A METHOD FOR THE DETERMINATION OF ALLANTOIN IN RABBIT URINE.

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INTRODUCTION.

During the past 10 years, the methods for the determination of uric acid have been gradually improved, so that short, accurate methods are now available for the estimation of this important catabolite. On the other hand, during the same period, there have been no marked improvements in the methods for allantoin, which are long, tedious, and probably not accurate, especially for small amounts of allantoin. As a consequence, most of the recent studies on purine metabolism have been made on human subjects, who excrete uric acid as the principal end-product of purine metabolism, rather than on those mammals in which allantoin is assumed to have a metabolic significance similar to that of uric acid.

One group of methods for the estimation of allantoin depends upon the precipitation of allantoin from solution as a metallic salt. The methods of this type, which were published prior to that of Wiechowski (1), in 1909, may be dismissed without further consideration, since they have been found to be unreliable. The method of Wiechowski, which is the standard method at the present time, depends on the fact that if urine is diluted so that the urea concentration is 1 per cent or less, and is freed from chlorides and substances precipitated by phosphotungstic acid and basic lead acetate, the allantoin may be completely precipitated by mercuric acetate in the presence of a large amount of sodium acetate. The allantoin may be isolated and determined gravimetrically, or the nitrogen content of the mercury allantoin compound may be estimated. Handovsky (2), in 1914, modified the latter part of the procedure recommended by Wiechowski by the precipitation of the allantoin with a measured amount of a standard mercuric acetate-sodium acetate solution. After removal of the mercury allantoin salt by filtration, the excess of mercuric acetate in the filtrate is determined by titration with standard ammonium thiocyanate.

The second group of methods is based on the fact that, under definite conditions, the allantoin nitrogen as well as the urea nitrogen of a urine may be hydrolyzed to ammonia. A separate determination of the pre-
formed ammonia and the ammonia resulting from the hydrolysis of urea enables one to calculate the allantoin by difference. In the early procedure of Folin (3) for urea, the urine was subjected to the action of hydrochloric acid in the presence of magnesium chloride, and the ammonia formed was considered to have come from urea. Benedict (4) carefully studied this reaction and concluded that in addition to the urea nitrogen, all of the allantoin nitrogen, 2 per cent of the uric acid nitrogen, and 1 per cent of the creatinine nitrogen is converted to ammonia. Plimmer and Skelton (5), in 1914, apparently disregarding the small amount of nitrogen resulting from the breakdown of uric acid and creatinine, followed the procedure of Folin for urea, and considered the ammonia nitrogen to be derived from preformed ammonia, urea, and allantoin. A separate determination of the urea and preformed ammonia was made by the urease method and the allantoin calculated by difference. Harding and Young (6) have simplified the method of Plimmer and Skelton by carrying out the hydrolysis with hydrochloric acid and magnesium chloride, in an Erlenmeyer flask fitted with a reflux tube.

During the course of the last 5 years the writer has made numerous determinations by the Harding and Young method and by the Handovsky modification of the Wiechowski method. It is felt that neither of these methods is satisfactory, especially if a urine contains small amounts of allantoin and normal or high amounts of the other urinary nitrogenous constituents. Since in the method of Harding and Young the allantoin is determined by difference, a small error in the urea determination makes a considerable difference in the value for allantoin. A second objection is the difficulty in exactly duplicating the conditions for the hydrolysis, even though the directions of Harding and Young are followed as closely as possible. It was often observed that the hydrolysis of four samples of the same urine would yield two pairs of checks, although the hydrolyses were carried out carefully under as nearly identical conditions as possible. Although the discrepancy between the pairs was not great, it was enough to cast doubt upon the accuracy of the method.

The titration of mercuric salts by ammonium thiocyanate involves a very difficult end-point, and this fact aside from the length of time required to carry out the determination constitutes a serious objection to the Handovsky method. It is unfortunate that small differences in the final titration with thiocyanate should make considerable variation in the value for allantoin. In our hands, satisfactory recoveries of allantoin.
added to rabbit urine could not be consistently obtained by the method of Handovsky. In view of these facts, a direct method for allantoin, which is shorter than the methods of Handovsky and Wiechowski and which will more accurately measure the allantoin content of biological fluids, is desirable. The method to be discussed in this paper, which, it is believed, will meet these qualifications, depends upon the hydrolysis of allantoin to oxalic acid, the precipitation of the latter as calcium oxalate, and its estimation by a permanganate titration.

Theory of the Determination.

