CCXLV. A WATER-SOLUBLE PRECURSOR OF CHOLINE FOUND IN THE KIDNEY AND OTHER TISSUES.

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(Received July 25th, 1935.)

In a recent publication [Booth and Milroy, 1935] brief reference was made to an extremely water-soluble ester of choline which contains in addition phosphoric acid and a compound with many of the characteristics of sphingosine but no fatty acid. It is found in the kidney, brain and in other tissues.

In this paper the method of isolation and the chemical nature of the body as found in the kidney are given in detail, then reference is made to its presence in the brain and liver, and lastly the biological properties are briefly discussed.

Method of isolation.

The fresh finely minced tissue is extracted with boiling water for five minutes. The mixture is then made acid to litmus with glacial acetic acid and boiled for three minutes. The warm extract after filtration and cooling to room temperature is precipitated completely with basic lead acetate and rendered alkaline to litmus with ammonia. The lead precipitate is allowed to settle for two hours and removed. The filtrate, after removal of the lead, is evaporated to dryness under reduced pressure at 40°. A little quartz sand is added and the residue is thoroughly extracted with 3-4 changes of hot absolute alcohol. The precipitate which forms after mixture of the alcoholic extracts is removed and the filtrate is evaporated to dryness at a low temperature under reduced pressure. The dry residue is frequently extracted with alcohol. The combined second alcoholic extracts sometimes give a precipitate which is discarded. The final clear alcoholic extract is precipitated with a saturated solution of HgCl_2 in absolute alcohol. The precipitate is separated, washed with absolute alcohol and extracted twice with cold water (6°). After removal of the mercury as sulphide, the extract is neutralised to litmus and concentrated under reduced pressure at 40°. The residue is exhaustively extracted with absolute alcohol containing 3-4 % HCl . Sufficient alcoholic mercuric chloride for complete precipitation is added to the clear alcoholic extract. Usually only a cloud appears. A saturated solution of fused sodium acetate in alcohol is now added until the supernatant fluid remains clear. The precipitate is separated and washed with absolute alcohol.

As a rule the second HgCl_2 precipitate is completely soluble in cold water and gives on analysis 2N:1P. If this ratio is still too high another precipitation with HgCl_2 is necessary. The cold-water extract of the second HgCl_2 precipitate contains about 50 % of the organic P originally present in the lead filtrate. It is advisable to estimate the amount of phosphorus at every stage. Large losses are usually due to faulty alcohol extraction. If the alcohol is definitely acid in reaction and the mixture thoroughly shaken with quartz sand, the extraction is much more complete. There is no evidence of hydrolysis of the substance by the acid alcohol if the extract is precipitated immediately and the mercury precipitate then separated and washed with alcohol.

After removal of the mercury from the cold-water extract of the final HgCl_2 precipitate the solution is evaporated to small bulk. The excess chloride in the concentrated solution is removed by silver carbonate and the dissolved silver by careful addition of hydrochloric acid.

Two experiments are quoted, the first in order to illustrate the method of separation and to furnish analyses of the final purified body and the second in order to study the products of acid hydrolysis. In all cases the total N was estimated by the micro-Kjeldahl method, the amino-N by the method of Van Slyke and the P by the method of Fiske and Subbarow [1925].

I. Horse kidney (2.07 kg.).

1. The original boiled extract contained, per kg. fresh tissue, 246 mg. ortho-P and 222 mg. organic P.

2. The basic lead filtrate contained 101 mg. organic P. per kg.

3. A portion of the alcoholic extract of the concentrated lead filtrate, containing 130 mg. organic P with a ratio of 30.8N : 1P, was taken for precipitation with HgCl_2 .

