CCCXXXII. A MODIFICATION OF YOUNG'S METHOD FOR THE DETERMINATION OF INOSITOL IN ANIMAL TISSUES.

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Since Scherer's isolation of inositol [1850], the attempts to devise satisfactory methods for its determination in animal tissues have been many; but the properties of the substance and the absence until recently of any chemical means of its estimation have rendered the problem difficult. The obstacles encountered necessitate the preliminary isolation of the inositol, and most workers have been obliged to follow this by weighing the isolated product. Young [1934, 1] modified the Fleury-Marque technique for the determination of polyhydroxy-compounds, making it suitable for the estimation of 1–5 mg. of inositol in pure solution with considerable accuracy; a development which made possible for the first time a quantitative study of the various isolation procedures and enabled a method to be produced giving 90% recovery of inositol from tissues [Young, 1934, 2]. This probably represents the highest accuracy to be expected from any method on the same general lines; but in common with all other existing procedures it possesses certain features that render it unsuitable for use in any but the simplest experiments on tissues, especially where only small amounts of material are available.

At the outset of investigations into *post mortem* and other changes occurring in the inositol content of animal tissues, it became evident that little headway would be be made until some trustworthy method could be found of determining the inositol contents of amounts of tissues of the order of 5–10 g., and employing also some method of extraction suitable for use in such experiments as the above. The work to be described represents an attempt to meet this need by a modification of the isolation process described by Young [1934, 2] combined with a micro-modification of the same author's method for the determination of inositol in pure solution. An amount of tissue containing not less than 5 mg. of inositol is used (about 3–5 g. of mammalian heart muscle) and the recovery by the process is about 90%.

Extraction of inositol. This has been accomplished either by the extraction of the minced tissue with aqueous acetone [Momose, 1916; Needham, 1923, 1, 2; Young, 1934, 2] or by solution of the tissue in alkali, followed by treatment with precipitating agents such as lead acetate [Rosenberger, 1908; Winter, 1934] to remove contaminating substances. Young [1934, 2] also found that treatment with alkali could be made the first stage of a method of determining inositol in tissues.

The use of acetone is inadvisable for two reasons. When small amounts of tissue are to be dealt with, the manipulative difficulties become great, for the

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extract must be freed from acetone and ether-extracted before proceeding; but more serious objections to its use are that it is a method of long duration, and that its completeness is open to question unless the sample is extracted at least twice [Young, 1934, 2].

Solution of the tissue in alkali has the important advantages that it is rapid, complete, and easy to use with small amounts of tissue. Winter's [1934] work indicates that the duration of heating in alkali may affect the amount of inositol obtainable from mammalian heart muscle. In the present work the tissues were treated by heating in 10 % KOH for the minimum time necessary to bring about their complete breakdown.

Removal of the alkali with a zinc salt has the advantage of removing in the precipitate a considerable amount of unwanted substances. A study was made of the behaviour of inositol in the Somogyi [1930] zinc hydroxide precipitation, using the iodomercurate method of determining inositol [Young, 1934, 1]. At least 98 % of the inositol was found to be present in the filtrate, and this procedure was therefore adopted as the method of treatment at this stage.

The filtrate after the above process is further clarified with the aid of the West-Peterson reagent [1932], which, as Young [1934, 2] has shown, is very effective for this purpose and permits a 98 % recovery of inositol. The filtrate is colourless and odourless and after removal of the mercury is finally subjected to Young's [1934, 2] baryta-alcohol precipitation. Recovery of the inositol at this stage is 96 %; and after removal of the barium as sulphate the solution is evaporated to small volume and the inositol crystallised from acetone-ether mixture [Young, 1934, 2].

Experimental.

Extraction. Into a large pyrex boiling-tube is measured 10 % potassium hydroxide from a burette, about 1 ml. per g. of tissue to be used. The tube is heated in a boiling water-bath and the weighed amount of tissue dropped in. The contents of the tube are stirred occasionally with a glass rod, and after heating for the minimum length of time to effect solution (30 min.) the hot solution is washed into a 50 ml. volumetric flask.

Zinc hydroxide precipitation. A solution of zinc chloride in dilute hydrochloric acid is used for neutralisation and has the following composition:

Zinc chloride (anhydrous)	...	126 g. per litre of solution
Hydrochloric acid (concentrated)		4·5 g. per litre of solution

1 ml. of 10 % potassium hydroxide is neutralised by 1·5 ml. of this solution. It should be checked before use by titration against the alkali, using phenolphthalein as indicator. It has been found preferable to use zinc chloride instead of zinc sulphate in this process; the chloride which passes through to the final product does not affect the determination of the inositol and by its bulk facilitates the collection of the final crystalline material.

