CXCII. THE DETERMINATION OF INOSITOL IN ANIMAL TISSUES¹.

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MANY attempts have been made to devise a satisfactory method of determining the inositol content of animal tissues. The nature of inositol and the absence of a simple method of estimating the substance have both contributed to the difficulties of the task. From necessity nearly all work has been directed towards isolating and weighing the inositol. The methods which were the outcome of early work were nearly all dependent upon the fact that inositol is precipitated by basic lead acetate. This principle was introduced by Marmé [1864] and more recently has constituted a stage in the methods of Rosenberger [1908], Starkenstein [1909], Needham [1923, 1] and Winter [1934]. The general procedure usually adopted involved the deproteinisation of the extract with a reagent such as normal lead acetate followed by precipitation of the inositol with basic lead acetate and ammonia. This lead precipitate was decomposed with hydrogen sulphide and the inositol thrown out of solution with alcohol. Needham [1923, 1] combined this process with an acetone extraction of the tissue and used a micro-combustion technique [1923, 2] for determining the carbon content of the isolated material. Winter [1934] destroyed the tissue with hot KOH solution, interposed a precipitation by baryta in alcoholic solution after the basic lead acetate precipitation, recrystallised the product obtained by decomposition of the baryta precipitate and weighed the pure inositol so obtained.

The development of the Fleury-Marque method of estimating inositol described in the preceding paper [Young, 1934] has made it possible to review the whole question of the determination of inositol in biological material. Primarily it must be recognised that any reagents capable of oxidising a compound as stable as inositol must inevitably be able to oxidise a wide range of substances. In this respect, however, potassium iodomercurate in an alkaline medium is much less catholic in its action than a reagent such as potassium permanganate. Thus Fleury and Marque [1929] found that although it could oxidise many organic hydroxylic compounds it showed either no action or only a feeble one in the case of compounds containing carboxyl groups as well as hydroxyl. In view of its lack of specificity it was felt that the iodomercurate method could only be applied with safety to the determination of inositol in tissues by combining it with an isolation process. The application of the iodomercurate method made it possible to check accurately the quantitative nature of all the processes involved

¹ A preliminary account of the work described in this and the preceding paper was made the subject of a paper given at the Biochemical Society meeting held on November 17th, 1933 [Young, 1933].

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in the isolation of inositol. Furthermore, it permitted the estimation of inositol in the presence of inorganic compounds, which is important on account of the insolubility of inositol in organic solvents.

The tissues chosen for detailed investigation were brain and heart-muscle, partly because of their widely different natures and partly on account of reports of their relatively high inositol contents.

THE ISOLATION OF INOSITOL.

The extraction process. Scherer [1850], who first isolated inositol, used water for extraction purposes, and this procedure was widely used by the earlier workers. By grinding brain tissues with normal lead acetate and water Müller [1857] made an extract from which he was able to isolate inositol. Rosenberger [1908] based his method on the stability of inositol to hot alkalis and destroyed the tissue with boiling potash solution. Momose [1916], acting on a suggestion of Rosenheim's, found that inositol can easily be extracted from fresh brain by means of acetone. Inositol is insoluble in acetone but is readily soluble in the aqueous acetone formed as the tissue is dehydrated. By combining the acetone extraction with the classical method of isolation Momose [1916] was able to obtain more inositol from brain than by applying the extraction procedures used by Scherer and Müller. Needham [1923, 1] used the acetone method and found that with butcher's beef-muscle no further amounts of inositol were obtained by a second extraction with acetone.

On the basis of the work of Momose [1916] and Needham [1923, 1] it was resolved to use the acetone method of extraction. It was found that when the tissue after acetone extraction was subjected to treatment with 70 % acetone it yielded an extract which contained inositol. The following experiments were therefore made with brain and heart-muscle. The minced tissue (50 g.) was first extracted with its own weight (1.25 vols.) of acetone for 24 hours. With this proportion of acetone and the water of the tissue an approximately 70 % concentration of acetone results. After filtration of the first extract (A) a second extraction with 1.25 vols. of aqueous acetone (70 % acetone) was made for 20 hours. This was filtered (B) and the tissue residue after being moistened with 70 % acetone was pressed on a small press, moistened with 70 % acetone, pressed again, and all the expressed liquid collected and filtered (C). The residue was then broken up and extracted again for 24 hours with 70 % acetone. This extract was filtered (D) and the tissue residue was finally extracted with water for 24 hours (E). The inositol content of each of these fractions was determined and yielded the following results:

Extract	mg. inositol found	
	50 g. brain (Ox)	50 g. heart-muscle (Sheep)
A	36.7	44.1
B	16.0	23.0
C	3.0	3.1
D	2.6	Trace
E	Trace	Trace

Thus, while extract A contained the greatest proportion of the inositol, considerable amounts were obtained in extract B. Extracts D and E showed only a trace of reduction on estimation, by far the greatest amount being obtained in the case of extract D from the brain. From these results it appeared that no serious inaccuracies would be introduced by confining the extraction to processes A, B and C.