4. The cold-water extract of the first HgCl_2 precipitate contained 118 mg. organic P and had a ratio of 8.1N : 1P.

5. The similar extract of the second precipitate contained 84.5 mg. organic P (2.8N : 1P).

6. A portion of the extract of the third precipitate, which gave a 2N : 1P ratio, was freed from excess chloride and dried to constant weight. On analysis the specimen (after deduction of the traces of chloride not removed) gave the following results:

	Found %	Calc. for $\text{C}_{23}\text{H}_{51}\text{O}_6\text{N}_2\text{P}$ %
Organic P	6.41	6.43
Total N	5.88	5.80
Amino-N	2.98	2.90

II. Ox kidney (1.61 kg.).

1. The basic lead filtrate contained 137.5 mg. organic P per kg.

2. The extract of the first HgCl_2 precipitate contained 90.6 mg. organic P (4.17N : 1P).

3. The extract of the second precipitate gave on analysis: 65.8 mg. organic P and 58.0 mg. total N (1.95N : 1P). Calculating the choline value from half the nitrogen, the amount of choline chloride in the bound form should be 288 mg. After acid hydrolysis of this extract 835 mg. chloroaurate (M.P. 233° , Au 44.44 %) were obtained. This corresponds to 263 mg. choline chloride. The chloroaurate was free from amino-N and P, and on testing biologically was found both qualitatively and quantitatively to be identical with the salt of choline.

Chemical nature.

The large amount of the acid-soluble organic phosphorus of the kidney which escapes basic lead acetate precipitation is evident from Tables I and II.

Table I.

Exp. no.	Source and moist weight of kidney tissue g.	Boiling water extract		Basic lead filtrate, mg. organic P per kg.	% organic P escaping precipitation
		mg. ortho-P per kg.	mg. organic P per kg.		
1	1000 horse	225	191	114	59.7
2	2070 horse	246	222	101	45.5
3	825 ox	340	240	127	53.0
4	1900 ox	313	243	143	58.8

On an average 54.2 % of the acid-soluble organic P of the kidney escapes basic lead precipitation.

Table II gives the absolute yields of organic P which escape basic lead precipitation.

Table II.

Exp. no.	Moist weight and source of kidney tissue (g.)	Basic lead filtrate mg. organic P per kg.
1	1500 horse	85.4
2	1250 "	90.6
3	2425 "	102.5
4	1260 "	93.0
5	2250 "	109.5
6	1280 "	100.8
7	1330 "	90.2
8	430 ox	155.6
9	650 "	132.9
10	530 "	128.3

On an average 108.8 mg. organic P per kg. escape precipitation by basic lead acetate.

The P-containing substance present in the lead filtrate of the kidney is extremely soluble in water. It can be extracted almost completely from dry residues of the lead-free filtrate by hot absolute alcohol. After precipitation with mercuric chloride, the substance is only slightly soluble in neutral alcohol but is readily dissolved by alcohol containing 3-4 % HCl. It is precipitated from acid alcohol by fused sodium acetate along with much nitrogenous impurity. It is insoluble in acetone, ether and chloroform. It can be precipitated from aqueous and alcoholic solution by a large excess of acetone but incompletely and along with nitrogenous impurity.

The Ba, Ca, Ag, Pb, Cu and Hg salts are all soluble in water. The double salt with gold and the periodide compound are also soluble in water. 25 % phosphotungstic acid in 5 % H_2SO_4 precipitates the P-containing substance incompletely and this method of separation is very unsatisfactory. The Ag and Ba salts are soluble in alcohol, but after baryta hydrolysis the Ba salt seems to lose its solubility. The double salts with cadmium chloride and mercuric chloride are both insoluble in alcohol.

The purified substance is yellow and resinous and on analysis shows approximately the composition of a compound ester of choline and sphingosine with phosphoric acid. The optical activity is apparently low as no rotation could be detected in a solution of the pure substance containing 4 mg. organic P per 1 ml. (=6.22 % choline-sphingosinephosphoric acid), when examined in a 1 dm. tube.

