A COLORIMETRIC METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHATE IN BLOOD SERUM.

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The methods most generally used for the determination of phosphates in blood are based upon the reduction of phosphomolybdic acid to the blue molybdic oxide, the color of which is compared to a solution containing a known amount of phosphate. The first method based upon this principle was introduced by Taylor and Miller (1) and was later modified by Bell and Doisy (2), Briggs (3), Benedict and Theis (4), and Fiske and Subbarow (5). However, the conditions necessary to obtain quantitative results have to be very carefully adjusted. Thus Roe, Irish, and Boyd (6) found the following conditions to influence greatly the production of the blue color: the concentration of the molybdic acid; the concentration of the reducing agent; the time allowed for the completion of the reaction; the hydrogen ion concentration; the presence of salts; and the amount of phosphate in the sample to be analyzed.

It is evident that a method in which most of these obstacles could be eliminated would be worth while. Such a method is made possible by using a soluble uranium salt for isolating phosphate from solution, since phosphate combines quantitatively with uranium to produce an insoluble salt. A volumetric procedure based upon this principle for the determination of phosphate in urine has been in vogue for many years. Gibson and Estes (7) have modified the method for urine into a colorimetric procedure by adding a solution of uranium acetate of known strength in excess of the amount necessary to precipitate the phosphate completely, and after removing the insoluble uranium phosphate by filtration, determining the excess uranium acetate colorimetrically. Sato (8) applied this technique to feces and urine, but instead of deter-
mining the excess uranium, he washed the uranium phosphate on a filter paper until all soluble uranium was removed; he then re-dissolved the uranium phosphate and determined the amount of uranium colorimetrically.

I have applied this procedure with a number of modifications to the determination of phosphate in blood serum with very good results. There is very little interference from the substances normally present in blood and from the reagents used in the performance of the test. The only factor that has to be carefully adjusted is the acidity of the serum filtrate. This point will be discussed fully later.

**Process.**—The serum proteins are removed with trichloroacetic acid and the phosphate precipitated as uranium phosphate in an acetic acid medium which contains ammonium acetate. The precipitate is washed free from uranium acetate, redissolved in trichloroacetic acid, and the color developed with potassium ferrocyanide.

**Method.**

Place 2 cc. of serum in a test-tube, and add 4 cc. of distilled water and 4 cc. of 20 per cent trichloroacetic acid. Shake well, let stand for 10 minutes, and filter through an ashless filter paper. When the filtration is complete a little more than 5 cc. of filtrate is obtained. Place 5 cc. of the filtrate into a conical 15 cc. centrifuge tube which is graduated at 10 cc. Into a similar tube place 5 cc. of the standard phosphate solution. Add to each tube a drop of brom-thymol blue indicator solution. Add dilute ammonium hydroxide until the solution turns blue (pH 7.6). Add 0.3 cc. more of the dilute ammonium hydroxide and then add 5 per cent acetic acid solution until the color just turns yellow (pH 6.0). Add 1 cc. of uranium acetate solution and 2 cc. of 95 per cent alcohol. Let stand for 1 minute, shake well, and let stand for 15 minutes. Centrifuge for about 3 minutes at moderate speed and pour off the supernatant fluid, letting the tube drain for a minute by touching its mouth against a clean filter paper in order to remove the last drop. Add 5 cc. of 20 per cent alcohol and break up the precipitate with a thin glass rod; add 5 cc. more alcohol, washing off the rod. Centrifuge and pour off the supernatant fluid as before. Wash once more with 10 cc. of 20 per cent alco-
hol and pour off the supernatant fluid. All of the excess uranium acetate is thus completely removed. Dissolve the precipitate in 1 cc. of 20 per cent trichloroacetic acid. This is best done by holding the tube at its mouth between the thumb and the index finger of the right hand and repeatedly hitting the bottom of the tube against the palm of the left hand. Hold up against the light to make sure that the precipitate is completely dissolved. Add 5 cc. of water and 1 cc. of a 10 per cent solution of potassium ferrocyanide, and fill up with water to the 10 cc. mark. Mix, let stand for about 2 minutes, and compare in the colorimeter, setting the standard at 20 mm.

Calculation of Results.

\[
\frac{S}{R} \times 5 = \text{mg. phosphorus in 100 cc. serum.}
\]

\[
S = \text{reading of standard in mm.}
\]

\[
R = \text{reading of unknown in mm.}
\]

Preparation of Reagents.

1. Trichloroacetic Acid.—This reagent is not obtained commercially in very pure state and may contain traces of phosphate. Since it boils at 195° without breaking down, it is advisable to purify it by distillation.

2. Standard Phosphate Solution.—Carefully weigh out 0.2193 gm. of pure dry KH₂PO₄ and dissolve in water in a 200 cc. volumetric flask, making up with water exactly to the mark. Place 20 cc. of this solution into a 500 cc. volumetric flask half filled with water, add 200 cc. of 20 per cent trichloroacetic acid, and make up to the 500 cc. mark with water. 5 cc. of this solution contain 0.05 mg. of phosphorus which is equivalent to 5 mg. per 100 cc.