As early as 1864, Baeyer (7) pointed out that the hydrolysis of allantoin with barium hydroxide yielded oxalic acid as one of the end-products. Claus (8) in 1874 came to the same conclusion, but differed somewhat from Baeyer with respect to the other products of the reaction. According to Baeyer, the alkaline decomposition of allantoin proceeds as follows:

\[
\begin{align*}
HN-CO & \quad NH_2 \\
OC & \quad CO \\
\rightarrow & \\
HN-CH-NH & \quad HN-CHOH \\
\text{Allantoin.} & \quad \text{Allanturic acid.}
\end{align*}
\]

Allanturic acid which is formed undergoes a simultaneous oxidation and reduction to hydantoinic and parabanic acid. The hydantoin is further hydrolyzed to hydantoic acid while parabanic acid is converted to oxalic acid and urea.

Thus, according to Baeyer, 2 molecules of allantoin yield 1 molecule of oxalic acid. Claus, however, considers that 3 molecules of allantoin yield 2 molecules of oxalic.
It is the purpose of this paper to present evidence which demonstrates that the hydrolysis of allantoin to oxalic acid may be utilized for the quantitative estimation of allantoin in urine. The method as applied to urine will first be given in detail, in order that the experiments, which it is believed show the principles of the determination to be sound, may be better understood.

Method for Urine.

Since urine is highly pigmented, and may contain varying amounts of oxalic acid, the direct application to the urine of the hydrolysis procedure for the conversion of allantoin to oxalic acid will not yield accurate results. Allantoin forms, under the proper conditions, an extremely insoluble compound with mercuric acetate, but the addition of mercuric acetate to a urine containing allantoin will not result in the quantitative removal of the latter, since the presence of chlorides, ammonia, and high concentrations of urea interfere with this precipitation. Evidence will be presented in this paper, which demonstrates that treatment of the urine with phosphotungstic acid, followed by the addition of basic lead acetate, will remove most of the interfering substances, and will give a solution favorable for the precipitation of the mercury-allantoin compound. After precipitation, the mercury-allantoin compound is decomposed with hydrogen sulfide and the mercuric sulfide removed. The filtrate containing the allantoin is hydrolyzed with sodium hydroxide and the oxalic acid which is formed is precipitated as calcium oxalate. The estimation is completed by the titration of the calcium oxalate with potassium permanganate in the usual manner. The procedure in detail, as it has been applied to the determination of allantoin in rabbit urine, is as follows: The urine is diluted, so that the concentration of the urea is approximately 0.5 per cent. As a rule, the dilution of the 24 hour sample of a rabbit urine to 300 or 350 cc. is satisfactory. Concentrations of urea greater than 0.5 per cent prevent a quantitative precipitation of the allantoin by the mercuric acetate in a later step of the procedure. For the removal of basic compounds, such as uric acid, creatinine, ammonia, and most of the urinary pigments, the urine is acidified and treated with phosphotungstic acid. An acid mixture, consisting of equal parts by volume of glacial acetic acid and 4 N sulfuric acid, is added to an aliquot of the urine in
such an amount that the final concentrations of the acetic and sulfuric acids are approximately 5 and 1 per cent respectively. Since it is extremely important that an excess of phosphotungstic acid be avoided, preliminary tests\(^1\) are made on small portions of urine, and then, on the basis of these tests, the calculated amount of solid phosphotungstic acid is added to the acidified aliquot of urine to be used in the determination. It has been found convenient to use 100 cc. of urine, which are measured into a 150 cc. Erlenmeyer flask and then acidified with 10 cc. of the acid mixture. After the addition of the calculated amount of solid phosphotungstic acid, a rubber stopper is inserted and the flask vigorously shaken to insure the complete solution of the phosphotungstic acid.

After 1 hour the precipitate is removed by filtration and solid lead oxide (PbO) is added, until the solution reacts faintly alkaline, due to the formation of basic lead acetate by the interaction of the lead oxide with the sulfuric and acetic acids. Usually 16 to 19 gm. of lead oxide are required for this neutralization, and since an excess of the lead oxide is to be avoided, the smaller amount is first added to the filtrate, which is preferably contained in a 150 cc. Erlenmeyer flask. The flask is gently rotated until the yellow lead oxide has been replaced by the white lead salts, and the flask has become appreciably warmer, due to the heat of neutralization. A rubber stopper is now inserted and the flask vigorously shaken. A small portion (2 cc.) is filtered into a test-tube and a drop of phenol red\(^2\) indicator added. If the color

\(^1\) To determine the correct amount of phosphotungstic acid required to precipitate the basic substances, 2 cc. portions of urine are measured into test-tubes and acidified with 0.2 cc. of the acid mixture (equal parts of glacial acetic and 4 N sulfuric acids). Measured portions of a 10 per cent aqueous solution of phosphotungstic acid are now added from a Mohr pipette to the test-tubes containing the acidified urine. At the end of 5 minutes, the contents of the tube are filtered, and small amounts (0.2 cc.) of the 10 per cent phosphotungstic acid solution are added. Those tubes in which no further precipitate is obtained in 3 minutes are considered to have had sufficient phosphotungstic acid for the removal of the basic compounds. From this preliminary test one calculates the smallest amount of solid phosphotungstic acid required for the desired aliquot of urine.

