CXXXV. THE ESTIMATION OF CHOLINE AND ACETYLCHOLINE

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THE most specific method of estimating choline consists in acetylation followed by biological assay of the acetylcholine formed [Hunt, 1915]. This method is, of course, as sensitive as that for the estimation of acetylcholine itself, but is very time-consuming. All other methods are chemical ones. Stanek [1904] precipitated choline as the periodide and estimated the iodine by titration with thiosulphate. Roman [1930] advised precipitating the choline as the enneaiodide with a stronger solution of iodine. This method was only applied to serum; I have applied it to other tissue extracts and have found that many substances other than choline are precipitated and the results were as much as ten times too great. In Beattie's [1936] method, which estimates acetylcholine as well as choline, the choline was precipitated as the reineckate and this was dissolved in acetone and estimated colorimetrically. Here again interfering substances increase the result. The method can only estimate 0.3 mg. and it is not possible to estimate acetylcholine in the presence of choline. Mention may also be made of the lengthy method of decomposing the choline to trimethylamine which is then estimated [Linzel & Monasterio, 1931].

The chemical methods for estimating acetylcholine (A.C.) are few and none can be described as a microdetermination. Ammon [1933] estimated A.C. in Warburg's apparatus by means of the carbon dioxide liberated by interaction of enzymically liberated acetic acid with sodium bicarbonate. Abdon & Uvnäs [1937] estimated the acetic acid liberated by choline esterase by adding ferric chloride and estimating the red colour so formed in a step-photometer. The limits of the method were not stated. It should be noted that the above methods depend upon the estimation of the acetic acid formed enzymically from A.C. and, in reality, were developed to study the action of choline esterase.

The method to be described below for the estimation of choline consists first in precipitation as reineckate [Kapfhammer & Bischoff, 1930] and then as the enneaiodide, with elimination of interfering substances at both stages, mainly by differences of solubility of the salts of choline and the interfering compounds. The choline enneaiodide is measured by a slight modification of the usual colorimetric technique.

Addition of iodine to very dilute solutions of choline (or A.C.) caused a deepening of the reddish brown colour of the iodine without formation of a visible precipitate. In the case of choline a visible precipitate did not form until the concentration of choline exceeded 35 μ g. per ml. In the case of A.C. the corresponding value was 20 μ g. It has been found that until a heavy precipitate formed the amount of light passing through the solution was inversely proportional to the concentration of the choline or A.C., provided that the ratio of standard to unknown was not > 2.

It has been noted by the author and also by Beattie [1936] that choline reineckate has a glistening appearance. This effect is characteristic of choline as it is not given by A.C., lecithin or the interfering substances in tissue extracts. Ammonium reineckate precipitates other substances besides choline from tissue extracts but it has been found that these interfering reineckates are either soluble in $N/10$ NaOH or are insoluble in water at 60° , at which temperature choline reineckate dissolves. Further, the iodides of the interfering substances are insoluble in 30 % alcohol, whereas that of choline is readily soluble.

In the estimation of A.C. it is first necessary to separate the A.C. from choline which is very often present. This presented some difficulties but the separation has been aided by differences in the solubilities of the reineckates and the enneaiodides. A.C. reineckate is soluble in 40 % alcohol in the presence of excess ammonium reineckate, while choline reineckate is only slightly soluble under these conditions. In the case of A.C. it is possible that there is formation of a double salt. On the other hand, choline iodide is more soluble than A.C. iodide in 10 % alcohol. Again in tissue extracts there are other substances which are also precipitated by iodine; most however are precipitated by phosphotungstic acid and the iodides of the remainder are insoluble in 30 % alcohol which, of course, dissolves A.C. iodide. To a certain extent A.C. is precipitated by phosphotungstic acid but only if the concentration exceeds $40\text{ }\mu\text{g. per ml.}$ and the danger is obviated further by carrying out the precipitation at 30° . Finally, it was found that A.C. required only half the concentration of iodine which was necessary for complete precipitation of choline.

EXPERIMENTAL

Reagents

(1) *Ammonium reineckate.* A saturated solution is prepared each day by shaking about 5 g. of ammonium reineckate with 100 ml. of water and keeping for 15 min. About 5 ml. of this should be freshly filtered before using.

(2) *Iodine.* 8 g. of iodine and 10 g. of KI are dissolved in 50 ml. of water and kept overnight before use. The solution will keep about a fortnight.

(3) *Approx. N NaOH.*

(4) *Phosphotungstic acid.* A 10 % solution.

(5) *40 % alcohol.*

(6) *Standard choline solution.* The choline is dried at 100° and a solution is prepared so that 1 ml. contains 1 mg.

