L. STUDIES ON INOSITOL.

I. A METHOD OF QUANTITATIVE ESTIMATION.

By JOSEPH NEEDHAM (*Benn Levy Student*).

From the Biochemical Laboratory, Cambridge University.

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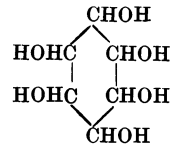
Inositol was first discovered in 1850 by Scherer [1850], who isolated it from Liebig's extract of meat and accurately determined its empirical formula. Between that date and 1864 it was studied by Cloetta [1856], Müller, W. [1857], Cooper-Lane [1861] and Marmé [1865] who gradually outlined the main features of the problem of its significance, ascertaining its distribution in plants and animals, and elaborating tests and methods of extraction. All the earlier workers regarded it as in constitution allied to the sugars, because of its empirical formula, $C_6H_{12}O_6$; and it was accordingly named "muscle-sugar," from one of its principal sources. Thus it was left for Maquenne to show in 1887 that inositol was not a sugar but a cyclic hexamethylene compound, or hexahydroxyhydrobenzene.



Ever since then, the obvious physiological implications of such a structure have attracted considerable interest, and the more so since the discovery of phytin, the calcium magnesium salt of inositolhexaphosphoric acid, in plants, by Palladin [1895], Winterstein [1897], Posternak [1903], and others. Throughout both periods all investigation has been greatly hampered by the lack of a reasonably accurate method of quantitative estimation, without which no real advance could be made. From this difficulty the plant chemists found a partial way out by determining the relative amounts of inorganic and organic phosphorus, though this could only give the most indirect knowledge of the metabolic significance of inositol. However, as far as the animal body was concerned, in spite of the lack of an estimation-method, Starkenstein [1909] concluded that inositol "has no special physiological significance in metabolism." Rosenberger [1910], from an experimental basis even more doubtful, put forward the contrary view that inositol subserved some most important function.

In view of such facts as these, it seemed desirable to continue the search for a method, as the really essential preliminary to an extended study of the general physiological meaning of the inositols.

ATTEMPTS TO EVOLVE A METHOD ON A NEW PRINCIPLE.

The first observation which seemed to be hopeful was found in the work of Neuberg [1908], who succeeded in obtaining furfural from inositol. Some such procedure as that used in the estimation of pentoses might have been possible, but on looking up the original paper, it was found that the change was anything but quantitative, necessitating dry distillation with phosphoric anhydride, and only yielding very small amounts of furfural. Rosenberger in 1910 had hoped that the hexacetate of inositol prepared by Maquenne in 1887 might be applicable to a quantitative method, but this also seemed useless, because of the tri- and tetra-acetates formed at the same time—at any rate for the minute quantities of inositol found in tissue extracts.

A colorimetric method would have been most desirable so the colour-tests for inositol were examined carefully. The most obvious thing about them is that they all depend on the reactions of the oxidation-products of inositol, rhodizonic acid, and other quinonoid bodies. Scherer's original test [1852], modified successively by Mayer [1907] and Salkoffski [1910], consists essentially of a rapid oxidation with strong boiling nitric acid, followed by the formation of calcium rhodizonate (?) which is brick-red or pink in colour. The reactions of Meillière [1906, 1, 2, 3] and of Gallois [1865], are exactly the same in principle, but the red mercury salt is formed instead; while Perrin's test [1909] depends on the formation of a reddish silver salt. Denigés [1907], after oxidation with nitric acid, adds potassium hydroxide, sodium nitrocyanide, and acetic acid; the solution is said to change from blue to brown to red; Seidel [1884, 1887] reported good results from the use of strontium acetate. Hugo Müller's procedure is the only one which does not utilise oxidation with nitric acid, for here hydrogen peroxide is used instead in the presence of a ferrous salt in traces [1907, 1912].

The writer's experiences with these tests were far from satisfactory. Müller's reaction could be ruled out at once for the purpose in hand, for the products were known to be many and various, oxalic and other acids being produced as well as quinones. In spite of the utmost care it was impossible to get Denigés' test to work at all, and the same applied to the test introduced by Seidel. Seidel's paper was unfortunately inaccessible to the writer, but he was informed by Dr Rosenheim that in the original description of the test, strontium acetate does not appear, and the text-books have described it wrongly. It is surprising that the "Analyse des Harns" of Neubauer and Vogel, usually so accurate, should have fallen into this mistake. What Seidel really used was sodium acetate and barium chloride, in which case the reaction is simply a variant of the Scherer test and is easy to perform.