\(^2\) The phenol red and brom-cresol purple indicators were made according to the directions given in Clark's "Determination of Hydrogen Ions," and a rough estimation of the pH was made by matching the colors of the solutions with the Clark color chart.
indicates that the pH of the solution is lower than 7.2, a drop of brom-cresol purple is added to the same tube. If a pH between 6.0 and 7.0 is indicated, it is necessary to add only a small amount of the lead oxide to the major portion of the solution to bring it to a pH of 7.2. Phosphates, sulfates, some of the urinary pigments, preformed oxalic acid, and any excess of phosphotungstic acid are precipitated by the basic lead acetate as insoluble lead salts. To obtain the greatest quantity of filtrate from the bulky lead precipitate, it is advisable to transfer the entire mixture to 50 cc. centrifuge tubes and throw out the major portion of the precipitate by centrifugation. The supernatant liquid is filtered through a small filter and a clear solution with a faint green tinge is obtained. Aliquot portions of this filtrate, which should have a pH of 7.2 to 7.4, are measured into 50 cc. centrifuge tubes, and a solution of 1 per cent mercuric acetate in 30 per cent sodium acetate is added. The final concentration of the mercuric acetate should not fall below 0.2 per cent. As a rule, if 100 cc. of urine are used for the analysis, the volume of the filtrate following the treatment with lead oxide is such that two 40 cc. portions may be taken for analysis. For this amount of filtrate, 10 cc. of the mercuric acetate-sodium acetate reagent are added. The mercuric acetate is thoroughly mixed with the solution by means of a stirring rod, and the tubes are set aside for ½ hour. At the end of this time, the precipitates are removed by centrifugation. If it is not convenient to centrifuge at the end of ½ hour, the period of precipitation may be extended to 1 hour, since numerous experiments have demonstrated that the differences in results obtained with a precipitation period of ½ hour, as compared to 1 hour, are within experimental error. The supernatant liquids are discarded and the precipitates are

3 The precipitate obtained when the mercuric acetate-sodium acetate solution is added to the filtrate, which is slightly alkaline because of the presence of basic lead acetate, is not composed entirely of the mercury allantoin compound. If 100 cc. of water are acidified with the acid mixture, neutralized with lead oxide, and this filtrate treated with the mercuric acetate-sodium acetate reagent, a similar precipitate is obtained, although allantoin is not present.

4 If the conditions are favorable for the precipitation of the allantoin, a portion of this supernatant liquid should give an immediate turbidity with 0.5 mg. of allantoin.
washed in the centrifuge tubes with 15 cc. of distilled water and centrifuged again. The supernatant liquids are again discarded and the precipitates are suspended in 15 cc. of water. 0.2 cc. of concentrated hydrochloric acid is added to each tube. With the use of a stirring rod to break up the larger particles, the greater part of the precipitate is dissolved, with the exception of a small residue of lead chloride. Hydrogen sulfide is now passed into the tubes until the heavy metals have been completely precipitated as sulfides. These are removed by centrifugation and the supernatant liquids which contain the allantoin are decanted into 150 cc. Erlenmeyer flasks, in which the hydrolysis of allantoin to oxalic acid is to be made. The precipitated sulfides are washed in the centrifuge tubes with 10 cc. of distilled water, and the washings added to the Erlenmeyer flasks. The excess of hydrogen sulfide is removed by aeration.

The solutions containing the allantoin are made slightly alkaline by the addition of 1 cc. of a solution of sodium hydroxide which contains 0.5 gm. of sodium hydroxide per cc. of solution. A glass bead is now added to each flask and the flasks are heated over a Bunsen burner until the volume has been reduced to about 12 cc. 9.25 cc. of the same strong alkaline solution are added to each flask and the volume made to 25 cc. with distilled water.

The excess of hydrogen sulfide is removed by aeration rather than by boiling, since heating the solution in the presence of the acid causes marked pigmentiation.

500 gm. of sodium hydroxide are dissolved in water and after cooling made to a volume of 1000 cc.

