LXIII. THE ESTIMATION OF ALLANTOIN IN URINE IN THE PRESENCE OF GLUCOSE.

By ROBERT HENRY ADERS PLIMMER
AND RUTH FILBY SKELTON.

From the Ludwig Mond Research Laboratory for Biological Chemistry, Institute of Physiology, University College, London.

(*Received Nov. 13th, 1914.*)

It has been shown by us in a previous communication [1914] that the urea and allantoin in urine can be estimated by Folin's magnesium chloride method; the urea is estimated by means of urease and the difference between the two values is the amount of allantoin present. Glucose in the urine does not affect the determination of urea by means of urease, as shown by Marshall [1913], but it causes a loss of from 9–30 per cent. of the nitrogen of the urea when Folin's magnesium chloride method is used, as shown by Mörner [1903]. The humin which is formed by heating the glucose in urine with hydrochloric acid contains nitrogen [Udransky, 1888]. The same error applies to the estimation of allantoin. Mörner overcame the difficulty in the case of urea by precipitating the glucose with baryta mixture, alcohol and ether, and estimating the urea in the filtrate, but this procedure does not serve for the estimation of allantoin, since it is precipitated with the glucose [Mörner, 1903; Haskins, 1906].

Wiechowski's method for the estimation of allantoin in urine depends upon the precipitation of allantoin as mercury compound after pigments, chlorides and other compounds have been removed, and is not affected by the presence of glucose, but for this method large amounts of urine are required and the estimation takes several days to perform.

The Folin method if it could be adapted to the estimation of allantoin would be of advantage, in that only small quantities of urine are required, which is important when other estimations, *e.g.* of "acetone bodies," have to be performed, and it would be of further advantage in that the results are quickly obtained.

In order to make use of the Folin method it is essential that the glucose be removed from solution. This removal of glucose was found to be a more troublesome process than was anticipated. Several methods were attempted before it was found that the glucose could be completely precipitated by basic lead acetate and sodium hydroxide.

(1) *Removal of Glucose by Fermentation.*

The simplest way of removing glucose seemed to be by fermentation by yeast. Mörner had already tried this method and found it not to be entirely satisfactory. Some preliminary experiments indicated that this method might be possible. The estimation of urea and allantoin was carried out on 5 cc. of urine, and on 5 cc. of urine to which 5 cc. of a 5 per cent. solution of glucose had been added and removed by fermentation. Our data in cc. of 0·1 N ammonia were:

Urine		Urine + Glucose		Urine		Urine + Glucose	
15·0	15·15	15·3	15·15	49·4	49·7	49·7	49·55
15·3		15·0		50·0		49·6	

Further experiments did not give such satisfactory results:

16·2	16·25	15·9	15·7	53·2	53·15	52·2	51·9
16·3		15·3		53·1		51·6	

It was possible that in these experiments the conditions for the fermentation were not suitable, and it seemed to us that if the conditions for complete fermentation could be found the removal of glucose by the action of yeast would be possible. Numerous experiments were made to test the rate of fermentation of glucose by yeast. 10 cc. of 1, 2, and 3 per cent. solutions of glucose in water were fermented with 1 to 10 cc. of 1 to 5 per cent. suspensions of yeast in water for periods of 6 to 20 hours; the solutions were filtered and the filtrates tested for glucose. The results were extremely variable, as is seen in the following summary:

Series of expts.	Per cent. of glucose solution	Per cent. of yeast suspension added	Number of cc. of yeast suspension required to ferment the glucose	Time of fermentation in hours
1	1	3	4	6
2	1	5	3	6
3	2	1	5	16
4	2	3	4	16
5	2	5	7	6
6	2	5*	5	17
7	2	5	7	17
8	3	3*	6	17
9	3	3	10	20
10	3	4*	6	17
11	3	4	6	20
12	3	5	6	20
13	3	5	10	17

* Small quantities of glucose still left unfermented.

The variability which is particularly noticeable in experiments 3 and 6, and 5 and 7, is mainly accounted for by the use of different samples of yeast, but even when the same sample of yeast was used and the fermentation carried out with the same quantity of glucose dissolved in urine the glucose was not always completely removed in the expected time.