The required amount of the above solution is added to the hot solution in the flask with gentle agitation, and a solid mass results. The flask is then heated in the water-bath for a few moments with gentle shaking, when the precipitate becomes lighter and granular in character and the contents of the flask become fluid again. When this has occurred, the flask is cooled under the tap and the contents are made up to volume. After standing a few moments, the contents are filtered on a coarse paper.

The West-Peterson precipitation. An aliquot of the above filtrate—30 ml. can usually be obtained—is transferred by pipette to a dry 250 ml. conical flask.

From a pipette are then added slowly with shaking 5 ml. of the acid-mercuric sulphate reagent [West and Peterson, 1932], made by dissolving 27 g. of mercuric sulphate in 100 ml. of 10 % (by weight) sulphuric acid at 5° and separating the solution from any precipitate which forms as room temperature is reached. The mixture is neutralised by the addition of solid barium carbonate (that sold as "pure precip. by soda") until a drop of the solution does not redden blue litmus paper, when the flask is stoppered, shaken for a short time, and filtered on a dry Büchner funnel into a dry flask. The whole of the filtrate is poured into a dry 100 ml. Phillip's beaker and H_2S passed in the cold without acidification. After filtering on a dry paper into a dry flask, an aliquot of the filtrate is transferred by pipette to a 50 ml. beaker and evaporated on the water-bath to less than 5 ml.

The baryta-alcohol precipitation. The solution is transferred to a 30 ml. centrifuge-tube and re-heated in the water-bath. To the hot solution (volume 5 ml.) are now added 2·0 g. of crushed crystalline barium hydroxide, the solution heated for 5 min. with occasional stirring and the tube then removed from the water-bath. Immediately, 20 ml. of absolute ethyl alcohol are added slowly with vigorous stirring, the rod removed and the tube allowed to stand, preferably in the ice-chest, for 2–3 hours.

After the tube has stood for this length of time, it is centrifuged at 3000 r.p.m. for 3 min. and the alcohol poured off. The precipitate is stirred up in 10 ml. of hot water, the sides of the tube being well washed down at the same time, and then from a graduated pipette is added with stirring the required amount of N H_2SO_4 to acidify the solution, a drop of methyl red being added as indicator when the solution is nearly neutralised. Finally a very small amount of norite charcoal is stirred into the solution, this then diluted to 25 ml. with hot water, the rod removed and the tube heated in the water-bath for 45 min. The purpose of the charcoal is to remove the indicator added. Inositol is not adsorbed by charcoal in acid solution, or by barium sulphate under the same conditions, so that no loss occurs. Care must be taken to heat the tube very gently at first, until the remains of the alcohol have been removed, or frothing will occur. When the heating is finished, the tube is centrifuged at 3000 r.p.m. for 5 min. and the solution collected in a 100 ml. beaker. The precipitate is then stirred up in 20 ml. of hot water, re-heated for 30 min., centrifuged and the washing added to the main solution.

The acetone-ether precipitation. The solution is concentrated on the water-bath to 5–10 ml., transferred to a 100 ml. flask and re-evaporated to 6 ml. or less. After cooling, 60 ml. of acetone and 30 ml. of ether are added slowly, the sides of the flask scratched with a glass rod to induce crystallisation and the flask stoppered and placed in the cold room for 24–36 hours.

The precipitate which forms is removed by filtration on a sintered glass micro-filter (Schott and Gen., Jena—12G3) or on asbestos on a small Gooch crucible and well washed with acetone and finally with ether. It is then dissolved in hot water, traces of ether being removed from the solution by heating on the water-bath, and then made up to 25 ml. in a volumetric flask. Portions of 5 ml. of this solution are taken for analysis by the micro-modification of the iodomercurate method described below.

Quantitative results.