The Scherer reaction itself, the oldest and most used of all, seemed at first likely to be of some use quantitatively. It appears that a chloride (Ba, Ca, Sr, Al) is necessary, as well as traces of a catalyst such as platinum or a mild oxidising agent like chloramine-T. With the aid of these sugges-

tions and of Mayer's modification, the comparative delicacy of the test was investigated, and it was found that only down to 1 in 3000 were the results uniform and constant. Below that dilution, unknown factors seemed to possess great influence. Furthermore, Mayer [1907] reported failure of the test with a sample of pure inositol, and it was found in this work that quite small quantities of lead salts completely inhibited the reaction. The reaction could not be applied, then, to the estimation of inositol in tissue extracts, or in any even moderately complex mixture.

In consequence, it was necessary to fall back on the classical method for the isolation of inositol; precipitation with basic lead acetate and subsequent decomposition with hydrogen sulphide. This has been used in the past by many workers, including Marmé [1865], who introduced it; Gallois [1864], Külz [1876], Rosenberger [1908, 1, 2] and Starkenstein [1909]. As the basis for a method of estimation it suffered from the fact that it had never been shown to be quantitative, though Meillière [1906, 2] assumed that it was, and Starkenstein [1909] referred to it as "fast quantitativ." Meillière [1907] had reported that the copper acetate compound of inositol studied by Thudichum was insoluble, and could be quantitatively precipitated: the writer's experiences with this are recorded below. In such attempts at quantitative work as had been done, the lead precipitation had been used in the course of "estimation by weighing the isolated product." This of course is an inaccurate procedure, and requires the taking of large amounts of initial material, thus effectively masking any small variations in the tissue; to say nothing of the time consumed in working up the resulting extracts. It therefore occurred to the writer that if the lead precipitation were united to a good method of extraction, and to a micro-carbon estimation at the final stage, some progress might be made with the general problem. The following experimental work was accordingly carried out.

The process of extraction.

All the earlier workers were accustomed to use extractions of hot and cold water. W. Müller [1857] departed from this practice, however, by grinding wet brain tissue with neutral lead acetate, and Rosenberger [1908, 2] by boiling the tissue with potash, and subsequent alternate treatment with nitric acid and baryta. Both methods were most unsatisfactory; the former because it gave very impure samples of inositol, and the latter because of its drastic nature—"any inositol, which has survived this prolonged treatment," as Momose [1916] says, "being precipitated by lead in the usual way." Momose, working on a suggestion of Rosenheim's, discovered that very good yields of inositol from brain tissue were to be obtained by working up the first watery acetone extract. By this means, inorganic salts, known to interfere with the lead precipitation, were much reduced in quantity, and, as very little else besides inositol was extracted, the crude product was purer.

To find whether this procedure was, or could be made, quantitative, 6 kilos. of butcher's beef muscle was carefully freed from fat, finely minced, and extracted with acetone in the proportion of 1·25 litres per kilo. This, of course, amounts to extraction with dilute acetone, since the muscle contains about 70 % water. After standing all night the strained and filtered liquid was evaporated under diminished pressure to remove all the acetone, and the aqueous residue was worked up for inositol in the usual manner, *i.e.* precipitation successively with neutral, then with basic, lead acetate; decomposition with hydrogen sulphide, and finally precipitation with alcohol and ether. During the stages of the process, qualitative tests were made to show the presence and behaviour of other possible extractives; these are given in Table I.

Table I.

	After removal of acetone	After precipitation with neutral lead acetate	After precipitation with basic lead acetate	After addition of alcohol (absolute)
Reducing sugar	–	–	–	–
Inositol	+	+	+	+
Creatinine	+ +	+ +	+	–
Creatine	+	+	–	–
Glutathione	+ +	–	–	–
Glycogen	–	–	–	–
Lactic acid	–	–	–	–
Cholesterol	–	–	–	–
Purine bases	–	–	–	–

Meanwhile the tissue was again extracted by shaking with acetone in the same proportions for about four hours and allowing it to stand thus overnight. The second acetone extract was treated in exactly the same manner as the first, except that 2500 cc. of water was added to compensate for the absence of the tissue water in the first instance. Then the tissue was extracted a third time, but with water. The results appear in Table II.

Table II.

		Amount of inositol isolated in g.
Extract 1.	Acetone and water	0·935
„ 2.	„ „	nil
„ 3.	Water only	nil

It was afterwards found that tissue should not be allowed to remain in the acetone longer than three days; for cholesterol begins then to be extracted in spite of the water present.

