ON THE COLORIMETRIC ESTIMATION OF URIC ACID IN URINE.

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About three years ago Folin and Macallum\(^1\) described a new micro method for the determination of uric acid in urine, which depends upon a color reaction between uric acid and a specially prepared solution of phosphotungstic acid. Subsequently, Folin and Denis\(^2\) modified the procedure somewhat, introducing a preliminary precipitation of the uric acid by Salkowski's method. These latter investigators also proposed a permanent standard solution for use in making the color comparison for uric acid determinations.

The so-called micro methods of analysis are particularly applicable to substances which occur in small quantities, because in such cases a relatively high percentage error, which is apt to occur in micro methods, is of little moment; and, furthermore, because our recognized methods for the determination of such substances are themselves usually subject to considerable error. It would seem, therefore, that a rapid micro method for uric acid determination should be of special service, since the older method for this determination is laborious and not above suspicion regarding its accuracy. Yet the colorimetric procedures suggested for uric acid estimation by Folin and his collaborators do not seem to have met with as general adoption as they appear to merit. Perhaps this is due partially to certain inaccuracies of presentation, and a few other small errors, such as are very apt to creep into the first presentation of a method.

In the present paper it is our purpose to discuss in some detail various aspects of the Folin-Denis method for uric acid estima-

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2. O. Folin and W. Denis: ibid., xiv, p. 95, 1913.
Folin and Denis concluded that the reaction between the uric acid and the phosphotungstic acid reagent takes place in acid solution, and that the compound formed dissociates into the colored compound upon addition of the alkali. Such a behavior of uric acid (reduction in acid solution) would be so at variance with the general reducing properties of this compound that we were led to study this point a little further than was reported by Folin and Denis. These authors arrived at their conclusion that the reduction occurs in acid solution because the addition of alkali to the reagent and subsequent treatment of this mixture with uric acid failed to develop any color. We have found that this fact is explained by a decomposition of the reagent by the carbonate, and should not be taken to indicate a reduction in acid solution by uric acid. If the reagent be made alkaline with carbonate, then reacidified with phosphoric acid, added to the uric acid, and then carbonate added, no color develops. If, on the contrary, the carbonate be added to the uric acid and then the reagent be added, the full color develops. These experiments furnish direct evidence that the reduction occurs only in alkaline solution.\(^2\) The point is obviously not of practical importance, and we mention it only to correct a possible misunderstanding regarding the reaction involved.

Another point in connection with the method is the question of the time required for the development of the maximal color. One would conclude from the Folin papers that the maximal color develops immediately on the addition of the sodium carbonate, and that, therefore, the results would not vary by waiting before adding the water. We have found that solutions which were diluted immediately after the addition of the sodium carbonate gave a reading about 3 mm. lower than those obtained after waiting forty to sixty seconds before diluting. In using the method this point should be taken into account, and one should wait one-half to one minute after adding the carbonate, before diluting with water. Longer waiting is to be avoided, because of a tendency to the development of turbidity if the solution stands too long before diluting.

The finding of a suitable standard solution for the uric acid comparison is obviously a most important point. As is well known, uric acid is readily soluble in alkalis, but in such solutions it rapidly undergoes decomposition or oxidation.

Folin and Macallum report that they devoted considerable time to trying to find a permanent standard. They found that colored glasses were not satisfactory as they transmitted too much or too little light; that some substances, including the aniline dyes, gave too bright a color to be used;

\(^2\) Some time ago Dr. Folin told one of us that he was well aware of this error in the early statement concerning the reaction and had intended to correct it subsequently, but had neglected to do so.
and that other substances gave blue solutions which were less bright and more greenish than the blue given by the uric acid. They stated that the uric acid reagent itself can be used as a standard by adding an excess of uric acid and standardizing against a lithium carbonate solution of uric acid, but Folin and Denis subsequently stated that this procedure was not satisfactory, because the color faded more rapidly than that obtained from solutions made with an excess of the reagent.

The standard which was first recommended by Folin and his collaborators was a solution of uric acid in lithium carbonate which was described as being made by washing 250 mgm. of Kahlbaum's uric acid into a flask with 25 to 50 cc. of water and adding 25 cc. of a 0.1 per cent lithium carbonate solution and shaking at intervals for an hour before diluting. In attempting to make up the lithium carbonate solution as directed, we have found that the uric acid fails to dissolve in the quantity of carbonate indicated within the course of an hour, or even much longer. We have sometimes started to prepare such a solution early in the afternoon, have shaken the mixture frequently for four hours and then left it standing over night, and still found an undissolved residue of uric acid the following morning. Even when obtained, the solutions of uric acid in lithium carbonate are not very stable. Folin and his collaborators stated that such a solution could be depended upon to remain unaltered for a week. This statement may hold for solutions kept cold, but certainly does not apply to solutions kept at room temperature in warm weather. Under such conditions we have noticed a 10 per cent loss of uric acid in two days, and a 25 per cent loss at the end of a week.

