phospholipid molecule. Experimental evidence for any increased rate of turnover of fatty acids in liver phospholipids under the influence of dietary choline, is not yet available, however, and hence a completely satisfactory explanation of lipotropic action must await further investigation.

There seems to be some confusion in the minds of certain recent writers on the subject of lipotropic action as to whether the labile methyl groups or choline *per se* is the ultimate lipotropic factor. The evidence is accumulating that the intact choline molecule is the effective lipotropic substance. Compounds such as betaine and methionine appear to exert their lipotropic effect by contributing labile methyl groups either for the synthesis or sparing of choline. The experiments of Welch & Landau (1942) have established that arsenocholine, which is lipotropic and does not possess labile methyl groups, when fed, is incorporated *in toto* into the phospholipid molecule. In the case of this choline analogue, it is the intact molecule and not a labile methyl group which exerts the biological effect.

The present experiments with the triethyl homologue of choline, which also is lipotropic and lacks labile methyl groups, supply another example of a lipotropically active substance which is utilized intact in the synthesis of new phospholipid molecules. The arguments advanced by Welch & Landau (1942) in support of the hypothesis that the intact choline molecule is the active lipotropic substance are substantiated by the findings reported above.

Several explanations may be offered for the failure of Channon *et al.* (1937) to detect the triethyl homologue of choline in the liver phospholipids of their experimental animals. The basal diet fed by

them was so low in protein (5% casein) that it probably did not permit metabolic processes to function normally. At this low protein intake, the quaternary ammonium chloride may have exerted a more toxic action than it did in the present experiments. Whatever the explanation, it has been possible to feed six times the daily dose used by them, and to give it for twice as long a period of time, without any greater weight loss or mortality than they observed. Furthermore, improvements in micro-analytical methods during the past 10 years have probably contributed to the present successful accomplishment of the project initiated by Channon and his colleagues. The establishment of the fact that the lipotropically active triethyl homologue of choline is biologically incorporated into the molecule of liver phospholipids, removes one of the impediments to general acceptance of the hypothesis that the lipotropic action of choline involves reactions which utilize the intact molecule.

SUMMARY

1. A significant difference between the micro-chemical and microbiological assays for choline in the hydrolysates of the phospholipids isolated from the livers of rats fed triethyl- β -hydroxyethyl-ammonium chloride, suggested that appreciable amounts of an unnatural base had been incorporated into the liver phospholipids. Fractionation procedures were devised which led to chemical proof that the triethyl homologue of choline had been incorporated into the phospholipids.

2. This finding provides further proof that the lipotropic effect of choline is associated with the intact molecule rather than its methyl groups.

We are indebted to Dr J. H. Ridout for the extraction of the liver lipids and some of the preliminary chemical analyses and to Miss J. M. Lang who performed the microbiological assays. The expenses of this investigation were defrayed, in part, by a grant from the Banting Research Foundation.

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