THE DETERMINATION OF URIC ACID IN BLOOD.

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The determination of uric acid has been usually regarded as the least accurate and the most tedious and exacting of any of the analyses commonly made upon the blood. Certainly the heat coagulation method followed by concentration and precipitation with silver magnesia mixture as proposed by Folin and Denis (1) and modified by the present writer (2) was a laborious process, and required relatively large amounts of blood and careful analytical work for satisfactory results. Folin and Wu (3) have recently suggested a very interesting procedure which provides for direct precipitation of uric acid from highly diluted blood filtrates. After decomposing the precipitate with sodium chloride in hydrochloric acid solution the uric acid is determined practically as formerly, but instead of using 10 to 20 cc. of blood for a determination, as in the older process, Folin and Wu made use of an equivalent of only 2 cc. of blood for the development of color in their method. The depth of color thus obtained in most bloods is exceedingly weak. To the mind of the present writer it is questionable whether the great advance made by Folin and Wu in precipitating the uric acid directly from the dilute blood filtrate is not more than offset by having the final solutions so weak in color that it is questionable whether they can be compared accurately in a colorimeter by most analysts.

The observation of the writer several years ago that cyanide caused an increase of the color given by uric acid and phosphotungstic acid in alkaline solution offered the possibility of greatly increasing the color obtainable from a given quantity of uric acid. With a large excess of cyanide (several cc. of a 5 per cent solution of potassium or sodium cyanide) the reaction was found to become
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exceedingly delicate, but advantage could not be taken of this fact for analytical purposes for two reasons. With the large excess of cyanide the color reaction was found not to be closely enough proportional to the quantity of uric acid present. Furthermore, turbidity always developed in such solutions, which was a very objectionable feature in analytical work, as the precipitate would continue to form slowly over a considerable period of time. The results obtained in this connection did, however, lead us to keep in mind the possibility of developing a more satisfactory technique for uric acid determination, based upon the use of large quantities of cyanide. It seemed possible that the reaction might be thus made more specific for uric acid, so that the determination could be made directly upon the blood filtrate, without preliminary precipitation of the uric acid. Experiments along this line have been carried on from time to time for the past few years, but the results obtained have hitherto been unsatisfactory for one reason or another. Last September experiments were begun in which the possible application of arsenic tungstic acid in uric acid determination was studied. Results obtained in this connection have proved very satisfactory, and the purpose of the present paper is to describe new uric acid reagents based upon the use of arsenic and phosphoarsenic tungstic acids, and to present a method for uric acid determination in blood which can be applied directly to small quantities of the Folin-Wu blood filtrate.

Arsenic tungstic acids have been described by Kehrmann (4), Fremery (5), and Gibbs (6). In the present work it was soon found that boiling sodium tungstate and arsenic acid together in solution would give a reagent which could be used for uric acid determination, but the condensation of the two acids under such conditions was very slow. Long boiling was required, and the solutions were apt to become quite strongly colored during the continued heating. We, therefore, added hydrochloric acid as a condensing agent. Using this acid, it was found that arsenic and tungstic acids will react very promptly in hot solution, so that the reaction is complete within 20 minutes or less, and the solution colors even less than do the phosphotungstic acids. Our very first experiments showed that reagents prepared in this way are definitely superior to the older phosphotungstic acid reagents, even when used in exactly the same way. Under such
conditions they develop about 20 per cent more color than do the phosphotungstic acid reagents, and are very much less apt to yield turbidity. It was found using 10 gm. of sodium tungstate, and condensing the tungstic acid derived from this by means of hydrochloric acid with 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 gm. of arsenic pentoxide and making to a final volume of 100 cc., that any quantity of the arsenic pentoxide above about 1.8 gm., yields a reagent with a color-yielding power equal to the solution obtained when any of the larger quantities of arsenic acid are used.

We shall describe the arsenic tungstic acid reagent which we first employed, though we no longer use it. It may be of service to those who wish to continue to use the old precipitation method for uric acid determination, since it is more easily prepared than is the old phosphoric acid reagent, and is remarkably free from any tendency to formation of turbidity. This reagent is prepared as follows: 100 gm. of sodium tungstate (J. T. Baker's c. p. is satisfactory, or the "Primos" brand may be used if obtainable) and 30 gm. of pure arsenic acid (As$_2$O$_5$) are placed in a liter flask and about 700 cc. of water added. 50 cc. of concentrated hydrochloric acid are then added and the mixture is boiled for about 20 minutes. It is then cooled and diluted to 1 liter.