Folin and Denis finally suggested a permanent standard solution made by combining uric acid with formaldehyde. It is described as follows:

"One gram of uric acid in a volumetric liter flask is dissolved by means of an excess of lithium carbonate (200 cc. of a 0.4 per cent solution), to the solution is added 40 cc. of 40 per cent formaldehyde solution and the mixture is shaken and allowed to stand for a few minutes. The clear solution is acidified by the addition of 20 cc. of normal acetic acid and the whole is diluted up to the liter mark with water. The solution should remain perfectly clear and the next day (but not before) it can be standardized against a freshly prepared lithium carbonate solution of uric acid."

This formaldehyde-uric acid solution was recommended as meeting the requirements of a permanent standard and was said to act like ordinary pure uric acid solutions with reference to the quality and stability of the color produced with an excess of the reagent.

Contrary to the experience of Folin and Denis, we have not found the formaldehyde-uric acid solution which they propose to be a satisfactory standard, and we believe that very erroneous results may follow its use, unless the strictest precautions are taken as to standardizing the solution for exactly the temperature of the carbonate solution and diluting water employed for any individual determination. It is true that the formaldehyde-uric acid solution undergoes no deterioration upon standing, but the color yielded by a definite volume of the solution is affected by the tem-
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perature of the carbonate solution and diluting water out of all proportion to the effect of these factors upon pure uric acid. This fact was first brought to our notice by finding a uric acid-formaldehyde solution on one day to give a value of 28.5 mm. for 5 cc. (compared with the color from 1 mgm. of uric acid set at 20 mm.), while the following day it read 24.5 mm. against the same quantity of uric acid. The temperature was the only variable factor involved, and this fact led us to try some experiments with the sodium carbonate solution and the diluting water at various temperatures, using the same carbonate solution and water for the pure uric acid as for the formaldehyde-uric acid solution.

To summarize our results in this connection it may be stated that the uric acid-formaldehyde solution showed variations in its colorimetric value of about 50 per cent when the temperature of the solutions used varied between 12 and 40 degrees. This temperature range is, of course, extreme, but so are the variations obtained. Other factors which affect the uric acid-formaldehyde solution besides the temperature are the samples of reagent used, (different samples of reagent prepared according to the same technique give very different color values), and the time elapsing before dilution.

As a result of our work on the formaldehyde-uric acid solution we were convinced that it is affected so differently from pure uric acid by many factors, that it is unreliable for use as a standard solution.

It was therefore desirable to find a suitable standard. Since temperature and time of standing before dilution affect pure uric acid as regards the quantity of color produced, we have worked altogether upon the assumption that the standard solution must be a solution of pure and unchanged uric acid. A standard solution which we employed for some time was one in which pyridin was used to effect solution of the uric acid. Although we have since found a more satisfactory solution, (and, we believe, an ideal standard solution), we shall describe the pyridin solution because it can be prepared very readily, is permanent for ten days under widely varying temperature conditions, and offers a convenient solution to check up another standard as occasion may require.

The pyridin-uric acid solution is made by washing 250 mgm. of uric acid (which should be powdered in a mortar before weighing) into an Erlenmeyer flask with 150 cc. of distilled water; then add 4 cc. of pyridin—Kahlbaum's is preferable—and warm the
mixture until clear (one-half minute to 65° to 70° is sufficient). Do not allow it to boil. Wash into a 250 cc. volumetric flask, cool, and dilute to the mark. One cc. of solution, of course, contains 1 mgm. of uric acid. As stated above, this solution loses no measurable amount of its color-yielding power within ten days, and is far more readily prepared, and more stable than the solution in lithium carbonate.