The use of boiling 10 % KOH solution for disintegration of the tissue in the recent work of Winter [1934] on the estimation of inositol in heart muscle is noteworthy. Preliminary experiments by the author have shown that as an alternative to Winter's treatment the destruction of the tissue by KOH solution (70 g. KOH, 100 ml. H₂O) at the temperature of the boiling water-bath can be made the first stage of a method of estimation.

The precipitation of contaminating substances present in the extract. Normal lead acetate was the reagent most commonly used in the past for the removal of nitrogenous material and other contaminating substances present in tissue extracts prepared for the isolation of inositol. During recent years considerable attention has been directed towards the development of reagents preliminary to the determination of sugar. Certain other reagents which have come into use for the precipitation of nitrogenous substances in tissue extracts, *etc.* were studied with a view to finding the most suitable.

The Patein and Dufau [1902] mercuric nitrate reagent was used by a number of workers for the removal of interfering substances before sugar estimation, and Benedict and Osterberg [1918] showed that the mercuric nitrate-sodium bicarbonate treatment of urine yielded filtrates containing almost no measurable nitrogen. The disadvantage of this reagent lies in the large increase in the salt content of the filtrate which it produces, and on account of this it was ruled out for the present purpose. Smith [1932], in her study of the determination of sugar in bacteriological culture media, found that precipitation by phosphotungstic acid removed a larger proportion of non-sugar reducing substances than tungstic acid or any of the recognised mercury reagents. West *et al.* [1929], working on the determination of true sugar in blood, described a method of preliminary treatment which involved the use of mercuric sulphate in sulphuric acid followed by neutralisation with solid barium carbonate. The procedure was recommended by the authors for the preparation of filtrates from hydrolysed tissues. The reagent was subsequently modified slightly when applied to urine [West and Peterson, 1932]. On the basis of these considerations it was decided to compare the extent to which the alkaline iodo-mercurate reagent was reduced by filtrates prepared from identical tissue extracts by means of the West *et al.* mercuric sulphate method, phosphotungstic acid in sulphuric acid, normal lead acetate and mercuric chloride [Johnson, 1887].

Complete extractions of weighed samples of tissue (ox-brain and sheep's heart-muscle) were performed by the acetone method described above, the acetone was removed by distillation, the residual solution was extracted with ether and the resulting aqueous solution was made up to a known volume. Measured volumes of this solution were then submitted to complete precipitation by the methods under consideration and after removal of the excess of reagents the filtrates were brought to a slightly acid condition and estimated by the modified Fleury-Marque method. (H₂S was used for precipitating excess of mercury and lead and excess H₂S removed subsequently; baryta solution was added to neutrality in case of the excess phosphotungstic acid.) The following results were obtained:

Method tested	Reducing power of filtrate (expressed as mg. inositol) equivalent to 100 g. of	
	Brain (Ox)	Heart-muscle (Sheep)
Mercuric chloride	344	649
Normal lead acetate	318	601
Phosphotungstic acid in sulphuric acid	291	553
Acid mercuric sulphate	257	437

Determinations of the inositol content of the extracts gave results of 111.4 mg per 100 g. of ox-brain and 140.4 mg. per 100 g. of sheep's heart-muscle.

The results showed the acid mercuric sulphate to be the most effective reagent for the process under consideration and it was therefore studied further. Nine experiments with aqueous solutions of inositol (10, 25, 50 and 100 mg.) subjected to this precipitation gave an average recovery in the filtrate of 98.5 % of the inositol taken (limits 97.6–100 %). Five experiments with inositol added to brain and heart-muscle extracts showed an average recovery of 98.8 % of added inositol (limits 94–104 %). On account of these results and the rapidity with which it could be performed the acid mercuric sulphate-barium carbonate precipitation was adopted as a stage in the isolation process.

