CCXLIV. A WATER-SOLUBLE PRECURSOR OF CHOLINE IN THE HUMAN PLACENTA.

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THE presence in the tissues of free choline and its water-soluble precursors has recently attracted attention. Strack *et al.* [1934] have studied the occurrence of choline in the human placenta. They found that aqueous extracts of the placenta contain free choline in amounts which vary according to the length of time during which autolysis is allowed to take place in the uterus before fixation of the tissue in boiling water. After precipitation of the choline in the extracts as a chloroaurate, there remains in solution a choline-containing substance which contains choline and phosphorus in a 1 : 1 ratio. The suggestion is made that this substance may be a sphingomyelin corresponding to a similar substance found in the liver by the same workers [1933]. Booth and Milroy [1935] have isolated from the kidney and more recently from the liver and brain a substance which seems to correspond to that of Strack *et al.* It contains nitrogen and phosphorus in the atomic ratios:

$$\text{Total N : Amino-N : P} = 2 : 1 : 1.$$

It yields choline on hydrolysis with strong acid.

The problem investigated here is whether the method described by Booth and Milroy can be used for the isolation of a similar substance from the placenta, and also to compare the results obtained for the placenta with those arrived at in the case of the kidney. It will be shown by using this method that there is obtainable from the human placenta a compound such as Booth and Milroy have described in the kidney. This would seem also to correspond to the substance found by Strack *et al.*

Experimental procedure.

For the qualitative detection of free choline the microcrystalline test of Florence was used. A drop of the solution to be tested is mixed with three drops of Florence's periodide solution and examined microscopically for choline periodide crystals. Precipitation with 50% gold chloride was also used. The quantitative assay of choline was done by means of the periodide method of Staněk [1905]. The solution after precipitation with Staněk's periodide solution is passed through a Jena filter. The precipitate is dissolved in aqueous NaOH, and the amount of choline determined by estimation of the nitrogen. Biological estimations of choline were also made after acetylation using as test objects the frog's heart, frog's rectus and mammalian intestine.

The method for isolating the substance containing the bound choline of the placenta is as follows. The placenta is minced in boiling water and the filtered extract precipitated with basic lead acetate. After removal of the lead the filtrate is concentrated to dryness at 37° and extracted with alcohol. The alcoholic extract is precipitated with mercuric chloride, the precipitate extracted with cold water and after removal of the mercury concentrated to dryness,

again extracted with alcohol and re-precipitated with mercuric chloride. A cold-water extract of this contains the bound choline and this can be further purified by a third precipitation with mercuric chloride.

Results.

The isolation of the choline-containing substance can be followed by estimations of the phosphorus and nitrogen at each stage of the procedure. In the case of the kidney, Booth and Milroy found that the basic lead filtrate contained about half the organic phosphorus of the kidney and they showed that this was firmly bound with choline. The phosphorus amounted approximately to 110 mg. per kg. of fresh kidney. In the case of the placenta the amount of phosphorus escaping precipitation with basic lead acetate is very much less and only amounts to 10–20 mg. in each placenta (500 g. of fresh tissue), so that it is evident that such a phosphorus-choline-containing compound as exists in the kidney can only be present in the placenta in very small amounts. If, however, the basic lead filtrate from the kidney be tested with Florence's reagent, it is found to contain no free choline, whilst in the case of the placenta free choline is always present up to about 100 mg. This suggests that the free choline of the placenta may have been derived from a water-soluble precursor such as has been described.

The basic lead filtrate is concentrated to dryness under reduced pressure at 37° and the residue extracted with alcohol. It is found that there is a loss of about 25–50 % of the organic phosphorus during the alcohol extraction. Some of this can be recovered by extracting with acid alcohol. The alcoholic or acid alcoholic extract is now precipitated with mercuric chloride after neutralisation, if necessary, with fused sodium acetate. This results in a copious white precipitate containing both free and bound choline. This precipitate, after washing with alcohol, is extracted with cold water. The mercury compound of the free choline is relatively insoluble in cold water, but if the solution is analysed after removal of the mercury it is found that about 90 % of the organic phosphorus is contained in it. This organic phosphorus, as will be shown later, is in combination with choline so that the mercury salt of the bound choline is readily soluble in cold water. The solution is now neutralised, evaporated at 37° to dryness, extracted with acid alcohol, as the compound is now relatively insoluble in neutral alcohol, and re-precipitated with mercuric chloride.

An aqueous extract of this second mercury precipitate in the case of the kidney contains the choline-containing substance in relatively pure form. On analysis, it is found to contain:

Total N : Amino-N : P in ratio of 2 : 1 : 1.

That the same does not hold in the case of the placenta is seen from the following sets of figures obtained in three different experiments by analysis of the aqueous extract of the second mercury precipitate.

P	:	N
1	:	4.03
1	:	8.20
1	:	3.10

It is evident that the procedure used does not yield a substance of fixed composition, and in all cases the nitrogen value is much higher than would be obtained from the kidney. Owing, however, to the very small amount of organic phosphorus obtainable from the placenta it was considered advisable to examine the nature of the solution obtained before attempting further purification.