Not only was the rate of fermentation unreliable, probably due to variations in the acidity of the urine, but also it was found necessary to make a correction for the amount of nitrogen introduced into the solution with the yeast.

Though the desired results can often be obtained, there is so much uncertainty in the removal of glucose by fermentation that the method is not a suitable one for practical use.

Some experiments were also made with zymin and with yeast dried according to v. Lebedeff's method and kindly given to us by Prof. Harden. The fermentation of the glucose was much slower, and a still greater correction for the amount of nitrogen introduced in the preparation had to be made.

(2) *Removal of Glucose by Benzoylation.*

Glucose and other carbohydrates give insoluble benzoyl esters when they are acylated by the Schotten-Baumann method. This method was therefore tested after adding glucose to urine and shaking the solution with varying quantities and excess of benzoyl chloride and sodium hydrate. In only one experiment out of 11 was the glucose completely removed. Further in the strongly alkaline liquid there is loss of ammonia from the urine. This method was therefore not serviceable.

(3) *Removal of Glucose by Precipitation with Cupric Hydroxide.*

Salkowski [1872, 1879] observed that glucose was completely precipitated in combination with cupric hydroxide when five molecules of copper sulphate and eleven molecules of sodium hydroxide were added for every molecule of glucose; Yoshimoto [1908] repeated these experiments and extended them to the precipitation of other carbohydrates. We can confirm the results with glucose; the glucose is completely precipitated if the proportions are exactly those given by Salkowski but not otherwise. A slight excess of alkali dissolves the copper hydroxide compound.

Owing to the sensitiveness of the precipitation towards alkali it was not anticipated that its application to the removal of glucose from urine of varying acidity would give the desired result. The precipitate with urine is of large bulk, the filtration is slow and the alkalinity of the solution leads to loss of ammonia during filtration. Glucose was generally found to be present in the filtrate. This method is again not applicable to urine.

(4) *Removal of Glucose with Lead Hydroxide.*

Glucose is not precipitated from solution by lead acetate; it is partially precipitated by basic lead acetate. It is also stated that glucose is removed from solution by basic lead acetate and ammonia, when these reagents are added to diabetic urine in the proportions used in the estimation of aceto-acetic acid and hydroxybutyric acid by Shaffer and Marriott's method [1913]. We have tested urine, to which glucose had been added, for glucose after such a precipitation and have found it still present, so that its precipitation under these conditions is not complete.

It would no doubt be possible to find the exact proportions of these reagents which would completely precipitate the glucose, but since for the purpose of estimating allantoin ammonia cannot be used we have used sodium hydroxide instead. Our experience with copper hydroxide suggested that the precipitation of glucose would be complete if the proportions were:

$$2C_6H_{12}O_6 : 5\,(Pb\,(OH)_2\,.\,Pb\overline{ac}_2) : 10NaOH,$$

and on testing these proportions it was found that the precipitation of glucose was complete. The filtrate from the precipitate shows no reduction or, in a few cases only, a very slight reduction of Fehling's solution.

This precipitation with lead hydroxide is not so sensitive to excess of sodium hydroxide as that with copper hydroxide. A slight excess of sodium hydroxide may be added, and in many experiments as much as 2·5 molecules

of sodium hydroxide were used, and the whole of the glucose removed. A larger excess of the sodium hydroxide leads to solution of the precipitate.

This method can therefore be applied to the estimation of allantoin in urine containing glucose.

The Effect of Basic Lead Acetate and Sodium Hydroxide upon Allantoin.

The estimation of allantoin in urine by the Folin method will be therefore possible if allantoin is not precipitated by the basic lead acetate and sodium hydroxide, or by the glucose lead hydroxide compound. Two series of experiments were therefore made to test if allantoin were so precipitated.

A. 25 cc. of approximately 0·1 N allantoin solution were treated with the amounts of basic lead acetate and sodium hydroxide required assuming that the solution contained 1, 2, 3 and 4 per cent. of glucose.