The correct estimation of this volume in a 150 cc. Erlenmeyer flask is rather difficult. A very close approximation to this volume can be made by adding 25 cc. of water to the dry flask to be used in the hydrolysis, placing it on a level surface and marking the water level with two strips of a label on opposite sides of the flask. By the use of this method, it is possible to gauge the volume to within 2 cc. of the desired amount. A second method is as follows: Add 25 cc. of water to a large number of 150 cc. Erlenmeyer flasks and then prepare a T tube of glass rod of such a size that when the short arm is placed across the neck of the flask, the long arm just touches the surface of the water. Better results will be obtained if the tip of the T tube is coated with high melting paraffin. Considerable time may be spent in the selection of a group of flasks which will meet this requirement, but since the same flasks may be used many times for the hydrolysis procedure, it is effort well spent. By following the second procedure, volumes slightly lower than 25 cc. are more likely
The amount of alkali which is added is sufficient to neutralize the small amount of acid present and to make the final concentration of the sodium hydroxide 20 per cent. The contents of the flasks are now heated over a micro burner for 1 hour. Later experiments will demonstrate that the hydrolysis of allantoin for 1 hour at the boiling temperature of 20 per cent sodium hydroxide yields an amount of oxalic acid which is in quantitative agreement with that demanded by Baeyer's theory of the reaction. It is essential for complete hydrolysis that vigorous boiling be maintained during the entire hour.

In order to keep the volume constant during the hydrolysis period each flask is fitted with a test-tube, which serves as a Hopkins condenser. The test-tubes, which should fit snugly but not tightly into the necks of the Erlenmeyer flasks, extend to within 1 inch of the surface of the liquid during the hydrolysis. A small stream of water sent through the condensers will prevent the loss of liquid from the flasks.

It has been our experience that there is very little spattering within the flask, but after the period of hydrolysis it is advisable to wash the portion of the condenser within the flask with a fine jet of water. The flasks are then cooled, 2 drops of methyl red added, and concentrated hydrochloric acid slowly run in from a burette with constant shaking, until the solution is definitely pink. 0.5 cc. of concentrated ammonium hydroxide is then added. It has been found that this treatment is the most efficient for the removal of the siliceous material that has resulted from the action of the alkali upon the glass. The contents of the flask are now transferred quantitatively to a 50 cc. volumetric flask and, after cooling to 20°C., made to volume with distilled water. After a thorough mixing, the contents of the flask are transferred to 50 cc. centrifuge tubes and the precipitate of siliceous material to result than volumes over 25 cc. Unless large errors in the estimation of the volume are made, the results will not be vitiated, since the hydrolysis of allantoin with 25 per cent sodium hydroxide yields practically the same results as the hydrolysis by 20 per cent sodium hydroxide. However, if error in the estimation of the volume is such that the concentration of the alkali during the hydrolysis is only 16 per cent, results about 10 per cent lower than theoretical are obtained. (See later experiment on pure solutions of allantoin.)
removed by centrifugation. Aliquot portions (usually 40 to 45 cc.) are measured into 50 cc. centrifuge tubes, and the pH adjusted to 5.8 to 6.0 by the addition of acetic acid. 2 cc. of a 20 per cent solution of calcium chloride are added to each tube, and the inside surface of the tube rubbed with a rubber tipped stirring rod until a fine crystalline precipitate of calcium oxalate is formed. The tubes are set aside for 1 hour and the calcium oxalate is thrown down by centrifuging for 5 minutes at 1500 r.p.m. The precipitate is washed once with 15 cc. of a cold saturated solution of calcium sulfate, which contains just enough ammonium hydroxide to make it faintly alkaline to phenolphthalein, and centrifuged. The washed precipitate is dissolved in 20 cc. of N sulfuric acid and titrated with a standard solution of potassium permanganate. The usual precautions for making a permanganate titration are observed. Clark (9) has discussed permanganate titrations of oxalic acid in detail, including the preparation and standardization of weak permanganate solutions.

The tube containing the oxalic acid to be titrated is placed in a boiling water bath, and a small thermometer inserted. When the contents of the tube are heated to 80° C., the tube is removed and the titration made. During the titration the tube is partially submerged in water kept at 90° C. in order that the temperature within the tube shall not fall below 65° C. before the completion of the titration. The hot water is conveniently held in a large porcelain casserole, which serves also to provide a white background for the titration. The permanganate solution should be measured from a burette graduated to 0.02 cc. The amount of the standard permanganate solution required to give 20 cc. of the N sulfuric acid the faint pink end-point, is subtracted from all titrations. The permanganate solutions which have been used in this study have varied in strength from 0.015 N to 0.019 N. If by hydrolysis, 2 molecules of allantoin yield 1 molecule of oxalic acid, 1 cc. of 0.01 N potassium permanganate is equivalent to 1.581 mg. of allantoin.

