CCXVIII. A COLORIMETRIC METHOD FOR THE DETERMINATION OF CHOLINE AND ACETYLCHOLINE IN SMALL AMOUNTS.

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WHILE the presence of small amounts of choline and acetylcholine can easily be detected by biological methods, their quantitative micro-chemical estimation presents considerable difficulty. The only method hitherto described for the micro-chemical estimation of choline appears to be that of Roman [1930], a modification of the macro-method of Stanek [1906] depending on the precipitation of choline as choline periodide. By this method quantities of choline from 0.001 to 5 mg. can be estimated, but its application is limited since creatinine, adenine and other nitrogenous bases are also precipitated. Furthermore, the method is complicated by the fact that the physical conditions of the experiment require very careful control. In the present paper a simple colorimetric method for the estimation of choline and acetylcholine is described.

The principle of the method is the same as that used by Kapfhammer and Bischoff [1930] for the isolation of choline and acetylcholine from tissues. It depends on the fact that choline and acetylcholine form reineckates which are insoluble in ice-cold water and in absolute alcohol but are easily soluble in acetone giving a bright red solution. In the method of Kapfhammer and Bischoff acetone was used on account of its selective solvent action on the reineckate, for the further purification of the precipitate after washing with ice-cold water and absolute alcohol. The acetone was then evaporated and after several extractions with ether the insoluble residue was dried and weighed.

In the present method the reineckate is dissolved in acetone, and by comparing the colour of this acetone solution with that of a standard acetone solution of choline or acetylcholine reineckate, the amount of choline or acetylcholine combined as reineckate can be determined. The depth of the red colour depends on the amount of the reineckate radicle present. The colours are the same in equimolecular acetone solutions of choline and acetylcholine reineckates, since 1 mol. of each base combines with 1 mol. of reineckate.

By this method quantities as small as 0.2 mg. of choline chloride and 0.32 mg. of acetylcholine bromide can be determined.

Method.

The solutions used were:

1. Choline chloride solutions containing 0.2–2 mg. choline chloride per ml.
2. Acetylcholine bromide solutions containing 0.3–3 mg. acetylcholine bromide per ml.

1 ml. of the solution is taken and to it 1 ml. of a freshly prepared saturated solution of ammonium reineckate (B.D.H.) is added. A precipitate of choline reineckate or acetylcholine reineckate separates out. These two reineckates differ in appearance and solubility. The choline reineckate has a glistening appearance;

its precipitation is complete in less than 10 min. and it can be quantitatively precipitated at temperatures up to 60°. The acetylcholine reineckate separates more slowly as a dull pink powder and for complete precipitation requires a much lower temperature (about 5°). To ensure that the precipitation is complete the container should be surrounded by ice for 20 min. A means of avoiding the greater difficulty in estimating the acetylcholine is to estimate the choline set free by alkaline hydrolysis. This hydrolysis can be effected in a few min. at room temperature by addition of NaOH to 1% concentration, the solution being neutralized before precipitation. After precipitation is complete the solution is filtered by suction through a small asbestos-packed crucible and the precipitate on the asbestos is washed once with 2 ml. of ice-cold water and twice with 2 ml. of absolute alcohol, or until the alcohol is no longer pink. The receiving flask is emptied and a test-tube is placed inside the flask to receive the acetone solution. Before applying suction 1 ml. of acetone is poured on the precipitate, which rapidly dissolves. The acetone solution is sucked into the receiving tube and successive small amounts of acetone are added until all the precipitate is dissolved and washed through. The tube is removed from the filtering flask and the contents are transferred to a graduated tube or small measuring cylinder which can be read to 0.05 ml. The receiving tube is rinsed with acetone and the washings added to the graduated tube. The volume of the acetone solution is carefully noted and the colour is compared with that of a standard solution containing 0.78 mg. of choline reineckate per ml. of acetone or with a methyl red solution of the same colour, the preparation of which as a standard is described later. The standard corresponds to 0.26 mg. choline chloride or to 0.42 mg. acetylcholine bromide per ml. When the total amount of choline is small (0.2–0.3 mg.) the total volume of the acetone should not be more than 1–2 ml. in order to have a colour comparable with that of the standard. The comparison of the colour in such a small volume can be carried out in a micro-colorimeter. When on the other hand the total amount of choline is large (2–3 mg.), dilutions up to 10 ml. are required to obtain a colour comparable with the standard. For colorimetric comparisons daylight was found to be more suitable than artificial light.