To determine the presence or absence of free choline the extract was concentrated at 37° and 50 % gold chloride added. This gave no precipitate. Another fraction was heated at 100° for 8 hours in a sealed tube with an equal volume of 50 % hydrochloric acid. On addition of gold chloride, after neutralisation, a precipitate formed. The melting point of this precipitate (210°) was much lower than that of pure choline chloroaurate. A part of the gold salt was suspended in water and the gold removed by hydrogen sulphide. The filtrate gave a strongly positive microcrystalline test for choline. The presence of choline was further confirmed by acetylating a part of the gold-free solution and testing biologically on the heart of frog, rectus of frog, mammalian blood pressure and intestine. On all these test objects a typical acetylcholine-like action was obtained. The action was inhibited by atropine and did not appear after mild alkaline hydrolysis. One may conclude therefore that the cold-water extract of the second mercury precipitate contains no free choline, but that choline can be set free from it by hydrolysis with strong acid.

In carrying out quantitative estimations of the amounts of free and bound choline by Staněk's periodide method, it was considered advisable to do control experiments in which a known amount of free choline was added to the solution before precipitation. 5 ml. of the extract were precipitated with Staněk's solution, and nitrogen and phosphorus values determined in the filtrate and precipitate. The same procedure was repeated on another sample of 5 ml. to which had been added 2 ml. of choline solution containing 1.736 mg. of choline-nitrogen. The results were as follows:

	5 ml. extract		5 ml. extract + 1.74 mg. choline-N	
	P	N	P	N
	mg.	mg.	mg.	mg.
Before precipitation	0.710	3.05	0.71	4.78
After precipitation: filtrate	0.678	2.88	0.07	2.79
precipitate	0.041	0.09	0.0	1.81

From these figures, it is seen that any free choline added is removed quantitatively, the original extract itself contains only the smallest traces of free choline, and that all the phosphorus and therefore the bound choline escapes precipitation.

The bound choline was estimated by precipitation with Staněk's solution after strong acid hydrolysis (25 % HCl at 100° for 8 hours). The nitrogen and phosphorus values in the periodide filtrate and precipitate in one case were as follows:

	P	N
	mg.	mg.
Extract before hydrolysis	0.70	2.72
Hydrolysed extract after precipitation: filtrate	0.70	2.44
precipitate	0.0	0.31

It is seen that the amount of nitrogen (0.31 mg.) in the form of choline set free by acid hydrolysis bears to the total phosphorus (0.70 mg.) an atomic ratio of 1 : 0.95.

In another case the figures obtained were:

	P	N
	mg.	mg.
Extract before hydrolysis	1.05	4.074
Hydrolysed extract after precipitation: filtrate	—	3.54
precipitate	—	0.56

Again, the amount of nitrogen (0.56 mg.) in the form of bound choline bears to the phosphorus (1.05 mg.) an atomic ratio of 1 : 1.1. It is evident, therefore, that the extract contains no free choline, but contains choline firmly bound which can be liberated by acid hydrolysis, the amount of choline in this form being in equal ratio with the phosphorus in the extract. There is also a large amount of nitrogen present which does not represent either free or bound choline.

The choline-containing substance can be freed from extraneous nitrogen by a third precipitation with mercuric chloride. In one sample after this treatment an analysis of the aqueous extract of the mercury precipitate gave the following figures: total N, 2.313 mg.; amino-N, 1.164 mg.; total P, 2.416 mg.; the atomic ratio being 2.11 : 1.07 : 1.0.

A portion of this extract was taken and after acetylation tested biologically on the frog's rectus. The assay showed the presence of choline and the amount of choline was estimated to correspond with half of the total nitrogen. The amounts present were insufficient for accurate chemical assay.

In contrast with the biological activity of this very small quantity of nitrogen-containing substance was the complete absence of any activity from the non-choline-nitrogen present in the periodide filtrate of the aqueous extract of the second mercury precipitate. In order to show this the filtrate (after removal of the iodine with molecular copper and removal of the copper as a sulphide) was acetylated and tested on the frog's heart, frog's rectus and mammalian intestine.

We have, therefore, obtained from the placenta a substance containing choline-N, amino-N and phosphorus in 1 : 1 : 1 ratio. The amount of the substance isolated has been insufficient for a determination of other possible constituents of the molecule, but it would appear probable that it is similar to that isolated by Booth and Milroy from the kidney, which they regard as a choline phosphoric acid ester of sphingosine, and is also of the same nature as the substance present in the extract obtained by Strack *et al.* from the placenta.

SUMMARY.

There is present in aqueous extracts of the human placenta a substance containing bound choline in a water-soluble form. Each molecule of this substance contains one molecule of choline, one of phosphoric acid and one amino-linkage.

The substance is present in only very small quantities in the normally born placenta.

The presence of free choline in the placenta can be readily demonstrated by Florence's microcrystalline test.

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