CCXLIII. A MICROCHEMICAL TEST FOR CHOLINE AND ITS ESTERS IN TISSUE EXTRACTS.

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ANY simple test, by means of which we can detect small amounts of free choline and distinguish between it and its water-soluble esters in tissue extracts, is of interest.

It has been known for a long time that periodide solutions are very delicate precipitants for choline. In 1897 Florence used the periodide reagent in testing for semen. In 1901 Bocarius showed that the test was due to the presence of choline in the semen. Staněk [1905; 1906, 1, 2], using his concentrated potassium tri-iodide reagent and determining the amount of nitrogen in the periodide precipitate, was able to estimate the amount of choline present in plant extracts. Roman [1930] worked with solutions of pure choline and used a modified Staněk's reagent. He claims to be able to determine accurately amounts of choline ranging from 5 γ to 5 mg. per 1 ml., by estimating the iodine in the enneaiodide precipitate. Carles and Leuret [1934] have studied the microcrystalline characters of the periodide compounds of choline, betaine and trimethylamine in pure solution.

Only scanty data, however, appear in the literature regarding the microscopic characters of choline periodide in tissue extracts. Moreover, there is no account of the microscopic appearances of the periodide compound of any of the choline esters. The present investigation therefore deals with the microcrystalline characters of the periodides of choline and its esters.

In all the experiments the solution to be examined was mixed with Florence's reagent on a clean dry microscopic slide in the proportions suggested by Carles and Leuret, *i.e.* 3 drops of the reagent to 1 drop of the unknown solution. Immediately after covering the preparation, the specimen was examined. The Florence's reagent used had the following composition: potassium iodide 1.65 g.; iodine 2.54 g.; distilled water 30.00 g.

All the solutions examined were neutral or acid to litmus.

Choline periodide.

The microscopic picture varies greatly with the strength of the choline solution:

(a) *1:100 dilution.* Intense precipitate of large black globules of reddish brown hue, presenting an oily appearance. See Fig. 1.

(b) *1:1000 dilution.* In this dilution the most characteristic crystals of choline periodide are seen. They are light brown monoclinic prisms with the characters shown in Fig. 2 and exhibit pleochroism from a pale yellow-brown to dark brown or almost black when examined in plane polarised light. Although the actual strength of the double refraction is indeterminable, it is presumably very low as no high order colours can be seen.

(c) *1:50,000 dilution.* Thin black rods as shown in Fig. 3. In greater dilutions choline cannot be detected. In working with tissue extracts, however, the

sensitivity of the test can be increased about 100-fold by concentration and re-extraction with suitable solvents.

The above findings confirm the admirable work of Carles and Leuret who worked with solutions of pure choline.

Albumin, glycogen and glucose in 0.5% solutions do not interfere with the sensitivity of the test. The influence of lipoids has not been studied.

Fig. 1. Choline periodide, 1 : 100.

Fig. 2. Choline periodide, 1 : 1000, obtained by HCl hydrolysis of the water-soluble precursor from sheep's brain.

Fig. 3. Choline periodide, 1 : 50,000.

Fig. 4. Acetylcholine periodide, 1 : 1000.

In filtered boiling-water extracts of brain, kidney and liver (two parts of boiling water to one part of fresh tissue) there is no free choline within the limits of the test, although choline when added to boiling-water extracts of these organs can be detected in dilutions up to 1 : 50,000. In diseases of the central nervous system such as syphilis and epilepsy the choline content of the cerebrospinal fluid is increased. The cerebrospinal fluid from two cases of general paralysis of the insane was tested. In each case a sample of 20 ml. was examined directly and again after concentration to 1 ml. *in vacuo*. No crystals of choline periodide were found, *i.e.* choline was excluded up to a dilution of 1 : 1,000,000. These results are in agreement with those of Page and Schmidt [1931], who estimated the choline content of the cerebrospinal fluid biologically. They give values ranging from 0.13 to 1.3 mg. choline per litre in syphilis of the nervous system. In the solutions examined up to the present there has been no microcrystalline deposit with Florence's reagent which might be confused with the periodide of choline.

Acetylcholine periodide.

Unlike that of choline the precipitate is never crystalline. A 1 : 100 solution gives large black globules similar to those of choline. In dilutions of 1 : 500, 1 : 1000 and 1 : 10,000 tiny black granules are seen, which become smaller, and

rarer the higher the dilution (Fig. 4). Above a dilution of 1 : 10,000 the granules are no longer seen.

After hydrolysis by 1% NaOH for two minutes at room temperature, making acid to litmus with HCl and then adding Florence's reagent, the typical crystals of choline periodide are seen, provided that the dilution is suitable. That the granules contain acetylcholine is proved by the fact that practically all the acetylcholine present can be found in the periodide precipitate, using Staněk's method for the estimation of choline. These granules are not specific for acetylcholine and the test if it is to be of value in the detection of acetylcholine must be carried out before and after alkaline hydrolysis.

The water-soluble precursor of choline.

This body was isolated from the kidney by Booth and Milroy [1935] and has since been obtained by Booth from the brain. This ester of choline is not precipitated by Staněk's reagent, as is evident from phosphorus and nitrogen determinations of the precipitate and supernatant fluid. Thus any precipitate, amorphous or crystalline, occurring in solutions of this water-soluble precursor treated with Florence's reagent, cannot be due to this choline-containing compound. Frequently there may be evidence of free choline along with the precursor as is shown by a study of the microcrystalline deposit which may appear, but this only constitutes a small fraction of the choline present. In order that the choline should be set free from this ester acid hydrolysis must be carried out (Fig. 2).

When the mother substance is acetylated it is broken down, the degree of decomposition varying with the vigour of the acetylation, and the choline set free is re-esterified. After removal of the acetyl chloride from the reaction mixture, it gives granules but no crystals with Florence's reagent. After hydrolysis with 1% NaOH for 2 min. at room temperature, however, the typical crystals of choline periodide are seen. The mother substance itself is not decomposed by this mild alkaline hydrolysis.

SUMMARY.

1. A micro-chemical test for the detection of small amounts of free choline is described. The test can detect 20 γ /100 g. free choline in the tissues, since the sensitivity of the test is 1 : 50,000 and this can be increased 100-fold by appropriate treatment of the extracts.
2. Acetylcholine can be detected as choline after hydrolysis with 1% NaOH for 2 min. at room temperature.
3. The water-soluble ester of choline referred to gives no precipitate with Florence's reagent.

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