In Table I are recorded the results of the analysis of rabbit urine for allantoin, before and after the addition of allantoin. Recoveries of allantoin ranging from 90 to 104 per cent have

* For the precipitation of the oxalic acid, it is advisable to use centrifuge tubes of the type recommended by Clark (9).
been obtained, with an average recovery of 98 per cent. Satisfactory recoveries of added allantoin are obtained only if an excess of phosphotungstic acid and lead oxide is avoided.

Rabbit urines have also been analyzed before and after the addition of egg albumin and glucose. The presence of albumin to the extent of 0.3 per cent was without effect upon the values obtained for allantoin. The influence of higher concentrations of albumin were not studied, since it is possible to remove the larger amounts by heat coagulation, prior to the analysis for allantoin. If the content of glucose in a urine is not more than 0.5 per cent, the values for allantoin are unaffected. Higher concentrations of glucose (0.7 to 1.0 per cent) give values for allantoin which are from 4 to 9 per cent lower than those obtained on the original urine.

Experiments have also been made which demonstrate that if the urea concentration of the urine is approximately 0.5 per cent, further dilution of the urine will not lead to higher results for allantoin. A typical experiment may be cited. 100 cc. of a urine containing approximately 550 mg. of urea were analyzed for allantoin as previously described. A 75 cc. and a 50 cc. portion of the same urine were diluted with water to 100 cc. and analyzed in the same manner. The values obtained for allantoin.

### TABLE I.

Recovery of Allantoin Added to Rabbit Urine.

<table>
<thead>
<tr>
<th>Allantoin per 100 cc. of urine.</th>
<th>Allantoin added to 100 cc. of urine.</th>
<th>Total allantoin found per 100 cc. of urine.</th>
<th>Allantoin recovered.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>40.5*</td>
<td>5.0</td>
<td>45.3*</td>
<td>4.8</td>
</tr>
<tr>
<td>23.2</td>
<td>10.0</td>
<td>33.6</td>
<td>10.4</td>
</tr>
<tr>
<td>10.9</td>
<td>15.0</td>
<td>24.4</td>
<td>13.5</td>
</tr>
<tr>
<td>34.7</td>
<td>22.0</td>
<td>56.8</td>
<td>22.1</td>
</tr>
<tr>
<td>31.0</td>
<td>24.0</td>
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<td>24.5</td>
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<td>32.5</td>
<td>25.0</td>
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<td>24.7</td>
</tr>
<tr>
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<td>25.0</td>
<td>51.0</td>
<td>22.6</td>
</tr>
<tr>
<td>34.5</td>
<td>37.5</td>
<td>70.0</td>
<td>35.5</td>
</tr>
<tr>
<td>44.5</td>
<td>50.0</td>
<td>96.5</td>
<td>52.0</td>
</tr>
</tbody>
</table>

* The figures given in Columns 1 and 3 are the averages of closely agreeing check determinations.
after calculation to 100 cc. of the original urine were 28.4, 28.1, and 28.9 mg., respectively. The greatest variation, it will be noted, is less than 3 per cent.

Rabbit urines have been analyzed both by this method and by the method of Handovsky. The method proposed in this paper gives values for allantoin which are 15 to 20 per cent higher than those obtained by the procedure of Handovsky. In the Handovsky method, prior to the precipitation of the allantoin as the mercury salt, it is necessary to remove the metallic ions of silver and lead from solution as the sulfides. The precipitate of the sulfides is very bulky, and since this precipitate is not washed, it is thought that considerable loss in allantoin may occur at this step of the procedure. In the new method that is proposed, the allantoin is precipitated without the removal of the lead ions from the solution. The precipitate is decomposed with hydrogen sulfide and the resulting precipitate of metallic sulfides, which is small in amount, is washed with water. It has been our experience that the recoveries of allantoin added to rabbit urine were consistently lower by the Handovsky method, than by the method described in this paper. While the recovery of added allantoin is not a final proof of the accuracy of the method, it is logical to assume, that of the two methods, the one which gives the higher value for allantoin in the original urine and also leads to a higher percentage recovery of the added allantoin is more nearly correct.

As an illustration of the exact procedure, the following experiment is given in detail: A 24 hour sample of rabbit urine was diluted to 350 cc. A 100 cc. portion of this urine was acidified with 10 cc. of the acid mixture and treated with phosphotungstic acid and lead oxide as previously directed. Two 40 cc. aliquots of the filtrate, which were obtained after the lead oxide treatment, were measured into centrifuge tubes and the allantoin precipitated. After hydrolysis of the allantoin, each solution was made to 50 cc. for the removal of silicates and then 45 cc. aliquots carefully measured for the precipitation of the oxalic acid. The titrations with 0.01639 N potassium permanganate for the two check determinations were 3.0 and 3.1 cc. A blank of 0.05 cc., which represents the amount of the permanganate required to give a pink tinge to 20 cc. of N sulfuric acid, is subtracted from the average of these two titrations. Since 3.0 cc. of 0.01639 N
potassium permanganate represent the allantoin of \( \frac{18}{55} \left( \frac{40}{110} \times \frac{45}{50} \right) \)
of the original 100 cc. of urine, \( 3.0 \times \frac{55}{18} \) or 9.17 cc. represent the allantoin content of 100 cc. of the urine. Since 1 cc. of 0.01639 N potassium permanganate is equivalent to 2.59 mg. of allantoin, the allantoin content of 100 cc. of the urine is 23.75 mg.

