THE PREPARATION OF URIC ACID REAGENT COMPLETELY FREE FROM PHENOL REAGENT.

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It had been known for a long time that ordinary phosphotungstic acid in alkaline solutions gives some blue color with certain reducing substances when, in 1912, it was shown by Folin and Denis that this property of phosphotungstic acid could be increased enormously by the introduction of suitable changes in the mode of preparing the solutions. The solution thus obtained from sodium tungstate and phosphoric acid was introduced as a uric acid reagent, while the solution produced by the action of phosphoric acid on sodium tungstate plus sodium molybdate became their phenol reagent. A systematic study of the subject, by Wu (1) in 1920, showed that the uric acid reagent is phospho-18-tungstic acid, whereas ordinary phosphotungstic acid consists almost entirely of the inactive phospho-24-tungstic acid. Many variations and some improvements in the preparation of the uric acid reagent have been described since 1912. But, while it was recognized from the beginning that the greatest attainable degree of specificity could be obtained only in the absence of molybdate, it was not until 1924 that the first attempt was made, by Folin and Trimble (2), to provide for the removal of the molybdates which are present in varying amounts in all available samples of sodium tungstate. Folin and Trimble described a very satisfactory test for the molybdates and also gave a method for their partial removal by means of H₂S, as a preliminary step in the preparation of the uric acid reagent. Manufacturers of sodium tungstate have in the meantime contributed nothing except to meet the demand for normal tungstates as distinguished from the

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less soluble $p$-tungstates. The brands now available, except Merck's best "reagent" tungstate, seem to contain even more of molybdates than was the case a few years ago.

Scheiner (3) an Italian, has recently come to the conclusion that there is practically very little difference between the phenol reagent and the uric acid reagent. Scheiner's paper deals exclusively with the alleged uselessness of the reagents, and he does not state what particular determination he was trying to learn. The title of his paper may be given as "Sources of Error in the Determination of Phenols, Tyrosine and Uric Acid by means of Phosphotungstic Acid [Uric Acid Reagent]."

One of the most exacting needs for a uric acid reagent which is free from phenol reagent is encountered in attempting to make cystine determinations in protein hydrolysates according to the method of Folin and Looney. To get a reagent which gives the maximum color with cysteine, yet gives no trace of color with tyrosine, and a negligible color with the sodium sulfite which must be used for reducing cystine to cysteine, it is quite essential that a reagent with the highest degree of selectivity be secured. Indeed it might be fairly questioned whether one is not here asking for too much in the way of colorimetry based on selective oxidation.

In the course of a proposed critical study of the colorimetric cystine determination, we found that the uric acid reagents prepared according to Folin and Trimble from the sodium tungstates now available could not be made to yield usable results. One old uric acid reagent made by one of us 4 years ago was very nearly satisfactory, but we were not able to reproduce it. In these circumstances we were led to revise the Folin-Trimble process and finally succeeded in developing a method which, from all brands of sodium tungstate now available, gives uric acid reagents quite free from phenol reagent. Our method is based on the following principles and observations:

1. Sodium molybdate does, sodium tungstate does not, combine with phosphoric acid at room temperatures.
2. After the addition of the right amount of phosphoric acid to a sodium tungstate solution, the uncombined molybdate reacts fairly rapidly with $H_2S$.
3. Most of the molybdenum is converted into brown insoluble
sulfides by the H_2S treatment, and these sulfides are removed at once by filtration.

4. A part of the molybdate is converted into soluble sulfur compounds by the H_2S treatment, and the longer this treatment is continued the greater will be the fraction of soluble molybdenum sulfur compounds.

5. The soluble sulfomolybdates are very soluble in alcohol, and can be quantitatively removed by a single 5 minute extraction with alcohol.

On the basis of these principles and observations 100 gm. of sodium tungstate can now be converted in the course of about 2 hours into 1 liter of uric acid reagent completely free from phenol reagent.

The process is as follows:

Transfer 100 gm. of sodium tungstate and 200 cc. of water to a 500 cc. Florence flask. Shake until the tungstate is dissolved. Add slowly, with shaking and cooling, 20 cc. of 85 per cent phosphoric acid. The solution must not be allowed to become warm from the heat of the reaction with the phosphoric acid. Pass H_2S into the phosphotungstate solution at a very moderate rate for 20 minutes. At the end of the first 3 or 4 minutes, add gradually and slowly another 10 cc. of 85 per cent phosphoric acid without interrupting the H_2S current. It would be simpler to add all of the phosphoric acid (30 cc.) before beginning the H_2S treatment, but by adding the phosphoric acid as described, one obtains the molybdenum sulfide in a somewhat less finely divided condition, so that it can be removed more easily by filtration. Incidentally, it may be remarked that the 30 cc. of phosphoric acid should be just sufficient to render the solution slightly acid to Congo red paper. At the end of 20 minutes, filter the solution through a good grade of quantitative filter paper. It is advisable to collect the first 40 cc. of filtrate in a 50 cc. cylinder, because the first portion may be a little turbid, and it may need to pass through the filter a second time.