In Fig. 1 the hydrolysis curves of the P-containing substance found in the lead filtrate of the kidney and liver are compared with that of calcium glycerophosphate (Merck's neutral). Hydrolysis was carried out with 1.53N H_2SO_4 at 100°. In all three cases the phosphoric acid is firmly bound.

Half the nitrogen of the pure substance is present in the form of choline. All the choline is set free by 20 % HCl at 100° in 8 hours. The chloroaurate obtained from the HCl hydrolysate melts at 233° (sharp), instead of 252°, but is otherwise identical with that of choline. The amount of choline obtainable from the substance can only be given approximately, but from 250 to 300 mg. choline chloride per kg. fresh kidney have been obtained by acid hydrolysis of the purified substance.

The N and P contents of this ester and the fact that half the nitrogen is in the amino-form suggest strongly the presence of sphingosine, although it has not been satisfactorily isolated. There is no fatty acid or reducing carbohydrate among the products of hydrolysis.

Fig. 1. A, $\circ$ — $\circ$ lead filtrate (kidney); B, $\times$ — $\times$ lead filtrate (liver); C, $\square$ — $\square$ calcium glycerophosphate.

All these facts agree with the suggestion that the substance is the choline ester of sphingosinephosphoric acid. The question as to whether all the organic P present in the basic lead filtrate is of the same type is still uncertain. Naturally traces of the well-known phosphoric acid esters will escape lead precipitation. About 50 % of the organic phosphorus originally present in the lead filtrate can be obtained in the pure ester form. How much of the remaining 50 % is in the same form it is impossible to say.

Strack *et al.* [1933; 1934] describe a similar substance which they found in the liver and placenta and from which choline is readily set free in acid solution. They regard the substance from the liver as a water-soluble sphingomyelin. They state that the quantity available did not allow them to identify the fatty acids but they conclude that the contents of P, total N, amino-N and choline-N correspond to a sphingomyelin of mol. wt. 800 [Thierfelder and Klenk, 1930]. They refer to half the nitrogen being in the amino-form (Van Slyke determination), although no such nitrogen is present when the sphingosine is linked to the fatty acid as in the ceramides, sphingomyelins and cerebrosides. They also state that it is possibly the same body as that described by Fränkel *et al.* [1933], although the sphingomyelin obtained from the liver by the latter authors was only slightly soluble in water, forming a colloidal solution.

Karrer and Salomon [1926] have obtained by partial baryta hydrolysis of lecithin a choline ester of glycerophosphoric acid. Unlike that of glycerophosphate the Ba salt of this ester is fairly soluble in alcohol.

Presence in the brain and liver.

The points on which the identity of the ester can be established with reasonable certainty are as follows:

- (1) It contains organic P in a form not precipitable by basic lead acetate and is soluble in water and alcohol.
- (2) The double salt with mercuric chloride is insoluble in alcohol but is extremely soluble in cold water.

(3) It is not precipitated by 50 % gold chloride.

(4) It shows a ratio of 2N : 1 P, half of the nitrogen being in the amino-form, and yields choline on hydrolysis.

Brain. In the following two experiments the brain tissue was worked up in the way described above. Unfortunately the amount of material available did not allow of complete purification, and so in the first experiment the second mercuric chloride precipitate was examined for bound choline by acid hydrolysis and precipitation with 50 % gold chloride and in the second by similar hydrolysis and precipitation with Florence's reagent. The values given are calculated for 1 kg. moist tissue.