This reagent may be employed exactly as is the phosphotungstic acid reagent of Folin and Denis, and is to be recommended in place of that reagent to anyone who may prefer to continue using the old method. We found, however, that with the new arsenic tungstic acid reagent it is possible to use an excess of sodium cyanide solution, and to dispense with the use of sodium carbonate. Under such conditions a much deeper color develops for a given quantity of uric acid than can be obtained under the old condition.

The color obtainable with the new reagent under the new condition was so great that it seemed probable that the new reaction would be much more specific for uric acid than was the old. Hence we were led to try the reaction directly upon blood filtrates and also compared the relative color given by a typical "interfering substance" in the new process. The results obtained here fell just short of our hopes. Resorcinol, used in minute quantities, would give about one-fourth to one-third as much color as
an equal weight of uric acid,¹ and when the reaction was applied
directly to blood filtrates, a considerable proportion of bloods
yielded distinctly high figures by the direct method, while others
gave figures which agreed closely with those obtained by the pre-
cipitation method of Folin and Wu employed as described later
in this paper.

As the results obtained in this connection so nearly fulfilled
our expectations we were led to investigate the possibilities further
before abandoning the hope of obtaining a reagent which could
be applied directly to blood filtrates.

We therefore tried, among other things, combining tungstic
acid with both arsenic and phosphoric acids. Curiously enough
a reagent was thus obtained which is certainly more specific for
uric acid than either arsenic tungstic or phosphoric tungstic acid
alone, and as we shall see below, we believe that the new reagent
fulfills reasonable demands for direct determination of uric acid
on the Folin-Wu filtrates from human blood.

The reagent used in the method described below is prepared as
follows: 100 gm. of pure sodium tungstate are placed in a liter
flask and dissolved in about 600 cc. of water. 50 gm. of pure
arsenic pentoxide are now added, followed by 25 cc. of 85 per cent
phosphoric acid and 20 cc. of concentrated hydrochloric acid.
The mixture is boiled for 20 minutes, cooled, and diluted to
1 liter. The reagent appears to keep indefinitely.

When used as described below this arsenic phosphotungstic
acid reagent yields nearly seven times as much color from a given
weight of uric acid as does the old phosphoric acid reagent as
formerly employed. The new reagent is scarcely affected by a
typical polyphenol such as resorcinol in the presence of uric acid.
If five times as much resorcinol as uric acid be present in a solu-
tion, the quantity of color obtained is increased by only about
15 per cent, and there is no influence on the shade or quality of
color produced. With fifty times as much resorcinol as uric acid,
there is about 66 per cent increase in the color obtained. It is

¹ It is not correct to generalize regarding the amount of color given by
resorcinol and uric acid with the old (or new) reagent, since under the
conditions employed for uric acid determinations the color given by
resorcinol is only very roughly proportional to the quantity of the sub-
stance present.
interesting to note that if no uric acid be present, resorcinol yields considerably more color than when even very minute amounts of uric acid are contained in the solution. This is probably best explained by assuming that the uric acid reacts first with the color-yielding substance in the reagent.

Since the substances in blood, other than uric acid which give color with the old reagent are unknown (though it is commonly assumed that they are probably polyphenols), it is obvious that the true specificity of a reagent for uric acid determination in blood can be determined only by comparing the results obtained when the reaction is applied directly to blood filtrates with those obtained by the use of some accepted precipitation method. We have made such comparison in some detail for human blood, and the findings will be discussed below.

The technique of the new method for uric acid determination in human blood is as follows.

**Standard Solutions.**—The color obtained in the new method from a given quantity of uric acid is so intense that the standard solutions employed have a concentration of uric acid considerably below the solubility of uric acid in pure water. For this reason we are able to employ as standard, solutions of uric acid which are strongly acid with hydrochloric acid. We shall report later upon the keeping quality of these solutions. At present we prepare them fresh once in 2 weeks by appropriate dilution of the phosphate standard solution described by Benedict and Hitchcock in a previous paper. Unless kept in an excessively warm room, the phosphate standard may be relied upon to keep about 2 months.

It is desirable to keep on hand two standard solutions, one of which contains 0.01 mg. of uric acid per cc., while the second contains 0.02 mg. of uric acid in 5 cc. of solution. The second standard is the one commonly employed, but the first may occasionally be of service, and is valuable in instances where it is desired to use the Folin-Wu procedure for comparison of results by the old and new procedures. For the preparation of the first standard, 25 cc. of the phosphate standard solution (containing 5 mg. of uric acid) are measured into a 500 cc. volumetric flask, and the flask is about half filled with distilled water. 25 cc. of dilute hydrochloric acid (1 volume of concentrated acid diluted to 10 volumes with water) are added, and the solution is diluted...
to 500 cc. This solution contains 0.01 mg. of uric acid in 1 cc. For preparation of the second standard (the one which is most frequently employed) the procedure is the same, except that instead of starting with 25 cc. of the phosphate solution, 10 cc. are employed and diluted after acidification exactly as for the other standard. It should be remembered that these standard solutions should be freshly prepared once in 2 weeks.