If the conditions have been right, the filtrate should be clear, and it will have a greenish color, because a little blue is produced by reduction of any uric acid reagent that may have formed, while the soluble sulfomolybdates are red.
Transfer the filtrate to a separatory funnel (capacity 1 liter) and add, with shaking, 300 cc. (1.5 volumes) of alcohol. The mixture separates at once into a reddish or slightly greenish supernatant solution, and a bluish, very heavy solution at the bottom. The latter contains all of the phosphotungstic acid in a supersaturated solution, and it is best to withdraw it rather soon into a weighed 500 cc. Florence flask. If left too long in the separatory funnel, it sometimes forms crystal deposits which block the exit through the stop-cock.

In so far as any insoluble molybdenum sulfide happens to be present, this will be floating between the two layers of liquid in the separatory funnel, and these solid aggregates must not be allowed to pass through the stop-cock and into the phosphotungstic acid solution. The mixture remaining in the separatory funnel is discarded. It contains not only the sulfomolybdates, and the greater part of the surplus H₂S, but probably also various other impurities.

Add water to the concentrated phosphotungstic acid in the 500 cc. flask until the weight of the contents amounts to 300 gm. Boil the solution over a micro burner for a few minutes, until a paper moistened with lead acetate solution shows that the H₂S has been removed. Then, but not until then, cut down the flame, and add 20 cc. of 85 per cent phosphoric acid. It is only with the addition of this last quantity of phosphoric acid that the optimum conditions are obtained for transforming the ordinary (1:24) phosphotungstic acid into the active (1:18) phosphotungstic acid, that is to say, into the uric acid reagent.

Insert a 10 cm. funnel into the 500 cc. flask to hold a 200 cc. flask filled with cold water, and boil gently for 1 hour. At the end of this time, the reaction is finished. Cut down the flame, remove the condenser (funnel and flask), filter, and add to the filtrate a few drops of bromine, and boil, to remove the blue color of the solution. When the blue color is gone, boil rapidly for a few minutes, to remove the bromine, then cover the mouth of the flask with a beaker and cool under running water.

Transfer 25 gm. of lithium carbonate to a liter beaker, add first 50 cc. of phosphoric acid, then add slowly 250 cc. of water and boil, to remove the CO₂. Cool the resulting lithium phos-
phate solution; and add it to the concentrated uric acid reagent in the 500 cc. flask and dilute to 1 liter.

The reagent which we obtain by the process described above is better than any uric acid reagent we have ever had before in this laboratory, not only for cystine determinations, but also for uric acid determinations. It is very active, yet possesses the highest obtainable degree of specificity, hence, gives the minimum of blanks, with sulfite, cyanide, etc., and does not give a trace of color with tyrosine. In addition, it shows less tendency to give disturbing precipitates than do our other uric acid reagents.

The blue color which this reagent gives with uric acid is of a different shade from the blue which is obtained with uric acid reagents which have been prepared in the ordinary way, that is, without the preliminary removal of the molybdate. The blue from the ordinary reagents has, by comparison, a distinctly violet tint. This point is rather suggestive in connection with Wu's detailed work on the active ingredients of the uric acid reagent. By fractional crystallization, Wu obtained two active compounds which he called the A and the B form of phospho-18-tungstic acid. These differed in chromogenic values, and they differed markedly in the shade of blue which they produced with uric acid. It seems altogether probable that the finding of these two different compounds represents the fact that the sodium tungstate from which they were prepared contained sodium molybdate.

While convenience and speed of preparation were considered of minor importance in developing the method described above, the process is so simple that all who have occasion to prepare and use the uric acid reagent should be able and willing to first remove the molybdate—until such time as some manufacturers can be induced to supply sodium tungstate which is completely free from molybdate.

BIBLIOGRAPHY.
CORRECTIONS.

On page 585, Vol. lxxxii, No. 3, June, 1929, lines 15 and 18, read *maximum* for *minimum*; lines 17 and 29 read *maxima* for *minima*.

On page 778, Vol. lxxxii, No. 3, June, 1929, line 5, read

\[ \text{pH}_{38^\circ} = \frac{\text{E.M.F.} - e}{0.06169} \text{ for } \text{pH}_{38^\circ} = \frac{\text{E.M.F.} + e}{0.06169}. \]

On page 110, Vol. lxxxiii, No. 1, July, 1929, line 6 from the foot of the page, read, *Sodium tungstate does, sodium molybdate does not*, for *Sodium molybdate does, sodium tungstate does not*. 
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