B. 25 cc. of the same allantoin solution were treated with the same quantities of basic lead acetate and sodium hydroxide in the presence of 1, 2, 3 and 4 per cent. of glucose.

In both cases the volumes were made up to 250 cc., the precipitates filtered off and the nitrogen estimated by Kjeldahl's method in 50 cc. (= 5 cc. of the original solution) of the filtrate. A total nitrogen estimation, C, was also made in 5 cc. of the original solution.

	A. cc. 0·1N NH_3	B. cc. 0·1N NH_3	C. cc. 0·1N NH_3
1 per cent.	2·6	—	2·7
2 „	2·6	2·2	
3 „	2·5	2·2	
4 „	2·5	2·2	
1 per cent.	2·5	2·45	2·75
2 „	2·35	2·3	
3 „	2·5	2·5	
4 „	2·45	2·4	

Though the amount of nitrogen in the filtrate was in all cases less than in the original solution, it cannot be concluded that allantoin is precipitated by basic lead acetate and sodium hydroxide. It is most probable that this difference is due to the precipitation of traces of impurity, such as uric acid, in the allantoin. It is unlikely that the same figure would have been obtained in the series of experiments in which quantities of basic lead acetate and sodium hydroxide varying from 14 to 56 cc. and 4 to 17 cc. respectively were used.

Application of the Method to Urine.

In applying this precipitation of glucose by basic lead acetate and sodium hydroxide to urine the ammonia present in the urine must be removed, as the reagent is alkaline and the ammonia is slowly evolved during filtration. The removal of ammonia presents no difficulty if it be effected by Folin's air current method. The solution which remains can be used for the allantoin estimation, the excess of sodium carbonate being neutralised with acetic acid, using phenolphthalein as indicator, and warming to remove the carbon dioxide before the precipitation of the glucose. Owing to the presence of phosphates, sulphates, pigments and other compounds in urine an excess of basic lead acetate above that required for the amount of glucose present must be added: 25 cc. of basic lead acetate and the corresponding amount of sodium hydroxide have been found to suffice for 25 cc. of dog's urine (when the 24 hours quantity has been diluted to 500 cc.).

In performing the hydrolysis by the magnesium chloride method with large quantities of liquid the water which distils into the special form of condenser is emptied out as required till the concentration is sufficient for the hydrolysis of the urea and allantoin.

The experimental details are as follows:

25 cc. of urine are treated with 1 gram of sodium carbonate and the ammonia estimated by Folin's method; it is not necessary to use paraffin or toluene to prevent frothing if tall cylinders are employed. The solution is washed into a 250 cc. measuring flask, and the sodium carbonate neutralised by carefully adding glacial acetic acid from a burette and warming. To the neutral solution 25 cc. of basic lead acetate + 14 cc. of basic lead acetate for every per cent. of glucose are added. These quantities require 6·2 + 3·4 cc. of 2N sodium hydroxide to precipitate the glucose and to remove any excess of lead acetate. It is advisable to determine the exact amount of 2N sodium hydroxide which is required to precipitate the lead hydroxide from the basic lead acetate by a special experiment, as in making up solutions of the basic lead acetate and 2N sodium hydroxide the solutions have never quite the same concentration.

The caustic soda is added slowly from a burette with constant shaking; the solution is made up to 250 cc. and the precipitate filtered off.

50 cc. of the filtrate should be tested for glucose before proceeding to the allantoin estimation, as it occasionally happens that the glucose is not completely removed. A slight reduction has no effect upon the result. If there

is considerable reduction the removal of the glucose must be repeated in another sample. We are unable to explain why the precipitation sometimes fails, but it seems most probable that it is due to improper neutralisation of the sodium carbonate.

50 cc. of the filtrate (= 5 cc. of urine) are used for the estimation of the urea + allantoin by Folin's magnesium chloride method.