In connection with the use of the standard solution it should be pointed out that in the new method, as with most colorimetric methods, results are most accurate when standard and unknown correspond closely in depth of color. Using 0.02 mg. of uric acid as standard, results are satisfactorily exact when the unknown reads between 10 and 24 mm., when the standard is set at 15 mm. This represents a quite satisfactory range for bloods, as one standard is applicable for bloods containing from about 2.5 to 6 mg. of uric acid per 100 cc. With 2 mg. of uric acid results may be about 10 per cent too high; i.e., we might obtain 2.1 or 2.2 mg. of uric acid per 100 cc. instead of the true value of 2 mg. Absolute results, where desired, can, of course, be obtained for bloods on the outer limit of any standard by a repetition of the determination, using a closer standard. Increased accuracy cannot be obtained by changing the dilution of either the standard or unknown after the reaction is completed. The determination is so simple, and the quantity of material required so small, that it is no hardship to repeat determinations on the few bloods which fall outside the limits of 2 to 6 mg. per 100 cc. In repeating the determination on such bloods it is desirable to correct by using more or less of the blood filtrate, as indicated by the determination, with corresponding change in addition of water to make a total volume of 10 cc. before addition of cyanide and reagent as described below. It is safer in such cases to repeat with 5 cc. of filtrate, using 0.01 mg. of uric acid in the standard. Where more than 5 cc. of blood filtrate are employed the reading should be made promptly, in order to avoid the development of turbidity.

It should be stated that the method described below yields a slight trace of color in a blank determination. To those who have been in the habit of reading the Folin-Wu uric acid solution this color may appear a serious matter. In the present method, where the color developed is relatively so much more intense, the
color from a blank is negligible, inside the limits above mentioned, since it is present in both standard and unknown.

The blood is precipitated with tungstic acid as described by Folin and Wu (3), the blood being allowed to stand at least 10 to 20 minutes after adding the tungstate and sulfuric acid, before filtration. This tends to insure complete protein precipitation. The use of excess of acid in the precipitation is to be avoided.

5 cc. of the water-clear filtrate (representing 0.5 cc. of blood) are transferred to a test-tube\textsuperscript{2} and 5 cc. of water are added. The standard solution, containing 0.02 mg. of uric acid (prepared as described above), is placed in another tube and the volume likewise made up to 10 cc. To both standard and unknown are added 4 cc. of 5 per cent sodium cyanide solution\textsuperscript{3} containing 2 cc. of concentrated ammonia per liter.\textsuperscript{4} To each tube is then added 1 cc. of the arsenic phosphoric acid tungstic acid reagent. The contents of each tube should be mixed by one inversion immediately after addition of the reagent, and placed immediately in boiling water, where the tubes should be left for 3 minutes after immersion of the last tube, but the time elapsing between immersion of the first and last tubes should not exceed 1 minute. There will be no difficulty here in getting the tubes all into the hot water within 1 minute, unless it is attempted to run more than about five unknown solutions in one series. A 3 minute sand-glass is very convenient in connection with the heating. After the 3 to 4 minute heating the tubes are removed and placed in a large beaker of cold water for 3 minutes and read in a colorimeter against the standard as soon as may be convenient. Long standing before reading may lead to development of turbidity. It is best to read the solution within

\textsuperscript{2} As will be seen from the description of the method the test-tubes used need not be graduated, since no dilution is ordinarily made prior to reading in the colorimeter. The test-tubes employed should be of uniform diameter (18 to 20 mm.).

\textsuperscript{3} On account of the high toxicity of cyanide solutions they should never be handled in pipettes, but should always be measured from a burette. The reagent is also best measured from a burette.

\textsuperscript{4} Some cyanide solutions were found to yield more color in blank determinations than did others. Experiments on this point showed that it is desirable to have a trace of ammonia present in the cyanide solution. It is desirable to prepare the cyanide solution fresh once in 2 months.
5 minutes after removing from the cold water. Where this is done we have never encountered turbidity. Where a large number of bloods are to be analyzed it is best to run not more than four at a time with one standard. This provides for greater uniformity in handling, does not cool the bath down too much, and makes it easy to finish reading before any turbidity may develop.\(^5\)

A few words of explanation concerning the adoption of heating in connection with the uric acid determination may be desirable. We adopted the immersion in hot water for a few minutes after a very careful study had demonstrated that it is not possible to obtain conditions where the reaction goes to even approximate completion in less than 15 minutes at room temperature. Such standing may lead to development of turbidity, and we have found it much better to adopt the short period of heating, which is certainly no hardship. Prolonged heating is to be avoided, since the color will fade under such conditions. There is no difficulty whatever if the tubes are placed in water which is within 10° of boiling and left there for 3 to 4 minutes, and then immersed in cold water as described above.