Calculation. With the standard set at 20 mm. and the unknown giving a reading of x , then $\frac{20}{x} \times 0.26$ (for choline) $\times$ volume of the unknown solution = mg. of choline chloride in amount of solution taken.

Preparation of standard solution.

A sample of choline reineckate was prepared according to the method of Kapfhammer and Bischoff, the only difference being the use of ammonium reineckate instead of reinecke acid for the precipitation. The total N of the dried choline reineckate determined by Kjeldahl's method was 22.8% (calc. 23.2%).

78 mg. of this salt were dissolved in 100 ml. of acetone. This solution was unsuitable as a permanent standard owing to evaporation of the acetone and fading of the colour. An artificial standard was therefore prepared by dissolving 0.0134 g. of methyl red in 5 ml. of $N/5$ NaOH and diluting to 500 ml. with distilled water; for use 4 ml. of this stock solution were transferred to a flask, 10 ml. of $N/100$ H_2SO_4 were added and the volume made up to 100 ml. A pink solution was obtained of the same colour as the 0.078% acetone solution of choline reineckate, corresponding to 0.26 mg. choline chloride per ml. or 0.42 mg. acetylcholine bromide per ml. The dilute standard remained unchanged for some days and the stock solution indefinitely. Several different samples of methyl

red were used in the preparation of standard solutions and the colours obtained in each case were the same.

Examples of the results obtained by this method for the choline estimation in choline chloride solutions are given in Table I, col. 2. The values are compared with those obtained by Roman's method [1930], col. 3, and with those calculated as choline chloride from the total N, col. 1.

Table I.

mg. choline chloride per ml.

1	2	3
Calculated from total N	Colorimetric estimation	Roman's method
2.35	2.35	—
1.56	1.52	—
0.90	0.92	0.93
0.88	0.87	—
0.76	0.70	—
0.72	0.71	0.71
0.70	0.67	—
0.62	0.62	—
0.54	0.54	0.59
0.46	0.48	—
0.35	0.38	—
0.31	0.32	—

Table II gives the results obtained by the estimation of acetylcholine precipitated at 5° as acetylcholine reineckate from acetylcholine bromide solutions.

Table II.

mg. acetylcholine bromide per ml.

Calculated from total N	Colorimetric estimation
1.90	1.87
1.26	1.28
0.99	0.96
0.49	0.43
0.40	0.38

Effect of temperature.

The comparative solubilities of choline and acetylcholine reineckates at different temperatures (5–60°) are shown by the following experiments.

1 ml. of acetylcholine bromide solution was precipitated with ammonium reineckate at room temperature (16°) before and after alkaline hydrolysis in 1% NaOH and the acetylcholine chloride values of the two solutions were compared with that obtained when 1 ml. of acetylcholine bromide solution was precipitated at 5°. The results are given in Table III.

Table III. *Effect of temperature on the estimation of acetylcholine bromide.*

Acetylcholine bromide. mg. per ml.

Temperature ° C.	(Before alkaline hydrolysis)	(After alkaline hydrolysis)
5	1.28	—
16	0.97	1.26
19	—	1.26
60	Nil	1.17

Using a choline chloride solution containing 1.35 mg. per ml. the choline reineckate precipitation was not affected by increasing the temperature up to 60°.

These values, typical of many, show that with a solution of acetylcholine reineckate low values are obtained if the temperature is allowed to rise, whilst the yield of choline reineckate is not diminished at any temperature up to 60°. By hydrolysing the acetylcholine to choline before precipitation temperature effects can be minimized and accurate determinations carried out at room temperature. It was noticed that acetylcholine when in the presence of choline chloride can also be quantitatively precipitated at room temperature.

Effect of dilution on precipitation of choline.

A series of estimations was made in solutions containing from 0.3 to 0.03 mg. choline chloride per ml. To each solution 1 ml. of saturated ammonium reineckate was added and the precipitate in each case was dissolved in 2 ml. of acetone. The full value was obtained at all these dilutions. The temperature was kept low (5°) during the procedure since the extreme range of the method was being tested. At greater dilutions than the above the choline values fell. It thus appears that a quantitative precipitation of free choline can be obtained in solutions containing as little as 0.03 mg. choline chloride per ml. Since the final precipitate must contain about 0.2–0.3 mg. choline chloride in order to be comparable with the standard, 7–10 ml. of such a solution must be taken for the precipitation. 1 ml. of saturated ammonium reineckate solution is sufficient for the precipitation of quantities of choline up to 3 mg. choline chloride.