**Experiments on Solutions Containing Known Amounts of Allantoin.**

Before an attempt was made to apply the method to urine, numerous experiments were made with pure solutions of allantoin and mixtures of allantoin with other urinary constituents. Solutions of allantoin were hydrolyzed by various concentrations of alkali for periods ranging from 30 to 120 minutes, and the oxalic acid, which resulted from this hydrolysis, was determined in the manner outlined in the method. For amounts of allantoin over 6 mg., the total volume of the hydrolysis mixture was 25 cc., while for amounts less than 6 mg., the hydrolysis was made in a volume of 12.5 cc. In the procedure for the removal of the siliceous material, these volumes were diluted to 50 and 25 cc. and 40 and 20 cc. aliquots respectively were used for the precipitation of the oxalic acid as calcium oxalate. The results of the alkaline decomposition of pure allantoin are given in Table II. In all cases the temperature of the reaction was the boiling point of the alkali employed in the hydrolysis. The amounts of allantoin in Column 4 of Table II are calculated from the amount of oxalic acid found in the reaction mixture, on the assumption that 2 molecules of allantoin yield 1 molecule of oxalic acid. In this table, as well as in succeeding tables, the results are the average of closely agreeing check determinations.

The results given in Table II indicate that the hydrolysis of allantoin by a boiling solution of 20 per cent sodium hydroxide for a period of 1 hour yields an amount of oxalic acid which is in quantitative agreement with that demanded by Baeyer's theory of the reaction. Apparently for concentrations of alkali stronger than 20 per cent, the course of the reaction is somewhat different, since recoveries of allantoin greater than 100 per cent are obtained. If the concentration of sodium hydroxide is 20 per cent or higher, periods of hydrolysis longer than 1 hour also lead to
results higher than theoretical. It cannot be definitely stated that when allantoin is hydrolyzed by 20 per cent sodium hydroxide for a period of 1 hour, that the products of hydrolysis are limited to oxalic acid, hydantoic acid, carbon dioxide, and ammonia. Quantitative recoveries of allantoin are obtained, however, if Baeyer's theory of the reaction is assumed to be correct.

<table>
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<tr>
<th>Concentration of sodium hydroxide (per cent)</th>
<th>Time of hydrolysis (min.)</th>
<th>Allantoin Present (mg.)</th>
<th>Recovered (mg.)</th>
<th>Percentage Recovered (per cent)</th>
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<td>120</td>
<td>10.0</td>
<td>11.6</td>
<td>116</td>
</tr>
</tbody>
</table>

It will be recalled that one step in the procedure for the determination of allantoin in urine is its precipitation as the mercury salt, which is removed by centrifugation. The precipitate is dissolved in weak hydrochloric acid and then decomposed with hydrogen sulfide. After removal of the mercuric sulfide the filtrate containing the allantoin is hydrolyzed with 20 per cent sodium hydroxide. Table III is a record of experiments with
pure allantoin solutions which have been precipitated by mercuric acetate and then treated precisely as outlined in the method for urine. In these experiments the final concentration of the mercuric acetate has varied from 2.5 to 0.25 per cent. The lower concentrations are apparently as efficient for the precipitation of the allantoin as the higher concentrations, providing there is an excess of mercuric ions. It is essential, however, that whatever strength of mercuric acetate is employed, the acidity due to its hydrolysis should be buffered by sodium acetate, so that the precipitating reagent is neutral. In most of our later experi-

<table>
<thead>
<tr>
<th>Concentration of sodium hydroxide</th>
<th>Time of hydrolysis</th>
<th>Allantoin</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>per cent</td>
<td>min.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>3.0</td>
<td>2.8</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>5.0</td>
<td>4.7</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>6.0</td>
<td>5.8</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>10.0</td>
<td>9.8</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>10.0</td>
<td>9.7</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>10.0</td>
<td>9.8</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>15.0</td>
<td>15.1</td>
</tr>
</tbody>
</table>

ments, a solution of 1 per cent mercuric acetate in 30 per cent sodium acetate was used. Since the recoveries in the experiments recorded in Table III are of the same order as those of Table II, it is evident that the precipitation of the allantoin as the mercury salt, and the subsequent removal of the mercuric ions prior to hydrolysis, has not resulted in the loss of allantoin.