**Calculation.**—Employing the standard solution containing 0.02 mg. of uric acid and using 5 cc. of the 1:10 blood filtrate, the calculation for the uric acid content of the original blood is as follows:

\[
\frac{S}{R} \times 4 = \text{mg. of uric acid per 100 cc. of original blood}
\]

in which \(S\) represents the height of the standard solution in millimeters, and \(R\) the reading of the unknown solution. If instead of using 5 cc. of blood filtrate in the determination, 2.5 or 10 cc. are employed, the final figure is multiplied or divided by 2 accordingly.

\(^5\) Excess of potassium salts in blood filtrates is undesirable in both the uric acid and the creatinine determinations. It would probably be advantageous for these determinations to employ sodium oxalate instead of the potassium salt to prevent coagulation. There is no apparent advantage in the almost universal habit of using potassium oxalate as an anticoagulant for blood.
Discussion of the Results by the New Method.

In Table I are given the results of determination of uric acid by the new method upon fifty samples of human blood. We have also included in the table figures for the uric acid on the same blood samples obtained by a slightly modified Folin-Wu procedure, together with figures for the non-protein nitrogen. Sufficient blood was not available for comparison by the older silver magnesium precipitation method, but Folin and Wu have stated that their method essentially duplicates the results by the older procedure. In using the Folin-Wu procedure our technique has been identical with that described by these writers up through the decomposition of the precipitate by means of 10 per cent sodium chloride in 0.1 N hydrochloric acid. From this point our procedure differed from that described by Folin and Wu, because these writers advocated the use of a sulfite-containing standard, which has not appealed to us. The use of the sulfite standard, together with the dilution employed by Folin and Wu led to such weak final colors that we were unable to read them. We shall describe our exact technique from the point of precipitation of the uric acid, as it may prove useful to others in connection with checking up results by the new method upon any particular samples of blood.

In using the Folin-Wu technique we have invariably employed 20 cc. of filtrate for the precipitation, except where the blood uric acid exceeded 6 mg. per 100 cc. In such cases 10 cc. of the filtrate were used. The measured volume of filtrate, contained in a centrifuge tube, is precipitated by means of the silver lactate-lactic acid solution as described by Folin and Wu. After cen-

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6 These bloods were obtained from the pathological laboratories of the Roosevelt Hospital through the courtesy of Dr. Wm. G. Lyle, Director. We are also indebted in this connection to Mr. Harry Osterberg, whose figures for non-protein nitrogen on these bloods we have included in the table.

7 Silver lactate purchased in the market is apt to contain considerable amounts of reduced silver, and the product is quite expensive, and often difficult to obtain. We have prepared our silver lactate-lactic acid solution starting from silver nitrate, as follows: Dissolve 22.5 gm. of silver nitrate in about 500 cc. of water in a liter stoppered cylinder. Add an excess (about 60 cc.) of approximately 5 N sodium or potassium hydroxide.
trifugation the residue in the tube is very thoroughly stirred with
the acid sodium chloride solution, using 1 cc. where 10 cc. of
blood filtrate were precipitated, and 2 cc. where 20 cc. were used.
A volume of water is now added to the centrifuge tube which
will bring the total volume of the solution to 10 cc. where the
quantity of uric acid expected does not exceed 0.08 mg., or to 12
cc. where a quantity between 0.08 and 0.12 mg. is expected.
(These figures were selected because they represent the maximal
concentration obtainable where we employed the standard solu-
tion containing 0.01 mg. of uric acid per cc.) The contents of
the centrifuge tube are again thoroughly stirred, and after cen-
trifugation are poured as completely as possible into a clean dry
test-tube. In two other tubes quantities of the standard solution
previously described (containing 0.01 mg. of uric acid per cc.)
are measured to give a total uric acid content in one tube of 0.05
mg. and in the second of 0.08 mg. of uric acid. If high uric acid
is expected it is desirable to have a third standard containing a
total of 0.1 mg. of uric acid. 2 cc. of the acid sodium chloride
solution are added to each of the tubes, and the volume made to
to 10 cc. for the weaker standards, and left at 12 cc. for the stronger
standard. To each standard and unknown tube are now added
3 drops of 5 per cent sodium cyanide solution, and 1 cc. of the Folin-
Denis uric acid reagent, followed by 2 cc. of 20 per cent sodium
carbonate solution. The contents of the tubes are mixed by a
single inversion, and read promptly in the colorimeter, setting
the standard at a height of 20 mm. Should any of the unknown

