CXCI. THE VOLUMETRIC DETERMINATION OF INOSITOL.

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THE lack of a simple method of estimation has greatly hindered the quantitative study of inositol, and most workers in this field have been obliged to adopt the laborious procedure of isolating and weighing the substance. A survey of the chemistry of inositol shows few reactions likely to be of value in attempting to evolve a method of estimation, and the difficulties encountered in endeavouring to apply quantitatively the colour tests and certain other reactions of inositol have been described by Needham [1923, 1]. One outstanding departure from the gravimetric method was due to Needham [1923, 1, 2], who determined the carbon content of the isolated material by a micro-combustion method.

Fleury and Marque [1929], as one of the analytical applications of potassium iodomercurate in alkaline solution, described a method of estimating polyhydric alcohols and related substances, including inositol. The oxidation of reducing sugars by potassium iodomercurate in hot alkaline solution was first applied analytically by Sacchse [1876], and Rupp [1905] made use of the reduction of iodomercurate in an alkaline medium to metallic mercury by the action of reducing compounds in his iodimetric method of estimating mercury. This procedure was in turn adapted by Bougault and Gros [1922] for the estimation of aldehydes. In the case of glucose the amount of mercury reduced indicated that the alcoholic groups were also being attacked. This led Fleury and Marque to study the action of alkaline solutions of potassium iodomercurate on certain hydroxylic compounds containing no aldehyde groups. They showed that the reaction was such that a number of these compounds, inositol among them, could be estimated by a modification of the technique used by Rupp [1905]. The course of the reaction was found to be dependent upon the concentration of potassium iodomercurate, the degree of alkalinity and the time of heating, for all of which they determined the optimum conditions.

The procedure adopted by Fleury and Marque [1929] involves heating the solution of the substance to be estimated at the temperature of the boiling water-bath with solutions of potassium iodomercurate and sodium hydroxide, with the addition of a suspension of barium sulphate. As the oxidation of the substance proceeds metallic mercury is deposited in the layer of barium sulphate at the bottom of the reaction flask. After a given period of heating the mixture is cooled and acidified, and standard iodine solution is added. Only a comparatively small excess of iodine is required to bring the mercury into solution on gently rotating the flask. This is due to the very finely divided form in which it has been maintained by the barium sulphate. Finally the excess of iodine is titrated with sodium thiosulphate solution.

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With their method Fleury and Marque found that 10 mg. of anhydrous inositol are equivalent to 7.34 ml. of 0.1 *N* iodine solution (calculated from all values obtained for different quantities of inositol from 1 to 20 mg.). The results which have been obtained by this method compared with those recorded by Fleury and Marque [1929] are as follows:

mg. of inositol	Equivalent titrations—ml. of 0.1 <i>N</i> sodium thiosulphate (or iodine) solution	
	Fleury and Marque	Present author
1	0.67	0.67
5	3.67	3.70
10	7.39	7.41
15	11.03	11.04
20	14.33	14.49

In making quantitative studies of inositol in biological material [Young, 1934] it became necessary to estimate small amounts of inositol with much greater accuracy than is permitted by the original procedure. On account of this the Fleury-Marque method was modified in order to estimate 1–5 mg. of inositol more accurately than was hitherto possible. The details of this method, which embodies essentially the same technique as that of Fleury and Marque, are described below (I).

Owing to the fact that the iodo-mercurate method can also be used for estimating glucose it was felt desirable to make an investigation of the determination of inositol in the presence of this substance. Since the two compounds fractionate in a similar manner, it was not found possible to separate them by precipitation methods. By subjecting the mixture of the two substances to yeast fermentation it was found possible to recover the inositol after breakdown of the glucose, and the details of this work are given in a later section of this paper (II).

I. THE MODIFIED METHOD OF ESTIMATING INOSITOL.

The quantities and strengths of reagents used in the modified method compared with those employed in the original are as follows:

	Original method ml.	Modified method ml.
Inositol solution	7.5	5.0
Potassium iodo-mercurate solution	7.5	3.0
Sodium hydroxide solution	10.0	4.0
Barium sulphate suspension (10 %)	10.0	—
Barium sulphate suspension (20 %)	—	2.0
Sulphuric acid solution	20.0	8.0
Iodine solution (0.1 <i>N</i>)	20.0	5.0

In place of the 0.1 *N* sodium thiosulphate solution used for titration of the excess of iodine by Fleury and Marque, 0.02 *N* solution is used in the modified method.

It will be seen that the same concentrations of iodo-mercurate and alkali have been retained. In order to determine the optimum period of heating a series of time experiments was performed with 2.5 mg. and 5.0 mg. of inositol. The results (Fig. 1) indicate that the reaction is almost complete at the end of 45 minutes' heating in a boiling water-bath. The rate of reaction after this period is so slow that small errors in the time of heating and subsequent rate of cooling of the reaction flask and its contents have no significant effect on the results of the estimation.

The equivalent titrations for 1–5 mg. of inositol obtained by three observers¹ using the modified method and working with independently prepared reagents are given below, each of the mean figures in the last column being obtained from at least twelve determinations.