Later we were led to try the solubility of uric acid in phosphates. As a result of these trials we have found what would seem to be an ideal standard solution, in that it is very readily prepared, does not need to be standardized, and appears to keep indefinitely. This solution is prepared as follows: 9 grams of pure crystallized disodium hydrogen phosphate, together with 1 gram of crystallized sodium dihydrogen phosphate are dissolved in 200 to 300 cc. of hot water and the solution is filtered, if it was not previously perfectly clear. The filtrate is made up to a total volume of about 500 cc. with hot water, and this hot or warm (and perfectly clear) solution is poured upon exactly 200 mgm. of pure uric acid suspended in a few cc. of water in a liter volumetric flask. The mixture is agitated for a moment or two until the uric acid completely dissolves and is then cooled. Exactly 1.4 cc. of glacial acetic acid are added, and the contents of the flask are diluted to the mark and mixed. About 5 cc. of chloroform are then added to prevent growth of bacteria or moulds in the solution.

5 cc. of this solution contain exactly 1 mgm. of uric acid. We have had samples of the solution for periods of over two months now, and none has shown any detectable diminution of uric acid content as measured by its color-yielding power. The mixture is very delicately balanced. It reacts acid to litmus paper, but there is no tendency whatever to a separation of any uric acid. Yet if 0.5 cc. more of acetic acid per liter is added than is called for in the above directions an abundant crystallization of uric acid will take place within a few hours.

We cannot, of course, guarantee the indefinite stability of this standard solution, and until further tested it would best be checked up against a fresh uric acid solution (either in phosphate, pyridin, or lithium carbonate) once every month or two.

Before describing the exact technique which we suggest for
the determination of uric acid in urine we desire to call attention to one other general point. This concerns the concentration of the carbonate solution which is employed as the alkali. Folin and his collaborators have recommended in every instance the use of measured volumes of "saturated" sodium carbonate. Since the solubility of sodium carbonate varies enormously with the temperature, and since the concentration of carbonate markedly affects the quantity of color developed, we are of the opinion that it is best to use always a solution of sodium carbonate of known and definite concentration. We have found a 20 per cent solution (200 grams of carbonate dissolved in warm water and made up to 1 liter) of anhydrous sodium carbonate to be the most satisfactory. Such a solution is practically saturated at 20°, but can be cooled 5° or 10° lower for some hours without deposition of the salt. During cold weather we keep this carbonate solution in large Erlenmeyer flasks. If a deposition of crystalline carbonate occurs at any time the slightest warming of the solution for a moment or two will effect complete solution. Using this solution, one is certain of always employing the same amount of carbonate, and results are more uniform and satisfactory.

The method for the estimation of uric acid in urine proposed by Folin and Denis is as follows:

"From 1 to 2 cc. of urine are measured into an ordinary centrifuge tube by means of a modified Ostwald pipette. A sufficient amount of distilled water is then added to bring the volume of the liquid in the tube to about 5 cc., six drops of 3 per cent silver lactate solution, two drops of magnesia mixture, and a sufficient amount (10 to 20 drops) of concentrated ammonium hydrate to dissolve the silver chloride are then added. The tube is now centrifuged for one or two minutes, the supernatant liquid poured off, and to the residue in the bottom of the tube are added five or six drops of freshly prepared saturated hydrogen sulphide water, and one drop of concentrated hydrochloric acid, and the tube is placed in a beaker of boiling water until all excess of hydrogen sulphide has been driven off. When the tube has been cooled, add 2 cc. of the uric acid reagent, 10 cc. of saturated sodium carbonate solution, transfer to a 50 cc. volumetric flask and make up to volume. The color comparison is then made in the usual manner against the color obtained from 5 cc. of the standardized uric acid-formaldehyde solution (or a freshly prepared pure uric acid solution)."

When properly carried out the above procedure of Folin and Denis yields accurate results. One point, however, has been omitted in their description, which is absolutely essential if results of any value are to be obtained. The point referred to is the necessity for thoroughly stirring up the pre-
cipitate containing the uric acid, after addition of the hydrogen sulphide water, so as to insure complete decomposition of the precipitate and liberation of the uric acid. If this simple step is omitted something over 50 per cent of the uric acid will be lost, whether the determination be applied to urine or to pure uric acid solution. The figures reported by Folin and Denis show that they must have adopted such a mechanical breaking up of the precipitate with the hydrogen sulphide water as a routine procedure, and simply neglected to mention it in the published description. It can be positively stated that results obtained by the Folin-Denis method without attention to the above mentioned point are totally worthless.