solution, then add water to about 1 liter and shake. Allow to stand for a
few minutes and decant the supernatant fluid. Add distilled water to
about 1 liter, shake, and pour off the supernatant fluid after a moment or
two. Repeat this washing by decantation until the wash water fails to
react alkaline to litmus paper. The total washing process need not take
longer than 10 minutes. After the last decantation make up to a volume
of about 200 cc. with distilled water, and add 35 cc. of lactic acid (sp. gr. 1.2).
Shake thoroughly and dilute to 500 cc. Filter through a dry folded filter,
returning the first portions until the filtrate appears as clear and colorless
as distilled water. Filter the entire solution through this filter, and pre-
serve in an amber, glass-stoppered bottle.

8 In the description it is assumed that either the 10 or 20 cc. of filtrate
has been precipitated in one centrifuge tube. Where two tubes have been
employed one-half the indicated quantity of chloride solution and of water
should be added to each of the two tubes.
tubes having a dilution of 10 cc. match the 0.1 mg. standard more closely than any of the weaker standards, this comparison may be made after adding 2 cc. of water to the unknown tube. In employing the Folin-Wu procedure it should be remembered that unknown and standard solutions must match quite closely for satisfactory results.

We shall now discuss the comparative figures obtained by the two methods. For facilitating this comparison we have tabulated in Column 4 the difference by the two methods in milligrams of uric acid in 100 cc. of blood, using the Folin-Wu figures as standard. Thus where the new method gives results higher than the old, figures in Column 4 are preceded by a plus, and where lower, by a minus sign.

Even a casual inspection of Table I reveals that the new method tends to give higher results than does the Folin-Wu procedure, at least on those bloods which contain less than 50 mg. of non-protein nitrogen per 100 cc. Thus out of the total of fifty bloods, we find that in thirty-one, or 62 per cent, the new method yields higher figures than the other. This fact in itself could not, however, lead us to question the accuracy of the new method, since in the new procedure we have eliminated precipitation, decomposition, and transfer of the final solution, which would theoretically tend to cause slight losses. For a proper consideration of the differences by the two methods it is, therefore, more correct to take account only of differences which exceed, let us say, 0.5 mg. per 100 cc. of blood, since duplicates by the same method will frequently vary by this amount, and plus differences up to the 0.5 figure can properly be credited in favor of the new method. Out of the total of fifty bloods we find that there are twenty, or 40 per cent, in which the new method exceeds the old by more than 0.5 mg. per 100 cc., eight (16 per cent) in which the figures lie between 1 and 1.5 mg., and none in which the new method exceeds the old by as much as 2 mg. of uric acid per 100 cc. of blood. We shall return later to a discussion of the higher figures by the new method.

From a general standpoint it would appear that if there are no cases where the figures by the new method exceed those by the old by as much as 2 mg. per 100 cc., that very probably the question is a theoretical rather than a practical one. But a very
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<td>3.6</td>
<td>2.5</td>
<td>+1.1</td>
<td>44</td>
<td></td>
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</table>
A crucial question arises at this point. If the tendency to higher results by the new method is due to an interfering substance, and this substance tends to increase in the blood in conditions of kidney inefficiency, then we would have to conclude that the new method could not be of service for determining uric acid. This point seems adequately covered by the results on the bloods which showed 60 mg., or more, of non-protein nitrogen per 100 cc. (Table I; Nos. 1, 5, 7, 11, 16, 25, 26, 38, 41, and 46). These bloods cover a wide range of nitrogen retention as indicated by figures from 60 to 277 mg. of non-protein nitrogen per 100 cc. There is excellent agreement by the two methods in five out of the ten bloods. In three of the remaining five, the new method gives lower results than the old, ranging from 0.4 to 2.1 mg. per 100 cc. of blood. In only one case of a blood with marked accumulation of non-protein nitrogen does the new method yield an appreciably higher figure than the old; viz., 1.1 mg. per 100 cc. in Blood 16.

We believe that there is definite basis for assuming that the lower figures which the new method gives in the majority of the retention cases are the more correct. Theoretically the lower