(7) *Standard acetylcholine.* 0.1 g. of A.C. (Hoffman La Roche) is dissolved in 100 ml. of $N/100$ HCl. This will keep in the cold about a week. It is diluted each day so that it contains $100\text{ }\mu\text{g. in 1 ml.}$

The tissue extracts were prepared by dropping the tissue into 10 % trichloroacetic acid (at least 1 ml. per g.), then cutting up finely. The filtered extract was extracted by shaking once with ether, twice with light petroleum and again twice with ether. It was now evaporated under reduced pressure at 40° till 1 ml. was equivalent to 1–2 g. of tissue.

The estimation of choline

A standard reineckate suspension was prepared by adding 0.3 ml. of ammonium reineckate to 1 ml. of a solution of choline containing approximately $20\text{ }\mu\text{g.}$ It was then centrifuged and the supernatant fluid poured off and the precipitate was suspended in 1 ml. of water. This should keep several hours. To

1–5 ml. of choline solution or tissue extract, which should be neutral or slightly acid, was added half the volume of ammonium reineckate and the solution was then shaken; in the case of tissue extracts 0.1–0.5 ml. of *N* NaOH was added (for 1–5 ml.). It was then centrifuged for 2 min. at 3000 r.p.m., the liquid was poured off and the tube was drained as much as possible, care being taken that none of the precipitate was lost. Water was now added, 1 ml. at a time, with shaking, till a suspension of the same density as the standard reineckate previously prepared was obtained. The volume added was noted and the tube was placed in a water bath at 60°. After 2 min. it was centrifuged for 2 min. and the fluid poured into another tube; 0.3 ml. of the iodine reagent was now added and the tube compared with a standard in the colorimeter. 0.3 ml. of alcohol was added to the unknown and was again compared. The correct value was obtained by subtraction. The standard was prepared by taking 1 ml. of a solution containing 25 μ g. of choline to which was added 0.5 ml. of ammonium reineckate. It was then shaken and centrifuged, the fluid was poured off and 1 ml. of water was added to the precipitate which was now heated to 60°, cooled and 0.3 ml. of iodine added as before. There are several points about this estimation of choline which may need explanation. The preparation of the standard suspension of 20 μ g. of choline reineckate was a device to obtain the choline of the unknown in a dilution at which the estimation by the method suggested was most accurate. The alcohol was added to the iodide to dissolve the choline iodide, but it did not dissolve iodides other than those of choline. It has been found that the colour due to a solution of about 20 μ g. of choline iodide in 1 ml. was reduced to that of the colour of the iodine alone by the addition of 0.3 ml. of alcohol.

Lastly, the method did not distinguish between choline and acetylcholine and so, strictly speaking, estimated total choline. Of course the acetylcholine, if present, could be estimated by the method to be described and the choline itself could be obtained by difference.

The estimation of acetylcholine

A. Pure solutions or extracts which contain a minimum of interfering substances such as serum, which should contain not more than 400 μ g. of choline.

It is first necessary to determine the approximate choline content by the above method.

In 1.5 ml. the total choline content was brought to 400 μ g. by addition of the necessary amount in the smallest volume possible (about 0.5 ml.). At the same time a control blank was carried out with 400 μ g. of choline alone. Half the volume of ammonium reineckate was added to both tubes. They were then shaken and centrifuged for 2 min. The supernatant liquid was poured off and the tubes were drained as much as possible, then 1.4 ml. of 40% alcohol and 0.2 ml. ammonium reineckate were added to the tubes which were shaken to disturb the precipitate and placed in a water bath at 57° for 3 min. They were centrifuged again and the liquid poured into other tubes which were now placed in a vigorously boiling water bath for 4 min. The tubes will boil easily if a “boiling” tube is added (a capillary sealed about 2.3 cm. from one end; these tubes should always be removed from the solution while it is boiling and then they will last indefinitely). The tubes were cooled and 0.1 ml. of the iodine reagent was added (a fairly heavy precipitate should form), followed by 0.05 ml. of alcohol and the tubes were inverted once. If the precipitate in the control tube did not almost completely dissolve a further 0.03 ml. of alcohol was added to all tubes,

and if this were not sufficient another 0.03 ml. Care must be exercised at this point. On addition of the alcohol to the control solution it should become almost, but not definitely, clear; it usually takes about 30 sec. for the alcohol to dissolve the choline iodide. If too much alcohol is added A.C. may be lost. The tubes were centrifuged and the liquid poured off, 0.04 ml. of alcohol was added to the tubes and the precipitates were dissolved by placing the tubes momentarily in hot water; 1 ml. of water and 0.15 ml. of iodine were added and the tubes were compared with a standard in the colorimeter. The reading for the blank was then subtracted.