Effect of dilution on precipitation of acetylcholine.

An acetylcholine bromide solution corresponding to the final dilution of choline chloride at which a quantitative precipitation was found possible was treated in the same way but at a lower temperature (1–2°). The precipitation was carried out before and after alkaline hydrolysis. The results are given in Table IV.

Table IV.

10 ml. solution containing 0.48 mg. acetylcholine bromide	mg. acetylcholine bromide found
Before hydrolysis	0.28
After hydrolysis	0.50

At this dilution the acetylcholine cannot be quantitatively precipitated even at low temperatures. This demonstrates the advantage of hydrolysis of the acetylcholine to choline before precipitation.

The results obtained by this method show that quantities of choline chloride from 0.2 mg. upwards can be determined with an error of $\pm 3\%$. Precipitation may be carried out at room temperature unless the solution is very dilute (containing 0.03–0.05 mg. per ml.), in which case a lower temperature (5°) is necessary for quantitative precipitation. For the estimation of acetylcholine it is advisable to hydrolyse to choline before precipitation since the precipitation of choline reineckate is less influenced by temperature than that of acetylcholine reineckate. In the same way after suitable hydrolysis the amount of choline in other choline compounds can be determined. As an example of this the results obtained on acid hydrolysis of a lecithin emulsion are given in Table V. The emulsion examined had total N 0.046 mg. per ml. It was hydrolysed in 7.8%

HCl at 110° and after different intervals the amount of free choline was determined. The results are expressed in terms of choline-N. These show the rate of liberation of choline and the percentage of the total N which can be accounted for by choline.

Table V. *Lecithin emulsion hydrolysed in 7.8% HCl at 110°.*

Time in hours	mg. choline-N per ml.
0	—
4	0.012
9	0.022
21	0.036

The emulsion used was made from a commercial egg lecithin preparation (Griffin & Tatlock). The maximum value of free choline was obtained after 21 hours' hydrolysis, at which time the choline-N amounted to 78% of the total N in agreement with Maclean and Maclean [1927].

The results given so far have dealt with the estimation of choline and its compounds in pure solution. To see how far the choline estimation may be interfered with by the presence of other substances such as occur in animal tissues and fluids, known amounts of choline chloride were added to extracts of different organs (rabbit's liver and kidney), to a tryptic digest and to urine, and the choline value was determined before and after addition. The results are given in Table VI. The kidney and liver extracts were prepared by extracting 1 vol. of liver or kidney with 3 vol. of 5% trichloroacetic acid, and subsequently filtering and neutralizing. The tryptic digest was acidified, boiled, cooled and filtered before precipitation.

Table VI.

	mg. choline chloride per ml.			
	1	2	3	4
	Originally present	Amount added	Total found	Recovered
Kidney extract	0.13	0.69	0.85	0.72
Liver extract	0.18	0.77	0.94	0.76
Tryptic digest	Nil	1.50	1.56	1.56
Urine	Nil	1.50	1.56	1.56

From these results it is seen that added choline can be recovered from crude extracts without appreciable loss. It follows that the method is applicable to the study of the liberation of choline from choline compounds in tissue extracts by chemical or enzymic hydrolysis. The original choline value of the reineckate precipitate from such an extract represents both choline and acetylcholine. Strack *et al.* [1935] have recently pointed out that the value obtained by Bischoff *et al.* [1932] for the acetylcholine content of skeletal muscle is probably a carnitine value, since this base also forms a reineckate with properties similar to those of acetylcholine reineckate. In order to find whether carnitine would also be estimated in the present method, a preparation of carnitine was made from a meat extract according to a method described by Strack *et al.* [1936]. The gold salt was prepared, the gold removed, and a reineckate precipitated which was found to be insoluble in water and alcohol, and soluble in acetone giving a pink colour. Carnitine therefore, if present, will also be estimated with choline in this method as in that of Bischoff and Kapfhammer. Since its amount in ordinary tissue extracts is small, no error will arise from this source in the study of the breakdown of choline compounds.

SUMMARY.

A micro-colorimetric method for the determination of small amounts of choline is described. Quantities of the order of 0.3 mg. choline in a concentration of 0.003 % can be estimated with an error of not more than 3 %.

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