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Per 100 cc. of blood</th>
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<tbody>
<tr>
<td></td>
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<tr>
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<tr>
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<td>49</td>
<td>2.9</td>
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<td>50</td>
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</table>
The silver lactate precipitation of uric acid from dilute blood filtrates as suggested by Folin and Wu is a remarkable example of accomplishing the seemingly impossible, and is not at all a simple ordinary precipitation as might be inferred from reading a description of the method. Folin and Wu provide for precipitating uric acid from the blood filtrate where the actual concentration of the uric acid may be even less than 1 mg. in a liter, or 1 part in 1,000,000. Even the most insoluble salts known, such as silver iodide cannot be simply precipitated from such great dilution. Furthermore, if one adds the silver lactate solution to pure uric acid solution of five or even ten times the concentration existing in normal blood filtrates there is no visible precipitation. Yet the fact remains that the uric acid is actually removed from the blood filtrate by the silver lactate precipitation. Hence we must infer that the uric acid (probably as the silver salt) is adsorbed by, or in some way dragged down with, other substances simultaneously precipitated. Probably chloride, oxalate, and tungstate (all of which are usually present in the Folin-Wu blood filtrate) contribute to this removal of uric acid from these dilute solutions. It is, however, doubtful upon theoretical grounds whether a precipitation accomplished in this way can be highly specific for any one compound. It is easy to demonstrate that in the case of blood filtrates other substances are carried down with the uric acid, and what is important for our present discussion, that most of the color-yielding substances (in the uric acid reaction) are so precipitated. Here we encounter findings which will impress many readers as not only difficult of interpretation, but as probably involving false assumptions or incorrect work. Yet the present writer has worked on this question in considerable detail and can offer only the conclusions given below, together with the facts upon which they are based.

It is notable that Folin and Wu, instead of dissolving their precipitated uric acid in cyanide, and making the determination directly upon this solution as they do in the case of the urine,

* Uric acid added directly to blood filtrates is recovered very satisfactorily in the new method, indicating that there is nothing in blood filtrates which inhibits the development of the color due to uric acid.
take the extra steps of decomposing the precipitate with a chloride solution and centrifuging off the silver chloride. No explanation is offered by Folin and Wu as to why this apparently unnecessary procedure is adopted in the case of the blood. But if we attempt to get along without it, we immediately get into trouble. If the “silver urate” precipitate is dissolved in enough cyanide to readily bring it into solution (about 10 drops of 5 per cent solution) and an equal quantity of cyanide added to the standard solution (employing, of course, the old phosphotungstic acid reagent for the final color production) we commonly obtain results which are from 150 to 300 per cent too high.

The high results under such conditions might be due to one of three factors: (1) The silver present in the solution where the precipitate has been directly dissolved might be responsible for the high result; or (2) other color-yielding substances may have been precipitated from the blood which dissolve unaltered in the cyanide, but which are decomposed by treatment with acid sodium chloride so that they no longer yield color with the uric acid reagent; or, (3) other color-yielding substances may have been precipitated from the blood which pass into solution when the whole precipitate is directly dissolved, but which remain in the precipitate when treatment with acid sodium chloride is employed.

It can be proved beyond a reasonable doubt that neither of the first two suppositions can explain the high results. If the same silver lactate solution employed in the precipitation is added to a standard solution of uric acid it has practically no effect upon the reading. If a few drops (1.5 drops for each cc. of solution) of 10 per cent sodium tungstate solution (the one employed in the original precipitation of the blood) are added to a standard solution of pure uric acid of about the concentration found in blood filtrates, and this solution is then treated with the silver lactate, the precipitate centrifuged off, and the procedure concluded as for the blood, it will be found that the uric acid taken is recovered almost exactly whether the precipitate be decomposed by the acid chloride solution, or whether it be dissolved directly in cyanide.

These results seem to demonstrate quite conclusively that the high results where cyanide is employed directly on blood filtrates
are not due to the presence of the silver. The demonstration is of importance and shows that the high figures where cyanide is employed directly, are not due to finely divided metallic silver (which would, of course, reduce the phosphotungstic acid reagents). That metallic silver plays no part in this connection is also indicated by the light color of all the precipitates obtained with the blood. The merest traces of metallic silver in a mixture will darken it perceptibly, and this effect is absent from the precipitates with properly prepared and kept silver lactate solution.

The second possibility above suggested, viz. that the high results may be due to a compound which is destroyed by the acid solution, is shown not to be correct by the fact that if the precipitate from blood is stirred with acid sodium chloride and then the entire precipitate dissolved in cyanide the results are still two or three times too high.

By exclusion then, we arrive at the conclusion that the silver lactate precipitates other compounds from blood filtrates which give the uric acid reaction with the old reagent. This conclusion is brought practically to the point of certainty by noting that the figures obtained by dissolving the "silver urate" directly in cyanide increase with increase of total interfering substances in the blood. Indeed for many bloods it seems that practically all the color-yielding substance present in the blood, uric acid, and non-uric acid, is precipitated by the silver lactate solution. Illustrative results in this connection for a few bloods are given in Table II.

In order to show the much greater specificity of the new process for uric acid determination, figures obtained by applying the new process directly to the same blood filtrates, are also included.