We have tested the method upon urine to which glucose had been added with the following results, a control (= NH_3 + urea + allantoin) having been previously made; the figures are in cc. of 0·1 N ammonia:

Glucose added %	Reduction of filtrate	Urea + allantoin	NH_3 per 5 cc.	NH_3 + urea + allantoin	Difference
1·3	0	42·7	3·0	45·8	−0·1
2·0	+	—	3·0	45·8	—
3·0	0	42·4	3·0	45·8	−0·4
4·0	0	42·9	3·0	45·8	+0·1
1	+	—	1·2	36·2	—
2	slight	35·0	1·2	36·2	0
3	0	35·1	1·2	36·2	+0·1
4	0	34·5	1·2	36·2	−0·5
1·2	very slight	32·3	2·0	34·5	−0·2
2	,,	32·5	2·0	34·5	0
3	0	32·6	2·0	34·5	+0·1
4	0	32·8	2·0	34·5	+0·3
1	very slight	32·4	2·0	34·5	−0·1
2	0	32·0	2·0	34·5	−0·5
3	0	32·3	2·0	34·5	−0·2
4	slight	32·6	2·0	34·5	+0·1
1	very slight	32·5	2·0	34·5	0
2	,,	32·4	2·0	34·5	−0·1
4	,,	32·6	2·0	34·5	+0·1
5	0	32·5	2·0	34·5	0
1	+	—	2·1	39·3	—
2	0	36·8	2·1	39·3	−0·4
3	0	37·0	2·1	39·3	−0·2
4	0	36·8	2·1	39·3	−0·4

The maximum difference is − 0·5 cc., but in many of the experiments there was no difference: a difference of 0·4 cc. is often obtained in two parallel experiments with normal urine; it cannot be regarded as a great discrepancy, as the Folin method is a difficult one to perform.

Analyses of Diabetic Dog's Urine.

We have devised this method for the analysis of diabetic dog's urine and we have been fortunate in being able to use it upon the urine of a dog rendered diabetic by Prof. Starling. The analyses have been performed by

Mr W. W. Reeve, who has carried them out according to our directions. No difficulty was experienced in the removal of the glucose, though the solutions sometimes showed a slight reduction. The output of allantoin of a diabetic dog is greater than that of a normal dog upon a similar diet. The data in cc. 0·1 N ammonia per 5 cc. urine are the following:

Amount of glucose present in %	NH_3	NH_3 + urea	Urea + allantoin	Allantoin
1·0	1·2	29·1	28·8	0·9
5·6	1·4	30·8	31·9	2·5
1·2	0·9	10·8	12·3	2·4
4·5	1·5	28·5	29·0	2·0
4·0	1·5	24·4	25·2	2·3
3·0	2·4	34·1	35·8	4·1
5·8	2·1	35·9	36·4	2·6
3·8	2·9	37·6	38·4	3·7
6·0	2·2	35·7	36·4	2·9
3·0	2·4	32·5	33·7	3·6
3·5	2·9	35·9	36·7	3·7

The fact that these analyses have been made by an independent worker shows that the method is satisfactory.

Summary.

The estimation of allantoin in urine containing glucose can be effected by Folin's magnesium chloride method if the glucose be first removed. The glucose can be removed by precipitation with basic lead acetate and sodium hydroxide if the proportions are $2C_6H_{12}O_6 : 5\,[Pb(OH)_2 . Pb\overline{ac}_2] : 10NaOH$.

The expenses of this research and of the previous one upon the estimation of urea and allantoin have been defrayed by a grant to one of us from the Government Grant Committee of the Royal Society, to whom we desire to express our thanks.

REFERENCES.

Haskins (1906). *J. Biol. Chem.*, **2**, 243.
Marshall (1913). *J. Biol. Chem.*, **14**, 283.
Mörner (1903). *Skand. Arch. Physiol.*, **14**, 297.
Plimmer and Skelton (1914). *Biochem. J.*, **8**, 70.
Salkowski (1872). *Pflüger's Arch.*, **5**, 220.
—— (1879). *Zeitsch. physiol. Chem.*, **3**, 79.
Shaffer and Marriott (1913). *J. Biol. Chem.*, **16**, 265.
Udransky (1888). *Zeitsch. physiol. Chem.*, **12**, 42.
Yoshimoto (1908). *Zeitsch. physiol. Chem.*, **56**, 425.