In the procedure for estimation of uric acid in urine which we wish to present we have combined the three solutions used in precipitating the uric acid into one, and have substituted a rapid and convenient procedure in place of the rather troublesome decomposition with freshly prepared hydrogen sulphide water. In this latter step we dissolve the precipitated uric acid in place of attempting to decompose it and precipitate the silver, as is done with hydrogen sulphide. The solvent employed is two drops of a 5 per cent solution of potassium cyanide. The cyanide instantly dissolves the precipitate and the silver exhibits no effect in any subsequent part of the procedure. While potassium cyanide has no power whatever of yielding any coloration with the uric acid reagent and alkali, still it exhibits a most interesting effect upon the color produced by a given quantity of uric acid. 1 mgm. of uric acid will give 18 per cent more color with the uric acid reagent and alkali, if previously treated with two drops of the cyanide solution, than is obtained when the cyanide is not present. This effect seems to be due chiefly to a marked diminution in the rate of the fading of the color from solutions containing the cyanide. Other factors may also be involved and we expect to study this point further. For practical purposes it will be sufficient to point out that the final results are unaltered providing the two drops of cyanide are added to both the standard solution and the unknown. The slower fading of the color under these conditions is a distinct advantage. If the cyanide solution be kept in a small dropping bottle the addition of two drops to the standard does not require a moment of time.

The modified technique which we wish to recommend for the determination of uric acid in urine is as follows:

Such a volume of urine as will contain from 0.7 to 1.3 mgm. of uric acid (2 to 4 cc. is usually the right amount) is measured into
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a centrifuge tube, diluted to about 5 cc. with water, and treated with 15 to 20 drops of an ammoniacal silver magnesium solution. The contents of the tube are now mixed with a small stirring rod and the tube is then centrifuged for one or two minutes. The supernatant solution is then poured off as completely as possible, the tube being inverted, and the inside of the lip touched with a towel or piece of filter paper. The residue in the tube is then treated with two drops of 5 per cent potassium cyanide solution, and the mixture thoroughly stirred with a narrow stirring rod for half a minute. A few drops (0.5 to 1.0 cc.) of water are then added and the solution is again stirred. 2 cc. of the uric acid reagent are then added and the mixture is stirred, after which

4 This solution has the following composition:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 per cent silver lactate solution</td>
<td>70 cc</td>
</tr>
<tr>
<td>Magnesia mixture</td>
<td>30 cc</td>
</tr>
<tr>
<td>Concentrated aqueous ammonia</td>
<td>100 cc</td>
</tr>
</tbody>
</table>

Shortly after being mixed the solution will develop a slight turbidity, which should be filtered off. The clear filtrate will keep indefinitely. The magnesia mixture is made as follows: dissolve 17.5 gm. of crystallized magnesium sulphate and 35 gm. of ammonium chloride in about 100 cc. of water; add 60 cc. of concentrated aqueous ammonia, and dilute to 200 cc.

Failure to pour off the supernatant fluid completely is apt to be followed by rapid development of turbidity in the final colored solution, owing to the presence of appreciable quantities of ammonia. Where directions are followed exactly as given in this paper, we have never encountered any trouble from turbid solutions. Should turbidity develop, filtration can be resorted to, but in such cases the standard solution must also be filtered into the colorimeter chamber. This is due to the fact that reoxidation of the colored compound is accelerated by filtration, and an unfiltered clear standard will read higher than the same solution after filtration.

Potassium cyanide solutions will keep for several months, but not for years. Old solutions which have become colored or which smell strongly of ammonia should not be employed.

At this point a perfectly clear solution is obtained where pure uric acid solution in carbonate or pyridin is used. In the case of urine some magnesium ammonium phosphate is precipitated (together with the uric acid) which does not dissolve in the cyanide solution. After adding the two subsequent reagents, however, a perfectly clear solution is obtained.

The uric acid reagent of Folin and Denis is prepared by boiling together 100 gm. of sodium tungstate, 80 cc. of 85 per cent phosphoric acid, and 750 cc. of water under a reflux condenser for an hour and a half, cooling the solution, and diluting to one liter.
10 cc. of 20 per cent sodium carbonate solution are added, the mixture is washed quantitatively into a 50 cc. flask at the end of about one-half minute, and diluted to the mark. This solution is compared in a colorimeter with a simultaneously prepared colored solution obtained by treating 5 cc. of the standard uric acid solution (described earlier in this paper) contained in a 50 cc. flask with two drops of the potassium cyanide solution, 2 cc. of the uric acid reagent, 10 cc. of 20 per cent sodium carbonate solution, and diluting to the mark at the end of about one-half minute. The standard solution is best set at a height of 15 mm. in the colorimeter.

The procedure described above yields quantitative results for pure uric acid solutions and for uric acid added to urine. The figures obtained for uric acid in various samples of urine agree within a few per cent with those obtained by the Folin-Shaffer method. We are inclined to regard the new procedure as perhaps more accurate than the titration method of Folin and Shaffer.
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