It seems clear from the results reported in Table II that there is a direct relationship between the total color-yielding compounds in the blood, and the color obtained where the "silver urate" is dissolved directly in cyanide. We must, therefore, conclude that while these substances may be largely precipitated by silver lactate in lactic acid, Folin and Wu found a means of decomposition for the precipitate which effects a remarkable separation between the uric acid and the other color-yielding compounds. It seems quite inexplicable to the present writer how, upon theoretical grounds, this separation can be effected, but the facts seem to admit of no other explanation. It seems reasonable,
however, to assume that the tendency to higher results by the Folin-Wu method for the bloods high in non-protein nitrogen is best explained upon the assumption that some of the precipitated non-uric acid color-yielding material may be set free during the decomposition of precipitates containing excessive quantities of this material. Certainly it appears that the lower results for such bloods by the new method are probably more nearly the correct ones.

The tendency to somewhat higher figures by the new method for ordinary bloods as exemplified in Table I can scarcely be definitely explained at present. It is possible that the excess

TABLE II.

Showing the results obtained with human bloods when the "silver urate" precipitate is dissolved directly in cyanide solution. Comparative figures are given by the modified Folin-Wu decomposition method, and by the old and the new reagents applied directly to the blood filtrate.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Per 100 cc. of blood.</th>
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<tbody>
<tr>
<td></td>
<td>mg.</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>10.2</td>
</tr>
<tr>
<td>3</td>
<td>2.4</td>
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<td>5</td>
<td>2.5</td>
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<td>6</td>
<td>15.7</td>
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figures represent an effect of some interfering substance which shows no tendency to increase with kidney inefficiency. This explanation is unlikely, especially since the difference between the two methods tends to disappear or is reversed as soon as the uric acid by the Folin-Wu method approaches a figure of 3.5 to 4.0 mg. The only evidence that we have that the figures by the new method represent essentially only uric acid in the bloods showing the discrepancy as well as in the others, is the fact that where the determinations are made by letting the filtrates stand in the cold, so that the maximal color develops slowly, it is found that where standard and unknown contain approximately the same
amounts of the reacting substance as measured by the final color, results are practically identical whether the readings be made early or late in the process of color development. This similarity in rate of color development in standard and unknown lends support to the view that the figures represent uric acid only. Conversely, since the exact conditions of salt content, acidity, etc., and other factors which may influence the adsorption precipitation of the silver urate in the blood filtrates cannot be readily duplicated upon solutions totally free from uric acid for tests upon complete recovery, there is some question as to whether the small increment of uric acid shown in the new direct method may not be lost in some way in the precipitation and decomposition method.

Another possible explanation which suggests itself concerning these differences is that in these bloods we may be dealing with the presence of some second form of uric acid—combined or altered in some way so that it reacts more strongly in the new method than in the old. We investigated this possibility, and soon became convinced that it deserves no consideration. When we applied the new process to the solution obtained by decomposing the silver precipitate as in the Folin-Wu method we invariably obtained figures identical with or lower than where the old reagent and procedure were employed. In no instance where the new and the old processes have been applied actually to portions of the same solution, whether the blood filtrate directly, the solution after sodium chloride decomposition, or the solution obtained by dissolving the silver precipitate directly in cyanide, have we obtained higher figures by the new process. They have usually been appreciably lower.

Hence it seems that there can be no question of a second form of uric acid which reacts differently with the two procedures in these bloods.

It seems that an impartial study of Table I should lead to the adoption of the new procedure for general work.\footnote{If one prefers to continue using the Folin-Wu precipitation and decomposition method, the new process can, of course, be applied to the solution thus obtained. 5 or 10 cc. of filtrate are precipitated and decomposed as in the Folin-Wu method, the volume being made to 10 cc. with water before the final stirring and centrifugation. The clear solution obtained after centrifuging is poured as completely as possible into a test-tube, and the process concluded as in the regular new method.}
S. R. Benedict

is more expeditious and requires the use of less blood than does any other procedure. It is simple and gives a satisfactory depth of color and is far safer in the hands of many analysts. The differences by the two methods are in many instances negligible, and in only one or two instances out of the fifty analyses reported, would acceptance of the new figures suggest a different interpretation as regards uric acid abnormality or retention. In one instance, Blood 13, the new method gives a figure above the accepted normal, while in another, No. 43, the interpretation would be reversed if the new figures are accepted. In the latter case the figure by the new process is almost certainly more correct. The difference by the two methods chiefly affects the bloods where the Folin-Wu method gives 2.5 mg. or below. Thus we find that out of the fifty bloods, the Folin-Wu method gives a figure of 2.5 mg. or below, per 100 cc. for fifteen bloods, while only five bloods give similarly low figures by the new process. Where the new process is used it is probable that the commonly accepted figures should be increased by about 0.5 mg. per 100 cc., so that the usual normal figures would probably be about 3.0 mg., while no evidence of definite uric acid retention would be evident before a figure of 4.0 mg. or over per 100 cc. was reached. The question as to whether the figure 4.0 mg. per 100 cc. of blood represents a true pathological retention must remain open, just as it is for quite similar figures by the old methods. Such border-line questions can be answered only by extensive data.