It seems justifiable, then, to conclude from these data, that the process of extraction with dilute acetone succeeds in removing from the tissue all the inositol which it contains. The sample obtained in the experiment given above melted at 221° and when examined in the polarimeter, in comparison with a sample of *i*-inositol prepared from phytin, failed to exhibit any optical activity. The conclusion is that the inositols of ox muscle, ox brain, human brain, and phytin, are identical, the last three having been shown to be so by Momose [1916].

The process of precipitation.

To prepare a supply of inositol, which could be drawn upon for blank experiments, a quantity of commercial phytin was procured and hydrolysed under pressure, bearing in mind the experiences of Plimmer and Page [1913] with organic phosphorus esters. Into hard glass tubes were placed 50 g. of phytin, with 200 cc. of 35 % sulphuric acid; these were sealed and heated for nine hours at 170°. Afterwards, the magnesium and calcium sulphates were filtered from the reaction mixture, the sulphuric acid removed as barium sulphate, excess of baryta as barium carbonate, and the inositol brought down by the addition of alcohol. From 50 g. of phytin, 8·4 g. of inositol were obtained; or about 84 % of the theoretical yield, assuming that Anderson's formula for phytin is correct [1912]. This inositol (M.P. 219°) was used for the following series of experiments. Since, as has been noted above, traces of lead interfere with the Scherer reaction, it was not possible to depend on it as an indication of the presence of inositol, and in each case it was necessary to evaporate the solutions to low bulk and precipitate the inositol, if it was present, with absolute alcohol or ether.

In order to approximate as much as might be to the actual conditions of tissue extracts, the concentration was made about the same as it would be expected to be from 200 g. of muscle. 8 cc. of a standard 0·5 % inositol solution were diluted to 140 cc. with distilled water, thus allowing for the water of the tissues. To this the saturated basic lead acetate solution was added, the conditions being varied. A preliminary test was done to see if the addition of 500 cc. of acetone, and its removal, had any effect on the inositol content. *A priori*, this was most unlikely, as inositol is insoluble in acetone; and colour-tests showed that it was not the case.

Table III.

	Inositol found in the filtrate after decomposition of lead compound with H_2S and precipitation by alcohol
Basic lead acetate, saturated solution boiled	
equal quantity, or less	+ + +
excess	+ +
excess + concentrated ammonia 3 cc.	+ +
excess, reprecipitated 3 times	+ +
excess left to stand three days	+ + +
Basic lead acetate, saturated solution unboiled	
equal quantity, or less	+ −
excess	+ −
excess + concentrated ammonia 3 cc.	−
excess + reprecipitated three times	−
excess left to stand three days	+ +

Inositol tends to be held, perhaps in an adsorbed state, by the precipitate of lead sulphide, requiring two extractions with water at about 70° to remove it completely. It is readily adsorbed by Fuller's earth, though not by kieselguhr. Several points are clear from the above results. In the first place, Momose's observation that the lead acetate compound dissociates on prolonged washing receives corroboration from the fact that the precipitation

seems to fail when the mixture is not filtered for three days. The ordinary hydrolysis of the basic salt to the hydroxide is presumably much intensified by boiling, hence the importance of using a cold unboiled saturated solution. Meillière showed that the presence of ammonia was important in the precipitation, and his observation was fully confirmed. The quantities of inositol appearing on the addition of absolute alcohol were too small to be weighed; and when no trace of cloudiness appeared, the result was recorded as negative.

To effect a further separation the well-known insolubility of inositol in alcohol was made use of. By evaporating to low bulk (less than 20 to 30 cc. if quantities of the order of 40 mg. as here, are present) and the addition of 300 cc. of absolute alcohol, the inositol present is all precipitated, and the resulting slight cloudiness flocculates well after half-an-hour's standing. In this connection the statement of Cooper-Lane [1861] could not be confirmed. He considered that if too much alcohol was added, the inositol failed to come out of solution; but this was never found to be the case if the watery solution was taken down to a sufficiently low bulk. Reprecipitation after adding a little water to the filtered liquid, and distilling off the alcohol, never gave any further cloudiness.

After standing overnight to ensure complete precipitation of the inositol, it is filtered off from the alcohol by passing it through a carefully prepared Gooch crucible, with very small holes, containing a layer of the cleanest asbestos about three millimetres thick. The air current drawn through by the filter-pump is allowed to pass for 10 minutes to make sure that the asbestos and inositol are quite free from alcohol; and the content of the crucible is then quantitatively transferred to a 500 cc. flask in which it is shaken with about 60 cc. of distilled water. At the end of 10 minutes the inositol is completely dissolved and the asbestos is then filtered off. The solution contains all the inositol from the tissue taken—and nothing else—and, after being made up accurately to 100 cc. is ready for estimation by the micro-carbon part of the method. Numerous tests, carried out on the various stages of this part of the whole method, showed that the inositol could be transferred thus from alcohol to water, without the least loss.