Recovery of inositol from pure solution. In tests of the accuracy of the method, 5 mg. of inositol were subjected to the whole process, being added to 5 ml. of 10 % potassium hydroxide in the boiling-tube, heated, and the "isolation"

proceeded with as above. The following are typical recovery figures obtained in the case of four experiments done at the same time:

Tube	Inositol found mg.	Recovery %
1	4·81	96·2
2	5·22	104·4
3	4·57	91·4
4	5·25	105·0

Recovery of inositol from heart muscle. This was tested in the following way, 20 g. of heart muscle were dissolved in 20 ml. of 10 % potassium hydroxide, the solution filtered through a plug of glass wool into a 100 ml. volumetric flask and made up to volume. Of this solution 25 ml. (equivalent to 5 g. of tissue) were transferred by pipette to a 50 ml. volumetric flask and 5 mg. of inositol added. The procedure thereafter was as detailed above. The control was prepared in the same way from the same solution, except that no inositol was added.

Exp. no.	Wt. tissue taken (*A* and *B*) g.	Wt. of inositol added to *B* mg.	Inositol found *A* mg.	Inositol found *B* mg.	Recovery of added inositol %
1. (Dog's heart)	5	5	7·95	12·45	90
2. (Dog's heart)	5	5	6·80	11·15	87
3. (Sheep's heart)	5	5	4·85	9·61	95
4. ,,	5	5	3·37	8·42	101
5. ,,	5	5	3·62	7·91	85

The average recovery of added inositol in the above five experiments was 91·6 %.

The estimation of 0·2–1·0 mg. of inositol in pure solution.

When amounts of heart muscle of the order of 5 g. are subjected to the isolation process just described, the final solution obtained for estimation will contain about 0·2–1·0 mg. of inositol in 5 ml. In order to estimate amounts of inositol of this order with the requisite accuracy, it is necessary to modify slightly Young's original procedure, which was intended for use with 1–5 mg. of inositol. The modifications consist of heating the solutions in the water-bath in 60 ml. pyrex boiling-tubes, and of using 0·02 *N* iodine and 0·01 *N* sodium thiosulphate solutions for the final titrations instead of the solutions used by Young [1934, 1]. A heating period of 30 min. has been employed, as Young's curves [1934, 1] show the reaction to be practically complete in this time.

Solutions required.

Potassium iodo-mercurate solution, made by adding a solution of 288 g. of potassium iodide in water to 108 g. of mercuric chloride in water with shaking, and diluting the solution to 1 litre. The greenish-yellow solution obtained should be filtered if not perfectly clear.

30 % sodium hydroxide solution, stored in a Schellbach burette.

Barium sulphate suspension. 20 g. of the material marketed as "pure for X-ray examinations" suspended in 80 ml. of water. This must be well shaken just before use.

Sulphuric acid. 200 ml. of the pure concentrated acid diluted to 1 litre.

0·02 *N* iodine solution.

0·01 *N* sodium thiosulphate solution.

Starch solution.

Standard inositol solution, prepared from anhydrous inositol, obtained by drying crushed crystalline hydrated inositol at 100–110° to constant weight.

Procedure. Into a dry pyrex boiling-tube are pipetted 5 ml. of the solution to be estimated, containing not more than 1·0 mg. of inositol. To this are added 3 ml. of the iodo-mercurate solution from a 10 ml. micro-burette, 4 ml. of the 30 % sodium hydroxide and 2 ml. of the 20 % barium sulphate suspension from a wide-tipped pipette. After mixing the contents of the tube by gentle rotation, its mouth is covered with a glass ball and the tube placed in a boiling water-bath for 30 min., at the end of which time it is removed with as little disturbance of the contents as possible to a bath of cold running water for 5 min. From a burette 8 ml. of the 20 % sulphuric acid are then run slowly into the solution, and the contents mixed by gentle rotation. At the end of a further 5 min., 5 ml. of the 0·02 *N* iodine solution are added from a standard pipette and the contents of the tube well mixed by rotation and by stirring with a glass rod left in the tube. At the end of a further 10 min., with occasional stirring of the solution, this is transferred to a 100 ml. beaker and the excess iodine titrated with 0·01 *N* thiosulphate from a 10 ml. micro-burette (0·05 ml. graduations), using starch solution as the indicator.

The following equivalent titrations were obtained:

mg. of inositol	Equivalent titrations ml. of 0·01 *N* sodium thiosulphate
0·2	1·70
0·4	3·26
0·6	4·70
0·8	6·27
1·0	7·77

SUMMARY.

A modification of Young's method for the determination of inositol in animal tissues is described, suitable for use with amounts of tissue containing 5 mg. or more of inositol. Used in conjunction with a micro-modification of the same author's method for the determination of inositol in pure solution which is also described, the method gives 90 % recovery of inositol from tissues.

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