3. Ammonium Hydroxide.—28 per cent NH₄OH diluted 1:5.

4. Brom-Thymol Blue.—0.5 per cent solution.

5. Uranium Acetate Solution.—Dissolve 20 gm. of uranium acetate in a liter flask half filled with water (do not heat). Add 30 cc. of glacial acetic acid and make up with water to a liter. Mix well until practically all of the uranium acetate is dissolved (some undissolved substance will remain). Let stand for 2 days and filter through an ashless filter paper. Any phosphate that may have been present in the uranium acetate is thus removed.
DISCUSSION.

The trichloroacetic acid in the serum filtrate and in the standard must be completely neutralized since uranium phosphate is highly soluble in this acid and no precipitation of phosphate will occur even in the presence of an excess of acetic acid. After the trichloroacetic acid is neutralized, an excess of ammonia is added in order to produce an excess of ammonium acetate upon neutralization with acetic acid. This is necessary because uranium phosphate is somewhat soluble in acetic acid and the ammonium acetate, by depressing the hydrogen ion, prevents the solution of uranium phosphate by the acetic acid.

The amount of acetic acid added is of extreme importance. If the alkali is not completely neutralized some uranium will precipitate as NH₄UO₂O₇. When very little acid is present more phosphate is precipitated than when acetic acid is present in greater concentrations. If too much acetic acid is used precipitation of uranium phosphate will be hindered or entirely prevented even in the presence of ammonium acetate. The influence of acidity is shown by the curve (Fig. 1) in the accompanying diagram where colorimetric readings are plotted against acidity.

Thirty-four tubes were set up, each containing 5 cc. of the trichloroacetic acid phosphate standard. They were divided into two sets of seventeen tubes each. One set was neutralized with N
NaOH and the other with $n \text{NH}_4\text{OH}$. Varying amounts of normal acetic acid were added to the tubes in each set and the amount of phosphorus determined colorimetrically as described in the method. One of the tubes showing a color of medium intensity was used as a standard, set in the colorimeter at 20 mm., and the rest of the tubes were read against it.

The curves obtained with the sodium and ammonium hydroxides show that the use of ammonia as a neutralizing agent is preferable to the use of sodium hydroxide. In the ammonia curve a maximum coloration occurs in the first four tubes. In this region the curve is very sharp. In the zone between 0.1 and 10 cc. of added normal acid there is no change in the intensity of the color produced. While the color in the first four tubes, where the acidity is low, is more than twice as deep as in the remaining tubes and should therefore be preferable in a colorimetric method, yet the disadvantage of the low margin of safety is too great to use low acidity. While the color is lighter in the remaining tubes, a straight line is obtained over a large range of acidity; thus by using higher acidity sources of error due to acidity are avoided. The mid-point of acidity on the straight line was chosen as the proper acidity to be used in the method.

In the beginning of the experiment NaOH was used as the neutralizing agent and it was thought that the deeper colors produced at the lower range of acidity were possibly due to some NaUO$_2$O$_7$ being precipitated since the NaOH, being a strong base, is easily dissociated and produces free OH$^-$ ions, thus precipitating uranium oxide. That such was not the case was easily proved by the following experiments.

Experiment 1.—Controls containing trichloroacetic acid and no phosphate, when made just acid to brom-thymol blue without further addition of acid, repeatedly failed to produce a precipitate, even after standing overnight.

Experiment 2.—Added phosphate was completely recovered from solutions containing both very low and high acidity. Two sets of six tubes each were set up to which increasing amounts of phosphate were added. In one set the reaction was adjusted just acid to brom-thymol blue, while to the other set were added 5 cc. of $n$ acetic acid. The amount of phosphorus was determined as described in the method. The first tube in each set was used as
the standard and set in the colorimeter at 20 mm. The rest of the tubes were diluted with water to approach the intensity of the color of the respective standards. Complete recoveries of phosphate were obtained in each set in spite of the fact that the amount of precipitate, and consequently the intensity of the color, in the tubes of the low acidity were more than double in amount than in the tubes of the high acidity. The increase in color was in each case proportional to the amount of phosphate. This is shown by Table I and Fig. 2.

These experiments show definitely that at the different levels of acidity the precipitates consist of uranium phosphate and no uranium oxide is being precipitated. The difference in the amount of precipitate is probably due to different combinations of the uranium with the phosphate. A search in the literature revealed the existence of some confusion as to the true nature of combination, some giving the formula UO₂HPO₄, and some (UO)₂HPO₄. Another possible combination is (UO₂)₃(P₄O₁₂). Simon (9) says that with the dibasic phosphate 2 molecules of uranium combine with 1 molecule of phosphorus, while with the monobasic phosphate 1 molecule of uranium combines according to the following reactions:

\[
\text{Na₃HPO₄} + 2\text{UO} \cdot \text{NO}_₂ = 2\text{NaNO}_₃ + (\text{UO})₂\text{HPO}_₄
\]
\[
\text{NaH₂PO₄} + \text{UO} \cdot \text{NO}_₂ = \text{NaNO}_₃ + \text{UOH}_₂\text{PO}_₄
\]