The standard was prepared by adding 0.15 ml. of iodine and 0.04 ml. of alcohol to 1 ml. of a solution containing 5–15 μg . of A.C. as required.

The solution to be tested should contain between 4 and 20 μg . of A.C. although amounts down to 1 μg . can be estimated. If the solution should contain more than 20 μg . the test should be repeated with a more dilute solution.

The standard deviation is $\pm 0.68 \mu\text{g}$.

It has been found empirically that small amounts of A.C. are only precipitated completely under the conditions employed if a definite amount of choline is present; 400 μg . have been chosen as this amount. In addition the temperature at which the mixed reineckates of choline and A.C. dissolve in 40 % alcohol varies with the amount of choline present and the age of the ammonium reineckate. If the solution contains more than 400 μg . of choline the temperature at which the A.C. reineckate is dissolved should be raised 3° for each additional 50 μg . of choline. If on the addition of 0.1 ml. of iodine to the control tube no precipitate forms it is best to repeat from the beginning, raising the temperature to 60°. If, on the other hand, the control solution does not almost clear on the addition of 0.11 ml. of alcohol the temperature should be reduced to 54°. However, in most cases 57° will be found to be most suitable. The need for varying the temperature has been found because the ammonium reineckate is not stable and various samples differ from one another. Apparently this affects the ease of solubility of the A.C. reineckate and so some samples require a higher temperature than others.

B. *Tissue extracts containing interfering substances, such as liver, stomach and intestinal extracts.*

The approximate choline content of the extract should be determined and if it is less than 30 μg . choline should be added to this amount. This prevents an excess of phosphotungstic acid which may precipitate some of the A.C. If there are more than 90 μg . of choline present the amount of phosphotungstic acid to be added should be increased by half. To 1 ml. of extract which had been in a water bath at 30° for 2 min., 0.1 ml. of phosphotungstic acid was added immediately and the tube was inverted but not shaken and then centrifuged for 2 min. The liquid was poured carefully into another tube and 0.15 ml. of iodine and 0.08 ml. of alcohol were added. It was then centrifuged for 2 min., the liquid poured off and 0.04 ml. of alcohol was added, the tube being warmed as before; then 1 ml. of water and 0.15 ml. of iodine were added. It was read in the colorimeter and then 0.25 ml. of alcohol was added and it was read again. The value of A.C. was obtained by subtraction.

The amount of light passing through the solution is inversely proportional to the concentration within the limits of 4–25 μg . of A.C. provided that the ratio of standard to unknown does not exceed 2. Below 4 μg . the standard should more nearly approach the unknown. As the standard takes only a few seconds to prepare it is not a difficult matter to make several to compare with the one unknown.

If a scum should form on the surface of any of the tubes it can be carried down by adding two drops of a suspension of aluminium hydroxide. This was prepared by precipitating 25 g. of potassium alum in water with 5.5 g. NaOH. It was filtered, washed and made into a suspension with 80 ml. of water.

Results with tissue extracts

The results in Table I give the choline and A.C. contents of various tissues. In addition are given the results obtained for A.C. by estimation on the frog's rectus abdominis or the leech muscle.

Table I

Tissue	Animal	Choline $\mu\text{g./g.}$	A.C. $\mu\text{g./g.}$	
			Chemical method	Biological method
Submaxillary gland	Dog	6.0	4.2	—
Small intestine	Dog	48	3.0	2.8
		62	3.2	3.1
	Rabbit	20	2.0	2.1
		32	2.5	—
	Cat	32	0.5	0.5
Stomach	Dog	21	0.5	—
		10	1.0	1.0
	Rabbit	12	1.5	—
		12	1.8	1.6
		14	1.2	1.0
	Cat	25	1.2	0.9
Liver	Dog	16	0.05	—
		70	—	0.1
	Rabbit	64	—	—
		30	—	—
		32	—	—
	Cat	95	0.5	0.2
Blood	Rabbit	74	—	0.1
		1.0	—	—

In addition some of the tissue extracts were acetylated and tested on the leech muscle. The results so obtained agreed with those for choline obtained by the chemical method.

Recovery of added A.C.

A.C. was added in varying amounts to the concentrated extract and the usual procedure carried out. In all cases, with the exception of some intestinal extracts, the recovery was 90–100 %. Often it was found impossible to obtain intestinal extracts which were perfectly clear; whether the particles were fat or breakdown products of protein is difficult to say. If the latter were true one might have expected adsorption to have taken place, for the recovery of A.C. varied from 70 to 90 %.

SUMMARY

Sensitive and specific methods for the estimation of choline and acetylcholine are described.

The sensitivity of the method for acetylcholine is 1 in 10^6 , of that for choline 5 in 10^6 .

The methods have been applied to tissue extracts and give results which agree with those obtained by biological methods.

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