In the method of Wiechowski (1) for allantoin, it is stated that correct results cannot be obtained unless the urine to be analyzed is so diluted that the concentration of urea is not more than 1 per cent. He has also pointed out the necessity for the complete removal of the chlorides. The effect of the addition of the urea,
sodium chloride, creatine, and creatinine on the recovery of allantoin from aqueous solutions has been studied and the results have been summarized in Table IV. In each experiment recorded in Table IV, the solution containing the allantoin and the added constituent was adjusted to a pH of 7.2 to 7.4 by the precipitating reagent (1 per cent mercuric acetate in 30 per cent sodium acetate). In these experiments, the mercury allantoin compound

TABLE IV.

Effect of the Presence of Some Common Urinary Constituents upon the Recovery of Allantoin from Mixtures of Pure Compounds.

The allantoin was precipitated in every case as the mercury salt, which was then decomposed with hydrogen sulfide, and the mercury free filtrate hydrolyzed for 1 hour with 20 per cent sodium hydroxide.

<table>
<thead>
<tr>
<th>Concentration of sodium chloride. per cent</th>
<th>Concentration of urea. per cent</th>
<th>Creatine present. mg.</th>
<th>Creatinine present. mg.</th>
<th>Allantoin. Present. mg.</th>
<th>Allantoin. Recovered. mg.</th>
<th>Allantoin. per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1*</td>
<td>*</td>
<td>10.0</td>
<td>7.4</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td></td>
<td>10.0</td>
<td>4.1</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td></td>
<td>10.0</td>
<td>2.2</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td>10.0</td>
<td>9.5</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td></td>
<td>10.0</td>
<td>9.4</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td></td>
<td>10.0</td>
<td>8.3</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td></td>
<td>10.0</td>
<td>8.0</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>10.0</td>
<td>9.4</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>10.0</td>
<td>9.4</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>10.0</td>
<td>9.5</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>10.0</td>
<td>8.9</td>
<td>89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>10.0</td>
<td>8.6</td>
<td>86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The concentration of the urea and sodium chloride is calculated from the volumes of the solutions after the addition of the mercuric acetate solution.

was not removed by centrifugation until 1 hour after the addition of the mercuric acetate reagent. The remaining procedure for the experiments given in Table IV was the same as for those recorded in Table III. From an inspection of Table IV it is evident that under the conditions of these experiments, chlorides even in small amounts markedly interfere with the recovery of the allantoin. If the concentration of the urea is over 0.5 per cent, the recoveries of allantoin are also unsatisfactory. The
presence of creatine is without effect, but in the tubes containing the creatinine, the recoveries are approximately 10 per cent lower than the recoveries that are usually obtained from pure solutions of allantoin. Creatine and creatinine were included in this series, since, according to Greenwald (10), creatine is oxidized by mercuric acetate to methylguanidinoglyoxylic acid, which is hydrolyzed to methyl guanidine and oxalic acid. Apparently the conditions are unfavorable for the oxidation of the creatine and the creatinine by the mercuric acetate, since high results are not obtained when these compounds are present, as one might expect if the methylguanidinoglyoxylic acid had been formed. The presence of creatinine leads to low recoveries of allantoin, but since in the method for urine, creatinine is precipitated by the phosphotungstic acid, no further study has been made of the influence of creatinine. Since uric acid is likewise removed from the urine by phosphotungstic acid, the effect of its presence upon the recovery of allantoin from aqueous solutions has not been studied.

It remains to be demonstrated that pure solutions of allantoin and mixtures of allantoin with other urinary constituents can be treated successively with each reagent, which is used in the method

<table>
<thead>
<tr>
<th>Allantoin present</th>
<th>Urea present</th>
<th>Sodium chloride present</th>
<th>Allantoin recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>35.0</td>
<td>0</td>
<td>0</td>
<td>33.7</td>
</tr>
<tr>
<td>37.5</td>
<td>0</td>
<td>0</td>
<td>35.8</td>
</tr>
<tr>
<td>35.0</td>
<td>500</td>
<td>100</td>
<td>33.5</td>
</tr>
<tr>
<td>35.0</td>
<td>500</td>
<td>200</td>
<td>33.6</td>
</tr>
<tr>
<td>35.0</td>
<td>500</td>
<td>400</td>
<td>33.2</td>
</tr>
<tr>
<td>35.0</td>
<td>500</td>
<td>1000</td>
<td>33.7</td>
</tr>
<tr>
<td>35.0</td>
<td>750</td>
<td>400</td>
<td>31.2</td>
</tr>
<tr>
<td>35.0</td>
<td>1000</td>
<td>400</td>
<td>29.5</td>
</tr>
<tr>
<td>35.0</td>
<td>2000</td>
<td>1000</td>
<td>25.6</td>
</tr>
</tbody>
</table>