If these reactions were true, then it would seem that at low acidity the phosphate existed in the dibasic form, thus combining with twice as much uranium. That such is not the case can be easily proved. An aqueous solution of Na₃PO₄ was divided into
three equal parts. One portion was acidified with acetic acid to phenolphthalein:

$$\text{Na}_3\text{PO}_4 + \text{H}_2\text{C}_2\text{H}_3\text{O}_2 + \text{phenolphthalein} = \text{Na}_2\text{HPO}_4 + \text{NaC}_2\text{H}_3\text{O}_7$$

Another portion was neutralized with normal acetic acid to methyl orange:

$$\text{Na}_3\text{PO}_4 + 2\text{H}_2\text{C}_2\text{H}_3\text{O}_2 + \text{methyl orange} = \text{NaH}_2\text{PO}_4 + 2\text{NaC}_2\text{H}_3\text{O}_7$$

No acid was added to the third portion. The three solutions were titrated with 2 per cent uranium acetate solution to which no acetic acid was added, cochineal being used as an indicator. The following amounts of uranium acetate were necessary to combine with the three phosphate solutions:

- $\text{Na}_3\text{PO}_4 = 8.24$ cc. uranium acetate.
- $\text{Na}_2\text{HPO}_4 = 16.54$ cc. uranium acetate.
- $\text{NaH}_2\text{PO}_4 = 16.46$ cc. uranium acetate.

This shows that the mono- and dibasic phosphates combine in the same proportions with uranium acetate. In the case of the tribasic phosphate it seems probable that the precipitate was
uranium oxide, since \( \text{Na}_3\text{P}O_4 \) is easily hydrolyzed by water, producing a molecule of sodium hydroxide:

\[
\text{Na}_3\text{P}O_4 + \text{H}_2\text{O} = \text{Na}_2\text{HPO}_4 + \text{NaOH}
\]

This question is being investigated at present, but since it has no direct bearing upon the method it is left out of this paper.

In the volumetric method for the determination of phosphate in urine by means of uranium, and in the colorimetric procedures of Gibson and Estes (7), and of Sato (8), the phosphate solution is first heated to about 95° before the uranium solution is added. In this method this was found unnecessary and even undesirable; better checks and better recoveries of phosphate were obtained in the cold. Moreover, when the solution is heated secondary calcium phosphate (\( \text{CaHPO}_4 \)) may be precipitated.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Added P per cc.</th>
<th>Serum plus P calculated per 100 cc. serum.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>Recovered.</td>
<td>Calculated.</td>
</tr>
<tr>
<td>1</td>
<td>None.</td>
<td>3.9</td>
<td>5.8</td>
</tr>
<tr>
<td>2</td>
<td>0.02</td>
<td>5.8</td>
<td>7.9</td>
</tr>
<tr>
<td>3</td>
<td>0.04</td>
<td>8.1</td>
<td>9.9</td>
</tr>
<tr>
<td>4</td>
<td>0.06</td>
<td>9.8</td>
<td>9.9</td>
</tr>
<tr>
<td>5</td>
<td>0.08</td>
<td>11.6</td>
<td>11.9</td>
</tr>
</tbody>
</table>

The precipitate obtained on centrifugation is very gelatinous and packs tightly to the bottom of the tube so that no danger is encountered in losing any phosphate during the process of decantation.

The 20 per cent alcohol used for washing the precipitate prevents any possible solution of uranium phosphate, since it is absolutely insoluble in such a medium while uranium acetate is very soluble in it.

The stability of the color of uranium ferrocyanide depends largely upon the acidity. The higher the acidity the less stable the color. When left standing for some hours, the yellow color changes to blue and a precipitate settles out. This blue color is not proportional to the amount of uranium present but to the ferrocyanide.
The yellow color of uranium ferrocyanide develops immediately and will last without any change for at least half an hour.

Sato (8) used HCl for dissolving the uranium phosphate. The use of inorganic acids, however, was found to be detrimental to the results, since the presence even of minute traces of iron will produce a blue color with the ferrocyanide, and the color produced in absence of iron is not so good since the quality of the color varies with slight changes in the amount of HCl used. No such effect is produced with trichloroacetic acid.

Good recoveries of added phosphate were obtained from blood serum. A sample of serum which was found by this method to contain 3.9 mg. of phosphorus per 100 cc. was divided into five equal parts. No phosphate was added to the first part; to the other parts were added increasing amounts of phosphate. The results are shown in Table II.

SUMMARY.

A method is described for the determination of inorganic phosphate in blood serum. The proteins are removed with trichloroacetic acid; the filtrate is alkalinized with ammonium hydroxide and reacidified with acetic acid, and the phosphate precipitated with uranium acetate. The uranium phosphate is washed free of uranium acetate with dilute alcohol. It is then redissolved in trichloroacetic acid, converted into uranium ferrocyanide, and is compared colorimetrically with a similarly treated solution containing a known amount of phosphorus. The color is proportional to the amount of uranium, thus to the amount of phosphorus.

BIBLIOGRAPHY.

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