The precipitation of inositol. Basic lead acetate and ammonia were almost invariably used for this operation in the past. This procedure was not included in the isolation process under investigation since the use of ammonia is undesirable in view of the nature of the iodo-mercurate reagent. Thudichum [1901] described the precipitation of inositol by copper acetate but this was not found to be quantitative by Needham [1923, 1]. Buston and Pramanik [1931] reported the isolation of inositol from a baryta and alcohol precipitate obtained in the course of their work. The quantitative studies of this method by the author, described below, showed that it could be adapted for the present purpose.

The addition of alcoholic solutions of baryta to aqueous inositol solutions was found to give incomplete precipitation under the conditions tested, and saturation of the cold solutions with crystalline barium hydroxide followed by the addition of alcohol likewise gave unsatisfactory results. The solubility of barium hydroxide in water rapidly increases with rise of temperature, and it was felt that if a relatively large amount of this substance were to be dissolved in the hot aqueous inositol solution and then thrown out of solution by the addition of cold alcohol the chances of inositol being carried down by the precipitate might be enhanced. This was found to be so. Replacement of the cold alcohol by hot alcohol had little effect on the results. The inositol-baryta precipitate was found to be stable and showed only a slight tendency to dissociate on long standing (48 hours). Washing the precipitate with small amounts of cold absolute alcohol caused no detectable loss of inositol. An average precipitation of 96.3 % (limits 94.0–98.0 %) was found in twelve experiments with solutions (10 ml.) containing 10, 25 and 50 mg. of inositol. A slight excess of sulphuric acid was used to decompose the precipitates, and when this was done in hot solution followed by washing of the barium sulphate precipitate by hot water no loss of inositol by adsorption on the barium sulphate could be detected. In all the above experiments the solutions obtained after filtration of the barium sulphate were evaporated to dryness (after the addition of a slight excess of sodium carbonate solution) in order to remove traces of alcohol before estimation.

The crystallisation of inositol. The final stage of the isolation process is the throwing down of the inositol from solution in crystalline form. The earlier workers were accustomed to do this by addition of a large excess of alcohol with or without ether. Inositol is insoluble in acetone and since this liquid is employed in the extraction process it seemed that a purer product might be obtained by the use of acetone instead of alcohol for the crystallisation. An investigation was made of the precipitation of 10 and 25 mg. of inositol from 3 ml. of aqueous solution, and it was found possible to obtain 100 % precipitation of inositol in crystalline form by the addition of 30 ml. of acetone and 15 ml. of ether followed by refrigeration. The method was found to be equally effective when instead of using 3 ml. of neutral aqueous solution experiments were con-

ducted with 2 ml. of inositol solution together with 1 ml. of 2*N* H₂SO₄ or 1 ml. of glacial acetic acid.

Summary of the process. The method of determining inositol in tissues which was the outcome of the above work is as follows:

Minced tissue extracted with pure and aqueous acetone. Acetone removed from extract by distillation. Aqueous residue ether-extracted and subjected to the acid-HgSO₄-BaCO₃ treatment. Filtered

The nature of the material isolated from tissues by the new method.

Application of the method to brain and heart-muscle yielded good crystalline precipitates in the final stage, which on estimation behaved typically as inositol. The material showed no reduction with the Hagedorn-Jensen [1923] sugar reagent and either gave no Nessler precipitate or only a faint trace with the alkaline iodomercurate reagent. It gave positive Scherer [1850] and Fischler-Kürten [1932] tests for inositol. The crystals were not free from inorganic material and usually yielded incomplete melts, melting commencing at various temperatures from 210 to 219°. The ash content of the product estimated was lower when A.R. barium carbonate was used for neutralisation in the acid mercuric sulphate precipitation instead of the variety sold as "pure precip. by soda." The latter substance has a greater neutralising power for sulphuric acid than the former and this is probably due to its being contaminated by sodium carbonate. Two tissue estimations were performed using A.R. barium carbonate in the acid-mercuric sulphate precipitation and the products obtained by the complete precipitation of the solutions estimated were analysed (Schoeller).

(Found: Sample from brain (sheep) C, 39.74; H, 6.53; residue, 1.41; sample from heart-muscle (sheep): C, 39.92; H, 6.59; residue, 4.92; C₆H₁₂O₆ requires C, 40.00; H, 6.66 %.)

These results show that when the method is applied to brain and heart-muscle the product estimated is inositol. The contamination of the inositol by small amounts of inorganic compounds does not impair the accuracy of the iodomercurate estimation.