I. Sheep brain (290 g.).

1. The boiling-water extract contained 247 mg. organic P.

2. The lead filtrate contained 77 mg. organic P.

3. The extract of the first HgCl_2 precipitate gave on analysis 49.2 mg. organic P (7.42N : 1P).

4. The extract of the second precipitate contained 36.3 mg. organic P (3.6N : 1P). After removal of the Hg and neutralisation to litmus, the extract was concentrated to 2 ml. and acidified (20 % HCl). A small sample was tested immediately with gold chloride. There was no precipitate. After hydrolysis for 9 hours at 100°, 486 mg. chloroaurate (m.p. 252°, Au 44.46 %) were obtained. This corresponds to 153 mg. choline chloride. A portion of the chloroaurate after removal of the gold was acetylated and tested biologically. It was found to have the same action as acetylcholine. The chloroaurate is therefore identical with that of choline. Assuming a ratio of 1P : 1 choline in the second sublimate precipitate, it follows that 93.6 % of the bound choline can be recovered as the chloroaurate after acid hydrolysis.

II. Horse brain (580 g.).

1. The lead filtrate contained 51 mg. organic P.

2. The cold water extract of the first HgCl_2 precipitate contained 35 mg. organic P.

3. The extract of the second precipitate gave on analysis 25 mg. organic P (4.3N : 1P). The extract gave no crystals of choline periodide but after hydrolysis these could be easily demonstrated.

There is therefore present in the brain a water-soluble P-containing substance which yields choline on hydrolysis. From the partly purified body 153 mg. choline chloride per kg. have been obtained by acid hydrolysis. The ester is probably identical with that found in the kidney. A third precipitation with HgCl_2 would almost certainly reduce the N : P ratio to 2 : 1.

Liver. There is definite evidence of the choline ester in the liver. In Fig. 1 the hydrolysis curve of the P-containing substance in the lead filtrate is compared with that of the similar substance in the kidney. The curves stand in good agreement. In one experiment a 2N : 1P ratio was reached in the second mercuric chloride precipitate and half the nitrogen was accounted for by choline, using Staněk's method of estimation [1905; 1906, 1, 2].

Biological properties.

The biological study has been confined to the action of the ester, before and after acetylation, and of the acetylated base derived from this ester by acid hydrolysis, gold precipitation and acetylation, on the usual choline test objects. The strengths of solutions used are given in terms of nitrogen values.

The mother substance when freshly prepared has no action on the eserinated frog's rectus. Sometimes it shows a slight activity in strong concentrations due

to the presence of free choline. The acetylated mother substance (Fig. 2) is qualitatively identical with acetylcholine in its action. Acetylcholine has been demonstrated in the reaction mixture by the periodide reagent and the degree of activity depends on the amount of decomposition during acetylation and the quantity of acetylcholine thus formed. The acetylated base (Fig. 3) behaves qualitatively and quantitatively exactly like acetylcholine. The action is prevented in all cases by atropine.

Fig. 2.

Fig. 2. Rabbit's intestine. *M*, acetylated mother substance, half N-value as acetylcholine = 1/1,748,000. *M*₁, same as *M* after atropine.

Fig. 3.

Fig. 3. Eserinised rectus of frog. 1. Acetylcholine chloride, 1/500,000. 2. Acetylated base from mother substance (brain) N-value = acetylcholine 1/450,000. 3. 2 after atropine.

SUMMARY.

1. An extremely water-soluble substance with chemical properties which indicate that it is the choline ester of sphingosinephosphoric acid has been isolated. Over 260 mg. choline chloride per kg. fresh kidney may be obtained by acid hydrolysis of the pure substance and over 150 mg. choline chloride per kg. fresh brain by acid hydrolysis of the partly purified substance.

2. The method of isolation depends on the precipitability of the ester from alcoholic solution as the double salt of HgCl_2 and the solubility of this double salt in cold water (6°). Purification is effected by repeated precipitation with HgCl_2 .

3. The general characters of the water-soluble ester of choline found in the brain and liver indicate that it belongs to the same class as the one met with in the kidney.

4. The ester has no action on the eserinised rectus of the frog. During acetylation it is decomposed and the acetylated derivative has all the chemical and biological properties of acetylcholine.

The author wishes to acknowledge that throughout the whole of this work he has had the invaluable guidance and co-operation of Prof. T. H. Milroy.

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