The lead precipitation described above proved satisfactory and could always be relied on. Thudichum [1864], considered precipitation by copper acetate to be quantitative if the inositol solution was evaporated to low bulk on a water-bath with about twice its volume of saturated copper acetate solution, until no more of the green flocculent precipitate was formed. The degree of accuracy which this method possesses can be gauged from the following typical example:

Calculated amount of inositol in the solution per cc. = 25 mg.
Found by the copper precipitation = 24·2 mg.

The method gives low results when checked against the lead precipitation, and is more limited in its application than the latter, since it fails in the presence of traces of sugar.

The process of microestimation of carbon.

Faced with the problem of estimating these small quantities of inositol in pure solution, it occurred to the writer that the quickest way of proceeding would be to ascertain the carbon-content by means of a micro-combustion method, and to calculate the inositol content from that. Evaporation and subsequent weighing in a platinum dish would of course have solved the difficulty, but the time required in drying to constant weight seemed to contraindicate this method. The method used [see Needham, 1923], cannot be termed a wet combustion in the strict sense, for that would imply one of the chromic acid oxidation methods; what was done was to evaporate a known amount of the solution in the actual boat of the combustion furnace.

The micro-methods collected and described by Pregl [1917] and others, were here of little use, for they all apply to combustions of the dry material, and to deal quantitatively with such small amounts of it as 20 mg. or less, would be very difficult. An expensive microchemical balance would be necessary for the preliminary weighing out, and the final measurement, being gravimetric, would estimate such small quantities of carbon dioxide less well than a burette measurement. These difficulties were overcome by the aid of a method of combustion from solution, and the measurement of the gas evolved, by volume.

In order to check the accuracy of the whole process, from beginning to end, it was necessary to carry out all the operations on a solution of known strength. For this purpose 8 cc. of a 0·4 % solution of inositol were taken, diluted to 140 cc. and the whole method proceeded with as described above. The calculated concentration of inositol in the final pure aqueous solution amounted to 32·00 mg. %. From 1 cc. of the liquid 0·231 cc. of CO_2 were given off on combustion. This, when translated into terms of concentration of inositol, is equivalent to 31·70 mg. %.

The method applied to some tissues of the rabbit.

Two rabbits, *A* and *B*, *B* freshly killed, and *A* dead two days before, were taken and estimations performed as described: on thigh-muscles, heart, kidney and liver. The following figures were obtained:

Tissue	Rabbit	Cc. of CO_2 finally evolved	Tissue originally taken in g.	mg. % Of final solution	mg. % Of original tissue	g. per kilo
Muscle	*A*	0·241	100·0	16·1	16·1	0·16
„	*B*	0·171	121·0	11·5	9·3	0·10
Heart	*A* and *B*	0·234	15·5	10·9	100·6	1·00
Liver	*B*	0·209	85·5	15·6	16·3	0·16
Kidney	*A* and *B*	0·163	35·5	14·0	30·6	0·30

Until more extended investigations have been made, it is impossible to appraise the exact significance of the above figures. For example, the individual variations of normal rabbits are as yet unknown. At the same time, while bearing this in mind, it is certainly interesting that the muscle from the

freshly-killed rabbit contained 50 % less inositol than that from the rabbit which had been dead two days. It recalls the view of Rosenberger [1908, 1, 1910], that tissues contain an "inositogen"; though his conclusions were drawn from the comparison of fresh rabbit muscle, and commercial beef; tissues perhaps hardly comparable. Unfortunately, there are no previous figures with which to compare the above. But it is worth remembering that Marmé [1864], reported specially good yields of inositol from heart muscle.

Obviously, the applicability of the method as a whole, may not be general. In extracts of plant tissues, for example, or of animal tissues, not yet investigated, there might well be substances present, which, extracted by dilute acetone, and carried down by basic lead acetate, would seriously affect the final result. But as far as the data go at the time of writing, the general principle seems safe.

Summary.

1. A method is described for the estimation of *i*-inositol in animal tissues.

2. Preliminary results, obtained by the aid of the method on some of the tissues of the rabbit, are given.

3. The inositol of ox muscle is identical with that which is produced by the hydrolysis of phytin.

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