Fig. 1.

Fig. 2.

mg. of inositol	Equivalent titrations—ml. of 0.2 N sodium thiosulphate solution			
	L. Y.	J. C.	F. G. K.	Mean
1	2.75	2.76	2.85	2.79
2	6.35	6.28	6.25	6.29
3	9.90	9.90	9.80	9.87
4	13.38	13.47	13.25	13.37
5	16.72	16.82	16.73	16.76

¹ The author is indebted to Dr J. Chapman and Mr F. G. Kent for their assistance in this matter.

The relationship between the equivalent titration and weight of inositol estimated is illustrated in Fig. 2. It yields a graph which closely approximates to a straight line over the range 1–5 mg.

Reagents. The following reagents are used in the estimation:

Potassium iodomercurate solution. A solution of 288 g. of KI in water is added with shaking to 108 g. of mercuric chloride in water and made to 1 litre. The greenish yellow-coloured solution obtained is filtered unless perfectly clear.

Sodium hydroxide solution. 300 g. of pure sodium hydroxide are made to a litre with water. This solution is stored in an automatic Schellbach burette with the usual precautions.

Barium sulphate suspension. 20 g. of finely powdered barium sulphate (the form marketed as "Pure for X-ray examinations") are suspended in 80 ml. of water. This must be well shaken immediately before use.

Sulphuric acid solution. 200 ml. of concentrated acid are made to 1 litre with water.

0.1 *N* iodine solution.

0.02 *N* sodium thiosulphate solution. Prepared by diluting 0.1 *N* solution daily as required.

Starch solution.

Inositol solutions. Owing to the fact that hydrated inositol ($C_6H_{12}O_6 \cdot 2H_2O$) is efflorescent, all standard inositol solutions are made from the anhydrous material. This is readily obtained from the crushed crystalline hydrate by heating it at 100–110° until constant in weight.

Procedure. Into a dry 100 ml. pyrex conical flask are pipetted 5 ml. of the solution to be estimated, containing not more than 5 mg. of inositol. Next, 3 ml. of the potassium iodomercurate reagent are introduced followed by 4 ml. of the 30 % NaOH solution. Lastly, 2 ml. of the well shaken barium sulphate suspension are added and the contents of the flask mixed by gentle rotation. The flask is firmly clamped in a level position in a boiling water-bath with the level of the water of the bath well above the level of the contents of the flask. As the reaction proceeds a red precipitate forms first and is soon replaced by a grey-black precipitate of mercury. After 45 minutes' heating the flask is removed and clamped in a bath of running water. After cooling for 5 minutes 8 ml. of the 20 % H_2SO_4 solution are added without moving the flask. After 5 minutes longer in the cooling bath the flask is removed and 5 ml. of 0.1 *N* iodine solution are measured into it. By carefully rotating the flask at intervals during a period of 10 minutes the mercury is readily brought into solution. Any undissolved mercury can be seen as a grey-black precipitate collected at the centre of the bottom of the flask on viewing the flask from underneath immediately after rotating it. At the end of the 10 minutes the excess of iodine is titrated with 0.02 *N* sodium thiosulphate solution, freshly prepared starch solution being added as the end-point is approached. The presence of the barium sulphate does not interfere with the detection of the end-point. The result obtained by direct titration of 5 ml. of 0.1 *N* iodine solution is used for the calculation of the equivalent titration, since it is found to be identical with the value obtained in a blank estimation.

Further experimental details. A series of experiments revealed that the slight errors of measurement resulting from the use of a 10 ml. micro-burette (0.05 ml. graduations) for measuring the potassium iodomercurate solution and a Schellbach burette (0.10 ml. graduations) for the NaOH solution did not have any detectable effect on the estimations. It is advisable to use a wide-tipped 2 ml. pipette for the addition of the barium sulphate suspension. The sulphuric acid solution is added from a burette and a standard pipette is used for the iodine solution. Care must be taken to avoid any undue disturbance of the contents of the reaction flask from the commencement of heating until the addition of the iodine solution. The reagents should be checked against standard inositol solutions periodically. Under ordinary conditions any deviations found are small and when necessary calculated corrections can be applied.

II. THE ESTIMATION OF INOSITOL IN GLUCOSE-INOSITOL MIXTURES.

Since glucose can be estimated by the iodomercurate method described above, the components of a glucose-inositol mixture must be separated before the inositol can be estimated. This separation is not readily brought about by chemical means and in consequence of this a study was made of the removal of glucose from such mixtures by fermentation methods.

The use of yeast fermentation by a number of workers for the determination of true sugar in blood has led to the recognition of certain precautions which must be observed in such quantitative investigations. The subtraction of the "residual reduction" value obtained after alcoholic fermentation of blood from the "apparent sugar" value was introduced by Otto [1885] for the determination of true sugar in blood. The causes of the conflicting results obtained by various authors have been reviewed by Hiller *et al.* [1925] and Somogyi [1927]. Baker and Hulton [1933] have recently studied the removal of sugars from dilute solutions by fermentation. Wholly satisfactory results were not obtained until Somogyi [1927] drew attention to the need for preliminary purification of the yeast suspensions used in the fermentation. Somogyi purified his yeast by suspending the fresh commercial product in water and repeatedly washing it by centrifuging until a supernatant liquid was obtained which yielded no reduction with the sugar reagent.