A few words covering the quantity of blood filtrate necessary or desirable to carry out the new procedure may be of value. As the method is above recommended it will be noted that we finally heat a rather large volume (15 cc.) of a very dilute solution. This solution may contain as low a concentration of uric acid as 1 part in 1,500,000 parts of solution or even less, and accurate results can still be obtained. It may seem that it would be better to carry out the reaction with more concentrated solutions, or with smaller volumes. We have found that higher concentrations are not so desirable, as turbidity may occur, and the results as a whole are not so uniform. It is, of course, perfectly possible to cut everything in half in the process as above described, using 2.5 cc. of filtrate, and having an ultimate volume of 7.5 cc. during the heating. This is permissible where circumstances may
require it, but the writer believes that the larger volumes are commonly measured with greater accuracy, and since 0.5 cc. of blood is certainly not excessive for a uric acid determination, it seems better to recommend starting with that equivalent unless there are reasons to the contrary.

There are, of course, certain instances where it may be desirable or necessary to work with very minute quantities of blood, such as can be obtained readily by puncture. In the case of infants, or where repeated examinations on the same individual are to be made, the use of blood obtained by puncture may be essential. For this reason a modification of the new procedure is here described which might perhaps be properly called an "ultra-micro" method, since it can be used to determine as little as 0.002 mg. of uric acid, and yields results very comparable with those obtained by the procedure described earlier in this paper. The modification requires the use of only 0.2 cc. of blood (0.1 cc. of filtrate), and is carried out as follows:

0.2 cc. of blood is carefully pipetted into a narrow pointed centrifuge tube. 1.4 cc. of water are added and the mixture is stirred with a fine glass rod. 0.2 cc. of 10 per cent sodium tungstate solution is added, followed by 0.2 cc. of 0.75 N sulfuric acid, and the mixture is thoroughly stirred and allowed to stand for 10 minutes. The tube is then centrifuged at high speed for a few moments and 1 cc. of the clear supernatant fluid is pipetted into a long narrow (1 cm.) test-tube. 1.8 cc. of 2.8 per cent sodium cyanide solution (containing 1.5 cc. of concentrated ammonia per liter) are then added, followed by 4 drops (0.2 cc.) of the arsenic phosphotungstic acid reagent. The mixture is then treated as is the unknown in the regular process, and can be compared with the 0.02 mg. regular standard solution. The 3 cc. of colored solution are readily read in the narrow form cup of the Bock-Benedict colorimeter up to a height of about 24 mm.

In using this modification it is, of course, essential that calibrated pipettes be employed, and that all the measurements be made with great care. Under such conditions we have obtained surprisingly close duplicates between the two modifications. Of ten bloods analyzed by both methods the maximal difference was 0.6 mg. of uric acid per 100 cc., and in six of the samples the variation did not exceed half of this figure.
In conclusion one or two points of general interest in connection with the new process may be mentioned. The increased specificity of the new procedure as regards uric acid lies probably chiefly in the reagent employed and only partly in the use of cyanide instead of carbonate for development of the alkalinity. A corresponding, though not so great, increase in color is obtained with the old phosphotungstic acid reagent through the use of large quantities of cyanide, but the reaction thus obtained is not very specific for uric acid. The cyanide acts in a dual capacity in causing the increased color. It has an effect upon the reaction, probably through combination with the uric acid as exemplified in the older procedures, and it is superior to carbonate as an alkali, because it causes a much slower decomposition of the reagent than does the stronger alkali, and hence gives more time for the reaction at the critical point when the linkage of the complex tungstic compound is being broken. That this view is correct is rendered probable by a fact observed by the writer some years ago that the use of borax in hot solution would markedly increase the color yield in the uric acid determination. The results were not reported because the color developed was not closely proportional to the quantity of uric acid present. In this instance the specificity of the reaction was not appreciably increased, although the color yield for a given quantity of uric acid was greater than in the carbonate process.

It is to be noted that the new process described in the present paper is now recommended only for application to the Folin-Wu filtrates from human blood. Studies of the new process under certain other conditions of precipitation, and for other bloods, are being carried on.

We shall shortly report upon the application of the new procedure to the determination of uric acid in urine.

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THE DETERMINATION OF URIC ACID IN BLOOD
Stanley R. Benedict


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