* The amounts of allantoin, urea, and sodium chloride given in Columns 1, 2, and 3 of this table are contained in 100 cc. of solution. Each 100 cc. of solution was carried through the entire procedure proposed for the analysis of allantoin in urine.
previously described for urine, without the loss of significant amounts of allantoin. Table V is a record of a number of experiments of this nature. 100 cc. portions of solutions containing either pure allantoin or allantoin with varying amounts of sodium chloride and urea were treated with 5 cc. of 4 N sulfuric acid and 5 cc. of glacial acetic acid. Then 1 gm. of phosphotungstic acid was added and put into solution with shaking. Since none of the compounds in these solutions (allantoin, urea, or sodium chloride) are precipitated by the phosphotungstic acid, there is an excess of 1 gm., which must be removed as the lead salt. The solution was now treated as described in the method for the estimation of allantoin in urine. It is important to note that the allantoin is precipitated by mercuric acetate from a solution which is faintly alkaline (pH 7.2 to 7.4) due to the presence of basic lead acetate. Apparently under these conditions, sodium chloride in large amounts does not interfere with the precipitation of allantoin by mercuric acetate. The concentration of urea must not greatly exceed 0.5 per cent if good recoveries of allantoin are to be obtained.

Mixtures of allantoin and creatine, allantoin and hippuric acid, and allantoin and uric acid have been studied in a manner similar to that just described. Recoveries of allantoin ranging from 94 to 98 per cent were obtained. In no case were the recoveries over 100 per cent. As a final test of the method, two solutions containing allantoin and many of the common urinary constituents were prepared. Solution 1 contained per 100 cc.:

- 25 mg. allantoin.
- 500 " urea.
- 300 " glucose.
- 50 " ammonium sulfate.
- 50 " sodium acid phosphate.

- 100 mg. creatine.
- 100 " creatinine.
- 200 " calcium chloride.
- 25 " uric acid.
- 50 " hippuric acid.

Solution 2 contained per 100 cc. the same amount of allantoin but 50 per cent more of each of the other constituents. The analysis of these solutions for the content of allantoin was made according to the procedures previously outlined for urines. 94 per cent of the allantoin was recovered from Solution 1, and 87 per cent from Solution 2. The lower recovery of allantoin from Solution 2 may be in part due to the high content of urea, which prevents the quantitative precipitation of the allantoin.
Although the present method was designed primarily for the estimation of allantoin in rabbit urine, the writer knows of no reason why it cannot be applied to the urine of other animals which excrete allantoin as the principal end-product of purine metabolism. From the results obtained with solutions containing known amounts of allantoin and from the analysis of urines before and after the addition of allantoin, it is believed that the method is more accurate than those which have been used prior to this time. This is particularly true of urines which contain small amounts of allantoin.

If it is advisable to use less than 100 cc. of urine for the analysis, the amount of acid mixture must be reduced in proportion. It is not necessary that the hydrolysis of allantoin to oxalic acid be made in a volume of 25 cc., and if one is dealing with a small amount of allantoin it is better to make the hydrolysis in a 100 cc. Erlenmeyer flask, with a final volume of 10 cc. For the removal of the silicates this volume is conveniently diluted to 25 cc., and the largest possible aliquot of this solution used for the precipitation of the oxalic acid. Again if a very dilute urine is to be analyzed it is advisable to use more than 100 cc. of urine for the analysis. The mercury allantoin precipitates from several portions of the basic lead acetate filtrate may then be combined prior to the decomposition with hydrogen sulfide, and the subsequent hydrolysis with alkali.

Although the present method requires from 6 to 7 hours for completion, approximately one-half of this time is required for the actual manipulation. This is much less time than that required for the original Wiechowski method, and somewhat less than that required for the Handovsky method. The original purpose, when the research was undertaken, was to provide a short, accurate method for the determination of allantoin. It was soon found, however, that accuracy was sacrificed for speed when an attempt was made to eliminate certain steps in the procedure, which are now employed.
A. A. Christman

SUMMARY.

A method for the determination of allantoin in rabbit urine is described, which is based upon the hydrolysis of allantoin to oxalic acid by alkali, followed by precipitation as calcium oxalate, and estimation of the latter by the usual permanganate titration.

BIBLIOGRAPHY.

A METHOD FOR THE DETERMINATION OF ALLANTOIN IN RABBIT URINE
A. A. Christman


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