In the present work it was decided to remove the yeast by filtration on a sintered glass filter and to follow this by washing the yeast under standard conditions. This procedure has the following advantages: (i) it avoids the need for correcting for the cell volume of the yeast, (ii) it yields the whole of the solution, (iii) it is rapid.

Procedure. The fermentation technique which was the outcome of the above considerations was as follows: 10 ml. of the solution to be fermented contained in a 30 ml. pyrex beaker covered by a watch glass were incubated at 37°, and 10 ml. of a 20 % yeast suspension at room temperature (prepared as described below) were added and the mixture incubated at 28°. The mixture was stirred at intervals by a glass rod left in the beaker, and after the requisite period the yeast was filtered by suction on a sintered glass filter (Schott and Gen., Jena. -1G4). The beaker was successively rinsed with three approximately equal portions of water, making 10 ml. in all, and the rinsings were passed in turn through the filter. The whole filtration process took about 5 minutes. The filtrate was evaporated to dryness on the water-bath to remove alcohol (which reduces the iodomercurate reagent), redissolved in a little water, evaporated dry again and finally dissolved in water and estimated.

Preparation of the yeast suspension. Fresh baker's yeast was suspended in five times its weight of water and centrifuged, and portions of the supernatant liquid were estimated by the modified iodomercurate method after filtration through the sintered glass filter. By this means it was found that a progressive decrease in the amount of reducing substances occurred during the first four or five washings and that after this the reducing value of further washings tended to approach an approximately constant value equivalent to about 0.1 mg. inositol per g. of yeast being washed. When this state in the washing process was reached the yeast was suspended in four times its weight of water and was ready for use. When not in use it was stored in the refrigerator and was allowed to come to room temperature before being employed in fermentation experiments. The water was centrifuged off and replaced at intervals as recommended by Somogyi [1927].

The rate of fermentation of glucose. In order to determine the rate of breakdown of glucose under the conditions described above a series of experiments was done with 10, 25, 50 and 100 mg. of glucose and 5, 10, 20 and 30-minute periods of fermentation. For each period a blank experiment was performed. The blanks were finally made to 50 ml. and 5 ml. portions estimated. The small reduction values which they gave are shown in the table below:

Time of incubation mins.	Equivalent titration— ml. of 0.02 <i>N</i> sodium thiosulphate
5	0.11
10	0.16
20	0.20
30	0.22

The residual reduction values obtained after fermentation of the glucose, corrected for the above blanks were as follows:

mg. of glucose taken	mg. of glucose found after fermentation period of			
	5 mins.	10 mins.	20 mins.	30 mins.
10	—	—	—	—
25	1.7	—	—	—
50	16.0	—	—	—
100	42.1	21.2	1.2	1.6

The residual reductions are expressed in terms of glucose, the equivalent titrations for 1–5 mg. of glucose by the modified iodo-mercurate method having been found to be as follows:

mg. of glucose	Equivalent titration— ml. of 0.02 <i>N</i> sodium thiosulphate
1	3.07
2	6.31
3	9.84
4	13.32
5	16.69

It will be noted that these values are very close to those obtained for the same weights of inositol.

The action of yeast on glucose-inositol mixtures. A recovery of 24.9 mg. was obtained in an experiment in which 25 mg. of inositol were incubated with yeast for 20 minutes under the conditions described above, and this showed that no significant loss of inositol occurred during the process. Experiments were then done in which solutions containing inositol (25 mg.) and varying amounts of glucose (10, 25 and 50 mg.) were submitted to the fermentation procedure for periods long enough to permit the breakdown of all the glucose. The results were as follows:

Time of fermentation mins.	mg. of glucose taken	mg. of inositol taken	Residual reduction (expressed as mg. inositol)
5	10	25	25.1
10	25	25	25.1
20	50	25	25.2

With a mixture of 25 mg. inositol and 100 mg. glucose (30 minutes' fermentation) the results were less satisfactory, a reduction equivalent to 26.1 mg. of inositol being obtained after correcting for the residual reduction resulting from 30 minutes' fermentation of this weight of glucose. In the case of glucose-inositol mixtures in which the glucose does not preponderate so largely the results indicate that accurate determinations of the amounts of inositol present can readily be performed by the application of a yeast fermentation technique such as that described above.

SUMMARY.

1. A modification of the Fleury and Marqué method of estimating polyhydric alcohols and related substances is described which permits 1–5 mg. of inositol to be estimated with greater accuracy than is possible by the original procedure.

2. The estimation of inositol in glucose-inositol mixtures is considered, and a technique for the rapid fermentation of the glucose by yeast is described which

gives accurate results when applied to the estimation of inositol in mixtures containing as much as twice its own weight of glucose.

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