EXPERIMENTAL.

The details about to be described are for the estimation of inositol in 20–40 g. of tissue.

Extraction. The finely minced tissue is weighed and extracted in a closed conical flask with its own weight (1.25 vols.) of acetone. After 24 hours' extrac-

tion with occasional shaking the liquid is poured off through a glass wool plug contained in a filter-funnel. The plug is washed with a few ml. of 70 % acetone and then transferred to the flask. After 20 hours' extraction the liquid is separated and the tissue residue pressed on a small press. It is then moistened with 70 % acetone and pressed again. The first and second acetone extracts and the expressed liquid are combined and filtered on a Büchner funnel. All washings are done with 70 % acetone.

Distillation and ether extraction. The acetone is removed from the extract by distillation on a gently heated water-bath. The aqueous extract which is left is evaporated and filtered (volume 100–150 ml.) and is extracted twice with equal volumes of ether, a few drops of alcohol being added when necessary to break the emulsions which may form. The combined ethereal extracts are extracted with half their volume of water. This washing is combined with the aqueous extract, heated gently to remove ether, concentrated by evaporation, filtered and made up to 100 ml. in a volumetric flask.

The acid mercuric sulphate precipitation. The aqueous extract is poured into a dry pyrex litre conical flask. The 100 ml. volumetric flask is then rinsed with two successive portions of 5 ml. of water and these in turn allowed to drain into the conical flask. From a burette are then added slowly with shaking 30 ml. of the acid-mercuric sulphate reagent [West and Peterson, 1932] prepared by dissolving 27 g. of mercuric sulphate in 100 ml. of 10 % (by weight) sulphuric acid at 5° and separating the solution from the slight precipitate which forms as room temperature is reached. Sufficient solid barium carbonate is then added to neutralise the mixture (60–70 g. of the material sold as "pure precip. by soda" or 160–170 g. of A.R. barium carbonate). The flask is rotated for a short time until most of the carbon dioxide has escaped. It is then stoppered and shaken vigorously, care being taken to release the pressure at intervals. When no more pressure develops the mixture is tested with blue litmus paper, and if necessary more barium carbonate added with shaking until the mixture does not turn blue litmus red. It is then filtered on a dry Büchner funnel into a dry flask under reduced pressure and a carefully measured amount (75–95 ml. of the filtrate, which is water-clear and colourless, is transferred to a 125 ml. Phillip's beaker. It is heated on the water-bath and made just acid with two or three small drops of concentrated sulphuric acid. A steady stream of H_2S is then bubbled through the mixture for one minute and the precipitate of BaSO_4 and HgS filtered into a 250 ml. conical flask. The precipitate is well washed with hot water and H_2S removed from the filtrate by adding a small fragment of porous pot and boiling on a sand-bath for a short time.

The baryta and alcohol precipitation. The solution in the conical flask is transferred to a 100 ml. beaker and concentrated on the water-bath to 10 ml. To the hot solution 3 g. of crushed crystalline barium hydroxide are added, the liquid is heated on the water-bath for 5 minutes with occasional stirring and 40 ml. of ethyl alcohol are then added slowly with vigorous stirring. At one point in the process the contents of the beaker appear to solidify. The precipitate is allowed to settle at room temperature for 2–3 hours and then filtered under reduced pressure on a sintered glass filter (Schott and Gen. Jena –3G3). The beaker is rinsed twice with 5 ml. of absolute alcohol, and the washings are passed in turn through the precipitate on the filter. When filtration is complete the precipitate is transferred by means of a small spatula to the original 100 ml. beaker. The small residue on the filter is dissolved in hot water and added to the main precipitate (volume 40–50 ml.). The beaker and its contents are heated on the water-bath, a slight excess of hot 2N H_2SO_4 is added and heating is con-

tinued for 45–60 minutes. The barium sulphate is filtered and washed with hot water until the volume of the filtrate (contained in a 250 ml. beaker) is about 125 ml.

The acetone-ether precipitation. The solution in the 250 ml. beaker is concentrated to 10–15 ml., transferred to a 30 ml. beaker, concentrated to about 3 ml., transferred to a 50 ml. pyrex conical flask and carefully concentrated on an electrically heated sand-bath to 3 ml. After cooling, 30 ml. of acetone and 15 ml. of ether are added, the sides of the flask are scratched with a glass rod to induce crystallisation, the flask is firmly stoppered and placed in the ice-chest for 24–36 hours. The precipitate which forms is filtered under reduced pressure on a sintered glass micro-filter (Schott and Gen. Jena. –12G3) and washed with acetone and ether. The conical flask and micro-filter are dried at 100–110° for a short time, and the material on the filter and also that adhering to the flask are dissolved in water, filtered if necessary and made up to a known volume.

The estimation. The volume of the solution for estimation should be such that the 5 ml. used for the modified iodo-mercurate estimation [Young, 1934] contain less than 5 mg. of inositol. The amount of inositol in the sample of tissue taken is obtained by multiplying the determined content of the final solution by the factor obtained by dividing 140 by the volume of filtrate taken after the acid-mercuric sulphate precipitation.

Quantitative results.

In order to trace the liberation of the inositol from contaminating substances by the various precipitations experiments were done in which iodo-mercurate estimations were performed after each stage. The results were as follows:

	Reducing value (expressed as mg. inositol per 100 g. tissue)	
	Brain (ox)	Heart-muscle (sheep)
After acid-mercuric sulphate precipitation	330	522
After baryta and alcohol precipitation	170	226
After acetone and ether precipitation (inositol)	149	154

It had been hoped that the isolation process might be shortened by the omission of the acetone and ether precipitation, but the above results showed that this was not possible.

As a test of the quantitative nature of the method an aqueous solution of 20 mg. inositol was mixed with acetone so as to give a 70 % concentration of acetone and this was submitted to the whole process. The estimation indicated 18.0 mg. as the amount of inositol originally taken. A series of experiments was then conducted with different tissues in order to find the loss of inositol which occurs during the process. Equal weights (A and B) of the sample of minced tissue were treated identically except that a known amount of inositol was added

Exp. no.	Tissue	Weights taken g. (A and B)	mg. inositol added to B	Inositol found		% recovery inositol
				A	B	
1	Brain (ox)	80	50	118.9	165.6	93.4
2	Brain (sheep)	40	25	70.3	93.7	93.6
3	Brain (sheep)	20	25	34.4	57.4	92.0
4	Heart-muscle (sheep)	50	50	76.9	121.0	88.2
5	Heart-muscle (dog)	20	25	31.1	52.7	86.4
6	Skeletal muscle (ox)	20	25	<1.0	21.5	—
7	Skeletal muscle (rabbit)	40	25	10.9	32.3	85.6

to one portion (B) of the tissue before extraction. The average recovery of the added inositol in the experiments recorded below was 89.9 % (Exp. 6 is excluded from this calculation).

The determinations of the inositol content of several tissues were as follows:

Tissue	mg. inositol per 100 g. tissue			
Brain (ox)	(1) 149	(2) 111		
Brain (sheep)	(3) 176	(4) 172		
Heart-muscle (sheep)	(5) 154	(6) 157	(7) 170	
		(8) 140	(9) 158	
Heart-muscle (dog)	(10) 156	(11) 174		
Skeletal muscle (rabbit)	(12) 16	(13) 27		
Skeletal muscle (ox)	(14) less than 5			

Exps. (3) and (4) were performed with different weights (20 and 40 g.) of the same specimen of brain and Exps. (5) and (6) with equal weights (50 g.) of the same specimen of heart-muscle. They serve to show the reproducibility of results which the method permits. The amounts of material obtained in the skeletal muscle, Exps. (12), (13) and (14), were insufficient for satisfactory qualitative study. The method was found to be unsuitable for application to liver.

In view of the work of Winter [1934] on the *post mortem* changes in the inositol content of dog's heart-muscle it should be pointed out that all the determinations recorded above were performed on tissues which had been removed from the animals at least several hours previously. Whether or not the inositol content of the tissues had reached a steady state before the determinations were started is not known. Winter reported values up to 185 mg. inositol per 100 g. dog heart-muscle and this maximum figure is close to the results of Exps. (10) and (11). In the case of brain very few quantitative data can be found in the literature, but it is of interest to note that Thudichum [1901] reported the inositol content of the grey matter of human brain to be 193 mg./100 g. and of the white matter to be 217.1 mg./100 g., results which are somewhat higher than those found for ox and sheep brains in the present work.

SUMMARY.

The modified Fleury-Marque method of estimating inositol has been used to study the behaviour of inositol with various precipitating reagents. From the results obtained a new method of determining the inositol content of tissues has been evolved and a detailed investigation of its application to brain and heart muscle has been made.

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